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ORIGINAL ARTICLE

Different Phenotypic Methods for Detection of Beta Lactam Resistance in *Escherichia coli*

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ABSTRACT

Background: Beta- lactam resistant Escherichia coli *is a major cause of both nosocomial and community-acquired infections. It is essential to detect the beta lactam resistance 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) beta lactamase and AmpC production among E.coli 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 in 5o isolates of E.coli were 6 (12%) isolates not resistant by screening with antibiotic sensitivity tests, 3 (6%) isolates were negative by the gold standard phenotypic tests, 12 (24%) were ESBL alone, 2 (4%) were ESBL and AmpC, 10 (20%) were AmpC alone, 8 (16%) were ESBL and Carbapenemases and 9 (18%) were carbapenemases alone. The combined disk test showed high sensitivity and specificity in detection of ESBL and MB, For AmpC detection; the disk approximation test showed higher sensitivity and specificity than boronic acid in detection of AmpC.* ***Conclusion:*** *The rate of Beta lactamases production in E.coli is seriously increased, the ESBL showed the highest percentage among E.coli isolates. The phenotypic confirmatory tests showed high sensitivity and specificity and proved to be reliable methods for detection of the beta lactamase resistance, genotypic tests are recommended to be a gold standard tests to increase the specificity of the phenotypic tests.*

INTRODUCTION

The incidence of nosocomial infections caused by resistant *E.coli* in the clinical settings is seriously increased. The β-lactam antibiotics are commonly prescribed, the inappropriately usage of them have led to appearance of various β-lactamases such as the ESBLs, metallo-β-lactamases and AmpC β-lactamases.[1] Enterobacteriaceae, especially *E. coli* producing ESBLs, have been established since the1980s as a major cause of hospital-acquired infections. ESBLs are enzymes capable of conferring bacterial resistance to the penicillins, third-generation cephalosporins, and monobactam, they are inhibited by clavulanic acid[2]**.** Additional resistance to cephamycins and carbapenems soon followed by the emergence of the AmpC β- and carbapenemases. AmpC enzymes are similar to ESBLs in that they show reduced susceptibility to penicillins, third-generation cephalosporins and monobactam .However, in contrast to ESBLs, AmpC also inactivate cephamycins (second- generation cephalosporins). These enzymes are poorly inhibited by the commercially available β-lactamase inhibitors such as clavulanic acid, sulbactam etc. but are inhibited by cloxacillin and phenylboronic acid.[3] Carbapenemases are a group of β-lactamases that confer resistance to multiple β-lactam antibiotics, including penicillins, cephalosporins, β-lactam-inhibitor combinations and carbapenems [4]**.**

The phenotypic confirmatory tests are highly sensitive and specific and more suitable to use as routine tests in clinical laboratories. [5]

The study was designed to detect the distribution of different beta lactamases among the *E.coli* isolates and to compare between different phenotypic methods for detection of B-lactamases.

METHODOLOGY

This prospective study was done in microbiology unit of clinical pathology department at Assiut university hospital. This study 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 Compact15system. [6]

Detection of ESBLs:

Isolates that showed resistance to third generation cephalosporin were suspected to be ESBL producers and 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 enterobacteria , *E. coli* appear as pink to burgundy colonies or translucent colonies with a pink to burgundy centre.
- ***Combined disk test (Oxoid):*** [6] 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.
- ***ESBL test of vitek2 compact 15 (BioMérieux):***[8] 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 clavulanic acid.
- ***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.

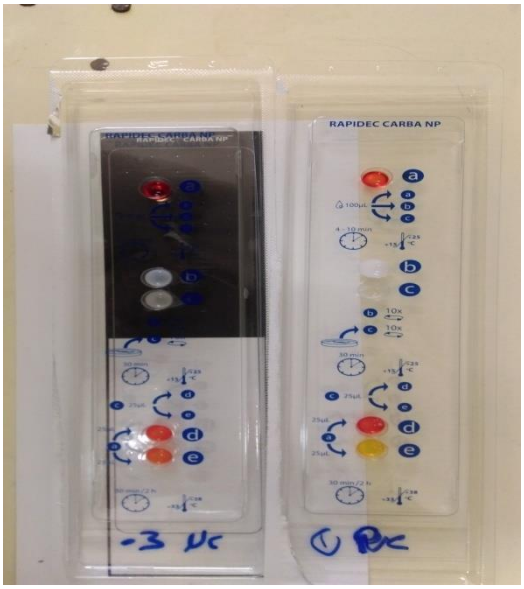
Detection of carbapenemases:

