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ORIGINAL ARTICLE

Phenotypic methods for detection of various β-Lactamases in Gram negative bacilli

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ABSTRACT

Background*: Infections caused by gram negative bacilli producing β-lactamase have serious implications for both public health and infection control practices. These infections are often associated with retardation in the management with effective therapy, as β-lactam resistance often challenges empirical treatment regimens.* ***Objectives:*** *The study aimed to review the rates of ESBL, MBL and AmpC beta lactamases production among Gram negative bacilli and to assess the best phenotypic method that detect the resistance.* ***Methodology:*** *This study included 200 isolates obtained from patients admitted to different departments in Assiut university hospital. Screening and phenotypic tests which are confirmatory for resistance were done.* ***Results:*** *The most common type of beta lactamases in G-ve isolates by confirmatory tests was the ESBL (46%) and most common in Salmonella spp (57%) then AmpC (44%) and mostly among Serratia marcescens (83%), and lastly the metallo beta lactamase (34%) and mostly in Proteus mirabilis and Burkholderia cepacia (40%)for each of them. For detection of ESBL, Vitek2 and the ChromID™ ESBL agar were the most sensitive while CDT was more in specificity. For AmpC, disk approximation test showed more sensitivity and less specificity than Boronic acid. While for carbapenemase, the ChromID® Carba smart agar detect the highest percentage, high sensitivity is detected in the combined disk test for MBL.* ***Conclusion:*** *The phenotypic confirmatory tests were highly sensitive and specific and proved to be reliable methods that detect the beta lactamase resistance, genotypic tests are recommended to be a gold standard tests for increasing the specificity of the phenotypic tests.*

INTRODUCTION

Gram-negative bacilli causing infections are on rise world over. The extensive use of broad-spectrum antibiotics is capable of causing colonization with resistant strains which increase morbidity, mortality. There is resistance to many classes of antibiotics production caused by Multidrug-resistant organisms (MDRO) of various β-lactamases particularly cephalosporins [1]. Extended spectrum β-lactamases (ESBLs) can induce resistance to many types of the newer β-lactam antibiotics, which include cephalosporins like ceftriaxone, cefotaxime, ceftazidime, and monobactams (e.g., aztreonam), but not the cephamycins (e.g cefotetan and cefoxitin) and carbapenems (e.g., imipenem, meropenem, and ertapenem) [2].

The transfer of chromosomal genes for the inducible AmpC β-lactamase onto plasmids was the cause of arise of Plasmid-mediated AmpC β-lactamases which result in appearance of AmpC β-lactamases in isolates of Klebsiella pneumoniae, Escherichia coli, Salmonella spp., Citrobacter freundii, Enterobacter aerogenes, and Proteus mirabilis [3]. the importantance of Metallo β-lactamases (MBLs) arise from their ability to hydrolyze most of drugs which include carbapenems, aminoglycosides and fluoroquinolones and their ability of rapid dissemination because they are plasmid mediated [4].

There are many aproblems in detecting various β-lactamases in many clinical laboratories. Various phenotypic methods should be used to detect various β-lactamases in microbiology laboratory on basis of day-to-day to prevent antimicrobial resistance by evidence-based use of antimicrobials [5]. The study aimed to detect the distribution of different beta lactamases among G-ve isolates and to compare between different phenotypic methods that detect B-lactamases.

METHODOLOGY

This prospective study was done in Microbiology Unit of Clinical Pathology Department at Assiut University Hospital and included 200 isolates obtained from different 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. Standard

microbiological tests have been used to identify all isolates. The antimicrobial susceptibility tests were done by the Kirby Bauer disc diffusion method according to the CLSI guidelines and by Vitek2Compact15system [6].

Detection of ESBLs:

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 [7].

ChromID™ ESBL agar (BioMérieux):

Which is a selective chromogenic medium used according to manufacture instructions for the detection of Extended Spectrum ß-Lactamase producing enterobacteriacea. Blue, brownish-green and Green colonies: *Klebsiella, enterobacter, Serratia* and *Citrobacter* (KESC) group. Light brown to dark brown colouration: *Proteus, Providencia, Morganella, Salmonella and Burkholderia*.

