PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF ESCHERICHIA COLI ISOLATES FROM INFANTS WITH DIARRHEA

Yahya J. Shahood1, Ahmed D. Jabar2
1,2Department of Biology, College of Science, Wasit University, Kut, Iraq

ABSTRACT:
Diarrheagenic Escherichia coli is still an important pathogen that causes diarrhea which lead to hospital admissions and death specially in children.

A total of 123 diarrheal samples were collected from children under two years and for both gender male and female suffering with watery or bloody diarrhea, in hospital and private clinics in Wasit province during a period from 15th of December 2020 to 15th of March 2021. According to culture, biochemical tests and API 20E, the results of 61 isolates were supposed to be E. coli. The IDNA was extracted from these 61 isolates from diarrheal cases.

All isolates studied for detection the virulence gene of three diarrheagenic Escherichia coli strains based on using Multiplex Polymerase Chain Reaction technique, i.e., amplified 6 primer (eae, bfp, stx1, stx2, stxII, lt), then detection the resistance genes by amplified 4 primer (TEM, SHV, CTXM2, CTXM1), the results showed the distribution of the strains and their susceptibility to antibiotics.

Results showed that Diarrheagenic E. coli were detected in 49.6% (61/123) among diarrheal children. The distribution of 61 DEC pathotypes isolates were, EPEC was found in 16% (10/61), ETEC in 100% (61/61), EHEC in 0% (0/61).

Through the results that we obtained in our study, it was shown that Diarrheagenic E. coli was present in formula-fed infants in a significant percentage 39/61 (it is the highest percentage). While in infants who were fed mixed feeding, the percentage was lower as 13/61, and in infants dependent on natural feeding (breastfed), the percentage showed the lowest where it was 9/61.

The antibiotic susceptibility test for 61 of diarrheagenic Escherichia coli isolates, were performed by disc diffusion method, using 15 antibiotics and their results showed different percentages of resistance to antibiotics as follows: the highest level of resistance was Amoxiclav (100%), Piperacillin (100%), Amoxicillin (90%) followed by Cefepime (89%), Cefotaxime (84%), Cefoxitin (87%), Nalidixic acid (84%), Ceftriaxone (85.7%), Cefixime (70%), Azithromycin (45%), Aztreonam (52%), Gentamicin (61%). The maximum E. coli sensitivity was to Amikacin (100%) followed by Ciprofloxacin (85.7%), Gentamicin (61%).

We can conclude in this study, that Multiplex PCR is a rapid and accurate procedure which can be used for identification and isolation of Diarrheagenic E. coli strains successfully. Enterotoxigenic E. coli and Enteropathogenic E. coli are the most dominant pathotype in the studied samples, tended to occur more in children less than 2 years of age.

Sensitivity tests showed higher resistance to different antibiotics in isolates collected from hospitalized children from isolates of diarrheal E. coli collected from non-breastfed children (formula-fed infants) compared to isolates collected from breastfed children natural. This study also revealed that the most active antibiotic against diarrheagenic E. coli was Amikacin followed by Ciprofloxacin, and Gentamicin.

Keywords: Antimicrobial Resistance Genes, Diarrheagenic Escherichia coli, EPEC, ETEC, EHEC, Multiplex PCR, Antibiotic susceptibility, Virulence gene.
I. INTRODUCTION:

Diarrhea is one of the major causes of morbidity and mortality especially in children less than 5 years (Walker et al., 2012). The infectious agents of diarrhea vary from bacteria (E. coli, Salmonella, Shigella, Vibrio), viruses (Rotavirus, Adenovirus, Hepatitis A and E, Norwalk) and protozoa (Giardia, Cryptosporidium, Cyclospora, Microsporidia, Isospora) are usually responsible for serious diarrheal disease outbreaks (O’Ryan et al., 2020).

Diarrheagenic Escherichia coli are one of the most significant etiologic agents of diarrhea that causing high morbidity and mortality, typically among children (Abd El Gany et al., 2020).

