Original Article

The Frequency of *bla*$_{OXA-48}$ and *bla*$_{NDM-1}$ Genes in the Enterobacteriaceae Strains Isolated from Clinical Samples

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Abstract

Carbapenems are β-lactam antibiotics used against enterobacterial isolates; the effectiveness of them has become seriously controversial. The most common genes encoding Carbapenemase include *bla*NDM-1 and *bla*OXA-48, which are transmitted through plasmids such as horizontal gene transfer. This study was conducted to trace these two genes in Enterobacteriaceae strains isolated from clinical samples in the southeast of Iran. A total of 200 Gram negative isolates were collected from hospitals in Zahedan, Iran, over one year. The minimum inhibitory concentration for imipenem and meropenem was assessed by using an Epsilometer (E-Test). The presence of a carbapenemase enzyme was also investigated using the Modified Hodge Test (MHT). The *bla*OXA-48 and *bla*NDM-1 genes were detected by PCR. Among the 200 isolates recovered, 60 carbapenem-resistant strains were identified. MHT also turned out positive for 31 of the samples. The *bla*NDM-1 gene was traced in 25 isolates. The *bla*OXA-48 gene was present in 20 strains, and *bla*OXA-48 and blaNDM-1 were simultaneously present in 15 isolates. Given the relatively high frequency of the studied resistant genes in this region of Iran, screening measures are mandatory for preventing the spread of these isolates to other parts of the country.

Keywords: Carbapenems, Enterobacteriaceae, *bla* $_{NDM-1}$, *bla* $_{OXA-48}$

Introduction

Antibiotic resistance in bacteria has the potential to lead to the black periods of history, before the emergence of modern medicine. This claim is supported by reports of superbug bacteria that are resistant to common antibiotics [1]. Except for the *Acinetobacter* and *Staphylococci* species, superbug bacteria belong mainly to the Enterobacteriaceae family. The members of this family are natural flora of clones and are the most important causes of hospital infections as well as severe bacterial infections among people. Since the colon is a suitable environment for the Horizontal Gene Transfer (HGT) of resistant genes, antibiotic-resistant strains, especially carbapenem-resistant ones are spreading [2]. Carbapenems including imipenem and meropenem are often proposed as "last-line agents" for the effective treatment of infections caused by large-scale cephalosporins-resistant Enterobacteriaceae. Carbapenems prevent cell wall synthesis and affect the efflux pump as well [3].

Although there are various mechanisms at play in Enterobacteriaceae resistance to carbapenems, the production of the enzymes hydrolyzing carbapenems has been regarded as an important mechanism of resistance among Enterobacteriaceae over the past decade and the impact of this last line of antibiotic treatment has become seriously controversial [4]. The ever-increasing number of carbapenemases in bacteria results in many concerns because the isolates that synthesize carbapenemases have resistance to a majority of antibiotics, such as cephalosporins, aminoglycosides, quinolones, sulfamethoxazole-trimethoprim, etc. [5].

According to Ambler classification, carbapenemases are classified in three classes in terms of their amino acid sequences, namely classes A, B, and D. Class B consists of metallocarbapenemase, such as VIM-1 and IMP-1. Class D contains oxacillinases such as OXA-48, while class A contains penicillinases and *Klebsiella pneumoniae* Carbapenemase [2, 6].

Recently, a new enzyme has been identified in Class B of Enterobacteriaceae called NDM-1. The *bla*NDM-1 gene which encodes this enzyme is found on plasmids or transposons [7]. These elements are transferred from one organism to another using processes such as conjugation or gene transduction. The probability of the global spread of this enzyme is very high [8].

NDM-1 and OXA-48 enzymes are found abundantly in *Enterobacteriaceae*, and their genes are located on plasmids that can be transferred among Enterobacteriaceae species. The size of these plasmids is about 52.5 kb [9-12]. OXA-48 is a carbapenemase that is encoded by the *bla*OXA-48 gene. It was first detected in *Klebsiella pneumoniae* in Turkey and then in France and Belgium. The enzyme has been identified

in Enterobacteriaceae isolates of non-European countries including Lebanon, Tunisia, Israel, India, and Iran [13]. In addition to *Klebsiella pneumoniae*, OXA-48 has also been identified in *Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii*, and *Proteus rettgeri* strains [4].

Moreover, due to the high prevalence of Enterobacteriaceae strains producing carbapenemase in India and Pakistan [5] and the proximity of Iran to these countries, the present study was conducted to evaluate the frequency of these two genes in Enterobacteriaceae isolates from clinical samples in southeast Iran.

