Prevalence of SHV β-lactamases in *Escherichia coli*

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Resistance to β-lactam antibiotics by Gram-negative bacteria, especially *Escherichia coli*, is a major public health issue worldwide, and is often caused by the production of β-lactamase enzymes. Specifically, extended-spectrum β-lactamases (ESBLs), which cannot be diagnosed by phenotypic tests recommended by the Clinical and Laboratory Standards Institute (CLSI), are often produced. In this study, the use of designed primers with high ability to detect the sub family SHV gene was preferred. We focused on evaluating the prevalence of extended-spectrum β-lactamases using the disk diffusion method and confirmatory test (combined disk), as well as polymerase chain reaction (PCR) for the following gene (SHV). Our findings indicate that the prevalence of ESBLs in Iran is rising, which may lead to the ineffective treatment of these infections using β-lactam antibiotics. The development of non-phenotypic diagnostic methods capable of detecting β-lactamase enzymes is essential for the control of resistant strains as well as successful treatment through the administration of an appropriate β-lactam drug.

**Key words:** *Escherichia coli*, beta lactamase enzymes, SHV-type extended spectrum beta-lactamases.

INTRODUCTION

Gram-negative pathogens have evolved several mechanisms to resist β-lactam antibiotics. However, the most predominant mechanism results from the production of β-lactamase enzymes, which inactivate the antibiotic by hydrolysis of the β-lactam ring (Stapleton et al., 1999; Rayamajhi et al., 2008). The widespread and unregulated use of β-lactam antibiotics in medicine has led to the evolution of novel β-lactamase enzymes in gram-negative pathogens, including the extended spectrum β-lactamases (ESBLs) (Bradford, 2001; Denton, 2007). The SHV-type-ESBLs are encoded in the chromosome of *Klebsiella* isolates, which can be transported to mobile elements such as plasmids and thereby promote the transfer to other microbial societies, in particular Enterobacteriaceae (Paterson and Bonomo, 2005). Detection of ESBLs is essential at the time of diagnosis, and a failure to detect these genes is the major cause of treatment failure using β-lactam antibiotics (Netzel et al., 2008).

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**Abbreviation:** ESBLs, Extended-spectrum β-lactamases.
2007). One of the current methods for ESBL detection is an initial screen for a series of cephalosporin indicators, especially ceftazidime and cefotaxime, and subsequent confirmatory tests with clavulanic acid (an inhibitor of ESBLs). In this test, if the zone of inhibition (ZOI) in the presence of clavulanic acid is greater than the ZOI in the absence of clavulanic acid, those organisms are classified as ESBL producers (Stürenburg and Mack, 2003; Pitout and Laupland, 2008). The prevalence of ESBL resistant strains amongst certain pathogens, particularly *Escherichia coli*, is increasing and therefore a broader spectrum of resistant strains is emerging (Goossens and Grabein, 2005; Deshpande et al., 2006).

Over the past decade, the appearance of novel β-lactamase enzymes among pathogens has led to inadequacies in the diagnosis of ESBL resistant strains using the disk diffusion method (Goossens and Grabein, 2005). As a consequence, the use of molecular techniques in conjunction with phenotypic tests is essential (Jacoby and Medeiros, 1991; Pitout and Laupland, 2008). In this study, we addressed the prevalence of ESBLs and also sought to detect SHV-type β-lactamase genes by PCR using designed primers.

### MATERIALS AND METHODS

**Bacterial strains**

Over 500 clinical samples were collected from hospitals in Tehran and 200 *E. coli* isolates were detected using standard biochemical tests including the indole, methyl red, Voges-Proskauer, and citrate (IMViC) tests. All strains of *E. coli* isolated from samples were stored in skim milk at -70°C until required for further tests.

