First report of TEM-104-, SHV-99-, SHV-108-, and SHV-110-producing *Klebsiella pneumoniae* from Iran

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Abstract

Introduction: Extended-spectrum beta-lactamases (ESBLs) are bacterial enzymes capable of hydrolyzing beta-lactams. The aim of this study was to describe the prevalence of TEM- and SHV-type ESBL-producing *Klebsiella pneumoniae* strains in Zahedan, Southeast Iran. **Methods:** A total of 170 non-repetitive *K. pneumoniae* strains were collected from patients referred to three teaching hospitals of Zahedan. Antibiotic susceptibility testing was determined for 17 antibiotics using the Kirby-Bauer disc diffusion method. The frequency of ESBL-producing strains was calculated, and minimum inhibitory concentrations of ESBL-producing strains were determined for cefotaxime, ceftazidime, ceftriaxone, and cefpodoxime. The presence of bla_{TEM} and bla_{SHV} genes was tested in all ESBL-producing strains using polymerase chain reaction and DNA sequencing. **Results:** Among the 170 *K. pneumoniae* clinical isolates, 55 (32.4%) were ESBL producers; 92.7% (n=51) and 72.7% (n=40) of the isolates carried the bla_{SHV} and bla_{TEM} genes, respectively, and 67.3% (n=37) carried both genes. The sequencing results showed that all bla_{TEM} types were bla_{TEM-1}, except for two isolates that were $bla_{TEM-104}$. The bla_{SHV} types were bla_{SHV-1}, bla_{SHV-11}, bla_{SHV-12}, bla_{SHV-99}, $bla_{SHV-108}$, and $bla_{SHV-110}$. **Conclusions:** The percentage of bla_{TEM} and bla_{SHV} among ESBL-producing *K. pneumoniae* isolates from Zahedan is relatively high, indicating the need for further surveillance and consideration in antibiotic use. To the best of our knowledge, this is the first report of TEM-104-, SHV-99-, SHV-108-, and SHV-110-type ESBLs among clinical isolates of *K. pneumoniae* from Iran, and TEM-1, SHV-1, SHV-11, and SHV-12 appear to be the dominant ESBLs in this region.

Keywords: *Klebsiella pneumoniae*. Extended-spectrum beta-lactamases. Antibiotic resistance.

INTRODUCTION

Klebsiella pneumoniae is an important opportunistic nosocomial pathogen responsible for a variety of infections, including urinary tract infections, pneumonia, septicemia, wound infections, and infections in intensive care units[1]. This bacterium is one of the leading causes of community-acquired and nosocomial infections and is associated with high mortality rates[2]. The prevalence of *K. pneumoniae* with multi-drug resistance, which commonly leads to systemic infections and death, has been increasing in recent years[3]. The emergence of infections caused by these multidrug-resistant strains is a clinical challenge, with limited and complex treatment options[4]. Beta-lactam antibiotics (including penicillins, cephalosporins, carbapenems, or monobactams) have been widely applied in the treatment of serious infections caused by Gram-negative

bacteria since the 1980s, contributing to the rapid spread of bacteria resistant to these antibiotics worldwide[5]. The main mechanism of resistance is the production of beta-lactamases, which can protect bacteria against the lethal effect of beta-lactam antibiotics on cell wall synthesis[6]. Hydrolysis of beta-lactam antibiotics by beta-lactamases is the most common mechanism of resistance for this class of antibacterial agents in clinically important Gram-negative bacteria[7]. Extended spectrum beta-lactamases (ESBLs) were first described in 1983, which are capable of hydrolyzing oxyimino-cephalosporins (e.g., cefotaxime, ceftazidime, and ceftriaxone) and monobactams (e.g., aztreonam), but not cephamycins or carbapenems[8]. Beta-lactamase inhibitors (such as clavulanic acid, sulbactam, and tazobactam) prevent the action of ESBLs[9] [10]. Most of the genes encoding ESBLs are plasmid-borne and are often located in the transposons and integrons, which facilitates their mobilization with other resistance determinants. Thus, the genes encoding ESBLs may be easily transferred between bacteria. The most prevalent ESBLs are included in three groups: temoneira (TEM), sulfhydryl variable (SHV), and cefotaximase (CTX-M). The SHV enzymes are named after the thiol variable active

