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Emergence of *bla*$_{CTX-M-15}$ Gene and Its Transferability in *Enterobacter* spp. Isolated From the Hospitals of Tehran, Iran

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Background: *Enterobacter* spp. is increasingly recognized as an important nosocomial pathogen and implicated in many episodes of hospital acquired infections.
Objectives: The current study aimed to describe distribution and transferability of *bla*$_{CTX-M-15}$ gene, and the antibiotic susceptibility pattern in the clinical isolates of *Enterobacter* spp.
Materials and Methods: A total of 110 *Enterobacter* isolates were collected from five hospitals in Tehran, Iran from May 2012 to April 2013. *Enterobacter* species were identified by API 20E system. Antibacterial susceptibility was determined by disk diffusion method, and extended spectrum beta lactamase (ESBL) production was confirmed by combined disk method. The *bla*$_{CTX-M-15}$ gene was identified by PCR with specific primers. The transferability of the *bla*$_{CTX-M-15}$was tested by conjugation with broth matting method.
Results: The prevalence of *Enterobacter* species was *E. cloacae* (78.2 %), *E. aerogenes* (6.13 %) and *E. sakazakii* (8.2%). They were from different clinical sources. Maximal resistance in *Enterobacter* isolates was noticed against Augmentin®, trimethoprim - sulfamethoxazole and cefoxitin 75.5%, 64.5%, and 59.1%, respectively. Fourteen isolates, showed ESBL phenotype. The *bla*$_{CTX-M-15}$ gene frequency in *Enterobacter* isolates was 11.8%. Three conjugative plasmids containing *bla*$_{CTX-M-15}$ were found in one *Enterobacter* isolate.
Conclusions: It was the first report on the *bla*$_{CTX-M-15}$ gene emergence in clinical *Enterobacter* spp. in Iran. The current study demonstrated the predominant presence of the gene encoding *CTX-M-15* among ESBL producing *Enterobacter* spp. commonly with a large plasmid, in the setting.

Keywords: Conjugation; Drug Resistance; ESBLs; *Enterobacter*

1. Background

The genus *Enterobacter* includes the facultative anaerobic gram-negative bacteria belonging to Enterobacteriaceae family and widely found in the environment. Recently, the *Enterobacter* spp. has taken on clinical significance and has emerged as nosocomial pathogens, especially, from intensive care units (1, 2). *Enterobacter* species are significant causes of nosocomial infections and are intrinsically resistant to aminopenicillins, cefazolin and cefoxitin due to the production of constitutive chromosomal AmpC β-lactamases (3).

ESBLs (Extended-Spectrum Beta-Lactamases) are typically inhibitor-susceptible β-lactamases that hydrolyze penicillins, cephalosporins and aztreonam and are mostly encoded by mobile genes. The most frequently encountered ESBLs belong to the *CTX-M*, SHV, and TEM families (4). In clinical strains, *CTX-M*-encoding genes are commonly located on plasmids which vary in size from 7 - 200 kb (5). Many of these plasmids are conjugative with transfer frequencies ranging from 10^{-2} - 10^{-7} (6). To date, more than 60 types *CTX-M* ESBLs belonging to five evolutionary groups are described. In most clinical isolates *CTX-M-15* is the most frequent *CTX-M* type, and is reported in Enterobacteriaceae isolates from many regions of the world (7-9). It is necessary to know the frequency of ESBL positive strains in hospitals to formulate a policy of empirical therapy in high risk units where infections due to resistant organisms are much higher (8-10).

2. Objectives

The current study aimed to describe the antibiotic susceptibility pattern of the clinical isolates and *bla*$_{CTX-M-15}$ gene distribution in clinical isolates of *Enterobacter* spp., and the transferability of the *bla*$_{CTX-M-15}$ by conjugation.

3. Materials and Methods

3.1. Bacterial Isolates and Identification

A total of 110 *Enterobacter* isolates were collected from hospitals in Tehran from May 2012 to April 2013. Isolates were identified by conventional methods or the API 20E system (bioMerieux, Inc., Hazelwood, MO).

