



Molecular Investigation of Some Virulence Factors in *Enteropathogenic E.coli* Isolated from Diarrhea Cases in Children Under Five Years Old in Al-Diwaniyah Province / Iraq

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Abstract

The results of the initial culture showed that there were 210 (65.01%) of the total samples of (323) samples that tested positive for E.coli, depending on the cultivar, morphological and microscopic traits of the MacConkey agar medium. Blood agar (Blood agar and Eosin Methylene Blue (EMB) differential medium, in addition to biochemical tests compliant with the urease test). The oxidase test and the catalase test. In addition to the IMViC group tests. Chromogenic media of EPEC bacteria was used for the purpose of distinguishing it from the rest of the E.coli species. When bacteria were grown on this medium, 37 out of 120 bacterial isolates (120/37) (30.83%) belonged to EPEC isolates. It was grown on this medium and appeared in the form of violet-colored colonies as a result of its production of β -glucuronidases. Fifteen EPEC isolates were selected for genetic investigation of some of their virulence genes, represented by the eaeA and bfp genes and (15/13) (86.66%) contained the bfp gene responsible for the formation of the pilin protein, which is included in the structure of the adhesion filaments of bacteria (Pillus).

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Key Words: Pathogenic Escherichia Coli, eaeA, bfp, Diarrhea.

DOI Number: 10.14704/nq.2022.20.8.NQ44189

NeuroQuantology 2022; 20(8): 1751-1758

Introduction

Enteropathogenic E. coli (EPEC) is one of the pathogens that cause the death of many children under five years of age as a result of diarrhea, especially in developing countries, and this bacteria is the basis for the emergence of summer epidemics that spread in infant centers and nurseries. The role of childbirth the infection of this type results in diarrhea of different severity that causes inflammation of the epithelial cells in the intestine, and the degeneration of the villi of the intestinal cells. (Ochoa and co., 2018). Diarrhea is defined as a change in the consistency of stool (fluid or containing mucus or blood) or an increase in the number of stools passed (to two or three times the usual number, although infants may pass stools 2-5

times a day normally). Diarrhea is not considered a disease, it is a symptom that accompanies many diseases. Diarrhea is usually common in children between the ages of six months and two years. It is the age of eating which is the main source of diarrhea, and children younger than six months rarely get diarrhea due to the immunity acquired from the mother through the placenta (UNICEF, 2018). Clinical symptoms of diarrhea include watery stools without blood or blood, vomiting, and fever in some cases, as well as loss of appetite, rapid weight loss, and damage to the intestinal mucosa caused by bacteria (Gatinu et al, 2016).

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Diarrhea caused by these bacteria is limited to infants and children under five years of age, who have severe and long-term watery diarrhea with mucus, fever, and dehydration, and is associated with a high average of mortality. The incubation period is estimated at less than 9 to 12 hours, and infection occurs when children eat food contaminated with bacteria. Diarrhea caused by these bacteria is still a health problem that represents a public health concern, where the number of deaths reaches 1.3 million deaths annually (Nair et al, 2020). The pathogenicity of this bacterium is due to its possession of many virulence factors, including iron chelates (siderophores), cytotoxic necrotizing factor and superficial structures such as flagella, capsule and LPS. lipopolysaccharides, Bacteria confer antigenic properties by producing somatic antigen O (O), flagellar antigen H, and capsular antigen K. They also possess cilia (Fimbriae) or (Pilli), which helps it to stick to the tissues of the host, giving it the ability to form biofilm (Terlizzi et al, 2017, Zowawi et al, 2015). In view of the lack of studies on these bacteria in Al-Diwaniyah province, this study was conducted to shed light on some virulence factors in pathogenic *Escherichia coli*.

Materials and Methods

Sample Collection

The 323 stool samples were collected from children aged five years and under suffering from diarrhea, for the period from 30/12/2019 to 1/5/2019 from the Maternity and Children Teaching Hospital and Al-Hussein Hospital in Al-Diwainah province. Each sample was immediately cultured on nutrient media MacConkey agar (Blood agar), then incubated at 37°C for 24 hours, for the purpose of diagnosing the developing bacteria (Macfaddin, 2000).