site and are commonly associated with *K. pneumoniae*. The TEM enzyme was named after the patient from whom it was isolated, Temoneira, and was first discovered in *Escherichia coli* in Greece. In addition, chromosomal-related CTX-M-type ESBL was reported in *Kluyvera ascorbata*[11]. Detection of ESBL production is important, because ESBL-positive strains are associated with increased mortality as compared to ESBL-negative strains. One major concern is the spread of ESBL-positive bacteria within hospitals, which may lead to outbreaks or to endemic occurrence. Another concern is the failure to treat infections caused by ESBL-positive organisms, as therapeutic choices are currently limited[1]. Detection of ESBLs by conventional antimicrobial susceptibility methods and delay in the detection and reporting of ESBL production by Gram-negative bacilli are associated with prolonged hospital stays, and increased morbidity, mortality, and healthcare costs[9]. Relatively little research on ESBL-producing bacteria has been conducted in the Southeastern region of Iran, and the resistance patterns of bacterial strains, especially the ESBL-producing mechanism, has not been determined in this region. Therefore, the aim of the present study was to evaluate the prevalence of TEM- and SHV-type ESBL-producing *K. pneumoniae* in Zahedan, Southeast Iran, using phenotypic and genotypic methods.

METHODS

Isolation of bacteria

Klebsiella pneumoniae isolates were obtained from 170 non-repetitive clinical samples, including urine, tracheal tube secretions, blood, wound secretions, urine catheter secretions, synovial fluid, and pharyngeal secretions, collected from patients referred to three teaching hospitals (Khatam al Anbiya, Ali ibn Abi Talib, and Buali) of Zahedan, Southeast Iran from January to December 2012. Collected samples were cultured on blood agar and MacConkey agar **(**Merck, Darmstadt, Germany) media. *K. pneumoniae* strains were identified based on standard microbiology methods such as Gram staining, oxidase and catalase tests, as well as biochemical tests with reference to standard tables[12].

Ethical considerations

This study was evaluated and approved by the Ethics Committee of Zahedan University of Medical Sciences (project No. 90-17).

Antimicrobial susceptibility testing

The resistance profiles and antibiotic susceptibility of isolates were determined using the disc diffusion method (Kirby-Bauer) based on Clinical and Laboratory Standards Institute (CLSI) guidelines[13]. The antibiotic discs (MAST, Merseyside, UK) used were imipenem (10μg), amikacin (30μg), gentamicin (10μg), ciprofloxacin (5μg), ceftazidime (30μg), cefotaxime (30μg), cefpodoxime (10μg), ceftriaxone (30μg), aztreonam (30μg), cefepime (30μg), nalidixic acid (30μg), nitrofurantoin (300μg), co-trimoxazole (25μg), tetracycline (30μg), chloramphenicol (30μg), streptomycin (10μg), and colistin sulfate (25μg). *Escherichia coli* ATCC 25922 was employed for the quality control of antibiotic discs**[14].**

Screening for ESBL-producing isolates

Based on the CLSI recommended method for screening of ESBL-producing isolates, the phenotypic confirmatory test was conducted with the disc diffusion method using discs containing cefotaxime (30μg) and cefotaxime/clavulanic acid (30/10μg), as well as those containing ceftazidime (30μg) and ceftazidime/clavulanic acid (30/10μg)[14].

