Evaluation of Substrate Profile Test for Detection of Metallobetalactamses among Imipenem Resistant Clinical Isolates of Gram Negative Bacteria

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Abstract: ***Background & objectives:*** Metallo-β-lactamases (MBLs) are a class of carbapenemases enzymes which play an important role in carbapenem resistance. Therefore, it is necessary to detect MBLs among bacterial isolates by a simple and inexpensive method. This study was undertaken to evaluate a substrate profile test (SPT) for detection of MBLs among imipenem resistant clinical isolates of gram negative bacteria (GNB). ***Methods:*** A total of 35 imipenem resistant isolates (IMPR) which were previously identified for harboring VIM and NDM metallobetalactamases genes by PCR were included in this study. Determination of minimum inhibitory concentration (MIC) of imipenem against tested IMPR isolates was performed by agar dilution method. Modified Hodge test (MHT), UV spectrophotometry, EDTA double disc synergy test (EDDST) and Substrate profile test were four methods used for screening MBLs production among these tested isolates. ***Results*:** The tested IMPR isolates included *K.pneumoniae* (n=16), *E. coli* (n=7), *P.aeruginosa* (n=6), *P.mirabilis* (n=4) and *E. aerogenes* (n=2). Determination of MIC of imipenem against tested IMPR isolates revealed that all tested IMPR isolates, except for 3 isolates of *K. pneumoniae*, were found to have MICs ≥16 µg/ml. Moreover, 50% of the tested *P.aeruginosa* showed MICs of 256 µg/ml. The tested two *E.aerogenes* isolates had MIC of 16 µg/ml. SPT results showed 100% concordance with the results of UV spectrophotometry but not MHT and EDDST for detection of MBLs. It was observed that among the 7 *E.coli*isolates that were positive for MBL production by UV spectrophotometry and substrate profile, 5 (14.3%) were positive for MBL production by DDST. In addition, among the 6 *P.aeruginosa* showed MBL activity by UV spectrophotometry and substrate profile only 3 (8.6%) isolates showed MBL production by DDST. Moreover, only one *P. mirabilis* did not show MBL activity by EDDST even though all *P. mirabilis* isolates were positive for production of MBL using spectrophotometry and substrate profile. All the tested *K.pneumoniae* (n=16) and *E.aerogenes* (n=2) isolates were MBLs producers by all the used tests. ***Interpretation & conclusions*:** the substrate profile test (SPT), was evaluated compared to other methods to detect MBLs enzymes in GNB. The substrate profile test (SPT) is better than EDTA double disc synergy test (EDDST) and modified Hodge test (MHT) for screening of metallo-β-lactamases (MBLs). Moreover, this method had the same efficacy of UV spectrophotometry for detection of MBLs. It could be introduced into the workflow of any clinical Microbiology laboratory that routinely performs antibiotic sensitivity by disc diffusion test.

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Key words: Metallo-β-lactamases (MBLs), UV spectrophotometry detection of metallobetalactamases, EDTA double disc synergy test (EDDST), substrate profile test (SPT)

1. Introduction

Carbapenem antibiotics are potent, broad-spectrum β-lactams and they resist hydrolysis by most β-lactamases like extended-spectrum β-lactamases and Amp C β-lactamases **[1]**. Carbapenems are often conserved as effective therapy of severe infections caused by multi-resistant organisms**[2]**. However, carbapenem-resistant Gram negativebacteria have appeared over the past decadedue to the emergence of carbapenemases, especially metallo-β-lactamases (MβLs), which limit their the clinical use**[3].**

Carbapenemases are a family of enzymes produced by many bacteria and hydrolyze not only carbapenems but also other β-lactams including penicillins and cephalosporins. Carbapenemases can be classified on the basis of function or structure. Ambler classification, which is based on molecular structure, is the most commonly used classification. In this classification, carbapenemasesdivide into three major classes A, B, or D. Class A and Dhave a serine amino acid at their active sites, like extended spectrum β-lactamases (ESBLs). In contrast, the class B enzymes are known as metallo-β-lactamases, because they require zinc as a cofactor**[4].**

