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# Prevalence of integrons and Antimicrobial Resistance Genes Among Clinical Isolates of *Enterobacter* spp. From Hospitals of Tehran

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**Background:** *Enterobacter* infections are increasingly recognized as an important nosocomial infection. Here we describe the prevalence of three classes of integrons in clinical isolates of *Enterobacter* spp. and the prevalence of antibiotic resistance genes among isolates with integron.

**Objectives:** Here we describe the prevalence of integrons genes among clinical isolates of *Enterobacter* spp. and antibiotic susceptibility pattern, ESBL production and the prevalence of resistance genes among clinical isolates of *Enterobacter* spp.

**Materials and Methods:** A total of 110 *Enterobacter* isolates collected from four hospitals in Tehran during 2012-2013. *Enterobacter* species were identified by using API 20E system. The existence of integron classes was investigated by PCR assay through the amplification of integrase genes. Then, antibacterial susceptibility and confirmation of ESBL phenotype was determined. Then, the bla groups, $bla_{TEM}$, $bla_{SHV}$, $bla_{CTX\text{-}M\text{-}1}$ and aminoglycoside modifying enzymes genes were identified by PCR with specific primers.

**Results:** The prevalence of *Enterobacter* species were *E. cloacae* (78.2 %), *E. aerogenes* (13.6 %) and *E. sakazakii* (8.2%). They were from different clinical sources. Forty five of *Enterobacter* isolates have integron but there was not detected class 3 of integrons. All isolates with integron were susceptible to imipenem. Ten isolates of *Enterobacter* with integron showed ESBL phenotype. The frequency of $bla_{TEM}$, $bla_{SHV}$ and $bla_{CTX\text{-}M\text{-}1}$ genes are 20%, 0% and 15.6%, respectively. The frequency of genes encoding ANT (2″)-Ia, APH (3´)-Ia, AAC (6´)-Ib and AAC (3)-IIa were 11.1%, 13.3%, 13.3 % and 20 %, respectively.

**Conclusions:** The high prevalence of integron-positive isolates in our MDR *Enterobacter* isolates indicates that these mobile genetic elements are common among different *Enterobacter* spp. and associate with reduced susceptibility to the first-line antimicrobial drugs. This so highlight the continued monitoring of drug resistance in clinical settings.

*Keywords:Enterobacter*; Integrons; Aminoglycosides

## 1. Background

Bacteria such as *Enterobacter* spp. exist in the human gut, as well as the environment. This bacterium represents an important class of opportunistic pathogenic microorganisms. Recently, the *Enterobacter* spp. have taken on clinical significance and have emerged as nosocomial pathogens, especially, from intensive care units (1-3). *Enterobacter* spp. is significant causes of nosocomial infections and is intrinsically resistant to aminopenicillins, cefazolin and cefoxitin due to the production of constitutive chromosomal AmpC β-lactamases (4). The production of β-lactamases is the most important mechanism responsible for β-lactam resistance in most of these species. Many other resistance determinants that are able to render ineffective almost all antibiotic families have been recently acquired. Mainly in this genus, Aminoglycoside resistance is due to the enzymatic modification (3). *Enterobacter* species have become increasingly resistant to antimicrobial agents, partly as a result of genes carried on integrons. Different integron classes have been described, and classes 1, 2, and 3 have been associated with antibiotic resistance (5, 6). The prevalence of multidrug resistance among isolates of the *Enterobacter* spp. in human infections is rising.

## 2. Objectives

The objectives of our study were to determine the prevalence of integrons genes, antibiotic susceptibility, confirm of ESBL production and the prevalence of resistance genes among integron-positive isolates.

## 3. Materials and Methods

### 3.1. Bacterial Isolates and Identification

A total of 110 *Enterobacter* isolates were isolated from five hospitals (Milad, Loghman, Ali-Asghar, Mofid and Imam Khomeini) in Tehran between May 2012 and April 2013. Isolates were identified by conventional methods and by the API 20E system (bioMerieux, Inc., Hazelwood, MO).