3.2. Antibiotic Susceptibility Testing

The antibiotic susceptibility of integron positive isolates, was determined by disk diffusion method on Mueller-Hinton agar plates (Merck, Darmstadt, Germany) as recommended by the clinical laboratory standards Institute (CLSI) (11). The disks contained the following antibiotics (Mast, UK): Augmentin® (30 μg), imipenem (10 μg), co-trimoxazole (25 μg), tetracycline (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), aztreonam (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefoxitin (30 μg), tobramycin (10 μg), amikacin (30 μg), gentamicin (10 μg), and cefepime (50 μg). They were obtained from mast Pharmaceutical Inc. U.K. *E.coli* ATCC 25922 was used as control for antimicrobial susceptibility test.

3.3. ESBL Confirmation by Combination Disk Method

The isolates showing reduced susceptibility to ceftazidime or cefotaxime were tested for ESBLs production by the combination disk method according to CLSI guidelines (CLSI). Combination disk method was performed using four disks: cefotaxime (CTX) (30 μg), cefotaxime (30 μg) + clavulanic acid (10 μg), ceftazidime (CAZ) (30 μg), and ceftazidime (30 μg) + clavulanic acid (10 μg). A 5 mm increase in a zone diameter for the tested antimicrobial agent (CAZ or CTX) in combination with clavulanic acid versus its zone when tested alone was considered as ESBLs positive. Quality control for ESBL production was performed using *E. coli* ATCC 25922 as negative control. Minimum inhibitory concentrations (MICs) of ceftazidime and cefotaxime were determined for ESBLs isolates by the E-test (AB Biodisk, Solna, Sweden) according to CLSI guidelines.

3.4. Polymerase Chain Reaction Analysis

The DNA of ESBL-producing isolates were extracted by boiling method and used as template in PCR assay. Amplification reactions were performed in a total volume of 25 μL of reaction mixture containing 5 μL of 10 × PCR buffer, 2.5 mM $Mgcl_2$, 200 mM dNTP, and 1.25 units of Taq polymerase, 10 pmol of each primer and 1 μL of the sample DNA. The following specific primers with 850 bp length were designed for the PCR reactions (F): 5'-AGAATAAGGAATCCCATGGTT and (R): 5'-GCAAGACCTCAACCTTTTCC (12). Cycling conditions were as follows: Initial denaturation at 94°C for 5 minutes; 35 cycles of 94°C for 1minute, 55°C for 45 seconds, and 72°C for 1 minute followed by a final extension at 72°C for 7 minutes. *Klebsiella pneumoniae* TMU4 was used as positive control.

3.5. Conjugation Experiments

The isolates with $bla_{CTX\text{-}M\text{-}15}$ gene were used as donor strains in conjugation experiments. Conjugation transfer assay was performed in broth culture with *E. coli* 15ARr (cefotaxime sensitive and rifampicin resistant) as the recipient. Before conjugation transfer assay, donor strains were tested for sensitivity to rifampicin and resistance to cefotaxime on nutrient agar containing rifampicin (50 mg/mL) and cefotaxime (100 mg/mL). Donor and recipient cells were mixed at a ratio of 1:10. The trans-conjugants were selected on nutrient agar containing cefotaxime (100 mg/mL) supplemented with rifampicin (50 mg/mL) (13).

3.6. Conjugation Frequency

Conjugation frequency was also expressed as the percentage of transconjugants per added donor cell in 1 mL (13-15). Colony forming unit per mL (cfu/mL) was used instead of the number of cells. The CFU of donors and transconjugants from the dilution plates were measured with selective antibiotics (cefotaxime and rifampicin) (13-15).

Donor number was determined by plating 10^{-4}, 10^{-5} and 10^{-6} dilutions. For transconjugants all dilutions (from 1 - 10^{-6}) were plated.

3.7. PCR Analysis and Determination of MIC of Transconjugants

DNA of trans-conjugants were obtained by the plasmid extraction kit (BIONEER®) and screened for $bla_{CTX\text{-}M\text{-}15}$ gene. MICs of ceftazidime and cefotaxime were determined for trans-conjugants by the E-test (AB Biodisk, Solna, Sweden) according to CLSI guidelines (CLSI 2012).

