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Emergence of OXA-10 and OXA-48 Like Carbapenemases among *Enterobacter* Isolates from Inpatients in Namazi Hospital in Shiraz

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ABSTRACT

Backgrounds: The global spread of carbapenemase-producing *Enterobacteriaceae* represents a public health concern. This study aimed to investigate the prevalence of carbapenem resistance and the presence of some oxacillinase types and class 1-3 integrons among *Enterobacter* clinical isolates from an Iranian inpatient population.

Materials & Methods: Ninety *Enterobacter* isolates from hospitalized patients were diagnosed by microbiological methods. Antibiogram pattern was also determined. The presence of class 1-3 integrons and four types of oxacillinase genes was assessed using PCR.

Findings: Among 90 *Enterobacter* isolates, the most common species was *E. aerogenes*, (45.6%), followed by *E. cloacae* (30%). The highest resistance rate was against ampicillin (96.7%). Multidrug resistance (MDR) was substantial (93%). Carbapenemase-producers were detected in 96% of carbapenem-resistant isolates by mCIM test. The frequency of evaluated genes was as follows: *intI1* = 50 (55.6%), *intI2* =12 (13.3%), bla_{oxa-1} =6 (6.7%), bla_{oxa-2} =5 (5.6%), bla_{oxa-10} =18 (20%), and bla_{oxa-48} =18 (20%).

Conclusion: Determinants of class 1 integron along with OXA-10 and OXA-48 like carbapememases are responsible for relatively considerable carbapenem resistance among isolates. This is the first report about the presence of OXA-10 and OXA-48-producing *Enterobacter* spp. in Iran, indicating that the prevalence of oxacillinases in the country might be on the rise.

Keywords: *Enterobacter,* Carbapenemase, Oxacillinase, Integron, Iran.

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Introduction

Enterobacter, as an important Gram-negative rod among Enterobacteriaceae members, accounts for a wide range of nosocomial infections, especially bloodstream (BSI), respiratory tract (RTI), and urinary tract infections (UTI). Among *Enterobacter* spp., *E. aerogenes* and *E. cloacae* have been described in several outbreaks of nosocomial infections in Europe during the last 30 years [1, 2]. The incidence of nosocomial infections due to *Enterobacter* spp. is associated with remarkable morbidity and mortality, especially among hospitalized patients in intensive care units (ICUs) [3, 4].

Beta-lactams, especially thirdgeneration cephalosporins and carbapenems, are frequently used to treat infections caused by *Enterobacter* spp. [5, 6]. However, *E. cloacae* and *E. aerogenes* as the most frequently isolated species are intrinsically resistant to many drugs [7]. Moreover, the acquisition of multidrug resistance (MDR) among *Enterobacter* spp. is increasing, severely compromising available therapies [8]. Nosocomial infections caused by carbapenem-resistant Enterobacteriaceae (CRE) are considered as a challenge for patients, physicians, and public health, and this issue is due to their ability to spread across the world. A mortality rate of 30-44% has been attributed to infections caused by CRE [9, 10]. The main mechanism of carbapenem resistance in Enterobacteriaceae, including *Enterobacter* spp., is carbapenemase production. According to the Ambler classification, molecular class D beta-lactamases (OXA-type carbapenemases) have recently been increasingly transmitted to *Enterobacteriaceae* and have become a considerable cause of carbapenem resistance [9, 11]. They are active on extended-spectrum cephalosporins, monobactams (ESBL), and carbapenems [12]. There are different types of OXA-type carbapenemases, including OXA-48 and its variants, which are widespread amongst *Enterobacteriaceae* [11]. Among different carbapenemases, OXA-48 is the most predominant type in Mediterranean and Middle East countries [13]. Furthermore, integrons as one of the mobile genetic elements play a major role in the spread of antibiotic resistance among bacteria, especially Gram-negative rods. To date, four general classes of integrons, namely classes 1 to 4, have been introduced, among which class 1-3 integrons are capable of acquiring gene cassettes through site-specific recombination [14, 15]. There are few reports of carbapenemase-producing *Enterobacter* spp. in Iran.

Objectives: The present study aimed to investigate the presence of some OXA-type carbapenemase genes and class 1-3 integrons among *Enterobacter* clinical isolates from inpatients in Namazi Teaching Hospital in Shiraz, southern Iran.

