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# Detection methods of Enterobacteriaceae producing extended spectrum betalactamase

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**ABSTRACT:** Production of extended-spectrum beta-lactamases (ESBL) considered one of the most important resistance mechanisms that impair antimicrobial treatment of infections caused by *Enterobacteriaceae*. Four phenotypic methods were compared to detect ESBL production; the tests based on the synergy between a third-generation cephalosporin and clavulanate. These tests are: the double-disk synergy test (DDST) 25 to 30mm, DDST (30mm), DDST (20mm) and the double-disk (Spanish- test). In our study, we worked on 81 strains of ESBL enterobacteriaceae. Synergy test 25 to 30 mm, could detect 90.12% of ESBLs strains. So if we had known the best distance which we will clearly detected the "champagne cork" appearance, we practiced synergy test at 30mm. This test highlighted ESBL production in only 17 (20.99%) strains. distance reduction between C3G disks and clavulanate disk to 20 mm led to detect 73 (90.12%). Double disk test (Spanish test) detected 81 (100%), this test confirms the presence of ESBLS. By comparison with other tests, this test had the highest rate of ESBLS. In most cases; standard disk diffusion tests are effective, and still recommended for ESBL detection in routine laboratories. Nevertheless, it is worth combining standard disk diffusion test with other approaches, such as modified disk tests or E tests.

**KEYWORDS:** ESBL, *Enterobacteriaceae*, DDST, phenotypic detection, C3G disks, clavulanic acid.

## 1 INTRODUCTION

Extended spectrum β lactamases (ESBL) are a group of enzymes which have the capability of hydrolyzing third-generation cephalosporins and aztreonam (but not cephamycins and carbapenems) and which are sensitive to inhibitors such as clavulanic acid, sulbactam and tazobactam [1]. Theses enzymes are spreading among Enterobacteriaceae.

They are usually associated with resistance to multiple unrelated antibiotics such as aminoglycosides, chloramphenicol, trimethoprim-sulfamethoxazole, tetracycline, and fluoroquinolones, leaving few therapeutic choices [2]. The first ESBL was detected in Germany in 1983, among different enterobacterial isolates recovered from inpatients at intensive care units (ICUs). It was recognized by the producer strains abnormal resistance to cefotaxime and ceftazidime, which was transferable by conjugation to Escherichia coli [3]. ESBLs are usually plasmid-mediated β-lactamases, most commonly found in Klebsiella pneumoniae but also increasingly reported in Escherichia coli, Proteusmirabilis and other Gram-negative bacilli [4].

Betalactamases are increasing in number and diversification of the group of enzymes is occurring that inactivates b-lactam type of antibacterial. These can be classified into two major approaches.

The first is based on the biochemical and functional characteristics of the enzymes and the second is based on the molecular structure of the enzyme. Ambler classification is the most classification used widely [5]. That divides betalactamases into four classes (A, B, C and D) based upon their amino acid sequences. Functional classification of the b-lactamases is based on spectrum of antimicrobial substrate profile, enzyme inhibition profile, enzyme net charge, hydrolysis rate and other parameters. Bush et al presented a classification based on 4 major groups (1-4) and subgroups (a-f). According

to this classification, most ESBLs belong to group 2 B e, which b-lactamases inhibited by clavulanic acid, and can hydrolyze penicillins, narrow and extended spectrum cephalosporins and monobactams [6].

Classical ESBLs have evolved from the widespread plasmid-encoded enzymes families TEM, SHV, cefotaxime (CTX-M) and oxacillin (OXA).

TEM (Class A): TEM 1 is capable of hydrolyzing penicillins and first generation cephalosporins but is unable to attack the oxyimino cephalosporin. The first TEM variant with increased activity against extended spectrum cephalosporins was TEM [7].

