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Distribution of IS*Aba1* and IS*Aba2* among OXA-type Carbapenemase-Producing Clinical Isolates of *Acinetobacter baumannii* from Hospitals in Tehran, Iran

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The purpose of this study was to determine the antimicrobial susceptibility patterns of *Acinetobacter baumannii* isolates and identify the presence of oxacillin-hydrolyzing (OXA) carbapenemase genes and their related insertion sequences (ISs), which have an important role in drug resistance. A total of 105 clinical isolates of *A. baumannii* were tested for their susceptibility to different antibiotics using a disc diffusion method. All isolates were evaluated for the presence of the $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, and $bla_{\text{OXA-58-like}}$ genes along with IS elements by polymerase chain reaction (PCR). The PCR analysis showed the presence of the $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-23-like}}$ genes and IS*Aba1* in all isolates. About 64.76% of the isolates were positive for the $bla_{\text{OXA-24-like}}$ gene, and 92.38% were positive for IS*Aba2*. All isolates were negative for the $bla_{\text{OXA-58-like}}$ gene. The lowest resistance rates were observed for trimethoprim/sulfamethoxazole (92.38%) and minocycline and amikacin (93.33% both). The presence of IS elements in the clinical isolates, detected in the current study, can explain the rapid dissemination of resistance genes. The high prevalence rates of OXA genes among the *A. baumannii* isolates show that it is necessary to control nosocomial infections and isolate patients with these infections.

Keywords: *A. baumannii*, β–lactamases, OXA genes, IS*Aba1*, IS*Aba2*.

Acinetobacter baumannii is a non-fermentative, Gram-negative coccobacillus associated with a variety of serious infections[1, 2]. *A. baumannii* is now recognized as one of the most common bacterial causes of infectious diseases in hospitals throughout the world[3]. This opportunistic pathogen causes infections that are not well treated because of its ability to survive in the presence of most classes of antimicrobial drugs[3]. The pathogen has many innate and acquired mechanisms for antibiotic resistance, such as alterations in the target site, overproduction of efflux pumps, low permeability of the outer membrane, and inactivation of enzymes including â-lactamases4. â-Lactamases, which are grouped into four classes (A, B, C, and D) according to amino-acid sequence similarities, are

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enzymes that degrade â-lactam antibiotics[5]. â-Lactam antibiotics, including penicillins, cephalosporins, and carbapenems, inhibit peptidoglycan transpeptidases. The group D or carbapenem-hydrolyzing class D â-lactamases are known as oxacillinases (OXA) because of their ability to hydrolyze oxacillin[6]. These enzymes are classified into four main OXA subfamilies: OXA-23-like, OXA-24/40-like, OXA-58-like, and OXA-51-like, with the latter chromosomally located in all *A. baumannii* strains. Thus, it is an important genetic marker for the identification of these bacteria[7, 8]. The OXA types have weak hydrolytic properties, but their genes are usually associated with genetic elements, such as insertion sequences (ISs), able to increase the expression of carbapenemases and also mobilize the genes[7, 9]. The most common IS is IS*Aba1*, although other IS elements, such as IS*Aba2*, are also associated with the resistance. When an IS element is located upstream of several OXA-type â-lactamase genes, such as the $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-51-like}}$, and $bla_{\text{OXA-58-like}}$ genes, it increases their expression to a level that confers resistance to carbapenems[5, 10, 11]. Therefore, it is very important to evaluate the prevalence of ISs as well as the OXA genes in *A. baumannii* from different hospitals and medical centers.

To our knowledge, there are no data regarding the occurrence of OXA genes, together with members of the IS family, especially IS*Aba2*, in *A. baumannii* isolates from different hospitals in Tehran, Iran. Since IS elements are transferable and can increase antibiotic resistance of bacteria, we aimed in this study to evaluate the antimicrobial activity of 14 antibiotics against clinical isolates of *A. baumannii*, which were collected from five hospitals in Tehran, Iran. Furthermore, the distribution of the four subgroups of OXA carbapenemase genes, as well as the presence of the insertion sequences IS*Aba1* and IS*Aba2*, were studied in *A. baumannii*.

MATERIALS AND METHODS

Bacterial isolates

In this study, a total of 105 clinical isolates of *A. baumannii* were collected from patients in five different hospitals in Tehran between 2014 and 2015. Phenotypic identification of the isolates as *A. baumannii* was performed using biochemical tests, including growth at 42 °C, culture on selective media, negative oxidase test, lack of lactose fermentation, etc. The PCR amplification and sequencing of the $bla_{\text{oxa-51-like}}$ gene were used to confirm strain identity as *A. baumannii*.

