

Original Research Article

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Evaluation of Aminophenyl boronic Acid for Detection of KPC Production in *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is the leading cause of nosocomial infection. The simultaneous detection of MBLs as well as the recent 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. 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. The aim of this work is to establish a phenotypic screening test for identification of KPC among *Pseudomonas aeruginosa* isolates by using carbapenem (Imipenem and Meropenem) disks supplemented with aminophenylboronic acid. Fifty clinical isolates of carbapenem resistant *Pseudomonas aeruginosa* were collected from different clinical specimens. Phenotypic screening test was made for detection of KPC carbapenamase among *Pseudomonas aeruginosa* isolates by using of modified hodge test and carbapenem (Imipenem and Meropenem) disks supplemented with aminophenylboronic acid Combined Disk Testing (CDT). Out of 50 *Pseudomonas aeruginosa* isolates, Bla-KPC gene was detected in 15 (30%) of isolates by real time PCR, 33 (66.0%) were positive by Modified Hodge test with sensitivity 100%. Out of 50 *Pseudomonas aeruginosa* isolates, CDT using (IPM+APB) and CDT (MEM+APB) positive results were present in 18% and 22% of samples respectively. Based on PCR as a reference test, the sensitivity, specificity, PPV, NPV and accuracy for CDT (IPM+APB) were 53.3%, 97.1%, 88.8%, 82.9%, and 84%. For CDT (MEM+APB) were 66.7%, 97.1%, 90.9%, 87.1% and 88% respectively. This phenotypic screening test is reliable for detecting *P. aeruginosa* isolates suspected of producing KPC carbapenemases.

Keywords

Aminophenyl boronic Acid, KPC, *Pseudomonas aeruginosa*.

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Introduction

Pseudomonas aeruginosa is known widely as opportunistic organism which causes outbreaks of hospital acquired infections of high morbidity and mortality. Also, it is involved in infections of immune suppressed patients (Zafer, 2014). Infections caused by it are difficult to be treated as it shows high resistance to many antimicrobials (Khan *et al.*, 2014).

The emergence of *Klebsiella pneumoniae* carbapenemase (KPC) has now become

global concern (Pasteran *et al.*, 2012). KPC producers are mostly *Enterobacteriaceae* but *Pseudomonas aeruginosa* have also been first reported in 2007. The KPC family has a great potential for spreading due to location of its gene (bla_{KPC-2}) on plasmids (Hirsch and Tam, 2010).

The simultaneous detection of MBLs as well as the recent 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 (Glupczynski *et al.*, 2010).

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 (Pasteran *et al.*, 2011).

Several inhibition-based tests have been developed for the detection of carbapenemase. Recently, a combination disk test (CDT) for the detection of MBL and KPC-producing *Pseudomonas aeruginosa* was developed with the use of imipenem disks supplemented with 3-aminophenylboronic acid (APB) (Pasteran *et al.*, 2011), dipicolinic acid (DPA) (Yong *et al.*, 2012), or cloxacillin (Fournier *et al.*, 2013).

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) (Nordmann *et al.*, 2012). 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 (Monteiro *et al.*, 2012).

The aim of this work is to establish a phenotypic screening test for identification Of KPC among *Pseudomonas aeruginosa* isolates using of carbapenem (Imipenem and Meropenem) disks supplemented with aminophenyl boronic acid.

Materials and Methods

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 20E system (bioMerieux, Marcy l Etoile, France). Then isolates were stored at -70°C until further processed. They were screened for the presence of carbapenemase by Modified Hodge test (MHT). Combined Disk Testing (CDT) using a carbapenem disk (imipenem and meropenem supplemented with Aminophenylboronic acid) was assessed for the ability of detection of KPC possessing isolates compared to PCR for detection of kpc gene (bla_{kpc}) as a reference method (Pasteran *et al.*, 2011).

Polymerase chain reaction

Real time Polymerase chain reaction (PCR) was performed for detection of KPC gene (bla_{KPC}) in the 50 isolates. Bacterial DNA was extracted using DNA extraction kit (Thermo Scientific GeneJET Genomic Purification Kit, Lithuania, EU). The reaction mixture included Maxima® SYBR Green qPCR Master Mix (2X) (Sigma, Germany), Oligonucleotide primers; bla_{KPC} :

Forward primer: 5'-TTGTTGATTGGCT AA AGGG-3'

Reverse primer: 5'- CCATACACTCCGC AGGTT-3' (Wang *et al.*, 2012).

