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Prevalence and Molecular Characteristics of Carbapenemase-Producing Enterobacteriaceae Among Intensive Care Unit patients: A Cross-Sectional Study in North of Iran

Mehrdad Gholami [1], Ali Haghi [2], Sadegh Ranjbari [3], Mohammad Ahanjan [1*]

1. Department of Medical Microbiology and Virology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

2. Department of Electrical and Computer Engineering, University of Tehran, Tehran, Iran

3. Department of Pathobiology, Division of Microbiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background: Carbapenems are often used as last-line agents for treating serious healthcare-associated infections (HCAIs) caused by multidrug-resistant strains. Moreover, increasing resistance to this class of antibiotics has been a major concern worldwide. In the present study, we report the prevalence and molecular characteristics of CPE among intensive care unit patients from university hospitals in Sari, North of Iran.

Materials and Methods: One hundred non-repeated clinical specimens were obtained from hospitalized patients in ICUs of four teaching hospitals in Sari, North of Iran. The API 20E system was used to identify the Enterobacteriaceae. Antibiotic susceptibility testing was performed by the disk diffusion and E-test methods. Subsequently, the MHT and CDT tests were used to identify carbapenemase-producing clinical isolates, and polymerase chain reaction (PCR) was used to detect the presence of NDM-1, VIM, and OXA-48 genes in the isolates' chromosomal DNA.

Results: The most enterobacterial isolates in the present study included Escherichia coli (37%), Klebsiella pneumoniae (21%), and Serratia rubidaea (10%), respectively. The most and least effective antibiotics against the clinical isolates were amikacin and meropenem, with resistance rates of 24% and 73%, respectively, while 51% of the isolates were imipenem-resistant. Also, 58 isolates were detected as multi-drug resistant (MDR) of which all of them were carbapenem-resistant. Among 73 carbapenem-resistant isolates, 35 (47.9%) and 33 (45.2%) of them were MHT and CDT positive, respectively. Moreover, 30.1%, 31.5%, and 38.3% of these isolates contained VIM, NDM-1, and OXA-48 carbapenemase encoding genes, respectively.

Conclusions: This work demonstrates that the MHT is not a reliable test for the study of strains producing OXA-48 or VIM carbapenemases. Additionally, there was a significant association between the presence of carbapenem resistance genes and *K. pneumoniae* isolates. It seems that the presence of at least two of these genes can be one of the main reasons for resistance to carbapenems in *Enterobacteriaceae*.

Keywords:
Enterobacteriaceae
Carbapenem
NDM-1
VIM
OXA-48

*** Corresponding Authors:**
Mohammad Ahanjan
Address: *Molecular and Cell Biology Research Center, Mazandaran University of Medical Sciences, Sari, Iran*
E-mail: hanjan2007@gmail.com

Introduction

Enterobacteriaceae are large and heterogeneous groups of gram-negative, motile or non-motile, and oxidase-negative bacilli [1]. These microorganisms cause infections such as urinary tract infections, wound infections, pneumonia, meningitis, bacteremia, and endocarditis [1,2]. Sulfonamides, ampicillin, cephalosporins, and aminoglycosides have certain antimicrobial effects against Enterobacteriaceae, while carbapenems are used as the last choice for the treatment of these organisms' infections [2,3]. Acquiring several resistance genes by carbapenemase-producing *Enterobacteriaceae* (CPE) causes many problems in the treatment of infectious diseases related to these bacteria [3,4]. Some carbapenemases are classified in the A group of the Ambler classification, and others belong to the B and D classes [5]. While clavulanic acid can inhibit class A and D carbapenemases such as GES, SME, and OXA types, the metallo-beta-lactamases (B class of Ambler) are not inhibited by this beta-lactamase inhibitor, and they are zinc chelating agents, such as ethylene-diamine tetraacetic acid (EDTA), that inhibit the production of these enzymes [5,6]. NDM-1 is a metallo-β-lactamase that confers resistance to many antibiotics in Enterobacteriaceae and has emerged as a global health threat, becoming endemic in the Middle East [7]. The *NDM*-1 gene is located on mobile plasmids and on two other plasmids that have not yet been typed and can be transmitted to different bacteria [8,9] the detection of Klebsiella pneumoniae (KLPN. The mortality rate associated with NDM-1 producer bacteria is about 18-67% in the world [10]. Although NDM-1 was identified in 2006, this enzyme was first described in 2008 [11], and water samples from New Delhi contain bacteria that produce this metallo-beta-lactamase [12]. Furthermore, the *bla*OXA-48 gene, which encodes a plasmid-mediated beta-lactamase, was first reported in Turkey in 2001 and has since become a major concern in Europe, North Africa, and the Middle East [13]. An outbreak caused by OXA-48-producing *Klebsiella pneumoniae* was reported in Turkey [13,14]. This enzyme, which belongs to the group D beta-lactamases, can hydrolyze carbapenems but displays weak activity against extended-spectrum cephalosporins, including ceftazidime and cefepime [15]. The metallo-β-lactamase encoded by the Verona Integron, VIM, which is often harbored by class 1 integrons [16], was detected for the first time in Pseudomonas aeruginosa --20 and later in other bacteria, including *Klebsiella pneumoniae*, *Escherichia coli,* and *Enterobacter* spp [17].

