

## Phenotypic Detection of Metallo $\beta$ -Lactamase in Carbapenem Resistant Gram Negative Bacilli Isolates

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### Abstract

**Background:** A rising threat of the rapid spread of acquired metallo  $\beta$ -lactamases (MBLs) among Gram Negative Bacilli (GNB) is a matter of public health concern worldwide. Hence, detection of MBLs producing clinical isolates via an accurate and cost effective technique is necessary to prevent their dissemination as well as regulation of antimicrobial stewardship policy. This study aimed to detect MBLs in Carbapenem Resistant Gram Negative Bacilli (CRGNB) using two phenotypic tests; Combined Disc Test (CDT) and Double Disc Synergy Test (DDST).

**Methods:** We conducted a prospective study on 130 GNB isolates recovered from different clinical specimens collected from different patients. Isolates were identified then antibiotic susceptibility profile was determined by VITEK® 2 compact system. GNB which showed resistance to meropenem were screened for MBLs production by CDT and DDST.

**Results:** Out of 36 CRGNB isolates, 23 isolates (63.9%) were MBLs positive by CDT, and 20 (55.6%) by DDST. With almost perfect agreement between CDT and DDST.

**Conclusion:** Our study validates two phenotypic methods (CDT and DDST) for the detection of MBLs production, making them highly applicable to routine clinical laboratories.

**Key Words:** MBLs – Combined disc test – Double disc synergy test – CRGNB.

### Introduction

RESISTANCE of pathogenic organisms to antibiotics is a worldwide problem with serious consequences on the treatment of infectious diseases. The increased use or misuse of antibiotics are major contributing factors that have resulted in economic losses to the consumer, hospital and society as more expensive drugs are used as second-line treatment, more tests need to be carried out

and invariably the patient has to stay in the hospital for longer duration [1].

The introduction of carbapenems into clinical practice represented a great advance in the treatment of serious bacterial infections caused by  $\beta$ -lactam resistant bacteria, due to their broad spectrum of activity and stability to hydrolysis by most  $\beta$ -lactamases [2]. Carbapenems have been used as the last resort of antimicrobials in the treatment of serious infections caused by GNB. However, the clinical use of carbapenem is in danger with the emergence of carbapenem resistance, which become a public health problem around the world in terms of increased mortality, longer hospital stays, and higher costs [3]. Resistance to carbapenem is due to decreased outer membrane permeability, increased efflux systems, alteration of penicillin-binding proteins and carbapenem hydrolyzing enzymes (carbapenemase) which is the most widespread cause of carbapenem resistance [4].

Carbapenemases can be divided into metallo-carbapenemases (zinc-dependent class B) and non metallo-carbapenemases (zinc-independent classes A and D) [5]. The MBLs was first detected in 1960, in *Bacillus cereus* which was chromosomal in location. Then, first plasmid mediated MBLs isolates was found in *Pseudomonas aeruginosa* in 1991 in Japan. Since early 1990s, MBLs encoding genes have been reported all over the world in clinically important pathogens, such as *Pseudomonas* spp., *Acinetobacter* spp., and members of the Enterobacteriaceae family [6].

Acquired MBLs is encoded by integron borne mobile gene cassettes, hence MBLs producing strains are often resistant to different classes of

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antimicrobial agents with transferable properties to various types of bacteria [7]. Thus, the detection of MBLs producing GNB is necessary to aid in appropriate treatment and infection control measures, and to prevent their dissemination [8].

Detection of genes coding for MBLs usually gives reliable and satisfactory results. However, because of the cost of that method, it is of limited practical use for routine diagnostic microbiology laboratories. Thus, a simple and inexpensive phenotypic testing methods are more practical for detection of MBLs producers [9]. Several phenotypic methods have been described for detection of MBLs among clinical isolates as DDST, CDT and the MBLs Epsilometer test (E-test) [10].

Microbiology laboratories must be prepared to screen for MBLs-producing isolates by a low cost, convenient and sensitive procedure. The present study was undertaken to detect MBLs in GNB using two phenotypic methods (CDT and DDST).

