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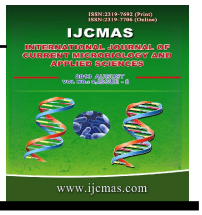


**Original Research Article**

# Detection and molecular characterization of extended spectrum of beta lactamase (ESBL) producing *Escherichia coli*

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**Keywords**

*E.coli;*
ESBL;
Beta-lactamases;
Double Disc Synergy Test;
PCR ;
RFLP;
Multidrug resistant *E.coli.*

**A B S T R A C T**

Extended spectrum beta - lactamases (ESBLs) are on the rise in hospital settings across the globe. Hence it is necessary to know the prevalence of ESBL so as to formulate a policy of empirical therapy in high risk units. The aim of the study is to assess the prevalence of ESBL positive strains isolated from the clinical samples from the intensive care unit patients of Sooriya hospital, Chennai, India and to analyze the presence of TEM gene and its Molecular characterization. A total of 100 non repetitive E.coli isolates were obtained during the period from February to November 2011 from clinical samples (urine, pus and blood) of intensive care unit patients. Standard biochemical tests were performed to confirm the organism. Antimicrobial assay was performed for all isolates by Antibiotic susceptibility testing by Kirby Bauer method, and the production of ESBL was determined by the Double Disc Synergy Test (DDST). PCR was used to demonstrate the presence of TEM gene. The isolates producing TEM gene were typed using the Restriction Fragment Length Polymorphism (RFLP).Out of 100 clinical samples 10 samples were potential ESBL producers. The presence of TEM gene was confirmed by PCR. In RFLP analysis, restriction cleavage by XbaI reveals six different banding patterns for the samples indicating six different source of origin. We conclude from this study that the clinical samples showed 10% ESBL. The analysis of TEM gene by PCR and RFLP typing showed the prevalence of ESBL from six different origin.

## Introduction

Antimicrobial resistance is a growing threat worldwide. Resistance mechanisms have been found for every class of antibiotic agents. In recent years, increased incidence of resistance to beta-lactams among members of the family *Enterobacteriaceae* has been reported worldwide (Jacoby and Medeiros, 1991, Samaha - Kfoury and Araj, 2003). The Extended Spectrum of β-Lactamases (ESBL) are plasmid-mediated enzymes which are capable of hydrolyzing and inactivating a wide variety of β -lactams including third generation cephalosporins (3GC), penicillins and aztreonam (Chaudhary and Aggarwal, 2004). .First

plasmid mediated β-lactamase in Gram negative organisms was reported in 1965 from an *Escherichia coli* isolate belonging to a patient in Athens, Greece, named Temnoniera (hence designated TEM). Another common plasmid mediated β-lactamase found in *Klebsiella pneumoniae* and *E. coli* is SHV-1 (named after the sulfhydryl "variable"active site) (Al-Jasser, 2006). ESBL-producing gram-negative bacteria are becoming a major global concern and usually harbor plasmid-mediated enzymes of the TEM, SHV, OXA, PER, and CTX-M types (Rodríguez-Baño *et al*., 2004; Quinteros *et al*., 2003). The TEM-1/2 and SHV-1 broad-spectrum β-lactamases are the most prevalent secondary β-lactamases among clinical isolates of *Enterobacteriaceae* worldwide (Bradford *et al.,* 1994) . Their evolutionary success is likely due to their efficient activities against penicillins and narrow- to intermediate-spectrum cephalosporins and to the fact that either the $bla_{TEM}$ or the $bla_{SHV}$ gene is often carried on self-transmissible or mobilizable plasmids capable of rapid horizontal spreading among different enterobacterial species (Chanal *et al*., 1996). The introduction in clinical practice of expanded-spectrum β-lactams resistant to the enzymes, such as oxyimino cephalosporins and monobactams, was a major breakthrough in the antimicrobial chemotherapy of gram-negative infections. However, it was soon counteracted by the appearance of secondary β-lactamases with an extended spectrum of hydrolytic activity (expanded-spectrum β-lactamases [ESBLs]) that also includes many expanded-spectrum β-lactams. Among emerging ESBLs, those most commonly encountered in *Enterobacteriaceae* are derivatives of the TEM and SHV prototypes, in which one or more amino acid substitutions are responsible for an extension of the substrate specificity that may include oxyimino cephalosporins and monobactams (Cha|$$|Ad|fibi *et al*., 1999). To date, carbapenems and cephamycins have remained resistant to these TEM- and SHV-type ESBL variants, while mechanism-based β-lactamase inhibitors (such as clavulanate and tazobactam) have retained a very efficient inhibitory activity (Knox**,** 1995). Several clinical microbiology tests that presumptively identify the presence of an ESBL has been reported but the task of identifying which specific ESBL gene is present in a clinical isolate is more complicated. This study is aimed at giving the molecular detection of the ESBL gene types prevailing in clinical isolates of *E coli* and describing the epidemiology.

