

Original Research Article

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Prevalence of Extended-Spectrum β -Lactamases in Uropathogenic *Klebsiella pneumoniae* and Characterization of the *bla* Genes in a Tertiary Care Centre

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ABSTRACT

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Urinary tract Infections caused by extended-spectrum β -lactamase (ESBL), producing *Klebsiella pneumoniae* are on a rise all over the world with high morbidity and mortality. This study was carried out to determine the presence of TEM, SHV and CTX-M genes in extended-spectrum β -lactamase (ESBL) producing *Klebsiella pneumoniae*. A total of 300 *Klebsiella pneumoniae* isolates were collected and identified using traditional culturing and biochemical tests. Antibiotic susceptibility testing was performed by disc-diffusion method according to the CLSI guideline. Isolates were screened for ESBL and confirmed by phenotypic confirmatory disc diffusion test (PCDDT). 100 randomly selected isolates tested for the presence of ESBL encoding genes using PCR with specific primers for the detection of CTX-M, SHV and TEM genes using a standard protocol. Imipenem showed the highest antibacterial activity against ESBL producing *K. pneumoniae*. Based on the results of PCR, the prevalence of TEM, SHV and CTX-M genes among ESBLs-positive isolates was 74%, 27%, and 44% respectively. In conclusion, the rate of ESBL-producing *K. pneumoniae* was high in the present study. The bacterial resistance to many classes of antibiotic leads to limited treatment options. Since the management of infections caused by these organisms is difficult, it is important to control such strains in order to prevent and reduce their spread.

Introduction

Urinary tract infections (UTIs) are among the most common infectious diseases encountered in the community and in the hospital; they result in high rates of morbidity and high economic costs associated with treatment (Arjunan *et al.*, 2001) (Rahman *et al.*, 2009) (Hryniewicz *et al.*, 2010). The extensive use of β -lactam antimicrobial agents in order to treat patients with UTI has recently led to the emergence of resistant strains all over the world. Beta-lactam resistance is mediated by extended spectrum β -lactamase (ESBL) genes that are mostly encoded by plasmid

(Topaloglu *et al.*, 2010). According to a study by Klevens *et al.*, (2002) among the various nosocomial infections urinary tract infections accounts for 15% of the infections with a mortality of 15,000 deaths every year.

The first plasmid-mediated β -lactamase in gram-negative organism was described in the early 1960s in TEM-1 gene (Datta and Kontomichalou, 1965). There are many types of ESBLs like TEM, SHV, CTX, OXA, AmpC, etc. but majorities of the ESBLs are derivatives of TEM or SHV enzymes, and

these enzymes are most often found in *E. coli*, *K. pneumoniae* and *Acinetobacter baumannii*. It has been seen that point mutation has formed the basis of resistance in *bla* genes (Jacoby and Medeiros, 1991). So far > 400 ESBLs have been reported that typically, have been derived by point mutation from the TEM, SHV and CTX-M groups (Barguigua *et al.*, 2011).

Until recently, TEM and SHV variants were the most ESBLs produced by *Klebsiella* spp., *Enterobacter* spp and *E. coli*, ESBLs have emerged as a major problem in hospitalized patients worldwide and have been involved in epidemic outbreaks.

Detection of common ESBL genes such as TEM, SHV and CTX-M by molecular methods in ESBL-producing bacteria and their pattern of antimicrobial resistance can provide useful information about its epidemiology and aid in rational antimicrobial therapy (Jain and Mondal, 2008). As very little information is available on molecular types of ESBL positive *Klebsiella* species from this part of North India this study was taken up. The current study aimed to identify the antibiotic susceptibility pattern of urinary isolates of *K. pneumoniae* within the community and within the hospital Subharti medical college, and determine the prevalence of TEM, SHV and CTX-M β -lactamase gene by phenotypic and genotypic (PCR) methods.