### 3.2. Detection of Integrons

Bacterial DNA were harvested by conventional boiling method (7). Two or three colonies of overnight culture of the bacteria on nutrient agar (Merck, Germany) were transferred into a 1.5 ml centrifuge tube with PBS and centrifuged at 12,000 rpm for 10 minutes. After removal of the supernatant, the sediment was suspended into 200 μL of distilled water. The tube was placed in a water bath at 95°C for 10 minutes and supernatant was used as template DNA. Determination of integron classes was performed by multiplex PCR using the primers described in Table 1. Polymerase chain reactions were performed in a 25 μL volume. Amplification reactions were performed in a total volume of 25 μL of reaction mixture containing 5 μL of 10 × PCR buffer, 2.5 mM MgCl2, 200 μM dNTPs, 1.25 units of Taq polymerase, 10 pmol of each primer and 1 μL of sample DNA. PCR was performed on a DNA Engine Dyad, Peltier Thermal Cycler (Bio-Rad, Hercules, CA). PCR condition was showed in Table 1. PCR products were electrophoresed in 1.5% agarose, stained by Gel Red dye.

### 3.3. 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) (8). The disks containing the following antibiotics were used (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), cefepime (50 μg) were obtained from mast Pharmaceutical Inc. U.K. *E. coli* ATCC 25922 was used as control for antimicrobial susceptibility test.

### 3.4. 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 antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was considered as a ESBLs positive. Quality control for the production of ESBL was performed using *E. coli* ATCC 25922 as negative control. Minimum inhibitory concentration (MIC) of ceftazidime and cefotaxime was determined for ESBLs isolates by the E-test (AB Biodisk, Solna, Sweden) according to the guidelines of CLSI.

### 3.5. PCR Analysis for Detection of Antimicrobial Resistance Genes

The DNA of ESBL-producing isolates or aminoglycoside-resistant isolates were extracted by boiling method. Amplification reactions were performed in a total volume of 25 μL of reaction mixture containing 5 μL of 10 × PCR buffer, 2.5 mM Mgcl2, 200 mM dNTP, and 1.25 units of Taq polymerase, 10 pmol of each primer and 1 μL of sample DNA. The specific primers for $bla_{TEM}$, $bla_{SHV}$, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}15}$ and aminoglycoside modifying enzyme genes were shown in Table 1. PCR was performed on a DNA Engine Dyad, Peltier Thermal Cycler (Bio-Rad, Hercules, CA) at the following setting: Initial denaturation at 94°C for 5 minutes; 35 cycles of 94°C for 1min, and annealing temperatures were show in Table 1 followed by a final extension at 72°C for 7 minutes. K.pneumoniae TMU1-3 for $bla_{TEM}$, $bla_{SHV}$, $bla_{CTX\text{-}M\text{-}1}$ and *P. aerogenes* TMU1-4 were used as positive control strains for other resistance genes. The negative control consists of all PCR reagents without DNA. Products were electrophoresed in 2% agarose, stained by Gel Red dye. Gels were viewed and photographed with Bio-Rad GelDoc XR (Bio-Rad, USA).

## 4. Results

### 4.1. Bacterial Isolates and Identification

A total of 110 Salmonella isolates were collected. It was observed that the prevalence of *Enterobacter* species were *E. cloacae* (78.2 %), *E. aerogenes* (6.13 %) and *E. sakazakii* (8.2%).