**Screening and phenotypic identification of ESBLs**

*E. coli* isolates were screened for susceptibility to antimicrobial agents using a standard Disk diffusion method on Muller-Hinton agar to test for ESBL producing strains. *E. coli* was streaked out on Muller-Hinton agar plates at the desired density and filter disks containing antibiotic were placed on the agar. The antibiotics used were as follows: cefotaxime (30 µg), ceftazidime (30 µg), gentamycin (10 µg), amoxicillin (30 µg), imipenem (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), cotrimoxazole (1.25 µg), ciprofloxacin (5 µg) and chloramphenicol (30 µg) (Mast Diagnostics Ltd., UK). After incubation for 24 h at 37°C, the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI). *E. coli* isolates resistant to cephalosporins were selected for confirmatory tests (combined disk method), which used ceftazidime (30 µg), ceftazidine/clavulanate (30/10 µg), cefotaxime (30 µg), and cefotaxime/clavulanate (30/10 µg) (Mast Diagnostics Ltd., UK). After incubation for 24 h at 37°C, production of ESBLs was confirmed by a ≥5 mm increase in the ZOI in the presence of clavulanic acid compared to samples without clavulanic acid (Clinical and Laboratory Standards Institute, 2005; Song et al., 2007). Isolates expressing ESBLs were tested for the *bla*<sub>SHV</sub> gene by PCR.

**Design of primers**

50 sequences related to the *bla*<sub>SHV</sub> gene of *E. coli* were identified in GenBank. These sequences were aligned using a MEGA 4 multiple-alignment program to identify analogous loci. These loci were used to design primers using Gene runner. The designed primers were then tested in *silico* for homology with submitted sequences using BLAST. Finally, a set of designed primers were evaluated using PCR.

**PCR and sequencing of the β-lactamase genes**

Genomic DNA was isolated from *E. coli* strains expressing ESBLs using the extraction kit (Bioneer, Seoul, Korea) according to the manufacturer’s instructions. Subsequently, the *bla*<sub>SHV</sub> gene was amplified by PCR using designed primers (Table 1) in the following conditions: one reaction contained 2.5 µl PCR buffer (10 X), 2 µl MgCl<sub>2</sub> (50 mM), 1 µl dNTP (10 mM), 1.5 µl of each primer (50 pmol/µl), 1 µl Taq polymerase (5 U/µl), 2 µl template DNA (50 pmol/µl), and sterile H<sub>2</sub>O (14.5 µl) in a final volume of 25 µl. Evidently, two negative control reactions were exercised. One of them contained the same material without DNA template and the other contained genomic DNA of a SHV negative clone which was confirmed by phenotypic assay. Moreover, *Klebsiella pneumoniae* ATCC 7881 was used as a positive control for *bla*<sub>SHV</sub> expression. PCR amplification was achieved using the following conditions: initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and elongation at 72°C for 1 min. The final elongation step (72°C) occurred for 10 min. Subsequently, the amplicons were detected by electrophoresis using a 0.8% agarose gel. PCR products were purified using a kit (Fermentas, Germany) according to the manufacturer’s instructions. PCR products that corresponded to the expected size of the SHV cluster were sent to Macrogen Research, Seol, Korea for sequencing analysis. The resulting sequences were aligned with known SHV sequences in the NCBI database.

**RESULTS**

In our study, 200 clinical isolates of *E. coli* were collected over six months. The samples were isolated from urine and urinary catheter (62.5%), stool (24%), blood (9%), wound tissue (2.5%), and other clinical samples (2%). The patterns of resistance of the 200 *E. coli* isolates to 10 antimicrobial agents are shown in Table 2. The majority of isolates showed a high degree of resistance to oxyimino cephalosporins but appeared to be susceptible.

### Table 1. Primers used for amplification.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5’ to 3’ as synthesized)</th>
<th>Expected amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV</td>
<td>SHVF</td>
<td>GCCTGTTATTATCTCCCTGTAGC</td>
<td>764</td>
</tr>
</tbody>
</table>
to imipenem as well. Up to 70% of the isolates exhibited a multidrug-resistance (MDR) phenotype.

128 (65%) of the ceftazidime and cefotaxime resistant strains of *E. coli* in the Disk diffusion method were classified as putatively positive for ESBLs and were selected for subsequent testing by the combined disk assay. In the combined disk assay, 115 (89.8%) isolates were found to express ESBLs among the 128 isolates screened. ESBL-expressing strains of *E. coli* were more common in urinary samples (80%).

PCR was performed on genomic DNA isolated from all 128 resistance strains using designed primers. These data shows that among these isolates, 7 (5.5%) were positive for the *bla*<sub>SHV</sub> (Figure 1).

