Screening of Tetracycline's Resistance Genes among *Enterobacter cloacae* and *Citrobacter spp.* isolates

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Abstract. There has been a growing prevalence of tetracycline-resistant bacteria across many species and genera. As a consequence, the efficacy of tetracycline treatment has diminished with time. So this work aimed to study of tetracycline-resistant genes among Enterobacter cloacae and Citrobacter spp. Out of a total of 364 clinical specimens, 135 (37.08%) exhibited bacterial growth. Out of the total, 22 (6.04%) were classified as gram-positive bacteria, 102 (28.02%) as gram-negative bacteria, and 11 (3.02%) as mixed growth (including both gram-negative and gram-positive bacteria). The Vitek-2 system findings indicated that the proportion of Enterobacter cloacae was 25 (6.86%), whereas Citrobacter spp. accounted for just 2 (0.54%) cases. The Enterobacter cloacae and Citrobacter isolates exhibited significant levels of resistance to several antibiotics, as shown by their drug susceptibility results. The PCR findings indicated that the tetrA gene was present in 8 out of 25 isolates (32%) of E. cloacae, and in 1 out of 2 isolates (50%) of Citrobacter spp. The tetrB gene was found in 92% of E. cloacae isolates (23 out of 25) and in 100% of Citrobacter spp. isolates (2 out of 2). In this study, the tetraC gene was identified in 6 out of 25 (24%) E. cloacae isolates and in 1 out of 2 (50%) Citrobacter spp. isolates. The TetraD and TetraE genes were not identified in the current study. The amino acid sequence analysis of the tetA and tetB genes revealed a similarity rate of 83% and 95% respectively.

1 Introduction

Enterobacter cloacae (E. cloacae) and Citrobacter spp. have become significant pathogens in recent years, causing many healthcare-associated diseases. It might be difficult to identify Enterobacter species since they have similar physical traits. The occurrence of multidrug-resistant strains of E. cloacae and Citrobacter spp. in healthcare facilities is a notable concern [1, 2].

Tetracycline is a potent antibiotic that has strong antibacterial effects against a broad range of pathogens. Tetracycline is a powerful antibiotic that works by blocking protein synthesis. It is considered a good medication due to its low toxicity and minimal risk of allergic reactions. Additionally, it is easily absorbed when taken orally and has a broad

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spectrum of effectiveness against disease-causing microorganisms. These factors contribute to tetracycline being highly regarded as an effective medication. The proliferation of tetracycline-resistant strains is a direct result of the extensive use of tetracycline in both the medical and food sectors. The development of resistance to tetracycline often occurs as a result of acquiring tet resistance genes. A total of 38 distinct tet genes have been identified in the resistant strains, each using various resistance mechanisms. The genes function via several ways such as expressing efflux pump proteins, destroying enzymes, and producing ribosomal protecting proteins [4, 5].

Antibiotics are pharmaceutical substances used for the prevention and treatment of bacterial illnesses. Antibiotic resistance, conversely, arises when bacteria undergo alterations due to the usage of antibiotics. An instance when bacteria undergo evolutionary changes that result in their resistance to medications, making infections harder to treat and increasing the likelihood of disease transmission, severe illness, and mortality. Bacterial resistance is the capacity of a bacteria to endure or accept the impact of antibiotics, meaning it can survive the growth-inhibiting or lethal effects of antimicrobial substances at concentrations that can be achieved in clinical settings. Antibiotic resistance is an escalating worldwide issue when medicines lose their effectiveness in treating infectious illnesses [6].

2 Materials and Methods

2.1 Ethical approval

Every the sample of urine utilized in this study was obtained with the consent and authorization of the patients and healthcare institutions [7].