4. Results

It was observed that 74.55% of the isolates were from the urine culture, 13.64% from the chip throat, 6.36% from wound, 3.64% from the blood culture and 0.91 % from the eye infection. *Enterobacter cloacae* (78.2%), *E. aerogenes* (6.13%) and *E. sakazakii* (8.2%) were determined. The frequency of clinical *Enterobacter* spp. isolates was higher in females (60%) than males (40%). Analysis of the antimicrobial susceptibility profile of the isolates showed that they were all susceptible to imipenem. Of the 110 isolates, 95.5% were resistant to Augmentin®, 64.5% resistant to trimethoprim-sulfamethoxazole, 22.7 % resistant to ceftazidime, and 23.6% resistant to cefotaxime (Table 1).

Of the 110 *Enterobacter* isolates, 1 (0.9%) was susceptible to all of the tested antimicrobials and 85 (77.3%) were multidrug-resistant and showed resistance to more than two antimicrobial families. Combined disc test was performed on 29 isolates. Fourteen *Enterobacter* isolates showed ESBL phenotype. All of the fourteen *Enterobacter* isolates had the $bla_{CTX\text{-}M\text{-}15}$ gene (Figure 1).

The transferability of the $bla_{CTX\text{-}M\text{-}15}$ was tested by conjugation. A conjugative plasmid containing $bla_{CTX\text{-}M\text{-}15}$ was found in three *Enterobacter* isolates. Antibiotic susceptibility profile of transconjugant strains and donor strains are showed in Table 2. Conjugation frequency was calculated by the number of transconjugants in 1 mL per the number of donor cells in 1 mL which was 0.9×10^{-5}.

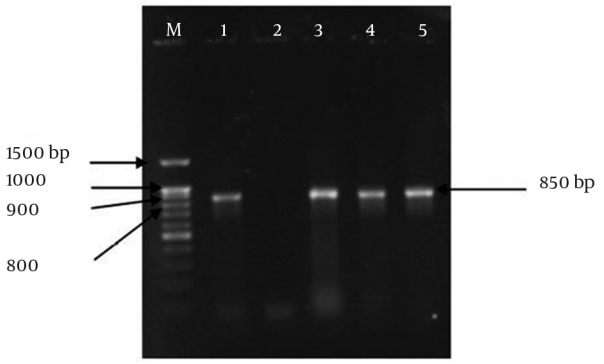
These results were confirmed by PCR. The MIC of parental isolates and transconjugants were similar and included cefotaxime 256 and for ceftazidime.

Table 1. Percentage of Isolates Susceptible, Moderately Susceptible, or Resistance to Each Antibiotic [a,b]

Antibiotic	Susceptible	Intermediate	Resistant
AUG	2 (1.8)	(2.7) 3	(95.5) 105
SXT	36 (32.7)	(2.7) 3	71 (64.5)
CHL	89 (81)	7 (6.4)	14 (12.8)
CIP	97 (88.2)	6 (5.5)	7 (6.4)
IPM	110 (100)	0 (0)	0 (0)
T	52 (47.3)	9 (8.2)	49 (44.5)
AN	(91) 100	0 (0)	10 (9.1)
TOB	89 (81)	1 (0.9)	20 (18.2)
GM	91 (77.8)	3 (2.7)	16 (14.5)
CTX	81 (73.6)	3 (2.7)	(23.6) 26
CAZ	83 (75.5)	2 (1.8)	25 (22.7)
ATM	87 (79.1)	1 (0.9)	22 (20)
CPM	93 (84.5)	5 (4.5)	12 (11)
FOX	50 (45.5)	0 (0)	60 (54.5)

[a] The valves are present as No. (%).
[b] Abbreviations: AN, amikacin; ATM, aztreonam; AUG, Augmentin®; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; CPM, cefepime; FOX, cefoxitin; GM, gentamicin; T, tetracycline; TOB; tobramycin; and SXT, trimethoprim-sulfamethoxazole.

