

PER, CTX-M, TEM and SHV Beta-lactamases in Clinical Isolates of *Klebsiella pneumoniae* Isolated from Tehran, Iran

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Abstract

Objective(s)

Different types of extended spectrum beta-lactamases (ESBLs) are encountered in the clinical settings worldwide. There are a few studies regarding the prevalence of ESBL genes among *Klebsiella pneumoniae* isolates at Tehran especially those of bla_{PER} and bla_{CTX} . The aim of this study was to determine the prevalence of bla_{SHV} , bla_{TEM} , bla_{PER} and bla_{CTX} genes among clinical *K. pneumoniae* of different hospitals in Tehran.

Materials and Methods

Two hundred isolates of *K. pneumoniae* were received from different clinical specimens. The susceptibility of the isolates to 10 different antibiotics was examined by disk diffusion test. The MICs for ceftazidime were also determined using micro-broth dilution assay. Isolates showing $MIC \geq 4 \mu\text{g/ml}$ for ceftazidime were screened for ESBL production by phenotypic confirmatory test (PCT) and subjected to PCR for studied genes. Variation among four amplified genes was evaluated using PCR-RFLP.

Results

By disk diffusion test, resistance to ceftazidime and cefotaxime were 34.7% and 33.5% respectively. However, all strains were susceptible to imipenem. Eighty isolates showed $MICs \geq 4 \mu\text{g/ml}$ for ceftazidime of which 77 (96%) were positive for ESBL in PCT. The prevalence of bla_{SHV} , bla_{CTX-M} , bla_{TEM} and bla_{PER} among these isolates were 26%, 24.5%, 18% and 7.5%, respectively. No variation was detected in the genes by PCR-RFLP.

Conclusion

As far as we know this is the first report of the bla_{PER-1} in *K. pneumoniae* in Iran. The bla_{CTX-M} was the second most common gene detected among the ESBL positive isolates of *K. pneumoniae*. For rapid identification of ESBL producing isolates it was recommended that clinical laboratories adopt simple test based on CLSI recommendation for confirming ESBL production in enterobacterial species.

Keywords: CTX-M PER, ESBLs, *Klebsiella pneumoniae*, SHV, TEM

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Introduction

Antimicrobial resistance is a growing problem in many bacterial pathogens and is of particular concern for hospital-acquired nosocomial infections (1). *Klebsiella pneumoniae* is an important pathogen that causes urinary tract infections (UTIs), pneumoniae, and intra-abdominal infections in hospitalized immunocompromised patients with severe underlying diseases (2). Resistance of *K. pneumoniae* to many antibiotics such as extended spectrum cephalosporins due to plasmid mediated enzymes (extended spectrum beta-lactamases: ESBLs) results in treatment failure of infections caused by these isolates (3). Difficult treatment of these infections may allow ESBL producing pathogens to remain within the environment and patients for the long period of time and to spread easily within and between hospitals. Within a few years of the commercial release of β -lactams, gram-negative bacilli (especially *K. pneumoniae*) that harbored mutated versions of the potent TEM and SHV enzymes were detected. These and other newly detected β -lactamases (for example CTX-M and PER types) hydrolyze β -lactam antibiotics containing the oxymino side-chain (4). CTX-M preferentially hydrolyze cefotaxime and based on the changes in amino acids sequences identities is divided into five groups (5). PER-1 is a clinically important enzyme with strong ESBL activity which can efficiently hydrolyzes penicillins and cephalosporins. It has first been detected in *Pseudomonas aeruginosa* and later in several bacterial species from various geographic areas of Europe and Asia (6). Because of inappropriate usage of antibiotic in treatment of infection caused by ESBL producing pathogens, it seems that studies about correct detection and antibiotic resistance pattern of these organisms are necessary. In recent years a few studies were done about the *K. pneumoniae* isolates producing ESBLs in our country (7-12). Also despite of importance of PER type β -lactamase, there isn't any information concerning *K. pneumoniae* isolates harboring *bla_{PER}* gene in Iran. Therefore, the present study was carried out to determine the prevalence of the genes encoding SHV, TEM,

CTX-M and PER responsible for ESBL production in the *K. pneumoniae* isolated from clinical specimens at Tehran hospitals.

