Original Article

Determination of the Activity of Antibiotic Resistance in Bacterial Causative Agents of Diarrhea among Children Under the Age of 5 Years

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Abstract

Background: *Escherichia coli* is one of the most important bacterial agents that cause diarrhea in children. **Objectives:** The present study was carried out to investigate the bacterial causative agents of diarrhea in children, as well as the genes that encoded for antibiotic resistance to beta-lactams in *E. coli* isolated from the feces of children under the age of 5 years with symptoms of diarrhea and its relationship to antibiotic resistance. **Materials and Methods:** One hundred stool samples were collected from the diarrhea patients. Individually 15 samples of stool were planted on several culture media, and then the bacterial isolates were diagnosed using the Vitek 2 technique. After that an antibiotic sensitivity test was conducted. Forty isolates of *E. coli* were subjected to molecular detection by polymerase chain reaction. **Results:** The results of the sensitivity test showed that most of bacterial isolates were resistant to the all tested types of antibiotic. In contrast three (7.5%) bacterial isolates were resistant to the antibiotic Amikacin, which is the lowest percentage of resistance. The results of molecular identification showed that 12 (66%) isolates were carried the blaCTX-M gene, which was significantly higher ($P \leq 0.05$) compared to the isolates carrying the blaTEM gene 6 (33%) isolates. **Conclusion:** It can be concluded that *E. coli* isolated from diarrhea children under of 5 years old conferred of antibiotics resistances against a wide range of antibiotics tested could be due to genes (such as TEM and CTX-M) which encoded to this resistance activity.

Keywords: Antimicrobial resistance, blaCTX-M, blaTEM, *E. coli*, β-lactamase genes

Introduction

Diarrhea is considered as one of the most important causes and deaths among children under the age of 5 years in a various regions of the world. It is caused by contaminated food and water sources. Repeated bouts of diarrhea can also lead to malnutrition, but this disease can be prevented and treated.[1] There are some factors which could cause diarrhea, including bacterial or viral agents, or factors related to the health habits of the individual. *Escherichia coli* is one of the most important bacterial agents that cause diarrhea in children.[2] *Escherichia coli* is a member of the intestinal family which lives harmlessly in the intestines and rarely causes diseases in healthy individuals. However, it is considered an opportunistic pathogenic bacterium when the host's immunity is weak. Its pathogenic strains cause many diseases such as diarrhea, urinary tract infections, blood poisoning, and so on.[3,4] *Escherichia coli* bacteria have the ability to resist antibiotics because they contain beta-lactam enzymes that confer antibiotic resistance to penicillin and cephalosporin, and these enzymes are encoded by genes such as (*CTX-M*, *TEM*, and *SHV*).[5,6] These genes are usually carried by plasmids, and plasmids can carry resistance genes to a number of different unrelated classes of antibiotics.[7] The first discovery of the beta-lactam enzyme was *TEM-1* in 1964 isolated from *E. coli* from a blood sample of a man suffering from septicemia, and

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soon the *SHV-1*β-Lactamase was discovered in strains of *Klebsiella* spp. resistant to environmental lactams.[8] The bla *CTX M-15* is the most prevalent beta-lactam gene, and this high prevalence raises public health concerns.[9] Resistance to β-lactam–β-lactamase inhibitor combinations in *E. coli* may be due to different mechanisms, including TEM-1 penicillinase hyperproduction, constitutive AmpC over-production or plasmid AmpC production, OXA-type β-lactamase production.[10] It was found that the random use of antibiotics led to an increase in bacterial strains that are resistant to multiple antibiotics.[11,12] Therefore, it is necessary to conduct a drug sensitivity test in order to determine the effective antibiotic, and thus it is possible to reduce the emergence of resistant strains and reduce the economic burden.[13] It is worthy for searching about the genes (*CTX-M*, *TEM*, and *SHV*) which encoded to the characteristic of beta-lactam antibiotic resistance, using modern molecular techniques that provided the best diagnostic methods, including the polymerase chain reaction (PCR) technique, which is the technique the most common and widely accepted with very high accuracy for correct diagnosis.[14,15] Therefore the present study was designed to investigate the presence of *E. coli* which causes diarrhea and has antibiotic-resistance due to beta lactam genes.

