

RESEARCH ARTICLE

Detection of Extended-Spectrum β -lactamases Production by *Escherichia coli*: A Phenotypic Comparative Study

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Abstract

Resistance to antibiotics is a serious concern and immediate steps are required to curb the resistance gained by microorganisms. Extended spectrum beta- lactamases (ESBLs) producing *E.coli* (*Escherichia coli*) is rapidly increasing and making antibiotics difficult to treat around the world. The screening of ESBL producers among *Escherichia coli* was done by method Double Disk Synergy Test (DDST), using ceftazidime and ceftriaxone disc and Combined Disc Test (CD) using ceftazidime-clavulanic acid according to Clinical and Laboratory Standard Institute (CLSI) protocol. In the DDST method, out of 60 clinical isolates of *Escherichia coli*, 22 were found ESBL positive isolates, and among 22 positive isolates 18 strains shows increase in zone of inhibition by both ceftazidime and ceftriaxone disc. The remaining 4 ESBL isolates, 3 of them showed zone of inhibition to ceftazidime and only 1 isolate showed inhibition by ceftriaxone disc. Similarly, in CD method apart from 22 isolates detected by DDST method it detected 1 more isolate as ESBL producers. Furthermore, the CD method detects inducible beta- lactamase in III generation of cephalosporin sensitive isolate. Our study showed 22 isolates were found by DDST method and 23 isolates by CD method. This study results showed the prevalence of ESBL in *E. coli* were 36.66% by DDST and 38.33% by CD tests and we conclude that combined disk test (CD) is better than DDST for detection of ESBL.

Keywords: Extended-spectrum β -lactamase, *Escherichia coli*, Antibiotics, India.

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INTRODUCTION

The beta lactam antibiotics are hydrolyzed by Extended-spectrum β -lactamases (ESBL) producing microorganism (*Klebsiella species*, *Acinetobacter*, *Pseudomonas aeruginosa*, *Citrobacter*, *Proteus*, *Enterobacter* and *Escherichia coli*) and is a major challenge in infectious diseases (Aminzadeh Z et al., 2008; Khanfar et al., 2009). Antibiotic resistant strains of *Escherichia coli* produce an enzyme known as ESBL and such strains are resistant to other class of antibiotic (Jacoby GA et al., 2005; Livermore DM et al., 2007). This makes the pathogen hard to treat. ESBL producing strains cause a wide range of infections from septicemia to urinary tract infection (Thakur S et al., 2013). The main target of ESBL producing pathogens are elderly people and those who have been recently admitted in the hospital and are taking antibiotics. The ESBLs are typically plasmid mediated enzymes that hydrolyze penicillin, third-generation aztreonam and cephalosporin. They are not active against cefotetan, ceftiofur and cephamycins but are susceptible to clavulanic acid. Other, Antibiotics classes like, fluoroquinolones and aminoglycosides the ESBL are resistant (Egea P et al., 2011; KOs et al., 2007; Yadav et al., 2015). Plasmids play an important role in resistance to cephamycins involving amp C gene. The oxyimino group addition to the C-7-amino position of second and third generation of cephalosporin results in the broad spectrum cover to Enterobacteriaceae. The attachment of beta lactamase to the antibiotic is prevented by oxyimino group (Kangh et al., 2005; Sachin et al., 2011). Furthermore, uncontrolled use of aztreonam and cephalosporin (Third generation cephalosporin) results in the mutation in the enzymes and which in turn cause the emerge of ESBL producing pathogens (Grover et al., 2006; Sharma et al., 2013; Kumar et al., 2014). The double disk test method is primarily used to detect the ESBL producing microorganism. The aim of this study is to find the ESBL producing *E. coli* from non ESBL producing strains.

MATERIALS AND METHODS

The clinical isolates of *E. coli* strains were obtained from specimens (urine, blood, wound, pus, and semen) from patients admitted in different wards of Fortis Hospital, Noida, India between February 2016 to May 2017 following

standard procedures CLSI. A total of 60 samples of *E. coli* were collected of all age groups. After receiving the clinical isolates in the laboratory they were immediately cultured on MacConkey agar for further study. Standard protocol was followed for the identification of *E. coli* (Collee et al., 1996; Bailey and Scott's 2007). Permission for work was approved by the Ethical committee of the institute and consent from patients.

