

PHENOTYPIC AND GENOTYPIC DETERMINATION
OF METALLO-BETA-LACTAMASES IN CLINICAL
ACINETOBACTER SPP ISOLATES

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Abstract

Carbapenem-resistant *Acinetobacter* isolates are increasingly isolated worldwide due to the intensive use of carbapenems. Metallo-beta-lactamases (MBL) are class B betalactamases and can hydrolyse all betalactams including carbapenems except aztreonam. It is necessary to detect carbapenem-resistant strains in routine microbiology laboratories to prevent the spread of antibiotic resistance and to choose the best treatment. Genotyping is reliable but expensive and complicated. For this reason, as an alternative to genotyping, cheap, reliable, and easily applicable phenotypic methods that can be used in daily practice are being developed. This study aimed to determine the presence of MBL causing carbapenem resistance in *Acinetobacter* isolates by genotypic and phenotypic methods for routine laboratory use. Double disc synergy test (DDST), combined disc diffusion test (CDDT), modified Hodge test (MHT) and carbapenemase inactivation method (CIM) were performed as phenotypic tests. Multiplex polymerase chain reaction (PCR) was performed to genotypically detect NDM, VIM, IMP, GIM, SIM, SPM gene regions responsible for MBL production. Among the phenotypic tests, the highest positivity rate was in the CIM test with 98 isolates (95.1%). CIM was followed by DDST with 94.1% and CDDT with 86.4%. The lowest positivity rate was found in MHT with 70.8%. NDM, VIM, IMP, SIM, SPM and GIM genes were not detected in the tested isolates. Despite high positivity in phenotypic tests, no positivity was detected in genotypic tests. There may be some reasons for this situation: Variants of these genes may not have been detected with the primers used. Due

to the bactericidal effect of EDTA, false positivity may be seen in DDST and CDDT. Carbapenemases other than MBL, penicillin binding protein changes, efflux pumps, porin loss, and decreased permeability may have caused higher positivity in MHT and CIM. In our study, it was concluded that phenotypic tests significantly detected carbapenemase production, but should be used more carefully for the detection of MBL production.

Key words: *Acinetobacter*, carbapenem, metallo-beta-lactamases, phenotypic, genotypic

Introduction. *Acinetobacter baumannii* is an opportunistic pathogen that frequently causes outbreaks of infection, mostly in intensive care units. *A. baumannii* mainly causes bacteraemia, pneumonia (especially ventilator-associated pneumonia), and urinary tract infection after hospitalisation of patients with more severe disease. In the last decade, the number of multidrug-resistant isolates of *A. baumannii* has been increasing, probably as a result of the widespread use of antimicrobial agents. Class B β -lactamases, or metallo- β -lactamases (MBLs), require zinc ions to catalyse the hydrolysis of β -lactams and have no sequence or structural homology with serine β -lactamases. They exhibit a broad spectrum substrate profile catalysing the hydrolysis of a wide range of β -lactam antibiotics including penicillins, cephalosporins, carbapenems, and cefamycins. MBLs efficiently hydrolyse all β -lactams in vitro except aztreonam [1]. Carbapenem resistance types should be determined in clinical microbiology laboratories in order to limit the spread of resistance and to determine effective treatment. Although genotypic methods are the most reliable, alternative, cost-effective and easy-to-use phenotypic tests are being developed.

The aim of this study was to determine the presence of MBL causing carbapenem resistance by genotypic and phenotypic methods for routine laboratory use.

Material and methods. This study included 103 carbapenem-resistant *Acinetobacter baumannii* isolates. Species-level identification was performed by Vitek MS (Biomérieux, France), and antibiotic susceptibility was determined by the Vitek 2 Compact (Biomérieux, France) automated system. Double disc synergy test (DDST), combined disc diffusion test (CDDT), modified Hodge test (MHT), and carbapenemase inactivation method (CIM) were used to determine carbapenemases. Multiplex PCR was performed using specific primers to determine IMP, VIM, GIM, SIM and SPM genes.

Double disc synergy test (DDST). *A. baumannii* isolates were inoculated on Mueller Hinton agar with 0.5 McFarland turbidity and an imipenem disc (10 μ g) and a blank disc were placed 12 mm (centre to centre) away. Ten μ l EDTA (0.5 M, pH 8) was added to the blank disc and evaluated after 18 h incubation. The test was considered positive if the zone of inhibition of the imipenem disc expanded towards the EDTA blank disc.

Combined disc diffusion test (CDDT). After *A. baumannii* isolates were inoculated on Mueller Hinton agar with 0.5 McFarland turbidity, two imipenem discs (10 µg) were placed on the medium surface with 22 mm between them. Ten µl EDTA (0.5 M, pH 8) was added to one of the imipenem discs and evaluated after 18 h incubation. The test was considered positive if the zone of inhibition of the imipenem/EDTA disc was ≥ 7 mm larger than the zone diameter of the imipenem disc alone [2].