Metallo-β-lactamases (MBLs) are a group of carbapenemases enzymes whichhydrolyse all β-lactams but not monobactamslike aztreonam. They are not susceptible to therapeutic β-lactams inhibitors such as clavulanate, sulbactam, and tazobactam. MBLs require zinc-ions to catalyze the hydrolysis of β-

lactam antibiotics, so MBLs activity is inhibited in the presence of metal chelatorslike ethylenediaminetetraacetic acid (EDTA)**[5]**. Verona integron-encoded MBLs (VIM) and active on imipenem (IMP) were the common MBLs identified in *Enterobacteriaceae***[6]**. New Delhi β-lactamase (NDM-1), which originated in India, was first reported in 2009 **[7]** and has been isolated in Europe, North America, Asia, Australasia, and Middle East **[8-12]**

Although there are different phenotypic methods for detecting MBLs, PCR is considered the gold standardmethod, but its accessibility is often limited to reference laboratories**[13].** Most of non-molecular based techniques depend on inhibition of MBLs activity by chelating agents e.g. EDTA, dipicolinic acid, and thiol compounds **[14]**. Ourstudy aims atevaluating the performance of different phenotypic methods for detecting MBLs production among IMPR Gram-negative bacterial isolates in comparison with the gold standard PCR for MBL genes (Bla_{VIM} and Bla_{NDM}), in order to select a rapid, reliable, economical, and easy to set up workflow method for detection of MBLs.

2. Materials and Methods

Isolates and culture media

This study was performed at the Department of Microbiology, Faculty of Pharmacy, Tanta University, Egypt. A total of 35imipenem resistant isolates (IMPR) isolated from blood, burn, sputum, urine, wound and tracheal secretions specimens from patients admitted to different wards in Tanta university hospital between April 2014 to October 2014. All 35 IMPR isolates were characterized to the species level using MALDI-TOF (Bruker Daltonics Gmb H, Bremen, Germany). All these isolates were previously identified for harboring VIM and NDM metallobetalactamases genes by PCR. Culturing of isolates was performed on Macconkey and Mueller-Hinton agar plates (Oxoid Ltd, Hampshire, United Kingdom)

Determination of minimum inhibitory concentration (MIC) of imipenem

The MIC of imipenem against IMPR isolates was determined by agar dilution method. Procedures were performed and results were interpreted according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [15]**.**

Modified Hodge test (MHT)

This test was performed according to CLSI guidelines (2015) as follows: an overnight culture of 0.5 McFarland standard suspension of *E. coli* ATCC 25922 was inoculated on the surface of Muller Hinton agar plate (MHA). After drying, 10µg imipenem disc was placed at the center of the plate and the test isolate was streaked at straight line from the edge of the disc to the periphery of the plate in one direction then. The plate was incubated overnight at 37°C for 18 h. A clover leaf-type indentation at the intersection of the test organism and the *E. coli* ATCC 25922, within the zone of inhibition around the imipenem disc is considered positive for carbapenemases production.[16]

EDTA double disc synergy test (EDDST)

EDTA double disc synergy test (EDDST) was performed according to lee *et al*[17]. Tested isolates were adjusted to a turbidity equivalent to that of a 0.5 McFarland standard and used to surface inoculate Mueller–Hinton agar (Oxoid –UK) plates. A 10 µg imipenem disc or a 10 µg meropenem disc (Oxoid-UK) was placed on the plate, and a blank filter paper disc (6 mm in diameter, Whatman filter paper no. 1) was placed at a distance of 10 mm (edge to edge). Ten microliters of a 0.5 M EDTA solution was added to the blank disc. After overnight incubation, the presence of any synergistic inhibition zone was interpreted as positive.

UV spectrophotometry

The IMPR isolates were subjected to UV spectrophotometry for detection of MBLs. The crude enzyme extract of each tested bacterial isolate was prepared according to Bernabeu *et al*[18]. Using imipenem as a substrate, the crude enzyme extract was tested for its hydrolysis activity by UV spectrophotometry at a wavelength of 297 nm. Analysis was performed in a quartz cuvette by adding 970 µL of phosphate buffer (0.1 mol/L, pH 7.0), 50 µmol/L of ZnSO4, and 15 µL of crude enzyme extracts with 15 µL of imipenem at 10 mmol/L (150 µmol/L final concentration). Hydrolysis was measured for 10min, and the gradual decrease of absorbance of imipenem due to the presence of MBLs was observed.

