Multicentre study of the main carbapenem resistance mechanisms in important members of the Enterobacteriaceae family in Iran

S. Armin¹, F. Fallah¹, A. Karimi¹, T. Azimi¹, H. S. Kafi³, S. S. Zahedani⁴, R. M. Ghanaiee¹ and L. Azimi¹
¹) Pediatric Infections Research Center, Research Institute of Children’s Health, Shahid Beheshti University of Medical Sciences, ²) Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, ³) Drug Applied Research Center, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz and ⁴) Department of Medical Microbiology, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

Abstract

Resistance to carbapenems has been increasingly reported from the Enterobacteriaceae family, with different mechanisms in different geographic parts of the world. This study investigated the mechanisms of carbapenem resistance in Escherichia coli, Klebsiella pneumoniae and Enterobacter spp. carried out as a multicentre study (n = 10). All third-generation cephalosporin-resistant E. coli, K. pneumoniae and Enterobacter spp. that had been recovered from the selected provinces were included. Modified Hodge test and Carba NP test were done as a phenotypical method for detection of carbapenemase; the most common carbapenemase was detected by PCR. We evaluated the presence of an active efflux pump by using cyanide 3-chlorophenylhydrazone. Overexpression of AcrA/B and presence of OqxAB was detected by real-time PCR and conventional PCR respectively. Microorganisms in this study included 58 E. coli, 95 K. pneumoniae and 60 Enterobacter spp. Modified Hodge test showed a sensitivity of 41% and a specificity of 83%, and the Carba NP test showed a sensitivity of 26% and a specificity of 92% for detection of carbapenemase. OXA-48 was the most frequently detected carbapenemase, followed by NDM-1. Thirty-nine percent and 27% of positive cyanide 3-chlorophenylhydrazone test organisms included active AcrA/B and OqxAB efflux pumps respectively. The result showed the Carba NP test was more specific than MHT. Data confirmed the involvement of AcrA/B and OqxAB efflux pump as a carbapenem resistance mechanism in selected bacteria. Similar to other reports from the Middle East, we found OXA-48 and NDM-1 to be the most frequent carbapenemase.
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Introduction

Enterobacteriaceae is a family of Gram-negative bacteria that are associated with a wide variety of community and nosocomial infections [1,2]. Antibiotic resistance may be chromosomal dependent or plasmid mediated so it is easily transferred between bacteria [1–4]. Carbapenems have a broad spectrum of activity against Gram-negative bacteria and are used as one of the last lines of antibiotic therapy, especially in cases of infection with extended-spectrum β-lactamase–producing organisms [4,5]. Unfortunately, resistance to carbapenems has been increasingly reported in the Enterobacteriaceae family from different geographic parts of the world. The first report of carbapenemase producer Enterobacteriaceae was reported in 1993 [1]. Multiple factors have contributed to an increase in rates of carbapenem resistance during the last decades; these factors include hospital policies like noncompliance with hand hygiene, inadequate isolation of infected patients and rising unnecessary carbapenem use along with inappropriate dosing and incorrect duration of antibiotic therapy [6].

Resistance to carbapenems can arise via different mechanisms, the most common being the production of carbapenemase (hydrolizing carbapenems and other β-lactam antibiotics), poor
binding to penicillin-binding proteins and efflux pumps [6,7]. Each of these mechanisms enables the organisms to resist to all or one of the carbapenem group (imipenem, meropenem, ertapenem, doripenem) and the third-generation cephalosporins, (ceftriaxone, cefotaxime, cefazidime) [8]. Because carbapenem-resistant Enterobacteriaceae (CRE) express high resistance to most available antibiotics such as cephalosporins, quinolones and aminoglycosides, physicians are left with limited treatment options [9]. CRE mostly affects patients with underlying conditions such as immunodeficiency, prolonged or recurrent hospitalization, intensive care unit admission, indwelling medical devices and frequent courses of antibiotic therapy, resulting in poor outcomes [10]. According to available data, CRE infections are associated with high morbidity and with mortality of up to 72% [11]. Among the Enterobacteriaceae isolates, Escherichia coli, Klebsiella spp. and Enterobacter spp. constitute the greatest risk for public health because of their prevalence, wide spectrum of clinical manifestations, multidrug resistance and rapid spread of resistance to other organisms [6]. In Iran, some studies have been done about CRE in several geographic areas on various sample types by different methods [1,2,12,13], but our study is to our knowledge the first report from ten provinces performed by uniform phenotyping and genotyping methods throughout the country.

