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Original Article

Prevalence of Extended Spectrum β-Lactamases *Klebsiella pneumoniae* in various Clinical Samples and Characterization of the bla Genes in a Tertiary Care Hospital of Western Maharashtra

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Abstract

Introduction: This study was carried out to determine the presence of blaTEM, blaSHV, and blaCTX-M genes in extended-spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae* at a tertiary care hospital of western Maharashtra. **Materials and Methods:** A total of 2312 clinically significant, non-repetitive isolates of *K. pneumoniae* isolated from outpatient and inpatients attending the hospital were included in the study. An antibiotic susceptibility test, screening, and phenotypic confirmation for ESBLs producers were performed to check for the presence of ESBL enzymes. A polymerase chain reaction was carried out for the detection of ESBL-producing genes CTX-M-15, TEM, and SHV. **Results:** Non-repetitive clinical specimens processed for culture and identification in our hospital revealed 931 (40.26%) were ESBL-producing *K. pneumoniae.* A total of 45 bacterial isolates were subjected to molecular genotyping for the production of extended broad spectrum β-lactamases. Of the 45 samples, 40 (88.9%) were positive for blaCTX-M gene, and 44 (97.8%) were positive for blaTEM and blaSHV genes. ESBL production in these samples is largely found to be contributed by CTX-M, TEM, and SHV genes as the prevalence for these genes was higher. **Conclusion:** This is among the premier report describing the simultaneous occurrence of blaTEM, blaSHV, and blaCTX-M genes and their dissemination. This raises concern and emphasizes a need for more molecular studies to search for the presence of these gene pools in this locality.

Keywords: *blaCTX-M, blaSHV, blaTEM, ESBL production, extended-spectrum β-lactamase, Klebsiella pneumoniae*

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Introduction

Extended-spectrum β-Lactamases (ESBL) producing *Klebsiella pneumoniae* are being identified as the most often isolated *species* from human infections and are of serious concern for the development of therapies.[1] This is mainly due to the enzymes produced by these organisms which hydrolyze the β-Lactam antibiotics such as penicillins, oxyimino-cephalosporins, and monobactams.[2] The center for Disease Control and Prevention reported that, among healthcare-associated infections, 8% of *Klebsiella* spp. were carbapenem resistant in 2007 compared to <1% in 2000.[2] *K. pneumoniae* is clinically the most important member of the *Klebsiella* genus and is responsible for a wide range of infections from septicemia, pneumonia, urinary tract, wound, skin, and soft tissue in community-acquired infections.[3] Since all these infections lead to prolonged hospitalization, it also increases treatment cost, morbidity, and mortality.[4]

In Indian hospitals, ESBL prevalence varies greatly. Most of these ESBLs are derived from the amino acid

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substitution of parent enzymes TEM and SHV. Among all the ESBLs, the predominant ones are TEM, SHV, and CTX-M types and those are of major clinical concern and have been involved in epidemic outbreaks. The production of these is mediated by blaTEM, blaSHV, and blaCTX-M genes, respectively.[5]

The present study is designed to ascertain the present status of ESBL production in clinical isolates of MDR *K. pneumoniae* and to indicate the frequency of *K. pneumoniae* isolates encoding TEM, SHV, and CTX-M-15 genes at a tertiary care hospital of western Maharashtra.

Materials and Methods

This study was carried out in the Department of Microbiology of a 2000 bedded tertiary care hospital in Western Maharashtra from June 2018 to March 2020. The study was approved by the institutional ethics committee (Ref no. DYPU/EC/01/07).

All non-duplicate isolates ESBL-producing multidrug resistant (MDR) *K. pneumoniae* from various clinical specimens from both outpatients and inpatients were included in this study. Standard microbiological techniques were used for the isolation and identification of the isolates.[6] Identification and confirmation of all the isolates were done by standard conventional methods. All blood culture samples were processed by Bact/Alert 3D system and VITEK 2 Compact was used on isolates recovered from intensive care unit (ICU) patients as it reduces the turnaround time for reports. Antibiotic susceptibility testing was performed using Kirby Bauer's disc diffusion technique on Mueller Hinton agar (MHA) and interpretations of susceptibility were done according to the Clinical Laboratory Standards Institute (CLSI) guidelines.[7]

