Emergence of OXA-10 and OXA-48 like carbapenemases among Enterobacter isolates from inpatients in southern Iran

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Research

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Abstract

Background

The global spread of carbapenemase-producing Enterobacteriaceae represents a public health concern. The aim of this study was to investigate prevalence of carbapenem resistance, oxacillinase types and the presence of class 1–3 integrons among *Enterobacter* clinical isolates from an Iranian inpatients' population.

Methods

Ninety *Enterobacter* isolates recovered from hospitalized patients were diagnosed by the standard microbiological methods. Antibiogram pattern was also determined. The presence of class 1–3 integrons and four types of oxacillinase genes were assessed using PCR.

Results

Of the 90 *Enterobacter* isolates, the most common species was identified as *E. aerogenes*, (45.6%), followed by *E. cloacae* (30%). The highest resistance rate was against to ampicillin (96.7%). Multi-drug 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

The determinants of class 1 integron with OXA-10 and OXA-48 like carbapememases have been responsible of relatively considerable of carbapenem resistance among isolates. This is the first OXA-10 and OXA-48-producing *Enterobacter* spp. in Iran, indicating that the prevalence of oxacillinases might be on the rise in country.

Background

Enterobacter as an important Gram-negative rod among Enterobacteriaceae members is responsible for wide range of nosocomial infections, in particular bloodstream (BSI), respiratory tract (RTI) and urinary tract infections (UTI) [1, 2]. The emergence of nosocomial infections due to *Enterobacter* spp. are associated with a remarkable of morbidity and mortality, especially among hospitalized patients at intensive care units (ICUs) [3, 4].

Betalactams, especially thirdgeneration cephalosporins and carbapenems are frequently used to treat infections caused by *Enterobacter* spp.[5, 6]. However, *Enterobacter cloacae* and *Enterobacter aerogenes* as the most frequently isolated species are intrinsically resistant to many drugs [7]. Moreover, acquisition of multi-drug resistance (MDR) among *Enterobacter* spp. is increasing, so that has severely been compromised the available therapies [8]. Nosocomial infections caused by carbapenem-resistant Enterobacteriaceae (CRE) are considered a challenge to patients, physicians, and public health, and this subject is due to capability of their widespread 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 or OXA-type carbapenemases as plasmid-encoded β-lactamases, more recently, have transmitted into the Enterobacteriaceae and are becoming a considerable cause of carbapenem resistance[9, 11]. They are active on extended-spectrum cephalosporins, monobactams (ESBL), and the carbapenems [12]. There are different types of carbapenem hydrolyzing OXA-type carbapenemases, including OXA-48 and its variants that are widespread amongst Enterobacteriaceae members and accounted one of the most concerning developments in CRE isolates in the last decade [11]. It is suggested that among different carbapenemases, OXA-48 is the most predominant in Mediterranean and Middle East countries [13]. Furthermore, integrons as one of the mobile genetic elements have a major role in wide spread of antibiotic resistance among bacteria. To date, four general classes of integrons, namely classes 1–4 have been introduced, in which classes 1–3 integrons are capable of acquiring gene cassettes through site-specific recombination and regarded as the frequent classes involved in therapeutic failure among Gram-negative rods[14, 15]. There are few reports of carbapenemase-producing *Enterobacter* spp. from Iran. The present study aimed to investigate some OXA-type carbapenemase genes and the presence of class 1–3 integrons among clinical isolates of *Enterobacter* from inpatients in southern Iran.

Methods

Clinical isolates

The study was carried out between August 2018 and April 2019 using 90 non-duplicated *Enterobacter* isolates obtained from hospitalized patients at Shiraz Namazi Teaching Hospital, southern Iran. Only one isolate was collected from per patient. Of these 90 isolates, 40 were belonged to a previous study [16]. The isolates were recovered from different clinical samples, including blood, wound, endotracheal tube aspirates, abdominal discharge, urine, and eye. Primary identification of *Enterobacter* isolates was performed by standard microbiological tests and confirmed in species level by Microgene™ GnA + B-ID system (Microgen Bioproducts Ltd, UK) diagnostic kit (Mast, UK) according to the manufacturer 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 following the declaration of Helsinki and approved by the Ethics Committee of Shiraz University of Medical Sciences (approval number IR.SUMS.REC.1397.816).

