

## Occurrence of *TEM* & *SHV* gene in extended spectrum $\beta$ -lactamases (ESBLs) producing *Klebsiella* sp. isolated from a tertiary care hospital

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**Background & objectives:** Extended-spectrum  $\beta$ -lactamases (ESBLs) are rapidly evolving group of  $\beta$ -lactamase enzymes produced by the Gram negative bacteria. These enzymes have been derived from *TEM* and *SHV* genes by mutations and have been well described in *Klebsiella pneumoniae*. Information on molecular types of ESBL positive *Klebsiella* sp. is lacking from India. We therefore undertook this study to look for the *TEM* and *SHV* genes in ESBL positive *Klebsiella* sp. isolated from the patients admitted to a tertiary care hospital in north India.

**Methods:** A total of 204 multidrug-resistant isolates of *Klebsiellae* obtained from clinical samples; blood (n=108), urine (n=15), pus (n=2) and sputum (n=79) were obtained and screened for resistance to 3<sup>rd</sup> generation cephalosporins (3GC). The ESBL status was determined by double disk diffusion test (DDDT) and further by ESBL E-test. Multiplex PCR specific for *TEM* and *SHV* genes was performed to distinguish four different genotypes: TEM-positive, SHV-positive, TEM- and SHV-positive and non-TEM non-SHV ESBL types.

**Results:** Eighty six per cent (175 of 204) of the isolates were found to be resistant to at least one of the 3GCs, of which 97.1 per cent (170) of *Klebsiella* sp. isolates were confirmed to be positive for ESBL. Of these 170 isolates, 95 were randomly selected for PCR of *TEM* and *SHV* genes. Isolates having both *TEM* and *SHV* genes were common (67.3%) whereas only 20 per cent isolates possessed *TEM* gene and 8.4 per cent *SHV* gene alone.

**Interpretation & conclusion:** Our findings showed that the majority of the ESBL positive clinical isolates of *Klebsiella* sp. carried both *TEM* and *SHV* genes followed by *TEM* alone. Such studies need to be done in various geographical regions of the country to know about the prevalent genotypes for better management of infection.

**Key words** ESBLs - *Klebsiella* sp.- SHV - TEM

Extended-spectrum  $\beta$ -lactamases (ESBLs) are the rapidly evolving group of  $\beta$ -lactamase enzymes produced by the Gram negative bacteria, which have the ability to hydrolyse all cephalosporins and aztreonam but are inhibited by clavulanic acid<sup>1</sup>. Most ESBLs are generally mutants of classical *TEM* and *SHV* genes<sup>2</sup>. *TEM* and *SHV* genes were first described in *Klebsiella pneumoniae* from Western Europe<sup>3</sup>.

Though presence of ESBLs amongst *K. pneumoniae* have been reported from India<sup>4-8</sup>, there is no information on their molecular types. The present study was carried out to determine the prevalence of mainly *TEM* and *SHV* genes responsible for ESBL production amongst the ESBL positive *Klebsiella* species isolated from the patients admitted to a tertiary care hospital in north India.

### Material & Methods

**Bacterial isolates:** A total of 204 consecutive non-duplicate clinical isolates of multidrug resistant *Klebsiella* sp. [blood (n=108), urine (n=15), pus(n=2) and sputum (n=79)] received in the clinical bacteriology laboratory, All India Institute of Medical Sciences (AIIMS), New Delhi, from August 2003 to July 2004 were included in this study.

**Antimicrobial susceptibility testing:** Isolates were tested by the disk diffusion method on Mueller Hinton agar (Hi-Media, Mumbai) following the zone size criteria recommended by the National Committee for Clinical Laboratory Standards (NCCLS)<sup>9</sup>. The antibiotic ( $\mu$ g) tested included ampicillin (10), piperacillin (100), piperacillin/tazobactam (100/10), cefoperazone/sulbactam (75/10), ceftazidime (30), cefotaxime (30), ceftazidime (30) and ciprofloxacin (5).

