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EXTENDED-SPECTRUM BETA-LACTAMASES IN CEFTAZIDIME-RESISTANT
ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE ISOLATES IN
TURKISH HOSPITALS

*S Hoşoğlu, S Gündeş, F Kolaylı, A Karadenizli, K Demirdağ, M Günaydın, M Altindis, R Çaylan, H Ucmak

Abstract

**Purpose:** To study the prevalence of TEM-, SHV- and GES-type β-lactamases among *Escherichia coli* and *Klebsiella pneumoniae* strains having ceftazidime MICs higher than 2 mg/L. **Methods:** A total of 63 *E. coli* and 41 *K. pneumoniae* isolated from five different university hospitals were studied for the existence of TEM-, SHV- and GES-type β-lactamases. Susceptibility tests were carried out according to the criteria of National Committee for Clinical Laboratory Standards. MICs were obtained by agar dilution method. Existence of extended-spectrum β-lactamases (ESBLs) were assessed by double-disc synergy test (DDST). Existence of the above-mentioned β-lactamase genes were studied both by PCR with specific oligonucleotide primers and isoelectric focusing methods. **Results:** None of the isolates were carbapenem-resistant. DDSTs were positive in 50 (79.3%) and 33 (80.5%) of *E. coli* and *K. pneumoniae*, respectively. TEM gene was detected in 41 (65.1%) and 19 (46.3%), whereas SHV gene in 18 (28.6%) and 20 (48.8%) of *E. coli* and *K. pneumoniae* strains, respectively. GES genes were not detected. **Conclusions:** TEM and SHV genes are highly prevalent among ESBL-producing *E. coli* and *K. pneumoniae*, whereas GES-type ESBLs are absent and found not to be responsible of ceftazidime resistance in Turkish hospitals.

**Key words:** Ceftazidime-resistant, *E. coli*, ESBL, Klebsiella, SHV, TEM, GES

Extended-spectrum β-lactamases (ESBLs) are frequently encountered among clinical *Enterobacteriaceae*, predominantly *Klebsiella pneumoniae* and to a lesser extent, *Escherichia coli* and other species.1,2 The genes coding for ESBLs are usually carried by plasmids, which strongly facilitate their spread among strains of many species of Gram-negative bacteria. ESBLs exhibit high degrees of diversity in their structures and activities and several families reflecting their evolutionary and/or functional similarities can be distinguished.3,5 The majority of the ESBLs arise by mutations that alter the hydrolytic activities of classical enzymes TEM-1, TEM-2 and SHV-1.6 The TEM and SHV ESBLs exhibit a considerable variety, mostly with respect to their ranges of substrate preferences and to their levels of hydrolytic activity.2,5

In early 1990s, ESBL-producing gram-negative bacteria, exhibiting a higher level of resistance to cefotaxime than to ceftazidime, were described in Germany (1990), France and Argentina (1992).7 These were the first reports of gram-negative isolates producing various non-TEM, non-SHV ESBLs, namely CTX-M and GES/IBC types. Such Ambler class A β-lactamases have <40% identity with the β-lactamases of the TEM and SHV series.7,8 In the two recently published articles, Wachino et al. designated a novel ceftazidime-hydrolysing class A ESBL (GES-a) as GES-3 and a new cephamycin-hydrolysing and inhibitor-resistant class A ESBL (GES-b) as GES-4. Actually, their articles have described different nomenclature of GES-type ESBLs and controversial conclusions on the relationship between β-lactamase inhibitor resistance and an amino acid substitution in the centre of the omega-loop region.9,10 Before Wachino et al. submitted their sequences for GES-3 and GES-4 genes to the GenBank nucleotide database, sequences for GES-3 and GES-4 genes had already been released by Vourli et al.11 As known, GES-a and GES-b genes are completely different from GES-3 and GES-4 genes. GES-3 and GES-4 were capable of hydrolysing imipenem, whereas GES-a could not hydrolyse imipenem and GES-b had a substrate profile extended to cephamycins as well as imipenem. Presently, the different GES-type ESBLs have been designated by identical names. It is suggested that GES-a and GES-b genes be renamed as GES-5 and GES-6 genes, respectively.

