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THE EMERGENCE OF *bla*$_{\text{OXA-48}}$ AND *bla*$_{\text{NDM}}$ AMONG ESBL-PRODUCING *KLEBSIELLA PNEUMONIAE* IN CLINICAL ISOLATES OF A TERTIARY HOSPITAL IN IRAN

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The aim of this study was to investigate the prevalence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and the most common types of carbapenemases, metallo-beta-lactamases (MBLs), and extended-spectrum beta-lactamases (ESBLs) among CRKP isolates in a tertiary hospital in Isfahan, Iran. Eighty non-repetitive clinical isolates of *K. pneumoniae* were obtained from different clinical specimens. Antibiotic resistance pattern of isolates was determined by disk diffusion method and production of carbapenemases and MBLs was confirmed using modified Hodge test and E-test, respectively. Molecular detection of the antibiotic resistance genes was performed using PCR. Fifty-one (63.8%) isolates have decreased susceptibility to carbapenems, of which 46 (90.2%) isolates were as carbapenemase producer and four (7.8%) isolates were positive for MBLs, phenotypically. The results of PCR showed that the prevalence of *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$, and *bla*$_{\text{TEM}}$ genes among CRKP isolates were 90.2%, 15.7%, 98%, 96.1%, and 90.2%, respectively. No isolates carrying the *bla*$_{\text{KPC}}$, *bla*$_{\text{GES}}$, *bla*$_{\text{IMI}}$, *bla*$_{\text{VIM}}$, and *bla*$_{\text{IMP}}$ genes were detected. This study showed that the production of OXA-48 is one of the main mechanisms of resistance to carbapenems in CRKP isolates in Isfahan. In addition, the dissemination of NDM-producing CRKP isolates is a potential risk for the health care system of this area in the near future.

Keywords: *Klebsiella pneumoniae*, CRKP, OXA-48, NDM, ESBLs

Introduction

Klebsiella pneumoniae is an important opportunistic bacterial pathogen associated with both community- and hospital-acquired infections and potentially

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causing mortality, especially in hospitalized patients [1]. An important part of hospital infections (4%–8%) is due to this bacterium. *K. pneumoniae* isolates are increasingly resistant to several antibiotics. Hospital outbreaks caused by *K. pneumoniae* isolates are frequent and the transmission of resistant strains between the hospital and community has been described previously [2]. Treatment of infections caused by drug-resistant *K. pneumoniae* isolates, especially strains producing extended-spectrum beta-lactamases (ESBLs), as well as isolates with multidrug-resistant and extensively drug-resistant phenotypes is very complicated and costly, and usually not associated with satisfactory results [3, 4]. Carbapenems are a group of beta-lactam antibiotics that have been used as the "last-line" treatment for *Enterobacteriaceae*-associated infections, especially those producing ESBLs [5, 6]. Compared to cephalosporins, penicillins, or beta-lactam/beta-lactamase inhibitors, carbapenems have a broader antimicrobial spectrum [7]. During the past decade, the emergence of resistance to carbapenems in glucose non-fermenting (e.g., *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) and glucose-fermenting (e.g., *Enterobacteriaceae*) Gram-negative bacilli has been reported from all around the world [8, 9]. Recently, the spread of carbapenem-resistant *Enterobacteriaceae*, especially carbapenem-resistant *K. pneumoniae* (CRKP), is the most important clinical problem in the field of antibiotic resistance that should be monitored seriously and prevented from its progress [10]. This study aims to investigate the prevalence of CRKP and the most common types of carbapenemases, metallo-beta-lactamases (MBLs), and ESBLs among CRKP in a tertiary hospital in Isfahan, Iran.

Materials and Methods

Bacterial isolates

During a 9-month period, April–December 2017, 80 non-repetitive *K. pneumoniae* isolates were obtained from various specimens of inpatients in Alzahra hospital, Isfahan, Iran. All isolates were identified in microbiology laboratory of medical school using the conventional methods, such as Gram staining and biochemical (oxidase, sugar fermentation, IMViC, Kliger's iron agar, nitrate reduction, motility, etc.) tests [11]. In addition, to confirm the species, a PCR detection based on the 16S–23S internal transcribed spacer sequence of *K. pneumoniae* was conducted [12]. This study was evaluated and approved by the Ethics Committee of Isfahan University of Medical Sciences (project no. 395891).

