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ORIGINAL ARTICLE

High prevalence of OXA-48-like and NDM carbapenemases among carbapenem resistant *Klebsiella pneumoniae* of clinical origin from Iran

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ABSTRACT

Background and Objectives: *Klebsiella pneumoniae* is increasingly developing resistance to last-resort antibiotics such as carbapenems. This study aimed to investigate the dissemination of common carbapenemase encoding genes among 48 clinical isolates of carbapenem-resistant *Klebsiella pneumoniae* (CRKP).

Materials and Methods: Antimicrobial susceptibility testing was performed by broth dilution and disc diffusion methods. The phenotypic evaluation of carbapenemase production was performed by using Modified Carbapenem Inactivation Method. Presence of carbapenemase encoding genes bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{IMP}, and bla_{VIM} was screened by PCR.

Results: Overall, carbapenemases were produced in all CRKP isolates. The $bla_{OXA\text{-}48\text{-}like}$ and bla_{NDM} were the most prevalent genes detected among all and 66.6% (n=32) of CRKP isolates respectively. The bla_{VIM} was detected in only one isolate co-harboring NDM and OXA-48-like carbapenemases. The bla_{KPC} and bla_{IMP} genes were not identified in any of the isolates. While tigecycline was the most active agent against CRKP isolates with low resistance rate (4.1%), high rate of resistance was observed to colistin (66.6%), amikacin (79%) and most of other tested antimicrobials.

Conclusion: Our results revealed predominant prevalence of OXA-48-like and NDM carbapenemases among CRKP clinical isolates. High rate of resistance to last-resort agents such as colistin among CRKP isolates is a source of great concern.

Keywords: *Klebsiella pneumoniae*; Carbapenem resistance; NDM; OXA-48-like; VIM

INTRODUCTION

Increasing antimicrobial resistance among bacteria and associated infections is a growing issue and a grave threat to human health. Carbapenems are often considered as the last-resort therapeutics for treatment of infections caused by multidrug-resistant (bacteria resistant to more than three antibiotic classes) *Enterobacterales* (1), due to their broad spectrum of activity and inherent stability against a variety of β-lactamases (2). However, the efficacy of these antimicrobials is hampered by emergence of carbapenem inactivating enzymes produced by major Gram negative pathogens. Among the most significant pathogens are carbapenem-resistant *Klebsiella pneumoniae* (CRKP), which leads to mortality rates as high as 40 to 50% (3). Therapeutic options for CRKP are limited, increasing the complexity of managing of infections caused by these superbugs (4).

K. pneumoniae accounts for about one third of all Gram-negative infections overall causing broad spectra of hospital and community acquired diseases including urinary tract, blood stream or surgical wound infections, pneumonia and septicemia (5). Together with other highly important MDR pathogens, *K. pneumoniae* has been classified as an ESKAPE organism (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii,*

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Pseudomonas aeruginosa, and *Enterobacter* species) which are the leading cause of nosocomial infections throughout the world.

The production of carbapenemases including Ambler Class A (KPC), Class B metallo-β-lactamases (MBLs) (NDM, VIM, IMP) and class D (OXA-48-like) is the most common resistance mechanism among carbapenem-resistant *Enterobacterales* (CRE). These Enzymes exhibit variable levels of carbapenem resistance through their carbapenem-hydrolyzing activity (4). The distribution of NDM, KPC and OXA-48-producing *Enterobacteriaceae* varies worldwide. Since its first identification in *K. pneumoniae* strain in the United States in 1996, the KPC carbapenemase and its variants have been increasingly reported from North America (6), Greece (7), Italy (8), and some regions of China (9). First detected in *Enterobacteriaceae* isolates in Turkey, the OXA-48 carbapenemase has disseminated regionally with the Middle East and North African countries being considered the principal reservoirs of this enzyme (10). NDM, a novel metallo-beta-lactamase was first reported in a Swedish patient traveled to New Delhi in 2008 (11). Currently Asian continent (mostly India and China) serves as the major reservoir of NDM producers, followed by United Kingdom which has reported the largest number of NDM-producing CRE cases among European countries (9, 12).

Since different carbapenemases have variable level of carbapenem-hydrolyzing activity and susceptibility to novel carbapenemase inhibitors such as avibactam, vaborbactam and taniborbactam, identification of prevalent carbapenemases among CRKP isolates circulating in each geographic region provides important insights into treatment and infection control strategies. In this study we aimed to characterize the prevalence of different carbapenemases (including KPC, NDM, OXA-48-like, IMP and VIM) and the drug susceptibility pattern in a series of clinical CRKP isolates.