Because of the high-speed spreading of metallo-beta-lactamases by integrons and other mobile genetic elements between the Gram-negative bacteria, recognition of a standard phenotypic technique for the encoding genes is essential. In the present study, we report the prevalence and molecular characteristics of CPE among intensive care unit patients at university hospitals in Sari, northern Iran.

Materials and Methods

Patients and samples

In this descriptive cross-sectional study, we examined patients with nosocomial infections who were hospitalized in Intensive Care Units (ICUs) within 48 hours prior to sample collection. In this study, the clinical samples included sputum and urine. These specimens were collected from patients referred to the teaching therapeutic centers of Mazandaran University of Medical Sciences (MAZUMS) during 9 months.

Bacterial isolates

Samples were cultured on Eosin methylene blue agar or MacConkey agar. After 24 hours of incubation in ambient air conditions at 37°C, standard microbiological tests such as consumption of citrate, production of SH2 and indole, motility, methyl red, Voges-Proskauer (MR-VP), and urease, along with the API 20E system, were used for the identification of *Enterobacteriaceae* clinical isolates [18].

Antimicrobial susceptibility testing and MIC

The susceptibility pattern of the isolates was assessed against cefepime (30 µg), ceftazidime (30 µg), gentamicin (10 µg), amikacin (30 µg), imipenem (10 µg), meropenem (10 µg), ciprofloxacin (5 µg), and levofloxacin (5 µg) (MAST, UK) with the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [19,20). Minimum inhibitory concentration (MIC) for imipenem and meropenem was determined using the E-test (MAST, UK) [20]. In antimicrobial susceptibility testing, *Escherichia coli* ATCC 25922 was used as a reference strain.

Modified Hodge Test (MHT) and Combined Disk Test (CDT)

The MHT and CDT tests were used as a phenotypic technique to distinguish carbapenemase-producing strains. Briefly, we first prepared a standard suspension containing 1.5×10^8 CFU/ml of E. coli ATCC 25922, a carbapenem-susceptible strain, and then diluted it 1:10 in saline. Then, inoculated it on Mueller Hinton agar (MHA) (Merck, Germany) with a sterile swab and allowed the plate to dry for 3–10 minutes. The 10 µg meropenem disc (MAST, UK) was placed in the center of the plate. Using a 10-µL loop, picked 3–5 grown overnight colonies of test and Quality Control organisms and inoculated them in a straight line (20–25 mm in length) out from the edge of the disk.

The plates were incubated at 37 °C in an ambient air condition for 20 hours. We then examined the MHA plate for enhanced growth around the test organism streak at the junction of the streak and the zone of inhibition. Enhanced growth of *E. coli* ATCC 25922, such as clover leaves at the intersection, indicates the positive result for carbapenemase production [21].

CDT was used to phenotypically diagnose metallo-beta-lactamase in clinical isolates [22]. To perform this test, we cultivated the bacterial isolates on MHA and then put a 10 µg meropenem disk in the center of the plate. After that, a meropenem-EDTA (10 µg/0.25 M) (*ROSCO* Diagnostica, Taastrup, Denmark) combined disk was placed at a distance of 20 mm (center-to-center) from the imipenem disk. An increase of ≥ 5 mm in inhibition zone diameter of the isolate around the imipenem-EDTA combined disk compared to the imipenem disk was regarded as a positive result for the production of metallo-beta-lactamase. A NDM-1 positive *Klebsiella pneumoniae* was chosen as the positive control in CDT.