Antimicrobial susceptibility tests

Antimicrobial susceptibility testing of the isolates against a panel of 14 agents was performed by the Kirby–Bauer disc diffusion method on the Mueller–Hinton agar (Merck, Germany) according to the current Clinical and Laboratory Standards Institute guidelines (2014). The following antimicrobials were used for the susceptibility tests: amikacin (AMK, 30 µg), ceftazidime (CAZ, 30 µg), cefepime (FEP, 30 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CRO, 30 µg), ciprofloxacin (CIP, 5 µg), gentamicin (GEN, 10 µg), imipenem (IMP, 10 µg), meropenem (MEM, 10 µg), piperacillin (PIP, 100 µg), piperacillin/tazobactam (TZP, 100 + 10 µg, respectively), tigecycline (TGC, 15 µg), minocycline (MCN, 30 µg), and trimethoprim/sulfamethoxazole (SXT, 1.25 + 23.75 µg, respectively). All antibiotics were purchased from Rosco Diagnostica, Denmark.

DNA extraction

Genomic DNA was extracted using a standard DNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions. A total of 5 ìL of extracted DNA was used for each reaction.

Molecular detection of OXA carbapenemase genes and IS elements and nucleotide sequencing

All isolates were subjected to PCR for the detection of the four main groups of OXA carbapenemase genes ($bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-58-like}}$, and $bla_{\text{OXA-24-like}}$), as well as the IS*Aba1* and IS*Aba2* elements. The primers used in this study are listed in Table 1.

The PCR mixture contained 1 µL of extracted DNA, 1 µL of each forward and reverse primer, 9.5 µL of water, and 12.5 µL of Master Mix (Ampliqon, Denmark). Amplification was performed in a thermocycler (Eppendorf, Germany) programmed for the following amplification conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 45 s at 94 °C (denaturation), 53–55 °C (annealing), and 45 s at 72 °C (extension), and final extension at 72 °C for 7 min (Table 2). In contrast to the other amplicons, the annealing temperature for IS*Aba2* was 48 °C. The amplified

PCR products were analyzed by 1.5% agarose gel electrophoresis at 100 V for 50 min in 1X Tris/borate/EDTA buffer containing RedSafe (iNtRON Biotechnology, Korea). The DM 100–2,300-base pair (bp) ladder (SMOBiO, Taiwan) was used as a molecular weight marker. Gels were photographed using a UVIdoc gel documentation system (Uvitec, UK). The PCR products were purified using a PCR purification kit (Bioneer), and sequencing was performed by the Bioneer Company (Korea).

RESULTS AND DISCUSSION

In this study, we investigated 105 clinical isolates of *A. baumannii* from five hospitals in Tehran. After strain identification by conventional methods, antimicrobial susceptibility testing was performed. The results of the susceptibility testing are presented in Table 3. The high rates of resistance shown in the table indicate that this is a phenotype often observed in *A. baumannii*. Indeed, 100% of the isolates were resistant to CTX, and the resistance to other cephalosporins was also very high. Carbapenems, such as IMP and MEM, also showed high rates of resistance. The lowest rate of resistance was observed for SXT.

The distribution of OXA-type genes and IS elements in the A. *baumannii* strains isolated from the hospitals in Tehran is shown in Table 4. All isolates were positive for the $bla_{\text{OXA-51-like}}$ gene, which confirmed their identity as *A. baumannii*, and negative for the carbapenemases belonging to the OXA-58 family. Furthermore, 100% of the isolates carried $bla_{\text{OXA-23-like}}$ genes, which are one of important resistance factors in this bacterium, and 64.76% of the isolates carried $bla_{\text{OXA-24-like}}$ genes. The IS*Aba1* sequences were found in all analyzed strains of *A. baumannii*. The IS*Aba2* sequences were detected in 92.38% of the isolates. The results of agarose gel electrophoresis of the PCR products of all OXA genes and the IS elements are presented in Fig. 1. It was confirmed that the $bla_{\text{OXA-51-like}}$ and IS*Aba1* sequences were present in all samples, but we did not find the $bla_{\text{OXA-58-like}}$ sequences in the samples obtained from the hospitals selected. The presence of the $bla_{\text{OXA-23-like}}$ and IS*Aba2* sequences was observed in most cases.

