

Prevalence of CTX-M-Type and PER Extended-Spectrum β -Lactamases Among *Klebsiella* spp. Isolated From Clinical Specimens in the Teaching Hospital of Kashan, Iran

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Abstract

Background: Extended-spectrum β -lactamases (ESBLs) is one of the most important mechanisms of resistance to β -lactams especially among Enterobacteriaceae family including *Klebsiella* spp. Different types of extended-spectrum β -lactamases including CTX-M-type and PER enzymes are identified among gram negative bacteria.

Objectives: The current study aimed to determine the prevalence of CTX-M-type and PER extended-spectrum β -lactamases among *Klebsiella* spp. isolated from clinical specimens in the teaching hospital of Kashan, Iran.

Patients and Methods: One hundred *Klebsiella* spp. were isolated from clinical specimens of hospitalized patients at Shahid-Beheshti hospital from December 2012 to November 2013. Disk diffusion method was used to determine the susceptibility of these isolates to 14 different antimicrobial agents; disks were purchased from MAST company (United Kingdom). The phenotypic double disk synergy confirmatory test was used to screen the isolates to produce extended-spectrum β -lactamase. DNAs of isolates were extracted using boiling method and PCR assay was used to characterize the *bla*_{CTX-M} type and *bla*_{PER} genes. The purified PCR products were sent to Macrogen research company (Korea) for sequencing.

Results: Of the total 100 *Klebsiella* isolates, 93% was susceptible to imipenem. Resistance to ampicillin, ceftazidime, ceftriaxone, aztreonam and cefotaxime was (92%), (67%), (65%), (64%) and (59%), respectively. The phenotypic confirmatory test (PCT) confirmed that 35% (n = 35) of the isolates were ESBL-producing *Klebsiella* strains. The prevalence of *bla*_{CTX-M} type and *bla*_{PER} genes among *Klebsiella* isolates were 28% (n = 28) and 9% (n = 9), respectively.

Conclusions: The prevalence of ESBL-producing *Klebsiella* strains in Shahid-Beheshti hospital in Kashan has increased. The study concluded that there was a high prevalence of the *bla*_{CTX-M} type gene among ESBL positive isolates.

Keywords: ESBL, Clinical Specimens, *bla*_{CTX-M}

1. Background

The extended-spectrum β -lactamases (ESBLs) including CTX-M-type and PER enzymes have increased among Enterobacteriaceae in many parts of the world (1). Until now 90 different CTX-M β -lactamases have been described and divided into five phylogenetic groups (2, 3). CTX-M type β -lactamases re classified under amber class A which induce high level resistance to ceftazidime, cefotaxime and aztreonam (4). PER enzymes, as a class A extended-spectrum beta-lactamase also confer resistance to oxyimino beta-lactams (5).

Due to extensive use of β -lactam antibiotic over the last several decades in the healthcare centers, various β -lactamases have emerged (5). Extended spectrum β -lactamases (ESBLs) are the enzymes produced by gram-negative pathogens hydrolyze β -lactam antibiotics containing an oxyimino group (third generation cephalosporins and aztreonam) and are inhibited by β -lactamase inhibitors such as clavulanic acid, sulbac-

tam and tazobactam (6, 7). ESBLs are prevalent in different parts of the world and are mainly identified among Enterobacteriaceae family such as *Klebsiella pneumoniae* strains (8, 9). Infections due to these ESBL-positive *Klebsiella* isolates cause increased morbidity and mortality (10). CTX-M-producing *Klebsiella pneumoniae* are increasingly involved in infections, especially among hospitalized patients. Furthermore, these bacteria seem to have been imported from the community into the clinical settings (11). The prevalence of ESBL producing *K. pneumoniae* varies in different countries, although CTX-M type β -lactamase enzymes are the predominant ESBLs in the most parts of the world (12-16). The prevalence of ESBL producing *K. pneumoniae* in Iran is reported to range from 19.6 to 75% (17-21). In Kashan, few studies have been performed to determine the prevalence of ESBL genes among *K. pneumoniae* isolates especially those of *bla*_{PER} and *bla*_{CTX-M}.