Screening and phenotypic confirmation for ESBL production

Based on the CLSI recommendations cephalosporin/clavulanate combination discs were used. Overnight growth in a broth of Gram-negative bacteria was adjusted to 0.5 McFarland Standard. Confirmation was done by the combination disc method. ESBL production was confirmed by placing a disc of cefotaxime (30 µg) and ceftazidime (30 µg) at a distance of 25 mm from a disc of cefotaxime/clavulanic acid (30/10 µg) and ceftazidime/clavulanic acid (30/10 µg), respectively, on a lawn culture of test strains on MHA. After overnight incubation at 37°C, the strain was considered ESBL positive if there was an increase in zone size of 5 mm in the zone size of Cephalosporin/Clavulanate combination disc when compared with cephalosporin alone.[7]

Detection of ESBL genotype by polymerase chain reaction (PCR)

Bacterial genomic deoxyribonucleic acid (DNA) was isolated using a Nucleospin Mini kit (REF 740952, MN, Mecherey-Nagel, Germany) as per the manufacturer's instructions. Five to six colonies were scraped off from the slants and were directly dissolved in lysis buffer from the Nucleospin kit. The final elution of DNA was performed in 100 µl of elution buffer. Five microliters of each DNA were checked on 1% agarose gel (w/v) spiked with Labsafe Nucleic Acid staining dye (G-Biosciences, USA) for visualization of DNA. PCR for various genes included in this project was performed using the PCR primers and conditions described in Table 1.

Following PCR mix was prepared for all the DNA samples. The final volume of each reaction was 25.0 µl and genomic DNA was added later to each tube [Table 2].

The bacterial MDR gene was amplified using standard PCR [Table 3]. The primer pairs CTX-M-15, TEM, SHV, Forward, and Reverse were used in a PCR with an annealing temperature of 55°C [Table 4]. The reaction was performed in 2720 Thermal Cycler (Thermo Fisher, USA). A total of 45 bacterial isolates were subjected to molecular genotyping for the production of extended broad spectrum β-lactamases. The ESBL strains confirmed phenotypically were tested for the presence of blaCTX-M, blaTEM, and blaSHV genes to identify the resistance mechanism. The expected amplified products of these genes were of sizes 995 bp, 717 bp, and 471 bp, respectively, as compared to the molecular weight marker. Molecular studies were done for 45 isolates taking into consideration of financial constraints.

Data Analysis: All the statistical analyses were carried out using Statistical Package for Social Sciences version 20.

Results

A total of 2312 *K. pneumoniae* were isolated during the study period. Out of these, 931 (40.26%) were

Table 1: PCR mix reagents

Materials	Volume (µl)	Final concentration
Genomic DNA	2.00	100-200 ng
10×PCR buffer	2.50	1×
50 mM $MgCl_2$	0.75	2.5mM
0.5 mM dNTP Mix	0.50	0.05 mM each dNTP
10 pmole primer solution (Mix of F and R)	1.00	0.2 mM
Taq DNA polymerase (5.0 units/µl)	0.20	1 unit/reaction
DMSO	1.25	5%
Nuclease Free water	16.80	

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Table 2: List of primers used for PCR amplification

Target Gene	Primer	Seq (5′-3 ′)	Bases	Amplicon size	Reference
blaCTX-M-15	Forward	CACACGTGGAATTTAGGGACT	21	995 bp	T1
	Reverse	GCCGTCTAAGGCGATAAACA	20		
blaTEM	Forward	CTTCCTGTTTTTGCTCACCCA	21	717 bp	T2
	Reverse	TACGATACGGGAGGGCTTAC	20		
blaSHV	Forward	TCAGCGAAAAACACCTTG	18	471 bp	T3
	Reverse	TCCCGCAGATAAATCACC	18		

Table 3: Thermal cycling program

PCR			
Stage	**Temperature (°C)**	**Time (min: sec)**	**Cycles**
Initial Denaturation	95	5:00	Hold
Denaturation	95	0:30	
Annealing	55	0:30	35 Cycles
Amplification	72	0:30	
Final Amplification	72	10:00	Hold
Hold	4	Until use	Hold
Amplification product size	As mentioned in the primer table		

Table 4: Age-wise distribution

Age group	Number of patients
1-20 years	168
21-40 years	244
41-60 years	286
60 years	233
Total	931

Table 5: Summary of total number of PCR product positive amplifications

Name of the gene	Total no of positive amplicons	% of positive samples
CTX-M-15	40	88.9
TEM	44	97.8
SHV	44	97.8

ESBL-producing *K. pneumoniae*. Out of these samples males were 576 (68%) and females were 355 (32%).

Age-wise distribution [Table 4] and Ward-wise distribution: Of the total 931 ESBL-producing *K. pneumoniae*, 196 were from ICU, 641 were from inpatient department, and 94 were from outpatient department. Out of these 931, ESBL-producing *K. pneumoniae* strains, 45 were purified and selected for genomic study (given the cost and financial constraints). These were mostly isolated from critically ill patients in ICU and septicemia patients.

