Prevalence of plasmid-encoded carbapenemases in multi-drug resistant Escherichia coli from patients with urinary tract infection in northern Iran

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ABSTRACT

Objective(s): Resistance to carbapenems as the last line for controlling resistant bacteria is increasing due to production of carbapenemase. The aim of this study was to detect the plasmid-encoded carbapenemases using phenotypic methods and multiplex PCR among the multi-drug resistant (MDR) isolates from patients with urinary tract infection (UTI) in northern Iran.

Materials and Methods: Antimicrobial susceptibility testing and extended spectrum β-lactamase (ESBL) production test were performed for 91 MDR Escherichia coli strains by disc diffusion and double disk synergy tests (DDST), respectively. Carbapenemases production was confirmed using Hodge test, EDTA double disk synergy test (EDST) and combined disk test (CDT). The isolates were subjected to PCR targeting blaIMP, blaVIM, blaKPC and blaOXA-48 β-Lactamase genes.

Results: Resistance of isolates to 1st, 2nd, 3rd, and 4th generations of cephalosporins, carbapenems, and penicillins were 73%, 84.5%, 62%, 37.5%, 37.5% and 16%, respectively. Based on CDT and Hodge test, 1 (3%) and based on EDST, 2 (6%) of 33 ESBL producers synthesize a type of carbapenemase. The frequency of blaIMP, blaVIM,blaKPC, and blaOXA-48 genes was 87.9%, 9.8%, 2.1%, and 15.3%, respectively. Existence of blaIMP conferred more resistance to cephalolin, fosfomycin, and piperacillin (P≤0.01) and carrying blaVIM caused more resistance to cephalotin, ceftazidime and ceftriaxone (P≤0.01). The presence of blaKPC conferred more resistance to ceftazidime and presence of blaOXA-48 caused more resistance to chloramphenicol and piperacillin (P≤0.05).

Conclusion: Identification and controlling of this nearly low frequent ESBL and carbapenemase producing strains are important due to the presence of plasmid genes encoding carbapenemase.

Introduction

Over the last decade, the emergence of resistance to carbapenems, has become a major public health crisis worldwide especially in developing countries, due to their rapid spread and the lack of development of new antimicrobial agents (1-3). Resistance to carbapenems was reported in 86% of gram-negative bacterial strains in Iran in 2010 (4).

Throughout 2006–2018, incremental trend of resistance to carbapenems was evident in Iran (5). The least rate of resistance was reported in year 2010 at Milad Hospital (6). During years 2012–2015, a study evaluated the trend of antibiotic resistance in Acinetobacter baumannii. Based on their study results, resistance rate in A. baumannii increased from 83% in year 2012 to 96% in year 2015. Also 100% of A. baumannii isolates during these years were resistant to carbapenem (7). No specific trend was followed by the other microorganisms’ resistance patterns. Most of the carbapenem-resistant strains were isolated from burn patients, and many studies which were conducted in this group were from Motahari Hospital, Tehran, Iran (5).

Since 1993, wide varieties of carbapenemases have been recognized that belong to three molecular classes: the Amblor class A, B, and D β-lactamases (3).

In this investigation, four carbapenemases including IMP, VIM, KPC, and OXA-48 were studied. KPC stands for Klebsiella pneumoniae carbapenemase and is a class A β-lactamase that has the ability to hydrolyze penicillins, cephalosporins, and carbapenems. KPC was initially reported from a K. pneumoniae strain isolated in North Carolina in 1996 (8).

Class B metallo-β-lactamases (MBLs) are mostly of the Verona integron-encoded metallo-β-lactamase (VIM) and IMP types and, more recently, of the New Delhi metallo-β-lactamases-1 (NDM-1) type (3, 9). MBLs can hydrolyze all β-lactams except monobactam (e.g., aztreonam). Their activity is inhibited by EDTA but not by clavulanic acid (9).

The IMP-type enzymes, initially reported in 1991 in a Serratia marcescens clinical isolate from Japan (2), have now been reported all over the world in Enterobacteriaceae, Pseudomonas aeruginosa, and A. baumannii (2). The most commonly found class B carbapenemases are of the VIM type, which has been identified in all continents (2). The death rates associated with MBL producers are high (18% to 67%) (10, 11).