E. coli are a rod-shaped, gram-negative, facultative anaerobic bacterium non-sporulation organism, the cells are about 0.3 μm in diameter and 1.0 - 6.0 μm long with a cell volume of 0.6 to 0.7μm (Kimmitt et al., 2000). It is motile due to peritrichous flagella and Some strains are non-motile, nearly all E. coli strains are lactose fermenting, but this function perhaps delayed or absent in some Escherichia spp (Hall, G. S., 2013). Temperature of Growth for E. coli ranges from (37-45) ° C, and around 37 ° C is the optimal growth temperature. E. coli In general, develops within the PH range of 4.4-9.0 (Duc et al., 2020).

II. AIMS OF THE STUDY:

1- Detection the distribution of Diarrheagenic E.coli pathotypes among children with diarrhea in Wasit province, Iraq by multiplex PCR.

2- Assessing the antimicrobial susceptibility profile of Diarrheagenic E.coli, in order to contribute to the establishment of a more effective empirical antibiotic therapy for the disease.

3- Detection of some antibiotic resistance genes and virulence genes using molecular methods.

III. MATERIALS AND METHODS:

Collection of Different Samples and Processing

In a cross sectional study a total of one hundred twenty-three diarrheal cases were collected from children under two years admitted to : Al-Zahraa teaching hospital hospital, Al-Kut hospital for Gynecology obstetrics and pediatrics and Al-Karamaa teaching hospital and from private clinics in Wasit province from both sex male and female during a period from 15th of December 2020 to 15th of March 2021, the data recorded for the cases were: name, age, Breastfeeding type, type of diarrhea, and Place of residence.

Isolate and identification of bacteria

The stool samples collected and transported on Carry Blair swabs (This medium has a low nutrient content to prevent replication of organisms while maintaining viability. Sodium thioglycollate is added to impede oxidation, with buffering agent to prevent overgrowth of enteric pathogens, the alkaline pH minimizes the destruction of bacteria due to acid formation).

Then cultured on MacConkey agar, EMB, Blood agar, and nutrient agar (for longer store) and incubated aerobically at 37°C for 24hours, the isolated bacteria was identified according to morphological, biochemical tests and API 20E kit.

Molecular Detection (PCR) of Resistances Genes and Virulence gene:

Primer Preparation

All primers used in this study were synthesized by (AlphDNA/ Canada) as a lyophilized form, prepared according to the manufacturer’s instructions of company. by dissolves in double deionized water, then vortex to give a final concentration of (100 pmol/ ul) as stock solutions, to prepare 10 pmol/ul concentration as work solution added10 ul of stock solution to 90ul of nuclease free water, Table (1).

<table>
<thead>
<tr>
<th>E.coli strain</th>
<th>Primers ( Sequence (5’ – 3’))</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (1): primers with their sequences

www.turkjphysiotherrehabil.org
### DNA Extraction

DNA extraction was done according to the procedure (Geneaid presto mini gDNA Bacteria Kit). The major steps of the procedure were:

- **First Step Cell Harvesting/Pre-lysis:**
  1. Inoculated the culture in 8ml brain heart infusion broth at 37°C overnight. Bacterial culture transfer at a 1.5ml appendorf tube.
  2. Centrifuge at 14000 rpm for 1 minute and the supernatant discarded.
  3. 180 µl GT buffer added and well mixed with pipette.
  4. 20 µl of Protinase K was added. The tube incubated in water bath for 10 minute at 60°C and inverted 3 times during incubation period.

- **Second Step / Lysis Step:**
  1. 200µl of GB buffer added to eppendorf and resuspended for 10 second by vortex.
  2. Reincubated in water bath for 10 minute in 70°C until the lysate cleared, at this step the elusion Buffer left in water bath at 70°C.

- **Third Step/ DNA Binding:**
  1. DNA binding that done by adding 200µl of absolute ethanol eppendorf and shacked vigorously to mix. All the mixture transfer to GD colum that putted up 2ml collection tube.
  2. Centrifuged 2 minutes at 16000 rpm, collection tube discarded and use new one with the same GD Colum.