**ESBL screening and confirmation by phenotypic methods:** The isolates showing resistance to one or more 3<sup>rd</sup> generation cephalosporins (3GCs) were tested for ESBL production by double disk-diffusion test (DDDT) using four discs cefotaxime (CTX)

(30  $\mu$ g) cefotaxime + clavulanic acid (10  $\mu$ g), ceftazidime (CAZ) (30  $\mu$ g), and ceftazidime +clavulanic acid (10  $\mu$ g). The inoculum and incubation conditions were same as for standard disk diffusion recommendations. A >5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was designated as ESBL positive<sup>9</sup>.

**ESBL E-test:** All the isolates positive by DDDT were further confirmed by ESBL E-test method (AB Biodisk, Sweden) using strip impregnated with gradient of different concentrations (0.5-32  $\mu$ g/ml) of CAZ at one side and at other side different concentrations of CAZ (0.064-4  $\mu$ g/ml) along with a fixed concentration of clavulanic acid (4  $\mu$ g/ml). Inoculum was prepared by emulsifying well isolated colonies from an overnight agar plate in saline to achieve a turbidity equivalent to 0.5 McFarland. A sterile cotton swab was dipped into the inoculum suspension. The entire agar surface was swabbed three times; rotating the plate approximately 90 degrees each time to ensure an even distribution of inoculum. With a pair of forceps the strip was applied to the inoculated agar plate in a way so that the whole length of the strip is in contact with the agar surface. Plates were incubated at 37°C for 16-18 h. The presence of ESBL was confirmed by the appearance of a phantom zone or deformation of CAZ ellipse or when CAZ MIC is reduced by >3 log<sub>2</sub> dilutions (ratio CAZ/CAZ+CA,  $\geq$  8) in the presence of clavulanic acid as per the manufacturer guidelines.

*K. pneumoniae* ATCC 700603 was used as positive control and *Escherichia coli* ATCC 25922 was used as negative control (obtained from American Type Culture Collection, USA).

### Multiplex PCR for TEM and SHV genes

(i) Primers for *TEM* and *SHV* - The primers of *TEM* and *SHV* gene were designed by downloading the 110 sequences of both TEM and SHV variants available from the GenBank Database (<http://>

[www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Display & DB=nucleotide](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Display & DB=nucleotide)). These variants were aligned by the use of ClustalX software (ver.1.83; available at: <http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html>)<sup>10</sup>. Consensus sequence was created from the aligned sequences using the BioEdit software (ver.7.0.5.2; available at <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Primers were designed from the consensus sequence and analyzed for hair-pin loop and primer dimer formation using OLIGO (ver 4.0) selection software (National Biosciences, Plymouth, MN, USA).

The primers used to amplify *TEM* gene (Table) corresponded to the position 55 to 75 and 752 to 771 respectively with 717 bp fragment size and to amplify *SHV* gene corresponded to position 509 to 526 and 962 to 979 respectively with a fragment size of 471 bp as obtained by the nucleotide-nucleotide BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST.html>).

(ii) Amplification of *TEM* and *SHV* gene: Initially the PCR for both *TEM* and *SHV* genes was carried out individually. Genomic DNA was prepared from overnight cultures grown on Luria-Bertani medium [LB] (Hi-Media, Mumbai) by inoculating a single colony into 100 µl of double distilled water, boiling at 95°C for 10 min. Equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added, centrifuged at 8000 g for 15 min. The upper layer was used as crude DNA. Amplification reactions for *TEM* and *SHV* genes were performed in a 25 µl volume containing 2.5 µl of 10X PCR reaction buffer with MgCl<sub>2</sub> (15 mM), 0.5 µl (200 µM)

deoxynucleoside triphosphates mix (dNTPs, 10 mM), 0.5 µl (each) primers (10pm/µl) with 0.5 µl (3 U/µl) *Taq* DNA polymerase (Bangalore Genei, India). Five microlitres of the template DNA preparation was added to the reaction mixture. PCR amplifications were carried out on a My Cyclor DNA thermal cyclor (BioRad, Australia).

