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ORIGINAL ARTICLE

Evaluation of Dipiclonic acid for detection of Metallo-beta lactamase in *Pseudomonas aeruginosa* clinical isolates

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ABSTRACT

Background: *Pseudomonas aeruginosa is an important pathogen able to cause severe infection and difficult to be treated due to resistance to multiple antimicrobial agents. Detection of carbapenemase producing strains by phenotypic or molecular tests in the clinical laboratory is of major importance for the determination of appropriate therapeutic schemes and the implementation of infection control measures.* ***The aim*** *of this work is to evaluate a screening test for detection of MBLs among Pseudomonas aeruginosa isolates using carbapenem (Imipenem and Meropenem) disks supplemented with dipiclonic acid (DPA).* ***Methodology:*** *Fifty clinical isolates of carbapenem resistant Pseudomonas aeruginosa were collected from different clinical specimens. They were screened for the presence of carbapenemase by Modified Hodge test (MHT). Dipiclonic acidwas assessed for the ability of detection of MBL possessing isolates compared to PCR for detection of MBL gene* ***(bla_{IMP})*** *as a reference method.* ***Results****: Out of the 50 isolates Bla-IMP gene was detected in 13(26.0%), 33 (66.0%) were positive by MHT. In reference to bla-IMP PCR, MHT has100% sensitivity and 77% specificity. Combined Disk Testing (CDT) using (DPA) with imipenem and meropenem were positive in 10 specimens (20%) and negative in 40 specimens (80%).Based on PCR as a reference method, the sensitivity, specificity, PPV, NPV and accuracy for each of (IPM+DPA) and (MEM+DPA) were 76.9%, 100%, 100%, 92.5% and 94% respectively for each.There was an almost highly significant agreement between PCR and CDT (IPM+DPA) and CDT (MEM+DPA), as 76.9 % of PCR positive samples were positive by CDT (IPM+DPA) and CDT (MEM+DPA) and 100% of negative PCR samples were negative by both tests.This phenotypic screening test for detection of MBL among P. aeruginosa isolates, based on the use of meropenem and imipenem disks supplemented with DPA is reliable for detecting Pseudomonas aeruginosa isolates producing MBL carbapenemases.*

INTRODUCTION

Pseudomonas aeruginosa is an important pathogen able to cause severe infection and difficult to be treated due to resistance to multiple antimicrobial agents [(1)].

Pseudomonas aeruginosa is the leading cause of nosocomial infection.Several *Pseudomonas aeruginosa* nosocomial outbreaks are caused by patient-to-patient transmission or contaminated devices[(2)].

The emerging threat of antimicrobial resistance in *Pseudomonas aeruginosa* arises from the extraordinary capacity of this microorganism to develop resistance to almost all classes of antimicrobial agents.This resistance occurs through selection of mutations in chromosomal genes along emergence and dissemination of transferable resistance determinants like metallo-β-lactamases (MBLs) (class B carbapenemase) which have the highest clinical impact on antimicrobial therapy in hospitals worldwide due to its ability to hydrolyze a wide variety of β-lactam antibiotics[(1)]. The genes coding for these enzymes are frequently located in mobile genetic elements facilitating the dissemination of resistance among different bacteria [(3)].

The simultaneous detection of MBLs as well as the emergence in *Pseudomonas aeruginosa* of extended-spectrum β-lactamases (ESBLs) with carbapenem-hydrolyzing activity such as the KPC enzymes, add further complexity and concern to the resistance pattern of this organism [(1)].

So, detection of carbapenemase producers either by phenotypic or molecular tests in the clinical laboratory is of major importance for the determination of appropriate therapeutic schemes and the implementation of infection control measures [(4)].

Several inhibition-based tests have been developed for the detection of carbapenemase (MBL or KPC) producers using a combination disk test (CDT). The use of carbapenem disks supplemented with 3-aminophenylboronic acid (APB)[(4)], dipicolinic acid (DPA) [(5)] or cloxacillin[(6)] was described.

