Int.J.Curr.Microbiol.App.Sci (2016) *5*(6): 105-114

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 6 (2016) pp. 105-114**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.506.013

Evaluation of a 12 Disc Test for Phenotypic Detection of β- lactamases Resistance in Gram Negative Bacilli

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Keywords

12 disc test, beta lactamases, AmpCβL, co producers.

Article Info

Accepted:
10 May 2016
Available Online:
10 June 2016

A B S T R A C T

Various mutated forms of β-lactamases pose a therapeutic challenge to the health care settings as they hydrolyze a wide range of β-lactam antibiotics. Detection of these enzymes and their co existence is still a diagnostic challenge. In this, we evaluated a novel 12 disc test as a screening test for the presence of any kind of beta lactamases or its co producers. A 12 disc test was put on 300 isolates that showed susceptibility pattern as Intermediate/Resistant to ceftazidime/ ceftriaxone or aztreonam on routine sensitivity testing by modified Kirby bauer method. Further, double disc synergy test, AmpC disc test, E test for MBL and modified Hodge test for KPC/ MBL (used as confirmatory tests) were also done on each of them. By 12 disc test, out of 300 isolates, 64 (21.3%) were ESβL, 8 (2.6%) K1βL, 76 (25.3%) high level AmpCβL, 08(2.6%) AmpCβL, 08 (2.6%) AmpCβL+ ESβL co producers, 108 (36%) Carbepenamases and 28 (9.3%) MβL+ ESβL producers. By confirmatory tests, 64 (21.3%) were ESβL, 08 (2.6%) AmpCβL, 64 (21.3%) High level AmpCβL, 08 (2.6%) AmpCβL + ESβL, 44 (14.6%) AmpCβL + MβL, 16 (5.3%) AmpCβL+ MβL+ ESβL, 52 (17.3%) MβL, 8 (2.6%) KPC and 12 (4%) ESβL+ MβL. 12 disc test is a good screening test for rapid identification of type of β lactamases.

Introduction

An alarming rise in the rates of the antibiotic resistance has now become a serious and an increasingly common public health concern, with severe implications, especially in the intensive care units. The β-lactam antibiotics are among the most frequently prescribed antibiotics world-wide because of their efficacy, broad spectra and low toxicity.

A variety of mutated forms of β-lactamases like the extended spectrum beta lactamases (ESβLs), AmpC β-lactamases (AmpCβL) and metallo-β-lactamases (MβL) have evolved due to the selective pressure generated by the indiscriminate use of these antibiotics. This has emerged as the most worrisome resistance mechanism posing a therapeutic challenge to the health care settings as they are capable of hydrolyzing a wide range of β-lactam antibiotics, notably the extended-spectrum penicillins, third and fourth generation cephalosporins, and the carbapenems (Oberoi L. *et al.*, 2013)

The confirmed ESβL-producing isolates should be reported as resistant to all penicillins, cephalosporins, and aztreonam to avoid therapy with antibiotics that may be clinically ineffective but bacteria producing these enzymes may not be recognized as they are falsely susceptible in routine tests as a result patients start receiving ineffective antibiotics which contributes to the spread of the pathogens with hidden resistance. Tests based on ceftazidime and cefotaxime tested alone and in combination with clavulanate are useful in ESBL detection but they may yield false positive results with *Klebsiella pneumonia* carbapenemase (KPCs) and hyper produced K1β-lactamaes (K1βL) and false negative results with isolates that co-produce a high level of AmpCβL. Thus, detection of AmpCβL especially in these has its own importance because they have been associated with false ESβL negative results. Carbapenems are generally used to treat ESβL/AmpCβL- associated infections, so it is also important that reduced carbapenem susceptibility of ESBL-positive isolates is not ignored simultaneously as it may indicate carbapenemase production, which contraindicates carbapenem therapy too.