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 [6].

ESBL test of vitek2 compact 15(BioMérieux):

It is a new tool to detect ESBL production rapidly which is based on simultaneous assessment of the inhibitory effects of cefepime, cefotaxime, and ceftazidime, alone and combined with clavulanic acid [7].

E-Test (BioMérieux):

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

Detection of carbapenemases:

Isolates that showed resistance to carbapenems were suspected to be carbapenemase producers and confirmed by phenotypic tests;(ChromID® CARBA SMART agar, Modified Hodge Test and Rapidec Carba NP Test).Sensitivity, specificity couldn't be calculated for these tests due to the inability to perform PCR which is the gold standard test[8]. Those isolates were also tested for metallo beta lactamases production by combined disk test and E-test. The E-test was the gold standard test [9].

ChromID® CARBA SMART agar (BioMérieux):

Which is a selective chromogenic medium used according to manufacture instructions to detect carbapenemase producing enterobacteria:

- Bluish-green to bluish-grey or purple colonies: **KESC group** *(Klebsiella, Enterobacter, Serratia, Citrobacter).* light brown to colonies *(proteus, salmonella, burkholderia).*

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 [10]. 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: (2) negative result, (1,3) positive result

-RapidecCarba NP Test(BioMérieux): It is a ready to use strip to detect carbapenemase production rapidly. The test was used according to manufacture instructions and based on detection of hydrolysis of carbapenem by carbapenemase as hydrolysis acidifies the medium which changes the PH indicator color. **Figure (2)**

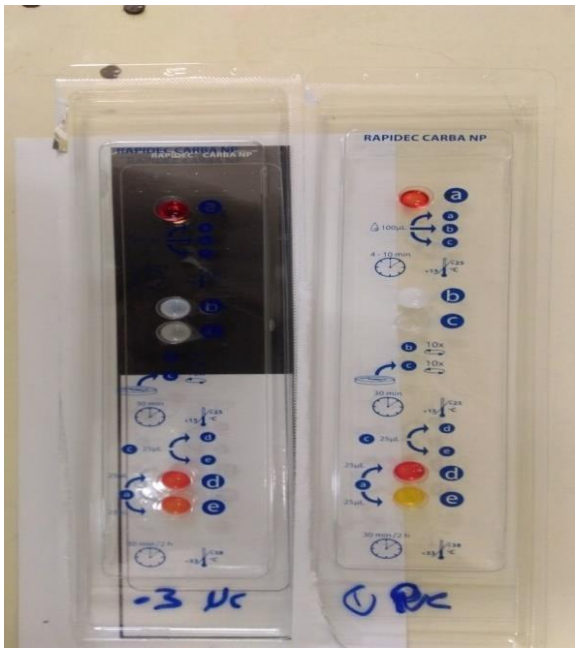

Fig. 2: RAPIDEC CARBA NP: a- Negative result, b- Positive result

- Detection of Metallo β-lactamases were done by *Combined disk test (Oxoid):*

The test evaluate the synergy between carbapenem and EDTA, Two disks - 10 µg meropenem and meropenem/ EDTA (10µg + 750µg) were used [11].

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

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

Detection of AmpC ß-Lactamases:

Isolates that showed resistance to cefoxitin were suspected as AmpC producers and subjected to phenotypic confirmatory tests;(Boronic acid test method, Disk approximation test, and three-dimensional test). Three-dimensional test was the gold standard test [12].

Three-dimensional test:

AmpC production is able to inactivate the cefoxitin that diffuses from the disk after the disk had 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[13]. **Figure (3)**

Fig. 3: Three-dimensional test

Disk approximation test:

30 µg ceftazidime disk was placed at center of Mueller Hinton Agar plate inoculated with the tested bacteria, then 30 µg cefoxitin, 10 µg imipenem and 20/10 µg amoxicillin-clavulanate disks were placed 20 mm away from ceftazidime disk. The flattening of the inhibition zone between the disk of ceftazidime and the inducing substrates (cefoxitin, imipenem and amoxicillin-clavulanate disk) was considered as a positive result [13]. **Figure (4)**

Fig. 4: Disk approximation test

Boronic acid disk test:

The test evaluated the synergy between cefoxitin and phenylboronic acid, Two 30 µg cefoxitin disks were used, 20 µl of 15 µg/ml phenylboronic acid was dispensed onto one disk[13]. **Figure (5)**

Fig. 5: Boronic acid disk test

RESULTS

From the 200 isolates that were involved in the study 50 different gram negative isolates were submitted to screening and phenotypic tests which is confirmatory for detection of different beta lactamases.