Molecular techniques remain the reference standard for identification and differentiation of carbapenemases. Most are based on PCR and may be followed by

sequencing if needed for precise identification of a carbapenemase, rather than its group (e.g. VIM-type, KPC-type, and IMP-type)[7]. They are either conventional PCR or multiplex real-time PCR techniques. Real-time PCR is fast and reliable method for rapid screening and identification of most relevant genes in carbapenemase positive clinical isolates [8].

The aim of this work is to evaluate a phenotypic screening test for detection of MBLs among *Pseudomonas aeruginosa* isolates using carbapenem (Imipenem and Meropenem) disks supplemented with dipiclonic acid.

METHODOLOGY

Fifty clinical isolates of carbapenem resistant *Pseudomonas aeruginosa* were collected from different clinical specimens that were referred for routine culture and sensitivity to the Central Microbiology Laboratory, Ain Shams University Hospitals,Cairo, Egypt. Identification of the isolates was confirmed by using the API 20 NE system (bioMerieux, France). They were tested for the presence of carbapenemase by Modified Hodge test (MHT). Dipiclonic acid was assessed for the ability of detection of MBL possessing isolates compared to PCR for detection of MBL gene (bla_{IMP}) as a reference method [4; 5].

Modified Hodge test

Testing for the presence of carbapenemase by Modified Hodge test (MHT) was done by inoculating the surface of a Muller-Hinton agar (Oxoid, UK) plate with a culture suspension of *E. coli* (ATCC-25922) adjusted to a one tenth turbidity of a 0.5 McFarland. A meropenem 10µg disc (Oxoid, UK) was placed at the center of the plate and the isolates to be tested were streaked from the edge of the disc to the periphery of the plate. The plates were incubated at 35°C overnight. A clover leaf-like indentation of the *E.coli* growing along the test organism growth streak within the disk diffusion zone indicates the presence of a carbapenemase producing organism. While no growth of the *E.coli* along the test organism growth streak within the disc diffusion indicates that this isolate is a non carbapenemase producing organism [8].

Phenotypic detection of MBL by Combined Disk Testing using dipiclonic acid disk

One hundred mg of dipicolinic acid powder (Sigma-Aldrich, Germany) was dissolved in one ml of sterile water to reach final concentration of 100 mg/ml**.** Ten microliter of dipicolinic acid stock solution was dispensed onto commercially available discs containing meropenem (10µg) (Oxoid, UK). The final amount of DPA on the meropenem discs was 1000 µg [4]. Eighty three and half mg of DPA (Sigma-Aldrich, Germany)was dissolved in one ml of sterile water to reach concentration of 83.5 mg/ml**.**Ten microliter of DPA stock solution was dispensed onto commercially available discs (Oxoid, UK.) containing imipenem (10µg). The final amount of DPA on the imipenem discs was 835 µg [5]**.**

A 0.5 Mcfarland inoculums was prepared and spread on MHA plates. Four discs were placed on each plate:meropenem 10ug,meropenem 10ug +DPA,imipenem 10ug and imipenem 10ug+DPA. The plates were incubated overnight at 37°C.

An increase in the zone size of ≥ 5 mm for meropenem and imipenem in the presence of DPA compared with that of the carbapenem substrate alone was considered a positive for MBL production(Figure 1) and no or an increase in the zone size of <5 compared with that of the carbapenem substrate alone was considered a negative result (Figure 2).

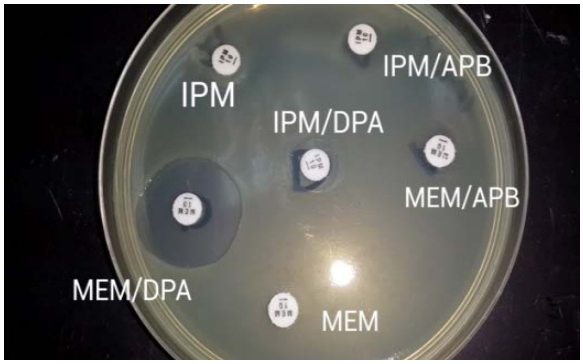

Fig. 1: Positive isolate for MBLs (increase in zone diameter ≥5mm with MEM+ DPA compared with MEM alone)