- **Fourth Step/ Washing Step**
  1. 400µl from W1 buffer was added to GD column.
  2. Centrifuge for 30 seconds at 16000 rpm.
  3. Flow-through discarded and back the GD column to its collection tube.
4- 600 μl wash buffer Added to GD colum. Centrifuged at 16000 rpm for 30 seconds. Flow-through discarded and back the GD colum to its collection tube. Another 3 minutes of centrifuged again for at 16000 rpm until the column dried.

- Fifth Step/DNA Elution:
  1- Replace the collection tube for the dried GD column.
  2- 100μl of elution buffer that preheated at previous step was added at the column center.
  3- After centrifuged for 30 seconds at 16000 rpm to purified DNA elute done
  4- The product collected in micro appendorf tube and saved at -20°C.

IV. RESULT AND DISCUSSION:

Culture results

The percentage of bacterial growth which was isolated from 123 samples: 49.6 % (n=61), were E. coli followed by other gram negative bacteria 27.6% (n=34) which include (Proteus, Klebsiella, Salmonella, Enterobacter, Pseudomonas, some isolates that could not be identified) and other samples that were no growth 22.8 % (n=28) as shown in table (2).

<table>
<thead>
<tr>
<th>NO. of samples</th>
<th>Valid Percent</th>
<th>Culture result</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
<td>49.6%</td>
<td>E. coli</td>
</tr>
<tr>
<td>34</td>
<td>27.6%</td>
<td>Other gram negative bacteria (17 Klebsiella, 3 Proteus, 4Pseudomonas, 2 Enterobacter, 1 Salmonella, 7 not be identified)</td>
</tr>
<tr>
<td>28</td>
<td>22.8%</td>
<td>No growth</td>
</tr>
<tr>
<td>123</td>
<td>100%</td>
<td>Total</td>
</tr>
</tbody>
</table>

P< 0.001

Distribution of Diarrheagenic E. coli pathotypes

Diarrheagenic E. coli were detected in 49.6 % (61 /123) among diarrheal children. The distribution of 61 DEC pathotype isolates were, EPEC was found in 0.16 %( 10/61), ETEC in 100% (61/61), EHEC in 0% (0/61), respectively as shown in table (3).

<table>
<thead>
<tr>
<th>Diarrheagenic E. coli pathotypes</th>
<th>Genes</th>
<th>Frequency of virulence gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPEC</td>
<td>Eae</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Bfp</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Lt</td>
<td>0</td>
</tr>
<tr>
<td>ETEC</td>
<td>StII</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Stx1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Stx2</td>
<td>0</td>
</tr>
<tr>
<td>EHEC</td>
<td>Stx1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Stx2</td>
<td>0</td>
</tr>
</tbody>
</table>

Results of antibiotic susceptibility test

Antibiotic susceptibility test results were showed: the highest level of resistance to Amoxiclav (100%), Piperacilline (100%), Amoxicillin (90%) followed by Cefepime ( 89 % ), Cefoxitin (87% ), Nalidixic acid (84%), Cefotaxime (70.8%), Cefixime (70%), Ceftriaxone(64%), Aztreonam (52%), Azithromycin (52%) , Levofloxac (51%), as shown in figure (1) and table 4.
The maximum E.coli sensitivity was to Amikacin (100%) flowed by Ciprofloxacin (85.7%), Gentamicin (61%).

There is high prevalence of resistance to antimicrobial agents among DEC isolated from children with diarrhea (Nguyen et. al., 2005 b).

Many factors responsible for an increase in rates of antimicrobial resistance include misuse/over use of antibiotic by healthcare professionals and general public (Konate et.al., 2017a; WHO,2014 ; Magiorakos et.al., 2012), and inadequate surveillance systems and independence on reliable microbiological techniques that leads to inappropriate prescription of antibiotics.