## Materials and Methods

### Study setting, patient demographics and clinical specimens

A total of 100 non repetitive *E.coli* isolates obtained from clinical samples (urine, pus and blood) of intensive care unit patients of age group between 30 - 60 years attending at the Sooriya hospital which is a 200 bed multispecialty hospital located at Vadapalani in Chennai. Patients of intensive care unit were reviewed for their demographics, including age, sex, and the type of clinical specimens (Table 1).

### Antimicrobial susceptibility testing

*E.coli* samples recovered from clinical specimens were confirmed by standard biochemical methods. Antimicrobial susceptibility was determined by modified Kirby Bauer disc diffusion method as per CLSI recommendations. Ten samples were tested against the following antibiotics

imipenem (10µg), amikacin (10µg), amphicillin (10µg), cefazolin (30µg), aztreonam (30µg), cefotaxime (30µg), ceftazidime (30µg), ceftriaxone (30µg), gentamycin (10µg), tetracycline (30µg), and kanamycin (30µg). According to the zone diameter breakpoints recommended by CLSI (Du *et al.*, 2002) (Table 2A Enterobacteriaceae M02 and M07 from CLSI M100-S21 Twenty first informational supplement) isolates showing appropriate inhibition zone size were identified as ESBL producers and shortlisted for confirmation of ESBL production (Table. 2).

**Double disc synergy test**

*E. coli* that exhibited resistance to third generation cephalosporins were screened to detect ESBL production by DDST. Cefotaxime 30µg was placed at a distance of 15 mm edge to edge from a centrally placed augmentin disk containing 20µg of amoxicillin+10 µg of clavulanic acid. Plates were incubated at 35°C for 18-20 h and the pattern of zone inhibition was noted. Isolates that exhibited a distinct shape/size with potentiation towards amoxicillin + clavulanic disc were considered potential ESBL producers (Clinical and Laboratory Standards Institute, 2011) (Figure.2, Table 3).

**Preparation of genomic DNA**

Genomic DNA was purified by phenol extraction method (Sambrook *et al.*, 2001). The DNA was stored at -20ºC. The samples were run on 0.8% Agarose gel and stained with Ethidium bromide. The stained gel was examined under UV light to look for the presence of DNA bands of particular size using a molecular weight marker; λ DNA *Hind III* double digest.

**PCR amplification for β-lactamase encoding genes**

PCR analysis for β-lactamase genes of the family TEM was carried out. Primers obtained from Medox, (Chennai) used for *bla* TEM amplification were 5′- CTTCCTGTTTTGCTCACCCA -3′ and 5′ TACGATACGGGAGGGCTTAC -3′. For PCR amplification, the parameters were, 94°C for 1 minute initial denaturation, and 30 cycles of 94°C for 30 seconds, 63°C for 1 minute, 72°C for 1 minute and a final extension in 72°C for 7 minutes. The amplified products were resolved in 1.5% agarose gel. The gel was visualized by staining with ethidium bromide. A 100bp ladder molecular weight marker was used to measure the molecular weights of amplified products.

**Molecular typing of *E. coli* DNA by RFLP**

The DNA samples were digested by using *Xba*I restriction enzyme. The digestion mix constitutes 5µl of Restriction enzyme, 7µl of Restriction assay buffer, 18µl of Sterile water . The mixture was distributed into each 0.5ml tubes. 10µl of the DNA sample was then added to each vials. The digestion tubes were spun again for a few seconds for complete settlement. The tubes were then kept at 37°C for 4 hours. The digested product were then loaded and run on 1.5% agarose gel containing ethidium bromide and the fragments were visualized under UV transilluminator.

