Evaluation of metallo-beta-lactamase production in multiple antibiotic-resistant *Pseudomonas* spp. and *Acinetobacter baumannii* strains

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ABSTRACT

This study aimed to evaluate the metallo-beta-lactamase (MBL) production in *Pseudomonas* spp. and *Acinetobacter baumannii* using phenotypic and genotypic methods and to determine the most appropriate phenotypic method. The study included 55 *Pseudomonas* spp. (53 *Pseudomonas aeruginosa*, 1 *P. fluorescens* and 1 *P. putida*) and 33 *A. baumannii* isolates which were resistant to imipenem (IMP) and/or meropenem (MEM). Six phenotypic and one genotypic (real-time polymerase chain reaction [RT-PCR]) methods were used. According to the phenotypic tests, the rates of MBL-positive *Pseudomonas* spp. and *A. baumannii* were, respectively: 25.5% and 39.4% by the gradient test; 21.8% and 21.2% by the Rosco rapid CARB screen test; 9.1% and 21.2% by the modified Hodge test (MHT); 32.7% and 66.7% by the combined EDTA disk diffusion test; 56.4% and 100% by IMP + EDTA and 49.0% and 72.7% by MEM + EDTA and 9.1% and 3.0% by IMP + dipicolinic acid (DPA) for the Rosco MBL confirm test; 36.4% and 61.0% by IMP + DPA and 54.5% and 61.0% MEM + DPA for the double disk synergy test. MBL genes were detected only in three *Pseudomonas* spp. (blaIMP in two *P. aeruginosa* isolates and blaVIM in a *P. fluorescens* isolate). For *Pseudomonas* spp., the MBL positivity rate did not significantly differ between the RT-PCR and MHT and between the RT-PCR and Rosco MBL confirm test (with IMP + DPA) (p > 0.10). In conclusion, the Rosco MBL confirm test (with IMP + DPA) phenotypically predicted the MBL positivity most closely to the RT-PCR method for both *Pseudomonas* spp. and *A. baumannii* isolates.

Introduction

*Pseudomonas aeruginosa* and *Acinetobacter baumannii* are among the most important causes of healthcare-associated infections. In these bacteria, production of carbapenemases plays an important role in antimicrobial resistance. Among the carbapenemases, metallo-beta-lactamases (MBLs) are becoming increasingly important [1].

In infections for which carbapenems are used, detecting the likely production of carbapenemases using a simple and reliable method has become equally important as the isolation of the agent itself [2]. Although molecular methods are the most reliable methods for detecting carbapenemases, their use in routine applications is impractical; thus, a phenotypic test with a high sensitivity and specificity is needed for daily use [3].

The present study aimed to investigate the presence of MBLs in *Pseudomonas* spp. and *A. baumannii* isolates using several phenotypic methods and real-time polymerase chain reaction (RT-PCR), as the molecular method, and to determine the most appropriate phenotypic method yielding the closest result with that of the molecular method.

Materials and methods

The present study included *Pseudomonas* spp. and *A. baumannii* isolates, which were isolated from various clinical samples sent to the Laboratory of Medical Microbiology Department in Ankara Dişkapı Yıldırım Beyazıt Training and Research Hospital between January and December 2014 and were found to be resistant to imipenem (IMP) and/or meropenem (MEM)
Table 1. Nucleotide sequences of primers used in polymerase chain reactions.

| Gene   | Primer sequence (5′–3′) |
|--------|-------------------------|
| IMP-F  | 5′-GGA ATA GAG TGG CTT AAY TCT C-3′ |
| IMP-R  | 5′-CCA AAC YAC TAS GTT ATC T-3′ |
| VIM-F  | 5′-GAT GTT TGG TCC CAT A-3′ |
| VIM-R  | 5′-CGA ATG CGC AGC ACC AG-3′ |
| GIM-F  | 5′-TGG ACA CAC CTT GTG CTG AA-3′ |
| GIM-R  | 5′-AAC TTC CAA CTT TGC CAT GC-3′ |
| SPM-F  | 5′-AAA ATC TGG GTA GCA AAA CG-3′ |
| SPM-R  | 5′-ACA TTA TCC GCT GGA ACA GG-3′ |
| SIM-F  | 5′-TAC AAG GGA TTC GGC ATC G-3′ |
| SIM-R  | 5′-TAA TGG CCT GTT CCC ATG TG-3′ |

according to the Clinical & Laboratory Standards Institute (CLSI) guidelines [4].