MATERIALS AND METHODS

Bacterial isolates and antimicrobial susceptibility testing (AST). A total of 48 clinical isolates of *Klebsiella pneumoniae* obtained from Abu Ali Sina Organ Transplant Center, from January to September 2022, were included in this study. The isolates had been obtained from urine (n=36), sputum (n=4), blood (n=2), abdominal fluid (n=2), CSF (n=2) and wound (n=2). Identification of isolates was performed by using conventional biochemical methods, including IMViC tests (indole test, methyl red test, Voges-Proskauer reaction, citrate utilization test), motility, reactions observed on Triple Sugar Iron (TSI) agar (H2S and gas production, carbohydrate utilization pattern), urease and ONPG (O-nitrophenyl-beta-D-galactopyranoside) tests (13). Testing susceptibility to imipenem, tigecycline and colistin was performed using broth dilution method using freshly prepared Mueller Hinton Broth from Difco (BD diagnostics, Sparks, MD) and antibiotic powders from Glentham Life sciences (UK). The susceptibility to other classes of antibiotics was determined by disk diffusion method (Kirby Bauer) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines using the following antibiotics: gentamicin, amikacin, ceftriaxone, cefepime, ciprofloxacin, chloramphenicol, tetracycline, doxycycline, nitrofurantoin (Condalab, Spain & Liofilchem, Italy). The AST results were interpreted according to CLSI guidelines except for tigecyclien and colistin for which Food and Drug Administration (FDA) breakpoints issued for *Enterobacteriaceae*, (susceptible ≤ 2 mg/L, intermediate = 4 mg/L, resistant ≥ 8 mg/L) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (colistin MIC>2mg/L resistant) were used respectively. *Escherichia coli* ATCC 25922 was used as quality control strain for antimicrobial susceptibility testing.

Phenotypic method for detection of carbapenemase production. Phenotypic detection of carbapenemase production, was performed using modified carbapenem inactivation method (mCIM) as described in CLSI. In brief, 1 µl of test organism was added into a tube containing 2 ml of tryptic soy broth (TSB). A 10-µg meropenem disk was then aseptically added into the bacterial suspension. Next, the tube was incubated for 4 h ± 15 min at 35°C ± 2°C. The disks were then transferred onto MH agar seeded with a 0.5McFarland suspension of *E. coli* ATCC 25922. Following overnight incubation, the zone of inhibition was measured to determine whether the meropenem had been hydrolyzed. A zone diameter of 6 to 15 mm was considered a positive result, a zone diameter of ≥19 mm was considered a negative result and a zone diameter of 16 to 18 mm was considered an indeterminate result (14).

Detection of resistance genes in carbapenem-resistant isolates. Pure colonies were selected for DNA extraction using a boiling method as described previously (https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/278_mcr-multiplex-pcr-protocol-v2-oct16.pdf). All the CRKP strains were examined for the presence of the most common carbapenemase encoding genes ($bla_{KPC,}$ bla_{NDM}, $bla_{OXA-48-like}$, bla_{IMP}, and bla_{VIM}) by polymerase chain reaction (PCR) with specific primers listed in Table 1. PCR reactions were performed using Taq DNA Polymerase 2× Master Mix RED (Ampliqon, Denmark) according to manufacturer's protocol. The PCR products were electrophoresed in a 1.5% agarose with 1× TAE (Tris/Acetate/EDTA) buffer and DNA safe stain (Yekta Tajhiz Azma, Iran) and visualized under UV light using a Uvitec Gel Documentation system (Cambridge, UK). *K. pneumoniae* and *Escherichia coli* carrying the studied genes (identified during previous studies and confirmed for carrying the genes by sanger sequencing or whole genome sequencing (15) were used as positive controls.

RESULTS

A total of 48 clinical isolates of *K. pneumoniae* isolates were studied with the majority being found as urinary isolates (75%). All isolates revealed positive results for mCIM phenotypic method of carbapenemase production and were characterized with imipenem MICs ranging from 4 mg/L to ≥64mg/L with about 68% (n=33) having MICs of ≥64mg/L (Table 2) as determined by standard broth dilution method. Only 33.3% of isolates had colistin MIC ≤2 mg/L and categorized as colistin susceptible with the remaining isolates displaying low and very high levels of colistin resistance respectively. Tigecycline with susceptibility rate of 79% was the most active agents against CRKP isolates (Table 2).

Table 2. The MIC values of the antibiotics tested for carbapenem resistant *K. pneumoniae* isolates

Antibiotic	Number of isolates with MICs (mg/L)								
	≤0.25	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**
Imipenem	-	-	-	-	11	2	0	2	33
Colistin	5	4	3	4	4	8	4	4	12
Tigecycline	-	2	13	23	8	1	1	-	-

According to disc diffusion results, high resistance rate to ceftriaxone (100%), cefepime (100%), ciprofloxacin (100%), gentamicin (89.5%) and amikacin (79%) was identified. Resistance rate to other antimicrobials is presented in Fig. 1.

