TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2017) 47: 1661-1666

doi:10.3906/sag-1607-91

Investigation of class-d beta-lactamases causing carbapenem resistance in clinical *Acinetobacter baumannii* isolates*

Iskandar DAVANDEH[1], Bayrı ERAÇ[1,], Sabire Şöhret AYDEMİR[2]**

[1]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Ege University, İzmir, Turkey
[2]Department of Medical Microbiology, Faculty of Medicine, Ege University, İzmir, Turkey

Received: 21.07.2016 • **Accepted/Published Online:** 02.06.2017 • **Final Version:** 13.11.2017

Background/aim: *Acinetobacter baumannii* is an important causative agent of nosocomial infections, and carbapenems have been frequently used in the treatment of these infections. This study was designed to investigate the prevalence of primary carbapenem hydrolyzing oxacillinase (CHO) types in clinical *A. bumannii* strains.

Materials and methods: Minimum inhibitory concentration (MIC) values of 76 imipenem nonsusceptible *A. baumannii* strains, isolated from a tertiary care hospital, were determined by microdilution method. The clonal relationship of the isolates was analyzed with enterobacterial repetitive intergenic consensus (ERIC)-PCR, and the presence of CHO major groups (OXA-23; OXA-24, OXA-51, and OXA-58 groups) was investigated with multiplex PCR.

Results: According to the ERIC-PCR patterns, the isolates were distributed in 13 different clones, the largest of which had 40 members. $bla_{OXA\text{-}51\text{-}group}$ was detected in representatives of all clones, whereas $bla_{OXA\text{-}23\text{-}group}$ was detected in representatives of all but two small clones. Additionally, the presence of $bla_{OXA\text{-}58\text{-}group}$ was discovered in the members of two small clones, whereas $bla_{OXA\text{-}24\text{-}group}$ was not encountered in any of the examined strains.

Conclusion: Molecular fingerprinting revealed that most imipenem-resistant *A. baumannii* strains were clonally related. $bla_{OXA\text{-}23\text{-}group}$ and $bla_{OXA\text{-}51\text{-}group}$ were mostly responsible for the imipenem resistance of the examined *A. baumannii* strains.

Key words: *Acinetobacter baumannii*, carbapenemase, enterobacterial repetitive intergenic consensus PCR, OXA-genes

1. Introduction

Acinetobacter baumannii is a gram-negative, nonfermentative coccobacillus. It is increasingly recognized as a major pathogen causing nosocomial infections, including ventilator-associated pneumonia, bacteremia, meningitis, urinary tract, and wound infections, particularly in patients admitted to burn therapy and intensive care units (1). One of the most alarming characteristics of this bacterium is its ability to develop resistance to the majority of available antibiotics, including carbapenems, which are drugs of choice in severe infections (2). Several mechanisms responsible for resistance to carbapenems have been described in *A. baumannii* isolates: decreased outermembrane permeability, efflux-pumps, target-site modifications, and synthesis of carbapenemases (3). Among these, production of carbapenem-hydrolyzing oxacillinases (CHOs) or class D carbapenemases stands out as the most common resistance mechanism (4,5). The Amber class D carbapenemases of *A. baumannii* can be divided into six subgroups: OXA-51-like (intrinsic-chromosomal), OXA-23-like, OXA-40/24-like, OXA-58-like, OXA-143-like, and OXA-235-like (6). Types and frequency of CHOs may vary among different countries and centers. The aim of this study was to investigate the prevalence of primary CHO types in clinical *A. baumannii* strains isolated from a 2000-bed regional university hospital.

2. Materials and methods

2.1. Bacterial strains and determination of imipenem minimum inhibitory concentration values

Seventy-six carbapenem nonsusceptible clinical *A. baumannii* isolates, one per patient, were selected from 122 clinical isolates collected between January and March 2012 by Ege University Hospital, Turkey. Isolates were obtained from blood (n = 18), tracheal aspirates (n = 28), bronchial

* This study was presented as a poster at the 11th Antimicrobial Chemotherapy Days Congress, İstanbul, Turkey, 18–20 April 2014.

** Correspondence: bayri.erac@ege.edu.tr

aspirates (n = 1), bronchoalveolar lavage (n = 7), urine (n = 9), bile (n = 1), wounds (n = 3), sputum (n = 7), and wounds (n = 2). *A. baumannii* isolates were identified with standard microbiological-biochemical methods and Vitek-2 (Biomerieux). Identification of the isolates was additionally confirmed with PCR amplification of the OXA-51-like gene.