Materials and Methods

Bacterial strains

Total of 200 isolates of *K. pneumoniae* were collected from different three general and two private hospitals in Iran during February 2006 to April 2007.

The specimens yielded these isolates included urine (n= 137), blood (n= 9), wound (n= 14), sputum (n= 22), CSF (n= 4), central venous line (CVL) (n= 2) intra abdominal abscess (n= 3), throat (n= 3), sperm (n= 1), stool (n= 1), and vaginal swab (n= 1), trachea (n= 2), dialysate solution (1). These isolates were recovered either from patients hospitalized at ICUs, urology, respiratory and surgery wards (n= 154) or from outpatients (n= 46). Among 200 collected isolates, 37 (18.5%) were isolated from children. Gender distribution among patients was 109 females and 91 males.

Antibiotic susceptibility test

All isolates were identified by conventional bacteriological tests (13). Antibiotic susceptibility testing was performed as NCCLS recommended using disk containing ceftazidime (CAZ: 30 μ g), ceftriaxone (CRO: 30 μ g), cefotaxime (CTX: 30 μ g), ceftizoxime (ZOX: 30 μ g), piperacilin (PIP: 100 μ g), piperacilin/tazobactam (PT: 110 μ g), gentamicin (GM: 10 μ g), amikacin (AN: 30 μ g), imipenem (IMP: 10 μ g), ciprofloxacin (CIP; 5 μ g) (BBL, USA). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as controls (14).

MICs for ceftazidime were determined by broth micro-dilution method (15). In brief, ceftazidime and imipenem were serially diluted at concentration ranging from 0.125 to 512 μ g/ml in Muller-Hinton broth supplemented with calcium and magnesium cations.

Screening for ESBL producing isolates

ESBL production ability of isolates showing MICs \geq 4 μ g/ml for ceftazidime was examined by using phenotypic confirmatory test (PCT). In brief, pairs of discs containing cefotaxime

(30 µg) and ceftazidime (30 µg) with and without clavulanic acid (10 µg) were placed on opposite sides (at a distance of 20-30 mm) of the same inoculated plate containing Muller Hinton agar (BBL-Becton Dickinson). A positive test result was defined as ≥ 5 mm increase in zone diameter compared to a disk without clavulanic acid (16).

DNA extraction and PCR

ESBL positive isolates were cultured in LB (Luria-Bertani) broth at 37 °C overnight and plasmid DNA was extracted according to the published method of Johnson and Woodford (17).

Specific primers and annealing temperature for amplifying the *bla*_{SHV}, *bla*_{TEM}, *bla*_{PER} and *bla*_{CTX} genes by PCR were shown in Table 1. PCR was carried out in solution containing 200 µM concentration of dNTPs, 10 Pmol of each primer, 0.8 mM/µl MgCl₂, 0.5 U *Taq* polymerase and 50 ng DNA template in a final volume of 25 µl. *K. pneumoniae* 7881 containing *bla*_{SHV}, *bla*_{CTX-M} and *bla*_{TEM} gene and *P. aeruginosa*, KOAS strain containing *bla*_{PER} gene (Kindly provided by Patrice Nordmann) were used as controls.

PCR-RFLP

The CTX, PER and TEM ESBL amplified genes were characterized by PCR-Restriction Fragment Length Polymorphism (RFLP). This analysis was performed using *Pst*I for TEM and PER and *Pvu*II for CTX-M ESBL amplified genes. Restriction fragments were analyzed using gel electrophoresis in a 1% (W/V) agarose gel.

