Egyptian Journal of Medical Microbiology Volume 28 / No.4 / October 2019 95-102 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Phenotypic methods for detection of Beta Lactam *resistance* in *Klebsiella pneumoniae*

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Key words:
phenotypic methods, Klebsiella pneumoniae, Beta lactam resistance

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ABSTRACT

Background*:Drug-resistant isolates remain an important hospital-acquired bacterial pathogen, add significantly to hospital stays and especially problematic in high-impact medical areas such as intensive care units. Klebsiella pneumoniae is the most common cause of nosocomial respiratory tract, and the second-most frequent cause of Gram-negative bacteraemia and urinary tract infections. It is essential to detect the beta lactam resistance in K. pneumoniae for proper antibiotic therapy and to limit the spread the infection.* ***Objectives:*** *The study was designed to review the rates of extended-spectrum β-lactamases (ESBL), metallo beta lactamases (MBL) and Ampicillin resistance gene group C (AmpC) beta lactamases production among K. pneumoniae isolates and to assess the best phenotypic method for detection of the resistance.* ***Methodology:*** *This study included 200 isolates obtained from patients admitted to different Departments in Assiut University Hospital. Screening and phenotypic confirmatory tests for resistance were done.* ***Results:*** *The Percentages of B –Lactamase enzymes producers from 50 isolates of K. pneumoniae were11 (22%) isolates not resistant; 6 (12%) by screening with antibiotic sensitivity tests and 5(10%) by the gold standard phenotypic tests. The remaining 39of 50 isolates (78%) were resistant and distributed as follow; Ten strains (20%) were ESBL alone, 2 (4%) were ESBL and AmpC, 4(8%) were ESBL and Carbapenemases, 13(26%) were AmpC producers alone and 10 (20%) were Carbapenemases producers alone. The combined disk test showed high sensitivity and specificity for detection of ESBL and MBL, For AmpC detection; the disk approximation test showed higher sensitivity and specificity than boronic acid.* ***Conclusion:*** *The rate of Beta lactamases production by K.pneumoniae is seriously increased, the most common type of beta lactamases in K.pneumoniae was the ESBL32% then AmpC30 %and lastly the Metallo beta lactamase (MBL) 28%. The phenotypic confirmatory tests showed high sensitivity and specificity and proved to be reliable methods for detection ofthe beta lactamase resistance, genotypic tests are recommended to be a gold standard tests to increase the specificity of the phenotypic tests.*

INTRODUCTION

Antibiotic resistance has become a serious global problem and affects almost every bacterial species. The nosocomial infections by multidrug resistant *K.pneumoniae* are emerging worldwide.[1]The incidence of ESBL, AmpC and MBL producing strains among *K.pneumoniae* isolates has been increasing alarmingly leading to limited therapeutic alternatives. These enzymes are either plasmid or chromosomally mediated and can be easily transferred to other bacteria lacking them. [2]

Extended spectrum beta lactamases (ESBL) are protective enzymes produced by Gram negative bacteria which make them resistant to third generation cephalosporin e.g. ceftazidime and cefotaxime. They also make the organism resistant to penicillins as well as other classes of antibiotics thus making treatment options difficult. ESBL do not destroy cephamycin and their effect is blocked by clavulanic acid .[3]

Ampicillin resistance gene group C (AmpC) enzymes are again produced by gram negative bacteria but inhibition by clavulanic acid is poor in them. They make the organism resistant to cephalosporin, cefoxitin and monobactam like aztreonam. They are inhibited by cloxacillin and phenylboronic acid. This different action on cephamycins and b-lactamase inhibitors differentiates AmpC enzymes positive organisms from the ESBL producers Chromosomal or plasmid mediated resistance.[4] Carbapenems were the only hope for treatment of infections caused by these resistant bacteria. However, this last barrier was also broken by carbapenemases (MBL) enzymes that started emerging worldwide and were able to destroy carbapenems. The metallo β-lactamase in Gram negative bacteria are a challenge to the clinicians as they destroy most of the known β-lactam antibiotics including carbepenems.[5]

The phenotypic confirmatory tests are highly sensitive and specific and more suitable to use as routine tests in clinical laboratories.[6] This study was designed to detect the distribution of different beta lactamases among the *K.pneumoniae* isolates and to compare between different phenotypic methods for detection of B-lactamases.

