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Original Article

Antimicrobial Susceptibility Patterns and Distribution of bla_{OXA} Genes among *Acinetobacter* spp. Isolated from Patients at Tehran Hospitals

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SUMMARY: Multiple drug-resistant strains of *Acinetobacter* have created therapeutic problems worldwide. This study was conducted to determine the antimicrobial susceptibility patterns and prevalence of $bla_{OXA\text{-}type}$ carbapenemases among isolates of *Acinetobacter* spp. obtained from Iranian patients. Here, 128 *Acinetobacter* isolates were identified at the species level, and their susceptibilities to different antibiotics were determined using disk agar diffusion testing. Isolates were then subjected to multiplex-PCR targeting bla_{OXA} genes. More than 50% of the isolates showed multidrug resistance to different antibiotics. The rates of susceptibility to imipenem, meropenem, piperacillin-tazobactam, and amikacin were 50.7, 50, 42.1, and 38.2%, respectively. The MICs of carbapenems for the resistant isolates ranged from 64 to ≥256 μg/ml. All strains of *Acinetobacter baumannii* possessed a $bla_{OXA\text{-}51\text{-}like}$ gene. The co-existence of $bla_{OXA\text{-}51\text{-}like}$/$bla_{OXA\text{-}23\text{-}like}$ and $bla_{OXA\text{-}51\text{-}like}$/$bla_{OXA\text{-}24\text{-}like}$ was detected in 25% (n = 32) and 17.9% (n = 23) of the isolates, respectively. Over 70% of carbapenem-resistant strains contained at least two genes encoding OXA-type carbapenemase. Resistance to carbapenems in the population of *Acinetobacter* strains in Iran is high, with the majority of isolates showing multidrug resistance. A wide diversity of OXA genes exists among the strains of *A. baumannii* in Iran. Detection of $bla_{OXA\text{-}51\text{-}like}$ can be used as a simple and reliable method to differentiate *A. baumannii* strains from other species.

INTRODUCTION

Acinetobacter baumannii and its phenotypically related species (*Acinetobacter calcoaceticus*, *Acinetobacter haemolyticus*, and *Acinetobacter lwoffii*) are important agents of nosocomial infections and pneumonia. Immunocompromised patients are at particularly high risk of being infected with these organisms (1,2).

Hospital strains of *Acinetobacter* are usually multidrug resistant. The problem is compounded by increasing rates of resistance to broad-spectrum antibiotics including carbapenems, the drugs of choice against infection with *Acinetobacter* (3,4). However, resistance to these antibiotics has emerged due to the production of carbapenem-hydrolyzing-β-lactamases (carbapenemases) among these organisms (3). Two classes of molecular carbapenemase, classes B and D, have been found among strains of *Acinetobacter*. The enzymes in class D (OXA enzymes) have emerged as the major carbapenemases in the world, although metalloenzymes are mainly prevalent in East Asia (5).

OXA enzymes (encoded by bla_{OXA} genes) can be subclassified into eight distinct subgroups, of which OXA-23-like, OXA-24-like, OXA-51-like, and OXA-58 have been identified in *Acinetobacter* spp. Recent reports from different countries have shown that $bla_{OXA\text{-}51}$-type genes are intrinsically harbored by *A. baumannii* isolates and they support the presence of a direct reservoir of β-lactam-resistance genes within the nosocomial environment (5,6). Determination of the genes encoding resistance to carbapenems would be helpful for gaining a better understanding of the mechanisms of resistance and transmission of resistance among the strains of *Acinetobacter* spp. The aims of this study were to determine the drug susceptibility patterns of *Acinetobacter* strains isolated from patients with nosocomial infections (conditions that develop 48 to 72 h after admission to a hospital) at Tehran hospitals and to identify the genes encoding the four subgroups of OXA-carbapenemases among the isolates.

