Genetic properties of $bla_{CTX-M}$ and $bla_{PER}$ $\beta$-lactamase genes in clinical isolates of Enterobacteriaceae by polymerase chain reaction

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**ABSTRACT**

**Objective(s):** $bla_{CTX-M}$ and $bla_{PER}$ are two genes that encode class A extended-spectrum $\beta$-lactamases (ESBLs) and can be responsible for therapeutic problems. This study was carried out to evaluate the molecular properties of these genes in clinical isolates of Enterobacteriaceae by polymerase chain reaction (PCR), restriction digestion and sequencing.

**Materials and Methods:** During six months, starting from January 2012, one hundred clinical isolates of Enterobacteriaceae were collected from urinary samples. The ESBL-producing isolates were detected by phenotypic confirmation test. After plasmid extraction, $bla_{PER}$ and $bla_{CTX-M}$ genes were detected using PCR by specific primers. The $bla_{CTX-M}$ PCR products were digested with Taq1, and two of the $bla_{CTX-M}$ genes were sequenced.

**Results:** Phenotypic tests showed that 27 (27%) isolates were ESBL producers with the highest frequency for Klebsiella pneumoniae (47.4%) and Escherichia coli (17.9%). Twenty six (26%) of Enterobacteriaceae isolates harbored the $bla_{CTX-M}$ gene, and none of them had $bla_{PER}$. The restriction analysis of PCR products showed that all $bla_{CTX-M}$ amplified products had the same patterns. Both sequenced bacteria were CTX-M-15 type ESBL carriers.

**Conclusion:** The results of this study showed the $bla_{CTX-M-15}$ gene in Enterobacteriaceae isolates for the first time in Mashhad, Iran. High degrees of associated resistance to co-trimoxazole and gentamicin were found in ESBL producers. Therefore, an integrated and regular management of antibiotic prescription need to be trained in our society.

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**Introduction**

Resistance to $\beta$-lactam antibiotics dates back to the first years of discovery of resistance to the first antibiotic, penicillin. The first $\beta$-lactamase was observed in Escherichia coli bacteria which hydrolyzed penicillin (1). The emergence of resistance to $\beta$-lactam antibiotics is related to $\beta$-lactamases coding by plasmids which are rapidly spread from strain to strain among most clinical isolates (2). Until now, over 200 different types of extended-spectrum $\beta$-lactamase (ESBL) have been identified worldwide, the majority of which are found in Enterobacteriaceae family (3). Most ESBLs are derivatives of TEM (Temoneira) or/and SHV (sulphhydryl variable) enzymes (4, 5). In recent years, a new family of plasmid-mediated ESBLs, called CTX-M (cefotaxime-hydrolyzing $\beta$-lactamase), has been arisen that preferentially hydrolyzed cefotaxime (3). CTX-M was reported in 1989 for the first time in Germany (6) and is often found in E. coli and Klebsiella pneumoniae as well as in other Enterobacteriaceae (3). PER-1 (Pseudomonas extended-resistant) was first detected in Pseudomonas aeruginosa (7) and later in Salmonella enterica and Acinetobacter isolates, PER-2, which shows 86% homology to PER-1, has been reported in S. enterica, E. coli, K. pneumoniae, Proteus mirabilis, and Vibrio cholera O1 El Tor (8, 9). Currently, microbial resistance through ESBL has been recognized globally and now ESBLs are a problem throughout the world (3). Although some studies have been carried out to detect ESBL-producing bacteria in Mashhad (10, 11), there are not enough data on molecular properties of $bla_{CTX-M}$ and $bla_{PER}$.
genes among Enterobacteriaceae bacteria in this area. Therefore, the present study was undertaken in two teaching hospitals of Mashhad University to determine genetic properties of ESBL-producing Enterobacteriaceae isolates related to bla\textsubscript{CTX-M} and bla\textsubscript{PER} genes.

Materials and Methods
Bacterial isolates and antibiotic susceptibility testing
During six months, from January to June 2012, 100 Enterobacteriaceae were collected from urinary samples of inpatients and outpatients referred to Qaem Hospital and 17-Shahrivar Hospital Laboratories in Mashhad. All isolates were identified by differential biochemical standard tests. Antibiotic susceptibility testing was applied by disk diffusion method for ampicillin (AP, 10 µg), cephaplothin (KF, 30 µg), nalidixic acid (NA, 30 µg), gentamicin (Gm, 10 µg), amikacin (AK, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), nitrofurantoin (NI, 300 µg), co-trimoxazole (TS, 25 µg), and imipenem (IMI, 10 µg) disks (MAST, UK) on Mueller-Hinton agar (Microbiology, Germany) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (12).

