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Original Article

High Prevalence of Multidrug Resistance and Metallo-beta-lactamase (MβL) producing *Acinetobacter Baumannii*Isolated from Patients in ICU Wards, Hamadan, Iran

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ABSTRACT

Background: *Acinetobacter baumannii* is gram- negative opportunistic coccobacilli, the most important agent in nosocomial infections with high mortality rate. Multidrug resistance in strains isolated from nosocomial infections, makingit difficult to treat and sometimes impossible. The aim of the present study was to investigate antibiotic resistance in *A. baumannii* isolates from Iranian patients in Hamadan, west of Iran.

Methods: In this cross sectional study 100 *A. baumannii* isolated from trachea, blood, urine, sputum and wound samples of patients bedridden in Intensive care unit (ICU) wards of three educational hospitals during June 2011 to October 2012 was included. Isolates confirmed at species level using biochemical tests and tracing bla_{OXA-51} gene using Polymerase chain reaction (PCR) and preserved frozen at -70 °C until examination. Their susceptibility to 17 antibiotics was performed using Kirby-Bauer disc diffusion method. Determination of minimum inhibitory concentration and Metallo-beta-lactamase production was carried out using E-test method.

Results: Resistance rate of isolates were 94%, 85%, 84%, 97%, 95% and 98% against meropenem, imipenem, amikacin, ciprofloxacin, piperacillin/tazobactam and cefotaxime, respectively. No resistant isolate was observed against tigecycline and also no sensitive isolate seen against aztreonam and cefotaxime. Results of E-test illustrated that 99% of all isolates were Metallo-beta-lactamase (MβL) producing, which were resistance to imipenem; also 85% of them were resistance to meropenem. MIC50 and MIC90 of the isolates were ≥256 and ≥32µg/ml for imipenem and meropenem, respectively.

Conclusions: The antibiotic resistance against most of the antibiotics, especially carbapenems is very high in Hamadan region. In addition colistin sulfate and tigecycline were most effective antibiotics and to be used in *A. baumannii* infections.

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Introduction

Acinetobacter baumannii is a non-motile, gram-negative, non-fermentative, oxidase-negative and aerobic bacilli, which is one of the most opportunistic pathogenic agents in humans[1,2]. The bacteria are widespread in environment, and considerably are resistant against most antibiotics, low nutrient and arid condition[3]. *Acinetobacter* spp. cause variety of nosocomial infections with high morbidity and mortality, including pneumonia, bacteremia, urinary tract, skin and soft tissue infections, especially in patients with severe illness [1,3,4]. Epidemiologically, outbreaks are mostly intra- and inter-hospitals, globally [2,5]. *Acinetobacter* spp. is skin's flora, and it is thought that more than 25% of healthy humans are its carrier. Additionally, hospitalized patients possess higher carriage potential, particularly when outbreaks occur [6].

The prevalence of multidrug resistant *A. baumannii* strainshave been increasing during recent years continually and causing of highly mortal hospital infections[7]. The increase in drug resistance of *Acinetobacter* to the most of antibiotics is resulting from abuse of antimicrobial agents [8]. The bacteria show a wide range of antimicrobial resistance mechanisms. They have the ability of acquiring plasmids, transposons and integrons and mostly, the production of carbapenemases[9]. Carbapenem resistance is now observed worldwide in *A. baumannii* isolates, leading to limited therapeutic options. Several carbapenem-hydrolyzing lactamases have been documented in *A. baumannii*. Lactamases from class B illustrate highly hydrolyzing activity of carbapenems [2]. Carbapenems, which were the drug of choice, today they are no longer using for treatment of *A. baumannii* infections [9].

In Iran like other countries several studies carried out about drug resistance of *Acinetobacter baumannii* and found high resistant rate to most of the antibiotics [10,11]. To understand the epidemiology of *Acinetobacter baumannii* drug resistance, it is necessary to study on drug resistance of the infection in each region of the country. The aim of the present study was to investigate multidrug resistance in *Acinetobacter baumannii,* isolated from Iranian patients in Hamadan City, west of Iran.

Methods

Sampling and bacterial isolates

In this cross sectional study totally 100 *Acinetobacterbaumannii* isolated from clinical specimens collected from patients bedridden in ICU wards of three educational hospitals of Hamadan City (June 2011 to October 2012).These isolates were cultured from 74 tracheal aspirate, 16 blood, 5 urine , 4 sputum and 1 wound samples. Biochemical tests have been done to identify *A. baumannii* isolates [12]. If the isolates had following conditions such as; non-fermentative, gram negative coccobacilli, citrate positive, Indole negative, TSI base/base, non-motile, H_2S negative, urease negative, oxidase negative and catalase positive, they considered *A. baumanni* and confirmed by detection of *bla*OXA-51-like carbapenemase gene by PCR as described previously [13]. The confirmed isolates kept preserved at -70 °C until sampling ended.

blaOXA-51- like carbapenemase detection by PCR

Single PCR was used for amplification of *A.baumani* internal gene for molecular detection of isolates at species level, using pair of $bla_{OXA\text{-}51}$ primers (Bioneer ® Korea); OXA-F 5´-TAATGCTTTGATCGGCCTTG-3´, and OXA-R 5´-TGGATTGCACTTCATCTTGG-3´ [13,14].