Of the 45 samples, 40 (88.9%) were positive for blaCTX-M gene and 44 (97.8%) were positive for blaTEM and blaSHV genes [Figures 1-3 and Table 5]. ESBLs production in these samples is largely found to be contributed by CTX-M, TEM, and SHV genes as the prevalence for these genes was higher. Annotated sequences are submitted to the Genbank database in National Centre for Biotechnology Information (NCBI) via BankIt tool under the accession numbers as mentioned: 24 [16 isolates] MZ504612, MZ504613 MZ504614

MZ504615, MZ504616, MZ504617, MZ504618, MZ504619, MZ504620, MZ504621,

MZ504622, MZ504623, MZ504624, MZ504625, MZ504626, MZ504627, MZ504628,

MZ504629, MZ504630, MZ504631, MZ504632, MZ504633, MZ504634, and MZ504635

Discussion

From India, Mirza *et al.*[2] have reported an increasing incidence of MDR *K. pneumoniae* infection in hospital and community and reported 29.32% of ESBL producers as *K. Pneumoniae.* Grover *et al.*[8] reported 30.15% isolates to be pure ESBL producers.

Reports on ESBL production rate in *K. pneumoniae* isolated from different countries also show significant variations. In a study in Japan and USA, the prevalence rate was reported as 40 and 44%, respectively. However, in Southeast Asia, the prevalence rates of ESBL reported varied from 20% to more than 60%. ESBL production in India, Taiwan, Thailand, Pakistan, and Korea was reported at 44, 8–29, 64, 36–56, and 22%, respectively.[9]

For genotyping, 45 *K. pneumoniae* strains were randomly selected. Out of these 45 isolates of *K. pneumoniae* studied for PCR to detect bla genes, 40 harbored blaCTX-M-15 gene (88%), 44 harbored blaSHV gene (97%), and 44 harbored blaTEM gene (97%). The PCR amplification of CTX-M-specific products without sequencing usually provides sufficient evidence that blaCTX-M gene is responsible for ESBL production in an isolate.[10]

Maleki *et al.*[11] reported the following prevalence rates as CTX-M-3 (56.5%), SHV (85.5%), and TEM (16.1%). Bora *et al.*[12] have reported 77.58% of blaTEM and

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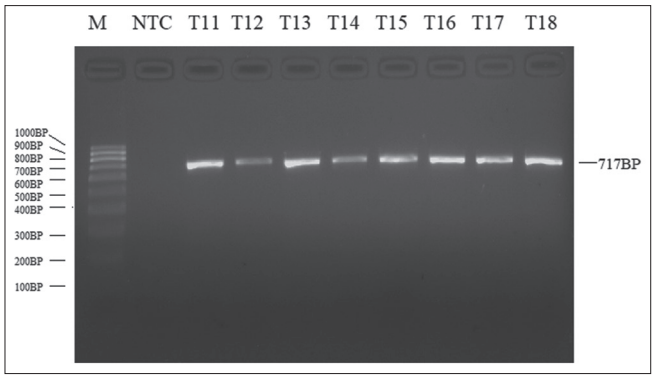

Figure 1: Agarose gel showing PCR amplified product of blaTEM gene, Lane M = 1 kb DNA ladder (APS, Lifetech, Pune), Lane NTC = No template control (no templated DNA added), Lane T11–T18 showing blaTEM positive amplicons (717bp)

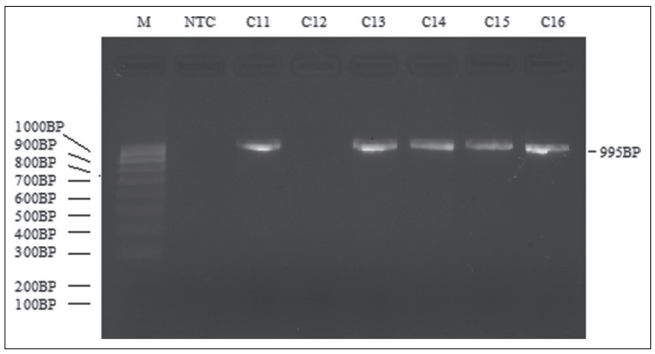

Figure 2: Agarose gel showing PCR amplified product of blaCTX-M-15 gene, Lane M = 1 kb DNA ladder (APS, Lifetech, Pune), Lane NTC = No template control (no templated DNA added), Lane C11, C13–C 16 showing blaCTX-M-15 positive amplicons (995bp)

