Araştırma Makalesi *Research Article*

Pamukkale Tıp Dergisi
Pamukkale Medical Journal

doi:https://dx.doi.org/10.31362/patd.1142048

Evaluation of risk factors and detection of metallo-beta-lactamase enzyme production in carbapenem-resistant *Pseudomonas* and *Acinetobacter* species

Karbapenem dirençli Pseudomonas ve Acinetobacter türlerinde metallo-beta-laktamaz üretiminin saptanması ve risk faktörlerinin değerlendirilmesi

Suna Seçil Öztürk Deniz, Nurcan Baykam

Gönderilme tarihi:07.07.2022 Kabul tarihi:09.08.2022

Abstract

Purpose: To investigate the frequency of MBLs in *Pseudomonas* and *Acinetobacter* species with E-test, to determine the risk factors and to evaluate the demographic and clinical features of infected patients.

Materials and methods: Imipenem or meropenem resistance of *Pseudomonas* and *Acinetobacter* isolated from several clinical samples with conventional methods were evaluated with imipenem EDTA E-test and the presence of Metallo-β-lactameses MBL was examined. Several isolates were screened for VIM-1, VIM-2, IMP-1, and IMP-2 with a PCR test.

Results: Of 46 carbapenem resistant *Acinetobacter* isolates, 41 (89%), as well as of 19 carbapenem resistant *Pseudomonas* isolates, 5 (26%) had MBL positivity with imipenem-EDTA E-test. A history of Intensive Care Unit stay, mechanical ventilation and cephalosporin use were found to be significant risk factors with respect to MBL production.

Conclusion: Detection of MBL production in *Acinetobacter* and *Pseudomonas* species especially in ICU patients is of prime importance to control infection rapidly and effectively, which contribute to prevention of outbreaks.

Key words: Metallo-beta-lactameses, *Acinetobacter* spp, *Pseudomonas* spp.

Ozturk Deniz SS, Baykam N. Evaluation of risk factors and detection of metallo-beta-lactamase enzyme production in carbapenem-resistant *Pseudomonas* and *Acinetobacter* species. Pam Med J 2022;15:814-823.

Öz

Amaç: *Pseudomonas* ve *Acinetobacter* türlerinde, E-test ile MBL sıklığının araştırılması, risk faktörlerinin belirlenmesi ve enfekte hastaların demografik ve klinik özelliklerinin değerlendirilmesi.

Gereç ve yöntem: Çeşitli klinik örneklerden konvansiyonel yöntemlerle izole edilen *Pseudomonas* ve *Acinetobacter* türlerinin imipenem veya meropenem direnci imipenem EDTA E-test ile değerlendirildilerek, Metallo-beta-laktamaz (MBL) varlığı incelendi. MBL varlığı saptanan izolatlardan örnekler alınarak, PCR testi ile VIM-1, VIM-2, IMP-1 ve IMP-2 taraması yapıldı.

Bulgular: 46 karbapenem dirençli *Acinetobacter* spp. izolatından 41'i (%89) ve 19 karbapenem dirençli *Pseudomonas* spp. izolatından 5'inde (%26) imipenem-EDTA E-testi ile MBL- pozitifliği saptandı. Yoğun bakımda yatış öyküsü, mekanik ventilasyon ve sefalosporin kullanımı MBL üretimi açısından önemli risk faktörleri olarak bulundu.

Sonuç: Özellikle yoğun bakım üniteleri hastalarından izole edilen *Acinetobacter* ve *Pseudomonas* türlerinde MBL üretiminin pratik yöntemlerle kısa sürede saptanması, enfeksiyonların hızlı ve etkin bir şekilde kontrol altına alınmasını kolaylaştırarak, salgınların önlenmesinde büyük önem taşımaktadır.

Anahtar kelimeler: Metallo-beta-laktamazlar, *Acinetobacter* spp, *Pseudomonas* spp.

Öztürk Deniz SS, Baykam N. Karbapenem dirençli *Pseudomonas* ve *Acinetobacter* türlerinde metallo-beta-laktamaz üretiminin saptanması ve risk faktörlerinin değerlendirilmesi. Pam Tıp Derg 2022;15:814-823.

