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ORIGINAL ARTICLE

Antibiotic susceptibility pattern and identification of extended spectrum β-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* from Shiraz, Iran

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ABSTRACT

Background and Objectives: *Klebsiella pneumoniae*, one of the important causes of nosocomial infections, is the most common extended spectrum β-lactamases (ESBLs) producing organism. ESBLs are defined as the enzymes capable of hydrolyzing oxyimino-cephalosporins, monobactams and carbapenems. The aims of this study were to identify ESBL-producing *K. pneumoniae* isolates and detect their antibiotic susceptibility pattern.
Materials and Methods: This cross-sectional study was conducted from December 2012 to May 2013 in teaching hospitals in Shiraz. Clinical specimens from the urine, sputum, wound, blood, throat, and body fluids were isolated and identified as *K. pneumoniae.* Antibacterial susceptibility testing was performed for 14 antibiotics using disk diffusion method according to CLSI guidelines. Isolates showing resistant to at least one of the β-lactam antibiotics were then evaluated for production of β-lactamase enzymes using E-test ESBL and combined disk Method. Also, MICs for ceftazidime and imipenem were determined using E-test. The presence of the bla_{SHV}, bla_{TEM}, bla_{PER} and bla_{CTX-M} genes was assessed by PCR.
Results: Of 144 *K. pneumoniae* isolates from different specimens, 38 (26.3 %) was identified as ESBL producer by phenotypic confirmatory test. All ESBL producing isolates were susceptible to imipenem and meropenem and resistant to aztreonam. The highest rate of resistance belonged to amoxicillin (100%), cefotaxime (50%) and gentamicin (42.3%) and the lowest rates were seen for meropenem (11.8%), imipenem and amikacin (both 15.9%). Sixty-two isolates had MICs≥ 4 µg/mL for ceftazidime, of which 38 were positive for ESBLs in phenotypic confirmatory tests (PCT). The prevalence of bla_{SHV}, bla_{CTX-M}, and bla_{TEM} genes among these isolates were 22.2%, 19% and 16%. bla_{PER} was not detected in the studied isolates.
Conclusions: Due to the relatively high prevalence of ESBLs-producing *K. pneumoniae* isolates in the studied population, it seems that screening of infections caused by ESBL producers can lead to the most effective antibiotics therapies.

Keywords: *Klebsiella pneumoniae,* ESBL, PCR, *E-test ESBL*, Combination disk

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INTRODUCTION

Antimicrobial resistance is a growing problem in many bacterial pathogens and it is one of the particular concerns for hospital-acquired nosocomial infections(1). *Klebsiella pneumoniae* is an opportunistic pathogen that causes a notable proportion of community and hospital acquired infections including urinary tract, pneumonia, septicemia and soft tissue infections. It is resistant to many antibiotics such as extended spectrum cephalosporins because of production of extended spectrum β-lactamases (ESBLs) (2, 3). The most widely used classification is Ambler classification which divides β-lactamases into four classes (A, B, C, D) based on molecular structure. ESBLs are Class A β-lactamases and may be defined as enzymes that hydrolyse and cause resistance to various types of β-lactam antibiotics, including the oxyimino-cephalosporins, monobactams and carbapenems, but not the cephamycins. They are inhibited in vitro by clavulanic acid (4). At first, ESBLs were described in *K. pneumoniae* isolates during 1983 in Europe (5) and in 1989 in the US (6). There are different genotypes of ESBLs. The most common ones are SHV, TEM, and CTX-M types. Their genes are located on chromosomes, plasmids or transposons. All have been increasingly described worldwide. bla_{PER} is a clinically important enzyme with strong ESBLs activity which can efficiently hydrolyze 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 (7). Because of inappropriate use of antibiotics in treatment of infections caused by ESBLs producing pathogens, it seems that studies about appropriate detection and antibiotic resistance pattern of these organisms are necessary (8-10). Therefore, the aims of this study were to characterize the drug susceptibility pattern of *K. pneumoniae* isolated from clinical specimens at five teaching hospitals in Shiraz, Iran, and to investigate the prevalence of ESBLs encoding genes among the isolates by phenotypic and genotypic methods.

