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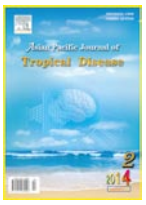

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Molecular epidemiology of hospital acquired OXA-carbapenemase-producing *Acinetobacter baumannii* in Western Iran

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ABSTRACT

Objective: To determine the antimicrobial susceptibility patterns and prevalence of bla_{OXAtype} carbapenemases among *Acinetobacter baumannii* (*A. baumannii*) isolates obtained from Iranian patients.

Methods: The isolates were identified from patients hospitalized between July 2011 and June 2013 in three hospitals in the Kermanshah region, Iran. All of the suspected colonies were assessed by using standard biochemical tests. Antimicrobial susceptibility testing was performed on all isolates by the Kirby-Bauer disk diffusion method. The main groups of OXA carbapenemase genes ($bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like/40-like}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$) were detected using PCR and finally carbapenem-resistant *A. baumannii* were genotyped by pulsed field gel electrophoresis.

Results: Eighty four clinical isolates were collected from the hospitalized patients. These isolates were confirmed as *A. baumannii*. All isolates were found to carry $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-24-like/40-like}}$ were seen in 56 and 24 isolates respectively and $bla_{\text{OXA-58-like}}$ was not detected in any of the isolates with similar in our previous study. Among 40 *A. baumannii* isolates that selected for pulsed field gel electrophoresis analysis, we obtained 6 pulsotypes (including 4 common types and 2 single types).

Conclusions: Our study and our previous study (2010-2011) indicated that multidrug-resistant *A. baumannii* strains are spreading and carbapenemase resistance are common in Iran. More researcher and greater emphasis on the prevention of health care-associated transmission of multidrug-resistant *Acinetobacter* infection are essential.

1. Introduction

Acinetobacter spp. are Gram-negative pathogens and an important cause of nosocomial infections in the intensive care units (ICUs)[1]. During the last decade reports of nosocomial infections caused by *Acinetobacter* spp., especially *Acinetobacter baumannii* (*A. baumannii*), have increased dramatically[2]. *A. baumannii* can colonize multiple sites of hospitalized patients and survive on inanimate surfaces. These aforementioned characteristics may have contributed to the prominent role of *A. baumannii* in nosocomial infections[3]. It causes a multitude of infections that include bacteremia, pneumonia, meningitis, urinary tract and wound infections[4,5]. In particular, carbapenem-resistant *A. baumannii* are emerging as a multidrug-resistant pathogen responsible for community- and hospital-acquired infections worldwide in recent years[6]. Carbapenem resistance in *A. baumannii* is due to a variety of combined mechanisms such as hydrolysis by beta-lactamases, alterations in the outer membrane proteins and penicillin-binding proteins and increased activity of efflux pumps[3]. Among them, resistance to

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carbapenems mediated by the Ambler class D OXA-type carbapenemases and Ambler class B metallo-beta-lactamases are of greatest concern as they are encoded by genes which are transmissible and account for most of the resistance to carbapenems in this species[7]. Four major families of OXA carbapenemase genes have been identified in *A. baumannii*: $bla_{OXA-23-like}$ (bla_{OXA-23}, bla_{OXA-27} and bla_{OXA-49}); $bla_{OXA-24-like}$ ($bla_{OXA-24-like/40-like}$, bla_{OXA-25}, bla_{OXA-26} and bla_{OXA-40}); $bla_{OXA-58-like}$ and $bla_{OXA-51-like}$[8]. Some of these genes ($bla_{OXA-23-like}$, $bla_{OXA-24-like/40-like}$ and $bla_{OXA-58-like}$) have been reported to be encoded on plasmids, which contributes to the spread of resistance[1]. We present here a molecular epidemiological and microbiological analysis of carbapenem-resistant *A. baumannii* strains isolated from patients hospitalized at Taleghani, Imam Reza and Khomeini hospitals in Western Iran.

2. Materials and methods

2.1. Bacterial isolates

The isolates were identified from patients hospitalized between July 2011 and June 2013 in three hospitals in the Kermanshah region, Iran. These strains were recovered from sputum, blood and urine. The isolates were stored at −70 °C in trypticase soy broth supplemented with 30% glycerol pending further analysis. All of the suspected colonies were assessed by using standard biochemical tests such as oxidase, thyroid stimulating immunoglobulin, motility and oxidative-fermentative test and then identified as *A. baumannii* using API 20NE (Version 6.0, bioMérieux, Marcy L'Etoile, France)[9].

