Pseudomonas aeruginosa - Modified Hodge Test (PAE-MHT) and ChromID Carba Agar for Detection of Carbapenemase Producing *Pseudomonas Aeruginosa* Recovered from Clinical Specimens

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Abstract

AIMS: This study aims to evaluate the ability of ChromID Carba agar, and *Pseudomonas aeruginosa* modified Hodge test (PAE-MHT) for detection of carbapenemase-producing *P. aeruginosa* and to determine the associated carbapenemase gene classes by PCR.

METHODS: One hundred Carbapenem-resistant *P. aeruginosa* (CRPA) isolates were tested for: i) carbapenemases production by ChromID carba agar, Modified Hodge test (MHT) and (PAE-MHT) and ii) detection of some carbapenemase genes by PCR.

RESULTS: All (100%) of the isolates showed growth on ChromID Carba agar with 100% sensitivity. Using MHT, 54% of isolates were positive, 3% were indeterminate, and 43% were negative, demonstrating 58.9% sensitivity and 80% specificity. On performing PAE-MHT, 91% of the strains were positive, 3% were intermediate, and 6% were negative, demonstrating 97.9% sensitivity and 80% specificity. The most prevalent gene was *blaKPC* (81%), followed by *blaVIM* (74%); *blaIMP* was detected in only one isolate, and *blaOXA-48* in 34% of the isolates.

CONCLUSIONS: We conclude that PAE-MHT and ChromID Carba are sensitive, specific, simple and cost-effective screening tests for detection of CRPA isolates compared to the traditional MHT.

Introduction

Carbapenemase-producing P. aeruginosa strains are resistant to almost all β-lactams. Carbapenem-resistant *P. aeruginosa* (CRPA) can be produced as a different consequence mechanism, such as decreased bacterial outer membrane permeability, overexpression of AmpCs or expression of carbapenemases [1].

Carbapenemases production in *P. aeruginosa* belong to the Ambler class A (KPC- and GES-type β-lactamases) and most commonly the Ambler class B (Metallo-β-lactamases) (MBLs) of the VIM, IMP, SPM, GIM, AIM, DIM, FIM, and NDM types [1], also OXA beta-lactamase genes in *Pseudomonas aeruginosa* have been identified [2].

Several inhibitor-based tests have been developed for the detection of carbapenemases in *P. aeruginosa*. However, misdetection of newly emerging isolates with a combination of carbapenemases could occur with these methods [3].

Using a novel indicator strain, *K. pneumoniae* ATCC 700603, the MHT was optimised for more accurate and reliable detection of carbapenemase production in *P. aeruginosa*, and this test was named the *P. aeruginosa* MHT (PAE-MHTs) [3]. Chromogenic media containing a carbapenem (chromID Carba, chromID KPC, chromID ESBL) are convenient tools for the screening and rapid detection of carbapenemase-producing Gram-negative bacilli (CPGNB). Among these media, chromID Carba demonstrated the highest sensitivity and specificity. Different genotypic methods have been applied for the detection of carbapenemases encoding genes in clinical isolates of *P. aeruginosa* [4].

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Thus, we performed ChromID Carba agar chromogenic medium, MHT and PAE-MHT to evaluate their ability for detection of CRPA and compared the results to PC.

Methods

The study included 100 carbapenem-resistant *P. aeruginosa* isolates (imipenem and/or meropenem) detected by disk diffusion method according to CLSI, 2015 [5] among different isolates recovered from different specimens referred to Central Microbiology Laboratory for routine culture and sensitivity. The selected CRPA isolates had been referred from the following departments of Ain Shams University Hospitals; burn unit (55%), ICU (22%), surgery department (16%), and from the internal medicine department (7%). They were recovered from {pus (71%), respiratory (17 %), urine (11%) and only one strain (1%) from blood specimens. All isolates were subjected to:

A) Phenotypic detection of carbapenemases producing P. aeruginosa

1. ChromID carba agar

After, overnight incubation of blood agar plates at 36 ± 1ºC, isolates were suspended in one mL of 0.9% sterile saline solution and the inoculum was adjusted to a density of a 0.5 McFarland standard (10^8 CFU/ mL). Then, ten µL (10^6 CFU/ mL) of this suspension was streaked by 10 µL standard loop onto ChromID Carba agar (bioMérieux, France).

