Original article

Detection of Metallo-Beta-Lactamase (MBL) in non-fermenter Gram-negative bacilli using combined disk and MBL-E-strip methods

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ABSTRACT

Background: The occurrence and spread of antibiotic resistant bacteria is one of the highest challenges, especially for developing countries. The rate of resistance among Gram-negative bacteria especially non-fermenters are increasing to all available antibiotics. One major concern is their resistance to the beta lactam antimicrobials. The aim of this study was to evaluate the effectiveness of combined disc method in comparison with MBL-E-strip method for the detection of Metallo-Beta-Lactamase (MBL) producing non-fermenter Gram-negative bacilli.

Materials and methods: In this study non-fermenter Gram-negative bacilli were isolated from different clinical specimens including blood, pus, urine, fluid aspirates and respiratory tract. Isolates were identified up to species level by API 20 NE kit. Antimicrobial susceptibility testing of non-fermenter Gram-negative organisms were achieved by modified Kirby Bauer disk diffusion method as recommended in Clinical and Laboratory Standards Institute (CLSI) guidelines for Antimicrobial Susceptibility Testing (M100-S28). Combined disc test and Metallo-Beta-Lactamase E-test strips were used for the detection of Metallo-Beta-Lactamases.

Results: Out of a total number of 51 isolates (non-fermenter Gram negative bacilli) which were resistant to imipenem were included in this study. Among total, 16 were *Acinetobacter baumanii*, 16 were *Burkholderia cepacia*, 7 were *Pseudomonas aeruginosa*, 5 were *Pseudomonas luteola*, 4 were *Stenotrophomanas maltophilia*, 2 were *Pseudomonas fluorescens* and 1 was *Pseudomonas stutzeri*. The comparison of two phenotypic methods showed that the combined disk test (CDT) detected MBL production in 80.3% isolates, whereas MBL-E- strip detected MBL production in 90.2%. The diagnostic accuracy of CDT was 78% in this study.

Conclusions: Combined disc test and MBL-E-strip tests have reliable sensitivity and specificity and were comparable for detection of MBL enzyme. CDT has 83% sensitivity and 40% specificity. The high sensitivity indicates that this test can be used as a good screening tool for MBL detection. These results can help to detect MBL production more effectively and efficiently.

Keywords:
Metallo-Beta-Lactamase, Gram negative, Non fermenter, Bacilli

INTRODUCTION

The occurrence and spread of antibiotic resistant bacteria causing infection is a major health issue for physicians. It is one of the most important public health issue of the 21st century.[1,2] Antibiotic resistance occurs when an antibiotic loses its capability to properly control or kill bacterial growth or the bacteria become "resistant" and continue to multiply in the presence of therapeutic levels of an antibiotic.[3] As the number of antimicrobial agents that are under development is very limited, the problem of multidrug resistant (i.e. resistant to at least three groups of antimicrobial agent) is becoming more and more threatening.[4,5]

The rate of resistance among Gram-negative bacteria especially non-fermenters are increasing to all available antibiotic groups.[6,7] Non-fermenter Gram-negative bacteria accounts for one fifth of all Gram-negative bacteria.[8] The most essential and frequent non-fermenting Gram-negative organisms are *Pseudomonas species*, *Acinetobacter species* and *Stenotrophomonas maltophilia*.[9-11] They play increasingly an important role as opportunistic healthcare associated infections in patients who are critically ill or who have impaired host defense.[12]

Beta lactams exert bactericidal activity primarily on cell wall synthesis in bacteria. The most important mechanism of resistance to beta lactams in bacteria are the production of hydrolytic enzymes termed beta lactamases which divide the beta lactam ring and inactivate the drug.[11,13,14]

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Metallo-Beta-Lactamase (MBL) belongs to class B enzyme that can hydrolyze carbapenem and is resistance to all available beta lactam inhibitors.[9] MBL enzyme needs zinc for its catalytic activity and is inhibited by metal ion chelators such as ethylene diamine tetra acetic acid (EDTA) and thiol based compounds.[15] Resistance to this class has started to emerge from 1990 and has been reported worldwide in non-fermenting Gram-negative bacilli with different frequencies.[10]

Currently there is no specific CLSI parameter for the detection and confirmation of MBL producing organisms.[16] Therefore several non-molecular techniques have been tried on the basis that MBL require zinc for its action. This activity can be inhibited by chelating agents such as EDTA, dipicolinic acid and thiol compounds.[2,17,18]

Combined disc test (CDT) and MBL-E-test strips were used by the researcher for the detection of MBL.[8] Both CDT and MBL-E-test strips are known to be effective for detection of MBL enzyme. They have good sensitivity. The early detection of MBL producers is important for therapeutic purposes and for effective infection control.[19] The aim of this study was to compare Combined Disc Test and Epsilometer (E)-Test method for detection of MBL in non-fermenter Gram-negative bacilli.