Escherichia coli ATCC 25922 and bla_{KPC} -carrying *K. pneumoniae* ATCC BAA-1705 were used as negative and positive controls, respectively. The amplification was done as described by (Alper Ergin *et al.*, 2011). The PCR conditions were with initial denaturation step at 95°C for 10 min and 38

cycles of amplification consisting of: denaturation at 95°C for 15s., annealing at 60°C for 30s, extension at 72°C for 30s. Detection of the PCR amplified product was done by SYBR Green (Sigma, Germany) (Muldrew, 2009).

Modified Hodge test

Screening 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* (ATCC25922) 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 (CLSI, 2015).

Phenotypic detection of KPC enzymes with Combined Disk testing by aminophenylboronic acid disk

Preparation of meropenem and imipenem discs containing APB

Preparation of stock solution of amino phenyl boronic acid for imipenem and meropenem disks is made as follows: Sixty mg of APB powder (Sigma-Aldrich, Steinheim, Germany) is dissolved in one ml of sterile water to reach final concentration 60mg/ml. Ten microliter of APB stock solution is dispensed onto commercially available discs (Oxoid, UK.) containing imipenem (10µg) and meropenem (10µg).

The final amount of APB on the discs is 600µg. APB discs were allowed to dry for 60 min then used immediately (Tsakris *et al.*, 2011).

The test strain was inoculated on MHA plates according to the CLSI guideline. Discs containing meropenem 10µg and imipenem 10µg plus 600 µg APB were placed apart from each other on the MHA plates. 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 APB compared with that of the drug alone was considered a positive result (Fig 1) and no or an increase in the zone size of < 5 compared with that of the drug alone was considered a negative result (Fig 2).

Results and Discussion

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 fifteen (15) *Pseudomonas aeruginosa* positive *bla*_{KPC} isolates are isolated from wound swabs (9), urine specimen (3) and sputum specimens (3).

Real time PCR

Out of 50 *Pseudomonas aeruginosa* isolates, *bla*_{KPC} gene was detected in 15 (30%) of isolates by real time PCR.

Modified Hodge test

Out of 50 *Pseudomonas aeruginosa* isolates, 33 (66.0%) were positive by Modified Hodge test. *bla*_{KPC} gene was detected in 15

(30%) out of the 50 isolates by PCR. In reference to *bla*_{KPC} PCR, MHT has 100% sensitivity.

Combined Disk Testing by aminophenyl boronic acid

Out of 50 *Pseudomonas aeruginosa* isolates, (9) 18% isolates were KPC producers by CDT using (IPM+APB) and (11) 22% of isolates were KPC producers by CDT using (MEM+APB) (table 1).

There was a highly significant agreement between PCR and CDT using (IPM+APB) as regard detection of *bla*_{KPC} ($\kappa=0.571$), as 53.3% of *bla*_{KPC} PCR positive isolates were positive for detection of KPC enzymes by (IPM+APB) and 97.1% of *bla*_{KPC} PCR negative isolates were negative for detection of KPC enzymes by CDT (IPM+APB).

As regard CDT using (MEM+APB), there was large significant agreement between PCR and (MEM+APB) for detection of *bla*_{KPC} as regard test result ($\kappa=0.69$), as 66.7% of isolates that were positive by *bla*_{KPC} PCR, were positive for detection of KPC enzymes by CDT (MEM+APB) and 97.1% of *bla*_{KPC} negative PCR isolates were negative by CDT (MEM+APB).

There was a highly significant agreement between CDT (IPM+APB) and CDT (MEM+APB) as regard test results ($\kappa=0.751$) as 88.9% of CDT (IPM+APB) positive isolates were positive by CDT (MEM+APB) and 92.7% of CDT (IPM+APB) negative isolates were negative by CDT (MEM+APB).

