Association of doripenem resistance with OXA-type carbapenemases in *Acinetobacter baumannii* isolates

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ABSTRACT

الأهداف: لتقييم النشاط في مختبر doripenem لراكدة بومانية (A. baumannii) ولعزل مختلف carbapenemases OXA ، وتقييم دور هذه الانزيمات في تطوير مقاومة carbapenem.

الطريقة: قد أجريت هذه الدراسة بأثر رجعي مع 25 ألف baumannii يعزل في جامعة ساكاريا للتدريب ومستشفى أبحاث، ساكاريا، تركيا خلال الفترة ما بين يونيو إلى أكتوبر2014م. وأجري اختبار الحساسية للمضادات الحيوية باستخدام النظام الآلي فيتيك 2 (bioMérieux، Marcy l'Etoile، France). تم تحديد تركيزات المثبطة الأدنى (MICs)باستخدام شرائط (bioMérieux، Marcy l'Etoile، France) . تم تنفيذ الكمية PCR في (Fluorion Instrument Iontek، Istanbul، Turkey) .

النتائج: تم تقسيم العزل إلى 5 مجموعات على أساس التشكيلات قابليتها وOXA من نوع carbapenemase الإيجابي.المجموعة 2 للعزل التي MIC كل الميروبينيم وdoripenem هي في حدود 4–32 ميكروغرام / مل كانت سلبية لكلا blaOXA–23 and blaOXA–58 . المجموعة 3 للعزل التي MIC من الميروبينيم وdoripenem هو في حدود 4–32 ميكروغرام / مل، blaOXA–23 هو إيجابي، blaOXA–58 هو سلبي. مجموعة 5 للعزل التي MIC من الميروبينيم هو >32 ميكروغرام / مل، وذلك من doripenem هو في حدود 16–32 ميكروغرام / مل كانت إيجابية لكلا blaOXA–23 and blaOXA–58 .

الخاتمة: تركيبات جين blaOXA–23 and blaOXA–58 قد تمنح المقاومة مع MIC أكبر بكثير من كل من الميروبينيم وdoripenem. ولكن وجود blaOXA–58 وحده لم يكن مرتبطا المقاومة doripenem.

Objectives: To evaluate the in vitro activity of doripenem in *Acinetobacter baumannii* (*A. baumannii*) clinical isolates that possess different OXA-type carbapenemases, and to evaluate the roles of these enzymes in the development of carbapenem resistance.

Methods: This retrospective study was conducted with 25 *A. baumannii* isolates at Sakarya University Training and Research Hospital, Sakarya, Turkey from June to October 2014. Antibiotic susceptibility testing was carried out using the Vitek-2 automated system (bioMérieux, Marcy l'Etoile, France). Minimum inhibitory concentrations (MICs) were determined using Etest strips (bioMérieux, Marcy l'Etoile, France). Quantitative polymerase chain reaction was performed in a Fluorion Instrument (Iontek, Istanbul, Turkey).

Results: Isolates were divided into 5 groups based on their susceptibility profiles and OXA-type carbapenemase positivity. Group 2 isolates whose MIC of both meropenem and doripenem are in the range of 4-32 μg/mL were negative for both bla_{OXA-23} and bla_{OXA-58}. Group 3 isolates whose MIC of meropenem and doripenem is in the range of 4-32 μg/mL, bla_{OXA-23} is positive, and bla_{OXA-58} is negative. Group 5 isolates whose MIC of meropenem is >32 μg/mL, and that of doripenem is in the range of 16-32 μg/mL were positive for both bla_{OXA-23} and bla_{OXA-58}.

Conclusion: The bla_{OXA-23} and bla_{OXA-58} gene combinations may confer resistance with a much greater MIC of both meropenem and doripenem. However, the presence of bla_{OXA-58} alone was not correlated with doripenem resistance.