Figure 1: *P. aeruginosa on ChromID Carba. Five strains are colourless, and five show brown pigmentation*

All plates were incubated in ambient air at 36 ± 1°C for 24 hours. Some carbapenemase producing Pseudomonas strains were colourless, and some showed brown pigmentation (Figure 1). Non-carbapenemase producing Pseudomonas strains showed no growth [4].

2. Modified Hodge test (MHT)

Modified Hodge test was performed according to CLSI [6]. An inoculum of the indicator organisms (E. coli ATCC 25922), was adjusted to a 0.5 McFarland turbidity standard then a 1/10 dilution was made by adding 1ml of 0.5 McFarland turbidity tube to 9ml sterile saline and inoculated on the surfaces of Mueller-Hinton agar plates by swabbing. After the plates had been allowed to stand for 10 min at room temperature, a disk of meropenem, 10 µg (Oxoid, UK) was placed in the centre of each plate. Subsequently, four colonies of the test organisms, grown overnight on blood agar plate, were inoculated onto the plate in a straight line from the edge of the disk to the periphery of the plate (without touching the disc) by swabbing. A positive result is indicated by the enhanced growth of the indicator strain towards a meropenem disk, clover leaf-type indentation at the point of intersection of the isolate with the indicator strain. Whereas, no enhanced growth of the indicator strain towards a meropenem disk (no clover leaf-type indentation at the point of intersection of the isolate with the indicator strain is considered negative. Indeterminate results showed inhibition of the growth of the indicator strain produced by the test isolate (Figure 2 & 3).
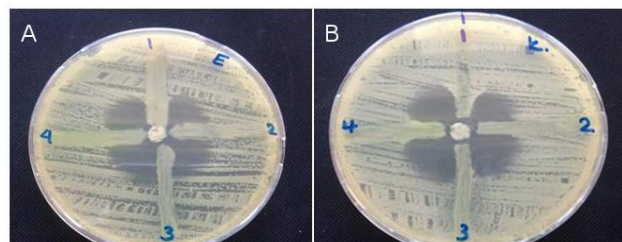
Figure 2: *A) MHT; isolates 1, and 4 show positive results, isolates 2, and 3 show negative results; B) PAE-MHT; isolates 1, 2, 3, 4 show positive results*

3. Pseudomonas aeruginosa modified Hodge test (PAE-MHT)

The same procedure as MHT was performed but *K. pneumonia* ATCC 700603 was used as indicator strain [7]. Interpretation of results was the same as in MHT (Figure 2 & 3).

B) Genotypic detection of carbapenemase encoding genes

All isolates were subjected to molecular identification of carbapenemase encoding genes using conventional PCR for (*blaKPC*) [8] and conventional multiplex PCR for (*blaVIM, blaIMP, and*

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blaOXA-48) [9]. DNA extraction was done using Boiling method [10].
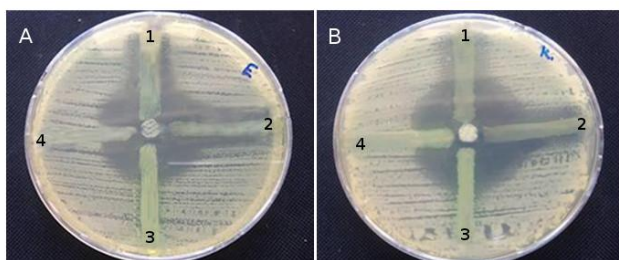

Figure 3: A) MHT; isolates 1, 2 show indeterminate results, isolate 3 shows a negative result, isolate 4 shows positive results; B) PAE-MHT; isolates 1, 2 show indeterminate result, Isolate 3 shows a negative result, isolate 4 shows a positive result

All PCR reactions were performed with My Taq HS Red mix (Bioline, UK) 2 X and carried out on a Thermal Cycler 2720 (Applied Biosystem, USA) instrument. The primers (Table 1) and the components of the master mix were added to templet DNA in a two separate reaction mixture; one for *blaKPC* gene and the other for *blaIMP, blaVIM* and *blaOXA-48* genes in a final volume of 25 µL for each reaction mixture.