### 4.2. Detection of Integrons

Of 110 isolates, 45 (41 %) *Enterobacter* isolates exhibited either a class 1 integron, or class 1 and 2 integron (Figure 1). However integron class3 was not found. Among *Enterobacter* isolates, class 1 integrons were common (45 isolates), whereas class 2 integrons were found only in 9 isolates, and all of them also carried a class 1 integron. Primers *Int1*F and *Int1*R were used to amplify a 160 bp fragment of the intI1 gene for the class1 integrase, the primers *Int2*F and *Int2*R amplified a fragment of 288 bp, specific for the *intI2* gene and primers *Int3*F and *Int3*R were used to amplify a specific *intI3* gene. Distribution of *Enterobacter* species among integrons positive of *Enterobacter* isolates is given in Table 2. The prevalence of species among integron positive isolates consists of *E. cloacae* 80% (n = 36), *E. aerogenes* 15.6% (n = 7) and *E. sakazakii* 4.4% (n = 2).

### 4.3. Antibiotic Susceptibility Testing and ESBL Confirmation

Analysis of the antimicrobial susceptibility profile of the 45 integron positive isolates showed that all were susceptible to imipenem. Of 45 isolates, 97.8 % of the isolates were resistant to augmentin, 71.1% were resistant

to cefoxitin and 62.2 %, were resistant to trimethoprim-sulfamethoxazole. Rate of resistance to ceftazidime, cefotaxime, cefepime and ciprofloxacin were 33.3%, 28.9%, 15.6% and 13.3%, respectively. 22.2%, 13.3% and 22.2% were resistant to amikacin, tobramycin and gentamicin, respectively (Table 3). Data also indicated an apparent association between the resistance panels of antibiotics including augmentin, trimethoprim-sulfamethoxazole, cefoxitin and tetracycline and class 1 and 2 integrons (Table 4). Ten isolates of integron positive isolates showed ESBL phenotype. Three isolates exhibited class 1 and 2 integrons and seven isolates exhibited class 1 integron.

**Table 1.** Primers, PCR Conditions, and Respective References

| Primers | Nucleotide Sequence (5´to3´) | PCR Condition | | | | References |
|---|---|---|---|---|---|---|
| | | Amplicon, bp | Denaturing | Annealing | Extension | |
| ***Int1*** | | 160 | 94°C, 30 s | 55°C, 30 s | 72°C, 30 s | (9) |
| F | CAGTGGACATAAGCCTGTTC | | | | | |
| R | CCCGAGGCATAGACTGTA | | | | | |
| ***Int2*** | | 788 | 94°C, 30 s | 55°C, 30 s | 72°C, 30 s | (9) |
| F | CACGGATATGCGACAAAAAGGT | | | | | |
| R | GTAGCAAACGAGTGACGAAATG | | | | | |
| ***Int3*** | | 979 | 94°C, 30 s | 55°C, 30 s | 72°C, 30 s | (10) |
| F | GCCTCCGGCAGCGACTTTCAG | | | | | |
| R | ACGGATCTGCCAAACCTGAC | | | | | |
| ***ctx-m-1*** | | 850 | 94°C, 1 m | 55°C, 45 s | 72°C, 1 m | (11) |
| F | GGT TAA AAA ATC ACT GCG TC | | | | | |
| R | TTG GTG ACG ATT TTA GCC GC | | | | | |
| ***tem*** | | 850 | 94°C, 1 m | 55°C, 1 m | 72°C, 1 m | (11) |
| F | ATG AGT ATT CAA CAT TTC CG | | | | | |
| R | CCA ATG CTT AAT CAG TGA GG | | | | | |
| ***shv*** | | 231 | 94°C, 30 s | 55.5°C, 30 s | 72°C, 1 m | (12) |
| F | AAG ATC CAC TAT CGC CAG CAG | | | | | |
| R | ATT CAG TTC CGT TTC CCA GCG G | | | | | |
| ***aph(3´)-Ia*** | | 632 | 94°C, 1 m | 55.5°C, 1 m | 72°C, 1 m | (13) |
| F | ATG GGC TCG CGA TAA TGT C | | | | | |
| R | CTC ACC GAG GCA GTT CCA T | | | | | |
| ***ant (2´´)-Ia-*** | | 700 | 94°C, 1 m | 51.9°C, 1 m | 72°C, 1 m | (13) |
| F | TCC AGA ACC TTG ACC GAA C | | | | | |
| R | GCA AGA CCT CAA CCT TTT CC | | | | | |
| ***aac(3)-IIa*** | | 740 | 94°C, 1 m | 50.3°C, 1 m | 72°C, 1 m | (13) |
| F | CGG AAG GCA ATA ACG GAG | | | | | |
| R | TCG AAC AGG TAG CAC TGA G | | | | | |
| ***aac(6´)-Ib*** | | 482 | 94°C, 1 m | 59°C, 1 m | 72°C, 1 m | (13) |
| F | TTG CGA TGC TCT ATG AGT GG | | | | | |
| R | CTC GAA TGC CTG GCG TGT TT | | | | | |