Table (4) The prevalence of antimicrobial resistance profiles of DEC

<table>
<thead>
<tr>
<th>مضادات</th>
<th>Clinical DEC</th>
<th>R</th>
<th>I</th>
<th>S</th>
<th>SUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRO</td>
<td>59% 1% 40% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK</td>
<td>0% 0% 100% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cip</td>
<td>11% 9% 80% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lev</td>
<td>51% 7% 42% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>82% 3% 15% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRL</td>
<td>100% 0% 0% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEP</td>
<td>86% 2% 12% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOX</td>
<td>79% 10% 11% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td>25% 6% 69% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZM</td>
<td>52% 17% 31% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFM</td>
<td>70% 0% 30% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATM</td>
<td>52% 2% 46% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMC</td>
<td>100% 0% 0% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX</td>
<td>71% 0% 29% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>84% 11% 5% 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure (1): The prevalence of antimicrobial resistance profiles of DEC
Molecular Detection of Antibiotic Resistance Genes in Diarrheagenic E. coli strains

61 Diarrheagenic E. coli isolates were identified out of 123 samples, it was detected by polymerase chain reaction for 61 isolates of resistance genes Cefotaxime (CTX-M), Aztreonam (TEM), and Sulf-hydryl variable (Bla-SHV), and the following results were obtained where 45 (73.7%) of the isolates positive for the CTX-M1 gene, 43 (70.4%) of the isolates are positive for the CTX-M2 gene, while 18 (29.5%) of the isolates are positive for the Bla-SHV gene, and 18 (29.5%) of the isolates are positive for the TEM gene, as show in table (5).

Table (5) Distribution of positive values for resistance genes of PCR results

<table>
<thead>
<tr>
<th>Genes</th>
<th>N. Positive</th>
<th>%</th>
<th>N. of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CTX-M1</td>
<td>45</td>
<td>61</td>
</tr>
<tr>
<td>2</td>
<td>CTX-M2</td>
<td>43</td>
<td>61</td>
</tr>
<tr>
<td>3</td>
<td>Bla-SHV</td>
<td>18</td>
<td>61</td>
</tr>
<tr>
<td>4</td>
<td>TEM</td>
<td>18</td>
<td>61</td>
</tr>
</tbody>
</table>

Our molecular study revealed that bacterial isolates contain CTX-M1 and CTX-M2 genes in a high percentage compared to Bla-SHV and TEM genes, and this fact was similar to previous studies (Shahcheragh et. al., 2009 and Hernández et. al., 2005), but it was different compared to the results of (Taşlı et. al., 2005 and...
Ramazanzadeh et. al., 2010), therefore the distribution pattern of TEM and SHV genes in isolated E. coli varies by geographic location, as show in Figure (2), (3),(4).

Figure (2): Gel electrophoresis of amplified (TEM and SHV) genes

Figure (3): Gel electrophoresis of amplified (CTXM1) genes

Figure (4): Gel electrophoresis of amplified (CTXM2) gene

Molecular Detection of Virulence gene in Diarrheagenic E. coli strains
Diarrheagenic *E. coli* were detected in 49.6% (61/123) among diarrheal children. The distribution of 61 DEC pathotype isolates were, EPEC was found in 0.16% (10/61), ETEC in 100% (61/61), EHEC in 0% (0/61), respectively as shown in table (3).

Among all the Diarrheagenic *E. coli* pathotypes, Enteropathogenic *E. coli* (ETEC) were found to be the most common pathotypes for children with (100%). Where the proportion of the gene *StII* was 100% , *StII* gene was appeared in all ETEC isolates detected in our study that mean all of them were typical ETEC, as shown in the table (6) and figure (5).

ETEC and EPEC were reported as the most common Diarrheagenic *E. coli* pathotypes (Blanton *et al*., 2018; Moyo *et al*., 2007; Wang *et al*., 2015).

Enteropathogenic *E. coli* 0.16% (10/61) isolates came second after Enteroaggregative *E. coli* as causative agent of diarrhea among Diarrheagenic *E. coli* pathotypes in our study, as shown in the table (6) and figure (5).

No genes were detected for Enterohemorrhagic *E. coli*, where was the ratio 0% 0/61 from isolates of diarrheagenic isolates, all 61 isolates in our study don’t produce nether stx1 or stx2.

Figure (5): Gel electrophoresis of amplified (*Bfp, Eae, StII*) genes

---

**REFERENCES**


www.turkjphysiotherrehabil.org 19644

