Genetic Characterization of blaSHV/VEB/PER Genes in ESBL-Producing MDR *Klebsiella Pneumonia* Strains Isolated from Patients in Isfahan, Iran

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Abstract

This study was conducted to detect three genetic variants of Extended-Spectrum Beta-Lactamase (ESBL, in which *Klebsiella pneumoniae* (*K.pneumoniae*) isolates were collected from sections of a teaching hospital in Isfahan and were detected using standard IMVIC biochemical tests and urease. These were confirmed by identification of the ureD gene. Antimicrobial susceptibility testing was performed using the standard Kirby-Bauer disk-diffusion method on Mueller-Hinton agar (Merck, Germany). The performance and interpretation were based on the guidelines from the Clinical Laboratory Standards Institute (CLSI, 2013). Screening and phenotypic identification of ESBL isolates were performed by DDST. The presence of genes responsible for ESBL resistance, such as SHV, PER and VEB type ESBL genes was identified by PCR and indicator isolates sequencing performed by Macrogen (Seoul, Korea). The nucleotide sequences were analysed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST), the Lahey database, and CromasPro-2 and Mega-4 software to determine the subvariants of the three variants of ESBL (SHV, PER, VEB). These were compared with blaSHV-11 gene from *K. pneumonia* (accession no. X98101), blaSHV-5 gene from *K. pneumonia* (accession no. X55640), blaPER-1 gene from *P. aeruginosa* transposed on Tn2345 (accession no. AF010416) and blaVEB-1 gene from *K. pneumonia* (accession no. AF010416). A total of 120 isolates (84%) were recognized as MDR. The highest rate of resistance was recorded for piperacillin (80%), ceftazidime (76%), and cefotaxime (73%) and the lowest rate was for ertapenem (47.3%), meropenem (50.8%), and imipenem (58.7%) following detection of ESBL isolates of *K. pneumoniae* (101 isolates; 71%). The ward and the clinical specimen with the most prevalence were ICU with 55(38.7%) and urine with 61(42.9%). The lowest prevalence was related to the neurosurgery ward with 8 (5.6%) samples and the clinical specimen with the lowest prevalence was cerebrospinal fluid (CSF) with 2 (1.4%) samples. PCR detection in ESBL-producing *K. pneumoniae* showed that, of the clinical isolates, 42.2% contained blaSHV (42/101), 2.9% contained blaVEB (3/101) and 2% contained blaPER (2/101). Sequencing of 10 selected PCR products of SHV genes showed that 7/10 isolates were similar to the strain SHV-11 and 3/7 isolates were similar to the strain SHV-5. The sequencing of two PCR products of the PER genes showed they were similar to the strain PER-1. Sequencing of three PCR products of the VEB genes showed they were similar to the strain VEB-1. The overall prevalence of ESBL-producers was found to vary greatly in different geographical areas; this may be the result of differences in the type and amount of antibiotics consumed and differences in the time of collection of isolates. The present study reflects an increase in the prevalence of ESBL-producers in Iran. The most common ESBL
type found in this study was SHV and that VEB and PER types were rare. In addition, sequence analysis results of our study show the rate of SHV-11, PER-1 and VEB-1 was maximum. This study showed that $\beta$-lactamase-producing *K. pneumoniae* strains are an emerging threat in ICUs. They should be monitored through the implementation of timely identification and strict isolation methods that will help to reduce adverse outcomes and mortality rates in patients.

**Keywords:** blaSHV/VEB/PER genes, ESBL-producing MDR, Klebsiella pneumonia strains

**Introduction**

Microbial resistance through extended-spectrum beta-lactamase (ESBL) was first reported in the early 1980s in Europe and then in the United States soon after the introduction of third-generation cephalosporins to clinical practice (Hassan and Abdalhamid, 2014). The enzyme $\beta$-lactamase continues to be the leading cause of resistance to beta-lactam antibiotics in gram-negative bacteria. There has been an increased incidence and prevalence of ESBL enzymes that hydrolyze and cause resistance to oxyimino-cephalosporins and aztreonam (Versalovic et al, 2011). More than 300 different ESBL variants have been described (Ahmed et al, 2013). Although temoneira (TEM) and sulphydryl variable (SHV) variants are the most common ESBLs, strains expressing CTX-M ESBL have begun to emerge (Ahmed et al, 2013).

