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Tetracycline Resistance Associated with *tet* Genes or Integrons among *Enterobacter cloacae* Strains Isolated from Patients with Urinary Tract Infections

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ABSTRACT

Background: This study aimed to evaluate the prevalence of *tet* genes and Class I and 2 integrons in *Enterobacter cloacae* strains isolated from patients with urinary tract infections (UTIs).

Materials & Method: A total of 50 *E. cloacae* isolates were collected. Antimicrobial susceptibility pattern and tetracycline MIC were determined. The presence of *tet* genes (tetA, tetB, tetC, tetD) and Class 1 and 2 integrons and the content of Class 1 integron were determined.

Findings: Tetracycline MIC pattern classified 36 % of the *E. cloacae* isolates as resistant. The most common *tet* gene was *tet*C (22%), followed by *tet*D, *tet*A, and *tet*B. Class 1 integron was detected in 64% of the isolates. Class 1 integron content analysis showed two variable gene cassettes (*aadA*1 and *aadA*5/*dfrA*17 genes). The frequency of *aadA*5/*dfrA*17 was 18.75%, which was more common than aadA1 gene (6.25%).

Conclusion: The most important genetic markers for tetracycline resistance in *E. cloacae* isolates were *tet*C and Class 1 integron. Harboring Class 1 integron and resistance to streptomycin and ciprofloxacin were significantly correlated.

Keywords: Integron, *Enterobacter cloacae*, Tetracycline resistance genes, Urine, Integron content, Class 1 and 2 integrons

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Introduction

Enterobacter is a genus of Gram-negative, facultative, anaerobic bacilli belonging to family *Enterobacteriaceae* which are widely found in nature [1]. The identification of this genus is routinely carried out using phenotypic and genotypic methods mainly based on the conventional biochemical tests and molecular based methods, respectively. These organisms are capable of causing opportunistic infections in hospitalized or debilitated patients [2-3]. Indiscriminate use of antibiotics in medical health and veterinary industries has led to an upward trend in antimicrobial resistance and the emergence of multidrug-resistant (MDR) strains among a massive variety of microorganisms [4-5]. Before the extensive use of antibiotics, *Enterobacter* species were rarely considered as pathogens. But nowadays, there are many reports about the role of these organisms in causing major clinical diseases including bacteremia and urinary tract infections [6]. The increase in antimicrobial resistance among the clinical isolates is considered as a principle threat to the global public health, which could lead to enhanced clinical failures in the treatment of infectious diseases [7]. Different kinds of resistance have been reported in *E. cloacae* strains, including resistance to extended-spectrum cephalosporins, tetracyclines, extended-spectrum β-lactamas (ESBLs) and integron-associated class 1 [8-10]. Tetracycline is a broad spectrum bacteriostatic antibiotic which is widely used for the treatment and also as a promoter in animal husbandry industry [11]. The emergence of tetracycline resistant strains is a consequence of widespread usage of tetracycline both in medical and food industries. Resistance to tetracycline is usually due to the acquisition of *tet* resistance genes. Approximately 38 different *tet* genes have been recognized among the resistant strains, applying different resistance mechanisms. The mechanisms of these genes include: encoding efflux pump proteins, inactivating enzymes, and ribosomal protection proteins [12-13]. Based on the *tet* genes characteristics and mechanisms, they have been classified in different groups named with alphabetic letters. The efflux pump-mediating *tet* genes, including *tet*A, B, C, D, and E, have been reported as the most frequent genes among *Enterobacteriaceae* [14-15]. Overabundant use of antibiotics in the treatment of bacterial infections has led to the selection of resistant strains and increased risk of vertical and horizontal transfer of resistance genes among bacterial isolates. Until recently, it was thought that antimicrobial resistance genes transfer in bacteria is mainly occurred through the conjugation and transduction by plasmids, phages, and transposons carrying resistance genes, but later on, another mechanism was identified for antibiotic resistance genes transfer, which is done by elements called "integrons". Integrons are genetic components which are able to integrate into the alien DNA and transmit animated genetic elements called gene cassettes. As the integrons have a promoter for the genes in their contents, they could express the gene packages which were captured. Therefore, integrons not only act as a vector of gene expression but also as a natural cloning system. Integrons possess two essential genes located in conserved segment (CS), named *int* genes which are able to transmit and insert gene cassettes. Based on the differences in *int* genes, integrons have been classified into 4 different classes. Class 1 integron is the most common integron in enterobacterial isolates. Class 1, 2, and 3 integrons in bacterial isolates have been determined to be associated with antimicrobial resistance in a variety of bacterial species [16-17].