### Material and Methods

This prospective study was conducted over a period of 8 months duration from October 2013 to June 2014 on 130 specimens (88 males and 42 females) of patients admitted to different wards/ICUs at Assiut University Hospitals including Chest, Emergency, General ICU, Neurology, Burn, ENT, Tropical Medicine, Internal Medicine, Pediatrics, Coronary, Gynecology and Surgery Units. Samples include 34 endotracheal aspirates, 31 sputum samples, 22 wound swabs, 18 blood samples, 15 throat swabs, 6 urine samples, 3 pleural fluid samples and one vaginal swab. Collection of samples were done under strict aseptic precautions according to standard protocols and processed at once [11].

#### *Laboratory processing of the samples:*

Samples were processed both at Assiut University Hospitals, Clinical Pathology Department, Microbiology Unit, Egypt and at Tokyo University, Institute of Medical Science, Faculty of Frontier Sciences, Department of Medical Genomics, Prof. Sumio Sugano laboratory, Japan.

All samples were cultured directly on MacConkey agar (BD Bacto™, France). Blood samples were inoculated into BACT/ALERT® PF culture bottles, then incubated in the BACT/ALERT 3D system (BioMérieux, USA) for periodic reading. Subcultures on MacConkey agar were done for all BACTEC culture bottles with positive growth.

GNB were identified by Gram staining, colony morphology, biochemical reactions, API RapiD 20ETM (Sysmex BioMérieux, Japan) and VITEK® 2 Compact (BioMérieux, France). Then identification of all samples was confirmed with 16S rRNA gene sequencing, where Bacterial DNA was extracted using Gentra® Puregene® kit (a product of Qiagen® USA). We used universal primers fD 1 (5' AGAGTTTGATCCTGGCTCAG 3') and rP2 (5' ACGGCTACCTTGTTACGACTT 3') as general primers for 16S ribosomal subunit amplification; supplied by Invitrogen®, Life Technologies®. The expected amplicon size was 1550 bp. PCR reactions were done using Thermo Scientific Phusion High-Fidelity PCR Master Mix (ref F-531L) according to product manual. Then agarose gel electrophoresis was done and correct size PCR product was extracted from agarose gel using QIAquick® gel extraction kit. Sequencing was done using Sanger method. The BigDye® Terminator v3.1 Cycle Sequencing Kit was used. Sequences obtained were merged using online service by EMBOSS merger (<http://emboss.bioinformatics.nl/cgi-bin/emboss/merger>) and then blasted using NCBI Blastn engine. Organisms with highest similarity was recorded.

#### *Antimicrobial susceptibility testing:*

VITEK® 2 compact system (AST-GNB card) (BioMérieux, France) was used for the automated quantitative susceptibility testing of isolated colonies for most clinically significant GNB. The following antimicrobials were included: Ampicillin, Ampicillin/Sulbactam, Piperacillin/Tazobactam, Cefazolin, Cefoxitin, Ceftazidime, Ceftriaxone, Cefepime, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Nitrofurantoin and Trimethoprim/Sulfamethoxazole.

#### *Phenotypic detection of MBLs in CRGNB isolates:*

##### *A- Combined Disc Test (CDT):*

The inoculum was adjusted to a 0.5 McFarland standard, and a Mueller-Hinton Agar plate (Oxoid Limited, England) was inoculated as recommended by CLSI for antibiotic sensitivity testing by the disc diffusion method. The presence of MBLs was determined by placing two 10 µg meropenem discs (Oxoid, England) on the inoculated plate, in which 10 µl of 0.5 M EDTA was added to one of the meropenem discs. After overnight incubation at 37°C, the inhibition zones of meropenem and meropenem-EDTA discs were compared. A zone difference of over 7mm between the meropenem and the meropenem-EDTA inhibition zones confirmed the isolate to be MBLs-positive [12].