Identification of isolates and antibiotic susceptibility testing

The study included 88 non-duplicate isolates, of which 55 were *Pseudomonas* spp. and 33 were *A. baumannii* isolates. The identification and antimicrobial susceptibility testing of the isolates were performed using the BD Phoenix 100 automated system (Becton Dickinson, USA). The minimum inhibitory concentrations (MICs) of IMP and MEM were determined using the gradient test (ETEST®, bioMérieux, France). *Escherichia coli* ATCC® 25922™, *P. aeruginosa* ATCC® 27853™ and IMP-, VIM-, and SPM-positive isolates from previous studies were used as controls.

Evaluation of metallo-beta-lactamase production by phenotypic methods

In the present study, six different phenotypic tests were used to evaluate MBL production in the isolates:

1. Gradient test: MBL gradient strips (ETEST®, bioMérieux, France) with IMP on one end and IMP-EDTA on the other end were used. MBL positivity was evaluated as ≥eighthfold reduction in IMP MIC values detected in the presence of EDTA.
2. Rosco Rapid CARB screen test: This test was performed using the Rapid CARB Screen (RCS) kit (Rosco Diagnostica A/S, Taastrup, Denmark) and the results were interpreted in accordance with the manufacturer’s instructions. Although the test was for *Enterobacteriaceae* and *P. aeruginosa* strains, it was also performed for *A. baumannii* in the present study.
3. Modified Hodge Test (MHT): This test is based on the principle of showing the degradation that occurs in the inhibition zone of susceptible bacteria in the presence of carbapenemase-producing bacteria. MHT was performed and interpreted as described previously by Lee et al. [5].
4. Combined EDTA disk diffusion test: This test is based on the demonstration of ≥7 mm enlargement of the zone diameter of IMP in the presence of 750 μg/disk EDTA due to inhibition of carbapenemase enzyme in the presence of the metal-chelating agent EDTA [6].
5. Rosco MBL confirm test: This test was performed using the Total MBL Confirm kit (98016; Rosco Diagnostica A/S, Taastrup, Denmark), which uses tablets developed to detect MBL-producing bacteria. The test is based on the inhibition of MBL by EDTA and DPA. The kit contains 10 μg IMP, 10 μg MEM, IMP+EDTA, IMP+DPA and DPA disks. The MBL test was considered positive if the inhibition zone diameters in the presence of EDTA were ≥7 mm larger than the inhibition zone diameters and if the inhibition zone diameters in the presence of DPA were ≥5 mm larger than the inhibition zone diameters.
6. Double disk synergy test with DPA: The synergy between DPA and IMP and MEM disks was investigated, as the indicator of presence of MBL, as described by Lee et al. [7].

Evaluation of metallo-beta-lactamase production by RT-PCR

The RT-PCR method was used to evaluate the MBL production using the following primers: IMP, VIM, GIM, SPM and SIM. The nucleotide sequences of the primers are presented in Table 1. DNA isolation for the isolates was performed according to the manufacturer’s instructions (Qiagen GmbH, Germany).

Statistical analysis

Data analysis was performed using the DOS Microstat statistics package. The results of the descriptive statistics were expressed as numbers and percentages. Comparison of the results from the RT-PCR method with those from the phenotypic methods for each species was performed using the dependent samples t-test. A p-value of <0.05 was considered statistically significant.

Results and discussion

Among the 88 isolates included in this study, 53 were identified as *P. aeruginosa*, one was identified as *P. fluorescens*, one was identified as *P. putida*, and the
remaining 33 ones were identified as *A. baumannii*. The rates of the isolates that were positive for MBLs according to the phenotypic methods and RT-PCR are summarized in Table 2. A representative result from each phenotypic test is presented in Figure 1.

According to the RT-PCR results, the presence of MBL genes was detected only in three *Pseudomonas* spp. (Table 2): *bla*<sub>IMP</sub> was detected in two *P. aeruginosa* isolates, while *bla*<sub>VIM</sub> was found in the *P. fluorescens* isolate.