METHODOLOGY

This prospective study was done in the Microbiology Unit of Clinical Pathology Department Assiut University Hospital. It included 200 isolates obtained from various clinical specimens (blood, urine, sputum and pus) in years from June 2016 to May 2017.The study was approved by the ethical committee of Faculty of Medicine, Assiut University.

All isolates were identified by the standard microbiological tests. The antimicrobial susceptibility tests were done by the Kirby Bauer disc diffusion method according to the CLSI guidelines and by Vitek2 Compact 15 system.[7]

Detection of ESBLs:

The isolates that showed resistance to third generation cephalosporin were suspected to be ESBL producers and were confirmed by phenotypic tests ; (chromID™ ESBL agar, ESBL test of vitek2, combined disk test and E-Test). We used the ESBL E-Test as a gold standard test .[8]

***- ChromID™ ESBL agar (BioMérieux)*:**

It is a selective chromogenic medium used according tomanufacture instructions for the detection of Extended Spectrum ß-Lactamase producing enterobacteriacae, *K.pneumoniae* appear as green, brownish green or blue colonies.

***- Combined disk test (Oxoid)*:**

The test evaluates the synergy between an oxyimino cephalosporin and clavulanic acid. A disc of ceftazidime (30 µg) alone and ceftazidime + clavulanic acid (30 µg/10 µg) were used.[7]

- ESBL test of vitek2 compact 15(BioMérieux):

It is a new tool for rapid detection of ESBL production which is based on simultaneous assessment of the inhibitory effects of cefepime, cefotaxime, and ceftazidime, alone and in the presence of clavulanicacid.[8]

- E-Test (BioMérieux):

Cefotaxime/cefotaxime + clavulanic acid(CT/CTL) and Ceftazidime/ceftazidime + clavulanic acid (TZ/TZL) were used according to manufacture instructions to detect the clavulanic acid inhibitable ESBL.

Detection of carbapenemases:

The Isolates that showed resistance to carbapenems were suspected to be carbapenemase producers and were confirmed by phenotypic tests;(ChromID® CARBA SMART agar, Modified Hodge Test and Rapidec Carba NP Test).

Sensitivity and specificity couldn't be calculated for these tests due toinability to perform PCR which is the gold standard test.[9] These isolates were also tested for metallo beta lactamases production by combined disk test and E-test.The Etest was taken as a gold standard test .[10]

- ChromID® CARBA SMART Agar (BioMérieux):

It is a selective chromogenic medium used according to manufacture instructions for the detection of carbapenemase producing enterobacteria, *K.pneumoniae* appear as green, brownish-green or blue colonies.

- Modified Hodge Test (MHT):

Carbapenemase production by the tested microorganism is able to inactivate the carbapenem that diffuses from the disk after the disk has been placed on the Mueller Hinton Agar. This allows carbapenem susceptible *E. coli* ATCC® 25922™ to grow toward the disk making a clover leaf-like indentation [11].(Figure.1).

- Quality control:

(1) *K. pneumoniae* ATCC BAA 1705, positive control.
(2) *K. pneumoniae* ATCC BAA 1706, negative control.

Fig. 1: Modified Hodge Test:, (1) positive result (2) negative result

- Rapidec Carba NP Test (BioMérieux):

It is a ready to use strip for the rapid detection of carbapenemase production. The test is used according to manufacturer instructions and based on the detection of carbapenem hydrolysis by carbapenemase as hydrolysis acidifies the medium which changes the color of the PH indicator (Figure 2).

