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First prevalence of metallo beta-lactamases producing *Enterobacteriacea* in Iranian cancer patients

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Key words: *Enterobacteriaceae, Metallo-beta-lactamases, Carbapenemases, Antibiotic resistance, Cancer patients*

Parole chiave: *Enterobacteriaceae, metallo-beta-lattamasi, Carbapenemasi, resistenza antibiotica, pazienti oncologici*

Abstract

Background. *Hospital-associated infections, recently renamed Healthcare-associated infections, are among the most common life-threatening complications of hospitalized patients, especially the immunocompromised patients. Regarding the significant role of Enterobacteriaceae in nosocomial infections and also the increasing trends of carbapenem-resistant strains, the present study aimed to evaluate the antibiotic resistance pattern and the occurrence of metallo-beta-lactamases (MBLs) in Enterobacteriaceae strains from Iranian cancer patients.*

Methods. *This hospital-based cross-sectional study was conducted in teaching hospitals of two cities in the central parts of Iran during the 6 months period from December 2015 to May 2016. The Enterobacteriaceae isolates were obtained from different clinical specimens and were identified using standard microbiological methods. Antimicrobial susceptibility pattern for the bacterial isolates was determined using the disk diffusion method. The presence of antibiotic resistance genes was determined by PCR method.*

Results. *The distribution of Enterobacteriaceae isolates were 74 (71.8%) E. coli, 23 (22.3%) Klebsiella spp., 3 (2.9%) Proteus spp., 2 (1.9%) Salmonella spp., and 1 (1%) Shigella spp. The results of antibiotic susceptibility revealed that all of the isolates were multiple-drug resistant (MDR) and 60% of them were (excluded Salmonella and Shigella) carbapenem-resistant. Of all the carbapenem-resistant isolates, 31.7% were MBL-positive. Meanwhile, fosfomycin and minocycline were the most effective antibiotics against MBL-positive bacteria. Moreover, none of the investigated carbapenemases genes were found in MBL-positive isolates.*

Conclusion. *This study highlights the importance of MBLs producing Enterobacteriaceae in causing nosocomial infections in cancer patients. However, carbapenem resistance was not associated with the presence of MBL genes such as IMP, VIM, and SPM.*

Introduction

Despite considerable preventive efforts, hospital-associated infections (HAIs) still are a constant and significant healthcare problem (1). HAIs are one of the most common life-threatening complications of hospitalized patients, especially the immunocompromised patients (2). Due to their compromised immune system,

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neutropenia altered gut flora because of frequent antibiotic administration, chemotherapy and radiotherapy (3).

Hospitalized cancer patients are exposed to various types of infections (4). Previous studies show that most common bacterial isolates from patients with cancer are Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant enterococci (VRE), multidrug-resistant Gram-negative bacteria, and *Clostridium difficile* (3). Among Gram-negative bacteria, particularly *Enterobacteriaceae*, are responsible for majority of HAIs in the United States and Europe (5). The emergence of multiple-drug-resistant (MDR) *Enterobacteriaceae* is currently considered as a major public health concern, particularly among cancer patients (6).

After extended spectrum beta-lactamases (ESBL), the most important β-Lactamases that cause resistance among Gram-negative bacteria are carbapenemases enzymes, which hydrolyze a group of antibiotics called carbapenem (7, 8). Carbapenem are important therapeutic agents for the treatment of serious infections in hospital settings (8). There are two main molecular families of carbapenemases in Gram-negative bacteria including serine carbapenemases which are based on the presence of serine in their active site and metallo-beta-lactamases (MBLs) that require the presence of zinc ion in the active site of the enzyme (7, 8). Their activity is inhibited by metal chelators like EDTA and thiol-based compounds but not by tazobactam, sulbactam and clavulanic acid (8). Among several types of MBL genes described throughout the world, VIM, IMP and SPM types are the most clinically significant carbapenemases which encoded by *bla* $_{IMP}$, *bla* $_{VIM}$ and *bla* $_{SPM}$ genes (8). These enzymes which belong to group B of Ambler classification, often confer high-level resistance to all b-lactams except aztreonam (8).

Regarding the significant role of *Enterobacteriaceae* in nosocomial infections and also the increasing trends of carbapenem-resistant strains which raise issues of serious concern in the treatment of Gram-negative bacilli infections, the present study aimed to evaluate the antibiotic resistance pattern and the occurrence of metallo-beta-lactamases in *Enterobacteriaceae* strains from Iranian cancer patients.