Substrate profile test

The substrate profile of each crude enzyme extract was performed by testing the hydrolysis activity of the enzymes on antibiotic discsas the following: *E. coli* ATCC 25922 was grown in BHI (Brain Heart Infusion) liquid medium at 37 °C. After 12 hours of growth, it was inoculated on the surface of Mueller-Hinton agar plates at a concentration of 10^6 cells/mL. Subsequently, 4 different antibiotic discs (meropenem-imipenem-ertapenem-aztreonam) were separately placed on the surface of inoculated plate at proper distance (the control plate). At the same time, the previous steps were repeated but the 4 different antibiotic discs were impregnated with 20 µl of crude enzyme extract before placing on the surface of inoculated plate (test plate). The plates were incubated at 37 °C for 1 h. Zones of growth inhibition were observed. absence of inhibition zone diameter of any of the tested antibiotic discs impregnated with the

crude enzyme extract compared to that of the same disc without extract was recorded.

Statistical analysis

All the statistical analyses were performed using IBM SPSS version 22. Qualitative data were described using number and percentage. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Comparisons between different groups regarding categorical variables were carried out using paired sample *t*-test

3. Results

The tested isolates included *K.pneumoniae* (n=16), *E. coli* (n=7), *P.aeruginosa* (n=6), *P.mirabilis* (n=4) and *E. aerogenes* (n=2). All these isolates (n=35) were resistant to imipenem and were previously identified for harboring VIM and NDM metallobetalactamases genes by PCR (data not shown).

Determination of minimum inhibitory concentration (MIC) of imipenem against tested IMPR isolates

For the determination of MIC, serial dilutions (0.25-256 µg/ml) of imipenem concentrations were prepared and the results were shown in figure 1. Except for 3 isolates of *K. pneumoniae*, all tested IMPR isolates were found to have MICs ≥16 µg/ml. Moreover, 50% of the tested *P.aeruginosa* showed MICs of 256 µg/ml. The tested two *E.aerogenes* isolates had MIC of 16 µg/ml.

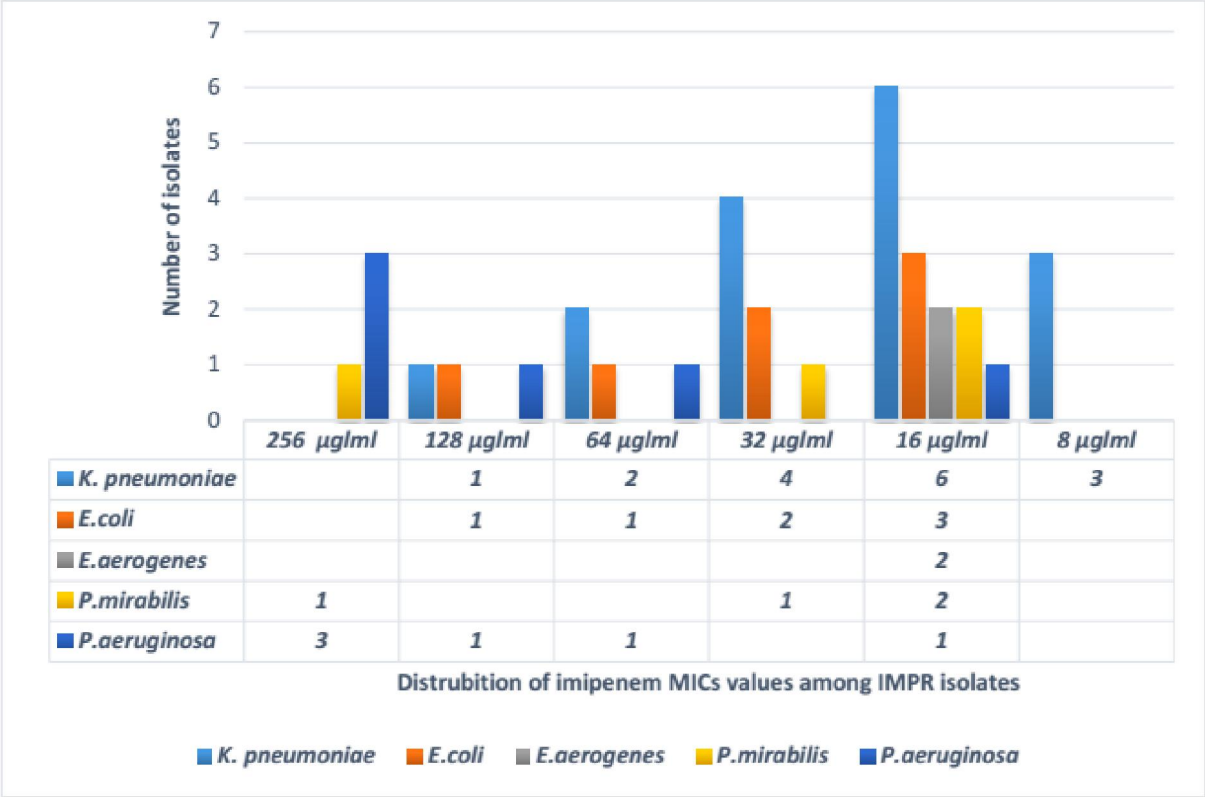


Figure 1: Histogram showing the distribution of MIC of imipenem among the tested IMPR isolates