Their detection is a crucial infection control issue because they are often associated with extensive, sometimes total, antibiotic resistance and more-resistant organisms can be vectors responsible for carbapenemase transmission to members of the families in which the resistance mechanism is not recognized (Kenneth *et al.*, 2010)

We conducted this study to detect different resistance phenotypes of beta lactamases among isolates of family *Enterobacteriaceae, Pseudomonas spp.* and *Acinetobacter spp.* in our set up and to evaluate a novel 12 disc test (a single plate method) as a screening test to detect the presence of any kind of beta lactamase or its co producers.

Material and Methods

The study was conducted in the department of Microbiology, SMS medical College, Jaipur from the period of March 2014 to April 2014. A total of 339 consecutive, non repetitive clinical isolates of gram negative bacilli (GNB), belonging to family *Enterobacteriaceae, Pseudomonas spp.* and *Acinetobacter spp* from different clinical samples, which were received from various wards and ICUs were identified by the standard microbiological tests (Collee, *et al.*, 1996). The routine antimicrobial susceptibility pattern of the isolates was determined by the Kirby Bauer disc diffusion method according to the CLSI guidelines (CLSI 11th edn, 2012). A 12 disc test (Schreckenberger *et al*) was put on 300 isolates that showed susceptibility pattern as Intermediate/Resistant to ceftazidime/ ceftriaxone or aztreonam. Though, Schreckenberger PC. *et al.*, recommend the 12 disc test for family *Enterobacteriaceae* only, but we have used it on *Pseudomonas* and *Acinetobacter spp.* too as they are also commonly isolated in our set up. The reference strains, ESβL positive *Klebsiella pneumonia* ATCC 700603 and *E. Coli* ATCC 25922 were included in the study as controls.

12 Disc Test

(Schrekenberger *et al*)**:** Mueller Hinton agar (MHA) in 150 mm diameter plate was used. Following antibiotic discs (Hi media labs Pvt Ltd) were placed as shown in fig 1:
Aztreonam (30), Ceftazidime (30), Ceftazidime + clavulante (30/10), Cefotaxime (30), Cefotaxime +clavulante (30/10), Cefoxitin (30), Cefotetan (30), Ceftriaxone (30), Cefepime (30), Ertapenem (10), Imipenem(10), Meropenem (10).

Interpretation criteria used for the screening test (Schrekenberger *et al*):

1. An isolate showing an increase in zone size of ≥5 mm with clavulanate compared with plain ceftazidime/cefotaxime disks is interpreted as ESβL positive.

2. AmpCβL is detected when the isolate shows resistance to first, second and third generation cephalosporins, the beta lactam inhibitor drug and cephamycins, but susceptibility to Cefepime disk. In addition to the above, high level AmpCβL producers are resistant to Monobactem (Aztreonam).

3. Carbapenemase production is indicated by resistance to the Carbapenems (Meropenem/ Imipenem / Etrapenem).

4. K1 βL: Sensitive to cefoxitin, No Clavulanic Effect, Resistant to Aztreonam and sensitive to ceftazidime.

After performing the 12 disc test, all the isolates were subjected to following CLSI recommended phenotypic tests to compare the results with the screening test. For ESBL detection, double disc synergy test (Giriyapur RS. *et al.,* 2011), for MBL/KPC detection, modified Hodge test (Amjad *et al.,* 2011) and E-test method using Ezy-MIC™ Strips (Hi Media Laboratories Pvt. Ltd.) were used (Mobashshera *et al.,* 2015). Along with these, AmpC disc test (Singhal S. *et al.,* 2005) for AmpCβL detection was used and all of them were considered as confirmatory tests in our study.

Result: Out of the 339 isolates of *Enterobacteriaceae, Pseudomonas spp and Acinetobacter spp,* 300 (88.4%) isolates were found to be producing any of the beta lactamases. Among these 300 isolates, 144(45%) were *E.Coli,* 56 (18.6%) *Enterobacter spp,* 32 (10.6%) *Pseudomonas spp,* 20(6.6%) were *Klebsiella spp,* 20 (6.6%) *Acinetobacter spp,* 12(4%) *Proteus spp*, 12(4%) *Citrobacter spp* and 4(1.3%) *Hafnia spp.*

Table1 shows organism wise distribution of various beta lactamases.