From the 50 isolates, the most common detected organisms were *Salmonella* followed by *Enterobacter cloacae*, *Enterobacter aerogenes* and *Serratia marcescens* then *Proteus mirabilis* and *Burkholderia capacia* as shown in table 1.

Table 1: Shows percentage of each organism:

Organism	**%**
Salmonella spp. (14)	28 %
Enterobacter cloacae (13)	26%
Enterobacter aerogenes (6)	12%
Serratia marcescens (6)	12%
Proteus mirabilis (5)	10%
Burkholderia capacia (5)	10%
Proteus vulgaris (1)	2%

Phenotypic screening Tests:

The antibiotic resistance pattern by vitek2 and disk diffusion method was almost the same; (there was a mild variation in resistance to different antibiotics), as shown in **Table 2**

Table 2: Antibiotic resistance pattern of *gram –ve bacilli* by disk diffusion method and vitek 2:

Antibiotic	**Resistantance (%) by disk diffusion method**	**Resistantance (%) by Vitek 2**
Ampicillin	39(78%)	42(84%)
Piperacillin/tazobactam	-	25(50%)
Cefazolin	47(94%)	48(96%)
Cefoxitin	48(96%)	47(94%)
Ceftazidime	39(78%)	37(74%)
Ceftriaxone	38(76%)	39(78%)
Cefepime	17(34%)	17(34%)
Meropenem	28(56%)	27(54%)
Amikacin	24(48%)	25(50%)
Gentamicin	-	28(56%)
Tobramycin	-	29(58%)
Ciprofloxacin	15(30%)	14(28%)
Levofloxacin	-	12(24%)
Trimethoprim sulfamethoxazole	-	19(38%)
Azetronam	16(32%)	-

Phenotypic confirmatory tests:

ESBL phenotypic confirmatory tests:

Among the phenotypic confirmatory tests, Vitek2 and the ChromID™ ESBL agar were the most sensitive while CDT was more in specificity**,** as shown in table 3

Table 3: Sensitivity, specificity, positive predictive value and negative predictive value of phenotypic confirmatory tests for ESBL detection:

Confirmatory test	**Sensitivity**	**Specificity**	**PPV**	**NPV**
Combined disk test	82%	88%	90%	78%
ESBL test of Vitek2	86%	82%	86%	82%
Chromogenic media	86%	52%	71%	75%

Carbapenemases phenotypic confirmatory tests:

Among the phenotypic confirmatory tests the ChromID® Carba smart agar detected the highest percentage, high sensitivity was detected among combined disk test for MBL. The results are shown in table 4 and table 5.

xTable 4: Percentage of carbapenemase detection by phenotypic confirmatory tests:

Confirmatory test	chromID® CARBA SMART	RapidecCarba NP Test	MHT
Percentage of carbapenemase detection	75%	67%	46%

Table 5: 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%	63%	80%	87%

Results of phenotypic confirmatory tests for AmpC

Among the phenotypic methods we noted that the disk approximation test showed more sensitivity and less specificity than Boronic acid, as shown in table 6

Table 6: Sensitivity, specificity, positive predictive value and negative predictive value of phenotypic confirmatory tests for AmpC detection:

Confirmatory test	Sensitivity	Specificity	PPV	NPV
Disk approximation test	81%	69%	69%	81%
Boronic acid disk	54%	88%	80%	69%

Distribution of different beta lactamases among the 50 Gram negative isolates:

These isolates were ESBL only, ESBL+Carbapenemases, ESBL+AmpC, AmpC only**,** Carbapenemases only**,** carbapenemases+Ampc and ESBL+Ampc+carbapenemases, **(Table 7and figure 6)**