Modified Hodge test (MHT) for detection of carbapenemases among the tested isolates

All IMPR isolates (n=35) were tested for the presence of carbapenemases using MHT. Positive results were detected by the appearance of distorted inhibition zone surrounding imipenem disc as shown in figure 2. In general, the carbapenemases activity was detected in 74.3% (26/35) of the IMPR isolates. The activity varied between *E.coli* (4/26; 15.4%), *K.pneumoniae* (12/26; 46.2%), and *P.mirabilis* (4/26; 15.4%) *E.aerogenes* (2/26; 7.8%) and *P.aeruginosa* (4/26; 15.4%).

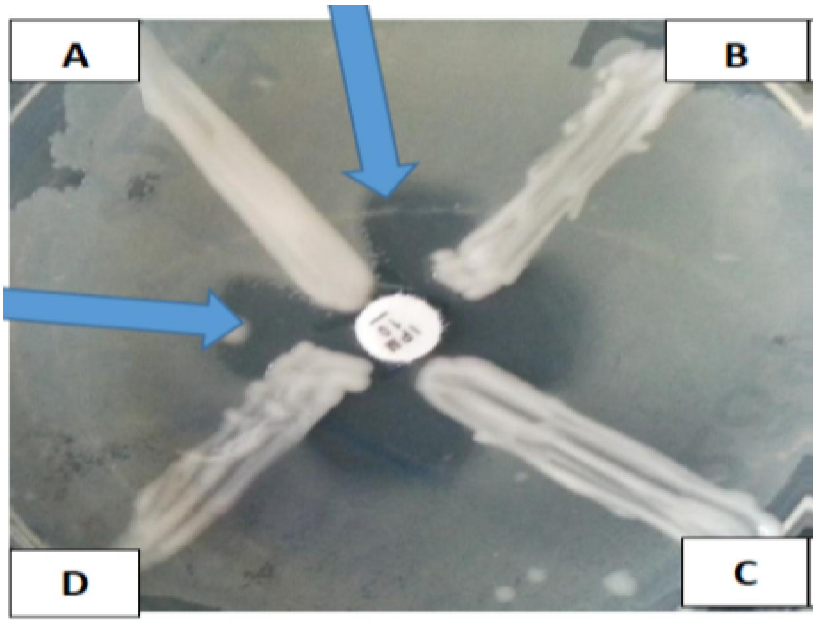


Figure 2: Modified Hodge Test for detection of carbapenemases. A photograph of a representative experiment of MHT. Positive result is indicated by the presence of a distorted or clover leaf-shaped inhibition zone due to carbapenemases production by the test strain as indicated by arrows. A. Positive result by *K. pneumoniae* (K1) B. Negative result by *E.coli* (E6) C. Negative result by *P. mirabilis* (Pr8) D. Negative control

EDTA double disc synergy test (EDDST)

A total of 35 IMPR isolates were subjected to DDST for detection of MBL enzymes. It was found that 29 (82.9%) isolates were positive for DDST as indicated by potentiation of the inhibitory zones between imipenem or meropenem discs and EDTA discs as shown in figure 3. Negative results were detected among one *P.mirabilis* isolate, two isolates of *E.coli* and three isolates of *P. aeruginosa.*

UV spectrophotometry

All IMPR isolates were positive for MBLs activity by UV spectrophotometry as indicated by the decrease of imipenem absorbance in the presence of crude extracts of enzymes of the tested isolates as shown in figure 4.