OXA stands for oxacillinase and is a diverse group of β-lactamases classified to class D. Some of OXA
β-lactamases additionally have the capability to hydrolyze carbapenems. OXA-48 was first found in a *K. pneumoniae* strain isolated in Turkey in 2001 (12). Its production mediates resistance to penicillins and carbapenems (especially imipenem), but not to cephalosporins. In Iran, OXA-48 was first reported in 2017 in the *Escherichia coli* isolates (13).

Among the uropathogenic bacteria, *E. coli* is predominant in both community and nosocomial urinary tract infection (UTI) (14–16). These resistance patterns have shown large inter-regional variability. Understanding the spectrum and resistance patterns may help guide effective empirical antibiotic therapies and decrease treatment failure and costs.

Contact precautions and outbreak detections require reliable detection of carbapenemases. However, detection of carbapenemase in Enterobacteriaceae is challenging, because carbapenemase-producing *K. pneumoniae* with low carbapenem MBCs have been described in the CLSI or EUCAST-susceptible range. Also, a difference between porin loss coupled with an ESBL or AmpC β-lactamase or carbapenemase is not feasible in carbapenem-resistant isolates alone on the basis of the antibiogram (17). Phenotypic tests such as the modified Hodge test are helpful to detect carbapenemases but have low sensitivity for NDM and low specificity (18). Phenotypic tests based on synergy with EDTA are available for detection of MBL, but can produce false-positive outcomes with certain strains and cannot distinguish between types of MBL (19). Class A carbapenemases such as KPC can be identified through synergy with boronic acid, but if AmpC β-lactamases are coproduced, false-positive synergy test findings occur (20). Thus, confirmation using molecular analysis is essential.

Due to limited information on carbapenemase in Iran (21), identifying the resistant strains is a major challenge for diagnostic laboratories. The carbapenemases that were surveyed in this study were encoded by plasmids and due to their transfer to other isolates, the purpose of this study was to identify types of carbapenemases using phenotypic methods and to determine the frequency of plasmid genes encoding carbapenemases (IMP, VIM, KPC, and OXA-48) among the MDR isolates causing UTI in northern Iran.

### Materials and Methods

#### Bacterial isolates

Urine samples of the patients (138 samples including 31 male and 107 female specimens with mean age of 43 for male and 41 for female) were collected from appropriate patients in early morning mid-stream using sterile, wide-mouthed glass bottles with screw cap tops between May and July 2017. Samples were maintained in an icebox until laboratory analysis. Sample collection and its analysis were no more than one hour apart.

The usual bacteriological methods were applied for cultivation, isolation and identification of the strains. Urine samples were cultured on Nutrient Agar, Blood Agar, Eosin Methylene Blue Agar (EMB), and MacConkey agar plates and incubated at 37 °C for 18–24 hr. Urine culture was considered positive, if it contained ≥10⁵ cfu/ml *E. coli* from positive urine cultures identified by their characteristic appearance on the media, Gram staining reaction, by the pattern of biochemical tests such as catalase, oxidase, ONPG, IMViC tests, lactose fermentation, H₂S and CO₂ production, urea hydrolysis, and lysine decarboxylase (22). The isolates were stored at -70 °C in a Tryptic Soy Broth containing 15% glycerol until processing.

#### Antibacterial susceptibility testing

To identify the susceptibility of the isolates to antibiotics, the disc diffusion test was used according to Clinical and Laboratory Standards Institute (CLSI) (23) guidelines; the following antibiotics were utilized: ampicillin (AMP)(10 μg), amoxicillin (AMX)(25 μg), oxacillin (OXA)(5 μg), fosfomycin (FOP)(200 μg), piperacillin (PIP)(100 μg), streptomycin (STR)(10 μg), ticarcillin (TET)(30 μg), chloramphenicol (CHL)(30 μg), ceftizime (CEP)(30 μg), ceftriaxone (CRO)(30 μg), ceftazidime (CAZ)(30 μg), cefalothin (CF)(30 μg), cefazolin (CFZ)(30 μg), ceftaxime (CTX)(30 μg), cefixime (CFM)(5 μg), cefuroxime (CXM)(30 μg), imipenem (IMP)(10 μg), meropenem (MEM)(10 μg), amoxicillin-clavulanic acid (AMC)(20/10 μg), and ciprofloxacin (CIP)(5 μg). *E. coli* ATCC 25922 and ATCC 35218 were used as the standard strains to control the quality of the applied antimicrobial agents. MDR is defined as resistance to three or more antibiotics.