All isolates of *Hafnia spp* (04) were found to be ESβL producers (100%), whereas maximum number of AmpCβL and carbapenemase producers (CPs) were from *Acinetobacter spp* (80% each) in this study.

Table 2 depicts the results of 12 disc test.

Distribution of different types of beta lactamases detected by the confirmatory tests used in the study is shown in table 3. Out of 300 screened isolates, total ESβL producers were found to be 104 (34.6%), K1βL 8 (2.6%), AmpCβL producers 156 (52%) and CPs were 132 (44%). Out of the 132 CPs, 80 (60.6%) strains were sensitive to Imipenem and showed resistance to Meropenem, whereas 8 (6%) strains which were sensitive to Meropenem exhibited resistance to Imipenem. 84 (24%) isolates were found to be co- producers of these beta lactamases.

When the results of 12 disc test were compared with the confirmatory tests from tables 2 and 3, ESβL, AmpCβL and high level AmpC βL producers were found to be exactly same by both the tests i.e 64 (21.6%), 8 (2.6%) and 76 (25.3%) respectively. Out of 108 (36%) CPs detected by 12 disc test, 60 (20%) were confirmed as CPs and 44 (14.6%) turned out to be co producers of AmpCβL+ CP by confirmatory tests. Chromosomal AmpCβL + ESβL co producers detected by 12 disc test were 8 (2.6%), whereas by confirmatory test 12 (4%).

12 disc test detected 28 (9.3%) as ESβL+ CP co producers, out of these, only 12 (4%) turned out to be ESβL+ CP and rest 16 (5.3%) were ESβL+AmpCβL+ CP by confirmatory methods..

8(2.6%) isolates which were reported as CP by 12 disc test were found to be KPC by confirmatory tests. K1βL could only be detected by 12 disc test and were 8 (2.6%). They were found to be negative for any of the beta lactamases by confirmatory tests.

Results and Discussion

Clinical laboratories have been facing problems in detection of the enzymes ESβLs, AmpCβL and MβLs since they were discovered. Also, the co expression of these enzymes makes their detection more complicated. Confusion still persists in the choice of optimal test methods, their reporting conventions and the importance of resistance mechanism. Failure of detection of these enzymes lead to therapeutic failures and uncontrolled spread further.

We have undertaken this study to detect different beta lactamases among isolates of *Enterobacteriaceae*, in our set up. Also, we have evaluated a novel 12 disc test (a single plate method) as a screening test to detect the presence of any kind of beta lactamase or its co producers and compared its results with standard phenotypic methods used as confirmatory tests.

In the present study, the prevalence of ESβL positive strains was found to be 104/339 (30.6%). Studies done previously had reported an overall prevalence of ESBL varying from 33.86% to 64.8%.(Valsan *et al.*, 2013, Wadekar *et al.*, 2013, Sinha *et al.*, 2008, Vijaya *et al.*, 2014) It is a well known fact that ESβL positive strains often show false susceptibility results on standard disc diffusion method. However, their spread is so extensive that all laboratories should include their detection by means of special tests, along with the routine testing by CLSI recommended methods.

The prevalence of AmpCβL positive strains in our study period was found to be 156/339 (46.0%). Others have reported 50.9% (Chatterjee *et al.*, 2010), 24% (Sinha *et al.*, 2008) 14.0% (Vijaya *et al.*, 2014) and 11.1% (Laghawe *et al.*, 2012). This shows a wide variation in the prevalence of beta lactamases from region to region or even from hospital to hospital. Prevalence of AmpCβL as found in our present study is almost double of what we had reported seven years back (24%) in a study done under same set up (Sinha P. *et al.*, 2008). Efforts to detect AmpCβL enzymes in GNBs are largely non-existent. Undoubtedly this is due in part to the lack of standard guidelines for detecting AmpCβL producing isolates (Laghawe *et al.*, 2012)

