Prevalence of \( \text{bla}_{\text{TEM}} \) AND \( \text{bla}_{\text{SHV}} \) genes in clinical Isolates of *Klebsiella pneumoniae* in a tertiary care hospital

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**ABSTRACT**

This study was carried out to determine the existence and to describe phenotypic and genotypic characteristics of \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \) genes in extended-spectrum \( \beta \)-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. We conducted this study to look for these genes in ESBL positive *Klebsiella pneumoniae* isolated from the patients from Govt. Medical College and Hospital, Aurangabad. A total of 300 strains of *Klebsiella pneumoniae* were selected for the study from Jan 2013-June 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 \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \) genes via Polymerase chain reaction (PCR). Multiplex PCR was also performed for the same. Phenotypic confirmatory test was able to detect ESBL production in 90.07% of *Klebsiella pneumoniae* isolates. Among the two ESBL genotypes, the most prevalent genotype was found to be \( \text{bla}_{\text{TEM}} \). Majority of ESBL producing isolates possess both ESBL genes. Multiplex PCR can be used as a rapid method to identify common genes (\( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \)) responsible for extended spectrum beta lactamase production in *Klebsiella pneumoniae*. Saving precious time and resources, more importantly early treatment. PCDDT results correlated with genotypic method in all the tested strains.

**Key words:** ESBL, *Klebsiella pneumoniae*, \( \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}} \).

**INTRODUCTION**

*Klebsiella pneumoniae* are emerging important agents of nosocomial infections in hospitals. The presence of invasive devises, contamination of respiratory support equipment, use of urinary catheters, and use of antibiotic greatly increases the likelihood of nosocomial infections in hospitalized patients. In addition, poor health status and treatment in an intensive care unit or nursing homes are other factors. Infections with *Klebsiella pneumoniae* have a worldwide distribution. Extensive use of broad – spectrum antibiotics in hospitals has contributed to an increased carriage of *Klebsiella* and subsequently, the development of multidrug – resistant strains that produce extended – spectrum beta lactamase.[1]

Because of their increased spectrum of activity especially against the oxyimino-cephalosporins,these enzymes were called extended-spectrum \( \beta \)-lactamases[ESBLs]. The prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution.[2]

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. [3]
We conducted this study to look for bla_TEM & bla_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.

**MATERIALS AND METHODS**

A total of 300 consecutive non duplicate clinical isolates of multi-drug resistant Klebsiella pneumoniae received in the clinical laboratory, during Jan 2013-June 2013 were included in the study.

The specimens included Pus (n=166), Blood(n=26), Urine(n=18), Sputum(n=12), Other fluids/Aspirates(n=78). All the samples were processed by standard methods. [4, 5, 6]

To determine their multi-drug resistance, antimicrobial susceptibility testing was performed by Kirby-Bauer’s disc diffusion method as per Clinical Laboratory Standards Institute-2012 (CLSI) recommendations. Antibiotic discs were procured from Hi-Media laboratories, Mumbai (India).

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 cefotaxime 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 Cefazidime 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 primers labelled bla_TEM and bla_SHV.

Detection of bla_TEM and bla_SHV genes from genomic DNA of Klebsiella pneumoniae was performed with a set of primers which were taken from previous published article [9] and synthesized by Merck, Bangalore, India. In addition multiplex PCR was also performed with these primers. (bla_TEM and bla_SHV). Table No.1

**Polymerase Chain Reaction (PCR)**

**Preparation of DNA**

The DNA extraction was done by procedure described by Lal P et al [8] 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°C.
- Single colony was picked with a sterile loop and suspended in 100µl double distilled water.
- The suspension was heated at 95°C for ten minutes.
- Following centrifugation at 10,000 rpm for 1 min, the supernatant was used as the crude DNA.

**Amplification of bla_TEM and bla_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]. Tables No.2 & 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.

It amplified 867 bp bla\textsubscript{TEM} and 930 bp bla\textsubscript{SHV} coding region in \textit{Klebsiella} sp. genome. The primers (Merck, Bangalore) used were:

Table 1: Showing Primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
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<tbody>
<tr>
<td>TEM F</td>
<td>ATAAAAATCTTGAAGACGAAA</td>
<td>867</td>
</tr>
<tr>
<td>TEM R</td>
<td>GACAGTTACCAATGCTTAATCA</td>
<td></td>
</tr>
<tr>
<td>SHV F</td>
<td>GGGTTATTCTTATTTGTCGC</td>
<td>930</td>
</tr>
<tr>
<td>SHV R</td>
<td>TTAGCGTTGCCAGTGCTC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Detection of bla\textsubscript{TEM} and bla\textsubscript{SHV} gene:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
<th>Cycle</th>
</tr>
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<tbody>
<tr>
<td>Initial denaturation</td>
<td>Initial denaturation</td>
<td>94°C</td>
<td>5 min</td>
<td>01</td>
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<tr>
<td>Amplification</td>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
<td>30</td>
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<tr>
<td>Am</td>
<td>Annealing</td>
<td>52°C</td>
<td>1 min</td>
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<tr>
<td>Extension</td>
<td>72°C</td>
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<tr>
<td>Final Extension</td>
<td>Extension</td>
<td>72°C</td>
<td>10 min</td>
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Reactions were performed in a DNA thermal cycler, according to the protocol followed above. PCR products were analyzed by electrophoresis with 1.5 per cent agarose gel. After staining with ethidium bromide the gel was photographed on an ultraviolet light illuminator by gel documentation system.

