



Original Article

A Study on Extended Spectrum B -Lactamases in Coliforms

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Abstract

This study was done to detect extended spectrum β –lactamases (ESBL) in Coliforms from various clinical samples collected in the department of Microbiology laboratory, Government Medical College Kozhikode. A total of 150 samples were tested for ESBL, 100 were found to be positive by double disk synergy test; out of these 100 strains, *E.coli* were 55% and *K.pneumonia* were 45%. Imipenem appears to be the drug of choice for serious infections confirmed to be caused by ESBLs.

Keywords: Double disk synergy test, Beta lactamases, Enterobacteriaceae.

Introduction

β -Lactam antibiotics are among the safest and most frequently prescribed antimicrobial worldwide; however, emergence of β – Lactam resistance in clinically important pathogens increasingly has limited their utility. To treat infections due to β – lactamase producing bacteria that were resistant to penicillin and early cephalosporin derivatives, new generations of relatively enzyme-stable and broad-spectrum cephalosporin derivatives were introduced in the late 1970s and 1980s. However, over the past decade, antibiotic-resistant mutants producing extended-spectrum β -lactamase (ESBL) emerged among gram-negative bacteria, predominantly *Escherichia coli* and *klebsiella pneumoniae*¹.

Emergence of ESBL – producing isolates has important clinical and therapeutic implications. First, in most bacterial isolates, resistance determinants for ESBL production are carried on

plasmids that can easily spread from organism to organism. Second, the spread of resistance toward extended – spectrum cephalosporins further limits the utility of the β – lactam class and may lead to increased prescription of more broad-spectrum and expensive drugs such as imipenem. More important, antibiotic selection for treatment of serious infections due to ESBL producing *E.coli* and *K.pneumoniae* is a clinical challenge due to the complex nature of in vitro susceptibility testing and vivo correlation. Perhaps the biggest challenge lies in overcoming widespread unawareness among clinicians regarding these resistant organisms due to under reporting from microbiology laboratories and lack of an obvious marker to indicate production of ESBL.¹

Extended spectrum β – lactamases are extremely broad spectrum to β – lactamase enzymes found in a variety of *Enterobacteriaceae*. When producing these enzymes, organisms become highly

effective at inactivating various to β – lactam antibiotics. In addition ESBL- producing bacteria are frequently resistant to many classes of antibiotics resulting in difficult to treat infections.² Emergence of resistance to β – lactam antibiotics began even before the first to β – lactam, penicillin, was developed. The first to β – lactamase was identified in *Escherichia coli* prior to the release of penicillin for use in medical practice³. The first isolate resistant to extended spectrum cephalosporins was found in Germany in 1983, and it produced an SHV – type β – lactamase (for sulphhydryl variable).⁴ The First Reported Outbreak of ESBL producing organism occurred in France in 1985.⁵ The first plasmid-mediated β – lactamase in gram-negatives, TEM-1, was described in the early 1960s.³ The TEM-1 enzyme was originally found in a single strain of *E.coli* isolated from a blood culture from a patient named Temoniera in Greece, hence the designation TEM.⁶ ESBLs are mutant plasmid mediated beta-lactamases (e.g. TEM-1, TEM-2,SHV-1), which have an extended substrate profile that permits hydrolysis of all cephalosporins, penicillins, and aztreonam.⁷ Being plasmid and transposon mediated has facilitated the spread of TEM-1 to other species of bacteria. Within a few years after its first isolation, the TEM-1 β – lactamase spread worldwide and is low found in many different species of members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*. Another common plasmid mediated β – lactamase found in *Klebsiella pneumoniae* and *E.coli* is SHV-1 (for sulphhydryl variable).⁸

The first of these enzymes capable of hydrolyzing the newer β – lactams, SHV-2, was found in a single strain of *Klebsiella ozaenae* isolated in Germany.⁹ Because of their increased spectrum of activity, especially against the oxyimino-cephalosporins, these enzymes were called extended spectrum β – lactamases (ESBLs).