**Table 2.** Distribution of *Enterobacter* Species Among Integrons Positive Isolates

| Integron | Spp. | | | |
|---|---|---|---|---|
| | *E. cloacae* (n = 36) | *E. sakazakii* (n = 2) | *E. aerogenes* (n = 7) | Total, No. (%) (n = 45) |
| **Class 1** | 30 | 1 | 6 | 37 (82.2) |
| **Class 2** | 0 | 0 | 0 | 0 (0) |
| **Class 1 and 2** | 6 | 1 | 1 | 8 (17.8) |
| **Class 3** | 0 | 0 | 0 | 0 (0) |

**Table 3.** Percentage of Isolates Susceptible, Moderately Susceptible or Resistance to Each Antibiotic [a,b]

| Antibiotic | Susceptible | Intermediate | Resistant |
|---|---|---|---|
| AUG | 0 (0) | 1 (2.2) | 44 (97.8) |
| SXT | 15 (33.3) | 2 (4.4) | 28 (62.2) |
| CHL | 35 (77.8) | 2 (4.4) | 8 (17.8) |
| CIP | 35 (77.8) | 4 (8.9) | 6 (13.3) |
| IPM | 45 (100) | 0 (0) | 0 (0) |
| T | 14 (31.1) | 5 (11.1) | 26 (57.8) |
| AN | 39 (86.7) | 0 (0) | 6 (13.3) |
| TOB | 35 (77.8) | 0 (0) | 10 (22.2) |
| GM | 35 (77.8) | 0 (0) | 10 (22.2) |
| CTX | 31 (68.9) | 1 (2.2) | 13 (28.9) |
| CAZ | 29 (64.4) | 1 (2.2) | 15 (33.3) |
| ATM | 30 (66.7) | 1 (2.2) | 14 (31.1) |
| CPM | 35 (77.8) | 3 (6.7) | 7 (15.6) |
| FOX | 13 (28.9) | 0 (0) | 32 (71.1) |

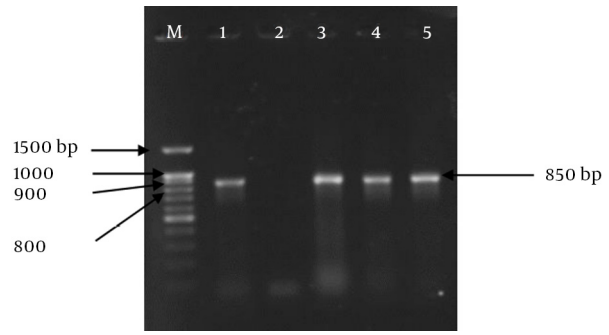
[a] Abbreviations: AUG, augmentin; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; CIP, ciprofloxacin; T, tetracycline; CPM, cefepime; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; FOX, cefoxitin; GM, gentamicin; AK, amikacin; TN, tobramycin.
[b] Data are presented as No. (%).

