Molecular Diagnosis of Diarrheagenic E. coli Infections Among the Pediatric Patients in Wasit Province, Iraq

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Abstract

Diarrheagenic Escherichia coli still an important pathogen that cause diarrhea which lead to hospital admissions and death specially in children. In order to identify the common pathotypes of E. coli via investigate different virulence genes. A total of 210 stool samples were collected from children under five years presented with diarrhea from different hospitals and private clinics in Wasit province, Iraq, on the other hand, 40 stool samples were collected from healthy children considered as control group. Regarding to culture, biochemical tests and API 20E results 100 isolates were supposed to be E. coli. The DNA were extracted to that 100 isolates from diarrheal cases and for 40 isolates of control, concentration of DNA samples were between (50-360 mg/µl ) and the purity between (1.8-2). All isolates studied for detection virulence gene of five Diarrheagenic Escherichia Coli strains based on using multiplex Polymerase Chain Reaction technique, by amplified 13 primer (eaeA, bfpB, aggR, astA, pic, hly, stx1, stx2, invE, ipaH, elt, estA, estIb), and showed the distribution of the strains and its susceptibility to antibiotics. The most frequent pathotypes was Enteropathogenic E.coli 19/42 (45.3%) with 9 typical and 10 atypical, followed by Enteroaggregative E. coli 17/42 (40.5%), Enterotoxigenic E. coli 3/42 (7.1%), Enteroinvasive E. coli 3/42 (7.1%), and 0/42 (0%) in Shigatoxin producing E.coli and no DEC in all control patients. The highest resistance to antibiotics was (95.2%) to Amoxicillin and Ampicillin, respectively, Sulfa-Trimethoprim 92.9%, followed by 85.7% for Tetracycline and Cephalothin, Ceftriaxone 81% and Cefotaxim "clavulanic acid 71.4%. While the lowest resistance was to Chloramphenicol (19%), Ciprofloxacin (16.7%), Amikacin (7.1%) and no resistance was detected toward Imipenem. We can conclude in this study, multiplex PCR is a swift, and accurate procedure can be used for Diarrheagenic E.coli identification and isolation successfully of strains.

Key words: Diarrheagenic E.coli, virulence genes, Multiplex PCR.

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INTRODUCTION

Diarrheal disease is still a global problem around the world, especially in children under five years in developing countries. According to the World Health Organization (WHO), diarrheal diseases are the second leading cause of death (~760,000 per year) in children. The microbial causes of diarrhea are a variety of bacterial, viral, and parasite. Among these pathogens, diarrheagenic E. coli plays a major role in causing diarrhea in children under 5 years.

When the microbial agent is bacteria, E. coli consider one of the major causes, especially to infantile diarrhea. Depending on specific virulence gene, clinical features, and serotypes, diarrheagenic E. coli divided into 6 stains: Enteropathogenic E. coli, Enteroaggregative E. coli, Enteroinvasive E. coli, Enterotoxigenic E. coli, Shiga toxin-producing E. coli, and Diffusely Adherence E. coli. Culture and biochemical test can’t distinguished between commensal or pathogenic strains of E. coli in stool, therefore PCR used to detect the virulence genes in pathogenic strains, multiplex PCR provide detection to many diarrheagenic E. coli strains virulence genes with high sensitivity, specificity.

The aim of this study was detecting the distribution of diarrheagenic E. coli pathotypes among children with diarrhea in Wasit province, Iraq by multiplex PCR, and 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.

