Original article

Phenotypic detection of Extended Spectrum Beta-Lactamases and Amp C Beta-Lactamases among nosocomial isolates in a tertiary care hospital

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Abstract

Background & objectives: Extended-Spectrum Beta-Lactamases (ESBLs) is an important resistance mechanism in Enterobacteriaceae infections. Lack of standard guidelines from Clinical Laboratory Standards Institute (CLSI) for Amp C beta-lactamase detection poses a problem. This study was undertaken to detect ESBLs by phenotypic tests and Amp C beta-lactamase by inhibitor based method. Material and Methods: 200 consecutive non-repetitive isolates of E.coli, Klebsiella and Proteus from clinical samples were screened for ESBLs as per CLSI guidelines and confirmed by PCDT, DDST and E-tests (AB Biodisk, Biomerieux). Amp C beta lactamases were screened by cefoxitin resistance and confirmed by inhibitor (Cloxacillin) based method. Simultaneous occurrence of Amp C and ESBLs was detected by combined disk test (Neo-Sensitabs and Diatabs). Descriptive and Kappa statistics were used. Results: Out of 200 isolates studied, 131 were initially screened as ESBL producers and later 114 (57%) were confirmed by phenotypic methods. E-Test was found most sensitive phenotypic test as compared to PCDT and DDST. 13 strains resistant to cefoxitin (30µg) were found to be pure Amp C producers. Combined disk test detected 36 to be ESBL and Amp C co-producers. Surprisingly, six isolates found sensitive to cefoxitin disk were confirmed as Amp C producers by cloxacillin disk inhibition test. Conclusion: 57% ESBLs and 27.5% Amp C producers were isolated from nosocomial pathogens showing significant resistance to 3rd generation cephalosporins. Phenotypic confirmation by E-test, PCDT & DDST were useful for ESBL identification and for detection of Amp C, cloxacillin was found to be an effective inhibitor.

Key words: Amp C beta-lactamases, beta- lactamase/ beta- lactamase inhibitor combinations, Extended spectrum beta-lactamases, inhibitor based tests.

Introduction

ß-lactam antibiotics are the most commonly used antimicrobials for empirical therapy. Production of ß-lactamases is one of the main mechanisms of developing bacterial resistance to these classes of antibiotics. Ampicillin hydrolyzing classical ß-lactamases (TEM-1, TEM-2 & SHV-1) became quite prevalent in gram negative pathogens soon after extended spectrum penicillins like ampicillin and amoxicillin started being used worldwide in 1970s. The total number of ESBLs characterized now exceeds 200.[1] The first report of plasmid encoded ß-lactamase capable of hydrolyzing the extended spectrum cephalosporins was published in 1983 from Germany.[2] ESBLs are plasmid mediated ß lactamases that are derived from the mutations in older ß-lactamases like TEM-1, TEM-2 & SHV-1 in one or more amino acids. These new ß-lactamases were

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coined as Extended-spectrum- ß-lactamases (ESBLs) because of their spectrum of activity against oxyiminocephalosporins (third generation cephalosporins). Thus, ESBLs are ß-lactamases capable of conferring bacterial resistance to the penicillins; first, second & third generation cephalosporins; and aztreonam by hydrolysis of these antibiotics (but not the cephamycins and carbapenems) and are inhibited by ß-lactamase inhibitors (BLI) such as clavulanic acid, sulbactam and tazobactam.[3]

