The Detection of Antibiotic Susceptibility Patterns of Multi-Drug Resistant Bacteria especially those Producing Extended-Spectrum Beta-Lactamases (ESBL)

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Over the last few decades, β-lactams are the most widely used and favored antimicrobials worldwide, because of their efficacy, broad spectra and low toxicity. They inhibit the bacterial penicillin-binding proteins (PBPs), which are the enzymes that catalyze the final cross-linking of the bacterial cell wall polymer, peptidoglycan. However, due to heavy use of β-lactams antibiotics, bacteria developed various mechanism of resistance. Modification or substitution of the PBPs is important mechanism in gram-positive ***cocci****, while production of β-lactamases is main cause of resistance among gram-negative* ***bacilli****. Mostly plasmid harbors the resistance gene and therefore, is crucial in disseminating resistance into previously susceptible species. Throughout the 1960s and 1970s there was a relentless rise in reports of resistance to β-lactams as a consequence of the selection of bacteria that produce β-lactamases.*

Keywords: Penicillin-Binding Protein (PBP), β-lactams antibiotics, Gram-positive *cocci*, Gram-negative *bacilli*.

1. INTRODUCTION:

Over the 20 years, many new β-lactam antibiotics have been developed that were specifically designed to resist the hydrolytic action of β-lactamases. However, with each new class that has been used to treat patients, new β-lactamases emerged that cause resistance to that class of drug.B-lactam antibiotics containing an oxyimino group (third generation cephalosporins and aztreonam) and are inhibited by β lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. ESBLs are usually plasmid-mediated β-lactamases, most commonly found in *Klebsiellapneumoniae*, but also increasingly in *Escherichia coli, Proteus mirabilis* and other Gram-negative *bacilliIn* the united State, ESBL production in *Enterobacteriaceae* varies from 0-25%, in France 40% and across the Europe, 20% non ICU isolates of *K. pneumoniae* are potential ESBL producers [1]. Production of β-lactamase enzyme by Gram-negative bacteria is the most important mechanism of resistance to β-lactam antibiotics. The enzyme was identified in *Escherichia coli* prior to the use of penicillin in medical practice [2]. Some Gram-negative bacteria possess a naturally occuring, chromosomal mediated β-lactamases, likely developed due to the selective pressure exerted by β-lactam producing soil organisms found in the environment [3]. Ambler proposed first molecular classification on the basis of sequence similarity. He divided the β-lactamases in to four classes A-D Class A, C and D comprise evolutionary different group of serine enzymes and class B comprises

zinc type [4] and which have remained the most prevalent ESBL type, in terms of both variant numbers and numbers of producer strain isolation. OXA enzymes are the only ESBLs of class D [5]. TEM-1 hydrolyzes ampicillin at a greater rate than carbenicillin, oxacillin and cephalothin and has negligible activity against extended spectrum cephalosporns. TEM-2 has same hydrolytic profile as TEM-1, but differs from TEM-1 by having a more active native promoter and by a difference in isoelectric point (5.6 compared to 5.4). TEM-13 also has a similar hydrolytic profile to TEM-1 and TEM-2 [6]. CTX-M type β-lactamases hydrolyze cefepime with higher MIC than produce by other ESBL types [7]. In the past this enzyme was predominantly found in three geographic areas: South America, the Far East and Eastern Europe. However now a days, many authors have reported the advent of this enzyme in most of the continents. Various reports are from the United Kingdom [8]. First report of this isolate in *P. aeruginosa* came from Ankara, Turkey. In France, some OXA derivative of ESBL as OXA-18 and OXA-13 were found in *P. aeruginos* [9]. By the early 1990s, prevalence of ESBL producing organisms in France became 25-30% in nosocomially-acquired infections [10]. There are many individual studies, reflecting the association of ESBL colonization, including the presence of gastrostomy or jejunostomytube [11]. Haemodialysis [12] is specific for gene family (e.g. TEM or SHV) but the test is labor intensive and it cannot distinguish between ESBLs and non-ESBL, also cannot distinguish between variants of TEM or SHV [13]. In *K. pneumonia*, decreased expression of outer-membrane porins often accompanies ESBL production and may allow a TEM- or SHV- type ESBL to express resistance to cefepime or allow an AmpCβ-lactamase to express resistance to imipenem [14]. Though ESBLs are major problems in our clinical setting, we do not have the exact data on different types of ESBLs prevalent in our hospital and also the characters of these strains that are necessary for formulating the antibiotic policy.