**B- Double Disc Synergy Test (DDST):**

The inoculum was adjusted to a 0.5 McFarland standard, and a Mueller-Hinton Agar plate was inoculated. One blank filter paper disc was treated with 10 μl of 0.5 M EDTA and placed on the center of inoculated plate. 10 μg meropenem disc (Oxoid, England) was placed 20mm away from the EDTA disc (measured center-to-center). After overnight incubation at 37°C, Enhancement of inhibition zone in the area between the EDTA disc and MER disc in comparison with inhibition zone on the far side of the drug was interpreted as a positive result [13].

**Results**

Out of 130 GNB studied, 40 were *Pseudomonas aeruginosa* strains, 27 *Proteus mirabilis*, 19 *Providencia vermicola*, 10 *Serratia marcescens*, 9 *Providencia rettgeri*, 8 *Escherichia coli*, 6 *Providencia stuartii*, 3 *Citrobacter koseri*, one *Acinetobacter baumannii*, one *Alkaligenes faecalis*, one *Enterobacter cloacae*, one *Escherichia fergusonii*, one *Proteus penneri*, one *Pseudomonas putida*, one *Salmonella enterica* and one *Serratia* species.

The resistance rates of GNB were found as 89.2% (no. 116) for ampicillin, 54.6% (no. 71) for ampicillin/sulbactam, 32.3% (no. 42) for piperacillin/tazobactam, 90.8% (no. 118) for cefazoline, 52.3% (no. 68) for cefoxitin, 43.8% (no. 57) for ceftazidime, 55.4% (no. 72) for ceftriaxone, 33.8% (no. 44) for cefepime, 20.8% (no. 27) for meropenem, 13.1% (no. 17) for amikacin, 19.2% (no. 25) for gentamicin, 27.7 (no. 36) for tobramycin, 58.5% (no. 76) for ciprofloxacin, 72.3% (no. 94) for levofloxacin, 85.4% (no. 111) for nitrofurantoin and 66.2% (no. 86) for trimethoprim/ sulphamethoxazole (Table 1) & Fig. (1).

Among 36 (27.7%) meropenem resistant isolates, 22 (61.1%) isolates were *Pseudomonas* spp., 6 (16.7%) were *Providencia* spp., 3 (8.3%) were *E. coli* spp., 2 (5.6%) were *Proteus* spp., one (2.8%) was *Acinetobacter* spp., one (2.8%) was *Enterobacter* spp. and one (2.8%) was *Serratia* spp.

The carbapenem resistant isolates were more frequently isolated from sputum samples 14 (3 8.9%), followed by endotracheal aspirates 7 (19.4%), followed by wound swabs 6 (16.7%), followed by urine samples 4 (11.1%), then throat swabs and pleural fluid equally 2 (5.6%) and one blood sample (2.8%).

The highest frequency of CRGNB isolates was found in Chest Department represented 9 isolates

(25.1%) from total number of CRGNB isolates, followed by both general ICU and Emergency Unit represented 7 (19.4%), Neurology Department represented 4 (11.1%), both Pediatric Department and Burn Unit which represented 3 (8.3%) and then ENT, Internal Medicine and Tropical Medicine Departments, one sample (2.8%) from each department of them.

Out of 36 CRGNB isolates, 23 isolates (63.9%) were MBLs positive by CDT Fig. (2), and 20 (55.6%) by DDST Fig. (3).

Kappa value of 0.83 ( $p < 0.001$ ), indicating almost perfect agreement between CDT and DDST [14] as shown in (Table 2) and Fig. (4).

Table (1): Antibiotic sensitivity testing results.