## Result and Discussion

Totally 100 *E. coli* isolates were isolated from different clinical specimens, out of which 30 samples were urine , 50 samples were blood and 20 samples were pus. Ten

isolates were confirmed to positive for ESBL production. Third generation cephalosporins showed 40% to 60% resistance. For these isolates gentamicin and kanamycin showed 30% resistance whereas amphicillin and tetracycline showed 50 to 80% resistance. Imipenem, aztreonam and amikacin depicted 100, 90, and 80% sensitive respectively (Table 4). Using the DDST method, ESBL was confirmed in 10 isolates (10%) of which one blood sample (2%) and the rest were urine samples (30%) (Figure 2).

**PCR and RFLP analysis**

Confirmed ESBL samples were checked for the presence of TEM gene by PCR amplification and it was found to be positive. PCR products of molecular size 717 bps showed that it was TEM gene and it resulted in 10% of isolates contained $bla_{TEM}$ genes (Figure 4; Figure 5). The results showed that 10% of isolates were positive for ESBL both by phenotypic and genotypic methods. Further Restriction fragment length polymorphism (RFLP) was carried out for genomic DNA with *Xba I* restriction enzyme to check for the polymorphism (Figure 6). RFLP analysis showed six different banding patterns for the ten samples among which five samples showed identical banding pattern and other two samples also had the similar banding pattern, indicating two different source of origin. Other samples had different patterns indicating that they are not from same region. Thus ten samples showed six different banding pattern concludes that the samples are not from same origin.

This study demonstrates the presence of ESBL mediated resistance of *E.coli* from clinical specimens collected from intensive care unit patients of Sooriya hospital. The prevalence of ESBLs is 10%. This study, aimed to detect and to do the molecular characterization of the types of ESBL genes in clinical isolates of *E coli*. In addition to DDST, PCR amplification assay for the detection of the ESBL genes in clinical isolates of *E coli* was used in this study because this assay has been shown to have the advantage of rapidly screening large numbers of clinical isolates (Woodford *et al*., 2006) and the DNA was used for further molecular epidemiological characterization (Bradford, 2001).

Blood stream infections caused by extended spectrum beta lactamases (ESBL) producing microorganisms are a major concern for clinicians, since they markedly increase the rates of treatment failure and death particularly in intensive care units in medical and surgical wards (Du, *et al*., 2002). Hence the detection of ESBL is necessary to formulate the treatment policy (David *et al*., 2005). There have been reports of considerable geographical differences in ESBLs worldwide and within countries (Coque *et al*., 2008). The hospital to hospital variability that occurs possibly reflects the direct proportionality between use and misuse of antibiotics (Deepti Rawat and Deepthi Nair, 2010). In India, prevalence rate of ESBLs varies from 28 to 84%. While earlier reports from North India have documented the prevalence of ESBLs ranging from 55 to 69%, a recent study from North India reported an alarming 64.8% of ESBL producing isolates. In Southern India, 21.6% ESBL producing bacteria have been documented in a study from Karnataka and another study from Coimbatore reported about 40% ESBL producers (Al- Agamy *et al*., 2009).

**Table.1** The details of intensive care unit patients

| Parameter | Value |
|---|---|
| Sex | Both Male & female |
| Age group (yr) | 30 - 60 |
| Specimen type | Blood (50 samples)<br>Urine (30 samples)<br>Pus (20 samples) |

**Table.2** Standard Zone diameter breakpoints (CLSI (Jan 2011) M100-S21 Twenty first informational supplement, Vol 31, No 1)

| Antimicrobial agent | Disk content | Zone diameter Breakpoints | | |
|---|---|---|---|---|
| | | S | I | R |
| Cefotaxime | 30µg | ≥26 | 23 - 25 | ≤22 |
| Ceftazidime | 30µg | ≥21 | 18 - 20 | ≤17 |
| Ceftriaxone | 30µg | ≥23 | 20 - 22 | ≤19 |
| Cefpodoxime | 10µg | ≥21 | 18 - 20 | ≤17 |
| Cefazolin | 30µg | ≥23 | 20 - 22 | ≤19 |
| Amphicillin | 10µg | ≥17 | 14 - 16 | ≤13 |
| Tetracycline | 30µg | ≥15 | 12 - 14 | ≤11 |
| Amikacin | 30µg | ≥17 | 15 - 16 | ≤14 |
| Gentamycin | 10µg | ≥15 | 13 - 14 | ≤12 |
| Imipenem | 10µg | ≥23 | 20 - 22 | ≤19 |
| Aztreonam | 30µg | ≥21 | 18 - 20 | ≤17 |
| Kanamycin | 30µg | ≥18 | 14 - 17 | ≤13 |