Based on PCR as a reference test, the sensitivity, specificity, PPV, NPV and accuracy for CDT (IPM+APB) were 53.3%, 97.1%, 88.8%, 82.9%, and 84%. For CDT (MEM+APB) were 66.7%, 97.1%, 90.9%,

87.1% and 88% respectively (table 2).

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). Aminophenylboronic acid was assessed for the ability of detection of KPC possessing isolates compared to PCR for detection of *kpc* gene (*bla*_{kpc}) as a reference method.

In the current study, out of 50 *Pseudomonas aeruginosa* isolates, 33 (66.0%) were positive by Modified Hodge test. *Bla*-KPC gene was detected in 15 (30.0%) out of the 50 isolates by PCR. In reference to *bla*-KPC PCR, MHT has 100% sensitivity. Similarly, In a study made by Lee *et al.*, 2001 in Yonsei University Seoul, Korea, a total of 530 imipenem-resistant *Pseudomonas* isolates were screened by the modified Hodge test, Sensitivities and specificities were 100% and 88%, respectively (Lee *et al.*, 2001).

However, in a study made by Pasteran *et al.*, (2011) evaluated the ability of the Modified Hodge Test (MHT) to discriminate between various carbapenemase-producing *Pseudomonas aeruginosa* isolates (n=149), the MHT resulted in a low sensitivity (78%) and specificity (57%). In another study made by Noyal *et al.*, 2009 in Puducherry, India, a total of 140 *Pseudomonas* spp. (103 *P. aeruginosa* and 37 other *Pseudomonas* spp.) were screened for meropenem resistance by Kirby Bauer disc diffusion method. Modified Hodge test, EDTA disk synergy (EDS) test and AmpC disk test were used for the detection of carbapenemases, MBLs and AmpC β -lactamases, respectively. Among the 32 meropenem resistant *P. aeruginosa*, 15 (46.9%) were AmpC β -lactamase producers, 16 (50.0%) MBL

producers by EDS test, but only 9 (28.1%) found positive for carbapenemases by modified Hodge test. This study concluded that Modified Hodge test may not be a useful screening test for carbapenemases as many MBL producing isolates were not detected by this test. Also, Lari *et al.*, 2015, performed MHT on 241 *Pseudomonas* spp, his results stated that MHT had an excellent sensitivity (100%) for detection of carbapenemases but low specificity (2.6%) for the detection of KPC among Iranian bacterial specimens.

In the current study, there was a substantial significant agreement between PCR and CDT (MEM+APB) as regard test result, as 66.7% of PCR positive samples were positive by CDT (MEM+APB) and 97.1% of PCR negative results were negative by CDT (MEM+APB). Similarly, Pasteran *et al.*, (2011) performed CDT using APB on *P. aeruginosa* (n=149) had excellent sensitivity (97%) and specificity (97%) for the detection of KPC enzymes.

However, Lari *et al.*, (2014), performed another study on Carbapenem-resistant *P. aeruginosa* strains (n=241) which were

isolated from wounds of hospitalized burn patients. Using 400 µg 3-amino phenyl boronic acid (APBA) as a KPC inhibitor, per disk plus Meropenem vs Meropenem alone. One hundred eighty-six strains were resistant to all tested carbapenems (Imipenem, Meropenem and Ertapenem).

Seventy-five strains had MHT-positive test results. A synergism effect between Meropenem and APBA was observed in 11(15%) *P.aeruginosa* strains with positive MHT test results. On the other hand, only 3 strains had synergism with APBA alone. PCR showed specific bands after gel electrophoresis in only two MHT-positive strains.

The proportion of positive results from molecular testing of carbapenem resistant *P. aeruginosa* is 0.01(1.07%). One of two strains had a synergistic effect with APBA. These findings indicate that the sensitivity and specificity of inhibitory test (use of boronic acid) could not be explained by these results, as the author proposed that there is high false positive results by CDT with meropenem and APBA.