Antimicrobial susceptibility testing

Antibiotic susceptibility of all isolates to amikacin, ampicillin, amoxicillin/clavulanate, imipenem, ciprofloxacin, nitrofurantoin, trimethoprim-sulfamethoxazole (SXT), ceftazidime, cefoxitin and gentamicin (Mast Co., UK) was performed on Muller-Hinton agar plates (Merck Co., Germany) using the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [17]. *E. coli* ATCC 25922 was used as the quality 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 and interpreted by the modified carbapenem inactivation method (mCIM) in accordance with CLSI recommendations [17]. Briefly, suspension of bacteria was suspended in 2 mL of TSB, and a 10 µg meropenem disk (Mast Co., UK) was added prior to incubation at 35°C for 4 h. Meropenem disk was then transferred onto on Muller-Hinton agar, inoculated with a 0.5 McFarland suspension of *Escherichia coli* ATCC 25922, and incubated at 35°C for 18 to 24 h. The presence of carbapenemase activity was recognized by an inhibition zone ≤ 15 mm in diameter or presence of pinpoint colonies within a 16–18 mm zone, and absence of carbapenemase activity was revealed by zones ≥ 19 mm (clear zone) in diameter [19].

Class 1, 2 and 3 integrons and oxacillinase resistance genes assay

Genomic DNA of *Enterobacter* spp. was extracted by the simple boiling method as previously described [20]. The presence of potential resistance genes encoding class 1, 2 and 3 integrons and oxacillinase, including *intI1*, *intI2*, *intI3* integrases and $bla_{\text{oxa-1}}$, $bla_{\text{oxa-2}}$, $bla_{\text{oxa-10}}$ and $bla_{\text{oxa-48}}$ were screened by PCR amplification using specific previously reported primers [21–25]. PCR reactions were carried out using a thermal cycler 5530 (Ependrof master, Germany) with 2 µL of each specific primer (1 µM), 1 µL DNA template (100 ng), and 12.5 µL Premix PCR buffer (Amplicon, Denmark) (1X), in a total volume of 25 µL. PCRs consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, primer annealing at 55°C for *intI1* and 58°C for *intI2* and *intI3* and extension step at 72°C for 60 sec, and a final extension at 72°C for 7 min. For the $bla_{\text{oxa-1}}$, $bla_{\text{oxa-2}}$, $bla_{\text{oxa-10}}$ and $bla_{\text{oxa-48}}$ genes, annealing temperature were chosen at 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 gel documentation system. The amplicons of $bla_{\text{oxa-48}}$-like gene-producing isolates were submitted for sequencing (Bioneer Co., Munpyeongseoro, Daedeok-gu, Daejeon, South Korea) and 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

The analysis was done using SPSS™ software, version 21.0 (IBM Corp., USA). Chi-square or Fisher's exact tests were used and differences were considered statistically significant when the p-value was less than

0.05.

Results

Study population and clinical characteristics of Enterobacter isolates

Our isolates were recovered from 90 individuals admitted as inpatients, consisting of 25 (27.8%) female and 65 (72.2%) males with a median age of 33 years (range = 8 days to 76 years). Among *Enterobacter* isolates, 58 (64.4%) isolates were obtained from the intensive care units (ICUs), 24 (26.7%) from the internal units and 8 (8.9%) from the surgery units. The isolates were obtained 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%).

3.2. Antimicrobial resistance of Enterobacter spp.

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

Table 1
Antibiotic resistance pattern of *Enterobacter* spp.