Parametric methods (t-test) were used for statistical analysis of the data obtained from drug susceptibility testing.

Results

All isolates were susceptible to imipenem in disk diffusion. Exception was an isolate that showed intermediate level of resistance to imipenem which was cultured from sputum. Using the same method, 68 (34.7%) and 66 isolates (33.5%) were resistant to ceftazidime and cefotaxime respectively.

The rates of resistance to other antibiotics were as follows: piperacilin (56%), piperacilin/tazobactam (12.2%), ciprofloxacin (18%), ceftizoxime, ceftriaxone (27%) and amikacin (17.5%). Antimicrobial susceptibility testing showed that 82 isolates (41%) were resistant to at least one of the third generation cephalosporines.

Eighty isolates showed MICs ≥ 4 µg/ml for ceftazidime of which 77 (96%) were positive for ESBL in PCT. The MICs of ceftazidime in ESBLs producing isolates ranged from 4 to > 512 µg/ml, 53% of which showed MICs ≥ 128 µg/ml. The ESBL-producing isolates were recovered mostly from urine (n= 46), sputum (n= 15) and wound (n= 8) specimens. Figure 1 showed the results of resistance to antibiotics in ESBL positive isolates compared to all of isolated strains.

In this study, 53 of 200 (26.5%) were isolated from out-patients, 4 (7.5%) of which were resistant to ceftazidime. Moreover, 87.5% of pediatric isolates (28 of 32) had been cultured from urine of which 42.5% were resistant to ceftazidime. According to our results ESBLs were more common in a private hospital (55%) than public hospitals (45%). Patients who underwent kidney transplantation were significantly infected (6 of 9) with ESBL phenotype.

Table 1. Primer sequences of the ESBL genes amplified by PCR.

Primer name	Sequence (5' to 3')	Size (bp)	Annealing temperature	Gene	Reffrences
CTX-MA	CGCTTTGCGATGTGCAG	550	63°C	<i>bla</i> _{CTX-M}	35
CTX-MB	ACCGCGATATCGTTGGT				
PER-A	ATGAATGTCATTATAAAAGC	925	45°C	<i>bla</i> _{PER}	36
PER-B	AATTTGGGCTTAGGGCAGAA				
TEM-A	GAGTATTCAACATTTCCGTGTC	848	43°C	<i>bla</i> _{TEM}	36
TEM-B	TAATCAGTGAGGCACCTATCTC				
SWSHV-A	AAGATCCACTATCGCCAGCAG	231	60°C	<i>bla</i> _{SHV}	36
SWSHV-B	ATTCAGTTCGGTTCCAGCGG				