Molecular detection of carbapenemases genes

Genomic DNA from the isolates was extracted using the SDS (Sodium dodecyl sulfate) method [23]. The primers used for the detection of interest genes are shown in Table 1. The PCR amplification reaction was completed in a total volume of 25 µl, including: 300 ng of extracted genomic DNA, 12.5 µL TEMpase Hot Start PCR Master Mix (2×) (Ampliqon, Copenhagen, Denmark), 10 pmol/ µL of each primer (Bioneer Co., South Korea), and sterile distilled water. The samples were amplified in a Techne TC-512 thermocycler (Eppendorf, Hamburg, Germany) as follows: initial denaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 95 °C for 40 s, annealing at 63 °C, 65 °C, and 63 °C for 60 s for *bla*OXA-48, *bla*NDM-1, and *bla*VIM, respectively, and extension at 72 °C for 40 s and a final extension at 72 °C for 10 min. Staining of PCR products was done with Gel Red™ (Biotium, Landing Pkwy, Fremont, CA, USA) and loaded on 1% agarose gel. Finally, the agarose gel was exposed to UV transilliumiator.

Statistical analysis

The data was analyzed using the Statistical Package for the Social Sciences (SPSS) software version 16. Comparisons of data across groups were performed using the *Chi*-square test. *P*-values less than 0.05 were considered statistically significant.

Results

Patients and bacterial isolates

We collected 180 clinical samples from hospitals affiliated with MAZUMS, of which 100 clinical isolates of *Enterobacteriaceae* (from 59 males and 41 females) were identified. Out of all strains, 38 and 62 of them were isolated from sputum and urine samples, respectively. The isolates (No.) included *Citrobacter diversus* [1], *Citrobacter freundii* [4], *Enterobacter aerogenes* [5], *Enterobacter gergoviae* [3], *Enterobacter cloacae* [5], *Escherichia coli* [37], *Klebsiella oxytoca* [6], *Klebsiella ozaenae* [1], *Klebsiella pneumoniae* [21], *Proteus mirabilis* [4], *Proteus vulgaris* [3], and *Serratia rubidaea* [10].

Antimicrobial susceptibility profile

According to the Antimicrobial Susceptibility Testing (AST), the most and the least effective antibiotics against the clinical isolates of *Enterobacteriaceae* were amikacin and meropenem, respectively. Table 2 displays the drug susceptibility pattern of the carbapenem-resistant isolates of the present study, and Table 3 compares the susceptibility profile of the total isolates and carbapenem-resistant ones. Out of 100 clinical isolates of *Enterobacteriaceae* in this study, 58% of them were detected as MDR, of which all of the MDR isolates belonged to the carbapenem-resistant groups. Interestingly, among 10 *Serratia rubidaea* isolates, 8 (80%) were MDR and resistant to meropenem, with MICs ranging from 4 to 2048 µg/ml. However, 6 (75%) MDR *Serratia rubidaea* isolates showed a MIC range of 4-2048 µg/ml, and the other two isolates were intermediate resistant and susceptible to this carbapenem. Other MDR isolates included: *E. coli* (N=20), *K. pneumoniae* (N=18), *K. oxytoca* (N=2), *C. freundii* (N=3), *P. mirabilis* (N=2), *E. aerogenes* (N=3), and *E. cloacae* (N=2).

The highest resistance to meropenem was observed among clinical isolates of *Serratia rubidaea* (100%), *followed by Klebsiella pneumoniae (76.1%)* and *Escherichia coli* (67.5%). Moreover, 90%, 71.4%, and 29.7% of these three enterobacterial species were resistant to imipenem, respectively, which was higher than the resistance rates of other species identified in this study. We detected no difference between disk agar diffusion and the E-test for AST of imipenem and meropenem. Moreover, among carbapenem non-resistant isolates of the present study, just 5 (18.5%) isolates were resistant to piperacillin, and 3 (11.1%) of them were identified as intermediate

Table 1. The primers used for the detection of carbapenemase genes in the clinical isolates of *Enterobacteriaceae*.