Amplification procedure performed with 25 µl of master mix containing 2.5 µl 10XPCR buffer with MgCl2, 2.5 µl dNTPs MIX(2Mm),1 µl of 10pM fromeach forward and reveres primers, 0.2 µl Taq polymerase 5U/ µl, 3 µl DNA template,14.8 µlDNase/RNase-FreeDistilledWater.

PCR reaction was performed in the thermal cycler using the following steps: initial DNA denaturation at 94 °C for 5 min, then 30 cycles of denaturation at 94 °C for 30 sec, annealing at 52 °C for 40 sec and extension at 72°C for 50 sec, followed by final extension at 72 °C for 5 min. Agarose gel electrophoresis of the amplified DNA with 100 bp size marker (Fermentas ®, Korea)have done for 2 h at 80 V in a 2% agarose gel stained with ethidium bromide to detect the 353 bp band, also contain positive and negative control (Figure 1). The *A. baumani* harboring *bla*OXA-51-like gene (obtained from Dr. Bahador, Tehran university of medical sciences, Iran) was used as positive control.

Antibiogram

The susceptibility of isolates to 17 antibiotics (Mast CO, UK) was performed using Kirby-Bauer disc diffusion method [12] and the procedure has been done according to the construction of manufacturer. In summary, 1.5×10^8CFU, equivalent to McFarland Turbidity Standard No. 0.5, transferred to Muller-Hinton agar (Merck, Germany) and Antibiogram discs containing 10 µg imipenem, 10 µg meropenem , 30 µg amikacin, 10 µg gentamicin, 30 µg aztreonam, 30 µg cefepime, 30 µg ceftazidime, 5 µg ciprofloxacin, 15 µg tigecycline, 30 µg doxycycline, 5 µg levofloxacin, 100 µg piperacillin, 10 µg tobramycin, 10 µg colistin sulphate, 20µg ampicillin/sulbactam, 110 µg piperacillin/tazobactam were placed on the medium and then incubated at 35 °C for 18 hours. Results interpreted using Clinical and Laboratory Standards Institute (CLSI) criteria[15] and reported as sensitive, intermediate and resistance. *Pseudomonas aeruginosa* ATCC 27853 was used as the control strain in susceptibility testing.

Minimal Inhibitory Concentration (MIC)

E-test strips (Lioflichem ® Italy), containing imipenem and meropenem were used to determination of minimal inhibitory concentration (MIC) according to the instruction of the company. In brief, 1.5×10^8CFU, equivalent to McFarland Turbidity Standard No. 0.5, transferred to Muller-Hinton agar (Merck, Germany) using sterile swab. Then E-test strips placed on the cultured medium and incubated at 35 °C for 18 hours. Results interpreted using CLSI criteria. Isolates which possess intermediate level of resistance rate considered resistance [16]. *Pseudomonas aeruginosa* ATCC 27853 was used as the control strain in susceptibility testing.

E–test MBL detection test

MIC Test Strip (Lioflichem® Italy), containing imipene/imipenem + EDTA were used to determination of phenotypic MβL enzyme production. E-test was performed according to the manufacturer’s instructions. In brief, 1.5×10^8CFU, equivalent to McFarland Turbidity Standard No. 0.5, transferred to Muller-Hinton agar (Merck, Germany) using sterile swab. Then E-test strips placed on the cultured medium and incubated at 35 °C for 18 hours. A reduction in the MIC of imipenem of ≥3 dilutions in the presence of EDTA is interpreted as a positive test. Additionally, a strain was considered

MβLproducer if a phantom zone or deformation of the ellipse is observed.

Data analyzed by SPSS v.16 using McNemar and Chi square tests regarding. *P*≤0.05as significance level.

Results

Totally 100 isolates of *A. baumanni* were identified by microbiology standard methods and confirmed with PCR (Figure 1).