**Table.3** Zone diameter breakpoints recommended by CLSI (M100-S21 Twenty first informational supplement, Vol 31, No 1) β - lactam/ β lactamase combination

| Antimicrobial agent | Disk content | Zone diameter Breakpoints | | |
|---|---|---|---|---|
| | | S | I | R |
| Amoxilin - clavulanic acid | 20µg/10µg | ≥18 | 14 - 17 | ≤13 |

**Table.4** Antibiotic susceptibility of ESBL Producers of *E.coli*

| Antibiogram | ESBL | positive | |
|---|---|---|---|
| | | n | % |
| Cefotaxime | Sensitive | 6 | 60 |
| | Resistant | 4 | 40 |
| Ceftazidime | Sensitive | 5 | 50 |
| | Resistant | 5 | 50 |
| Ceftriaxone | Sensitive | 4 | 40 |
| | Resistant | 6 | 60 |
| Cefpodoxime | Sensitive | 4 | 40 |
| | Resistant | 6 | 60 |
| Cefazolin | Sensitive | 3 | 30 |
| | Resistant | 7 | 70 |
| Amphicillin | Sensitive | 2 | 20 |
| | Resistant | 8 | 80 |
| Tetracycline | Sensitive | 5 | 50 |
| | Resistant | 5 | 50 |
| Amikacin | Sensitive | 8 | 80 |
| | Resistant | 2 | 20 |
| Gentamycin | Sensitive | 7 | 70 |
| | Resistant | 3 | 30 |
| Imipenem | Sensitive | 10 | 100 |
| | Resistant | 0 | 0 |
| Aztreonam | Sensitive | 9 | 90 |
| | Resistant | 1 | 10 |
| Kanamycin | Sensitive | 7 | 70 |
| | Resistant | 3 | 30 |

**Figure.1** Percentage of Resistance for ESBL producers

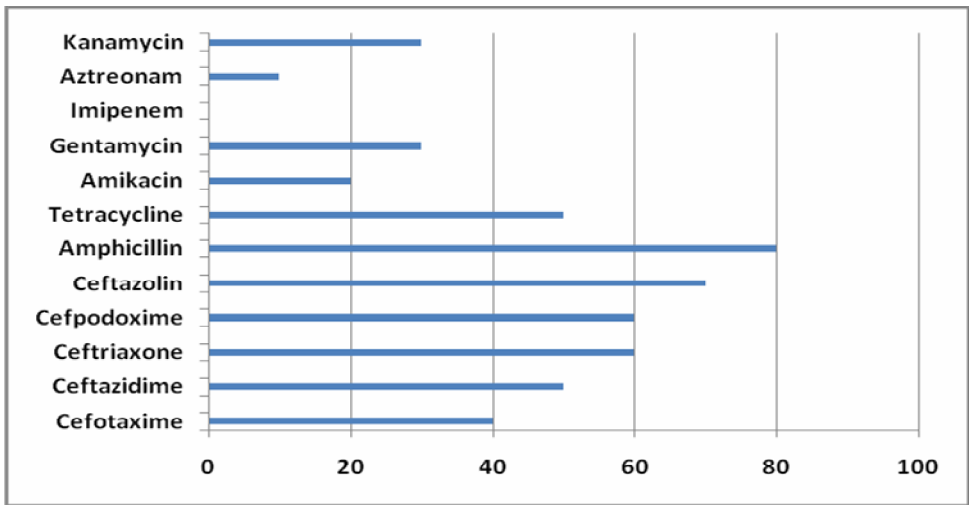



**Figure.2** Double disc synergy test.(Augmentin Disc (20µg of amoxicillin + 10µg of clavulanic acid) with 30µg of Cefotaxime )