Objectives: This study aimed to evaluate the antibiotic resistance pattern, the prevalence

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of tetracycline resistance genes, and the distribution and content of Class I and 2 integrons in the *E. cloacae* strains isolated from patients with urinary tract infections (UTIs).

Materials and Methods

Ethics approval and consent to participate: The study began after ethical approval by Medical Ethics Committee of Tarbiat Modares University (Code: IR.MODARES.REC). The participants provided their written informed consent in order to participate in this study.

Bacterial strains and samples: A total number of 21600 urine specimens were collected from patients with UTIs from three large academic hospitals in Tehran, Iran, during 2016-2018. Patients with symptomatic UTIs were characterized with bacteriuria (direct observation of bacteria in urine specimens) and pyuria (urinary leukocyte excretion rates of ⩾400,000 leukocytes/h, which correlates with a hemocytomer count of ⩾10 leukocytes/ mm^3) [18]. Among 7200 culture-positive urine samples, a total of 50 *E. cloacae* strains were isolated. Urine samples were cultured on BHI agar medium and incubated at 37 °C for 18-24 hrs, discrete colonies were subjected to Gram staining. The Gram negative bacilli were subjected to biochemical tests for the presence of *E. cloacae* strains using API 20E identification kit in accordance with the manufacturer's manual (BioMérieux SA, Lyon, France). The standard strain of *E. cloacae* ATCC 13048 was used as positive control.

Resistance pattern of *E. cloacae* isolates: Disc diffusion and minimum inhibitory concentration (MIC) [2] methods were used to determine resistance to different antimicrobial agents. Results were evaluated and interpreted according to CLSI (Clinical and Laboratory Standards Institute) guidelines (2017). Antibiotic discs including tetracycline (T, 30µg), minocycline (MIN, 30µg), docetaxel (TS, 25µg), streptomycin (ST, 10µg), gentamicin (GM, 10µg), ampicillin (AP, 10µg), and ciprofloxacin (CIP, 5µg) were placed on cultured media. After incubation at 37°C for 18-24 hrs, inhibition zones diameter was accurately measured using a millimeter ruler. According to CLSI guidelines (2018), the antibiogram test results for each antibiotic were reported as susceptible, resistant, or intermediate.

Determining the tetracycline MIC by agar dilution method: The 0.5 McFarland concentration of the isolates was prepared and cultured on Muller Hinton agar containing different concentrations of tetracycline, ranging from 0.5 to 256 $\mu g.ml^{-1}$, and incubated at 37 °C for 18 hrs. The minimum concentration of each antibiotic inhibiting bacterial growth was considered as MIC of that antibiotic [19].

Prevalence of tetracycline resistance genes and Class I and 2 integrons: The genomic DNA of all the isolates was extracted using boiling method. The specific primers and PCR programs used in this study are shown in Table 1. The presence of four different genes including *tet*A, *tet*B, *tet*C, and *tet*D, as tetracycline resistance genes, was evaluated among the isolates. The *int*1 and *in* primers were used for determining Class 1 integron and its genetic content, respectively. The presence of intergron Class 2 was determined by *hep* primer. The PCRs were performed in a 25µL mixture consisting of 12.5 µL Amplicon master mix (superTaq DNA polymerase, dNTPs and Taq-buffer), 10 pmol from each primer, 5 µL DNA template, and sterile distilled water up to 25 µL as a final volume of PCR mixture. The PCR products were electrophoresis on 1% agarose gel and visualized using Gel Documentation system. PCR program was set as follows: an initial denaturation step