2. Objectives

The current study aimed to determine the prevalence of *bla_{PER}* and *bla_{CTX-M}* genes among ESBL producing *Klebsiella* strains which can provide useful epidemiological information that might aid the management of antimicrobial therapies.

3. Patients and Methods

3.1. Bacterial Isolates

One hundred *Klebsiella* isolates were collected from clinical specimens of hospitalized patients at Shahid-Beheshti hospital in Kashan from December 2012 to November 2013. The species were isolated from patients of both genders (64% female, 36% male). The *Klebsiella* strains were identified by standard microbiological tests (22).

3.2. Antibiotic Susceptibility Test

The isolates were tested for antimicrobial susceptibility pattern by the disk diffusion method according to the CLSI guidelines (23). Antimicrobial susceptibility testing was performed on Mueller-Hinton agar. The following antibiotic disks were used: ampicillin (30 Mg), aztreonam (30 Mg), amoxicillin/clavulanic acid (20 Mg), cephalothin (30 Mg), cefixime (30 Mg), nalidixic acid (30 Mg), trimethoprim-sulfamethoxazole (25 Mg), imipenem (10 Mg), ceftazidime (30 Mg), ceftoxitin (30 Mg), cefteteraxion (30 Mg), gentamicin (10 Mg), ciprofloxacin (5 Mg) and nitrofurantoin (300 Mg). The antibiotic disks were obtained from MAST company (Mast Companies, UK) and the quality control organism was *Escherichia coli* ATCC 25922.

3.3. ESBL Detection by Double Disk Synergy Test

ESBL production of all the 100 *Klebsiella* strains was confirmed by ceftazidime (30 Mg) and cefotaxime (30 Mg) antibiotic disks with and without clavulanic acid (10 Mg), by double disk synergy test (DDST). Briefly, the organisms were swabbed on to Mueller-Hinton agar plates (as performed for disc diffusion), then an amoxicillin-clavulanate disk (20 + 10 Mg) was placed in the center of the plate and ceftizoxime (30 Mg), cefotaxime (30 Mg) and cefteteraxion (30 Mg) discs were placed 15 mm away from the central disk (24, 25). The plates were incubated at 37°C for up to 24 hours. An increase of > 5 mm inhibition zone for antibiotics around the amoxy-clav disk compared to that of the cephalosporin disk alone was considered ESBL production. ESBL producing strain *K. pneumoniae* ATCC 700603 and non-ESBL producing strain *E. coli* ATCC 25922 were used as positive and negative controls respectively (23).

3.4. Detection of *bla_{CTX-M}* and *bla_{PER}* Genes by PCR

DNAs of isolates were extracted using boiling method and PCR amplification was carried out using specific

primers for *bla_{CTX-M}* gene including: 5'-CGCTTTGCGATGTGAG-3', and 5'-ACCGCGATATCGTTGGT-3' to amplify a 590 bp fragment and *bla_{PER}* gene specific primers including: 5'-GTAAATTGGGCTTAGGGCAG-3', and 5'-CAGCGCAATCCCCACTGT-3' to amplify an 855 bp fragment (26). PCR was performed in 25 ML volume reaction mixtures containing 10 pM of each primer, 200 Mm dNTP, 1.5 Mm MgCl₂, 1.5 ML of template DNA and 1 U Taq DNA polymerase in the reaction buffer provided by the manufacturer (CinnaGen, Tehran, Iran). The following thermo-cycling program was carried out for PCR experiments: initial denaturation at 94°C for 5 minutes; 30 cycles of 94°C for 25 seconds, 52°C for 40 seconds and 72°C for 50 seconds; and a final elongation at 72°C for 6 minutes in a thermal cycler (Eppendorf master cycler®, MA). The amplified products were electrophoresed and separated on 2% agarose gel. The gel was visualized by staining with ethidium bromide (0.5 mg/mL) in a dark room for 30 minutes. A 100 base pair ladder was used as molecular weight marker to measure the molecular weight of the amplified products (100 bp DNA ladder, MBI Fermentas). The images of ethidium bromide stained DNA bands were analyzed using a gel documentation system (Biorad, UK).