PCR screening of medically important carbapenemase genes revealed presence of at least one carbapenemase gene in all studied CRKP isolates. The $bla_{OXA-48-like}$ and bla_{NDM} were the most prevalent genes detected among all (100%) and 66.6% (n=32) of CRKP isolates respectively (Fig. 2). Among the clinical isolates, 16 harbored only OXA-48-like (33.3%), 31 co-harbored NDM+OXA-48-like (64.5%) and one co-harbored NDM+OXA-48-like+VIM (2%). The bla_{KPC} and bla_{IMP} genes were not detected in any of the isolates.

Table 1. Nucleotide sequences of primers used in this study

Primer name	Sequence (5′ to 3′)	Product Size (bp)	Annealing temperature (°C)	Reference(s)
NDM-F	GCCCAATATTATGCACCCGGTC	803	59	(30)
NDM-R	AGCGCAGCTTGTCGGCCAT			
KPC-F	CCGTCTAGTTCTGCTGTCTTG*	799	58	
KPC-R	CTTGTCATCCTTGTTAGGCG			
OXA-F	GCGTGGTTAAGGATGAACAC	438	56	
OXA-R	CATCAAGTTCAACCCAACCG			(16)
VIM-F	CGATGGTGTTTGGTCGCATA*	391	56	
VIM-R	CGAATGCGCAGCACCAG			
IMP-F	GGAATAGAGTGGCTTAAYTCTC	232	54	
IMP-R	GGTTTAAYAAAACAACCACC			

* differs from primer in reference 16 only by addition of one C nucleotide at 5' end.

While OXA-48 as the only carbapenemase was commonly detected in CRKP isolates with low level carbapenem resistance (MIC=4, 8 mg/L), the NDM and VIM were only detected in CRKP isolates with high level carbapenem resistance (n=1, MIC=32mg/L; n=31, MIC ≥64mg/L) (Fig. 3).

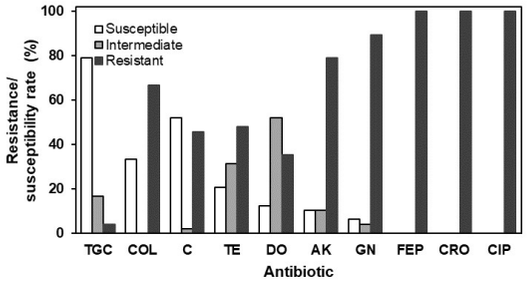

Fig. 1. Antimicrobial susceptibility testing results for all tested antibiotics determined by broth dilution and disc diffusion methods. TGC, tigecycline; COL, colistin; C, chloramphenicol; TE, tetracycline; DO, doxycycline; AK, amikacin; GN, gentamicin; FEP, cefepime; CRO, ceftriaxone; CIP, ciprofloxacin

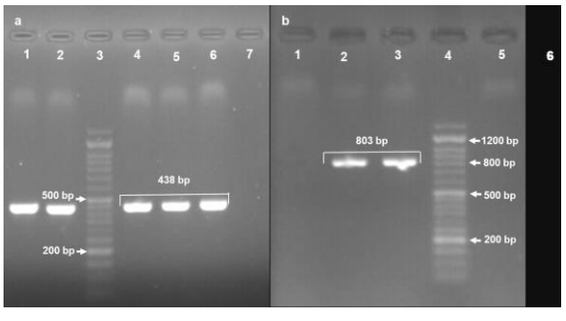

Fig. 2. Agarose gel electrophoresis of PCR products of different carbapenemase encoding genes. a) Lanes 1,2,4-6, $bla_{\text{OXA-48-like}}$ positive; Lane 3, 50 bp DNA ladder (GeneDirex, 50-1500bp); Lane7, no-template control (NTC). b) Lanes 1&5, bla_{NDM} negative; Lanes 2&3, bla_{NDM} positive; Lane 4, DNA ladder