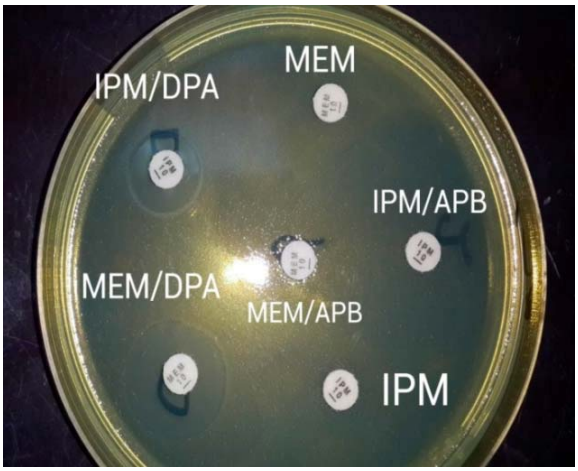

Fig. 2: Positive isolate for MBLs (increase in zone diameter ≥ 5mm with IPM+DPA compared with IPM alone)

Real time Polymerase chain reaction

Real time Polymerase chain reaction (PCR) was performed for detection of MBL gene (***bla*$_{IMP}$**) in the 50 isolates using QiagenRoter gene 5plex(Germany). Bacterial DNA was extracted using DNA extraction kit

(ThermoScientific, Lithuania) according to manufacturer's instructions. DNA amplification was done using Maxima SYBR Green qPCR Master Mix (2X) (Sigma, Germany).Primers used in the amplification were designed according to[3]. A reaction master mix was prepared by adding the components described in table (1) for each 25µl reaction in a tube at room temperature. Sterile distilled water was used as negative control.

Table 1: Components of reaction mixture for each 25 ul reaction

Reaction component	*Concentration*	*Volume*
Maxima® SYBR Green qPCR Master Mix (2X), no ROX	12.5 ul	12.5 µl
Forward Primer (5'-GAGTGGCTTAATTCTCRATC-3')	0.3 µM	0.75 µl (1:10)
Reverse Primer (5'-AACTAYCCAATAYRTAAC-3')	0.3 µM	0.75 µl (1:10)
ROX Solution	10 nM/ 100 nM	0.05 µl (1:10)
Template DNA	≤500 ng	5 µl (1:10)
Water, nuclease-free	to 25 ul	6 µl
Total reaction volume	25 ul	25 µl

Reaction tubes were then loaded onto Rotor Gene and the amplification program was adjusted as follows: initial denaturation at 95°C for 10 minutes, followed by 38 cycles of amplification consisting of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. The amplification program was followed immediately by a melt program consisting of 1 minute at 95°C, 30 sec at 55°C then again to 95°C for 30 sec.

Statistical analysis:

Data was presented and suitable analysis was done according to the type of data obtained for each parameter.

A. Descriptive statistics:

Frequency and percentage of non numerical data.

B. Analytical statistics:

The diagnostic test evaluation; sensitivity, specificity and the positive and negative predictive values and Cohen's Kappa coefficient (k) for agreement between two tests were calculated for determining the diagnostic validity of the test. Kappa's 0.81-1 is excellent, 0.61 to 0.80 is substantial, 0.41-0.60 is moderate,0.21-0.40 is fair, 0-0.20 is fair and below 0 is no agreement. All the analyses were performed with commercially available software (SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, USA, 2001).

RESULTS

Clinical isolates

Out of 50 *Pseudomonas aeruginosa* carbapenem resistant isolates: 22 (44%) are from wound swabs, 10 (20%) from urine, 8 (16%) from sputum,4 (8%) from blood, 3(6%) from pus, 2(4%) from drains and 1(2%) from oral ulcer. The thirteen (13) *Pseudomonas aeruginosa* positive *bla-IMP* isolates are isolated from wound swabs (5), urine specimen(3), sputum specimens, blood culture, oral ulcer, pus, and drain (1).