The minimum inhibitory concentrations (MICs) of imipenem against 76 *A. baumannii* isolates were determined with the microdilution method, according to the Clinical Laboratory Standard Institute (CLSI) recommendations (7). The final concentrations of imipenem (Santa Cruz, USA) were adjusted between 0.125 and 128 mg/L. *Pseudomonas aeruginosa* ATCC 27853 was used for quality control. The lowest antibiotic concentration that inhibits growth is considered the MIC value.

2.2. Enterobacterial repetitive intergenic consensus PCR

The genetic relationship of 76 clinical *A. baumannii* isolates was determined with enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR). All primers used in this study are listed in Table 1. DNA was extracted by boiling the suspension of a colony for 10 min. The supernatant of this suspension was used as the PCR template. All reactions were performed in a 25 µL-volume, containing 1.5 U of hot-start Taq DNA polymerase (Thermo Scientific, USA). Cycling conditions were 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 40 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. DNA band patterns were analyzed with an imaging device (Vilber Lourmat fusion FX-7) following electrophoresis on agarose gels (1.5% w/v). To evaluate similarity between the strains, Jaccard coefficients [SJ = nAB × 1/(nAB + a + b)] were calculated according to the banding patterns. When the calculated SJ was <0.8, two isolates were accepted as 'clonally distinct'. Isolates were classified as 'clonally related' when the calculated value was 0.8 ≤ SJ < 1.0; when it was 1.0, the isolates were considered as 'identical'. Dendrograms were formed using Jaccard coefficients and MEGA 4 with UPGMA method (8). Representatives of each ERIC-PCR pattern were used for the detection of basic CHO types with multiplex-PCR.

2.3. Detection of basic carbapenem hydrolyzing oxacillinase types with multiplex-PCR

CHO group determinants were detected in a total of 22 strains, which were representatives of each clone and subtype determined by ERIC-PCR. The presence of OXA-51-like, OXA-23-like, OXA-40/24-like, and OXA-58-like genes was investigated with multiplex PCR, as previously described (4). PCR amplifications were performed in 50-µL reaction volumes containing 0.2 mM of primer mix, 0.2 mM dNTP (Thermo Scientific), 5 µL of genomic DNA extract, 1 U of hot start Taq DNA polymerase (Thermo Scientific), and 1x of supplied PCR buffer. In addition, separate PCRs were performed with primers specific to each CHO group. The 50-µL reaction mixture was composed of 1.75 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol of each primer, and 1.5 U of hot start Taq DNA polymerase. The amplification conditions were initial denaturation at 95 °C for 5 min, 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a final elongation at 72 °C for 10 min. *A. baumannii* strains confirmed to be positive for the CHO group determinants in a previous study were used as positive controls in this study (9).

All positive results were confirmed by direct sequencing of the PCR products (Macrogen Inc., Korea). Sequence analysis results were compared with the reference sequences at the level of both nucleotide and amino acid, using European Bioinformatics Institute (EBI) and National Center for Biotechnology Information (NCBI) web-based services. Reference sequences used for bla_{OXA-23}, bla_{OXA-51}, and bla_{OXA-58} were CP001182, AJ309734, and AY665723, respectively.

Table 1. Primers used in this study.

Primer	Sequence (5' to 3')	Tm	Amplicon size (bp)
OXA-23-F	5'-GAT CGG ATT GGA GAA CCA GA	58 °C	501
OXA-23-R	5'-ATT TCT GAC CGC ATT TCC AT	54 °C	501
OXA-51-F	5'-TAA TGC TTT GAT CGG CCT TG	56 °C	353
OXA-51-R	5'-TGG ATT GCA CTT CAT CTT GG	56 °C	353
OXA-58-F	5'-AAG TAT TGG GGC TTG TGC TG	58 °C	599
OXA-58-R	5'-CCC CTC TGC GCT CTA CAT AC	63 °C	599
OXA-24-F	5'-GGT TAG TTG GCC CCC TTA AA	58 °C	246
OXA-24-R	5'-AGT TGA GCG AAA AGG GGA TT	56 °C	246
ERIC-2	5'-AAG TAA GTG ACT GGG GTG AGC G	-	-

3. Results

IMP MIC values of the studied 76 clinical isolates of *A. baumannii* ranged from 8 to 128 mg/L (Table 2). Seventy-two (94.74%) of the isolates were resistant (MIC ≥ 16 mg/L) and four (5.26%) isolates were intermediately resistant (8 mg/L) to IMP.