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The rise of antibiotic resistance is an increasingly important threat, particularly for infections caused by *Acinetobacter baumannii (A. baumannii)*. The use of carbapenems to treat *A. baumannii* infection has resulted in outbreaks of infection with carbapenem-resistant *Acinetobacter spp*.[1] We are now faced with more problematic drug-resistant pathogens that threaten to move us into what some consider the post-antibiotic era of infectious diseases. Regardless, a potential strategy is to focus on the relation of molecular characterization of the isolates and the response to antimicrobial theraphy.[2] Carbapenem resistance in *Acinetobacter spp*. has been ascribed to the recruitment and production of carbapenem-hydrolyzing class D β-lactamases (CHDLs), and to a lesser extent metallo-β-lactamases. In *A. baumannii*, the CHDLs can be intrinsic (OXA-51-like), or acquired (OXA-23-like, OXA-24-like, and OXA-58-like).[3] Although these enzymes weakly hydrolyze carbapenems, they can confer strong resistance when bla_{OXA} genes are overexpressed, as a result of their association with mobile elements, such as IS*Aba1*, which carries a strong promoter.[4] The bla_{OXA-23} gene, in association with IS*Aba1* is spread by *A. baumannii* worldwide, and is the most prevalent OXA allele in isolates from Turkey.[3,5] However, the carbapenem against, which antibiotic resistance emerges according to OXA gene status is unknown. The purpose of this study was to evaluate the in vitro activity of doripenem in a collection of *A. baumannii* clinical isolates that possess different OXA-type carbapenemases, and to evaluate the roles of these enzymes in the development of carbapenem resistance.

Methods.

Bacterial isolates and antibiotic susceptibility testing. Twenty-five *A. baumannii* isolates were obtained from clinical samples sent to Sakarya University Training and Research Hospital Microbiology Laboratory, Sakarya, Turkey from June to October 2014. Species identification was performed using conventional methods. Antibiotic susceptibility testing was carried out using the Vitek-2 automated system (bioMérieux, Marcy l'Etoile, France). The isolates were divided into 5 groups: 1) carbapenem-susceptible and bla_{OXA-51}-positive; 2) carbapenem-resistant and bla_{OXA-51}-positive; 3) carbapenem-resistant and bla_{OXA-51}- and bla_{OXA-23}-positive; 4) carbapenem-resistant and bla_{OXA-51}- and bla_{OXA-58}-positive; and 5) carbapenem-resistant and bla_{OXA-51}-, bla_{OXA-23}-, and bla_{OXA-58}-positive.

Minimum inhibitory concentration (MIC) testing. The MICs of imipenem and meropenem were determined using the Vitek-2 automated system. The Densi-Check 2 system (bioMérieux, Marcy l'Etoile, France) was used to calibrate the turbidity of samples to 0.5 McFarland standard. The MIC values of ≥32 μg/ml for ceftazidime, ≥16 μg/ml for gentamicin, ≥128 μg/ml for piperacillin, ≥4 μg/ml for ciprofloxacin, ≥8 μg/ml for imipenem, and ≥8 μg/ml for meropenem were defined as resistant, based on the Vitek-2 automated system.

Minimum inhibitory concentration testing by Epsilometer test (Etest). The MICs of imipenem, meropenem, and doripenem were also determined using Etest strips with Imipenem IP 32, Meropenem MP 32, and Doripenem DOR 32 (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's guidelines. Suspensions of each isolate in Mueller-Hinton broth, adjusted to the density of a 0.5 McFarland standard, were swabbed in 3 directions to ensure uniform growth on Mueller-Hinton agar plates. Etest imipenem, meropenem, and doripenem strips (0.002 to 32 μg/ml) were applied to each plate when the agar surface was completely dry, and the plates were incubated at 35°C for 16-20 hours. The MIC was read at the point of complete inhibition of all growth, including hazes. The MIC values of ≥8 μg/ml for imipenem, meropenem and doripenem were defined as resistant.[6]

Quantitative polymerase chain reaction (qPCR). The DNA was extracted from fresh cultures of *A. baumannii* colonies according to the protocol included with the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). The qPCR was performed using a Fluorion Instrument (Iontek Laboratory, Istanbul, Turkey) with primers specific for Oxa_{51}, Oxa_{23}, and Oxa_{58} (**Table 1**). Amplification was carried out in triplicate using 3 samples for all isolates under the following conditions: initial denaturation for 10 minutes at 95 °C, followed by 45 cycles of denaturation at 95 °C for 20 seconds, annealing at 68 °C for 10 seconds, and elongation at 72 °C for 15 seconds. A final melting curve analysis was performed from 70 to 90 °C with a slope of 0.5 °C/second. Transcription data were analyzed using the Fluorion

Table 1 - List of primers used for amplification of different OXA-type carbapenemasess genes.