Materials and Methods

Clinical isolates: The present study was carried out on 90 non-duplicated *Enterobacter* isolates obtained from hospitalized patients in Namazi Teaching Hospital in Shiraz during August 2018 to April 2019. Only one isolate was collected from each patient. Of these 90 isolates, 40 isolates belonged to a previous study [16]. The isolates were recovered from different clinical samples. *Enterobacter* isolates were primarily identified by microbiological tests and then confirmed at the species level by Microgene™ GnA+B-ID system (Microgen Bioproducts Ltd, UK) diagnostic kit (Mast, UK) according to the manufacturer's instructions. All the confirmed isolates were stored in tryptic soy broth (TSB) (Merck Co., Germany) containing 20% glycerol at -70 °C until further use. This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (approval number: IR.SUMS. REC.1397.816).

Antimicrobial susceptibility testing: Antibiotic susceptibility testing was performed for the isolates against 10 antimicrobial agents (Mast Co., UK) (shown in Table 1) on Muller-Hinton agar plates (Merck Co., Germany) using the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [17]. *Escherichia coli* ATCC 25922 was used as

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the control strain. MDR was defined as non-susceptibility to ≥1 agent in ≥3 different antibiotic classes [18].

Carbapenemase phenotypic detection: Detection of carbapenemase-producing *Enterobacter* spp. was performed by the modified carbapenem inactivation method (mCIM) in accordance with CLSI recommendations [17]. Briefly, the bacterial suspension was suspended in 2 mL of TSB, and then a 10 µg meropenem disk (Mast Co., UK) was added to the medium prior to incubation at 35 °C for 4 hrs. The meropenem disk was then transferred onto Muller-Hinton agar, inoculated with a 0.5 McFarland suspension of *E. coli* ATCC 25922, and incubated at 35 °C for 18 to 24 hrs. The presence of carbapenemase activity was recognized by an inhibition zone diameter of ≤15 mm or the presence of pinpoint colonies within a 16-18 mm zone; also, the absence of carbapenemase activity was revealed by inhibition zone diameters of ≥19 mm (clear zone) [19].

Evaluation of class 1, 2, and 3 integrons and oxacillinase resistance genes: Genomic DNA of *Enterobacter* spp. was extracted using the boiling method as previously described [20]. The presence of potential resistance genes encoding class 1, 2, and 3 integrons and oxacillinases, including *intI1*, *intI2*, and *intI3* integrases and bla_{oxa-1}, bla_{oxa-2}, bla_{oxa-10}, and bla_{oxa-48}, was screened by PCR amplification using previously reported primers [21-25]. PCR reactions and conditions were performed using a thermal cycler 5530 (Eppendorf master, Germany) in a total volume of 25 µL as reported previously. For *intI1*, *intI2*, *intI3*, bla_{oxa-1}, bla_{oxa-2}, bla_{oxa-10}, and bla_{oxa-48} genes, annealing temperatures were set at 55, 58, 58, 48, 59, 54, and 60 °C, respectively. PCR products were electrophoresed on 1.5% agarose gel, stained with KBC power load dye (CinnaGen Co. Iran), and visualized in a gel documentation system. The amplicons of $bla_{oxa-48-}$like gene-producing isolates were submitted for sequencing (Bioneer Co., Munpyeongseo-ro, Daedeok-gu, Daejeon, South Korea), and the results were analyzed using the GenBank database of the National Center for Biotechnology Information through BLAST network service (http://www.ncbi.nlm.nih.gov/BLAST/).

Statistical analysis: Data analysis was done using SPSS™ software, Version 21.0 (IBM Corp., USA). Chi-square and Fisher's exact tests were used where appropriate, and the differences were considered statistically significant when the *p*-value was less than 0.05.

Findings

Study population and clinical characteristics of *Enterobacter* isolates: The studied isolates were recovered from 90 hospitalized individuals

Table 1) Antibiotic resistance pattern of the studied *Enterobacter* spp.

