AN INEXPENSIVE METHOD OF PREPARING EDTA DISKS FOR THE DETECTION OF METALO-BETA-LACTAMASES IN UROPATHOGENIC *E COLI*

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ABSTRACT

Objective: The aim of this study was to determine the frequency of Metalo-beta lactamases in uropathogenic *Escherichia coli* and to check the efficiency of in house prepared EDTA disks against them.

Study Design: Descriptive and Quasi experimental.

Place and duration of study: The Microbiology Department, Combined Military Hospital, and Institute of Dentistry Lahore, Pakistan from February 2016 to August 2016.

Material and Methods: Uropathogenic *E coli* were isolated in 83 urine specimens, 12 isolates had shown resistance to Carbapenems (Imipenem and Meropenem), by Kirby-Bauer disk diffusion method. These isolates were further subjected to Modified Hodge Test as recommended by clinical laboratory standard institute. Isolates which were resistant to carbapenem discs one or both with positive MHT were further subjected to Double disk synergy test and combine disk test using in house prepared EDTA disks 750µg for the detection of Metalo-beta lactamases.

Result: Frequency of Carbapenemases in uropathogenic *E. coli* was 13.25 % with a frequency of Metalo-β-lactamases to be 9.64%. Double disk synergy test and combine disk test both prove highly sensitive (100%) for the detection of Metalo-beta lactamases.

Conclusion: Double disk synergy test and combine disk test, using in house prepared EDTA disks is an inexpensive technique and improves reporting of Metalo-beta lactamases.

Key Words: Modified Hodge test, Double disk synergy test, Carbapenemases.

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INTRODUCTION

Studies from all over the world are alarming Microbiologists that a great number of gram-negative bacilli (GNB) have acquired Metallo-β-lactamases (MBLs) [1,2,3,4,5]. These bacteria are now capable of rendering all extended spectrum beta lactams drugs and carbapenems ineffective [6]. In tertiary care hospitals where control and spread of infection is the primary goal of all clinical practices, efficient detection techniques for MBL producing GNB are required not only for optimal treatment of patients but to keep infection under control [7].

As a general rule Molecular method are always considered as the “gold standard” to detect the MBL genes; but surprisingly in this case clinical laboratory standard institute (CLSI) and European centre for antibiotic susceptibility testing (EUCAST) are no longer supporting/ recommending this method.8 Furthermore, CLSI recommends that if an isolate gives a zone diameter of <19mm on disk diffusion against Carbapenems (Imipenem, Meropenem) or has a raised Carbapenem MIC i.e. >4 µg/ml8 or the one that falls in the susceptible range but raised MIC i.e. >2-4 µg/ml [8], Should be subjected to a phenotypic test i.e. Modified Hodge Test (MHT) to confirm resistance generated by carbapenemases or not. Modified Hodge Test (MHT) involving less technical expertise is considered as a simple testing tool for the detection of carbapenemases in a laboratory [8].

In a resource constraint Microbiological set ups simple phenotypic method for the detection of MBL, have always been a requirement. In recent years many phenotypic screening tests for the detection of various classes of Carbapenemases (A,B,D) have been presented in various studies. These include use of β-lactamase inhibitors Clavulanic acid or Boronic acid or Ethylene Diamine Tetra acetic Acid (EDTA) in combination with Carbapenems (Imipenem/Meropenem) to detect MBLs. The double-disk synergy test (DDST) or combined disk test (CDT) serves as an example. [9,10,11].

The aim of this study was to determine the frequency of Metalo-beta lactamases in uropathogenic Escherichia coli (E coli) and to check the efficiency of in house prepared EDTA disks in their detection by comparing them with commercially prepared EDTA disks (Oxoid, UK).

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MATERIAL AND METHODS

The study was carried out at CMH Lahore, which serves as Primary Referral Centre for central and southern Punjab and adjacent areas of Sindh and Baluchistan. The study was conducted from Feb 2016 to August 2016 for a period of 07 months.

Out of 1103 non-repetitive urine samples, 83 E. coli were recovered. All the isolates were identified by colony morphology, gram's staining and biochemical methods using API 20E. Antimicrobial susceptibility testing was done using Kirby-Bauer disk diffusion method and MICs were interpreted as per CLSI guidelines [8].

Isolates with a zone size of <19 mm for Meropenem (MEM) or Imipenem (IMP) 10 µg disk were tested for the presence of Carbapenemases through MHT in accordance with the CLSI guidelines (CLSI, 2016)8. All MHT positive isolates were subjected to DDST12,13 and CDT13, for the detection of class B, Metalo-β-lactamases.

First of all a preparation of 0.5M solution of EDTA was made by adding100 ml of distilled water, in 18.6 gms of EDTA (disodium salt, dehydrate Sigma-Aldrich, Germany). NaoH was added drop by drop to this solution to adjust the PH to 8.0.

