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To Assess Genotyphic Methods in the identification of ESBL producing Uropathogenic Escherichia coli

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Introduction: Urinary tract infections (UTIs) are the most common bacterial infections affecting approximately 11% of adult women each year globally, with approximately 60% of women experiencing UTI during their lifetime. Extended-spectrum β-lactamases (ESBLs) are extremely broad spectrum β-lactamase enzymes, which can be produced by Gram-negative bacteria. They are mainly found in a family of Enterobacteriaceae. ESBLs are produced by the mutation of the TEM-1, TEM-2, and SHV-1 β-lactamases.

Materials and Methods: This prospective study was conducted in the Department of Microbiology, Index Medical College, India. A total of 300 consecutive urine samples were screened from patients with symptomatic UTI. Clean-catch midstream urine samples were collected in sterile disposable container and processed within one hour.

Genotypic Assay

- The Boiling method was used to extract DNA from bacterial samples.
- SHV, TEM, and CTX-M beta-lactamase genes were detected by PCR. PCRs were carried out using thermal cycler in a total volume of 25 µl containing 10 pmol of each three pair of primers, 25 µmol of dNTPs, 5 µl of template DNA, 2.5 µl of 10X Taq buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3)], 2 mM MgCl2 and 2.5 U of Taq polymerase.

Results:

In our study, of the 300 samples 211 were female and 89 males, which correspond to 70.4% of female and the 29.6% male. The genotyping results of ESBL producing isolates obtained by PCR amplification of SHV, CTX-M and TEM genes. Of 137 ESBL positive isolates, 1 (7.6%) were carrying SHV, 1 (0.7%) CTX-M and 8 (5.8%) TEM genes, while 39 (28.4%) isolates included all three genes together.

In addition, 36 (26.2%) isolates included SHV and TEM, 30 (21.8%) TEM and CTX-M, and 21 (15.3%) SHV and CTX-M genes together. Out of 137 isolates shown susceptible to the third generation Cephalosporins by combined disc test were also ESBL positive by genotypic method as they were carrying SHV and TEM genes.

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Conclusion:
The blaCTX-M and blaTEM genes predominated in this study, with the coexistence of multiple genes in single isolates indicating increased transmission of genetic determinants and the likely increase of ESBL pathogens in Indian hospitals. However, the AmpC genes typically associated with cefoxitin resistance were not observed in this study, a possible indication of other cefoxitin-resistant mechanisms that warrant further investigation.
Keywords: Genotyphic methods, ESBL, Escherichia coli

Introduction

Urinary tract infections (UTIs) are the most common bacterial infections affecting approximately 11% of adult women each year globally, with approximately 60% of women experiencing UTI during their lifetime. [1] Sporadic studies done on the prevalence of UTIs in Bangladesh and an investigation of 200 UTI patients, including men and women of various age groups, found females to be more susceptible to UTIs (80% positive) than males. In both genders, the prevalence rate was highest among those in the age group of 21–40 years (33%). The study also showed *E. coli* to be the predominant etiological agent, contributing to 57.38% of infections. [2]

The rapid emergence of antibiotic resistance threatens effective prevention and treatment of an increasing range of infections. Some bacteria are naturally resistant to certain antibiotics and others can acquire resistance through mutations in some of their genes when they are exposed to an antibiotic. [3] This acquired resistance can spread to other bacterial species. The main mechanism of antibiotic resistance mostly found is enzyme production such as β-lactamase enzymes. The β-lactamase enzymes produced by some bacteria provide resistance to β-lactam antibiotics by hydrolyzing β-lactam rings. [4]

Among antimicrobial resistant bacteria, *Escherichia coli* is one of highly concerned bacteria in the family Enterobacteriaceae. *E. coli* is a common cause of urinary tract infection and intra-abdominal infection in humans and is the second most common Gram-negative bacteria causing community-acquired bloodstream infection, accounting for 7.3% of all bloodstream infection isolates. [5] ESBL-producing *E. coli* isolates have become an importance in community-onset infections, as well as nosocomial infections. The prevalence of resistance to fluoroquinolones and extended-spectrum cephalosporins in *E. coli* had highly increased over the past decade rendering severely limited therapeutic options for these infections. [6]