2. MATERIALS AND METHODS

A total of 200 consecutive non-duplicate clinical isolates of the family *Enterobacteriaceae*from various clinical specimens such as blood, respiratory, wound swab/pus and urine were subjected to further study. These clinical isolates were *E. coli, K. Pneumoniae, Enterobacter*species,*Citrobactor*species, and *Proteus* species. These isolates were collected from infected patients admitted to various medical and surgical wards and intensive care units. Various specimens with culture are positive for the family *Enterobacteriaceae* are given below:

Blood	18
Sputum/endo-tracheal (ET) aspirate	23
Urine	73
Pus/infected surgical wound swab	56
Body fluids (Bile, Peritoneal dialysis fluid,CSFetc.)	30

2.1. Sample Collection and Transport

2.1.1. Blood

Venous blood (5 ml) was collected aseptically and inoculated directly into 50 ml biphasic brain heart infusion (BHI) medium (HiMedia) with sodium polyanethol sulphonate/BACTAC medium. Repeat blood culture was performed to rule out external contamination.

2.1.2. Sputum

Sputum samples were collected from deep cough of patient having lower respiratory tract infection. Samples were collected in a sterile, screw-capped container directly from mouth. These samples were transported to the laboratory within 2 hr at room temperature.

2.1.3. Urine

Clean voided mid stream urine were collected from patient having urinary tract infection. Samples were taken in sterile wide mouth, screw capped container after cleaning genital area with soap and water. These samples were transported to the laboratory with in half hour.

2.1.4. Pus/infected surgical drain

Abscess pus was aspirated directly or ultrasound guided as indicated from patient having wound infection. The specimen was taken directly or through sterile swabs (at least two) from wound aseptically.

2.1.5. Body fluids

Various peritoneal, bile and CSF fluids were collected aseptically in sterile, screw-capped container. These samples were transported to the laboratory immediately at room temperature.

2.2. Identification of Bacterial Isolates

All bacterial isolates from various specimens were identified by recommended tests [15] as given in Table 1.

Table 1: Standard identification test for *Enterobacteriaceae*.

Biochemical reaction	***E. coli***	***Klebsiella Sp***	***Enterobactor sp***	***Citrobactor sp***	***Proteus sp***
Motility	+	-	+	+	+
Glucose(gas)	+	+/-	+	+	+
Lactose (acid)	+	+/-	+	+/-	-
Indole	+	-	-	-/+	+/-
Voges-Proskauer	-	+/-	+	-	-
Citrate	-	+/-	+	+	+/-
Phenylalanine	-	-	-	-	+
Urease	-	+/-	-	-	+
Hydrogen sulphide	-	-	-	+/-	+

2.3. Preservation of Isolates

The isolated strains were stored in stock vials having brain heart infusion broth and 20% glycerol at -40°C, until tests were performed for the detection and characterization of ESBL.

2.4. Quality Controls

The control organism was used routinely with every test to compare with test results.

ESBL producer (positive control)	*K. pneumoniae* ATCC 700603
ESBL non-producer (negative control)	*E. coli* ATCC 25922

For this we used all these tests given below:

2.4.1. Disk potentiation test

This test was carried out for all Enterobacteriaceae isolated against ceftazidime (30 µg) and cefotaxime (30 µg) antibiotic disks with and without clavunalic acid (10 µg). This is a confirmatory test to see the ESBL production by bacterial isolates as recommended by CLSI, 2006.