Aims and objectives

This study was conducted to determine the prevalence of ESBL genes in *Klebsiella pneumoniae* isolated from patients visiting the outpatient departments and also who were admitted to various wards of the hospital as well to know the antibiogram profiles of the ESBLs producing *Klebsiella pneumoniae* isolates.

Materials and Methods

Sample collection

Fresh mid-stream urine samples and catheterized urine samples were collected at Chatrapati Shivaji Subharti Medical College Teaching Hospital. Both male and female patients between the age group of > 10 yrs up to 85 years were included in the study. Proportion of males (46%) and females (54%) are depicted in figure 1 indicating more complaints of UTI in females.

Immediate processing of the samples after collection was done to avoid contamination. These urine samples were inoculated on CLED agar and MacConkey's agar incubated at 37^oc for 18-24 hours as per CLSI guidelines to study their cultural characteristics. Isolates were confirmed as *Klebsiella pneumoniae* as per CLSI guidelines using the standard biochemical identification tests (CLSI, 2012).

Antimicrobial susceptibility testing in the presence of any potential growth was determined using the disc diffusion method according to the CLSI guidelines.

The antimicrobial which were tested included: Amikacin (30 μ g), Aztreonam (30 μ g), Ceftazidime (30 μ g), Cefotaxime (30 μ g), Ceftriaxone (30 μ g), Co-trimoxazole (25 μ g), Gentamicin (10 μ g), Imipenem (30 μ g), Ciprofloxacin (5 μ g), Nalidixic acid (30 μ g), Norfloxacin (10 μ g), and Nitrofurantoin (300 μ g).

Mueller Hinton Agar and antibiotic discs were procured from Hi-Media India. All assays included ESBL positive control standard strain of *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 as negative control.

Detection of Extended Spectrum β -Lactamases

Screening for ESBL production using disc diffusion method

All isolates showing resistance to one or more 3rd generation cephalosporins, namely ceftazidime, ceftriaxone and cefotaxime were considered a probable ESBL producer. Out of 300 strains 226 strains were suspected to be ESBL producers. These were then subjected to phenotypic confirmation.

Phenotypic confirmatory tests for ESBL production

Combination disc method

Based on the CLSI recommendations Cephalosporin / Clavulanate combination discs were used to assure the suspected ESBL strains by the combination discs diffusion method. Briefly, the overnight growth in broth of Gram negative bacteria was adjusted to 0.5 McFarland Standard. Confirmation was done by combination disc method as per the CLSI guidelines. ESBLs production was confirmed by placing disc of cefotaxime(30 μ g) and ceftazidime (30 μ g) at a distance of 20mm from a disc of cefotaxime/clavullinic acid (30/10 μ g) and ceftazidime/ clavullinic acid (30/10 μ g) respectively on a lawn culture of test strains (0.5 Mc Farland inoculum type) on Mueller Hinton Agar (Fig. 1).

After overnight incubation at 37⁰C the strain was considered ESBL positive if there was an increase in zone size of > 05 mm in the zone size of Cephalosporin / Clavulanate combination disc when compared with cephalosporin alone. All the 226 strains were subjected to the combination disc method of which 179 strains were phenotypically confirmed as ESBL producers.

DNA extraction and characterization of bla genes

Of the 179 strains phenotypically confirmed as ESBL positive 100 randomly picked non repetitive strains of *K. pneumoniae isolates* were then analyzed at their gene level.

The plasmid DNA was isolated from bacterial cells by using Plasmid Purification Kit based on the principle of alkaline lysis according to the manufacturer's instructions. The DNA extracted was stored at -20°C.

PCR amplification of *bla* genes, including *bla*TEM, *bla*SHV and *bla*CTX-M was performed with *Taq* master mix DNA polymerase.

Individual amplification for every gene was carried out on 2720 Thermocycler Applied Biosystems, primer sequences that were used for the detection of *bla*TEM, *bla*SHV and *bla*CTX-M genes in this study, which are listed in table 1 along with their sources.