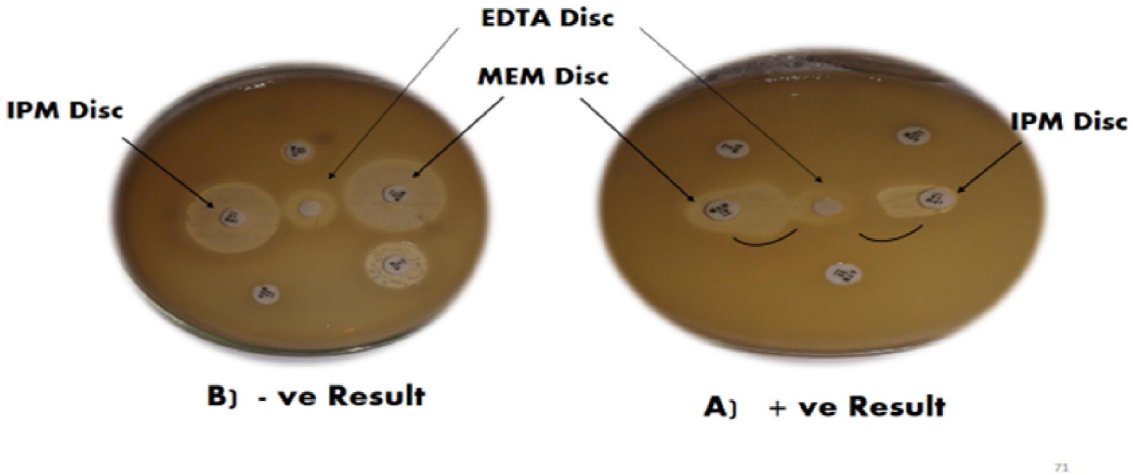


Figure 3: The double-disk synergy test (DDST) for detection of MBL. A) Positive result is indicated by potentiation of zones of inhibition between imipenem or meropenem and EDTA discs. B) Negative result is indicated by absence of potentiation of zones of inhibition between imipenem or meropenem and EDTA discs

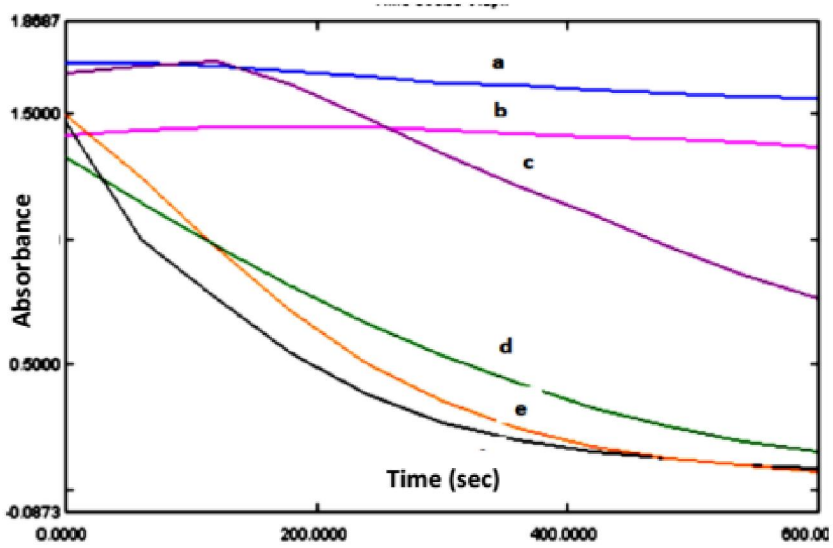
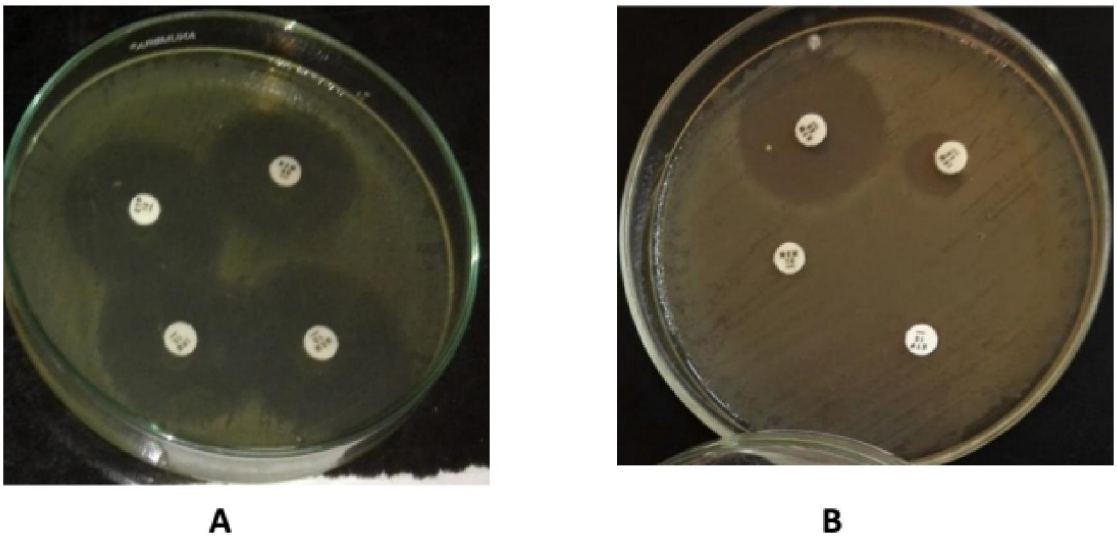