2.2. Antimicrobial susceptibility testing

A suspension of each isolate was prepared; then cultured onto Mueller-Hinton agar. Antimicrobial susceptibility testing was performed on all isolates by the Kirby-Bauer disk diffusion method for the following antimicrobial agents[10]: amikacin (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), trimethoprim/sulfamethoxazole (30 μg), tigecylcine (30 μg) gatifloxacin (5 μg), colistin (10 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), piperacillin (100 μg), piperacillin-tazobactam (100/10 μg), polymyxin B (300 units), levofloxacin (5 μg), minocycline (30 μg), mezlocillin (75 μg), tetracycline (30 μg), tobramycin (10 μg), cefepime (30 μg), cefpodoxime (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), rifampicin (5 μg) (MAST, merseyside, UK). Phenotypic identification of extended-spectrum beta-lactamase (ESBL) and metallo-beta-lactamase (MBL) production was performed by the double disk synergy test and Etest MBL, respectively[11].

2.3. Molecular typing

Detection of the main groups of OXA carbapenemase genes ($bla_{OXA-23-like}$, $bla_{OXA-24-like/40-like}$, $bla_{OXA-51-like}$, $bla_{OXA-58-like}$) were performed using PCR[12]. The primers used are shown in Table 1. DNA extraction was carried out using the phenol-chloroform method. Amplification reaction was performed in a final volume of 50 μL. Each reaction mixture contained 5 μL of 10X PCR buffer, 1.6 IU of *Taq* DNA polymerase, and 2 μL of mixed deoxyribonucleoside triphosphates, 2 mmol/L $MgCl_2$, 500 ng of primers and 10 ng of bacterial DNA. The conditions used for PCR were 94 °C for 5 min, followed by 30 cycle of denaturation at 94 °C for 25 seconds, annealing at 53 °C for 40 seconds, and extension at 72 °C for 50 seconds and final extension at 72 °C for 6 min. PCR products were separated by electrophoresis on a 1% agarose gel.

Table 1
Primers of polymerase chain reaction.

Primers	Nucleotid sequences	Expected amplicon size (bp)
OXA-23-F	GAT CGG ATT GGA GAA CCA GA	501
OXA-23-R	ATT TCT GAC CGC ATT TCC AT	
OXA-24-F	GGT TAG TTG GCC CCC TTA AA	246
OXA-24-R	AGT TGA GCG AAA AGG GGA TT	
OXA-51-F	TAA TGC TTT GAT CGG CCT TG	353
OXA-51-R	TGG ATT GCA CTT CAT CTT GG	
OXA-58-F	AAG TAT TGG GGC TTG TGC TG	599
OXA-58-R	CCC CTC TGC GCT CTA CAT AC	

The gel was stained with ethidium bromide. A 100 bp ladder molecular weight marker (Roche Applied Sciences) used as a molecular size marker and patterns were photographed with a Gel Doc instrument (BIO-RAD, USA).

2.4. Pulsed-field gel electrophoresis (PFGE)

These strains were collected from three hospitals where most of collected isolate came from. Forty carbapenem-resistant *A. baumannii* were analysed by PFGE using methods (plug preparation, cell lysis, cell washing and digestion) as described Mohajeri *et al.*[13], with slight differences. We used *A. baumannii* ATCC 19606 for reference. Genotyping of all organisms were performed with *SmaI* (Fermentase) digestion. The lambda ladder PFG marker (NEB, US) was used as a molecular size marker. Electrophoresis in a pulsed-field electrophoresis system (Chef Mapper; Bio-Rad Laboratories, Hercules, CA, USA) by program Multi state with conditions: temperature 14 °C; voltage 6 V/cm; switch

angle 120°, switch ramps in first block: initial switching time: 1 seconds; final switching time: 20 seconds for 18 h and switch ramps in second block: Initial switching time: 5 seconds; final switching time: 50 seconds for 8 h. The gels were stained with ethidium bromide and patterns were photographed with UV gel Doc (BIO-RAD, USA) (Figure 1). The DNA banding patterns were analyzed using Bionumeric 7.0 software (Apllied maths NV, St-Martens-Latem Beligum) and using dice similarity index for cluster analysis and the unweighted pair group average for clustering. Patterns were compared with 1.0% optimization and 0.5% tolerance in this software. The PFGE DNA patterns obtained were compared as described by Tenover *et al*[14].