Materials and Methods

Study design

This hospital-based cross-sectional study was conducted in teaching hospitals of two cities in the central part of Iran during the the 6 months period from December 2015 to May 2016. A broad spectrum of medical services is provided by these hospitals, including treatment for cancer patients with radiotherapy, chemotherapy and hormonal therapy. For each patient demographic informations including age, sex, admission date, hospitalization unit, cancer type, and site of infection were collected. This study was in accordance with the declaration of Helsinki and has been approved by the regional Ethics Committee. Also, we only record medical information and the details of the patient were kept strictly confidential.

Sampling and bacterial isolation

The *Enterobacteriaceae* isolates were obtained from clinical specimens such as urine, wound, blood and sputum samples from cancer patients. All clinical specimens were cultured on blood agar and MacConkey agar and incubated aerobically at 37° C for 24 h. The identification of Gram-negative bacteria was performed using standard microbiological methods including Triple Sugar Iron agar (TSI), Simmons' citrate agar, Christensen's urea agar, Indole test, Methyl red and Voges-Proskauer tests. The confirmed isolates were stored at -70 °C in trypticase soy broth containing 20% glycerol.

Antimicrobial susceptibility testing

Antimicrobial susceptibility pattern for the bacterial isolates was determined using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) recommendation (9). The tested antibiotic disks were imipenem (10 µg), meropenem (10 µg), cefepime (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefotetan (30 µg), cefazolin (30 µg), co-trimoxazole (75 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), tobramycin (10 mg), aztreonam (30 mg), tetracycline (30 µg), doxycycline (30 µg), minocycline (30 µg), fosfomycin (200 µg), cefuroxime (30 µg), ampicillin (10 µg), and nitrofurantoin (FM) (300 µg) disks (Padtan Teb, Iran). *Escherichia coli* ATCC 25922 was used as a control strain for susceptibility testing. Based on CDC definition the resistance to at least 1 agent in 3 antimicrobial categories was considered as MDR (10). Combined disk test (CDT) was used to determine the MBLs production as described by Yong et al. (11).

DNA extraction and PCR assay

Total DNA of the isolates were extracted by boiling methods according to the protocol as described previously (12). All MBLs-positive isolates were screened for the presence of *bla* $_{IMP}$, *bla* $_{VIM}$, and *bla* $_{SPM}$ genes by the previously described primers (13). All used primers in the current study are listed in Table 1. PCR conditions were as follow: 10 min initial denaturation at 94° C, followed by 30 cycles of denaturation (94° C/40 seconds), annealing 50 s at 55° C for *bla* $_{IMP}$ and *bla* $_{VIM}$, at 52 °C for *bla* $_{SPM}$, and extension (72° C/60 sends), and a final extension at 72° C for 5 min. PCR products were analyzed using 1.5% agarose gel with KBC loading dye (Cinna Gen Co. Iran).

Statistical Analysis

Analyses were performed using SPSS™ software, version 21.0 (IBM Corp., USA). The results are presented as descriptive statistics in terms of relative frequency. Values were expressed as the percentages of the group (categorical variables). Chi–square or Fisher's exact tests were used to determine the significance of differences. A difference was considered statistically significant if the p value was less than 0.05.

Results

During the six month study, a total of 103 non-duplicated Gram-negative bacteria were collected from clinical specimens in the studied hospitals in central parts of Iran. Out of the 100 positive cultures, 44 (42.7%) belonged to males and the remaining 59 (57.3%) to females samples.

The distribution of *Enterobacteriaceae* isolates were 74 (71.8%) *E. coli*, 23 (22.3%) *Klebsiella* spp., 3 (2.9%) *Proteus* spp., 2 (1.9%) *Salmonella* spp., and 1 (1%) *Shigella* spp.