There are hospitals in which GES-type β-lactamases are the most prevalent, but it seems that in the majority of countries, the isolation of GES-producing strains or outbreaks caused by these organisms remain sporadic.7 There are a number of reports from Turkey about these enzymes. The β-lactamase types confirmed in Turkey were mostly TEM- and SHV-derived ESBLs.12,13 In this study, we evaluated TEM, SHV and GES types of ESBLs among phenotypically ceftazidime-resistant *K. pneumoniae* and *E. coli* strains from five different Turkish university hospitals.
Materials and Methods

Study design

Sixty-one E. coli isolates and 43 K. pneumoniae isolates resistant to ceftazidime were collected from five university hospitals located in different cities throughout Turkey (Diele University Hospital in Diyarbakir, Ondokuzmayis University Hospital in Samsun, Kocatepe University Hospital in Afyon, Firat University Hospital in Elazig and Farabi Hospital in Trabzon). The clinical microbiology laboratories of the hospitals performed identification of the species of the isolated strains and preliminary determinations of their susceptibility patterns. All bacteria were transferred to Infectious Diseases Research Laboratory at Kocaeli University Hospital, Kocaeli, Turkey.

Microbiological methods

The bacterial isolates potentially harbouring ESBLs were those with a positive phenotypic confirmatory test for ESBLs according to current National Committee for Clinical Laboratory Standards (NCCLS) criteria.14

Antibiotic susceptibility testing

All strains were re-identified before the study. The strains were inoculated into MacConkey’s agar and initially identified by glucose and lactose fermentation and oxidation, citrate utilization, urea hydrolysis, indole and oxidase production and motility tests. Selected isolates were further identified by the VITEK identification system (bioMérieux, Lyon, France). Antibiotic susceptibility tests were performed on Mueller-Hinton (MH) agar (Oxoid). Antibiotic disks were obtained from Oxoid. The susceptibility and MIC values of the bacteria were tested against ceftazidime, ceftazidime-clavulanate, cefotaxime, aztreonam, imipenem-cilastatin, meropenem, cefoxitin, amikacin, gentamicin and cefotaxime-clavulanate. MICs were determined by replicating approximately 10^4 CFU of bacteria per spot by the aid of a multipoint inoculator onto freshly prepared MH agar plates containing serial twofold dilutions of the related antibiotics. Agar plates were evaluated after 18 hours of incubation at 37 °C.15

Screening for ESBL producers

Isolates inhibited by ≥2 mg/L of at least one of the oxyimino-β-lactam ceftazidime, cefotaxime and aztreonam were considered as putative ESBL producers. ESBL production was confirmed by double-disc synergy test (DDST). DDST was done to determine synergy between a disc of amoxicillin/clavulanic acid (20 µg/10 µg) and 30 µg disc of each 3GC antibiotics earlier mentioned. MH agar plates were prepared and inoculated with standardized inoculum (0.5 McFarland tube) to form a pure culture. Disc (30 µg) of each 3GC antibiotics was placed on the agar at a distance of 15 mm centre to centre from amoxicillin/clavulanic acid disc. ESBL production was interpreted if the inhibition zone around the test antibiotic disc increased towards the amoxicillin/clavulanic acid disc or if neither discs were inhibitory alone but bacterial growth was inhibited where the two antibiotics diffuse together.

Isoelectric focusing of β-lactamases

β-lactamases were released by freezing and thawing a dense suspension of bacteria in 0.1 m phosphate buffer (pH 7.0) 10 times. After centrifugation for 15 min at 12,000×g, supernatants were subjected to an ampholine gel with a pH range of 3.5-10. Ampholine gels were prepared according to the formulation of Matthew et al.16 but were supplemented with 10% sucrose. After focusing at 10 W for 90-120 min at 4 °C on an isothermal-control electrophoresis apparatus (model CWS-2000; ISOLAB, Inc, Akron, Ohio), the enzymes were located with 1 mL nitrocefin in 0.1 m phosphate buffer (pH 7.0). Estimations of pI values were made by comparison with standards TEM-1 (5.4), TEM-2 (5.6), TEM-3 (6.3) and SHV-1 (7.6). Screening for ESBL gene(s) was performed by PCR using the primers for bla TEM, bla SHV and bla GES. On the isoelectric focusing gel, β-lactamase activities with pIs of 5.4, 5.6 and 7.6 were detected in most isolates. On the basis of DNA sequencing and pI values, the β-lactamase activity of pI 5.4 corresponds to that of TEM-1 β-lactamase, the pI value of 6.3 represents TEM-3 β-lactamase and the β-lactamase activity of pI 7.6 corresponds to that of SHV-1 β-lactamase.