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. [9] Those isolates were also tested for metallo beta lactamases production by combined disk test and E-test. The E-test was taken as a gold standard test.[10]

- ***ChromID® CARBA SMART Agar (BioMérieux):*** Which is a selective chromogenic medium used according to manufacture instructions for the detection of carbapenemase producing enterobacteria, *E. coli* appear as pink to burgundy colonies or translucent colonies with a pink to burgundy centre.
- ***Modified Hodge Test (MHT):***[11] 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. 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: (26) negative result, (27) positive 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 manufacture 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)



a- Negative result b- Positive result

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

Etest (IP/IPI) imipenem and imipenem-EDTA (BioMérieux):

Strips are 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 taken as a gold standard test.[13]

– ***Three-dimensional test:***[14] 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. Figure (3)

Fig. 3:Three-dimensional test

– ***Disk approximation test:***[13] 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 blunting or flattening of the inhibition zone between the ceftazidime disk and the inducing substrates (cefoxitin, imipenem and amoxicillin-clavulanate disk) was considered as a positive result. Figure (4)

Fig. 4: Disk approximation test

– ***Boronic acid disk test:***[15] The test evaluates 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. Figure (5)

Fig. 5: Boronic acid disk test

RESULTS

From the 200 isolates that included in the study there were 50 *E-coli* isolates which 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 to Ampicillin, Meropenem and Ciprofloxacin. Table (1)

Table 1: Antibiotic resistance pattern of *E-Coli* by disk diffusion method and vitek 2:

Antibiotic	Resistantance (%) by disk diffusion method	Resistantance (%)by Vitek 2
Ampicillin	47(94%)	49(98%)
Ampicillin/sulbactam	-	40(80%)
Piperacillin/tazobactam	-	33(66%)
Cefazolin	45(90%)	45(90%)
Cefoxitin	28(56%)	28(56%)
Ceftazidime	44(88%)	44(88%)
Ceftriaxone	44(88%)	44(88%)
Cefepime	44(88%)	44(88%)
Meropenem	20(40%)	22(44%)
Amikacin	5(10%)	5(10%)
Gentamicin	-	22(44%)
Tobramycin	-	28(56%)
Ciprofloxacin	34(68%)	35(70%)
Levofloxacin	-	37(74%)
Trimethoprim /sulfamethoxazole	-	41(82%)
Azetronam	42(8٤%)	-

Results of phenotypic confirmatory tests:

ESBL phenotypic confirmatory tests for:

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:

Cofermatory test	Sensitivity	Specificity	PPV	NPV
Comined disk test	95%	96%	95%	96%
ESBL test of Vitek2	91%	95%	95%	91%
Chromogenic media	91%	43%	64%	82%

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	91%	82%	77%

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%	100%	100%	83%

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
Disk approximation test	83%	94%	91%	88%
Boronic acid disk	67%	62%	57%	71%

Distribution of different beta lactamases among the 50 Ecoli isolates:

The Percentages of B –Lactamase enzymes in 5o isolates of *E.coli* were 6 (12%) isolates not resistant by screening with antibiotic sensitivity tests, 3 (6%) isolates were negative by the gold standard phenotypic tests, 12 (24%) were ESBL alone, 2 (4%) were ESBL and AmpC, 10 (20%) were AmpC alone, 8 (16%) were ESBL and Carbapenemases and 9 (18%) were carbapenemases alone. Table (6) Figure (6)

Table 6: Distribution of different beta lactamases among the 50 *E.coli* isolates:

Type of enzyme	Positive (n=50)	Positive (%=100%)
ESBL	12	24%
ESBL+AmpC	2	4%
AmpC	10	20%
ESBL+ CARBA	8	16%
CARBA	9	18%
No resistance by screening tests	6	12%
No resistance by Standard tests	3	6%

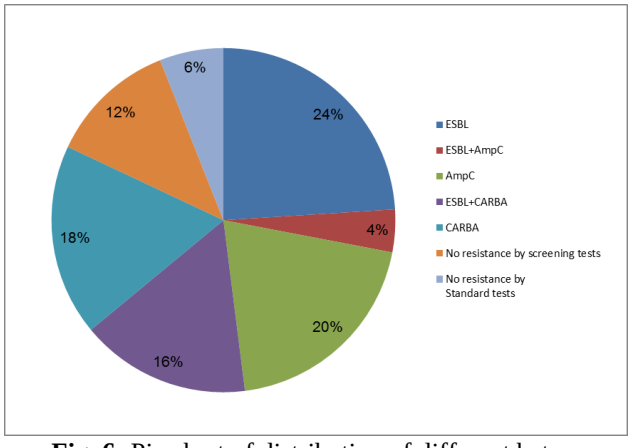


Fig. 6: Pie chart of distribution of different beta lactamases among the 50 E.coli isolates