1. Laboratory Diagnosis

1-1. Diagnosis of *E.coli*

The phenotypic characteristics of Mtn bacteria were studied by culturing stool samples on culture media, then pure colonies of bacterial growth were taken and stained by gram stain to study the phenotypic traits of the isolated bacterial species. The study included bacterial colonies of shape, texture, edges, growth, or non-growth on Differential Media, including MacConkey agar and Selective Media. Where they were grown on eosin-

methylene blue (EMB) agar medium. The phenotypic characteristics of bacterial cells, when stained with Gram stain, included the shape of the bacterial cell, the regularity of the cells together, and the nature of their interaction with the pigment.

1-2. Biochemical Tests

A. Oxidase Test

The assay was conducted by transferring a quantity of bacterial growth using sterilized wooden sticks to a filter paper saturated with the reagent (Tetramethyl- ρ -henylenediaminedihydrochloride). The coloration of the bacterial colonies in purple after ten seconds indicates the positive result of the detection. (Forbes and co., 2007).

B. Catalase Test

Catalase is an enzyme that catalyzes the release of oxygen from hydrogen peroxide. A small amount of bacterial growth was transferred by a sterile wooden stick onto the surface of a clean, dry glass slide, One drop of 3% H₂O₂ was added to it. The formation of gas bubbles indicates a positive result (Forbes, et al, 2007).

C. Indole Production Test

The pure colony was cultured in peptone water and then incubated at 37°C for 24 hours and some drops of Kovacs reagent were added to it. The presence of the pink ring after adding the reagent is considered a positive result of the test (Forbes et al, 2007).

D. Methylene-Red Test

The assay was carried out by inoculating the culture tubes containing Medium VP-MR with the bacterial culture, and incubating at 37°C for 48-24 hours, after that 5 drops of the previously prepared methyl red reagent were added to each tube with shaking. The appearance of the red color within 15 minutes indicates the complete dissolution of sugars and the production of acid (Collee et al, 1996).

E. Test Voges-Proskauer

The assay was conducted by inoculating Medium VP-MR with the bacterial culture and incubated at 37°C for 48-24 hours. After that, 1 ml of the previously prepared reagent was added to each



tube with shaking. The appearance of the pink color within 2-5 minutes, which becomes dark cherry within 30 minutes with continuous shaking, expresses the positive result (Collee et al, 1996).

F. Citrate Test

The test was conducted by inoculating the slanted jacket medium with the bacterial culture to be examined and incubated at 37 °C for 24-48 hours. The change of the color of the green medium to blue and the appearance of growth on the planting lines indicate the positivity of the test (Forbes et al, 2007).

G. Detection of Urease Test

This enzyme was detected by inoculating the urea agar medium in the bacterial culture, which was incubated at a temperature of 37 °C for a period ranging from 24 hours to seven days. The appearance of the pink color indicates a positive result (Collee et al, 1996).

1-3. DNA Extraction of Bacterial DNA

DNA was extracted from EPEC bacteria, using the ready-made kit, according to the company's instructions, as follows:

1. Transfer 1 ml of suspended EPEC isolate onto heart-brain infusion broth medium and put into sterile 1.5 ml Eppendorf tubes.
2. Transferred to a refrigerated centrifuge at 15000 r/min to collect the bacterial cells and then dispose of the supernatant liquid.
3. The mixture was incubated at room temperature for 10 minutes. During the incubation period, the tubes were stirred to ensure complete analysis of cells in the mixture.
4. 200 µl of GB Buffer solution prepared from the kit was added to the mixture of lysed cells and mixed well with the mixture. vortex for 5 seconds
5. The mixture was incubated at a temperature of 70 C for 10 minutes using a water bath.
6. 200 microliters of absolute ethyl alcohol were added to the dissolved mixture and the mixture was mixed well with the Vortex mixer for 10 seconds.
7. Transfer the mixture from the Alpendorf tube to collection tubes measuring 2 ml containing columns containing filters for

purification of DNA (GD filter column) that are equipped with the kit.