3.5. DNA Sequencing

The purified PCR products were sequenced using the ABI capillary system (Macrogen Research, Seoul, Korea). Sequences were compared using online BLAST software (<http://www.ncbi.nlm.nih.gov/BLAST/>).

4. Results

A total of 100 *Klebsiella* isolates were obtained from patients at the hospital. They were from urine (75%), sputum (7%), blood (6%) and tracheal aspirate (12%). The mean age of the infected patients was 45 years (aged between 0 - 90 years old) and the median length of hospitalization was seven days. Samples were isolated from patients of both genders (64% female, 36% male) (Table 1).

The antibiotic susceptibility profile measured by disk diffusion is shown in Table 2. The highest rate of resistance was to ampicillin (92%) and the lowest to imipenem (7%). The resistance rate among ESBL producing *Klebsiella* isolates was higher than that of non-ESBL producing *Klebsiella* isolates (Table 3).

Of all the isolates, 64% showed resistance to more than three antimicrobial families and identified as multidrug-resistant (MDR). The phenotypic confirmatory test (PCT) confirmed that 35% (n = 35) of isolates were ESBL-producing *Klebsiella* strains.

PCR using universal primers (Figures 1 and 2) indicated that among 35 ESBL positive strains, 28 isolates carried *bla_{CTX-M}* gene and 9 isolates had *bla_{PER}* gene. GenBank accession numbers obtained for the purified PCR products were KJ803828 and KJ803829 respectively.

Table 1. Comparison of Demographic and Laboratory Data Among ESBL Positive and ESBL Negative *Klebsiella* Isolates^a

Factors	ESBL Negative	ESBL Positive	P Value
Age, y			0.306
≥ 50	11 (16.9)	29 (82.9)	
< 50	54 (83.1)	6 (17.1)	
Gender			0.540
Male	22 (33.8)	14 (40)	
Female	43 (66.2)	21 (60)	
Type of admission			0.140
Hospitalization	49 (75.4)	27 (77.1)	
Outpatient	16 (24.6)	8 (22.9)	
Length of hospitalization, y			0.002
> 7	6 (9.2)	6 (17.1)	
≤ 7	59 (90.8)	29 (82.9)	
Ward			0.013
ICU	14 (21.5)	17 (48.6)	
Medical	8 (12.3)	0	
Infectious	28 (43.1)	12 (34.3)	
Surgery	0 (0)	0	
Obstetrics and gynecology	0 (0)	0	
Children	11 (16.9)	4 (11.4)	
Emergency	0	0	
CCU	4 (6.2)	2 (5.7)	
Sample type			0.004
Urine	46 (70.8)	29 (82.9)	
Sputum	7 (10.8)	0	
Catheters	0	0	
Blood	6 (9.2)	0	
Wound	0	0	
Tracheal aspirate	6 (9.2)	6 (17.1)	
Resistance pattern			0.105
Drug resistance	36 (55.4)	0	
Multi drug resistance	29 (44.6)	35 (100)	

Abbreviation: ESBL, Extended-spectrum β-Lactamase.

^aValues are expressed as No. (%).**Table 2.** The Antibiotic Susceptibility Profile of *Klebsiella* Isolates Measured by Disk Diffusion Method^a

