Molecular characterisation of extended spectrum beta lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from urinary specimens

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ABSTRACT

Extended spectrum beta lactamase (ESBL) production is a major resistance mechanism of Gram-negative bacteria. Phenotypic tests for ESBL detection only confirm whether an ESBL is produced or not, but it cannot detect the ESBL subtype. There are various genotypes of ESBLs. Of these, the most common are the TEM, SHV and CTX-M types. Fifty phenotypically ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* urinary isolates were subjected to polymerase chain reaction to look for the presence of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. Forty-one isolates were positive for TEM gene, eleven isolates were positive for SHV gene and 10 isolates were positive for CTX-M gene. Both TEM and SHV genes were present in six isolates. Two isolates showed the simultaneous presence of CTX-M and TEM genes. All the three genes were present in two isolates.

Key words: CTX-M, extended spectrum beta lactamase, polymerase chain reaction, SHV, TEM

INTRODUCTION

The prevalence of extended spectrum beta lactamase (ESBL) producing bacteria varies worldwide. They are reported in almost all parts of the world and are increasing at an alarming rate. The situation in India is not different. The prevalence of ESBLs in India has now reached epidemic proportions, ranging from 62% to 100% in *Escherichia coli* and Klebsiella spp. as observed in the 10 Indian medical centre SENTRY study.[1] High rates of ESBL-positive isolates were observed in nine study centres in India, each representing distinct cities in various regions of India: New Delhi, Lucknow, Indore, Mumbai, Hyderabad, Bengaluru, Chennai, Tamil Nadu and Kolkata indicating that ESBL-positive strains in the country are not restricted to any single city or region.[2]

ESBL-producing strains of Enterobacteriaceae have emerged as a challenge in hospitalised as well as community-based patients.[3] There are several genotypes of ESBLs. Of these, the most common genotypes are the TEM, SHV and CTX-M.

In the 1980s and 1990s, TEM- and SHV-ESBLs were the predominant ESBL types whereas the CTX-Ms were less prevalent. CTX-M enzymes, though first discovered in 1989, began to be detected at a higher rate only after 2000. They were confined not only to the hospital setting but also to the community, with *E. coli* being the most important pathogen-producing CTX-M enzymes.[4]

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TEM- and SHV-ESBLs, with over 100 mutations being reported, have become a major cause of hospital-acquired infection, particularly in the Intensive Care Unit. It has been suggested that the naturally occurring TEM-type ESBLs are the result of fluctuating selective pressure from several beta-lactams within a given institution rather than selection with a single agent.[5] There are over 100 variants of CTX-M to date and they have been associated with numerous outbreaks of infections both in hospitals and in the community.

Molecular characterisation of ESBL-producing isolates can be a useful epidemiological tool in the surveillance of these multidrug-resistant (MDR) organisms. Such data can help us know the transmission of these organisms in hospitals, which is useful to make effective and timely interventions. In this background, a study was conducted on fifty ESBL-producing MDR *E. coli* and *Klebsiella pneumoniae* urinary isolates to check for the presence of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes using polymerase chain reaction (PCR).

MATERIALS AND METHODS

The study was conducted in the Department of Microbiology, Government Medical College, Thrissur, during the period of August 2011 to July 2012.

According to the recent studies in India, the prevalence of ESBL ranges from 40% to 100%.[1,6-8] From this data, the sample size of the study was calculated as 150, taking a power of 80% and allowable error as 20% at 5% confidence level. The presence of ESBL production was tested by double disc diffusion test described by Jarlier *et al*.[9] and phenotypic confirmatory test (PCT) recommended by Clinical and Laboratory Standards Institute (CLSI), 2010.[10]

Molecular characterisation of fifty MDR isolates determined as ESBL producers by PCT was done at the Department of Agricultural Microbiology, College of Horticulture, Kerala Agricultural University. The sample size for doing PCR was restricted to fifty due to financial constraints. A random selection of fifty MDR *E. coli* (n = 41) and *K. pneumoniae* (n = 9) was made by lot method from the 150 isolates, on which ESBL production was detected by PCT. PCR was carried out to detect the presence of the most common ESBL genes – bla_{TEM}, bla_{SHV} and bla_{CTX-M}. PCR was done in Eppendorf Mastercycler, Gradient (Eppendorf, Germany).

DNA template preparation was done as follows: Using a sterile loop, a medium-sized colony of a fresh culture of the organism on nutrient agar plate was transferred to PCR vial and suspended in 10 μl distilled water. Denaturation was done at 98°C for two min. The vial was then centrifuged at 11,000 rpm for one min. The supernatant (1 or 2 μl) was taken as the template to do the PCR.

The master mix was prepared by adding 2.5 μl Taq buffer, 1 μl dNTPs, 2 μl Taq polymerase, 16.5 μl distilled water and 1 μl each of forward primer and reverse primer. Finally, after dispensing 24 μl of the master mix in the individual amplification tubes, 1 μl of the template DNA was added in the corresponding tubes to make up the total volume to 25 μl. The PCR primers used (Sigma Aldrich, Bengaluru) and the expected amplicon sizes are shown in Table 1.

