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Molecular Characterization of *Bla* CTX-M, *Bla* TEM, *Bla* SHV-Beta Lactamase Produced by Uropathogenic *Escherichia coli* Isolates

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Abstract: CTX-M type enzymes are rapidly spreading among *Enterobacteriaceae* and are now the most prevalent Extended Spectrum β-Lactamase in many parts of the world. This study was concerned with the molecular characterization of the *bla* CTX-M, *bla* TEM and *bla* SHV- β-Lactamase produced by Uropathogenic *Escherichia coli* (UPEC) isolates. A collection of 324 UPEC isolates from urinary tract infected patients attending the Raja Muthiah Medical College and Hospital (RMMCH), Annamalai University at Chidambaram, India was investigated. The antimicrobial susceptibility assay by disc diffusion technique according to Clinical and Laboratory Standards Institute (CLSI) guidelines was investigated using these isolates obtained during 2012-2013. Selected 20 UPEC isolates were screened for *bla* CTX-M subgroups gene like CTX-M-14, 15, 24, 27, *bla* SHV subgroups gene like SHV-11, 12 and *bla* TEM-1 by using uniplex PCR. Out of 14 different antibiotic discs tested, the rates of antibiotic resistance were calculated as 55, 49, 48, 47, 47, 46, 45, 44, 39, 38, 33 and 32% for amikacin, nalidixic acid, ampicillin, co-trimoxazole, tetracycline, gentamicin, tobramycin, chloramphenicol, norfloxacin, nitrofurantoin, piperacillin/tazobactam and meropenem, respectively. UPEC isolates in Double Disk Approximation (Synergy) test (DDAT) showed 62% were positive and CLSI combination disk method 38% was positive to ESBL production. Among 20 UPEC strains CTX-M-14 gene was detected in 85%, CTX-M-15 gene was detected in 100%, CTX-M-24 gene was detected in 90% and CTX-M-27 gene was detected in 55%, TEM-1 gene was detected in 100% strains and SHV-11 gene was detected in 100% & SHV-12 gene was detected in 85% strains. This study found that acute uncomplicated UTI affects a large proportion of the population. The study confirmed *Escherichia coli* to be a major uropathogen. There is a high prevalence of ESBL production in the UPEC isolates studied.

Key words: Extended Spectrum Beta-Lactamase · Urinary Tract Infections · *E. coli* · CTX-M · SHV · TEM Genes

INTRODUCTION

Escherichia coli (*E.coli*) is the most predominant pathogen causing 80-90% of community-acquired urinary tract infections (UTIs) and 30-50% of nosocomial-acquired UTIs. Recurrent UTIs (RUTIs) are reported in 25% of women within 6 months of an acute UTI episode and pose a major problem [1].

E. coli is the predominant facultative anaerobe of the human colonic flora. The organism typically colonizes the infant gastrointestinal tract within hours of life and, thereafter, E. coli and the host derive mutual benefit [2]. The ever-increasing bacterial resistance to antibiotics is one of the most challenging tasks of all the current medical issues. A single mutation in bacteria, which leads to a new resistance mechanism against various drugs are like undoing within moments, great efforts in developing these drugs [3].

The Extended Spectrum Beta Lactamases (ESBLs) are enzymes which are produced by Gram negative bacilli that mediate a resistance to the penicillins, cephalosporins and the monobactams and are commonly recognized in

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Enterobacteriaceae. Gram negative bacteria are the major pathogens in hospitalized patients with weakened host defenses, due to malignancy, malnutrition, debilitation, trauma, a prolonged antibiotic use and surgery. The most frequently occurring bacteria are *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter and Acinetobacter baumanii*. Once these organisms are established as the source of the infection, they are aggressively treated with antimicrobial drugs [4].