DISCUSSION

In the current study we found that the results of antibiotic susceptibility tests by vitek2 automated system and disk diffusion method were almost the same; there were mild variation in resistance to: Ampicillin, Meropenem and Ciprofloxacin, but the vitek2 automated system required less technical time per test, and provided earlier results than disk diffusion method.

This agreed with Jorgensen et al study which proved that vitek2 and disk method produced very similar overall susceptibility category agreements.[16]

On the other hand Rechenchoski et al study compared the Vitek 2® and disc diffusion method, with using the broth microdilutionas gold standard and reported that the Vitek 2® automated system was more sensitive than Disc diffusion method.[8]

As regard the comparison between different ESBL phenotypic confirmatory tests, we found that the combined disk test was the most sensitive and specific test as its sensitivity and specificity were 95% and 96% respectively ,then of ESBL test of Vitek2 were 91% and 95% respectively, and lastly the chromID™ ESBL agar showed 91% sensitivity and 43% specificity

This agrees with Singh, 2014 study which reported that the combined disk test showed 93% sensitivity and 100% specificity which were higher than ESBL test of Vitek2that showed 91,8% sensitivity and 97,2% specificity.[17]

Also agree with Kenneth et al study in which the sensitivity of ESBL test of Vitek2 was 91% and the specificity was 89%, and agrees with Yves et al study in which the sensitivity of combined disk test (CDT) was 89% and the specificity was 88%.[18,19]

The chromID ESBL agar detected ESBL-producing *E.coli* 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 24h. 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%.[20]. Färber et al is

another study agree with the current study as it reported a high sensitivity (94%) and low specificity (42%) for chromID™ ESBL agar.[21]

In the current study; we found that among the phenotypic confirmatory tests for carbapenemase detection The chromID® CARBA SMART agar detect the highest percentage of carbapenemaseas as it detected 20 isolates of the total 22 isolates (91%), then the Rapidec Carba NP test detect 18 isolates (82%) and lastly Modified Hodge Test (MHT) detect 17 isolates (77%).

We found that the chromogenic media was a reliable method for detection of carbapenemase and this agrees with Papadimitriou et al study which reported that chromID CARBA was found to be an easily performed and very accurate method for CPE detection.[22]

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 detect higher percentage of carbapenemas and was time saving, this agrees with Lifshitz et al study.[23]

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 100% specificity,This agree with Omair et al., 2012 study in which the sensitivity and the specificity were (97%, 100%).[12]

The detection of AmpC mediated resistance was a problematic as there are no guidelines of Clinical and Laboratory Standards Institute for phenotypic techniques to investigate AmpC-producing organisms.

In the present study we found that the Disk approximation test (DAT) detected AmpC beta-lactamase more reliably than Boronic acid test, as sensitivity and specificity of DAT were 83% and 94% respectively and those of Boronic acid test were 66% and 62% 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]

As regard Boronic acid test; the present study agree with Helmy and Wasfi study; an Egyptian study in which sensitivity and specificity of Boronic acid test were 65% and 73%, and reported that the cloxacillin was a better inhibitor than Boronic acid particularly among AmpC-positive E. coli and P. mirabilis isolates.[15]

In the present study we noted that not all cefoxitin resistant isolates are AmpC β-lactamase producers. This can be explained by that cefoxitin resistance could be due to extended spectrum beta lactamases (ESBLs) and metallo beta lactamase (MBL).

As regard distribution of different beta lactamases among E –coli; The ESBLs rate was the highest among the E –coli isolates and this agreed with other Egyptian studies conducted at Assiut, Banha, Alexandria, Sohag and Menoufia University hospitals. [24,25,26,27,28]

Future studies may be carried out in larger sample size with application of genotypic methods which considered the gold to increase the sensitivity of the tests. Implementation of antibiotic policies stewardship and strict implementation of infection control measures to decrease antibiotic resistances.

In this study there were no personal benefits or any conflict of interests, this study wasn't published at any other journal.

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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