Suna Seçil Öztürk Deniz, Ass. Prof. Pamukkale University Faculty of Medicine, Department of Infectious Diseases and Clinical Microbiology, Denizli, Turkey, e-mail: susede20@yahoo.com (https://orcid.org/0000-0002-5440-5383) (Corresponding Author)

Nurcan Baykam, Prof. Hitit University Corum Erol Olcok Training and Research Hospital, Department of Infectious Diseases, Corum, Turkey, e-mail: nbaykam@yahoo.com (https://orcid.org/0000-0002-2398-8686)

Introduction

Multi-drug resistant (MDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are the most commonly isolated bacteria among the agents of healtcare infections. These agents leading to severe infections and outbreaks especially in the intensive care units have become increasingly resistant to many antibiotics as well as carbapenem, with a significant threat to public health across the world [1-4].

Carbapenemases, released by bacteria, is one of the important mechanisms involved in the development of resistance to carbapenems (imipenem and meropenem). Metallo-β-lactameses (MBLs) that make up Ambler Molecular Class B compose the most significant group among carbapenemases due to their ability to hydrolyze beta lactamases except for aztreonam. Among a variety of gram-negative bacilli, the resistance genes are likely to become highy disseminated. It is essential to rapidly detect Metallo-β-lactamase (MBL)-positive gram-negative bacilli in order to control infection and to prevent their spreading [5, 6].

MBL producing bacteria are difficult to identify clinically with the use of routine antibiotic sensitivity tests, therefore, molecular techniques or enzyme tests are required. Straightforward phenotypic methods, such as double-disk synergy (DDS) tests using a ceftazidime disk and imipenem/imipenem+EDTA (IP/IPI) E-test that limits the MBL activity by chelating agents such as EDTA have been introduced to the laboratory practice. Due to the technical problems confronted with the disc diffusion method, the use of IP/IPI E-test, which is rapid, specific, repeatable, can be beneficial to surveillance studies to monitor the emergence of MBL [6, 7].

The current study aimed to investigate the incidence of MBL in MDR *Acinetobacter* and *Pseudomonas* species isolated from hospitalized patients with E-test, and to evaluate the demographic and clinical features.

Materials and methods

Setting and data acquisition

This study was based on a thesis in medical specialty. Data involved the period before 2020. Approval of the ethics committee was not required when the study was carried out. This study was performed for a mean period of one year in a tertiary hospital with 1200 beds in Turkey. Patients most of whom stayed in ICU and who were followed in the internal diseases and surgery clinics, and carbapenem resistant *Acinetobacter* spp and *Pseudomonas* spp. were evaluated.

All methods were carried out in line with the current guidelines and regulations. Demographic (age, gender) and clinical data (the clinic where patients were followed, previous antibiotic use, underlying diseases, undergoing invasive procedures, a history of ICU stay) were obtained from the patient's files.

Isolation of *Acinetobacter* spp. and *Pseudomonas* spp.

Isolates from blood, urine, tracheal aspirates, wound, sputum, cerebrospinal fluid (CSF), and tips of the catheters were identified by means of conventional methods. Antimicrobial susceptibility tests were carried out by the Bauer-Kirby disc diffusion method. Species with inhibition diameters around imipenem (IMP-10 µg) or meropenem (MEM-10 µg) disc ≤13 mm were considered to be resistant, those with inhibition diameters around imipenem (IMP-10 µg) or meropenem (MEM-10 µg) disc, ≥16 as susceptible, those with inhibition diameters around imipenem (IMP-10 µg) or meropenem (MEM-10 µg) dics to be between 14 and 15 mm to be intermediately susceptible. *Pseudomonas spp.* and *Acinetobacter spp*. resistant to, and intermediately susceptible to imipenem/meropenem were included in the study. The isolates had been kept in buyyon-glycerin at -20°C until they were included in the study.

The identification MBL resistance with E-test

Isolates resistant to, and intermediately susceptible to imipenem/meropenem by the disc diffusion method were evaluated with imipenem E-test (AB Bio Disc/Sweden) and imipenem minimum inhibitory concentration (MIC) values were identified, which were analyzed with the use of imipenem-EDTA (IMP-EDTA) E-test MBL strips containing 456 µg/ml imipenem (IP) on one end and 1-64 µg/ml IP and constant level of EDTA on the other end (Figure 1). So, MBL positivity was defined in line with the criteria established by producer based on variability of MIC values (Figure 2).

