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Antimicrobial Susceptibility Patterns and Distribution of bla$_{kpc}$ Genes among *Acinetobacter baumannii* Isolated from Patients at Tehran - Iran Hospitals

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Some strains of *Acinetobacter* which show multiple-drug resistance have produced therapeutic difficulties in worldwide. This is also a new problem in Iran. The purpose of current study was to define the antimicrobial susceptibility patterns and prevalence of bla$_{kpc}$ gene in *Acinetobacter baumannii* isolates which had been isolated from patient in Tehran- Iran. This study was performed on 70 isolates of *Acinetobacter* which were isolated from patients. After identification of isolates in species level using cultural and biochemical methods, the susceptibility tests were carried out on 50 isolates of *A. baumannii* using disk diffusion method. Then isolates were considered for presence of bla$_{kpc}$ gene by PCR. In this study 50 isolates of *A. baumannii* and 20 isolates of *Acinetobacter lwoffii* were isolated from patients. More than 55% of isolates showed multiple-drug resistance and also above 40% resistance to imipeneme and meropeneme was recorded. The MIC of isolates which were resistant to carbapenemes was above 32 µg/ml. The PCR results showed that 8 cases (16%) of isolates had bla$_{kpc}$ gene which most of them had been isolated from patients who were hospitalized in the ICU. Carbapenem and multiple-drug resistant A. *baumannii* is expanding in Iran and it is considered as an important hazard for hospitalized patients. Moreover regarding to existence of bla$_{kpc}$ gene in this bacterium and possibility of transformation of these genes to the other bacteria, reconsideration in antibiotics consumption patterns and more attention to nosocomial infections control criteria are inevitable.

Key Words: *Acinetobacter baumannii,* Nosocomial infections, Multiple-drug resistance, Carbapenem, bla$_{kpc}$, PCR.

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Acinetobacter spp. are gram-negative, oxidase -negative, nonmotile and strictly aerobic coccobacilli that distributed widely in nature and nowadays are considered as an opportunistic pathogen relating to life-threatening nosocomial infections[1-3]. This genus includes several species. *A. baumannii* is the most common species which have been isolated from patients. Other species such as *A. lwoffii, A. johnsonii and A. haemolyticus* are rarely isolated from the patients. During the

last few decades *A. baumannii* is considered as one of the life- threatening microorganisms due to its ability to acquire drug resistance[4,5]. Various researches show that different strains of *A. baumannii* have been resisted to broad- spectrum antibiotics and now carbapenems are the selective antibiotic for treating of *A.baumannii* infections, although resistance to carbapenems is also increasing worldwide[6,7]. Various studies have illustrated that resistance to carbapenems in *Acinetobacter* spp. is due to the production of a type of beta lactamase enzyme that is called carbapenemase. Ambler has classified beta lactamases in different classes[8-10] . Class B (metalo-beta- lactamases) and class D (oxacillinase) are considered as a mechanism of resistance to carbapenems in *A.baumannii* strains that have been also reported in Iran[11-13].

bla$_{kpc}$ family with eight various bla$_{kpc}$ variants (bla$_{kpc}$-2 to -9) are also a group of powerful carbapenemases belong to â lactamases class A which identified initially in an isolate of klebsiella in the United States and then in other geographical regions worldwide and in other bacteria. Different studies have demonstrated that variants 2, 3, 4 and 10 are more prevalent in *A.baumannii*[14-17] . Regarding to this note that the presence of the bla$_{kpc}$ gene has not been described in clinical isolates of *A.baumannii* strains in Iran, this study was performed for the first time in Iran to determine drug-resistance pattern and existence of bla$_{kpc}$ gene in multiple-drug resistant *A.baumannii* by PCR method.

METHODOLOGY

Bacterial isolates

500 samples including blood, respiratory secretions, urine, skin ulcer and oral mucosa were obtained from hospitalized patients in Imam Khomeini, Baqyatallah and Milad hospitals between years of 2010 to 2011. 50 isolates of *A.baumannii* from blood culture (n= 19, 38%), mucosa (n=15, 30%), ulcers swabs (n=6, 12%), urine (n=4, 8%) and unknown origin (n=5, 10%) were obtained by cultivation method. All of the isolates were identified by conventional biochemical and microscopic procedures. The isolates were maintained in nutrient broth medium containing 50% glycerol at - 80°C.

Antimicrobial susceptibility testing

Antibiotic susceptibility tests were performed using disk diffusion method on Mueller Hinton Agar medium as recommended by the Clinical and Laboratory Standards Institute [18] . The standard strain of *E.coli* ATCC 25922 was used as a negative control and the standard strain of *A.baumannii* ATCC 19606 was also used as a positive control. The antibiotics (Mast Diagnostics, Mast group Ltd., Merseyside, UK) of ampicillin-sulbactam (10/10 µg), aztreonam (30 µg), amikacin (30 µg), cefepime (30 µg), ceftazidime (30 µg), gentamicin(10 µg), imipenem (10 µg), meropenem (10 µg), norfloxacin (10 µg), ofloxacin (1 µg), ciprofloxacin (5 µg), piperacillin-tazobactam (10/100 µg) and tobramycin (10 µg) were selected for use in antibiotic susceptibility tests. The MICs of piperacillin-tazobactam, imipenem and meropenem were determined by broth microdilution assay. According to the various researches the multiple-drug resistant *A.baumannii* is referred to those strains of this bacterium which are resistant to three or more than three classes of antibiotics including quinolones (ciprofloxacin), broad-spectrum cephalosporins (ceftazidime and cefepime), Beta-lactam-beta-lactamase inhibitor combinations (ampicillin-sulbactam), aminoglycosides (amikacin and tobramycin) and carbapenems (imipenem and meropenem).

