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FALSE POSITIVE PHENOTYPIC DETECTION OF METALLO-BETA-LACTAMASES IN *ACINETOBACTER BAUMANNII*

Branka Bedenić^{1,2}, Ranko Ladavac³, Mirna Vranić-Ladavac⁴, Nada Barišić⁴, Natalie Karčić⁴, Katherina Bernadette Sreter⁵, Slobodan Mihaljević⁶, Luka Bielen⁷, Haris Car⁸ and Nataša Beader^{1,2}

¹Department of Microbiology, School of Medicine, University of Zagreb, Zagreb, Croatia;

²Department of Clinical and Molecular Microbiology, Zagreb University Hospital Centre, Zagreb, Croatia;

³Department of Nephrology, Pula General Hospital, Pula, Croatia; ⁴Department of Microbiology, Public Health Institute of Istria County, Pula, Croatia; ⁵Department of Clinical Immunology, Pulmonology and Rheumatology, Sestre milosrdnice University Hospital Centre, Zagreb, Croatia;

⁶Department of Anesthesiology, Zagreb University Hospital Centre, Zagreb, Croatia;

⁷Department of Internal Medicine, Zagreb University Hospital Centre, Zagreb, Croatia;

⁸Zagreb Secondary Medical School, Zagreb, Croatia

SUMMARY – Phenotypic detection of metallo- β -lactamases (MBLs) in *Acinetobacter (A.) baumannii* is a serious challenge to clinical microbiologists. MBLs are inhibited by metal chelators such as ethylenediaminetetraacetic acid (EDTA). Production of MBLs cannot be recognized based on resistance phenotype. Therefore, phenotypic tests using EDTA are recommended. The aim of this study was to investigate the sensitivity and specificity of inhibitor based tests (EDTA) for detection of MBL. A total of 172 *A. baumannii* strains (123 carbapenemase positive and 49 carbapenemase negative) were analyzed. Phenotypic detection of MBLs was performed by the combined disk test with EDTA (CDT-EDTA) and EPI-dilution test (EPI-DT). Both tests were positive in all 11 isolates possessing VIM-1 MBL, showing 100% sensitivity. However, false positive results were observed in strains with class D carbapenemases using both tests, i.e. all OXA-23 and OXA-24/40 producing organisms and most OXA-58 positive strains (77% with CDT-EDTA vs. 65% with EPI-DT). False positive results can occur because oxacillinases are converted to a less active state in the presence of EDTA, leading to augmentation of the inhibition zone around the carbapenem disk or reduction of carbapenem minimum inhibitory concentrations. This study showed high sensitivity but low specificity of phenotypic methods in the detection of MBLs.

Key words: *Beta-lactamases; Imipenem; Meropenem; Acinetobacter baumannii; Edetic acid*

Introduction

Acinetobacter (A.) baumannii is a glucose non-fermentative gram-negative coccobacillus, considered a relevant nosocomial pathogen occurring particularly

in intensive care units (ICUs), as well as in burn therapy units¹. The most frequent healthcare-associated infections are urinary tract infections, bacteremia, surgical site infections, and ventilator-associated pneumonia. *A. baumannii* strains become resistant to almost all antimicrobial agents, including carbapenems². Beta-lactamase-mediated resistance is the most common mechanism of carbapenem resistance in this species. The most important mechanism of carbapenem resistance is the production of carbapenemases of class A

Correspondence to: Branka Bedenić, MD, PhD, Department of Clinical and Molecular Microbiology, Zagreb University Hospital Centre, Kišpatićeva 12, HR-10000 Zagreb, Croatia;

E-mail: bbedenic@mef.hr, branka.bedenic@kbc-zagreb.hr

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(KPC)³, class B (metallo-beta-lactamases: metallo-β-lactamases (MBLs) of IMP, VIM or SIM family)⁴⁻⁸ or class D carbapenem-hydrolyzing class D carbapenemases (CHDL)⁹. However, *Acinetobacter* may develop resistance to carbapenems through various combined mechanisms, including decreased permeability, altered penicillin binding proteins (PBPs), and rarely, efflux pump overexpression¹⁰. The production of MBLs cannot be detected based on conventional susceptibility testing. Isolates co-harboring CHDL and MBLs have also been reported¹¹⁻¹⁴, complicating the phenotypic detection. The genes encoding MBLs are located on integrons that frequently contain genes encoding extended spectrum beta-lactamases (ESBLs) and other resistance determinants, thus reducing therapeutic options¹⁵. Therefore, simple screening and inexpensive non-molecular methods need to be implemented in routine practice of diagnostic clinical laboratories. MBLs are inhibited by chelating agents such as ethylenediaminetetraacetic acid (EDTA) and thiol compounds that are frequently used in inhibitor based detection methods¹⁶. Several tests have been proposed for the detection of MBLs in gram-negative bacteria with Epsilon test (E-test) and combined disk test most commonly used^{8,16}. However, there are concerns about the specificity of inhibitor-based tests, particularly in *Pseudomonas aeruginosa*¹⁷. False positive results have been reported because the chelating agents themselves increase membrane permeability and enhance the bactericidal effect. Recently, false positive phenotypic tests have also been described in *A. baumannii*^{14,18}. However, there are no systematic studies to date on the reliability of inhibitor based tests for detecting MBLs in *A. baumannii*. The aim of this study was to assess the sensitivity and specificity of inhibitor based tests in the detection of MBLs in *A. baumannii* possessing VIM and CHDL, as well as to determine the accuracy of these non-molecular tests in distinguishing between MBLs and other carbapenemases.