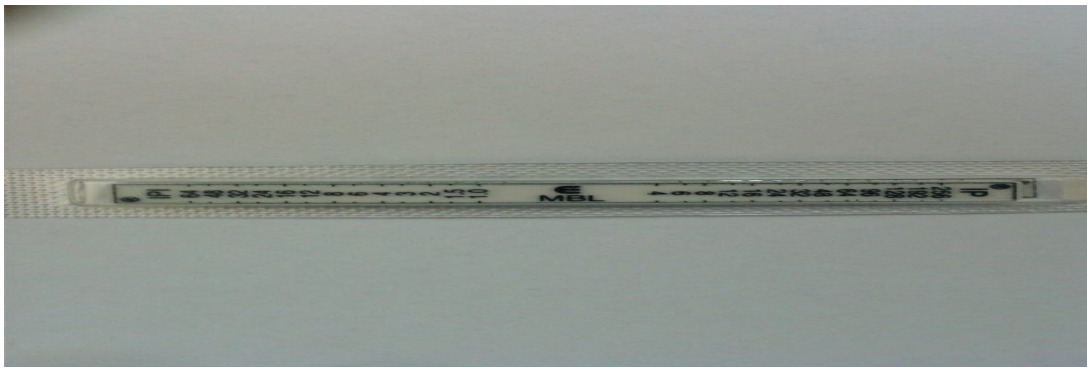

Figure 1. IPM-EDTA E-test (E-test MBL) strip

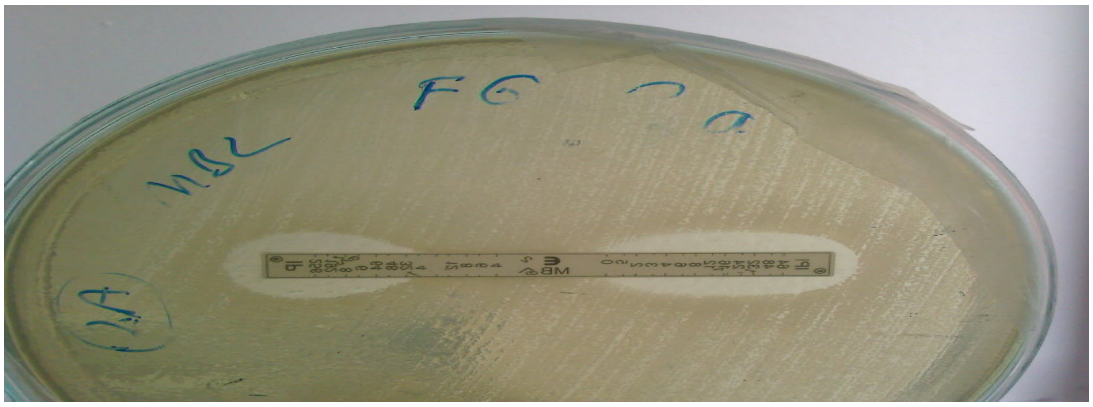

Figure 2. Detecting of MBL with E-test based on Mueller Hinton agar

Genotypic evaluation by PCR test

VIM-1, VIM-2, IMP-1 and IMP-2 were screened with a PCR test using 'Oligo Yap 3.0' software.

Statistical analysis

Data were processed with the Stata statistic program (STATA 10.0, Texas, USA). The chi square test was used for categoric variables and t-test was used for continues variables. A *p* value of <0.05 was considered significant. Logistic regression model was used, and MBL was considered to be dependent variable, independent variables included age, gender, ICU stay, cephalosporins as well as mechanical ventilation, total parenteral nutrition. In addition, mortality was considered to be dependent variable and independent variables included age, ICU stay, cephalosporins as well as mechanical ventilation, total parenteral nutrition.

Results

The current study included 63 infected/ colonised patients and evaluated 65 carbapenem-resistant nonfermentative gram-negative isolates. Of isolates, 46 were *Acinetobacter* spp. (15 *A. baumanni*, 31 *Acinetobacter* spp.), 19 were *Pseudomonas* spp. (16 *P. aeruginosa*, 3 *Pseudomonas* spp.). At the time of obtaining cultures, 18 patients were staying at the surgery clinic, 7 were at the internal diseases clinic and 40 were in the intensive care unit.