Among gram negative bacteria, enzymatic resistance to β – lactams is mediated predomi-

nantly by two major types of β – lactamases; chromosomally encoded enzymes produced in high quantity either constitutively or after induction. Ambler class C, e.g. in *Citrobacter*, *Enterobacter*, *Serratia*, *Morganella morganii*, or plasmid encoded enzymes of Ambler class A detected mainly in *Klebsiella pneumoniae* and *E.coli*, *salmonella spp*, and *shigella spp*. These species usually do not produce Amp C β – lactamases in high amounts to reach clinical resistance.¹⁰

Currently numerous β – lactamases are known, and sequencing allows them to be divided into four classes A to D according to their amino acid homologies,¹² with classes A and C being the most important clinically^{6,7}

A successful approach to the control of the spread of ESBL producing organisms involved switching to different classes of broad spectrum antibiotics for the treatment of serious infections. The most successful replacement antibiotic have been imipenem and piperacillin tazobactam.¹¹

Aim of the Study

To detect Extended Spectrum β – lactamases in Coliforms

Materials and Methods

This study was conducted in the Department of Microbiology, Medical College Calicut, during a period of one year. Resistant and Sensitive strains of *E.coli* and *Klebsiella pneumoniae* for the study were obtained from samples such as pus, blood, cerebrospinal Fluid, sputum, urine, bronchioalveolar lavage, tracheostomy site swab, central venous catheter tip and other bodyfluids. A clinical work up of the patients was done and the samples were obtained from patients admitted in post operative surgical ward, Institute of Chest Diseases Paediatrics, surgical medical and the intensive care units. Extended Spectrum beta lactamase production (ESBL) was tested by the following methods.

olates of resistant *E.Coli* and *Klebsiella pneumoniae* by the double disk approximation test

.MIC to Ceftazidime in the range 1 microgram/ml to 64 microgram/ml was done on the 100 resistant *E.Coli* and *Klebsiella pneumoniae*. MIC to Ceftazidime was also done on 50 Sensitive *E.Coli* and *Klebsiella pneumoniae*.

Antibiotic sensitivity is done on Mueller – Hinton Agar routinely in the laboratory by the Kirby Bauer disk diffusion method with antibiotics Ampicillin (10 µg), cefazolin (30 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Amikacin (30 µg), Ceftazidime (30 µg) and Cefotaxime (30 µg).

Detection of Extended Spectrum β – Lactamases (ESBL) (Jarlier et al 1988)

Double Disk Synergy Test

ESBL detection test was done by the double disk approximation test (DDST) described by Jarlier (1988). The turbidity of the inoculum was standardized by comparing with 0.5 McFarland standard.

For determining the ESBL phenotype, by the DDST the organism was swabbed on to Mueller – Hinton Agar and β – lactam antibiotic disks like Ceftazidime ((30 µg), Cefotaxime (30 µg), Cefpodoxime(30 µg), Aztreonam (30 µg) and cefoxitin (10 µg) were applied surrounding a disk containing Cefta-clavulanate (30-10 µg) which was placed in the centre of the plate such that centre to centre distance between this and β – lactam antibiotics was 25mm. after overnight incubation at 37⁰C, enhancement of the zone of inhibition between the cefta-clavulanate disk and any one of the β – lactum disk indicated the presence of an ESBL.

Another Method for ESBL detection was also done as per NCCLS recommendations. In this disk diffusion testing ≥ 5 mm increase in a zone diameter for an antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL producing organism.

ESBL was also detected by determining the minimum inhibitory concentration of both resistant and sensitive *E.coli* and *Klebsiella pneumoniae* to Ceftazidime. The procedure was

the standard microdilution broth method using Ceftazidime in the concentrations ranging from 1 microgram/ml to 64 microgram/ml

Tube Dilution Method

Materials used for minimum inhibitory concentration (MIC)

Sterile test tubes 7.5 x 1.3cm size ,Over night test agar culture of 50 sensitive and 100 resistant *E.coli* and *Klebsiella pneumoniae*.Ceftazidime in pure drug form (From Smithkline Beecham Pharmaceuticals) Sterile distilled water 500ml Nutrient broth medium 500ml *E.coli*; ATCC 25922 was used as the control strain.