Traditionally, *E. coli* phylogroups B2 and D have been understood to cause the majority of ExPEC infections, while phylogroups A and B1 were associated with comensal extraintestinal strains. [7] However, recent reports have revealed higher percentages of phylogroup A strains in UTI cases. [8] A strong association has also often been detected between a particular multilocus sequence type (MLST) with a pathology, such as the correlation of globally dominant *E.coli* ST131 and

extraintestinal infections, especially in India. [9] Like ST131, many other successful clonal lineages of different sequence types (ST), including 410, 95 and 10 have disseminated globally due to their relatively higher virulence, fitness, and metabolic capabilities, along with acquisition of antibiotic resistance genes. [10]

Extended-spectrum β-lactamases (ESBLs) are extremely broad spectrum β-lactamase enzymes, which can be produced by Gram-negative bacteria. They are mainly found in a family of Enterobacteriaceae. ESBLs are produced by the mutation of the TEM-1, TEM-2, and SHV-1 β-lactamases, which have been discovered since 1980–1990 and first detected in Western Europe. [11] To date, more than 350 different natural ESBL variants are known, which have been classified into nine distinct structural and evolutionary families based on amino acid sequence comparisons such as TEM, SHV, CTX-M, PER, VEB, GES, BES, TLA, and OXA. [12]

The main types of ESBL variants include TEM, SHV, CTX-M, and OXA. Interestingly, $bla_{\text{CTX–M}}$ has rapidly increased and is now widely found in clinically isolated *E. coli* across the world. [13] ESBLs, especially of the CTX-M type, are strongly associated with specific clonal *E. coli* strains. To date, little is known about the epidemiology of ESBL variants in India. Moreover, it is critical to provide up-to-date resistance pattern which affect the treatment decision in this region. Therefore, this study was aimed to investigate the phenotypic characteristic and variation of genetically related ESBL-producing *E. coli* in Indore.

Materials and Methods

This prospective study was conducted in the Department of Microbiology, Index Medical College, India. A total of 300 consecutive urine samples were screened from patients with symptomatic UTI. Clean-catch midstream of urine samples were collected in sterile disposable container and processed within one hour.

Genotypic Assay

- The Boiling method was used to extract DNA from bacterial samples.
- SHV, TEM, and CTX-M beta-lactamase genes were detected by PCR. PCRs were carried out using thermal cycler in a total volume of 25 µl containing 10 pmol of each three pair of primers, 25 µmol of dNTPs, 5 µl of template DNA, 2.5 µl of 10X Taq buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3)], 2 mM MgCl2 and 2.5 U of Taq polymerase.

The Primer sequences and cycling conditions used for three different PCRs are shown in Table 1.

PCR products were separated by gel electrophoresis on 1% agarose gel. In order to confirm the accuracy of genes amplified in this study, a PCR product of each gene result was confirmed by NCBI Blast Tool.

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Table 1. Primers and cycling conditions used for amplification of SHV, CTX-M and TEM genes

Resistance gene	Sequence (5' to 3')	Size (bp)	Cycling conditions
SHV	GATGAACGCTTTCCCATGATG CGCTGTTATCGCTCATGGTAA	214	95°C for 5 min; 35 cycles of 95°C for 60s, 61°C for 60s, 72°C for 60s; 72°C for 5 min
CTX-M	TTTGCGATGCATACCAGTAA CGATATCGTTGGTGCCATA 5	590	95°C for 5 min; 35 cycles of 95°C for 60s, 60°C for 30s, 72°C for 60s; 72°C for 5 min
TEM	ATGAGTATTCAACATTTCCG GTCACAGTTACCAATGCTTA	847	95°C for 5 min; 35 cycles of 95°C for 60s, 58°C for 60s, 72°C for 60s; 72°C for 5 min

Statistical analysis

The results of the study was statistically analyzed using SPSS v 25.0 software wherever suitable. The Chi- square test was done to analyze statistical significance. The p-value of less than 0.05 was considered statistically significant.