2.4.2. Double disk approximation (DDA) method

This test was carried out for bacterial isolates, which were positive in disk potentiation test or showed resistant zone without any enhancement in zone size with addition of clavulanic acid [16] he antibiotic disks used in this method were ceftazidime (30 µg) cefotaxime (30 µg) cefpodoxime (10 µg) and ceftriaxone (30 µg) from HiMedia. This is a confirmatory test to see the ESBL production by bacterial isolates.

2.4.3. MIC by agar dilution method

This test was also carried out for all *Enterobacteriaceae* isolates to detect MIC values of the ESBL producing. This was carried out following CLSI guidelines, 2006.

2.4.4. Molecular method

All ESBL producer strains were characterized genotypically to know the ESBL type such as TEM, SHV and CTX-M.

2.4.5. Characterization of DNA

The integrity of high molecular weight DNA is an important factor, which should be considered during extraction steps. Integrity was checked by electrophoresis on 0.8% agarose gel (made in 1×TAE buffer) containing ethidium bromide (5µl of 10mg/stock for every 50 ml of agarose). The plasmid DNA appeared as three bands near the well as shown in Table 2. Storage of DNA is done at 4°C for daily use and at -70°C for several years.

Table 2: Primer sequence of ESBL genes.

bla TEM [17]	Forward primer	5'-AGAGTATGAGTATTCAACATT-3'
	Reverse primer	5'-ATCTCAGCGATCTGTCTAT-3'
bla SHV [17]	Forward primer	5'-GGGTTATTCTTATTTGTCGCT-3'
	Reverse primer	5'-TAGCGTTGCCAGTGCTCG-3'
bla CTX [18]	Forward primer	5'CGCTTTGCGATGTGCAG-3'
	Reverse primer	5'ACCGCGATATCGTTGGT-3'

2.5. PCR Procedure

Final reaction volume of 25µl was prepared with H_2O (Mili-Q grade), 20 pmol of both primers, 1 mM of each Dntp, 1X Taqpolymeras, 10X PCR buffer, 1.5 mM $MgCl_2$ 5 µl DNA template denaturation at 96°C for 3min and after annealing for 35 cycle then we get:

bla TEM	bla SHV	bla CTX
96°C for 30 sec	96°C for 30sec	96°C for 30sec
50°C for 30 sec	58°C for 30 sec	57°C for 30 sec
72°C for 30 sec	72°C for 30 sec	72°C for 30 sec

2.6. Electrophoresis Condition

The amplified PCR product was analyzed on 1.5% agarose containing 1 µl/ml ethidium bromide. The amplified product was examined for the presence of specific bands. The

size of bands was compared with 50 bp ladder under UV transillumination (UVS System, USA).

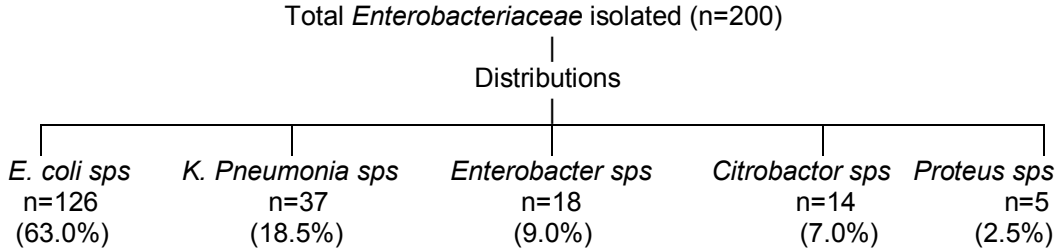
The PCR products were of following size-

bla	TEM	837bp
bla	SHV	929bp
bla	CTX	544bp

3. RESULTS AND DISCUSSION

All ESBL isolates were also characterized by molecular methods to know the common ESBL types in the hospital.