Molecular characterization of uropathogenic *E. coli* (UPEC) has become an important objective in the epidemiological investigation of infectious agents. CTX-M- producing *Escherichia* are becoming increasingly involved in urinary tract infections, seemingly imported from the community into the hospital setting [5]. The first *bla* CTX-M was detected in a clinical *E. coli* isolate in Germany in 1990 [6] and then the CTX-M-producing *Enterobacteriaceae* has globally been detected. CTX-M is named after their higher hydrolytic activity against cefotaxime than ceftazidime and the place of first isolation. *Bla* CTX-M is 291 amino acids encoding enzyme and the change in any one of them results in a new CTX-M variant [7]. The first plasmid mediated beta lactamase in gram negative bacteria, TEM-1, was described in the early 1960s. Being plasmid and transposon mediated, it quickly spread to various species of bacteria. SHV-1 was the next plasmid mediated beta–lactamase discovered. Most of the ESBLs are mutants of the TEM and the SHV enzymes [8, 9]. In the view of this, the present work aimed at the molecular characterization of uropathogenic strains of *E. coli* using the PCR technique.

MATERIALS AND METHODS

Bacterial Isolates: A set of 478 urine samples was collected from patients attending the Raja Muthiah Medical College and Hospital (RMMCH) at Chidambaram during November 2012 to August 2013. The test organism was confirmed as *E.coli* as per standard isolation and identification procedures [10] in 324 samples.

Antibiotic Susceptibility Assay: The above isolates were tested for antimicrobial susceptibility assay by the disc diffusion technique according to Clinical and Laboratory Standards Institute (CLSI) guidelines [11], with commercially available discs (Hi-Media, Mumbai). The following antibiotic discs (Drug concentration in µg) were used: amikacin (30), ampicillin (10), co-trimoxazole (25), chloramphenicol (30), tetracycline (30), tobramycin (10), gentamicin (10), imipenem (10), norfloxacin (10), piperacillin/ tazobactam (100/10), meropenem (10), nitrofurantoin (300), nalidixic acid (30) and levofloxacin (5). *E.coli* MTCC 443 was used as the reference strain.

Detection of ESBL: All the 324 *E.coli* isolates were subjected to ESBL production identified by a number of tests including Double-disk approximation test [12]. The combination-disk test using both cefotaxime and ceftazidime, alone and in combination with clavulanic acid, was performed for the detection of ESBL according to the CLSI guidelines [13]. *E. coli* MTCC 729 was used for the quality control of the ESBL detection methods.

Preparation of Genomic DNA Extraction: Genomic DNA was isolated from bacterial cells using DNA purification kit (Pure Fast® Bacterial Genomic DNA purification kit, HELINI Biomolecules, Chennai) and stored at 20°C. The samples were run on agarose gel and stained with ethidium bromide. The stained gel was examined for the presence of bands under UV-light using molecular weight marker (HELINI Biomolecules, Chennai).

Detection of β-lactamase *bla* $_{CTX-M}$, *bla* $_{TEM}$ and *bla* $_{SHV}$ genes by PCR Technique: Among 122(38%) ESBL producing uropathogenic *E. coli*, 20 strains were taken for molecular detection of *bla* CTX-M, *bla* TEM and *bla* SHV which was performed by using uniplex PCR.CTX-M subgenes 14,15, 24 and 27 were detected by this method. The primers used for detection of CTX-M, *bla* TEM and *bla* SHV genes were obtained from Helini Biomolecules, Chennai. For PCR amplifications, about 1 ml of DNA was added to 25µl mixture containing 1µl of 10mM dNTPs mixer, 1µl of each primer and 2U of Taq DNA polymerase (Helini Biomolecules, Chennai) in 10X PCR buffer. Amplification was performed in a thermocycler (Corbett Research, Australia) with cycling parameters comprising initial denaturation at 94ºC for 3 min followed by 35 cycles each of denaturation at 94ºC for 30 sec, annealing at 56°C for 30 sec, amplification at 72°C for 30 sec and final extension at 72°C for 5 min, for the amplification of *bla* CTX-M, *bla* TEM and *bla* SHV.