Phenotypic ESBL detection
Screening for ESBL producers was carried out by double disk approximation test and ESBL confirmative test according to CLSI standards. Double disk approximation test was performed by augmentin (20 + 10 µg), cefotaxime and ceftazidime disks, and ESBL confirmation test was tried out by cefotaxime/clavulanate (10 + 30 µg) combination disk and ceftazidime (13, 14). A positive test defined as ≥5 mm increase in zone diameter in comparison to a disk without clavulanic acid (13, 14). Genus and species of ESBL-producing bacteria were confirmed by Microgen kit and Microgen identification system software (Microgen Bioproducts GNA- ID UK).

PCR amplification of bla\textsubscript{CTX-M} and bla\textsubscript{PER} genes
Pure bacteria was inoculated in Lauria Bertani broth (Biomark, India) containing 100 µg/ml ampicillin (Sigma, Germany) and incubated at 37°C, shaking 185 rpm for 16-18 hr. Plasmid extraction was done by Perfect Prep Spin Mini Kit-5 Prime-USA according to procedure instructions. A volume of 5 µl of extracted plasmid (contained 18-20 ng/µl of DNA) was used to perform PCR. Identification of bla\textsubscript{CTX-M} gene was conducted with a pair of primer CTX-M (5′- ATG TGC ACC AGT AAR GT-3′) and CTX-MU2 (5′- TGG GTR AAR TAR GTS ACC AGA-3′) which amplified a 593 bp fragment (15). The specific primers which were used for amplification of bla\textsubscript{PER} were PER-forward (5′-AATTGGGCTTGGGCCAGAA-3′) and PER-reverse (5′-ATGAATCTGTATTAAAGGC-3′) to amplify a 924 bp fragment (16). PCR was performed in 30 µl mixture of 3 µl 10X buffer, 1 µl of 10 mM MgCl\textsubscript{2}, 0.25 µl of 5 U/µl Taq DNA polymerase (Fermentas-Lithuania), 0.5 µl of 10 mM of each deoxynucleotide triphosphate, 1 µl of 10 µM of each primer and 5 µl of plasmid extracts in a thermal cycler (Kyratec-Korea). The amplification was performed according to conditions which are shown in Table 1.

Pseudo. aeroginosa containing bla\textsubscript{PER}, and E. coli containing bla\textsubscript{CTX-M} received from Pasteur Institute, Iran, were used as positive controls. The PCR products were evaluated after electrophoresis on 1% gel and staining with ethidium bromide. A 100 bp ladder standard (Fermentas, Lithuania) was used as molecular weight ladder.

Restriction analysis of PCR products and confirmation of the amplified products
Following PCR, the bla\textsubscript{CTX-M} PCR products were digested with Taq1 (Fermentas, Lithuania) for 3 hr at 65°C. PCR products were extracted with Agarose Gel Extract Mini Kit-50 Prep (5 Prime, USA) according to procedure guidelines. For restriction enzyme digestion, 6 µl of each PCR products were mixed with 25 µl buffer, 1 µl distilled water and 2 µl Taq1 restriction enzyme. The restriction enzyme was selected by using CLC Main workbench 5 software.

DNA sequencing analysis
The PCR products of two samples that showed the highest multi-resistance to tested antibiotics were sequenced on both strands by using CTX-MU-1 and CTX-MU-2 primers with an ABI 3730 XL automated DNA sequencer (Macrogen, Korea). The nucleotide sequences were analyzed using the Sequencher sequence software alignment (Version 4.10.1) and were compared to the identified β-lactamase: CTX-M-15 gene from K. pneumoniae plasmid pMRC151 in the GenBank nucleotide database under accession no. AY995205 available on the Internet at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Table 1. PCR conditions for amplifying bla\textsubscript{CTX-M} and bla\textsubscript{PER} genes by polymerase chain reaction

| Stage          | Temperature (°C) | Time     | No. cycle |
|----------------|-----------------|----------|-----------|
| Hot start      |                 |          |           |
| Denaturation   | 94              | 5 min    | 1         |
| Annealing      | 94              | 30 sec   |           |
| Extension      | 55 (bla\textsubscript{CTX-M}) | 30 sec  |            |
|                | 48 (bla\textsubscript{PER}) |          | 35        |
| Final extension| 72              | 3 min (bla\textsubscript{CTX-M}) | 1         |
|                | 72              | 5 min (bla\textsubscript{PER}) |           |
Table 2. Antibiotic susceptibility of isolated Enterobacteriaceae family bacteria to various antibiotics

| Antibiotics      | Susceptible | Intermediate | Resistance |
|------------------|-------------|--------------|------------|
| Ampicillin       | 12          | 30           | 58         |
| Cefotaxime       | 29          | 43           | 28         |
| Cephalothin      | 45          | 9            | 46         |
| Ceftazidime      | 48          | 38           | 14         |
| Co-trimoxazole   | 54          | 2            | 44         |
| Nalidixic acid   | 58          | 7            | 35         |
| Gentamicin       | 75          | 12           | 13         |
| Amikacin         | 93          | 7            | 0          |
| Nitrofurantoin   | 96          | 2            | 2          |
| Imipenem         | 100         | 0            | 0          |

Statistical analysis

Statistical analysis was carried out by using Statistica software. Chi-square test was used for determination of significance of association. The P-value ≤ 0.05 was considered significant.