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Table 1) Primers sequences used in this study

Gene	Primer Sequence 5'.....3'	Amplification Condition	References
tet A	GTAATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	927 bp	(20)
tet B	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	659 bp	(21)
tet C	CTTGAGAGCCTTCAACCCAG ATGGTCGTCATCTACCTGCC	418 bp	(21)
tet D	AAACCATTACGGCATTCTGC GACCGGATACACCATCCATC	717 bp	(21)
int 1	TGCGTGTAAATCATCGTCGT CAAGGTTCTGGACAGTTGC	900 bp	(22)
hep	CGGGATCCCGGACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	Variable	(23)

at 95°C for 5 min, 30 cycles of denaturation at 95°C for 1 min, annealing for 1 min (for *tet*A, *tet*D, and *int*1 at 53°C; for *tet*B and *tet*C at 55°C; for *in* and *hep* at 56°C), extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

Nucleotide sequence accession numbers: One representative of each of the two gene cassettes contained in Class 1 integron was deposited in the GenBank database under Accession Numbers MN052647 and MN052648.

Statistical analysis: The sample size of this study was calculated using the sample size formula based on the prevalence of *E. cloacae* strains in urine samples collected from patients with UTIs.

Findings

A total of 2173 out of 7243 urine samples were positive for bacterial infection, of which 50 (2.3%) cases were associated with *E. cloacae* as the main pathogen according to the biochemical tests results. The resistance, intermediate, and susceptibility rates of *E. cloaceae* strains to different antibiotics used in this study are shown in Table 2. The highest resistance was observed to ampicillin (92%) (46 out of 50 isolate), and the lowest resistance was observed to streptomycin and minocycline (12%). The tetracycline MIC was also evaluated by agar dilution method to validate the results of disc diffusion method. According to the MIC results, 18 strains (36%) were resistant to tetracycline, and only 1 strain showed susceptibility (2%); however, the tetracycline resistance rate was only 6% based on the disc diffusion method results, which was significantly different from the MIC results (P value < .05). Eleven different resistance patterns were detected among the *E. cloacae* isolates, and the most common one (56%) (28 isolates out of 50) belonged to the resistance to minocycline, tetracycline, and ampicillin (Table 3). Most of the isolates showing this resistance pattern also harbored Class 1 integron (53%); however, only 1 isolate harbored *tet*A gene, and 8 isolates harbored *tet*C gene, other *tet* genes were not detected in this resistance pattern.

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Table 2) Antibiogram results of *E. cloacae* isolates using the disc diffusion method

Antibiotic Agents	ST (10mg)* N(%)	TS (25mg) N(%)	CIP (5mg) N(%)	AP (10mg) N(%)	MIN (30mg) N(%)	GM (10mg) N(%)	T (30mg) N(%)
Resistance	6 (12%)	18 (36%)	9 (18%)	46 (92%)	6 (12%)	8 (16%)	3 (6%)
Intermediate	12 (28%)	0	2 (4%)	3 (6%)	46 (92%)	0	0
Susceptible	30 (60%)	32 (64%)	39 (78%)	1 (2%)	0	42 (84%)	47 (94%)

*ST: streptomycin; TS: co-trimoxazol; CIP: ciprofloxacin; AP: ampicillin; MIN: minocycline; GM: gentamicin; T: Tetracyclin

Table 3) Resistance pattern among the *E. cloaceae* isolates

Antibiotics	No. of Strains (n=50)	No. of Strains Harboring Integron Class 1 (n=32)
AP, T	1	1
AP, MIN	1	1
AP, MIN, T	28	15
ST, AP, MIN, T	6	4
CIP, MIN, T	1	0
ST, AP, MIN, GM, T	1	0
ST, CIP, AP, MIN, T	2	1
ST, TS, AP, MIN, GM,T	1	1
ST, TS, CIP, AP, MIN,T	3	3
ST, CIP, AP, MIN,GM,T	3	3