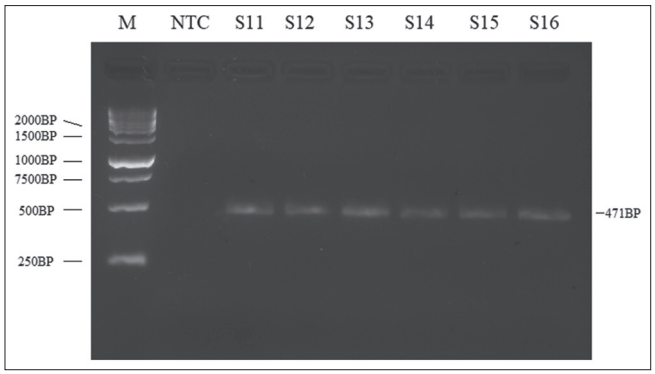

Figure 3: Agarose gel showing PCR amplified product of blaSHV gene, Lane M = 10 kb DNA ladder (APS, Lifetech, Pune), Lane NTC = No template control (no templated DNA added), Lane S11-S16 showing blaSHV positive amplicons (417bp)

blaSHV as the less prevalent ESBL genotype showing 50.57%. Sharma *et al.*[13] have reported 72% of blaSHV production. Nasehi *et al.*[14] in their study found that the blaSHV, blaCTX-M, and blaTEM genes were detected in 23, 22.5, and 16% of strains, respectively. This study also showed that 26.25% (n = 21) of isolated strains contained three genes. Many other researchers have reported similar results. Hassan *et al.*[15] found that ESBL genotypes showed a remarkable increase in the CTX-M (97.4%) compared to SHV (23.1%). The predominant ESBL was CTX-M-15 (92.1%). No TEM ESBL was detected in this study. Shahid *et al.*[16] have reported CTX-M in 11 (39.2%), TEM in 5 (17.9%), and SHV in 7 (25.0%). Out of 73 isolates studied, the presence of any of the bla genes (blaSHV, blaCTX-M, and blaTEM) was noticed in a total of 28 (38.4%) isolates.

It was observed that all but one of the isolates harbored only the SHV gene, while TEM and CTX-M-15 genes were not present. Another isolate harbored only TEM gene, while SHV and CTX-M-15 genes were not present. Kotekani *et al.*[17] in their study found 75.51% blaCTX-M-15 gene and 77.58% blaSHV gene. They have reported 61.29% isolates harboring CTX-M and SHV genes.[17] While a few studies have shown that CTX-M type ESBLs have emerged as the most common type of ESBL showing the highest predominance among *Klebsiella spp* and are responsible for sporadic, in our study the presence of TEM and SHV gene was found more than CTX-M-15.

Forty of total 45 samples, that is, 88% of the strains harbored all three genes: CTX-M-15, TEM, and SHV (44,45). Hassan *et al.* found that the co-existence of the blaSHV and blaCTX-M was detected in 24 isolates (20.5%).[15] Bora *et al.* also reported that the co-existence of blaSHV, blaCTX-M, and blaTEM was found predominantly in their study (42.6%).[13] Shahid *et al.* in their study reported that none of the isolates showed the presence of all these three bla genes (blaSHV, blaCTX-M, and blaTEM) in a single bacterial cell.[16] The presence of TEM, SHV, and CTX-M genes highlights the growing complexity of antibacterial resistance problems.

Conclusion

This study highlights the need for monitoring the spread of the MDR clonal complex among the *Klebsiella spp.* and a better understanding of its dissemination can prevent its emergence and spread. Antimicrobial resistance is promoted by two factors: lapses in infection control and antibiotic selective pressure. From the results, it can be clearly concluded that there is an increase in ESBL-producing *K. pneumoniae* isolates as compared to previous studies. Though the study is limited by the small sample size, it highlights the presence of CTX-M-15, TEM, and SHV in multiple isolates of *K. pneumoniae,* indicating that this is probably disseminated horizontally through the high occurrence of ESBL organisms and transmissible resistance genes. Detection of common ESBL genes

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such as TEM, SHV, and CTX-M in ESBL-producing bacteria and their pattern of antimicrobial resistance can provide useful information about its epidemiology and aid in rational antimicrobial therapy. Genotypic identification of resistant genes is of utmost importance as it can help any clinician also for appropriate therapy. More molecular studies are needed in different regions to find the predominant ESBL enzymes in that particular area for the epidemiological purpose.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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