#### Detection of ESBL

In order to identify ESBL, double disk synergy test (DDST), which depends on comparing the inhibition zone given by CAZ (30 μg) and CAZ-plus-clavulanate (30 μg/10 μg) was used. A difference of ≥5 mm between the zone of CAZ-plus-clavulanate and CAZ alone was taken to indicate ESBLs production as advocated by CLSI (23).

#### Hodge test

Briefly, a 0.5 McFarland bacterial suspension of *E. coli* ATCC 25922 as control or susceptible strain was inoculated on the surface of a Mueller-Hinton agar (MHA) as a lawn culture. After brief drying, a 10 μg imipenem disk was placed at the center, and the test isolate was streaked from the center to the periphery of the plate and the plate was incubated overnight. Isolates which produced a cloverleaf-shaped inhibition zone were recognized as producers of carbapenemase (24).

#### Imipenem-EDTA combined disk test (CDT)

As recommended by CLSI, the control strain was cultured as a lawn on the MHA plate along with test isolates (turbidity of 0.5 McFarland). Then, two 10-μg meropenem discs were located on the lawn culture with 20 mm distance from center to center of the discs. In one of the meropenem disks, a 10 μl 0.5 M EDTA was added and incubated overnight. Isolates indicating a rise of ≥7 mm in the meropenem+EDTA disc’s inhibition zone size compared to the meropenem disc alone were known MBL producers (24).

#### EDTA-disk synergy test (EDST)

An overnight broth culture of the test isolate was suspended to the turbidity of 0.5 McFarland and used to swab inoculate a MHA. A 10-μg meropenem disc and a blank disk (Whatmann filter paper no. 2, 6 mm in diameter) were located 10 mm apart from edge to edge. 10 μl EDTA solution 0.5 M was then used as the blank disc. The plates were incubated at 37 °C overnight and an expanded inhibition zone was interpreted as positive EDS (24).

#### Multiplex PCR technique

DNA extraction was performed with suspending
one colony in 100 µl of distilled water (95 °C for 10 min) followed by centrifugation of the cell suspension. The DNA concentration and purity were determined by spectrophotometric measurement of absorbance at 260 and 280 nm by a UV spectrophotometer. All DNA samples were dissolved in water and stored at -20 °C.

The PCR reactions were carried out using a 96-well mini PCR System Thermal Cycler (BioRad, USA) in a final volume of 25 µl containing 200 ng of each primer, 50 ng genomic DNA, 1.5 mM MgCl₂, 200 µM dNTPs, and 1.0 U of Taq DNA Polymerase in the buffer provided by the manufacturer (25). The sequences of specific primers were designed based on relevant DNA sequences available in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank) using Oligo-primer analysis software (Version 7.54, Molecular Biology Insights, USA). Primers sequences were listed in Table 1.

Amplifications were carried out with the following thermal cycling conditions: 10 min at 94 °C and 30 cycles of amplification consisting of 94 °C for 40 sec, 60 °C for 40 sec, and at 72 °C for 1 min, with 7 min at 72 °C for the final extension. For the multiplex PCR analysis, the annealing temperature was at 55 °C for amplification of bla<sub>IMP</sub>, bla<sub>VIM</sub>, and bla<sub>IMP</sub> genes, and 57 °C for amplification of bla<sub>OXA</sub> gene. The PCR products were subjected to electrophoresis on a 2% agarose gel and observed under UV after staining with ethidium bromide (25).

**Statistical analysis**

The data were statistically analyzed using One-way analysis of variance (ANOVA) and differences among the means were determined at P≤0.01 using Duncan’s multiple range tests (by SAS, 9.1).