The dominant enzyme variants until the late 1990s were TEM, SHV and OXA (Livermore and Hawkey, 2004; Livermore et al, 2007). New families of ESBLs have emerged (6), including families of *pseudomonas* extended-resistance (PER) and Vietnamese extended-spectrum beta-lactamase (VEB) (Stürenburg and Mack, 2003). VEB and PER variants are rare and are valuable for study. PER-1 was first detected in *Pseudomonas aeruginosa* (Nordmann et al, 1993) and later in *Salmonella enteric* and *Acinetobacter* isolates. PER-2, which shows 86% homology to PER-1, has been reported in *S. enterica*, *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Vibrio cholera* O1 El Tor (Bauernfeind et al, 1996; Petroni et al, 2002).

The blaSHV genes are responsible for the production of SHV $\beta$-lactamases, large families of enzymes with evolutionary affinity. Of the 172 enzyme types in the SHV family, 45 are extended-spectrum $\beta$-lactamases on transferable elements such as plasmids and transposons (Al-Jasser, 2006; Shah et al, 2004). This localization of bla genes can facilitate horizontal spreading of antibiotic resistance among bacterial strains (El Salabi and Walsh, 2013).

*K. pneumoniae* resides in the intestinal tract of about 40% of humans and animals. It is an opportunistic human pathogen, meaning that under certain conditions it can cause disease. For example, nosocomial infections are those contracted by hospitalized patients in a weakened state (Manikandan and Amsath, 2013). *K. pneumoniae* causes recurrent cough and acute exacerbation of chronic obstructive pulmonary disease defined as presence of increased sputum volume, purulence and dyspnea and is responsible for 30-50% of exacerbations (Madhavi, 2012). *K. pneumoniae* is naturally resistant to ampicillin, but not to extended-spectrum $\beta$-lactam antibiotics. Many factors can contribute to the resistance of bacteria towards antibiotics. Adaptation of bacteria to stressful environments can result in bacteria adaptation, survival and growth. This could ultimately result in the production of ESBL producers in *K. pneumoniae* (Puspanadan et al, 2013).

**Materials and Methods**

**Sampling**

The samples tested comprised 500 clinical samples from urine, the trachea and bronchi, blood, sputum, ascetic fluid, CSF, abscesses and wounds were collected from sections of a teaching hospital in Isfahan (pediatrics, internal medicine, ICU, surgery, emergency, other). *K. pneumoniae* isolates were detected in 28% (142) of the samples using standard IMVIC biochemical tests (indole,
methyl red, Voges- Proskauer, citrate) and urease. These were confirmed by identification of the ureDgene (243bp), which is responsible for hydrolysis of urea (Mashouf et al, 2014).*K. pneumoniae* ATCC 700603 was used as the positive control. The target gene primers were lyophilized as listed below:

```
ure –D F: 5’_ -CCCGTTTTACCCGGAAGAAG - 3
ure –D R: 5’ - GGAAAGAAGATGGCATCCTGC -3
```

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility testing was performed using the standard Kirby-Bauer disk-diffusion method on Mueller-Hinton agar (Merck, Germany). Antibiotic discs (Mast, England) were used for early detection of *K. pneumoniae* isolates that produce ESBLs. The performance and interpretation were based on the guidelines from the Clinical Laboratory Standards Institute (CLSI, 2013). The following antimicrobial agents were tested: ceftazidime, cefepim, aztreonam, levofloxacin, ticarcillin, cefotaxim, amoxicillin/clavulanicacid, piperacillin/tazobactam, piperacillin, gentamicin, amikacin, and ciprofloxacin. *E. coli* ATCC 25922 was used as a control.

**Screening and phenotypic identification of ESBLs by DDST**

In initial detection, isolates showing resistance or decreased sensitivity to more than one of the third generation cephalosporins (3GC-ceftazidime, cefotaxime) were selected as probable ESBL producers (Anjali et al, 2013). The phenotype was confirmed by DDST where the tested strains were lawn cultures (0.5 McFarland std.) on Mueller-Hinton agar. Antibiotic disks of ceftazidime and ceftazidime-clavulanic acid were placed 15 mm apart and the plates were placed under aerobic incubation at 37°C. An increase of more than 5 mm in zone diameter for either antimicrobial agent tested in combination with clavulanic acid over the initial zone diameter of the agent when tested alone was considered proof of ESBL. *E. coli* ATCC 25922 was used as a negative control and *K. pneumonia* ATCC700603 as a positive control.

**Genotypic identification of ESBLs**

**Preparation of DNA template**

The DNA template for *K. pneumonia* was prepared from freshly cultured isolates by suspending 3-5 colonies from the plate in 500 μl of transposon buffer and boiling it for 10 min. After boiling, the samples were cooled at -20°C for 5 min before centrifuging at 10,000 rpm for 10 min (Amjad et al, 2011).

