Iranian Journal of Basic Medical Sciences

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Genetic properties of *bla*$_{CTX-M}$ and *bla*$_{PER}$ β-lactamase genes in clinical isolates of *Enterobacteriaceae* by polymerase chain reaction

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Jul 3, 2013
Accepted: Dec 25, 2013

Keywords:
CTX-M-15
Enterobacteriaceae
Extended-spectrum beta-lactamase
PER

ABSTRACT

Objective(s): *bla*$_{CTX-M}$ and *bla*$_{PER}$ are two genes that encode class A extended-spectrum β-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.

► *Please cite this paper as:*
Nakhaei Moghaddam M, Hashemi Beidokhti M, Amel Jamehdar S, Ghahraman M. Genetic properties of *bla*$_{CTX-M}$ and *bla*$_{PER}$ β-lactamase genes in clinical isolates of *Enterobacteriaceae* by polymerase chain reaction. Iran J Basic Med Sci 2014; 17:378-383.

Introduction

Resistance to β-lactam antibiotics dates back to the first years of discovery of resistance to the first antibiotic, penicillin. The first β-lactamase was observed in *Escherichia coli* bacteria which hydrolyzed penicillin (1). The emergence of resistance to β-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 β-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 (sulphydryl variable) enzymes (4, 5). In recent years, a new family of plasmid-mediated ESBLs, called CTX-M (cefotaxime-hydrolyzing β-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*$_{PER}$

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

Stage		Temperature (°C)	Time	No. cycle
Hot start		94	5 min	1
	Denaturation	94	30 sec	
Cycle	Annealing	55 (*bla*$_{CTX-M}$), 48 (*bla*$_{PER}$)	30 sec	35
	Extension	72	30 sec (*bla*$_{CTX-M}$), 60 sec (*bla*$_{PER}$)	
Final extension		72	3 min (*bla*$_{CTX-M}$), 5 min (*bla*$_{PER}$)	1

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*$_{CTX-M}$ and *bla*$_{PER}$ 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), cephalothin (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 employed by augmentin (20 + 10 µg), cefotaxime and ceftazidime disks, and ESBL confirmation test was tried out by ceftazidime/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$_{CTX-M}$ *and bla*$_{PER}$ *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*$_{CTX-M}$ gene was conducted with a pair of primer CTX-MU1 (*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*$_{PER}$ were PER-forward (*5′-AATTTGGGCTTAGGGCAGAA-3′*) and PER-reverse (*5′-ATGAATGTCATTATAAAAGC-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 $MgCl_2$, 0.25 µl of 5 U/µl Taq DNA polymerase (Fermentas-Lituania), 0.5 µl of 10 mM of each deoxynucleaotide 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*$_{PER}$, and *E. coli* containing *bla*$_{CTX-M}$ 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*$_{CTX-M}$ 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 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%).

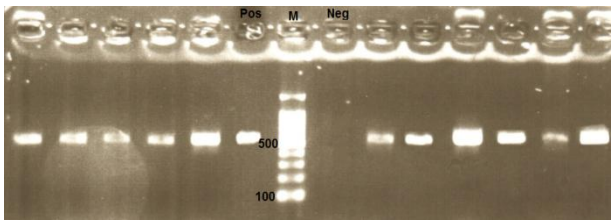

Figure 1. PCR products of CTX-M type extended-spectrum β-lactamases producing isolates on 1% agarose gel (Pos: positive control, Neg: negative control, M: marker 100 bp)

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 bla_{CTX-M} gene and none of them contained 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

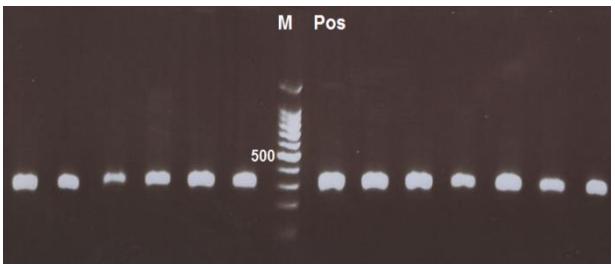

Figure 2. Digestion pattern of the *bla*$_{CTX-M}$ amplified products on 1% agarose gel (M: marker, Pos: positive control)

showed that all of the *bla*$_{CTX-M}$ amplified products had similar patterns (Figure 2). According to the sequence presented in NCBI website, after enzyme excision by TaqI on *bla*$_{CTX-M}$, 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 *bla*$_{CTX-M}$ 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 (*bla*$_{CTX-M-15}$ 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).

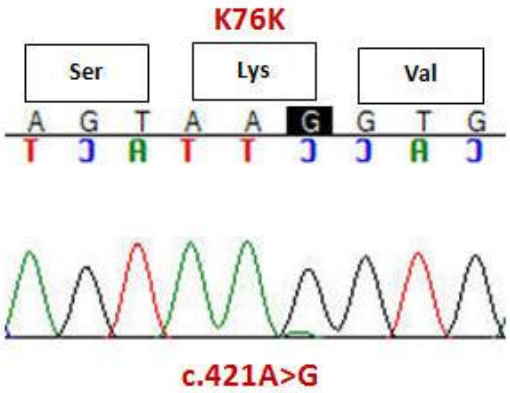

Figure 3. The homozygous A to G transition substitution at position 421, leading to a silent mutation at codon 76 in β-lactamase CTX-M-15 gene

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 *bla*$_{PER}$ 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 *bla*$_{PER}$ gene has been reported among ESBL-producing strains of *Pseudo aeroginosa* isolated from burn patients (24). The *bla*$_{PER-1}$ gene has been detected mainly in glucose-nonfermenting 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 possi-bility of further dissemination of *bla*$_{PER-1}$ 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 *bla*$_{CTX-M-15}$ 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.

Acknowledgement

This work was partly supported by the Research Deputy of Islamic Azad University- Mashhad Branch and carried out in the Department of Biology, Faculty of Basic Sciences, Islamic Azad University Mashhad Branch, Iran.

Conflict of Interests

All authors declare that they have no conflicts of interest.

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