MATERIALS AND METHODS

In this lab based study, non-fermenter Gram-negative bacteria were isolated from various clinical specimens including blood, pus, urine, fluid aspirates and respiratory tract specimens. Non-fermenter Gram-negative bacilli was identified by the basic microbiology tests i.e. colony morphology, Gram staining, catalase test and oxidase test. All non-fermenter Gram-negative bacteria were recognized up to species level by basic laboratory procedures.[20-22] Susceptibility testing for all non-fermenters were done by modified Kirby Bauer disk diffusion method as prescribed in Clinical and Laboratory Standards Institute (CLSI) guidelines for antimicrobial susceptibility testing (M100-S28).[20,23] All those organisms which were resistant to imipenem (10µg) were selected for Metallo-Beta-Lactamases detection. Detection of Metallo-Beta-Lactamase was done by Combined Disc Test and Epsilometer (E)-Test.[24,25]

The combined disk test was done by using antimicrobial disks of imipenem (10µg) and ceftazidime (30µg) alone and in combination with EDTA (750µg) on Mueller Hinton agar plates. The combined disk test was done on all imipenem resistant non-fermenter isolates. All isolates showing raise in the zone of inhibition of ≥7 mm for either antibiotics agent in combination with EDTA versus its zone when tested alone was labeled as confirmed MBL producer.[25] E-Test Metallo-Beta-Lactamase strips (Ab Biodisk, Solna, Sweden) consisting of imipenem (IP) and imipenem + EDTA (IPI) were designed to detect the Metallo-Beta-Lactamase enzyme. These MBL-E-test strips consists of thin, plastic carrier calibrated with reading scale in µg/ml on one side, whereas the other side of strip carries two predefined gradients. IP stands of imipenem (1 to 64µg/ml) and IPI imipenem + constant level of EDTA (4 to 256µg/ml). The test was performed by applying E-strip on Mueller Hinton agar plates according to instruction given by kit (Ab Biodisk, Sweden). The presence of MBL was reflected by reduction of IP MIC by ≥8 or >3 log 2 dilution in the presence of EDTA or appearance of phantom zone or deformation of IP ellipse.[24]

RESULT

Among total 51 clinical isolates of non-fermenter Gram-negative bacteria, 16 (31.4%) were identified as *Acinetobacter baumannii*, 16 (31.4%) were *Burkholderia cepacia*, 7 (13.7%) were *Pseudomonas aeruginosa*, 5 (9.8%) were *Pseudomonas luteola,* 4 (7.8%) were *Stenotrophomanas maltophilia*, 2 (3.9%) were *Pseudomonas fluorescence* and 1 (1.96%) was *Pseudomonas stutzeri*. Table 1 shows the frequency of non-fermenter Gram-negative bacilli isolated from different clinical samples. The high frequency of resistance to multiple antibiotics were observed as cefepime (100%), cefoperazone (98%), ceftriaxone (96%), chloramphenicol (94%), ciproxin and levofloxacin (96%) each, tetracycline (96%), aztreonam (94%), piperacillin (94%), tazobactam (92%), septran (90%), amikacin (88%) and ceftazidime (88%).

The combined disk test detected Metallo-Beta-Lactamase production in 41 (80.3%) isolates out of 51, whereas MBL-E-strip detected Metallo-Beta-Lactamase production in 46 (90.2%) isolates. Figure 1 shows the comparison of two phenotypic methods, the combined disk test and Metallo-Beta-Lactamase-E-strip test for production of Metallo-Beta-Lactamase in non-fermenter Gram-negative bacteria.