3. Results

Overall, we collected 84 clinical isolates from the hospitalized patients, between July 2011 and June 2013 from different wards in three hospitals of Kermanshah, Iran. These isolates were confirmed as *A. baumannii* by conventional biochemical testing and the API 20NE kits. The mean age of the men with *A. baumannii* isolates were (32.76±23.31) years and the women were (28.23±27.40) years. These samples were recovered from sputum 55 (65.48%), blood 27 (32.14%), and urine 2 (2.38%). Fifty five isolates (55.5%) were from patients in ICUs, 8 (9.5%) were from those admitted in pediatric wards, 15 (17.8%) were from the emergency wards and 6 (7.2%) were from patients in infectious disease wards (Table 2).

Table 2

Distribution of OXA-genes of *A. baumannii* from the hospitalized patients in Kermanshah, Iran.

Wards & Antibiotic	No. of isolates	OXA-23	OXA-24/40	OXA-23 & OXA-24/40
ICU	55	41	13	12
Children	8	3	5	2
Emergency	15	6	3	2
Infection	6	6	3	3
Imipenem	68	48	22	18
Meropenem	63	46	19	16

The rate of resistance was 97.6% for cefpodoxime, 96.4% for cefotaxime, 96.4% for ampicillin, 92.9% for ceftriaxone, 83.3% for rifampicin, 82.1% for mezolocilin, 80.1% for imipenem, 78.6% for piperacillin, 75.0% for meropenem, 72.6% for cefepime, 71.4% for ceftazidime, 70.2% for tetracycline, 69.0% for ciprofloxacin, 63.1% for gentamicin, 59.5% for levofloxacin, 54.8% for co-trimoxazole, 50.0% for amikacin, 46.6% for gatifloxacin, 44.0% for tobramycin, 14.3% for minocycline, 13.1% for polymyxin B, 10.7% for colistin and 3.6% for tigecycline (Table 3). The multi-drug resistent rate was 47.6% (n=40) and the pan drug resistant (PDR) rate was 11% (n=10), In addition, this study did not exhibit low resistance against polymyxin B and colistin (13.1% and 10.7%, respectively). Etest MBL and double disc synergy test methods showed that 83.8% (n=62) and 45.9% (n=34) of isolates were positive, respectively.

Table 3

Antimicrobial-susceptibility pattern of *Acinetobacter* spp. isolates in Kermanshah hospitals according to disk agar gel diffusion method. *n* (%).

Antimicrobial	Susceptibility		
	Susceptible	Intermediate	Resistant
Amikacin	30 (35.7)	12 (14.3)	42 (50.0)
Ceftriaxone	1 (1.2)	5 (6.0)	78 (92.9)
Ciprofloxacin	26 (31.0)	0 (0.0)	58 (69.0)
Trimethoprim-sulfamethoxazole	37 (44.0)	1 (1.2)	46 (54.8)
Gatifloxacin	39 (46.4)	6 (7.1)	39 (46.4)
Colistin	75 (89.3)	0 (0.0)	9 (10.7)
Gentamicin	27 (32.1)	4 (4.8)	53 (63.1)
Imipenem	11 (13.1)	5 (6.0)	68 (81.0)
Meropenem	16 (19.0)	5 (6.0)	63 (75.0)
Piperacillin	11 (13.1)	7 (8.3)	66 (78.6)
Polymyxin B	73 (86.9)	0 (0.0)	11 (13.1)
Ceftazidime	23 (27.4)	1 (1.2)	60 (71.4)
Levofloxacin	27 (32.1)	7 (8.3)	50 (59.5)
Minocycline	63 (75.0)	9 (10.7)	12 (14.3)
Mezlocillin	11 (13.1)	4 (4.8)	69 (82.1)
Tetracycline	23 (27.4)	2 (2.4)	59 (70.2)
Tobramycin	44 (52.4)	3 (3.6)	37 (44.0)
Tigecylcine	79 (94.0)	2 (2.4)	3 (3.6)
Cefepime	20 (23.8)	3 (3.6)	61 (72.6)
Cefpodoxime	2 (2.9)	0 (0.0)	82 (97.6)
Cefotaxime	2 (2.4)	1 (1.2)	81 (96.4)
Rifampicin	9 (10.7)	5 (6.0)	70 (83.3)
AMP-Sulbactam	54 (64.3)	3 (3.6)	27 (32.1)
Ticarcillin	12 (14.3)	3 (3.6)	69 (82.1)
Piperacilin-tazobactam	26 (31.0)	3 (3.6)	55 (65.5)
Ampicillin	3 (3.6)	0 (0.0)	81 (96.4)