Table 1: Primers sequences of carbapenemase encoding genes

Primer name	Primer sequence	PCR product size (bp)	Refrences
KPC (forward) KPC (reverse)	5′-ATGTCACTGTATCGCCGTCT-3′; 5′-TTTTCAGAGCCTTACTGCCC-3′	893-bp	[8]
IMP-A (forward) IMP-B (reverse)	5′-GAA GGY GTT TAT GTT CAT AC-3′ 5′-GTA MGT TTC AAG AGT GATGC-3′ (where Y = C or T and M = A or C)	587-bp	[9]
VIM2004A (forward) VIM2004B (reverse)	5′-GTT TGG TCG CAT ATC GCA AC-3′ 5′-AAT GCG CAG CAC CAG GATAG-3′	382-bp	[9])
Oxa 48A (forward) Oxa 48B (reverse)	5'-TTGGTGGCATCGATTATCGG-3′ 5′-GAGCACTTCTTTTGTGATGGC-3'	744-bp	[31]

Stock solution of primers = 100 pmol/1 µL; Working solution of primers = 4 pmol/1 µL.
Stock solution of primers = 100 pmol/1 µL; Working solution of primers = 4 pmol/1 µL.

The amplification program for *blaKPC* was composed of initial denaturation at 95°C for 15 min, 38 cycles of denaturation for one minute at 94°C, annealing at 62°C for one minute, and an extension step at 72°C for one minute, then final extension for ten minutes at 72°C. As for *blaIMP, blaVIM* and *blaOXA-48*, it was composed of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturation at 94°C for one minute, primer annealing at 54°C for one minute, and primer extension at 72°C for one and half minute.

For detection of the amplified Products; ten µl were examined by 1.2% agarose gel in Tris-boric acid EDTA buffer (TBE). A DNA size marker was included for comparison. Electrophoresis was performed at 80 volts, and the gel was then stained by ethidium bromide (0.5 µg/ml) for 30 minutes. Then, it is visualised using a UV transilluminator and photographed. *BlaKPC, blaIMP, blaVIM,* and *blaOXA-*

48 genes gave bands at 893, 587, 382, and 744 bp. Respectively (Figure 4).
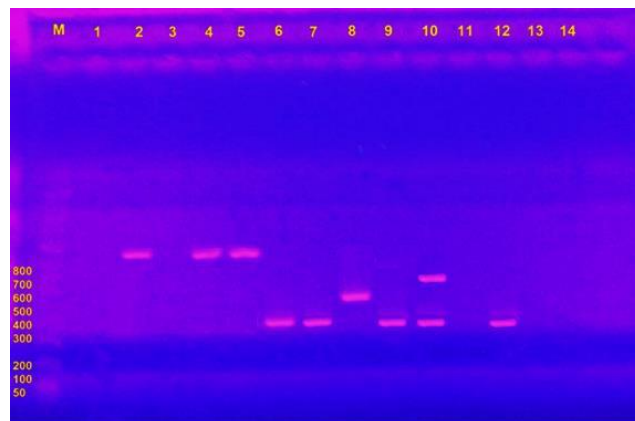

Figure 4: Gel electrophoresis of PCR products; Lane M is DNA ladder; Lanes 2, 4 & 5 are positive for KPC (893 bp); Lanes 6, 7, 9 & 12 are positive for VIM (382 bp); Lane 8 is positive for IMP (587 bp); Lane 10 is positive for for both oxa 48 (744 bp) with VIM (382bp); Lanes 1, 3, 11, 13 & 14 are negative

Results

All (100%) of the CRPA isolates showed growth on ChromID Carba agar after overnight incubation at 36 ± 1°C indicating carbapenemase production. Detection of carbapenemase by MHT showed that; (54%) of isolates were positive, three (3%) produced indeterminate results and (43%) were negative. On performing PAE-MHT, (91%) of the strains were positive, three (3%) produced indeterminate results and (6%) were negative. False positive results were 20%, and false negative results were 2.1%.

