First report of TEM-104-, SHV-99-, SHV-108-, and SHV-110-producing \textit{Klebsiella pneumoniae} from Iran

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\textbf{Abstract}

\textbf{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 \textit{Klebsiella pneumoniae} strains in Zahedan, Southeast Iran. \textbf{Methods}: A total of 170 non-repetitive \textit{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 \textit{bla}_{TEM} and \textit{bla}_{SHV} genes was tested in all ESBL-producing strains using polymerase chain reaction and DNA sequencing. \textbf{Results}: Among the 170 \textit{K. pneumoniae} clinical isolates, 55 (32.4\%) were ESBL producers; 92.7\% (n=51) and 72.7\% (n=40) of the isolates carried the \textit{bla}_{TEM} and \textit{bla}_{SHV} genes, respectively, and 67.3\% (n=37) carried both genes. The sequencing results showed that all \textit{bla}_{TEM} types were \textit{bla}_{TEM-1} except for two isolates that were \textit{bla}_{TEM-4}. The \textit{bla}_{SHV} types were \textit{bla}_{SHV-1}, \textit{bla}_{SHV-9}, \textit{bla}_{SHV-11}, \textit{bla}_{SHV-90}, \textit{bla}_{SHV-101}, \textit{bla}_{SHV-108}, \textit{bla}_{SHV-110}, \textit{bla}_{SHV-111}. \textbf{Conclusions}: The percentage of \textit{bla}_{TEM} and \textit{bla}_{SHV} among ESBL-producing \textit{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 \textit{K. pneumoniae} from Iran, and TEM-1, SHV-1, SHV-11, and SHV-12 appear to be the dominant ESBLs in this region.

\textbf{Keywords}: \textit{Klebsiella pneumoniae}. Extended-spectrum beta-lactamases. Antibiotic resistance.

\section*{INTRODUCTION}

\textit{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\textsuperscript{(1)}. This bacterium is one of the leading causes of community-acquired and nosocomial infections and is associated with high mortality rates\textsuperscript{(2)}. The prevalence of \textit{K. pneumoniae} with multi-drug resistance, which commonly leads to systemic infections and death, has been increasing in recent years\textsuperscript{(3)}. The emergence of infections caused by these multidrug-resistant strains is a clinical challenge, with limited and complex treatment options\textsuperscript{(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\textsuperscript{(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\textsuperscript{(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\textsuperscript{(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\textsuperscript{(8)}. Beta-lactamase inhibitors (such as clavulanic acid, sulbactam, and tazobactam) prevent the action of ESBLs\textsuperscript{(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 Klebsiella 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 is not easy due to the appearance of amplicon-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. 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, sputum, 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), cefazidime (30µg), cefotaxime (30µg), ceftriaxone (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 ceftazidime (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 antibiotics, such as amikacin, cefotaxime, ceftriaxone, and cefpodoxime, were determined for isolates with an ESBL-positive phenotype using the E-Test method (Liofichem 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 blaTEM and blaSHV 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 blaTEM 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 blaSHV gene. The primers and PCR conditions described by Nasheh et al. (16) were used to amplify the blaTEM and blaSHV 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 blaTEM and blaSHV genes were sequenced by Bioneer Company, and the results were analyzed using the BLAST program (www.ncbi.nlm.nih.gov 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
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_ genes. Figure 1 and Figure 2 show 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...
only two samples showing a different subtype, bla\text{TE4M-104}. Among the samples positive for the bla\text{SHV} gene, 17 samples were bla\text{SHV-11} subtype, 16 samples were bla\text{SHV} subtype, 14 samples were bla\text{SHV-4} subtype, 2 samples were bla\text{SHV-108} subtype, 1 sample was bla\text{SHV-9} subtype, and 1 sample was bla\text{SHV-104} 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. 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. in Milad Hospital, but higher than those reported in Lebanon (20%) in 2007, Al-Agamy et al. showed that 214 (97.3%) of 220 strains isolated in Saudi Arabia carried the bla\text{SHV} gene, which is higher than that found in our study. A study conducted by Ghalifourian et al. in 2011 in Iran determined that 94% of *K. pneumoniae* strains carried the bla\text{SHV} gene, which is in parallel with our results. In recent years, the high relative prevalence of the bla\text{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*. In the present study, 72.7% of the strains carried the bla\text{TE4M} 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. 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. In a subsequent study, Feizabadi et al. further identified that all of the *K. pneumoniae* isolates from a Labbafinejad Hospital that carried the bla\text{TE4M} 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. Overall, it seems that the majority of ESBLs in *K. pneumoniae* strains of Zahedan are derived from the bla\text{SHV} and bla\text{TE4M} 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.

**Conflict of interest**

The authors declare that there is no conflict of interest.

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