Genes	The primer sequences (5' to 3')	Product size (bp)	References
*bla*OXA-48	GGGGACGTTATGCGTGTATT GAGCACTTCTTTTGTGATGGC	783	[36]
*bla*NDM-1	GGTGCATGCCCGGTGAAATC ATGCTGGCCTTGGGGAACG	661	[39]
*bla*VIM	GATGGTGTTTGGTCGCCATA CGAATGCGCAGCACAG	390	[40]

Table 2. The results of antimicrobial susceptibility testing along with the phenotypic and genotypic detection of carbapenemases in the clinical isolates of Enterobacteriaceae.

Isolates No.	Antimicrobial susceptibility results											Phenotypic tests		Carbapenemase genes		
	Disk Agar Diffusion									MIC (µg/ml) by E-Test						
	PIP	CFZ	CFM	TOB	GM	AM	CIP	MER	IMI	MER	IMI	MHT	CDT	*OXA-48*	*VIM*	*NDM-1*
1	R	R	R	R	R	R	R	R	R	2048	2048	+	+	-	+	+
2	R	R	R	R	R	R	R	R	R	1024	2048	+	+	-	+	+
3	R	R	R	R	R	R	R	R	R	256	256	-	+	-	-	+
4	R	R	R	R	R	R	R	R	R	256	256	+	+	-	-	+
5	R	R	R	R	R	R	R	R	R	128	128	-	+	-	+	-
6	R	R	R	R	R	R	R	R	R	256	256	-	+	-	-	+
7	S	S	S	S	S	I	S	R	I	32	2	+	-	-	-	-
8	S	S	S	S	S	S	S	R	R	32	32	+	-	+	-	-
9	R	R	R	S	S	S	R	R	R	1024	1024	+	+	+	+	-
10	R	R	R	S	S	S	R	R	R	256	512	+	-	+	-	-
11	R	R	R	R	S	S	R	R	R	8	8	-	-	-	-	-
12	R	R	R	S	S	S	R	R	R	2048	2048	-	+	+	+	-
13	R	R	R	S	S	S	R	R	R	2048	2048	+	+	+	+	-
14	R	S	R	S	S	S	I	R	R	2048	2048	+	+	+	+	-
15	R	R	R	S	S	S	R	R	R	32	64	-	-	+	-	-
16	I	R	I	S	S	S	R	R	R	32	64	-	+	+	-	-
17	R	R	R	R	R	R	R	R	R	16	16	-	-	-	-	-
18	S	S	S	S	S	S	S	R	R	1024	512	+	+	-	+	+
19	I	S	S	S	S	S	S	R	R	256	128	-	+	-	-	+
20	R	R	R	R	R	R	R	R	R	16	16	-	-	-	-	-
21	R	R	R	R	R	R	R	R	R	16	16	-	-	-	-	-
22	R	R	I	S	S	S	R	R	R	512	256	+	+	+	-	+
23	S	S	S	S	S	S	S	R	S	16	8	-	-	+	-	-
24	S	S	S	S	S	S	S	R	R	4	4	-	-	-	-	-
25	R	R	R	S	S	S	R	R	R	32	16	-	-	+	-	-
26	R	R	R	S	R	S	R	R	R	4	8	-	-	-	-	-
27	R	R	R	I	S	S	S	R	S	4	0.5	-	-	-	+	-
28	R	R	R	I	S	S	S	R	R	32	8	+	-	-	-	+
29	R	R	R	R	S	I	R	R	R	64	128	+	+	-	-	+
30	R	R	R	R	S	I	R	R	R	16	16	+	-	-	-	-
31	R	R	R	S	S	S	R	R	R	128	256	+	+	+	-	+
32	R	R	R	R	R	R	R	R	R	16	16	+	-	-	-	-
33	R	R	R	S	S	S	R	R	R	16	32	+	-	-	-	-
34	R	R	R	S	S	S	S	R	S	32	0.1	-	-	+	-	-
35	R	R	R	R	R	R	R	R	R	64	64	-	-	-	+	-
36	R	R	R	R	R	R	R	R	R	64	32	-	+	-	-	+
37	R	R	S	I	S	S	R	R	S	16	0.25	+	-	-	-	+
38	R	I	S	S	S	S	R	R	R	64	8	+	+	-	-	+
39	S	S	S	S	R	S	S	R	S	16	0.25	+	-	-	-	+
40	R	I	I	I	R	S	S	R	S	8	0.25	-	-	-	-	-
41	R	R	R	R	R	R	R	R	R	64	32	-	+	-	-	+
42	R	R	R	S	R	S	R	R	R	256	128	+	+	+	-	+
43	R	R	S	S	I	S	S	R	I	16	2	-	-	-	-	-
44	R	R	S	S	S	S	R	R	S	16	0.5	-	-	+	-	-
45	S	S	S	S	S	S	R	R	S	16	0.25	-	-	-	-	-
46	R	R	I	S	S	S	R	R	I	16	2	-	-	-	-	-
47	I	I	I	R	S	S	R	R	S	16	0.25	-	-	-	-	-
48	R	I	I	R	R	I	S	R	I	16	2	-	-	-	-	-
49	S	R	R	R	R	R	R	R	R	2048	2048	+	+	+	+	+
50	S	R	R	S	S	R	R	R	I	16	2	-	-	-	-	-
51	S	S	S	S	S	S	S	R	S	4	0.1	-	-	-	-	-
52	R	R	R	R	R	S	S	R	I	32	2	-	-	-	+	-
53	R	R	R	S	S	S	R	R	I	16	2	-	-	-	-	-
54	S	S	S	S	S	S	S	R	S	4	0.1	-	-	-	-	-
55	R	R	R	S	S	S	R	R	R	64	32	+	+	-	+	-
56	R	R	R	R	R	R	R	R	R	256	128	+	+	-	+	+
57	R	R	S	R	R	R	R	R	R	16	16	-	+	-	-	-
58	S	S	S	S	S	S	S	R	I	64	2	+	-	+	+	-
59	S	I	S	S	S	S	S	R	S	16	0.25	-	-	+	-	-
60	S	S	S	S	S	S	S	R	S	4	0.25	+	-	-	+	-
61	R	R	I	R	R	R	S	R	R	16	16	-	-	-	-	-