Materials and Methods

Bacterial Strains

Over one year from May 2016 to 2017, clinical specimens were collected from hospitalized patients in Zahedan. The samples were first inoculated into MacConkey agar and Eosin Methylene Blue agar. The identification of isolates was carried out by using the API20E system (BioMerieux, Marcy-1, Etoile). The bacterial strains were stored in *Trypticase Soy Agar* (TSA) with glycerol 20% at -70 °C for the subsequent steps.

Antibiotic Susceptibility

The carbapenem resistance of the isolates was assayed by disc diffusion as per the CLSI using Imipenem (10 µg) and Meropenem (10 µg) discs (Mast Diagnostics, Derby Road, Bootle, UK); [14]. The Minimum Inhibitory Concentration (MIC) was determined for antibiotics including Imipenem, Meropenem, and Colistin using an Epsilometer (E-test) with MBL strips (Rosacea Degli, Abruzzo, Italy) and the isolate susceptibility was identified by the CLSI 2017 guidelines [15]. For detection of metallo-β-lactamase phenotypes in the isolates, Etest MBL strips (Rosacea degli, Abruzzo, Italy) was used.

Modified Hodge Test (MHT)

The phenotypic detection of beta-lactamase production in resistant isolates was performed by MHT based on the CLSI recommendations and quality control guidelines. A 24-hour culture of the *E. coli* strain *ATCC 25922* was prepared, equal to 0.5 McFarland, diluted at 1:10 and cultured on a plate surface using a cotton swab. The 10-µg Ertapenem disc (Mast Diagnostics, Derby Road, Bootle, UK) was placed in the center of the plate. A perpendicular line was then drawn from the edge of the antibiotic disc to the border of the plate from a 16-hour culture of the organism using a loop. After 24 hours incubation, the plates were evaluated for the presence of shoal leaf niches at the intersection of the sample and the standard specimen [16].

Genotypic Assay of β-Lactamases

The molecular detection of *bla*OXA-48 and *bla*NDM-1 genes was performed in the isolates using PCR. The bacterial DNA was extracted using a special Kit (Sinaclon Co., Iran) as well as two primers for *bla*OXA-48 and *bla*NDM-1 carbapenemase genes [17, 18]. The PCR products were sent to Macrogen Co. for sequencing, and the results of the sequencing analysis were then blasted using Chromas software on the NCBI website.

Results

A total of 200 isolates were collected from clinical samples in Zahedan. Out of these 200 isolates, 120 were *E. coli* (60%), 67 were *Klebsiella pneumoniae* (33.5%), five *Proteus mirabilis* (2.5%), three *Citrobacter* spp. (1.5%), and five were *Pseudomonas aeruginosa* (2.5%).

Antibiotic Resistance Profile

A total of 60 isolates (30%) were resistant to imipenem and meropenem as per the disc diffusion findings. Overall, 66% of the *E. coli* isolates, 22% of the *K. pneumoniae*, 6 % of the *P. mirabilis*, and 6% of the *P. aeruginosa* were resistant to carbapenems. All of them were sensitive to colistin.

To evaluate the production of carbapenemase, the MHT was performed on 60 isolates resistant to imipenem and meropenem. The results turned out positive for 31 (51.6%) isolates out of the 60 resistant to imipenem and meropenem. Figure 1 illustrates the percentages of Modified Hodge Test (MHT)-positive isolates.

The MIC value was more than 16 µg/ml for all the Meropenem-resistant isolates. In the phenotypic assessment of NDM-1 production, 5% of the isolates were found to contain Metallo-β-lactamase.

DNA PCR Amplification

Sixty carbapenem-resistant isolates were identified by the MHT. The *bla*NDM-1 gene was detected in 25 (41.6%) of these 60 isolates. The *b*laOXA-48 gene was traced in 20 isolates (33.3%). The simultaneous presence of *bla*OXA-48 and *bla*NDM-1 was detected in 15 isolates (25%). Table 1 shows the frequency of OXA-48- and NDM-1-producing isolates as per the phenotypic and molecular tests.

Discussion

The transfer of *bla*NDM-1- and *bla*OXA-48- encoding plasmids occurs between different species of bacteria by Horizontal Gene Transfer (HGT). The rapid diagnosis of bacteria harboring the plasmids is, therefore, essential [19, 20].