The cycling conditions for amplification were as follows: for *SHV* gene, initial denaturation at 94°C for 2 min and 30 cycles of 1 min at 94°C, 30 sec at 52°C and 45 sec at 72°C, followed by 5 min at 72°C and for *TEM* gene initial denaturation of 2 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, followed by 7 min at 72°C.

After individually amplifying the genes, conditions were standardized to amplify both the genes in a single reaction mixture. The cycling conditions for amplifying both the genes were as follows: initial denaturation at 2 min at 94°C and 30 cycles of 45 sec at 94°C, 45 sec at 52°C, and 45 sec at 72°C, followed by 7 min at 72°C. The resulting PCR products were analyzed by electrophoresis with 1.5 per cent agarose gels in Tris-borate-EDTA buffer (TBE; Gibco, NY, USA). The gels were stained with ethidium bromide and a band observed at desired position was photographed on an ultraviolet light transilluminator. A molecular weight standard (100 bp ladder, Promega, Madison, USA) was included on each gel.

## Results & Discussion

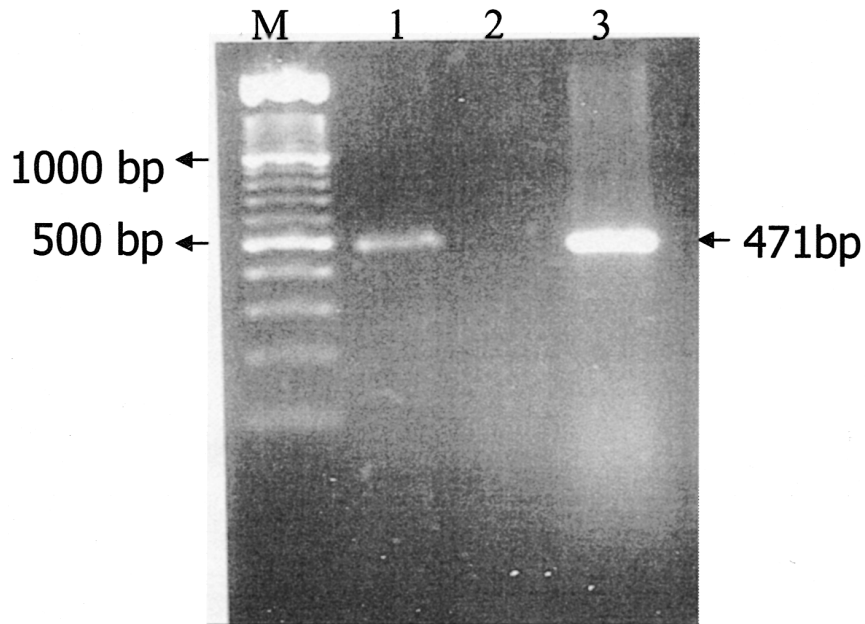
Of the 204 isolates, 175 (85.8%) were found to be resistant to at least one of the 3GCs. Among these 175 isolates, 97.1 per cent (170) were found to be ESBL positive by DDDT as well as by ESBL E-test method.

A total of 95 ESBL positive *Klebsiella* isolates were randomly selected to detect the