Overall 132/339 (38.9%) isolates were found to be MβL producers in our study. This is higher in comparison to some studies (Valsan *et al.*, 2013, Wadekar MD. *et al.*, 2013, Vijaya *et al.*, 2014), but correlates well with the study done by Chatterji SS. *et al.*, (2010) who had reported a positivity rate of 41.7%. Production of MBL has tremendous therapeutic consequences since these organisms also carry multidrug resistance genes and the only viable option remains the potentially toxic Polymyxin B and Colistin (Wadekar *et al.*, 2013). In the present study, we found large number of MβL/KPCs to be sensitive to Imipenem but resistant to meropenem i.e. 80 (60.6%), whereas 8 (6%) showed vice versa results. Renu *et al.*, (2010) had reported 20% Imipenem sensitive MβLs in her study. These carbapenem susceptible organisms with hidden MβL genes can spread unnoticed in hospitals if such isolates are

reported as sensitive without screening for the presence of MβLs. Screening of only Imipenem resistant organisms for MβL is insufficient and screening of all the Imipenem susceptible isolates creates unnecessary work with a lower yield. Hence, some criterion is needed to select out Imipenem susceptible isolates for MβL screening. In 12 disc test, three carbapenems i.e. Imipenem, Meropenem and Ertapenem are used so as not to miss any of the MβLs.

Co existence of ESβL and AmpCβL was seen in 12 (4%) of the 300 isolates. Others have reported 8% (Sinha P. *et al.,* 2008) and 16.7% (Vijaya *et al.,* 2014). ESβL with MβL /KPC were seen in 12 (4%) isolates and this was similar to the study done by Laghave *et al.,* 2012, who has reported 4.86% of co existence. Vijaya *et al.,* (2014) and Salimi *et al.,* (2013) have also reported coexistence of 1.5% and 12.5% respectively. AmpCβL+ MβL were seen among 44 (14.6%) isolates in our study, whereas Salimi F. *et al.,* (2013) and Vijaya *et al.,* 2014 have reported in 81% and 1.5% isolates respectively. All three i.e. ESβL+ MβL+ AmpCβL were seen in 16 (5.3%) isolates in our study. Others have reported 23.7% (Chatterjee *et al.,* 2010) and 1.5% (Salimi *et al.,* 2013). Thus, there is high level of coexpression of these beta lactamases as resistance mechanism, which cannot be ignored and their timely detection needs to be stressed upon.

In 12 disc test, two sets of double discs (CAZ – CAZ + Clav and CTX - CTX + Clav) for ESβL and K1βL screening, two discs (Cefotetan and Cefoxitin) for AmpCβL screening and three drug discs (Imipenem, Meropenem and Ertapenem) for screening of MβL /KPC were used so that none of the beta lactamase is missed in screening. When the results of 12 disc test were compared with the confirmatory tests as shown in tables 3 and 4, no discrepancy was found in detection of ESβL, AmpCβL and high level AmpCβL.

There are currently no CLSI approved methods to detect chromosomal or plasmid mediated AmpC gene resistance but as per the recommendations of 12 disc test, 84/92 (91.3%) plasmid mediated AmpCβL and 8/92 (8.6%) chromosomal AmpCβL were detected in our study. Chromosomal AmpC beta-lactamases can be produced inducibly or constitutively and inducible expression of the AmpC gene occurs when the enzyme is produced at a high level for example under exposure to inducing agents, such as Cephamycins (ie. Cefoxitin), Ampicillin and Carbapenems (i.e. Imipenem, Meropenem, Ertapenem). Induction is temporary and may be reversed when the antibiotic inducer is removed. In some organisms, mutations occur that cause the AmpCβL gene to become permanently expressed at high levels. These organisms are termed permanently de-repressed mutants (Schreckenberger *et al.*,)

Out of 108 (36%) pure CPs, detected by 12 disc test, only 60 (20%) came out to be CPs by confirmatory methods and 44 (14.6%) turned out to be AmpCβL+ CP. Similarly 12 disc test detected 28 (9.3%) isolates as ESβL+ CP, out of which only 12 (4%) turned out to be ESβL+ CP and rest 16 (5.3%) were ESβL+ AmpCβL+ CP by confirmatory tests. Such discrepancy may not change the therapeutic outcome as the treatment given for AmpCβL+ CP and for ESβL+ AmpCβL+ CP is same as given for CP alone.