Modified Hodge test (MHT). The suspension of *E. coli* ATCC 25922 strain prepared at 0.5 McFarland turbidity was diluted 1:10 and inoculated on Mueller Hinton agar. A meropenem disc (10 µg) was placed in the centre of the medium. *A. baumannii* isolates to be tested were inoculated in a straight line from the centre of the plate to the periphery and evaluated after 18 h of incubation. The test was considered positive in the case of a disturbed zone of inhibition [3].

Carbapenem inactivation method (CIM). A suspension was made by suspending a full 10 µl inoculation loop of culture, taken from blood agar plate in 400 µl water. Meropenem (10 µl) disc was added to the suspension and incubated for 2 h. At the end of the 2 h, the disc in the suspension was removed and placed on Mueller Hinton agar inoculated with carbapenem sensitive *E. coli* ATCC 25922 standard strain and evaluated after 24 h of incubation. The test was considered positive in case of growth around the meropenem disc (no inhibition zone) [4].

Molecular methods. Primers defined by WOODFORD [5] for the determination of IMP, VIM, GIM, SIM, SPM genes and ZARFEL et al. [6] for the determination of NDM gene were used (Table 1). For PCR reaction, 10X PCR buffer (5 µl), 25 mM MgCl₂ (4 µl), dNTP mixture (10 mM) (1 µl), Taq DNA polymerase (5 U/µl) (1 µl), primers (1 µl), template DNA (4 µl), purified water (23 µl) were added. Initially, pre-denaturation was performed at 95 °C for 5 min, followed by 30 cycles of amplification. Each cycle consisted of 45 s target DNA denaturation at 95 °C, 45 s primer binding at 60 °C, 1 min primer elongation at 72 °C. Finally, the amplification programme was terminated with a final extension of 8 min at 72 °C. After amplification, DNA bands were separated according to their molecular size under 120 V electric current for 75 min. After staining the gel in distilled water containing 0.5 µg/ml ethidium bromide for 20 min, the gel was analysed in an imaging device. In PCR tests, positive strains containing NDM, VIM, IMP, SIM, SPM, GIM genes were used as positive controls.

Due to the nature of the study, informed consent was not obtained, only isolates were tested in the study and electronic data of patients were used without identifying information. The study was approved by the directorate of hospital of medical faculty.

Results. Tracheal aspirate samples (*n*: 49, 47.5%) constituted the majority of the clinical samples. Tracheal aspirate samples were followed by blood (*n*: 24, 23.3%) and wound (*n*: 12, 11.6%) samples. All isolates are sensitive to colistin. After colistin, the least resistance was found against amikacin (*n*:

T a b l e 1

SIM, SPM, GIM, NDM, IMP, VIM genes primer sequence

Primer name	Primer sequence
IMP-F	5'-GGAATAGAGTGGCTTAATTCTC
IMP-R	5'-CCAAACCACTACGTTATCT
SPM-F	5'-AAAATCTGGGTACGCAAACG
SPM-R	5'-ACATTATCCGCTGGAACAGG
VIM-F	5'-GATGGTGTTTGGTTCGCATA
VIM-R	5'-CGAATGCGCAGCACCAG
GIM-F	5'-TCGACACACCTTGGTCTGAA
GIM-R	5'-AACTTCCAACTTTGCCATGC
SIM-F	5'-TACAAGGGATTCGGCATCG
SIM-R	5'-TAATGGCCTGTTCCCATGTG
NDM-F	5'-ACC GCC TGG ACC GAT GAC CA
NDM-R	5'-GCC AAA GTT GGG CGC GGT TG

51, 49.5%). Amikacin was followed by gentamicin (n : 74, 71.8%) and trimethoprim/sulfamethoxazole (n : 82, 79.6%). Levofloxacin and ciprofloxacin were found to be resistant in all isolates.

Among the phenotypic tests, the highest positivity rate was in the CIM test with 98 isolates (95.1%). CIM test was followed by DDST with 97 isolates (94.1%), CDDT with 89 isolates (86.4%), MHT with 73 isolates (70.8%) (Table 2).

T a b l e 2

Results of phenotypic tests

	DDST(%)	CDDT(%)	MHT (%)	CIM(%)
Positive	97 (94.1)	89 (86.4)	73 (70.8)	98 (95.1)
Negative	6 (5.8)	14 (13.5)	30 (2.9)	5 (4.8)

DDST: Double disc synergy test

CDDT: Combined disc diffusion test

MHT: Modified Hodge test

CIM: Carbapenem inactivation method

In all PCRs, no positivity was detected in NDM, VIM, IMP, SIM, GIM, SPM gene regions, although the bands belonging to the gene regions used as positive control were detected.