Antimicrobial agent	Intermediate No. (%)	Resistant No. (%)	Susceptible No. (%)
• Ampicillin	0 (0%)	116 (89.2%)	14 (10.8%)
• Ampicillin/Sulbactam	0 (0%)	71 (54.6%)	59 (45.4%)
• Piperacillin/Tazobactam	5 (3.8%)	42 (32.3%)	83 (63.8%)
• Cefazoline	0 (0%)	118 (90.8%)	12 (9.2%)
• Cefoxitin	3 (2.3%)	68 (52.3%)	59 (45.3%)
• Ceftazidime	0 (0%)	57 (43.8%)	73 (56.2%)
• Ceftriaxone	1 (0.8%)	72 (55.4%)	57 (43.8%)
• Cefepime	5 (3.8%)	44 (33.8%)	81 (62.3%)
• Meropenem	9 (6.9%)	27 (20.8%)	94 (72.3%)
• Amikacin	6 (4.6%)	17 (13.1%)	107 (82.3%)
• Gentamicin	11 (8.5%)	25 (19.2%)	94 (72.3%)
• Tobramycin	12 (9.2%)	36 (27.7%)	82 (63.1%)
• Ciprofloxacin	13 (10%)	76 (58.5%)	41 (31.5%)
• Levofloxacin	5 (3.8%)	94 (72.3%)	31 (23.8%)
• Nitrofurantoin	1 (0.8%)	111 (85.4%)	18 (13.8%)
• Trimetho/Sulphamethoxazole	0 (0%)	86 (66.2%)	44 (33.8%)

Table (2): Agreement between CDT and DDST (n=36).

	DDST		p-value
	Negative	Positive	
<b>CDT:</b>			
Negative	13 (36.1%)	0 (0%)	<0.001*
Positive	3 (8.3%)	20 (55.6%)	
<b>Kappa</b>	83%		<0.001

\* Chi-square test was used to compare the difference in proportions between groups.  
\* % calculated from total number.

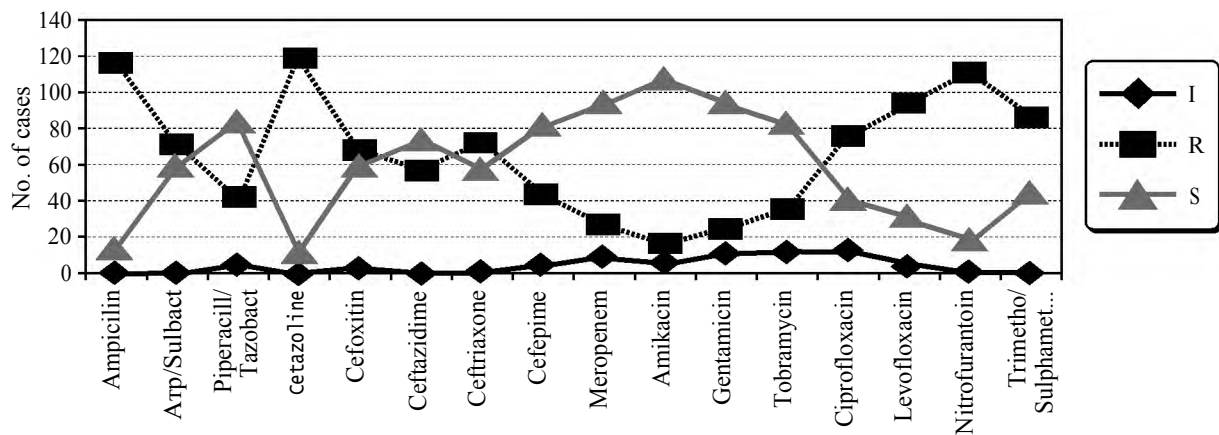
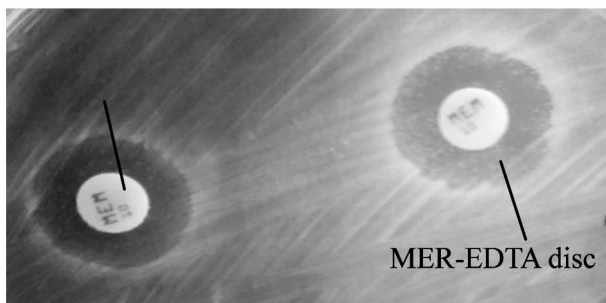
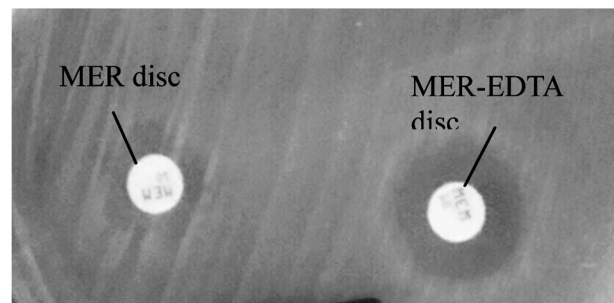


Fig. (1): Antibiotic sensitivity testing results.