Four to ten bands with sizes ranging from 250 to 1500 bp were formed with ERIC-PCR. According to the ERIC-PCR band patterns, seven main groups (I–VII) and 13 different clones (A–M) were determined among the studied *A. baumannii* strains (Figure 1). The largest clone (clone A) had three subtypes and 40 members, corresponding to 52.63% of all isolates. Aside from clone A, nine-membered clone B, 14-membered clone C, and two-membered clones G, K, and L were identified. In addition, seven clones with only one member were observed.

Basic CHO groups ($bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}40/24\text{-}like}$, and $bla_{OXA\text{-}58\text{-}like}$) were sought in a total of 22 strains, which were representatives of each clone and subtype determined by ERIC-PCR. Chromosomal $bla_{OXA\text{-}51\text{-}like}$ was detected in all studied isolates. $bla_{OXA\text{-}23\text{-}like}$ was determined in representatives of all clones, except clones K and L. Instead, $bla_{OXA\text{-}58\text{-}like}$ was found in members of these clones (K and L) (Figure 2). $bla_{OXA\text{-}40/24\text{-}like}$ was not observed in any of the examined strains. Distribution of CHOs among 22 *A. baumannii* isolates, which are representatives of each clone and subtype, and IMP MIC values are shown in Table 3. To confirm the results obtained by multiplex PCR, separate PCRs were performed with primers specific to each CHO group, and PCR products were sequenced for both directions. Bioinformatic data were consistent with the multiplex PCR results.

4. Discussion

Carbapenems are among the most effective beta-lactam antibiotics and are frequently used in the treatment of infections caused by nosocomial gram-negative pathogens such as *A. baumannii*. The positive selective pressure caused by increased use of carbapenems has resulted in emergence and expansion of resistance in common gram-negative pathogens (10). Although metalloenzymes are particularly common in East Asia, carbapenemases belonging to molecular class D (CHO) are considered to be the main mechanism responsible for carbapenem resistance in *A. baumannii* isolates (4,5).

IMP MIC values were ≥32 mg/L in 71.05% of the examined strains in this study, which suggested that IMP resistance was high and widespread among *A. baumannii* isolates in our hospital. The results of ERIC-PCR have shown that 63 (82.9%) of 76 isolates belonged to one of three major clones, and the rest (17.1%) were distributed among 10 other small clones. Many studies carried out in Turkey have revealed that CHO-detected *A. baumannii* isolates were mostly clonally related in medical centers (11,12). For example, in a study investigating the prevalence of OXA-type carbapenemases in carbapenem-resistant *A. baumannii* strains isolated from two major medical centers in Turkey, resistance was reported largely due to the strains showing clonal relationship in both centers (13). Moreover, certain clones (international clones-IC I–III) have been found responsible for spreading imipenem nonsusceptible *A. baumannii* isolates throughout the world, and they often carry CHOs (14). $Bla_{OXA\text{-}23}$-producing *A. baumannii* strains that belong to IC-II are also reported to be prevalent in the Mediterranean region (6). It is necessary to apply a further molecular typing method, such as multilocus sequence typing (MLST), for international comparison of our strains.

Table 2. IMP MIC values of 76 clinical *A. baumannii* isolates.

MIC (mg/L)	128	64	32	16	8
Number of isolates	4	22	28	18	4

As expected, $bla_{OXA\text{-}51\text{-}group}$ was detected in representatives of all clones in this study. While $bla_{OXA\text{-}23\text{-}group}$ was found in representatives of all but two small clones (two-membered clones), $bla_{OXA\text{-}58\text{-}group}$ was identified only in members of these two clones. In accordance with the results of our study, $bla_{OXA\text{-}23\text{-}group}$ dominance is seen in many studies conducted in Turkey. For example, in a study investigating CHO prevalence in 834 clinical *A. baumannii* strains, isolated from 13 Turkish university and state hospitals between 2008 and 2011, the rates of $bla_{OXA\text{-}23\text{-}group}$ and $bla_{OXA\text{-}58\text{-}group}$ positive strains were determined to be 74.4% and 17.3%, respectively (15). Moreover, $bla_{OXA\text{-}24\text{-}group}$ was not encountered in any isolate examined in that study. Although $bla_{OXA\text{-}58}$ has long been the most common type of CHO in carbapenem-resistant *A. baumannii* isolates in Mediterranean countries, it is reported to be substituted by $bla_{OXA\text{-}23}$ since 2009 (6). $Bla_{OXA\text{-}23\text{-}group}$-producing, carbapenem-resistant *A. baumannii* isolates are detected in many countries, suggesting that this gene is common worldwide (16,17). In another Turkish study, $bla_{OXA\text{-}23\text{-}group}$ was detected in 26 (59.1%) of 44 carbapenem-resistant *A. baumannii* isolates collected from two hospitals in İstanbul and Ankara (13). Twenty-five of these $bla_{OXA\text{-}23\text{-}group}$-positive strains were isolated from İstanbul, which reveals that the prevalence of CHOs may show huge variability between medical centers.