Measurement of the minimum inhibitory concentration of the isolates

The minimum inhibitory concentration (MIC) values of four antibiotics, cefotaxime, ceftazidime, ceftriaxone, and cefpodoxime, were determined for isolates with an ESBL-positive phenotype using the E-Test method (Liofilchem MIC test strips, Italy). The concentration gradient for cefotaxime was 0.002-32μg/ml, and that for ceftazidime, ceftriaxone, and cefpodoxime was 0.016-256μg/ml. The interpretative criteria were applied following the E-test manufacturer's guidelines and according to CLSI recommendations. *E. coli* ATCC 25922 was used as a quality control for the MIC test strips[14] [15]**.**

DNA extraction and polymerase chain reaction amplification

After performing the MIC measurements and determining the resistant and ESBL-producing samples, the samples deemed resistant in the phenotypic confirmatory test were selected to evaluate the presence of the beta-lactamase genes bla_{TEM} and bla_{SHV} using polymerase chain reaction (PCR). The plasmid deoxyribonucleic acid (DNA) of ESBLs-positive isolates, which was extracted with AccuPrep Plasmid Nano-Plus Plasmid Mini Extraction Kit Cat. No.: K-3112 (Bioneer, Daejeon, South Korea), was used for detection of the bla_{TEM} gene. In addition, the chromosomal DNA of isolates with an ESBLs-positive phenotype was isolated with the Molecular Biological System Transfer (MBST) DNA extraction kit (Iran), and used for detection of the bla_{SHV} gene. The primers and PCR conditions described by Nasehi et al.[16] were used to amplify the bla_{TEM} and bla_{SHV} genes[16].

Sequencing of PCR products

To confirm the accuracy of the test as well to determine the subtypes of the *bla* genes detected, samples testing positive for the bla_{TEM} and bla_{SHV} genes were sequenced by Bioneer Company, and the results were analyzed using the BLAST program (www.ncbi.nlm.-nih.gov5 BLAST).

Statistical analysis

The data were qualitatively described (percentage and frequency) using SPSS Software (version 18).

RESULTS

In this study, 170 isolates of *K. pneumoniae* were collected from three major hospitals of Zahedan: 128 (75.3%) isolates from Khatam al Anbiya Hospital, 25 (14.7%) isolates from Ali ibn Abi Talib Hospital, and 17 (10%) isolates from Buali Hospital. **Table 1** displays the pattern of antibiotic resistance of all isolates for the 17 different antibiotics tested.The rates

TABLE 1

Antimicrobial susceptibilities of the *Klebsiella pneumoniae* isolates (n=170).

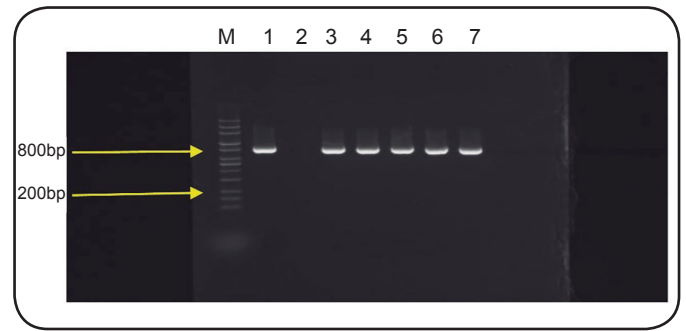| Antibiotic	Sensitive n (%)	Intermediate n (%)	Resistant n (%)
Cefepime	118 (69.4)	15 (8.8)	37 (21.8)
Cefotaxime	99 (58.2)	1 (0.6)	70 (41.2)
Ceftriaxone	99 (58.2)	2 (1.2)	69 (40.6)
Ceftazidime	100 (58.8)	11 (6.5)	59 (34.7)
Cefpodoxime	98 (57.6)	1 (0.6)	71 (41.8)
Aztreonam	97 (57.1)	10 (5.8)	63 (37.1)
Imipenem	156 (91.8)	6 (3.5)	8 (4.7)
Gentamicin	126 (74.1)	0 (0.0)	44 (25.9)
Amikacin	161 (94.7)	2 (1.2)	7 (4.1)
Streptomycin	115 (67.7)	6 (3.5)	49 (28.8)
Tetracycline	105 (61.8)	5 (2.9)	60 (35.3)
Ciprofloxacin	126 (74.1)	10 (5.9)	34 (20.0)
Nalidixic acid	108 (63.5)	22 (13.0)	40 (23.5)
Co-trimoxazole	98 (57.6)	0 (0.0)	72 (42.4)
Chloramphenicol	154 (90.6)	0 (0.0)	16 (9.4)
Nitrofurantoin	60 (35.3)	13 (7.6)	97 (57.1)
Colistin sulfate	168 (98.8)	0 (0.0)	2 (1.2)