In double disc synergy test (EDTA –DDST), after adjusting test strains to 0.5 McFarland standard they were streaked on the Mueller-Hinton agar plates (MH agar). A MEM disk (Oxoid, Basingstoke, UK) 10-µg was placed on the MH agar plate along with a filter paper disk (6 mm in diameter, Whatman filter paper no. 2) containing 750µg of EDTA at a distance of 10-12mm from each other (Figure-2). After an incubation of 16-18 hrs at 35^{o}C, if there was a zone enhancement of MEM disk towards EDTA disk, it was interpreted as positive [13].

In EDTA combined disc test (EDTA-CDT) after adjusting test strains to 0.5 MacFarland standard and streaking them on MH agar plate, two MEM disks 10 µg each were placed on MH agar plate, again 10µl of 0.5 M EDTA solution containing 750 µg of EDTA was dropped on one disk of MEM (Figure-2). After an overnight incubation of 16-18 hrs at 35^{o}C, if on comparison the zone size of the MEM+ EDTA disk was enhanced i.e. ≥4-7 mm than zone size of MEM disk alone, it was interpreted as positive [13].

Fitness of in-house prepared EDTA disks was checked by applying them along with commercially prepared EDTA disks against 12 isolates which were screened positive for carbapenemase production.

Evaluation of storage effects on filter paper disks containing EDTA was carried out by dividing them into batches and storing them at two different temperatures. One batch at 4°C and other at -20°C. Stored EDTA disks from each batch were tested every week against same control strain and zone size was noted and compared with initial results [12].

SPSS version 21 was used to analyse the data. Keeping results of Commercially prepared EDTA disk as Gold Standard, results of two methods DDST and CDT were compared using Chi-Square test. Sensitivity, specificity, PPV and NPV along with overall efficiency of each method was also calculated.

RESULTS

Out of 83 E coli, isolated from 1103 samples for urine culture. 12 uropathogenic E coli (14.45%) were found to be resistant to Carbapenems with high MICs and considered as probable Carbapenemase producers (Table-1). However, MHT was positive in 11/12, isolates.

Presence of class B Carbapenamases (MBL) was detected in 8/12 (72.7%) isolates through DDST and CDT using homemade disks (Figure-1&2). 02 isolates had shown enhancement of zone between Meropenem and Amoxicillin + clavulanic acid disks, confirming the presence of Class A or D carbapenemases. MICs by E-test were >04µg/ml were seen in isolates resistant to both the carbapenems in our study. Commercially prepared EDTA disk had similar results against these 08 isolates.

The MHT when compared with disk diffusion method with commercially prepared EDTA disk and clavulanic acid disk had shown an overall efficiency of 98.80% with a 100% sensitivity and 98.63% specificity. NPV and PPV in case of MHT was found to be 100% and 90.91%.

In our E coli isolates frequency of Carbapenemases was 12.04% with 9.64% detected through CDT and DDST to be having MBL enzymes. Class A or D was detected in 02/12 (2.4%) isolates through DDST. Class of carapenemase in 01 isolate remained unknown, so while calculating sensitivity, specificity etc it was not considered.

On comparing DDST and CDT with commercial EDTA disk results, both the methods had given a sensitivity and specificity of 100 %, with 95% CI 63.06-100% respectively. Both the methods had PPV, NPV and overall efficiency of 100%.

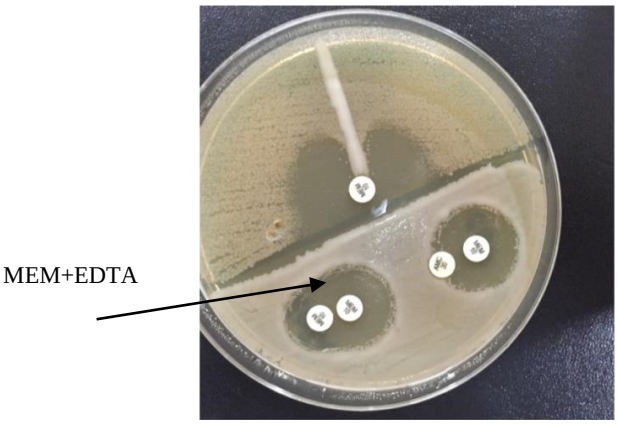

Figure-1: Positive Modified Hodge Test (MHT) along with double disk synergy tests (DDST) showing presence of class 'B' Metalo-Beta Lactamase (MBL).