As in many studies conducted in Turkey, $bla_{OXA\text{-}24\text{-}group}$ was not observed in any of the isolates examined in our study. However, in a study conducted in İzmir, $bla_{OXA\text{-}72}$, a variant of group OXA-24/40, was identified in an *A. baumannii* isolate for the first time in Turkey (18). These

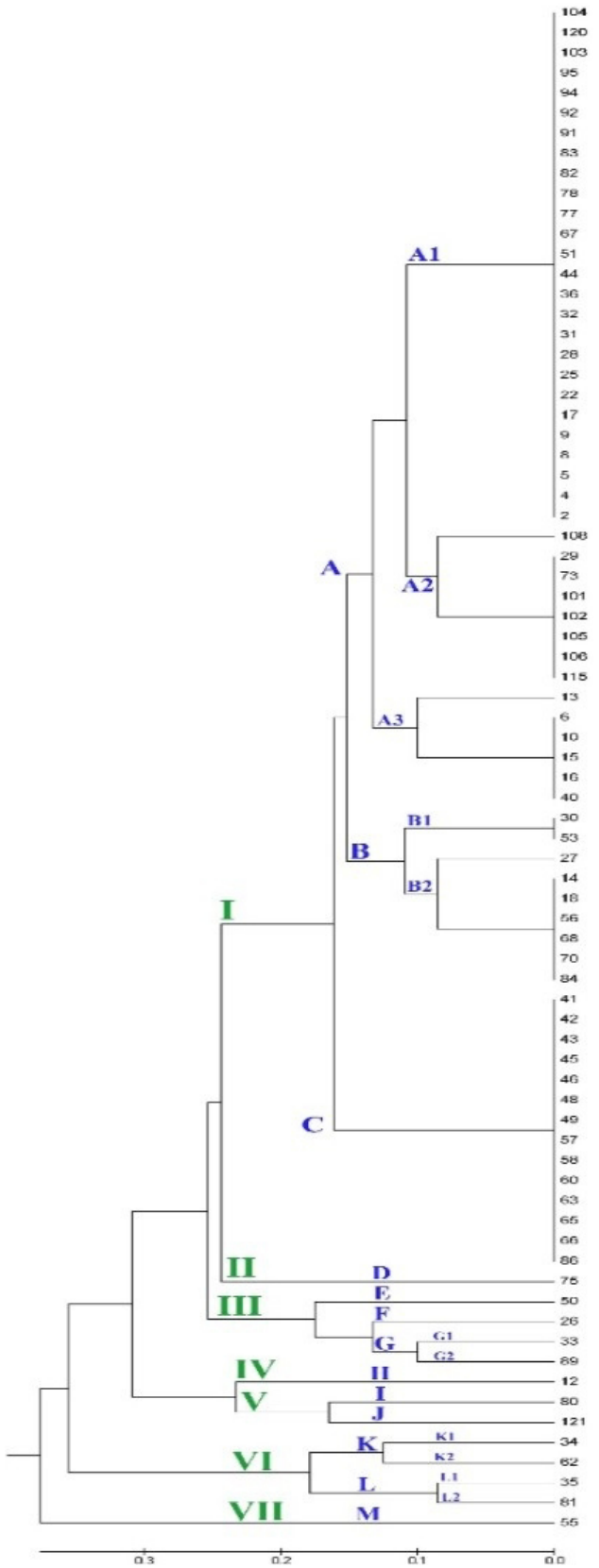

Figure 1. Dendrogram of *A. baumannii* isolates (I–VII: main groups, A–M: clones).