The amplified products were separated using 2% agarose. 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 (Helini Biomolecules, Chennai) was used to measure the molecular weight of amplified products. The images of ethidium bromide stained DNA bands were digitized using a gel documentation system (Helini Biomolecules, Chennai).

Table 1: Antibiotic susceptibility pattern of isolated uropathogenic *E. coli* (n=324)

S. No	Antibiotics	R (%)	I (%)	S (%)
1	Amikacin (30µg)	55	26	19
2	Ampicillin(10µg)	48	32	20
3	Chloramphenicol(30µg)	44	27	30
4	Co – Trimoxazole(25µg)	47	32	22
5	Gentamicin(10µg)	46	33	21
6	Imipenem(10µg)	07	42	51
7	Levofloxacin(5µg)	12	32	56
8	Meropenam(10µg)	32	41	27
9	Nalidixic acid(30µg)	49	31	20
10	Nitrofurantoin(300µg)	38	32	30
11	Norfloxacin(10µg)	39	33	29
12	Piperacillin/Tazobactam (100/10µg)	33	39	28
13	Tetracycline(30µg)	47	26	27
14	Tobramycin(10µg)	45	35	21

(R- Resistant; I- Intermediate resistance; S- Sensitive)

Table 2: Primers sequences and PCR cycles performed for ESBL genes

bla genes	Primer sequences	PCR cycles
CTX-M-14	5'-TTATGCGCAGACGAGTGCGGTG-3'	1 cycle of 10 minutes (min) at 94°C; 30 cycles of [1min at4, 1min at 48°C, 1min at 72°C]; 1 cycle of 7min at 72°C.
	5'-TCACCGCGATAAAGCACCTGCG-3'	
CTX-M-15	5'-GAGCCGACGTTAAACACCGCCA-3	
	5'-GCTGCACCGGTGGTATTGCCTT-3'	
CTX-M-24	5'-TTATGCGCAGACGAGTGCGGTG-3'	
	5'-GCGTCATTGTGCCGTTGACGTG-3'	
CTX-M-27	5'-TTATGCGCAGACGAGTGCGGTG-3'	
	5'-GCCACCGAGCTGGGCAATCAAT-3'	
TEM-M-01	5'- CCAAACGACGAGCGTGACACCA-3'	1 cycle of 5 minutes (min) at 96°C; 35 cycles of [1min at 96°C, 1min at 58°C, 1min at 72°C]; 1 cycle of 10min at 72°C.
	5'-AGCGCAGAAGTGGTCCTGCAAC-3'	
SHV-11	5'-CGCCGCCATTACCATGAGCGAT-3'	1 cycle of 5 minutes (min) at 96°C; 35 cycles of [1min at 96°C, 1min at 60°C, 1min at 72°C]; 1 cycle of 10min at 72°C.
	5'-CCGGGAAGCGCCTCATTCAGTT-3'	
SHV-12	5'-CGCCGCCATTACCATGAGCGAT-3'	
	5'-ACCCGATCGTCCACCATCCACT-3'	

RESULTS AND DISCUSSION

Out of the 478 urine samples 406 (86.93%) sample showed positive and the rest 72 (15.06%) showed negative for microorganisms. Among the isolates, 324 (79.80%) were found to be *E.coli* and the remaining 82 (20.19%) were harboring other microorganisms. The *E. coli* isolates were resistant to the antibiotics such as amikacin, nalidixic acid, ampicillin, cotrimoxazole, tetracycline, gentamicin, tobramycin, chloramphenicol, norfloxacin, nitrofurantoin, piperacillin/tazobactam and meropenem which were found to be in the order of 55, 49, 48, 47, 47, 46, 45, 44, 39, 38, 33 and 32%, respectively. The intermediate resistance of imipenem was found to be 42%. The highest sensitivity was observed in the levofloxacin 56%. The results are shown in Table 1. From the 79.80% of *E.coli* isolates, 38% were confirmed of ESBL producer by double disk approximation test.