**Comparison of RT-PCR with phenotypic methods for metallo-beta-lactamase detection**

For the *Pseudomonas* spp. isolates, there was a significant difference between the rates of MBL positive isolates detected by RT-PCR and those detected by all phenotypic methods, except for the Rosco MBL confirm test (IMP + DPA) and MHT ($p > 0.10$). Although no MBL gene was detected in *A. baumannii* by RT-PCR, there was no significant difference between the results obtained using RT-PCR with the Rosco MBL confirm test (IMP + DPA) and the double disc synergy test (IMP/DPA) ($p > 0.10$ for both). There were no significant differences between the rates of MBL positive isolates detected by other phenotypic methods and by RT-PCR. Both for the *Pseudomonas* spp. and the *A. baumannii* isolates, false positivity was highest with Rosco MBL confirm (IMP + EDTA; MEM + EDTA) and combined EDTA disk diffusion tests.

*P. aeruginosa* and *A. baumannii* are very resistant bacteria, particularly causing infections in the intensive care units, and their antibiotic resistance tends to increase in recent years [8–10]. As the carbapenem group of antibiotics are the last available option, carbapenem resistance has a particular importance. In the 2014 report of the European Antimicrobial Resistance Surveillance Network (EARS-Net), carbapenem resistance was reported as 4%–43% in *Pseudomonas* spp. and as 0%–93% in *Acinetobacter* spp.
spp. which were isolated from invasive specimens [11]. In the 2016 report of the EARS-Net, carbapenem resistance was reported as 2.4%–51.6% in Pseudomonas spp. and as 0%–95.4% in Acinetobacter spp. isolated from invasive specimens [12]. In the 2014 report of the Central Asian and Eastern European Surveillance of Antimicrobial Resistance (CAESAR), 10%–87% of P. aeruginosa isolates and 11%–93% of A. baumannii isolates from invasive specimens were reported to be carbapenem resistant [13]. In the more recent 2016 report of the CAESAR, 12%–86% of P. aeruginosa isolates and 11%–95% of A. baumannii isolates from invasive specimens were reported to be carbapenem resistant in 2015 [14]. In the 2013 annual report of the National Antimicrobial Resistance Surveillance System of Turkey (NARSS), the carbapenem resistance rates of P. aeruginosa isolated from blood and cerebrospinal fluid samples were 33.4% and 26.1% for IMP and MEM, respectively [15]. Although the total carbapenem resistance rate was unknown during the collection of the isolates, the IMP and MEM resistance rates were 95.5% and 35% respectively, in blood isolates of A. baumannii and 96.1% and 27.5% respectively, in blood isolates of P. aeruginosa which were collected in 2017 in our hospital (data not shown). In the present study, isolates resistant to either IMP or MEM were included among all the clinical isolates collected during the study period.

Since MBLs play an important role in the resistance against carbapenem group antibiotics, detecting MBL production in bacterial isolates by a simple and reliable laboratory method is of great importance. Although the most reliable methods are molecular methods, such as PCR and sequence analysis, they are not practical options for routine use in laboratories, since they are expensive, require special equipment, and are labor intensive. Thus, phenotypic tests with high sensitivity and specificity to be used in routine laboratory practice are crucial [16,17].

Although Walsh et al. [18] detected a sensitivity of 94% and a specificity of 95% for the Etest MBL strip (gradient test) in P. aeruginosa strains, false positivity has been reported to be still high both in Pseudomonas spp. and A. baumannii strains [18–21], as was also determined in our study. For the Rosco rapid CARB screen test, while AbdelGhani et al. [22] reported a sensitivity of 99% and a specificity of 100%, Dortet et al. [23] reported a sensitivity of 89.5% and a specificity of 71%. In the present study, 12 Pseudomonas spp. were detected as carbapenemase positive by the Rosco rapid CARB screen test; however, only three isolates were found to be MBL positive by RT-PCR. Nine isolates were positively identified by the Rosco rapid CARB screen test but negatively by the molecular method. Therefore, in the present study, it can be assumed that the Rosco rapid CARB screen test was positive because of the presence of other carbapenemases which were not investigated. MHT is a simple method; currently, however, it is not widely used due to its subjectivity and high false positivity [5]. In the present study, MHT was positive in 9.1% of Pseudomonas spp. and 21.2% of A. baumannii isolates. Another test used for the phenotypic determination of MBLs is the combined EDTA disk diffusion test [14]. The sensitivity of this test is between 76% and 96% in different studies [6, 21, 24]. In the present study, the rate of MBL positivity by the combined EDTA disk diffusion test was 32.7% in Pseudomonas spp. isolates and 66.7% in A. baumannii isolates. As compared with the RT-PCR results, the combined EDTA disk diffusion test had a high rate of false positivity both in Pseudomonas spp. and in A. baumannii isolates.