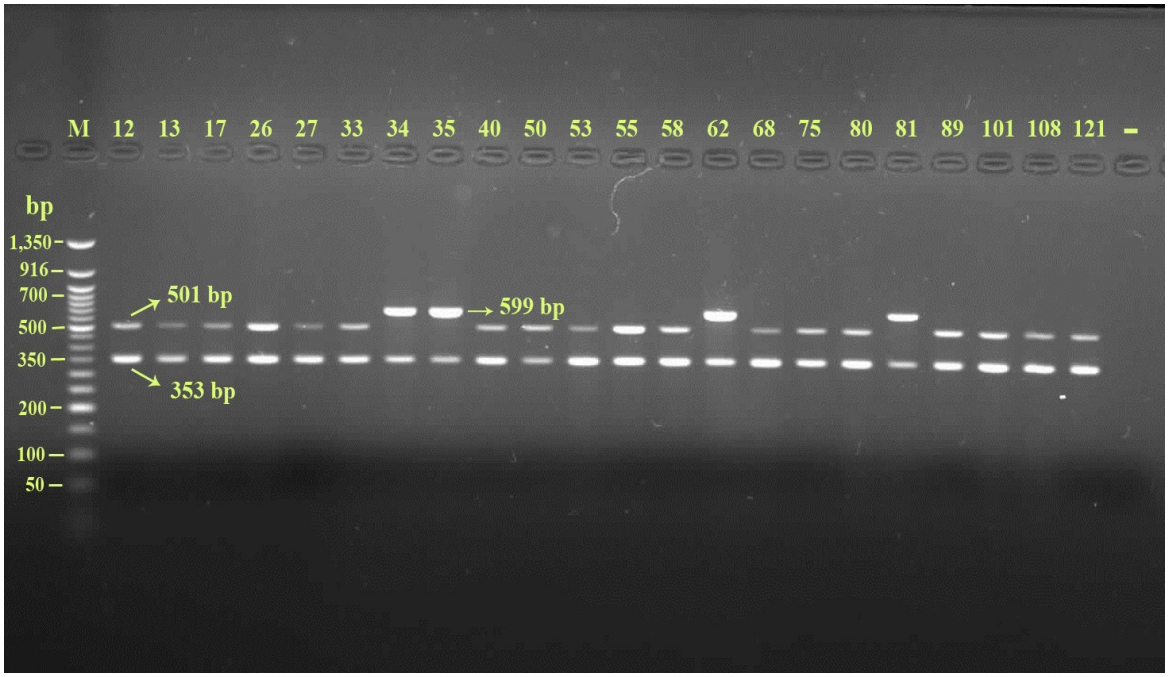

Figure 2. Multiplex PCR products of 22 *A. baumannii* isolates representing all clones and subtypes (M: 50-bp marker, -: negative control, OXA-51: 353 bp, OXA-23: 501 bp, OXA-58: 599 bp).

Table 3. Carbapenem-hydrolyzing oxacillinases (CHOs), clones, and imipenem (IMP) MIC values of 22 *Acinetobacter baumannii* isolates, which are representatives of each clone and subtype.

Strain No	Clone	IMP MIC (mg/L)	Detected CHO genes			
			OXA-51-like	OXA-23-like	OXA-58-like	OXA-24-like
12	H	16	+	+	–	
13	A3b	16	+	+	–	
17	A1	16	+	+	–	
26	F	32	+	+	–	
27	B2b	64	+	+	–	
33	G1	16	+	+	–	
34	K1	64	+	–	+	
35	L1	64	+	–	+	
40	A3a	16	+	+	–	
50	E	64	+	+	–	
53	B1	16	+	+	–	
55	M	64	+	+	–	
58	C	32	+	+	–	
62	K2	64	+	–	+	
68	B2a	32	+	+	–	
75	D	64	+	+	–	
80	I	64	+	+	–	
81	L2	64	+	–	+	
89	G2	64	+	+	–	
101	A2a	128	+	+	–	
108	A2b	32	+	+	–	
121	J	16	+	+	–	
Total 22			22	18	4	0

findings suggest that the $bla_{OXA\text{-}24\text{-}group}$ is not yet common in Turkey, although its prevalence should be monitored carefully.

Our results indicate that most imipenem-resistant *A. baumannii* isolates were clonally related in our hospital, and two CHO groups ($bla_{OXA\text{-}51\text{-}group}$ and $bla_{OXA\text{-}23\text{-}group}$) were responsible for imipenem resistance. It has been suggested that infection control measures should be applied strictly, and appropriate training should be provided to hospital staff to prevent the spread of carbapenem-resistant *A. baumannii* isolates. It is essential to carry out such molecular epidemiological studies to take the necessary measures against pathogens such as carbapenem-resistant *A. baumannii* strains.