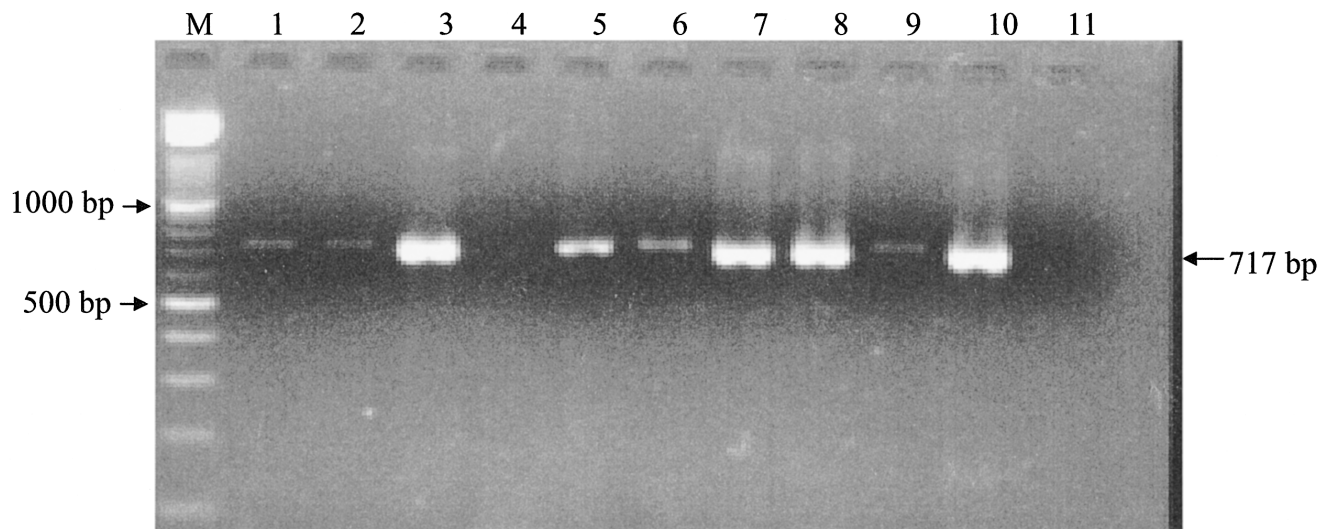
**Table.** Primers used for detection of *SHV* & *TEM* genes

Target Primer	Sequence(5'-3')	Product size (bp)
SHV	<i>SHVF</i> TCAGCGAAAAACACCTTG	471
	<i>SHVR</i> TCCCGCAGATAAATCACC	
TEM	<i>TEMF</i> CTCCTGTTTTTGCTCACCCA	717
	<i>TEMR</i> TACGATACGGGAGGGCTTAC	

F, forward; R, reverse



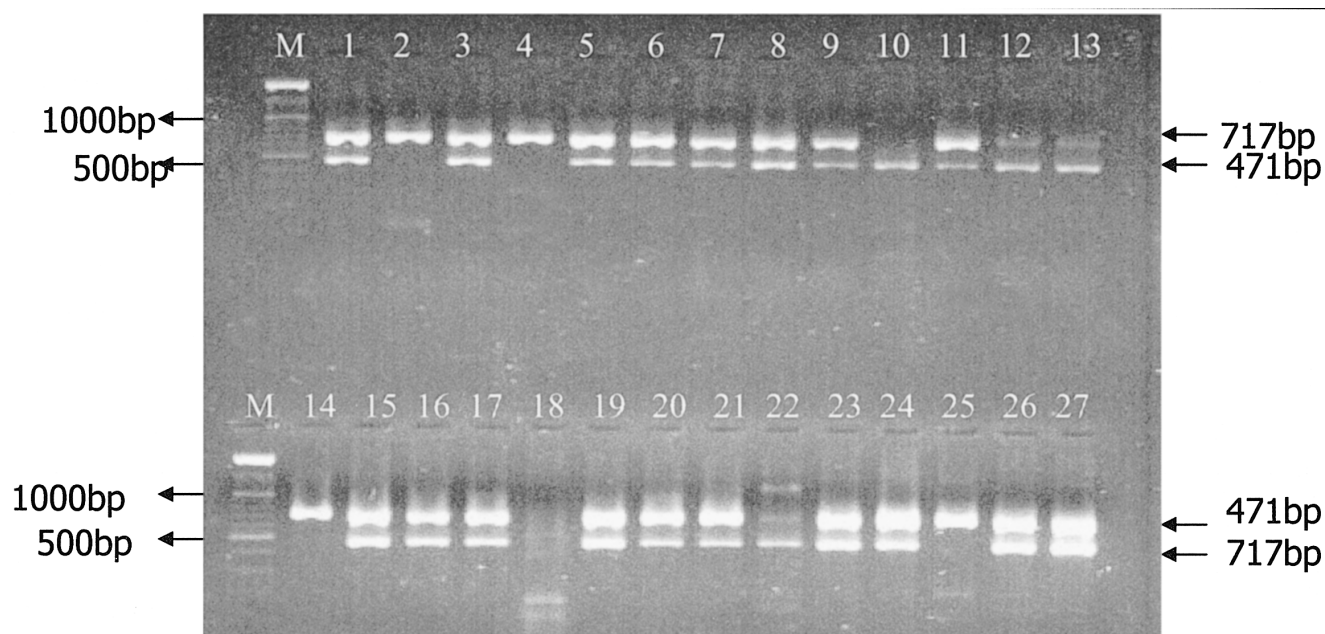
**Fig. 1.** PCR amplification of *SHV* gene, lane M: 100 bp ladder, Lane 1: *K. pneumoniae* clinical isolate, lane 2: *E.coli* ATCC25922 negative control, lane 3: *K. pneumoniae* ATCC700603 positive control.



