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Metallo-βeta-Lactamase Detection Comparative Evaluation of Double-Disk Synergy versus Combined Disk Tests

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ABSTRACT

Objective: To establish (i) if carbapenemases are a major cause of carbapenem resistance in *Enterobacteriaceae* and belong to metallo- β-lactamases (ii) Which one is the best phenotypic method for the detection of metallo- β-lactamases.

Patients and Methods: This cross-sectional study was conducted at pathology department, Punjab Institute of Cardiology Lahore. Samples were randomly enrolled from daily lab work and analysed. During the period of September 2016 to January 2017, a total of 2970 clinical samples were enrolled and processed for bacterial culture. Every isolate of *Enterobacteriaceae* was processed for detection of carbapenem resistance and for the detection of carbapenemases producers by modified Hodge test. Metallo- β-lactamases detection (MBL) was done by three different phenotypic techniques, (i) Combined disk technique (0.1 M EDTA), (ii) (0.5 M EDTA). (iii) Double disk synergy technique (DSST).

Results: Out of total n=2970 samples, 38.7% (n=1150) were culture positive of which 40.5% (n=550) were Enterobacteriaceae. Among these, 9.0 % (n=50) were carbapenem-resistant; 98% (49/50) were carbapenemase producers (modified Hodge test -Positive). According to (i) Combined disk technique (0.1 M EDTA), 98% (48/49) were metallo- β-lactamases positive (ii) Combined disk technique (0.5 M EDTA), 86% (42/49) were metallo- β-lactamases, 2% (1/49) were non-determinable (iii) Double disk synergy technique (DDST) showed 100% (49/49) isolates were metallo- β-lactamases positive. Chloramphenicol and Tigecycline were found sensitive in 28% and 16% respectively; all other antimicrobials were highly resistant against carbapenem-resistant isolates.

Conclusion: Carbapenemases are a major cause of carbapenem resistance in *Enterobacteriaceae*. Double-disk synergy technique is good for the detection of MBL as compared to other phenotypic methods. Each carbapenem-resistant isolate of Enterobacteriaceae should be process for the detection of Carbapenemase especially MBL.

Key words: Combined disk technique, Double disk synergy technique, Metallo-β-lactamases.

Author`s Contribution
[1] *Conception, synthesis, planning of research and manuscript writing*
[2] *Interpretation and discussion*
[3,4] *Data analysis, interpretation*
[5,6] *manuscript writing, Active participation in data collection*

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Introduction

Despite of multiple solid steps taken to control drug resistance it is still emerging a dilemma of the civilized world. The emergence of drug resistance is credited to over and inappropriately use of antimicrobial drugs.

Carbapenems are the most commonly used drugs against multi-drug resistant (MDR) and extended spectrum beta-lactamases (ESBL) strains of Enterobacteriaceae.[1, 2] The emergence and spread of resistance in *Enterobacteriaceae* are complicating the treatment of serious nosocomial infections and threatening to create species resistant to all currently available agents. The vast majority of *Enterobacteriaceae*, including ESBL producers, remain susceptible to Carbapenems, and these agents are considered preferred empirical therapy for serious *Enterobacteriaceae* infections. Carbapenem resistance, although rare, appears to be increasing.[3] Carbapenemases (Metallo ß-lactamases or MBLs) have been emerged and spread from *P. aeruginosa* to *Enterobacteriaceae.*[4] Better antibiotic stewardship and strict infection control programs are needed to prevent further spread of ESBLs and other forms of resistance in *Enterobacteriaceae* throughout the world.[3]

Resistance to Carbapenems develops when bacteria acquire or develop structural changes within their Penicillin Binding Proteins (PBP), when they acquire MBL that are capable of rapidly degrading Carbapenems, or when changes in membrane permeability arise as a result of the loss of specific outer membrane porins.[5] Several resistance mechanisms occur to evade the efficacy of Carbapenem and the Carbapenemases are the most prominent enzymes that neutralize Carbapenem.[6]