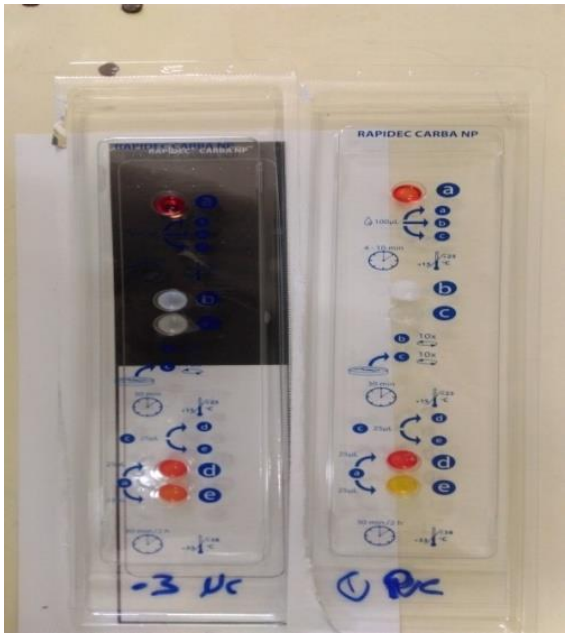

Fig. 2: Rapedic carbanp: a- Negative result, b- Positive result

Detection of Metallo β-lactamases:

-Combined disk test (Oxoid):

The test evaluates the synergy between carbapenem and EDTA, Two disks - 10 µgmeropenem and meropenem/ EDTA (10µg + 750µg) were used.[12]

- E-test (IP/IPI) imipenem and imipenem-EDTA (BioMérieux):

Strips were used according to manufacturer instructions to confirm the presence of EDTA inhibitable MBL (Metallo ß-Lactamase) enzymes.

Detection of AmpCß-Lactamases:

The Isolates that showed resistance tocefoxitin were suspected as AmpC producers and were subjected to phenotypic confirmatory tests;(Boronic acid test method, disk approximation test, and three-dimensional test).Three-dimensional test was taken as a gold standardtest[13].

Three-dimensional test:

AmpC production is able to inactivate the cefoxitin that diffuses from the disk after the disk has been placed on the Mueller Hinton Agar,This allows cefoxitin susceptible *E. coli* ATCC® 25922™ to grow toward the disk making a clover leaf-like indentation[14].(Figure. 3).

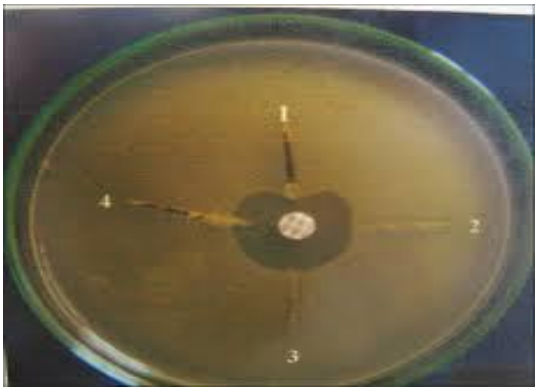

Fig. 3: Three-dimensional test

***Disk approximation test*:**

Thirty µg ceftazidime disk was placed at center of Mueller Hinton Agar plate inoculated with the tested bacteria, then 30 µgcefoxitin, 10 µgimipenem and 20/10 µg amoxicillin-clavulanate disks were placed 20 mm away from ceftazidime disk. The blunting or flattening of the inhibition zone between the ceftazidime disk and the inducing substrates (cefoxitin, imipenemaamoxicillin-clavulanate disk) was considered as a positive result[14]. (Figure 4) .

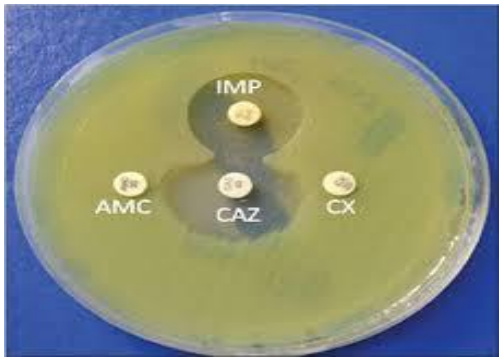

Fig .4: Disk approximation test

-Boronic acid disk test:

The test evaluates the synergy between cefoxitin and phenylboronic acid, Two 30 µgcefoxitin disks were used, 20 µl of 15 µg/ml phenylboronic acid was dispensed onto one disk. [14]. (Figure. 5).