Results

One hundred bacterial isolates of the Enterobacteriaceae family were detected from urinary samples of patients. There were 67 E. coli, 19 K. pneumoniae, 5 K. oxytoca, 5 Enterobacter cloacae, 2 Proteus mirabilis, 1 Pro. vulgaris, and 1 Citrobacter diversus. Among them, E. coli was the most common isolated microorganism followed by K. pneumoniae.

Fifty seven urinary samples were from inpatients and 43 samples were from outpatients. Table 2 shows the results of disc diffusion susceptibility test for isolated bacteria. As Table 2 indicates, there is high prevalence of resistance and intermediate to ampicillin (88%) and cefotaxime (71%). Moreover, the most susceptibility was for imipenem (100%) and nitrofurantoin (96%) among the tested antibiotics.

Compared to K. pneumoniae, a higher percentage of E. coli isolates were resistant to nalidixic acid (81.8% vs. 9.1%), co-trimoxazole (6.1% vs. 33.4%), cefotaxime (75% vs. 25%) and cephalothin (6.2% vs. 26.1%); while resistance to ceftazidime was higher in K. pneumoniae in comparison to E. coli (60% vs. 40%).

The antimicrobial susceptibility results of inpatients and outpatients are shown in Table 3. Antimicrobial resistance among hospitalized patients was higher than that of outpatients.

ESBL production was observed in 27% of isolated bacteria by phenotypic confirmatory test. Twelve (27.9%) of 43 isolated bacteria from inpatients and 15 (26.3%) of 57 bacteria from outpatients were ESBL-producing. Among isolated Enterobacteriaceae, the frequency of ESBL production for K. pneumoniae and E. coli was 47.4% and 17.9%, respectively. A high percentage of ESBL producers were resistant to co-trimoxazole, gentamicin and nalidixic acid (Table 4). Resistance to gentamicin and co-trimoxazole was associated with ESBL production (P-value < 0.01).

Among seven bacteria with multi-resistance to gentamicin, co-trimoxazole and nalidixic acid, 5 (71.4%) were ESBL producers, 4 were K. pneumoniae, and three were E. coli.

In the present study, 26 isolates harbored the \textit{bla}_{CTX-M} gene and none of them contained \textit{bla}_{PER}. Figure 1 shows PCR products on 1% agarose gel.

The restriction enzyme digestion analysis

Table 3. Antimicrobial susceptibility test results of clinical isolates of inpatients and outpatients (S: susceptible, R: resistant)

| Antibiotics      | Outpatients (n=57) No. (%) | Inpatients (n=43) No. (%) |
|------------------|---------------------------|--------------------------|
|                  | S  | R  | S  | R  |
| Co-trimoxazole   | 34 (59.6) | 22 (38.5) | 21 (46.6) | 21 (46.6) |
| Nalidixic acid   | 41 (71.9) | 13 (22.8) | 17 (39.6) | 22 (51.3) |
| Nitrofurantoin   | 56 (98.3) | 0 | 40 (93.1) | 2 (4.6) |
| Amikacin         | 51 (89.5) | 0 | 42 (97.6) | 0 |
| Gentamicin       | 48 (84.2) | 5 (8.7) | 27 (62.8) | 8 (18.6) |
| Ceftazidime      | 29 (50.8) | 3 (5.4) | 19 (44.3) | 11 (25.5) |
| Ampicillin       | 4 (7.1) | 22 (47.3) | 8 (18.6) | 31 (72.1) |
| Cefotaxime       | 28 (49.1) | 10 (17.6) | 1 (2.3) | 18 (41.9) |
| Cephalothin      | 29 (50.8) | 21 (36.8) | 16 (37.2) | 25 (58.2) |
| Imipenem         | 57 (100) | 0 | 43 (100) | 0 |

Table 4. Antimicrobial susceptibilities of clinical isolated bacteria

| Antibiotics      | Outpatients (n=57) No. (%) | Inpatients (n=43) No. (%) |
|------------------|---------------------------|--------------------------|
|                  | S  | R  | S  | R  |
| Co-trimoxazole   | 9 (33.3) | 18 (66.7) | 45 (61.7) | 26 (35.6) |
| Nalidixic acid   | 14 (51.8) | 11 (40.8) | 44 (60.2) | 44 (60.2) |
| Nitrofurantoin   | 27 (100) | 0 | 69 (94.6) | 69 (94.6) |
| Amikacin         | 27 (100) | 0 | 66 (90.4) | 66 (90.4) |
| Gentamicin       | 17 (62.9) | 8 (29.7)* | 58 (79.4) | 58 (79.4) |
| Imipenem         | 27 (100) | 0 | 73 (100) | 73 (100) |

* P-value < 0.01
showed that all of the blaCTX-M amplified products had similar patterns (Figure 2). According to the sequence presented in NCBI website, after enzyme excision by TaqI on blaCTX-M products would be 270 and 323 kbp being located nearly in one area on the gel.