Amplification

For PCR amplification for TEM, SHV and CTX-M genes the following reaction mixture was prepared: - 1 μ l of template DNA+ 12.5 μ l of master mixture (containing 10X PCR buffer+DNTP's+Taq DNA polymerase 1 μ l each of TEM(F)+TEM(R), SHV(F)+SHV(R) and CTX-M(F)+ CTX-M (R) primers for detection of TEM, SHV and CTX-M gene respectively.

Finally the volume was made up to 25 μ l by adding 9.5 μ l nuclease free water. The cycling conditions applied are illustrated in table 2.

Gel electrophoresis

The amplified products were separated in 1.5 per cent agarose gel. The gel was visualized

by staining with ethidium bromide (0.5 mg/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 visualized using a under ultraviolet illumination (Alphaimager TM 3400, USA).

Statistical analysis of the data was analyzed using the chi square tests.

Results and Discussion

In this study the antibiotic resistance pattern of *K. pneumoniae* isolates to different β -lactam and non- β -lactam antibiotics are found to vary widely. Majority of the *K. pneumoniae* isolates were found to be multi-drug resistant (MDR) *i.e.*, resistant to three or more antibiotics used in the study. A total of 82.0 % of *K. pneumoniae* isolates (*i.e.* 246 of 300 isolates) exhibited the MDR phenotypes. 4 isolates (all from inpatients) of the total 300 (1.3%) showed resistance to all the antibiotics used. Out of 300 *K. pneumoniae* isolates, a total of 226 isolates (75.3%) showed positive results in initial screening test of for ESBL production while the phenotypic confirmation showed a total of 179 isolates (59.6%) as positive for ESBL production. The result of PCR detection of ESBL genotypes, all the 100 isolates *K. pneumoniae* were found to possess one or more ESBL genes tested in this study. Overall, 86% (86/100) of *K. pneumoniae* isolates were positive for one or more ESBL genes. Agarose gel showing PCR amplified product of *bla* genes are depicted in figures 1–3. Among the 100 isolates the number of ESBL producing *K. pneumoniae* with TEM, SHV and CTX-M were 74%, 27% and 44% respectively. Some strains harboured one or more than one ESBL genes and in few all the three were detected as depicted in table 3. Urinary tract infections (UTIs) is one of the most common infections and the annual

global incidence is estimated to be around 250 million cases (Ronald *et al.*, 2001). The extensive use of antimicrobials has led to high percentage of ESBL producing *Klebsiella* sp. In the recent past there has been an increase in the acquisition of extended spectrum β -lactamase (ESBL) enzymes among gram negative bacteria rendering an overall resistance to third generation cephalosporins (3GC's). The prevalence rate of ESBLs producing *Klebsiella* spp in india is reported to vary between 6-87% which correlates well with other studies from adjoining parts of North India (Hansotia *et al.*, 1997; Sheemar *et al.*, 2016; Oberoi *et al.*, 2012; Sharma *et al.*, 2013; Mathur *et al.*, 2005 and Jain *et al.*, 2005). Reports from ESBL prevalence worldwide in community and hospital varies widely reported between 3%-100% (Der *et al.*, 2005; Chanwit 2007; Bean *et al.*, 2008 and Cristina 2011). In this prospective study it was observed that almost 82% of the total isolates of *Klebsiella pneumoniae* were resistant to third generation cephalosporins and other antibiotics similar to studies by (Fauzia and Damle, 2015; Bora *et al.*, 2014), making them MDR strains. One important factor seen is the high usage of antibiotics in the intensive care units which also is an important factor in imposing potential for patient to patient transmission of organisms. ESBL producing gram negative bacteria have been responsible for numerous outbreaks of infection globally imposing a great challenge in infection control. So it becomes crucial to identify ESBLs as a routine in the hospitals and laboratories (Vandana and Honnavar, 2009). Out of 300 *K. pneumoniae* isolates, a total of 226 isolates (75.3%) showed positive results in initial screening test of for ESBL production, later only 179 (79%) of 226 were phenotypically confirmed. Among the phenotypically confirmed 179 strains 114 were IPD samples and 65 were OPD samples. Most of the ESBL producing *K. pneumoniae* isolates in this study were susceptible to