Table.1 Description of PCR, MHT, CDT (IPM+APB) and CDT (MEM+APB) results

		N	%
PCR	Positive	15	30.0%
	Negative	35	70.0%
MHT	Positive	33	66.0%
	Negative	17	34.0%
CDT (IPM + APB)	Positive	9	18.0%
	Negative	41	82.0%
CDT (MEM + APB)	Positive	11	22.0%
	Negative	39	78.0%

Table.2 shows sensitivity, specificity, PPV and NPV of CDT (IPM+APB) and CDT (MEM+APB)

	Sensitivity	Specificity	PPV	NPV	Accuracy
CDT (IPM + APB)	53.3%	97.1%	88.8%	82.9%	84%
CDT (MEM + APB)	66.7%	97.1%	90.9%	87.1%	88%



Fig.1 Positive results for MEM and IMP+ APB **Fig.2** Negative results for MEM and IMP+ APB

In this study, there was a moderate significant agreement between PCR and CDT (IPM+APB) as regard tests results as 53.3% of PCR positive isolates were positive by CDT (IPM+APB) and 97.1% of negative PCR isolates were negative by CDT (IPM+APB). Similarly Maurer et al., (2015) screened for KPC in Enterobacteriaceae using CDT (IPM+APB) which had sensitivity 75% and specificity 99.4%. Tsakris *et al.*, (2011) used CDT (IPM+APB) with 82.1% sensitivity and 97.6% specificity.

There was a strong highly significant agreement between CDT (IPM+APB) and CDT (MEM+APB) for detection of KPC producers as 88.9% of CDT (IPM+APB) positive isolates were positive by CDT (MEM+APB) and 92.7% of negative CDT (IPM+APB) isolates were negative by CDT (MEM+APB). Similarly, Hansen et al., (2012) used commercial disks in CDT (MEM+APB) which identified all KPC producing strains and he concluded that meropenem is preferable for detection of

class A carbapenemases than ceftazidime (CAZ) or cefotaxime (CTX). Similarly, Hansen *et al.*, (2012) used commercial disks MEM+APB which identify all KPC producing strains and he concluded that meropenem is preferable for detection of class A carbapenemases than ceftazidime (CAZ) or cefotaxime (CTX). Similarly a study by Tsakris *et al.*, (2011) found that among 108 CDT (MEM+APB) positive KPC isolates, 92 were positive by CDT (IPM+APB). He used different concentrations of APB and concluded that 600µg of APB identify more KPC producers than 300µg and meropenem is more sensitive substrate for detection of KPC irrespective of boronic acid compound or concentration. He concluded from his comparative study that APB is the most effective inhibitor of KPC enzymes, and its use in combined-disk tests with meropenem may give the most easily interpreted results.

On the other hand, Borba *et al.*, (2012) screened for KPC among *K.pneumoniae*

isolates using CDT using MEM+APB and IPM+APB with sensitivity 33.3% and 0% respectively. This may be due to using 30µg of MEM and IPM disks and after preparation of CDT, the disks are stored at -20°C for three weeks which could affect solution stability and antibiotic when subjected to storage. He also suggested decreasing cut-off to 3mm in order to optimize results.

Also, APB might interfere more with AmpC enzymes compared with other derivatives like phenyl boronic acid (PBA) for class A enzymes like KPC which may explain differences in combined disk tests with the use of two different compounds that suggests that inhibitory activity of APB is lower against KPC enzymes when compared to PBA (Pournaras *et al.*, 2010). This can be solved by simultaneous use of cloxacillin which inhibit AmpC type β-lactamases (Hrabak *et al.*, 2014).

In this study, based on PCR as a reference test, PPV, NPV and accuracy for each of IPM+APB and MEM+APB was 53.3%, 100%, 100%, 92.5% and 94% respectively. Similarly Tsakris *et al.*, (2011) CDT (MEM+APB) and (IPM+APB) showed that PPV 96.7%, 97.3% and NPV 83.2%, 96.9% respectively. Upon detection of KPC, methods with boronic acid compounds showed great sensitivity in detecting KPC positive strains but specificity requires further evaluation. False positive results occur mainly in strains with overproduction of AmpC cephalosporinases and porin modifications. While false negative results were proven to occur in strains which coproduce VIM-type enzymes (Flonta and Almas, 2011).

In conclusion, this phenotypic screening test for identification of KPC enzymes among *P. aeruginosa* isolates, based on the use of meropenem and imipenem disks

supplemented with APB is reliable for detecting *P. aeruginosa* isolates suspected of producing KPC carbapenemases. This test will enable routine laboratories to identify these isolates with high confidence levels.

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