8. The collection tubes were placed with the columns containing the mixture in the refrigerated centrifuge and rotated at a speed of 15000 rpm to get rid of the decomposing products.
9. The precipitated solution of lysed cells was removed and transferred to column GD on the DNA into a new collection tube.
10. 400 µl of W1 Buffer solution prepared with the kit was added to the column containing the DNA to wash the DNA, then the tubes were placed in the centrifuge at a speed of 15,000 cycles for 30 seconds.
11. The precipitate was removed, then 600 microliters of the washing buffer containing absolute ethyl alcohol, which was prepared with the kit, was added to the column containing the DNA to get rid of the fat, and the tubes were placed in a centrifuge at a speed of 15,000 cycles for 30 seconds.
12. The sediment was removed and the tubes were returned to the refrigerated centrifuge again to dry the columns at a speed of 15,000 cycles for 3 minutes.
13. The columns containing the DNA were transferred to sterile Eppendorf tubes with the addition of 50 µl of the Elution Buffer solution prepared with the kit to the center of the column and left for 5 minutes, then the tubes were placed in a refrigerated centrifuge at 15000 cycles for 30 seconds to dissolve the DNA and preserve At a temperature of -20°C until the polymerase chain reaction test is performed.

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1-4. Preparation of agarose gel

Prepare according to the method of Russell and Sambrook (2001) and my agencies:

1. Dissolve 1.5 g of agarose gel in 100 ml of TBE Buffer buffer solution at a concentration of 1 x and using a hot plate for 15 minutes at a temperature of 60° C.
2. Leave the gel to cool to a temperature of 51°C, then add 5 microliters of ethidium bromide dye and mix well with the gel.
3. Pour the agarose gel into the transfer plate (Tray) after fixing the comb in it, then leave it to solidify at room temperature for 31 minutes. Then carefully remove the comb from the gel to get the pits.



1-5. Preparation of a Single Polymerase Enzyme Reaction Mixture

Prepare a single polymerase enzyme reaction mixture using the AccuPower® PreMix PCR kit according to the company's instructions as follows:

1. The polymerase enzyme reaction mixture was prepared in (PCR) tubes equipped with

the kit and container of the polymerase enzyme reaction components, with the addition of the other components of the reaction mixture in Table (1).

Table 1. Components and volumes of a single polymerase chain reaction mixture

No.	Components		Volume in microliters
1	AccuPower® PreMix PCR solution		5
2	Template DNA		3
3	Primer	Forward primer	1.5
4		Reversed primer	1.5
5	Deionized Nuclease Free Water		14
6	Total volume		25

2. After completing the preparation of the polymerase enzyme reaction mixture, the tubes were closed and carefully mixed with a vortex mixer for 5 seconds.
3. The tubes were transferred to the Thermocycler for the reaction of the polymerase enzyme to perform the process of amplifying the DNA according to the optimum conditions for the Thermo cycling conditions represented in the process of separating the DNA strand (denaturation) and linking the primers with the separated

strand (annealing) and lengthening the chain (DNA Extension.)

1-6. Thermal Cycles Program for DNA Amplification

The polymerase enzyme reaction was conducted using the thermal amplifier of the PCR Thermocycler. The device was programmed for the genes under study according to the reaction, as shown in Table (2).

Table 2. The heat cycle program for the single polymerization reaction

Number of cycles	Temperature/time					gene
	Final elongation	Conditions cycles			Scan start	
		Elongation	Correlation	Scan		
36	10 min.72 /	1min.72/	sec.94/ 30	min.94/1	94/5	<i>eaeA</i>
30	72/7 min.	72/1min.	63/60 sec.	94/30 sec.	94/5	<i>bfp</i>

1-7. Electrophoresis in Agarose Gel

1. Transfer 5 µl of amplified DNA (PCR) product to pits in 1.5% agarose gel.
2. Transfer 2 microliters of a DNA ladder of size 100-1000 base pairs into the first hole to measure the volume of the amplified DNA, by comparing the volume of the amplified gene with the volumes of the volume index.
3. The surface of the gel was immersed in TBE buffer at a concentration of x1, and the DNA samples were traveled bypassing it at a voltage of 100 volts for a period of 80 minutes, after which the gel containing the PCR product was examined using a UV-

transilluminator with a wavelength of 291 nm, and images were photographed by a machine. Gel Documentation Unit, to identify the bands and measure their molecular weights when compared with the positions of the bands belonging to a DNA ladder with known molecular weights 100-1000 base pairs.