Deep tracheal aspirates (20) and wound tissue specimens (17) made up the majority of samples 31%, and 26%, respectively; specimens of urine accounted for 18% of blood 11%, of abscess 3%, of sputum 3%, CSF 3%, of nasal swab 2%, of catheter tip 2%, of ear drainage 2%. *Acinetobacter* spp. were mainly isolated from tracheal aspiration (37%) and from wound tissue specimens (24%); *Pseudomonas* spp. from urine samples (32%) and from wound tissue specimens (32%).

Phenotypic detection of MBLs and Antimicrobial susceptibility testing

Of 65 isolates, all were resistant to imipeneme with the disc diffusion method, (the zone diameters less then 13 millimeters). 62 were also resistant to meropeneme (the zone

diameters ≤12 mm). Of the 3 isolates susceptible to meropeneme, 2 were *Acinetobacter* spp, one was *Pseudomonas* spp., the zone diameters of which were 16, 17 and 22 millimeters.

According to the criteria of CLSI (The Clinical and Laboratory Standards Institute**),** the isolates whose imipeneme MIC ≤4 µg/ml have been defined as susceptible, and those whose imipeneme MIC ≥16 µg/ml have been defined as resistant, and those whose imipeneme MIC 4 to16 µg/ml as intermediately susceptible. Of the isolates found to be imipenem-resistant with the use of disc diffusion method, 57, whose imipeneme MIC ≥16 µg/ml, were found to be imipeneme resistant, 7, whose imipeneme MIC 6-12 µg/ml, moderately sensitive, 1 whose imipeneme MIC ≤4 µg/ml, was found to be sensitive with the imipenem E-test.

When evaluating with IP-EDTA E-test with respect to MBL production, 46 isolates (70,7%) were MBL positive, 19 isolates (29.2%) were MBL negative. Of *Acinetobacter* spp. isolates, 41 (89%) were MBL positive, 5 (11%) were MBL negative; of *Pseudomonas* spp. isolates, 5 (26%) were MBL positive, 14 (74%) were MBL negative.

All MBL positive isolates were found to be resistant to imipenem with E test, of which 44 had a MIC value of ≥32 µg/ml, two had a MIC value of ≥16 µg/ml. Of MBL negative isolates, one was susceptible to imipenem, six were intermediately susceptible and 12 were resistant with IP-E-test.

When performing PCR test on several isolates that had been examined with MBL-E test, VIM-1, VIM-2, IMP-1 and IMP-2 MBLs yielded no positive results.

All MBL-positive isolates were determined to be resistant to amoxicillin/clavulanic acid (AMC), aztreonam, ceftriaxone, cefixime and nitrofurantoine. MBL-negative isolates were all resistant to (100%) AMC, cefixime and nitrofurantoine, while having a resistance rate of between 84 and 89% to aztreonam and ceftriaxone. MBL-negative isolates had a considerable higher rate of resistance to netilmycin, trimethoprim-sulfamethoxazole (TMP - SXT) and to tetracycline as compared with MBL-positive isolates. However, MBL positive isolates had a higher rate of resistance to cephoperazone/ sulbactam, piperacillin/tazobactam, amikacin sulfate, ciprofloxacin, cefepime, which are more commonly prescribed antibiotics (Table 1).