All isolates were found to carry $bla_{\text{OXA-51-like}}$ (n=84) by PCR. Most isolates also carried acquired OXA carbapenemase genes, including $_{\text{blaOXA-23-like}}$ (n=56), $bla_{\text{OXA-24-like}}$ (n=24). $bla_{\text{OXA-58-like}}$ was not detected in any of the isolates, similar to our previous study. Among carbapenem-resistant *A. baumannii* isolates, 70.3% (n=52) contained $bla_{\text{OXA-23-like}}$ and 32.4 % (n=24) contained $bla_{\text{OXA-24-like}}$.

Among 40 *A. baumannii* isolates that selected for PFGE analysis in this study, 40 were resistant to both antibiotics: imipenem and meropenem (co-resistant). We obtained 6 pulsotypes (including 4 common types and 2 single types) that the clone A was dominant and widespread in different wards and hospitals and similarity in this clone was about 97%. Other clones especially clone C were in next dominant clones after clone A (Figure 1). Clone D and clone E exist only in male. Most pan drug resistant isolates existed in clones A and C. Carbapenem-resistant isolates of clone A

showed high resistance rates to polymyxin B and colistin, whereas all isolates of clone C were susceptible to polymyxin B and colistin. Most of isolates in clone A were positive for both OXA-genes (oxa-23 and oxa-24/40).

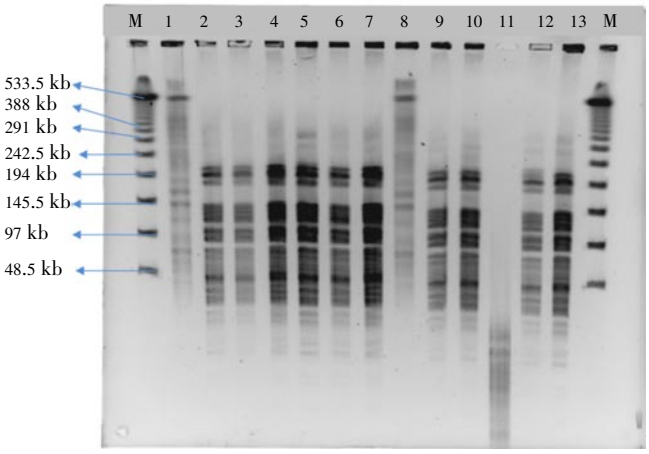

Figure 1. CHEF profiles of *A. baumannii* strains isolated from different wards.

M: Lambda ladder PFG marker; Lane: 2, 3, 4, 5, 6, 7, 9, 10, 12 and 13: Clone A and A1; Lane 1 and 8: Clone C.