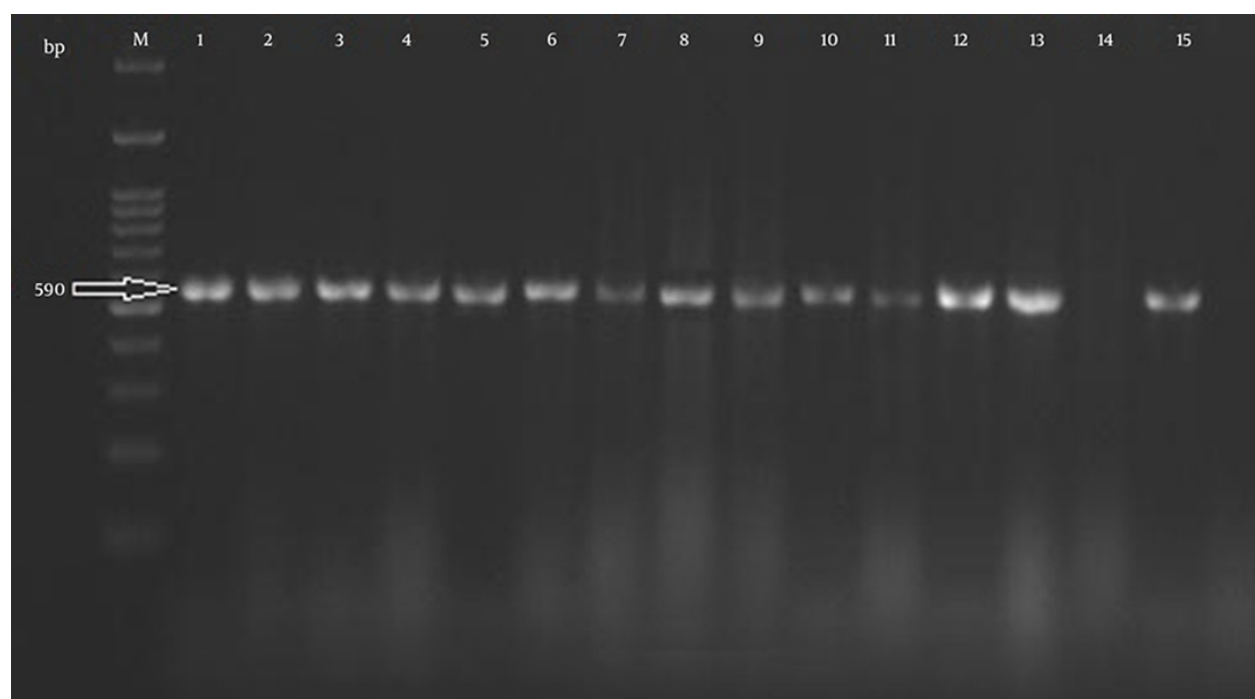
Antibiotic	Isolates	
	Sensitive	Resistant
Ampicillin	8 (8)	92 (92)
Nalidixic acid	49 (49)	51 (51)
Cotrimoxazole	75 (75)	25 (25)
Ciprofloxacin	67 (67)	33 (33)
Ceftriaxone	65 (65)	35 (35)
Aztreonam	64 (64)	36 (36)
Ceftazidime	67 (67)	33 (33)
Cephalothin	56 (56)	44 (44)
Gentamicin	80 (80)	20 (20)
Nitrofurantoin	64 (64)	36 (36)
Amoxicillin-clavulanic acid	51 (51)	49 (49)
Cefoxitin	74 (74)	26 (26)
Cefotaxime	59 (59)	41 (41)
Imipenem	93 (93)	7 (7)

^aValues are expressed as No. (%).

Table 3. The Antibiotic Susceptibility Patterns Among ESBL Producing *Klebsiella* Isolates^a