Extended-spectrum β -lactamase Screening of the isolates

An inoculum of 0.5 McFarland dilutions of all test strains, negative control and positive control were prepared in 3 ml of normal saline. Streak a lawn of all dilution of all test strains, negative control and positive control to a Mueller Hinton agar plate and allow to dry 3–5 minutes. After drying, an amoxicillin-clavulanic acid (20/10 μ g) disc was placed at the center of the plate. A disc of ceftazidime (30 μ g) and ceftriaxone (30 μ g) were placed one on either side of amoxicillin-clavulanic acid (20/10 μ g) disc at a distance of 15 mm from center to center. All plates were incubated at $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in incubator for 16–24 hours. *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 700603) producing beta-lactamase were used as Negative control and positive control. After the incubation of 24 hr the culture plate was studied for zone of inhibition in an area between ceftriaxone and amoxicillin clavulanic acid in comparison with the zone of inhibition on the far side of these drugs was taken as a positive.

Double disc synergy test (DDST)

Muller Hinton Agar culture plate was used as standard disk diffusion assay. The ceftriaxone of 30 microgram and ceftazidime of 30 microgram (15mm apart), including a disk of augmentin 20 +10 micrograms) disks were used for screening of ESBL producing microorganism (Wayne, PA, 2013). The zone of inhibition of either ceftriaxone or ceftazidime towards amoxicillin and clavulanic acid is considered as ESBL producing pathogen and is known as confirmatory phenotypic test.

Combined Disc Test (CD)

The ESBL producing group I (class A) microorganism are inhibited by clavulanic acid and for such type of strains third generation of cephalosporins along with clavulanic acid can be used as confirmatory test (Kang HY et al., 2005). If the zone of inhibition restricts to third generation

of cephalosporin only, than it can be considered as confirmatory test for ESBL. An inoculum of 0.5 McFarland dilutions of all test strains, negative control and positive control were prepared in 3 ml of normal saline with the help of 0.5 McFarland standards. Streak a lawn of all dilution of all test strains, negative control and positive control to a Mueller Hinton agar plate and allow to dry 5 minutes, than aseptically a disk of ceftazidime (30µg) -clavulanic acid (10µg) and ceftazidime (30µg) were placed at a 30 mm of distance. All plates were incubated at 35°C in incubator for 24 hours. Non-ESBL producing organism *E.coli* and an ESBL-producing organism *K. pneumoniae* were used as negative and positive controls respectively. After 18 hr of incubation the culture plate is examined for the zone of inhibition. The 5mm zone of inhibition of (Ceftazidime-Clavulanic acid) is greater than that of ceftazidime than it will be taken as positive for ESBL production.

RESULTS

Double Disk Synergy Test (DDST)

A total of 60 non duplicate isolates of *E. coli* were included in the study. Out of 60 strain, 46 (76.66%) were isolated from urine, 4(6.66%) from wound, 4 (6.66%) from pus, 2 (3.33%) from semen and 4 (6.66%) from blood (Figure 1). All 60 non duplicate isolates of *E. coli* isolates were tested by DDST, detected 22 (36.66%) strains as ESBL producers (Figure 2), among which 15(25%) isolates from urine, 2 (3.33%) from pus, 2 (3.33%) from wound, 2 (3.33%) from blood and 1 (1.66%) from semen (Figure 3). Out of 22 ESBL positive isolates, 18 isolates produced increase zone of inhibition by both ceftazidime and ceftriaxone disc (Figure 4). While in remaining 4 isolates, 3 isolates produced increase zone of inhibition by only ceftazidime and 1 isolates by ceftriaxone disc.

Combined Disc Test (CD)

All 60 non duplicate isolates of *E. coli* isolates were again tested by Combined Disc Test, detected 23(38.33%) strains as ESBL producers, among which 16 (26.66%) isolates from urine, 2 (3.33%) from pus, 2 (3.33%) from wound, 2 (3.33%) from blood and 1 (1.66%) from semen (Figure 3). The isolates exhibited a significant zone size enhancement with the combination disk (ceftazidime -clavulanic acid) when compared to the plain antibiotic (ceftazidime) discs (Figure 5).

The CD detected the same 22 strains of DDST as well as additional 1 strain as ESBLs producers. The CD test also detects inducible B- lactamase in 3rd generation cephalosporine sensitive isolates. Only one isolate is sensitive to ceftazidime but it is detected as ESBL producer.

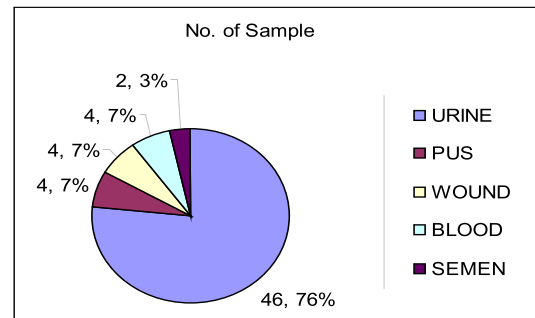


Fig. 1. Distribution of sample collection.