MATERIALS AND METHODS

Study area and bacterial isolation. This cross-sectional study was performed at five teaching hospitals in Shiraz, Iran during 6 months from December 2012 to May 2013. *K. pneumoniae* isolates (N=144) were collected from different clinical samples. The identification of isolates were confirmed using standard microbiological techniques. The specimens included the urine (n= 108), sputum (n= 9), wound (n= 7), body fluids (n= 6), blood (n= 5), throat (n= 5) and other samples (n= 4). These isolates were recovered either from hospitalized patients (n= 85) or from outpatients (n= 59). Of total patients, 63 were men and 81 were women. Hospitalized patients defined as they undergo invasive examinations, treatments, and care practices, so the hospital environment may facilitate the transmission of microorganisms among such patients.

Antibiotic susceptibility test. Antimicrobial susceptibility testing was performed using agar disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (11). The antimicrobial disks tested were ceftazidime (CAZ: 30µg), ceftriaxone (CRO: 30µg), cefotaxime (CTX: 30µg), ceftizoxime (ZOX: 30µg) ، amoxicillin (A:10µg), cotrimoxazole (TS: 25µg), cefepime (CPM: 30µg)، cefpodoxime (CPD:30µg), aztreoname (ATM: 30µg), gentamicin (GM: 10µg), amikacin (AN: 30µg)، levofloxacin (LEV: 5µg), imipenem (IMP: 10µg), and meropenem (MEM; 10µg) disks (MAST, UK). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as controls (13). Also, MICs for ceftazidime and imipenem were determined using E-test (Liofilchem, Italy) method (11).

Screening for ESBL producing isolates. The combination disk method was used to detect ESBL producing isolates (12). In brief, pairs of disks contained cefotaxime (30µg), cefpodoxime (30µg) and ceftazidime (30µg) were used with and without clavulanic acid (10µg) on the same inoculated plate containing Muller Hinton agar (Himedia, India). A positive test result was defined as a ≥5 mm increase in the zone diameter compared to a disk without clavulanic acid (11). Also, ESBL E-test (CAZ/CAL) was used for ESBL producing isolates (11).

DNA extraction and molecular assays. Bacterial whole DNA was extracted from ESBLs producing isolates using the small-scale phenol-chloroform extraction method (15) and the extracted DNA was

used as PCR template.

Specific primers, annealing temperature and other details for amplifying the bla_{SHV}, bla_{TEM}, bla_{PER} and bla_{CTX-M} genes by PCR method are shown in Table 1(16). PCR was carried out in 25 µl volume reaction mixtures containing 10 pM of each primer, 200 µM dNTP, 1.5 mM $MgCl_2$, 1.5 µl of template DNA and 1 U *Taq* polymerase in the reaction buffer provided by the manufacturer (CinnaGen, Tehran). The following thermo-cycler program was carried out for PCR experiments: initial denaturation at 94 °C for 4 min and 35 cycles of 1 min at 94 °C, 45 sec at the annealing temperature (62 °C for bla_{SHV}, 63 °C for bla_{CTX-M}, 48 °C for bla_{PER} and 55 °C for bla_{TEM}), 1min at 72 °C and 10 min at 72 °C was considered for the final extension. Then, PCR products were analyzed by agarose gel electrophoresis.

K. pneumoniae ATCC 7881 containing bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes and *P. aeruginosa*, KOAS strain containing bla_{PER}, gene (Kindly provided by Pasteur Institute of Iran, Tehran) were used as the positive controls.

RESULTS

In this study, 59 out of 144 isolates (41%) were from outpatients. The antimicrobial susceptibility testing showed that 79 isolates (54.8%) were resistant to at least one of the third generation cephalosporines. All ESBL-producing isolates were shown to be susceptible to imipenem and meropenem in disk diffusion method. Using the same method, 43.7%، 45.8% and 50% were resistant to ceftazidime، cefpodoxime and cefotaxime, respectively. The rates of resistance to other antibiotics were as follows: 144 (100%) of the *K. pneumoniae* isolates were resistant to amoxicillin, 63 (43.7%) to cotrimoxazole,72 (50%) to cefotaxime, 63 (43.7%) to ceftazidime, 66 (45.8%) to cefpodoxime, 61 (42.3%) to cefteriaxon, 58 (40.2%) to ceftizoxime, 54 (37.5%) to aztreonam, 52 (36.1%) to cefepime, 31 (21.5%) to levofloxacin, 28 (19.4%) to gentamicin, 23 (15.9%) to both amikacin and imipenem, and 17 (11.8%) to meropenem. Sixty-two isolates showed MICs≥ 4 µg/mL for ceftazidime, of which 38 (61.3%) were positive for ESBLs by E-Test ESBL method (Tables 2-3); however, by using combined disks, ceftazidime/clavulanic acid, cefotaxime/clavulanic acid and cefpodoxime/clavulanic acid 35 (%92.1), 34 (%89.4) and 31 (81.5%) of the isolates were identified as β-lactamase producing isolates respectively. 17 (11.8%) isolates were resistant to all antibiotics tested. The ESBL-producing isolates were recovered mostly from the urine (n= 29), blood (n=2), throat (n=1), fluid (n=2), wound (n= 2), sputum (n= 1), and other (n=1) specimens. The resistance rate to the tested antibiotics in ESBLs positive isolates compared to all isolates are shown in Fig. 1.

