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**Original Research Article**

# Genotypic detection of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in a Tertiary care hospital

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## Abstract

**Aim**: This study was carried out to determine the presence of TEM and SHV genes in extended-spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae*. The study was also aimed to compare results of PCDDT and genotypic methods. Information on molecular types of ESBL positive *Klebsiella pneumoniae* is less from India; lesser still from this area of Marathwada, a part of Maharashtra state.

**Materials and Methods:** A total of 340 strains of *Klebsiella pneumoniae* were selected for the study from June 2013-December 2013. Kirby – Bauer disk diffusion method was performed to determine the antibiotic resistance pattern. Screened for ESBL and confirmed by phenotypic confirmatory disc diffusion test (PCDDT). 100 randomly selected isolates were investigated for the presence of TEM and SHV genes via Polymerase chain reaction (PCR). Multiplex PCR was also performed for the same.

**Results:** Phenotypic confirmatory test was able to detect ESBL production in 90% of *Klebsiella pneumoniae* isolates. Among the two ESBL genotypes, the most prevalent genotype was found to be TEM. Majority of ESBL producing isolates possess both ESBL genes.

**Conclusion:** Multiplex PCR can be used as a rapid method to identify common genes (TEM and SHV) responsible for extended spectrum beta lactamase production in *Klebsiella pneumoniae*. It will prove valuable for surveillance and for determining the line of treatment against drug resistant organisms, thus saving precious time and resources. PCDDT results correlated with genotypic method in all the tested strains.

**Keywords:** ESBL, *Klebsiella pneumoniae*, TEM, SHV

## 1.Introduction

Extended-spectrum β-lactamases (ESBLs) continue to be a major problem in clinical setups the world over, conferring resistance to the expanded-spectrum cephalosporins.[1] The vast majority of *Klebsiellae* infections are associated with hospitalization. As opportunistic pathogens, *Klebsiella* species primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases. Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*, the medically most important species of the genus.[2]

Extended spectrum β-lactamases (ESBL's) are rapidly evolving group of β-lactamase enzymes produced by Gram negative bacteria. These enzymes have been derived from TEM and SHV genes by mutations and have been well described in Enterobacteriaceae especially *Klebsiella pneumoniae*.[3] Information on molecular types of ESBL positive *Klebsiella* species is less from India; lesser still from this area of Marathwada, a part of

Maharashtra state. We conducted this study to look for TEM & SHV genes in ESBL positive *Klebsiella pneumoniae* isolated from the patients from Govt. Medical College and Hospital, Aurangabad. The study was also aimed to compare results of PCDDT and genotypic methods.

## 2. Material and Methods

A total of 340 multidrug –resistant isolates of *Klebsiella pneumoniae* were obtained from various clinical samples, received in the clinical laboratory, Government Medical College and Hospital, Aurangabad, Maharashtra, during June 2013-Dec 2013 were included in the study.

The specimens included Pus (n=186), Blood (n=37), Urine (n=25), Sputum (n=16), other fluids/Aspirates (n=76). All the samples were processed by standard methods.[4][5][6] Samples obtained were screened for resistance to 3rd generation Cephalospoirins (3GC).The ESBL status was determined by PCDDT. PCR and multiplex PCR specific for TEM and SHV genes were performed. As a screening test, isolates showing inhibition zones ≤ 27 mm for Cefotaxime, ≤ 22 mm for ceftazidime were selected as potential ESBL producers.

Phenotypic Confirmatory Disk Diffusion Test [PCDDT]: Extended spectrum beta lactamase detection in *Klebsiella pneumoniae* isolates was done by phenotypic confirmatory disk diffusion test [PCDDT]. Disk of cephotaxime and ceftazidime alone and those containing a combination of Clavulanic acid with these antibiotics were used as per CLSI guidelines. Following control strains were used for ESBL detection.

Positive control for ESBL -*Klebsiella pneumoniae* ATCC: 700603.

Negative control for ESBL- *Escherichia coli* ATCC: 25922

**Interpretation:** Organism was considered ESBL producer if there was more than 5mm increase in zone diameter for Ceftazidime and Cefotaxime tested in combination with Clavulanic acid versus its zone when tested alone.[7]

For detection of ESBL genes, PCR was performed using the following set of primer, labelled as TEM and SHV. (Table No.1)

Primers for TEM and SHV gene were taken from previous published article[8] and synthesized by Merck-Bangalore, India. In addition multiplex PCR was also performed with these primers (TEM and SHV).