Table 2. Continued

62	S	S	S	S	S	S	S	R	R	64	64	+	+	+	+	-
63	S	S	S	S	S	S	I	R	S	16	0.25	-	-	+	-	-
64	R	R	R	S	S	S	R	R	R	64	128	+	+	+	+	-
65	S	R	R	S	S	S	R	R	R	128	128	+	+	+	-	+
66	R	R	R	S	S	S	R	R	R	128	64	+	+	+	+	-
67	R	R	R	S	S	S	R	R	R	16	32	-	+	+	-	-
68	R	R	R	R	R	R	R	R	R	512	512	+	+	+	-	+
69	R	R	R	R	R	R	R	R	R	2048	2048	+	+	+	+	+
70	R	R	R	R	R	R	R	R	R	16	32	+	-	-	+	-
71	R	R	R	R	R	R	R	R	R	16	16	-	+	+	-	-
72	R	R	R	R	R	R	R	R	R	512	256	+	+	+	+	-
73	R	R	R	R	R	R	R	R	R	32	64	+	-	-	-	+

Abbreviations: PIP, Piperacillin; CFZ, Ceftazidime; CFM, Cefepime; TOB, Tobramycin; GM, Gentamicin; AM, Amikacin; CIP, Ciprofloxacin; MER, Meropenem; IMI, Imipenem; MIC, Minimum Inhibitory Concentration; MHT, Modified Hodge Test; CDT, Combined Disk Test; R, Resistant; I, Intermediate Resistant; S, Susceptible.

Table 3. The comparison of antimicrobial susceptibility pattern between all clinical isolates and carbapenem resistant isolates.