Results of PCR for detection of the four carbapenemases encoding genes (*blaKPC, blaVIM, blaIMP, blaOXA-48*) on 100 CRPA isolates showed that; *blaKPC, blaVIM, blaIMP* and *blaOXA-48* were detected in 81%, 74%, 1%, and 34% of the isolates respectively. The four carbapenemase genes were positive in (95%) of CRPA isolates. Five strains were negative for all the four carbapenemase genes included in the study.

PCR results showed that 19% isolates were positive for only one gene; nine (%) isolates for *blaKPC* and ten (10%) isolates for *blaVIM*. Fifty seven (57%) strains were positive for two genes; *blaKPC + blaVIM* 41 (41%) strains, *blaKPC + blaIMP* one (1%) strain, *blaKPC + blaOXA-48* 11 (11%) strains and *blaVIM + blaOXA-48* four (4%) strains. Nineteen strains (19%) were positive for three genes (*blaKPC + blaVIM + blaOXA-48*) collectively. No strains were positive for the four genes collectively (Table 2).

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Table 2: The distribution of carbapenemase encoding genes among 95 PCR-positive isolates (Per isolate)

Carbapenemase genes	n	(%)
One gene only detected:		
KPC only	9	(9.5)
VIM only	10	(10.5)
Total	**19**	**(20)**
Two genes detected:		
KPC + VIM	41	(43.2)
KPC + IMP	1	(1)
KPC + OXA-48	11	(11.6)
VIM + OXA-48	4	(4.2)
Total	**57**	**(60)**
Three genes detected:		
KPC + VIM + OXA-48	19 (20)	
Total	19 (20	

In our study, the performances of MHT, PAE-MHT and ChromID Carba were compared to PCR, which is recommended as a standard gold method for detection of carbapenemase production [11]. MHT gave 58.9% sensitivity, 80% specificity, 20% false positive rate and 41.1% false negative rate. On the other hand, PAE-MHT showed higher sensitivity (97.8%), specificity was 80%.

When results of ChromID Carba were compared to PCR results, 95 strains, that were PCR positive, showed growth on the ChromID Carba. The remaining five isolates that showed growth on ChromID Carba were negative by PCR. Thus, the sensitivity of ChromID Carba was 100% (compared to PCR), but the specificity could not be calculated as the test didn't show any negative results.

Regarding the five isolates that were PCR negative but showed growth on the ChromID Carba, three of them gave positive results by both MHT and PAE-MHT while the remaining two were negative.

In the present study, 3% of the CRPA isolates gave indeterminate results by both MHT and PAE-MHT; 2% were only MBL producers (*blaVIM*), and 1% was both KPC and MBL (*blaVIM*) producer.

Discussion

Carbapenemase-producing *P. aeruginosa* strains are resistant to almost all β-Lactams. So, their spread in hospital settings is extremely dangerous. Also, carbapenemase genes are usually located on transferable genetic determinants such as plasmids, leading to their rapid dissemination. Hence, detection of carbapenemases is important for early implementation of infection control measures [1].

On performing MHT, 54% of our strains were positive, 3% of showed indeterminate result and 43% were negative. In a previous study, among the 32 meropenem resistant *P. aeruginosa*, only 9 (28.1%) were positive for carbapenemases by MHT. However, 16 (50.0%) were MBL producers by EDTA disk synergy test [12].

In our study, the sensitivity of MHT was 58.95%, and specificity was 80%. False positive results were detected in 20% of the strains, and 41.1% were a false negative. The false positive results might be due to the co-production of CTX-M and hyperproduction of AmpC which may result in the minor hydrolysis of carbapenems [13].