The aim of the present study was the detection of different carbapenem resistance mechanisms in E. coli, Klebsiella pneumoniae and Enterobacter spp. isolated from both inpatients and outpatients at selected hospitals.

Materials and methods

In the present cross-sectional study, we gathered all third-generation cephalosporin-resistant E. coli, K. pneumoniae and Enterobacter spp. recovered from patients seeking care at or admitted to ten different hospitals in ten provinces in Iran from September 2016 to September 2017. The study was approved by the ethics committee of the National Institutes for Medical Research Development (NIMAD) (reference IR.NIMAD.REC.1396.001).

Antibiotic susceptibility testing

Carbapenem susceptibility was determined by disc diffusion agar methods according to the Clinical and Laboratory Standards Institute (CLSI) guideline [14]. Carbapenem discs were obtained from MAST in the United Kingdom. The term ‘carbapenem-resistant Enterobacteriaceae’ was designated to refer to Enterobacteriaceae that are resistant to meropenem and or imipenem.

Phenotypical carbapenemase detection

Modified Hodge test (MHT) was done according to CLSI by using ertapenem discs (10 μg) and E. coli ATCC 25922. Carba NP test was conducted by phenol red solution and imipenem powder [14].

Molecular detection of carbapenemase genes

Carbapenem-resistant organisms were evaluated for the presence of seven carbapenemase enzymes by PCR: K. pneumoniae carbapenemase (KPC), New Delhi metallo-β-lactamase (NDM-1), Verona integron-encoded metallo-β-lactamase (VIM), oxacillinase-48–type carbapenemase (OXA-48), imipenem (IMP), metallo-β-lactamase, Guiana extended spectrum β-lactamase (GES) and São Paulo metallo-beta-lactamase (SPM-1). Primers and PCR conditions have been described previously [15–17].

Phenotypical screening of active efflux pump

The cyanide 3-chlorophenylhydrazone (CCCP) as an efflux pump inhibitor could recognize active efflux pumps phenotypically. In this study, imipenem MIC was determined by micro broth dilution assay with and without 25 μg/mL CCCP [20]. The presence of active efflux pumps was confirmed by at least a 4-fold decrease in MIC of the tested antibiotics in presence of CCCP [18].

Evaluation of efflux pump gene expression

Gene overexpression of efflux pump was investigated on all strains with a phenotypically positive test. We used semi-quantitative real-time PCR for the evaluation of AcrA and AcrB. In the first step, RNA extraction was done by Thermo RNA extraction kit (cat. no. K0732); then complementary DNA synthesis was done with a Thermo kit (cat. no. K1622). Finally, the overexpression of AcrA and AcrB genes was arranged by semiquantitative real-time PCR. We used 16S ribosomal RNA as a housekeeping gene. Gene overexpression was calculated by the formula of Pfafi et al. Primers and PCR conditions have been described previously and are listed in Table 1 [18–20].

Plasmid-borne efflux pump genes

Plasmid-encoded oqxA and oqxB efflux pump genes conferring resistance to olaquindox in selected bacteria were detected by conventional PCR with the primers listed in Table 1 [19].