**Table 4.** Antibiotic Resistance Pattern Among Integron Positive Isolates [a]

| Antibiotic | Only Class 1 (n = 37) | Class 1 and 2 (n = 8) | Total (n = 45) |
|---|---|---|---|
| AUG | 1 | - | 1 |
| AUG, T | 1 | - | 1 |
| SXT, AUG | 4 | - | 4 |
| AUG, FOX | 5 | 2 | 7 |
| SXT, AUG, T | 1 | 1 | 2 |
| SXT, AUG, FOX | 1 | - | 1 |
| AUG, CHL, FOX | 2 | - | 2 |
| AUG, T, FOX | 4 | - | 4 |
| SXT, AUG, T, FOX | 4 | 1 | 5 |
| AUG, CIP, T, FOX | 1 | 1 | 2 |
| AUG, T, AN, GM | 1 | - | 1 |
| AUG, CTX, CAZ, FOX | 1 | - | 1 |
| SXT, AUG, CHL, T, FOX | 1 | - | 1 |
| SXT, AUG, TOB, CTX, ATM | 1 | 1 | 2 |
| SXT, AUG, CIP, T, GM | 1 | - | 1 |
| SXT, TOB, GM, CTX, CAZ | 1 | - | 1 |
| SXT, AUG, T, GM, FOX | 1 | - | 1 |
| AUG, CIP, CAZ, ATM, CPM | 1 | - | 1 |
| SXT, AUG, T, CTX, CAZ, ATM | 1 | - | 1 |
| SXT, AUG, T, CTX, CAZ, ATM, FOX | 2 | - | 2 |
| SXT, AUG, CHL, CIP, T, AN, TOB, CAZ, ATM, FOX | 2 | - | 2 |
| SXT, AUG, T, AN, TOB, GM, CTX, CAZ, ATM, FOX | 1 | - | 1 |
| SXT, AUG, CHL, T, TOB, GM, CTX, CAZ, ATM, CPM, FOX | 1 | - | 1 |
| SXT, AUG, CHL, T, TOB, GM, CTX, CAZ, ATM, CPM, FOX | 1 | - | 1 |
| Sensitive | - | - | - |

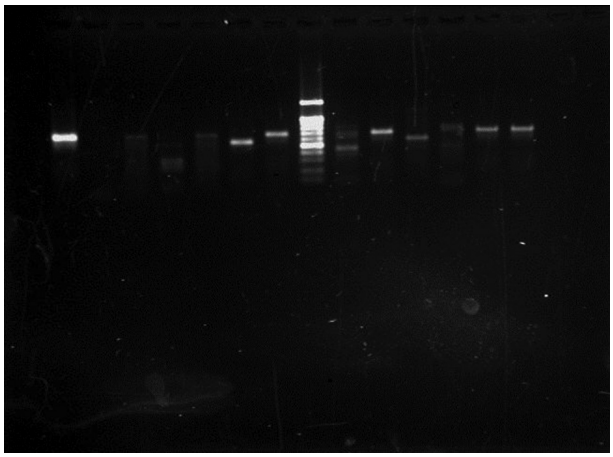
[a] Abbreviations: AUG, augmentin; T, tetracycline; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; FOX, cefoxitin; CIP, ciprofloxacin; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; GM, gentamicin; AK, amikacin; TN, tobramycin; CPM, cefepime.

**Figure 1.** Detection of CTX-M enzyme by amplification of blaCTX-M Gene




Lane M: 100 bp Plus Blue DNA ladder (GeneON); lane 1: control; Positive; lane 2: control negative; lanes 3-5: clinical isolates.

**Figure 2.** Detection of ANT (2″)-Ia, APH (3′)-Ia, AAC (3)-IIa and AAC (6′)-Ib Enzymes by amplification of aph (3′)-Ia, ant (2′′)-Ia, aac (6′)-Ib genes



Lane M: 100 bp Plus Blue DNA ladder (GeneON); lane 1, 5: aph (3′)-Ia; Positive (632 bp); lane 2, 4, 7, 8: ant (2′′)-Ia positive; Lane 3: aac (6′)-Ib Positive (482 bp).