Table 7: Distribution of different beta lactamases among the 50 Gram negative isolates:

Type of enzyme	Positive (n=50)	Positive (%=100%)
ESBL	11	22%
CARBA	4	8%
AmpC	9	18%
ESBL+AmpC	2	4%
ESBL+ CARBA	3	6%
CARBA+AmpC	3	6%
ESBL+CARBA+AmpC	7	14%
No resistance by screening tests	2	4%
No resistance by Standard tests	9	18%

The most common type of beta lactamases in Gram negative isolates as detected by the confirmatory tests was the ESBL most common in *Salmonella* spp (57%) then AmpC was mostly among *Serratia marcescens (83%)*, and lastly the metallo beta lactamase was mostly detected in *Proteus mirabilis* and *Burkholderia cepacia (40%) for each genus.*

Table 8: The most common type of resistance among each organism

	Type of organism (no.)	The most common type of resistance.
1.	Salmonella spp. (14)	ESBL(8) (57%)+ no resist (6) (43%).
2.	Enterobacter cloacae (13)	Ampc(7)(54%)+ ESBL(3) (23%)+ MBL (3)(23%)
3.	Enterobacter aerogenes (6)	ESBL(3)(50%)+ MBL (2)(34%)+ Ampc(1)(16%)
4.	Serratia marcescens (6)	Ampc(5)(83%)+ no resist (1)(17%).
5.	Proteus mirabilis (5)	MBL (2)(40%)+ no resist (3)(60%).
6.	Burkholderia cepacia (5)	Ampc(2)(40%)+ MBL (2)(40%)(+ ESBL(1)(20%)
7.	Proteus vulgaris (1)	ESBL(1)(100%)

DISCUSSION

Antimicrobial resistance had become a serious problem and affects nearly all bacterial species. Many species of bacteria produce Beta-lactamase enzyme that disrupts the four-membered ring of β-lactam of penicillin and cephalosporin groups of antibiotics, which destroy their antimicrobial activity. The production of a β-lactamase by an organism may be a plasmid-associated acquired property or chromosomal and constitutive [14].

In the current study the results of Vitek 2 compact system and disk diffusion method were almost the same; but there was a mild variation in resistance to some antibiotics. The VITEK 2 compact requires less technical time per test, and provided earlier results than disk diffusion method. This agrees with Jorgensen et al.,[15] study which proved that vitek2 and disk method produced very similar overall susceptibility category agreements.

Also Rechenchoski et al.,[8] study reported that the Vitek 2® automated system was more sensitive than Disc diffusion method as compared the by the *broth microdilution* method as a gold standard.

As regard the comparison between different ESBL phenotypic confirmatory methods, we found that Vitek2 and the ChromID™ ESBL agar were the most sensitive (86%) for each of them while CDT was higher in specificity(88%). Färber et al.,[16] study agreed with the current study where the sensitivity and the specificity for the chromogenic agar were (94%), (42%) respectively.

Also Carrër et al.,[17] reported that The ChromID ESBL medium showed good sensitivity; but its disadvantage is the inability to detect OXA-48-like producers which are susceptible to cefpodoxime in the absence of ESBL coproduction and also this medium lacks specificity, because of coselection of widespread ESBL producers which may occur on that medium.

In the current study, CDT showed highest specificity and lowest sensitivity results, This was against with De Gheldre et al.,[18] study which reported that the sensitivity of CDT was 89% and the specificity was 88% and also Thomson et al.,[19] study which reported that sensitivity of ESBL test of Vitek2 was 91% and the specificity was 89%.

Garrec et al.,[20] study which reported a low ability of the Vitek2 system as a routine method in detection of ESBL production, that was below 80% when considering all species and specificity was low (50% to 79%) due to a rather high frequency of in_determinate results.

In the current study; the phenotypic tests for carbapenemase detection, the chromID® CARBA SMART agar detected the highest percentage of carbapenemase (75%), then the Rapidec Carba NP test (67%) and lastly Modified Hodge Test (MHT) (46%).