Table 1. Primer sequences and other details of the ESBL genes used for amplification by PCR method.

Primer name	Sequence (5' to 3')	Amplicon size (bp)	Annealing temperature	Gene	References
CTX-MA	CGCTATTGCGATGTGCAG	550	63 °C	bla_{CTX-M}	16
CTX-MB	ACCTGCGATATCGTTGGT				
PER-A	ATGAATGTCATTATAAAAGC	925	45 °C	bla_{PER}	16
PER-B	AATTTGGGCTTAGGGCAGAA				
TEM-A	GAGTATTCAACATTTCCGTGTC	848	43 °C	bla_{TEM}	16
TEM-B	TAATCAGTGAGGCACCTATCTC				
SWSHV-A	AAGATCCACTATCGCCAGCAG	231	60 °C	bla_{SHV}	16
SWSHV-B	ATTCAGTTCCGTTTCCCAGCGG				

Table 2. Comparison of the MICs of ceftazidime and imipenem among ESBL-producing and other isolates in this study

Antibiotics	ESBL Positive	ESBL Negative	Isolates were resistant to all antibiotics*
Ceftazidime	MIC ≥ 4	MIC ≤ 3	MIC > 32
Imipenem	MIC ≤ 2	MIC ≤ 2	MIC ≥ 6

***** 17 isolates were resistant to all antibiotics

Table 3. The number of *K. pneumoniae* isolates in each studied hospital and the isolates with ESBLs genes

Hospital	Number of isolates	Patients	Isolates with ESBLS genes
Namazi	67 (46.5%)	Outpatient	5
		Hospitalized	16
Faghihi	34 (23.6%)	Outpatient	4
		Hospitalized	7
Ali Asghar	7 (4.9%)	Outpatient	0
		Hospitalized	1
Shooshtari	22 (15.3%)	Outpatient	2
		Hospitalized	1
Mottahari (clinic)	14 (9.7%)	Outpatient	2
		Hospitalized	0
Overall	144 (100%)	Outpatient	13
		Hospitalized	25

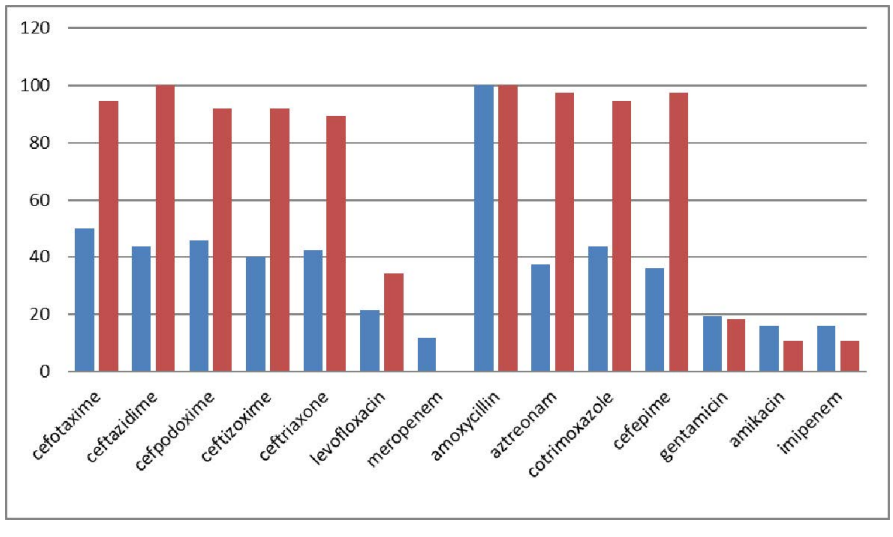

Fig. 1. Comparison of antibiotics resistance rates among ESBL positive isolates (red columns) with all isolates of *K. pneumoniae* (blue columns).

