Phenotypic Detection of ESBL, MBL (IMP-1), and AmpC Enzymes, and Their Coexistence in Enterobacter and Klebsiella Species Isolated From Clinical Specimens

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Abstract

Background: Bacterial resistance to beta-lactam is a major problem in all developed and developing countries. The genera of Klebsiella and Enterobacter are associated with opportunistic and nosocomial infections. All beta-lactamase genes can cause dissemination of resistance to beta-lactams.

Objectives: The present study aimed to investigate the phenotypic detection of beta-lactamases in Enterobacter and Klebsiella species isolated from clinical specimens.

Materials and Methods: This cross-sectional study was performed on 59 Klebsiella spp. and 49 Enterobacter spp. isolated from clinical samples. They were confirmed using API 20E. These bacteria were evaluated for the production of extended-spectrum beta lactamase (ESBL), metallo-beta-lactamase (MBL) (IMP-1), and the pAmpC and iAmpC enzymes. This was done using the Clinical and Laboratory Standards Institute (CLSI) method, 2-mercaptopropionic acid, the cefoxitin method, and the use of imipenem as an enzyme inducer, respectively. Mask-ESBL production was also identified, using different concentrations of 3-amino-phenyl boronic acid compound. Data were analyzed with SPSS version 22.

Results: In total, 38 (64.4%) Klebsiella spp. and 41 (83.7%) Enterobacter spp. produced at least one type of beta-lactamase. AmpC-producing Enterobacter spp. (71.4%), and ESBL-producing Klebsiella spp. (42.4%) had the highest prevalence of beta-lactamase types in each genus. There were two bacteria in both types that were resistant to all antibiotics without producing any type of beta-lactamase.

Conclusions: According to our findings, it is necessary to pay special attention to ESBL production in Klebsiella spp., while in Enterobacter spp., it is essential to search for AmpC production (chromosomal and plasmid). In addition, the genotypic evaluation of beta-lactamase variety in these bacteria may be necessary in different geographical areas.

Keywords: Klebsiella, Enterobacter, AmpC beta-Lactamase

1. Background

The genera of Klebsiella and Enterobacter, of the Enterobacteriaceae family, are associated with opportunistic and nosocomial infections, such as wound and urinary tract infections. K. pneumoniae is a common cause of lower respiratory tract infections among hospitalized patients, and the prevalence of infection is increased with long hospital stays. In humans, K. oxytoca can produce infections similar to K. pneumoniae. E. cloacae and E. aerogenes have been isolated from wounds, urine, blood, and CSF more often than the other Enterobacter spp. E. sakazakii has been identified in brain abscesses and in respiratory and wound infections (1).

Bacterial resistance to beta-lactam antibiotics is a major problem in all developed and developing countries (2). Antimicrobial resistance in the Enterobacteriaceae family has increased in recent years; the most severe type is probably carbapenemase-producing K. pneumoniae (1). The main mechanism of resistance to beta-lactams (penems, cefems, carbapenems, and monobactam) in gram-negative bacteria is the production of beta-lactamase enzymes (3).

Beta-lactamases are divided into A, B, C, and D molecular classes in the Ambler classification system (1, 4). The extended spectrum beta-lactamase (ESBL) enzyme is a type of beta-lactamase that hydrolyzes penicillins, cephalosporins, and aztreonam, and that is coded by movable genes (5). ESBLs play a major role in the creation of multidrug resistance (MDR) among gram-negative bacilli. Rapid diagnosis of ESBL-producing bacteria can be important in limiting the spread of MDR pathogens and infections (6). The CLSI (Clinical and Laboratory Standards Institute) has provided a protocol to identify ESBLs (A and D Ambler classes) in the Enterobacteriaceae family...
Plasmid-mediated metallo-beta-lactamases (MBLs, belonging to Ambler class B) are classified into three main groups: IMP, VIM, and SPM (8). The CLSI recommends a method for the detection of small quantities of MBL, which has not yet been approved (9). There are different techniques for the isolation of MBL-producing bacteria, including the double-disk synergy test (DDST) using EDTA, or 2-mercaptopropionic acid with imipenem or ceftazidime (10). Among these compounds, 2-mercaptopropionic acid (2MPA) is able to detect even small amounts of the enzyme (11).