**DISCUSSION**

The prevalence of β-lactamase expressing *E. coli* strains varies significantly over time as well as geographic location (Al-Jasser, 2006). For example, among 7,054 strains of *E. coli* isolated between 1994 and 1996 in Barcelona, the prevalence of ESBL-producing strains was 0.14% (Sabaté, 2002). In 2001, however, the prevalence increased to 2.1% (Kenny et al., 2003). Similarly, other studies have shown that the prevalence of ESBL-expressing bacteria in Iran is rising according to the resistant rate of *E. coli* to cephalosporin indicators in this study (89.8%) compared with another study like Nakhaei et al. (32.11%) which is reported from Iran (Nakhaei et al., 2009) and could possibly be due to the irregular use of β-lactam antibiotics, especially expanded cephalosporins in Iran (Al-Jasser, 2006). Our study shows that among the 128 *E. coli* isolates screened using the Disk diffusion method, only 115 isolates were found to express ESBLs using the CLSI-recommended confirmatory assay (Combined Disk assay). Today, a significant challenge for diagnostic laboratories is the detection of extended spectrum β-lactamases, especially with the discovery of novel β-lactamase enzymes such as AmpC. These novel β-lactamases often register as false negatives when they are screened for using conventional ESBL phenotypic assays (Hanson, 2003). The misdiagnosis of β-lactam resistant bacteria by diagnostic laboratories can lead to the prescription of unsuitable drugs and can result in the failure to cure the infection or even death of the patient (Paterson et al., 2001). In order to verify the strains that were negative in the confirmatory test, we performed further evaluation using molecular methods as recommended by the CLSI (Walther-Rasmussen and Heiby, 2002; Song et al., 2007). PCR was performed on all 128 resistant isolates, and seven (5.5%) of the strains tested were shown to express *bla*<sub>SHV</sub> (Figure 1). Our study identified one sample with a negative ESBL phenotype that expressed the *bla*<sub>SHV</sub> gene, showing that possession of an ESBL gene may not always be detected by ESBL expression assays. The *bla*<sub>SHV</sub> gene was only detected in 5.5% of the isolates tested. Other ESBL genes such as TEM, OXA, and CTX-M may be responsible for the ESBL phenotype (Zhang et al, 2009).

The prevalence of the *bla*<sub>SHV</sub> gene in this study was very similar to that reported in a Swedish hospital (6%, 2001 to 2006) (Fang et al., 2008) as well as the incidence reported at the Seoul National University Hospital in Korea (8.7%, 1995 to 1999) (Kim and Lee, 2000). In addition, our data was slightly higher than that reported in Thailand (3.8%, 2005) (Kiratisin et al., 2008) and was lower than that reported at a University Hospital in Salamanca, Spain (15.6%, 2001 to 2004) (Romero et al., 2007) as well as at several Turkish hospitals (28.6, 2007) (Hosoglu et al., 2007).

ESBLs were first described in 1983 in Germany. Since then, a variety of these enzymes have been found worldwide in a broad spectrum of pathogens, in particular Klebsiella sp. and *E. coli*, and their expression has been associated with failures in treatment and increased mortality (Thomson, 2001; Denton, 2007). Previous investigations have not taken into account the expression of novel β-lactamases and the inability to detect them in phenotypic tests. Additionally, several clinical institutes do not screen for ESBL expression in bacteria. Our data supports the need for clinical diagnostic laboratories to

**Table 2.** Pattern of resistance among 200 *E. coli* isolates to 10 antimicrobial agents.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Resistance</th>
<th>Intermediate</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>1 (0.5%)</td>
<td>0</td>
<td>199 (99.5%)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>109 (54.5%)</td>
<td>16 (8%)</td>
<td>75 (37.5%)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>78 (39%)</td>
<td>4 (2%)</td>
<td>118 (59%)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>61 (30.5%)</td>
<td>36 (18%)</td>
<td>103 (51.5%)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>143 (71.5%)</td>
<td>29 (14.5%)</td>
<td>28 (14%)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>128 (64%)</td>
<td>7 (3.5%)</td>
<td>65 (32.5%)</td>
</tr>
<tr>
<td>Cepazidin</td>
<td>111 (55.5%)</td>
<td>11 (5.5%)</td>
<td>78 (39%)</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>161 (80.5%)</td>
<td>3 (1.5%)</td>
<td>36 (18%)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>148 (74%)</td>
<td>13 (6.5%)</td>
<td>39 (19.5%)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>189 (94.5%)</td>
<td>3 (1.5%)</td>
<td>8 (4%)</td>
</tr>
</tbody>
</table>
improve diagnostic methods that can unequivocally detect ESBL-expressing organisms.

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REFERENCES