Results

During the study period, 360 isolates of E. coli, 230 isolates of K. pneumoniae and 137 isolates of Enterobacter spp. were received at our centre as third-generation cephalosporin-resistant isolates. (The total numbers of third-generation–susceptible and –resistant E. coli, K. pneumoniae and Enterobacter spp. isolates were 1222, 696 and 621 respectively.) Resistance to third-generation cephalosporins was detected in 360, 230
and 137 colonies of *E. coli*, *K. pneumoniae* and *Enterobacter* spp. respectively. Based on the inclusion criteria, 58 colonies of *E. coli*, 95 of *K. pneumoniae* and 60 of *Enterobacter* spp. which were not susceptible to at least one of the carbapenem antibiotics were considered for further evaluation. The results of MHT and Carba NP tests are shown in Table 2.

OXA-48 was the most frequent resistance gene in the studied strains, followed by NDM-1 (Table 3). One important finding was the detection of specificity and sensitivity of MHT and Carba NP, as follows: MHT: sensitivity 41%, specificity 83%, positive predictive value 70%, negative predictive value 60% and accuracy rate 62%; and Carba NP test: sensitivity 25%, specificity 83%, positive predictive value 70%, negative predictive value 65% and accuracy rate 60%.

By adding CCCP we evaluated the presence of active efflux pump in 19 (33%) of *E. coli*, 37 (39%) of *K. pneumoniae* and ten (17%) of *Enterobacter* spp. in the carbapenem-resistant strains.

### Table 1. Primers used in this study

| Genes   | Primer sequencing (5′ - 3′) | PCR product size (bp) | Reference |
|---------|-----------------------------|-----------------------|-----------|
| KPC     | CTGCTCTGCTCTGCTTGGCC         | 636                   | [12]      |
| KPCr    | GCCGCGGAGGAATTATG             | 573                   |           |
| GESF    | GCGCCTGTCCTGCTGCTGCTCAGCAC  | 371                   | [13]      |
| GESR    | TGCCATAGCAATAGCCGCTGAT       | 390                   | [14]      |
| VIMF    | GATGATGCTTTGCTGCTAC          | 139                   | [14]      |
| VIMR    | CGATGATGCTTTGCTGCTAC         | 139                   | [14]      |
| IMPF    | TTAGATGCTTTGCTGCTAC          | 139                   | [14]      |
| IMPR    | GATGAGGAAATAGACCACTGCT       | 129                   | [15]      |
| NDM-1L  | CCCGCGGAGGAATTATG             | 447                   | [15]      |
| NDM-1R  | GCGGCGGAGGAATTATG             | 447                   | [15]      |
| SPM-1L  | ACGGCTTCTTCTGCGAGGAGA        | 389                   | [16]      |
| SPM-1R  | GTGAGGAGAATTATG               | 389                   | [16]      |
| 48F     | CGGAGCGGAGGAATTATG            | 392                   | [17]      |
| 48R     | CACGAGGAGAATTATG              | 392                   | [17]      |
| aqA     | CTGCGATGCTTTGCTGCTAC          | 512                   | [17]      |
| aqB     | ACTACGATGCTTTGCTGCTAC         | 512                   | [17]      |
| AcrK    | CTGAGGAAATTATG                | 107                   | [18]      |
| AcrR    | TGAGGAAATTATG                 | 107                   | [18]      |
| AcrB    | GCTGCGATGCTTTGCTGCTAC         | 107                   | [18]      |

### Table 2. MHT and Carba NP test results

| Bacteria            | Positive for: | Only MHT | Only Carba NP | Both |
|---------------------|---------------|----------|---------------|------|
| *Escherichia coli*  | 16 (28%)      | 4 (7%)   |               |      |
| *Klebsiella pneumonia* | 21 (22%)      | 16 (17%) | 10 (11%)      |      |
| *Enterobacter* spp. | 8 (13%)       | 2 (3%)   | 1 (2%)        |      |

### Table 3. Prevalence of carbapenemase among surveyed Gram negative bacteria

| Bacteria            | IMP | KPC | NDM-1 | OXA-48 |
|---------------------|-----|-----|-------|--------|
| *Escherichia coli*  | 1   | 1   | 5     | 9      |
| *Klebsiella pneumonia* | —   | 29  | 46    |        |
| *Enterobacter* spp. | —   | 19  | 7     |        |

The number of bacteria (with positive results in the CCCP test) with gene overexpression which was detected by real-time PCR among efflux pump inhibitor–positive strains is shown in Table 4.