Gene	Primer	Sequence (5'-3')	GenBank no.
bla_{OXA-23}	OXA-23-F	CGGTCTTGATCTCATGCAAA	GQ849192.2
	OXA-23-R	CCCAACCAGTCTTTCCAAAA	
bla_{OXA-51}	OXA-51-F	TCAGCAAGAGGCACAGTTTG	EU255296.1
	OXA-51-R	GCTGAACAACCCATCCAGTT	
bla_{OXA-58}	OXA-58-F	AATTGGCACGTCGTATTGGT	EU131095.1
	OXA-58-R	CCCCTCTGCGCTCTACATAC	

Relative Quantification Software (Iontek Laboratory, Istanbul, Turkey). *Acinetobacter baumannii* ATCC 19606 was used as a standard isolate. Primer dimers and other artifacts were evaluated by melting curve analysis. To confirm that specific amplification had occurred, melting curves for each amplicon were assessed and compared with T_m values obtained using type isolate DNA as the template.

Study design. This work was performed as part of a laboratory based study, with no direct involvement with the affected patients. Data were investigated retrospectively and isolates that provided to in vitro were examined for further tests. There is no need for ethical approval.

Statistical analyses. Descriptive statistics are presented as mean ± standard deviation. One way ANOVA test was used for comparing the data. Statistical significance was assumed when $p<0.05$. All statistical evaluation was performed using Statistical Package for Social Sciences version 20 (IBM Corp., Armonk, NY, USA) (**Table 2**).

Results. The clinical isolates were divided into 5 groups based on their drug susceptibility profiles and OXA-type carbapenemase positivity. Twenty isolates were resistant to imipenem and meropenem with Vitek-2 system, while 5 isolates were susceptible to imipenem and meropenem. Carbapenem resistance reported on the basis of Vitek-2 system and Etest. The MIC values were between 4 and >32 for carbapenem-resistant isolates with Etest. All of the isolates were positive for bla_{OXA-51}. The isolates were divided into 5 groups according to their OXA-type carbapenemase positivity (**Table 3**).

Group 2 isolates whose MIC of both meropenem and doripenem are in the range of 4-32 μg/mL were negative for both bla_{OXA-23} and bla_{OXA-58}. Group 3 isolates whose MIC of meropenem and doripenem is in the range of 4-32 μg/mL, bla_{OXA-23} is positive and bla_{OXA-58} negative. Group 5 isolates whose MIC of meropenem is >32 μg/mL and that of doripenem is in the range of 16-32 μg/mL were positive for both bla_{OXA-23} and bla_{OXA-58}.

The isolates in the first group had a doripenem MIC of between 0.5 and 0.75 μg/mL. The percentage of the carbapenem-resistant isolates with a doripenem MIC of ≥8 μg/mL in group 2 was 80% (4/5), group 3 = 80% (4/5), group 4 = 20% (1/5), and group 5 = 100% (5/5). Doripenem MICs were high in groups 2, 3, and 5, also in all bla_{OXA-23}-positive isolates. By contrast, doripenem MICs were low in bla_{OXA-58}-positive isolates. Decrease in the doripenem MIC level was found to be significant in group 4 isolates ($p<0.001$). Carbapenem MIC ranges were above the limit values in isolates that possessed both bla_{OXA-23} and bla_{OXA-58} genes. The MIC ranges for imipenem were high in all carbapenem resistant isolates.