A. baumannii has globally emerged as one of the most troublesome pathogens for healthcare institutions[12]. The ability of this microorganism to survive in both dry and wet conditions and its adherence to plastic and metal materials lead to a failure in hygiene prophylaxis. Changes in growth conditions may affect the transposition efficiency of several mobile elements. Those in turn may increase the resistance by upregulating gene expression or by conferring different resistance mechanisms. In addition, resistance genes can be expressed on plasmids and chromosomes, particularly genes of resistance to carbapenems[13]. Carbapenems are antibiotics of choice for the treatment of infections caused by *A. baumannii* when this bacterium is resistant to other â-lactam antibiotics. However, resistance to carbapenems has increased, and this can limit the choice of antibiotics for physicians. Generally, carbapenem resistance is associated with production of OXA enzymes, but metallo-â-lactamases can also confer resistance to carbapenems in *A. baumannii*[14, 15].

There are four main OXA subgroups associated with *A. baumannii*, and their respective genes are named $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-58-like}}$, and $bla_{\text{OXA-24–like}}$. The largest subgroup is OXA-51-like, which corresponds to chromosomal-encoded enzymes, and the PCR performed in our study showed that all 105 clinical isolates were positive for the $\text{bla}_{\text{OXA-51-like}}$ gene. A survey conducted by Visca *et al.*[16] also demonstrated the presence of the $bla_{\text{OXA-51-like}}$ gene among 117 strains of *A. baumannii*. Similar results were obtained in other previous studies as well[17-20]. Our study confirmed that the detection of the $bla_{\text{OXA-51–like}}$ gene can be used as a simple and reliable way to identify *A. baumannii*[18, 19, 21-23]. The antimicrobial susceptibility profiles of the 105 isolates are shown in Table 1, and most of them showed wide resistance spectra, a phenotype often observed in *Acinetobacter*. All isolates were resistant to CTX, and the rates of resistance to the other cephalosporins were high. These results are in accordance with those of other studies[19, 20, 24, 25]. Unfortunately, high resistance rates were found among *A. baumannii* isolates not only to â-lactams, including penicillin, third-generation cephalosporin, and carbapenems, but also to other drug classes, including aminoglycosides and fluoroquinolones[21]. These high rates are shown in Table 1, and the rates of resistance to antibiotics

such as PIP, TZP, GEN, MCN, and CIP were also high. In this study, the rates of resistance to IMP and MEM were 95.23 and 98.09%, respectively. These results showed that the high prevalence rates of resistance to carbapenems and the frequency of multiple resistance genes, such as OXA genes, are the causes of antibiotic resistant among these bacteria. The $bla_{\text{OXA-23}}$ carbapenemase gene has increasingly been reported worldwide[19]. The OXA-23 â-lactamase was first identified in an *A. baumannii* isolate from Edinburgh, United Kingdom, and the resistance phenotype was transferable, indicating a plasmid location[10]. OXA-23-like enzymes are able to hydrolyze oxyimino-cephalosporins, aminopenicillins, piperacillin, oxacillin, and aztreonam in addition to carbapenems[10]. In this study, the $bla_{\text{OXA-23-like}}$ gene was detected in 100% of the isolates, which is in accordance with other studies in the world[17, 26-29].

Another OXA-type carbapenemase is encoded by the $bla_{\text{OXA-24-like}}$ gene, and its prevalence in the isolates tested in the present study was 64.76%, with most of the isolates obtained from one hospital. The frequency of the $bla_{\text{OXA-24-like}}$ gene in Tehran was only 15% in 2009[30] and 17.3% in 2012[31, 32]. The last OXA-type carbapenemase investigated in our study is encoded by the $bla_{\text{OXA-58-like}}$ gene. None of the 105 clinical isolates in this study were positive for the $bla_{\text{OXA-58-like}}$ gene, and these results are similar to those from other studies conducted in Iran[30]. ISs are frequently identified in association with OXA â-lactamase genes[10], and the most prevalent is IS*Aba1*. IS*Aba1*, which has 11-bp inverted repeat sequences flanked by 9-bp direct repeats of the target sequence, has been identified in *A. baumannii* and, like many IS elements, contains promoters that play a role in the expression of antibiotic resistance genes[19]. In our study, all 105 clinical isolates were PCR-positive for IS*Aba1*, a finding similar to the data from previous studies[18,33]. Turton and other authors[17, 18] have proposed that insertion of IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$