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MATERIALS AND METHODS

Bacterial strains: Conventional biochemical tests were used for identification at the species level in 128 non-replicative, Gram-negative, short rods showing both a negative reaction on oxidase testing and a lack of lactose fermentation (7). These isolates were cultured from wounds (n = 47, 36.7%), the trachea (n = 30, 23.4%), blood (n = 23, 17.9%), cerebrospinal fluids (n = 11, 8.5%), urine (n = 10, 7.8%), and other tissues (n = 7, 5.4%). These strains were isolated from 7 different wards during the years 2005 - 2006. The majority of isolates were obtained from patients in intensive care units (n = 54, 42.1%), burned patients (n = 42, 32.8%), patients with infectious diseases (n = 11, 8.5%), and surgery patients (n = 8, 6.2%). The remaining isolates were from general admission patients (n = 8, 6.2%) and internal medicine wards (n = 5, 3.9%).

Susceptibility testing: Antimicrobial susceptibility testing was determined by the disk agar diffusion (DAD) method as recommended by the Clinical and Laboratory Standards Institute (8). In brief, an inoculum containing 10^6 CFU was placed on Muller-Hinton agar, and disks containing ampicillin (10 μg), ampicillin-sulbactam (10/10 μg), amoxicillin-

clavulanic acid (20/10 μg), piperacillin-tazobactam (100/10 μg), ticarcillin-clavulanic acid (75/10 μg), cefotaxime (30 μg), cefotetan (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), imipenem (10 μg), meropenem (10 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), amikacin (30 μg), netilmicin (30 μg), tobramycin (10 μg), and gentamicin (10 μg) were then placed on the agar plates. The treated plates were then incubated at 37°C for 24 h. Isolates showing intermediate levels of susceptibility were classified as resistant (9). The MICs of imipenem, meropenem, piperacillin, piperacillin-tazobactam, and cefotaxime (MAST, Merseyside, UK) were determined using microbroth dilution assay. *Escherichia coli* ATCC 25922 and ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality controls in each susceptibility determination.

PCR amplification of bla_{OXA} alleles: DNA was extracted from the strains by boiling one to three colonies in 100 μl of sterile ultrapure water for 10 min followed by centrifugation for 1 min at 14,000 rpm (10). To amplify the genes encoding carbapenemases, a multiplex-PCR assay was run using the primers $bla_{OXA\text{-}51\text{-}like}$ (353 bp: 5′-TAA TGC TTT GAT CGG CCT TG-3′ and 5′-TGG ATT GCA CTT CAT CTT GG-3′), $bla_{OXA\text{-}23\text{-}like}$ (501 bp: 5′-GAT CGG ATT GGA GAA CCA GA and 5′-ATT TCT GAC CGC ATT TCC AT), $bla_{OXA\text{-}24\text{-}like}$ (246 bp: 5′-GGT TAG TTG GCC CCC TTA AA and 5′-AGT TGA GCG AAA AGG GGA TT) and $bla_{OXA\text{-}58\text{-}like}$ (599 bp: 5′-AAG TAT TGG GGC TTG TGC TG and 5′-CCC CTCTGCGCTC TACATAC) (11).

Amplification was performed in a final volume of 50 μL containing reaction buffer 1×, 2 mM $MgCl_2$, 2 mM dNTP, 500 nM primers, 1.6 U *Taq* polymerase (Metabion, Martinsried, Germany), and 10 - 100 ng of DNA templates.

The thermocycler (Eppendorf, Hamburg, Germany) was programed at 94°C for 5 min followed by 30 cycles of 25 s at 94°C, 40 s at 53°C, 50 s at 72°C, and a final cycle of 6 min at 72°C.

The *A. baumannii* reference strains NCTC 13304, NCTC 13302, NCTC 12156, and NCTC 13305 were used as positive controls for the $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$, and $bla_{OXA\text{-}58\text{-}like}$ genes, respectively. A pool of DNAs extracted from reference strains were used as positive controls in all multiplex-PCR assays. The PCR products were separated by agarose gel electrophoresis. DNA from a clinical isolate of *P. aeruginosa* was used as a negative control in the amplification study.

RESULTS

Biochemical and conventional methods enabled the identification of 108 isolates as *A. baumannii*. The remaining isolates were identified as non-*A. baumannii* strains (*A. calcoaceticus*, $n = 10$; *A. lwoffii*, $n = 5$; and *A. haemolyticus*, $n = 5$). The susceptibility of isolates to different antibiotics is shown in Table 1.