Molecular Characterization of *bla* $_{CTX-M}$, *bla* $_{TEM}$ and *bla* $_{SHV}$ Genes: The molecular characterization of the ESBL producing *E. coli* was determined in this investigation. Among 122 (38%) ESBL producing uropathogenic *E. coli*, only 20 strains were selected from the highly ESBL production with biofilm formation for molecular detection of *bla* CTX-M, *bla* TEM and *bla* SHV which was performed by using uniplex polymerase chain reaction (PCR). The *bla* genes, primer sequences are given in Table 2. Further it is determined that the presence of CTX-M was found in all strains of UPEC included in this study. This data highlighted that all the UPEC strains may contain the CTX-M gene. The TEM was found in 11 strains (55%) and SHV genes were found among 12 strains (60%). The images of ethidium bromide stained DNA bands were digitized using a gel documentation system.

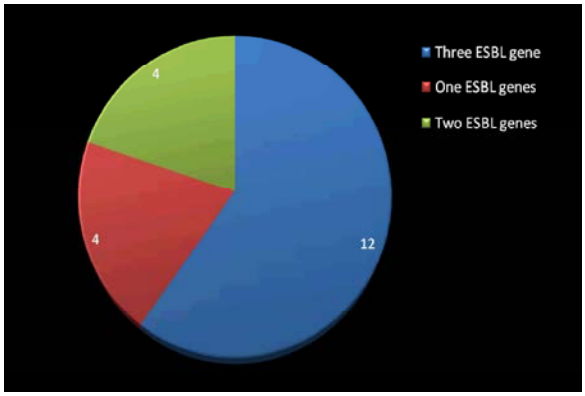

Fig. 1: Occurrence of ESBL *bla* genes by PCR method

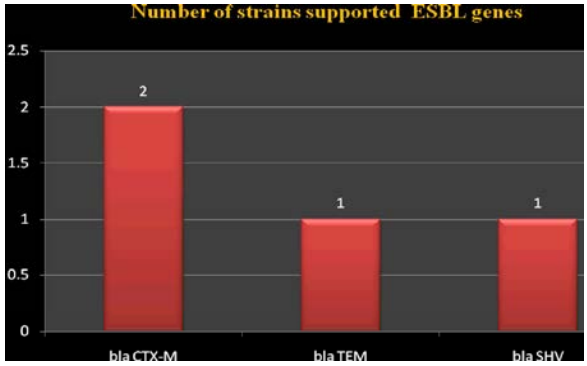

Fig. 2: Occurrence of single ESBL *bla* genes by PCR method

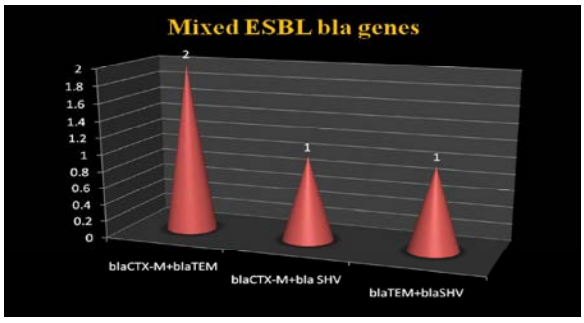

Fig. 3: Occurrence of mixed ESBL *bla* genes by PCR method

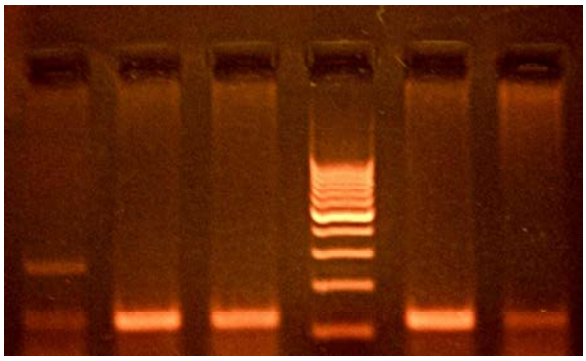

Fig. 4: PCR for *bla* CTX-M - 14 gene

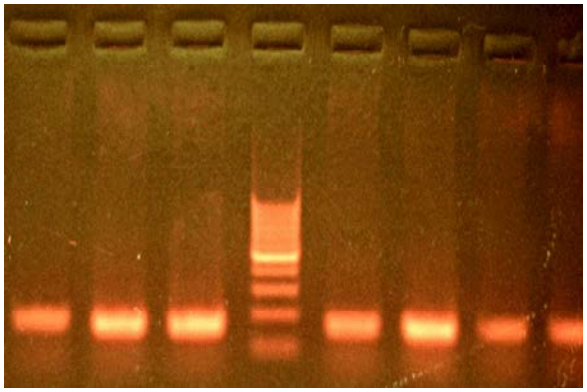

Fig. 5: PCR for *bla* CTX-M - 15 gene

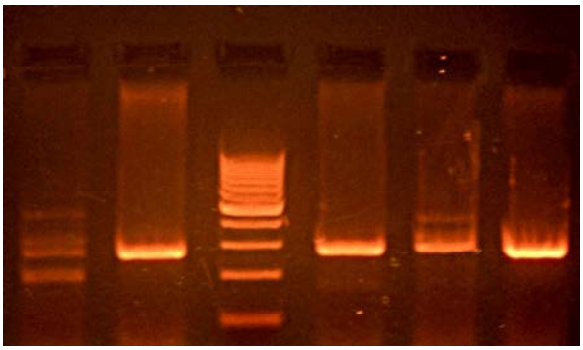