PCR amplification of the bla$_{KPC}$ gene

Total DNA was extracted using high pure PCR template Preparation Kit (Roche Co, Germany). For amplify the gene of bla$_{kpc}$, the primers used were those as previously described by Poirel *et al.*[15] (Table 1). Each PCR reaction mixture contained 15µl Master mix 1X (Ampliqon III Co, Denmark) that contained 2X PCR buffer, 1.5 mM MgCl2, 1µl template DNA (0.5 µg) , 0.15 mM dNTP, 1.25 U Taq DNA polymerase, 20 pmol of each forward and reverse primers and sterile distilled water up to 30 µL. PCR were performed in a GenAmp PCR system

Table 1. Primers for amplify the gene of bla$_{kpc}$ in *Acinetobacter baumannii* isolates

Nucleotide sequence (5‘to 3‘)	Primer
CGTCTAGTTCTGCTGTCTTG	Kpc-F
CTTGTCATCCTTGTTAGGCG	Kpc-R

Table 2. Frequency of multi- drug resistance in *Acinetobacter baumannii*

	Resistance to one or several antibiotics					Total
Number of antibiotics	1	2	3	4	>4	
Number of isolates resistant *A. baumannii*	7	16	13	5	9	50

Table 3. Antibiotic susceptibility patterns of *Acinetobacter baumannii* strains isolated clinical samples

Antimicrobial agents	*A. baumannii* (n=50) Resistance No.(%)	IntermediateNo.(%)	SusceptibilityNo.(%)
Meropenem	22 (44)	1(2)	27(54)
Imipenem	39 (78)	1(2)	10(20)
Ampicillin-sulbactam	31 (62)	3(6)	16(32)
Piperacillin-tazobactam	24 (48)	1(2)	25(50)
Aztreonam	49 (98)	0	1(2)
Cefepime	50 (100)	0(0)	0(0)
Ceftazidime	50 (100)	0 (0)	0(0)
Gentamicin	32 (64)	3(6)	15(30)
Norfloxacin	48 (96)	0	2(4)
Ofloxacin	46 (92)	0	4(8)
Ciprofloxacin	46 (92)	0	4(8)
Amikacin	45 (90)	0	5(10)
Tobramycin	14 (28)	2(4)	34(78)

(Eppendorf , Harburg, Germany) according to the following program: predenaturation for 10-min at 95^{0}C followed by 30 cycles each containing denaturation at 94^{0}C for 30 sec, annealing at 62^{0}C for 30 sec and extension at 72^{0}C for 40 sec, followed by final extension at 72^{0}C for 5 min. All of the PCR reactions were performed as duplicate. Then, The PCR products were analyzed using the electrophoresis technique on 1.5% agarose gel for 1 hour at 85 Volt and 25mA, stained by SYBERgreen and visualized under UV transilluminator (Fig. 1). Finally, amplification product was further evaluated by sequencing and restriction digestion procedures. Extracted genomes of reference strains of *Klebsiella pneumonia* ATCC BAA-1705 and *Klebsiella pneumoniae* ATCC BAA-1706 were used as positive and negative control in PCR reactions respectively

RESULTS

In the present study, 70 isolates of *Acinetobacter* were isolated from 500 collected samples which 50 (71.4%) samples were identified as *A.baumannii,* 12 (17.1%) samples as *A.lwoffii* and 8 (11.4%) samples were belong to other *Acinetobacter* species. Overall, 82% of the isolates were multidrug- resistant (Table 2). As in the table 1 show, 54% of the isolates were resistant to three or more than three classes of antibiotics and 32% of the isolates were resistant to two classes of antibiotics (Table 1). At least 44% and 78% of the isolates were respectively resistant to meropenem and imipenem (Table 3). These isolates of resistant to carbapenems showed MIC ≥ 32. Also, this isolates have high resistance to piperacillin-tazobactam. The most resistance to tested antibiotics was observed for cefepime and ceftazidime and the most susceptibility was observed for piperacillin-tazobactam, meropenem and tobramycin. Moreover in this research was not detected any isolates of *A.baumannii* that was resistant to all the tested antibiotics.

