

**Original Article**

## MOLECULAR CHARACTERISATION OF EXTENDED SPECTRUM BETA LACTAMASE PRODUCING STRAINS FROM BLOOD SAMPLE

DHAYA RANI VARKEY<sup>1</sup>, V. BALAJI<sup>2</sup> AND JAYANTHI ABRAHAM<sup>3, 2</sup>

<sup>2</sup>Department of Microbiology, Christian Medical College and Hospital, Vellore, India, <sup>1,3</sup>Microbial Biotechnology laboratory, School of Bio Sciences and Technology, VIT University, Vellore, India. Email: jayanthi.abraham@gmail.com

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### ABSTRACT

**Objective:** Extended spectrum beta lactamases (ESBLs) have been observed virtually in all the species belonging to the family of *Enterobacteriaceae*. The enzymes are predominantly plasmid mediated and are derived from broad-spectrum beta lactamase genes TEM, SHV, CTX-M. This study was undertaken to characterize ESBL producers in Trichy, Tamilnadu.

**Methods:** Total of 361 blood samples were screened for ESBL producing isolates. The organisms isolated were subjected to morphological and biochemical test and the isolates identified were predominantly *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae*. Antibiotic susceptibility test was performed employing Kirby Bauer's disc diffusion method.

**Results:** PCR analysis for β-lactamases genes of the family TEM, SHV and CTX-M was carried out. Among the isolates obtained 138 ESBL positive for *E. coli* harbour 75% TEM gene, 66% SHV gene and 71% CTX-M gene, followed by *K. pneumoniae*, and *E. cloacae*.

**Conclusion:** The results show an emergence of ESBL positive strains out of 361 isolates 250 samples were confirmed as ESBL producers.

**Keywords:** ESBL producing strains, β-lactamases genes, TEM, SHV and CTX-M.

### INTRODUCTION

Antimicrobial resistance is proven to develop when an organism is exposed to an antimicrobial substance for a given period of time as they develop adaptability to the compound. The resistance acquired by ESBL producing strains stems from genetic point mutation and it is an unsolved, growing problem. In the near future both clinicians and microbiologist will be battling against ESBL producing strains due to their dynamic evolution and epidemiology in preventing and controlling them. One of the alarming natures of the ESBL producing strains is that they are resistant to antibiotics like cephalosporins, imipenem, aztreonam etc. Bali et al. [1] reported that ESBLs are undergoing continuous mutations, causing the development of new enzymes showing expanded substrate profiles. One of the major reasons for their resistance is inappropriate usage of antibiotics in treatment of infection and rapid detection of the antibiotic pattern is crucial for treatment [2]. The prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution. The most prevalent gene is *bla<sub>SHV-15</sub>* which is often associated with a widely distributed variant of *Escherichia coli* [3]. Most reports on ESBL genes *bla<sub>TEM</sub>* and *bla<sub>SHV</sub>* types are related to cross infection in hospitals. In an investigation conducted by Reddy et al. [4] in Chicago the rate of ESBL gene carriage among high risk, hospitalized patients increased from 1.3% to 3.2%, and bacteraemia developed in 8.5% of all previously identified ESBL gene carriers during hospitalization. In Spain, an increase was also observed in faecal carriage in hospitalized patients from 0.3% in 1991 to 11.8% in 2003[5]. Murat et al. [5] detected ESBL producing *E. aerogenes* clinical isolates recovered from blood sample of patient admitted to a tertiary care hospital of Nigeria. In earlier works scientist [6,7,8] have reported that members belonging to Enterobacteriaceae family evade antibacterial treatment by the over expression of chromosomal AmpC beta lactamase and further complications are emerging with Class A ESBL producing strains. Among isolates of *K. pneumoniae*, the percentage of ceftazidime resistance ranges from 5 to 10% for non-intensive care unit (non-ICU) and ICU isolates, respectively some hospitals with low levels of ESBLs producing isolates may not find it cost-effective to test for ESBL producing isolates on a routine basis [9]. ESBL producing strains are mutant, acquired plasmid mediated β-lactamases which demonstrate unique hydrolytic properties. They have the potential enzyme to digest β-lactamase antibiotic, which possess oxyimino group such as oxyimino-cephalosporins. However, they are inhibited by