### Results

#### Isolates and their resistance patterns

From 138 patients that enrolled in this study, 112 urinespmeces were infected with bacteria, from which 91 were positive for *E. coli*. The remaining 21 strains included 15 *Enterobacter* sp. isolates, 5 *Proteus mirabilis* isolates and 1 of Group B *Streptococcus* isolates. *E. coli* isolates characterized with distinctive metallic green sheen on EMB and pink colored colonies on MacConkey’s agar, while white or creamy-colored colonies appeared on the nutrient agar. All 91 isolates of *E. coli* had given positive test for catalase, O, ornithine decarboxylase, indole, methyl-red test, CO₂ production, and for lactose fermenting, and negative biochemical test for oxidase, Voges Proskauer, citrate, urease, and H₂S production; there we confirmed that these isolates belonged to *E. coli*. It should be noted that the patterns of resistance of these *E. coli* isolates to 20 antibiotics were completely different and therefore all isolates were distinctive. The results of antibiotic susceptibility tests were depicted in Figure 1. The sensitivity of isolates to different antibiotics was different, with meropenem being effective on 91.99±1.89, piperacillin on 78.94±3.41, cephaprolin on 67.77±2.20, imipenem and chloramphenicol on 65.34±1.39 and 64.07±3.15, respectively, and cephepine on 58.82±2.37 of the tested isolates. All *E. coli* isolates were identified as MDR bacteria.

#### ESBL and carbapenemase detection

Of total 91 *E. coli* isolates, the synthesis of ESBL was detected in 33 isolates. In this study, phenotypic and genotypic tests were carried out for detection of different types of carbapenemases including IMP, VIM, KPC, and OXA-48. Based on the Hodge test, 3 (3%) of the 33 ESBL-producing *E.coli* isolates produced imipenemase. Using CDT and EDST methods, 1 (3%) and 2 (6%) of the 33 ESBL-producing *E. coli* isolates produced MBL (IMP, VIM, or both and/or other MBLs), respectively. Therefore, these two methods have somewhat different performances in MBL detection. It was interesting that

![Figure 1. The Susceptibility patterns of various antibiotics against 91 uropathogenic Escherichia coli strains isolated from urine samples](image)
by applying the phenotypic methods in this study, it was proven that none of the non-ESBL producing *E. coli* isolates were able to produce different types of carbapenemases.

**Multiplex PCR**

After optimizing the amplification conditions, amplicons with the desired sizes were obtained from the studied isolates and confirmed the specificity of the primers (Figure 2). The results of multiplex PCR analysis showed that frequency of carbapenemases genes including IMP, VIM, KPC, and OXA-48 in ESBL-producing *E. coli* isolates were 24% (8/33), 27% (9/33), 6% (2/33), and 42% (14/33) and in all isolates 8.7% (8/91), 9.8% (9/91), 2.1% (2/91), and 15.3% (14/91) respectively. Therefore, *bla* _imp_ and *bla* _vim_ genes had the highest and the lowest abundance among the *E. coli* isolates, and none of the carbapenemases genes were detected in non-ESBL producing *E. coli* isolates. Also, the results of phenotypic and multiplex PCR tests were consistent in non-ESBL producing *E. coli* isolates.

**Carbapenemases genes and resistance patterns**

In relation with the simultaneous presence of two or more carbapenemase genes in one isolate, only two strains (1%) included *bla* _imp_ and *bla* _vim_ genes and one strain (3%) included *bla* _vim_, *bla* _oxa-48_, and *bla* _imp_ genes. There was no simultaneous presence of *bla* _imp_ and *bla* _oxa-48_, *bla* _imp_ and *bla* _vpa_[a](#A) genes, or *bla* _imp_ and *bla* _vim_ genes in any of the isolates. Only one isolate (0.5%) carried all