Amp C ß-lactamases first appeared in the late 1970's and are usually resistant to penicillins, cephalosporins including the cephamycins and monobactams but are usually sensitive to the carbapenems. Unlike ESBLs, these enzymes are not inhibited by BLI (clavulanic acid, sulbactam, tazobactam) but by inhibitors, such as boronic acid and cloxacillin. This lack of inhibition by cephamycins and BLI differentiate Amp C ß-lactamases from ESBLs and is confirmed by using inhibitor based tests. ESBLs are grouped by the Ambler structural classification as molecular class A and by the Bush-Jacoby-Medeiros functional classification as functional class 2be. Amp C ß lactamases are classified in Ambler molecular class C and Bush-Jacoby-Medeiros functional class 1. Plasmid mediated Amp C ß-lactamases have arisen through the transfer of chromosomal genes for the inducible Amp C ß-lactamases onto plasmids as seen in isolates of E.coli, Klebsiella pneumoniae, Salmonella spp., Citrobacter freundii, Enterobacter aerogenes and Proteus mirabilis.[4,5] Gram-negative organisms that produce both ESBLs and Amp C beta-lactamases are increasingly being reported worldwide.[6] These organisms usually exhibit multidrug resistance. Detection of such complex resistance phenotypes is a serious challenge faced by clinical microbiology laboratories thereby contributing towards the uncontrolled spread of ESBL producing organisms and related treatment failures. Their early detection by laboratory methods allows selection of targeted antibiotics thereby minimizing the risk of further development of resistance.

Materials and Methods

A total of 200 isolates of E. coli, Klebsiella sp and Proteus spp isolated from clinically significant samples received from hospitalized patients during Jan-Apr 2012 were included. Antimicrobial susceptibility testing was done by using the Kirby Bauer method. Ethical approval was taken from local ethical committee before study.

ESBL Detection: E. coli, Klebsiella and Proteus isolates with reduced susceptibility to any of the third generation cephalosporins (3GC) in the initial screening tests as recommended by CLSI guidelines 2010, were processed for confirmation of ESBL production. The standard strains of E. coli ATCC 25922 (ESBL negative), and K. pneumoniae 700603 (ESBL positive) were used as controls. One or more of the following antimicrobials were used to screen ESBL producers i.e. cefpodoxime (30µg), ceftazidime (30µg), aztreonam (30µg), cefotaxime (30µg) and ceftriaxone (30µg) according to the zone size diameters in millimeters (mm) as per CLSI guidelines (Table1).

Antibiotics	Zone diameters
Cetazidime (30µg)	≤22mm zone
Cefotaxime(30µg)	≤27mm zone
Ceftriaxone (30µg)	≤25mm zone
Cefopodoxime (10µg)	≤17mm zone
Aztreonam (10µg)	≤27mm zone

Table 1: CLSI screening criteria for ESBL detection

Isolates initially suspected to be ESBL producers by screening test were further subjected to phenotypic confirmatory tests such as Phenotypic confirmatory disk diffusion test (PCDT), Double Disk Synergy test (DDST) and E-test. Any isolate found positive by any of the three confirmatory tests were considered to be an ESBL producer.

Confirmatory Tests for ESBL Producers

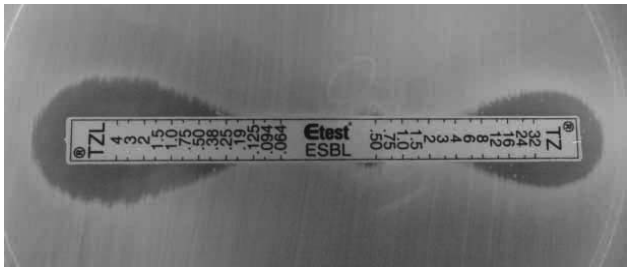
Phenotypic confirmatory disk diffusion test (PCDT): A standard inoculum of each isolate was made and a lawn culture was obtained by a suspension adjusted to a turbidity equivalent of 0.5 McFarland. A ceftazidime (30 µg) antimicrobial disk and a ceftazidime-clavulanic acid (CAZ-CLA, 30/10µg) antimicrobial disk were applied to the surface of the plate with minimum distance of 25mm from center to center. The plate was then incubated over night at 37°C in ambient air for 16-20 hours. A =5mm increase in the zone size diameter for 3GC tested in combination with clavulanic acid versus its zone when tested alone was suggestive of ESBL producer. Similar tests were performed using cefotaxime (30 µg) and cefotaxime with clavulanic acid (30/10µg) to further substantiate the results.

