Molecular study of carbapenemase genes in clinical isolates of Enterobacteriaceae resistant to carbapenems and determining their clonal relationship using pulsed-field gel electrophoresis

Fereshteh Shahcheraghi, Mohammad Mehdi Aslani, Hassan Mahmoudi, Zahra Karimitabar, Hamid Solgi, Abbas Bahador and Mohammad Yousef Alikhani

Abstract

Purpose. Enterobacteriaceae is a large family of Gram-negative bacteria that are considered as normal gut flora. They are the most common human pathogens. The main objective of this study was to investigate the carbapenemase genes in clinical isolates of Enterobacteriaceae resistant to carbapenem antibiotics and determine their clonal relationship using pulsed-field gel electrophoresis (PFGE).

Methodology. In the present study, bacteria were isolated and identified via conventional biochemical tests and API 20NE. Antibiotic susceptibility was evaluated by using the disc diffusion method and MIC was carried out using the E-test. Phenotypic determination of carbapenemases was performed by employing a modified Hodge test (MHT). Carbapenemase genes including IMP, VIM, KPC, NDM and OXA-48 were amplified by PCR. The relationships between their clonal types with l restriction enzyme were examined using PFGE.

Results. Out of 40 isolates that were resistant or moderately susceptible to carbapenem antibiotics, 29 (72.5 %) strains were positive for carbapenem enzymes phenotypically. Moreover, six isolates contained carbapenemase genes including IMP, VIM, NDM and OXA-48, but the KPC gene was not found in any of the isolates. PFGE results showed that E. coli strains in our area were clustered into eight pulsotypes (A–H), Klebsiella spp. isolates five pulsotypes (A–E) and Proteus spp. had two pulsotypes (A, B). The high resistance to antimicrobial agents in the A, B and F pulsotypes was attributed to E. coli clinical isolates.

Conclusions. Our results could reflect some hospital multidrug-resistant strains in nosocomial infections. The widespread emergence of carbapenem-resistant isolates has caused increasing concern in recent years. Therefore, specific strategies should be designed and evaluated for the control of resistant strains.

INTRODUCTION

The Enterobacteriaceae are rod-shaped, Gram-negative bacteria, which are considered as normal gut flora. These bacteria are the commonest cause of nosocomial infections and are considered the most common human pathogens [1]. Nosocomial infections, a major public healthcare problem, are more prevalent in developed countries because of the related mortality and socioeconomic costs [2]. Carbapenem antibiotics are subcategories of beta lactam antibiotics, which have a key role in treating severe infections. Due to their broad-spectrum activity, these antibiotics are commonly used in the treatment of life-threatening infections such as sepsis and are widely used as a last line of antibiotics in treating infections with multidrug-resistant Gram-negative bacilli such as Pseudomonas spp., Acinetobacter spp. and Enterobacteriaceae spp. Nowadays, due to excessive and inappropriate use of these drugs, the resistance to carbapenems has increased. This issue is one of the main causes of the expression of carbapenemase genes such as IMP, VIM, NDM, OXA-48 and KPC among the members of this family [3]. Carbapenemases are beta lactamase enzymes that can cause resistance to carbapenems. Most of these enzymes can produce resistance to other beta lactam antibiotics, such as a broad spectrum of...
cephalosporins. The choice of treatment for severe infections caused by resistant bacteria is considerably limited [4, 5]. Monitoring and identifying the source of microbial infections in polluted areas is very important. Quickly identifying and distinguishing the different bacterial strains causing these infections are the most important goals in epidemiological studies to identify the main source of pollution [6]. Molecular typing of bacteria is currently documented as a critical feature for infectious diseases [7]. Pulsed-field gel electrophoresis (PFGE) is one of the best methods for typing in epidemiological studies and it is used in various fields, such as biology, biotechnology, medicine, and clinical diagnostics in different areas. Due to the high discriminative power and reproductive ability of this method, it can be used for all human pathogens and finding polymorphisms in parts of the genome [8, 9]. The main objective of this study was to investigate the presence of carbapenemase genes in Enterobacteriaceae clinical isolates resistant to carbapenem antibiotics and determine their clonal relationship using the PFGE method.

**METHODS**

**Specimen collection**

In this cross-sectional study, over 9 months (August 2015 to May 2016), 500 clinical samples such as ascites, blood, bedsores, catheters, cerebrospinal fluid (CSF), pus, mucus, faeces, tracheal and urine were collected from hospitalized and outpatients in different wards of three educational hospitals affiliated to Hamadan University of Medical Sciences, Hamadan, Iran.