Imipenem (85.6%) followed by Amikacin (74.6%) and Nitrofurantoin (64.6%), indicating them as probable efficient drug for treating UTI caused by ESBL producing *K. pneumoniae*. Susceptibility of Imipenem among ESBLs (83.6%) and among non ESBLs (91.8%), the susceptibility of the isolates to other antibiotics were as follows: Gentamicin(44.3%), Ceftriaxone(41.6%), Aztreonam (38.3%), Co-trimoxazole(35.0%), Cefotaxime(29.6%), Ciprofloxacin(29.0%), Norfloxacin (28.3%), Ceftazidime (25.0%) and Nalidixic acid (18.6%). Least sensitivity was seen to Nalidixic acid. One can co-relate the high incidence of multi drug resistance to

the increase in cephalosporin consumption in India (Chaudhary and Aggarwal, 2004; Thomson, 2010). It is well known that indiscriminate and excessive antibiotic use ultimately results in resistant bacteria and this in itself is a driving force for clinically significant increase in the incidence of ESBL producing bacteria (Medeiros, 1997). Most of these MDR strains were isolated from in-patients, indicating probable hospital acquired infection. In this study a high prevalence of ESBLs is reported from ICU, gynaecological, surgical and medical wards. The reason for this could probably be the drug prescribing habits of these wards.

Table.1 Primers used for detection of TEM, SHV and CTX-M genes

	Bla Gene	Primer used in the study (5'-3')	Product Size(bp)
1	Bla TEM*	OT-1: 5'TTGGGTGCACGAGTGGGTTA3' OT-2: 5'TAATTGTTGCCGGGAAGCTA3'	504
2	Bla SHV*	OS-1: 5'TCGGGCCGCGTAGGCATGAT3' OS-2: 5'AGCAGGGCGACAATCCCGCG3'	626
3	Bla CTX-M#	CTX-M: F 5'-TTTGCATGTGCAGTACCAGTAA-3' CTX-M: R 5'-CGATATCGTTGGTGGTGCCATA-3'	544

(F): Forward base

(R): Reverse base

Reference: * Ozgumus et al 2008, #Edelstein et al 2003

Table.2 Cyclic conditions during PCR

Genes	Initial denaturation	No. of cycles	Amplification cycles	No. of cycles	Final extension	No. of cycles
bla TEM	94°C for 5 min	1	Denaturation at 95°C for 30 s	35	72°C for 7 minutes	1
bla SHV	94°C for 3 min		Annealing at 55°C for 45 s		72°C for 7 minutes	
bla CTX-M	94°C for 5 min		Elongation at 72°C for 45 s		72°C for 5 minutes	

Table.3 Distribution of ESBL genes among the isolates

ESBL Genes (Single/ in Combination)	NUMBER OF STRAINS (n=100)
bla TEM	74
bla SHV	27
bla CTX-M	44
bla TEM+ bla SHV	09
bla TEM+ bla CTX-M	25
bla SHV+ bla CTX-M	03
bla TEM+ bla SHV+ bla CTX-M	11
ONLY bla TEM	29
ONLY bla SHV	06
ONLY bla CTX-M	05

Fig.1 Proportion of male and female patients

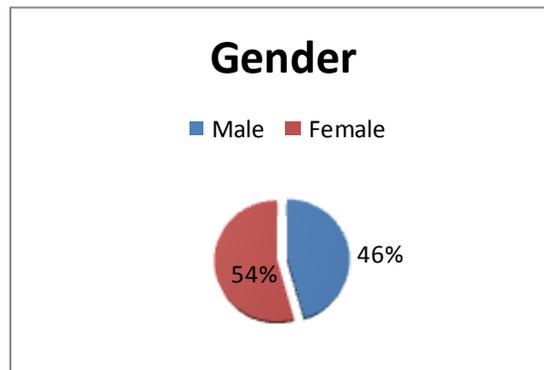


Fig.2 Showing 504 bp fragment bands of all *bla* TEM genes detected by PCR (lane 1: negative control, 2-positive control and 3-7 = ESBL positive isolates, M = 100 bp DNA ladder)