Fig. 5: Boronic acid disk test

RESULTS

From the 200 isolates included in the study there were 50 *Klebsiella pneumoniae* isolates were submitted to screening and phenotypic confirmatory tests for detection of various beta lactamases.

Results of phenotypic screening Tests:

The antibiotic resistance pattern by vitek2 and disk diffusion method was almost the same; there were mild variation in resistance toAmpicillin, Meropenem and Ciprofloxacin. (Table 1) .

Table 1: Antibiotic resistance pattern of *Klebsiella pneumonie* by disk diffusion method and vitek 2:

Antibiotic	Resistance (%) by Disk diffusion method	Resistance (%) by Vitek 2
Ampicillin	47 (94%)	49(98%)
Ampicillin/sulbactam	-	42(84%)
Piperacillin/tazobactam	-	44(88%)
Cefazolin	46(92%)	47(94%)
Cefoxitin	26 (56%)	30(60%)
Ceftazidime	45(89%)	46(91%)
Ceftriaxone	44 (88%)	44(88%)
Cefepime	44 (88%)	44(88%)
Meropenem	37(74%)	37(74%)
Amikacin	13(25%)	13(25%)
Gentamicin	-	22(44 %)
Tobramycin	-	40 (79%)
Ciprofloxacin	37(74%)	37(74 %)
Levofloxacin	-	32(64%)
Trimethoprim /sulfamethoxazole	-	40 (80%)
Azetronam	42(84%)	-

Results of phenotypic confirmatory tests:

ESBL phenotypic confirmatory tests:

Among the phenotypic confirmatory tests the combined disk test showed the highest sensitivity and specificity followed by ESBL test of vitek2 and lastly the chromogenic media which showed the lowest specificity. (Table 2).

Table 2: Sensitivity, specificity, positive predictive value and negative predictive value of phenotypic confirmatory tests for ESBL detection:

Confirmatory test	Sensitivity	Specificity	PPV	NPV
ESBL test of Vitek2	89%	81%	80%	89%
Comined disk test	90%	84%	86%	89%

Carbapenemases phenotypic confirmatory tests:

Among the phenotypic confirmatory tests the chromID® CARBA SMART agar detected the highest percentage of carbapenemase producer among the phenotypic confirmatory tests, then Carba NB and lastly MHT (Table 3).

The combined disk test showed high sensitivity (94%) and high specificity (100%) as a phenotypic confirmatory test for the detection of the metallo beta lactamases. (Table 4).

Table 3: Percentage of carbapenemase detection by phenotypic confirmatory tests:

Confirmatory test	chromID® CARBA SMART	RapidecCarba NP Test	MHT
Percentage of carbapenemase detection	76%	64%	62%

Table 4: Sensitivity, specificity, positive predictive value and negative predictive value of combined disk test for metallo beta lactamase detection:

Confirmatory test	Sensitivity	Specificity	PPV	NPV
Combined disk test	94%	89%	88%	94%

Results of phenotypic confirmatory tests for AmpC:

Among the phenotypic methods we noted that the disk approximation test showed higher sensitivity and specificity for detection of AmpC than Boronic acid disk test (Table 5).

Table 5 :Sensitivity, specificity, positive predictive value and negative predictive value of phenotypic confirmatory tests for AmpC detection:

Confirmatory test	Sensitivity	Specificity	PPV	NPV
Boronic acid disk	58%	78%	70%	68%
Disk approximation Test	84 %	92%	91%	86%

Distribution of different beta lactamases among Klebsiella pneumonie isolates:

The Percentages of B –Lactamase enzymes from 50 isolates of *K .pneumonie.* were 6 (12%) isolates not resistant by screening with antibiotic sensitivity tests, 5 (10%) isolates were negative by the gold standard phenotypic tests, 10 (20%) were ESBLalone, 2 (4%) were ESBL and AmpC, 13 (26%) were AmpC alone,4 (8%) were ESBL and Carbapenemases and 10 (20%) were carbapenemases alone.(Table 6andFig.6).