Prevalence of tetracycline resistance determinants and integrons among the *E. cloaceae* isolates: In the present study, four different classes of *tet* genes, including *tet*A, B, C, and D, were investigated among the isolates using specific primers. The PCR products with the band sizes of 927, 659, 418, and 787bp belonged to *tet*A, *tet*B, *tet*C, and *tet*D, respectively. The highest prevalence rate was associated with *tet*C gene (22%) (11 out of 49), followed by *tet*D (6%), *tet*A, and *tet*B (2%). Harboring *int*I and *hep* gene is the representative of Class 1 and 2 integrons. Although Class 2 integron was not found, Class 1 integron was common among the isolates (64%) (Table 4). Class 1 integron content analysis showed two variable gene cassettes at the sizes of 1000 and 1500 bp, belonging to *aadA1* and *aadA5/dfrA17* genes, respectively (Figure 1). The frequency of *aadA5/dfrA17* was 18.75%, which was more common than *aadA1* gene (6.25%).

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Table 4) Tetracycline resistance genes and Class 1 and 2 integrons in the isolates

Gene	Strains No. (%)
*tet*A	1 (2%)
tet B	1 (2%)
tet C	11 (22%)
tet D	3 (6%)
hep	0
Int 1	32 (64%)

Discussion

Increasing frequency of antimicrobial resistance among clinically isolated bacteria is considered as a global health problem. The same trend has been reported for *E. cloacae* strains [24]. Various factors could influence the resistance pattern of *E. cloacae* strains, including antibiotic regimen, geographic conditions, and the year of sampling [25]. Many studies have demonstrated the upward trend of antibiotic resistance among *E. cloacae* strains. In a study conducted by Mokracka and colleagues (2011), the tetracycline resistance was reported to be over 60% in Poland, while in the present study, the tetracycline resistance was lower [22]. As mentioned, one of the reasons for differences in antibiotic resistant patterns among *E. cloacae* strains is antibiotic regimen, which could vary in different geographical regions. For instance, the highest resistance rate in Iran has been reported against β-lactams, which is in agreement with the present study results [1], whereas another study by Bayani and colleagues (2013) demonstrated the highest resistance of *E. cloacae* isolates against cephalosporins; in their study, no MDR strain was detected [23]. In the present study, 5 strains were classified as MDR strains, underlining the treatment of MDR *E. cloacae* strains. In other studies, resistance to other antibiotic families, including tetracyclines and quinolones, was also reported with high resistance rate [28-29]. In the present study, the most prevalent *tet* gene among the *E. cloacae* isolates was *tet*C,