Discussion. Doripenem is a carbapenem antibiotic with activity similar to that of imipenem and ertapenem against Gram-positive cocci, and similar to that of meropenem against Gram-negative pathogens.[7] In a large study of nonfermentative bacilli, including 3,844 *A. baumannii* complex (ABC) isolates, 69.4% of the ABC isolates were susceptible to imipenem, 66.6% to meropenem, and 49.9% to doripenem, supporting the increased activity of imipenem and the decreased activity of doripenem.[8] By contrast, an earlier study found doripenem to be more active than imipenem and meropenem against 24 carbapenem-resistant ABC isolates (20.8% of the isolates were susceptible to doripenem, 16.7% to imipenem, and 4.2% to meropenem) by broth microdilution (BMD).[9] In this study, previous data verified on doripenem that was found to have higher activity against *A. baumannii* isolates than imipenem and meropenem.

β-lactamase production is the most prevalent mechanism of emerging resistance to β-lactam antibiotics, including carbapenems in *Acinetobacter spp*. as well as in other Gram-negative bacteria. Acinetobacter isolates that produce OXA-type β-lactamases have been reported to be resistant to carbapenems and to other antibiotics used extensively in clinics.[10] Of 539 *A. baumannii* clinical isolates investigated by the SENTRY antimicrobial surveillance program, OXA-type carbapenemases were found in 70% of the carbapenem-resistant isolates.[11] It was shown that bla_{OXA23} was the most prevalent gene (prevalence 95%) among the OXA-type carbapenemase-encoding genes, followed by bla_{OXA58} (11.9%) and $bla_{OXA24-40}$ (5.6%). In a multicenter study of 602 carbapenem-resistant *A. baumannii* isolates, high-level bla_{OXA-23}-like gene positivity was found at a rate of 74.4% (448/602) by

Table 2 - The mean minimum inhibitory concentration values with standard deviation for antibiotics tested by Etest.

Group	IPM	MEM	DOR
1	0.75 ± 0.00	0.65 ± 0.14	0.55 ± 0.11
2	>32 ± 0.00	22.8 ± 12.78	19.2 ± 12.45
3	>32 ± 0.00	26.8 ± 11.62	20 ± 12.01
4	>32 ± 0.00	10 ± 4.00	5.6 ± 1.67
5	>32 ± 0.00	>32 ± 0.00	28.8 ± 7.15
P-value*	1.000	0.009	<0.001

IPM - imipenem, MEM - meropenem, DOR - doripenem,
**p*-value was calculated excluding the first group

multiplex qPCR.[5] Based on year-on-year increases in positivity rates, Ciftci et al[5] concluded that bla_{OXA-23}-like gene positivity was responsible for carbapenem resistance in *A. baumannii* isolates. In our study, the percentage of carbapenem-resistant and bla_{OXA-23}-positive isolates (groups 3 and 5) with a doripenem MIC of ≥8 μg/mL was 90% (9/10). The mean MIC values for doripenem in groups 3 was 20 μg/ml and group 5 was 28.8 μg/ml. This suggests that bla_{OXA-23} may contribute to high doripenem resistance. However, isolates in group 2 with only bla_{OXA-51} had doripenem MIC values, indicating that other mechanisms such as outer membrane permeability, penicillin-binding protein modifications, and efflux pump systems may play an important role in doripenem resistance.

The OXA-58 was first identified in France in 2003 and confers reduced susceptibility to carbapenems.[1] Since then, this oxacillinase has been reported in hospitals from several European countries, including Spain, Turkey, Romania, Greece, Austria, the UK, and Italy.[1] Kulah et al[10] investigated the mechanisms of resistance to carbapenems in 145 carbapenem-resistant *A. baumannii* (CRAB) isolates. They found dominant bla_{OXA-58} positivity, but did not detect bla_{OXA-23} and bla_{OXA-24} positivity. The authors concluded that multiple clones of CRAB isolates producing OXA-58-type oxacillinase were responsible for a sustained CRAB outbreak in their hospital. In our study, of the carbapenem-resistant isolates, decrease in the doripenem MIC level was found to be significant in group 4 isolates ($p<0.001$). Additionally, doripenem resistance rate was found 20% (1/5), with a mean MIC value of 5.6 μg/ml for the carbapenem-resistant and bla_{OXA-51}- and bla_{OXA-58}-positive isolates (group 4). Therefore, we considered that bla_{OXA-58} was not correlated with doripenem resistance.