Table 6: Distribution of different beta lactamases among the 50 *K. pneumonie* isolates:

Type of enzyme	Positive (n=50)	Positive (%=100%)
ESBL	10	20%
ESBL+AmpC	2	4%
AmpC	13	26%
ESBL+ CARBA	4	8%
CARBA	10	20%
No resistance by screening tests	6	12%
No resistance by Standard tests	5	10%

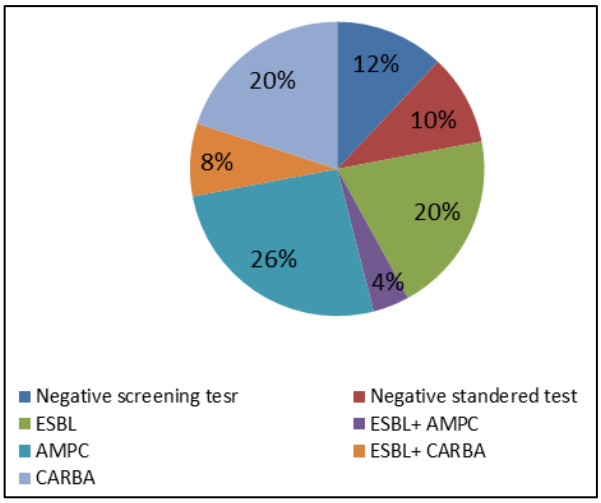

Fig .6: Distribution of different beta lactamases among the 50 *Klebsiella pneumoniae* isolates

DISCUSSION

Multidrug resistant *Klebsiella pneumoniae* are emerging worldwide, challenging the clinicians, public health professionals, and hospital infection-control teams. There is a lower level of awareness of ESBLs and other enzymes like AmpC and MBL produced by *Klebsiella pneumoniae* among the clinicians as well as laboratory technicians or pathologists conducting the tests in the laboratories. Confusion persists regarding the test to be put up for their detection as well as regarding the available treatment options against them .[1]

In the present study anumber of tests have been done for the detection of beta lactamases. These tests are further divided into screening and confirmatory tests.

Screening methods done by Vitek 2 compact system, disk diffusion method and they almost showed the same result;but there were mild variation in resistance to: Ampicillin, Meropenem and Ciprofloxacin. The VITEK 2 compact required less technical time per test, and provided earlier results than disk diffusion method.

This agrees with Jorgensen etal study which proved that vitek2 anddisk method produced very similar overall susceptibility category agreements.[15]

Rechenchoski et al., is another study that compared the Vitek 2® automated system and disc diffusion method, with using the *broth micro dilutionas* gold standard and reported that the Vitek 2® automated system was more sensitive than Disc diffusion method.[16]

As regard the comparison between different ESBL phenotypic confirmatory, we found that the combined disk test (CDT) was the most sensitive and specific phenotypic confirmatory test as its sensitivity and specificity were 90% and 84% respectively , then of ESBL test of Vitek2were 89% and 81% respectively, and lastely the chromID™ ESBL agar showed89% sensitivity and 60% specificity

This agree with Singh study which reported that the combined disk test showed 93.44% sensitivity and 100% specificity which were higher than ESBL test of Vitek2 that showed91,8% sensitivity and 97,2% specificity. [17]

Thomson et al., study reported that the sensitivity of ESBL test of Vitek2 was 91% and the specificity was 89% .[18], and also agree with De Gheldre et al., study in which the sensitivity of Combined disk test (CDT) was 89% and the specificity was 88%.[19]

Kumar and Kalyana is another study reported a high sensitivity (95%) and low specificity (44%) for chromID™ ESBL agar.[20]

The chromID ESBL agar detected ESBL-producing *K. pneumoniae* isolates with high sensitivity but showed the lowest specificities. The main advantage of the chromID ESBL agar is its sensitivity, which enables the recovery and identification of most ESBL-producing

organisms within 24 h. A previous study by Glupczynski et al. reported a sensitivity of 97.7% and a specificity of 89.0% for the chromogenic agar. In our evaluation, the chromogenic agar showed a comparable high sensitivity of 91% but a specificity of only 43%.[21]