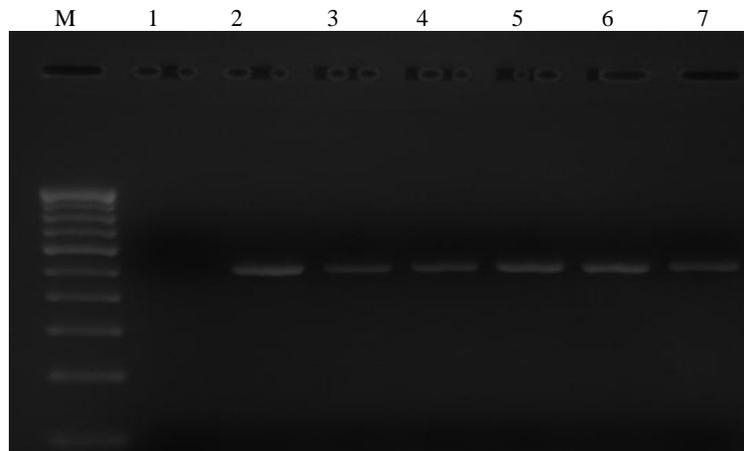


Fig.3 Showing 626 bp fragment bands of *bla* SHV genes detected by PCR (lane 1: positive control; 2: ESBL positive isolate and 3-6 ESBL negative isolates; 7: negative control; M = 100 bp DNA ladder)

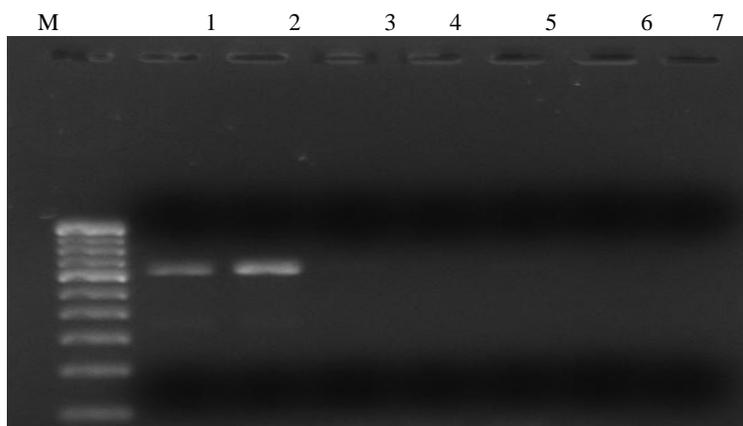


Fig.4 Showing 544 bp fragment bands of all *bla* CTX-M genes detected by PCR (lane 1: negative control; 2-positive control; 3-negative sample; 4-7 = ESBL positive isolates, M = 100 bp DNA ladder)