Out of the total 16 (31.4%) *A. baumannii*, MBL production was detected by combined disk test in 13 (81%) and by MBL-E-Strip in 14 (88%) samples. In total 16 (31.4%) isolates of *B. cepacia* MBL production was detected by CDT in 13 (81%) and MBL-E-strip in 16 (100%) samples. Similarly, in 7 (13.7%) isolates of

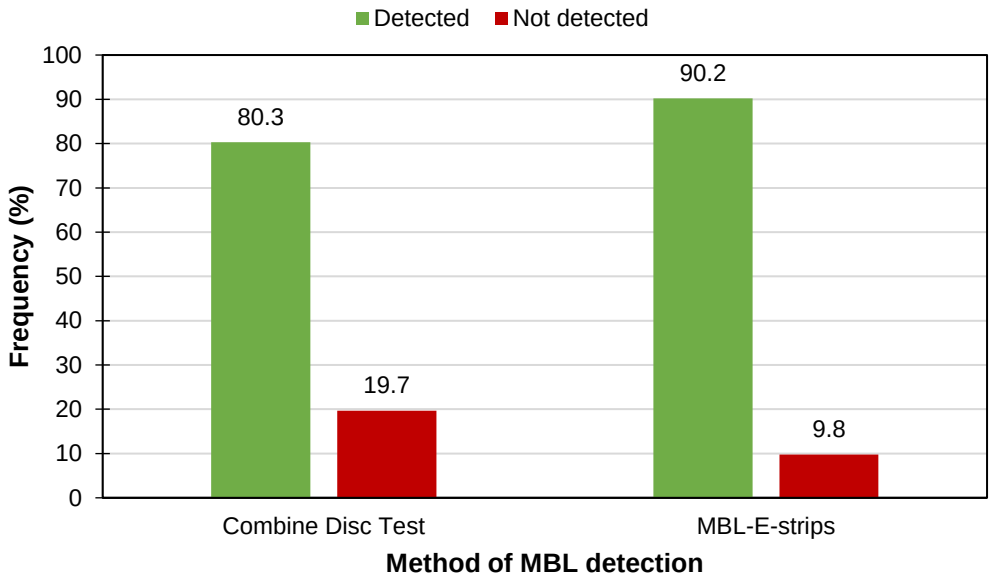

Figure 1. Comparison of two phenotypic methods combined disc test and MBL-E-test Strip for detection of Metallo-Beta-Lactamase in non-fermenter Gram-negative bacilli isolates (n = 51)

P. aeruginosa, CDT detected MBL production in 6 (86%) and MBL-E-strip was detected in 5 (71%). In one isolate of *P. stutzeri* (1.96%) MBL production was detected by both CDT (100%) and MBL-E-strip (100%). In out of 5 (9.8%) species of *P. luteola,* MBL production was detected by CDT in 4 (80%) and by MBL-E-strip in 5 (100%) samples. In 2 (3.92%) species of *P. fluorescence,* MBL production was detected by both CDT (100%) and MBL-E-strip (100%). In 4 (7.84%) species of *S. maltophilia*, MBL production was detected by CDT in 2 (50%) and MBL-E-strip in 3 (75%) samples. Table 1 shows the comparison of two phenotypic methods, CDT and MBL-E-strip for detection of MBL enzyme in species of non-fermenter Gram-negative bacilli.

When compared with MBL-E-Strip test, CDT showed a sensitivity of 83% and a specificity of 40%. The positive predictive value of the test was 93% and the negative predictive value was 20%. The overall diagnostic accuracy of the test was 78% (Table 1). Chi-square test showed a non-significant difference between combined disc test and MBL-E- Strip method in detection of Metallo-Beta-Lactamase enzyme.

DISCUSSION

Multi drug resistance bacterial pathogens are an emerging global threat in health care settings.[26] Infections due to Metallo-Beta-Lactamase producing organisms are associated with high mortality and morbidity.[15] The first case of MBL detection in Pakistan was reported by Butt and colleagues.[19] MBL has been isolated from clinical samples causing a serious issue for infection control management.[27] Moreover, the treatment options for MBL production organisms are expensive, toxic with poor outcome, and limited drugs. Therefore rapid detection is necessary that can help in modifying the treatment and initiate efficient infection control measures to prevent its dissemination.[28]