clavulanate and tazobactam which are β-lactamase inhibitors. At present there are 300 different ESBL producing strains clustered into nine different structural and evolutionary families based on amino acid sequence [1]. The first plasmid-mediated β-lactamase in gram-negative organism was described in the early 1960s in TEM-1 gene [10]. There are many types of ESBLs like TEM, SHV, CTX, OXA, AmpC, etc. but majorities of the ESBLs are derivatives of TEM or SHV enzymes, and these enzymes are most often found in *E. coli*, *K. pneumoniae* and *Acinetobacter baumannii*. This results from point mutation in *bla<sub>TEM</sub>* and *bla<sub>SHV</sub>* genes due to single amino acid changes Gly238 → Ser, Glu 240 → Lys, Arg 164 → ser, Arg164 → His, Asp179 → Asp and Glu (Asp) 104 → Lys which has formed the basis of this resistance [11]. Cohen et al. [12] screened for ESBL genes using microarray to detect single nucleotide polymorphism essential *bla<sub>TEM</sub>*, *bla<sub>SHV</sub>*, genes and *bla<sub>CTX-M</sub>* group and confirmed that DNA sequence were discordant. In yet another study conducted by Fereshteh et al.[13] the *bla<sub>VEB</sub>* gene was detected in almost all ESBL producing isolates from burn victims admitted to Kerman University general hospital of Iran. Survey regarding ESBL producing isolates detection is crucial for infection control measure and the choice of antimicrobial therapy chosen there upon. The detection of ESBL-producing bacteria in laboratories is a vital step for appropriate management of patients, but genotypic identification of these enzymes provides essential information for infection prevention and control efforts [14]. Keeping in view about the complexity of ESBL producing strains, the current study was undertaken to examine the prevalence of ESBL producing strains in Trichy district and occurrence of TEM or SHV gene among *Enterobacteraceae* members.

### MATERIALS AND METHODS

**Bacterial strains:** A total of 250 ESBL producing isolates were isolated from 361 blood samples received from hospitals in Trichy, Tamilnadu, for a period of two years (2010-2012). Written consent was obtained from the patients.

### Phenotypic confirmatory test

Morphological characterisation was studied by Grams staining, followed by biochemical tests such as indole, methyl red, Voges-Proskauer (VP), citrate, Triple sugar iron, urease, motility glucose, sucrose, lactose, mannitol and gelatin.

Kirby Bauer's disc diffusion method was employed for antibiotic susceptibility testing of the organism with cefotaxime (30 $\mu$ g), ceftazidime (30 $\mu$ g), amikacin (30 $\mu$ g), levofloxacin (5 $\mu$ g), imipenem, meropenem (10 $\mu$ g), ciprofloxacin (10 $\mu$ g), cefoxitin (30 $\mu$ g), ertapenem (10 $\mu$ g), tigecycline (30 $\mu$ g), piperacilline (30 $\mu$ g).

**Detection of ESBL producing strains:** ESBL producing strains detection was carried out using control strains ; ATCC *E. coli* 35218, and ATCC *Klebsiella pneumoniae* 700603 which was used following the Clinical and Laboratory Standards Institute CLSI guidelines [15] recommended method for screening and confirmation using cefotaxime and ceftazidime as substrates. Cefepime was also tested as substrate following the same method. A >5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was taken as positive result for ESBL production [9].

#### Genomic plasmid and DNA analysis

Plasmid DNA was isolated from bacterial cells by alkaline lysis method. Genomic DNA was purified by phenol extraction and ethanol precipitation method [16]. The DNA was stored at -20°C. The samples were run on 1.5 per cent agarose gel and stained with ethidium bromide. The stained gel was examined under UV light to detect the presence of plasmid bands of particular size using a molecular weight marker 1000bp (Roche, USA).

**PCR for  $\beta$ -lactamase encoding genes:** PCR analysis for  $\beta$ -lactamase genes of the family TEM and SHV was carried out. Primers obtained from Sigma, USA used for *bla<sub>TEM</sub>* were 5'- AAAATTCTTGAAGACG-3' and 5'-TTACCAATGCTTAATCA-3' and for *bla<sub>SHV</sub>* were 5'-TTAACTCCCTGTTAGCCA-3' and 5'-GATTGCTGATTTGCC-3' and *bla<sub>CTX-M</sub>*/F: 5'-TTTGCAGATGTGCAGTACCACTAA-3' and CTX-M/R1:5'-CTCCGCTGCCGGTTTATC-3') primers [17].

**Amplification of ESBL producing Genes:** Amplification was done using thermal cycler with cycling parameter comprising initial denaturation at 95°C for 2 min followed by 30 cycles each of denaturation at 95°C for 45sec, annealing at 62°C for 45 sec, amplification at 72°C for 1 min and post extension at 72°C for 5min, for the amplification of *bla<sub>TEM</sub>* and *bla<sub>SHV</sub>* amplifications conditions for thermal cycling remained the same except for temperature of 55°C. The amplified products were separated in 1.5 per cent agarose gel. The gel was visualized by staining with ethidium bromide (0.5 mg/ml) in a dark room for 30 min. A 100 bp ladder molecular weight marker (Roche, USA) was used to measure the molecular weights of amplified products. The images of ethidium bromide stained DNA bands were digitized using a gel documentation system.

#### RESULTS AND DISCUSSION

During the two year period, a total of 361 blood samples were collected from in and around Trichy district, of Tamilnadu. The organism were isolated from blood samples of patients and characterised by morphological and biochemical test. The isolates obtained were Gram negative. The biochemical profile were as follows Gram negative bacilli and non motile or motile indicated a positive reaction for Methyl Red, VP, citrate, urease, glucose, sucrose, lactose, Mannitol and a negative reaction for indole, TSI and gelatin test indicated the isolate to be *Klebsiella pneumoniae*. Gram negative, nonmotile organism positive for Indole, MR, glucose, sucrose, lactose and mannitol.