Antibiotics	Resistance rate No. (%)		Intermediate resistance rate No. (%)		susceptibility rate No. (%)		*P*-value
	Total	Carbapenem resistant	Total	Carbapenem resistant	Total	Carbapenem resistant	
PIP	58 (58)	53 (72.6)	3 (3)	3 (4.1)	39 (39)	17 (23.2)	
CFZ	53 (53)	53 (72.6)	5 (5)	5 (6.8)	42 (42)	15 (20.5)	
CFM	46 (46)	46 (63.01)	7 (7)	7 (9.5)	47 (47)	20 (27.3)	
TOB	29 (29)	29 (39.7)	5 (5)	4 (5.4)	66 (66)	40 (54.7)	
GM	29 (29)	29 (39.7)	2 (2)	1 (1.3)	69 (69)	43 (58.9)	
AM	24 (24)	24 (32.8)	5 (5)	4 (5.4)	71 (71)	45 (61.6)	
CIP	50 (50)	50 (68.4)	2 (2)	2 (2.7)	48 (48)	21 (28.7)	
MER	73 (73)	73 (100)	3 (3)	-	24 (24)	-	
IMI	51 (51)	51 (69.8)	8 (8)	8 (10.9)	41 (41)	14 (19.1)	

Note: The total number of isolates was 100, while the number of isolates resistant to carbapenems was 73.
Abbreviations: PIP, Piperacillin; CFZ, Ceftazidime; CFM, Cefepime; TOB, Tobramycin; GM, Gentamicin; AM, Amikacin; CIP, Ciprofloxacin; MER, Meropenem; IMI, Imipenem.

Table 4. Relation between the presence of carbapenemase encoding genes among the carbapenem resistant clinical isolates and the results of MHT and CDT phenotypic methods.

Phenotypic Test results	Only MHT positive isolates (N=13)	Only CDT positive isolates (N=11)	Both MHT and CDT positive isolates (N=22)	Both MHT and CDT negative isolates (N=27)	Total (N=73)	*P*-value
No. (%) of only *OXA*-48 positive isolates	2 (15.3)	3 (23.07)	-	7 (53.8)	12	< 0.05
No. (%) of only *VIM* positive isolates	2 (28.5)	1 (14.2)	1 (14.2)	3 (42.8)	7	NS
No. (%) of only *NDM*-1 positive isolates	4 (33.3)	5 (41.6)	3 (23.07)	-	12	NS
No. (%) of both *OXA*-48 and *VIM* positive isolates	1 (11.1)	1 (11.1)	7 (77.7)	-	9	< 0.05
No. (%) of both *OXA*-48 and *NDM*-1 positive isolates	-	-	5 (100)	-	5	< 0.05
No. (%) of both *VIM* and *NDM*-1 positive isolates	-	-	4 (100)	-	4	< 0.05
No. (%) of *OXA*-48, *VIM* and *NDM*-1 positive isolates	-	-	2 (100)	-	2	< 0.05
No. (%) of *OXA*-48, *VIM* and *NDM*-1 negative isolates	4 (20)	1 (5)	-	17 (85)	22	< 0.05
Total	13	11	22	27	73	

Abbreviations: MHT, Modified Hodge Test; CDT, Combined Disk Test; NS, Not statistically significant.

resistant to meropenem, and 3 different isolates were intermediate resistant against gentamicin, amikacin, and tobramycin. All other carbapenem-nonresistant isolates were susceptible to the other antibiotics tested in this study (P < 0.05).

MHT and CDT phenotypic testing

The MHT was performed to phenotypically detect carbapenemase production among 73 carbapenem-resistant clinical isolates. According to this test, 35 (47.9%) carbapenem-resistant isolates were detected as carbapenemase producers. The CDT results showed that 33 (45.2%) clinical isolates of *Enterobacteriaceae* were positive as metallo-beta-lactamase producers. Table 4 shows the number of carbapenem-resistant isolates detected as MHT- or CDT-positive. Among 73 carbapenem-resistant isolates, 27 (36.9%) isolates showed negative results in both MHT and CDT, while 22 (30.1%) isolates were detected as both MHT and CDT positive.

Molecular prevalence of carbapenemases genes

The PCR results of 73 carbapenem-resistant clinical isolates showed that 28 (38.3%), 22 (30.1%), and 23 (31.5%) of them contained the blaOXA-48, blaVIM, and blaNDM-1 carbapenemase genes, respectively. Table 4 shows the number of carbapenem-resistant isolates carrying these genes and the relation between their presence and the results of phenotypic tests. According to this Table, there was a statistically significant association between the presence of two or all three studied carbapenemase-encoding genes and positive results for both MHT and CDT (P < 0.05).