### 2.1 Polymerase Chain Reaction (PCR)

PCR was performed to detect for the presence of TEM [Table No.2] and SHV genes[Table No.3]. Multiplex PCR was also performed with these primers.[Table No.4]

#### 2.1.1 Preparation of DNA

The DNA extraction was done by procedure described by Lal *et al*[3] with some modifications. Genomic DNA was prepared by the following procedure:

- Luria - Bertani medium was inoculated to obtain isolated colonies of pure growth.
- The plate was incubated overnight at $37^0$C.
- Single colony was picked with a sterile loop and suspended in 100µl double distilled water.
- The suspension was heated at $95^0$C for ten minutes.
- Following centrifugation at 10,000 rpm for 1 min, the supernatant was used as the crude DNA.

#### 2.1.2 Amplification of TEM and SHV genes

- Amplification of TEM and SHV genes were performed in a 25µl volume PCR tube.
- PCR master mix (Merck, Bangalore) 3.5 µl
- Forward and Reverse primers 0.5 µl
- Template DNA 5.0 µl
- Quality Control: *Klebsiella pneumoniae* ATCC: 700603 was used as Positive Control and *Escherichia coli* ATCC : 25922 was used as Negative Control for PCR.

PCR amplifications were carried out in a Peltier Thermal Cycler [PTC-200, MJ Research, USA].

**Table 1: Showing primers used for detection of SHV and TEM genes**

| Target | Primer sequence(5'-3') | Product size (bp) |
|---|---|---|
| SHV F | TCAGCGAAAAACACCTTG | 471 |
| SHV R | TCCCGCAGATAAATCACC | |
| TEM F | CTTCCTCCTGTTTTTGCTCACCCA | 717 |
| TEM R | TACGATACGGGAGGGCTTAC | |

F: Forward; R: Reverse

The primers used to amplify TEM gene correspond to the position 55 to 75 and 752 to 771 respectively, with 717 bp fragment size and to amplify SHV gene correspond to position 509 to 526 and 962 to 979 respectively with a fragment size of 471 bp.

**Table 2: Amplification of TEM gene**

| Stage | Step | Temp | Time | Cycle |
|---|---|---|---|---|
| Initial denaturation | Initial denaturation | 94°C | 2 min | 01 |
| Amplification | Denaturation | 94°C, | 1min | 30 |
| | Annealing | 58°C | 1min | |
| | Extension | 72°C, | 1min | |
| Final Extension | Extension | 72°C, | 7 min | 01 |

**Table 3: Amplification of SHV gene**

| Stage | Step | Temp | Time | Cycle |
|---|---|---|---|---|
| Initial denaturation | Initial denaturation | 94°C | 2 min | 01 |
| Amplification | Denaturation | 94°C, | 1min | 30 |
| | Annealing | 52°C | 30 sec | |
| | Extension | 72°C, | 45 sec | |
| Final Extension | Extension | 72°C, | 5 min | 01 |

### 2.1.3 Electrophoresis

The resulting PCR products were analyzed by electrophoresis with 1.5% agarose gels in Tris - borate – EDTA buffer. The gels were stained with ethidium bromide and a band was observed at desired position was photographed on an ultraviolet light transilluminator. A molecular weight standard (100 bp ladder-Merck, Bangalore) was included on each gel.

**Table 4: For Multiplex PCR using SHV & TEM forward and reverse primers**

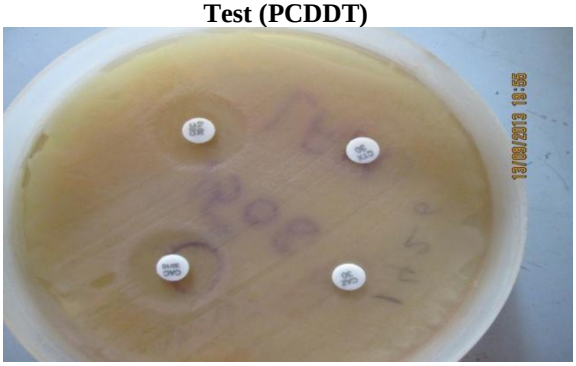
| Stage | Step | Temp | Time | Cycle |
|---|---|---|---|---|
| Initial denaturation | Initial denaturation | 94°C | 2 min | 01 |
| Amplification | Denaturation | 94°C | 45 sec | 30 |
| | Annealing | 52°C | 45 sec | |
| | Extension | 72°C | 45 sec | |
| Final Extension | Extension | 72°C | 7 min | 01 |

## 3. Results

Total 340 strains of *Klebsiella pneumoniae* were studied. It was observed that many (≥80%) of our isolates of *Klebsiella pneumoniae* were resistant to third generation Cephalosporin's and other antibiotics.