Table 3 - Carbapenem resistance pattern and presence of blaOXA genes in *Acinetobacter baumannii*.

Group	IPM*	MEM*	IPM†	MEM†	DOR†	OXA-51	OXA-23	OXA-58
1	S	S	0.75	0.75	0.75	+	-	-
1	S	S	0.75	0.5	0.5	+	-	-
1	S	S	0.75	0.75	0.5	+	-	-
1	S	S	0.75	0.75	0.5	+	-	-
1	S	S	0.75	0.5	0.5	+	-	-
2	R	R	>32	>32	>32	+	-	-
2	R	R	>32	6	4	+	-	-
2	R	R	>32	>32	>32	+	-	-
2	R	R	>32	>32	12	+	-	-
2	R	R	>32	12	16	+	-	-
3	R	R	>32	>32	>32	+	+	-
3	R	R	>32	6	4	+	+	-
3	R	R	>32	>32	16	+	+	-
3	R	R	>32	>32	16	+	+	-
3	R	R	>32	>32	>32	+	+	-
4	R	R	>32	16	4	+	-	+
4	R	R	>32	8	8	+	-	+
4	R	R	>32	8	4	+	-	+
4	R	R	>32	6	6	+	-	+
4	R	R	>32	12	6	+	-	+
5	R	R	>32	>32	>32	+	+	+
5	R	R	>32	>32	>32	+	+	+
5	R	R	>32	>32	>32	+	+	+
5	R	R	>32	>32	16	+	+	+
5	R	R	>32	>32	>32	+	+	+
ATCC	S	S	0.75	0.75	0.75			

*Vitek-2 antibiotic susceptibility results, †Etest results, IPM - imipenem, MEM - meropenem, DOR - doripenem

Chromosome-borne bla_{OXA-51}-like genes, which are ubiquitous in *A. baumannii*, play a role in carbapenem resistance when *ISAba1* precedes the gene; however, they have little effect on carbapenem susceptibility in the absence of this insertion sequence.[12] Our results indicate that bla_{OXA-51}-like genes were present in all of the clinical isolates of *A. baumannii* tested in this study. This result is in agreement with recent reports that an OXA-51-like oxacillinase occurs naturally in *A. baumannii*.[13]

Markelz et al[14] investigated the accuracy of carbapenem antimicrobial susceptibility tests for *A. baumannii-calcoaceticus* complex (ABC) isolates. They concluded that automated systems are less accurate than manual methods for determining susceptibility to imipenem and meropenem. Both automated and manual methods were inaccurate compared with BMD for doripenem using FDA breakpoints.[14] In our study isolates in group 4 were identified as meropenem-resistant using the Vitek-2 system, but Etest showed that only one isolate had a high meropenem MIC. This is remarkable considering studies related to the accuracy of automated systems. Therefore, for the study of meropenem resistance, Etest seems to be better than Vitek-2 system.

This study had certain limitations. Given the small sample size, the generalizability of our results is unclear. Further studies will be necessary to understand the mechanisms underlying this concordance between OXA enzymes and carbapenem resistance. However, an important limitation of this study was the failure to detect the presence of the IS*Aba1* gene. In statistical analyses, *p*-value was calculated excluding the first group.

In conclusion, our data support the idea that the basic mechanism underlying carbapenem resistance in *A. baumannii* isolates involves bla_{OXA} genes. Presence of bla_{OXA-23} is an important resistance mechanism of doripenem. But bla_{OXA-23} and bla_{OXA-58} gen combinations may confer resistance with a much greater MIC of both meropenem and doripenem. But bla_{OXA-58} presence alone was not correlated with doripenem resistance. Additionally, to improve accuracy, manual susceptibility testing with Etest confirmation, in addition to the Vitek-2 method, may be recommended.