Antibiotic susceptibility testing

Susceptibility to antibiotics was determined using Kirby–Bauer's disk diffusion method according to the recommendations of Clinical Laboratory Standard Institute (CLSI) [13]. For this purpose, 18 antibiotics (MAST, UK and Liofilchem, Italy) including imipenem (10 μg), meropenem (10 μg), ertapenem (10 μg), gentamicin (10 μg), ceftaroline (30 μg), piperacillin/tazobactam (100/10 μg), cefazolin (30 μg), cefuroxime (30 μg), ceftazidime (30 μg), cefepime (30 μg), cefoxitin (30 μg), ciprofloxacin (5 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), tigecycline (15 μg), aztreonam (30 μg), amoxicillin/clavulanic acid (20/10 μg), chloramphenicol (30 μg), and tetracycline (30 μg) were used. *Escherichia coli* ATCC 25922 was used for quality control of disk diffusion method [13].

Carbapenemase and MBL screening assays

To determine carbapenemase activity, the modified Hodge test (MHT) using ertapenem disk (MAST) was performed according to CLSI guidelines [13]. Moreover, to detect MBL activity, E-test method using strips containing meropenem/meropenem + EDTA (Liofilchem) was carried out based on manufacturer's guidelines.

PCR for detection of antibiotic resistance genes

DNA was extracted using the boiling method and used as template for PCR [14]. To detect antibiotic resistance genes including bla_{KPC}, bla_{GES}, bla_{IMI}, bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{OXA-48}, bla_{SHV}, bla_{TEM}, and bla_{CTX-M}, separate PCR reactions were performed. The list of all target genes and corresponding primers is presented in Table I [15–21]. PCR was performed using commercially available PCR Master Mix (AMPLIQON, Denmark) according to the manufacturer's instructions. Briefly, 1 μl template DNA (~100 ng/μl), 1 μl of each primer (10 pmoles/μl), and 9.5 μl DNase-free distilled water were added to 12.5 μl of Master Mix in a final volume of 25 μl. Thermocycling was carried out with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing for 45 s at primer-specific temperatures (Table I), extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR products were resolved by standard electrophoresis on 1.5% agarose gel containing DNA safe stain.

Table I. List of primers, expected amplicon size, and annealing temperatures

Target gene	Primer sequence (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)	References
bla_{KPC}	F: GATACCACGTTCCGTCTGG R: GCAGGTTCCGGTTTTGTCTC	58	246	[15]
bla_{GES}	F: GTTTTGCAATGTGCTCAACG R: TGCCATAGCAATAGGCGTAG	53	371	[16]
bla_{IMI}	F: ATGTCATTAGGTGATATGGC R: GCATAATCATTTGCCGTACC	50	879	[17]
bla_{VIM}	F: TTTGGTCGCATATCGCAACG R: CCATTCAGCCAGATCGGCAT	66	500	[18]
bla_{IMP}	F: GTTTATGTTCATACATCG R: GGTTTAACAAAACAACCAC	45	440	[18]
bla_{NDM}	F: GGGCAGTCGCTTCCAACGGT R: GTAGTGCTCAGTGTCGGCAT	52	475	[19]
bla_{OXA-48}	F: GCGTGGTTAAGGATGAACAC R: CATCAAGTTCAACCCAACCG	60	438	[20]
bla_{SHV}	F: ATGCGTTATATTCGCCTGTG R: TGCTTTGTTATTCGGGCCAA	60	753	[18]
bla_{TEM}	F: AAACGCTGGTGAAAGTA R: AGCGATCTGTCTAT	45	752	[18]
bla_{CTX-M}	F: TTTGCGATGTGCAGTACCAGTAA R: CGATATCGTTGGTGGTGCCATA	51	544	[21]

Results

Bacterial isolates

Table II shows demographic information and clinical characteristics of patients infected with *K. pneumoniae*. The mean age of patients was 52.7 years.