The production of AmpC enzymes (Ambler class C) in gram-negative bacteria is controlled by genes on the chromosome (inductive type) or by a plasmid (permanent type) (12). They confer resistance to cephalosporins, oxyimino groups, and 7-α-methoxy cephalosporins, and are not inhibited by ESBL inhibitors, such as clavulanic acid (13).

Generally, production of AmpC enzymes in Enterobacter species is controlled by chromosomal AmpC genes (3) and are considered in serious infections dependent on E. aerogenes and E. cloacae (14). Plasmid-mediated AmpC strains of K. pneumoniae and K. oxytoca are widely associated with MDR phenotypes (13). Plasmid genes (transferable) have been acquired by bacterial species, such as E. coli and K. pneumoniae, which lack chromosomal genes (14).

Various methods have been introduced for the detection of chromosomal AmpC using different inducers, such as imipenem, tazobactam, and cefoxitin (15, 16). Plasmid-mediated AmpC beta-lactamases are detected in enzyme extracts of bacteria or with techniques based on using enzyme-inhibitor compounds, such as boronic acid (17).

### 2. Objectives

Since all beta-lactamase genes can be distributed by transmissible genetic elements in bacteria and can cause dissemination of resistance to beta-lactam antibiotics, the present study aimed to investigate the phenotypic detection of different kinds of beta-lactamase enzymes in Enterobacter and Klebsiella species isolated from clinical specimens.

### 3. Materials and Methods

This cross-sectional study was performed from November 2011 to December 2014 on 108 Klebsiella and Enterobacter spp. isolated from urine (77, 71.3%), blood (2, 1.9%), wounds (2, 1.9%), secretions (7, 6.5%), feces (5, 4.6%), and other samples (sputum, CSF, discharge, Sheldon catheter) (15, 13.9%). The samples were taken from patients who were referred to the microbiology labs of the affiliated hospitals at Babol University of Medical Sciences in northern Iran.

#### 3.1. Diagnosis of Bacteria and Antimicrobial Susceptibility Testing

The isolates were identified with API 20E (bioMerieux SA, France) and differential tests, including KIA, SIM, and MR (Himedeae). Antimicrobial susceptibility testing (AST) was done based on the CLSI 2014 protocol. Antibiotics were used for the disk diffusion method on MHA medium (7), including cefotaxime (CTX), ceftazidime (CAZ), cepodoxime (CPD) (cephalosporin III), cefepime (CPM) (cephalosporin IV), ertapenem (ETP) (carbapenem), and aztreonam (ATM) (monobactam) (MASTDiscs, UK).

#### 3.2. Phenotypic Methods

##### 3.2.1. ESBL

The bacteria that were resistant to at least two antibiotics (CAZ ≤ 22 mm, CTX ≤ 27 mm) were suspected to produce ESBL enzymes. They were examined by confirmatory testing (7).

##### 3.2.2. Mask-ESBL

The Mask-ESBL test was performed on the isolates for which the confirmatory test of ESBL was negative, despite being resistant to two antibiotics, or if the growth-inhibitory zone was not increased around only one type of combination disk (18).

##### 3.3. Evaluation of pAmpC using Cefoxitin Agar Medium (CAM AmpC)

#### 3.3.1. Enzyme Extraction

For enzyme extraction, 25 µL of bacterial suspension (equal to 0.5 McFarland) was inoculated into 6 mL of Trypticase Soy Broth and incubated for 4 hours at 35°C. Then, it was centrifuged at 3,000 rpm for 15 minutes, and seven freeze/thaw cycles were performed on the precipitate (19).