2.2 Patients and Specimens Processing

The current research included a total of 364 individuals gathered from various clinical sources, selected randomly, who were suffering from different disorders. The treatment of burn injuries, wounds, blood-related conditions, and urinary tract infections took place in prominent hospitals and specialised burn centres in Al-Najaf City and Babil City, Iraq. Additionally, clinical labs in Al-Najaf City were involved in the diagnostic process. This medical intervention spanned several months, from October 2023 to February 2024. Each specimen was streaked on MacConkey Agar using cotton swabs and incubated aerobically at 37oC overnight in a sterile environment [8].

2.3 Diagnosis *of Enterobacter cloacae* and *Citrobacter spp.* Isolates

The suspected gram-negative bacteria, including Enterobacter cloacae and Citrobacter spp., were streaked on MacConkey Agar Medium. This was done based on microscopic, morphological, oxidase, and motility features, as well as certain important biochemical assays. The ultimate identification was carried out with the automated Vitek-2 compact system employing ID-GP cards [9, 10].

2.4 Antimicrobial agents susceptibility of *Enterobacter cloacae* and *Citrobacter spp.* Isolates

The Kirby-Bauer method was used to test the antibacterial medicines doxycycline, aztreonam, meropenem, fosfomycin, kanamycin, ciprofloxacin, and ticarcillin-clavulanate on 27 clinical isolates of Enterobacter cloacae and Citrobacter spp. The testing was done on

Mueller-Hinton agar [11]. To prepare the inoculation of all isolates, the overnight growth of each tested isolate was suspended in sterile normal saline that had been adjusted to a 0.5 McFarland standard tube. An analysis was conducted on antibacterial discs available for purchase at retail establishments. The zone diameters were interpreted following the directions given in the Clinical Laboratory Standards Institute (CLSI) guidance [12].

2.5 DNA extraction and PCR assay

Using the guidelines from the manufacturing firm, we used a genomic DNA extraction micro kit (Favorgen, South Korea) to gather all the nucleic acids from 27 clinical isolates of Enterobacter cloacae and Citrobacter spp. This was executed in compliance with the manufacturer's methodology. After confirming the integrity of the whole DNA sample by putting it in a deep freezer set to -20 degrees Celsius, a PCR analysis was conducted to examine the genes indicated in Table 1. The gel documentation equipment was used to analyse the migration of PCR amplification bands on a 1% agarose gel. Subsequently, the bands were stained with ethidium bromide at a concentration of 0.5 g/ml. The TetA and TetB genes from Enterobacter cloacae and Citrobacter spp. were delivered to Macrogen firm in South Korea for sequencing. The website (http://www.ncbi.nlm.nih.gov/) was used to compare the TetA and TetB gene sequences of local Enterobacter cloacae and Citrobacter spp isolates with those of foreign strains [13].

Table 1. Primer Sequence and condition

target	Sequence of primer 5' to 3'	annealing (°C)	Product (bp)	Reference
Tet A-F	TTGGCATTCTGCATTCACTC	55	494	[14]
Tet A-R	GTATAGCTTGCCGGAAGTCG			
Tet B-F	CAGTGCTGTTGTGTCATTAA	55	571	[14]
Tet B-R	GCTTGGAATACTGAGTGTAA			
Tet C-F	CTTGAGAGCCTTCAACCCAG	58	418	[15]
Tet C-R	ATGGTCGTCATCTACCTGCC			
Tet-D-F	ATTACACTGCTGGACGCGAT	57	1070	[15]
Tet-D-R	CTGATCAGCAGACAGATTGC			
Tet E-F	TATTAACGGGCTGGCATTTC	55	544	[16]
Tet E-R	AGCTGTCAGGTGGGTCAAAC			

2.6 Statistical analysis

The findings were analysed and computed based on numerical data and percentages utilising computer tools [14, 15, 16].