MATERIALS AND METHODS

Collection of samples

During the period from middle of September 2017 to middle of December 2017, a total of 210 stool samples were collected from children.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Primers</th>
<th>Primers (Sequence 5’ – 3’)</th>
<th>Product size (bp)</th>
<th>References</th>
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<tr>
<td>aggR</td>
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<tr>
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<td>astA</td>
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<td></td>
<td></td>
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<tr>
<td>pic</td>
<td>pic-F: AGCCGTTCGCCAGAAAGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hly</td>
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<td>stx2a-F: ATGCCCAGCTTCGCCCAAGATG</td>
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<td>elt</td>
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<tr>
<td>estlb</td>
<td>estlb-F: TGTCTTTTACCTCTTGCT</td>
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</table>

Table 1. Primers used for multiplex PCR reaction
(males and females) with an ages under five years
presented with diarrhea had been admitted at
hospitals and attended at private clinics in Wasit
province, Iraq. Otherwise, 40 stool samples were
collected from healthy children considered as
control. The stool samples transported on Carry
Blair swabs and cultured on MacConkey agar,
XLD, EMB, Blood agar, and CHROMagarSTEC and
incubated aerobically at 37 °C for 24 hours, the
isolated bacteria was identified according to
morphological, biochemical tests and API 20E kit.

**Antibiotic susceptibility test:** performed
by Kirby-Bauer procedure on Muller Hinton agar
and results interpreted according to Clinical and
Laboratory Standards Institute.

**DNA extraction:** was performed
according to the procedure (Geneaid Genomic
DNA extraction Kit).

**Multiplex PCR technique:** was used
for amplifying the genes. The mixture reaction
was performed in a total volume 50 μl of PCR
Mastermix Gold Multiplex 50x (DNA Template 4
μl, Forward primer 1 μl for each primer, Reverse
primer 1 μl for each primer, free water ddH2O
20μl). PCR cycling program parameters used in
this reaction for detection of (bfpB, eaeA, pic,
aggR, astA, invE, ipaH, hly, stx1, stx2, elt, estla,
estlb) genes as shown in table (1), the thermal
cycling program(Initial denaturation 95°C for 5
min. 1 cycle), (Denaturation 94°C for 30 sec 35
cycle), (Annealing 58°C for 30 sec. 35 cycle), (Extension 72°C for 1 min. 35 cycle), (Final extension
72°C for 7min. 1 cycle) (Holding 4°C 1 cycle). The
amplification products were electrophoresed
through a 2 % agarose gel and visualized with UV
transilluminator after ethidium bromide staining.
A 100 bp DNA ladder was used as a molecular
size marker in gel. The statistical analysis of all
the evidence was done using the system SPSS IBM
version 20 software, Chi-squire test. P-value ≤ 0.05
was considered statistically significant.

**RESULTS**

*E. coli* were isolated in 100 (47.6 %) of 210
collected samples followed by 78 (37.2%) of other
gram negative bacteria (Salmonella, Klebsiella,
Proteus, Pseudomonas) and 32 (15.2 %) samples
that were no growth. The results of primary
diagnosis to these 100 *E. coli* isolates by selective
and differential culture media were consistent with
the microscopic and biochemical tests results.

Multiplex applied on theses 100 and 40
control samples and the results showed that DEC
were detected in 42/ 100 (42 %) among diarrheal
children compared with 0/100 (0%) among control
children. The distribution of 42 DEC pathotype
isolates were: EPEC was found in 19 (45.3%), EAEC
in 17 (40.5% ), ETEC in 3 (7.1%), EIEC in 3 (7.1%)
and 0 (0% ) in STEC and controls.

From 19 isolates detected as EPEC which
was watery diarrhea 10 (52.6%) isolates of them
are atypical EPEC showed eaeA gene found without
bfpB gene, and 9 (47.4 %) were typical EPEC which
showed eaeA gene together with bfpB gene. All 19
isolates in our study don't produce nether stx1 or
stx2, in addition one of aEPEC showed astA gene.