Out of 50 *Pseudomonas aeruginosa isolates*,33 (66.0%) were positive by Modified Hodge test. *Bla-IMP* gene was detected in 13(26.0%) out of the 50 isolates by PCR.Combined Disk Testing (CDT) using dipiclonic acid (DPA) with imipenem and meropenem were positive in 10 isolates (20%) and negative in 40 specimens (80%).

Modified Hodge test

In the current study**,** out of 50 *Pseudomonas aeruginosa isolates*,33 (66.0%) were positive by Modified Hodge test (carbapenemase producers). In reference to *bla-IMP* PCR, MHT has sensitivity of 100% and specificity of 77%.

There was a fair agreement between Modified-Hodge test results and *bla-IMP* gene PCR results (kappa=o.307).

Combined Disk Testing (MEM+DPA)

In the current study, Combined Disk Testing (CDT) using dipiclonic acid (DPA) with meropenem were positive in 10 isolates (20%) and negative in 40 specimens (80%). Based on PCR as a reference method, the sensitivity, specificity, PPV, NPV and accuracy forCDT(MEM+DPA) were 76.9%, 100%, 100%, 92.5% and 94% respectively.There was an almost highly significant agreement between PCR and CDT (MEM+DPA) as regard tests results(kappa=0.831), as 76.9% of PCR positive samples were positive by CDT (MEM+DPA) and 100% of negative PCR samples were negative by CDT (MEM+DPA) test (table 2).

Table 2: Agreement between PCR results and MEM+DPA test results

		PCR (*bla-IMP*)				***Kappa***	***P(Sig)***
		Positive		**Negative**			
		N	**%**	**N**	**%**		
MEM + DPA	Positive	10	76.9%	0	0.0%	0.831	0.001HS
	Negative	3	23.1%	37	100.0%		

Combined Disk Testing (IMP+DPA)

In the current study, Combined Disk Testing (CDT) using dipiclonic acid (DPA) with imipenem were positive in 10 isolates (20%) and negative in 40 isolates (80%). Based on PCR as a reference method, the sensitivity, specificity, PPV, NPV and accuracy for each of IPM+DPA were 76.9%, 100%, 100%, 92.5% and 94% respectively.There was an almost highly significant agreement between PCR and CDT (IPM+DPA) as regard tests results, as 76.9% of PCR positive samples were positive by CDT (IPM+DPA) and 100% of negative PCR samples were negative by CDT (IPM+DPA) test (table 3).

Table 3: Agreement between PCR results and IPM+DPA test result

		PCR (*bla-IMP*)				***Kappa***	***P(Sig)***
		Positive		**Negative**			
		N	**%**	**N**	**%**		
IPM + DPA	Positive	10	76.9%	0	0.0%	0.831	0.001
	Negative	3	23.1%	37	100.0%		HS

DISCUSSION

Similar to our result of MHT, Amjad and colleagues 2011 in Armed Forces Institute of Pathology Rawalpindi Pakistan found that Out of 200 gram negative isolates, 138 (69%) were positive for carbapenemase production by Modified Hodge test. Out of 138 MHT positive organisms, the frequency of *E. coli* was 38%, followed by *P. aeruginosa (30%), Klebsiella pneumoniae (17%), Acinetobacter baumannii (12%), Citrobacter diversus (2%) and Enterobacter agglomerans (1.4%).* They concluded that Modified Hodge test is a simple test which can be performed in the routine lab.for detection of carbapenemases [(9)]. On the other hand, Jesudason et al. from India found that the Modified Hodge test was positive in (56%) of 50 imipenem resistant gram negative isolates (28 of them are *Pseudomonas*)[(10)]. Another study by Noyal et al. from India found that the Modified Hodge test was positive in (28.1%) of meropenem resistant *Pseudomonas isolates* .They explained this low detection rate due to relatively small sample size[(11)]. Also, Pasteran et al., used the algorithm of CDT using the meropenem-APB, meropenem-DPA and meropenem-Cloxacillin combination disk test for discriminating carbapenamase producing *p. aeruginosa* and non-producers. Then compared the results to Modified-Hodge test results for detection of carbapenamase producing *p. aeruginosa*. They found that the MHT resulted in a low sensitivity (78%) and specificity (57%) as there were high proportion of indeterminate results [(4)].