Among the carbapenem-resistant isolates containing the *bla*OXA-48 gene, only 4 (14.2%) isolates were susceptible to imipenem, while others showed a MIC range of 8-64 and 16-32 µg/ml against imipenem and meropenem, respectively. Moreover, among 22 blaVIM-positive isolates, only 2 (9.09%) and 1 (4.5%) were susceptible and intermediately resistant to imipenem, respectively. The range of MICs in *VIM-containing isolates against imipenem and meropenem was 32-128 and 4-128 µg/ml, respectively*. While 1 (4.3%) isolate carrying *bla*NDM-1 was sensitive to imipenem, all others showed a range of 8-256 and 32-256 µg/ml in MICs against imipenem and meropenem, respectively. Surprisingly, except one isolate, which was both *OXA*-48 and *VIM* positive, all other isolates which contained a combination of two carbapenemase encoding genes, including *OXA*-48 and *VIM*, *OXA*-48 and *NDM*-1, or *VIM* and *NDM*-1, were resistant to imipenem (MIC ranges of 64-2048 µg/ml) and meropenem (MIC ranges of 64-2048 µg/ml). Also, both isolates carrying a combination of three *OXA*-48, *VIM,* and *NDM*-1 carbapenemase genes showed a MIC range of 2048 µg/ml against both imipenem and meropenem (*P*-value < 0.05). Moreover, among 22 isolates containing any of the studied carbapenemase genes, all were meropenem-resistant, with MICs ranging from 4 to 32 µg/ml. However, 11 (50%) isolates in this group were resistant to imipenem, with MICs ranging from 4 to 32 µg/ml, and 17 (77.2%) were both MHT- and CDT-negative.

The most prevalent NDM-1 gene was shown in the isolates of Serratia rubidaea, of which 6 (60%) were positive, while 3 (30%) isolates contained a combination of *VIM* and *NDM*-1. The arrangement of *OXA*-48 and *NDM*-1 was more prevalent in *E. coli* isolates, while the combination of *OXA*-48 and *VIM* was more commonly detected in the isolates of *K. pneumoniae*. Additionally, both isolates carrying all three OXA-48, VIM, and NDM-1 carbapenemase-encoding genes were identified as *K. pneumoniae*.

Discussion

The spread of carbapenem resistance genes among *Enterobacteriaceae* has been a global concern [3,4]. In this study, 73% of isolates were resistant to at least one of the tested carbapenems, indicating a significant threat to patients with nosocomial enterobacterial infections. We found that 35 (47.9%) carbapenem-resistant isolates were positive in MHT, of which 2 (5.7%) isolates contained the *bla*OXA-48 gene alone. According to previous studies, MHT is not a reliable indicator of the presence of blaOXA-48, whereas this phenotypic method can reliably detect the presence of blaIMP and blaVIM carbapenemase genes in clinical isolates of *Enterobacteriaceae* [24–27]. A limitation of this method is that it cannot distinguish among different carbapenem classes, potentially resulting in a false-positive or false-negative response [26]. Moreover, the use of imipenem-EDTA is an appropriate test for recognition of metallo-beta-lactamase genes in *Enterobacteriaceae* [22]. The present study confirmed this idea, as 33 (45.2%) isolates were positive for CDT; of these, 16 (48.4%) and 19 (57.5%) contained *bla*VIM and *bla*NDM-1 genes, respectively. However, 18 (54.5%) CDT-positive isolates possessed the *OXA*-48 gene. The challenge of this issue is that the most isolated carrying these carbapenemase-encoding genes belonged to the group of isolates that showed both MHT and CDT-positive results. 14 (50%), 14 (63.6%), and 14 (60.8%) isolates, which contained blaOXA-48, blaVIM, and blaNDM-1, respectively, were positive in both phenotypic tests. It seems that these methods are more reliable for the detection of blaNDM-1 than other detected carbapenemase genes because all blaNDM-1-carrying isolates showed a positive result in MHT, CDT, or both of them. Additionally, we found that the use of both MHT and CDT was more effective for detecting isolates containing combinations of two or three carbapenemase genes, with 19 (90.4%) isolates that contained more than one carbapenemase gene yielding positive results in both phenotypic tests.