Results and Discussion

• Isolation and Identification

The results of the initial culture showed that there were 210 (65.01%) samples out of the total samples of (323) samples that tested positive for the E.coli Escherichia coli bacterium, depending on



the morphological and microscopic characteristics, biochemical tests and my criteria:

1. Diagnosis Using Morphological and Morphological Characteristics

Its colonies appeared on MacConkey agar as fermented to sugar lactose and gave pale pink colonies with sharp edges, which is a characteristic of this bacteria. The spread of growth outside the limits of the stab was observed when planted on the medium of the motility test, indicating that it is mobile as a result of possessing flagella (MacFaddin, 2000). E.coli bacteria were cultured on EMB (Eosin methylene blue) medium, which gave a metallic luster on this medium, and this characteristic is characteristic of E.coli bacteria from other members of the intestinal family, which indicates that the bacteria are able to ferment the sugars lactose and sucrose and the production of organic acids, which led to a change in the pH of the medium to acidic, and this led to the binding of the eosin and methylene dyes, which are deposited in the acidic medium, giving a metallic green shine (Levinson et al, 2004).

Microscopic Diagnosis

The results of the microscopic testing of the slides prepared from these colonies at the age of (18-24) hours, using Gram staining, showed that they are short Gram-negative bacilli (Holt et al, 1994).

Biochemical Diagnosis

Table (1) shows the results of the biochemical tests that were conducted on all studied bacterial isolates, as all isolates were negative for the two urease tests because the color of the medium did not change, which confirms that the bacteria do not consume urea because they do not possess the urease enzyme and negative for the detection of the oxidase test, by not turning the color of the colonies to purple when adding the reagent, which indicates that the bacteria do not have the enzyme (Cytochrome Oxidase) as a hydrogen acceptor. It was positive for the catalase test if it analyzed hydrogen peroxide (H₂O₂) into water and oxygen. Because the results of the above tests are specific to E.coli in general and cannot distinguish between its pathological types, chromogenic media of EPEC was used. When bacteria were grown on this medium, (37) out of 120 bacterial isolates belonging to E. coli grown on this medium (37/120) (30.83%) and

As for the results of the IMViC group tests, the bacteria were positive for the indole test, due to their ability to analyze the tryptophane acid in the test medium for their possession of the tryptophase enzyme, as the appearance of a red ring on the surface of the medium in the layer of isoamyle alcohol indicates the positiveness of the test and this test is important in differentiating between E.coli and other members of the intestinal family, as well as positive for the Methyl Red test as a result of the presence of acid resulting from the consumption and fermentation of glucose and peptose by bacteria, which led to a change in the pH of the medium and its color turned red. The bacteria were also negative for the Voges-Proskauer test as a result of the appearance of yellow to brown color in the liquid medium, which is caused by the bacteria not converting glucose sugar to acetylmethylcarbinol (Acetone), which leads to the non-reaction of the two reagents VP1 (alphanaphthol) and potassium hydroxide (VP2) with the resulting acetone and it was negative for the citrate test, which indicates that bacteria do not use citrate as the sole carbon source due to their lack of citrate permease enzyme. The result was that the color of the medium did not change to a bluish-green color due to the absence of citric acid production and the pH did not change. This is consistent with what was stated in Brown and Smith, 2017; Tille, 2017).

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Table 3. Phenotypic, biochemical and microscopic culture tests of isolated E.coli

No.	Test	Result
1	Gram stain	—
2	The appearance	Bacillus
3	oxidase test	—
4	Catalysts	+
5	indol	+
6	the movement	+
7	fermentation of lactose	+
8	GROW ON EMB	+Metalic green
9	MR	+
10	VP	—
11	urea test	—

it appeared in the form of violet-colored colonies as a result of their production of β-glucuronidases enzymes that break down the conjugate chromogenic and release the β-glucuronid chromogenic substrate or the so-called chromophore, which gives the bacterial colonies a