The results of antibiotic susceptibility revealed that all of the isolates were MDR,

Table 1 - Oligonucleotide sequences of primer sets for PCR

Genes	Sequence	Ref.	PCR Product Size (bp)
IMP	F: GGAATAGAGTGGCTTAAYTCTC R:GGTTTAAYAAAACAACCACC	13	233
VIM	F:GATGGTGTTTGGTCGCATA R:CGAATGCGCAGCACCAG	13	390
SPM	F: AAAATCTGGGTACGCAAACG R: ACATTATCCGCTGGAACAGG	13	271

Table 2 - The antibiotic susceptibility pattern of isolated pathogens from cancer patients

Antibiotic	MBLs-Positive No. 19			Total No. 100			p value
	R	I	S	R	I	S	
CXM	89.5	5.3	5.3	77	5	18	0.2
FOS	73.7	.0	26.3	35	5	60	0.001
MNO	73.7	10.5	15.8	29	8	63	<0.001
CPT	94.7	5.3	.0	75	5	20	0.01
FOX	89.5	.0	10.5	69	6	25	0.005
CTT	78.9	5.3	15.8	57	8	35	0.6
CZO	89.5	5.3	5.3	67	11	22	0.05
DOX	84.2	5.3	10.5	26	10	62	<0.001
ATM	84.2	.0	15.8	72	11	17	0.9
TOB	89.5	.0	10.5	78	13	19	0.5
TE	84.2	.0	15.8	56	11	33	0.1
AMP	84.2	10.5	5.3	62	15	23	0.04
FM	89.5	.0	10.5	40	12	48	<0.001
MEN	84.2	5.3	10.5	31	8	61	<0.001
AN	84.2	5.3	10.5	40	12	48	<0.001
CTX	89.5	.0	10.5	51	13	36	0.01
CAZ	89.5	.0	10.5	35	10	55	<0.001
FEP	84.2	.0	15.8	46	8	46	0.004
SXT	89.5	.0	10.5	52	5	43	0.002
CP	84.2	5.3	10.5	35	16	39	0.004
GM	89.5	.0	10.5	49	13	38	0.008
OFX	94.7	5.3	.0	45	16	39	<0.001
IMP	94.7	.0	5.3	44	7	49	<0.001

and 60% of them were (excluded *Salmonella* and *Shigella*) carbapenem-resistant. Of total of carbapenem-resistant isolates, according to the results of CDTs 19 (31.7%) isolates were considered as MBL-positive. The prevalence of MBL-positive isolates in separate of bacterial type were 27.7% in *E. coli*, 28.6% in *K. pneumoniae*, and 66.7% in *Proteus* spp. Among the MBL-positive bacteria, the most prevalent cancer type was blood and skin cancer each with (21%), followed by prostate cancer (15.8%). Moreover, the most of MBLs producing isolates were recovered from UTIs (79%) followed by SSTIs (21%).

Out of the 100 tested isolates, 60 (60%) were found to be resistant to carbapenem and were considered as carbapenem-resistant *Enterobacteriaceae*. The results of susceptibility pattern showed fosfomycin and minocycline were the most effective antibiotics against MBL-positive bacteria, while among them, the highest rate of resistance was to ofloxacin and cefotetan each with 95%. Furthermore, among the MBL-negative isolates, the highest rate of sensitivity was towards doxycycline (76.5%) followed by minocycline (74%). Meanwhile, among the MBL-negative isolates, 74% and 70% were found to be resistant to cefuroxime

and cefotetan, respectively. The full results of the antimicrobial susceptibility pattern of tested isolates are shown in Table 2.

Furthermore, two *Salmonella* isolates were susceptible to ampicillin, cefotaxime, cefepime, ciprofloxacin, and trimethoprim/sulfamethoxazole and only resistant to nalidixic acid. *Shigella* isolate were also susceptible to cefepime, ciprofloxacin, and nalidixic acid. The presence of MBL genes including *bla* $_{IMP}$, *bla* $_{VIM}$, and *bla* $_{SPM}$ were investigated by PCR on MBL-positive isolates. None of the investigated carbapenemases genes were found in MBL-positive isolates.

Discussion

The management and prevention of nosocomial infections in patients with malignancy can be challenging since cancer patients are exposed to a wide range of infections, mostly bacterial infections (14). The important concern that must be considered in the management of nosocomial infections, particularly those caused by Gram negative bacilli are periodic surveillance to identify the MDR strains for optimizing available infection control policies and treatment options (1). Here, we have studied the distribution and antibiotic resistance pattern of MBLs producing isolates of *Enterobacteriaceae* in Iranian cancer patients.

The results of current survey showed that *E. coli and K. pneumoniae* were the most frequent *Enterobacteriaceae* isolated from cancer patients, consistent with other studies that introduced them as one the most common cause of hospital associated infections (15, 16). However, the etiology of nosocomial infections may be varied due to differences in sample size, the source of infections, and the geographical distribution.