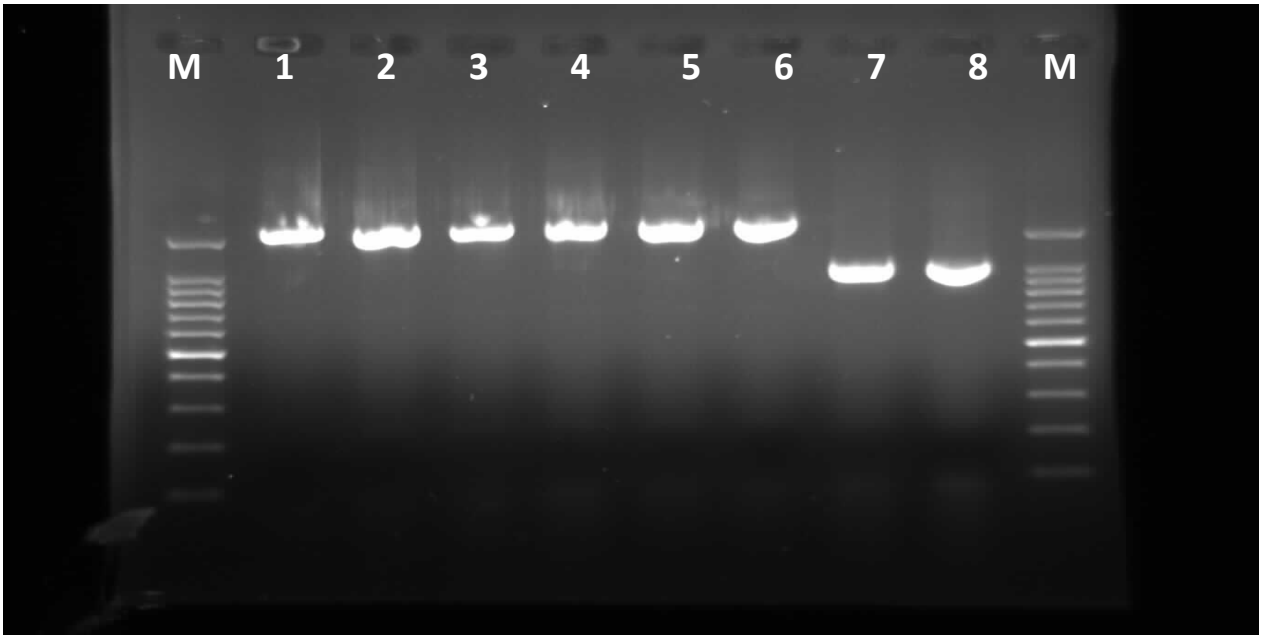

Figure 1) Class 1 integron content among integron positive strains. M:1kb DNA size marker; lane 1-6: clinical *E. cloacae* strains harboring *aadA5/dfrA17* gene cassette with 1500 bp size; lane 7 and 8: clinical *E. cloacae* strains harboring *aadA1* gene cassette with 1000 bp size.
*ST(10mg): streptomycin; TS(25mg): co-trimoxazol; CIP(5mg): ciprofloxacin; AP(10mg): ampicillin; MIN(30mg): minocycline; GM(10mg): gentamicin; T(30mg): Tetracyclin

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and all the isolates harbored only one type of *tet* genes; no evidence of heterogeneity was observed in the *tet* genes. It is noteworthy that all the isolates harboring *tet*B and *tet*D showed the same resistance pattern against tetracycline and minocycline; thus, the probable relationship between *tet* genes and resistance to minocycline could be hypothesized. The isolates with *tet*D gene had the highest tetracycline MIC value (≥128 $\mu g.mL^{-1}$), which proved that *tet*D might have a role in intensifying the tetracycline resistance. Low prevalence of tetracycline resistance genes in phenotypically resistant isolates could be due to the presence of other resistance mechanisms, including integrons or genes which has not yet been detected in *E. cloacae* strains. The prevalence of MDR *E. cloacae* strains demonstrated an upward trend in the emergence of MDR strains in the clinical samples in Iran in such a way that the rate has increased from zero in 2012 to 75% in 2017 according to the published data [27, 30-31]. Integrons are mostly responsible for the transmission of resistance determinants within species or among different bacterial species or genera. The frequency of Class 1 integron was 64% in this s1tudy, which is supported by the results of other studies conducted in Iran, while Class 2 integron was not detected. There is no evidence about the presence of Class 2 integron among *E. cloacae* isolates in published studies [1, 30]. According to the statistical analysis, the correlation between harboring Class 1 integron and resistance to streptomycin and ciprofloxacin was significant (p value< .05), while resistance to other antibiotics was not correlated with harboring Class 1 integron, and other resistance mechanisms should be responsible for resistance.

Conclusion

There is a growing trend in the diagnosis of *E. cloacae* in clinical samples. The increase in resistance to antibiotics is due to the proliferation of integrons and their resistance gene cassettes. Forasmuch as MDR *E. cloacae* strains causing nosocomial infection have increased, and the mechanisms of resistance are complicated, the prevalence, resistance profile, and genotype of *E. cloacae* strains should be routinely investigated in health care units.

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