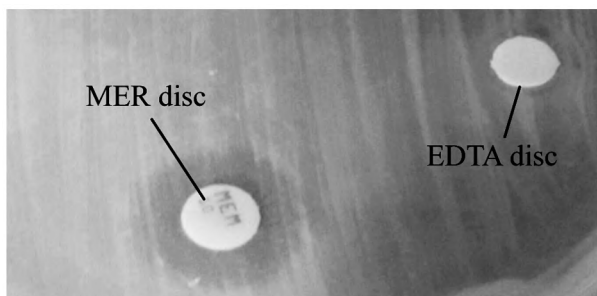


(A)

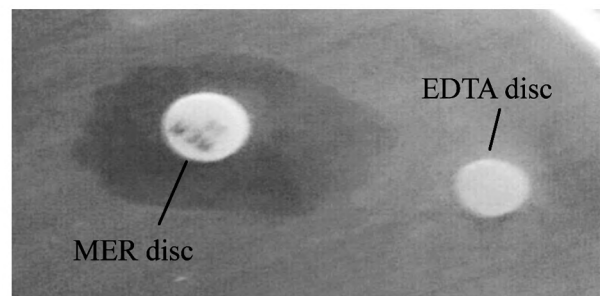


(B)

Fig. (2): CDT (A) Negative (B) Positive.



(A)



(B)

Fig. (3): DDST (A) Negative (B) Positive.

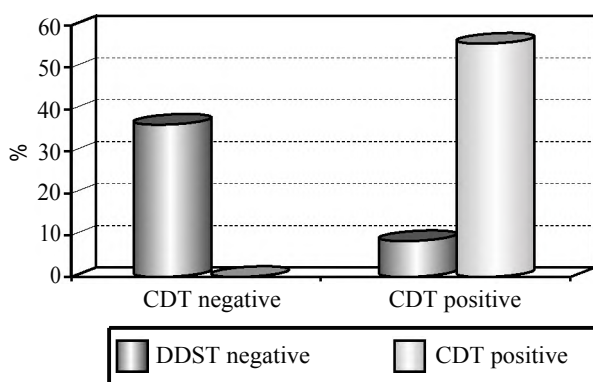


Fig. (4): Agreement between CDT and DDST.

### Discussion

Recent events indicate an increasing occurrence of antimicrobial resistance in GNB. Progressive antimicrobial resistance to most classes of antibiotics, including carbapenems, has made treatment of infection caused by these bacteria particularly difficult, even with combination therapy [15]. The clinical impact of carbapenem resistance has become a public health problem around the world in terms of increased mortality, longer hospital stays, and higher costs [3]. In the Middle East, the occur-

rence of CRGNB is increased, this increase could be due to extensive use of carbapenems which has likely generated a selective antibiotic pressure [16].

The isolated GNB in our study were most resistant to cefazoline (90.8%), while amikacin was found to be the most sensitive drug. Piéboji et al., [17], Lockhart et al., [18], Sharif et al., [19], Panchal et al., [9] and Zaki et al., [10] reported that isolated GNB were most resistant to Amoxicillin, ampicillin-sulbactam, ampicillin, Tetracycline and gentamicin then trimethoprim respectively.

