Detection of TEM & SHV genes in *Escherichia coli* & *Klebsiella pneumoniae* isolates in a tertiary care hospital from India

Jyoti Sharma*, Meera Sharma & Pallab Ray

Department of Medical Microbiology, Postgraduate Institute of Medical Education & Research, Chandigarh, India

Received August 4, 2009

**Background & objectives:** Extended spectrum beta lactamases (ESBLs) have been observed in virtually all the species of family *Enterobacteriaceae*. The enzymes are predominantly plasmid mediated and are derived from broad-spectrum beta lactamase TEM-1, TEM-2 or SHV-1 by a limited number of mutations. This study was undertaken to characterize ESBL producers among *Escherichia coli* and *Klebsiella pneumoniae* by PCR-RFLP, which were initially screened by phenotypic method.

**Methods:** A total of 100 isolates of each species (*E. coli* and *K. pneumoniae*) were screened for ESBL production. PCR analysis for β-lactamase genes of the family TEM and SHV was also carried out. PCR products of TEM and SHV genes were subjected to digest with three different restriction enzymes. The digested products were run on 1.5 per cent agarose gel, stained and examined for DNA bands.

**Results:** PCR carried out on plasmid DNA alone detected 30 per cent ESBL positive isolates using TEM primer and 38 per cent using SHV primer, whereas PCR for both plasmid and chromosomal DNA showed 56 per cent positivity for TEM and 60 per cent positivity for SHV.

**Interpretation & conclusion:** RFLP yielded homogeneous band pattern, suggesting that there may be a point source or a common evolutionary origin for all the ESBL isolates.

**Key words** ESBL - PCR - prevalence - SHV - TEM

In the battle against β-lactamase mediated bacterial resistance, development of third generation cephalosporins in early 1980s was a major breakthrough. But soon, a new plasmid encoded β-lactamase capable of hydrolyzing the extended spectrum cephalosporins was reported. These extended spectrum β-lactamases (ESBLs) are mutant, plasmid-mediated β-lactamases derived from older, broad-spectrum β-lactamases (*e.g.*, TEM-1, TEM-2, SHV-1), have an extended substrate profile which allows hydrolysis of all cephalosporins, penicillins, and aztreonam. These enzymes are most commonly produced by *Klebsiella* spp. and *Escherichia coli*. Failure to detect ESBL production by routine disk-diffusion tests has been well documented.

*Present address:* Senior Lecturer, Department of Microbiology, Dr HSJ Institute of Dental Sciences & Hospital, Panjab University, Chandigarh 160 014, India

e-mail: contactjyotisharma@yahoo.co.in
Moreover, many clinical laboratories are not fully aware of the importance of ESBLs and how to detect them; laboratories may also lack the resources to curb the spread of these resistance mechanisms. This lack of understanding or resources is responsible for a continuing failure to respond appropriately to prevent the rapid worldwide dissemination of pathogens possessing these β-lactamases. The consequence has been avoidable therapeutic failures in patients who received inappropriate antibiotics and outbreaks of multi drug-resistant, Gram-negative pathogens that required expensive control efforts. Clinical outcome data indicate that ESBLs are clinically significant in terms of complication rates and mortality and when detected, these indicate the need for the use of appropriate antibacterial agents. It is not treated adequately, ESBLs infected patients are at risk of having fatal outcome.

There are so many types of ESBLs like TEM, SHV, CTX, OXA, AmpC, etc. But majority of the ESBLs are derivatives of TEM or SHV enzymes and these enzymes are most often found in E. coli and K. pneumoniae. Keeping in view this scenario, the current study was investigated upon E. coli and K. pneumoniae to look for the presence of TEM or SHV gene.

**Material & Methods**

**Bacterial strains**: A total of 100 isolates of each species were screened to get 25 ESBL producing isolates each of E. coli and K. pneumoniae. Bacterial isolates were collected in the Department of Medical Microbiology from pus, sputum and blood culture of patients admitted to Nehru Hospital from May-September 2002, a tertiary care hospital attached to the Postgraduate Institute of Medical Education and Research, Chandigarh, India.

Isolates showing increased zone of inhibition to third generation cephalosporins i.e., ceftazidime (30 μg), cefotaxime (30 μg) and to fourth generation cephalosporins, cefepime (30 μg), were screened for ESBL production.

**ESBL detection**: ESBL detection was carried out following the CLSI (former NCCLS) recommended method for screening and confirmation using cefotaxime and ceftazidime as substrates (Clinical Laboratory Standards Institute, 2006)\(^1\). 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.

**Reference strains**: Three strains, E. coli J53 R1, E. coli C\(^{00}\)PUD16 and K. pneumoniae ATCC 700603 were used as standard ESBL-positive strains. E. coli J53 R1 harboured TEM-ESBL and the remaining strains carried SHV-ESBL. A non ESBL-producing organism (E. coli ATCC 25922) was used as negative control.

**Preparation of plasmid and genomic DNA**: Plasmid DNA was isolated from bacterial cells by alkaline lysis method\(^1\). Genomic DNA was purified by phenol extraction and ethanol precipitation method\(^1\). The DNA was stored at -20°C. The samples were run on 0.8 per cent agarose gel and stained with ethidium bromide. The stained gel was examined under UV light to look for the presence of plasmid bands of particular size using a molecular weight marker; λ DNA hind III double digest (Roche, USA).