**Fig. 2.** PCR amplification of *TEM* gene, lane M: 100 bp ladder, lane 1-3 & 5-10: *K. pneumoniae* clinical isolates, lane 4: *E. coli* ATCC25922 negative control, lane 11: negative control (no template DNA added).

presence *SHV* (Fig. 1) and *TEM* gene (Fig. 2). The amplified product of *SHV* gene with a fragment size 471 bp corresponded to 100 bp molecular weight marker in *K. pneumoniae* clinical isolate and *K. pneumoniae* ATCC 700603 (Fig. 1). Amplified product of *TEM* gene in *K. pneumoniae* clinical isolates had a fragment size of 717 bp (Fig. 2). In multiplex PCR most of the

isolates showed both *TEM* and *SHV* genes corresponding to position 717 bp and 471 bp respectively and some showed amplified product of only *TEM* gene (Fig. 3). The overall data revealed that *TEM* gene was present in 19 (20%), *SHV* in 8 (8.4%) and both *TEM* and *SHV* genes in 67.3 per cent (64) of the isolates (Fig. 3).



**Fig. 3.** Multiplex gene-specific PCR showing amplified product of *TEM* & *SHV* gene, Lane M: 100 bp ladder, lane 1-9, 11-17 & 19-27: *K. pneumoniae* clinical isolates, lane 10: *K. pneumoniae* ATCC700603 positive control, lane 18: *E. coli* ATCC25922 negative control. Some lanes (1, 3, 5-9, 11-13, 15-17, 19-24, 26, 27) showed amplified product of both *TEM* and *SHV* genes and some lanes (2, 4, 14, 25) showed amplified product of only *TEM* gene.

*Klebsiella* species has rapidly become the most common ESBL producing organism, making it difficult to eradicate this organism from the high risk wards such as intensive care units. Antimicrobial susceptibility testing showed that 86 per cent of isolates were resistant to at least one of the 3GCs in our hospital. This resistance is mainly associated with the production of enzymes called Temoniera (*TEM*) and sulphhydryl variable (*SHV*) that are generally plasmid-mediated<sup>11</sup>. The phenotypic expression of plasmid mediated ESBLs producing strains is resistant to 3GC.  $\beta$ -lactamase inhibitors such as clavulanic acid can restore susceptibility to inactive cephalosporin. The disk diffusion method does not allow routine differentiation of strains producing these enzymes. Molecular methods, like PCR need to be used for the differentiation of  $\beta$ -lactamase-producing isolates. Our findings showed that majority of isolates carried both *TEM* and *SHV* in contrast to *TEM* or *SHV* alone. Among the single gene carrier isolates the proportion of *TEM* was higher than the

*SHV*. Studies from other parts of the world reported that *SHV-5* gene was common in *K. pneumoniae* isolates<sup>12-15</sup>. Similar studies need to be done in different parts of the country to know the genotypes of ESBL enzymes in a particular geographical area for epidemiological purpose<sup>16</sup>.