Antibiotic	*E. aerogenes* (total = 41)	*E. cloacae* (total = 27)	*E. gergoviae* (total = 14)	*C. sakazakii* (total = 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)

Characterization of integrase and oxacillinase genes

PCR analysis of the three classes of integron genes showed that 50 (55.6%) and 12 (13.3%) isolates were carried *intI1* and *intI2* genes, respectively. Class 3 integron (*intI3*) was not found in any of the isolates. The antimicrobial resistance patterns of classes 1 and 2 integron-positive and negative isolates are presented in Tables 2 and 3, correspondingly. A statistically significant difference was determined between the presence of class 2 integron and higher rate of antimicrobial resistance related to ceftazidime and trimethoprim-sulfamethoxazole (Table 3).

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

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

Moreover, 18 (20%) isolates were harbored both bla_{oxa-10} and bla_{oxa-48} -like genes with different distributions among *Enterobacter* spp., and 6 (6.7%) and 5 (5.6%) ones were positive for the bla_{oxa-1}, and bla_{oxa-2} genes, respectively (Table 4). On the other hand, only 4 (16.6%) mCIM positive isolates were carried bla_{oxa-48} -like gene and other oxacillinase genes were not detected among these isolates. A statistically significant difference was diagnosed between the presence of bla_{oxa-10} and bla_{oxa-2} genes with higher rate of antimicrobial resistance related to ceftazidime (data not shown). Meanwhile, sequencing results confirmed that bla_{oxa-48} –like positive isolates were bla_{oxa-48} variant.

Table 4
Distribution of studied genes according to *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)

The frequency of *intI1*, *intI2* and bla_{oxa-1}, bla_{oxa-2}, bla_{oxa-10} and bla_{oxa-48} genes were more prevalent among *C. sakazakii, E. gergoviae, C. sakazakii, E. gergoviae, C. sakazakii*, and *E. cloacae*, respectively.

Discussion

An increase in the emergence of CRE, including *Enterobacter* spp. leaving limited treatment options, therefore are related to high morbidity and mortality [3, 4, 8]. In the current study, we determined antimicrobial resistance pattern and the presence of integrase and oxacillinase genes among 90 *Enterobacter* clinical isolates obtained from a tertiary care hospital from south of Iran. According literature, *E. cloacae* and *E. aerogenes* are the most prevalent clinical isolates of *Enterobacter* [8]. In consistent with literature, in our survey *E. aerogenes* and *E. cloacae* were also the most common species with frequencies 45.6 and 30%, respectively. On the contrary, in the study of Khashei *et al*., *E. gergoviae* (54.2%) was found the most frequently isolated species [16]. *Enterobacter* isolates have been the leading cause of nosocomial infections all over the world [1, 2]. In our work, most of isolates (37.8%) were recovered from respiratory tract samples. In agreement with our survey, in two study from China and Germany 91 and 37.8% of isolates were obtained from RTIs, respectively [10, 26]. In contrast, in several investigations blood and abdominal samples were the most frequent sites of *Enterobacter* isolation [8, 27, 28]. In this study, 64.4% of isolates were recovered from ICU ward. Likewise, some studies nationwide shared similar findings [26, 29]. This issue indicates the importance of long-term hospitalization for acquisition of these infections.

In susceptibility testing, all isolates were resistant to all of the tested antimicrobials, with 93% of strains showing MDR phenotype, making them a major therapeutic treat in our area. Based on CLSI, resistance to one or more carbapenems is considered as carbapenem resistance [27]. Hence, 27.8% our isolates were non-susceptible to imipenem (carbapenem resistant). The result is coinciding with two previously reported works from Iran and China with prevalence's of 29.2 and 25.7%, respectively [16, 30]. Moreover, among carbapenem-resistant isolates, 96% were phenotypically carbapenemase producers by mCIM test.

The mCIM is a reliable and simple method and has a sensitivity of 98–100% in comparison with the modified Hodge test and the carbapenemase Nordmann-Poirel (carba NP) test with sensitivities of 93–98% and 73–100%, respectively. Furthermore, due to being expensive of PCR, mCIM could be a suitable alternative method for detecting of carbapenemase producers [12, 31–33].