Results

Table 1: Distribution of the Percentage of patients according to age group

Age group in Years	Frequency	Percentage
<20	41	13.6
21-30	83	27.6
31-40	69	23.0
41-50	53	17.6
51-60	35	11.6
>60	19	6.3
Total	300	100

In table 1, maximum number of patients were in the age gathering of 21-30 years age gathering which were 34% (n =68) of complete followed by age group 31–40 years having 26.5% (n = 200).

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Table 2: Gender wise distribution

Gender	Frequency	Percentage
Male	**89**	**29.6**
Female	**211**	**70.4**
Total	**300**	**100**

In table 2, of the 300 samples 211 were female and 89 males, which correspond to 70.4% of female and the 29.6% male.

Table 3: Distribution of various uropathogens in culture positive samples

Name of the organism	Frequency	Percentage
Escherichia coli	137	45.6
Klebsiella pneumonia	86	28.6
Pseudomonas aeruginosa	8	2.6
Acinetobacter spp.	1	0.3
Enterococcus faecalis	2	0.6
Proteus spp.	29	9.6
Staphylococcus aureus	26	8.6
Staphylococcus saprophyticus	1	0.3
Citrobacter spp.	10	3.3
Total	200	100

In table 3, the present study shows the pathogens causing UTIs and their antibiotic susceptibility pattern. Escherichia. coli 45.6% was the predominant pathogen followed by Klebsiella pneumoniae 28.6%, Proteus spp. 9.6%, Staphylococcus aureus 8.6%, Citrobacter spp. 3.3%, Pseudomonas aeruginosa 2.6%, Enterococcus faecalis 0.6%, Staphylococcus saprophyticus 0.3% and Acinetobacter spp. 0.3%.

Table 4: Presence of SHV, CTX-M and TEM genes in samples resistant/susceptible to the third generation Cephalosporins by PCR

ESBL genotypes	Frequency	Percentage
SHV	1	0.7
CTX_M	1	0.7

TEM	8	5.8
SHV & CTX_M	21	15.3
SHV & TEM	36	26.2
CTX_M & TEM	30	21.8
CTX_M & TEM & SHV	39	28.4

In table 4, the genotyping results of ESBL producing isolates obtained by PCR amplification of SHV, CTX-M and TEM genes. Of 137 ESBL positive isolates, 1 (7.6%) were carrying SHV, 1 (0.7%) CTX-M and 8 (5.8%) TEM genes, while 39 (28.4%) isolates included all three genes together. In addition, 36 (26.2%) isolates included SHV and TEM, 30 (21.8%) TEM and CTX-M, and 21 (15.3%) SHV and CTX-M genes together. Out of 137 isolates shown susceptible to the third generation Cephalosporins by combined disc test were also ESBL positive by genotypic method as they were carrying SHV and TEM genes.

Discussion

Urinary tract infection (UTI) continues to be the common clinical entity among the patients of the outpatient department and also represents one of the common nosocomial infections in our hospitals. However, the reported incidences and their epidemiology in India are not consistent enough to reveal the actual scenario regarding the etiological spectrum and antimicrobial susceptibilities [14]. In this laboratory based study, we examined the organisms causing urinary tract infections and their antibiograms along with the production of extended-spectrum beta-lactamase enzymes by phenotypic and genotypic approaches. To the best of our knowledge, this report represents the first genotypic characterization of ESBL producing uropathogens from the cases of urinary tract infections from Indore.