#### 3.3.2. Evaluation

In this method, an MHA medium containing 6 µg/mL of cefoxitin (TAB/0.4 mg, Mast Diagnostics, UK, 325304) was used. A suspension of E. coli ATCC 25922 (equivalent to 0.5 McFarland) was cultured using the bacterial lawn technique. A well (5 mm) was created in the agar, to which 30 µL of bacterial extract was added. After one night incubation at 35°C, the bacteria that grew around the well were considered plasmid AmpC enzyme producers (Figure 1) (20).

#### 3.4. Evaluation of Chromosomal AmpC (iAmpC) by the Disk Method

In this method, an MHA medium containing 6 µg/mL of cefoxitin (TAB/0.4 mg, Mast Diagnostics, UK, 325304) was used. A suspension of E. coli ATCC 25922 (equivalent to 0.5 McFarland) was cultured using the bacterial lawn technique. A well (5 mm) was created in the agar, to which 30 µL of bacterial extract was added. After one night incubation at 35°C, the bacteria that grew around the well were considered chromosomal AmpC enzyme producers (20).
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Figure 1. Positive Plasmid AmpC Producing Isolate

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3.4.1. Metallo-Beta-Lactamase (IMP-1)

The isolates that were resistant to at least two antibiotics in the first stage (screening) (CAZ ≤ 22 mm, CTX ≤ 27 mm), were suspected to produce IMP-1 enzymes, and were examined for confirmatory testing (18).

The results were analyzed with SPSS version 22. Descriptive statistics were used.

4. Results

Fifty-nine isolated Klebsiella spp. bacteria and 49 Enterobacter spp. bacteria from the clinical samples (Table 1) were confirmed with API 20E and with differential tests, including KIA, SIM, and MR.

Among the 59 Klebsiella spp., 52 (88.1%) and seven (11.9%) were K. pneumonia and K. oxytoca, respectively. There were also 37 (75.5%) E. cloacae, 10 (20.4%) E. aerogenes, and two (4.1%) E. sakazakii among the 49 Enterobacter spp.

On the first AST, Klebsiella spp. were resistant to cefpodoxime (50.8%), ertapenem (13.6%), aztreonam (54.2%), cefazidime (52.5%), cefotaxime (55.9%), and ceftipime (45.8%). The Enterobacter spp. were resistant to cefpodoxime (65.3%), ertapenem (28.6%), aztreonam (46.9%), cefazidime (57.1%), cefotaxime (63.3%), and ceftipime (32.7%). In total, seven (11.9%) of the Klebsiella spp. and four (8.2%) of the Enterobacter spp. showed resistance to all of the beta-lactam antibiotics. Overall, 38 (64.4%) of the Klebsiella spp. and 41 (83.7%) of the Enterobacter spp. produced at least one type of beta-lactamase enzyme.

As seen in Table 2, ESBL production was identified in 17 (34.7%) Enterobacter spp. and 25 (42.4%) Klebsiella spp. (MESBL + ESBL).

Of the Enterobacter spp., seven (14.3%) bacteria produced iAmpC, pAmpC, and IMP-1 enzymes simultaneously. In addition, AmpC/MESBL co-production was seen in four (8.2%) of the Enterobacter spp. and in one (1.7%) of the Klebsiella spp. (Table 3).

It is noteworthy that among the 21 non-lactose-fermenter Enterobacter species, 12 (57.1%) produced at least one kind of beta-lactamase enzyme. There were also two bacteria in each genus that were resistant to all antibiotics, without producing any type of beta-lactamase.

5. Discussion

MDR microbial strains may occur via mutations of beta-lactamase due to the use of antimicrobial agents (22). The present study showed that 17 (34.7%), 21 (42.8%), 19 (38.8%), and 14 (28.6%) of the Enterobacter spp. produced ESBL, pAmpC, IMP-1, and iAmpC, respectively. However, the frequency of beta-lactamase-producing Klebsiella spp. were 25 (42.4%), 9 (15.2%), and 12 (23.1%), respectively. Other studies carried out in the different parts of the world, such as India, Nigeria, and Iran, confirm these results, with slight differences (12, 22-25).