Enteroaggregative *E. coli* 17 (40.5%)
isolates came second after Enteropathogenic
*E.coli* as causative agent of diarrhea among
Diarrheagenic *E. coli* pathotypes in our study, aggR

**Table 2. Antibiotic susceptibility test**

<table>
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<tr>
<th>Antibiotics</th>
<th>CEC</th>
<th>AMC</th>
<th>CTR</th>
<th>TE</th>
<th>SXT</th>
<th>AMP</th>
<th>CTL</th>
<th>C</th>
<th>CIP</th>
<th>IPM</th>
<th>AK</th>
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<td>1</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>33</td>
<td>42</td>
<td>36</td>
<td>85.7%</td>
<td>100</td>
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<td></td>
<td>19%</td>
<td>2.4%</td>
<td>16.7%</td>
<td>9.5%</td>
<td>4.8%</td>
<td>4.8%</td>
<td>73.8%</td>
<td>78.6%</td>
<td>31</td>
<td>78.6%</td>
<td>73.8%</td>
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<td>Intermediate</td>
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<td>1</td>
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<tr>
<td></td>
<td>9.5%</td>
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<td>0%</td>
<td>7.1%</td>
<td>7.1%</td>
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<td>7.1%</td>
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<tr>
<td>Resistance</td>
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<td>40</td>
<td>34</td>
<td>36</td>
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<td></td>
<td>71.4%</td>
<td>95.2%</td>
<td>81%</td>
<td>85.7%</td>
<td>92.9%</td>
<td>95.2%</td>
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<td>95.2%</td>
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<tr>
<td>Chi squire</td>
<td>28</td>
<td>72.4</td>
<td>44.14</td>
<td>52</td>
<td>67</td>
<td>34.38</td>
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<td>31.8</td>
<td>39.57</td>
<td>34.38</td>
<td>51.8</td>
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</table>

AK=Amikacin, AMC=Amoxiclav, AMP=Ampicillin, CTR=Ceftriaxone, CTL=Cephalothin, C= Chloramphenicol,
CIP=Ciprofloxacin, CEC=Cefotaxime/Clavulanic, IPM=Imipenem TE=Tetracycline, STX=Sulfa-Trimethoprim
gene was appeared in all EAEC isolates detected in our study that mean all of them were typical EAEC. Enterotoxigenic E. coli account 3 isolates (7.1%) and Enteroinvasive E. coli was detected in 3 isolates (7.1%) that suggested these pathotype maybe play a lessimportant role in childhood diarrhoea in developingcountries. When age stratification was analysed high incidence of DEC E. coli recorded in first and second age group flowed by third and fourth age group, and there were no cases recorded in fifth age group.

The prevalence of Enteropathogenic E. coli infection infections was high in first and second years, also all Enteroaggregative E. coli infections were detected under 2 years, while Enteroinvasive E. coli were high between 2-3 years also cause infection in first age group, in time all Enterotoxigenic E. coli infections were all above 1 year as shown in figure (1).

E. coli pathotypes, in our study were identified and isolated successfully by using Multiplex PCR. PCR products visualized to measured product size results from amplification the primers in compared with (100 bp) ladder as shown in figures (2, 3, 4, 5, 6, 7, 8, 9).

Antibiotic susceptibility test results were showed in table (2): The highest level of resistance were to Amoxiclav (95.2%), Ampicillin (95.2%), Sulfas -Trimethoprim (92.9%) followed by Tetracycline (85.7%), Cephalthin (85.7%) Ceftriaxone (81%) Cefotaxime/clavulanicacid 71.4%. The maximum E. coli sensitivity was to Imipenem (100%) flowed by Amikacin (85.7%), Ciprofloxacin (78.6%) Chloramphenicol (73.8%).

**DISCUSSION**

The distribution of DEC in our study was 42 (20%) among 210 diarrheal cases. Our result concur to other study in Iraq reported by Hamada et al. in Kirkuk (36%) and globally with other studies in Iran Heidary et al. (28 %), while our result contrast with other studies were showed less prevalence to Diarrheagenic E. coli Konateet al. revealed (7.4%) in Burkina Faso, Salmani et al. in Iran who showed (88%). These differences reflecting the difference in distribution of geographical areas, quality of sanitation.