3.1. Bacteria Isolated from Clinical Specimens



3.2. Patients Demography

The mean age of infected patient was 42.02 years (range- 1 to 76 years) and male: female ratio was 1:86:1 The system involve due to basic disease in these patients were gastrointestinal (37.5), renal (37.5), neurological (12.5%), endocrinal (6.5%), immunological (3.5%), and cardiac (2.5%), The duration of median hospital stay was 16.95 days (range 1-150 days). The overall clinical specimens were nearly same from medical (97) and surgical wards (103). Overall mortality rate among patient group was 12%.

3.3. Presumptive ESBL Production by Screening Test (Disk Diffusion Method)

For third generation cephalosporin antibiotic disks were used to screen the potential ESBL producers from bacterial isolates. Cefpodoxime detected the highest number of potential ESBL producers among the bacterial isolates followed by cefotaxime and ceftriaxone and lowest by ceftazidime. Out of 200 bacterial isolates, screening test by any of the four disks detected 110 isolates as potential ESBL producers and their distributions in different members of the family *Enterobacteriaceae* are given in Table 3 and 4.

Table 3: Presumptive ESBL production by different antibiotic screening test.

Antibiotic disk	No. of isolates with resistant zone (%)	No. of isolates with sensitive zone (%)
Ceftazidime	91 (45.5)	109 (54.5)
Cefotaxime	100 (50.0)	100 (50.0)
Ceftriaxone	95 (47.5)	105 (52.5)
Cefpodoxime	104 (52.0)	96 (48.0)
Either of the above disks	110 (55.0)	90 (45.0)

Table 4: Presumptive detection of ESBL production in different organisms by screening test.

Organisms	Presumptive ESBL production (%)			
	Ceftazidime	Cefotaxime	Ceftriaxone	Cefpodoxime
E. coli (n=126)	57 (45.2)	64 (50.8)	60 (47.6)	69 (54.8)
K. pneumoniae (n=37)	17 (45.9)	18 (48.6)	19 (51.4)	17 (45.9)
Other *Enterobacteriaceae* (n=37)	17 (45.9)	18 (48.6)	16 (43.2)	18 (48.6)

3.4. ESBL Production by Confirmatory Test

The increase of ≥5mm zone of inhibition with addition of clavulanic acid on cephalosporin disks was considered as positive ESBL producer. Cefotaxime detected more bacteria isolate as ESBL producer than ceftazidime. Out of 200 bacteria isolate, disk potentiation method by either of the two disks detected ESBL production in 129 isolate (64, 5).

3.5. Evaluation of Various Parameters with ESBL Infection

The ESBL producing strains were isolated from all types of infective sources. The prevalence of ESBL production varied in various clinical samples with highest rate from surgical wound infections; followed by respiratory, blood stream, urinary tract and other miscellaneous infections. Miscellaneous infectious specimens included peritoneal dialysis (PD) fluids, bile and CSF. The infectious specimens included peritoneal dialysis (PD) fluids, bile and CSF. The ESBL production in bacterial isolates from septicemia patients was 71.4%.

3.6. Subtypes of ESBL Strains by PCR

Plasmid isolates from 129 ESBL producing organisms were amplified by PCR; 82 isolates were positive either for bla_{TEM}, bla_{SHV}, or bla_{CTX}. A total of 47 isolates contained multiple β-lactams genes, while 35 isolates were positive for single gene, bla_{CTX} was the

commonest gene identified among ESBL producers as shown in Table 5. The PCR positivity was less in ESBL producing *K. pneumonia*e isolates in comparison to other organisms as given in Table 6.

Table 5: Subtypes ESBL producing strains by PCR.