PCR amplification (Fig.1) of *KPC* gene revealed that 8 (16%) of the isolates contained *bla*$_{KPC}$. This is also the first report of *bla*$_{KPC}$ in

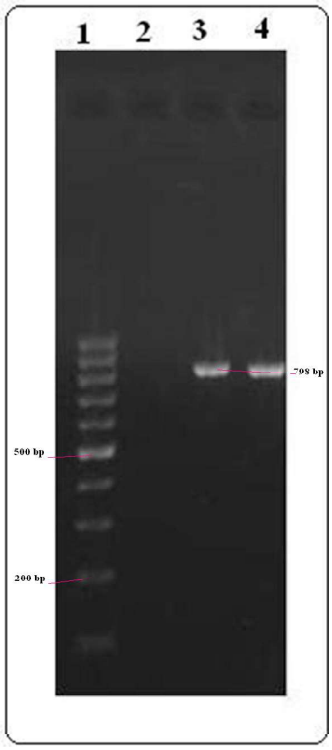

Fig. 1. Agarose gel electrophoresis of PCR amplified products generated from DNA samples. Lanes 1 DNA size marker (100bp DNA ladder, SM#333), Lane 2 negative control (*Klebsiella pneumoniae* ATCC BAA-1706), Lane 3 Positive control (*Klebsiella pneumonia* ATCC BAA-1705) , and Lane 4 show 798 bp bla$_{KPC}$ amplification product in *A. baumannii* strains isolated clinical samples.

A.baumannii in the Iran. Most of these isolates were multidrug-resistant such as meropenem and imipenem (MIC ≥ 16µg/ml). Meanwhile four cases of these strains had been isolated from blood and two cases from trachea of the hospitalized patients in the intensive care units who were treated with multiple antibiotic courses.

DISCUSSION

Acinetobacter baumannii is an opportunistic pathogen and one of the agents of nosocomial infections in the past 30 years. This bacterium especially multidrug-resistant isolates cause serious infectious in hospitalized patients mostly in intensive care units[19]. Due to expanded resistance to antimicrobial drugs, treatment of these infections is often difficult and carbapenems are currently the antibiotics of choice. Although resistance to carbapenems is also increasing. One of the ways of resistance to carbapenems is often associated with acquired carbapenemase production that is under control of the bla$_{kpc}$ gene which is now observed increasingly worldwide[9-11] . As regards the presence of the bla$_{kpc}$ gene in clinical isolates of *A.baumannii* in Iran has not been determined, so the present study was performed in Iran for the first time In order to determine the presence of the KPC gene in clinical isolates of *A.baumannii* in Tehran by PCR method.

In this study, 71.5% of isolates were identified as *A.baumannii* and 28.5% were *A.lwoffii* and other species of *Acinetobacter.* These results are approximately similar to Constantiniu and *et al*. findings at 2001 to 2004 who isolated 24 clinical isolates of *Acinetobacter* which 71% and 29% of them were *A.baumannii* and *A.lwoffii* respectively[20].

The present study like the Bayugo *et al*. studies at 2002 and Joshi *et al*. studies at 2003 showed that antibiotic resistance is seriously increasing so that 82% of the isolated *A.baumannii* strains showed multiple-drug resistance (MDR) phenotype. They in their studies, the rate of isolation of multidrug-resistant *A.baumannii* strains about 45% to 75% have been reported[21,22].

Like the Feizabadi *et al*.[11] study in Ian, this study, susceptibility rate to meropenem and imipenem was reported above 40% which is in contrariety with the findings of Hujer *et al*. [23] who reported a resistance of about 20% to the mentioned antibiotics. In another research which was carried out by Cisneros *et al* in Spain susceptibility rate to the imipenem has been reported about 43%. Anyway according to the various studies like the Zarrilli *et al*. study resistance rate to the carbapenems is increasing worldwide[10,24].

Although a lot of researches have been performed about determining of amount of the bla$_{kpc}$ gene in different isolates of *Klebsiella, Pseudomonas* and *E. coli* but the performed studies on *A.baumannii* in this case are low[24,25]. However the isolation percentage of this gene in various areas is different. for example in a study which carried out by Robledo *et al.,* in Puertorico the isolation rate of *A.baumannii* containing the carbapenemase encoding gene has been reported

about 3.8% which this is less than 16% that was obtained in the present study[24]. In another study that by Zulueta *et al*.[26] was performed in Mexico, the isolation rate of bla_{kpc} gene were reported about 22% which is almost similar to our report in this study. This study like the Poirel *et al*. study a rapid technique based on PCR by a pair of primers were used for identifying of carbapenemases encoding genes in *A.baumannii*[15].

As regards this is the first report of multidrug-resistant *A.baumannii* clinical isolates harboring the bla_{kpc} gene in Iran and as this genes on the transportable elements, It is possible that be transferred from one bacterium to another and in the community are distributed. Presence of the bla_{kpc} gene in *A.baumannii* strains with several acquired and innate mechanisms for resistance can be lead to the emerging of carbapenem resistant *A.baumannii* strains (carbapenems are the selective antibiotic for treating of the infections caused by multiple-drug resistant *A.baumannii* strains)[6,23]. Therefore reasonable consumption of antibiotics in hospitals for controlling of infections especially in ICU departments can has an important preventing role in appearance of these resistant strains and their related infections. This clearly emphasizes the importance of reasonable consumption of antibiotics in hospitals for controlling of infections especially in ICU -and reduce the possibility of nosocomial transmission and potential outbreaks.

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