3 Results

3.1 Bacterial growth and patients

Results of this study showed among 364 clinical specimens analyzed, 135 (37.08%) showed positive bacterial growth. Specifically, 22 (6.04%) were identified as gram-positive bacteria, 102 (28.02%) as gram-negative bacteria, and 11 (3.02%) as mixed growth (both gram-negative and gram-positive). The remaining 229 specimens did not show any bacterial growth. Table 2 presents the data based on their sex, which indicates that out of the total 232 (100%) clinical specimens, all were collected from female patients, while 132 (100%) were received from male patients who were experiencing various diseases.

Table 2. Rates of bacterial growth from different clinical source

Status	**Number (%)**	**Total Bacterial growth (%)**
Gram positive	22 (6.04 %)	135 (37.08%)
Gram negative	102 (28.02 %)	
Mix growth (both)	11(3.02%)	
No growth	229 (62.91 %)	
Total	364 (100%)	

3.2 Identification of Enterobacter cloacae and Citrobacter spp. isolates

The results listed in Table 3, confirmed by Vitek-2 system results, showed that the percentage of Enterobacter cloacae isolated from patients suffering from urinary tract infections was 21 (5.76%) compared to 2 (0.54%) return to Citrobacter spp. (1 (0.27%) C. freundii isolate and 1 (0.27%) C. farmer isolate). At the same time, the percentage of Enterobacter bacteria isolated from blood sources and burns was 1 (0.27%) and 3(0.82%), respectively.

Table 3. Number of Enterobacter cloacae and Citrobacter spp. from different clinical source

Source	Number	growth	No growth	E. cloacae	Citrobacter spp.
UTIs	273(75%)	83(22.80%)	190(52.19%)	21(5.76%)	1(0.27%) C. freundii 1 (0.27%) C. farmer
Blood	31(8.51%)	4(1.09%)	27 (7.41%)	1 (0.27%)	0 (0%)
Burn and wound	60 (16.48%)	48(13.18%)	12(3.29%)	3(0.82%)	0 (0%)
total	364 (100%)	135(37.08%)	229(62.91%)	25(6.86%)	2 (0.54%)

3.3 Antibiotic susceptibility of Enterobacter cloacae and Citrobacter spp. isolates

The current results in table 4 showed the high effectiveness 24(96%) of *Enterobacter cloacae* isolates were resistance to the antibiotic of aztreonam, while the resistance percentage reached 23(92%), against antibiotics of kanamycin and Ticarcillin-clavulanate, while resistance rates were 10(40%), 9(36%), 18(72%) and 8(32%) against antibiotics of doxycycline, meropenem, fosomycin and ciprofloxacin respectively.

Table 4. Antibiotic susceptibility of 25 *Enterobacter cloacae* isolates

Antibiotic name	Resistance No. (%)	Intermediate No. (%)	Sensitive No. (%)
doxycycline	10 (40%)	4 (16%)	11 (44%)
aztreonam	24(96%)	1 (4%)	0 (0%)
meropenem	9 (36%)	11 (44%)	5 (20%)
fosomycin	18 (7%2)	4 (16%)	3(12%)
kanamycin	23(92%)	2 (8%)	0 (0%)
ciprofloxacin	8 (32%)	9 (36%)	8 (3%2)
Ticarcillin-clavulanate	23(92%)	2 (8%)	0 (0%)

Data of antimicrobial agents of Citrobacter spp. were absoluted resistance 2(100%) agaist antibiotics of aztreonam, meropenem, kanamycin, ciprofloxacin and Ticarcillin-clavulanate while the rate of rasistance were 1(50%) toward doxycycline and fosfomycin antibiotics among Citrobacter spp. (Table 5).