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**Table 2. Comparison of the resistance rates between ESBL, non-ESBL producing, and total *Escherichia coli* isolates against 20 antibiotics and association between carbapenemase genes with resistance patterns among ESBL-producing isolates**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>ESBL (n=33)</th>
<th>non-ESBL (n=58)</th>
<th>Total (n=91)</th>
<th>IMP (n=8)</th>
<th>OXA-48 (n=14)</th>
<th>KPC (n=2)</th>
<th>VIM (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF</td>
<td>20 (60.60)</td>
<td>13 (22.41)</td>
<td>27 (30)</td>
<td>6 (75)</td>
<td>1 (7.14)</td>
<td>2 (100)</td>
<td>7 (77.77)</td>
</tr>
<tr>
<td>AMC</td>
<td>21 (63.63)</td>
<td>5 (8.62)</td>
<td>75 (83)</td>
<td>6 (75)</td>
<td>1 (7.14)</td>
<td>0 (0.00)</td>
<td>8 (88.89)</td>
</tr>
<tr>
<td>FEI</td>
<td>13 (42.42)</td>
<td>14 (41.37)</td>
<td>35 (38.5)</td>
<td>4 (50)</td>
<td>4 (28.5)</td>
<td>0 (0.00)</td>
<td>5 (55.55)</td>
</tr>
<tr>
<td>PIP</td>
<td>12 (36.36)</td>
<td>26 (44.82)</td>
<td>40 (43.5)</td>
<td>6 (75)</td>
<td>3 (21.42)</td>
<td>1 (50)</td>
<td>4 (44.44)</td>
</tr>
<tr>
<td>TET</td>
<td>20 (60.60)</td>
<td>11 (18.68)</td>
<td>37 (40.78)</td>
<td>5 (62.5)</td>
<td>6 (42.85)</td>
<td>1 (50)</td>
<td>5 (55.55)</td>
</tr>
<tr>
<td>CIP</td>
<td>10 (30.30)</td>
<td>33 (54.89)</td>
<td>47 (51.84)</td>
<td>5 (62.5)</td>
<td>6 (42.85)</td>
<td>1 (50)</td>
<td>5 (55.55)</td>
</tr>
<tr>
<td>STR</td>
<td>22 (66.66)</td>
<td>41 (69.87)</td>
<td>63 (69.23)</td>
<td>3 (37.5)</td>
<td>12 (85.71)</td>
<td>0 (0.00)</td>
<td>7 (77.77)</td>
</tr>
<tr>
<td>CHI</td>
<td>3 (9.09)</td>
<td>22 (37.93)</td>
<td>30 (32.96)</td>
<td>2 (25)</td>
<td>7 (50)</td>
<td>0 (0.00)</td>
<td>3 (33.33)</td>
</tr>
<tr>
<td>PIP</td>
<td>1 (3.03)</td>
<td>13 (22.41)</td>
<td>17 (18.68)</td>
<td>3 (37.5)</td>
<td>4 (28.5)</td>
<td>0 (0.00)</td>
<td>3 (33.33)</td>
</tr>
<tr>
<td>AMP</td>
<td>30 (90.90)</td>
<td>53 (91.37)</td>
<td>89 (97.80)</td>
<td>7 (87.5)</td>
<td>14 (100)</td>
<td>1 (50)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>AMX</td>
<td>30 (90.90)</td>
<td>53 (91.37)</td>
<td>84 (92.30)</td>
<td>7 (87.5)</td>
<td>14 (100)</td>
<td>1 (50)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>OXA</td>
<td>31 (93.93)</td>
<td>56 (96.55)</td>
<td>88 (96.70)</td>
<td>8 (75.7)</td>
<td>14 (100)</td>
<td>1 (50)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>BCO</td>
<td>18 (54.54)</td>
<td>39 (65.51)</td>
<td>58 (63.71)</td>
<td>5 (62.5)</td>
<td>11 (78.57)</td>
<td>1 (50)</td>
<td>5 (55.55)</td>
</tr>
<tr>
<td>CTX</td>
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<td>37 (63.79)</td>
<td>54 (59.34)</td>
<td>6 (75)</td>
<td>12 (85.71)</td>
<td>1 (50)</td>
<td>6 (66.66)</td>
</tr>
<tr>
<td>CFI</td>
<td>19 (57.57)</td>
<td>43 (74.13)</td>
<td>65 (74.21)</td>
<td>5 (62.5)</td>
<td>11 (78.57)</td>
<td>1 (50)</td>
<td>4 (44.44)</td>
</tr>
<tr>
<td>CAZ</td>
<td>16 (48.48)</td>
<td>32 (55.17)</td>
<td>49 (53.84)</td>
<td>4 (50)</td>
<td>7 (50)</td>
<td>1 (50)</td>
<td>7 (77.77)</td>
</tr>
<tr>
<td>IMP</td>
<td>7 (21.21)</td>
<td>20 (34.48)</td>
<td>30 (32.96)</td>
<td>3 (37.5)</td>
<td>2 (14.29)</td>
<td>0 (0.00)</td>
<td>3 (33.33)</td>
</tr>
<tr>
<td>MEM</td>
<td>5 (15.15)</td>
<td>2 (34.48)</td>
<td>6 (65.93)</td>
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<td>1 (7.14)</td>
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<td>0 (0.00)</td>
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<tr>
<td>CAZ</td>
<td>18 (54.54)</td>
<td>45 (77.58)</td>
<td>68 (74.72)</td>
<td>7 (87.5)</td>
<td>41.42 (50)</td>
<td>1 (50)</td>
<td>6 (66.66)</td>
</tr>
<tr>
<td>CXM</td>
<td>29 (87.87)</td>
<td>51 (87.93)</td>
<td>77 (84.61)</td>
<td>8 (100)</td>
<td>78.57 (80)</td>
<td>1 (50)</td>
<td>7 (77.77)</td>
</tr>
</tbody>
</table>