violet color, and this is identical to what the manufacturer of the culture medium mentioned. The average of isolation varied in the studies targeted to isolate EPEC. In the study conducted by Hasony (1996), the percentage of EPEC isolation in his study reached (52%), as well as with what was reached by Al-Abbas, (2018), where the percentage of EPEC isolation in his study was (33.6%) and in Nigeria The researcher Nweze (2010) isolated these bacteria with a percentage of (49.2%). In Fallujah Al-Ani was found (2007). The percentage of EPEC was (20.46%), and in Najaf, Al-Mohana and Al-Hilali (2011) found that the percentage of isolation of the above bacteria was (3.4%), while Alikhani et al, (2006) in Iran found the percentage of EPEC isolation was (44.9%). Turki et al (2011) indicated that he obtained 140 isolates of *E.coli* from cases of diarrhea in children, and that (36) (25.71%) isolates of them were related to the enteropathogenic *Escherichia coli* (EPEC). The reason for this difference in percentages may be due to the difference in the different environmental conditions of the sites of this study, the time period for completing the research, the difference in the number of samples, type of nutrition, health, and immune status of the body.

Deaths in children under five years of age are often associated with EPEC, and its isolation may be due to the infant not having acquired immunity from the mother and to the fact that the process of preparing children's food takes place under unsanitary conditions (Besser et al, 1993). Continuing to breastfeed children for six months reduces up to 55% of child deaths around the world that are caused by diarrhea, respiratory diseases, and other diseases (Chantry et al, 2006). Breast milk contains many compounds that protect

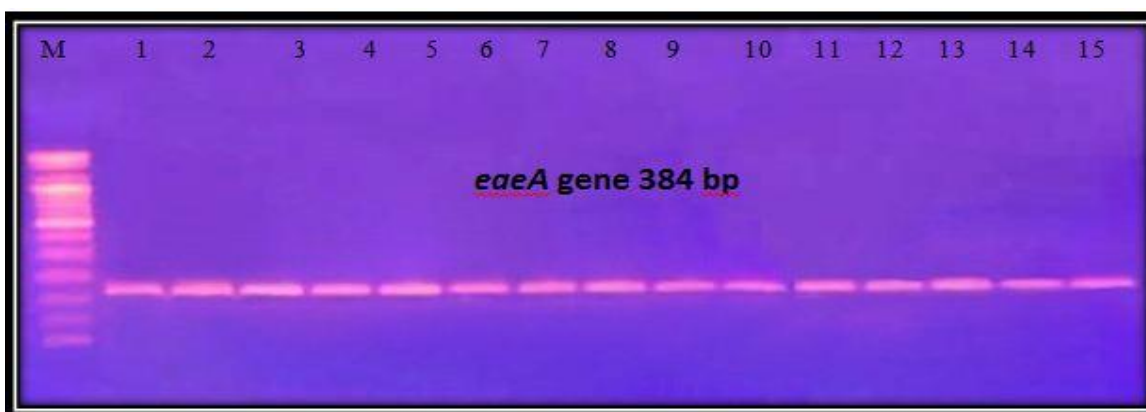
the infant from diseases; Because the immune system of children is immature (Newburg et al, 2000) or the reason may be due to the start of giving children some foods at this period of the child's life, which in turn is an appropriate medium for the transmission of pathogens.

Molecular Detection of Some Virulence Genes in EPEC. Bacteria

1. Escherichia Coli Attaching and Effacing Gene (eaeA)

The results of electrophoresis on agarose gel (Picture 1) for the detection of amplification products using a pair of specialized primers to amplify the *eaeA* gene sequence showed the appearance of bands resulting from the association of this primer with its complementary sequence in the genome of 15 (15/15) isolates (100%) of Bacteria EPEC isolated from children with diarrhea (Blanco et al, 2003) indicated the presence of this gene by 45% in the isolated strains of humans, and another study indicated its presence in all (100%) strains isolated from human disease samples (Paton et al, 1996). Many researchers have confirmed the strong link between the *eaeA* gene and the ability of EPEC to cause infection. Some types of *E.coli*, including EPEC, have a site on the chromosome that encodes some virulence factors called the locus enterocytes effacement (LEE) and within this region is located The *eaeA* gene that encodes the protein Intimin, which works to document the association between EPEC bacteria and the host intestinal cells, so this gene is an important virulence factor for these bacteria, and this gene is also a diagnostic gene for EPEC bacteria (Kaper, 1994; Gannon et al, 1993).