Antibiotic susceptibility testing

Table III shows the results of antibiotic susceptibility testing in considered isolates. The highest resistance rate was observed to cefazolin (87.5%), followed by amoxicillin/clavulanic acid (78.8%), cefuroxime (77.5%), tetracycline (77.5%), and aztreonam (76.2%). Tigecycline and chloramphenicol were the most effective antibiotics with 13.7% and 18.7% resistance rate, respectively. About 62.5% of isolates were resistant to meropenem and ertapenem as well as overall, 51 isolates showed the decreased susceptibility to carbapenems.

Table II. Demographic information and clinical characteristics of patients infected with *K. pneumoniae* ($n = 80$)

Demographics	Number (%)
Age range (years)	1–92
Sex	
Male	45 (56.3)
Female	35 (43.7)
Hospital wards	
Intensive care unit	48 (60.0)
Internal medicine	12 (15.0)
Surgery	8 (10.0)
Emergency	7 (8.8)
Coronary care unit	3 (3.7)
Hematology/oncology	2 (2.5)
Specimens	
Urine	29 (36.3)
Trachea	21 (26.3)
Blood	8 (10.0)
Cerebrospinal fluid	7 (8.8)
Wound	6 (7.5)
Sputum	3 (3.7)
Abscess	2 (2.5)
Catheter	2 (2.5)
Bronchoalveolar lavage	1 (1.2)
Drainage fluid	1 (1.2)

Table III. Antibiotic susceptibility of *K. pneumoniae* isolates ($n = 80$)

Antibiotic	Susceptible [*n* (%)]	Intermediate [*n* (%)]	Resistant [*n* (%)]
Imipenem	31 (38.8)	3 (3.7)	46 (57.5)
Meropenem	30 (37.5)	0 (0.0)	50 (62.5)
Ertapenem	30 (37.5)	0 (0.0)	50 (62.5)
Gentamicin	31 (38.8)	1 (1.2)	48 (60.0)
Ceftaroline	20 (25.0)	1 (1.2)	59 (73.8)
Piperacillin/tazobactam	24 (30.0)	4 (5.0)	52 (65.0)
Cefazolin	7 (8.8)	3 (3.7)	70 (87.5)
Cefuroxime	16 (20.0)	2 (2.5)	62 (77.5)
Ceftazidime	17 (21.3)	3 (3.7)	60 (75.0)
Cefepime	17 (21.3)	4 (5.0)	59 (73.7)
Cefoxitin	24 (30.0)	2 (2.5)	54 (67.5)
Ciprofloxacin	19 (23.8)	1 (1.2)	60 (75.0)
Trimethoprim/sulfamethoxazole	37 (46.3)	1 (1.2)	42 (52.5)
Tigecycline	57 (71.3)	12 (15.0)	11 (13.7)
Aztreonam	17 (21.3)	2 (2.5)	61 (76.2)
Amoxicillin/clavulanic acid	13 (16.2)	4 (5.0)	63 (78.8)
Chloramphenicol	36 (45.0)	29 (36.3)	15 (18.7)
Tetracycline	17 (21.3)	1 (1.2)	62 (77.5)

Carbapenemase and MBL screening assays

The MHT was performed on 51 isolates with decreased susceptibility to carbapenems and following results were obtained: 46 (90.2%) isolates were positive, 4 (7.8%) isolates were negative, and 1 (2%) isolate was indeterminate. Similarly, phenotypic MBL production testing on desired isolates identified 4 (7.8%) isolates as positive, 4 (7.8%) isolates as negative, and 43 (84.4%) isolates with non-determinable results.