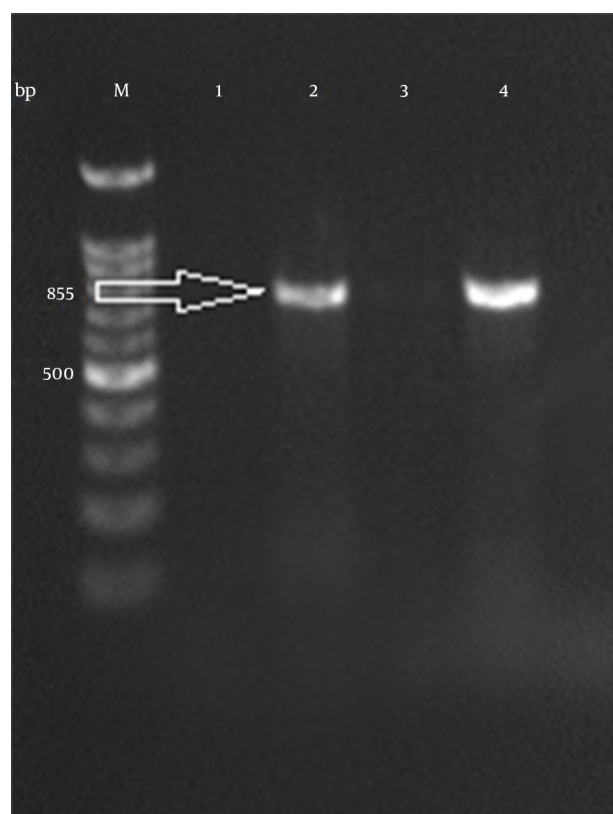
Antibiotic	Isolates	
	Sensitive	Resistant
Ampicillin	35 (100)	0
Nalidixic acid	27 (77.1)	8 (22.9)
Cotrimoxazole	24 (68.6)	11 (31.4)
Ciprofloxacin	9 (25.7)	26 (74.3)
Ceftriaxone	25 (71.4)	10 (28.6)
Aztronam	30 (85.7)	5 (14.3)
Ceftazidime	26 (74.3)	9 (25.7)
Cephalothin	18 (51.4)	44 (48.6)
Gentamicin	9 (25.7)	26 (74.3)
Nitrofurantoin	10 (28.6)	25 (71.4)
Amoxicillin-clavulanic acid	31 (88.5)	4 (11.5)
Cefoxitin	17 (48.6)	18 (51.4)
Cefotaxime	28 (80.0)	7 (20.0)
Imipenem	2 (5.7)	33 (94.3)

^aValues are expressed as No. (%).

Figure 1. Amplification of *bla*_{CTX-M} Gene Among ESBL Positive *Klebsiella* Strains

Lane M, 100-bp DNA ladder as the molecular size marker; lanes 1 - 13, *bla*_{CTX-M} positive *Klebsiella* isolates; lane 14, negative control; lane 15, positive control.

Figure 2. Amplification of *bla_{PER}* Gene Among ESBL Positive *Klebsiella* Strains



Lane M, 100-bp DNA ladder as the molecular size marker; lane 1, negative control; lane 2, positive control; Lane 3, *bla_{PER}*-negative *Klebsiella* isolate; Lane 4, *bla_{PER}* positive *Klebsiella* isolate.

5. Discussion

Klebsiella spp. is considered as the most common ESBL producing organism (8, 27). ESBL producing *Klebsiella* strains are a major concern for clinical therapeutics; therefore, some supplementary tests are needed to determine ESBL producing bacteria (28). In the current study, 35% of the *Klebsiella* strains isolates were ESBL producer by PCT test. A frequency of 46.9% ESBL production was reported by Raei et al. (9) among urinary isolates of *K. pneumoniae* in Tehran. In contrast, lower rates of ESBL production were detected in comparison to other studies in Iran and some parts of Middle East (15, 17, 18). The results show that the prevalence rates of ESBL production are different in different countries or different parts of each country (14, 15, 17-21). This could be due to different antibiotic policies in each region.

In accordance with other reports, the resistance rate among ESBL producing *Klebsiella* isolates was higher than that of non-ESBL producing *Klebsiella* isolates (29-31).

The current study findings showed a high rate of resistance to the third generation cephalosporins including cefotaxime (%80), ceftazidime (%74.3) and ceftriaxone (%71.4) among ESBL producing *Klebsiella* isolates. In a report from

Imam Hussein hospital in Tehran, 95.6%, 89.1% and 96.7% of ESBL producing *K. pneumoniae* isolates were resistant to cefotaxime, ceftazidime and ceftriaxone, respectively (9). High rates of resistance to third generation cephalosporins in CTX-M producers are also documented from China (29) and Croatia (30). These antibiotics are third generation cephalosporins commonly used in Iranian hospitals and resistance genes could be selected easily due to selection pressure.