Table 5. Antibiotic susceptibility of Citrobacter spp. isolates

Antibiotic name	Resistance No.(%)	Intermediate No. (%)	Sensitive No. (%)
doxycycline	1(50%)	1(50%)	0(0%)
aztreonam	2(100%)	0(0%)	0(0%)
meropenem	2(100%)	0(0%)	0(0%)
fosomycin	1(50%)	0(0%)	1(50%)
kanamycin	2(100%)	0(0%)	0(0%)
ciprofloxacin	2(100%)	0(0%)	0(0%)
Ticarcillin-clavulanate	2(100%)	0(0%)	0(0%)

3.4 Detection of tetracycline genes among *Enterobacter cloacae and Citrobacter spp.* isolates

The PCR results showed the percentage of tetrA gene was 8/25(32%) among isolates of E. cloacae, while it was 1/2(50%) among *Citrobacter spp.*isolates (Figure 1). At the same time, PCR results showed a high frequency of tetrB gene, reaching 23/25(92%) and 2/2(100%) among E. cloacae isolates and Citrobacter spp. isolates Respectively (Figure 2). However tetraC gene was detected in this work at rate 6/25(24%) among E. cloacae isolates and it recorded 1/2(50%) among Citrobacter spp. Isolates (Figure 3). At same repects,

tetraD and tetrE genes were not detected among E. cloacae and Citrobacter spp. isolates in present work.

The amino acid sequence analysis of tetA and tetB genes showed some differences when compared with the NCBI site. The rate of similarity in the amino acid sequence was 83% and 95% respectively (Figure 4,5).

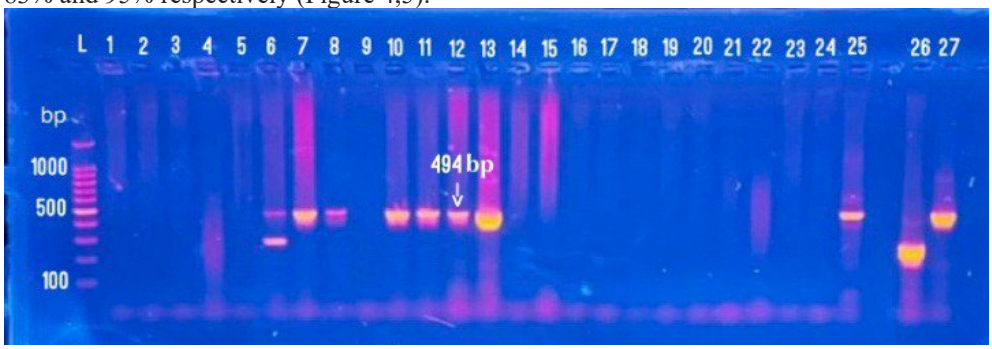


Fig. 1. Frequency of *tetA*, gene among 27 isolates (1 to 25 E.clocaea, 26 C.freundii, 27 C. farmer)

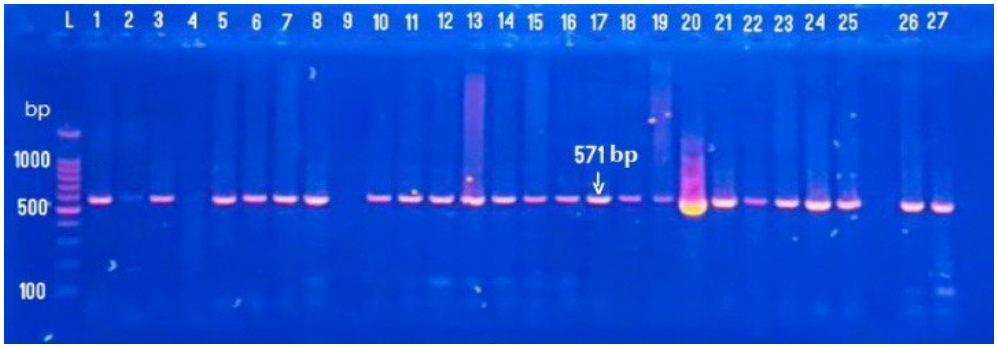


Fig. 2. Frequency of *tetB* gene among 27 isolates (1 to 25 E. clocaea, 26 C. freundii, 27 C. farmer)