Table 1. PCR primers to detect genes encoding OXA Carbapenemase and IS elements

Primers	Nucleotide sequences (5¹ to 3¹)	Amplicon size (bp)
OXA 51F	TAATGCTTTGATCGGCCTTG	353
OXA 51R	TGGATTGCACTTCATCTTGG	
OXA23F	GATCGGATTGGAGAACCAGA	501
OXA23R	ATTTCTGACCGCATTTCCAT	
OXA24F	GGTTAGTTGGCCCCCTTAAA	246
OXA24R	AGTTGAGCGAAAAGGGGATT	
OXA58F	AAGTATTGGGGCTTGTGCTG	599
OXA58R	CCCCTCTGCGCTCTACATAC	
ISAba1F	GTGCTTTGCGCTCATCATGC	435
ISAba1R	CATGTAAACCAATGCTCACC	
ISAba2F	AATCCGAGATAGAGCGGTTC	1100
ISAba2R	TGACACATAACCTAGTGCAC	

Table 2. The PCR cycles conditions to amplify the OXAs genes and ISA elements

Steps	OXA-51	OXA-23	OXA-24	OXA-58	ISAba1	ISAba2	Repeats
Activation	94°c/5min	94°c/5min	94°c/5min	94°c/5min	94°c/5min	94°c/5min	1 cycle
Denaturation	94°c/45s	94°c/45s	94°c/45s	94°c/45s	94°c/45s	94°c/45s	30 cycles
Annealing	54°C	53°C	54°C	54°C	55°C	48°C	
Extension	72°c/45s	72°c/45s	72°c/45s	72°c/45s	72°c/45s	72°c/45s	
Final extension	72°c/7min	72°c/7min	72°c/7min	72°c/7min	72°c/7min	72°c/7min	-

gene may provide the promoter to enhance gene expression, potentially contributing to increased levels of carbapenem resistance. IS*Aba2* was the last genetic element that we investigated. This element, similar to IS*Aba1*, can affect the expression of OXA carbapenemases. In the present study, the prevalence rate of IS*Aba2* was 92.38% (Table 3), and the high rate of this IS can account for enhanced promoter activity associated with resistance genes.

In conclusion, *A. baumannii* is an important pathogen in many countries. Based on the results obtained in this study, it can be considered as a red-alarm microorganism in hospitals, which causes high rates of mortality and morbidity due to its multiple mechanisms of resistance and fails to be treated with common antibiotics or is difficult to treat. This microorganism can cause serious and long-lasting infections, especially in children and immunodeficient patients. Our study was done on some of the genetic elements that can have a major role in resistance to antimicrobial drugs, particularly on mobile elements, which can be transferred between species and transform their antimicrobial patterns to increase resistance. Identification of these factors can help control the infection and reduce the prevalence rate of this microorganism.

Table 3. Antimicrobial susceptibility testing of A. *baumannii* isolated from hospitals in Tehran

Antibiotics	Concentrations	Sensitive	Resistant
Minocycline	30µg	7 (6.66%)	98 (93.33%)
Trimethoprim/ Sulfamethoxazole	1.25 µg + 23.75µg	8 (7.61%)	97 (92.38%)
Piperacillin	100 µg	2 (1.9%)	103 (98.09%)
Ceftazidime	30µg	4 (3.8%)	101 (96.19%)
Cefotaxime	30µg	0 (0%)	105 (100%)
Gentamicin	10µg	4 (3.8%)	101 (96.19%)
Ciprofloxacin	5µg	1 (0.95%)	104 (99.04%)
Amikacin	30µg	7 (6.66%)	98 (93.33%)
Tigecycline	15µg	2 (1.9%)	103 (98.09%)
Imipenem	10µg	5 (4.76%)	100 (95.23%)
Cefepime	30µg	1 (0.95%)	104 (99.04%)
Ceftriaxone	30µg	1 (0.95%)	104 (99.04%)
Meropenem	10µg	2 (1.9%)	103 (98.09%)
Piperacillin/tazobactam	100µg + 10µg	4 (3.8%)	101 (96.19%)

Table 4. Distribution of OXA type genes and IS elements of A. *baumannii* isolated from hospitals in Tehran

Genes	Percentage					
	Total	Loghman	Motahari	Taleghani	Milad	Mofid
OXA 51	105 (100%)	17 (100%)	33 (100%)	3 (100%)	48 (100%)	4 (100%)
OXA23	103 (98.09%)	17 (100%)	31 (93.93%)	3 (100%)	48 (100%)	4 (100%)
OXA58	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
OXA24	68 (64.76%)	17 (100%)	31 (93.93%)	3 (100%)	14 (11.53%)	3 (75%)
ISAba1	105 (100%)	17 (100%)	33 (100%)	3 (100%)	48 (100%)	4 (100%)
ISAba2	97 (92.38%)	12 (70.58%)	32 (96.96%)	3 (100%)	46 (95.83%)	4 (100%)