Figure 1. Detection of CTX-M-15 Enzyme by Amplification of *bla*$_{CTX-M}$ Gene



Lane M, 100 bp plus blue DNA ladder (Gene ON); lane 1: control. positive; lane 2: control negative; lanes 3 - 5: clinical isolates.

Table 2. Antibiotic Susceptibility Profile of the Donor and Transconjugant Strains [a,b]

Code	Antibiotic Susceptibility Profile (Donor Strains)	Antibiotic Susceptibility Profile (Transconjugant Strains)
1	SXT^R, AUG^R, CHL^R, CIP^R, IPM^S, T^R, AN^S, TN^R, GM^R, CTX^R, CAZ^R, ATM^R, CPM^R, FOX^R	SXT^R, AUG^R, CHL^R, CIP^I, IPM^S, T^R, AN^S, TN^R, GM^R, CTX^R, CAZ^R, ATM^R, CPM^R, FOX^R
2	SXT^R, AUG^R, CHL^S, CIP^S, IPM^S, T^R, AN^R, TN^R, GM^R, CTX^R, CAZ^R, ATM^R, CPM^R, FOX^R	SXT^R, AUG^R, CHL^R, CIP^R, IPM^S, T^R, AN^S, TN^R, GM^R, CTX^R, CAZ^R, ATM^R, CPM^R, FOX^R
3	SXT^R, AUG^I, CHL^S, CIP^S, IPM^S, T^S, AN^S, TN^R, GM^R, CTX^R, CAZ^R, ATM^R, CPM^R, FOX^R	SXT^R, AUG^I, CHL^S, CIP^S, IPM^S, T^S, AN^S, TN^R, GM^R, CTX^R, CAZ^R, ATM^R, CPM^S, FOX^R

[a] Abbreviations: AN; amikacin; ATM, aztreonam; AUG, Augmentin®; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CPM, cefepime; CTX cefotaxime; FOX, cefoxitin; GM, gentamicin; IMP; imepenem; SXT, trimethoprim-sulfamethoxazole; T, tetracycline; TN, tobramycin;.
[b] The letters I, R, and S refer to intermediate, resistant and susceptible strains, respectively.

5. Discussion

Enterobacter spp. is increasingly identified as a cause of serious nosocomial infections. In recent years, ESBL production among these isolates is becoming a major clinical concern because of its ability to develop resistance to several classes of antimicrobial agents and has high potential for transmission of resistance to other bacterial species (16). In the current study, the most prevalent clinical *Enterobacter* species was *E. cloacae* (78.2 %) that showed the important role of this species in human infections, which was in agreement with other reports (17-20).

In the current study, the frequency of ESBL-producing clinical *Enterobacter* spp. was 12.73% which was different from the other results in Pakistan (50%, 14.93%) (21, 22), Nigeria (37.5%) (10) and Pennsylvania, the USA (33.33%) (9).

Three of the fourteen isolates with *bla*$_{CTX-M-15}$ gene (21.4%), had a conjugative large plasmid. The low frequency is perhaps due to the type of conjugation method employed in in the current study.

Based on these findings, larger multi-center studies are needed to determine the molecular epidemiology of *Enterobacter* isolates, the distribution of CTX-M ESBL as well as the presence of conjugative plasmids among Enterobacteriaceae in the hospital populations. The current study results showed that the MIC of the transconjugants and parental strains to CAZ and CTX were similar and the resistance determinants to CAZ and CTX were transferred on a conjugative large plasmid. As a result, third-generation of cephalosporin, fluoroquinolones, and imipenem are suggested to be used as frontline remedial antibiotics to treat *Enterobacter* infections. Careful monitoring and employing appropriate infection control policy are necessary to prevent further emergence and spread of resistant organisms in the hospitals.

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Authors' Contributions

Kobra Salimian Rizi performed the laboratory work. Shahin Najar Peerayeh designed the work study, and Bita Bakhshi advised the work study.

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