**Bacterial isolation and identification**

The clinical samples were cultured on blood agar and MacConkey agar (Merck) and bacteria isolated and identified via conventional biochemical tests, and API 20NE (bioMérieux) was used in doubtful cases. Triple Sugar Iron (TSI), Simmons citrate, SIM (Sulfide, Indole, Motility), Methyl Red & Vogues-Proskauer (MR-VP) and urease can be mentioned as the biochemical tests used for identifying the family Enterobacteriaceae and species [10, 11].

**Confirmation of isolates using PCR**

In order to confirm the isolated bacteria as Enterobacteriaceae, all isolates were screened for the presence of the rpoB gene using a PCR technique with a fragment length of 512 bp [12].

**Antibiotic susceptibility test**

Antibiotic susceptibility of isolates to imipenem (10 µg), meropenem (10 µg) and ertapenem (10 µg) discs (MAST) was investigated using the disc-diffusion method as described in Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. MIC to imipenem was determined by E-test strips (Liofilchem), according to the manufacturer’s instructions and CLSI 2015 guidelines. The isolates with MIC values ≥4 µg ml⁻¹, 2–3 µg ml⁻¹ and ≤1 µg ml⁻¹ for imipenem were considered as resistant, intermediate and susceptible, respectively [13].

**Phenotypic determination of carbapenemases using the modified Hodge test (MHT)**

First, 0.5 McFarland (1.5×10⁸ c.f.u. ml⁻¹) of Escherichia coli ATCC 25922 in 5 ml of BHI broth (Merck) was prepared. The suspension was diluted (1:10) and was cultured uniformly on a Mueller–Hinton agar (Merck) plate. The carbapenem discs were placed in the centre of the plate, and the studied organisms were cultured from the edge of the disc to the edge of the plate in a direct line. The plate was incubated for 16–24 h at 37°C. A positive MHT test generated a clover-leaf shape of E. coli ATCC 25922 across the organism in the zone of inhibition. A negative MHT test generated no growth of E. coli ATCC 25922 across the organism in the zone of inhibition [14].

**Detection of genes encoding carbapenemase enzymes**

In order to detect the diversity of genetic regions among identified bacteria, genomic DNA was extracted using a phenol chloroform method [15]. The gene fragments

<table>
<thead>
<tr>
<th>Gene</th>
<th>3′-5′ Primer</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB-F</td>
<td>CAGGTCGTCACGGTAAACAG</td>
<td>512</td>
<td>[12]</td>
</tr>
<tr>
<td>rpoB-R</td>
<td>GTGGTTCAGTTTACAGTGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMP-F</td>
<td>GGAATAGATGGCTTAAYTCTC</td>
<td>188</td>
<td>[16]</td>
</tr>
<tr>
<td>IMP-R</td>
<td>CCAACCTACTASGTATCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIM-F</td>
<td>GATTTGGTGGTTGGCTCAT</td>
<td>390</td>
<td>[16]</td>
</tr>
<tr>
<td>VIM-R</td>
<td>CGAATTGCCAGCCACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KPC-Fm</td>
<td>CGCTAGTTCTCTGTCCTTG</td>
<td>798</td>
<td>[17]</td>
</tr>
<tr>
<td>KPC-Rm</td>
<td>CTGTGATCTCTGTAGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDM-F</td>
<td>GGTGTTGGGATCTGGTTTTC</td>
<td>621</td>
<td>[17]</td>
</tr>
<tr>
<td>NDM-R</td>
<td>CGAATGGGTCATCCAGATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA-48-F</td>
<td>CCGTGATTAAGGATGAACAC</td>
<td>438</td>
<td>[17]</td>
</tr>
<tr>
<td>OXA-48-R</td>
<td>CATCAAGTCAAGCCACCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
were examined using specific primers and PCR amplification according to previous studies (Table 1) [16, 17].

Typing of isolates using the PFGE method
Clinical isolates of *E. coli*, *Klebsiella* spp. and *Proteus* spp. were characterized by PFGE as described previously [18]. The intact chromosomal DNA from isolates was extracted for PFGE. The genomic DNA in agarose plugs was digested with *Xba*I restriction endonuclease (Fermentas) and the fragments were separated by CHEF-DR III apparatus (Bio-Rad Laboratories). The system was set up at 6 V cm⁻¹ for 19 h with pulse time ramped from 5 to 35 s. *Salmonella braenderup* H9812 was used as a molecular weight marker. The gels were stained with ethidium bromide and DNA patterns were photographed with UVP gel (UVP) (Figs 1, 2 and 3). The DNA banding patterns were interpreted as instructed by Tenover *et al.* [19].