**Genotypic detection of bla genes by PCR**

The supernatant obtained was used as the DNA template in polymerase chain reaction (PCR) analysis. The strains were documented as ESBL by molecular detection of bla<sub>SHV</sub>, bla<sub>VEB</sub>, bla<sub>PER</sub> by PCR. The primers used for the detection of these genes are shown in Table 1.

PCR was performed in 30 μl mixture of 3 μl 10X buffer, 1 μl of 10 mM MgCl2, and 0.25 μl of 5 U/μl Taq DNA polymerase (Fermentas, Lithuania), 0.5μl each of 10 mM deoxynucleoatide triphosphates, 1 μl each of 10 μM primers and 5 μl of plasmid extract in a thermal cycler (Kyratec, Korea).

**DNA sequencing analysis**

DNA sequencing for the 15 strains was performed to identify the bla genes. The PCR products of the genes were further purified using PCR purification kits (Fermentas) and subjected to direct sequencing performed by Macrogen (Seoul, Korea) as described previously (Shacheraghi et al, 2010). The nucleotide sequences were analysed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST), the Lahey database, CromasPro-2 and Mega-4 software to determine the subvariants of the three variants of ESBL (SHV, PER, VEB). These were compared with bla<sub>SHV-11</sub> gene from *K. pneumonia* (accession.no.X98101), bla<sub>SHV-5</sub> gene from *K. pneumonia*
(accession no. X55640), bla\textsubscript{PER-1} gene from \textit{P. aeruginosa} transposed on Tn2345 (accession no. AY866517.2) and bla\textsubscript{VEB-1} gene from \textit{K. pneumonia} (accession no. AF010416).

\textbf{Table 1: Primers used for amplification of ESBLs genes}

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequences</th>
<th>Expected Size</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV-F</td>
<td>ATGCCTATATCCGAGGCAAA</td>
<td>753</td>
<td>95°C, 5 min; 34 cycles of 94°C, 30 sec, 54°C, 30 sec, 72°C, 45 sec, 72°C, 10min</td>
</tr>
<tr>
<td>SHV-R</td>
<td>TGTG TGCTTTGTATCCGAGGCAAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| VEB-F  | CGACTTCCATTCCGGATGCCGGACTCTGCAACCAATAGC | 643           | 95°C, 5 min; 34 cycles of 94°C, 30 sec, 58°C, 30 sec, 72°C, 45 sec, 72°C, 10min |
| VEB-R  | ATGC GGACTCTGCAACCAATAGC                |               |                                       |

| PER-F  | AAAGAGCAAATTGAATCCATAGTCGGTTAATTTGGGCTTAGGCAG | 835           | 95°C, 5 min; 34 cycles of 94°C, 30 sec, 56°C, 30 sec, 72°C, 45 sec, 72°C, 10min |
| PER-R  | CCATAGTGGTTATTTGGGCTTAGGCAG              |               |                                       |

\textit{Statistical analysis}

Statistical analysis was performed using Whonet(v. 5.6) software on the data according to CLSI2013.

\textbf{Results}

The 142 clinical isolates of \textit{K. pneumoniae} were identified by biochemical methods and confirmed by PCR for the ure\textsubscript{D} gene. All isolates were positive for ure\textsubscript{D} gene, which confirmed their identity as \textit{K. pneumonia}.

\textbf{Figure 1.} Agarose gel showing \textit{ureD} gene. Lane 1: Ladder, Lane 2: positive control (\textit{K. pneumoniae} ATCC700603), Lane 3-5: test isolates. Lane 6: Negative control (Escherichia coli ATCC 25922)
Of the 142 clinical isolates of *K. pneumonia*, 57% were from males and 43% were females. In this study multi-drug resistant (MDR) *K. pneumonia* was defined as an isolate resistant to at least three classes of antimicrobial agents (Ben et al, 2006). A total of 120 isolates (84%) were recognized as MDR. The breakdown of the resistance rates between antibiotics is given in Figure 1 and 2.