The Rosco MBL confirm test is based on inhibition of MBLs with EDTA and DPA. Bartolini et al. [25] found that the Rosco MBL confirm test had a sensitivity of 95% and a specificity of 99% and emphasized that it was the most appropriate phenotypic test for routine use.

In their study investigating the presence of MBLs in P. aeruginosa and A. baumannii isolates, Hansen et al. [26] reported that the sensitivities of all carbapenem inhibitor combinations of the Rosco total MBL confirm kit were >80%, whereas the specificities and positive predictive values were low except for the combination of IMP-DPA in A. baumannii isolates. In another study, the sensitivity and specificity of the Rosco MBL confirm test were 89% and 49% respectively [27]. In the present study, the rate of MBL positivity with the Rosco MBL confirm test varied for Pseudomonas isolates. The highest rate was 56.4% with the combination of IMP+EDTA, followed by 49% with the combination of MEM+EDTA. In the A. baumannii isolates, the MBL positivity (100%) was also highest with the combination of IMP+EDTA. Due to its inhibitory property, EDTA has a high false positivity rate when combined either with IMP or with MEM.

In their study, Yong et al. [28] used the double disk synergy method for phenotypically demonstrating the presence of MBLs in Pseudomonas and Acinetobacter isolates, which were previously detected to be MBL positive. They reported that the combinations of IMP+DPA and MEM+DPA showed positive results in 93.2% and 86.4% of the isolates respectively. Picão et al. [29] suggested that the double-disk synergy test
was suitable for routine use but emphasized that the best screening method was dependent on the isolate type and common carbapenemases in that area. In the present study, for the double disk synergy test, the rate of MBL positivity in *Pseudomonas* isolates was 36.4% with the combination of IMP + DPA and 54.5% with the combination of MEM + DPA. The rate of MBL positivity in *A. baumannii* isolates was 6% both for these two combinations.

Küçükbasmacı et al. [30] did not detect any MBL resistance gene by multiplex PCR in any one of the 51 isolates of *P. aeruginosa*. Aksoy et al. [31] did not detect any MBL gene in 52 IMP resistant *A. baumannii* isolates but reported OXA-23-like and OXA-51-like resistance genes. In a study from northern Turkey, among 100 *P. aeruginosa* isolates studied by Ozgumus et al. [32], VIM-type MBL was detected in one strain, IMP-1 type MBL was detected in nine strains, and clonal analysis of the IMP-1 type positive isolates revealed that all the strains were clonally related. The results from our study were similar to those reported in the study by Ozgumus et al. [32]. In the present study, the Rosco MBL confirm test (combination of IMP + DPA) phenotypically predicted the MBL positivity most closely to the RT-PCR method for both *Pseudomonas* spp. and *A. baumannii* isolates.

In our study, the presence of IMP-, VIM-, SPM-, SIM- and GIM-type MBL encoding genes in the isolates of *A. baumannii* and *P. aeruginosa* was also investigated and, similar to the results of a recent multiyear multinational study, the IMP- and VIM-type MBL encoding genes were found to be the most frequent [33]. The present study did not examine the presence of OXA-type carbapenemases. It is known that carbapenem resistance can occur due to different carbapenemases other than MBLs, which might affect the positivity of the results of some of the tests used in the present study.

**Conclusions**

Although molecular tests are gold standard tests for detection of MBLs, they are very labor intensive, expensive, and unavailable for all routine laboratory applications. Identification of *Pseudomonas* spp. and *A. baumannii* strains with the most accurate and reliable methods and implementing early isolation measures are very important. In the present study, among the examined phenotypic tests, the Rosco MBL confirm test (combination of IMP + DPA) predicted the MBL positivity most closely to the RT-PCR method for both *Pseudomonas* spp. and *A. baumannii* isolates. In order to determine the sensitivities and specificities of phenotypic tests on different species with different carbapenem resistance mechanisms, further studies should be performed on previously detected isolates with already known resistance mechanisms and genes.

**Acknowledgments**

We are cordially grateful to Prof. Zerrin Aktaş from the Department of Medical Microbiology of Medical Faculty in Istanbul University for supplying the positive control strains used in our molecular studies.

**Disclosure statement**

The authors report no conflicts of interest.

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