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Picture 1. The products of amplification of the *eaeA* gene (384 base pairs) for EPEC bacteria using Single PCR technique and electrophoresed on agarose gel at a concentration of 1.5% (%) and a potential difference of 100 volts for a period of 80 minutes, where M Ladder DNA = (100-1500) pairs / Base, (1-15) all EPEC isolates examined on the *eaeA* gene responsible for the formation of the protein (Intimin)

2. BFP (Bundle-Forming Pili) Gene

The results of the study in figure (2) showed that 15/13 isolates (86.66%) of EPEC isolates possess this gene, and that its absence in isolates No. (5 and 11) is an indication of the absence of this gene in these isolates and may be a result of the fact that this gene encodes for a protein extramembrane that may be subjected to selective stress leading to mutations in the gene (Wang et al., 2002) Nathalia et al (2015) indicated that 152/20 (13.2%) of EPEC isolates possess this gene, while Tobe et al (1996) indicated that 75/31 (41.33%) of EPEC isolates possess this gene. The difference in ratios may be due to the number of isolates used in the study, the electoral pressure that occurs to the genes and exposes them to mutations that make them lose

these genes, in addition to the laboratory conditions of the study. This gene is also one of the important virulence genes of EPEC bacteria. It is located on a plasmid called EPEC Adherence Factor (EAF) that encodes for a protein (Pilin), which is included in the structure of the filaments that attach bacteria to the epithelial cells of the host intestine and then facilitate the entry of bacteria to intestinal cells, It was found that a mutation in this gene leads to a lack of coding for the pilin protein, which is included in the composition of these capillaries, and thus the non-occurrence of the process of adhesion to the epithelial cells. (Stone et al, 1996).

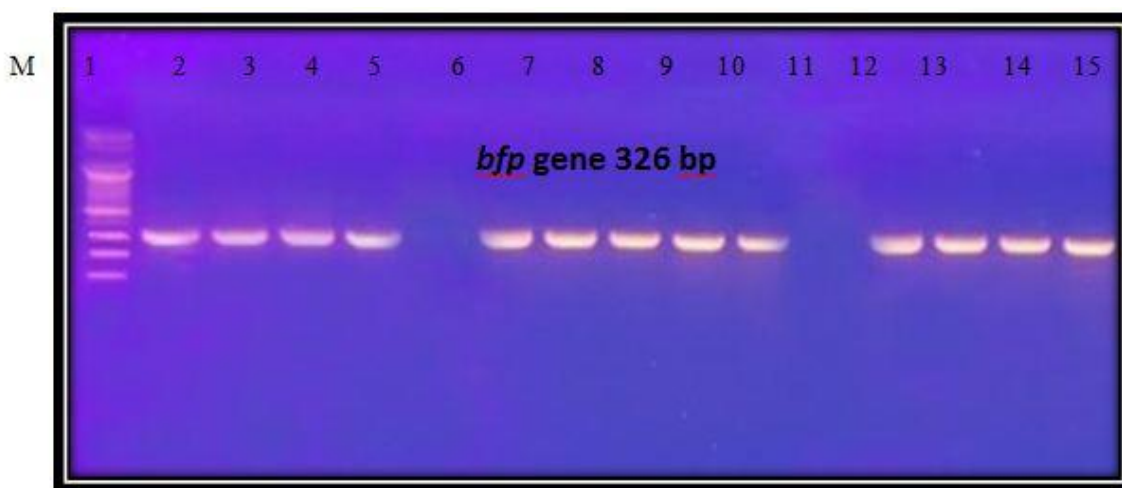


Figure 2. The amplification products of the *bfp* gene (326 base pairs) of EPEC bacteria using Single PCR technique and electrophoresed on agarose gel at a concentration of 1.5% (%) and a potential difference of 100 volts for a period of 80 minutes, where M Ladder DNA = (100-1500) pairs Base, 13 isolates (1,2,3,4,6,7,8,9,10,12,13,14,15) contain the *bfp* gene responsible for pilli filament formation

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