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### References

1. Kapil A. The challenge of antibiotic resistance: need to contemplate. *Indian J Med Res* 2005; 121 : 83-91.
2. Bush, K, Jacoby GA, Medeiros AA. A functional classification scheme for  $\beta$ -lactamases and its correlation with the molecular structure. *Antimicrob Agents Chemother* 1995; 39 : 1211-33.
3. Jacoby GA, Madeiros AA. More extended spectrum  $\beta$ -lactamases. *Antimicrob Agents Chemother* 1991; 35 : 1697-704.

4. Mathur P, Kapil A, Das B, Dhawan B. Prevalence of extended spectrum  $\beta$ -lactamase producing gram negative bacteria in a tertiary care hospital. *Indian J Med Res* 2002; *115* : 153-7.
5. Khurana S, Taneja N, Sharma M. Extended spectrum  $\beta$ -lactamase mediated resistance in urinary tract isolates of family *Enterobacteriaceae*. *Indian J Med Res* 2002; *116* : 145-9.
6. Manchanda V, Singh NP, Goyal R, Kumar A, Thukral SS. Phenotypic characteristics of clinical isolates of *Klebsiella pneumoniae* & evaluation of available phenotypic techniques for detection of extended spectrum  $\beta$ -lactamases. *Indian J Med Res* 2005; *122* : 330-7.
7. Grover SS, Sharma M, Pasha ST, Singh G, Lal S. Antimicrobial susceptibility pattern and prevalence of extended spectrum  $\beta$ -lactamase (ESBLs) producing strains of *Klebsiella pneumoniae* from a major hospital in New Delhi. *J Commun Dis* 2004; *36* : 17-26.
8. Abigail S, Mathai E, Jesudason MV, John TJ. Ceftazidime resistance among *Klebsiella pneumoniae* in south India. *Indian J Med Res* 1995; *102* : 53-5.
9. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Approved Standard M2/A7. National Committee for Clinical Laboratory Standards, Wayne, PA; 2002.
10. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The Clustal\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997; *25* : 4876-82.
11. Philippon A, Ben Redjeb S, Fournier G, Ben Hassen A. Epidemiology of extended spectrum  $\beta$ -lactamases. *Infection* 1989; *17* : 347-54.
12. Bauernfeind AE, Rosenthal E, Eberlein MH, Schweighart S. Spread of *Klebsiella pneumoniae* producing SHV  $\beta$ -lactamase among hospitalized patients. *Infection* 1993; *21* : 18-22.
13. Bingen EH, Desjardins P, Arlet G, Bourgeois F, Mariani-Kurkdjian P, Lambert-Zechovsky NY, *et al*. Molecular epidemiology of plasmid spread among extended broad spectrum  $\beta$ -lactamase producing *Klebsiella pneumoniae* isolates in a pediatric hospital. *J Clin Microbiol* 1993; *31* : 179-84.
14. Xiang X, Shannon K, French G. Mechanism and stability of hyperproduction of the extended-spectrum  $\beta$ -lactamases SHV-5 in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 1997; *40* : 525-32.
15. Perilli M, Amico ED, Segatore B, Rosaria de Massis M, Bianchi C, Luzzaro F, *et al*. Molecular characterization of extended-spectrum  $\beta$ -lactamases produced by nosocomial isolates of *Enterobacteriaceae* from an Italian Nationwide survey. *J Clin Microbiol* 2002; *40* : 611-4.
16. Pena CM, Pujol C, Ardanuy A, Ricart R, Pallares R, Linares J, *et al*. Epidemiology and successful control of a large outbreak due to *Klebsiella pneumoniae* producing extended-spectrum  $\beta$ -lactamases. *Antimicrob Agents Chemother* 1998; *42* : 53-8.

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