In the current study, 25.7% of the ESBL producing *Klebsiella* isolates were resistant to ciprofloxacin. This is in contrast with the results documented by Vranic-Ladavac in Croatia showing that all (ESBL)-producing *Klebsiella pneumoniae* associated with a nosocomial outbreak were resistant to ciprofloxacin (31). High rates of resistance to ciprofloxacin in CTX-M producer *Klebsiella* isolates were also reported from Canada and Austria (32, 33). Another finding indicated that rates of resistance to imipenem were 5.7% among ESBL producing *Klebsiella* isolates. In a study in Karaj all *K. pneumoniae* isolates were susceptible to imipenem (34). Du et al. (29) reported 3.3% resistance to imipenem among *Klebsiella pneumoniae* isolates. In another study conducted by Edelstein et al. (26) in Russian hospitals 100% sensitivity to imipenem was reported. Imipenem is the drug of choice for complicated infections caused by ESBL-producing *Klebsiella* strains; therefore, emerging 5.7% resistance to imipenem is alarming.

In the present study a high frequency of MDR was found among *Klebsiella* isolates and all ESBL producing *Klebsiella* isolates were identified as MDR strains. In a study conducted by Raei et al. (9) multidrug-resistance was observed in 96.7% of ESBL *K. pneumoniae* isolates. Similar findings were reported in other studies (29, 35). This result could be due to the point that plasmids encoding ESBLs also contain resistance genes to other antibiotics.

The global dissemination of *bla_{CTX-M}* ESBL genes over the years are described as the CTX-M pandemic; and CTX-M producing *Klebsiella* strains emerged as significant cause of hospital acquired infections (36). The current study results showed that 28% of *Klebsiella* isolates carried *bla_{CTX-M}* type gene. The frequency of *bla_{CTX-M}* type gene in *Klebsiella* strains in the present study was lower than that of Russian hospitals (26), but was in agreement with the results of studies conducted by Nasehi et al. (35) and Paterson et al. (37), which reported gene frequencies of 22.5% and 23.3% among *K. pneumoniae* isolates respectively.

In the current study of 35 ESBL producer *K. pneumoniae* isolates 80% carried *bla_{CTX-M}* gene. Kiratisin et al. (38) documented a strikingly high prevalence of *bla_{CTX-M}* gene (99.2%) among ESBL producing *K. pneumoniae* isolates at two university hospitals in Thailand. The *bla_{CTX-M}* genes are the predominant ESBL-related *bla* genes, in most parts of the world and high prevalence of *bla_{CTX-M}* gene in this study showed that CTX-M-type ESBL is endemic in the region.

The study identified 9% presence of *bla_{PER}* in *Klebsiella* isolates which was in agreement with the reports by Nasehi et al. (35) and Celenza et al. (39). In contrast, the preva-

lence of 55% and no occurrence were reported from Turkey and Italy respectively (35). Finally, the study found that of the 35 ESBL producing *Klebsiella* isolates 22.9% carried both *bla*_{CTX-M} and *bla*_{PER} genes. It was reported that MDR *Klebsiella* strains may be associated with several different betalactamases (29). The coexistence of *bla*_{CTX-M} type and *bla*_{PER} β-lactamases may have also contributed to the high rate of antimicrobial resistance in these isolates.

In conclusion, the current study result showed high ESBL occurrence with CTX-M type among *Klebsiella* isolates in Shahid-Beheshti hospital in Kashan that could be a major problem to treat *Klebsiella* infections.

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Footnotes

Authors' Contribution: Atena Amiri contributed to the writing and data collection. Farzaneh Firoozeh contributed to the writing, study design, technical and material support, concept, and editing the manuscript. Rezvan Moniri contributed to the drafting of the manuscript, and Mohammad Zibaei contributed to the edition and submission of the manuscript.

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