RESULTS
Five hundred clinical samples were collected from patients. Results showed that urine (203 samples; 66.1 %), tracheal (55 samples; 17.9 %) and blood (20 samples; 6.5 %) were the most collected samples. In total, 307 Enterobacteriaceae spp. were isolated. Moreover, in this study *E. coli* 207 (67.4 %), *Klebsiella* spp. 65 (21.2 %), *Proteus* spp. 21 (6.8 %), *Enterobacter* spp. 5 (1.6 %), *Citrobacter* spp. 4 (1.3 %), *Salmonella* spp. 3 (1 %) and *Shigella* spp. 2 (0.6 %) were the most common genera of the isolated family Enterobacteriaceae.

Antimicrobial susceptibility test
Out of 307 Enterobacteriaceae spp. isolated in this study, 40 (13 %) isolates were found resistant or moderately susceptible to one of the carbapenems (imipenem, meropenem and ertapenem) by using the disc diffusion method. The results showed that most strains resistant to carbapenems were isolated from urine (45 %) and tracheal (32.5 %) samples. Moreover, *E. coli* (45 %), *Klebsiella pneumoniae* (20 %) and *Proteus mirabilis* (15 %) had the highest frequency among the strains resistant to carbapenems.

Based on the imipenem MIC results, 21 (52.5 %) strains were susceptible, nine (22.5 %) resistant and 10 (25 %) isolates were semi-susceptible to imipenem. Four strains (10 %) had MIC₃₂ µg ml⁻¹.

MHT phenotypic testing
Forty carbapenem-resistant and semi-susceptible strains were examined using MHT for the presence of carbapenemase enzymes. Results showed 29 (72.5 %) strains were phenotypically positive for carbapenemase enzymes. The isolates producing carbapenemase enzymes included 15 (51.7 %) *E. coli*, six (20.7 %) *P. mirabilis*, three (10.3 %) *K. pneumoniae*, two (6.9 %) *Salmonella* spp., one (3.4 %) *Citrobacter freundii*, one (3.4 %) *Proteus vulgaris*, and one (3.4 %) isolate of *Enterobacter aerogenes*.
PCR results

In this study, six (15%) isolates were positive in terms of carbapenemase enzyme-encoding genes and their characteristics are shown in Table 2. The results of multiplex PCR in 40 strains of carbapenem-resistant and semi-susceptible strains of Enterobacteriaceae showed that two (5%) strains of K. pneumoniae contained IMP gene, two (5%) strains of Salmonella spp. contained VIM gene, one (2.5%) strain of K. pneumoniae contained NDM gene, and one (2.5%) strain of K. pneumoniae contained OXA-48 gene. KPC gene was not found in any of the isolates (Table 2).

PFGE analysis

According to the dendrograms (Figs 1–3), the PFGE results showed that E. coli strains isolated from hospitalized patients in our area were clustered into eight pulsotypes (A–H), isolates of Klebsiella spp. had five genetic profiles (A–E) and isolates of Proteus spp. had two genetic patterns (A, B).

In general, G (strain no. 163) and H (strain no. 84) pulsotype of E. coli which were isolated from tracheal samples had little similarity with other genetic patterns. The A and F genetic patterns were found among E. coli strains isolated...
The genetic patterns of D (strain no. 224) and E (strain no. 503) of *Klebsiella* spp. isolated from tracheal samples had a high genetic similarity to other groups. B, C and D genetic patterns were observed among the *Klebsiella* isolates in Sina hospital. The genetic pattern of B in the *Klebsiella* strains (strain nos 500 and 501) had superior resistance to imipenem, meropenem and ertapenem, and both strains were positive for carbapenemase genes (NDM and IPM).

The genetic pattern of A (with five isolates, including strain nos 141, 228, 232, 241 and 244) of *P. mirabilis* isolated from tracheal and wound samples in Sina and Besat hospitals was the most common pattern. Also, all strains were resistant to imipenem, meropenem and ertapenem. The genetic pattern of B (strain no. 155) is related to *P. vulgaris*, which was isolated from urine samples in Besat hospital and had superior resistance to carbapenem antibiotics.