Thermal cycling conditions used were initial denaturation done at 94°C, two min and followed by thirty cycles of denaturation at 94°C, 45 s; annealing temperature kept at 58°C one min for bla_{TEM}, bla_{SHV} and 50°C one min for bla_{CTX-M}; extension at 72°C, two min and final extension at 72°C, 10 min.

PCR products were analysed by agarose gel electrophoresis with 1% agarose gels in 50X Tris-acetate-EDTA buffer. The gels were stained with ethidium bromide. PCR products were visualised under ultraviolet light [Figures 1-3].

RESULTS

The total number of urinary specimens received during this period was 4550, out of which 812 samples yielded a significant growth of Gram-negative Bacilli. Among this 812 Gram-negative isolates, 451 (55.5%) were MDR, which means they were resistant to three or more antimicrobial classes. MDR *E. coli* (n = 368) and MDR *K. pneumoniae* (n = 76) were the predominant MDR Gram-negative urinary isolates.

Table 1: Primers used and amplicon sizes

Target gene	Primers used	Amplicon sizes (kb)	Annealing temperature (°C)
bla_{TEM}	GTATCCGCTCATGGAGACAATAACCCTG CCAATGCTTAATCAGTGGAGGCACC	900	58
bla_{SHV}	CGCCTGTGTATTATCTCCCTGTTAGCC TTGCCAGTGCTCGATAGAC	850	58
bla_{CTX-M}	CGCTTTGCGATGCGAG ACCGCGATATCGTTG	550	50

Based on the sample size calculated, 150 MDR urinary isolates of *E. coli* ($n = 117$) and *K. pneumoniae* ($n = 33$) were tested for the presence of ESBL production by double disc diffusion test described by Jarlier *et al.*[9] and PCT recommended by CLSI, 2010.[10]

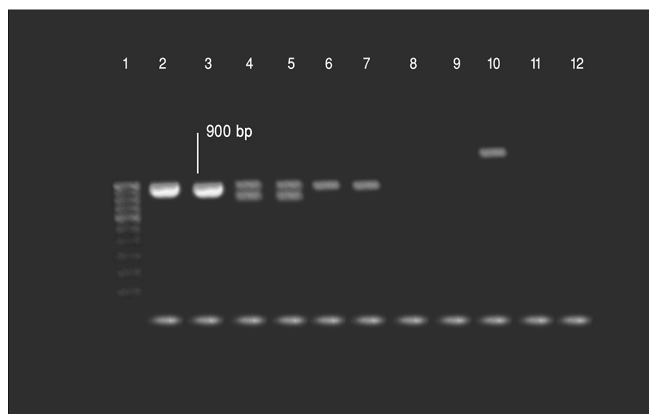

Figure 1: Polymerase chain reaction to detect bla_{TEM} (900 bp) lane 2, 3, 4, 5, 6 and 7 – positive for bla_{TEM}

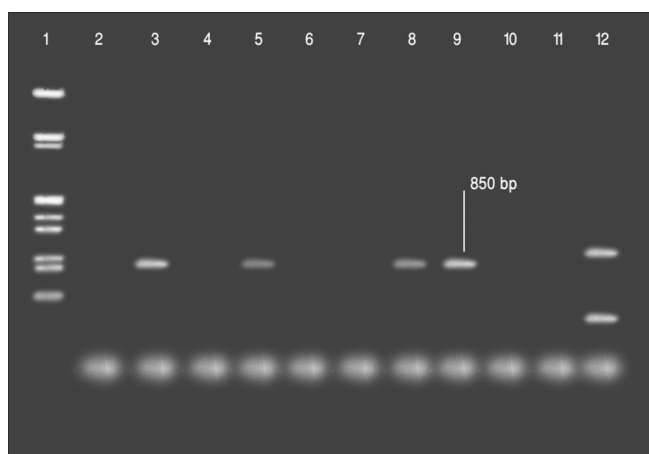

Figure 2: Polymerase chain reaction to detect bla_{SHV} (850 bp) lanes 3, 5, 8 and 9 – positive for bla_{SHV}

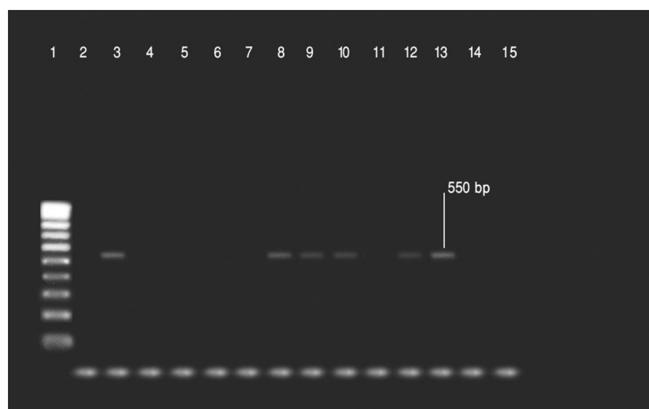

Figure 3: Polymerase chain reaction to detect $bla_{CTX\text{-}M}$ (550 bp) lanes 3, 8, 9, 10, 12 and 13 – positive for $bla_{CTX\text{-}M}$

Out of the 150 MDR isolates, 91% (136/150) were ESBL producers, 9% were negative for ESBL. Out of 117 *E. coli* studied, 107 (91.5%) were ESBL producers. Out of the 33 *K. pneumoniae* isolates under study, 29 (87.9%) were ESBL producers.