The carbapenem resistance rate among GNB in our study was 20.8%. This resistance rate is in accordance with many other investigators, as Zaki et al., [10] reported that carbapenem resistance among GNB isolated from Mansoura University Hospital in Egypt was 28%. Panchal et al., [9] reported 28% carbapenem resistance rate among GNB isolates in a rural hospital in India. Wattal et al., [20] reported high prevalence of resistance to carbapenems ranging from 13 to 51% in *E. coli* and *Klebsiella* spp. from ICUs and wards from a tertiary care hospital in Delhi. Gupta et al., [21] also reported high prevalence of resistance varying from 17 to 22% to various carbapenems among Enterobacteriaceae strains. While carbapenem resistance among NFGNB isolates according to Das et al., [22] and Rathod et al., [23] was 63% and 28%, respectively. Misuse and abuse of antibiotics in Egypt has adversely contributed to the rate of antibiotic resistance in the study and might also be the cause of the emergence of carbapenemase enzymes. However, our results are different from some investigators, Navaneeth et al., [24] reported a prevalence of 12% carbapenem resistance among 50 strains of GNB isolated from various clinical specimens. Also, Mohammadzadeh et al., [2] found that carbapenem resistance rate in GNB isolates was 7%, which means a low prevalence of carbapenem resistance in last two studies in comparison with our study. This difference may be attributed to different patient population with different underlying diseases, different environment and different numbers of investigated specimens.

In our study, the carbapenem resistant isolates were more frequently isolated from sputum samples 14 (38.9%), followed by endotracheal aspirates 7 (19.4%), followed by wound swabs 6 (16.7%), followed by urine samples 4 (11.1%), then throat swabs and pleural fluid equally 2 (5.6%) and one blood sample (2.8%). Among these; 22 (61.1%) isolates were *Pseudomonas* spp., 6 (16.7%) were *Providencia* spp., 3 (8.3%) were *E. coli* spp., 2 (5.6%) were *Proteus* spp., one (2.8%) was *Acinetobacter* spp., one (2.8%) was *Enterobacter* spp. and one (2.8%) was *Serratia* spp., Omair et al., (2012) found that out of 52 CRGNB, 23 (44%) were from nasobronchial lavage, 10 (19%) were from pus, 6 (11%) from urine, 5 (10%) from catheter tips, 3 (6%) from blood, 2 (4%) from sputum, 2 (4%) from ear swabs and only one (2%) from high vaginal swab. Among these 52 isolates, 33 (63%) were *Acinetobacter* spp., 10 (19.5%) were *Pseudomonas* spp. and 9 (17.5%) were *Escherichia coli*, and Mohammadzadeh et al., [2] reported that among 62 imipenem resistant isolates 35 (56.5%), 24 (38.7%) and 3 (4.9%) isolates were *Acinetobacter* spp., *Pseudomonas* spp. and Enterobacteriaceae respectively, where 50 (80%) were sputum samples, 12 (19%) were blood samples, 4 (0.4%) were wound samples and 6 (0.9%) were other samples. While in a study by Diwakar et al., [25], 30 out of 110 CRGNB strains were *Klebsiella pneumoniae* strains, 26 *Escherichia coli* strains, 15 *Citrobacter freundii*, 13 *Pseudomonas aeruginosa* strains, 13 *Acinetobacter Baumannii* strains, 5 *Citrobacter koseri* strains, 4 *Enterobacter* spp. Strains, 3 strains of *Proteus mirabilis* and 2 strains of *Proteus vulgaris* isolated. Maximum number of sample was urine 29 (26.4%) followed by pus 27 (24.5%), blood 22 (20%) and 32 (29.1%) from other infections.

Resistance to carbapenems is often mediated by production of carbapenem hydrolyzing enzymes; carbapenemases which have been reported in several countries and have emerged as the most important mechanism of carbapenem resistance. carbapenemases can be divided into metallo-carbapenemases and non metallo-carbapenemases [5]. Infections due to MBLs producing GNB are a cause of high mortality and morbidity. Early detection by an economic and accurate method may aid in appropriate treatment and infection control measures [26].

The emergence of MBLs producing GNB is challenge to microbiology laboratories because there are no standardized guidelines available to detect them. PCR usually gives reliable and satisfactory results; however, because of the cost of that method, it is of limited practical use for routine diagnostic microbiology laboratories. Thus, a simple and inexpensive testing method for detection of MBLs producers is necessary [9].