Figure 4: A representative Time course graph of spectrophotometric detection of metallo-β-lactamases among IMPR isolates. This figure illustrated a decrease in absorbance of imipenem due to hydrolysis by carbapenemases in crude cell lysates at wavelength 297 nm during 10 minutes represented by lines (c, d, e, f) which represent isolates En2, E2, K1 and Ps3; respectively. On the other hand, no decrease in the absorption due to absence of carbapenemases in the crude extract of (E8 and Pr6) which were represented by lines (a and b) respectively**.**

Substrate profile test (SPT)

Substrate profile of each crude extract of IMPR isolates (n=35) was determined by observing the abscence of any inhibition zones of meropenem, imipenem, ertapenem or aztreonam upon addition of the crude enzyme extract of the tested isolate. The substrate profile test was repeated 4 times for each crude enzyme extract. It was observed that all isolates (n=35) used meropenem, imipenem and ertapenem as substrates as indicated by abolishing of inhibition zone after the addition of the crude extract. On the other hand, aztreonam was not used as a substrate by any of the enzyme extract of the tested isolates



A B

Figure 5: A representative result of substrate profile tests. A) Negative control (*E. coli* ATCC 25922) showing no change in the inhibition zone after the addition of crude extract B) A positive result indicated by the absence of the inhibition zone after the addition of crude extract of *E.coli* (E2)

For the aim of comparison, all data of modified Hodge test (MHT), UV spectrophotometry, EDTA double disc synergy test (EDDST) and substrate profile test (SPT) tests are collected and presented in table 1. As shown in this table, all SPT results showed 100% concordance with the results of UV spectrophotometry but not MHT and EDDST for detection of MBLs. *E.coli* (E6) and *P. aeruginosa* (Ps3) were negative for MBLs activity by both MHT and EDDST but they showed positive results by SPT and UV spectrophotometry. Moreover, EDTA double disc synergy test (EDDST) detected MBL production in additional one *P.mirabilis* isolate, two *E.coli* isolates and four *K.pneumoniae* isolates, which were missed by the modified Hodge test. On other hand, one *E.coli*, one *P.mirabilis* and two *P.aerugnosa* showed positive MBL production by MHT but not by EDDST.

Table 1: Comparison between different phenotypic tests for detection of MBLs

Isolates code	MHT	EDDST	UV spectrophotometry	SPT
En1	+	+	+	+
En2	+	+	+	+
E1	+	-	+	+
E2	+	+	+	+
E3	-	+	+	+
E4	+	+	+	+
E5	-	+	+	+
E6	-	-	+	+
E7	+	+	+	+
k1	+	+	+	+
k2	+	+	+	+
k3	+	+	+	+
k4	+	+	+	+
k5	+	+	+	+
k6	+	+	+	+
k7	+	+	+	+
k8	+	+	+	+
k9	+	+	+	+
k10	-	+	+	+
k11	+	+	+	+
k12	-	+	+	+
k13	-	+	+	+
k14	-	+	+	+
k15	+	+	+	+
k16	+	+	+	+
Pr1	+	+	+	+
Pr2	+	+	+	+
Pr3	+	-	+	+
Pr32	+	+	+	+
ps1	+	-	+	+
ps2	+	-	+	+
ps3	-	-	+	+
ps4	+	+	+	+
ps5	+	+	+	+
ps6	-	+	+	+

Incidence of MBL producers isolates among IMPR isolates using the four phenotypic tests are presented in table 2. It was observed that among the 7 *E. coli* isolates that were positive for MBL production by UV spectrophotometry and substrate profile, 5 (14.3%) and 4 (11.4%) were positive for MBL production by DDST and MHT; respectively. In addition, among the 6 *P.aeruginosa* showed MBL activity by UV spectrophotometry and substrate profile only 3 (8.6%) and 4 (11.4%) isolates showed MBL production by DDST and MHT; respectively. Moreover, only one *P. mirabilis* did not show MBL

activity by EDDST even though all *P. mirabilis* isolates were positive for production of MBL using spectrophotometry and substrate profile.