Double Disk Synergy test (DDST): MHA plates were inoculated with standardized inoculum (0.5 McFarland) of the isolates to form a lawn culture. 30 µg disc of each ceftazidime (30µg) was placed on the agar at a distance of 30 mm centre to centre from

Amoxyclav (20/10µg) disc. ESBL production was interpreted if the inhibition zone around the test antibiotic disk showed zone of potentiation towards a source of BLI.

E-test: The ceftazidime/ceftazidime-clavulanate (TZ-TZL) ESBL E-test strip with stable concentration gradient of ceftazidime (MIC test range, 0.5-32 mg/L) on one end and a gradient of ceftazidime (MIC test range, 0.064-4mg/L) plus 4 mg/L clavulanic acid at the other. The E-test procedure, reading and interpretation were performed according to the manufacturer's instructions (ABO Biodisc, Biomerieux). MIC is interpreted as the point of intersection of the inhibition ellipse with the E-Test strip edge. A more than eight fold reduction in the MIC values of ceftazidime-clavulanate combination as compared to the ceftazidime alone (Fig.1)

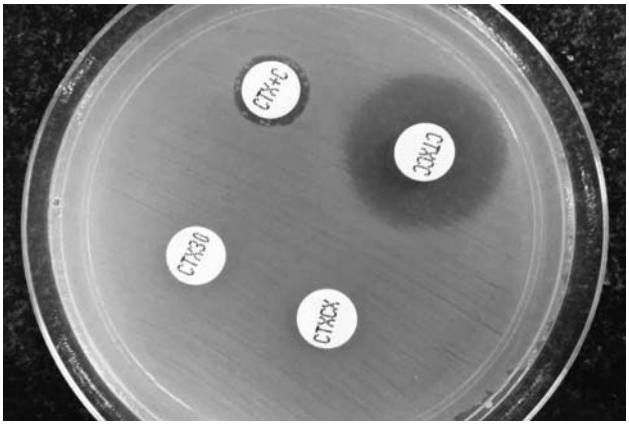
Figure 1: E-Test with ceftazidime (TZ)/ceftazidime



clavulanic acid (TZL) strip showing MIC of TZ/TZL = 8 in an ESBL isolate along with a small "Phantom zone".

or observation of "phantom zones" or deformation of inhibition ellipses were indicative of ESBL production. Phenotypic Tests for Amp C-Production: All isolates resistant to cefoxitin (30µg) with inhibition zone <16mm, were considered to be putative Amp C producers and were further confirmed using inhibitor based tests with cloxacillin (500µg) using Neo-sensitabs and Diatabs (Fig. 2).

Figure 2: Organism showing enhanced zone of sensitivity (=5mm) with ceftazidime/ clavulanate/cloxacillin disk as compared to ceftazidime/clavulanate disk and ceftazime cloxacillin disk on Combined disk test showing ESBL and Amp C production respectively



Combined disk test (ESBL+Amp C) (Rosco Diagnostica) was used for detection of ESBLs and Amp C simultaneously and the results were interpreted as per guidelines from European Committee on Antimicrobial susceptibility testing (EUCAST). An increase in zone size of =5mm for cefotaxime+cloxacillin (CTXCX) as compared to cefotaxime (CTX30) alone or for Cefotaxime+Clavulanic acid+Cloxacillin (CTXCC) as compared to Cefotaxime+Clavulanic acid (CTX+C) was indicative of Amp C production (Table 2).

CTX-Cefotaxime, CTX+C-Cefotaxime+Clavulanic acid, CTXCX- Cefotaxime+Cloxacillin, CTXCC-Cefotaxime+Clavulanic acid+Cloxacillin.