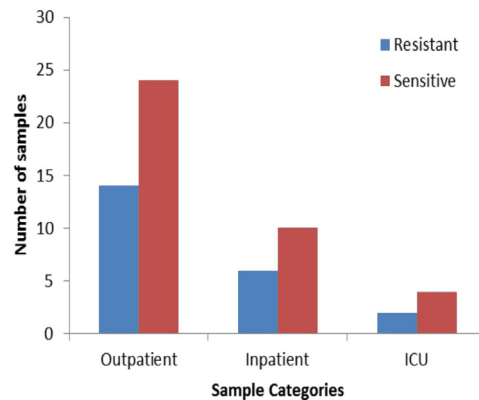


Fig. 2. Distribution of antibiotic resistance pattern of Escherichia coli isolated from patients

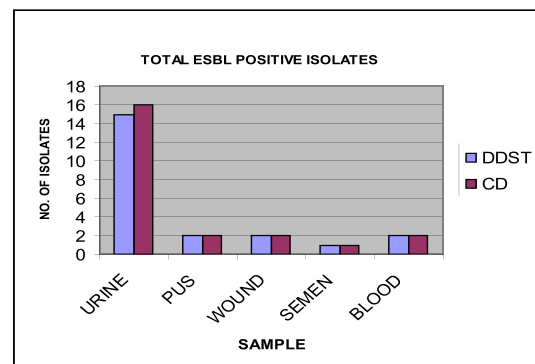


Fig. 3. Extended spectrum beta- lactamases producing isolates from various samples using phenotypic methods Double Disk Synergy Test(DDST) and Combined Disc Test (CD) method.

Among 23(42%) metallo beta lactamase (MBLs) producing isolates, 16(30%) isolates *E. coli* exhibited zone of inhibition difference between combination disk (ceftazidime -clavulanic acid) and ceftazidime disc alone were 6 to 10 mm. Remaining 7 (12 %) isolates exhibited e" 10 mm difference zone. Remaining 47 *E. coli* isolates had a smaller difference (less than 5 mm) in zone of inhibition around the combined disk than around the ceftazidime disc alone and thus they were considered to be non ESBL producer.

Comparison between double disk synergy test and combined disc test

The possible presence of ESBL-producing strains was tested among 60 *E. coli* strain by Double Disk Synergy Test (DDST) and Combined

Disc Test (CD) (Figure 3). Above two methods DDST and CD detected ESBLs producer isolates were 22 (36.66%) and 23 (38.33%) respectively (Table 1). The CD detected the same 22 strains of DDST as well as additional 1 strain as ESBL producers. When procedures of DDST were repeated for above 1 (firstly negative) strain these become positive for ESBL in repeated DDST test. The phenotypic methods CD and DDST are most reliable for ESBL detection in *E. coli*. However, the DDST has a disadvantage as distance varies with the strains. Our study confirms that the CD is far more accurate than DDST for ESBL producing bacteria. This method can be used a key to monitor ESBL emergence.