Similarly to the result of CDT using meropenem Giske et al. screened for MBL in *K. pneumoniae* isolates using in house discs CDT (MEM+DPA) and commercial diagnostic tablets from ROSCO with specificity 100%, 98% respectively and sensitivity 100% for both. He found that both type of discs performed well for detection of MBL **(12).** Also, Pasteran et al. conducted his study on *Pseudomonase aeruginosa* isolates where CDT (MEM+DPA) showed specificity 81% and sensitivity 97% [(4)]. Also, Van Dijk et al. used ROSCO diagnostic discs have 90% sensitivity and 96% specificity [(13).] Heinrichs et al. performed a study on *Pseudomonas aeruginosa* where CDT (MEM+DPA) (ROSCO Diagnostica) showed 97% sensitivity and 88% specificity PPV 78% and NPV 99% [(14)]. Another study by Yong et al., performed study on isolates of *Pseudomonas* spp. and *Actinobacter* spp, CDT was done using DPA with MEM with sensitivity 86.4% and specificity 94.1% and found that DPA is better than EDTA as MBL inhibitor [(5)].

On the other hand, Hansen et al. performed his study on *Pseudomonas aeruginosa* using total MBL confirm kit (ROSCO) which includes MEM and DPA which showed 89% sensitivity and 14% specificity. Insufficient inhibiting action due to suboptimal tablet

concentration of DPA [15]. Also a study by Shin et al. was performed on *Pseudomonas aeruginosa* and *Acintobacter* spp. He concluded that MEM disks were inappropriate for use in the DPA-based disk test because of the significant shortfall in their ability to detect VIM-2–producing isolates of *Pseudomonas* spp. and *Acinetobacter*spp [16]. Yong et al. suggested that hyperexpression of efflux pumps could influence meropenem combination testing (as opposed to imipenem) which is a substrate of the major efflux pumpthatmay result in lower specificity with Meropenem [5].

Similarly to our result of CDT using imipenem Heinrichs et al. screened for MBL by CDT (IPM+DPA) by ROSCO Diagnostica discs showed sensitivity 99% and specificity 95%, PPV 89%, and NPV of 99% [14]. Another study by Yong et al. using commercial (ROSCO) IPM+DPA tablets with sensitivity 93.2% and specificity 97.1% [5]. Peter et al. performed his study on *P.aeruginosa* using ROSCO discs, had sensitivity of 84.4% and specificity of 81.8% [17]. In South Korea Shin et al. found that CDT had specificity 100% and sensitivity 98.7% [16]. In a survey done in Belgian acute care hospitals by Glupczynski et al. done on MDR *P.aeruginosa* using IPM+DPA supplied by (ROSCO) had sensitivity 98% and specificity 95% [1]. Hansen et al. used CDT (IPM+DPA) (ROSCO) in *P.aeruginosa* showed sensitivity 83% and specificity 67% [15].

Several studies had recommended that detection of MBL in *K. pneumonaie, P.aeruginosa, and Actinobacter*by DPA-IPM disk is best phenotypic method due to their high efficacy [18]. On the contrary, in Iran Lari et al. performed his study on *P.aeruginosa, A.baumannii*, and *K.pneumoniae*, CDT showed 35% specificity and 100% sensitivity [19].

CONCLUSION

The prevalence of carbapenem-resistant organisms (CROs) is increasing worldwide, mainly due to carbapenemase production. So accurate detection of carbapenemase-producing *Pseudomonas* species is particularly important due to the rise of carbapenem resistance in these organisms. Also, CDT using meropenem or imipenem is a sesnsitive and specific test for detection of MBL in *Pseudomonas aeruginosa* isolates.

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