Among all the Diarrheagenic E. coli pathotypes, Enteropathogenic E. coli (EPEC) were found to be the most common pathotypes for children with (45.3%), our result compatible with localstudy by Sakhi who showed EPEC as most than other pathotypes (63%), and in contrast with Khalil and Al-Dulaimi where they show it came second after EAEC. Our finding was, however, similar to globally studies with Zhou et al., Thakur et al. and Chellapandi et al. that also reported a high frequency of EPEC pathotypes associated with pediatric diarrhea.

EPEC are sub-grouped into typical (tEPEC, eae+ bfpA+) and atypical (aEPEC, eae+ bfpA-) strains that differ in several respects Naji and Nasser. From 19 isolates detected as EPEC which was watery diarrhea 10 (52.6%) isolates of them are atypical EPEC showed eaeA gene found without bfpB gene, and 9 (47.4 %) were typical EPEC which showed eaeA gene together with bfpB gene.

![Fig.1. Prevalence diarrheagenic E. coli with age groups.](www.microbiologyjournal.org)
Fig. 2. Gel electrophoresis of amplified (eaeA, bfpB, aggR, astA, pic, hly, stx1, stx2, invE, ipaH, elt, estla, estlb) genes, the product size (482, 910, 400, 102, 1, 111, 688, 244, 324, 766, 437, 655, 157, 171 (bp) respectively), of E. coli strains using conventional PCR. Agarose 2%, and TBE (1X) at (75 V/cm for 90 min., stained with Ethydium bromide dye and visualized on a UV transilluminator. Lane (L): DNA ladder (100-3000 bp), Lanes: (1-12) stool samples.

Fig. 3. Gel electrophoresis of amplified (eaeA, bfpB, aggR, astA, pic, hly, stx1, stx2, invE, ipaH, elt, estla, estlb) genes, the product size (482, 910, 400, 102, 1, 111, 688, 244, 324, 766, 437, 655, 157, 171 (bp) respectively), of E. coli strains using conventional PCR. Agarose 2%, and TBE (1X) at (75 V/cm for 90 min., stained with Ethydium bromide dye and visualized on a UV transilluminator. Lane (L): DNA ladder (100-3000 bp), Lanes: (1-12) stool samples.

All 19 isolates in our study don’t produce nether stx1 or stx2, in addition one of aEPEC showed astA gene. Our study is close to study by Arif and Salih in Sulaimani, Iraq, and global reports by Malvi in India, that showed the distribution of atypical EPEC was higher than typical EPEC. Ochoa and Contreras report that atypical EPEC (aEPEC) are more prevalent than typical-EPEC (tEPEC).
Fig. 5. Gel electrophoresis of amplified (eaeA, bfpB, aggR, astA, pic, hly, stx1, stx2, invE, ipaH, elt, estla, estlb) genes, the product size (482, 910, 400, 102, 1, 111, 688, 244, 324, 766, 437, 655, 157, 171 (bp) respectively), of E. coli strains using conventional PCR. Agarose 2%, and TBE (1X) at (75 V/cm for 90 min., stained with Ethidium bromide dye and visualized on a UV transilluminator. Lane (L): DNA ladder (100-3000bp), Lanes: (1-12) stool sample.

Fig. 6. Gel electrophoresis of amplified (eaeA, bfpB, aggR, astA, pic, hly, stx1, stx2, invE, ipaH, elt, estla, estlb) genes, the product size (482, 910, 400, 102, 1, 111, 688, 244, 324, 766, 437, 655, 157, 171 (bp) respectively), of E. coli strains using conventional PCR. Agarose 2%, and TBE (1X) at (75 V/cm for 90 min., stained with Ethidium bromide dye and visualized on a UV transilluminator. Lane (L): DNA ladder (100-3000 bp), Lanes: (1-12) stool samples.