The carbapenem-resistant isolates detected by DAD testing showed a high degree of resistance to these antibiotics in the microbroth dilution assay (MIC range, ≥64 to ≥256). The MICs of carbapenem against 25 isolates (19.5%) that were identified as susceptible by DAD testng were in the intermediate range (MIC = 8). Moreover, the carbapenem-resistant isolates showed a high level of resistance to piperacillin, piperacillin-tazobactam (MIC range, ≥128 to ≥1,024), and cefotaxime (MIC ≥ 256). The MIC_{50} and MIC_{90} of these antibiotics are shown in Table 2.

All *A. baumannii* ($n = 108$) isolates tested positive for $bla_{OXA\text{-}51\text{-}like}$ by PCR, and 34 (26.5%) of these 108 isolates lacked the other OXA genes. Species other than *A. baumannii* ($n = 20$) were negative for the $bla_{OXA\text{-}51\text{-}like}$ gene. The co-existence of two different bla_{OXA} genes in the samples was observed, and their relative amounts were in the following order: $bla_{OXA\text{-}51\text{-}like}$ plus $bla_{OXA\text{-}23\text{-}like}$ 25% ($n = 32$), $bla_{OXA\text{-}51\text{-}like}$ plus $bla_{OXA\text{-}24\text{-}like}$ 17.9% ($n = 23$), $bla_{OXA\text{-}51\text{-}like}$ plus $bla_{OXA\text{-}58\text{-}like}$

Table 1. Antimicrobial susceptibility pattern of *Acinetobacter* spp. isolates in Tehran hospitals according to disk agar diffusion method

Antimicrobial	*A. baumannii* ($n = 108$)		Non-*A. baumannii* ($n = 20$)		Total ($n = 128$)	
	Susceptible no. (%)	Resistant no. (%)	Susceptible no. (%)	Resistant no. (%)	Susceptible no. (%)	Resistant no. (%)
Ampicillin	0 (0)	108 (100)	1 (5)	19 (95)	1 (1.27)	127 (97.3)
Ampicillin-sulbactam	18 (16.7)	90 (83.3)	12 (60)	8 (40)	30 (23.4)	98 (76.6)
Amoxicillin-clavulanic acid	0 (0)	108 (100)	8 (40)	12 (60)	8 (6.2)	120 (93.8)
Piperacillin	38 (35.2)	70 (64.8)	6 (30)	14 (70)	44 (24.4)	84 (65.6)
Piperacillin-tazobactam	41 (38)	67 (62)	13 (65)	7 (25)	54 (42.1)	74 (57.9)
Ticarcillin-clavulanic acid	0 (0)	108 (100)	9 (45)	11 (55)	9 (17)	119 (93)
Cefotaxime	8 (7.5)	100 (92.5)	3 (15)	17 (85)	11 (8.5)	117 (91.5)
Cefotetan	0 (0)	108 (100)	1 (5)	19 (95)	1 (2.7)	127 (97.3)
Ceftazidime	5 (4.7)	103 (95.3)	6 (30)	14 (70)	11 (8.5)	117 (91.5)
Ceftriaxone	7 (6.5)	101 (83.5)	5 (25)	15 (75)	12 (19.3)	116 (90.7)
Imipenem	53 (49.1)	55 (50.9)	12 (60)	8 (40)	65 (50.7)	63 (49.3)
Meropenem	52 (48.2)	56 (51.8)	12 (60)	8 (40)	64 (50)	64 (50)
Ciprofloxacin	13 (12.1)	95 (87.9)	12 (60)	8 (40)	25 (18.5)	103 (81.5)
Levofloxacin	18 (16.7)	90 (83.3)	15 (75)	5 (25)	33 (35.7)	95 (74.3)
Amikacin	38 (35.2)	70 (64.8)	11 (55)	9 (45)	49 (48.2)	79 (61.8)
Netilmicin	16 (14.9)	92 (85.1)	2 (10)	18 (90)	18 (14)	110 (86)
Tobramycin	16 (14.9)	92 (85.1)	10 (50)	10 (50)	26 (20.3)	102 (79.7)
Gentamicin	14 (13)	94 (87)	10 (50)	10 (50)	24 (18.7)	104 (81.3)