First, high-level production of chromosomal AmpC cephalosporins combined with decreased outer membrane permeability due to loss or alteration of porins can result in carbapenem resistance. This has been shown for *Enterobacter cloacae, Enterobacter aerogenes, Proteus rettgeri, Citrobacter freundii, Escherichia coli* and *K. pneumonia*. The second mechanism is the production of a β-lactamase, that is capable of hydrolysing carbapenems (e.g., IMI-1, IMP-1, Nmc-A, Sme-1, and CFIA). The third mechanism of resistance involves changes in the affinity of the target enzymes, the penicillin-binding proteins, for Carbapenems.[7] The rapid global spread of *K. pneumoniae* that produces *K. pneumoniae* carbapenemase (KPC) is of major concern. The most common mechanism of resistance for the β-lactam antibiotic in clinically important gram-negative bacteria is hydrolysis of this group by β-lactamases.[8] Carbapenemases are β-lactamases with versatile hydrolytic capacities. They have the ability to hydrolyse penicillins, cephalosporins, monobactams, and carbapenems. Bacteria producing these β-lactamases may cause serious infections in which the carbapenemase activity renders many β-lactams ineffective. Carbapenemases are members of the molecular class A, B, and D β-lactamases. Class A and D enzymes have a serine-based hydrolytic mechanism, while class B enzymes are metallo-β-lactamases that contain zinc in the active site.[9] Rapid detections of carbapenemases especially MBL producing gram-negative pathogens is crucial to prevent their widespread dissemination.[10] KPC genes are typically located on mobile genetic elements, especially a particular transposon known as Tn4401, which helps transfer between plasmids and across bacterial species. Tn4401 and related transposons have been detected in many species from different continents.[11] Several methods including modified Hodge test, double disc synergy method using imipenem-EDTA discs, EDTA- impregnated imipenem disc and EDTA impregnated meropenem discs and imipenem- EDTA impregnated E- test strips have also been recommended.[12, 13]

As carbapenemase mediated carbapenem resistance is more of a challenge for infection control than other forms of carbapenem resistance in *Enterobacteriaceae*, so this study will help in establishing a method for early detection of carbapenemase and this, in turn will lead to prompt measures to check their dissemination and will have a valuable importance in infection control.

Patients and Methods

This cross-sectional analytical type of study was carried in the pathology department of Punjab Institute of Cardiology Lahore. Samples were randomly enrolled from daily lab work during the period of January 2016 to January 2017 and analysed. A total of 2970 clinical samples were enrolled, every sample was processed for bacterial culture. Bacterial identification was done by colonial morphology, Gram stain and standard biochemical profile API-20 E. Every *Enterobacteriaceae* isolate was processed for antimicrobial susceptibility testing for the detection of carbapenem-resistant, and all those isolates that were carbapenem-resistant were further analyzed for the detection of carbapenemases

producers by modified Hodge test (MHT). Moreover, every carbapenemase producer (MHT-positive) isolate was tested for the detection of MBL produced by three different phenotypic methods. Only carbapenem-resistant isolates of *Enterobacteriaceae* were included. Repeat isolates of *Enterobacteriaceae* from all the specimens of the same patients were excluded.

Modified Hodge Test: A 0.5 McFarland (1:10) dilution of E. coli ATCC 25922 was prepared and inoculated on mueller Hinton agar, A 10-µgram meropenem disk was placed in the centre and in a straight line, test organism was streaked from the edge of the disk to the edge of the plate and was incubated at 35±2 in ambient air for 16-24 hours. After incubation, the plates were examined for a cloverleaf-type indentation at the intersection of the test organism and the E. coli 25922, within the zone of inhibition of the carbapenem susceptibility disk. MHT Positive test had a clover leaf-like indentation of the E.coli 25922 growing along the test organism growth streak within the disk diffusion zone. While MHT Negative test had no growth of the E.coli 25922 along the test organism growth streak within the disc diffusion. For quality control purpose *Klebsiella pneumonia* ATCC® BAA-1705 were used as positive control and Klebsiella *pneumonia* ATCC® BAA-1706 were used as negative control.