Materials and Methods

Bacteria

A total of 172 (123 carbapenem-resistant and 49 carbapenem-susceptible) *A. baumannii* strains were collected in this multicenter study (Pula General Hospital, Zagreb University Hospital Centre, Varaždin

General Hospital, Sisak General Hospital, Merkur University Hospital, Dr Fran Mihaljević University Hospital for Infectious Diseases, Bjelovar General Hospital and Godan Nursing Home from Zagreb) during the 2009-2014 period from various clinical specimens and analyzed in our previous studies^{14,19-20}.

Susceptibility testing

Susceptibility to imipenem and meropenem was tested by disk diffusion and broth microdilution test, and interpreted according to the CLSI criteria²¹⁻²³.

Detection of metallo-β-lactamases

Phenotypic detection of MBLs was performed by combined disk test with the addition of EDTA and by EPI-broth microdilution test with the addition of 1,10-phenanthroline and EDTA (0.5 mM)^{17,24-25}. Augmentation of the inhibition zone around imipenem and meropenem disk by at least 7 mm in the presence of EDTA was considered to be a positive result in disk diffusion, whereas an 8-fold (three dilutions) reduction in the minimum inhibitory concentration (MIC) of imipenem or meropenem by EDTA was indicative of the production of MBL in dilution test. The genes encoding MBLs (IMP, VIM, NDM, SIM) and carbapenem-hydrolyzing oxacillinases were detected by polymerase chain reaction (PCR) and sequencing in previous studies^{14,19,20}.

Results

Susceptibility testing

All carbapenemase positive strains, including MBL and CHDL were found to be resistant or intermediately susceptible to carbapenems with MICs of imipenem and meropenem ranging from 8 to >128 mg/L. The carbapenemase negative strains were predominantly susceptible or intermediately susceptible to carbapenems with 10 strains exhibiting resistance to meropenem due to the insertion sequence *ISAba1* upstream of *bla*_{OXA-51} gene.

Characterization of beta-lactamases

Twenty-seven strains were positive for *bla*_{OXA-23}-like, 74 for *bla*_{OXA-40}-like, and 22 for *bla*_{OXA-58}-like genes.

Eleven of the *bla*_{OXA-23}-like positive isolates co-harbored *bla*_{VIM-1} gene. Sequencing of representative *bla* genes revealed the presence of *bla*_{OXA-23}, *bla*_{OXA-72}, *bla*_{OXA-58}, and *bla*_{VIM-1} genes. Forty-nine strains were negative for acquired carbapenemases and were used as negative control strains.

Detection of metallo-β-lactamases

Combined disk test with EDTA and EPI-dilution test were positive in all 11 isolates possessing VIM-1 MBL, showing sensitivity of 100%, as illustrated in Table 1. However, false positive results in inhibitor based disk tests were observed in all OXA-23 and OXA-24/40 producing organisms with both disks, and in 77% and 73% of OXA-58 positive strains with imipenem and meropenem disk, respectively. Thus, the specificity of inhibitor based disk test was 0% for both OXA-23 and OXA-24/40, and 23% and 27% for OXA-58 producing strains, depending on the indicator disk. EPI dilution method yielded false positive results in all OXA-23 positive and OXA-24/40 positive strains and 68% of OXA-58 organisms (Table 1). For EPI-dilution test, the specificity was 0% for OXA-23 and OXA-24/40 positive isolates and 32% for OXA-58 producers. The carbapenemase negative strains yielded false positive results in disk test in 4 (8%) and 5 (10%) out of 49 strains with imipenem and meropenem disk, respectively. Only one strain demonstrated a false positive result in dilution method (2%).

The augmentation of the inhibition zone around imipenem disk in the presence of EDTA ranged from 8 to 16 mm with a median of 11 mm for OXA-23 MBL negative strains, 8 to 17 mm for OXA-24/40 (median 14 mm), and 4 to 18 mm for OXA-58 producers, as shown in Table 1. MBL positive strains showed an increase of the inhibition zone around imipenem disk of 8 to 15 mm (median 13 mm), as seen in Table 1. No significant increase of the inhibition zone was observed in carbapenemase negative strains (0 to 10 mm, median 5 mm).