[a] Data are presented No. (%), n = number of isolates. ESBL = extended spectrum β-lactamase IMP: imipenem; OXA: oxacillin; KPC: Klebsiella pneumoniae carbapenemase; VIM: Verona integron-encoded metallo-β-lactamase
Discussion

Resistance to carbapenemases is due to carbapenemase and other resistance mechanisms, such as ESBLs, efflux pumps, and/or porin mutations (10). The current emergence of carbapenemase-producing bacteria especially Enterobacteriaceae is of concern because it is often associated with the occurrence of multidrug-resistant isolates, where there are very few drug options available for them, if any (10). Therefore, detection and initial identification of carbapenemase-producing bacteria are important. In some cases, due to the low sensitivity or specificity of phenotypic methods, In some cases, molecular approaches may also be used due to the low sensitivity and specificity of the phenotypic methods. By phenotypic methods, but also by molecular approaches (26). Reliable identification of carbapenemases is essential for the implementation of contact precautions and the detection of the outbreak.

In the current study, from the 112 urine specimens from UTI patients infected with bacteria, 91 were positive for E. coli. Carbapenems and pipiracillin were the most effective antibiotics and all cephalosporins other than 4th cephalosporin affected more than 50% of isolates. Compared to the ESBL producing isolates, resistance of non-ESBL isolates was higher than different carbapenemases. Generally, the frequency of ESBL producing isolates and the types of carbapenemase genes among them were low and also in the detection of carbapenemases, there was no correlation between the results of phenotypic and molecular analyses. Due to the similarity of the results of the Hodge and CDT tests, it seems that in the Hodge test, the addition of EDTA did not have any effect on the improvement of the test. Also, the EDST test is comparison with CDT could detect more MBLs and therefore, EDST probably is more reliable. The results of current study in the detection of carbapenemases types in ESBL producing E. coli showed that multiplex PCR is both more sensitive and also more reliable than phenotypic methods due to detection of different carbapenemases and more positive samples.

Given that the resistance of the isolates to imipenem and meropenem was 29% and 6%, respectively, the multiplex analysis identified fewer resistance genes to carbapenems (frequency of OXA-48, KPC, IMP, and VIM genes was 7% (10/33), 1% (2/33), 4% (6/33), and 4% (6/33), respectively), which was similar to that of Gheitani and Fazeli (21). The possible reason for this may be the presence of other types of resistance genes among them were low and also in the detection of carbapenemases, there was no correlation between the results of phenotypic and molecular analyses. Due to the similarity of the results of the Hodge and CDT tests, it seems that in the Hodge test, the addition of EDTA did not have any effect on the improvement of the test. Also, the EDST test is comparison with CDT could detect more MBLs and therefore, EDST probably is more reliable. The results of current study in the detection of carbapenemases types in ESBL producing E. coli showed that multiplex PCR is both more sensitive and also more reliable than phenotypic methods due to detection of different carbapenemases and more positive samples.