The resistance rate of isolates is available in Table 1. Highest resistance rate of isolates observed 97%, 98%, 97%, 98% and 98% against aztreonam, ceftazidime, ciprofloxacin, piperacillin and cefotaxime, respectively. In additionno sensitive isolate seen on aztreonam and cefotaxime, just few isolates were in intermediate level of susceptibility, and no resistant isolate observed against tigecycline. Interestingly 99% of isolates were MβL producing, which can be one of the causes of high resistance to carbapenems in the present study. Significant relationship observed between the prevalence of imipenem resistance *A.baumanni* isolates among MβL producing ones (*P*=0.010). Furthermore there was statistically significant relationship between the prevalence of meropenem resistance *A .baumanni* isolates among MβL producing ones (*P*=0.040).

Resistance rate against imipenem using MIC was 99%, which is significantly higher comparing to 87% in Kirby-Bauer disc diffusion method (*P*<0.001) (Table 2). Also MIC of 97% of isolate was ≥256 μg/ml (Table 3). The MIC test was used for meropenem too, and the results showed 96% resistance rate, which relatively equal comparing to 95% resistance rate observed in Kirby-Bauer disc diffusion method. In addition MIC of 69% of isolates was ≥32 μg/ml (Table 3). For comparison of Kirby-Bauer disc diffusion method with MIC test, the intermediate resistance rate of isolates considered as resistance [16] (Table 4).

Beside all, both MIC 50 and MIC 90 of imipenem was ≥256 μg/ml, also both MIC 50 and MIC 90 of meropenem was ≥32 μg/ml (Table 2).

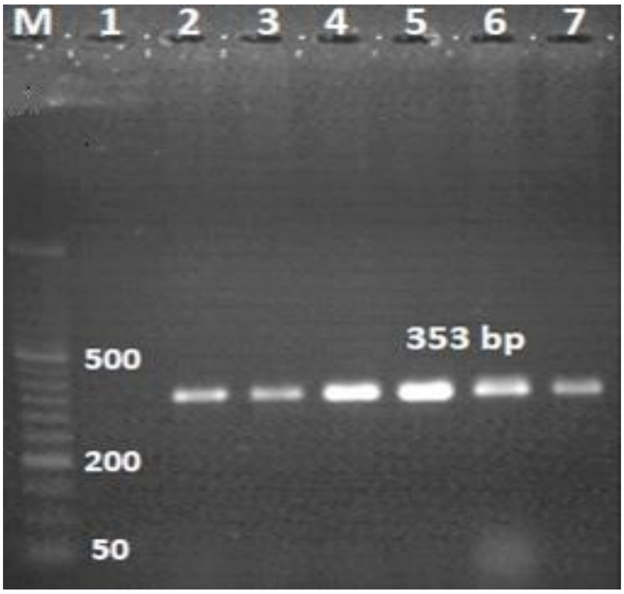

Figure 1: PCR resultsfor amplification of *blaOXA*-51 gene in *A. baumanni* isolates; M, 50 bp DNA ladder; Lane 1, negative control; Lane 2, Positive control; Lanes 3-7 different isolates containing *blaOXA*-51-like gene

Table 1: Susceptibility of *Acinetobacter baumannii* isolates to 17 antibiotics

	Antibiotic susceptibility			
Antibiotic	**Sensitive %**	**Intermediate %**	**Resistance %**	**Total %**
Meropenem	5	1	94	100
Imipenem	13	2	85	100
Amikacin	7	9	84	100
Gentamicin	11	1	88	100
Aztreonam	0	3	97	100
Cefepime	2	6	92	100
Ceftazidime	2	0	98	100
Ciprofloxsacin	2	1	97	100
Tigecycline	0	12	88	100
Doxicylin	34	37	29	100
Levofloxacin	3	6	91	100
Piperacillin	1	1	98	100
Tobramycin	20	1	79	100
Colistin sulfate	99	0	1	100
Ampicillin/sulbactam	46	23	31	100
Piperacillin/tazobactam	3	2	95	100
Cefotaxime	0	2	98	100

Table 2: Antimicrobial resistance of *Acinetobacter baumani* isolates by E. test (n = 100)

Antibiotic	Break point (μg/ml)	Resistant (%)	Intermediate (%)	Sensitive (%)	MIC-50 (μg/ml)	MIC-90 (μg/ml)	MIC Range (μg/ml)
Imipenem	Sensitive ≤4 Resistance ≥16	99	0	1	≥256	≥256	4-256
Meropenem	Sensitive ≤4 Resistance ≥16	87	9	4	≥32	≥32	0.38-32

Table 3: Distribution of susceptibility rate of *Acinetobacter baumannii* isolates to different concentrations of imipenem and meropenem using MIC test