SHV-type β-lactamases: Another family of β-lactamases is the SHV (sulfydryl variable) enzymes. The progenitor of the SHV enzymes, SHV-1, was first described in Klebsiella pneumoniae. SHV-1 confers resistance to broad-spectrum penicillins [8].

CTX name reflects the powerful hydrolytic activity of these betalactamases against cefotaxime [3]. They were isolated first in Munich. CTX-M is able to hydrolyze cephalothin better than benzyl-penicillin and cefotaxime better than ceftazidime [10].

OXA type (hydrolysis-oxacillin) enzymes are widespread and have been described mainly in Enterobacteriaceae and in P. aeruginosa. They usually confer resistance to the amino- and ureidopenicillin [11].

Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBL) has been found all over the world, and risk factors for acquiring these bacteria involve hospital care and antibiotic treatment. Surveillance studies are present in Europe, North America, and Asia, but there is no summarizing research published on the situation in Africa [12]. The prevalence of ESBLS in Europe is higher than in the USA but it is lower in Asia and South America [13].

## 2 MATERIALS AND METHODS

### 2.1 BACTERIAL ISOLATES

A total of 81 isolates of Enterobacteriaceae ESBLS from various samples of urine, blood, pus, came from five regions (A, B, C, D, E) of the medical care units, surgery, Gynecology and obstetrics, and Pediatrics, have been chosen in order to compare the different methods of ESBLS detection and determine the safest method. The study included patients of all ages and both sexes. The isolates were identified by following the standard laboratory procedure.

### 2.2 DETECTION OF ESBLS

Several tests for phenotypic detection based on the synergy between a third-generation cephalosporin and clavulanic acid, have been designed: double disc synergy test (DDT), ESBL E-tests, and the method of combined disk test and disk approximation Test.

#### 2.2.1 DOUBLE DISK SYNERGY TEST (25 AT 30MM)

The test was done to find a synergy image between antibiotic disk containing betalactamases inhibitor and a C3G (ceftriaxone, ceftazidime and cefotaxime) disc or a monobactam (aztreoname), this synergy image in champagne cork is characteristic of the presence of ESBL.

An Inoculum was prepared according to the technical N.C.C.L.S. of the antibiogram from a culture of 18 hours.

Mueller-Hinton agar was seeded according to the N.C.C.L.S. of the antibiogram technique, then two disks; one containing the association amoxicillin - clavulanic acid and the other a third generation cephalosporin were placed side-by-side at 3 cm distance measured Center to Center. Boxes of Petri dishes were incubated for 18 hours at 37 ° C.

The synergy image can be characteristic of the ESBL champagne Cork, or funnel corresponding to a proliferation of clavulanic acid-sensitive chromosomal betalactames.

#### 2.2.2 DOUBLE DISK TEST (SPANISH TEST)(FIG.1)

Detection of beta-lactamases spectrum expanded (or extended) can be confirmed by the double disk test. This test is gone to find an increase in the inhibition zone of a disk of C3G, preceded by the application of a disk that contains the association amoxicillin - clavulanic acid (AMC), compared to another drive on the same cephalosporin and placed side by side on the Mueller Hinton agar [14].

A bacterial suspension of opacity equal to 0, 5 MC Farland was prepared starting from 18 h culture, Mueller-Hinton agar is then seeded according to the antibiogram technique. Two disks were used, one containing an AMC, and the other containing a third generation cephalosporin (CTX). The broadcast was made at the ambient temperature of the laboratory for an hour, and then AMC disk is replaced by a disc containing the same third generation cephalosporin. Boxes petri were incubated for 18 hours at 35 ° C.