There were 22 (42.2%) and 8 (15.4%) ESBL and pAmpC enzymes, respectively, produced by K. pneumoniae in the current study, which was similar to the results of research into on ESBL-producing K. pneumoniae in Mashhad, northeastern Iran, which found 55 (43%) such species (24), and in Ahvaz, southern Iran, which identified 26 (47.3%) such species (26). However, in Zabol, southeastern Iran, Saide reported 20 (66.6%) ESBL-producing K. pneumonia in urine samples (27). Yusuf reported the production of ESBL in 10 (20%) and of pAmpC in two (4%) K. pneumoniae spp. in Nigeria (25), while Varsha reported production rates of these enzymes at 56 (56%) and 32 (32%), respectively, in India (13).

Paterson reported a high percentage of ESBL-producing K. pneumoniae and stated that infections caused by these bacteria must be considered as risk factors for bacterial infections due to ESBL producers in the community (28). Therefore, it seems that performing routine screening tests to determine their prevalence is necessary.

Among the Enterobacter spp. isolated in the present study, the AmpC-producing bacteria had the highest preva-
Figure 2. Positive Inducible AmpC Producing Isolate

Table 1. Frequency of Klebsiella and Enterobacter spp. in Clinical Samples

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Clinical Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
</tr>
<tr>
<td><strong>Klebsiella spp.</strong></td>
<td>47</td>
</tr>
<tr>
<td><strong>Enterobacter spp.</strong></td>
<td>30</td>
</tr>
</tbody>
</table>

*Values are expressed as No. (%).

Table 2. Frequency of ESBL, MESBL, pAmpC, iAmpC, and IMP-1 Production in Isolated Klebsiella and Enterobacter spp.*

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>ESBL</th>
<th>MESBL</th>
<th>pAmpC</th>
<th>iAmpC</th>
<th>IMP-1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. pneumonia</td>
<td>20 (38.5)</td>
<td>2 (3.4)</td>
<td>8 (15.4)</td>
<td>0</td>
<td>12 (23.1)</td>
<td>52 (100)</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>3 (42.9)</td>
<td>0</td>
<td>1 (44.3)</td>
<td>0</td>
<td>0</td>
<td>7 (100)</td>
</tr>
<tr>
<td>E. aerogenes</td>
<td>4 (40)</td>
<td>0</td>
<td>3 (30)</td>
<td>1 (10)</td>
<td>5 (50)</td>
<td>10 (100)</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>6 (16.2)</td>
<td>7 (18.9)</td>
<td>18 (48.6)</td>
<td>12 (32.4)</td>
<td>14 (37.8)</td>
<td>37 (100)</td>
</tr>
<tr>
<td>E. sakazakii</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (50)</td>
<td>0</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

*Values are expressed as No. (%).

In this study, the prevalence of E. cloacae isolates produced an AmpC enzyme in bacteria isolated from clinical specimens is the mutation of AmpD enzyme inside the cytoplasm of the bacterial cell (20). The transfer of AmpC genes to plasmids can increase pAmpC-producing bacteria.

In addition, 39.3% of the Enterobacter spp. were susceptible to cephalosporins. Some cephalosporins lead to the production of mutant strains that contain the constitutive production of high levels of AmpC enzyme (5, 28, 29). The most common cause of AmpC overexpression was the mutation of AmpD enzyme inside the cytoplasm of the bacterial cell (20). The transfer of AmpC genes to plasmids can increase pAmpC-producing bacteria.
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Table 3. Frequency of ESBL, MESBL, pAmpC, iAmpC, and IMP-1 Co-Producing Klebsiella and Enterobacter spp.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Bacteria</th>
<th>E + IM</th>
<th>E + pA</th>
<th>ME + pA</th>
<th>IA + ME</th>
<th>pA + IM</th>
<th>IA + pA</th>
<th>E + pA + IM</th>
<th>IA + pA + IM</th>
<th>IA + IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella spp.</td>
<td>1 (1.7)</td>
<td>1 (1.7)</td>
<td>1 (1.7)</td>
<td>0 (0)</td>
<td>3 (5.1)</td>
<td>0</td>
<td>0</td>
<td>1 (1.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>1 (2)</td>
<td>3 (6.1)</td>
<td>3 (6.1)</td>
<td>1 (2)</td>
<td>0</td>
<td>2 (4)</td>
<td>2 (4.1)</td>
<td>0</td>
<td>0</td>
<td>7 (14.3)</td>
</tr>
</tbody>
</table>