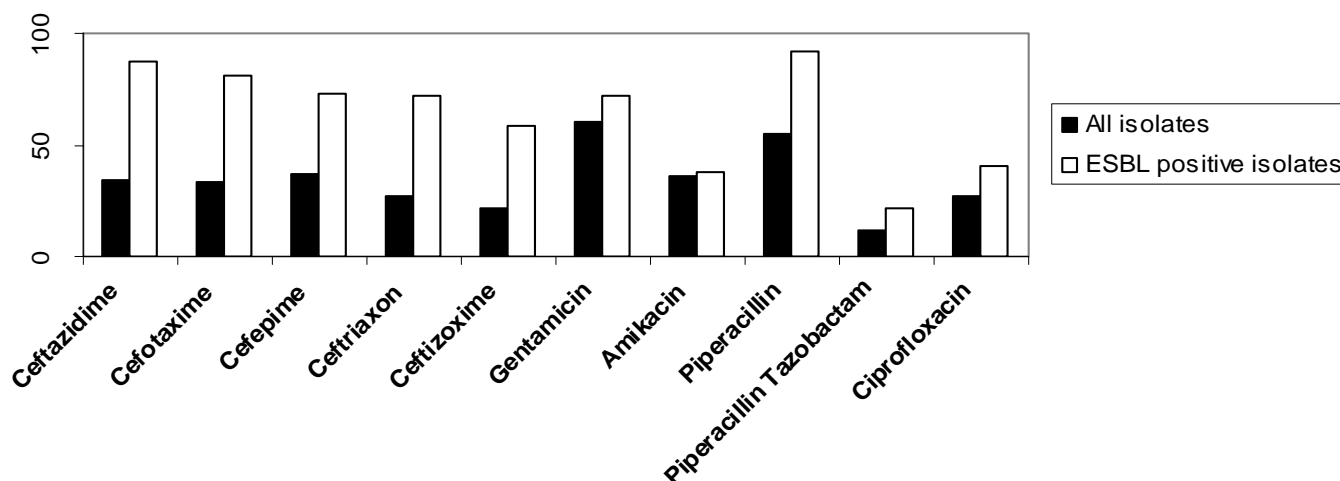


Figure 1. Comparison of resistance rates to antibiotics in ESBL positive (white columns) and all isolated strains (black columns) of *Klebsiella pneumoniae*.

The *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{PER} genes were detected in 23%, 22.5%, 16% and 7.5% of strains respectively. Table 2 showed distribution of *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX} and *bla*_{PER} genes based on the clinical specimens in ESBL positive strains of *K. pneumoniae*. Only one strain isolated from blood contained four beta lactamase genes that were resistant to all studied antibiotics except for imipenem, gentamicin and amikacin. This isolate showed MICs= 32 µg/ml to ceftazidime. In addition, 26.25% (n= 21) of isolated strains contained three genes, 41.25% (n= 33) two genes and 28.75% (n= 23) contained only one gene.

The amplicons obtained for *bla*_{TEM}, *bla*_{CTX-M}, and *bla*_{PER} genes were characterized by PCR-

RFLP. All TEM and PER producing isolates digested with *Pst*I gave amplimers with identical RFLP profiles. CTX producing isolates digested with *Pvu*II also gave amplimers with identical RFLP profiles (Figure 2).

Fifty six percent of isolates were resistant to piperacillin and 8% showed intermediate level of resistance to this antibiotic. Combination of this antibiotic with tazobactam reduced the resistance rate to 12%. Resistance to ciprofloxacin was found among 32% of ESBL producing *K. pneumoniae* strains in this study. Statistical analysis showed that there was significant correlation between the existence of ESBL and resistance to ciprofloxacin ($P < 0.05$).

Table 2. Distribution of *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX} and *bla*_{PER} genes based on the clinical specimens in ESBL positive strains of *Klebsiella pneumoniae*.

sample	total	ESBL (+) N (~ %)	<i>bla</i> _{PER} frequency	<i>bla</i> _{CTX} frequency	<i>bla</i> _{TEM} frequency	<i>bla</i> _{SHV} frequency
Urine	137	46 (33)	8	25	10	25
Sputum	22	15 (68)	4	8	12	12
Wound	14	8 (57)	1	6	5	4
Blood	9	2 (22)	1	2	2	1
CVL	2	--	-	-	-	-
Abscess	3	1 (33)	-	1	1	1
Throat	3	--	-	-	-	-
Dialysis	1	--	-	-	-	-
Stool	1	--	-	1	-	-
Vaginal	1	--	-	-	-	-
Sperm	1	--	-	-	-	-
Trachea	2	1 (50)	-	1	-	-
CSF	4	4 (100)	1	1	2	3
Total	200	77	15	45	32	46

