## ORIGINAL ARTICLE

# DETECTION OF TEM & SHV GENES IN EXTENDED SPECTRUM BETA LACTAMASE (ESBL) PRODUCING E.COLI & KLEBSIELLA PNEUMONIAE ISOLATED FROM A TERTIARY CARE CANCER HOSPITAL

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## ABSTRACT

**Introduction:** Extended-spectrum beta-lactamases (ESBLs) are rapidly evolving group of beta-lactamase enzymes commonly produced by the Gram negative bacteria, these enzymes have been derived from TEM and SHV genes by mutations. This study was done to look for TEM and SHV genes in ESBL positive *E.coli* and *K. pneumoniae* isolated from the cancer patients suffering with infections and admitted in a tertiary care cancer hospital.

**Methods:** A total of 156 clinical isolates of *E. coli* (*n*=80) and *K. pneumonia* (*n*=76) isolated from various clinical samples received in microbiology laboratory over a period of six months from May to October 2012. All the isolates were tested for ESBL production as recommended by the Clinical Laboratory Standard Institute (CLSI) by double disc diffusion screening test and by ESBL E-Test. Multiplex PCR was performed specific for TEM and SHV genes.

**Results:** In this study 64.10% (100/156) of the isolates were ESBL producers with highest(65.7%) incidence among *K. pneumoniae*. All the ESBL positive isolates were selected for multiplex PCR for TEM and SHV genes of which 44% *E.coli* were positive for TEM gene alone, 4% positive for both TEM and SHV genes, 52% of *E. coli* were negative for both TEM and SHV genes. In case of *K. pneumoniae* 76% were positive for both TEM and SHV genes, 20% for SHV gene alone and 4% were positive for TEM gene alone.

**Conclusion**: Our findings showed that majority of *E.coli* were negative for both TEM and SHV genes probably associated with other genes producing ESBL. Such molecular studies should be done in different regions of India to find the common ESBL enzymes present in that geographical area for epidemiological purposes.

**Key words:** ESBL, TEM, SHV, Multiplex PCR

### INTRODUCTION

Extended spectrum beta-lactamases are plasmid mediated beta-lactamases that confers resistance to all cephalosporins and aztreonam, except to cephamycins and carbapenems and are inhibited by clavulanic acid[1].The widespread use of third generation cephalosporins and aztreonam is believed to be the cause of mutations in the classical TEM and SHV genes[2].

Presence of ESBL among *E.coli* and *K.pneumoniae* is reported from every part of India but there was no information available on the molecular types of ESBL enzymes prevalent in this part of country. In our study we used multiplex PCR to find mainly TEM and SHV genes producing ESBL *E.coli* and *K.pneumoniae.*

### METHODS

A total of 156 consecutive non-repetitive clinical isolates of *E.coli* and *K.pneumoniae* isolated in the microbiology laboratory from a variety of clinical samples like, urine, sputum, blood, pus swabs, frank pus and other body fluids, over a period of six months from May 2012 to Oct 2012, were included in the study. The organisms were identified based on the colony morphology and the routine biochemical reactions.

Antimicrobial susceptibility testing: Antimicrobial susceptibility test was performed by using the Kirby Bauer disc diffusion method as per the CLSI guidelines2012. Antimicrobial discs used in this study were levofloxacin (5μg) ciprofloxacin (5μg) Piperacillin (100 μg) Piperacillin-tazobactum (100/10μg), ceftriaxone (30 μg) aztreonam (30 μg), Cefepime (30 μg) Gentamicin (10 μg), imipenem (10 μg). All the discs were obtained from Hi-Media, Mumbai, India.

All the isolates under study, irrespective of their susceptibility to ceftriaxone ($3^{rd}$ gen cephalosporin) and aztreonam, were tested for the ESBL production by the phenotypic confirmatory tests using Double disc diffusion test (DDDT) and ESBL E-test.

The Detection of ESBL by the phenotypic confirmatory Tests

Double Disc Diffusion Test (DDDT): This test was done using two combination of antibiotics along with clavulanic acid i.e. Ceftazidime (30µg)/ ceftazidime+clavulanic acid (30/10µg) (CAZ/CAZC) and Cefotaxime (30µg)/cefotaxime + clavulanic (30/10µg) (CTX/CTXC) acid. Both the discs were placed 25 mm apart centre to center, on a lawn culture of the test isolate on Muller Hinton agar plate and incubated overnight at 37$^0$C. A ≥5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulinic acid versus its zone when tested alone was designated as ESBL positive.