Fig. 7. Gel electrophoresis of amplified (eaeA, bfpB, aggR, astA, pic, hly, stx1, stx2, invE, ipaH, elt, estla, estlb) genes, the product size (482, 910, 400, 102, 1, 111, 688, 244, 324, 766, 437, 655, 157, 171 (bp) respectively), of E. coli strains using conventional PCR. Agarose 2%, and TBE (1X) at (75 V/cm for 90 min., stained with Ethidium bromide dye and visualized on a UV transilluminator. Lane (L): DNA ladder (100-3000bp), Lanes: (1-12) stool samples.

Enteroaggregative E.coli 17 (40.5%) isolates came second after Enteropathogenic E. coli as causative agent of diarrhea among Diarrheagenic E. coli pathotypes in our study, that agree with reports by Sakhi in Dhi-Qar city, also Globally with Thakur et al. But EAEC considered the major cause of diarrhea between diarrheagenic E. coli pathotypes in local studies by Khalil and Al-Dulami, also Globally, Rajendranet et al in India, Ali et al in Egypt.
and Konate et al. 20 in Burkina Faso.

EPEC and EAEC were reported as the most common diarrheagenic E. coli pathotypes Bueris et al. 29; Moyo et al. 30; Wang et al. 31.

Enterotoxigenic E. coli account 3 isolates (7.1%) of diarrheagenic cases, the detection of ETEC is in consonance with previous local findings by Hamada et al. 12, and concur with global study by Raghavan et al. 32 in India. Our study differ from other reports by Chiyangi et al. 33 in Zambia which suggested high prevalence of ETEC 40%.

Enteroinvasive E. coli was detected in 3 isolates (7.1%) of diarrheagenic isolates. Our study is nearly close with local study by Hamada et al. 12 (10%), and globally with (0.5%) by Moshtagian 34, and (12.9%) by Konate et al. 20, and (3.7%) by Zhou et al. 1.

Vieira et al. 35 also showed low rate of prevalence Enteroinvasive E. coli and suggested that this pathotype may play a less important role in childhood diarrhea in developing countries. In this study, no Shiga toxin-producing E. coli were detected this result is similar to local reports of Sakhi 22 and Hamada et al. 12. Globally, Ali et al. 28 and Cazalez-Roman 36, also showed no isolates of STEC were detected in children with diarrhea. STEC appears to be more frequent in adults than children Okeke et al. 37. These difference between our results and other studies may be attributed to rout of infection, virulence factors, pathogen strains, difference in population selection, time of collection and size of samples.

Antibiotic susceptibility test

Resistance to Amoxiclave (95.2%) agreement with another study had been reported high resistance in studies done by Al-Hilali 38 83.4% in Al-Najaf. Our result; disagreed with previous study in Alkut by Shamkhi 39 who recorded low
resistance to Amoxiclav with 7.1% Al-Shuwaikh et al.\textsuperscript{39} in Baghdad reported 33.3%. Increased Amoxiclav resistance coincided with growing Amoxiclav consumption at the community level, similarly, the isolated Diarrheagenic \textit{E. coli} pathotypes showed high resistance rates to Ampicillin and Sulfa-Trimethoprim Konate et al.\textsuperscript{41}, as mentioned in study by Goossens et al.\textsuperscript{42}; Llor and Bjerrum\textsuperscript{43} about antibiotic resistant, there is high resistant in the most consumption antibiotic. Sulfa-Trimethoprim is widely used in developing countries to treat diarrhea because of their availability over the countries Nguyen et al.\textsuperscript{44}. Attention should be given while prescribing Amoxiclav, Ceftriaxone, and Ampicillin to avoid increasing resistance pathotype by \textit{E. coli}. Similar study conducted by Konate et al.\textsuperscript{41} in Burkina Faso revealed that 85% of \textit{E. coli} isolates were resistance to Tetracycline. Our study for Cefotaxime was agreement with previous local studies by, AL Hilali\textsuperscript{38} 68%, Sakhi\textsuperscript{22} 85.7%, Ugwa et al.\textsuperscript{45}, also reported resistance to Ceftriaxone (91%) by \textit{E. coli} isolates. Rajeshwari et al.\textsuperscript{46} reported similar finding for the high resistance of Ceftriaxone 75% and cefotaxime (77.5%) in Indian children with diarrhea, while disagreement Khalil\textsuperscript{11} 4% and Hamada et al.\textsuperscript{12} 10%. Antibiotic susceptibility testing of isolates showed high resistance rate to Cephalothin (85.7%). The emergence of multidrug resistance especially in \textit{E. coli} has become a critical public concern, which was designated as resistance to one agent in three or more antibiotic classes. Kamwati\textsuperscript{47}; Alizadi\textsuperscript{48}. Many factors responsible for an increase in rates of antimicrobial resistance include misuse/over use of antibiotic by healthcare professionals and general public Magiorakos et al.\textsuperscript{49}; WHO\textsuperscript{50}; Konate et al.\textsuperscript{41}, and inadequate surveillance systems and independence on reliable microbiological techniques which leads to inappropriate prescription of antibiotics Wellington et al.\textsuperscript{51}. 