Method

The standard inoculum was prepared from a fresh overnight pureculture of 50 sensitive and 100 resistant *E.coli* and *Klebsiella pneumoniae*. Standardised bacterial suspensions of resistant *Klebsiella* and *E.Coli* were made that matches the turbidity of the 0.5 McFarland standard (I.e. 1.5 x 10⁸ Cfu/ml). series of doubling dilutions of Ceftazidime ranging from 1microgram/ml to 64 microgram/ml was prepared, into which 50 micro litre of test organism from the standard inoculums was added to each of the serial antibiotic solutions The tubes were examined for turbidity after incubation at 37⁰C for 16 to 18 hr. the lowest concentration of the antibiotic inhibiting bacterial growth as evidenced by lack of turbidity in the medium was taken as minimum inhibitory concentration (MIC). Interpretive MIC for sensitive coliforms to ceftazidime is <8µgm/ml and for resistant, the MIC is >32µgm/ml (NCCLS guide lines).

Results

One hundred and fifty isolates of resistant *E.coli* and *Klebsiella pneumoniae*, obtained during a period of one year were tested for the presence of ESBL by the double disk Synergy test. ESBLs were detected in 100 isolated (66.6%), of them 55% were *E.coli* and 45% were *K.pneumoniae*.

Table – 1 Prevalence of ESBLs in *E.coli* and *Klebsiella Pneumoniae*

| Total Isolates Tested | Isolates positive for ESBL | | | | | | Isolates negative for ESBL | |
|-----------------------|----------------------------|-------|--------------|----|--------|----|----------------------------|------|
| | Total | | K.pneumoniae | | E.coli | | | |
| | No. | % | No. | % | No. | % | No. | % |
| 150 | 100 | 66.6% | 45 | 45 | 55 | 55 | 50 | 33.4 |

The distribution the 100 ESBL positive isolates recovered from various specimens are given in Table.2. Maximum number of ESBL isolates

(39%) were found from wound infection sites, followed by 28% from aspirated pus from different sites.

Table 2 Distribution of 100 ESBL positive isolates from various specimens

| Sl. No | Samples | Total No. | | K.pneumoniae | | E.coli | |
|--------|---------------------------|-----------|-----|--------------|----|--------|----|
| | | No. | % | No. | % | No. | % |
| 1 | Aspirated pus | 28 | 28 | 7 | 7 | 21 | 21 |
| 2 | Blood culture | 12 | 12 | 8 | 8 | 4 | 4 |
| 3 | CSF | 2 | 2 | 1 | 1 | 1 | 1 |
| 4 | Other fluids | 4 | 4 | 3 | 3 | 1 | 1 |
| 5 | Swabs from Infected sites | 39 | 39 | 16 | 16 | 23 | 23 |
| 6 | Sputum | 12 | 12 | 9 | 9 | 3 | 3 |
| 7 | CVP tip | 3 | 3 | 1 | 1 | 2 | 2 |
| | Total | 100 | 100 | 45 | 45 | 55 | 55 |

Table.3 Sensitivity pattern of 100 ESBL isolates

| Isolates | % | Resistant to A, Cf, M, CAZ, Az, Cn, Cep | | Resistant to A, Cf, CAZ, Az, but sensitive to M | | Resistant to A, Cf, CAZ, Az, but sensitive to Cn | |
|----------------------|----|---|------|---|------|--|------|
| | | No. | % | No. | % | No. | % |
| K. Pneumoniae (n=45) | 45 | 29 | 64.4 | 16 | 35.6 | - | - |
| E.coli (n=45) | 55 | 28 | 50.9 | 26 | 47.3 | 1 | 1.8% |

A – Ampicillin, Cf - Cefazolin , M - Amikacin , CAZ - Ceftazidime , Az - Aztreonam, Cn – Cefoxitin, Cep- Cefpodoxime.

Mic to Ceftazidime

Table.4 MIC of 100 ESBL Positive *E.coil* & *Klebsiella pneumonia* to ceftazidime

| Isolates | No | MIC to ceftazidime (ug/ml) | | | | | | | | |
|--------------|----|----------------------------|---|---|----|------|----|------|----|------|
| | | 2 | 4 | 8 | 16 | % | 32 | % | 64 | % |
| E.coil | 55 | - | - | - | 24 | 43.5 | 23 | 42 | 8 | 14.5 |
| K. pneumonia | 45 | - | - | - | 20 | 44.5 | 19 | 42.2 | 6 | 13.3 |

Mic to Ceftazidime with sensitive isolates

The Mic to Ceftazidime done for the 50 sensitive isolates of *E.coil* and *Klebsiella pneumonia* isolates.