Table 2: Incidence of MBL producing isolates according to phenotypic methods.

Bacterial isolates	MHT	Spectrophotometry No (%)*	DDST No (%)*	Substrate profile No (%)*
Enterobacteraerogenes (n=2)	2 (5.7%)	2 (5.7%)	2 (5.7%)	2 (5.7%)
Escherishia coli (n=7)	4 (11.4%)	7 (20%)	5 (14.3%)	7 (20%)
K.pneumoniae (n=16)	12(34.3%)	16 (45.7%)	16 (45.7%)	16 (45.7%)
Proteus mirabilis (n=4)	4 (11.4%)	4 (11.4%)	3 (8.6%)	4 (11.4%)
Pseudomonas aeruginosa (n=6)	4 (11.4%)	6 (17.2%)	3 (8.6%)	6 (17.2%)
Total	26(74.3%)	35 (100%)	29 (82.9%)	35 (100%)

***Percentage is calculated according to the total number of carbapenemases producers (n=35)**

4. Discussion:

Adequate detection of MBLs producing Gram negative bacteria (GNB) is important for proper selection of antimicrobial therapy and applying infection control measures [19]. Detection of carbapenemases production in GNB involves a screening step followed by a genotypic and optional phenotypic confirmatory step. However, if genotypic confirmation is not immediately available, phenotypic confirmation tests should be performed to avoid any delay in reporting carbapenemase producers [16]. In this study, we attempted to evaluate substrate profile test (SPT) as a convenient test for detection metallo-β-lactamase (MBL) production in GNB compared to modified Hodge test (MHT), EDDST and UV spectrophotometry.

The concept of using crude enzyme extract in substrate profile testing dates back to the 80s [20, 21]. However, to our knowledge, this is the first study to assess the efficacy of SPT method to detect major types of mobile MBLs described in Egypt (NDM and VIM). The SPT uses only four antibiotic discs (meropenem-imipenem-ertapenem-aztreonam) which are globally available at low cost and have long shelf lives. The choice of antibiotics for the SPT test was made specifically on the basis of previously published properties of different classes of carbapenemases [5]

Our results demonstrated that MHT detected only 74.3% of MBLs producers. This result was compatible with Thomson (2010) who reported that the detection of MBL producers by the modified Hodge test lacks sensitivity [22]. Girlich *et al.* addressed this issue in a study on carbapenem non susceptible *Enterobacteriaceae* where its sensitivity to detect NDM-producers was only 50%. This was illustrated by a possible secretion of a substance such as colicin, a bacteriocin-peptide released by certain bacteria, which may inhibit the growth of the indicator strain and interfere on results of the test [23].

The study demonstrated that SPT increases the likelihood of picking up such isolates that may be missed by the EDDST. This can be explained on the basis that β-lactamases of gram-negative bacteria are normally cell-bound in the periplasm[24], so it is important to obtain enzyme extract by sonication because enzymes appear in the culture medium only in a small portion. Moreover, EDTA itself can inhibit some bacteria due to increased permeability of the outer membrane[25].

In comparison to the UV spectrophotometry, SPT has similar high performance (100% concordance), but by UV spectrophotometry is expensive, requires specialized technicians and instruments. In addition, the cost of implementing this technique might not be justified in medical centers that have a low prevalence of MBL producers[19]. These factors make the implementation of SPT by routine clinical microbiology laboratories more favorable. However, one drawback of SPT method is the preparation of crude enzyme extract and their addition to antibiotic discs is a time-consuming process.

Conclusion:

On the basis of our study findings, it was concluded that substrate profile test (SPT) is better than EDTA double disc synergy test (EDDST) and modified Hodge test (MHT) for screening of metallo-β-lactamases (MBLs). Moreover, this method had the same efficacy of UV spectrophotometry for detection of MBLs. It could be introduced into the workflow of any clinical Microbiology laboratory that routinely performs antibiotic sensitivity by disc diffusion test.

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