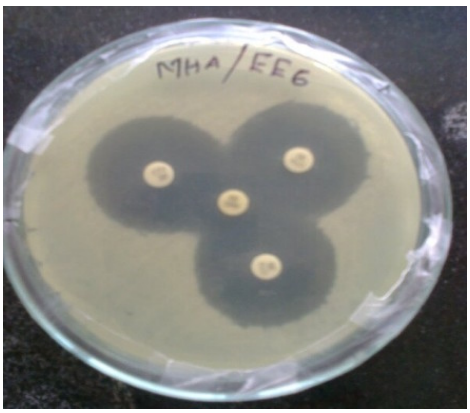



**Figure. 3** Genomic DNA of ESBL producing *E.coli* samples

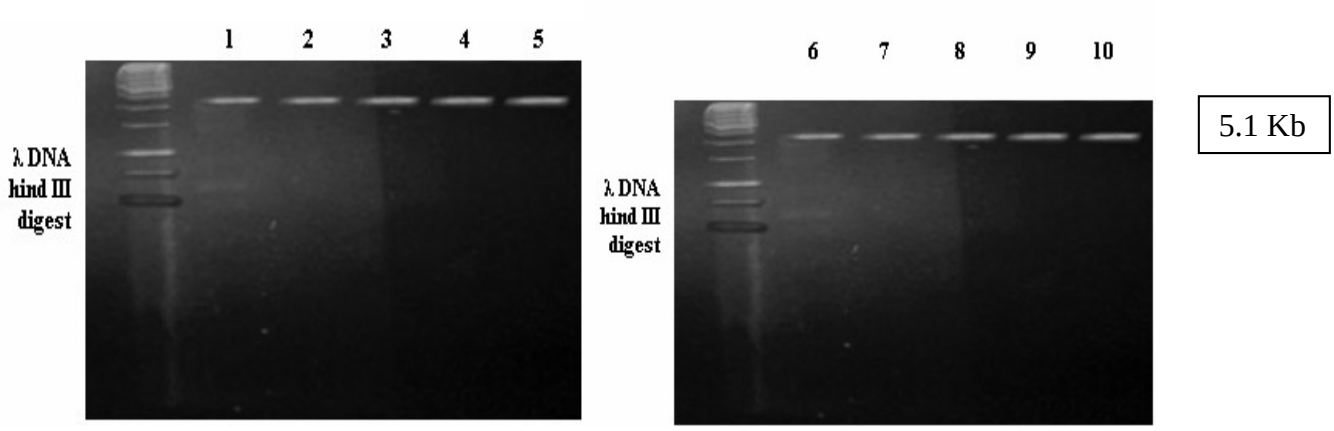


**Figure. 4** PCR products showing *Tem* gene of 717bp molecular weight

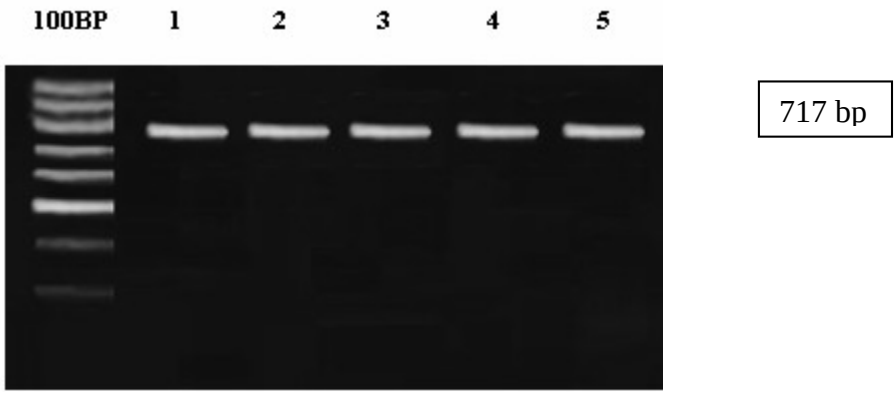



**Figure. 5** PCR products showing *Tem* gene of 717bp molecular weight

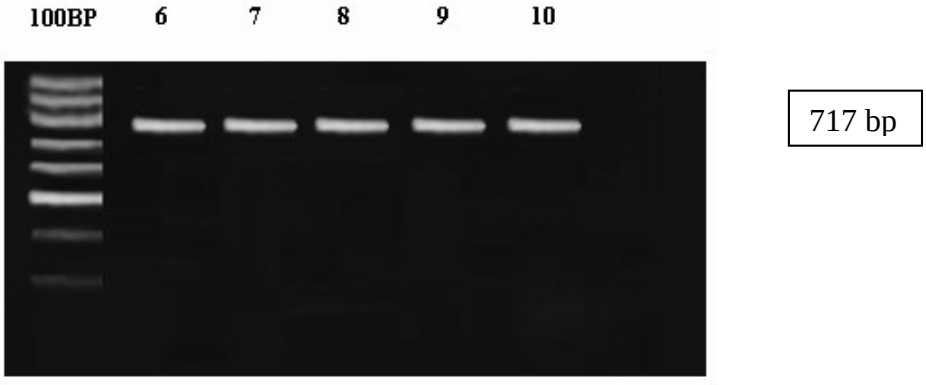
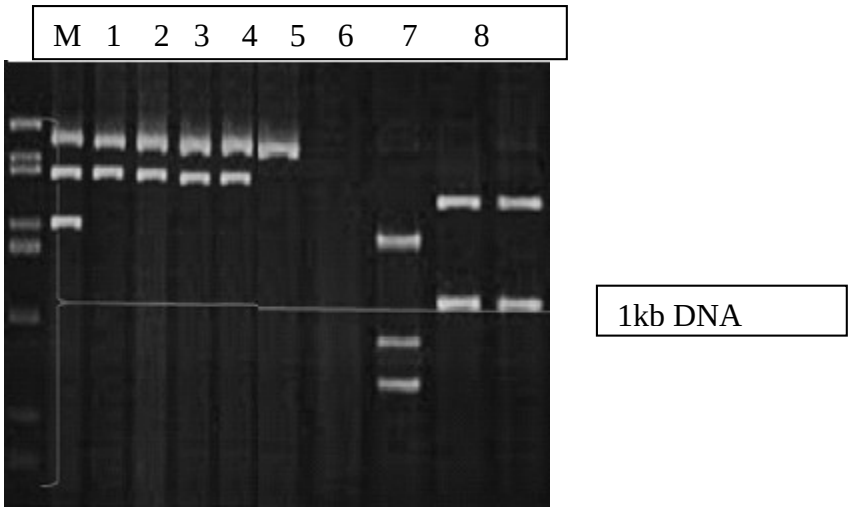



**Figure. 6** Digested DNA of *E. coli* samples



One similar report from Chennai found ESBL mediated resistance to 3GCs in 6.6% of the isolates recovered (Tanhkiwale *et al*., 2004) . Our study also reported that the prevalence of ESBL in Vadapalani area in Chennai is 10% which is similar to that of the other study conducted by Sri Ramachandra medical college in Porur, Chennai.

The population of India of over one billion represents a potentially vast reservoir of antimicrobial resistance genes including those ESBLs (Tsering *et al*., 2009). ESBLs are undergoing continuous mutations, causing the development of new enzymes showing expanded substrate profiles. At present, there are more than 300 different ESBL varients and these have been clustered into nine different structural and evolutionary families based on aminoacid sequence (Muzaheed *et al*., 2008). In the present study ESBL positive isolates screened by Double disc synergy test were also screened by PCR technique. All the ten isolates were found to contain TEM gene.

The limitation of this study, in nature, was that it was performed at a single hospital. Thus, the study results may not reflect the epidemiology of different centers and/or different geographic areas. From this study it was concluded that the occurrence of ESBLs in the isolates emphasized the need to adopt appropriate control measures for treatment to reduce the ESBL burden and thereby drug resistance. The isolates are multidrug resistant and become resistant to available antibiotics since it is plasmid mediated resistance the transfer of this resistance is very quick and also will be induced by treating the patients by cephalosporin drugs. The quick detection of these strains in microbiology laboratories is very important. The molecular typing would determine the type of ESBL that are present in each isolates.

Molecular detection and identification of β - lactamase would be essential for a reliable epidemiological investigation of antimicrobial resistance. Therefore ESBL producing organism should be identified quickly so that appropriate antibiotic usage and infection control measures can be implemented.

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