Out of fifty ESBL-producing MDR *E. coli* and *K. pneumoniae* isolates, 41 showed the presence of bla_{TEM}. Eleven isolates were positive for bla_{SHV} and 10 isolates for $bla_{CTX\text{-}M}$. Hence, bla_{TEM} predominated in the isolates, followed by bla_{SHV} and $bla_{CTX\text{-}M}$ [Table 2].

DISCUSSION

Phenotypic tests for ESBL detection only confirm whether an ESBL is produced, but it cannot detect the ESBL subtype. The phenotypic tests need to be evaluated periodically as their performance may change with the introduction of new enzyme. There are various genotypes of ESBLs. Of these, the most common genotypes are the TEM, SHV and CTX-M. Other clinically important types include OXA, VEB, PER, BEL-1, BES-1, SFO-1, TLA and IBC. Identification to this level is very difficult and is not available in all routine microbiology laboratories.

CTX-M beta-lactamases are more active against cefotaxime and ceftriaxone than against ceftazidime, but point mutations can increase their activity against ceftazidime as well. CTX-M now accounts for most of the ESBLs found in Enterobacteriaceae. The CTX-M group of ESBLs are diverse, with alleles divided into five distinct phylogenetic groups. In most countries, there are mixtures of CTX-M types, with ESBL isolates from India being completely dominated by the presence of CTX-M-15.[1,11]

In a study by Sharma *et al.* from Chandigarh on molecular detection methods of beta-lactamases, 84% of the ESBL producers belonged to TEM- and SHV-ESBL types.[12] The study further concluded that the rest 16% of ESBL producers may belong to other ESBL subgroups. Another

Table 2: Distribution of TEM, SHV and CTX-M genes among 50 multidrug-resistant isolates

ESBL genotype	ESBL producing GNB from urine (N)	Percentage (%)
TEM	31	62
SHV	3	6
CTX-M	6	12
TEM and SHV	6	12
TEM and CTX-M	2	4
TEM, SHV and CTX-M	2	4
Total	50	100

study by Jemima and Verghese from Chennai found that 15.83% of ESBL-positive isolates had bla_{CTX-M} gene.[13] They also noted that among those CTX-M-producing isolates, CTX-M-1 gene was positive in 47.3% isolates. Sekar *et al*. reported that 44.4% of *E. coli* and *K. pneumoniae* strains were found to be positive for bla_{CTX-M} gene by PCR.[14]

In another study by Manoharan *et al*., distribution of TEM, SHV and CTX-M ESBL types among 121 study isolates was noted.[15] They observed that TEM and CTX-M were predominantly seen in *E. coli* (39.2%), while among Klebsiella spp., TEM, SHV and CTX-M occurred together (42.6%). Another finding made by them was that higher resistance was noted among isolates carrying all the three (TEM, SHV and CTX-M) ESBL types.

The molecular characterisation of randomly selected ESBL-carrying isolates in this study showed a predominance of TEM-ESBLs followed by SHV-ESBLs and CTX-M ESBLs. This finding does not correlate with the current trend that CTX-M ESBLs are emerging as the predominant ESBL type. This may be because all the ESBL-carrying isolates were not studied for ESBL genes. Data from the past 10 years establish CTX-M genotype as the predominant ESBL in Europe and East Asia. Not many studies are available from different parts of our country checking the presence of the three genes – TEM, SHV and CTX-M together among the ESBL-producing pathogens. Therefore, the observations in this study indicate the need for a more detailed surveillance of the MDR isolates in this region, with special attention to molecular level detection. Although molecular characterisation of ESBLs is not very important in deciding the treatment, they provide useful epidemiological information which can make relevant contributions in formulating antibiotic policy of the area.

CONCLUSION

This study found TEM followed by SHV as the major genotype in a study group of 50 ESBL-producing *E. coli* and *K. pneumoniae* urinary isolates. This is in contrast to current global and Indian scenario where CTX-M is the major genotype. The sample size of this study was restricted due to financial constraints, which is a limitation of the study. This fact points to the need for a large scale genotypic study to understand the epidemiologic pattern of drug-resistant isolates. The control measures include strict implementation of antibiotic policy, proper hand hygiene protocol and long-term surveillance of the antibiotic susceptibility pattern of the microbial isolates.

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Conflicts of interest

There are no conflicts of interest.

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