Plasmid-mediated carbapenem resistance mechanism was evaluated by PCR on efflux pump inhibitor–positive strains. Two types of coexisting resistant mechanisms (pump and enzyme) were detected in three *E. coli*, 20 *K. pneumoniae* and five *Enterobacter* spp.

### Discussion

One of the phenotypical carbapenemase detection tests used in different studies is MHT. In our multicentre study, MHT was found to have 41% sensitivity and 83% specificity for the detection of carbapenemase. Doyle et al. [21] found that the sensitivity and specificity for MHT were 61% and 93% respectively. The results of the Doyle et al. study from Canada showed that MHT is less reliable in detecting NDMs, VIMs and IMPs, though it can be useful for detecting KPC and OXA-48 [21–23]. A study from the Middle East reported the overall sensitivity and specificity for the MHT as 89.6% and 96.77% respectively [24]. A single-centre survey from Iran indicated that MHT had 63% sensitivity and 60% specificity for the detection of carbapenemase [25]. This large difference in sensitivity and specificity from different studies may be due to different consumed material in MHT and also different primer and quality of PCR master mix as the reference-standard test. Reported sensitivity and specificity of MHT in our study were based on the utility of this test for detection of all of the most common carbapenemase, but in some studies, only specific enzymes had been tested [18,22].

The MHT test predicts the existence of another class of carbapenemase enzyme, such as the metallo-β-lactamases. On the basis of the CLSI recommendation, the reference standard
test for the detection of the presence of the KPC enzyme is the amplification of the KPC gene using PCR-based assays. Moreover, CLSI suggested the MHT test for the confirmation of the presence of the KPC enzyme in Gram-negative bacteria [26]. Results of our study revealed that the prevalence of KPC enzyme among surveyed Gram-negative bacteria is very low. Therefore, the main reason for the low sensitivity of the MHT test could be the low prevalence of KPC in the study. The 2018 CLSI guideline has removed MHT from the detection of carbapenemase as a result of its low sensitivity [27].

In the current study, the sensitivity of the Carba NP test was low (25%), but specificity was acceptable (94%) compared to another study [28,29]. In the case of the Carba NP test, researchers from Canada showed 100% specificity and 72.5% sensitivity for the detection of carbapenemase [28]. Another study from Switzerland reported 90.2% and 100% sensitivity and specificity respectively [29]. The difference in sensitivity may be attributable to the difference in the tested strains [29,30]. As mentioned in another study, Carba NP did not detect the OXA class of enzymes [31]. In this regard, more than half of bacteria (52.5%) in the current study were OXA-48 producing, which points to the low sensitivity of this test.

Overall, the Carba NP test was more specific than MHT. The low sensitivity of MHT, its low specificity and its time-consuming nature are all reasons why CLSI 2018 omitted this test.

The overexpression of efflux pumps is another important mechanism because it can mediate multidrug-resistant organisms and may lead to carbapenem resistance associated with resistance to other drugs (quinolones, penicillins, cephalosporins, aminoglycosides) [18,30]. In the present study, real-time PCR documented overexpression of at least one component of the AcrAB efflux pump in 58% of bacteria that were phenotypically positive for active efflux pump. Surveys show that overexpression of AcrA and AcrB causes resistance to fluoroquinolones [32,33], although there is no complete agreement about its effect on carbapenem resistance. Padilla et al. [32] reported that this system is responsible for increasing MIC for erythromycin, tetracycline, chloramphenicol, aminoglycosides and β-lactams but not carbapenems. However, other researchers have reported this system to be an effective mechanism for resistance to carbapenems [33,34].