Genes of the bla TEM, bla SHV and bla GES types identified by PCR assays, using as a template of total bacterial DNA and specific primers are given in Table 1. PCR screenings were accomplished in a final volume of 50 µL with a 5-µL DNA extract. The master mixture was composed of 1× buffer (supplied with DNA polymerase; Fermentas, Lithuania), 1.5 mM MgCl₂, 0.8 mM dNTPs, 50 pmol primers each and 1.5 U DNA polymerase. Amplification was accomplished after a 5 min denaturation at 95 °C by 40 cycles of 1 minute at 55 °C, 1.5 minute at 72 °C and 1 minute at 94 °C. PCR products were run on a 1.5% agarose gel at constant 12 V/cm and visualized on a UV lamp. PCR assays for bla SHV genes coding for enzymes containing the 278U19 and 733L20 substitutions were performed as described by Palucha et al.17

Table 1: Primers used in detecting extended-spectrum β-lactamases

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV-type</td>
<td>5'-CggTCAGCgAAAAAACACCT-3'</td>
<td>5'-TCCCgCAgATAAATCCACCCAC-3'</td>
<td>308-782</td>
</tr>
<tr>
<td>TEM-type</td>
<td>5'-ATgAgTTACCAcATTTCCgTg-3'</td>
<td>5'-TTAACAATgCTTAATCAgTgAg-3'</td>
<td>1-861</td>
</tr>
<tr>
<td>GES-type</td>
<td>5'-gCgTTTTgCAATgTgCCTAACC-3'</td>
<td>5'-gCgCCgCCATAgAggACTTTAg-3'</td>
<td>114-517</td>
</tr>
</tbody>
</table>

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Results

A total of 63 *E. coli* and 41 *K. pneumoniae* isolated from five different university hospitals were studied for the existence of TEM-, SHV- and GES-type β-lactamases. None of the isolates were found carbapenem-resistant. DDSTs were positive in 50 (79.3%) and 33 (80.5%) of *E. coli* and *K. pneumoniae*, respectively (Table 2).

Overall, 75 (72.1%) isolates, (47 *E. coli* and 28 *K. pneumoniae*) were characterized as ESBL producers. TEM gene was detected in 41 (65.1%) and 19 (46.3%), whereas SHV gene in 18 (28.6%) and 20 (48.8%) of *E. coli* and *K. pneumoniae* strains, respectively. In total, 47 *E. coli* isolates (74.6%) and 28 *K. pneumoniae* (68.3%) were detected as ESBL producer. GES genes were not detected (Table 2).

The distribution of ESBL producer strains was shown in Table 3. There were no significant differences between the hospitals in species distribution (chi-square = 1.8, *P* > 0.05) and rates of ESBL producers within each species (chi-square = 0.08, *P* > 0.05). There was no significant difference between the *E. coli* and *Klebsiella* strains for producing ESBL. The most active antibiotic was meropenem (none of the isolates was resistant to this drug) followed by piperacillin-tazobactam, considering all isolates or ESBL producers only (Table 4).

### Table 2: ESBLs detected by clavulanate synergy test and PCR

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Total number</th>
<th>DDST positive (%)</th>
<th>SHV</th>
<th>TEM</th>
<th>GES</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (strains)</td>
<td>63</td>
<td>50 (79.4)</td>
<td>18 (28.6)</td>
<td>41 (65.1)</td>
<td>0</td>
</tr>
<tr>
<td>ESBL positive</td>
<td>47</td>
<td>36 (76.6)</td>
<td>18 (38.3)</td>
<td>41 (87.2)</td>
<td>0</td>
</tr>
<tr>
<td>ESBL negative</td>
<td>16</td>
<td>14 (87.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>41</td>
<td>33 (80.5)</td>
<td>20 (49.8)</td>
<td>19 (46.3)</td>
<td>0</td>
</tr>
<tr>
<td>ESBL positive</td>
<td>28</td>
<td>22 (78.6)</td>
<td>20 (71.4)</td>
<td>19 (67.9)</td>
<td>0</td>
</tr>
<tr>
<td>ESBL negative</td>
<td>13</td>
<td>11 (84.6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>75</strong></td>
<td><strong>72 (86.8)</strong></td>
<td><strong>38 (50.7)</strong></td>
<td><strong>60 (80.0)</strong></td>
<td><strong>0</strong></td>
</tr>
<tr>
<td>ESBL positive</td>
<td><strong>29</strong></td>
<td><strong>22 (72.4)</strong></td>
<td><strong>0 (0)</strong></td>
<td><strong>0 (0)</strong></td>
<td><strong>0</strong></td>
</tr>
</tbody>
</table>

### Table 3: The distribution of ESBL producer strains according to centres

<table>
<thead>
<tr>
<th>Hospitals</th>
<th>DUH (n = 37)</th>
<th>Firat UH (n = 24)</th>
<th>OMUH (n = 32)</th>
<th>AKUH (n = 4)</th>
<th>Farabi H (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospitals’ total positive strains (%)</td>
<td>29 (78.4)</td>
<td>17 (70.8)</td>
<td>19 (59.4)</td>
<td>4 (100)</td>
<td>6 (85.7)</td>
</tr>
<tr>
<td><em>E. coli</em> (63 strains)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESBL positive (47)</td>
<td>23</td>
<td>7</td>
<td>10</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>ESBL negative (16)</td>
<td>4</td>
<td>3</td>
<td>8</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (41 strains)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESBL positive (28)</td>
<td>6</td>
<td>10</td>
<td>9</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>ESBL negative (13)</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