Out of 340 strains of *Klebsiella pneumoniae* 18 strains were sensitive to 3GC. All others i.e. 322 strains were subjected to PCDDT. Of these 322 strains, 306 strains showed increase in zone diameter of 5mm to the combination of Cefotaxime+Clavulanic acid when compared to Cefotaxime alone. Same 306 strains gave similar results with Ceftazidime+Clavulanic acid when compared to Ceftazidime alone (90%). All these 306 strains were reported as ESBL producers. (Figure 1)

**Figure 1: Phenotypic Confirmatory Disc Diffusion Test (PCDDT)**

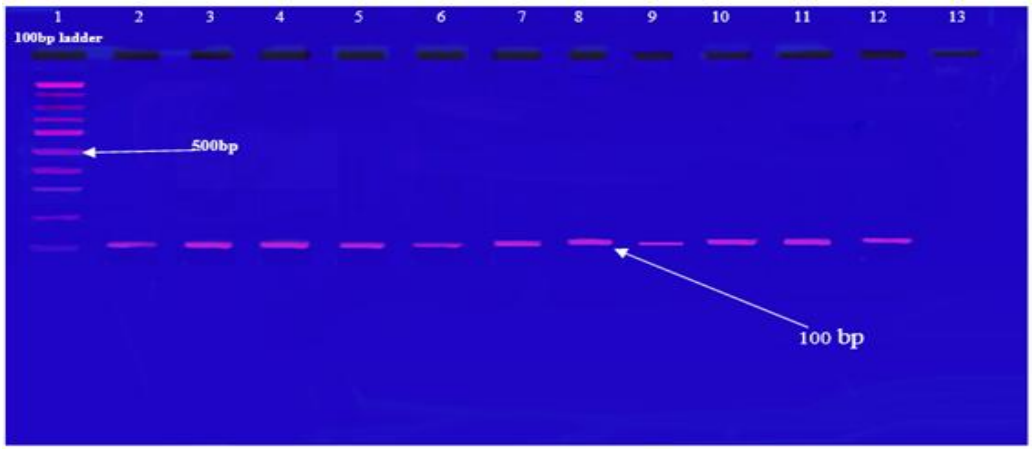


Ceftazidime and Cefotaxime showing an increase in zone diameter of >5mm with the addition of Clavulanic acid, indicative of ESBL production in a *Klebsiella pneumoniae* isolate.

Of the 306 ESBL positive clinical isolates, 100 random isolates were subjected to genotypic characterisation by PCR for presence of TEM and SHV gene. All the hundred isolates (100%) showed presence of TEM gene Figure 2, while forty isolates (40%) showed presence of SHV gene Figure 3. Thus forty isolates contained both TEM and SHV genes (40%) (Figure 4). Sixty isolates (60 %) had only TEM gene. No isolates showed SHV gene alone.