Antibiotic	E. aerogenes (n = 41)	E. cloacae (n = 27)	E. gergoviae (n = 14)	C. sakazakii (n = 8)	Total (No., %)
Ampicillin	39(95.1)	26(96.3)	14(100)	8(100)	87 (96.7)
Amoxicillin-clavulanate	39(95.1)	24(88.9)	13(92.9)	8(100)	74 (82.2)
Cefoxitin	34(82.9)	25(92.6)	11(78.6)	8(100)	78 (86.7)
Ceftazidime	34(82.9)	20(74.1)	11(78.6)	8(100)	73 (81.1)
Ciprofloxacin	16(39)	9(33.3)	4(28.6)	3(37.5)	32 (35.5)
Amikacin	13(31.7)	3(11.1)	2(14.3)	4(50)	22 (24.4)
Gentamicin	19(46.3)	10(37)	6(42.9)	3(37.5)	38 (42.2)
Trimethoprim/ sulfamethoxazole	23(56.1)	10(37)	9(64.3)	4(50)	46 (51.1)
Nitrofurantoin	34(82.9)	23(85.2)	9(64.3)	6(75)	72 (80)
Imipenem	14(34.1)	6(22.2)	2(14.3)	3(37.5)	25 (27.8)

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(inpatients), including 25 (27.8%) females and 65 (72.2%) males with a median age of 33 years (ranging from 8 days to 76 years). Among *Enterobacter* isolates, 58 (64.4%) isolates were obtained from the intensive care unit (ICU), 24 (26.7%) isolates from the internal ward, and eight (8.9%) isolates from the surgical ward. The isolates were recovered from RTI (n=34, 37.8%), UTI (n=20, 22.2%), BSI (n=16, 17.8%), skin and soft tissue infection (SSTI) (n=14, 15.6%), and other sources (n=6, 6.6%). All 90 clinical isolates of *Enterobacter* were classified as *E. aerogenes* (n=41, 45.5%), *E. cloacae* (n=27, 30%), *E. gergoviae* (n=14, 15.6%), and *Cronobacter* (*E*) *sakazakii* (n=8, 8.9%).

Antimicrobial resistance of *Enterobacter* spp.: The results of antimicrobial susceptibility testing are represented in Table 1. All the isolates showed resistance to all antimicrobials tested with different proportions. According to these results, the highest resistance rate (non-susceptible isolates) was against ampicillin (96.7%), whereas the lowest resistance was toward amikacin (24.4%). Among different species, *C. sakazakii* isolates revealed the highest (68.7%) resistance to antimicrobial agents, followed by *E. aerogenes* (64.6%). The majority of the isolates (n=84, 93%) exhibited multidrug resistant (MDR) phenotype. Out of 90 *Enterobacter* isolates, 25 (27.8%) isolates were phenotypically non-susceptible to carbapenems (imipenem as representative of carbapenems, Table 1). Among 25 carbapenem-resistant isolates, 24 (96%) isolates showed positive results in mCIM test. All of the mCIM-positive isolates were MDR.

Characterization of integrase and oxacillinase genes: PCR analysis of integron genes showed that 50 (55.6%) and 12 (13.3%) isolates carried *intI1* and *intI2* genes, respectively. Class 3 integron (*intI3*) was not found in any of the isolates. Antimicrobial resistance patterns of class 1 and 2 integron-positive and negative isolates are presented in Tables 2 and 3, respectively. A statistically significant association was found between the presence of class 2 integron and higher rates of antimicrobial resistance to ceftazidime and trimethoprim-sulfamethoxazole (Table 3).

Moreover, 18 (20%) isolates harbored both $bla_{\text{oxa-10}}$ and $bla_{\text{oxa-48}}$-like genes with different distributions among *Enterobacter* spp., and six (6.7%) and five (5.6%) isolates were positive for $bla_{\text{oxa-1}}$ and $bla_{\text{oxa-2}}$ genes, respectively (Table 4). On the other hand, only four (16.6%) mCIM-positive isolates carried the $bla_{\text{oxa-48}}$-like gene, and the other oxacillinase genes were not detected among these isolates. A significant correlation was observed between the presence of $bla_{\text{oxa-10}}$ and $bla_{\text{oxa-2}}$ genes and a higher

Table 2) Antibiotic susceptibility pattern of *Enterobacter* isolates according to the presence of class 1 integron.