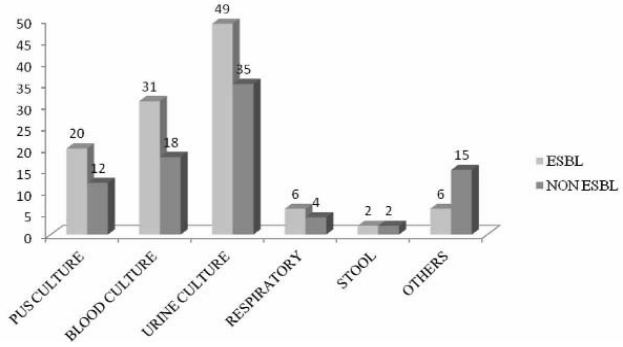
Table 2: Combined disk test (ESBL + Amp C)

		Cefotaxime CTX30	Cefotaxime+ Clavulanate CTX+C	Cefotaxime +Cloxacillin CTXCX
ESBL	CTX+C	≥5 mm	-	-
	CTXCC	-	< 4 mm	≥5 mm
Amp C	CTXCX	≥5 mm	-	-
	CTXCC	-	≥5 mm	< 4 mm
ESBL+ Amp C	CTX+C	< 4 mm	-	-
	CTXCC	-	≥5 mm	≥5 mm

Descriptive analysis and percentages for categorical variables were worked out. Kappa statistics including Cohen's unweighted Kappa, Kappa with lineage weighting, Kappa with quadratic weighting along with frequencies and proportion of agreement. Strength of agreement between 0.8-1 was taken as perfect, 0.6-0.8 as substantial and 0.4-0.6 as moderate.

Results

A total of 200 clinical specimens which constituted urine, stool, blood and body fluids (Fig. 3) were collected from medical wards, surgical wards,



ICU, transplant centers and burn center (Table 3).
The predominant isolates were E coli, Klebsiella

Table 3: ESBL distribution in wards

Ward	No of isolates	ESBL Producers	Percentage (%)
Medical	47	23	48.93
Surgical	73	32	43.83
ICU	50	37	74
Transplant Centre	19	15	78.94
Burn Center	11	7	63.63
Total	200	114	54

pneumonia and Proteus (Table 4).
131 ESBL producers found initially positive by

Table 4: Distribution of isolates

Microorganism	No of isolates screened	ESBL producing organism	Suspected AmpC producers	Confirmed AmpC producers
E. coli	129	77	31	28
Klebsiella	67	37	28	26
Proteus	4	0	2	1
Total	200	114	61	55

screening test (CLSI 2010) were further subjected to confirmatory tests such as E-Test, PCDT and DDST. 114 isolates were found as confirmed positive by these tests (Table 5).

Table 5: Comparative analysis of various tests for ESBL production

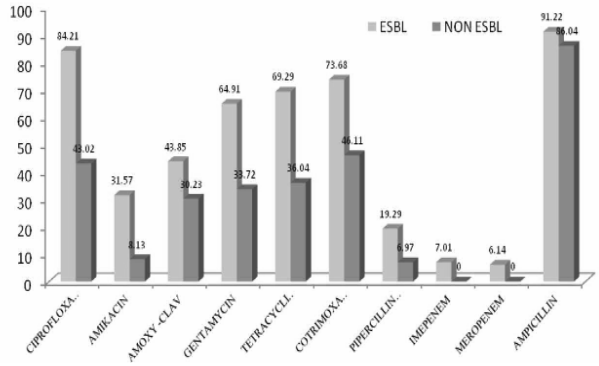
E-Test strips (AB Biodisk, Biomerieux) identified

	Screen positive	E-Test	PCDT	DDST	Confirmed ESBL
Isolates	131	112	109	92	114
Percentage	65.5%	56%	54.5%	46%	57%

112 ESBL producers, 109 were identified by PCDT which included 2 isolates not identified by E-Test and 92 were identified by DDST. There were 17 isolates which were resistant to one or the other 3GC but were not found to be ESBL producers by confirmatory tests.