The increase of the inhibition zone around the meropenem disk ranged from 9 to 17 mm for OXA-23 MBL negative strains (median 13 mm), 8 to 17 mm for OXA-24/40 positive strains (median 15 mm), and 5 to 19 mm for OXA-58 (median 13 mm). The true MBL positive strains showed enlargement of the inhibition zone around imipenem disk ranging from 12 to 18 mm (median 16 mm), as depicted in Table 1. Car-

Table 1. Number and percentage of positive isolates in dilution and disk method with imipenem or meropenem for metallo-β-lactamase (MBL) positive strains (true positive), carbapenem-hydrolyzing class D carbapenemases (CHDL) producing strains (false positive) and carbapenemase negative strains. Reduction of minimum inhibitory concentrations (MICs) and increase of the inhibition zone in the presence of EDTA is shown (range and median)

Type of carbapenemase	Number of positive strains /number of strains tested* n (%)	Range of imipenem MIC (mg/L) reduction (X-fold) †	Number of positive strains /number of strains tested n (%)	Range of meropenem MIC (mg/L) reduction (X-fold) †	Number of positive strains /number of strains tested ‡ n (%)	Range of inhibition zone enlargement (mm) around the imipenem disk (median)	Number of positive strains /number of strains tested ‡ n (%)	Range of inhibition zone enlargement (mm) around the meropenem disk (median)
VIM+OXA-23	11/11 (100%)	16->64 (32)	11/11 (100%)	16-256 (64)	11/11 (100%)	8-15 (13)	11/11 (100%)	12-18 (16)
OXA-23	16/16 (100%)	8-32 (16)	16/16 (100%)	8-64 (16)	16/16 (100%)	8-16 (11)	16/16 (100%)	9-17 (13)
OXA-24/40	74/74 (100%)	4->128 (16)	74/74 (100%)	8-256 (16)	74/74 (100%)	8-17 (14)	74/74 (100%)	8-17 (15)
OXA-58	15/22 (68%)	4->128 (48)	15/22 (68%)	4-512 (32)	17/22 (77%)	4-18 (10.5)	16/22 (73%)	5-19 (13)
Carbapenemase negative	1/49 (2%)	0-4 (2)	1/49 (2%)	0-8 (4)	4/49 (8%)	0-10 (5)	5/49 (10%)	0-8 (5)

*broth microdilution method with imipenem as indicator; †EDTA and 1,10-phenanthroline; ‡combined disk test with imipenem or meropenem disk as indicator

bapenemase negative strains yielded an increase of the inhibition zone around the imipenem disk ranging from 0 to 8 mm (median 5 mm).

The median enlargement of the inhibition zone for imipenem was highest with true MBL positive strains and OXA-24 producers (14 mm), followed by OXA-23 positive strains (11 mm) and OXA-58 (10.5 mm). The median increase of the meropenem inhibition zone in the presence of EDTA was most pronounced with true MBL producers (16 mm) and OXA-24/40 positive strains (15 mm), followed by OXA-58 and OXA-23 (13 mm). EDTA embedded blank disks produced growth inhibition zones ranging from 4 to 7 mm due to the bactericidal effect of EDTA causing increased membrane permeability.

The positive predictive value of the combined disk test with imipenem disk among the carbapenemase positive isolates was very low (9.48%) due to the very high number of false positive results. However, the negative predictive value was 100% with carbapenem-resistant strains.

Discussion

Taken all together, the specificities of the combined disk test and EPI dilution test among OXA positive strains were very low (4.46% and 6.25%, respectively). Their specificities for MBL detection were much higher among *A. baumannii* strains that were carbapenemase negative (91.8% and 98%, respectively). The difference between false positive rates of MBL detection with imipenem as indicator among OXA positive and carbapenemase negative *A. baumannii* strains was found to be statistically significant with both combined disk test and dilution method ($p < 0.001$).

High sensitivity of inhibitor based tests was previously reported in *A. baumannii*²⁶. However, specificity is a problem. EDTA caused a significant increase of the imipenem and meropenem inhibition zone, not only for MBL producers, but also for the CHDL positive isolates. False positive results in inhibitor based tests occur because in the presence of EDTA, oxacillinases are converted to a less active state leading to augmentation of the inhibition zone around the carbapenem disk and reduction of carbapenem MICs. This phenomenon was previously reported in *A. baumannii*¹⁸. The rate of false positive results in our study

was highest among OXA-40 and OXA-23 positive isolates and much lower but still very high in OXA-58 positive isolates. This result can be explained by better hydrolysis of carbapenem substrates by OXA-40-like and OXA-23-like beta-lactamases and less efficient by OXA-58.