As shown in Figure 3, sequencing of the PCR products for two tested isolates revealed one variation in the β-lactamase blaCTX-M gene from one isolate at position 421 (c.421 A>G; p.K76K). This mutation is silent due to coding the same amino acid as lysine. The sequence of the other isolate was similar to the compared strain (blaCTX-M-15 gene from K. pneumoniae with accession no. AY995205).

**Discussion**

Identification of common etiologic organisms of nosocomial pathogens and their pattern of antibiotic resistance is of great importance for controlling the diseases and reducing the costs. Since the members of Enterobacteriaceae are the main factors of nosocomial and community-acquired infections, establishing a new strategy in diagnosis and treatment of ESBL-producing bacteria is essential. ESBL-carrying Enterobacteriaceae is an increasing problem in the urinary tract infections throughout the world. The prevalence of ESBL-producing bacteria differs greatly worldwide and mainly depends on the extensive use of β-lactam antibiotics in the communities (17-19).

The occurrence of ESBL producers in urinary isolates of Enterobacteriaceae in our study was found to be 27% and the percentage of ESBL-producing strains among isolates of E. coli and K. pneumoniae was 17.9% and 47.4%, respectively. The resistance of bacteria isolated from hospitalized patients, particularly for β-lactam antibiotics, was higher than that of outpatients probably representing a more rapid transfer of antimicrobial resistance genes among hospitalized patients.

Our findings suggest that CTX-M type β-lactamases are widespread in the studied community (96.3%). We found higher prevalence of CTX-M type β-lactamase than that reported in many developed countries such as Spain and France (17).

The occurrence and distribution of ESBL varies among different species and countries. It demonstrates important geographical differences in Europe, ranging from a percentage below one (Estonia) to 41% (Romania) for E. coli, and from 0% (Iceland) to 91% (Romania) for K. pneumoniae (17). In Poland, the proportion of ESBL producers in hospitals (11.1%) varies in different species from 2.5% for E. coli to 40.4% for K. pneumoniae and 70.8% for Serratia marcescens, the two latter having a higher prevalence due to outbreak situations (17).

CTX-M-15 is frequently detected in Enterobacteriaceae in several countries like the UK, Turkey, Spain, Norway, Italy, Portugal (17), Korea (20), and India (21). Possibly similar to other societies, the prevalence of CTX-M-15-producing type of Enterobacteriaceae is increasing in our society. Since its first description in 2001, CTX-M-15 has been identified in multiple locations in Asia and Europe (22).

This study documented the emergence of CTX-M-15-producing Enterobacteria for the first time in Mashhad, Iran. CTX-M-15 differs from CTX-M-3 by an Asp-240 ➔ Gly substitution that increases activity against ceftazidime (23). Moreover, our results showed that none of the isolated Enterobacteriaceae carried blaPER gene, which is similar to what has been reported for E. coli isolates by Shahcheraghi et al from Tehran, Iran (16). Much higher (49.25%) prevalence of blaPER gene has been reported among ESBL-producing strains of Pseudo aeruginosa isolated from burn patients (24). The blaPER-1 gene has been detected mainly in glucose-fermenting Gram-negative bacilli, such as Pseudo aeruginosa (25) and Alcaligenes faecalis (26); however, it has been recently found in Enterobacteriaceae and Aeromonas media as well (29-31). This gene has been lately reported for the first time from K. pneumoniae. The authors are of the opinion that there is a possibility of further dissemination of blaPER-1 gene in Enterobacteriaceae (30). Our study also showed that a higher percentage of ESBL-positive isolates were resistant to gentamicin and nalidixic acid. Co-resistance to gentamicin and ciprofloxacin has been reported in India (31), and to aminoglycosides and fluoroquinolones in Uruguay (32) and Portugal (33).

**Conclusion**

This is the first report of blaCTX-M-15 gene in E. coli isolates from Mashhad hospitals. From the
results of the present study it may be concluded that CTX-M-producing *Enterobacteriaceae* and perhaps the CTX-M-15 type of ESBL producers are increasing in our community. Control on the antimicrobial prescription as well as an integrated and regular management of antibiotic healing need to be practiced in our society.

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**Conflict of Interests**

All authors declare that they have no conflicts of interest.

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