	Imipenem					Meropenem							
	Sensitive	Resistance				Sensitive			Intermediate		Resistance		
Break point	≤4µg/ml	≥16µg/ml				≤4 µg/ml			5-15 µg/ml		≥16 µg/ml		
MIC (µg/ml)	4	16	32	64	≥256	0.38	1.5	4	8	12	16	24	32
Isolates	1	0	1	1	97	1	2	1	3	6	16	2	69
Total	1	99				4			9		87		

Table 4: The frequency of Antimicrobial Resistance pattern of *Acinetobacter baumannii* isolates by disk diffusion agar and E. test (n= 100) using McNemar test

Antibiotic	Disk Diffusion Agar	E-Test	*P* value
Imipenem			0.001
Resistance [a]	87	99	
Sensitive	13	1	
Meropenem			1.000
Resistance	95	96	
Sensitive	5	4	

[a] Intermediate antibiotic susceptibility regarded as resistant

Discussion

One of the carbapenem resistance mechanisms is production of carbapenem-hydrolyzing β-lactamases, which is called carbapenemase, one of which, Methalo β-lactamase (MβL) is got higher importance in drug resistance against carbapenems[8,17]. Considerably high prevalence (99%) of the isolates was MβL producing which can be a main cause of high carbapenem resistance among *A.baumanni* isolates in our study. Interestingly 99% of isolates were resistant against imipenem using E-test method. Significant relationship observed between the prevalence of imipenem resistance *A.baumanni* isolates among MβL producing ones. Also there was statistically significant relashinship between the prevalence of meropenem resistance *A.baumanni* isolates among MβL producing ones. This finding suggests the important role of MβL in carbapenem resistance of *A.baumanni*.

In the present study drug resistance in *A.baumanni* isolates from ICU wards of three educational hospitals were very high, especially against aztreonam, ceftazidime, ciprofloxacin, piperacillin and cefotaxime, rate of 97%, 98%, 97%, 98% and 98%, respectively. Additionally majority of tested antibiotics were inefficient except tigecycline and colistin sulfate which showed a better sensitive rate, 88% and 99%, respectively, comparing to the other tested antibiotics. Globally and in Iran, several studies carried out about drug resistance of *A. baumannii* recently and similarly found high resistant rate to most of the antibiotics [10,11]. In addition our results illustrated even higher resistance rate in Hamadan region comparing to other studies in Iran[10,11].

In a relatively similar study, Feizabadi et al. 2008 reported prevalence of susceptibility of *A. baumanni* to imipenem, meropenem, piperacillin-tazobactam and amikacin rate of 50.7%, 50%, 42.1% and 38.2%, respectively [10], which shows higher sensitive isolates comparing to ours, rate of 5%, 13%, 3% and 7%, respectively. In the other study carried out by Hadadi et al. 2008, they reported 80.5%, 22%, 24.4% and 24.4 of susceptibility rate of *Acinetobacter* spp. for imipenem, cefepime, ciprofloxacin and ceftazidime, respectively, which illustrate notably higher rate of antibiotic sensitivity than ours[18] rates of 5%, 2%, 2% and 2% sensitive isolates.

In 2009 Morovat et al. reported susceptibility of *A.baumanni* to cefotaxime, imipenem, meropenem, piperacillin, piperacillin-tazobactam, and tigecycline rates of 7.5%, 42.5%, 42.5%, 21.2%, 28.7% and 91.2% using E-test method [19]. Our results shows lower sensitive isolates comparing to their report except in case of tigecycline, which our results illustrated slightly higher susceptibility. Our results shows lower sensitive rate and higher resistance rate of *A.baumanni* against most of the studied antibiotics comparing to other studies carried out throughout Iran[20,21].

From 17 studied antibiotics tigecycline and colistin sulfate were effective enough to be used in treatment of the infection, by considering of their side effects. Other antibiotics we studied were inefficient, indeed. Imipenem and meropenem have been reported effective antibiotic with lower resistance rate in most of studies in Iran[18,20,22], which our results do not support this idea. In arecent study in Iran,imipenemresistancein enteric gram-negative bacteriaisolated fromhospital infections have been reported equal to33%, which is not comparable with our results[23]. We found meropenem and imipenem ineffective against *A. baumanni* isolates in both E-test and Kirby-Bauer disc diffusion methods.

Our results showed that detection of *bla*OXA-51-like can be used as a simple and reliable way of identifying *A.baumannii*. We have found *bla*OXA-51-like in all isolates of *A.baumannii* we have investigated in this study.

Conclusion

Prevalence of drug resistance among *Acinetobacter baumannii* against most of the antibiotics in Hamadan City is very high, and alerting, even higher than any other part of the country. Permanent monitor of changes in *Acinetobacter baumannii* resistance will help determine national priorities for local intervention efforts.

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Conflict of interest statement

The authors have no conflict of interest.

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