Table 1. Antibiotic susceptibility of isolates

Antibiotics	**MBL-positive isolates**		**MBL-negative isolates**		***p***
	Suscebtible n (%)	Resistant n (%)	Suscebtible n (%)	Resistant n (%)	
Amikacin	5 (10)	41 (90)	7 (37)	12 (63)	**0.014**
AMC	0 (0)	46 (100)	0 (0)	19 (100)	
Aztreonam	0 (0)	46 (100)	3 (16)	16 (84)	**0.006**
Gentamicin	13 (28)	33 (72)	4 (21)	15 (79)	0.548
Chloramphenicol	2 (4)	44 (96)	2 (11)	17 (89)	0.346
Colistin sulphate	45 (98)	1 (2)	18 (95)	1 (5)	0.512
Levofloxacin	7 (15)	39 (85)	4 (21)	15 (79)	0.568
Netilmicin	36 (78)	10 (22)	7 (37)	12 (63)	**0.001**
Nitrofurantoin	0 (0)	46 (100)	0 (0)	19 (100)	
Ofloxacin	6 (13)	40 (87)	2 (11)	17 (89)	0.779
Piperacillin/tazobactam	3 (7)	43 (93)	8 (42)	11 (58)	**0.001**
Cefaperazon/sulbactam	1 (2)	45 (98)	3 (16)	16 (84)	**0.038**
Cefepime	1 (2)	45 (98)	3 (16)	16 (84)	**0.038**
Cefixime	0 (0)	46 (100)	0 (0)	19 (100)	
Ceftazidime	12 (26)	34 (74)	6 (32)	13 (68)	0.653
Ceftriaxone	0 (0)	46 (100)	2 (11)	17 (89)	**0.025**
Ciprofloxacin	3 (7)	43 (93)	7 (37)	12 (63)	**0.002**
Tetracycline	26 (57)	20 (43)	2 (11)	17 (89)	**0.001**
TMP-SMZ	21 (46)	25 (54)	3 (16)	16 (84)	**0.023**
Tobramycin	27 (61)	17 (39)	7 (37)	12 (63)	0.073

Evaluation of clinical data

Three participants assessed to have colonization were excluded from the evaluation of clinical data. The remaining 62 participants were compared with respect to the demographic and clinical features that may likely to affect MBL production (Table 2).

The mean age of 62 patients was 53 years (19 to 89 yrs.), the MBL-positive group and the MBL-negative group were similar with respect to age.

Forty-four patients (68%) had a history of ICU stay during hospitalization, which promoted MBL-positivity (p=0.003) with a risk as high as six times. Forty patients (65%) had undergone mechanical ventilation at some stages of hospital admission before samples for culture were obtained (p=0.035).

Table 2. Comparison of risk factors in MBL-positive and negative patients

	MBL-positive patients		**MBL-negative patients**		***p***
	n=44	**(%)**	**n=18**	**(%)**	
Age	54	-	49	-	0.396
Males	30	68	16	89	0.091
Prior antibiotics	43	98	15	83	**0.036**
Concomitant diseases	32	73	9	50	0.086
ICU stay	36	82	8	44	**0.003**
Undergoing surgery	28	64	10	56	0.553
A prior surgery	3	7	1	6	0.854
Invasive procedures	28	64	10	56	0.553
Total parenteral nutrition	32	73	10	56	0.189
Hemodialysis	5	11	1	6	0.483
Mechanical ventilation	32	73	8	44	**0.035**
Central venous catheter	39	89	16	89	0.977
Nasogastric tube	30	68	13	72	0.754
Arterial catheter	43	98	17	94	0.507
Urinary catheter	41	93	18	100	0.256
Tracheostomy	17	39	6	33	0.695
Intraabdominal drainage	13	30	2	11	0.124

*** Invazive procedures:** Gastroscopy, ERCP, PEG, bronchoscopy, tracheostomy, colostomy, colonoscopy and nephrostomy

When MBL-positivity and MBL-negativity were evaluated with respect to antibiotic use before cultures grew pathogens, the use of antibiotic was identified to be significant risk factor for MBL-positivity (p=0.036).

Similarly, use of cephalosporins was a significant risk factor for MBL-positivity (p=0.016). However, the use of carbapenem was not significant with respect to a risk factor for MBL-positivity with a mean duration of 18 days (17 days in the MBL positive group; 18 days in those with MBL negative, (p>0.05)).

Patients were admitted to the hospital mainly because of diseases of the central nervous system such as intracranial hemorrhage, hypoxemia, infections of central nervous system, bacteremia, skin-soft tissue, and organ/space surgical site infections (organ/space SSI), urinary tract infections, and traffic accidents, falls from a height, with no significant difference with respect to the reasons for hospitalization. There was no significant difference between the two groups with respect to the mean duration of hospital stay (60 days).

Of participants, 28 were discharged home, however, 34 participants had (55%) died. Mortality rates in the MBL positive and negative groups were found 55% and 56%, respectively, with no significant association with mortality (p>0.05).