In this research, of the 200 Enterobacteriaceae strains, 60 were carbapenem-resistant. The MHT results were positive for 31 samples (51.6%). The *bla*NDM-1 gene was present in 25 isolates (41.6%). The prevalence of the *bla*NDM-1 gene was higher in the present research setting compared to other regions of Iran. In a study by Shahcheraghi et al. in 2012 on clinical samples in Tehran, of the 11 carbapenem-resistant isolates, only one contained *bla*NDM-1 [20]. In the study by Fazeli in 2015 on clinical isolates in Isfahan, the frequency of strains with *bla*NDM-1 was 12.2% [21]. Afrugh et al. in 2016

in Ahvaz, of the 708 gram-negative isolates, did not detect the *bla*NDM-1 gene [22]. Due to the proximity of the southeast of Iran to Pakistan and the high prevalence of these strains in that country, the frequency obtained was expected compared to the rest of the country [23]. The results of the present study are consistent with some studies in other parts of the world [5, 24]. In research by Lascols et al., the frequency of NDM-1 β-lactamase was investigated in India and carbapenemase genes were identified in 66 isolates. The results showed the presence of *bla*NDM-1 in 33 isolates (50%) and blaOXA-48 in three isolates (4.5%) [5]. Radha et al. in 2015, evaluated the prevalence of NDM-1 and OXA-48 producing Enterobacteriaceae in Indian hospitals, and from a total of 425 samples of Meropenem-resistant Enterobacteriaceae, 264 strains (62%) were found to contain the *bla*NDM-1 gene [25].

In this research, the prevalence of Enterobacteriaceae strains with *bla*OXA-48 was 33.3%. In the study conducted by Azimi et al., carbapenemase *bla*OXA-48 was found in 27 out of the 28 *Klebsiella pneumoniae* isolates, and it was the first to report the presence of this gene in Iran, but *bla*NDM-1 gene was reported in none of the 28 isolates [13]. In a study by Pfeifer, of the nine isolates of Enterobacteriaceae resistant to carbapenem, six produced carbapenemase OXA-48 [12]. In a study by Radha et al., of the 425 samples of meropenem-resistant Enterobacteriaceae, *bla*OXA-48 isolates were reported in 4% [25].

In the present study, *bla*OXA-48 and *bla*NDM-1 coexisted in 15 isolates (25%). The simultaneous presence of these two genes in Enterobacteriaceae was higher in the present study than in previous reports [5]. Since these two genes are carried by plasmids that are easily transmitted to other bacteria through HGT, and since plasmid can also contain other resistant genes to antibiotics, the rapid screening of bacteria producing NDM-1 and OXA-48 seems essential.

A significant difference was observed in this research between the phenotypic methods of carbapenemase production using the E-test and the genotypic methods. Only 5% of the isolates had MBL enzymes as per the phenotypic E-test. Nonetheless, 50% of them contained NDM-1 and 33.3% contained OXA-48 as per the results of molecular methods.

Based on the present findings and the results of some similar studies, phenotypic methods for identifying β-lactamase, such as the E-test and MHT, cannot be well tolerated, because not only they can increase the likelihood of negative false results, but they also lead to delays in the effectuation of infection control measures and facilitate the outbreak of these strains. Further studies are needed to develop preventive strategies for controlling the release of isolates containing NDM-1 [26].

To the researchers' knowledge, the present study is the first to screen for NDM-1 and OXA-48 genes in Enterobacteriaceae strains in Iran (except for *Klebsiella pneumoniae*) and given the limited therapeutic options available, these findings are a serious warning about the possibility of the rapid spread of bacteria carrying these genes.

Conclusion

Because of the overuse of antibiotics in Iran, there is an increasing spread of extended-spectrum β-lactamase (ESBL)-producing isolates among Enterobacteriaceae. These isolates were identified in this study using phenotypic and genotypic methods. Nonetheless, more precise methods should be designed for the rapid identification of these isolates, and therapeutic alternatives for preventing their spread may also be essential.

A precise understanding of the public health threats caused by the bacteria carrying blaOXA-48 and blaNDM-1 is only possible by studies conducted to identify these strains in different geographical regions.

Funding Disclosure

No funding sources.

Conflicts of Interest Disclosure

No conflicts of interest.

Acknowledgments

No Acknowledgments.

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Table 1. Frequency of OXA-48- and NDM-1-producing isolates as per the phenotypic and molecular tests.