Table 5 MIC of sensitive strains of 50 *E.coil* & *Klebsiella pneumonia* to Ceftazidime

| Total No. (50) | | MIC to ceftazidime (ug/ml) | | | | | |
|----------------|----|----------------------------|-------|----------|------|----------|----------|
| | | 1 µgm/ml | % | 2 µgm/ml | % | 4 µgm/ml | 8 µgm/ml |
| E.coil | 16 | 16 | 100% | Nil | Nil | Nil | Nil |
| Klebsiella | 34 | 32 | 94.2% | 2 | 5.8% | Nil | Nil |

Enhanced zones with cefta-clavulanate with the 100 ESBL positive isolate

Table.6 Tests to detect ESBL and their results

| No. | Methods used | Total isolates tested | No.of Positive ESBL | % |
|-----|-----------------------|-----------------------|---------------------|-------|
| 1 | Double disk synergy | 150 | 100 | 66.67 |
| 2 | Broth dilution method | 100 | 100 | 100 |
| 3 | Enhanced Zone (>5mm) | 100 | 52 | 52 |

Discussion

Prevalence of ESBL producing strains in various species of enterobacteriaceae varies in different countries in different hospitals and it varies worldwide from <1% to 74% (1996).¹² In the present study out of the 150 resistance isolates tested ESBLs were detected in 100 isolates (66.67%)

ESBL producing gram negative organisms were first reported in Europe in 1983 and 1984. now it has been reported in all continents except Antarctica.¹³ usually one of the –three species *klebsiella pneumonia*, *Escherichiacoli* and enterbacter cloacae is at the top of the list.¹⁴

In Asia, the percentage of ESBL production in *E.coil* and *klebsiella* varied from 4% in korea to 8.5% in Taiwan and upto 12% in honkong.¹³ in one of the studies conducted in Pondicherry, india, 58.06% of *E.coli*, 43.7% of *klebsiella pneumonia* were found to be ESBL producers.¹⁵ In the present study, 55% of *E.coli* and 45% of *klebsiella pneumoniae* are found to be ESBL producers. In a teaching hospital in france, the ESBL isolates were 2.7% in 1990 and 2.9% in 1994.

In this study maximum number of ESBLs were isolated from wound infection site 39(39%) and from aspirated 28(28%) and 12(12%) of ESBL produced were isolated from blood jarlier et al (1988) has reported 18% of the ESBL +ve organisms from blood

In a study conducted al pakistan in the year 2003 out of the 200 nosocomial isolates, 75 (37.5%) were ESBL producers. Out of these, 25(33.33%) were obtained from pus and other body fluids. (6.67%) from blood, while no ESBL +ve isolates was obtained from CSF (1996)

Philippon et al (1989) reported 9% of all *klebsiella pneumoniae* isolates to be ESBL producers in 16 different French hospital and 8.6%ESBL producing *klebsiella pneumonia* were reported in USA.¹⁶ while in this study, a very high percentage of *E.coli* (55%) were found to be ESBL producers.

As per the NCCLS recommendation to confirm an isolate as ESBL producer using MIC as the

criteria, it was ≥ 2 ug/ml as to any one of the following antibiotics like cefpodoxime, ceftazidime, aztreonam or ceftriaxone. In the present study, 44 (44%) of the ESBLs had an MIC of 16 ug/ml to Ceftazidime, 42 (42%) had 32 ug/ml and 14 (14%) had 64 ug/ml.

