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Molecular Detection of *CTX-M* and Some Folate Bathway Inhibitors Resistance Genes among Gentamicin-Resistant *Escherichia coli* Isolates

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Abstract
Urinary tract infections (UTIs) are one of the most common bacterial infections found in humans after respiratory tract infections because used broad-spectrum antibiotics and the indiscriminate use of antibiotics, so this study aimed to investigate genes responsible for resistance of antibiotic among gentamicin- resistant *Escherichia coli* isolates. Results of this study showed among 573 samples collected from urine of urinary tract infection patients, among 573 clinical specimens 102(17.80%) were male patients while 471(82.19%) were female patients pointed that 169 (35.88%) of patients had gram negative and 44 (9.34%) are gram positive. while recorded 258(82.19%) as no-growth. While males record the growth rate of gram-negative bacteria are 40(35.88%), while the gram-positive bacteria were 11 (10.78%) also recorded 51(50%) from patients as non-growth status. Results of antibiotic susceptibility showed that the highest bacterial resistance was Amikacin 29(100.0%), while Imipenem 2(6.9%) had the least resistance. Results of polymerase chain reaction (PCR) amplification showed that *CTX-M* gene 29/29 (100%), while folate pathway inhibitors genes among gentamicin-resistant *E. coli* isolates demonstrates that 82.75 percent of gentamicin-resistant *E. coli* isolates contained positive bands at the correct position for *drf-G* genes, as determined by the molecular analysis of this study. In this study, the *drf-A, drf-B, and drf-K* genes were not detected, according to PCR results.

Keywords: *Escherichia Coli*, *CTX-M* Gene, Folate Pathway Inhibitors Genes, Antibiotic Resistance and PCR

Introduction

Urinary tract infection (UTI) is a broad term that describes a bacterial infection or inflammation of any region of the urinary system. One of the most predominant bacterial disorders encountered by doctors globally is a UTI. If a urinary tract infection (UTI) is not discovered and treated early enough, it can lead to chronic sickness and long-term kidney impairment. Patients with uncomplicated UTIs can be treated because the causal bacteria are relatively predictable*: E. coli* causes 80–90% of UTIs [1].

Escherichia coli has been implicated in most hospital and community-acquired infections, including several intestinal and extraintestinal infections, urinary tract infection, and some fatal infections that develop in immunocompromised patients. Even worse, the treatment of *E. coli* infections has become extremely challenging because of the increasing multidrug-resistance to antibiotics [2].

Females are more likely than males to develop UTI due to a shorter urethra, facilitating the bacteria to enter into the bladder more easily, closer proximity to the anus and the absence of prosthetic secretions. The pattern of antibiotic resistance may vary over time and also depends on the site of isolation and environmental conditions. The majority of the problems associated with antimicrobial resistance have been shown to be due to the presence of transferable plasmids encoding multidrug resistance and their dissemination among different bacterial species [3].

Antibiotic-resistant bacterial infections are usually associated with high morbidity and mortality as conventional antibiotics become less valuable. Antibiotic resistance is increasing globally at an alarming rate, especially in the last decade [4].

Gentamicin is commonly recommended for treating children with UTIs that require parenteral therapy. However, recent pediatric series have reported increasing rates of nosocomial and CA-UTI caused by extended-spectrum beta-lactamases (ESBL) strains, many of which may associate gentamicin resistance [5].

Materials and Methods

Collection of the Specimens

In the present investigation, 573 clinical specimens were collected at random from patients with urinary tract infections (UTIs). During the period from September to December 2022, all patients were committed to Al-Najaf City's leading medical facilities, such as the Central Public Health Laboratory, Al-Sadr Medical City, and a number of leading clinical laboratories. For sample collection, consent was obtained from all patients.

Isolates and Bacterial Diagnosis

After cleaning the genitals of patients with urinary tract infection and collecting urine samples in sterile containers, midstream urine was centrifuged at 2000 rpm for two minutes. The sediment was incubated with a brain heart infusion broth at 37 C overnight and streaked on Blood agar and MacConkey agar surfaces, which were then incubated aerobically at 37 C overnight [6]. Using ID-GN cards and the automated Vitek-2 compact system, final identification was conducted.