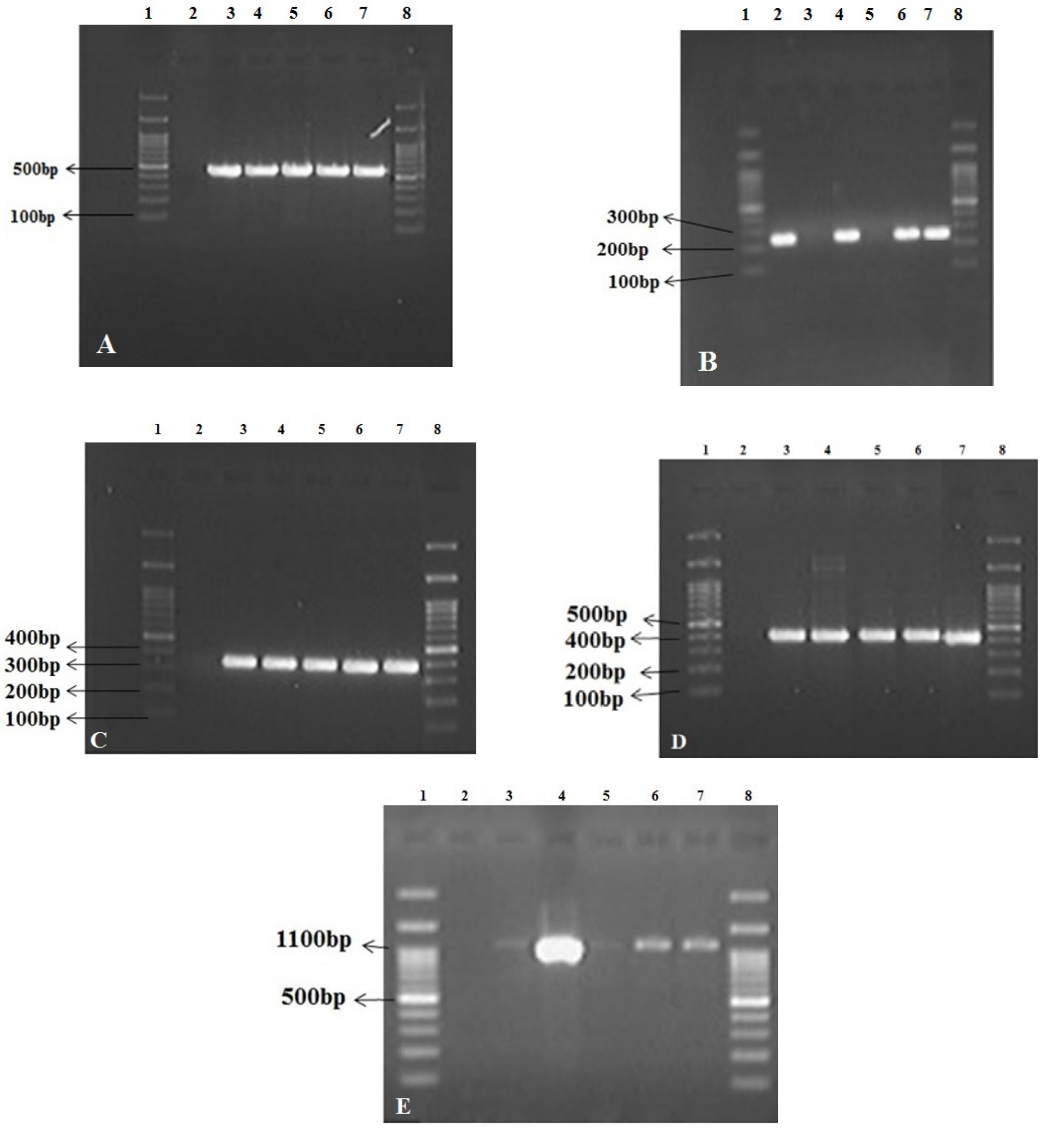

Fig. 1. Agarose gel electrophoresis of PCR products of OXAs genes and IS elements. A: OXA23; B: OXA24; C: OXA51; D: ISAba1; E: ISAba2. Band 1 and 8: Ladder; Band 2: negative; Band 7: positive; Bands 3-6: PCR products of related genes

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