The chromogenic media was a reliable method that detects carbapenemase and this agrees with Vrioni et al.,[21] study which reported that chromID CARBA was an easily performed and very accurate method for CPE detection and agrees with Olivgeris et al.,[22] studies which approved that chromID® CARBA SMART agar is a reliable and accurate method that detect carbapenemase.

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

As regard using Rapidec Carba NP test and MHT for detection of carbapenemase we found that Rapidec Carba NP test was better than MHT as it detected a higher percentage of carbapenemas and was time saving, this agrees with Lifshitz et al.,[23] study which reported that the Rapidec Carba NP was accurate, performed easily and faster than MHT.

In the current study the combined disk test is a reliable test for detection of metallo beta lactamases as it showed 94%sensitivity but its specificity was 63%.

Chu et al.,[24] study reported that false positive results may occur with combined disk test as EDTA may possess their own bactericidal activity resulting in expansion of zone of inhibition without true MBL production. On the other hand Picao et al.,[25] reported false negative results might arise from carbapenem hydrolysis or inactivation caused by EDTA .

Our study agreed with Omair et al.,[11] Pournaras et al.,[26] and Maurer et al.,[27] studies about the sensitivity of CDT which were (97%, 94.8%, 100% respectively) but disagreed with them about specificity that were (100%) for all of them.

As regard AmpC resistance we found that the detection of AmpC mediated resistance is problematic due to absence of Clinical and Laboratory Standards Institute (CLSI) guidelines for phenotypic methods that investigate AmpC-producing organisms.

In the current study we found that the disk approximation test shows more sensitivity and less specificity than Boronic acid as DAT shows sensitivity and specificity of 81% and 69% respectively and those of Boronic acid test were 54% and 88% respectively.

This disagrees with Saad et al.,[12] study as DAT show sensitivity and specificity 88% and 92% ,but agreed with Helmy and Wasfi study;[13] an Egyptian study in which sensitivity of Boronic acid test was 65% and specificity was 73%, and reported that cloxacillin was a better inhibitor specially among AmpC-positive *E. coli* and *P. mirabilis* isolates when it compared the inhibitory effect of cloxacillin and boronic acid on AmpC enzymes effects.

The explaination of the phenyl boronic acid test had low specificity was due to that the boronic acid can inhibit class A carbapenemase (KPC) β-lactamase besides AmpC [28].

In the current study we noted that not all cefoxitin resistant isolates were AmpC β-lactamase producers. This can be explained by resistance to cefoxitin not only caused by AmpC β-lactamase production but also other enzymes like extended spectrum beta lactamases (ESBLs) and metallo beta lactamase (MBL) or non-enzymatic mechanism like porin channel mutation[29].

For the distribution of different beta lactamases among gram negative bacilli, the rate of ESBL in the current study was the highest (46%) followed by AmpC (44%) and lastly MBL (34%), This result was corresponding to other Egyptian studies which was conducted at Hospital of Assiut University[30], Benha University Hospital[7] and Alexandria University Hospital[31].

As regard the most common type of beta lactamases in G-ve isolates by the confirmatory tests was the ESBL 46% and most common in *Salmonella* spp (57%) then AmpC 44% and mostly among *Serratia marcescens (83%)*, and lastly the metallo beta lactamase 34% and mostly in *Proteus mirabilis* and *Burkholderia cepacia (40%)for each of them.*

Ziech et al., study [32] reported that ESBL production was detected in 45% (44/98) of salmonella strains. On the other hand Clemente et al., study [33] analyzed 1120 isolates of *Salmonella* spp. and found only five ESBL-producing strains.

Lange et al.,[34] reported that carbapenemase-producers in *Proteus mirabilis* in only 8 (21.6%) strains.

MacDougall study[35] reported that the genes encoding for AmpC β-lactamases are common in the chromosomes of organisms such as *Serratia*, *Pseudomonas*, *Acinetobacter*, *Citrobacter*, and *Enterobacter*.

CONCLUSION

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

The limitation of this study was the small size of the samples and that PCR could not be used as the gold standard for some tests 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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