A negative reaction was observed towards citrate, TSI, urease and gelatin which was confirmed as *Escherichia coli*. *Enterobacter cloacae* was motile, Gram negative organism showing a positive reaction for Methyl red, TSI, glucose and mannitol and a negative reaction for indole, VP, sucrose, lactose and gelatin. Out of 361 isolates 250 samples were found to be ESBL producing organisms. *E. coli* showed 138 positive samples for ESBL producing strains among which TEM type ESBL producers were found in 75% of *E. coli*, 67% of *K. pneumoniae* and 89% of *Enterobacter cloacae*. SHV gene was confirmed in 66% of *E. coli* and 55% in *K. pneumoniae* and 18 % of *Enterobacter cloacae*. 71% CTX-M genes was harboured by *E. coli*, 85% in *K. pneumoniae* and 3 % of *Enterobacter cloacae*.

Thus, 250 of the 361 isolates were confirmed as potentially ESBL producers using ceftazidime/clavulanate and cefotaxime/clavulanate disks. Occurrences of ESBL production in isolates were as follows: 163 (65.2%) of *E. coli*, 111(35.6%) of *K. pneumoniae*, 83 (33.2%) of *Enterobacter cloacae*. Our findings support earlier findings of Bali et al. [1] that TEM and SHV beta lactamases are mainly found in *E. coli* and *K. pneumoniae* but can occur in other members of the family Enterobacteriaceae. The plasmid DNAs were isolated from 250 isolates of potentially ESBL producing strains *E. coli*, *K. pneumoniae*, which were screened by PCR using *bla<sub>TEM</sub>*, *bla<sub>SHV</sub>*, *bla<sub>CTX-M</sub>* specific primers.

Asma (2006) reported that CTX-M type  $\beta$  lactamases may be the most frequent type of ESBL producing strains worldwide predominantly found in three geographic areas such as South America, the Far East and Eastern Europe, which also reported in China, Japan, India, North America and Western Europe.

All the *E. coli* isolates were found to be resistant to ampicillin, cefotaxime and ceftazidime, Cefepime and 96.71% were resistant to levofloxacin followed by 81.69% resistant for piperacillin. They were found to be 93.89% sensitive to tigecycline followed by 87.67% to imipenem, 89.20% sensitive to meropenem and 87.32% sensitive to ertapenem. A similar trend was recorded in the resistant pattern of *K. pneumoniae*. Sensitivity to imipenem was found to be 75.53%, followed by meropenem 74.10%. Bazzaz et al. [2] have mentioned that the prevalence of ESBL producing strains of *E. coli* and *K. pneumoniae* was 59.2% in Iran. Total resistance was recorded with ampicillin, cefotaxime, ceftazidime, and cefepime again with *E. cloacae* followed by 97.0% cefeprome and 94.11% with cefoxicetine. Sensitivity was found to be around 70% against *E. cloacaea* with imipenem, meropenem, ertapenem and tigecycline.

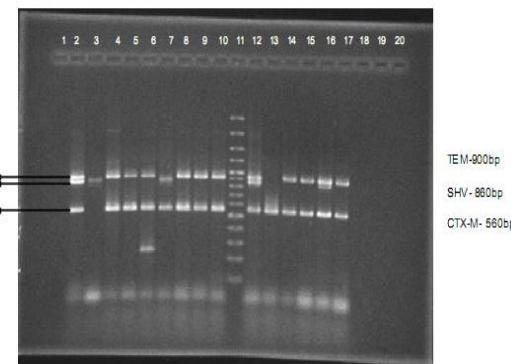


Fig.1: PCR bands for ESBL producing isolates

The investigation reveals an alarming percentage of ESBL producing strains within the *Enterobacteriaceae* strain. The ESBL producing genes are mostly found to be coded within the plasmid and hence readily transferred to other organisms, which could be the reason for high percentage of ESBL genes harbouring organisms. In an earlier investigation regarding a outbreak in Chicago by ESBL producing *K. pneumoniae* and *E. coli* it was concluded that a common plasmid harbouring TEM-10 was isolated from patients admitted to several hospitals and nursing homes and subsequently plasmid expressing TEM-10 was transferred to the normal flora of other patients [19]. In the present study the PCR bands yielded a homogeneous band pattern, clearly suggesting that there may be a point source or a common evolutionary origin for all the ESBL producing isolates (Fig. 1). This pattern results from point mutation in *bla<sub>TEM</sub>* and *bla<sub>SHV</sub>* genes due to single amino acid changes.

#### CONCLUSION

The result of the epidemiology analysis reveals an alarming percentage of ESBL producing strains occurrences in Trichy,

Tamilnadu. ESBL producing isolates have emerged as a major challenge which is due to over use of expanded-spectrum cephalosporins in the hospitals and nursing homes. As the genes are mostly plasmid mediated the spread of ESBL producing strains has become more rapid. This study helps us to understand the need for more precaution in use of antibiotics and the alarming rate of resistance seen in ICU and neonatal care units. Future studies will be focused on sequencing of TEM, SHV and CTX-M gene.

#### CONFLICT OF INTEREST

There is no conflicts of interest to declare.

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