The false negative results might be related to the presence of NDM type of MBLs [11]. This was consistent with our results, where 4/10 (44%) strains carrying only MBL genes, gave negative result by MHT. Also, MHT does not distinguish between carbapenemase types and lack sensitivity for MBL detection unless it is performed on MacConkey's agar or Zn^{+2} added to the carbapenem disk [14]. Moreover, it noted that the sensitivity and specificity of the test for detecting low-level Metallo-β-lactamase production are not known, and no data exists on the usefulness of this test for the detection of carbapenemase production in non-fermenting gram-negative bacilli like Pseudomonas [5]. On the other hand, our study revealed that six out of nine strains carrying *blaKPC* (66%) were negative for MHT and all the eleven strains (100%) carrying both *blaKPC* and blaOXA-48 were also negative for MHT.

A study carried on 64 *P. aeruginosa* isolates (42 carbapenemase producers and 22 carbapenemase non-producers) revealed that the MHT showed 78% sensitivity and 57% specificity. When the test was repeated on 15 strains, ten carbapenemase producers and five carbapenemase non-producers, nine strains out of 15 showed variable results over the test period (repeatability; 40%) [7].

In our study, 3% of the CRPA isolates produced indeterminate results by MHT; 2% were MBL producers (*blaVIM*), and 1% was both KPC and MBL (*blaVIM*) producer. This could be justified by a possible secretion of a substance such as colicin, a bacteriocin-peptide released by some GNB, which may inhibit the growth of the indicator strain and interfere with the results of the test [15].

Indeterminate results by MHT in 12% of MBL *P. aeruginosa* producers, which were not obtained for KPC producers, were observed in a previous study. Similarly, they reported a high proportion of indeterminate results (22% and 43% of carbapenemase and non- carbapenemase producers respectively) which occurred at a rate ten times higher in *P. aeruginosa* than that for Enterobacteriaceae. They optimised the MHT for more accurate and reliable detection of carbapenemase production in *P. aeruginosa* by using a novel indicator strain, *K. pneumoniae* ATCC 700603, and named this test the *P. aeruginosa* MHT (PAE-MHT). First, the test was performed using 64 *P. aeruginosa* isolates (42 carbapenemase producers and 22 carbapenemase non-producers), and it demonstrated 100% sensitivity and 98% specificity for detection of carbapenemase activity without indeterminate results [7].

In the present study, 91% of the strains of CRPA isolates were positive by PAE-MHT, 3%

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demonstrated indeterminate results and 6% were negative. The sensitivity of PAE-MHT was (97.81%), specificity was (80%), false positive results were 20%, and false negative results were 2.1%.

The false positive results in our study might be explained as previously mentioned in MHT by the overproduction of cephalosporinases as AmpC enzymes or some ESBL enzymes as (CTM-X) that may have minor hydrolytic activity on carbapenemases [7], [13].

In the current study, two strains showed false negative results with PAE-MHT; one was carrying *blaKPC* only while the other was carrying both *blaKPC* and *blaOXA-48*. This may be explained by the possibility of failure of the phenotypic expression of these genes [16].

On performing PAE-MHT, 3% of our CRPA isolates showed indeterminate results which may be due to the production of substances by the tested isolates that inhibit the growth of the indicator strain as reported for MHT [17]. This was discordant with Pasteran et al., who reported no indeterminate results [7].

All of the (100) CRPA isolates, in our work, showed growth on ChromID Carba and 95% of isolates that were proved to carry carbapenemase genes by PCR showed growth on ChromID Carba, so the estimated sensitivity of the ChromID Carba was 100%. However, the test showed false-positive results in five isolates that were negative by PCR.

False positive results with ChromID Carba have different explanations. Simner et al. reported that growth on a chromogenic medium only signifies carbapenem resistance (e.g. AmpC producer with porin loss may grow) but does not confirm the production of a carbapenemase. In their study, ChromID Carba agar demonstrated 89.8% sensitivity in detecting carbapenemase producing GNB but still could grow carbapenemase negative GNB (included 16.7 % of ESBL producers and 6.7% of AmpC producers) [4].

The second explanation could be the presence of other carbapenemase genes not included in our study as out of our five PCR negative isolates (positive by ChromID Carba), three strains were positive in both MHT and PAE-MHT, but the remaining two strains were negative by both tests.