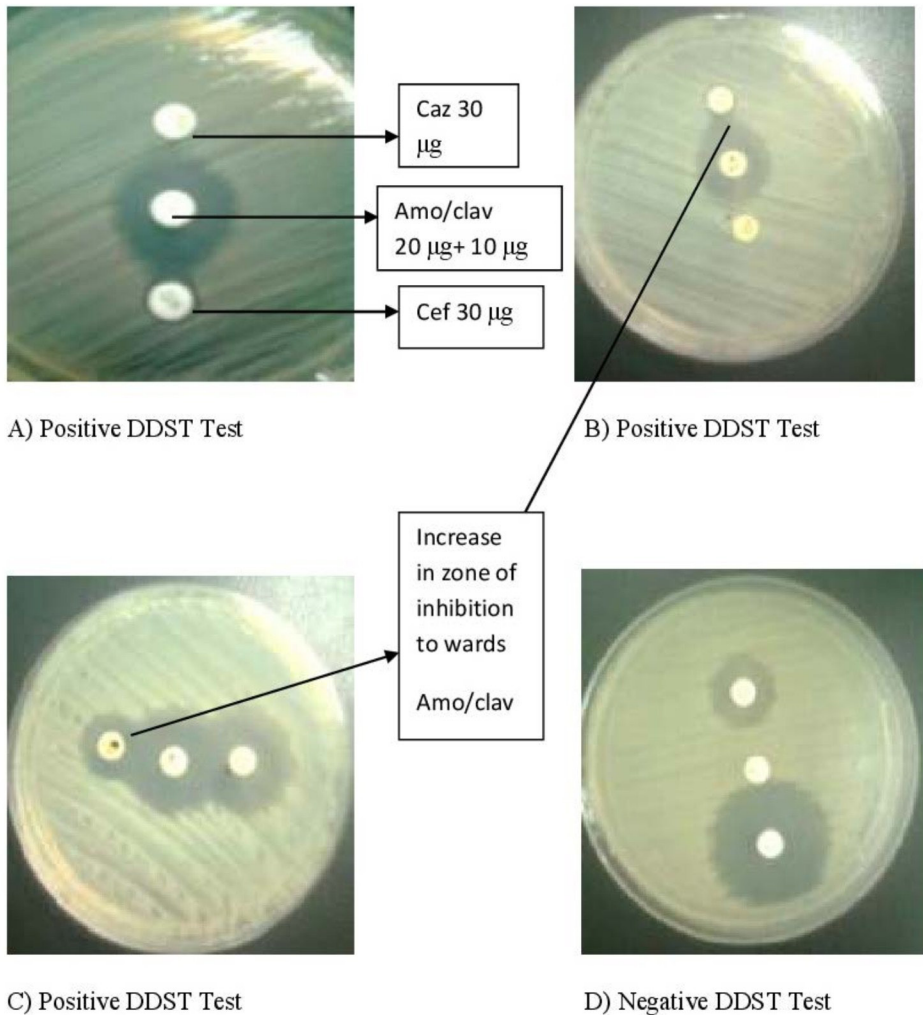


Fig. 4. Extended-spectrum β-Lactamase (ESBL) Positive Isolates by Double Disc Synergy Test (CD)

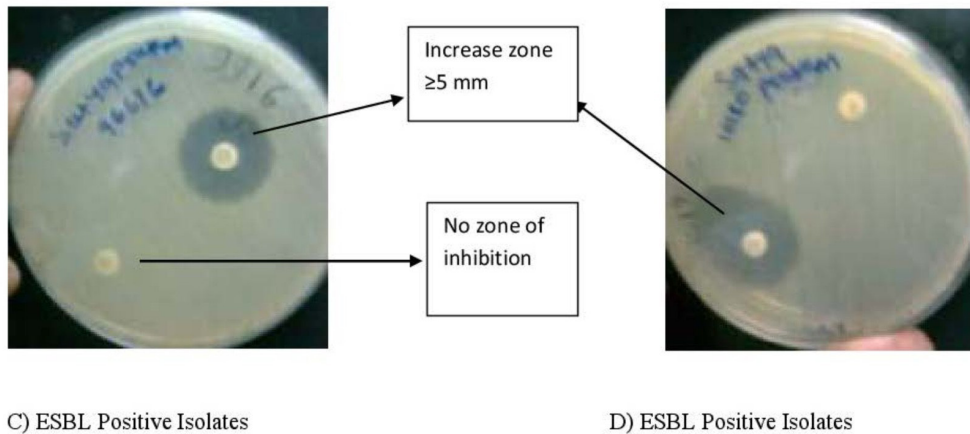
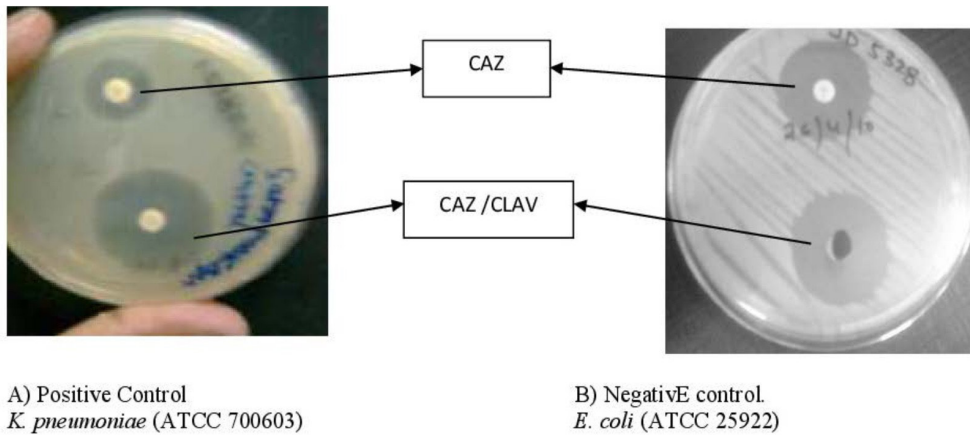