MBL Detection: MBL detection was done by phenotypic techniques, using single agar plate (Mueller-Hinton agar) inoculated with test organism and comprised of three components.

Combined Disk Technique (0.1 M EDTA): Two Imipenem disks (10 µg), one containing 10µl of 0.1M (292µg) anhydrous EDTA, were placed 25 mm apart. 10 µl of 0.1 M (292 µg) EDTA was chosen, as higher concentration led to inhibitory effects with the EDTA alone. An increase in zone diameter of > 4mm around the IPM-EDTA disk compared to that of the IPM disk alone was considered positive for an MBL.

Combined Disk Technique (0.5 M EDTA): A 0.5 M EDTA solution was prepared (pH= 8.0). Two disks of 10 µg imipenem were placed on mueller-Hinton agar and to one of them, 4 µL of EDTA solution was added. One blank disk with EDTA was also added as an EDTA control. Inhibition zones of imipenem alone and imipenem plus EDTA disks were read after 18-24 hours' incubation at 35^{0}C. For MBL-positive organisms, addition of EDTA to the imipenem disk (imipenem plus EDTA) increased the inhibition zone by 8-15 mm (mean 10.5 mm), while the increase for MBL-negative isolates was 1-5 mm (mean) 3.8 mm

Double Disk Synergy Technique: In DDST, an imipenem (10µg) disk was placed 20mm apart (center to center) from a blank disk containing 10µl of 0.1 M EDTA. Enhancement of zone of inhibition in the area between two disks was considered positive for an MBL. Statistical Package for Social Sciences (SPSS) version 21.0 was used for statistical analysis of the data, Descriptive statistics were applied.

Results

Out of total 2970 samples, 38.7% (n=1150) were culture positive of which 40.5% (n=550) were Enterobacteriaceae. Figure: 1.

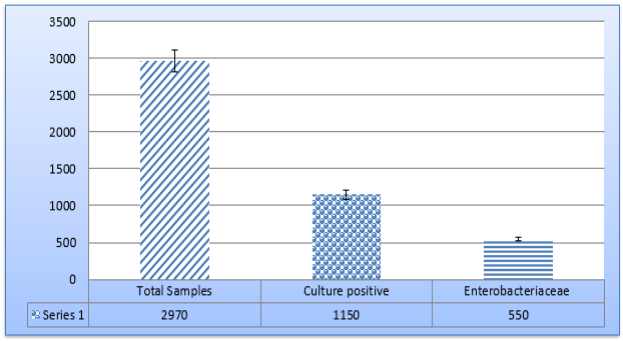

Figure 1: Breakup of culture positive isolates (n=2970)

Among this 9.0 % (n=50) were carbapenem-resistant isolates of which 98% (49/50) were found to be positive for carbapenemase production (MHT-Positive) Figure: 2.

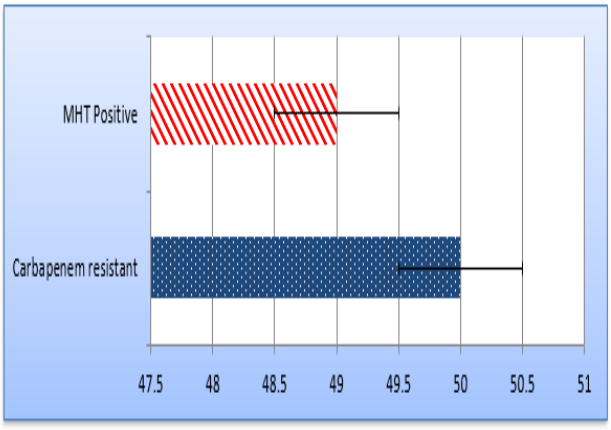

Figure 2: Frequency of Carbapenamases producers (n=50)