Discussion. The increasing incidence of multiple antibiotic resistance, including carbapenem resistance, and its ability to cause epidemics, especially in intensive care units (ICUs), has led to an increase in the importance of *A. baumannii* and has been constantly on the agenda. CESUR et al. [7] reported that *A. baumannii* isolates were most frequently isolated from tracheal aspirates with 42.5% and from wounds with 20%. ZER et al. [8] reported that 44.6% of *A. baumannii* isolates were isolated from respiratory tract samples and 34.7% from wound swabs in 2024. In our study, *A. baumannii* isolates were most frequently isolated from tracheal aspirate samples (n : 49, 47.5%). To find relevant articles, BOSTANGHADIRI et al. [9] searched MEDLINE (PubMed), Embase and Web of Science for relevant studies published in English language from 2000 to 25 April 2023. Subgroup meta-analysis of continents showed that Western Europe had the highest resistance to colistin at 7% and Eastern Europe had the lowest resistance to colistin at 1%. SHOJA et al. [10] in their 2017 study with *A. baumannii* isolates, found 97.5% resistance to ciprofloxacin, 92.5% to cefepime and ceftazidime, 80% to amikacin and 85% to gentamicin, but they did not detect resistance to colistin as in our study.

In a study conducted in European countries in 2008, carbapenem resistance among *Acinetobacter* strains was reported as 27% on average [11]. In the study conducted by Shoja et al. in 2017, 92.5% of the isolates were resistant to imipenem and meropenem. In the study of VURAL et al. [12] in 2024, it was determined that 75 (97.4%) strains were resistant to imipenem, while all strains were resistant to meropenem. Carbapenem resistance is gradually increasing in *Acinetobacter* isolates. Chromosomal or plasmid-transferred carbapenemases, modification of penicillin-binding proteins, efflux systems, loss of outer membrane proteins, absence of the CarO gene may cause development of resistance to carbapenems in *Acinetobacter* isolates [13]. In recent years, the most emphasised mechanism of carbapenem resistance is the presence of MBL enzymes. Although OXA-type carbapenemases are more common than MBL-type enzymes in *Acinetobacter* isolates, the carbapenemase activity of MBLs is significantly more potent (100–1000-fold) than OXA-type carbapenemases. SAIKIA et al. [14] detected 70.34% blaNDM and 51.16% blaIMP in genotypic tests to detect MBL production in 172 *A. baumannii* isolates. AKSOY et al. [15] determined the specificity and sensitivity of CIM as 100% and 45%, respectively, and TIJET et al. [16] determined the specificity and sensitivity of CIM as 100% and 98.8%, respectively. DEMIRAY et al. [17] detected positivity in 54 (90%) isolates with MHT and 52 (86.6%) isolates with CIM in 60 *Klebsiella* isolates in which carbapenemase production was detected by molecular method. When the molecular method was considered the gold standard, the sensitivities of the MHT and CIM were 85% and 80%, and the specificities were

95% and 93%, respectively. ASLAN et al. [18] found positivity in 34 isolates with DDST, 46 isolates with CDDT and 88 isolates with MHT in 100 carbapenem resistant *A. baumannii* isolates. AKSOY et al. [19] in 52 carbapenem resistant *A. baumannii* isolates, they detected 96% carbapenemase positivity with MHT, 21% MBL positivity with DDST and 98% MBL positivity with CDDT, but they did not detect IMP, VIM, GIM, SIM, SPM and NDM gene positivity. In the study of Shoja et al. conducted in 2017, MBL positivity was found in 55.6% and 97.3%, respectively, in *A. baumannii* isolates isolated from burn patients, according to the DDST and E-test results, while they could not detect any of the gene regions of NDM, IMP, VIM, SPM by PCR [10]. Similarly, although we detected positivity rates of 70.8% in MHT, 95.1% in CIM, 94.1% in DDST, 86.4% in CDDT, we could not detect IMP, VIM, NDM, GIM, SIM, SPM genes in PCR. Although carbapenemase production was detected at a high rate in phenotypic tests, MBL genes could not be detected by molecular methods. Variants of these genes may not have been detected with the primers used. Phenotypic tests such as CDDT and DDST are based on the inhibition of MBL by EDTA. EDTA can increase the permeability of the bacterial cell membrane at high concentrations and can be bactericidal. This situation can lead to false positives [20]. Since MHT and CIM detect the presence of carbapenemases, carbapenemases other than MBL may have caused the high positivity rate in these tests. PCR can be performed to detect these carbapenemases in a further study. In addition, change of penicillin binding protein, efflux pumps, loss of porin and decreased permeability may also explain the high positivity of these tests. In our study, it was concluded that phenotypic tests showed carbapenemase production significantly, but should be used more carefully for MBL production.

Carbapenem resistance is increasing in *A. baumannii* isolates in our country as in the whole world. Most of the genes encoding resistance-causing enzymes are localised in highly mobile genetic elements. Detection of carbapenem resistance and MBL production is important in terms of preventing the spread of resistant isolates. In addition, it is thought that the determination of the resistance mechanisms in bacteria will be useful in the selection of appropriate antibiotics, and may guide hospitals in determining empirical treatment options and taking preventive measures.

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