Antibiotic	Integron-1 positive n=50 No. (%)	Integron-1 negative n=40 No. (%)	p-value
Ampicillin	49 (98)	38 (95)	0.5
Amoxicillin-clavulanate	46 (92)	38 (95)	0.6
Cefoxitin	42 (84)	36 (90)	0.5
Ceftazidime	43 (86)	30 (75)	0.2
Ciprofloxacin	16 (32)	16 (40)	0.5
Amikacin	10 (20)	12 (30)	0.3
Gentamicin	20 (40)	18 (45)	0.6
Trimethoprim/ sulfamethoxazole	22 (44)	16 (40)	0.1
Nitrofurantoin	38 (76)	34 (85)	0.4
Imipenem	12 (24)	13 (32.5)	0.4

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Table 3) Antibiotic susceptibility pattern of *Enterobacter* isolates according to the presence of class 2 integron

Antibiotic	Integron-2 positive n=12 No. (%)	Integron-2 negative n=78 No. (%)	p-value
Ampicillin	12 (100)	75 (96.2)	1.00
Amoxicillin-clavulanate	10 (83.3)	74 (94.9)	0.1
Cefoxitin	8 (66.7)	70 (89.7)	0.05
Ceftazidime	7 (58.3)	66 (84.6)	0.04
Ciprofloxacin	1 (8.3)	31 (39.7)	0.05
Amikacin	2 (16.7)	20 (25.6)	0.7
Gentamicin	4 (33.3)	34 (43.6)	0.5
Trimethoprim/ sulfamethoxazole	2 (16.7)	44 (56.4)	0.01
Nitrofurantoin	11 (91.7)	61 (78.2)	0.4
Imipenem	2 (16.7)	23 (29.5)	0.5

Table 4) Distribution of studied genes according to the studied *Enterobacter* spp.

Gene	*E. aerogenes* (n=41)	*E. cloacae* (n=27)	*E. gergoviae* (n=14)	*C. sakazakii* (n=8)
Int1	17 (41.5)	17 (63)	9 (64.3)	7 (87.5)
Int2	5 (12.2)	3 (11.1)	4 (28.6)	-
oxa-1	2 (4.9)	2 (7.4)	-	2 (25)
oxa-2	-	3 (11.1)	2 (14.3)	-
oxa-10	9 (22)	5 (18.5)	2 (14.3)	2 (25)
oxa-48	7 (17.1)	7 (25.9)	3 (21.4)	1 (12.5)

rate of antimicrobial resistance to ceftazidime (data not shown). Meanwhile, the sequencing results confirmed that the $bla_{\text{oxa-48}}$ –like positive isolates were $bla_{\text{oxa-48}}$ variants.

The prevalence of *intI1*, *intI2*, $bla_{\text{oxa-1}}$, $bla_{\text{oxa-2}}$, $bla_{\text{oxa-10}}$, and $bla_{\text{oxa-48}}$ genes was higher among *C. sakazakii, E. gergoviae, C. sakazakii, E. gergoviae, C. sakazakii,* and *E. cloacae* species, respectively.

Discussion

In the current study, we determined the pattern of antimicrobial resistance and the presence of integrase and oxacillinase genes among 90 *Enterobacter* clinical isolates obtained from a tertiary care hospital. *E. cloacae* and *E. aerogenes* have been reported to be the most prevalent clinical isolates of *Enterobacter* [8]. Consistent with the literature, *E. aerogenes* (45.6%) and *E. cloacae* (30%) were also the most common species among the isolates evaluated in this study. Conversely, *E. gergoviae* (54.2%) was previously reported in a study to be the most prevalent species in Iran [16]. In the present study, most of the isolates (37.8%) were recovered from respiratory tract samples. In line with this study result, in two studies conducted in China and Germany, 91 and 37.8% of the isolates were obtained from RTIs, respectively [10, 26]. In contrast, in several investigations, blood and abdominal samples have been reported to be the most common sites of *Enterobacter* isolation [8, 27, 28]. In this study, 64.4% of the isolates were recovered from the ICU ward. Likewise, some studies nationwide have shared similar findings [26, 29]. This issue indicates the importance of long-term hospitalization in acquiring these infections. In antimicrobial susceptibility testing, 93% of the isolates showed MDR phenotype, making them a major therapeutic threat in this area. Nearly 28%