Positive by PCR for ESBL subtypes	Number amplified, n=82 (%)
bla_{TEM} + $blas_{SHV}$+ bla_{CTX}	13
bla_{TEM} + bla_{CTX}	23
bla_{TEM} + bla_{SHV}	2
bla_{SHV}+ bla_{CTX}	9
bla_{TEM} only	7
bla_{SHV}only	3
bla_{CTX}only	25
Overall ESBL subtypes	
bla_{TEM}	45 (54.9)
bla_{SHV}	27 (32.9)
bla_{CTX}	70 (85.4)

Table 6: Organism wise distribution of ESBL subtypes by PCR.

ESBL strain	Positive by PCR for either ESBL Subtypes (%)
E. coli (n=79)	51 (64.6)
K. pneumoniae (n=25)	11 (44.0)
Other *Enterobacteriaceae* (n=25)	20 (80.0)

3.7. Co-Resistance for other Antimicrobial Agents Exhibited by ESBL Producing Isolates

The co-resistance of ESBL producing bacterial isolates against other antimicrobial agents tested is shown in Table 7. These organisms were antimicrobial agents in different frequencies. All the strains were sensitive to meropenem. 89% percent strains were sensitive to Ertapenem. The resistance to amikacin varied from 7.6 to 32.0% among various organisms. ESBL producing organisms showed high level of resistance against gentamicin, trimethoprim-sulfamethoxazole and ciprofloxacin.

Table 7: Co resistance exhibit by ESBL producing isolates.

ESBL producing Organisms	Co-resistance to following antibiotic (%)				
	MEM*	**AK***	**CN***	**SXT***	**CIP***
E.coli (n=79)	0.0	6 (7.6)	56 (70.9)	64 (81.0)	77 (97.5)
K. pneumoniae (n=25)	0.0	5 (20.0)	16 (64.0)	20 (80.0)	23 (92.0)
Other *Enterobacteriaceae* (n=25)	0.0	8 (32.0)	14 (56.0)	18 (72.0)	21 (84.0)

*MEM-Meropenem, AK-amikacin, CN-gentamicin, SXT-trimethoprim-sulfamethoxazole, CIP-ciprofloxacin.

Various observations in the present study on ESBL production in the present hospital set up could be concluded as follows:

High prevalence of ESBL (64.5%) in the hospital emphasizes urgent need to adopt appropriate control measures to reduce the ESBL burden. For the detection of ESBL at least two third generation cephalosporin disks should be used. Phenotype confirmatory method preferably disk potentiation recommended by CLSI should be used in all clinical microbiological laboratories for confirmation of ESBL. To best of our knowledge, this is among the few studies in India to find the ESBL. Most common ESBL prevalent in the setup was CTX-M (85.5%), 57.3% of our genotypically confirmed ESBL isolates contained multiple β-lactamase genes indicating that CTX-M type with multiple β-lactamase genes would likely to emerge the dominant ESBL types in hospital setup. Significant association with prior antibiotic therapy support the hypothesis that selection pressure related to overuse of broad spectrum antibiotics especially third generation cephalosporins is responsible for emergence of high level resistance in the family *Enterobacteriaceae.* High co-resistance with other antimicrobial agent such as aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole minimizes the choice of antibiotic therapy to carbapenams for ESBL associated infections. Carbapenem is considered to be the last therapy of last resort against ESBL producing members of *Enterobacteriaceae*. But the newest carbapenem, Ertapenem has shown very good efficacy against ESBL producing organisms. Ertapenem has several advantages over the older carbapenem drugs (Imipenem and Meropenem) like less cost, once daily dosing and less risk of selecting multidrug resistant bacteria (e.g. MDR *Pseudomonas aeruginosa* and *Acinetobacter species)*. The observations in the present study highlight the need of an antibiotic policy in hospital setup and appropriate infection control measure to overcome the problems associated with ESBL infection. Although it may not possible to totally eradicate ESBL producing strains from hospital but appropriate use of antibiotics and adoption of preventive measure will certainly help to minimize ESBL associated infections and complications.

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