The purpose of this study was to evaluate the effectiveness of combined disc test for Metallo-Beta-Lactamase detection in comparison with confirmatory MBL E test. These methods were used to detect MBL production in non-fermenter Gram-negative bacilli, from various clinical specimens in our setup.[29] Table 1 shows distribution of frequency of species of non-fermenter Gram-negative bacteria. Out of a total number of 51 isolates, the most frequently isolated non fermenters were *A. baumannii* and *B. cepacia* (31.7%) each followed by *P. aeruginosa* (13.7%), *P. luteola* (9.8%), *S. maltophilia* (7.8%), *P. fluorescence* (3.9%) and *P. stutzeri* (1.96%). The results are in line with a number of studies, like a study carried out by Gupta[30] also showed that *A. baumannii* was mainly isolated organism from non-fermenter Gram-negative bacilli followed by *P. aeruginosa*, *P. stutzeri*, *B. cepacia,*

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Table 1. Frequency of the non-fermenter Gram-negative isolates and comparison of diagnostic accuracy of Combined Disc and Metallo-Beta-Lactamase-E-Strip tests

Organisms	Total *n* (%)	MBL detection method	
		Combined disc test *n* (%)	MBL-E-strip test *n* (%)
Acinetobacter baumannii	16 (31.37)	13 (81)	14 (88)
Burkholderia cepacia	16 (31.37)	13 (81)	16 (100)
Pseudomonas aeruginosa	7 (13.73)	6 (86)	5 (71)
Pseudomonas fluorescence	2 (1.96)	2 (100)	2 (100)
Pseudomonas luteola	5 (3.92)	4 (80)	5 (100)
Pseudomonas stutzeri	1 (9.80)	1 (100)	1 (100)
Stenotrophomonas maltophilia	4 (7.84)	2 (50)	3 (75)
Diagnostic accuracy of combined disc test			
Sensitivity (95% CI)		83% (68.58 to 92.18)	
Specificity (95% CI)		40% (5.27 to 85.34)	
Positive predictive value (95% CI)		93% (85.95 to 96.33)	
Negative predictive value (95% CI)		20% (6.72 to 46.46)	
Diagnostic accuracy (95% CI)		78% (64.68 to 88.71)	

S. maltophilia and *P. fluorescence*. Goel and colleagues[31] from India and El-Mosallamy and colleagues[18] from Egypt have also reported that *A. baumannii* was the most commonly isolated non fermenter pathogen. These non-fermenter organisms are considered as a major cause of hospital acquired infections.[32] However, some of studies are in contrast to these findings. A study done by Irfan and colleagues in Pakistan[28] showed that *Pseudomonas* species was the most frequently isolated non-fermenter Gram-negative bacilli and was 100% MBL producer. A study which was conducted by Malini and colleagues[33] in India, also reported that *P. aeruginosa* (53%) was major pathogen isolated among non-fermenters, followed by *A. baumannii* (22.3%), *P. flouresence* (10.8%) and *S. maltophilia* (2.6%). Meghna and colleagues[34] also pointed out similar result that *Pseudomonas* species were more commonly isolated among non-fermenter Gram-negative bacilli.

In this study we performed CDT for detection of MBL production and compared it with MBL-E-test strip method which is considered as standard phenotypic method. In CDT, 41 non fermenter Gram-negative bacilli isolates were MBL producers by both imipenem (IMP) + EDTA (ethylenediamine tetraacetic acid) and ceftazidime(CAZ) + EDTA. While 6 isolates were positive with IMP + EDTA and 4 were positive with ceftazidime + EDTA. Although both are effective but imipenem showed to be more sensitive for detecting MBL producer. The mean zone of inhibition by both IMP + EDTA and CAZ + EDTA were not significantly different. Manoharan and colleagues[35] and Sakshi and colleagues in 2009[36] also showed a sensitivity of IMP + EDTA, was better than CAZ+ EDTA. However a study carried out by Niranjan and colleagues[2], reported that MBL detection by both IMP+EDTA and CAZ + EDTA were equally effective.