References

1. Kohlenberg A, Brümmer S, Higgins PG, Sohr D, Piening BC, de Grahl C, et al. Outbreak of carbapenem-resistant *Acinetobacter baumannii* carrying the carbapenemase OXA-23 in a German university medical centre. ***J Med Microbiol*** 2009; 58: 1499-1507.
2. Terzi HA, Kulah C, Ciftci IH. The effects of active efflux pumps on antibiotic resistance in *Pseudomonas aeruginosa*. ***World J Microbiol Biotechnol*** 2014; 30: 2681-2687.
3. Zowawi HM, Sartor AL, Sidjabat HE, Balkhy HH, Walsh TR, Al Johani SM, et al. Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* isolates in the Gulf Cooperation Council States: dominance of OXA-23-type producers. ***J Clin Microbiol.*** 2015; 53: 896-903.
4. Elabd FM, Al-Ayed MS, Asaad AM, Alsareii SA, Qureshi MA, Musa HA. Molecular characterization of oxacillinases among carbapenem-resistant *Acinetobacter baumannii* nosocomial isolates in a Saudi hospital. ***J Infect Public Health*** 2015; 8: 242-247.
5. Ciftci IH, Aşık G, Karakeçe E, Oksüz L, Yağcı S, Sesli Çetin E, et al. [Distribution of blaOXA genes in *Acinetobacter baumannii* strains: a multicenter study]. ***Mikrobiyol Bul*** 2013; 47: 592-602. Turkish
6. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing - Twenty-Fourth Informational Supplement, M100S24E. CLSI: Wayne (PA); 2014.
7. Dedhia HV, McKnight R. Doripenem: position in clinical practice. ***Expert Rev Anti Infect Ther*** 2009; 7: 507-514.
8. Castanheira M, Jones RN, Livermore DM. Antimicrobial activities of doripenem and other carbapenems against *Pseudomonas aeruginosa*, other nonfermentative bacilli, and *Aeromonas spp*. ***Diagn Microbiol Infect Dis*** 2009; 63: 426-433.
9. Jones RN, Sader HS, Fritsche TR. Comparative activity of doripenem and three other carbapenems tested against Gram-negative bacilli with various beta-lactamase resistance mechanisms. ***Diagn Microbiol Infect Dis*** 2005; 52: 71-74.
10. Kulah C, Mooij MJ, Comert F, Aktas E, Celebi G, Ozlu N, et al. Characterisation of carbapenem-resistant *Acinetobacter baumannii* outbreak strains producing OXA-58 in Turkey. ***Int J Antimicrob Agents*** 2010; 36: 114-118.
11. Mendes RE, Bell JM, Turnidge JD, Castanheira M, Jones RN. Emergence and widespread dissemination of OXA-23, -24/40 and -58 carbapenemases among *Acinetobacter spp*. in Asia-Pacific nations: report from the SENTRY Surveillance Program. ***J Antimicrob Chemother*** 2009; 63: 55-59.
12. Li H, Liu F, Zhang Y, Wang X, Zhao C, Chen H, et al. Evolution of carbapenem-resistant *Acinetobacter baumannii* revealed through whole-genome sequencing and comparative genomic analysis. ***Antimicrob Agents Chemother*** 2015; 59: 1168-1176.
13. Lee YT, Kuo SC, Chiang MC, Yang SP, Chen CP, Chen TL, et al. Emergence of carbapenem-resistant non-baumannii species of Acinetobacter harboring a blaOXA-51-like gene that is intrinsic to *A. baumannii*. ***Antimicrob Agents Chemother*** 2012; 56: 1124-1127.
14. Markelz AE, Mende K, Murray CK, Yu X, Zera WC, Hospenthal DR, et al. Carbapenem susceptibility testing errors using three automated systems, disk diffusion, Etest, and broth microdilution and carbapenem resistance genes in isolates of *Acinetobacter baumannii-calcoaceticus* complex. ***Antimicrob Agents Chemother*** 2011; 55: 4707-4711.