Detection of Gentamicin Resistance among Escherichia coli Isolates and Antibiotic Susceptibility Testing

All *Escherichia coli* isolates were initially tested against the gentamycin antibioti using gentamicin disk (10µg) (Bioanalyse,Turkey) and employed on sterile media of Mueller Hinton agar (England) The suspension of all tested isolates were achieved based on 0.5 McFarland standard. However, only gentamicin-resistant *Escherichia coli* isolates were tested on several antibiotics (Bioanalyse, Turkey), including Cefixime (CFM 5mg), Ceftazidime(CAZ 30 mg), Amikacin (AK 10mg), Netilmicin (NET 30 mg), Tetracycline (TE 10 mg), Levofloxacin (LEV 5 mg), Norfloxacin (NOR 30 mg), Trimethoprim/ Sulfamethoxazole (SXT 25mg). Imipenem (IPM 10 mg), using the disc diffusion technique according to the Kirby–Bauer [7]. The zone diameter were applied base on instructions of the Clinical and Laboratory Standards Institute (CLSI) [8].

DNA Extraction and PCR Assay

The instructions provided by a manufacturing company, a genomic DNA extraction micro kit (Favorgen, South Korea) was used to collect all of the nucleic acid for 29 clinical isolates of *Escherichia coli*. This was done in accordance with the manufacturer's protocol. After ensuring the integrity of the whole DNA sample by storing it in a deep freezer set to-20 degrees Celsius, a PCR analysis was carried out in order to test for the genes listed in Table 1. The equipment for gel documentation was employed for the migration of PCR amplification (bands) at 1% agarose, and then the bands were dyed with ethidium bromide at a concentration of 0.5 g/ml [9].

Table 1: Primers and condition used in the study

Primer target	Sequence (5' to 3')	Product size	Annealing (ºC)	Reference
DfrA-F	CACTTGTAATGGCACGGAAA	270	57	10
DfrA-R	CGAATGTGTATGGTGGAAAG			
dfrB-F	AATTGTGTTAAATTAAAGATAACTT	572	43 ºC	10
dfrB-R	TAAGTATTCTTTAGATAAATCGGAT			
dfrK-F	GCTGCGATGGATAATGAACAG	321	49 ºC	10
dfrK-R	GGACGATTTCACAACCATTAAAGC			
dfrG-F	TGCTGCGATGGATAAGAA	405	57 ºC	10
DfrG-R	TGGGCAAATACCTCATTCC			
CTX-M-F	AACCGTCACGCTGTTGTTAG	766	57 ºC	11
CTX-M-R	TTGAGGCGTGGTGAAGTAAG			

Result and Discussion

Patients and bacterial growth

Results of this study showed among 573 samples collected from urine of urinary tract infection patients. Furthermore, the outcomes in a Table 2 described the frequency of bacterial growth and non-growth among the human sex, among 573 clinical specimens 102(17.80%) were male patients while 471(82.19%) were female patients pointed that 169 (35.88%) of patients had gram negative and 44 (9.34%) are gram positive while recorded 258(82.19%) as no-growth. While males record the growth rate of gram-negative bacteria are 40(35.88%), while the gram-positive bacteria were 11 (10.78%) also recorded 51(50%) from patients as non-growth status.

Table 2: Distribution of gram negative, gram positive and no growth according to Sex

Sex	Status	Number	within Sex%
Female	Gram negative	169	(35.88%)
	Gram positive	44	(9.34%)
	no-growth	258	(54.77%)
Total		471	(100%)
Male	Gram negative	40	(39.21%)
	Gram positive	11	(10.78%)
	no-growth	51	50%
Total		102	100%

The current study showed that clinical bacteria isolated from patients and belonging to the Gram-negative category grew at a higher rate than Gram positive bacteria. Gram negative organisms are largely responsible for UTIs and *E. coli* being the most common etiological agent [12].