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of the isolates were non-susceptible to imipenem (carbapenem resistant). This result is consistent with the results of two previous studies conducted in Iran (29.2%) and China (25.7%) [16,30]. Moreover, among the carbapenem-resistant isolates, 96% were phenotypically carbapenemase producers. The mCIM test is a reliable and simple method with a sensitivity of 98-100% compared to the modified Hodge test and the Carba NP test [12,31-33]. The main mechanism of resistance to carbapenems among *Enterobacter* isolates is carbapenemase production, mainly OXA-type carbapenemases, including bla_{oxa-1}, bla_{oxa-2}, bla_{oxa-10}, and bla_{oxa-48}-like genes, as well as metallo-beta-lactamases with a lower prevalence [22]. In the current study, the presence of different bla_{oxa} genes was detected. The prevalence of oxacillinase genes in *Enterobacter* spp. is lower than in other *Enterobacteriaceae* members, and their prevalence varies across the world. The presence of bla_{oxa-48} gene along with other oxacillinase genes such as bla_{oxa-10}-like genes has not been reported so far among *Enterobacter* isolates in this region. To the best of our knowledge, this is the first report about the presence of *Enterobacter* spp. harboring bla_{oxa-10} (20%) and bla_{oxa-48}-like genes (20%) in Iran.

In three studies conducted in Russia, Turkey, and Germany, the prevalence of bla_{oxa-48} among *Enterobacter* isolates has been reported to be 20, 34.8, and 10.7%, respectively [34-36]. In this study, 6.7 and 5.6% of the isolates harbored bla_{oxa-1} and bla_{oxa-2}-like genes, respectively. These results are slightly lower than the results obtained by Ramezanzadeh et al. (2016), showing a prevalence of 7.7 and 11.8% for bla_{oxa-1} and bla_{oxa-2}, respectively [37]. In the present study, only 16.6% of mCIM-positive isolates carried the bla_{oxa-48}-like gene, indicating that other mechanisms such as metallo-beta-lactamases or the *OmpK* gene are also involved in carbapenem resistance. These discrepancies in phenotypic and genotypic results have also been reported by other researchers [13,33,38,39].

Moreover, the presence of class 1, 2, and 3 integrons was detected among the isolates. It has been reported that class 1 integron is found in 40-70% of Gram-negative pathogens and plays a major role in antibiotic resistance [14,15]. Consistent with the literature, the prevalence rate of *intI1* was the highest (55.6%) among the isolates, followed by *intI2* (13.3%); also, 8.9% of the isolates were positive for the simultaneous presence of both classes of integron genes. However, a significant relationship was revealed between the presence of the *intI2* gene and higher rates of drug resistance against only two antibiotics (Table 3). In line with these results, Mortazavi et al. (2018) showed that 58.3% of *E. cloacae* isolates harbored class I integron; however, none of them had class II integron [40]. In the present research, the *intI3* gene was not detected in any of the isolates. It has been reported that the distribution of class 3 integron is limited to a few Gram-negative bacteria (except *Enterobacter* spp.) and varies from 0-10%, this result confirms the present study findings [15].

This investigation had several limitations. First, the sample size was relatively small. Second, due to the lack of temocillin disk (30 µg), it was not possible to phenotypically identify OXA-48-producing isolates. Third, we could not evaluate the presence of other OXA-type carbapenemase genes to better assess oxacillinase resistance genes among the isolates.

Conclusion

In summary, OXA-10 and OXA-48-type carbapenemases were detected in 20% of carbapenem-resistant *Enterobacter* isolates. Dissemination of antibiotic-resistant isolates co-producing oxacillinase and integrase genes may become an important therapeutic challenge in the future. Finally, the identification and prevalence of different integron classes and oxacillinase types among Gram-negative rods involved in nosocomial infections require further investigations.

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Ethical permissions: This study was conducted in accordance with the institutional Ethics Committee of Shiraz University of Medical Sciences (approval number: IR.SUMS.REC.1397.816). However, since only leftovers of clinical specimens were used in this study, the local ethics committee waived the need for informed consent.

Conflicts of iinterests: The authors declare that they have no competing interest.

Authors' contributions: Conceptualization: RK; Data Curation: MM, YM, RK; formal analysis: MM, YM, Funding acquisition: RK; Investigation: MM, RK; Methodology: MM, YM; Project administration: RK; Resources: RK; Software: MM; Supervision: RK, JS; Writing of the original draft: MM, RK; Writing—review and editing: RK. All authors read and approved the final manuscript.

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