Table 2. Distribution of bla_{OXA} alleles and determination of MICs for imipenem, meropenem, piperacillin, piperacillin-tazobactam and cefotaxime among carbapenem resistance *A. baumannii* and non-*A. baumannii* strains showing resistance to these antimicrobial agents in disk agar diffusion

Acinetobacter spp.	bla_{OXA} allele	No. of resistant isolates	Antibiotic	MIC (μg/ml)		
				Range	MIC_{50}	MIC_{90}
A. baumanniii	bla_{OXA-51} only	4	Imipenem	16 - ≥256	16	64
			Meropenem	16 - ≥256	16	64
			Piperacillin	1 - ≥2,046	512	1,024
			Piperacillin-tazobactam	1 - ≥2,046	512	1,024
			Cefotaxime	1 - ≥2,046	512	1,024
	$bla_{OXA-51/OXA-23}$	22	Imipenem	16 - ≥256	16	128
			Meropenem	16 - ≥256	16	128
			Piperacillin	1 - ≥2,046	512	1,024
			Piperacillin-tazobactam	1 - ≥2,046	512	1,024
			Cefotaxime	1 - ≥2,046	512	1,024
	$bla_{OXA-51/OXA-24}$	10	Imipenem	16 - ≥256	16	128
			Meropenem	16 - ≥256	16	128
			Piperacillin	1 - ≥2,046	512	1,024
			Piperacillin-tazobactam	1 - ≥2,046	512	1,024
			Cefotaxime	1 - ≥2,046	512	1,024
	$bla_{OXA-51/OXA-58}$	5	Imipenem	64 - 128	64	128
			Meropenem	32 - 128	64	128
			Piperacillin	512 - ≥2,046	512	≥2,046
			Piperacillin-tazobactam	128 - 1,024	128	1,024
			Cefotaxime	128 - 1,024	128	1,024
	$bla_{OXA-51/OXA-23/OXA-58}$	5	Imipenem	128 - ≥256	128	≥256
			Meropenem	128 - ≥256	128	≥256
			Piperacillin	128 - 512	128	512
			Piperacillin-tazobactam	64 - 128	64	128
			Cefotaxime	512 - 1,024	512	1,024
	$bla_{OXA-51/OXA-23/OXA-24}$	6	Imipenem	128 - ≥256	128	≥256
			Meropenem	128 - ≥256	128	≥256
			Piperacillin	512 - 1,024	512	1,024
			Piperacillin-tazobactam	512 - 1,024	512	1,024
			Cefotaxime	512 - 1,024	512	1,024
	$bla_{OXA-51/OXA-24/OXA-58}$	1	Imipenem	128	128	128
			Meropenem	64	64	64
			Piperacillin	512	512	512
			Piperacillin-tazobactam	512	512	512
			Cefotaxime	1,024	1,024	1,024
	$bla_{OXA-51/OXA-23/OXA-24/OXA-58}$	2	Imipenem	64 - 128	64	128
			Meropenem	32 - 64	32	64
			Piperacillin	128 - 512	128	512
			Piperacillin-tazobactam	128 - 512	128	512
			Cefotaxime	512 - 1,024	512	1,024
Non-*A. baumannii*	bla_{OXA-23} only	1	Imipenem	32	32	32
			Meropenem	16	16	16
			Piperacillin	512	512	512
			Piperacillin-tazobactam	512	512	512
			Cefotaxime	1,024	1,024	1,024
	$bla_{OXA-23/OXA-24}$	2	Imipenem	128 - 128	128	128
			Meropenem	64 - 128	64	128
			Piperacillin	512 - 512	512	512
			Piperacillin-tazobactam	128 - 512	128	512
			Cefotaxime	512 - 512	512	512

9% (n = 5), and $bla_{OXA-23-like}$ plus $bla_{OXA-24-like}$ 1.5% (n = 2).