![Figure 2: Pattern of Resistance to Antimicrobial agents among all *K. pneumonia*. Strains](image)

<table>
<thead>
<tr>
<th>Abbreviations: CAZ, ceftazidime; CTX:Cefotaxime; ATM:aztreonam; AMC, amoxicillin clavulanic acid; IPM, Imipenem; MEM, Meropenem; ETP, Ertapenem; GEN; gentamicin; CIP, ciprofloxacin; FEP, cefepime; AMK, amikacin; PIP, piperacillin; TZP, Piperacillin/tazobactam; LVX, Levofloxacin; TET, tetracycline; R, Resistance; S, Sensitive; I, Intermediate.</th>
</tr>
</thead>
</table>

![Figure 3: Result of DDST of *K. pneumoniae* strain CE: cefotaxime (30 g), CEC, cefotaxime +clavulanic acid (30/10 g)](image)
The highest rate of resistance was recorded for piperacillin (80%), ceftazidime (76%), and cefotaxime (73%) and the lowest rate was for ertapenem (47.3%), meropenem (50.8%), and imipenem (58.7%) following detection of ESβL isolates of K. pneumoniae (101 isolates; 71%). Figure 3 shows the results of DDST of a K. pneumoniae strain isolated from an eviscerated material along with the positive and negative control strains.

The prevalence of the enzyme in different clinical specimens (urine, the trachea and bronchi, blood, sputum, ascetic fluid, CSF, abscess, and wound) in different sections of the hospital (pediatrics, internal medicine, ICU, surgery, emergency, other) are showed in Tables 2 and 3.

**Table 2. Prevalence of K. pneumoniae ESBL among different clinical specimens**

<table>
<thead>
<tr>
<th>ESBL</th>
<th>- (%)</th>
<th>+ (%)</th>
<th>Number of patients (%)</th>
<th>Specimen type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>5(3.5%)</td>
<td>ascetic fluid</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>3(2.1%)</td>
<td>Abscess</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3(2.1%)</td>
<td>Blood</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>14</td>
<td>14(9.8%)</td>
<td>Bronchi</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>7</td>
<td>7(4.9%)</td>
<td>Catheter</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>2(1.4%)</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>4(2.8%)</td>
<td>Sputum</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>23</td>
<td>28(19.8%)</td>
<td>Trachea</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>36</td>
<td>61(42.9%)</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8</td>
<td>14(9.8%)</td>
<td>Wound</td>
</tr>
<tr>
<td></td>
<td>31(29%)</td>
<td>101(71%)</td>
<td>142(100%)</td>
<td>Total</td>
</tr>
</tbody>
</table>

The highest proportion of K. pneumonia was isolated from urine samples (61; 42.9%) and in the ICU (55; 38.7%). The lowest proportion was from CSF (2; 1.4%) and neurosurgery (8; 5.6%). PCR detection in ESBL-producing K. pneumoniae showed that, of the clinical isolates, 42.2% contained blaSHV(42/101), 2.9% contained blaVEB(3/101) and 2% contained blaPER (2/101) cases contained blaSHV, blaVEB and blaPER (Figures 3, 4 and 5).

**Table 3. Prevalence of K. pneumoniae ESBL among different sections of the hospital**

<table>
<thead>
<tr>
<th>ESBL</th>
<th>Number of isolates(%)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>9(6.3%)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>27(19%)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>55(38.7%)</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>27(19%)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>16(11.2)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8(5.6)</td>
</tr>
<tr>
<td></td>
<td>31(29%)</td>
<td>101(71%)</td>
</tr>
</tbody>
</table>

The prevalence of the enzyme in different clinical specimens (urine, the trachea and bronchi, blood, sputum, ascetic fluid, CSF, abscess, and wound) in different sections of the hospital (pediatrics, internal medicine, ICU, surgery, emergency, other) are showed in Tables 2 and 3.
SHV-type ESBL was the most common and PER-type ESBL was least common. The distribution of the \textit{bla}_{SHV}, \textit{bla}_{VEB} and \textit{bla}_{PER} is shown in Table 4.

Table 4: distribution of ESBL genes among different samples

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>PER</th>
<th>VEB</th>
<th>SHV</th>
<th>ESBL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal fluid</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>3.5%</td>
</tr>
<tr>
<td>Abscess</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0.7%</td>
</tr>
<tr>
<td>Blood</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1.4%</td>
</tr>
<tr>
<td>Bronchial</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>9.8%</td>
</tr>
<tr>
<td>Catheter</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.9%</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1.4%</td>
</tr>
<tr>
<td>Sputum</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>2.1%</td>
</tr>
<tr>
<td>Tracheal</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>16.1%</td>
</tr>
<tr>
<td>Urine</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>25.3%</td>
</tr>
<tr>
<td>Wound</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>5.6%</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>3</td>
<td>42</td>
<td>71%</td>
</tr>
</tbody>
</table>
Sequencing of 10 selected PCR products of SHV genes showed that 7/10 isolates were similar to the strain SHV-11 (blaSHV-11 gene from K. pneumonia; accession no. X98101) and 3/7 isolates were similar to the strain SHV-5 (blaSHV-136 gene from K. pneumonia accession no. X55640).