Data were evaluated with the logistic regression test in which MBL was considered to be the dependent variable using multiple variables. ICU stay was found to be statistically significant. When the effects of MBL positivity, ICU stay, mechanical ventilation, the prescription of cephalosporins, parenteral nutrition and age on mortality were evaluated with multivariable logistic regression analyses, mechanical ventilation and age were found to be statistically significant.

Discussion

Being substantially virulent pathogens, *P. aeruginosa* and *A. baumannii were* once recognized as opportunistic pathogens, however; these pathogens were associated with a serious threat to public health across the World, leading to Healthcare Associated Infections (HAI) such as ventilator-associated pneumonia, catheter-associated urinary tract infections, bacteremia, soft tissue infections especially after the emergence of Multidrug Resistant (MDR) isolates after 1990s. Studies performed especially in ICUs showed that *A. baumannii* and *P. aeruginosa* were ranked high as infectious agents and that the most common samples from which isolates were obtained included tracheal swabs, sputum and urinary samples [8-10].

The SENTRY program in which 52022 isolates were examined between 1997 and 2016 found that the most common infection was pneumonia (44.6%) [10] from which P. aeruginosa was isolated, from more than 400 medical centers, including Turkey. According to the Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing standards, the rates of carbapenem resistance were 17.4 and 10.9%, respectively [10]. The rates of *Acb* complex commonly isolated from individuals hospitalized with pneumonia and blood stream infection were 42.9% 37.3%, respectively [11, 12].

Although there are isolates susceptible to polymyxin B, colistin phosphate and tigecycline, among *Acinetobacter* species, a wide range of isolates are seen to be resistant to multiple antibiotics including carbapenems. In this study, most pathogens (68%), of which, 31% were isolated from tracheal aspirates from ventilator-associated pneumonia and 26% from soft tissue infections were obtained from patients with a history of ICU stay, which is consistent with the literature. We found that the isolates were resistant to multiple antibiotics as well as carbapenems [13, 14].

Although nonfermentative bacteria such as *Pseudomonas* spp. and *Acinetobacter* spp. have an inherent resistance, they have the ability to readily acquire resistance to many antibacterial agents. Despite carbapenems being the most effective antibiotics in the treatment of infections caused by MDR *Pseudomonas* spp., increased carbapenem resistance have resulted in challenges in the management of these infections. Multiple mechanisms are responsible for the resistance, carbapenemases as well. The most important mechanisms of *Pseudomonas* spp. resistance to carbapenems comprise the inability of carbapenems to easily diffuse into bacterial cell wall due to loss of the outer membrane purines (Opr D), increased production of MDR efflux (MEX) pumps and excessive production of beta-lactamases. Similarly, although *A. baumanni* has either inherent or acquired mechanisms, carbapenemases, particularly MBL and OXA-types are associated with carbapenem resistance. Early detection of outbreaks caused by species producing clonal and polyclonal MBL makes it easy to administer proper antibiotics and to take appropriate measures with respect to controlling infections [9, 10, 15-17].

Conventional susceptibility tests are neither sensitive nor specific in establishing MBL-producing species. However, as adjunct tests, simple phenotypic tests can be used to detect such species, particularly diffusion or dilution methods based on increasing synergism between MBL inhibitors (including either EDTA or thiol compounds) and oximinocephalosporins or carbapenems. Phenotypic tests such as double disk synergy (DDS) and E-MBL strip tests consisting of EDTA-IMP and EDTA and 2-mercaptopropionic acid have been used DDS tests were followed by EDTA and imipenem-EDTA disc-diffusion microdilution methods both of which were confirmed to be reliable in detecting MBLs in carbapenem resistant *Pseudomonas* and *Acinetobacter* species [18-22].

Walsh and colleagues showed that imipenem+imipenem-EDTA tests performed in

Mueller-Hinton agar plate had a sensitivity of 94% and a specificity of 95%, suggesting that such tests would be appropriate to detect MBL for diagnostic purposes. Studies have reported variable sensitivities of the same tests 100% and 36.7% and specificity 86.4%. Studies performed in Turkey using such methods established MBL positivity of *Pseudomonas* isolates and *Acinetobacer* species 24%, 67% and 21%, respectively. *Pseudomonas* spp. outbreaks from multiple sources resulted in challenges in detecting of IMP and VIM positive isolates because of wide variabilities in imipenem MIC values. This study found a MBL positivity of 70.7%, with *Pseudomonas* and *Acinetobacter* species 26% and 89%, respectively. Discrepancies in these studies were considered to result from the differences in the numbers of isolates examined [16, 18, 22-25].