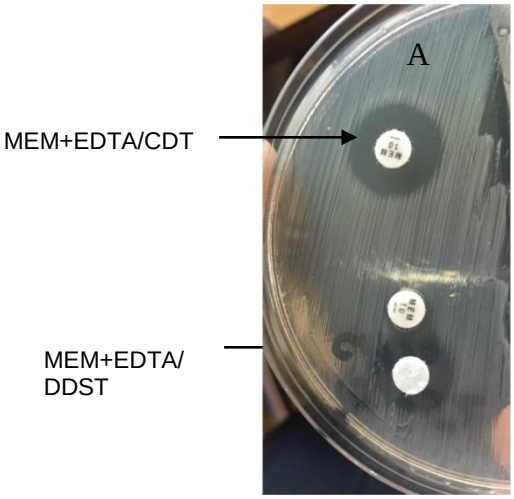

Figure-2: A; Combined disk test (CDT) & Double Disk Synergy Test (DDST) showing presence of class B, Metalo Beta Lactamase (MBL).

Table-1: Antibiotics Susceptibility Pattern of Carbapenem resistant isolates n=12

Antibiotics	Susceptible %		Resistance %	
Amox-clav	0	0%	12	100%
Co-trimoxazole	0	0%	12	100%
Cefoxitin	0	0%	12	100%
Ceftazidime	0	0%	12	100%
Ceftriaxone	0	0%	12	100%
Gentamycin	5	41.7%	7	**58.3%**
Amikacin	8	66.7	4	**33.3%**
Ciproxin	0	0%	12	100%
Imipenem	5	41.7%	7	**58.3**%
Meronem	0	0%	12	100%
PIP-TAZO	2	17%	10	**83%**
Cefoperazone sulbactam	0	0%	12	100%
Colistin	12	**100%**	0	0%
Fosfomycin	12	100%	0	0%
Nitrofurantoin	7	58.3%	5	**41.7%**

DISCUSSION

The genotypic detection of MBLs is always considered as "Gold Standard" to make a definitive diagnosis. However, every isolate resistant to Carbapenems being tested by genotypic methods is not justifiable. The modified Hodge test though, simple to perform and may detect the presence of carbapenemases, is unable to detect MBLs. Phenotypic tests requiring less technicalities and less expensive to detect MBLs is need of the hour.

In our study we have tried out two simple disk diffusion techniques DDST and CDT which were proved 100% efficient and 100% sensitive. When compared with other studies e.g Pandaya *et al* the sensitivity of DDST and CDT was 81.48% and 96.30% [14]. Another study by Galani *et al* shown sensitivity of DDST and CDT as 100% and 94.7% [13]. Bora *et al* reported a sensitivity of 100% with CDT, while in our study sensitivity was found to be 100% for both DDST and CDT [15].

As is obvious from the susceptibility picture of these 12 E. coli isolates producing MBLs or other carbapenemases by disc diffusion susceptibility testing, that most of them were resistant to beta lactams, beta lactams -lactamase combinations drugs, Imipenem, Meropenem and even Aztreonams thus fell in the category of multi drug resistant (MDR) isolates i.e. resistant to three or more classes of antimicrobials [15]. Susceptibility patterns differ in case of non-betalactam drugs like Aminoglycosides, Fosfomycin, Nitrofurantoin and Colistin. Furthermore, eight isolates were pan drug resistant being resistant to 07 or more than 07 drugs this trend of pan drug resistance is in concordance with other studies as well [15,16]. However, Colistin emerged as a single drug against which no resistance was seen in any of the MBL producing isolate. 2.4% of our isolates were sensitive to Pipracillin-tazobactam, which was in concordance with a study [15].

Frequency of MBLs in urinary isolates of E. coli was found to be 9.64% through DDST and CDT. 01 isolate that had gone undetected through DDST and CDT, although it was MHT positive require further probing through PCR for its definitive diagnosis as whether it had Carbapenemases or not or MBL gene or not. 08/12 isolates mean 72.7% uropathogenic carbapenemase producing E coli were having MBL enzymes, that was in concordance with the study conducted by Iman F *et al* where MBL were present in 70% of the isolates5. In our study most of the Carbapenem resistant isolates were multi drug resistant (Table-1) and were yielded from urinary samples of prolonged bed ridden patients with

permanent indwelling catheters. All these patients positive for MBL later treated with oral Nitrofurantoin and intravenous Amikacin according to susceptibility picture and responded very well.

The clinical scenario of ITC patient or patients with history of prolonged hospitalisation having being infected with multi drug resistant isolates is not a new one and rather talk of the scientific meetings now a days promulgate the need of measures to be taken as early as possible to curb the menace of such isolates in ITCs and high-risk settings in Army hospitals.

CONCLUSION

DDST and CDT, using in house prepared EDTA disks is an inexpensive technique and improves reporting of MBLs.

AUTHORS CONTRIBUTION

Qanita Fahim: Entire research work & write up
Ayesha Khalid: Helped in sample analysis
Fatima Hameed: Helped in data collection
Muhammad Saeed Anwar: Overall supervision

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