Isolates containing 3 or 4 carbapenamase genes were also identified, i.e., bla_{OXA-51} plus bla_{OXA-23} plus $bla_{OXA-24-like}$ (n = 6, 4.6%), bla_{OXA-51} plus bla_{OXA-58} plus bla_{OXA-23} (n = 5, 3.9%), bla_{OXA-51} plus bla_{OXA-23} plus bla_{OXA-24} plus $bla_{OXA-58-like}$ (n = 2, 1.5%), and bla_{OXA-51} plus bla_{OXA-58} plus bla_{OXA-24} (n = 1, 0.7%).

According to the observed MIC values, more than 70% of the carbapenem-resistant *Acinetobacter* spp. isolates possessed at least two genes encoding OXA-type enzymes. The co-existence of $bla_{OXA-51-like}$/$bla_{OXA-23-like}$ and $bla_{OXA-51-like}$/$bla_{OXA-24-like}$ genes was more common among carbapenem-resistant strains than among the other strains evaluated (Table 2).

Of 3 carbapenem resistant *A. calcoaceticus* isolates, 2 were

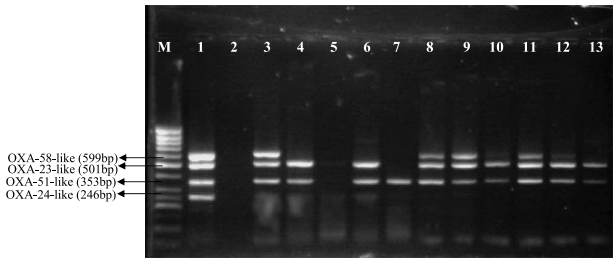

Fig. 1. Detection of genes encoding OXA carbapenemase by multiplex PCR. M, 100 bp DNA ladder; Lane 1, a pull of all DNAs from reference strains as positive control; 2, negative control (*Pseudomonas aeruginosa* DNA, field strain); 5, *Acinetobacter* spp. lacking any OXA gene; 3-4 and 6-13, different isolates containing various OXA genes.

positive for the $bla_{\text{OXA-23}}/_{\text{OXA-24}}$ allele and one was positive for the $bla_{\text{OXA-23}}$ allele (Table 2). No $bla_{\text{OXA-type}}$ genes were detected among 5 carbapenem-resistant strains of non-*A. baumannii*; these strains included *A. calcoaceticus* (n = 2), *A. lwoffii* (n = 2), and *A. haemolyticus* (n = 1).

The distribution of bla_{OXA} alleles among *A. baumannii* isolates with an intermediate level of resistance (MIC = 8) was follows: $bla_{\text{OXA-51-like}}$ (n = 2), $bla_{\text{OXA-51-like}}/bla_{\text{OXA-23-like}}$ (n = 10), and $bla_{\text{OXA-51-like}}/bla_{\text{OXA-24-like}}$ (n = 13).

Figure 1 shows the variance in the size of amplicon products of the multiplex-PCR assay for different OXA genes.

DISCUSSION

A. baumannii accounts for a substantial proportion of endemic nosocomial infections. Recent reports have indicated that the antimicrobial resistance of *Acinetobacter* isolates is increasing, which consequently poses an increased threat to hospitalized patients (12-14). The spread of antimicrobial resistance among *Acinetobacter* spp. in Iran has emerged as an important challenge for Iranian infectious disease specialists. Unfortunately, no data are available on the antimicrobial susceptibility of Iranian isolates of *Acinetobacter* or on the distribution of genes involved in resistance to carbapenems. In addition, little is known about larger patterns of isolate resistance in the Middle East in general.

The susceptibility of *A. baumannii* isolates to different antibiotics at our hospital was lower than that reported in other countries (15). With a susceptibility rate of 39%, we found piperacillin-tazobactam to be the most effective β-lactam after the group of carbapenems against *Acinetobacter* isolates. Our finding is similar to reports from Western countries (16).