The ESBL producers usually carry multiple resistant plasmid. In our study 4 isolates of Escherichia coli bla TEM + bla CTX-M and bla SHV+ bla CTX-M. Similar results have been reported by Yazdi M et al., and Yuan et al., [15]. Most ESBLs are derived from plasmid mediated penicillinases belonging to the TEM and SHV families. Recently the CTX-M group with a typical ESBL resistant phenotype which does not originate from TEM and SHV families has been described. The CTX-M group is a new family of plasmid mediated ESBL they preferably hydrolyse cefotaxime have been recognized by Xiong et al., [16].

In our stuyd, the genotyping results of ESBL producing isolates obtained by PCR amplification of SHV, CTX-M and TEM genes. Of 137 ESBL positive isolates, 1 (7.6%) were carrying SHV, 1 (0.7%) CTX-M and 8 (5.8%) TEM genes, while 39

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Similar results have been obtained in many studies in India and other countries [17]. In a study conducted from 10 Indian sites in six Indian cities by Welsh et al., [18] bla CTX-M was the most common genotype isolated. Similar results have been documented in Europe, Latin America and other countries [19]. The CTX-M genotype, originating from chromosomally encoded enzymes of the Kluyvera spp, has risen in predominance especially in Escherichia coli and Klebsiella pneumoniae they have greater ability to spread and cause outbreaks [20].

In our previous report too, we found the dominance of the bla-CTX-M gene among ESBLproducing enterobacteriaceae from various clinical specimens [21]. Moreover, multiple occurrences of genes in a same organism were also noted, where bla-TEM + bla-CTXM (54.8%) was common. These genes are usually present on the large plasmids accompanied with the genetic determinants conferring resistance towards various antimicrobials [22]. In this study too, ESBL-producing isolates were more resistant to cephalosporins and fluoroquinolones. However, nitrofurantoin and aminoglycosides proved to be the optimal first-line drugs in the cases of UTI caused by ESBL E coli in our study. This may be due to the restricted use of these drugs in our hospital setting, and nitrofurantoin is usually reserved to be prescribed only in cases of UTIs since it is excreted and concentrated in urine [23]. Carbapenems can be reserved for severe cases of UTI where primary therapy is ineffective [24].

The presence of more than one gene type in some of the isolates like bla TEM+ bla CTX-M means that the ESBL producing strains may be related to complex antimicrobial resistance. bla TEM producing TEM-1 is a broad spectrum β-lactamase that is always combined with CTX-M in the same plasmid [25]. It is important to continuously monitor resistance trends and enhancing the infection control of these pathogens in health care units.

In contrast, the genotypic method using specific PCR amplification of resistance genes seems to have 100% specificity and sensitivity. The cost of molecular method is particularly reduced for the bacteria belonging to the enterobacteriaceae family as their DNA is easily extractable by boiling method, a quick and cost effective DNA extraction method. Our study also showed that all ESBL positive samples comprised either SHV or TEM genes.

Infections caused by ESBL-producing organisms are a global problem. Mobile genetic elements contained in the bacterial species are easily transferable to other organisms in the vicinity [26]. Timely detection of the resistant strains along with

their antimicrobial susceptibilities is very important for the effective management of UTI in the endemic regions. However, limited facilities of detection and poor understanding of such bugs in the developing counties are responsible for global dissemination of such pathogens [27].

Conclusion

The blaCTX-M and blaTEM genes predominated in this study, with the coexistence of multiple genes in single isolates indicating increased transmission of genetic determinants and the likely increase of ESBL pathogens in Indian hospitals. However, the AmpC genes typically associated with cefoxitin resistance were not observed in this study, a possible indication of other cefoxitin-resistant mechanisms that warrant further investigation. In the current study, three isolates that were phenotypically positive by DDST method lacked TEM, SHV, and/ or CTX-M genes. This may be explained by that these three isolates may carry other ESBLs encoding genes, which coud not be detected by the used primers, or it could be chromosomally mediated AmpC β-lactamases.

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