DUH - Dicle University Hospital, OMUH - Ondokuzmayis University Hospital, AKUH - Afyon Kocatepe University Hospital, FUH - Firat University Hospital, Farabi H - Farabi Hospital

### Table 4: Antimicrobial resistant patterns (MIC as µg/L) of ESBL positive and ESBL negative strains

<table>
<thead>
<tr>
<th>Isolates</th>
<th>CAZ ≥ 2</th>
<th>CAZ ≥ 128</th>
<th>CAZ/CL ≥ 4</th>
<th>AM/CL ≥ 32/16</th>
<th>IMP ≥ 16</th>
<th>MEM ≥ 16</th>
<th>FOX ≥ 32</th>
<th>CTX ≥ 64</th>
<th>AZT ≥ 32</th>
<th>CEP ≥ 32</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (63 strains)</td>
<td></td>
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<tr>
<td>ESBL positive (47)</td>
<td>44 (94)</td>
<td>36 (77)</td>
<td>14 (30)</td>
<td>37 (79)</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>18 (38)</td>
<td>39 (83)</td>
<td>36 (77)</td>
<td>18 (38)</td>
</tr>
<tr>
<td>ESBL negative (16)</td>
<td>13 (81)</td>
<td>6 (38)</td>
<td>4 (25)</td>
<td>8 (50)</td>
<td>2 (13)</td>
<td>0 (0)</td>
<td>8 (50)</td>
<td>12 (75)</td>
<td>13 (81)</td>
<td>9 (56)</td>
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<td><em>K. pneumoniae</em></td>
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</tr>
<tr>
<td>ESBL positive (28)</td>
<td>1 (4)</td>
<td>24 (86)</td>
<td>8 (29)</td>
<td>22 (79)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>9 (32)</td>
<td>26 (93)</td>
<td>24 (86)</td>
<td>10 (36)</td>
</tr>
<tr>
<td>ESBL negative (13)</td>
<td>0 (0)</td>
<td>8 (62)</td>
<td>1 (8)</td>
<td>6 (46)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (23)</td>
<td>10 (77)</td>
<td>10 (77)</td>
<td>4 (31)</td>
</tr>
</tbody>
</table>


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Discussion

ESBL antibiotics are commonly included in the empirical antibiotic regimens for treatment of nosocomial infections. Especially, the selection of ESBL-producing de-repressive microorganisms in infection site is increasing by the clinical usage of broad-spectrum cephalosporins. Since then, several outbreaks have been reported in many European countries and the USA, the epidemiology of ESBLs has showed that this type of resistance problem is endemic in several places worldwide, with rates exceeding 50% in some countries.23 The prevalence of ESBL-producing Klebsiella and E. coli was found high in different studies in Turkey.19 In a three-year study, the rates of production of ESBLs were found to be 20.9% for E. coli and 50% for K. pneumoniae, using ceftazidime and ceftazidime/clavulanic acid E-test strips, at a Turkish university hospital. In this study, ESBL production increased each year (21.7%, 22.1% and 45.5%).20 According to Özgünün et al., the prevalence of ESBL-producing K. pneumoniae was 47% and E. coli was 12%; the ESBL-producing isolates were from intensive care units in 50% (14/28), from wards in 36.1% (35/97) and from outpatient clinics in 13.3% (10/75).21

Currently, there are many different methods for detection of ESBLs in laboratory settings but controversies exist regarding the clinical importance of such resistance, the choice of optimal laboratory methods to detect it. ESBL-mediated resistance could be determined by the combined disk methods, the DDST, the three-dimensional agar test, rapid automated systems using commercial cards, E-test ESBL strip and PCR detection methods.22 Some studies suggest that clinical microbiology laboratories should not rely on the rapid automated systems for method for screening ESBL producers but use another more reliable system such as the E-test.22 In our study, we used DDST and results were confirmed by PCR with specific oligonucleotide primers and further confirmed by isoelectric focusing of β-lactamase enzymes from the bacterial extracts.

The varieties of ESBLs are very rich in Turkey. Different types of SHV and TEM are endemic in Turkey. GES, CTX-M and PER types also were reported from Turkey in recent years.23 In our study since we did not detect GES types among E. coli and Klebsiella strains, we believe that this type of β-lactamases is not endemic or very rare in Turkey.

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References


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