**DISCUSSION**

This study was designed based on PFGE typing and molecular analysis of carbapenemase genes in 40 clinical isolates of *Enterobacteriaceae* strains resistant or moderately susceptible to carbapenem antibiotics. Antibiotic resistance prevents the effective treatment of infectious diseases, especially in hospitalized patients. Monitoring and periodic inspection of antibiotic susceptibility in bacterial pathogens have been taken into consideration due to the prevalence and importance of *Enterobacteriaceae* as pathogens in hospitalized patients, transmitting the organisms from patient to patient, and transferring resistance factors (such as plasmids and transposons) among different isolates [20]. In the present study, of 40 isolates resistant or semi-susceptible to carbapenems, 29 isolates (72.5 %) were MHT positive. This indicated the possible presence of carbapenemase genes in these isolates; nevertheless, this method is not able to distinguish KPC and MBL enzymes. In this regard, there were strongly positive MHT isolates containing only IMP or VIM enzymes and KPC was not found. However, the ertapenem antibiotic disc had the highest sensitivity for evaluation of the KPC enzyme and the strains containing KPC enzyme had the highest activity to the ertapenem antibiotic disc [21]. In this study, 14 isolates were MHT positive with the ertapenem antibiotic disc, and there was no KPC enzyme in any of the strains. However, the specificity of ertapenem has not yet been determined, because the *Enterobacteriaceae* producing Extended-spectrum beta-lactamases (ESBLs) and purine mutations may also cause resistance to ertapenem [22]. In a recent study conducted in Europe, only two of 171 isolates were resistant to ertapenem and produced carbapenemase enzymes, but none of them was KPC [23]. MHT results of the present study showed that 11 isolates were positive to antibiotic discs of imipenem, meropenem and ertapenem. So, there is a high probability for the presence of carbapenemase genes in these isolates. Of 40 isolates, six strains (15 %) contained carbapenemase genes. Also, MHT is a good method to identify carbapenemase genes except OXA-48. In general, then, OXA-48 gene causes no resistance against carbapenems, and this phenomenon has led to the loss of this gene in many strains. The OXA-48 gene may be present in susceptible isolates that have not yet been studied by molecular methods. Despite the resistance of some isolates to ertapenem antibiotic, no KPC gene was found, which indicated the low specificity of this disc for finding KPC enzyme [24]. Glupczynski et al. [24] studied the rapid emergence and spread of OXA-48-producing carbapenem-resistant *Enterobacteriaceae* isolates in Belgian hospitals in 2012; they reported 51 isolates among 147 clinical isolates of *Enterobacteriaceae*, indicating a reduced susceptibility to meropenem. In the mentioned study, the OXA-48 gene was identified as the most prevalent carbapenemase-encoding gene among the isolates. They reported that 19 (12.9 %) collected isolates included nine strains of *Enterobacter cloacae*, seven of *K. pneumoniae* and three of *E. coli* that contained this gene and these were identified in nine different hospitals. Other carbapenemase-encoding genes included NDM-1, VIM-1 and KPC-2 genes and were also were identified in *Enterobacteriaceae* isolates [24]. In 2013, the first NDM-1 gene-producing *K. pneumoniae* was reported in Iran [25]. In this report, of 360 *Enterobacteriaceae* isolates, 23 (6.3 %) were resistant to meropenem, 11 (3 %) were resistant to

**Table 2. The characteristics of isolates containing carbapenemase genes**

<table>
<thead>
<tr>
<th>Key</th>
<th>ID of isolates</th>
<th>Bacteria spp.</th>
<th>Imipenem MIC (µg ml⁻¹)</th>
<th>DAD result</th>
<th>MHT result</th>
<th>Carbapenemase gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ipm Mem Etp</td>
<td>Ipm Mem Etp</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>115</td>
<td><em>Salmonella</em> spp.</td>
<td>Resistance (≥32)</td>
<td>I S S</td>
<td>+ + –</td>
<td>VIM</td>
</tr>
<tr>
<td>2</td>
<td>119</td>
<td><em>Salmonella</em> spp.</td>
<td>Resistance (4)</td>
<td>I S S</td>
<td>+ – +</td>
<td>VIM</td>
</tr>
<tr>
<td>3</td>
<td>142</td>
<td><em>K. pneumoniae</em></td>
<td>Sensitive (1)</td>
<td>I S S</td>
<td>– – –</td>
<td>OXA-48</td>
</tr>
<tr>
<td>4</td>
<td>412</td>
<td><em>K. pneumoniae</em></td>
<td>Resistance (≥32)</td>
<td>R R R</td>
<td>+ + +</td>
<td>IMP</td>
</tr>
<tr>
<td>5</td>
<td>500</td>
<td><em>K. pneumoniae</em></td>
<td>Resistance (≥32)</td>
<td>R R R</td>
<td>+ + +</td>
<td>NDM</td>
</tr>
<tr>
<td>6</td>
<td>501</td>
<td><em>K. Pneumoniae</em></td>
<td>Intermediate–sensitive (2)</td>
<td>R R R</td>
<td>+ + +</td>
<td>IMP</td>
</tr>
</tbody>
</table>