ESBL E-Test: All the isolates which were positive for ESBL by DDDT were further confirmed by ESBL E-Test method (Biomerieux). E-Test is a plastic strip impregnated with a stable gradient of different concentration of cefotaxime(0.25-16µg/ml) at one end and the remaining end of which generates a gradient of cephalosporin (i.e., cefotaxime 0.016-1 µg/ml) plus a constant concentration of clavulanate (4 µg/ml). ESBL production is inferred if the MIC of cefotaxime is ≥ 0.5µg or ratio for cephalosporin upon cephalosporin plus clavulanate is ≥ 8 or there is deformation of ellipse or phantom zone as per the manufacturer guidelines.

*K.pneumoniae* ATCC 700603 was used as a positive control for ESBL production and *E.coli* ATCC 25922 was used as a negative control for ESBL production.

The detection of TEM & SHV genes was done by multiplex PCR: Multiplex PCR is a technique were two or more unique target DNA sequences can be amplified simultaneously in a single reaction mixture.

Extraction of plasmid DNA: Fresh culture of test organism , grown on nutrient agar (Hi-Media, Mumbai) were suspended in 500 µl of autoclaved distilled water and vortexed to get a uniform suspension. The bacterial cells were lysed by heating the suspension at 100$^0$C in water bath for 10 minutes. The cellular debris was removed by centrifugation at 8000 rpm for 5 minutes and the supernatant was used as the template DNA.

**Table 1: Primers used in this study**

| Target gene | Primer used | Sequence (5'-3') | Product Size(bp) |
|---|---|---|---|
| SHV | SHV(F*) | TCAGCGAAAAACACCTTG | 471 |
| | SHV(R*) | TCCCGCAGATAAATCACC | |
| TEM | TEM(F*) | CTTCCTGTTTTTGCTCACCCA | 717 |
| | TEM(R*) | TACGATACGGGAGGGCTTAC | |

(F*): Forward base; (R*): Reverse base

Amplification of TEM and SHV genes: Amplification reaction for TEM and SHV genes was performed in a 25µl of volume reaction mixture containing 12.5 µl master mix (10X PCR buffer+DNTP's+Taq DNA polymerase) as supplied by Qiagen, India. 1 µl each of SHV(F), SHV(R), TEM(F), TEM(R) primers, 3.5 µl DNase free water and finally 5 µl of extracted plasmid DNA template.

The thermal cycling conditions for amplifying both TEM and SHV genes in a single reaction mixture was as follows,

Initial denaturation at 95$^0$C for 15 min and then 30 cycles of 94$^0$C for 1 min, 58$^0$C for 90 sec, 72$^0$C for 1 min, followed by a final extension at 72$^0$C for 7 min. The resulting PCR products were analyzed by gel electrophoresis with 1% agarose gel in Tris-Borate-EDTA buffer. The gels were stained with ethidium bromide and the bands were observed and photographed using an automated Gel documentation system Alpha Imager.

It was performed using the amplicons of E.coli&K.pneumoniae from the clinical samples, DNA ladder 100bp(molecular weight standard), positive control of ATCC 700603K.pneumoniae and negative control ATCC 25922E.coli (fig: 1).

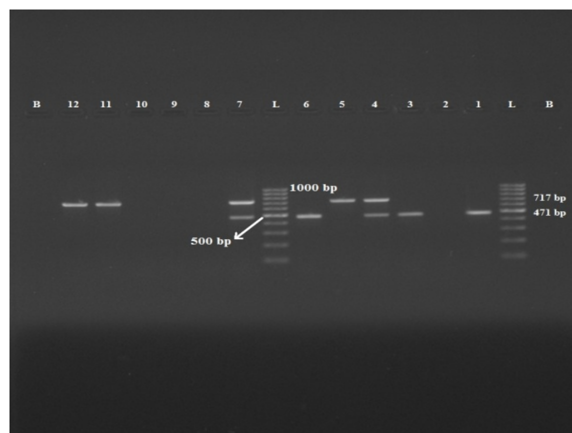


**Fig1 : Gel electrophoresis**

B=Blank;L=Ladder (100bp);1=ATCC *Klebsiella pneumonia 700603* (+ve control for SHV);2=ATCC *E.coli 25922* (-ve control for TEM &SHV);3 to 7 = *Klebsiellapneumoniae* (Test organisms); 8to12= *E coli* (Test organisms).

## RESULTS

Of the 156 isolates of *E.coli* (n=80) and *K.pneumoniae* (n=76) included in our study, it was seen that majority

of E.coli 30(37.5%) were isolated from urine samples, 23(28.7%) of them were isolated from pus swab, followed by blood samples 14 (17.5%), 6(7.5%) from sputum, 3(3.75%) from body fluids, 1(1.25%) each from frank pus, BAL, catheter tips samples.