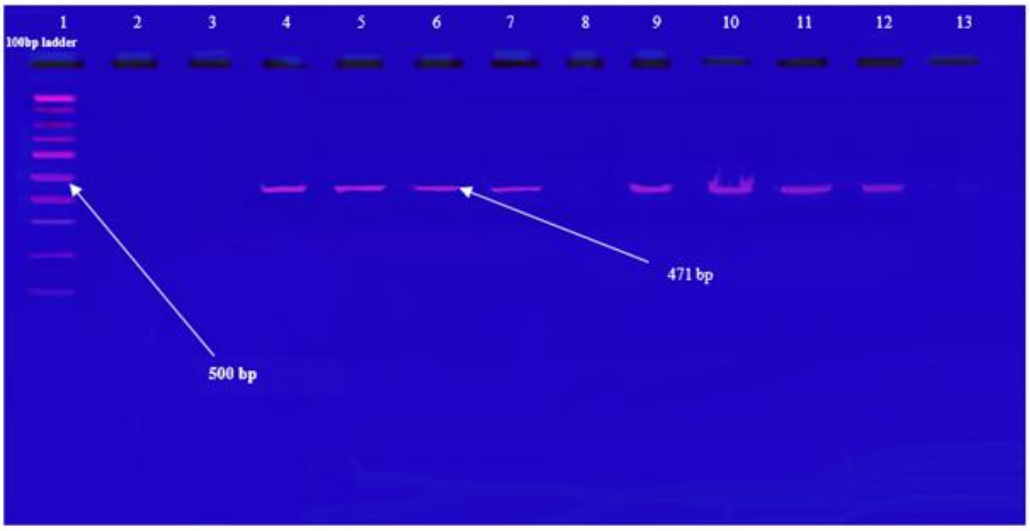
**PCR with TEM**

**Figure 2: Lane 1 is 100 bp ladder, lane 2-11 are samples lane 12 Positive control and lane 13 Negative control**



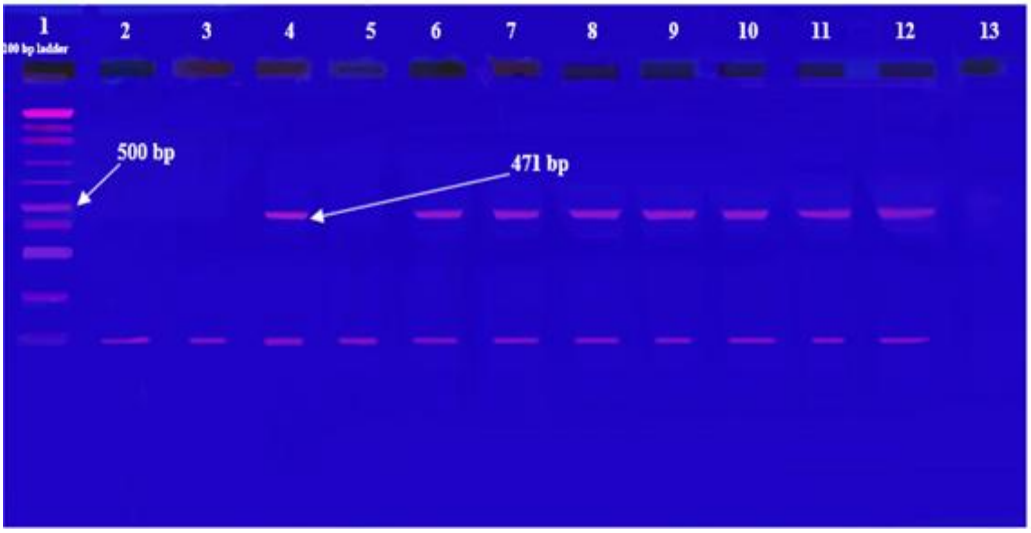
**PCR with SHV**

**Figure 3: Lane 1 is 100 bp ladder, lane 2-11 are samples and lane 12 Positive control,lane 13 Negative control.**




**Multiplex PCR using TEM & SHV primer**

**Figure 4: Lane 1 is 100bp ladder, Lane 2-11 are samples, lane 12 Positive control and lane 13 negative control**




## 4. Discussion

The high percentage of ESBL producing *Klebsiella* sp. may be due to the selective pressure imposed by extensive use of antimicrobials. Intensive care unit, in which antibiotic use is heaviest and the potential for patient to patient transmission of organisms is greatest, is an important factor. The infection control implications of ESBL producing *Klebsiella* spp. are under recognised. In most of the studies, molecular genetics evidences indicated patient to patient transmission of ESBL producing strains of *Klebsiella* sp.[9]

Resistance to third generation cephalosporins due to acquisition of extended spectrum β-lactamase (ESBL) enzymes among Gram negative bacteria is on the increase. Presence of ESBL producing organisms has been reported to significantly affect the course and outcome of an infection. Therefore infections due to ESBL isolates continue to pose a challenge to infection management worldwide.[10]

It was observed that many (≥80%) of our isolates of *Klebsiella pneumoniae* were resistant to third generation Cephalosporin's and other antibiotics, making them MDR strains. Most of these were isolated from in-patients, indicating probable HAI.

Patients having infections caused by ESBL producing organism are at increased risk of treatment failure with broad spectrum β-lactam antibiotic. Therefore, it is recommended that any organism confirmed for ESBL production be reported as resistant to all broad-spectrum β-lactam antibiotic, regardless of the susceptibility test result.[7]

In our study ESBL producing strains of *Klebsiella pneumoniae* were present in high percentage (90%) in almost all the specimens. Highest number of ESBL producers was found in Urine, followed by fluids/Aspirates, Blood and Pus. Prevalence of ESBLs is reported from medicine ICU, Surgery wards and NICU/Paediatric wards. The exact reason for this cannot be pointed out. But probably it relates to drug prescribing habits of these wards.