FIGURE 1. PCR amplification of the bla_{TEM} gene. Lane M: 100 bp-3k b ladder, lane 1: *E. coli* ATCC 35218 as a positive control; lane 2: *E. coli* ATCC 25922 as a negative control; lanes 3-7: bla_{TEM}-positive clinical isolates of *K. pneumoniae*; PCR: polymerase chain reaction; **E.:** *Escherichia;* **K.:** *Klebsiella.*

TABLE 2

Antimicrobial susceptibilities of ESBL-producing *Klebsiella pneumoniae* isolates (n=55).

Antibiotic	Sensitive n (%)	Intermediate n (%)	Resistant n (%)
Cefepime	18 (32.7)	14 (25.5)	23 (41.8)
Cefotaxime	0 (0.0)	0 (0.0)	55 (100.0)
Ceftriaxone	0 (0.0)	1 (1.8)	54 (98.2)
Ceftazidime	1 (1.8)	11 (20.0)	43 (78.2)
Cefpodoxime	0 (0.0)	0 (0.0)	55 (100.0)
Aztreonam	0 (0.0)	10 (18.2)	45 (81.8)
Imipenem	52 (94.5)	0 (0.0)	3 (5.5)
Gentamicin	25 (45.5)	0 (0.0)	30 (54.5)
Amikacin	51 (92.7)	0 (0.0)	4 (7.3)
Streptomycin	15 (27.3)	3 (5.5)	37 (67.2)
Tetracycline	15 (27.3)	1 (1.8)	39 (70.9)
Ciprofloxacin	27 (49.1)	8 (14.5)	20 (36.4)
Nalidixic acid	16 (29.1)	15 (27.3)	24 (43.6)
Co-trimoxazole	6 (10.9)	0 (0.0)	49 (89.1)
Chloramphenicol	44 (80.0)	0 (0.0)	11 (20.0)
Nitrofurantoin	34 (61.8)	5 (9.1)	16 (29.1)
Colistin sulfate	55 (100.0)	0 (0.0)	0 (0.0)

ESBL: extended-spectrum β-lactamase.

of resistance in *K. pneumoniae* isolates to ceftazidime, cefotaxime, ceftriaxone, cefpodoxime, and aztreonam antibiotics were 34.7%, 41.2%, 40.6%, 41.8%, and 37.1%, respectively.

Table 2 shows the pattern of antibiotic resistance of the ESBL-producing isolates. The maximum level of resistance was observed against cefpodoxime and cefotaxime (100%), followed by ceftriaxone (98.2%), co-trimoxazole (89.1%), aztreonam (81.8%), ceftazidime (78.2%), tetracycline (70.9%), streptomycin (67.2%), gentamicin (54.5%), cefepime (41.8%), and nalidixic acid (43.6%). Colistin sulfate, imipenem, amikacin, and chloramphenicol were associated with susceptibility values of 100%, 94.5%, 92.7%, and 80%, respectively, and were thus identified as the most effective antibiotics in the ESBL-producing isolates.