This result is comparable to that study was conducted in Turkey, and the study showed that the total number of 9556 positive bacterial cultures was common in females, at a rate of 70.6, compared to 53.4% in males [13]. Since females have a short and wider urethra, hence, the chances of colonization of microbes are greater. The cause of the disease is mainly due to catheterization, anatomical abnormalities, and behavioral factors [14]. Whereas a study was conducted for men, where they found that the rate of urinary tract infection in men was (0.9 to 2.4 cases per1000 men per year), and thus it varies according to age and is uncommon. Also, it was found that the infection is more common in elderly men, but it remains less common compared to infection in women [15].

Antibiotic Susceptibility of Gentamicin-Resistance *Escherichia coli*

Results showed from among 110 (100%) *Escherichia coli* isolate and by using disk diffusion According to Kirby–Bauer methods for gentamicin antibiotic result only 29(26.36%) *Escherichia coli* isolates were resistance to gentamicin, while 39 (35.45%) and 42 (38.18%) of isolates were intermediate and sensitive to this antibiotic respectively.

There were several previous studied showed elevation of gentamicin resistance among *E. coli*, however, In a previous study conducted by Abdelwahab *et al*., [16], they recorded that 139 (85%) of 165 *E. coli* isolates were discovered to be resistance to gentamicin.

Antimicrobial Susceptibility Testing of Gentamicin-Resistant E. coli Isolates

The results in Table 3 show that 25 (86.2%) isolates of gentamicin-resistant *E. coli* were resistance to Cefixime, This is consistent with a study conducted in Tunisia by 22(75.9%) of isolates was resistance to Ceftazidime, while the members of Amikacin and Netilmicin revealed resistance rate 29(100.0%) and 20(69.0%) respectively. The results showed that 20(69.0%) of Tetracycline was resistance and there is a close study conducted in Iran, showed that the percentage of resistance was 65% [17]. Rate of resistance Levofloxacin was 25(86.2%), this study is similar to the result obtained by Gururaju, *et al* [18] in India was recorded 79 % rate of Levofloxacin resistance. Rate of *resistance* Imipenem was 2(6.9%) this result is similar to the result obtained by Mohammed, *et al* [19] in southern Iraq where, he also noticed a decrease in the percentage of resistance in rate 5%. The resistance of bacteria toward Trimethoprim / Sulfamethoxazole was 21(72.4%), This is consistent with a Trimethoprim/Sulfamethoxazole, and these resistance may be related with Integrons which are bacterial genetic elements involved in the spread of antibiotic resistance genes among gram negative bacteria that several studies have looked at the epidemiology of integrons in the particular settings of bloodstream infections and UTIs caused by *Enterobacterales* [21]. while rate of Norfloxacin resistance was 23(79.3%), but a study Dehbanipour, *et al* [22] mention among 135 *E. coli* isolates were 45.2% of resistance to Norfloxacin.

Table 3: Antibacterial agent susceptibility of gentamicin-resistance *E. coli* isolates

Antimicrobial agent	Sensitive	Intermediate	Resistance
Cefixime	2(6.9%)	2(6.9%)	25(86.2%)
Ceftazidime	7(24.1%)	0(0.0%)	22(75.9%)
Amikacin	0(0.0%)	0(0.0%)	29(100.0%)
Netilmicin	6(20.7%)	3(10.3%)	20(69.0%)
Tetracycline	7(24.1%)	2(6.9%)	20(69.0%)
Levofloxacin	0(0.0%)	4(13.8%)	25(86.2%)
Norfloxacin	5(17.2%)	1(3.4%)	23(79.3%)
Trimethoprim / Sulfamethoxazole	7(24.1%)	1(3.4%)	21(72.4%)
Imipenem	23(79.3%)	1(3.4%)	2(6.9%)

Molecular Detection of CTX-M Genes among Gentamicin-Resistant E. coli Isolates

The spread of *CTX-M* has become widespread among *Escherichia coli* isolates globally, this gene is one of the main genes responsible for the ESBL enzymes, and the importance of this gene in antibiotic resistance, the current research, recorded that the percentage of gentamicin-resistant *E. coli* isolates which harbored CTX-M genes were 29/29 (100%) as shown in the Fig 1.