In the present study 67.74% isolates were sensitive to Meropenem, which correlates with the study of Rampure *et al*. They found 68.36% of their isolates to be sensitive to Meropenem.[11] Meropenem is a substrate for the multidrug efflux system. Overexpression of this efflux system raises the MIC of Meropenem and other substrate antibiotics, but not Imipenem. In the presence of a β – Lactamase or reduced permeability from downregulation of a critical outer membrane protein, frank resistance to Meropenem can occur.[12]

From early this decade, strains that produce *Klebsiella pneumoniae* carbapenemases (KPC) were reported in the USA and subsequently worldwide. These KPC-producing bacteria are predominantly involved in nosocomial and systemic infections. KPC β lactamases (KPC-1 to KPC-7) confer decreased susceptibility or resistance to virtually all β lactams. Carbapenems (imipenem, meropenem, and ertapenem) may thus become inefficient for treating enterobacterial infections with KPC-producing bacteria, which are, in addition, resistant to many other non-β-lactam molecules, leaving few available therapeutic options.[13]

The emergence of *Klebsiella pneumoniae* carbapenemases (KPCs) producing bacteria has become a significant global public health challenge while the optimal treatment remains undefined. In a review article by, Grace Clee and David Burgass observed that majority of infections in their review were due to *Klebsiella pneumoniae* (89%) producing KPC. Other agents producing KPC were *Pseudomonas sp, E. Coli, Enterobacter sp*. Their review was targeted to recommend treatment for KPC producers. They recommended the three most common antibiotic-class combinations for KPC: polymyxin plus carbapenem, polymyxin plus tigecycline, polymyxin plus aminoglycoside .They concluded, combination therapy is recommended for the treatment of KPC infections.[14]

Upto the end of the 1990s, clinical infections caused by ESBL-producing bacteria were associated with nosocomial outbreaks, where the chief ESBL producer was *Klebsiella pneumoniae* but not *E. coli*. The ESBL genotypes detected in the nosocomial setting were almost always TEM and SHV.[15]

In the last 12 years ESBL have gone from being an interesting scientific observation to a reality of great medical importance. Initially restricted to the hospital acquired infections, they have also been isolated from infections in outpatients. Major outbreaks have been reported from all over the world thus making them emerging pathogens.

In some centers like Ujjain, Bijapur, Kanchipuram the incidence of ESBLs is still very low 41%, 46% and 14% respectively.[16][17][18]

But in metropolitan cities like New Delhi there is gradual increase in ESBL producers, from 80% in 2002, 91% in 2005, 97% in 2007 and 100% in 2010.[8],[19][20][21] This probably relates to rampant and inadvertent use of third generation Cephalosporin's. Over the counter availability could be another cause when patients resort to self medication.

In a study by Lal *et al* using the same sequence of primers, they found majority (67.3%) of the ESBL positive clinical isolates of *Klebsiella* sp. carried both TEM and SHV genes followed by TEM (20%) and SHV (8.4%) separately. In multiplex PCR most of isolates showed both TEM and SHV genes and some showed TEM gene.[8]

Our PCR study is in accordance with the study of Rastogi *et al* with TEM 100%.[21] We found that multiplex PCR gave almost same results as PCR separately for TEM and SHV gene. This means that by using multiplex PCR, we can save our time, chemicals and cost. So we recommend using multiplex PCR for better time management and fast results.

It can be helpful in providing explanations for unusual complex antibiograms, confirming specific resistance mechanisms and epidemiological studies in a geographical area. From India high prevalence of ESBL producing *Klebsiella* strains has been reported by various groups. Reported frequency of ESBL producing *Klebsiella* species in India ranged from 14 % -- 100% [22][23]. Prevalence of ESBL producing *Klebsiella* around the world varies between 3%-8% to 100%.[23][24]

Detection of TEM and SHV genes by molecular methods in ESBL producing bacteria and their antimicrobial resistance can provide useful information about its epidemiology and risk factors associated with these infections.[25]

## 5. Conclusion

Reporting of ESBL producing isolates from clinical samples is useful for the clinicians to select appropriate antibiotics for the treatment of ESBL producing strains and to take proper precaution to prevent the spread of these resistant organisms to other patients.

The routine susceptibility test done by clinical laboratories fail to detect ESBL positive strains and can erroneously detect isolates sometimes to be sensitive to any of the third generation cephalosporin's, hence a special phenotypic confirmatory test is indispensable for detecting ESBLs.

Thus the high level of ESBLs among *Klebsiella pneumoniae* isolates is alarming and warrants special attention from clinicians and microbiologists. We as microbiologists should readily identify these isolates, so that proper therapy can be instituted to avoid misuse or overuse of antibiotics.

Multiplex PCR can be used as a rapid method to identify common genes (TEM and SHV) responsible for extended spectrum beta lactamase production in *Klebsiella pneumoniae*, isolated from different clinical samples without the need for phenotypic characterization.

It will prove valuable for surveillance and for determining the line of treatment against drug resistant organisms, thus saving precious time and resources.

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