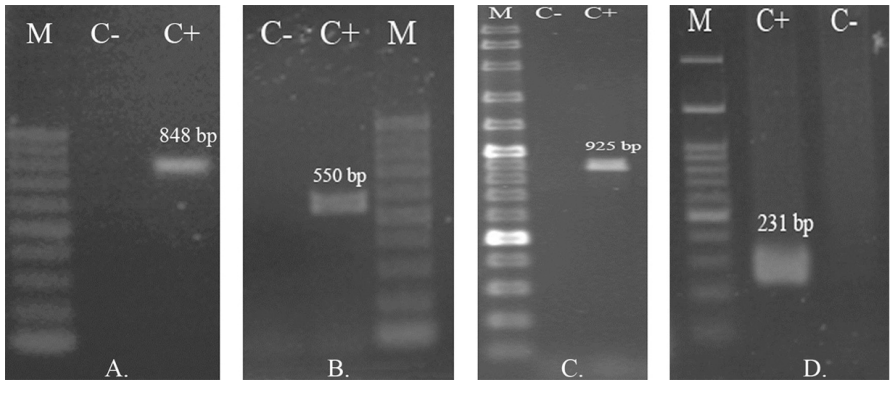

Fig. 2. PCR amplification of β-Lactamase genes of *K. pneumoniae*. M, 100 bp DNA ladder, C-, negative control, C+, positive control.(A)TEM, (B) CTX-M, (C) bla_{PER}, (D) SHV

The bla_{SHV}, bla_{CTX-M}, bla_{TEM}, and bla_{PER} genes were detected in 22.2%, 19%, 16% of our isolates respectively but the bla_{PER} was not detected (Fig. 2). Of 38 isolates identified by ESBLs confirmatory tests, 9 (23.7%) contained all the three genes (SHV, TEM, CTX-M), 17 (44.7%) isolates contained two genes and 12 (31.6%) isolates contained only one gene.

DISCUSSION

In our study, the susceptibility of 144 *K. pneumoniae* isolates to 14 antibiotics was assayed. 17 isolates of these were resistant to all antibiotics. That could be a serious risk for the people who were infected with such isolates. Multi-drug resistant strains (MDR) are those strains of a bacteria which are resistant to three classes of antibiotics (13). The present study showed a remarkable prevalence of ESBL producing *K. pneumoniae* isolates, 21% of *Klebsiella pneumoniae* isolates from patients were MDR. In the meantime, bla_{SHV} type of β-Lactamase was more frequently observed. There was not found any significant relationship between a particular sample and resistance rates (>05) but resistance was significantly increased among hospitalized patients ($p < 05$). It appears that the resistant isolates among the admitted patients are transferred to other patients through the personnel or medical equipment. Also, it is possible that these isolates are increasing through the inappropriate use of antibiotics in patients during the natural selection and can be dangerous, causing serious infections. In this study, the highest resistance rate was observed against β-lactam antibiotics, particularly penicillin and cephalosporins. The probable reason for this high resistance rate is inappropriate use of such antibiotics. MIC of ceftazidime for the majority of ESBL positive isolates (n=38) was >32µg/mL. However, these isolates were susceptible to imipenem. So, the best treatment coverage against ESBL-producing isolates was obtained with imipenem and meropenem. In our study, resistance rates to aminoglycoside antibiotics such as amikacin & gentamicin were 16% and 19.4%, respectively; this rates were close to imipenem. Similar to our study, combination treatment (imipenem + amikacin) has been used to treat infections caused by ESBL producing bacteria in other studies. The reason for this is that the bactericidal activity of this combination is more than imipenem alone, for the treatment of life threatening infections such as sepsis, hospital acquired pneumonia and intra-abdominal abscess. In this study, the resistance frequency of *Klebsiella pneumoniae* isolates to ceftazidime and cefotaxime was 43.7% and 50%, respectively. Like our study, in a study conducted by Nasehi et al. in (2010), such resistance rates were 34.7% and 33.5%, respectively. Also, this resistance rate in the study of Eftekhar et al. in 2012 was 49% and 37.2%, respectively (11, 16). From results of this study and mentioned studies, it is found that the resistance to broad-spectrum cephalosporins is increasing in recent years (except for the particular cases where studies have been carried out in certain wards of the hospital). Among the different antibiotic classes (except for cephalosporins) one antibiotic such as aztreonam, aminoglycosides, flouroquinolons, were chosen as representative; the maximum resistance was observed to aztreonam and minimum resistance was seen to amikacin, which is consistent with the studies conducted in the recent years (14, 15). In relation to the third-generation cephalosporins, the highest resistance rate was to cefotaxime (50%) and the lowest rate of resistance of isolates was to ceftizoxime (40.2%); the reason for this is probably lower use of this antibiotic. Of a total of 144 isolates, 38 (26.3%) showed ESBLs phenotype by combined disk Method and E-test ESBL which is different from the rate of ESBL producing isolates reported from other countries such as India (97.1%), Turkey (57%) and South Korea (30%), (14). About 38 (100%) isolates were identified as ESBL producer by using E-test ESBL ceftazidime. However, using the combined disks, ceftazidime/clavulanic acid, cefotaxime/clavulanic acid and cefpodoxime/clavulanic acid, were 35 (92.1%), 34 (89.4%) and 31 (81.5%) of the isolates were identified as β-lactamase producing isolates respectively. However, previous studies from Iran about the prevalence of ESBL positive strains of *K. pneumoniae* report different results; Feizabadi et al. in 2006 detected 44.5% ESBL positive rate among *K. pneumoniae* isolates from clinical specimens in Tehran (15). The rate of ESBL producing *K. pneumoniae* isolated from Tehran reported by Aminzadeh et al. was 52.5% in 2008 (16). Ramazanzadeh et al. reported a rate of 34.8% of ESBL producers among Gram-negative bacteria isolated from Kurdistan (17). In 2009, Bazzaz et al. also indicated that the prevalence of ESBL producing strains of *E. coli* and *K. pneumoniae* was 59.2% (18). Of 38 ESBL positive isolates, 37 were resistant to aztreonam; this reflects the high sensitivity of this antibiotic in differentiation