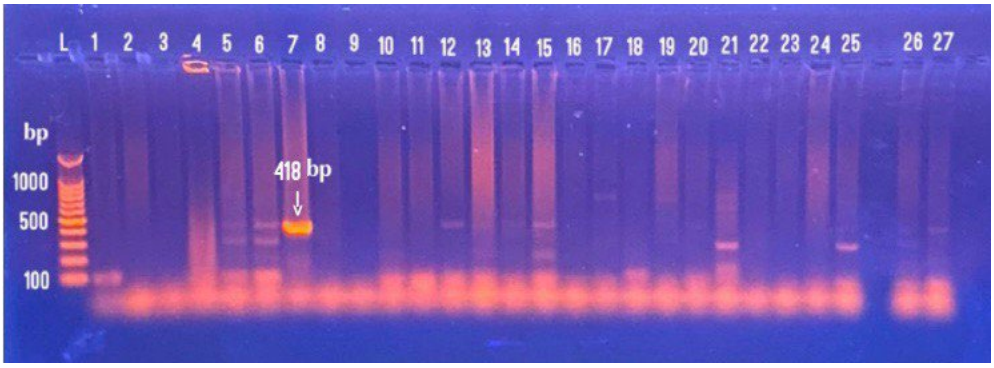
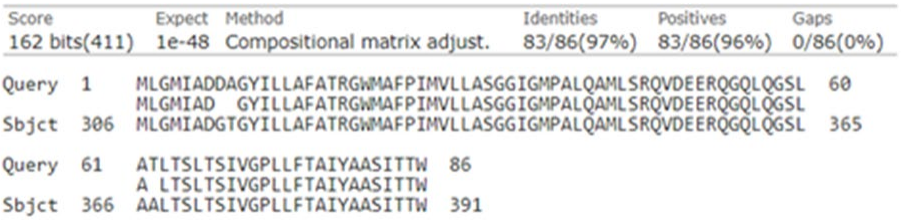


Fig 3. Frequency of *tetC* gene among 27isolates (1 to 25 E. clocaea, 26 C. freundii, 27 C. farmer)



Score	Expect	Method	Identities	Positives	Gaps
162 bits(411)	1e-48	Compositional matrix adjust.	83/86(97%)	83/86(96%)	0/86(0%)

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Query  1    MLGMIADDAGYILLAFATRGWMAFPIMVLLASGGIGMPALQAMLSRQVDEERQGQLQGSL  60
            MLGMIAD  GYILLAFATRGWMAFPIMVLLASGGIGMPALQAMLSRQVDEERQGQLQGSL
Sbjct  306  MLGMIADGTGYILLAFATRGWMAFPIMVLLASGGIGMPALQAMLSRQVDEERQGQLQGSL  365

Query  61   ATLTSLTSIVGPLLFTAIYAASITTW  86
            A LTSLTSIVGPLLFTAIYAASITTW
Sbjct  366  AALTSLTSIVGPLLFTAIYAASITTW  391
```

Fig. 4. Alignment aminoacid sequence of tetraA gene of local local *E. cloacae* isolate no.8 with E. cloacae strain WVX99925.1

Score	Expect	Method	Identities	Positives	Gaps
322 bits(824)	5e-108	Compositional matrix adjust.	162/171(95%)	164/171(95%)	0/171(0%)

```
Query  5    FVSGLWMLYLVRLLSGLSGANAAVAASVIADTTSASQRVKWFGWLGASFGLGLIAGPIIG  64
            F S LWMLYL RLLSG++GA  AVAASVIADTTSASQRVKWFGWLGASFGLGLIAGPIIG
Sbjct  14   FSSALWMLYLGRLLSGITGATGAVAASVIADTTSASQRVKWFGWLGASFGLGLIAGPIIG  73

Query  65   GFAGEISPHSPFFIAALQNIVTFLVVMFWFRETKNTRDNADTEVGVETQSNSVYITLFKT  124
            GFAGEISPHSPFFIAAL NIVTFLVVMFWFRETKNTRDNADTEVGVETQSNSVYITLFKT
Sbjct  74   GFAGEISPHSPFFIAALLNIVTFLVVMFWFRETKNTRDNADTEVGVETQSNSVYITLFKT  133