Fig. 5. Extended-spectrum β -Lactamase (ESBL) Positive Isolates by Combined Disc Test (CD)

Table 1. The number of ESBL producing *E. coli* in various samples by phenotypic method.

| Sample | DDST | CD |
|--------|------|----|
| Urine | 15 | 16 |
| Pus | 2 | 2 |
| Wound | 2 | 2 |
| Semen | 1 | 1 |
| Blood | 2 | 2 |

DISCUSSION

In the developing nations the emergence of ESBL producing pathogens is high and causing serious issues worldwide (KMendelsonG et al., 2005; Kumar et al., 2006; Bazzaz et al., 2009; ronenberg et al., 2013). In United States the *E. coli*

and *Klebsiella pneumoniae* are associated with ESBL production, hence; efficient methods are required to rapid detection of the ESBL producing pathogens of Enterobacteriaceae family (Kumar et al., 2006). Conventional susceptibility testing methods, on their own, fail to offer reliable susceptibility results for β -lactam antibiotics when testing those species that harbor ESBLs. 30-90 % of ESBL producers are found in various hospitals (Yadav KK et al., 2015). Our study shows the occurrence of ESBL was 36.66% in *E. coli* by DDST method and 38.33% by CD method, we are in agreement with DDST method which shows 95.65% as compared to CD test. The antibiotic resistance is a major issue to public health and patient care. The *E. coli* is highly resistant to penicillin and less

resistant to imipenem (Zhan et al., 2008). ESBL producing pathogens created a significant issue as these microbes are resistant to a wide range of antimicrobials (third generations of cephalosporins) and also have plasmid borne resistance to carbapenem (Lautenbach et al., 2001; Villa et al., 2000). Multidrug resistance has complicated the antibiotic choice (Rath S et al., 2014). Our study shows there is an increase in ESBL producing pathogens with time and there is a need of control measures to be taken to stop the misuse of antibiotics. Amikacin remains a drug of choice to treat the diseases caused by ESBL producing pathogens. This class of drug can be used to prevent the spread of ESBL resistance frequency. Molecular work is required to know more about the genes in the ESBL producing pathogens.

Poor sensitivity in DDST is mainly due to the lack of optimum disc space. Results in the impaired ESBLs inhibition by clavulanate leads to the inaccurate identification of chromosomal cephalosporinases producing ESBLs strain in the Muller-Hinton agar medium. Earlier studies report various alternative methods to overcome this disadvantage of DDST such as reduction of disc space from 20 mm to 30 mm to obtain more accurate results (Thomson KS et al., 1992) whereas, in our study we placed the ceftazidime and ceftriaxone disks at a distance of 15 mm from AMC disk to obtain more sensitive results as reported by Coudron PE et al., 1997).

The primary initial screen test for ESBL positive *E. coli* isolates is the CLSI method in many hospital based microbiology laboratories. The delayed results from this CLSI method may adversely affect the patient treatment; hence, there is a need to develop a reliable, sensitive, and rapid ESBL detection method. In this regard, in this study we tested an accelerated hybrid protocol to overcome the cons of the CLSI method. In our study we screen the ESBL strains by incorporating the combined disk test in the standard KB (Kirby-Bauer) disk diffusion antimicrobial susceptibility test to obtain the more accurate and rapid sensitive results. This new practiced hybrid protocol allowed us to detect the ESBL producing strain same day along with the first line antimicrobial susceptibility test and aided to improve the patient care.

In our study, we found that the combined disk test is reliable, convenient, cheap and easy

to perform than double disk synergy test. It also reduces the time of reporting and antimicrobial therapy. By placing a disc of ceftazidime / clavulanic acid as an extra disc for ESBL detection in *E. coli* routine susceptibility on MHA plate, the comparison between zone of inhibition difference ceftazidime and ceftazidime / clavulanic acid gives early detection of ESBL producing isolates. The sensitivity and specificity of the combined disc test is higher than that of the DDST as described above. Thus, the combined disk test (CD) can be easily used in routine laboratory of developing countries including India.

CONCLUSION

Our study validates a simple and highly sensitive phenotypic method for the detection of ESBL production in *E. coli*. Based on the findings of our study we conclude that CD is better than DDST for detection of ESBL. As, the CD test was able to detect more than one strain whereas, DDST test is limited to single strain only. Furthermore, CD test has high specificity and sensitivity than DDST. In our study the prevalence of ESBL in *E. coli* was 36.66% by DDST and 38.33% by CD tests.

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