Some previous studies in Iran indicated different outbreaks of ESBL producing E. coli. Contrary to our research, in another study, 115 (89.8%) E. coli strains were recognized as ESBL producers (27). Zaniani et al. (2012) reported that 43.9% of E. coli isolates were ESBL producers (28). Another study identified ESBL

### Table 3. Association between two carbapenemase genes with resistance pattern in 33 ESBL-producing Escherichia coli isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>IMP and KPC</th>
<th>IMP and OXA-48</th>
<th>IMP and VIM</th>
<th>KPC and OXA-48</th>
<th>KPC and VIM</th>
<th>OXA-48 and VIM</th>
</tr>
</thead>
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<tr>
<td></td>
<td>(n=0)</td>
<td>(n=0)</td>
<td>(n=0)</td>
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<td>(n=0)</td>
<td>(n=0)</td>
</tr>
<tr>
<td>CF</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>2 (100)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>AMC</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>2 (100)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>PEP</td>
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<td>0 (0.00)</td>
<td>1 (50)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
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<tr>
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<td>0 (0.00)</td>
<td>1 (50)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>TET</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
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a Data are presented No. (%), n = number of isolates
b ESBL = extended spectrum β-lactamases
IMP: imipenem; OXA: oxacillin; KPC: Klebsiella pneumoniae carbapenemase; VIM: Verona integron-encoded metallo-β-lactamase
producing E. coli in 44.1% and 21.2% of inpatients and outpatients isolates, respectively [29].

Most of the studies have used EDST, CDT, MHT, and E-test for detection of MBL. According to their findings, MBL production varied from 7% to 65%. Some studies recorded the use of EDST as one of the suitable methods to detect Ambler Class B MBL production and the positivity ranged from 14.8% to 72% (16-18). Their results demonstrate that EDST is more reliable and reproducible with elevated positivity rates. In our research like studies of Arakawa, Jesudasan, and John, EDST has shown the highest positivity (30-32).

Similar to previously published data, in the current study low positivity of Hodge test compared with other tests was shown, which varied from 14.8%-56.16% (30-34). Contrary to our study, in most studies, CDT is more robust than EDST and MHT (35).

Given that phenotypic tests may be false positive and/or low sensitive or specific, confirmation by molecular methods is required. In one study, 183 Enterobacteriaceae were identified from 442 patients with UTI and of them 160 (87.4%) were MDR. The most common isolates were K. pneumoniae and E. coli. Similar to our study, in their study the prevalence of carbapenemase was a low 2.73% (5/160) and all carbapenemase producing Enterobacteriaceae (CPE) produced ESBL. In their study, the most effective antibiotic was ciprofloxacin. Significant drug resistances were detected among CPE compared to other MDR Enterobacteriaceae (36). In another study, detection of IMP carbapenemase in 600 Enterobacteriaceae clinical isolates was determined by the PCR method. The most common isolate was E. coli. 52% (315/600) and the highest rates of resistance were towards ertapenem and imipenem. In combined disk tests by using of the ertapenem or imipenem, 25 isolates were confirmed positive for MBL enzymes based on the PCR method. The most common isolate was E. coli. The prevalence of OXA-48 and VIM carbapenemas was detected. The most common isolate was E. coli. The prevalence of OXA-48 and VIM carbapenemas was detected. The most common isolate was E. coli. The prevalence of OXA-48 and VIM carbapenemas was detected. The most common isolate was E. coli. (9) were found resistant to meropenem and only 17 isolates were confirmed positive for MBL enzymes based on the combination disk diffusion test (CDDT), but PCR did not detect any blabinp or blabim genes in MBL-producing strains (39).

According to the study by Gheirani and Fazeli (2018) on 183 K. pneumoniae isolates, the highest and lowest rates of resistance were detected against cefotaxime (98.2%) and gentamicin (43.6%), respectively. Among the 183 isolates, 134 (73.2 %) were positive based on MHT. Also, in accordance with our study, the prevalence of blabim and blabinp genes were low (21).

In a different study, 111 CPE were isolated from different clinical samples. Fifty isolates (55%) were resistant to imipenem and/or meropenem. All the study isolated exhibited a positive MHT. MBL and KPC screen tests were positive in 54 and 36 isolates, respectively phenylboronic acid, respectively. By using multiplex PCR, carbapenemase-encoding genes were detected in 63 isolates including 58 NDM, 1 VIM, 2 OXA-181, and 6 both NDM and VIM (40). In this study, similar to our study, the prevalence of OXA-48 and VIM carbapenemas was low.