The phenotypic confirmatory test showed that among the 170 isolates, 55 (32.4%) were ESBL producers. According to the CLSI criteria, if the MIC of cefpodoxime is ≥8 or if the MICs of cefotaxime, ceftazidime, and ceftriaxone are ≥2 for a bacterial strain, it can be considered as an ESBL producer[14]. Using these criteria, all 55 isolates were identified as ESBL producers to the four antibiotics mentioned above according to the MIC results.

The PCR assay of the ESBL-producing isolates indicated that 40 (72.7%) isolates were positive for the bla_{TEM} gene, and 51 (92.7%) isolates had the bla_{SHV} gene. Moreover, 37 (67.3%) of the isolates had both the bla_{TEM} and bla_{SHV}. **Figure1** and

Figure 2 shows the electrophoretic patterns of the bla_{TEM} and bla_{SHV} genes, respectively.

Overall, 91 positive PCR products, including 40 samples related to the bla_{TEM} gene and 51 samples related to the bla_{SHV} gene, were sequenced and analyzed. The majority of those positive for the bla_{TEM} gene were of the bla_{TEM-1} subtype, with
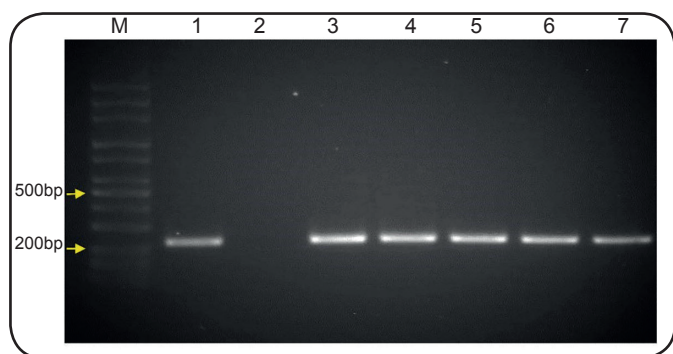
FIGURE 2. PCR amplification of the bla_{SHV} gene. **Lane M:** 100 bp-3k b ladder; **lane 1:** *K. pneumoniae* ATCC 700603 as a positive control; **lane 2:** *E. coli* ATCC 25922 as a negative control; **lanes 3-7:** bla_{SHV}-positive clinical isolates of *K. pneumoniae*; **PCR:** polymerase chain reaction; **E.:** *Escherichia*; **K.:** *Klebsiella*.

only two samples showing a different subtype, $bla_{TEM-104}$. Among the samples positive for the bla_{SHV} gene, 17 samples were bla_{SHV-11} subtype, 16 samples were bla_{SHV-1} subtype, 14 samples were bla_{SHV-12} subtype, 2 samples were $bla_{SHV-110}$ subtype, 1 sample was bla_{SHV-99} subtype, and 1 sample was $bla_{SHV-108}$ subtype.

DISCUSSION

Resistance in *Klebsiella pneumoniae* strains to a wide spectrum of antibiotics is rapidly increasing. Therefore, awareness toward the prevalence and extent of resistant strains to antibiotics can help to determine the effectiveness of different antibiotics for the treatment of infections. The prevalence rate of infections caused by organisms that are resistant to beta-lactam antibiotics is increasing. Therefore, identification of ESBL-producing strains is a great necessity in both hospitals and the community[17]. In this study, the rates of resistance in *K. pneumoniae* isolates to ceftazidime, cefotaxime, ceftriaxone, cefpodoxime, and aztreonam antibiotics were higher than those reported by Ghafourian et al[18], from isolates in Milad Hospital of Tehran (the capital of Iran), which were 65.8%, 46.4%, 60.4%, 52%, and 51%, respectively, indicating a trend toward increasing resistance to these antibiotics in Iran. Furthermore, we identified an overall frequency of 32.4% ESBL producers, which is less than those reported in Turkey (69%)[10], Taiwan (97%)[11], India (97%)[19], Saudi Arabia (55%)[20], and Nepal (62.7%)[21], but higher than those reported in Lebanon (20%)[22], the United States (7.6%), and Canada (4.9%)[23]. Al-Agamy showed that the rates of resistance of ESBL-producing isolates to cefotaxime, ceftazidime, aztreonam, cefepime, gentamicin, amikacin, and ciprofloxacin were 97.7%, 95.5%, 97.7%, 47.8%, 88.9%, 77.3%, and 11.4%, respectively, which supports our findings for cefotaxime, but indicates a higher resistance rate to the other antibiotics. Furthermore, Al-Agamy identified that all ESBL-producing isolates were susceptible to imipenem[20], whereas in our study, 5.5% of the ESBL-producing strains were resistant to imipenem. Although this is currently a small proportion, this finding can be considered a warning for the future, highlighting the necessity of further surveillance. In the present study, 100% of the ESBL-positive strains were