Studies have shown that bla IMP/VIM associated genes identified with phenotypic methods were not detected by means of a PCR test, which was suggested to be due to the fact that EDTA increases susceptibility to antimicrobials of microorganisms by raising cell membrane permeability. Although phenotypic methods may lead to false MBL-positivity, IMP-EDTA disc and E-test methods have been suggested to be used as the first-line tests because they do not yield false negative results. Furthermore, an algorhythm was structured taking account of inhibition zone diameter around imipenem/meropenem discs. Isolates with an inhibition zone diameter of <13 mm were considered to be MBL producer, and PCR was recommended to be performed for sequence typing. However, isolates with a zone diameter of 13 to 15 mm were considered to be likely MBL producer, so, PCR testing was recommended following MBL E-test. MBL E-test has been recommended to be an appropriate means of confirming the production of MBL in the presence of higher carbapenem resistance [26, 27-29].

Demographic and clinical features

The current study assessed the demographic and clinical features of patients. Studies showed differences in the risk factors for the development of infections of *Acinetobacter* spp. and *Pseudomonas* spp.

While several studies found female gender and young age to be significant risk factors for MBL positivity this study found no significant difference with respect to sex and mean age. Urinary catheters and administration of antibiotics, undergoing surgery and staying at the hospital with more than 500 beds have been considered the most important risk factors for *Acinetobacter* spp infections, however, prolonged hospitalization, antineoplastic chemotherapy, corticosteroids, permanent urinary catheters have been reported to be the risk factors for *Pseudomonas* spp. infections. The current study identified the rates of comorbidities and malignancy to be higher, which was not statistically significant in terms of MBL positivity. However, undergoing mechanical ventilation, ICU stay which accounted for the risk factors as high as six times were significant risk factors in terms of MBL-positivity [30-35].

This study identified the use of cephalosporin to be the significant risk for MBL- positivity consistent with studies suggesting that prolonged carbapenem use constituted an independent risk factor for imipenem resistant *Acinetobacter* spp. bacteremia, and that prolonged use of cephalosporin was associated with MBL producing *Acinetobacter* spp. bacteremia. The use and duration of carbapenems did not significantly differ between the groups with respect to MBL-positivity [13, 30-33].

It has been known that carbapenemase-encoding genes and other antibiotic-resistant genes are found in the same plasmids and transposons and that MBL positive isolates are not only resistant to beta-lactams but also to other groups of antibiotics. The current study found that the MBL-positivity group had a higher rate of resistance in to cefoperazone/sulbactam, piperacillin-tazobactam, amikacin, ciprofloxacin, cefepime, and that the MBL-negative groups had a higher rate of resistance to Netilmisin, TMP-SXT and tetracycline, which was thought to be associated with the frequency of drug use [22, 25].

Studies reported that MBL-positivity was associated with higher mortality rates, however, the current study showed that mechanical ventilation and age were associated with increased mortality, irrespective of MBL-positivity [31, 36].

In conclusion, MBL production may account for carbapenem resistance in *Acinetobacter* spp. and *Pseudomonas* spp. isolated especially from ICU patients. The use of phenotypic methods, being easy and available, in detecting MBL-

positivity may be a guide to perform infection control programs and administer antibiotics empirically.

Study limitations

The current study is a single center study, included a small sample size; due to unavailability of technical equipment, genome sequencing of antibiotic resistant genes in all isolates could not be performed by molecular analyses.

Conflict of interest: No conflict of interest was declared by the authors.

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Acknowledgment

This study was presented as an oral presentation at 15th National Internal and Surgical Scientific Intensive Care Congress, 21-24 November 2018.

Ethics committee approval: This study was based on a thesis in medical specialty. Data involved the period before 2020. Approval of the ethics committee was not required when the study was carried out.

Authors' contributions to the article

S.S.O.D. and N.B. have constructed the main idea. S.S.O.D. developed the theory and arranged the material and method section and have evaluated the data in results section. Written by S.S.O.D., reviewed, corrected by S.S.O.D. and N.B. Two authors discussed the entire study, reviewed the final version of the manuscript.