Färber et al,. is another study agreed with the current study as it reported a showed high sensitivity (94%) and low specificity (42%) for chromID™ ESBL agar.[22]

In the current work; we found that among the phenotypic confirmatory tests for carbapenemase detection The chromID® CARBA SMART agar detected the highest percentage of carbapenemase as it detected 26 isolates of the total 34 isolates (76%),the Modified Hodge Test (MHT) detected 22 isolates of all 34 isolates (64%)and the Rapidec Carba NP test detect 21 isolates of all 34 isolates (62%).

We reported that the chromogenic media was a reliable method for detection o f carbapenemase and this agrees with Vrioni et al., study which reported that chromID CARBA was found to be an easily performed and very accurate method for CPE detection [23.] and agrees with Olivgeris et al studies which approved that chromID® CARBA SMART agar is a reliable and accurate method for detection of carbapenemase.[24]

The major drawbacks we met at usage of chromogenic media were; the short half life of the media and its high cost, which may be the cause of limitation of its usage as a routine method for screening of resistance.

By using Rapidec Carba NP test and MHT for detection of carbapenemase, we reported that Rapidec Carba NP test was less than MHT as it detected mildly lower percentage of carbapenemase but it was time saving and accurate in its results and this agrees with Lifshitz et al study which reported that the RapidecCarba NP was easily performed and accurate and had a faster turn around time than MHT [25] .

In the present study we found that the combined disk test is a reliable test for detection of metallo beta lactamases as it showed 94%sensitivity and 89% specificity, and this agrees with Omair et al study in which the sensitivity was 97% and the specificity was100% 26 ,In another., study sensitivity of the combined disk test was 94.8% and the specificity was 100%.[27] while Maurer et al. reported that the sensitivity and specificity of the test were 100% . [28]

For AmpC resistance we reported that the detection of AmpC mediated resistance is problematic as there are no guidelines of Clinical and Laboratory Standards Institute (CLSI) for phenotypic techniques to investigate AmpC-producing organisms.

In our work we showed that the disk approximation test (DAT) detected Amp C beta-lactamase carrying bacteria more reliably than Boronic acid testas sensitivity and specificity of DAT came out to be 84% and 92% respectively and those of Boronic acid test were 58% and 78**%** respectively.

As regard DAT; the present study agrees with Saad et al. study in which sensitivity and specificity of DAT were 88% and 92%.[13]

For Boronic acid test; the present study agrees with Helmy and Wasfi study; an Egyptian study which reported that sensitivity and specificity of Boronic acid test were 65% and 73 respectively .[14]

Josephand Mathias is another study in which sensitivity and specificity of Boronic acid test were72% and 45**%**[29], in which the phenylboronic acid test low specificity was explained by that the boronic acid can inhibit class A carbapenemase (KPC) β-lactamase besides AmpC. [4]**.**

In the preset work we noted that not all the cefoxitin resistant isolates are AmpC β-lactamase producers. This can be explained by the cefoxitin resistance is not only due to AmpC β-lactamase production, but also could be due to some other enzymatic mechanism as extended spectrum beta lactamases (ESBLs) and metallo beta lactamase (MBL) . [30]

As regard distribution of different beta- lactamases among *Klebsiella pneumoniae***:**The ESBL rate was the highest among *K. pneumoniae* isolates and this agreed with other Egyptian studies conducted at Assiut University Hospital .[31] and at Benha university hospital .[8]and at Alexandria university hospital. [32]. On the other hand this disagreed with other Egyption studies conducted at Suez canal university.[33] and at Mansoura university[34].

CONCLUSION

The difference in the percentage rates of beta lactamase production might be attributed to different antibiotic policies which may aid in selection of certain antibiotic resistant pathogens than another, and/or strict implementation of infection control measures.

The limitation of this study was the small sample size and that PCR could not be used as the gold standard due to its unavailability and it high cost.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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