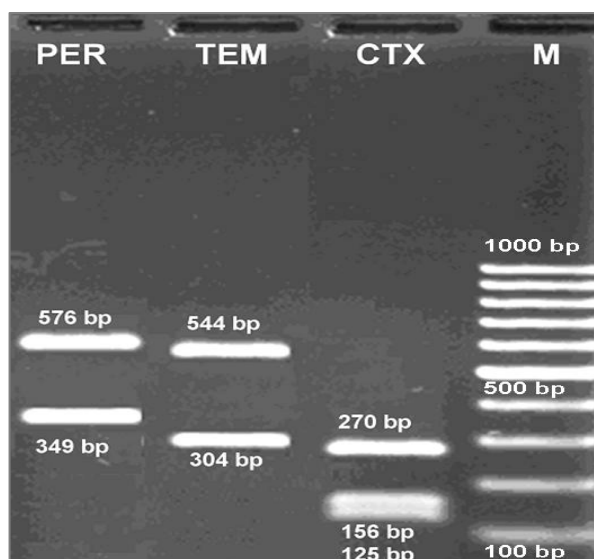


Figure 2. PCR-RFLP patterns of *bla*_{CTX} amplimers digested with *pvuII* and *bla*_{TEM}, *bla*_{PER} amplimers digested with *pstI*. Lane M, 100bp DNA ladder.

Discussion

K. pneumoniae is an important nosocomial pathogen that has the potential to cause severe morbidity and mortality. In recent years following extensive use of the expanded spectrum cephalosporines, outbreaks of infection caused by extended spectrum beta lactamase producing *K. pneumoniae* have been widely reported throughout the world (18). The production of ESBLs is a major threat to the use of new generation of cephalosporins (19, 20). Long hospitalization, diabetes, age over 60 and previous antibiotic treatment have been reported as the risk factors to acquire infections with ESBL strains (21).

In our study antimicrobial susceptibility testing showed that 41% of isolates were resistant to at least one of the third generation cephalosporines. Of totally 200 isolates, 77 isolates (38.5%) showed ESBL phenotype by PCT which is different from the rates of ESBLs in other countries in our region such as India (97.1%), Turkey (57%) and South Korea (30%) (22-24). However, previous studies from Iran about ESBL positive strains of *K. pneumoniae* is rare; Feizabadi *et al* in 2006 detected 44.5% ESBL positive rate among clinical *K. pneumoniae* isolated from clinical specimens in Tehran (8). The rates of ESBL producing *K. pneumoniae* isolated from Tehran reported by Aminzadeh *et al* was

52.5% in 2008 (9). Ramazanzadeh *et al* reported 34.8% of ESBL producing strains of Gram-negative bacteria isolated from Kurdistan (10). In 2009 Bazzaz *et al* also indicated that the prevalence of ESBL-producing strains of *E. coli* and *K. pneumoniae* was 59.2% in our country (11).

As 77 of 80 ceftazidime resistant isolates were ESBL positive in this study, it appears that ESBL production has a significant role in resistance to cephalosporines rather than other mechanisms of resistance such as loss of porins and efflux pumps in our research (25-26).

The MICs of ceftazidime for the majority of ESBL positive isolates (n=72) was >32µg/ml. However, these isolates were susceptible to imipenem. So, the best coverage against ESBL-producing isolates was obtained with imipenem (0% resistance), followed by piperacillin/tazobactam (22% resistance). Since ESBL encoding genes are generally found on plasmids, many of the organisms that harbor ESBLs also are resistant to other classes of antibiotics such as aminoglycosides, flouroquinolons, tetracyclines, chloramphenicol and sulfonamides (27). We obtained the same results in our study: the rate of resistance to cephalosporines, aminoglycosides and flouroquinolons in ESBL positive strains is higher compared to all of studied isolates (Figure 1). In accordance with our results, 41% of ESBL positive strains ($P < 0.05$) were resistant to ciprofloxacin which is lower than Lautenbach's report (60%) (28).

Our result show that replacing of cephalosporins with antibiotic containing β lactamase inhibitors (piperacillin-tazobactam) may help to reduce the occurrence of ESBLs producing organism as recommended by the others (29, 30). However high rate of intermediate resistant isolates is concerning since they probably become fully resistant with continuous use of piperacillin/tazobactam. We recommend that the prescription of piperacillin/tazobactam be restricted to the susceptible isolates only.