In a study conducted at the Center for Disease Control and Prevention, Atlanta, Georgia in the year 2003 for 131 isolates, the broth microdilution (BMD) MIC was taken as the confirmatory test for ESBL detection if at least one extended-spectrum cephalosporin was ≥ 2 micro g/ml. For 21 of 131 (16%) isolates, the ESBL confirmatory test was positive; i.e. the BMD MICs of Ceftazidime or cefotaxime decreased by ≥ 3 doubling dilutions in the presence of clavulanic acid (CA) or the disk diffusion zone diameters increased by ≥ 5 mm around Ceftazidime or cefotaxime disks in the presence of CA.¹⁷

The modal MICs for 30 selected isolates were 2 ug/ml for cefotaxime and ceftriaxone and 4ug/ml for ceftazidime. Three patients (two with mediastinitis and one with empyema), two of whom had bacteremia, were treated with an expanded spectrum cephalosporin – aminoglycoside combination, without success. Caellas and Goldberg (1989)¹⁸ identified 46 ESBL producing strains (44 *K. pneumonia* and 2 *E.coil* strains) in Argentinean hospitals. The MICs by agar dilution ranged from 1 to 256 ug/ml for cefotaxime and ceftriaxone and from 0.5 to 256 ug/ml for ceftazidime (ie. Some strains tested susceptible and others, tested resistant). In this study, 50 sensitive strains of *E.coil* and *Klebsiellae* were tested for MIC to ceftazidime. 48(96%) isolates were not ESBL producers as their MIC was <1ug/ml. 2 isolates (4%) had MIC of >2ug/ml showing ESBL production.

A strain of *K. pneumonia* resistant to cefotetan and cefoxitin was first isolated at New York Hospital Medical Centre of Queens (NYHMCQ)¹⁹ in March 1994, additional cefoxitin – resistant *K. pneumonia* and *E.coli* organisms were isolated from patients' blood, sputum, wounds, urine and body fluids from August to October 1994. The

patients were from different units within the hospital. Cefotetan and imipenem usage had remained constant during 1993 and 1994, and no other cephamycin or carbapenems were used at NYHMCQ. In October 1994, it was noted that three of the cephamycin-resistant *K. pneumonia* isolates were also resistant to imipenem.¹⁹

In this study, ninety nine ESBL isolates were cefoxitin resistant and only 1 ESBL isolates was sensitive to cefoxitin. As all the strains were sensitive to ceftaclav, the isolate cannot be either inhibitor resistant TEM or hyper producer type of β – lactamase. It can also be concluded that since the 99 ESBL isolates were sensitive to Cetfaclav it could represent TEM ESBL type.

Clinical Outcome

The clinical information about patients infected or colonized with ESBL producing isolated were also studied. ESBL producing bacteria were isolated from blood cultures of 12 patients with PUO and infective endocarditis, 7 of these patients were treated with expanded spectrum cephalosporins and aminoglycosides. In 7 patients, therapy was switched to ceftazidime plus amikacin, a response consisting with in vitro susceptibility testing. 3 patients were given imipenem and recovered which 2 patients died of burns septicemia in spite of treatment with third generation Cephalosporins and Amikacin.

ESBLs were isolated from the central venous catheter tip of 3 patients in the Medical ICU who had mitral valve prolapsed and RHD. These patients were treated with imepenam and were discharged.

Of 2 isolates from CSF, one of the patient with Piptazobactum and was cured. The other patient was from Neurosurgery ICU, with CP Angle tumour who was initially on ceftazidime and later was treated with imipenem and was cured. The remaining 88 patients from whom ESBL was isolated were treated with 3rd generation Cephalosporin and Amikaciin and were discharged.

Conclusions

Out of 150 samples tested for Extended Spectrum Beta Lactamases (ESBL), 100 were found to be ESBL positive by the Double disk synergy test (DDST). In the study 55% *E.coli* and 45% *K. pneumoniae* were ESBL producers. MIC to Ceftazidime was done for all the 100 ESBL producers, 24 *E.coil* and 20 *K. pneumonia* had MIC of >16 μ g/ml, 23 *E.coil* and 19 *K. pneumonia* had MIC of >32 μ g/ml. ,high MIC (>2 μ g/ml) detected the presence of ESBLs. The development and spread of ESBLs have most likely been caused by the over use of expanded spectrum cephalosporins in the hospital setting. The emergence of ESBL producing *K. pneumonia* and other ESBL producing enterobacteriaceae is a dilemma for clinicians because of the multi drug resistance in the organisms Imipenem appears to be the drug of choice for serious infections confirmed to be caused by ESBLs. Therefore, the therapeutic options are limited, making control and preventive measures particularly important.

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