Of total 114 confirmed ESBL producers, 42 isolates were found to be resistant to cefoxitin and hence were considered putative Amp C producers but on confirmatory tests using cloxacillin, only 36 isolates were finally confirmed as Amp C-ESBL co-producers. 78 isolates were found to be pure ESBLs and 13 isolates to be pure Amp C producers. Surprisingly, six Amp C producers were identified by combined disk test amongst the cefoxitin sensitive isolates. Hence the percentage of Amp C producers was found to be 27.5%. A comparative analysis of the antimicrobial susceptibility amongst the ESBL and non ESBL isolates was done (Fig. 4).

Figure 4: Comparative resistance among ESBL



and Non ESBL isolates

Statistical correlation of these tests was done by Kappa analysis. E-test was able to give exact MICs and hence was considered as reference test and other tests were compared against it. Sensitivity, specificity, positive and negative predictive values (PPV and NPV) of PCDT against E-Test was 95.53%, 97.73%, 98.17% and 94.51% respectively while that of DDST against E-Test was 82.14%, 100%, 100% and 81.48% respectively. Strength of agreement was perfect between PCDT and E-test and substantial between DDST and E-test.

Discussion

Emerging multidrug resistant nosocomial pathogens are of great concern in patients hospitalized for prolonged period. Third generation cephalosporins are the mainstay in empirical treatment of patients admitted in critical care centres, but alarmingly high number of cases resistant to these antibiotics are now being reported.[7] Recent studies revealed that patients with infection such as septicaemia with ESBLs producing organisms had significantly higher fatality rate than those with non-ESBL isolates.[8] The occurrence of ESBL among clinical isolates varies greatly worldwide and geographically and is changing rapidly over time.[9] Present study was undertaken to study the prevalence of ESBL producing strains of E.coli, Klebsiella and Proteus at our tertiary health care centre with a view to update the existing antibiotic policy of the hospital. The study predominantly included referred patients who had already received antimicrobial therapy; high risk patients at extremes

of age and having underlying diseases and patients who have received multiple antimicrobial chemotherapy. The prevalence of ESBLs in our study was found to be 57%. Maximum number of ESBL producers were isolated from clinical samples of patients from transplant centre and ICU, especially those with invasive devices such as indwelling catheters, endotracheal or nasogastric tubes, gastrostomies and tracheostomies. Non-judicious use of third generation cephalosporins is the other likely factor contributing towards this high prevalence of ESBLs.10,11 A similar study done earlier also found the increased isolation rate from such wards with patients who have received prior multiple antimicrobial treatments with high end antibiotics and have undergone multiple admissions.[12] The presence of ESBL in the present study was found to be 57% which is much lower than that found in most other tertiary care centers. Various studies from different parts of the country showed varying prevalence of ESBLs ranging from 66.66% to 91.77%.[10,13-17] The lesser ESBL isolation rate from our hospital may be attributed to the stringent adherence of the infection control measures by the hospital staff and proper institution and execution of the hospital antibiotic policy.

PCDT test was found to be a more sensitive alternative to the DDST for the detection of ESBLs. The DDST showed lower sensitivity of test result due to technical error in spacing of discs or improper storage. Different studies have also reported DDST to be less sensitive than PCDT.2,[18] E-Test strips were found to be the most sensitive of all with additional advantage of giving the exact MIC for each isolate.[19] Use of cefotaxime disc could detect more number of ESBLs, reflecting the presence of large number of CTX-M variants prevalent in nosocomial isolates. CTX-M types is a large group of ESBLs which have potent hydrolytic activity to cefotaxime.3 CTX-M enzymes are characterized by their ability to cause resistance to cefotaxime but remain sensitive to ceftazidime.[20] A notable exception to this is CTX-M-15, the most common CTX-M variant, which can also cause resistance to ceftazidime in addition to cefotaxime. Over the past decade CTX-M enzymes and the organisms producing them (most commonly E. coli) have emerged around the world and have become the most prevalent ESBLs.[21]