The prevalence of ESBLs varied from one hospital to another. It is uncertain whether this is due to the differences in infection control

practices among hospitals or to differences in use of new cephalosporins. The percentage of ESBLs belonging to private and general hospitals in this study was high and approximately the same. It seems that there are poor infection control practices and treatment policies in both private and general hospitals in Iran.

We detected six isolates with ESBL phenotype from out-patients. The rarity in isolation of ESBL phenotype from out-patients has already been reported by others (31).

K. pneumoniae with ESBL phenotype was found in 6 of 9 isolates which were recovered from patients who underwent kidney transplantation. It appears that ESBLs producing strains create major therapeutic problems in transplant wards. Importantly, 4 isolates from these patients showed resistance to amikacin and gentamicin too.

The prevalence of *bla*_{SHV} and *bla*_{TEM} genes in this study was 23% and 16% respectively which are different from the results of the multi-national study group (67% and 16% respectively) (32). In Iran, Feizabadi *et al* in 2009 showed that 69.7% of *K. pneumoniae* isolated from Tehran were ESBL positive and the prevalence of *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M-I} and *bla*_{CTX-M-III} among these isolates was 54%, 67.4%, 46.51% and 29%, respectively (12).

The CTX-M β -lactamases are now widespread in both nosocomial and community-acquired pathogens (33). In our study 22.5% and 7.5% of strains contained *bla*_{CTX-M} and *bla*_{PER} respectively. The number of CTX-M type ESBLs is rapidly expanding. They have been detected in some geographic areas and are now the most frequent ESBL type worldwide (6).

Different reports of PER type of ESBL in *K. pneumoniae* are available from all over the world. For example in 2003 prevalence of *bla*_{PER} gene in Turkey was 55.5% and in 2006 there was no occurrence in Italy (32, 34). Our data represent the first report on the existence of *bla*_{PER-1} gene among *K. pneumoniae* in Iran. This indicates that ESBLs other than SHV, TEM and CTX-M enzymes may also emerge in *Klebsiellae* in the near future. The results of antibiotic susceptibility testing showed that the

rate of resistant isolates harboring both *bla*_{SHV} and *bla*_{TEM} genes to ceftazidime (91.7%), cefotaxime (79.2%) and piperacillin (90.9%) is lower than the rate of resistant isolates harboring both *bla*_{CTX-M} and *bla*_{PER} genes to these three antibiotics (100%). It indicates the importance of PER to hydrolysis the β -lactam antibiotics. In our study the results of RFLP-PCR showed that all the *bla*_{PER-1}, *bla*_{TEM} and *bla*_{CTX-M} positive strains had a same restriction patterns and thus no new variants was detected in these genes.

Conclusion

The emergence and spread of ESBL-producing *K. pneumoniae* strains is worrisome and usage of cephalosporins against these isolates is ineffective. As imipenem is the drug of choice for serious infection disease nowadays, extensive use of this drug in treatment of infection caused by resistant isolates will enhance. Because of this problem prudent use of β -lactam antibiotics containing an oxyimino group and consistent application of basic infection control procedures in treatment centers is necessary and care should be taken to use imipenem just in critical conditions. Due to importance of ESBL producing organisms and difficult treatment of infections caused by these bacteria, for rapid identification of ESBL producing isolates clinical laboratories should adopt simple test based on CLSI recommendation for confirming ESBL production in enterobacterial species. Laboratory services should be available to support every infection control program. Unfortunately there is rare co-operation between clinical settings and laboratories in Iran. We should always remember that effective treatment of serious infections will only be achieved by close co-operation between clinical and laboratory staff.

Acknowledgment

We thank Professor Patric Nordmann for giving the standard β -lactamase producer strains and we also thank staffs from department of microbiology, Pasteur Institute of Iran specially Miss Shooraj for technical assistance.

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