In addition to rapid phenotypic tests, genotypic identification of carbapenemases among Gram-negative rods is an important step for infection control and prevention. The major 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 and metallobetalactamases with a less prevalence [22]. In the current work, the presence of bla_{oxa-1}, bla_{oxa-2}, bla_{oxa-10} and bla_{oxa-48} -like genes were diagnosed with frequencies 6.7, 5.6, 20, and 20%, respectively. The presence of oxacillinase genes in *Enterobacter* spp. are less than other Enterobacteriaceae members and their prevalence varies across the world. The bla_{oxa-48} first time identified among *Klebsiella pneumoniae* isolates in 2004 from Turkey. Afterward, the gene reported in some other Enterobacterales, including *E. cloacae* in Middle East, Africa and Europe [34, 35]. But this gene along with other oxacillinase genes such as bla_{oxa-10}-like has not been reported among *Enterobacter* isolates in our region, yet. To the best of our knowledge, this will be the first reporting on *Enterobacter* spp. harboring bla_{oxa-10} and bla_{oxa-48}-like genes from Iran.

In three studies conducted in Russia, Turkey and Germany, the prevalence of bla_{oxa-48} among *Enterobacter* isolates was reported as 20, 34.8 and 10.7%, respectively [36–38]. In addition, our results revealed that 6.7% and 5.6% of isolates were positive for bla_{oxa-1} and bla_{oxa-2}-like genes, correspondingly. The result was less slightly than those observed by Ramezanzadeh and colleague with prevalence's of 7.7% (bla_{oxa-1}) and 11.8% (bla_{oxa-2}) [39]. In this survey, we found only 16.6% of mCIM positive isolates were carried bla_{oxa-48} -like gene, indicating other mechanisms such as metallobetalactamases or *OmpK* gene that regulates expression of mutated efflux pump are involved in carbapenem resistance. These discrepancies on phenotypic and genotypic results are also cited by some authors [13, 33, 40–42].

On the other hand, we identified the presence of class 1, 2 and 3 integrons among our isolates. Carbapenemase genes are harbored on mobile genetic elements and can be disseminated to other bacteria, therefore contributing to the origin of antimicrobial resistance among Enterobacteriaceae members [32]. It is reported that class 1 integron is found in 40–70% of Gram-negative pathogens and has had a major role in widespread of antibiotic resistance [14, 15]. In consistent with literature, *intI1* was found in the highest rate (55.6%), followed by *intI2* (13.3%) and 8.9% of isolates were positive for both classes of integron genes, simultaneously. However, a significant difference was determined between isolates harboring *intI2* gene and higher rate of drug resistance with only two antibiotics (Table 3). In accordance to our results, Mortazavi *et al.* showed that 58.3% of *E. cloacae* isolates, harbored class I integron; however, none of them had class II integron [43]. In the present work, the *intI3* gene was not detected in any of the isolates. It is mentioned that distribution of class 3 integron has been limited within

a few Gram-negative bacteria, with the exception of *Enterobacter* spp. ranged from 0–10%, which is according to our findings [15].

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

Conclusion

In the current study, OXA-10 and OXA-48-type carbapenemases were detected in 20% of carbapenem resistant *Enterobacter* isolates, indicating that these oxacillinase genes are probably the most common mode of resistance to carbapenems in our geographical region. Dissemination of antibiotic resistant isolates co-producing an oxacillinase and integrases increases clinical concern and may become an important therapeutic challenge in the future. Finally, identification and prevalence of different classes of integron among Gram-negative rods involved in nosocomial infections, as well as different types of oxacillinase genes require further investigation.

Declarations

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Authors' contributions

RK designed the study, MM and YM conducted the experiments, MM and YM analyzed the data, RK and MM wrote the manuscript which was corrected by RK, RK and JS were supervisors of the study. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was in accordance with the declaration of Helsinki and an ethical permission was sought from the institutional Ethics Committee of Shiraz University of Medical Sciences (Approval No. IR.SUMS.REC. 1397.816). As we only used leftovers from clinical specimens, the institutional ethics committee waived the need for informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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