**PCR for β-lactamase encoding genes**: PCR analysis for β-lactamase genes of the family TEM and SHV was carried out. Primers obtained from Sigma, USA used for bla TEM were 5’ AAAATTCTTGAAGACG 3’ and 5’ TTACCAATGCTTAATCA 3’ and for bla SHV were 5’ TTAACCTCCTTGAAGACG 3’ and 5’ GATTTGCTTGGTAGGCC 3’. For PCR amplifications, about 500 pg of DNA was added to 50 μl mixture containing 200 μM of dNTPs, 0.4 μM of each primer and 2.5 U of Taq polymerase (Roche diagnostics) in 1x PCR buffer. Amplification was performed in a Techne® Genius Thermocycler (Cambridge, UK) with cycling parameters comprising initial denaturation at 94°C for 3 min followed by 35 cycles each of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, amplification at 72°C for 2 min and final extension at 72°C for 10 min, for the amplification of bla TEM. For bla SHV amplifications conditions for thermal cycling remained the same except for Tm 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 μg/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 (Alphaimager™ 3400, USA).

**Restriction digestion analysis**: The 1080 bp and 768 bp PCR products of TEM and SHV genes respectively were digested with restriction enzymes BSeG1, Pst I and EcoR I (Amersham Pharmacia Biotech, USA) according to manufacturer’s recommendations. After digestion, the products were run on 1.5 per cent agarose gel and stained with ethidium bromide. The
stained gel was examined under UV light to look for the DNA bands of particular size using a molecular weight marker of 100 bp ladder.

**Results & Discussion**

**ESBL detection by disk diffusion:** Out of 200 samples 70 per cent of *E. coli* and 60 per cent of the *K. pneumoniae* isolates were shown to produce ESBLs as investigated by disk diffusion test.

Two sets of primers were used to amplify TEM and SHV genes. PCR amplification of TEM and SHV was carried out on plasmid DNA and chromosomal DNA of *K. pneumoniae* and *E. coli*.

Of the 25 ESBL positive *K. pneumoniae* isolates 15 harboured TEM gene and 18 harboured SHV genes as detected by PCR and of the 25 ESBL positive *E. coli* isolates, 13 carried TEM gene and 12 possessed SHV gene (Table). When PCR was done for plasmid DNA alone, it detected 30 per cent ESBL positive isolates using TEM primer and 38 per cent using SHV primer, whereas PCR with both plasmid and chromosomal DNA showed 56 per cent positivity for TEM and 60 per cent positivity for SHV.

PCR products were digested using restriction enzymes *Bse*G1, *Eco*R1 and *Pst*1. (Fig. a, b and c). SHV amplicons digested with *Eco* R1 and *Pst*1 yielded two bands of molecular weight 550 and 150 bp app. TEM amplicons digested with *Bse*G1 and *Pst*1 yielded three bands and two bands (750 and 150 bp) respectively.

While some ESBLs confer high-level resistance to all oxyimino-cephalosporins, for other ESBLs, resistance may only be slightly increased or selectively affected in certain β-lactams. This creates problem for the clinical laboratory. Some ESBLs may fail to reach a level to be detectable by disk diffusion tests but result in treatment failure in the infected patient. The lack of correlation between ESBL production and disk-diffusion susceptibility results was evident in the present study. These types of discrepancies between susceptibility data and disc diffusion results have increased the need for an improved method of ESBL detection and to incorporate it into routine susceptibility procedures.

Phenotypic tests for ESBL detection only confirm whether an ESBL is produced but cannot detect the ESBL subtype. Nuesch & Hachler\textsuperscript{14} reported that although molecular methods appear sensitive, but are expensive, time consuming and require specialized equipment and expertise. Definitive identification is possible only by molecular detection methods. The techniques, which are necessary for the task of identifying the exact ESBL subtype, are available only in research facilities. A study by Shiri et al\textsuperscript{15} indicated that these phenotypic tests needed to be evaluated periodically, as their performance may change with the introduction of new enzyme.

For amplification of beta lactamases we used two sets of primers for TEM and SHV genes. PCR amplification of TEM and SHV was carried out on plasmid DNA as well as chromosomal DNA of isolates of *K. pneumoniae* and *E. coli*. PCR could detect 84 per cent ESBL positive isolates in *E. coli* and *K. pneumoniae* with either TEM or SHV primer. A study by Grover et al\textsuperscript{16} on phenotypic and genotypic methods of ESBL detection concluded PCR to be a reliable method of ESBL detection\textsuperscript{16}.

PCR products digested using restriction enzymes *Bse*G1, *Eco*R1 and *Pst*1 yielded homogeneous band pattern, suggesting that there may be a point source or a common evolutionary origin for all the ESBL isolates.

In this study we targeted the amplification for TEM and SHV gene DNA. Amplification of whole genomic DNA increased the positivity of detection, compared to amplification of plasmid DNA alone, suggesting β-lactamase expression controlled by both chromosomal and plasmids DNA.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>TEM</th>
<th>SHV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasmid DNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>11 (60)</td>
<td>18 (72)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>13 (52)</td>
<td>12 (48)</td>
</tr>
</tbody>
</table>
In conclusion, the ESBL producing isolates detected positive by CLSI method, PCR detected ESBL genes in 84 per cent of the isolates. Multiplex PCR, incorporating the primers for commonly prevalent ESBLs may be a valuable clinical and research tool for characterization of ESBLs. Moreover, detection of TEM and SHV both on plasmid and chromosome gave better understanding of ESBL production.

Acknowledgment

Authors acknowledge Dr. Reddy’s Laboratories, Hyderabad, for providing ESBL standard strains.

References


*Reprint requests*: Dr Jyoti Sharma, Senior Lecturer, Department of Microbiology, Dr HSJ Institute of Dental Sciences & Hospital Panjab University, Chandigarh 160 014, India e-mail: contactjyotisharma@yahoo.co.in