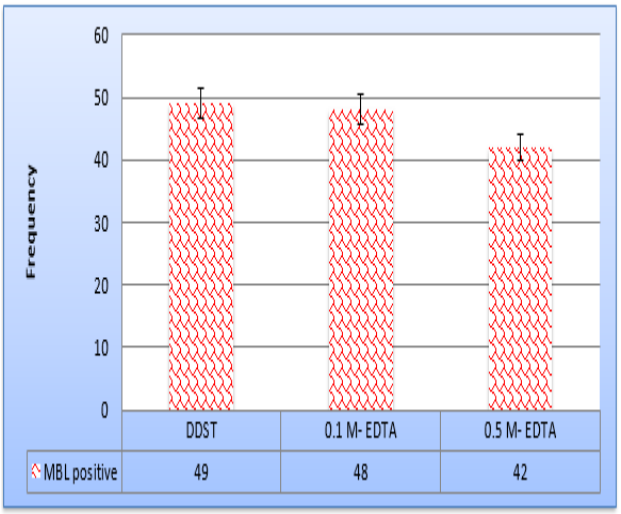

Figure 3: Breakup of three different modalities for the detection of MBL producers' (n=49)

Table:1 Antimicrobial resistant pattern of carbapenem resistant isolates

Antimicrobial drugs	Frequency	Percentage
Chloramphenicol	36	72
Tigecycline	42	84
Amikacin	47	94
Nalidixic Acid	49	98
Ciprofloxacin	49	98
Moxifloxacin	49	98
Cefoperazone+sulbactam	49	98
Co-Trimaxazole	49	98
Meropenem	50	100
Imipenem	50	100
Cefotaxime	50	100
Ceftazidime	50	100
Augmentin	50	100
Cefepime	50	100
Tazobactam+Piperacillin,	50	100
Tetracycline,	50	100
Piperacillin+sulbactam,	50	100
Ticarcillin+Clavulanic Acid	50	100
Gentamicin	50	100

According to (i) Combined Disk Technique (0.1 M EDTA), 98% (48/49) were MBL positive (ii) Combined disk technique (0.5 M EDTA), 86% (42/49) isolates were found to be positive for MBL production, and 2% (1/49) were placed in a non-determinable category. (iii) Double disk synergy technique (DDST)100% (49/49) isolates were found to MBL positive. Figure:3

Antimicrobial resistant pattern of carbapenem-resistant isolates is presented in Table:1

Discussion

The emergence of carbapenemases producers possesses alarming challenges as MDR infections around the globe. In the last 10 years, it has become a significant problem. These β-lactamases are able to hydrolyze the carbapenem and provide resistance to a broad spectrum of antibiotics. [9] Carbapenems are commonly used to treat infections caused by multidrug-resistant *Enterobacteriaceae* and they are one of the antibiotics of last resort for many bacterial infections, such as *E. coli* and *K. Pneumonia* ,[14] but now the clinical use of this group is under threat with the emergence of acquired carbapenemase, particularly Ambler class B metallo-β-lactamase (MBL) and worldwide spread of the resistance gene is becoming a potentially frightening scenario.[15] The present study was planned to evaluate the major source of carbapenem resistance in clinical isolates of *Enterobacteriaceae*, whether it is due to enzyme production or any other mechanism. Detection of carbapenemase was carried out by modified Hodge method according to the guidelines recommended by CLSI-2016.[16] The study further focuses on the detection of metallo-β-lactamase (MBL).Three different methods were used for detection of metallo-β-lactamase (MBL) which included double disc synergy method, combined disc method by using two different concentration of EDTA. This is the simplest, highly sensitive and specific method used in a number of widely published studies. Present study reported that of total n=2970 samples, 38.7% (n=1150) were culture positive, of which 40.5% (n=550) were Enterobacteriaceae. Among this 9.0 % (n=50) were carbapenem-resistant isolates of which 98% (49/50) were found to be positive for carbapenemase production (MHT-Positive). According to (i) Combined disk technique (0.1 M EDTA), 98% (48/49) were MBL positive (ii) Combined disk