To the best of our knowledge, this is the first report of MBLs producing *Enterobacteriaceae* in Iranian cancer patients, estimated 19%. Despite the great discrepancy in rate of MBLs producing carbapenem-resistant *Enterobacteriaceae*, our finding was consistent with the median values (range 7% to 93.1%) reported in different regions of Iran (12, 17-20). Prevalence of MBLs producing *Enterobacteriaceae* varies around the world and has been reported around 1.2% to 97.8% in developing countries (21-26). The differences may mostly arise from variation in detection methods, bacterial species, sample size, and infection control policies.

Unfortunately, all of our tested *Enterobacteriaceae* were MDR and subsequently the rates of antibiotic resistance were remarkable. However, **fosfomycin** showed the promising susceptibility against our tested isolates, particularly MBLs producing isolates. Previous experience with fosfomycin showed the significant efficacy and safety for the treatment of MDR organisms causing UTIs (27, 28), and since the majority of our isolates originated from UTIs, this antibiotic agent can be the effective last-line treatment option for related infections in our region. Moreover, despite the several comparable results with other studies from Iran and other countries, antibiotic susceptibility patterns can be variable, based on geographical distribution or source of infections (12, 17-19, 24, 26).

Hopefully none of our MBLs producing isolates contained investigated carbapenemases encoding genes *bla* $_{IMP}$, *bla* $_{VIM}$ or *bla* $_{SPM.}$ It seems that, based on previous reports, the mentioned genes are not the main mechanism of MBLs production in *Enterobacteriaceae*, while *bla* $_{NDM-1}$ is the most prevalent gene (12, 17-19, 25).

This study highlights the importance of MBLs producing *Enterobacteriaceae* in causing nosocomial infections in cancer patients. However, carbapenem resistance was not associated with the presence of MBL

genes such as IMP, VIM, and SPM. There is a need of a systematic surveillance of MBL-producing *Enterobacteriaceae* because this may provide a better understanding of how these isolates acquire resistance genes and spread worldwide.

Riassunto

Primi risultati di uno studio di prevalenza delle Enterobacteriaceae beta-lattamasi positive in pazienti oncologici iraniani

Premesse. Le Infezioni nosocomiali, recentemente ridenominate "Infezioni Associate all'Assistenza", rappresentano le più comuni tra le complicanze che mettono a rischio la vita dei pazienti ospedalizzati, particolarmente di quelli con problemi di immunodepressione. Per quanto concerne il significato del ruolo delle *Enterobacteriaceae* nelle infezioni nosocomiali, nonché in particolare dei ceppi carbapenem-resistenti, il presente studio intendeva valutare il profilo di antibiotico-resistenza e la frequenza delle metallo-beta-lattamasi nei ceppi di *Enterobacteriaceae* isolati dai malati oncologici in Iran.

Metodi. Uno studio trasversale ospedaliero è stato condotto negli ospedali di insegnamento di due città dell'Iran centrale nei sei mesi tra il Dicembre 2015 ed il Maggio 2016. Gli isolamenti di *Enterobacteriaceae* ottenuti dai diversi campioni clinici sono stati identificati con i metodi microbiologici standard. I loro profili di antibiotico-resistenza sono stati determinati con la tecnica della diffusione da dischi, e la presenza di geni dell'antibiotico-resistenza è stata rilevata con il metodo PCR.

Risultati. Gli isolamenti erano rappresentati da 74 (71.8%) *E coli*, 23 (22.3%) *Klebsiella* spp, 3 (2.9%) *Proteus* spp, 2 (1.9%) *Salmonella* spp a da 1 (1%) *Shigella* spp. Tutti gli isolamento si sono rivelati multiresistenti, ed il 60% di loro (con l'eccezione di *Salmonella* e *Shigella*) carbapenem-resistenti. Il 31.7% dei ceppi carbapenem-resistenti è risultato metallo-beta-lattamasi positivo. Si è però anche osservato che, su questi ultimi ceppi, erano fosfomicina e minocyclina ad essere gli antibiotici maggiormente efficaci. Inoltre, nessuno dei geni della carbepenemasi ricercati è stato ritrovato nei ceppi metallo-beta-lattamasi positivi.

Conclusioni. Questo studio ha evidenziato l'importanza delle *Enterobacteriaceae* producenti metallo-beta-lattamasi nel provocare infezioni nosocomiali nei pazienti oncologici; tuttavia, la carbapenem-rersistenza non è risultata associata alla presenza di geni MBL, quali IMP, VIM e SPM.

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Conflict of interest
There are no conflicts of interest

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