Table.1 Organism wise prevalence of different kind of beta lactamases.

	ESβL	**AmpC**βL	**M**βL **/KPC**	**K1** βL
***E. coli* (144)**	56 (38.8%)	64 (44.4%)	52 (36.1%)	0
***Citrobacter* (12)**	0	8 (66.6%)	4 (33.3%)	0
***Enterobacter* (56)**	12 (21.4%)	40 (71.4%)	28 (50%)	4(7.1%)
***Pseudomonas*(32)**	16 (50%	20 (62.5%)	20 (62.5%)	0
***Acinetobacter* (20)**	4 (20%)	16 (80%)	16 (80%)	0
***Klebsiella* (20)**	4 (20%)	8 (40%)	12 (60%)	0
***Proteus* (12)**	8 (66.6%)	0	0	4 (33.3%)
***Hafnia* (4)**	4 (100%)	0	0	0
Total (300)	104 (34.6%	156 (52%)	132 (44%)	8 (2.6%)

ESβL, AmpCβL, MβL /KPC as detected by confirmatory tests and K1 βL by 12 disc test.

Table.2 Results of 12 Disc Test (n=300).

Type of beta lactamase	**Number (percentage)**
ESβL	64 (21.3%)
K1βL	8 (2.6%)
Plasmid mediated AmpC	
High level AmpCβL	76 (25.3%)
AmpCβL	08 (2.6%)
Chromosomal AmpCβL**+ES**βL	
With high level AmpCβL	08 (2.6%)
With AmpCβL	0
Carbapenemase	108 (36%)
ESβL + CP	28 (9.3%)

Table.3 Results of confirmatory tests (n=300).

Type of beta lactamase	**Number (percentage)**
ESβL	64 (21.3%)
High level AmpCβL	76 (25.3%)
AmpCβL	08 (2.6%)
MβL	52 (17.3%)
KPC	8 (2.6%)
ESβL+AmpCβL	12 (4%)
ESβL +MβL	12 (4%)
AmpCβL+ MβL	44 (14.6%)
ESβL+ AmpCβL+ MβL	16 (5.3%)

Fig.1 Template for 12 disc test

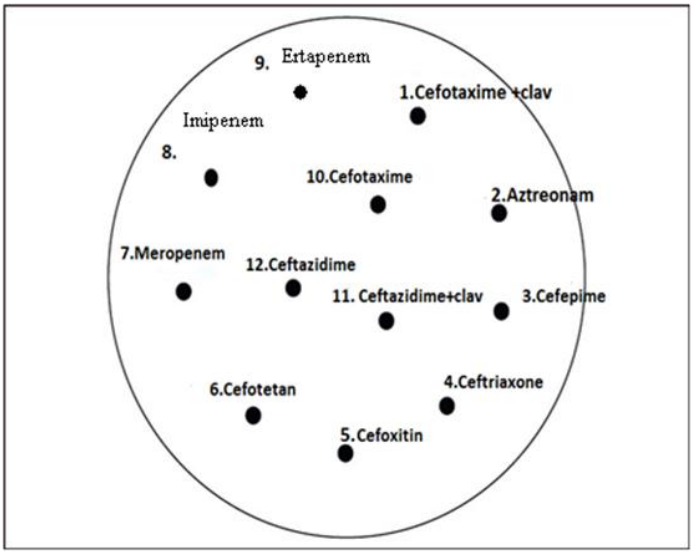
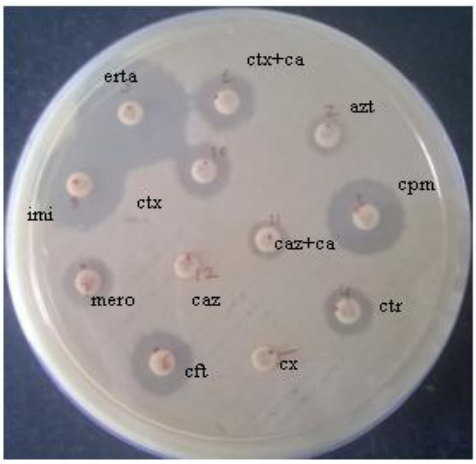