Figure 1 of this study shows the comparison Combined Disc Test and MBL-E-test strip for detection of MBL in non-fermenter Gram-negative bacterial isolates. It shows, MBL detection by CDT was 80.3% and by MBL-E-test strip was 90%. According to a study conducted by Young and colleagues[37], CDT test using IMP + EDTA was simple to perform and highly sensitive in detecting MBL enzyme. Various studies carried out on evaluation of MBL detection method by CDT and showed it was considered as best method. It is cheap, non-toxic and easily accessible.[18,38] Behera and colleagues[26] and Gupta and colleagues[39] have reported that MBL-E-test strip method was excellent but due to cost constraints, CDT could be used as simple screening test in the clinical microbiology laboratories.[23,26] A study done by Mittal and colleagues in 2014[40] in India reported that MBL-E-test strip gave 100% positive results. In another study E-test had sensitivity of 96% and specificity of 91%.[41]

Various studies conducted in different parts of world showed that for MBL detection among imipenem resistant isolates done by CDT and MBL-E-test strips were equally effective screening methods.[16,29,35, 41] Table 1 shows the comparison of CDT and MBL-E-strip test, for MBL enzyme detection among non-fermenter Gram-negative bacilli. It shows that MBL detection was observed in *A. baumannii* by CDT was 81% and by MBL-E-test strip was 88%, whereas in *P. aeruginosa* detection of MBL by CDT was 86% and by MBL-E-test strip was 71%. Kaleem and colleagues[42] in Pakistan reported, 84% MBL detection in *Acinetobacter* species

and 78% in *P. aeruginosa* by MBL-E-test strip. While CDT showed MBL percentage of 79.8% for *Acinetobacter* species and 83.8% for *P. aeruginosa* which were somewhat similar to results of this study. The percentage of MBL detection by CDT for *P. luteola* and *B. cepacia* has come out be 80% and 81% respectively in this study. While with MBL-E-test strip the percentage of detection of MBL for both organisms is 100%. *P. fluorescence* and *P. stutzeri* have given 100% detection of MBL by both methods. While in *S. maltophilia* the detection of MBL by CDT is only 50% and by MBL- E- strip is 75%.

Walsh and colleagues[43] reported that MBL-E-test strip has sensitivity and specificity of 100% for detection of MBL among Gram-negative bacteria as is given by *P. fluorescence*, *P. stutzeri, P. luteola* and *B. cepacia* in our study. It is the need of the day to detect MBL enzyme with a method that has high sensitivity and specificity. Some researchers have reported that CDT is an excellent method for MBL detection. Franklin and colleagues in 2006[44] showed the sensitivity and specificity of CDT was 100% and 98%. Whereas by Berges and colleagues in 2007[45] reported the sensitivity and specificity of CDT was 100% and 72.7% respectively. Current study showed that MBL-E-test strip can be taken as standard phenotypic method but is not cost effective. While CDT has given sensitivity 83%, specificity 40%, positive predictive value 93%, negative predictive value 20% and diagnostic accuracy 78% (Table 1). Studies conducted by Galani and colleagues in Greece[46] showed the sensitivity of CDT was 80% and by Monaharan and colleagues (20) it was 87% which is similar to our results. A study carried out by Omair and colleagues[16] in Pakistan showed CDT has sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy were found to be 97%, 100%, 100%, 92% and 98% respectively which is discordant to our findings.

The gold standard method for the detection of Metallo-Beta-Lactamase production is polymerase chain reaction (PCR).[16] PCR is a genotypic method to detect the genes[47], therefore it is also considered as a confirmatory test. However, due to its high cost, it is not available in routine microbiology laboratories.[2,39] Performance of PCR method requires special instrumentation and trained workers. That is why it is done only at certain reference laboratories.[9] Owing to the high costs of PCR, and the difficulty in performing this test, identification of a cost effective as well as sensitive and specific method for the detection of MBL producers is crucial. Our study is an endeavor in this direction as we have compared MBL-E-test strip with CDT method for the detection of MBL enzyme in non-fermenter Gram-negative bacilli. Both procedures have given reliable results, although MBL-E-test strip is comparatively more expensive. Sensitivity by CDT is 83% while specificity is 40%. The high sensitivity indicates that this test can be employed as a reliable screening tool for MBL detection. The cost effectiveness of CDT over MBL-E-test strip gives it a further advantage.

Thus the present study highlights the usefulness of CDT method for MBL detection in non-fermenter Gram-negative bacilli. Although both methods give consistent results but as these methods are used in developing country like Pakistan, the combined disc test is cheap and easy to perform. Therefore CDT method should be used routinely in all clinical microbiology laboratories for detection of MBL in non-fermenter Gram-negative bacilli to improve the health management of patients. There is an urgent need of further studies to compare CDT with MBL-E-test strip and other methods for accurate and reliable detection of MBL in non-fermenter Gram-negative bacilli to take infection control measures on time.

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