Carbapenems have been the drug of choice for the treatment of infections with *Acinetobacter* spp. However; in recent years, the number of isolates showing resistance to these antibiotics has increased (17,18). The main enzymes involved in resistance are OXA-carbapenemases. Outbreaks of genes encoding OXA-type carbapenemases have occurred globally, but have only arisen sporadically. Nosocomial outbreaks with *Acinetobacter* strains producing these enzymes have been reported in Brazil, French Polynesia, Spain, Southern Europe, the Balkans, Turkey, Korea, and Argentina (19-26). This is the first report of the existence of OXA-type carbapenemases among Iranian isolates of *Acinetobacter*.

Regardless of their susceptibility to carbapenems, all 108 isolates of *A. baumannii* in this study produced a 353-bp amplicon, which corresponded to the $bla_{\text{OXA-51-like}}$ gene in a PCR study. This gene was not detected among any other *Acinetobacter* spp. The present results support those of a previous report, suggesting that $bla_{\text{OXA-51-like}}$ is species-specific to *A. baumannii* (10-26). The relationship between $bla_{\text{OXA-51-like}}$ and the resistance of *A. baumannii* isolates to carbapenem still needs to be investigated; however, this relationship may not represent a true correlation, as already reported by other sources (10,11,27). Other groups have reported that IS*Aba1* (insertion sequence *A. baumannii*), which is adjacent to $bla_{\text{OXA-51-like}}$, plays a major role in the development of resistance to carbapenems. In previous studies, carbapenem resistance was associated only with isolates in which IS*Aba1* was upstream of $bla_{\text{OXA-51-like}}$, suggesting that IS*Aba1* provids the promoter for this gene (28).

The genes encoding the OXA-23/OXA-51 conjugate and the OXA-51/OXA-24 conjugate were predominant among the carbapenem-resistant isolates evaluated here (Table 2). Isolates with these patterns showed a high degree of resistance to β-lactam antibiotics such as carbapenems (imipenem and meropenem) and cephalosporines (ceftazidime and ceftriaxone). Nearly 80% of the isolates were resistant to cephalosporines such as ceftazidime. These isolates were also resistant to the aminoglycoside group of antibiotics, as only amikacin was found to have a slight effect on the *Acinetobacter* isolates (37.5% susceptibility rate). Based on the results obtained in this study and reports from other countries, alleles encoding OXA-23-like, OXA-24-like, OXA-58-like, or a combination of these alleles, were consistently associated with resistance or at least with reduced susceptibility to antibiotics (20,22,23,25). In the present study, the presence of certain *A. baumannii* (n = 25) isolates showing intermediate susceptibility to carbapenem might be attributed to the simultaneous presence of different bla_{OXA} alleles. Here, we did not observe any correlation between existence of any bla_{OXA} alleles and a resistance to other antibiotics such as piperacillin, piperacillin-tazobactam, or cefotaxime; here, all carbapenem-resistant strains showed resistance to these antimicrobials, and the distribution patterns of bla_{OXA} alleles among these multidrug-resistant strains varied (Table 2).

In conclusion, our findings indicate that multidrug-resistant *A. baumannii* strains are spreading and that carbapenemase resistance is increasingly common in Iran. We also identified at least one gene encoding OXA carbapenemase in 47 carbapenem-resistant *Acinetobacter* isolates; nevertheless, with exception of $bla_{\text{OXA-51-like}}$, no other carbapenemase-encoding alleles were detected in 5 carbapenem-resistant *Acinetobacter* spp. isolates. These results may suggest that various mechanisms of carbapenem resistance could have contributed to the carbapenem resistance in these isolates (e.g., an unknown OXA carbapenemase, increased efflux of β-lactam antibiotics, reduced affinity of penicillin-binding proteins for carbapenems, decreased permeability of the outer membrane, a combination of reduced permeability and high-level production of a β-lactams, etc.) (29).

Our findings also emphasize the importance of making updated susceptibility data for *A. baumannii* available to physicians in developing countries such as Iran. Continuous monitoring of changes in *Acinetobacter* spp. resistance will help determine national priorities for local intervention efforts.

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