However, these findings agree with many studies that observed showed high the prevalence of this gene among *E. coli* isolates [23, 24]. A previous work was conducted in southern Iran by Naziri, *et al* [25]. They observed that distribution of *CTX-M* gene among *E. coli* isolates were at rate of 37.2% *.*

At same respect, the rates of *CTX-M* gene among *E. coli* infection varies may be based on the regional rate and pattern of antimicrobial resistance and also predicts the severity of UTIs in that region. Additionally, another previous study, where a study was conducted in Nepal by Chaudhary, *et al*, [26], they showed that the proportion of the *CTX-M* gene was lower than the current study, as it was recorded at a rate of 36.6%*.*

Plasmid-mediated *CTX-M* type ESBLs, are detected mostly in community-acquired pathogens and are associated mainly with *Escherichia coli*. These β-lactamases compromise the efficacy of all β-lactams, except Carbapenems and Cephamycins, and are associated with many non–β-lactam resistance markers because of their locations on plasmids. Therefore, they may constitute a real threat for treating community-acquired *E. coli* –mediated urinary tract infections [27].

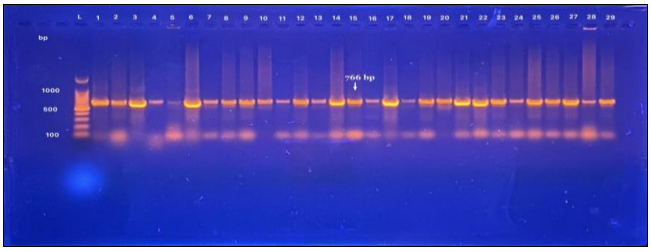

Fig 1: PCR amplification of *CTX-M* gene among 29 gentamicin-resistant Escherichia coli isolates

Molecular Detection of Folate Pathway Inhibitors Genes among Gentamicin-Resistant E. coli Isolates

Trimethoprim and sulfonamides drugs have been extensively utilized in the clinical treatment of infection due to their increased efficacy and minimal cost. Therefore, resistance to trimethoprim is becoming more prevalent in *Enterobacteriacea* [28, 29].

Fig 2 displays that 82.75 percent of gentamicin-resistant *E. coli* isolates contained positive bands at the correct position for *drf-G* genes, as determined by the molecular analysis of the current study. PCR data indicated that *drf-A*, *drf-B*, and drf-K genes were not detected in this study.

There were limited studies about spreading of antimicrobial agents resistance gene (dfr-type) among E. coli isolates. However, a previous international study achieved in Iran done by Torkan *et al*, [30] they observed that dfr-A1 gene was exist at rate 35.7% of *E. coli* isolates.

A recent work done by Al-Ghazaly and Tuwaij [10]. In Al-Najaf City/Iraq, they mention that a total of 28 (5.4%) Acinetobacter spp. isolates were found. 100% of Acinetobacter species contained *drf-G* alleles. 21 (75%) of the isolates contained *drf-A* genes, but neither *drf-B* nor *drf-K* genes were observed.

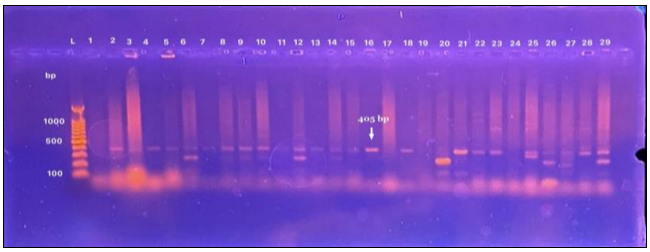

Fig 2: PCR amplification of dfr-G gene among 29 gentamicin-resistant Escherichia coli isolates

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