Materials and Methods

Bacterial sample collection

Stool samples (100) were collected from children under the age of 5 years, and 80 samples were collected from children suffering from symptoms of diarrhea and 20 samples were belonged to healthy children. All these children attended at the Children's Teaching Hospital in the Holy Karbala Governorate for the period from February 2, 2022 to June 15, 2022. Samples were transferred with a swab with (Cary-Blair) medium, and then the samples were cultured on several different differential media.[16]

Identification of bacterial isolates

In order to obtain a preliminary diagnosis of the bacterial isolates based on the phenotypic characteristics of the bacterial colonies, swabs were cultured and purified on several different culture media (MacConkey agar medium, EMB medium, X.L.D medium, TCBS medium, MacConkey sorbitol medium, and Chromium medium). The phenotypic characteristics of the isolated bacterial colonies, included color, shape, size, edges, and heights.[16]

Smears were taken from overnight bacterial isolates growing on the media of the MacConkey agar which were prepared using the Gram stain technique; after which they were examined using a light microscope to show the shape and color of the cells and the pattern of their clusters.[17]

Vitek2 system

The bacterial suspension was prepared according to the manufacturer's instructions, Bionarieux, by transferring a quantity of colonies growing on the medium of the congee at the age of (18–24) h to a tube containing 3 mL of sterile saline solution. The turbidity of the bacterial suspension was controlled compared to the standard McFarland's solution 0.5 using the Densi apparatus—check and this tube was placed in the device with the diagnostic card for bacteria Salicram GN-ID placed. The results of the diagnosis were displayed on the computer screen after about 4–12 h.[18]

Antibiotic susceptibility testing

The Kirby and Baure method was used to test the sensitivity of bacterial isolates (disk diffusion) as illustrated. Around (3–5) growing colonies on the medium of MacConkey agar at the age of (18–24) h were transferred to a tube containing 5 mL of normal saline and mixed well using the vortex mixing device. Then, suspension bacterial was spread on the surface of the dishes contain Mueller-Hinton agar using a sterile cotton swab. All dishes were left for 10–15 min. Antibiotic disks were placed on the surface of the cultured plate by using a sterilize forceps at equal distances and the discs were pressed gently. Then the plates were incubated at 35°C for 24 h. hours. The diameters of the inhibition zones around the discs were measured in mm using a ruler, and the measurement results were compared with standard tables[19] This method was used according to Brown and Smith.[17]

Molecular identification

Genomic DNA extraction Genomic DNA was extracted from pure isolates of *E. coli* bacteria using a Kit according to the manufacturer's instructions (Addbio for the kit). The concentration of DNA was determined spectrophotometrically at 260 nm (Thermo Scientific Nano Drop 1000) and sequenced by GATC-Biotech Laboratories.

Amplification of genes encoding for antibiotics resistance

Primer sequences used to amplify fragments of *TEM, SHV*, *and CTX-M* genes are illustrated in Table 1.

The following reagents were included in each PCR tube: 1 µL of primer F and 1 µL of R (10 pmol), 3 µL DNA template, 12.5 µL of ReadyMix Taq PCR Reaction and the final volume of 25 µL was completed by deionized water. The tubes were placed in a thermocycler (Labnet Edison, NJ, USA) and PCR cycling was performed under conditions as listed in Table 2.[20,21]

The DNA amplicons were separated on a 1.5% agarose gel for 45 min at 90 mV and compared with (100–1000) bp ladder (Promega).

Table 1: β-Lactamase genes and primers

Gene		Primers sequences	(bp)	Reverence
blaSHv	F	ATGCGTTATATTCGCCTGTG	753	(20)
	R	TGCTTTGTTATTCGGGCCAA		
blaTEM	F	AAACGCTGGTGAAAGTA	822	(20)
	R	AGCGATCTGTCTAT		
blaCTX-M	F	CGCTTTGCGATGTGCAG	550	(21)
	R	ACCGCGATATCGTTGGT		

Table 2: Optimum thermal conditions for PCR

Genes	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles number
TEM	95°C/5 min	95°C/1 min	58°C/30 s	72°C/1 min	72°C/10 min	30
SHV	95°C/5 min	95°C/1 min	60°C/30 s	72°C/1 min	72°C/10 min	30
CTX-M	95°C/5 min	95°C/1 min	58°C/30 s	72°C/1 min	72°C/10 min	30

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 20220137 on June 26, 2022.

Results

Identification of *Escherichia coli* isolates

Escherichia coli isolates were initially diagnosed based on their phenotypic characteristics, after growing them on MacConkey agar (MA), Eosin methylene blue agar (EMB), and MacConkey sorbitol agar. MS, and HiCrome EC 0157: H7 and this study was based on the diagnosis according to what was mentioned in Procop *et al.*[18]

After staining with a Gram stain, the swab was examined using a microscopic light microscope. All the cells of *E. coli* are short rods negative for Gram stain, and these results are consistent with what was mentioned in Jawetz and Adelbergs.[5]

Diagnosis of bacteria using the Vitek2 system

The isolates out of 80 were selected for the purpose of diagnosis using the Vitek 2 system. The results indicated that 40 isolates belong to *E. coli*.