### *4.4. PCR Analysis for Detection of Antimicrobial Resistance Genes*

The frequency of the genes encoding the enzymes TEM ،SHV، CTX-M, ANT (2″)-Ia, APH (3′)-Ia, AAC (3)-IIa and AAC (6′)-Ib in *Enterobacter* isolates with integron are 20%, 0% and 15.6 %, 11.1%, 13.3%, 13.3% and 20%, respectively. Figure 1 demonstrates the presence of the gene encoding the enzyme $_{\text{CTX-M-1}}$ and Figure 2 demonstrates the isolates of *Enterobacter* with genes encoding AMEs.

## 5. Discussion

Resistance to antimicrobial agents is often associated with the spread of transmissible plasmids and integrons which can be located on the chromosome or plasmids. The ability of integrons to integrate resistance gene cassettes makes them prime pools for the further dissemination of antibiotic resistance among clinical isolates of gram-negative bacteria, including *Enterobacter* isolates (13). Our findings indicated that the 41 % of isolated *Enterobacter* were integron positive, this rate of integron positive in our isolates are similar with published reports that *Enterobacter* harbors high prevalence of integron class 1, lower class 2 and no class 3 (14-16). Of course, in study in France a new class 3 integron, In3-5, detected in an *Enterobacter* cloacae isolate retrieved from a random French hospital effluent sample (17). Perhaps, the lack or poor role of integron class 3 may indicate its null role in antibiotic resistance. As mentioned above, the prevalence of class 1 integron, as compared to class 2 may imply that class 1 integron is more important in capturing resistant determinants. Among the published reports, the resistance of *Enterobacter* strains with integrons varies depending on geographical location and year of testing (18). We noted higher levels of resistance, described as a percentage of resistant strains, than those reported earlier (1, 17-21). Thirty-three of integron positive isolates (73.3%) were resistant to more than three unrelated antibiotics. The resistance for each antibiotic, except imipenem, was significantly associated with the presence of integrons. Class 1 integrons were detected in 41 % of 110 isolates. Due to the presence of class I of integrons in drug resistance to antibiotics such as beta-lactams, aminoglycosides and tetracycline, it is essential more attention to them. The variable presence of integrons among extended-spectrum beta-lactamase (ESBL)-producing *Enterobacter*iaceae species (0 to 66) is described (22). The frequency of ESBLs among integron positive isolates in this study was 22.2 % and the TEM-type beta-lactamases were more prevalent than two other types of ESBLs among integron positive isolates. As regards other types of ESBLs that were not detected among resistant isolates of this study, so the frequency of ESBLs among our integron positive isolates was different with other studies (17-19, 21, 22). Resistance to aminoglycosides among *Enterobacter* spp. has been associated with integrons (23). The gentamicin resistance among 15 resistant isolates that were tested for aac (3)-IIa gene was detected in 6 isolates. So, gentamicin resistance among other 9 isolates may be because of other gentamicin-modifying enzymes such as AAC (3)-I, AAC (3)-VI, AAC (2)-I, AAC (3)-IV, Ant (2′)-I or impermeability. The most prevalence of AMEs was tested in this study, belonged to AAC (6)-Ib enzyme that is similar with other studies (24, 25). This enzyme can modify amikacin, tobramycin, kanamycin, and netilmicin but not gentamicin. The high prevalence of integron-positive isolates in our MDR *Enterobacter* isolates indicates that integrons are common among different *Enterobacter* spp. and associate with reduced susceptibility to the first-line antimicrobial drugs. Nevertheless, to genetically confirm this association, sequencing and amplification of class 1 and 2 integrons cassette regions should to be performed. Moreover, integron-mediated resistance can be silenced in a bacterial population at no biological cost, which means that resistant bacteria persist even if the selective pressure imposed by antibiotic usage is reduced.

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