Table 3: For Multiplex PCR using bla\textsubscript{TEM} & bla\textsubscript{SHV} forward and reverse primers

<table>
<thead>
<tr>
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<tr>
<td>Amplification</td>
<td>Denaturation</td>
<td>94°C</td>
<td>01 min</td>
<td>35</td>
</tr>
<tr>
<td>Am</td>
<td>Annealing</td>
<td>52°C</td>
<td>01 min</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>01 min</td>
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<td>Extension</td>
<td>72°C</td>
<td>10 min</td>
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</tr>
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</table>

RESULTS AND DISCUSSION

Total 300 strains of \textit{Klebsiella pneumoniae} were studied. It was observed that many (≥80%) of our isolates of \textit{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.

Out of 300 strains of \textit{Klebsiella pneumoniae}, except 18 strains that were sensitive to 3GC, all others i.e. 282 strains were subjected to PCDDT. Of these 282 strains 254 strains showed increase in zone diameter of 5mm to the combination of Cefotaxime+Clavulanic acid when compared to Cefotaxime alone. Same 254 strains gave similar results with Ceftazidime+Clavulanic acid when compared to ceftazidime alone (90.07%). All these 254 strains were reported as ESBL producers. Fig No.1

In our study ESBL producing strains of \textit{Klebsiella pneumoniae} were present in high percentage (90.07%) in almost all the specimens. Highest number of ESBL producers was found in Urine, followed by fluids/Aspirates, Blood and Pus. ESBL producers were common in Surgery, Medicine and Paediatric wards followed by ObGy, ENT and Orthopaedics.

Of the 254 ESBL positive clinical isolates 100 random isolates were subjected to genotype characterisation by PCR for presence of bla\textsubscript{TEM} and bla\textsubscript{SHV} gene.

Amplification with bla\textsubscript{TEM} and bla\textsubscript{SHV} seventy isolates (70%) showed presence of TEM gene with bla\textsubscript{TEM} primers Fig No.2, while fifty isolates (50%) showed presence of SHV gene with bla\textsubscript{SHV} primersFig No.3 . Twenty isolates (20%) had both bla\textsubscript{TEM} and bla\textsubscript{SHV} gene. Fig No.4
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.

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]

The emergence of multidrug-resistant (MDR) and extended-spectrum β-lactamase producing Klebsiella pneumoniae pose serious antibiotic management problem as resistance genes are easily transferred from one organism to another.[11]

Extended spectrum β-lactamases (ESBLs) are plasmid mediated TEM and SHV derived enzymes, first isolated in Western Europe in mid 1980s, most commonly in Klebsiella sp., followed by Escherichia coli.[12]

Two studies from Chennai in 2006 show different rates of ESBLs-21.2% & 42.85%. [13, 14]

Similarly two studies in 2012 from Davangere show different rates of ESBLs-26.53% & 37.23%. It has been already reported that incidence of ESBLs differs not only across the country but from institute to institute. [15, 16]

The incidence of ESBLs in Manipal has actually decreased over the years. From 41% in 2007; it has decreased to 27.39% in 2009.[17, 18]

They developed quick screening methods to assess the different mechanisms of ESBL production, so that the patients can be treated with appropriate antibiotics.

In a study by Amita Jain & Rajesh Mondal using the same set of primers, they found presence of blaTEM gene in ESBL producing Klebsiella sp. was more common (48.4 %) than blaSHV (20.3%) gene. (26.5%) isolates had both TEM and SHV genes. [9]

Using the same set of primers, we got similar results also in multiplex PCR. We could find 20% strains with blaTEM and blaSHV together. These were the same strains detected when tested for these genes separately.

Our results clearly indicate high degree of prevalence of blaTEM gene in ESBL producing isolates.

We found that multiplex PCR gave almost same results as PCR separately for blaTEM and blaSHV gene. This means that by using multiplex PCR, we can save our time, chemicals and cost. So we recommend using multiplex PCR.

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.

With the spread of ESBL producing strains in hospitals all over the world, it is necessary to know the prevalence of ESBL positive strains in a hospital so as to formulate a policy of empirical therapy in high risk units where infections due to resistant organisms is much higher.[19]

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% [20, 21]

Prevalence of ESBL producing Klebsiella around the world varies between 3%-8% to 100%.[22, 23]

Genetic methods have a great potential in examination of strain involved in disease out breaks and in identification of the epidemic clones. Currently the gold standard in microbiological determination of these enzymes.[24]
Fig. 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.

Fig. 2: Amplification with blaTEM lane 1: 100 bp ladder, lane 2-11 samples, lane 12 positive control and lane 13 negative control.
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.
Multiplex PCR can be used as a rapid method to identify common genes (bla<sub>TEM</sub> and bla<sub>SHV</sub>) responsible for extended spectrum beta lactamase production in <i>Klebsiella pneumoniae</i>, isolated from different clinical samples. It will prove valuable for surveillance and for determining the line of treatment against drug resistant organisms, thus saving precious time and resources.

Thus the high level of ESBLs among <i>Klebsiella pneumoniae</i> 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.

Rapid diagnosis can be done by using the PCR amplification without the need for phenotypic characterization.

**REFERENCES**