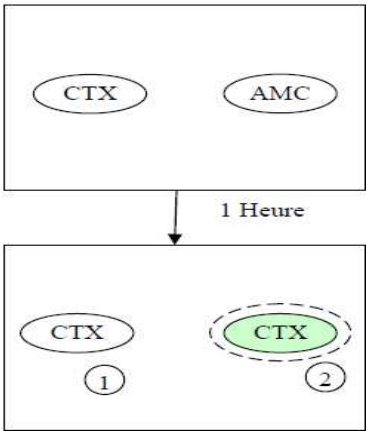


***Fig. 1. Schema of detection of ESBL in the Spanish test (Rahal 1999)***

#### 2.2.3 DOUBLE DISK SYNERGY TEST (30MM) [16]

A 0.5 McFarland of test isolate was swabbed on a Mueller-Hinton agar plate and 30 µg antibiotic disks of ceftazidime, cefotaxime, and aztreonam, were placed on the plate, 30 mm (center to center) from the amoxicillin/clavulanate (20 µg/10 µg) disk and incubated at 35°C for 18-24 hours.

A clear extension edge of the antibiotic's inhibition zone toward the disk containing clavulanate was interpreted as synergy, indicating the presence of an ESBL.

#### 2.2.4 DOUBLE DISK SYNERGY TEST (20 MM) [16]

An amoxicillin-clavulanate disk was placed at 20 mm, center to center, of ceftazidime, cefotaxime, and aztreonam disks on a Mueller-Hinton agar plate. Interpretation criteria for ESBL production were similar as those described above for the double disk synergy test (30 mm).

### 2.3 QUALITY CONTROL

Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, Proteus mirabilis ATCC 29753, Pseudomonas ATCC27853 and Escherichia coli ESBL used as a quality control of the laboratory have used as references strains.

## 3 RESULTS AND DISCUSSION

### 3.1 DISTRIBUTION OF ENTEROBACTERIACEAE SPECIES

In our study, 81 strains of enterobacteriaceae ESBL were used, the isolated organisms were as: Escherichia coli (n = 38), Klebsiella pneumoniae (n = 31), Enterobacter cloacae (n = 9), Proteus mirabilis (n = 2), and k. oxytoca (n = 1) fig.2.

Detection of ESBL Enterobacteriaceae is the challenging to microbiologists. We compared four phenotypic methods to detect ESBL production.

We worked on 81 strains of enterobacteriaceae ESBL, whose 73 (90.12%) could be using double disk synergy test (25 to 30 mm), 17 (20.99%) through double disk synergy test (30mm) (tab.1), 73 (90.12%) through double disk synergy test (20mm) (tab.2) and 81 (100%) by Double disk test (Spanish test) (fig.2).

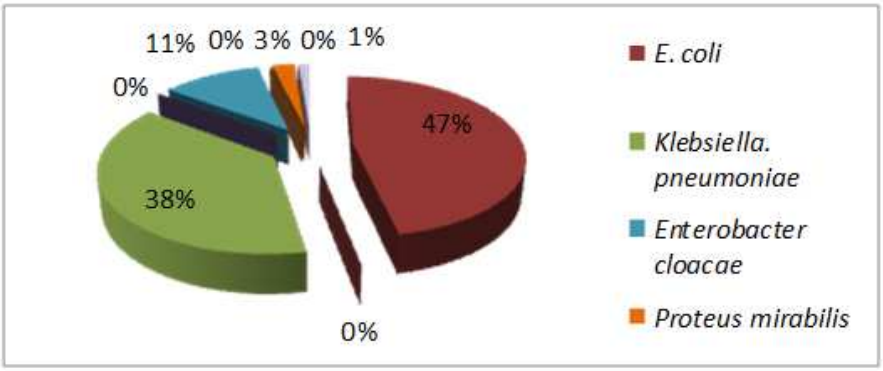


***Fig. 2. Distribution of Enterobacteriaceae species and their percentage***

***Table 1. Double disk synergy test (30mm) for 81 isolates***

| Numbers of strains tested | Results | | |
|---|---|---|---|
| | Presence of the synergy image | Description of the synergy image | Interpretation |
| 64 | - | - | High-level penicillinase |
| 17 | + | In champagne Cork | ESBL + High-level penicillinase |

***Table 2. Double disk synergy test (20mm) for 81 isolates***

| Numbers of strains tested | Results | | |
|---|---|---|---|
| | Presence of the synergy image | Description of the synergy image | Interpretation |
| 73 | + | In champagne Cork | ESBL + High-level penicillinase |

DDST: Double disk synergy test

### 3.2 DOUBLE DISK SYNERGY TEST (25 AT 30MM)

In our study we started ESBL strains detection by double disk synergy test (25 to 30mm); we could detect 90.12% of ESBL isolates.