Acknowledgments

We thank Prof Dr Safar Farajnia for kindly providing the positive control strains.

This study was supported by the Scientific Research Council of Ege University (Grant No. 12/ECZ/027).

References

1. Nowak P, Paluchowska P, Budak A. Distribution of bla_{OXA} genes among carbapenem-resistant *Acinetobacter baumannii* nosocomial strains in Poland. New Microbiol 2012; 35: 317-325.
2. Garnacho-Montero J, Amaya-Vıllar R. Multiresistant *Acinetobacter baumannii* infections: epidemiology and management. Curr Opin Infect Dis 2010; 23: 332-339.
3. Poirel L, Nordmann P. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. Clin Microbiol Infect 2006; 12: 826-836.
4. Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, Amyes SGB, Livermore DM. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int J Antimicro Ag 2006; 27: 351-353.
5. Opazo A, Domínguez M, Bello H, Amyes SGB, González-Rocha G. OXA-type carbapenemases in *Acinetobacter baumannii* in South America. J Infect Dev Countr 2012; 6: 311-316.
6. Djahmi N, Dunyach-Remy C, Pantel A, Dekhil M, Sotto A, Lavigne JP. Epidemiology of carbapenemase-producing enterobacteriaceae and *Acinetobacter baumannii* in Mediterranean countries. BioMed Res Int 2014; Article ID: 305784, http://dx.doi.org/10.1155/2014/305784.
7. Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Wayne, PA, USA: Clinical and Laboratory Standards Institute, 2000.
8. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24: 1596-1599.
9. Sohrabi N, Farajnia S, Akhi MT, Nahaei MR, Naghili B, Peymani A, Amiri Z, Rezaee MA, Saeedi N. Prevalence of OXA-type b-lactamases among *Acinetobacter baumannii* isolates from northwest of Iran. Microb Drug Resist 2012; 18: 385-389.
10. Coelho J, Woodford N, Afzal-Shah M, Livermore D. Occurrence of OXA-58-like carbapenemases in *Acinetobacter* spp. collected over 10 years in three continents. Antimicrob Agents Chemother 2006; 50: 756-758.
11. Ergin A, Hascelik G, Eser OK. Molecular characterization of oxacillinases and genotyping of invasive *Acinetobacter baumannii* isolates using repetitive extragenic palindromic sequence-based polymerase chain reaction in Ankara between 2004 and 2010. Scand J Infect Dis 2013; 45: 26-31.
12. Köseoğlu Eser Ö, Ergin A, Tunçkanat F, Hasçelik G. In vitro activity of tigecycline as a therapeutic option against multidrug-resistant *Acinetobacter* spp. New Microbiol 2008; 31: 535-542.
13. Gur D, Korten V, Unal S, Deshpande LM, Castanheira M. Increasing carbapenem resistance due to the clonal dissemination of oxacillinase (OXA-23 and OXA-58)-producing *Acinetobacter baumannii*: report from the Turkish SENTRY Program sites. J Med Microbiol 2008; 57: 1529-1532.
14. Karah N, Sundsfjord A, Towner K, Samuelsen Ø. Insights into the global molecular epidemiology of carbapenem non-susceptible clones of *Acinetobacter baumannii*. Drug Resist Update 2012; 15: 237-247.
15. Çiftci İH, Aşık G, Karakeçe E, Öksüz L, Yağcı S, Sesli Çetin E, Özdemir M, Atasoy AR, Koçoğlu E, Gül M et al. Distribution of bla_{OXA} genes in *Acinetobacter baumannii* strains: a multicenter study. Mikrobiyol Bul 2013; 47: 592-602.
16. Mugnier DP, Poirel L, Naas T, Nordmann P. Worldwide dissemination of the bla_{OXA}-*23* carbapenemase gene of *Acinetobacter baumannii*. Emerg Infect Dis 2010; 16: 35-40.
17. Zahedi Bialvaei A, Kafil HS, Leylabadlo HE, Asgharzadeh M, Aghazadeh M. Dissemination of carbapenemases producing Gram negative bacteria in the Middle East. Iran J Microbiol 2015; 7: 226-246.
18. Nur Sarı A, Biçmen M, Gülay Z. The first report on the outbreak of OXA-24/40-like carbapenemase-producing *Acinetobacter baumannii* in Turkey. Jpn J Infect Dis 2013; 66: 439-442.