Query  125  MPILLIIYFSAQLIGQIPATVWVLFTENRFGWNSMMVGFSLAGLGLLHSDF  175
            MPILLIIYFSAQLIGQIPATVWVLFTENRFGWNSMMVGFSLAGLGLLHS F
Sbjct  134  MPILLIIYFSAQLIGQIPATVWVLFTENRFGWNSMMVGFSLAGLGLLHSVF  184
```

Fig. 5. Alignment amino acid sequence of tetraB gene of local *Citrobacter farmeri* isolate no.27 with Enterobacter cloacae strain

5 Discussion

There were many a previous works achievement that indicated ability of Enterobacter spp. and Citrobacter spp. to cause disease to human, however, In a prior local study conducted by Farhani [17] in Al-Najaf City, the researchers found that out of 778 individuals suffering from various infections, 21 (7.3%) of the isolates were identified as belonging to the genus Enterobacter spp. additionally 376 (48.3%) had positive bacterial growth, 89 (23.7%) returned to gram-positive, and 287 (76.3%) returned to gram-negative. In contrast, 402 (51.7%) of the patients did not have any growth. Generally, in a prior local study conducted by Hayder and Aljanaby [18] the researchers reported that 461 urine samples were obtained from patients infected with urinary tract infections. These patients ranged in age from 18 to 60 years old, and there were 30 isolates (6.5%) that were identified as *Citrobacter freundii*. A research performed in Thi-Qar city by Jawad et al., [19] mention that out of 60 non-duplicate urine samples from patients with urinary tract infections, 35n (58.33%) specimen swere positive for the growth of Gram-negative bacteria. The frequency of E.clocae isolates were 5 (14.28%) while isolates of *Citrobacter* spp. were 2 (5.71%) generally, gram negative bacteria showed 13.37% resistance to ciprofloxacin. A previous global work done by Lee et al., [20] during period 2007 to 2017, they observed the percentages of *Citrobacter* species susceptible to ampicillin, amikacin, aztreonam, cefazolin, cefoxitin, cefotaxime, cefepime, piperacillin-tazobactam, ciprofloxacin, and imipenem were 9.5%, 97.6%, 73.8%, 9.5%, 14.3%, 71.4%, 92.9%, 83.3%, 83.3% and 100%, respectively.

6 Conclusion

The drugs of β-lactams constitute 65% of the total number of prescriptions for injectable antibiotics in the United States. Cephalosporins account for about 50% of the prescribed β-lactams. The β-lactams have good tolerability, effectiveness, and are often given [21, 22]. Shallin and tuwaij [23] mention the results of PCR amplification showed high spreading of *Tet- A* and *Tet-B* among *Burkholderia cepacian* isolates which reached 24/31(77.41%) respectively, while *tet-D* and *tet-E* were absent among *B. cepecia* isolates in this study. In a 2016 worldwide research done by in Iran Noorbakhsh *et al*., [24] 120 isolates of Enterobacteriaceae revealed that tetA and tetB gene were 25 and 36 isolates have these genes respectively, whereas the other isolates did not.

A recent research done in Iran by Ahmadi *et al*., [25] found that 100 isolates of Enterobacteriaceae revealed the presence of the tetA, tetB and tetC genes were 31, 8 and 21isolates that have this genes resectively, whereas the other isolates did not. A worldwide investigation was carried out in Iran by Banihashem et al. [26] to examine 50 samples of enterobacter spp. observed the presence of the Tetb gene was identified. Out of the total of 25 bacterial isolates, 22 possess the tetb gene whereas the remaining 3 do not have it. it while tetC gene was existed only in 11 isolates, whereas the other isolates did not exhibit it.

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