We found a significant association between

enterobacterial species and the presence of blaOXA-48 and blaVIM ($P < 0.05$), with the majority of strains carrying these genes identified as *Klebsiella pneumoniae*. This was in accordance with a study conducted in Turkey, where most of their *bla*OXA-48 positive *Enterobacteriaceae* were identified as *Klebsiella pneumoniae,* while the rate of resistance to carbapenems in their study was lower than our research [28,29]. This could be due to the country's proximity to northern Iran and the high level of cross-border travel between the two countries. Additionally, the prevalence of VIM-producing clinical isolates of Enterobacteriaceae was higher among Klebsiella pneumoniae than among other species in our and other studies worldwide [30]. Studies in other countries indicated the high prevalence of VIM-producing *Enterobacteriaceae* [31,32]. The high prevalence of the *VIM* metallo-beta-lactamase gene in enterobacterial isolates may be due to its facile spread via integrons among bacteria [33]. The high spread of metallo-beta-lactamases is the cause of high resistance to carbapenems in Enterobacteriaceae, as the study conducted by Heller *et al.* in Australia showed that the increased resistance of *E. cloacae* isolates against imipenem from less than 1% in 2001 to 20% and 50% in Non-ICU and ICU isolates in 2006 [34].

In our study, there was no significant relationship between the presence of only one of the carbapenem resistance genes, such as blaOXA-48, blaVIM, or blaNDM-1, and different enterobacterial isolates that were resistant to imipenem or meropenem. We found that the concurrent presence of at least two of these genes was associated with the emergence of carbapenem resistance and the MDR phenotype in clinical isolates of *Enterobacteriaceae*. Another study conducted in Italy [35] confirmed this issue, showing that the simultaneous presence of blaVIM and blaCTX-M-15 genes in clinical isolates of Klebsiella pneumoniae can lead to the emergence of an MDR phenotype. Additionally, the presence of blaNDM-1 is a major cause of carbapenem resistance; only 1 isolate carrying this gene in our study was susceptible to imipenem, and all were resistant to meropenem. This was consistent with another study conducted in Turkey [36], in which blaNDM-1 was detected in numerous Enterobacterial clinical isolates that were resistant to multiple important antibiotics, particularly carbapenems. They showed that most of their isolates containing the *bla*NDM-1 gene were also positive for blaOXA-48, indicating that it is a major concern in Turkey [36].

We found that the co-presence of multiple beta-lactamases in enterobacterial isolates could be a cause of MDR phenotype emergence, as almost all isolates contained combinations of two or three carbapenemase-encoding genes. On the other hand, several other mechanisms, such as the presence of other carbapenemase resistance genes, reduced numbers or absence of 36 kDa purines due to decreased expression, and increased expression of efflux pumps, can be major causes of the emergence of carbapenem resistance [37,38]. Additionally, the simultaneous presence of blaNDM-1 with other carbapenemase genes was a strong indicator of carbapenem resistance and the emergence of an MDR phenotype in this study. This combination can result in high MIC values; such isolates in our study showed MICs ranging from 256 to 2048 µg/ml. Moreover, we found that none of the isolates that were blaNDM-1-positive showed MHT- or CDT-negative results, indicating that a positive result with either phenotypic method could be an important indicator of the presence of the *bla*NDM-1 gene.

Using MIC testing of imipenem and meropenem, we found a significant association between enterobacterial species and resistance to these carbapenems ($P < 0.05$). There was a surprising result about the *Serratia rubidaea* isolates, which were all resistant to meropenem, and 80% of the *Serratia rubidaea* isolates were detected as MDR. These two properties were higher than those detected in clinical isolates of *K. pneumoniae* and *E. coli*. Because these bacteria are opportunistic pathogens, these issues can become a global concern. On the other hand, there was no significant association between carbapenem resistance and the prescription of beta-lactams such as cefazolin, ceftriaxone, or piperacillin in hospitalized patients included in this study ($P > 0.05$).

Conclusions

The high prevalence of carbapenemase-encoding genes in tested Enterobacteriaceae isolates is a significant concern worldwide, given the spread of these genes via class 1 integrons and the role of carbapenems as last-line therapeutic agents for infections caused by MDR Gram-negative bacteria. Therefore, the performance of phenotypic tests for detecting the possible presence of carbapenemase genes in all Gram-negative bacteria in clinical settings is an important issue that appears necessary in hospitals.

Declarations

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While preparing this work, the author used ChatGPT to improve the manuscript's language. After using this tool/service, the author reviewed and edited the content as needed and assumed full responsibility for the publication.

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