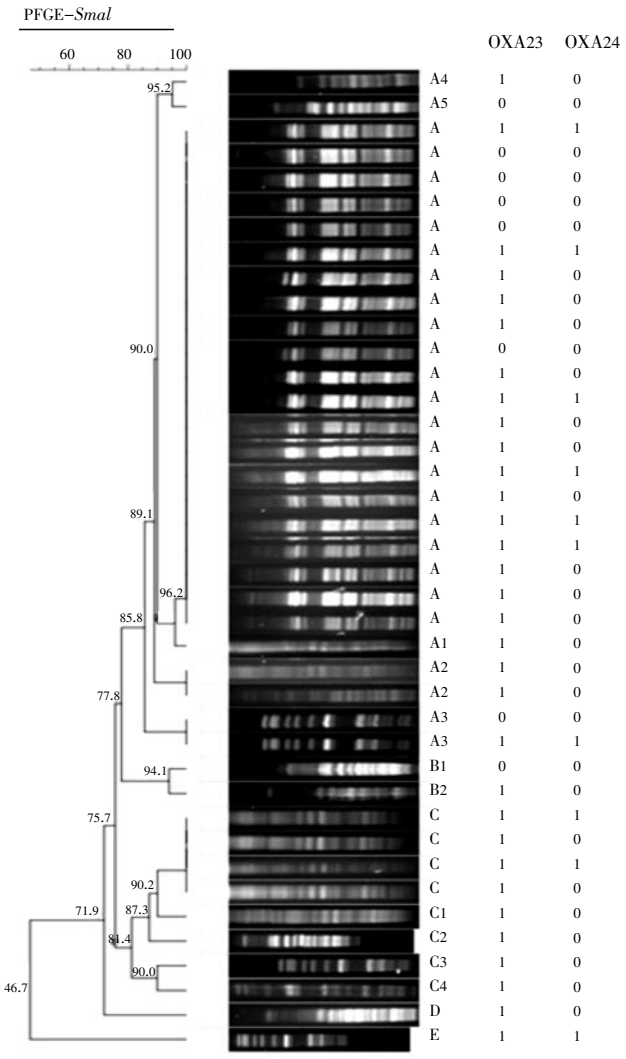

Figure 2. PFGE dendrogram of *A. baumannii* isolates.

4. Discussion

A. baumannii accounts for a substantial proportion of endemic nosocomial infections. A substantial increase in the rates of multidrug resistance and carbapenem-resistant *A. baumannii* has been reported in the hospitalized patients in the worldwide. As carbapenems are the first choice for treatment of *A. baumannii* infections, resistance to carbapenems have severely limited therapeutic options.

The high resistance rates found in this study may be associated with the high frequency at which these antimicrobial drugs were used for both prophylactic and therapeutic treatment of the hospitalized. Therefore, preventive antibiotics should be used as little as possible, while therapeutic antibiotics should be specific and used as short period of time as possible. The main enzymes involved in resistance are OXA-carbapenemases. All isolates were found to carry $bla_{\text{OXA-51-like}}$ (n=84) by PCR. The present result support those of a previous report, suggesting that $bla_{\text{OXA-51-like}}$ is a species specific to *A. baumannii*[13,15,16]. Studies have reported $bla_{\text{OXA-23-like}}$ to be the most common type of carbapenemase identified among carbapenem-resistant *A. baumannii*, followed by $bla_{\text{OXA-24-like/40-like}}$[17].

The present study showed that most of the isolates of *A. baumannii* were obtained from ICU. Our study with similar result with our previous study showed that $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-24-like/40-like}}$ were the two major determinants of carbapenem resistance among *A. baumannii* in our region[13]. As the P value was less than 0.001, we can conclude that there is a significant association between the $bla_{\text{OXA-23-like}}$ and resistance to carbapenems (imipenem and meropenem). This result was obtained in a similar study in Iran[13,18]. This result may suggest that various mechanisms of the carbapenem resistance could have contributed to the carbapenem resistant in this isolate. PFGE analysis is used with excellent data in epidemiological and molecular studies of numerous bacterial outbreaks and is gold standard for molecular epidemiologic in many bacterial especially *A. baumannii*[19]. This approach allows detecting pollution sources and epidemic organisms, genetic diversity and genetic distance between strains and also can clearly show facilitate communication between bacterial isolates in different part[20]. Two enzymes *SmaI* and *ApaI* have been proposed for the analysis of *Acinetobacter* isolates and in this study we used *SmaI* for PFGE analysis. Isolates in this study with PFGE profile A was believed to be endemic in the ICU, infant, infection and emergency wards and also remained predominant in the hospitals of Kermanshah in Western Iran, and the previous study authors have mentioned this problem[13]. In this study, a total of forty isolates of carbapenem-resistant *A. baumannii* were analyzed by PFGE, of which 32 isolates carried $bla_{\text{OXA-23-like}}$ and 11 isolates carried $bla_{\text{OXA-24-like/40-like}}$ genes. In addition, isolates that

were positive for $bla_{OXA-24-like/40-like}$ gene were also positive for $bla_{OXA-23-like}$ gene as well. We didn't detected bla_{OXA} genes (23 or 24/40) in some isolates resistant to carbapenems (resistant to imipenem or meropenem) in this study, so other genes must be involved in the resistance to carbapenems. The present study reveals that resistant rates of *A. baumannii* to available antibiotics are very high. It seems that infection control strategies may help to control the evolving problem of *A. baumannii* infections and prevent an epidemic of life-threatening nosocomial infections.

Overall, our study and our previous study (2010-2011) indicated that multidrug-resistant *A. baumannii* strains are spreading and carbapenemase resistance are common in Iran.

Conflict of interest statement

We declare that we have no conflict of interest.

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