In case of K. pneumonia, 27(35.5%) strains were isolated from pus swab, 18(23.68%) from sputum samples, 11(14.47%) from urine samples, 6(7.89%) from blood samples, 3(3.94%) from catheter tips, 2(2.63%) each from frank pus and body fluids, and 1(1.31%) was isolated from BAL sample. Among all 156 test strains 64.10% (100/156) were ESBL producers, ESBL production among *K.pneumoniae* was 65.7% (50/76) and among *E.coli* 62.5% (50/80).All these isolates were found to be ESBL positive by Double disc diffusion test as well as by ESBL E-test method.

All ESBL positive *E.coli* (n=50) and *K.pneumoniae* (n=50) were selected for the presence of SHV and TEM genes by multiplex PCR.In multiplex PCR 52% of *E.coli* were negative for both TEM and SHV genes. 44% were found to carry TEM gene alone 4% positive for both TEM and SHV genes, none of the isolates were positive for SHV gene alone.

In case of *K.pneuomoniae* (n=50) 76% were positive for both SHV and TEM genes, 20% for SHV genes alone and only 4% isolates positive for TEM gene alone (fig: 2).

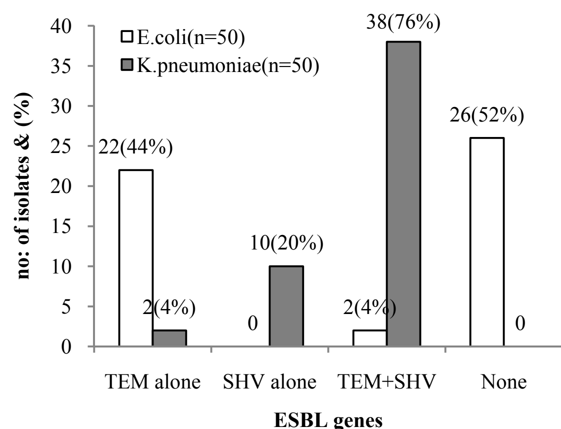


**Fig 1: Frequency distribution of TEM & SHV genes in ESBL positive isolates**

## DISCUSSION

Extended spectrum beta-lactamases (ESBL) are rapidly evolving group of beta-lactamase which share the ability to hydrolyse third generation cephalosporins and aztreonam, yet are inhibited by clavulanic acid. They are derived from genes for TEM-1, TEM-2 or SHV-1 by mutations that alter the amino acid sequence around the active site of these beta-lactamases[2]. SHV-2 and TEM-3 beta-lactamases are termed ESBls. There are around 200 TEM & 162 SHV types. Other types of ESBLs are CTX, OXA, AmpC etc. CTX-M (ESBL) is derived from chromosomal cephalosporinase (Cephotaximase, Munich). This enzyme is predominantly found in E.coli.

It is difficult to make valid comparison of the prevalence of ESBLs, because of variations in study design by different scientists. The incidence of ESBL in major hospitals of India has been reported to be in a range of 6%-87%[3-8].Out of the 156 isolates included in our study 64.10% showed ESBL production with highest incidence among *K.pneumoniae* (65.7%) followed by *E.coli*(62.5%). In India high prevalence of ESBL producing *K.pneumoniae* strains has been reported by various Groups[3, 7, 8]. In the present study we also noticed a high prevalence of ESBL producing *K.pneumoniae* as compared to *E.coli.*

Many studies have been conducted for the detection and prevalence of genes responsible for ESBLs production in India. In our study majority of ESBL positive isolates of E.coli (52%) were negative for both TEM and SHV genes, 44% of *E.coli* carried TEM gene alone and 4% isolates were positive for both TEM and SHV genes, none of the isolates were positive for SHV alone. In case of the ESBL positive isolates of *K.pneumoniae* 76% were positive for TEM and SHV genes, 20% for SHV alone and 4% isolates were positive for TEM gene alone. CTX-M gene is now the most common ESBL gene among *E.coli*in community and it may be due to overuse of ceftriaxone or due to faecal carriage and transfer of gene by horizontal transmission according to Cavaco et al. 2008[9].Our results corroborated with other studies done in India. Manohar et al[10] in 2006 showed TEM and CTX-X genes to be predominantly found in *E.Coli* (39.2%), while among the *Klebspp*, TEM and SHV and CTX-M genes occurred together in 42.6% of the isolates. According to PrabhaLal et.al[8] in 2007 at AIIMS New Delhi , majority of the ESBL positive isolates of *K.pneumoniae* carried both TEM and SHV genes (67.3%) followed by TEM gene (20%) and SHV in 8.4 % isolates.

*E.coli* and *K.pmeumoniae* are the most common ESBL producing organisms, making difficult for the clinicians to treat them particularly in ICUs. The resistance in these organisms is due to production of enzymes TEM and SHV that are generally plasmid mediated. Such molecular studies should be done in different regions of India to find the common ESBL enzymes present in that geographical area for epidemiological purposes.

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