Ciprofloxacin showed low resistant similar with local studies by Hamada et al.\textsuperscript{12} 10%, Khalil\textsuperscript{11} 8%. Globally Kamwati\textsuperscript{47} 4%, Canizalez-Roman et al.\textsuperscript{36} 21%. Ciprofloxacin was one of the most active antimicrobial agent which currently recommended to treated diarrhea in children Ayatollahi et al.\textsuperscript{52}. Amikacin showed low resistant agreement with 3.3% reported by Hamada et al.\textsuperscript{53} and Al-Hilali\textsuperscript{38} 0%, and globally with Zhou et al.\textsuperscript{1} 7.4%.

Imipenem with no resistant agreement with 0% resistant from Al-Hilali\textsuperscript{38} and Shamkhi\textsuperscript{39}. The Imipenem was the most effective antibiotic against DEC followed by Amikacin, Ciprofloxacin and Chloramphenicol. Imipenem has been highly effective against gram negative bacteria Mohammed et al.\textsuperscript{53}, Alam et al.\textsuperscript{54}. They should be used in life threatening multidrug resistance infections where there is no other alternative.

The statistical analysis to susceptibility test results in this study showed high resistance among Diarrheagenic \textit{E. coli} isolates which were collected from hospitalized children than isolates collected from private pediatric clinics (without history of hospital admitted) as shown in table (3). This result goes with report of Kamwati\textsuperscript{47} who showed isolates from children who had been hospitalized were more resistant than those isolated from children not previously hospitalized, and he conclude that recent history of antimicrobial use and hospitalization is a serious predisposing factor to carriage of Multi Drug Resistant strains. Fox-Lewis\textsuperscript{55} also mention that hospital-acquired \textit{Escherichia coli} isolates were multidrug resistant than isolates were community-acquired. Multi drug resistance MDR may be acquired from other patients who have received antibiotics. Infections caused by Multi drug resistance gram negative bacteria are difficult to treat and so may cause more prolonged symptoms in the site of infection Hawkey et al.\textsuperscript{56}.

**CONCLUSION**

Enteropathogenic \textit{E. coli} and Enteragggregatorgative \textit{E. coli} was the most common types of Diarrheagenic \textit{E. coli} among children less than 2 years of age presented with diarrhea in Wasit province. Enterotoxigenic \textit{E. coli} and Enteroinvasive \textit{E. coli} were more common in children more than 2 years of age in Wasit province. This study highlights the Using of multiplex PCR in identifying and successful isolation of Diarrheagenic \textit{E.coli} from normal flora and can be used as a rapid and accurate method for the isolation of pathogenic strains of \textit{E. coli}, this will greatly help pediatricians to decrease the use of antibiotic in treatment of diarrhea in...
children and decreasing the problem of increasing antibiotic resistance. The results of antibiotic sensitivity test revealed that the most active compound against Diarrheagenic E. coli isolates was Imipenem followed by Amikacin, Ciprofloxacin and Chloramphenicol.

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