With combined disk test, differences in the percentages of false positive results between OXA-23 and OXA-58 and between OXA 24/40 and OXA-58 were statistically significant ($p < 0.05$ and $p < 0.001$, respectively), as were those with EPI-dilution test ($p < 0.05$ and $p < 0.001$, respectively). The study showed high sensitivity but low specificity of phenotypic methods in the detection of MBLs in *A. baumannii* and pointed to the necessity of molecular detection of MBL genes in *A. baumannii* by PCR. Low specificity was found only for CHDL positive organisms. The carbapenemase negative strains yielded only a few false positive results, demonstrating that the effect of EDTA on oxacillinases rather than the bactericidal effect of EDTA itself was responsible for the false positive results. There was no difference in the sensitivity and specificity of both methods with regard to the indicator antibiotic. Similar results were obtained with both carbapenems, imipenem and meropenem. According to our results, inhibitor based tests can distinguish MBL positive from carbapenemase negative, but not from CHDL positive organisms.

Unlike in *A. baumannii*, EDTA based disk test was shown to have high specificity in *Enterobacteriaceae* in distinguishing MBLs from KPC and OXA-48²⁷. Some authors recommend double-disk synergy test using two ceftazidime disks and a filter disk containing EDTA adjacent to one CAZ disk for detection of MBLs in *Acinetobacter* spp., *Pseudomonas* spp. and *Enterobacteriaceae*. Expansion of the inhibition zone between two ceftazidime disks indicates production of MBL²⁴. According to some studies, supplementation of the EDTA disks with mercaptiopropionic acid as a thiol compound increases the sensitivity and specificity of the double-disk synergy test for detection of MBLs in *A. baumannii* and other gram-negative bacteria²⁸.

The difficulty to detect MBLs in *A. baumannii*, variable MICs of carbapenems, together with their potential to disseminate, highlights the need for an accurate screening method. E-test MBL is an easy and simple but expensive method¹⁶. Inhibitor based test

with EDTA, although inexpensive and simple to perform, lacks specificity. Dilution method is both laborious and time consuming, and was also shown to have low specificity. Molecular methods are expensive and require well-equipped laboratories and educated staff. Therefore, detection of MBLs in *A. baumannii* is a huge challenge to clinical microbiologists, particularly in isolates with multiple carbapenem-resistance mechanisms, such as those co-harboring CHDL and MBL. The difficulties with phenotypic detection have also been reported for OXA-48 β -lactamase in *Enterobacteriaceae*²⁹.

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Sažetak

LAŽNO POZITIVNA FENOTIPSKA DETEKCIJA METALO-BETA-LAKTAMAZA U *ACINETOBACTER BAUMANNII*

B. Bedenić, R. Ladavac, M. Vranić-Ladavac, N. Barišić, N. Karčić, K.B. Sreter, S. Mihaljević, L. Bielen, H. Car i N. Beader

Fenotipska detekcija metalo- β -laktamaza (MBL) u *Acinetobacter (A.) baumannii* je velik izazov kliničkim mikrobiolozi- ma. MBL su inhibirane metalnim kelatorima kao što je etilendiamin tetraoctena kiselina (EDTA). Produkcija MBL ne može se prepoznati na temelju fenotipa rezistencije. Zbog toga se preporuča primjena fenotipskih testova koji rabe metalne kela- tore kao što je EDTA. Cilj istraživanja bio je utvrditi osjetljivost i specifičnost testova s inhibitorom (EDTA) u otkrivanju MBL u *A. baumannii*. Analizirana su 172 izolata *A. baumannii* (123 karbapenemaza pozitivnih i 49 karbapenemaza negativ- nih). Fenotipska detekcija MBL je provedena metodom kombiniranih diskova (EDTA CDT-EDTA) i EPI-dilucijskom metodom (EPI-DT). Oba testa su bila pozitivna u svih 11 izolata pozitivnih na VIM-1 MBL, što odgovara osjetljivosti od 100%. Lažno pozitivni rezultati su dobiveni u karbapenem-rezistentnim izolatima pozitivnim na klasu D karbapenemaza u oba testa: svi OXA-23 i OXA-24/40 kao i većina OXA-58 pozitivnih sojeva (77% u CDT-EDTA i 65% u EPI-DT). Lažno pozitivni rezultati u testovima s inhibitorom nastaju zbog toga što oksacilinaze prelaze u stanje manje aktivnosti u prisustvu EDTA, što dovodi do smanjenja minimalne inhibitorne koncentracije karbapenema ili do uvećanja zone oko diska karbape- nema. Studija je pokazala visoku osjetljivost, ali nisku specifičnost fenotipskih testova za dokaz MBL u *A. baumannii*, što ukazuje na neophodnost korištenja molekularnih metoda.

Ključne riječi: *Beta-laktamaze; Imipenem; Meropenem; Acinetobacter baumannii; Etilen-diamin tetraoctena kiselina*