Research showed out of the 100 carbapenem resistant isolates (E. coli (25), K. pneumoniae (36), P. aeruginosa (18), and A. baumannii (22)), 70 isolates were MHT positive, while 65 isolates were CDT positive. In five isolates which were MHT positive but CDT negative, none of the 4 genes including blaind, blaind, blainp, and blainp were detected. The results of the multiplex PCR for four target genes showed only 5 strains with the blainp gene, 1 strain with the blainp gene, and none of the strains produced blainp. Out of 100 carbapenem resistant isolates, 65 isolates were harboring one or more than one genes, while in 35 isolates none of the genes was detected. The lowest common resistance gene was blainp (59/100) followed by blainp (15/100) while the blainp gene was the least frequent (6/100). Contrary to our research, the blainp gene did not detect in any of the isolates. Comparison of multiplex PCR with MHT and CDT among carbapenem producing isolates is observed (41).

In the study by Pavelkovich et al. in the Baltic States and St. Petersburg, Russia on CPE, of all 9757 strains in 1983 K. pneumoniae and 7774 E. coli isolated from intensive care patients and different clinical samples, 77 isolates (73 K. pneumoniae and 4 E. coli) (37%) were resistant to carbapenem. In this study unlike our study, of 77 strains, in 15 strains the blainp gene was detected and in the other 62 strains blainp, blainp, blainp, blainp, blainp, and blainp genes were not detected (42).

In a recent study, 210 MDR Gram-negative bacilli were obtained from different specimens such as urine (n=108) screened for carbapenem resistance. They used Uniplex PCR for detection of blainp, and blainp genes in E. coli and Klebsiella and applied multiplex PCR for detection of blainp and blainp genes in P. aeruginosa and A. baumannii isolates. Twenty three (11%) isolates (E. coli (6), K. pneumoniae (3), P. aeruginosa (5), and A. baumannii (9)) were found resistant to meropenem and imipenem by disc diffusion. The results of MHT showed that out of the 23 carbapenem-resistant isolates, 17 (74%) produced MBL. These were further confirmed by the E-test. MHT was negative for all isolates. All 17 isolates were subjected to PCR and found to contain at least one carbapenemase gene. Unlike our study, none of blainp, blainp, or blainp genes were detected in Enterobacteriaceae isolates (43).

In a study on frequency of carbapenemase genes including VIM, IMP, NDM, KPS, and OXA-48 in 227 MDR Gram negative bacteria, similar to our study, the most effective antibiotic was meropenem, and 80 strains (35%) were positive for one or more carbapenemase genes. Contrary to our study, IMP-types were the most predominant gene followed by VIM, in 49 (21.59%) and 28 (12%) isolates, respectively. Carbapenemase genes were most detected in K. pneumoniae (24, 11%), followed by P. aeruginosa (23, 10%), and E. coli with 19
isoles (8%)(44).
In one study, 60 bacteria were isolated from urine specimen including 26 strains of E. coli and 34 strains of K. pneumoniae. It was determined that meropenem and amikacin were the most effective antibiotics on E. coli, and imipenem the most effective antibiotic on K. pneumoniae. Four E. coli and 23 K. pneumoniae isolates were positive for carbapenemase production by using the MHT test. Although some results of phenotypic assays matched with the definite PCR identification, some results were misleading. Out of the 29 positive PCR samples, three samples of K. pneumoniae were negative for MHT and one E. coli sample was MHT positive but negative for PCR. Nine samples were positive for PCR but were determined as carbapenem sensitive by MicroScan (45).

In a study to detect MBL among 100 A. baumannii strains, 30 (30%) of the strains were positive for the blaTEM gene, but the blaIMP gene was not detected in any of the strains (46).

Conclusion
Although the prevalence of the ESBL producing strains and the simultaneous presence of several carbapenemases genes in the studied population were nearly low, these low prevalent strains and genes are responsible for resistance to some antibiotics. Thus, identification and controlling of these strains is important due to the presence of plasmid genes encoding carbapenemases and their easy transferability to other clinical isolates.

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Conflicts of Interest
The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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