It is an easy test, but it is the disadvantage in the fact that the synergy phenomenon become unnoticed when the inoculum is too dilute or when discs are placed far from the other [17], but also in the case of association, the same bacterium, an ESBL and a cephalosporinase [18]. Probably, that explains the 9.88% strains not detected.

"Champagne Cork" due to a synergistic effect between a disc containing the clavulanic acid, C3G disks and aztreonam. This synergy is one of the first tests to be described by Jarlier et al. [19].

So we can know the best distance which we will clearly detect the "champagne Cork" appearance, we practiced double disk synergy test at 30mm. This test has highlighted ESBL production in only 17 (20.99%) strains which is expressed by "champagne Cap" aspect, because these strains secrete their penicillinases with a high level which hides the characteristic synergy image of the ESBL.

### 3.3 DOUBLE DISK SYNERGY TEST (30MM)

The high sensitivity of the dissemination disc method when using two or more extended spectrum cephalosporins were reported previously [20, 21]. According to a study conducted in India, the combination of cefotaxime, and ceftazidime reached 81.97% sensitivity for correctly detect ESBL production, a distance of 30 mm was maintained between amoxicillin clavulanic acid and cephalosporin discs, but the inclusion of cefepime increases the sensitivity to 83.6% [16].

However, if ESBL-producing clinical isolates also have other mechanisms of resistance to β-lactams as proliferation of cephalosporinase, detection of the synergy image can be facilitated by the approximation of cephalosporin discs to the disc containing the clavulanic acid or by practicing a standard antibiogram on Mueller-Hinton agar added to 250 mg / L of cloxacillin (cephalosporinase-inhibitor) [20].

### 3.4 DOUBLE DISK SYNERGY TEST (20 MM)

To improve the sensitivity test, some authors suggested that reduction the distance between the C3G disks and the disk containing 20 mm clavulanic acid [18]. So, this test allowed us to detect 73 (90.12%) strains.

Among two tests (TSDD to 30mm, and 20 mm TSDD), we concluded when we closed cephalosporin disk to clavulanic acid disc, we increased the chances of better visualize the synergy image in "champagne Cork" (Fig. 3 and 5).