Antibiotic resistance profile of *Escherichia coli* isolates

The susceptibility of the bacterial isolates against ten types of antibiotics was selected, and the choice of antibiotics was based on the common use of them in previous studies. The bacterial isolates varied in their resistance to these antibiotics as follows: The results showed that 40 (100%) bacterial isolates were resistant to Ampicillin, which is the highest percentage of resistance, followed by 34 bacterial isolates representing 85% that were resistant to Co-Trimoxazole; then 33 bacterial isolates with a ratio of 82.5% were resistant to Cefotaxime, 29 (72.5%) of the bacterial isolates were resistant to the antibiotic Ceftriaxone, and 26 bacterial isolates were 65% resistant to Tetracycline. Further 21 bacterial isolates were resistant to Azithromycin which represent 52.5%, followed by 20 (50%) bacterial isolates which were resistant to the antibiotic Doxycycline, and 15 bacterial isolates with a ratio of 37.5% resistant to Ciprofloxacin, 6 (15%) bacterial isolates resistant to Gentamycin, and finally 3 bacterial isolates representing a ratio of 7.5% resistant to Amikacin, which represents the lowest number of isolates resistant to antibiotics, as shown in Figure 1.

Molecular identification

Isolates of *E. coli* bacteria were selected for the purpose of molecular investigation using the PCR technique, and the selection of bacterial isolates was based on examining a stool sample under a light microscope, which contained red blood cells or inflammatory cells. The results showed that 12 (66%) of the isolates were carried the *blaCTX-M* gene which was significantly higher ($P \leq 0.05$) than the *blaTEM* gene-carrying isolates with 6 (33%) isolates, and the blaSHV gene was not determined in any of the isolates.

The results of electrophoresis of PCR products to detect the genes under study showed that the size of the *CTX-M* gene found in *E. coli* bacteria is (550) bp, and the *TEM* gene found in *E. coli* had a size of (822 bp), and the *blaSHV* gene was not appeared during electrophoresis, which is consistent with previous studies in this regard, as shown in Figures 2 and 3.

Discussion

The results of the current study with regard to the antibiotic Ampicillin agreed with the results of previous study.[22] In contrast, the results of the current study had disagreement with Webale *et al.*[23] It could be the resistance to antibiotics

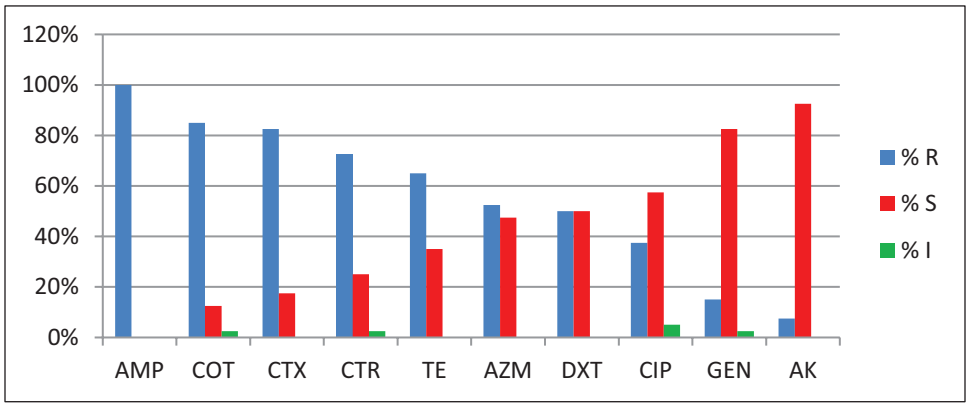

Figure 1: Antibiotic susceptibility of *E. coli* to different antibiotics using Kirby Bauer diffusion method. AMP = Ampicillin, AK = Amikacin, AZM =Azithromycin, CTX = Cephotaxime, CTR=Ceftriaxone, COT = Co-Trimoxazole, DXT = Doxycycline, GEN = Gentamycin, TE = Tetracycline, CIP = Ciprofloxacin