PCR for detection of antibiotic resistance genes

All 51 isolates with decreased susceptibility to carbapenems were tested in PCR for the presence of the selected antibiotic resistance genes. Forty-six (90.2%) isolates harbored $bla_{\text{OXA-48}}$ and eight (15.7%) isolates were positive for bla_{NDM}. Moreover, seven (13.7%) isolates had both $bla_{\text{OXA-48}}$ and bla_{NDM}, simultaneously. ESBLs-encoding genes including bla_{SHV}, $bla_{\text{CTX-M}}$, and bla_{TEM} were identified in 98%, 96.1%, and 90.2% isolates, respectively. Similarly, these (ESBLs) genes were detected concurrently in 45 (88.2%) isolates. In addition, six (11.8%) isolates were positive for the presence of $bla_{\text{OXA-48}}$, bla_{NDM}, bla_{SHV}, $bla_{\text{CTX-M}}$, and bla_{TEM} genes, together. The results of PCR were negative for bla_{KPC}, bla_{GES}, bla_{IMI}, bla_{VIM}, and bla_{IMP} genes.

Discussion

One of the main problems in dealing with clinical strains of *K. pneumoniae* is the increasing incidence of resistance to antibiotics. These resistant strains are rapidly expanding and have caused many problems with the treatment of infections in various health care settings [22]. Carbapenems are a group of beta-lactam antibiotics that are used to treat serious infections in hospitals. The constant uses of these antibiotics and the selective pressure resulting from it have led to resistance to these antibiotics in Gram-negative bacteria, especially *K. pneumoniae* [5]. Usually, infections caused by CRKP threaten patients in hospitals, nursing homes, and other health care centers and do not occur in immunocompetent persons [23, 24]. In this study, more than half of patients were over 56.5 years of age. In addition, almost half of CRKP isolates (45%) were obtained from hospitalized patients in intensive care unit, which highlights the importance of aging and long-term hospitalization in these infections. PCR results showed that 90.2% of CRKP isolates were positive for $bla_{\text{OXA-48}}$. OXA-48-producing *Enterobacteriaceae* was first identified in Turkey in 2001, subsequently reported from several countries

in the Middle East, North Africa, and Europe [25, 26]. The first report of OXA-48-producing *K. pneumoniae* strains in Iran was presented by Azimi et al. [27]. In two separate studies conducted in Tehran hospitals (capital of Iran), the prevalence of $bla_{\text{OXA-48}}$ among CRKP isolates was reported as 96.4% [27] and 4.1% [28]. Another study in southwestern Iran showed that 6.9% of CRKP isolates were positive for $bla_{\text{OXA-48}}$ [29]. The results of this study regarding the prevalence of $bla_{\text{OXA-48}}$-harboring CRKP is in parallel with the study by Solgi et al. [30] between 2014 and 2016 in our region. In their study, all (100%) CRKP isolates harbored $bla_{\text{OXA-48}}$. The results of the phenotypic test for detection of MBLs showed that only four (7.8%) CRKP isolates produced MBLs, while in the PCR assay, eight (15.7%) CRKP isolates were identified as bla_{NDM}-positive. This finding suggests that the phenotypic test alone is not sufficient for the definitive detection of MBLs, and a molecular test such as PCR should be used along with it. ESBLs-encoding genes including bla_{SHV}, $bla_{\text{CTX-M}}$, and bla_{TEM} with 98%, 96.1%, and 90.2% were the most prevalent *bla* genes among CRKP isolates, respectively. In a meta-analysis article that we previously published in Iran, the overall relative frequency (RF) of $bla_{\text{CTX-M}}$ gene among ESBLs-producing *K. pneumoniae* clinical isolates was 56.7% [31]. Our results show that the RF of $bla_{\text{CTX-M}}$ among CRKP isolates in Isfahan is higher than its RF in Iran. Solgi et al. [30] in their study from Isfahan have reported that 95.8%, 94.8%, and 96.9% of CRKP isolates were positive for $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV}, respectively. In this case, our results were fully consistent with their results. Similar to other studies that were carried out previously in Isfahan, we have not detected class A carbapenemases (KPC, GES, and IMI) among CRKP isolates [30, 32, 33]. In conclusion, this study showed that the production of class D carbapenemases (OXA-48) is one of the main mechanisms of resistance to carbapenems in CRKP isolates in Isfahan. Moreover, the dissemination of NDM-producing CRKP isolates is a potential hazard for the health care system of this area in the future that should be controlled.

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Conflict of Interest

The authors declare no conflict of interest.

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