Fig. 6: PCR for *bla* CTX-M - 24 gene

Out of 20 *E. coli* strains included, presence of all the three *bla* genes (bla_{CTX-M}, bla_{TEM} and bla_{SHV}) was noticed in 12 (60%) strains (Fig. 1). Occurrence of single *bla* gene namely bla_{CTX-M}, bla_{TEM} and bla_{SHV} was noticed in 4 (20%) strains (Fig. 2). Combination of mixed *bla* genes bla_{CTX-M}+ bla_{TEM}, bla_{CTX-M}+ bla_{SHV} and bla_{TEM}+ bla_{SHV} were detected in 4 (20%) strains (Fig. 3).

PCR based replicon typing revealed that, the majority of strains of CTX-M-producing *E. coli* were identified. The replicon types of the plasmids of two strains failed to be detected by this method. The following plates exhibited agarose gel electrophoresis of PCR amplified products showing the presence of CTX-M (550bp), SHV (842bp) and TEM (918bp) genes in test and control strains.

Among 20 UPEC strains CTX-M-14 gene was detected in 85% strains (Fig.4). Among 20 UPEC strains CTX-M-15 gene was detected in 100% strains (Fig. 5). Among 20 UPEC strains CTX-M-24 gene was detected in 90% strains (Fig.6). Among 20 UPEC strains CTX-M-27 gene was detected in 55% strains (Fig.7). Among 20 UPEC strains TEM-1 gene was detected in 100% strains (Fig. 8). Among 20 UPEC strains SHV-11 gene was detected in 100% strains (Fig. 9). Among 20 UPEC strains SHV-12 gene was detected in 85% strains (Fig. 10).