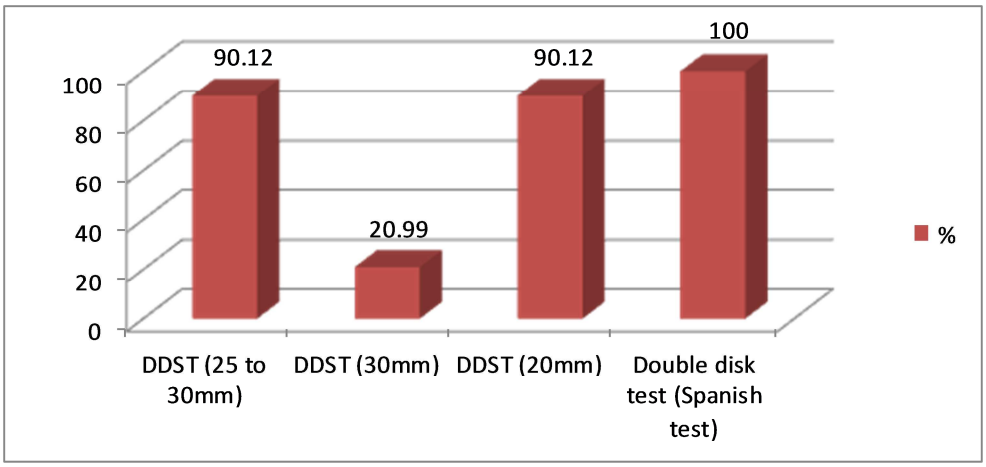


***Fig. 3. Comparison of the ESBLS identification methods***

However, by reducing the distance between disks (Centre) to 20 mm, the combination sensitivity of cefotaxime and ceftazidime is passed to 85% and even improved 88.5% due to the cefepime combination [22].

To get over the problem of the optimal disk spacing, Thomson and Sanders used recommended disk spacing to30 mm and then repeated at 20 mm to see if the old disk spacing was negative [23].

Another study conducted by Hélène Garrec and GER indicates that the combination of two or three disks CTX, CAZ or CPD allowed detecting 100% ESBL isolates [24].

Double disk synergy method by any of the four disks with a disk spacing of 20 mm detected ESBL production in 95% of enterobacteriaceae [25].Vercauteren et al found 97% sensitivity in detecting ESBL producing blood isolates and MacKenzie et al reported the sensitivity of the test > 90%, with a disk spacing <25 mm. Shukla et al from India also showed the similar results withsensitivity of 90.6% using three drugs cefotaxime, ceftriaxone and ceftazidime [26, 27, 28].

We have then passed to double disk test (Spanish test) in order to confirm the presence of ESBLS. This test has detected 81 (100%) strains. He gave us the highest rate of ESBLS in comparison with other tests.

It should be noted that the increase in diameter around the disk of cefotaxime preceded by the dissemination of the AMC was observed higher in some strains (e.g. strain No. 33) in which we noted a difference of 13 mm between two disks. This is in relation to the variation of phenotypic expression in vitro of ESBL and the level of their productions (Fig.4).

The affirmation of extended-spectrum betalactamases detection was observed in 35 strains by the double disk test in a study conducted in Algeria [14].

Our methods found certain complementarity: the first method (DDST 25 to 30mm) is a valid technique for enterobacteriaceae producing ESBL non-producing cephalosporinases. Other methods are complementary to the synergy test in the situations described above.

So, it turned mandatory the use of the cephalosporin disks approximation to the disk containing clavulanic acid (TSDD 20mm) and double disk test (Spanish test) in order to detect ESBL strains that can escape from one of the two methods.


*Fig. 4. Strain No. 33 positive double disk Test: the strain produces an ESBL. (The diameter of the inhibition zone around the CTX disc preceded by dissemination of the AMC disc = 33mm compared to the CTX = 20mm)*


*Fig. 5. Strains No 69: TSDD 30mm and TSDD 20mm. Synergy 'in champagne Cap" is clearer for the 20mm while she is absent for 30mm*

## 4 CONCLUSION

1. In conclusion, phenotypic confirmatory method preferably disk potentiation recommended by Clinical and Laboratory Standards Institute (CLSI) with at least two third generation cephalosporin disks should be used in clinical microbiology laboratories for the accurate detection of ESBL.
2. Considering the challenging nature of the isolates, the four phenotypic methods were highly sensitive and specific at ESBL detection, with the double disc test (Spanish test) (100 % sensitivity) being the most sensitive.
3. Our methods found certain complementarity: the first method (DDST 25 to 30mm) is a valid technique for enterobacteriaceae producing ESBL non-producing cephalosporinases. Other methods are complementary to the synergy test in the situations described above.
4. So, it turned mandatory the use of the cephalosporin disks approximation to the disk containing clavulanic acid (TSDD 20mm) and double disk test (Spanish test) in order to detect ESBL strains that can escape from one of the two methods.
5. High prevalence of ESBL emphasizes the need to adopt appropriate control measures to reduce the ESBL burden.

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