The OqxAB plasmid gene encodes a resistance–nodulation–division family multidrug efflux pump which enables resistance to antimicrobials like chloramphenicol, cefoxitin and fluoroquinolones [35].

Bialek-Davenet et al. [35] reported the effect of the OqxAB efflux pump on resistance to carbapenems for the first time in 2017. Present research indicated 68% of phenotypically positive bacteria carried the OqxAB efflux pump, which may be one of the responsible mechanisms for carbapenem resistance, similar to the study of Bialek-Davenet et al.

One of the important carbapenem resistance mechanisms is producing carbapenemase enzymes. The prevalence of carbapenemase-producing Enterobacteriaceae (CPE) is growing globally. Until 2012, no CRE had been reported from Iran, Jordan or Syria [36]. In our research, OXA-48 was the most common carbapenemase, followed by NDM-1.

Studies from the region show that NDM-1 has been detected in bacterial strains from Israel, Oman and Pakistan [33]. In addition, an OXA-48 outbreak occurred in Turkey, and there are sporadic reports from Israel [33]. In 2015 Leylabadi et al. [33] reported sporadic NDM-1-producing CPE from Iran and emerging outbreaks in Iraq, Afghanistan and the United Arab Emirates. In the current study, NDM-1 was found to be the second most common carbapenemase. On the one hand, the important aspect of these data is the high detection rate of this enzyme (45%) from border cities, so these findings highlight the significance of travelling or of medical tourism [33,37]. On the other hand, 28% of NDM-1-producing strains were detected from referral hospitals that admit patients from different cities, so we could not follow the geographical origin of the strains we found in this study.

OXA-48–producing CPE is common in Turkey and other surrounding countries. KPC was the commonest detected enzyme in CPE from the United States, whereas metallo-β-lactamase–producing CPE is frequently detected in India, Romania, Denmark, Spain and Hungary [38].

Clinically, detection of carbapenemase is a critical issue because they are accompanied by wide-ranging antibiotic resistance, treatment failure and mortality. These enzymes are usually encoded by mobile DNA elements with a high capacity for dissemination. Most other life-threatening groups are metallo-β-lactamases including IMP, VIM and NDM, as well as plasmid-mediated KPC, GES and OXA-48, as these enzymes can hydrolyze almost all β-lactams, including the carbapenems. Moreover, they can be unpredictably transmitted to other important nosocomial pathogens such as Pseudomonas aeruginosa or Acinetobacter baumannii. The US Centers for Disease Control and Prevention estimate that carbapenem-resistant K. pneumoniae and Escherichia species are responsible for more than 9000 healthcare-associated infections in the United States each year [36]. Current treatment options include tigecycline as well as the older antibiotics colistin and fosfomycin, which have been revitalized, and aminoglycosides, but the insufficient data regarding efficacy and toxicity are major obstacles to their use. Aztreonam is the only drug for the treatment of metallo-β-lactamase and some other compound (OXA-48)-producing strains, but it could be hydrolyzed by KPC-like enzyme [39,40].
We detected molecular mechanisms for carbapenem resistance in 118 (55%) of all phenotypical resistance strains. This result shows that there are other mechanisms for carbapenem resistance that were not investigated in this study, such as other efflux pumps, porin loss and overexpression of AmpC β-lactamases, as some strains have more than one resistance mechanism.

In conclusion, the presence of carbapenem-resistant bacteria is a threat to healthcare systems because different mechanisms are responsible for this phenomenon. Detailed knowledge of epidemiology and molecular characteristics of CPE is essential to stop the spread of these pathogens [36,41]. One of the most effective mechanisms is efflux pump overexpression because it can cause cross-resistance and generate multidrug-resistant and extensively drug-resistant strains. We found enzymatic carbapenem resistance and the presence of an active efflux pump (AcrA/B, OqxAB) to be the two major detected carbapenem resistance mechanisms.

Conflict of interest

None declared.

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