technique (0.5 M EDTA), 86% (42/49) isolates were found to be positive for MBL production, and 2% (1/49) were placed in a non-determinable category (iii) Double disk synergy technique (DDST)100% (49/49) isolates were found to MBL positive. We suspect that the pre-dominant MBL among these isolates is most probably new delhi metallo-β-lactamase (NDM-1) A similar study from India [17] reported that 107 clinical isolates of gram negative rods (GNR) were processed for the detection of MBL, by using (EDTA) as MBL inhibitor. Four phenotypic techniques were used (i) Combined disk synergy test (CDST) with 0.5M EDTA (ii) CDST with 0.1 M EDTA (iii) DDST with 0.5M EDTA (iv) DDST with 0.1 M EDTA. Out of 107 only 30 isolates were carbapenem resistant of which 21 (70%) isolates were MBL positive by CDST-0.1 M EDTA, 19 (63.3%) by CDST-0.5M EDTA, 17 (56.6%) by DDST-0.1 M EDTA, and 16 (53.3%) by DDST-0.5M EDTA. Every MBL-producer isolate was resistant to ampicillin/sulbactam while Polymyxin B was the only choice of drug with high sensitive rate. Therefore, CDST-0.1 M EDTA was reported as the best technique for the detection of MBL producers. Furthermore sensitivity of CDST-0.1 M EDTA and DDST-0.1 M EDTA technique have reported 100% and 79% respectively.[18] In our view, the best method to detect MBL production in *Enterobacteriaceae* is double disk synergy method because this method detected all the MBL producers. If we are using combined disk method, then we should perform it by both methods by using two different concentrations of EDTA until and unless one of these is recommended as gold standard after confirmation by molecular genetic analysis. Moreover, it is suggested that the method to be used as gold standard for detection of MBL should be confirmed by molecular genetic analysis of the MBL producers. In this study, we also tried to establish the sensitivity pattern of meropenem resistant *Enterobacteriaceae* to alternative antibiotics. Almost 20% isolates were found sensitive to chloramphenicol and 16% were susceptible to tigecycline. The sensitivity of all other antimicrobial tested in this study was poor against these organisms. Present study reflects that once an isolate is declared as carbapenemase producer, we will be left with a very limited choice of antibiotics because genes encoding these enzymes are clustered with those encoding to resistance to aminoglycosides and fluoroquinolones, thus further compromising our antibiotic choice for these isolates.[3] Similarly Walsh et al [15] mentioned very high resistant of MBL-producers against all Beta-lactams, aminoglycosides, tetracycline, and fluoroquinolones group of antibiotics. Moreover, present study strongly supports the inevitable need to differentiate carbapenemases producers *Enterobacteriaceae* from other strains to limit their spread. Clinical microbiology laboratories should be able to distinguish MBL producer strains because the uncontrolled spread of these organisms will result in treatment failure eventually. Confirmation of these enzymes requires molecular analysis, by PCR or DNA sequencing. At present, there is not enough data available from our country about the prevalence of carbapenemases including MBL. Avoidance of unnecessary use of antimicrobials should be a part of the recommended drug therapy in hospitalized patients, especially in ICU. Regular surveillance programs should be conducted to check the drugs susceptibility, their usage pattern and resistance mechanism. Molecular genetic analysis of these enzymes by DNA probing and PCR is suggested for further studies.

Conclusion

Double-disk synergy technique is good method for the detection of MBL producers as compared to other phenotypic methods. Making it highly applicable to routine clinical laboratories, each carbapenem-resistant isolate of Enterobacteriaceae should be processed for the detection of Carbapenemase especially MBL.

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