Isolate No.	Isolates	Sex	Sample	MIC (µg/ml)	MHT	E-test (MBL)	*NDM-1*	***OXA-48***
9a	*E.Coli*	F	Urine	>256	+	+	+	+
42a	*E.Coli*	M	Urine	>256	+	+	+	+
30a	*E.Coli*	F	Ulcer	>256	+	+	+	-
40a	*E.Coli*	F	Blood	>256	+	+	-	+
46a	*K.pneumoniae*	F	Urine	>256	+	+	+	+
42c	*P. mirabilis*	F	Urine	>256	+	+	+	+
68a	*E.Coli*	M	Urine	>256	+	+	+	-
39b	*E.Coli*	F	Urine	>256	+	+	+	+
43b	*E.Coli*	M	Blood	>256	+	+	-	+
54a	*K.pneumoniae*	F	Urine	>256	+	+	+	+
43c	*P. mirabilis*	F	Urine	>256	+	+	-	+
17c	*P.aeruginosa*	M	Urine	>256	+	-	-	+
47c	*P. mirabilis*	M	Urine	>256	+	+	+	+
67a	*E.Coli*	F	Urine	>256	+	+	+	+
19a	*E.Coli*	F	Urine	>256	+	+	+	-
52a	*E.Coli*	F	Urine	>256	+	+	+	-
12a	*E.Coli*	F	Urine	>256	+	+	+	+
51a	*E.Coli*	F	Urine	>256	+	+	+	-
62a	*E.Coli*	F	Urine	>256	+	+	+	+
35a	*E.Coli*	M	Urine	>256	+	+	+	-
47a	*E.Coli*	M	Urine	>256	+	+	+	-
45c	*E.Coli*	M	Blood	>256	+	+	+	+
46c	*P. mirabilis*	F	Blood	>256	+	+	+	-
53b	*E.Coli*	M	Urine	>256	+	+	-	+
43a	*E.Coli*	F	Urine	>256	+	+	+	+
36a	*E.Coli*	M	Urine	>256	+	+	+	+
38a	*E.Coli*	F	Urine	>256	+	+	+	-
23a	*E.Coli*	M	Ulcer	>256	+	+	+	+
26a	*E.Coli*	F	Urine	>256	+	+	+	+
55a	*K.pneumoniae*	M	Urine	>256	+	+	+	-
29a	*E.Coli*	F	Urine	>256	+	+	-	+

MHT: Modified Hodge Test; MBL: Metallo-β- lactamases.

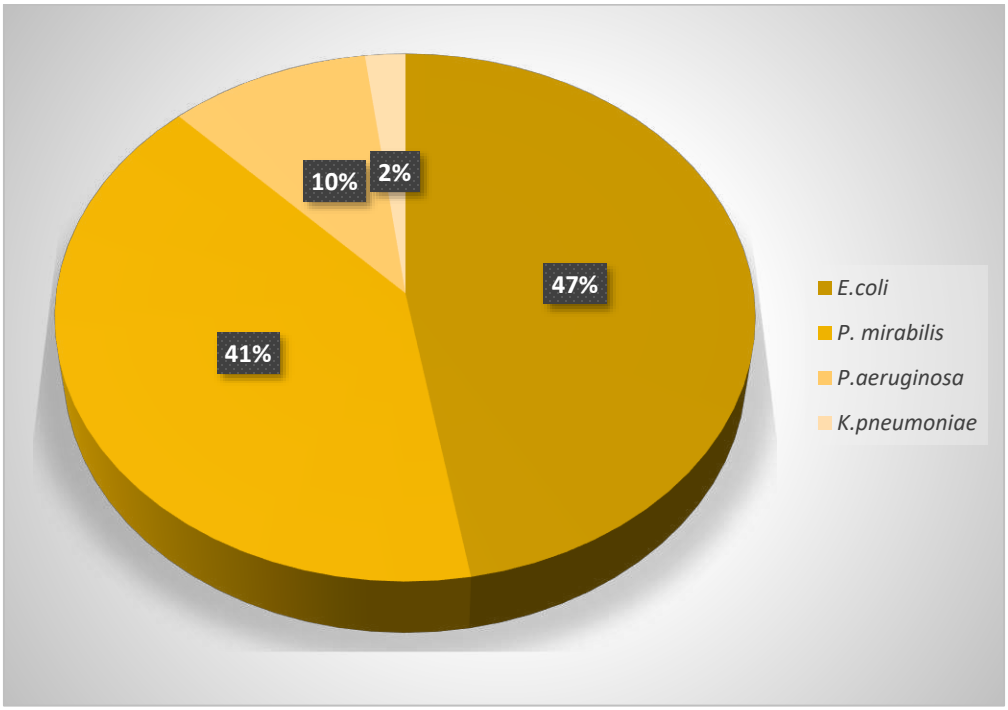

Figure 1: The percentages of isolates were positive for carbapenemase production by Modified Hodge test.