Clavulanic acid inhibits the ESBL enzyme activity and BL/BLI combinations are the treatment of choice for ESBL producers. Carbapenems seem to be the most effective alternative treatment for ESBL producers because ESBLs cannot hydrolyse carbapenems nor (unlike Amp C types) cephamycins such as cefoxitin and cefotetan. In the present study 17 isolates were found resistant to any of the 3GC but not found to be ESBL producers by confirmatory tests. This may be due to inhibitor resistant mutants which cannot be picked up on the tests employing ß lactam/ß-lactamase inhibitor (BL/BLI) combinations or may be due to ß-lactamase hyperproducer which are ESBL negative but 3GC resistant or because of presence of Amp C producers which give false negative results for ESBLs. Alternatives of treatment of severe ESBL-producing infections could be carbapenems, amikacin, tigecycline, and b-lactam/b-lactamase inhibitor combinations.

Of the 114 ESBL isolates, 42 isolates were found to be resistant to cefoxitin whereas on the contrary, ESBL producers belonging to class A of Ambler classification are generally sensitive to cephamycins such as cefoxitin. This could be due to the probability of Amp C co-producers along with ESBLs. 36 Amp C co-producers were confirmed by cloxacillin based test. Combined disk test showed 78 to be pure ESBLs and only 13 isolates to be pure Amp C producers amongst 16 cefoxitin resistant non-ESBLs. Three-dimensional test and a new Amp C disk test can distinguish between cefoxitin resistant non-Amp C producers and cefoxitin resistant Amp C producers.[22,23] In addition, resistance to BL/BLI combination can help to identify possible Amp C producing organisms.[24] Surprisingly amongst the cefoxitin sensitive isolates also, 6 were found to be Amp C producers on combined disk test. This may be due to newer Ambler class C (ACC) type of enzymes which have relatively lower activity to cefoxitin and hence appear susceptible.[25] Inhibitor based confirmatory method appears promising for Amp C detection as it increased the sensitivity of the test by detecting additional Amp C ß-lactamase.

To conclude, there is a need for a correct and reliable phenotypic test to identify Amp C ß-lactamases and to discriminate between pure ESBL, pure Amp C and ESBL-AmpC producers. Inhibitor based method using cloxacillin appears to be simple and effective in discriminating this type of resistant isolates. A more comprehensive study with a greater number of samples would further augment the findings of this

lates. A more comprehensive study with a greater number of samples would further augment the findings of this study. The detection of complex resistance mechanisms on a periodic basis is mandated in tertiary care set ups. Increasing rate of ESBL and Amp C producing gram-negative infections and their high resistance against most of the extended spectrum antibiotics makes detection of ESBL producing strains necessary and this detection should be included in routine programs of microbiology laboratories. Moreover, an antibiotic prescription committee should be constituted to supervise antibiotic prescription seriously in hospitals to prevent the emergence of such strains. Infection control precautions like barrier nursing, cohorting of patients and nurses, contact precautions through the use of disposable gloves, gowns, and strict attention to hand washing are essential to limit its spread. Development of an infection control policy and hospital antibiotic prescribing guide should follow next. Education of medical and nursing staffs, patients, visitors and medical students could play an important part. ESBL producers are intrinsically resistant to all cephalosporins and aztreonam (even if they exibit in vitro suscsptibility).[26] Interpretative comments can accompany microbiology reports to underline this fact. Co-resistance to quinolones and aminoglycosides are common.[27] Quinolone antibiotics are strong selectors of ESBL producers and their use should be restricted as far as possible.

Judicious use of third generation cephalosporins and empirical antibiotic therapy can lead to the withdrawal of the selective pressure and thus will limit the emergence of drug resistant bacteria especially gram negative bacilli belonging to Enterobacteriaceae group.

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