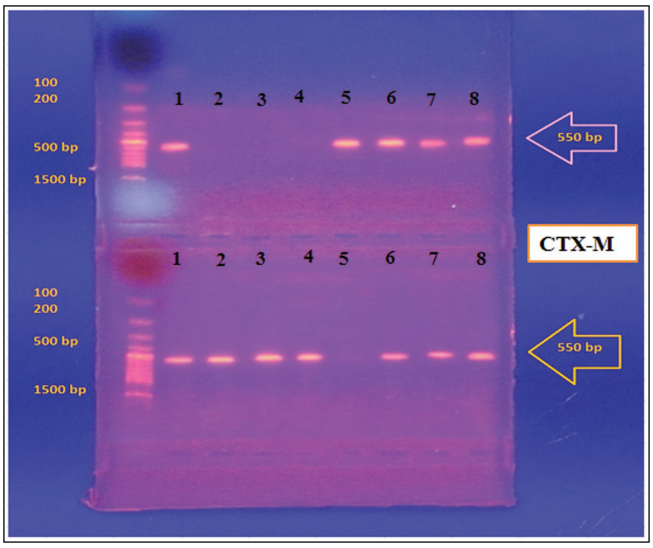

Figure 2: Electrophoresis of the PCR product of the CTX-M (550 bp) of *E. coli* isolates in 1.5% agarose and 100 V for 30 min

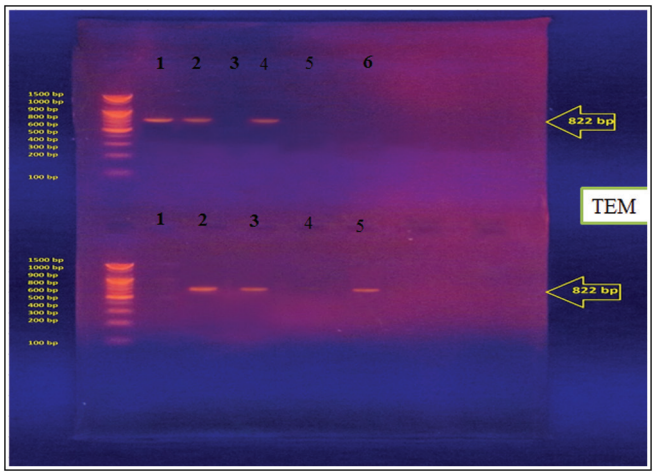

Figure 3: Electrophoresis of the PCR product of the TEM (822 bp) of *E. coli* isolates in 1.5% agarose and 100 V for 30 min

attributed to such reasons including the group of beta-lactams such as penicillin, cephalosporins, and presence of mutations in the penicillin-binding proteins (PBPs), or a change in the cell wall cytotoxicity by mutations in the outer membrane proteins, or the PBP systems.[24] The reason behind the contrary in rates of resistance of bacteria to the same antibiotic in above studies may be due to the use of herbs and medicinal plants commonly used in some regions as an alternative to the use of antibiotics, and thus will diminishing the emergence of resistance or its transmission between bacteria.[25]

The results of the current study regarding the antibiotic Cefotaxime and Ceftriaxone, of the beta-lactam group was consistent with the results of previous studies.[26,27] On the other hand, the results of the present study are in disagreement with some studies.[23,28] There are a number of possible suggestions which may explain the variation in ratio of bacterial resistance to the same antibiotic including the use of alternative methods to the use of antibiotics and the use of substances that may be toxic to humans which are considered as alternative treatment instead of using antibiotic, thus lead to decreasing the emergence of resistance and the transmission of the characteristic of resistance between bacteria.[29] All isolates have been found resistant to at least one β-lactam antibiotic, the current results are in disagreement with study of.[30] The data obtained in the present study regarding to Tetracycline and Doxycycline antibiotic are consistent with the study.[31,32] On the contrary, the current results are in disagreement with study.[33] The reasons for resistance to tetracycline antibiotics is attributed to the occurrence of mutations which could encoded for antibiotics resistance and their transmission between bacteria or a change in the permeability of the outer membrane by changing the permeability of proteins from purines that prevent the entry of some antibiotics.[34] Finally, present data regarding the antibiotics of Amikacin and Gentamycin of the aminoglycoside group, are consistent with the results of previous studies.[35,36] On the other hand, these results differed with Shatub *et al.*[22] and Hasan *et al.*[37] One of

the most important factors behind bacterial resistance is biofilms, as it is a key factor that contributes to giving bacteria the characteristic of antibiotic resistance and tolerance. Efflux pumps have a direct role in antibiotic resistance, which is present in the structures of biofilms of Gram-negative bacteria.[38] Biofilms play an important role in the acquisition of multi-antibiotic resistance by bacteria, and are found in Gram-positive and Gram-negative bacteria.[39] The results of the current study regarding the genes (*CTX-M, TEM, SHV*) are consistent with the study,[40] whereas these data are differed with a local study.[41]

Conclusion

It can be concluded that bacterial isolates which conferred of antibiotics resistances against a wide range of antibiotics tested could be due to some genes including (*TEM*, *CTX-M*) which encoded to this resistance activity. In future work, sensitivity test of antibiotics must be conducted for any inflammatory condition in order to obtain the appropriate antibiotic. In addition, searching for alternative model of treatment such as using of probiotics is required.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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