Abbreviations: E, ESBL; IA, inducible AmpC; IM, IMP-1; ME, mask-ESBL; pA, pAmpC.

Table 4. Frequency of Pure ESBL, MESBL, pAmpC, iAmpC, and IMP-1 Production in Isolated Klebsiella and Enterobacter spp.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Bacteria</th>
<th>ESBL</th>
<th>IMP-1</th>
<th>pAmpC</th>
<th>Mask ESBL</th>
<th>iAmpC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella spp.</td>
<td>21 (35.5)</td>
<td>7 (11.9)</td>
<td>3 (5.1)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>6 (12.2)</td>
<td>8 (16.3)</td>
<td>4 (8.2)</td>
<td>2 (4)</td>
<td>2 (4.1)</td>
<td></td>
</tr>
</tbody>
</table>

According to the present study, Enterobacter spp. co-production of iAmpC along with other beta-lactamases was found in 12 (24.5%) strains. These bacteria can lead to an increased rate of treatment failure in infected patients (5).

Another notable finding of this study was the fairly remarkable percentage of IMP-1-producing bacteria in combination with other beta-lactamase enzymes (Klebsiella 8.5%, Enterobacter 20.4%). The simultaneous production of carbapenemase and pAmpC can occur in strains that may lack outer membrane porin (3). Identifying the production of each mentioned enzyme in a bacterium can help physicians in choosing the appropriate antibiotic for treating infections caused by these organisms.

There were several contemplative findings in this study, including the presence of a significant percentage (57.1%) of beta-lactamase production in negative-lactose-fermentation Enterobacter spp. Further research should be carried out on the probable relationship between β-lactamase production and the inability of lactose consumption in bacteria. Furthermore, it was observed that two non-beta-lactamase-producing bacteria of each genus were resistant to all antibiotics, which drew attention to other resistance mechanisms, such as efflux pump activity or the lack of outer membrane proteins (18).

In addition to the findings described above, 26 bacteria of each genus showed resistance against cefotaxime disks (without a growth inhibition zone), which could not be detected by the iAmpC disk method; therefore, it appears this method is not suitable for the detection of the iAmpC enzyme (Figures 3 and 4).

Another important finding of this study was the presence of bacteria that simultaneously produced various beta-lactamases (Enterobacter 38.7%, Klebsiella 11.9%). These bacteria can limit clinical use of beta-lactam antibiotics and lead to laboratory diagnostic problems (31). This means that the identification of bacteria producing single enzymes or combinations of them is necessary for the control and treatment of infections (20).

According to the present study, Enterobacter spp. co-production of iAmpC along with other beta-lactamases was found in 12 (24.5%) strains. These bacteria can lead to an increased rate of treatment failure in infected patients (5).
5.1. Conclusions

According to the findings of this study, it is necessary to pay special attention to ESBL production in *Klebsiella* spp., while in *Enterobacter* spp., it is essential to search for AmpC production (chromosomal and plasmid), and there should be evaluation of multiple beta-lactamases produced by a bacterium in order to choose the proper treatment. In addition, the genotypic evaluation of bacteria producing a variety of beta-lactamases will help to prevent the spread of these genes among gram-negative bacteria in certain geographic areas.

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References


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**Figure 4. Non Detectable Isolate by Inducible Method**

1. imipenem; 2. CTX + boronic acid; 3. CTX.