Ipm, Imipenem; Mem, meropenem; Etp, ertapenem; DAD, Disk Agar Diffusion.
ertapenem, and four (1.1%) isolates were resistant to imipenem. It showed that MHT was positive in 11 (47.8%) carbapenem-resistant isolates, of which, only one K. pneumoniae isolate contained the NDM-1 gene and was resistant to antibacterics such as third-generation cephalosporin, carbapenem, ciprofloxacin, amikacin, kanamycin, trimethoprim sulfamethoxazole and aztreonam, while it was susceptible to colistin. This NDM-1-positive isolate was weakly positive or negative by MHT. According to our results, the NDM-1-positive isolate was also MHT weakly positive for imipenem, meropenem and ertapenem. The isolates containing IMP and VIM carbapenemase genes in the MHT test were observed as a strongly positive [25]. In this study, PFGE results showed that 15 genetically different strains of E. coli (eight patterns), K. pneumoniae (five patterns) and Proteus strains (two patterns) (named from A to H) were responsible for different infections in hospitals in Hamadan, Iran. Results showed that 50% of these bacteria were isolated from tracheal and wound samples of patients hospitalized in intensive care unit wards of the hospitals. In a similar study conducted by Anvarinejad et al. on typing with the PFGE method for 30 uropathogenic strains of E. coli, 26 genetic patterns were obtained. In this regard, isolated strains from pylonephritis and cystitis samples had similar patterns [26]. The D and E genetic patterns of K. pneumoniae strains that were isolated from tracheal samples did not have high genetic similarity with other groups. The B, C and D patterns of K. pneumoniae were observed among isolates collected from different parts of Sina hospital. Klebsiella strains with a B genetic pattern had a high resistance to all carbapenems such as imipenem, meropenem and ertapenem. Both strains were positive for carbapenemase genes (NDM and IPM). In Hashemi et al., a study on K. pneumoniae using enzymatic digestion with Xba1, 15 patterns of PFGE were identified and were classified into three main clusters comprising A, B, C. In the mentioned study, the largest PFGE pattern was observed in cluster isolated from two different hospitals in Tehran [27]. In general, a genetic pattern of P. mirabilis isolates (with five strains) was the most common pattern and all of the isolates were resistant to carbapenems. These isolates were also isolated from tracheal and ulcer samples in Sina and Besat hospitals. The B genetic pattern in these bacteria was related to P. vulgaris isolates, which were isolated from urine samples in Besat hospital and had superior resistance to carbapenem antibiotics.

**Conclusions**

Our results could reflect some hospital multidrug-resistant Enterobacteriaceae strains in nosocomial infections. Over the past decade, carbapenem-resistant Enterobacteriaceae have emerged and spread throughout the world. Widespread emergence of carbapenem-resistant isolates has been an increasing concern in recent years because carbapenem antibiotics (ertapenem, imipenem, meropenem) are often used as the last line of treatment for severe infections caused by resistant Gram-negative bacilli including the family Enterobacteriaceae. The MHT can be used as a simple method for identifying carbapenemase-producing strains in Gram-negative bacteria in routine microbiology laboratory tests as it showed intermediate or non-susceptible zones for imipenem, meropenem and ertapenem by disc diffusion. Using PFGE, diversity among strains was observed in Hamadan, but no epidemic strain was detected among them.

**Funding information**

The authors would like to acknowledge Hamadan University of Medical Sciences for providing financial support for this study.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**

15. Bloom MV, Freyer GA, McRick DA. Laboratory DNA Science: an Introduction to Recombinant DNA Techniques and Methods of Genome