The MBLs-producing GNB have now been reported in many geographic regions. In our study, prevalence of MBLs production in GNB was 63.9% using CDT and 55.6% using DDST. Similar study conducted by Das et al., [20] showed that 67% of

The MBLs-producing GNB have now been reported in many geographic regions. In our study, prevalence of MBLs production in GNB was 63.9% using CDT and 55.6% using DDST. Similar study conducted by Das et al., [20] showed that 67% of

isolates were MBLs positive by CDT and 58% were positive by DDST. The results of present study also correlate with the results of study conducted by Panchal et al., [9] in which 63% of the carbapenem resistant strains were MBLs producers by CDT and 53% by DDST. Diwakar et al., [23] also reported that 47% of GNB isolates were MBLs producers by CDT. While by using DDST Zaki et al., [10] found that 46.8% of isolates were MBLs positive. Our results were lower than those obtained by Pandya et al., [6] who found that 96.3% of isolates were MBLs positive by CDT and 81.5% were positive by DDST. Galani et al., [27] also reported that 94.7% of the carbapenem resistant strains were MBLs producers by CDT and 100% by DDST. Similar results were obtained by the study conducted by Picao et al., [28] in which 80% of isolates were identified as MBL producers by CDT and 82% by DDST. To the contrary, Thapa et al., [29] reported that MBLs production among GNB was 5.8%. Also, Mishra [30] found that 1.3% of the isolates were MBLs positive. This difference may be attributed to different patient population and different environment. In the present study there is almost perfect agreement between CDT and DDST.

#### Conclusion:

Our study validates two phenotypic methods (CDT and DDST) for the detection of MBLs production. Phenotypic methods are simple to perform, and the materials used are cheap, nontoxic, and easily accessible, making them highly applicable to routine clinical laboratories. Genetic confirmation by PCR may be used for positive isolates screened by phenotypic tests.

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## تقييم الميتالو بيتا-لاكتاميز في عينات العصيات السالبة الجرام والمقاومة للكربابينيم

أجريت هذه الدراسة على مدى ٨ أشهر على ١٣٠ عينة (٨٨ ذكور و٤٢ إناث) من المرضى المحجوزين في مختلف الأقسام بمستشفيات جامعة أسيوط، حيث شملت الدراسة أقسام الصدر والطوارئ ووحدة العناية المركزة العامة وقسم الأمراض العصبية وقسم الأنف والأذن والحنجرة وقسم الأمراض المتوطنة وقسم الباطنة العامة وقسم طب الأطفال وقسم أمراض القلب وقسم أمراض النساء وقسم الجراحة. وإشتملت العينات على ٣٤ عينة من القصبه الهوائية و٣١ عينة بلغم و٢٢ مسحة من الجروح و١٨ عينة دم و١٥ مسحة من الحلق و٦ عينات بول و٣ عينات من السائل البلوري ومسحة واحدة من المهبل.

بعد التعرف على العزلات المختلفة قمنا بعمل إختبار حساسية للمضادات الحيوية المختلفة، حيث كانت العصيات سالبة الجرام المعزولة أكثر مقاومة للسيفازولين (٩٠.٨٪)، في حين وجد أن أميكاسين هو الدواء الأكثر حساسية. وكان معدل المقاومة للكربابينيم في العزلات ٢٠.٨٪.

في هذا العمل، تم الكشف عن إنزيم الميتالوبيتا لكتاميز في العصيات سالبة الجرام والمقاومة للكربابينيم باستخدام طريقتين مختلفتين من الطرق المظهرية المستخدمة للتعرف على هذا الإنزيم، حيث أعطت طريقة CDT نتيجة إيجابية في ٦٣.٩٪ من العزلات، بينما كانت النتيجة إيجابية في ٥٥.٦٪ من الحالات باستخدام طريقة DDST. مع وجود إتفاق مثالي تقريبا بين الإختبارين.