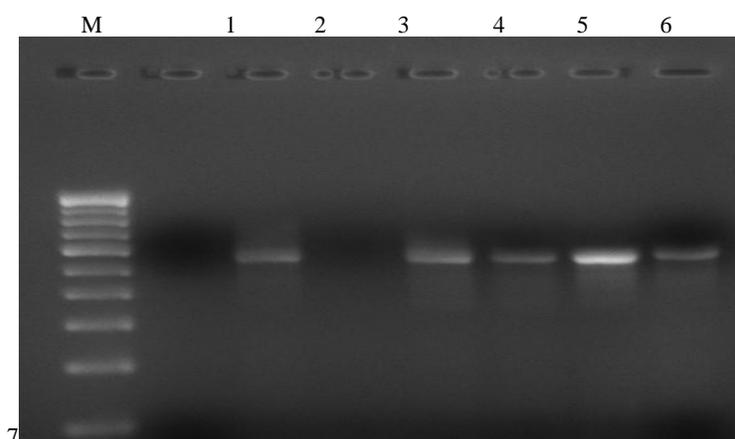
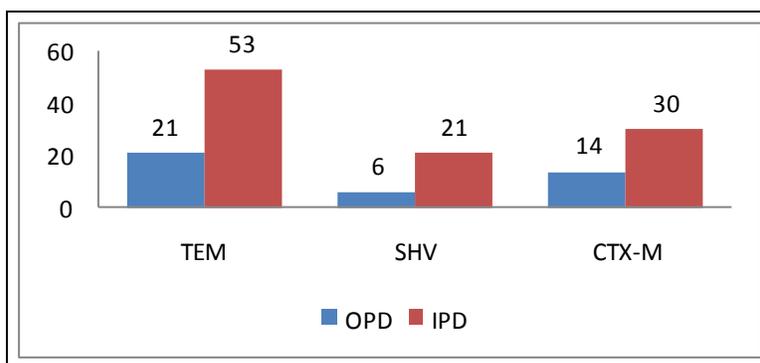


Fig.5 Prevalence of the respective *bla* genes in OPD/IPD



Apparently the phenotypic tests for ESBL detection can only confirm whether an ESBL

is produced but it cannot determine the ESBL subtype. Also some ESBLs may fail to reach a level to be detected by disk diffusion tests and then may lead to treatment failure. Although molecular methods appear sensitive, but are expensive, time consuming and require specialized equipment and expertise but it definitely aids in knowing the predisposition factors and epidemiological studies (Nuesch and Hachle, 1996). For molecular characterization 100 strains were randomly picked (69 were from IPD and 31 were from OPD samples).

It was found that out of the 100 uropathogenic *K.pneumoniae* isolate tested 74 isolates were positive for blaTEM, 27 isolates were positive for blaSHV, 44 were positive for a blaCTX-M, 09 isolates were positive for blaTEM and blaSHV, 25 isolates were positive for both blaCTX-M and blaTEM, 03 isolates were positive for both blaCTX-M and blaSHV, and 11 isolates was positive for all the three blaTEM, blaSHV and blaCTX-M. The old members TEM and SHV of ESBL which were responsible for nosocomial infections have been now replaced by a new type, CTX M which has gained prominence and is the predominant gene in the hospital settings as understood from our study where of the 100 isolates, PCR assay revealed that 74%, 27% and 44% were positive for TEM, SHV and CTX-M genes respectively. Prevalence of TEM gene in the isolates was similar to a study from Gujarat (Varun and Parijath, 2014). A study by Varkey *et al.*, (2014) reports a prevalence of 75% TEM gene, 66% SHV gene and 71% CTX-M gene. Here although the TEM gene is prevalent at almost the same rate both SHV and CTX-M are slightly more. another study by Sharma *et al.*, 2013 reported TEM (75%), SHV (60%) and CTX-M (85%) in *Klebsiella* spp. *K.pneumoniae* is one of the most common ESBL producing organisms, making difficult for the clinicians to treat them particularly in

the hospital settings. The resistance in these organisms is due to a plasmid mediated *bla* genes. There is definitely a need for more such molecular studies to be done in different regions of India to find the common ESBL enzymes present in that geographical area for epidemiological purposes.

From the above results, it can be concluded that there is an alarming percentage of ESBL producing *K. pneumoniae* isolates in urinary tract infections. Periodic surveillance of antibiotic resistance patterns, monitoring and judicious usage of extended spectrum Cephalosporin and enforcement of infection control practices should also be strengthened in all our tertiary health centers.

Prompt use of carbapenems instead of extended-spectrum cephalosporins, quinolones and aminoglycosides should be incorporated in practice.

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