Fig.2 12 disc test: Clavulanate enhancement present - ESβL positive. Resistance to ertapenem and meropenem indicate carbapenemase production and Cefoxitin, cefotetan and cefepime are resistant. **Confirmatory test**: ESβL+ AmpC βL + MβL.

Ctx: cefotaxime, caz: ceftazidime, caz+ clavulanic acid, Ctx+ca: cefotaxime + clavulanic acid, azt: aztreonam, cpm: cefepime, ctr: ceftriaxone, Cx: cefoxitin, cft: cefotetan, mero: meropenem, Erta: ertapenem,

Fig.3 12 disc test: Keyhole towards clavulanic acid indicates ESβL. Cefepime sensitive and Cefoxitin/Cefotetan resistant indicate AmpCβL; Imipenem and Meropenem both are sensitive but Etrapenem is resistant, indicates probability of carbapenemase production.
Confirmatory tests: ESβL+ AmpCβL



Ctx: cefotaxime, caz: ceftazidime, caz+ clavulanic acid, Ctx+ca: cefotaxime + clavulanic acid, azt: aztreonam, cpm: cefepime, ctr: ceftriaxone, Cx: cefoxitin, cft: cefotetan, mero: meropenem, Erta: ertapenem,

12 disc test detected 8 (2.6%) strains as Chromosomal AmpCβL + ESβL co producers whereas it was 12 (4%) by confirmatory test. This little discrepancy may be the result of masking effect of AmpCβL on clavulanate enhancement which is used for detection of ESβL.

KPCs which could be detected only by the confirmatory tests were 8(2.6%) and were reported as CP by 12 disc test. With 12 disc test, we are unable to differentiate KPC from MβL. We could detect K1βL 8(2.6%) which were considered as negative for any of the beta lactamases by confirmatory tests. The K1 enzyme is predominantly a penicillinase that can also significantly hydrolyze Aztreonam, Cefuroxime and Ceftriaxone and has weak activitiy against Cefotaxime or Ceftazidime. A distinctive feature of hyperproducers of K1 is its greater activity against Ceftriaxone over Cefotaxime and against Aztreonam over Ceftazidime (Schreckenberger P. *et al*) Unfortunately, there are currently no CLSI approved methods to detect K1 beta lactamases too.

In conclusion, microbiology laboratories must be able to detect resistant pathogens in a timely manner, especially those that are falsely susceptible *in vitro* to drugs that may be considered for therapy of infected patients so as to modify the treatment of the patient and prevent the dissemination of resistant strains further. We found that the 12 disc test is a very useful, sensitive and cost effective test which can be used in routine clinical microbiological laboratories for screening family *enterobacteriaceae* and the routinely isolated non fermenters, especially in the set ups where the automated systems or molecular facilities are not available. This will reduce the chances of missing any type of beta lactamases thereby reporting them as susceptible instead of resistant. The drawback of our study is that we have not

confirmed our results with molecular methods but except for AmpCβL and K1βL detection, we have used only CLSI recommended methods as confirmatory tests.

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How to cite this article:

Parul Sinha, Pallawi Goyal, Rajni Sharma, Aruna Vyas and Maheshwari, R.K. 2016. Evaluation of a 12 Disc Test for Phenotypic Detection of β- lactamases Resistance in Gram Negative Bacilli. *Int.J.Curr.Microbiol.App.Sci.* 5(6): 105-114.
doi: http://dx.doi.org/10.20546/ijcmas.2016.506.013