The sequencing of two PCR products of the PER genes showed they were similar to the strain PER-1 (blaPER-1 gene from P. aeruginosa transposon Tn2345; accession no. AY866517.2). Sequencing of three PCR products of the VEB genes showed they were similar to the strain VEB-1 (blaVEB-1 gene from K. pneumonia; accession no. AF010416).

**Discussion**

Identification of common etiologic organisms of nosocomial pathogens and their patterns of antibiotic resistance is of great importance for controlling disease and reducing medical costs. Since members of Enterobacteriaceae are the main contributors to nosocomial and community-acquired infections, establishing a new strategy for diagnosis and treatment of ESBL-producing bacteria is essential. The prevalence of ESBL-producing bacteria differs greatly worldwide and mainly depends on the extent of use of beta-lactam antibiotics in a community (Moghaddam et al, 2014).

Infection with MDR strains increase the duration of hospitalization and medical and surgical cost for a patient. In this study, about 84% (120/142) of all isolates were MDR. This finding is in accordance with studies conducted in Iran, Pakistan, Mexico and India that reported the incidence of MDR K. pneumoniae (Al-Zarouni et al, 2008).

The prevalence of ESBL-producing K. pneumoniae was 71% (101/142), which is higher than in India (66.7%) (Hoda Hassan, 2014) and Turkey (54.7%-61%) (Al-Zarouni et al, 2014). It is similar to other studies in Iran (72.1%) (Feizabadi et al, 2010) and much higher than reports from Saudi Arabia (25.2%), United Arab Emirates (41%) (Al-Zarouni et al, 2008) and Kuwait (31.7%) (Mokaddas et al, 2008). The overall prevalence of ESBL-producers was found to vary greatly in different geographical areas; this may be the result of differences in the type and amount of antibiotics consumed and differences in the time of collection of isolates. The present study reflects an increase in the prevalence of ESBL-producers in Iran. In Iran, the majority of ESBL-producing isolates were recovered from urine, which is similar to results of other studies (Fam et al, 2011).

The most common ESBL type found in this study was SHV, which is similar to that reported by Harada et al (2013). In Japan and different from the results reported by Jikun Du et al (2014). In China sequence analysis results show that the prevalence of SHV-11 was similar to results by Feizabadi et al in Tehran (2010) and that VEB and PER types were rare.

The results showed that two of the isolated K. pneumoniae carried the blaPER gene. This statistic was about 55% in Turkey (Paterson et al, 2003) and was nil in other studies, which is similar to what has been reported for E. coli isolates by Shahcheragh et al. (2010) in Tehran, Celenza et al. (2006) in Italy and Barguiguia et al. (2011) in Morocco. The much higher prevalence (49.25%) of blaPER gene has been reported among ESBL-producing strains of P. aeruginosa isolated from burn patients. The blaPER-1 gene has been detected mainly in glucose-non-fermenting gram-negative bacilli such as P. aeruginosa and Alcaligenes faeaealis; however, it has been recently found in Enterobacteriaceae and Aeromonas media (Picão et al, 2008; Lee et al, 2011; Umadevi et al, 2011). This gene has recently been reported for the first time in K. pneumoniae. The authors are of the opinion that there is a possibility of further dissemination of the blaPER-1 gene in Enterobacteriaceae.

Some ESBL isolates have none of these three genes (blaSHV, blaVEB, blaPER). This may result from the presence of other genes responsible for ESBL resistance, such as blaTEM, blaCTX-M.
and bla_{OXA}). None of the isolates in this study showed the presence of two or three ESBL-producing genes together.

The prevalence of β-lactamase-producing isolates and their isolation in life-threatening infections has increased dramatically worldwide. Intense pressure by patients to use PER-1 antimicrobial drugs has resulted in the eradication of normal flora and substitution with MDR isolates.

Phenotypic methods are the only screening methods available for the detection of ESBLs in a routine laboratory. Genotypic methods help confirm the genes responsible for ESBL production. This study showed that β-lactamase-producing *K. pneumoniae* strains are an emerging threat in ICUs. They should be monitored through the implementation of timely identification and strict isolation methods that will help to reduce adverse outcomes and mortality rates in inpatients.

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**References**