resistant to cefotaxime and cefpodoxime, 98.2% were resistant to ceftriaxone, 81.8% were resistant to aztreonam, and 78.2% were resistant to ceftazidime, which is indicative of the overall high rate of antibiotic resistance in this region. Therefore, to prevent a further increase in the resistance to these antibiotics, prescriptions must be carefully considered in each case. According to our study, colistin sulfate, imipenem, amikacin, and chloramphenicol are more appropriate choices for the treatment of infections induced by ESBL-producing isolates, regardless of their side effects. Since these drugs are more expensive and can cause more complications, the accurate control and logical prescription of cephalosporins might result in better outcomes. The present study further showed that the bla_{SHV} gene is highly (92.7%) prevalent in clinical ESBL-producing strains, and the most frequent subtypes were SHV-11, SHV-1, and SHV-12. In 2007, Al-Agamy et al.[20] showed that 214 (97.3%) of 220 strains isolated in Saudi Arabia carried the bla_{SHV} gene, which is higher than that found in our study[20]. A study conducted by Ghafourian et al.[24] in 2011 in Iran determined that 94% of *K. pneumoniae* strains carried the bla_{SHV} gene, which is in parallel with our results[24]. In recent years, the high relative prevalence of the bla_{SHV} gene in ESBL-producing *K. pneumoniae* strains has been reported worldwide. The prevalence of ESBL-producing *E. coli* and *K. pneumoniae* isolates (TEM- and SHV-type ESBLs) was less than 1% in 1997 in the Netherlands, which is a country known to employ antibiotics minimally; however, more recent studies indicate not only an increasing rate of resistance but also the emergence of new types such as SHV-31 in *K. pneumoniae*[25]. In the present study, 72.7% of the strains carried the bla_{TEM} gene, and the major subtype was TEM-1. These results are similar to those reported in India and Turkey, with a prevalence of 73% and 73.4%, respectively[10] [26]. Feizabadi et al. conducted a similar study on *K. pneumoniae* strains in Hospitals of Tehran in 2007, and also found that TEM-1, SHV-12, and SHV-1 were the most prevalent subtypes[27]. In a subsequent study, Feizabadi et al.[27] further identified that all of the *K. pneumoniae* isolates from a Labbafinejad Hospital that carried the bla_{TEM} gene were the TEM-1 subtype, and TEM-1, SHV-11, SHV-5, and SHV-12 were reported as the dominant ESBLs in resistant *K. pneumoniae* strains in Iran[28]. Overall, it seems that the majority of ESBLs in *K. pneumoniae* strains of Zahedan are derived from the bla_{SHV} and bla_{TEM} genes, with SHV-1, SHV-11, SHV-12, and TEM-1 as the most common subtypes. Moreover, the TEM-104, SHV-99, SHV-108, and SHV-110 subtypes were reported for the first time in Iran in this study. In conclusion, the prevalence of ESBLs is relatively high in the Southeast region in Iran, indicating that further studies are warranted to collect comprehensive epidemiological data of resistant strains.

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