One limitation of the ChromID Carba agar which is mentioned by the manufacturer and several studies [18], [19], [20], is the limited detection of OXA-48 producers, because of the low hydrolysing activity of this enzyme on carbapenem. However, in our study, this was not observed because all OXA-48 producing strains showed growth on Chrom ID Carba. This could be attributed to the finding that all OXA-48 isolates in our study were co-producer of other carbapenemases (KPC or VIM).

Diene and Rolain reported that the VIM carbapenemases enzymes, composed of around 33 variants, have been widely described in *P.aeruginosa* and lesser found in enterobacterial species [21].

Carrër et al. reported that most carbapenemases in clinical isolates of GNB including *P. aeruginosa* are IMP/VIM and KPC [22].

Among our 100 CRPA isolates, carbapenemase genes (*blaKPC, blaVIM, blaIMP, blaOXA-48*) were detected in 95% of the isolates. Only five isolates produced negative results for all of these four carbapenemases. PCR results revealed that *blaKPC* was the most prevalent gene as it was detected in 81% of the isolates, followed by *blaVIM* which was detected in 74% of the isolates, blaOXA-48 in 34% of the isolates, while *blaIMP* was found in one per cent of the isolates. Fifty seven (57%) of the strains was positive for two genes*; (41%: blaKPC + blaVIM), (1%: blaKPC + blaIMP), (11%: blaKPC + blaOXA-48) and (4%: blaVIM + bla OXA-48).* Nineteen strains (19%) were positive for three genes *(blaKPC + blaVIM + blaOXA-48).*

The five isolates with negative PCR results may carry other carbapenemase genes not included in our study (as mentioned previously) or may be due to the presence of another mechanism of carbapenem resistance, e.g. efflux pump and porin loss [23].

Comparable to our results Pasteran et al., reported that in 74 carbapenemase-producing *P. aeruginosa* isolates, 36 (48.6%) isolates carried *blaKPC*, 21 (28.37%) *blaVIM*, 9%) *blaIMP* and 8 (10.8%) carried *blaSPM* [3]. Huang et al. found that out of 135 Enterobacteriaceae isolates, 72 isolates (53%) were carbapenemase producers, and out of 221 *P. aeruginosa* isolates, 55 (25%) isolates were carbapenemase producers confirmed by PCR. *BlaOXA-48* carbapenemase was the predominant carbapenemase gene (82%) found in Enterobacteriaceae, while *blaVIM* gene was largely predominated (93%) in *P. aeruginosa* [20].

In contrast to our results, some studies reported that in Pseudomonas species, carbapenemases are mostly MBLs of VIM, IMP, SPM, GIM, AIM, DIM and NDM types and to lesser extent Ambler class A carbapenemases of the KPC and GES types[24], [25], [26].

Koutsogiannou et al. reported that all of MBL positive CRPA isolates (49 isolates) were carrying the *blaVIM* gene and were spread in all hospital wards, especially among the non-ICU patients [27].

Simner et al. found that out of 49 carbapenemases producing GNB (Enterobacteriaceae, *P. aeruginosa* and Acinetobacter) 16 isolates carried the *blaKPC* gene (one *P. aeruginosa*), 12 carried the *blaVIM* gene (five *P. aeruginosa*), and four isolates carried *blaIMP* (one

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P. aeruginosa). One of these GNB isolates (E. cloacae) carried both _blaVIM_ and _blaOXA-48_ [4].

Previous studies revealed association of MBLs (VIM/IMP) with KPC (_blaKPC + blaVIM and blaKPC + blaIMP_) in certain CRPA clones (ST564) in different areas of the world, indicating that this clone is a successful, worldwide multidrug-resistant clone with the ability to acquire relevant carbapenemases [24], [28].

Peshattiwar and Peerapur reported that the percentage of MBL (detected by a phenotypic method; IMP-EDTA DDST) in the imipenem resistant _P. aeruginosa_ isolates was 62.5%. They concluded that carbapenem resistance in _P. aeruginosa_ is predominantly via MBL production [29]. A nearly comparable rate was noted by Noyal et al., that attributed 50% of imipenem resistant _P. aeruginosa_ isolates to the production of MBL [12]. Upadhyay et al. reported a high prevalence of MBL 56 (46.6%) among 120 AmpC producing isolates [30].

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