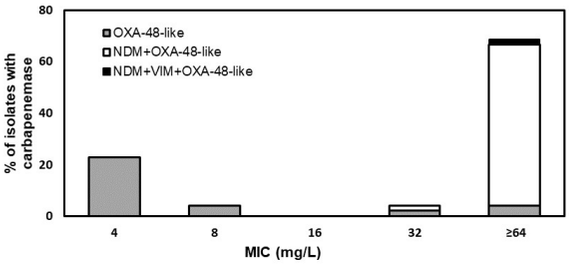

Fig. 3. Distribution of difference carbapenemases associated with imipenem MICs

DISCUSSION

CRE have spread rapidly around the world in the past few years posing great challenges to human health. The production of carbapenemases is the principle mechanism underlying carbapenem resistance in CRE throughout the world. The widespread distribution of CRE can be attributed to the plasmid-mediated horizontal transmission of carbapenemase encoding genes. CRE infections are resistant to nearly all antibiotics and are associated with poor clinical outcomes (17). In our study 75% of CRKP isolates were obtained from UTI cases. Urine has also been found as the predominant source of CRKP in other studies (18, 19). Our results demonstrated that all CRKP isolates carried carbapenemase genes. The OXA-48-like was found as the most prevalent carbapenemase being identified in all CRKP isolates followed by NDM and VIM enzymes. This is in accordance with recent studies which reported OXA-48-Like and NDM as the most frequent carbapenemases among CRKP isolates in Iran (20, 21). Since the first recognition of OXA-48 enzyme in 2001, several variants of OXA-48 carbapenemases have emerged, collectively forming the "OXA-48-like" subfamily including OXA-181, OXA-232, OXA-204, OXA-162, OXA-163, OXA-244, OXA-245, OXA-370 and OXA-405. All members of this superfamily differ from OXA-48 by 1 to 5 amino acid substitutions and/or deletions, leading to modified β-lactam hydrolyzing capabilities (22). Compared to other carbapenemases such as NDM and KPC, the OXA-48-like has been found to have weaker carbapenemase activity conferring only low-level *in vitro* resistance to carbapenems but resulting in carbapenem treatment failure (23). Similarly, 81.25% (13 out of 16) of isolates harboring the OXA-48-like as the only detected carbapenemase in this study were characterized with imipenem MIC of 4 (n=11) or 8 mg/L (n=2) indicating a low-grade imipenem hydrolyzing activity for this type of carbapenemase. Higher imipenem MICs identified (32 and ≥64mg/L) in the remaining 3 $bla_{\text{OXA-48-like}}$ positive isolates (as the only detected gene) can be attributed to presence of other carbapenemases or other carbapenem resistance mechanisms such as efflux overexpression and/or porin alterations (24). While OXA-48-like enzymes do not significantly hydrolyze third- and fourth- generation cephalosporins, some members of this family such as OXA-163 and OXA-

405 display an increased ability to hydrolyze cefotaxime and cefepime over OXA-48 (25). Moreover, significant proportion of OXA-48-like producers have been found to co-harbor ESBLs on additional plasmids mediating their resistance to later-generation cephalosporins (25). All OXA-48-like positive (as the only detected enzyme) CRKP isolates in this study, showed resistance to cefepime and ceftriaxone indicating presence of other ESBLs or carbapenemases with ESBL-like activity in these isolates. Coexistence of OXA-48-lik+NDM and OXA-48-like+NDM+VIM enzymes was identified in 64.5% (n=31) and 2% (n=1) of CRKP isolates respectively, all of which presented high level imipenem resistance. Several studies have reported the association between OXA-48-like and NDM in enterobacterial species from different countries (26-28) including Iran (29). The bla_{NDM} with prevalence of 66.6% was recognized as the second most prevalent carbapenemase encoding gene detected among CRKP isolates. This is in accordance with previous reports introducing Asian continent as the major reservoir of NDM producers (12). It's been found that KPC enzymes can confer higher carbapenem MIC values because of its better expression in *Klebsiella pneumoniae* (6). In this study KPC and IMP genes were not detected in any of the isolates. Similar results about the prevalence of KPC and IMP carbapenemases among CRKP isolates have been previously reported from Iran (29, 30). Currently, treatment options for CRE infections remain very limited with polymyxins, tigecycline, fosfomycin, aminoglycosides and combination of beta-lactams with novel beta-lactamase inhibitors (avibactam, vaborbactam, and relebactam) being listed as drugs of choice (4). According to AST results, tigecycline was found as the most active agent against CRKP isolates presenting the lowest resistance arte (4%, MIC>4mg/L). However, 66.6% and 79% of CRKP isolates were identified as colistin and amikacin resistant respectively. While similar studies from Iran have reported tigecycline as the most effective antibiotic against CRKP with resistance rate of 3.3-3.9% (20, 21), the identified resistance rates for amikacin in these studies were found to be 50.9% (20) and 38% (21). There have been increasing numbers of reports on colistin resistance among CRKP isolates worldwide (31, 32), particularly from Iran (30) making this old agent less effective for treatment of CRE infections in the country.

CONCLUSION

In summary our study reports high prevalence of OXA-48-like and NDM carbapenemases as well as co-existence of both among the studied clinical CRKP isolates. Also a very high rate of resistance to colistin and aminoglycosides was observed among the CRKP isolates which raises the concerns about the future outbreaks of infections caused by these difficult-to-treat superbugs. With limited therapeutic options, development of new antibiotics or novel inhibitors of carbapenemase for treatment of NDM/VIM positive bacterial infections is needed. Early identification of carbapenemase producers, in hospital settings or among asymptomatic carriers may contribute to restriction of their spread to community.

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