between ESBL isolates. So, all ESBL-positive isolates were resistant to at least one or two cephalosporins while the majority of the isolates were resistant to aztreonam antibiotic. In the present study, to compare the sensitivity and specificity of confirmatory phenotypic test, we also used ESBL E-test strips in which 3 isolates was added to the ESBL positive isolates (in the confirmatory phenotypic test by the combined disk method, 35 isolates were ESBL positive, in total ESBL positive isolates increased to 38), However, the ESBL E-test strips also confirmed 35 isolates identified by combined disks. These results were consistent with the results of Mohanty et al. in 2010 (19). The PCR results for the four types of ESBL, respectively, were as follows: 28 (%19.4) of isolates contained CTX-M gene, 32 (% 22.2) of isolates had SHV gene, 23 (% 16) of isolates contained TEM gene, but we did not find the PER gene in any isolate. The prevalence of three genes, CTX-M, TEM and SHV was consistent with a study done by Feizabadi et al. and also a study carried out by Nasehi and et al; in these studies, the most prevalence was related to SHV gene (20), while the prevalence of *PER* gene in Tehran was 7.5%. like present study in a study conducted in Mashhad in 2012, this gene showed no prevalence (21). In studies conducted in most European countries, American Latina and Eastern Asia, the most prevalence rate was related to SHV, TEM, and CTX-M types. However, TEM variants and SHV gene has been replaced by CTX-M in Gram negative bacteria including *K. pneumoniae* as the dominant β- Lactamase gene (22). In a study conducted in Canada during 2000- 2008, it was shown that the number of ESBL producing isolates until 2003 has remained very low. Then, there was a relative increase in 2004 to 2008 which remained stable. In 2009, a sharp increase in the number of ESBL producing isolates occurred. But the majority of ESBL producing bacteria had genotype of CTM-M-14 and 15. After 2005, the genotype SHV-12 was replaced with that (22). The studies conducted suggest that the SHV gene has more transmission power than other genes so that in most areas of the world this gene has been replaced by other genes. At the end, the main limitations of this study were small sample size, as well as the number of studied centers so; the results may not be generalized to all health centers in our region. Also, the isolates resistant to carbapenems may contain broad spectrum β-lactamase enzyme (KPC-type) or MBL enzymes whose phenotypic detection method is different from other genes.

In conclusion, considering such prevalence rate of ESBLs producing bacteria found in our study, screening for infections caused by ESBL-producing bacteria is important in the treatment and selection of the drugs of choice. It seems necessary for clinicians and health care systems to be fully aware of ESBLs producing microorganisms. Also, monitoring of the ESBLs production is recommended to avoid treatment failure and effective infection control in Iran.

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