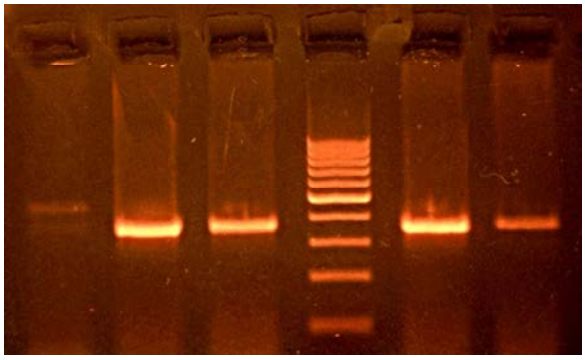

Fig. 7: PCR for *bla* CTX-M - 27 gene

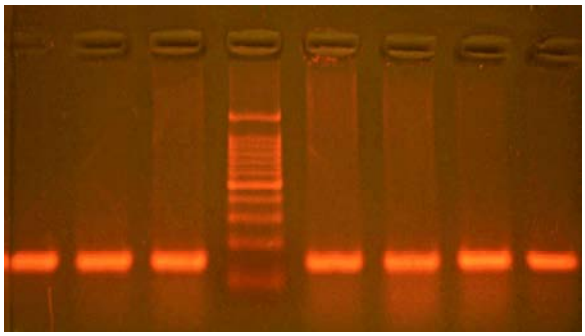

Fig. 8: PCR for *bla* TEM-1gene

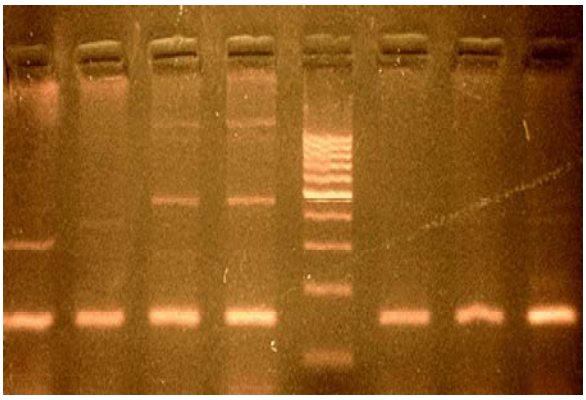

Fig. 9: PCR for *bla* SHV-11 gene

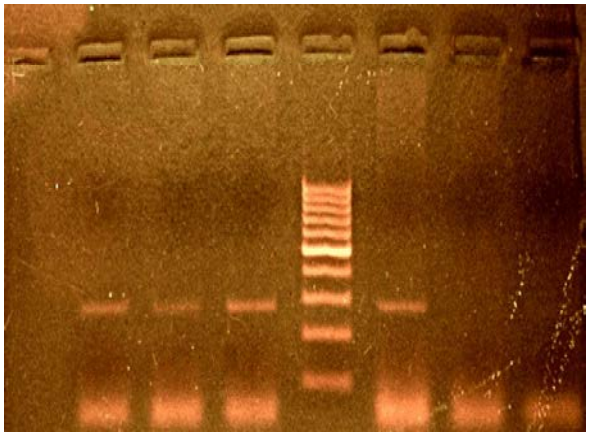

Fig. 10: PCR for *bla* SHV-12 gene

This study was undertaken to evaluate the bacterial strains isolated from patients diagnosed with Urinary tract infections. Urine samples were collected from 478 patients (Both male and female) for analysis. 324 (79.80%) samples showed positive to UTIs by *Escherichia coli*. The present study showed that *E. coli* is the predominant microorganism among the uropathogens. Various organisms have been reported to be isolated from patients with UTI, among which *E. coli* was reported as the most common organisms causing UTI [5, 14, 15]. Similarly, Murugan *et al*. [16] and Gaurav [17] have reported 71 and 72.2% of isolates, respectively to be as *E. coli* among the uropathogenic organisms.

Suman *et al*. [18] have reported that *E.coli* strains were sensitive to ampicillin (20%) followed by tetracycline (18%) and amikacin (14%). In the present study also UPEC strains are found to be sensitive to ampicillin (20%) followed by tetracycline (27%) and amikacin (19%).

Murugan *et al*. [16] have reported that *E. coli* strains were resistant to tobramycin (53.01%) and gentamycin (50%). The present study confirmed their results, in which UPEC strains were resistant to tobramycin (45%) and gentamycin (46%). The present study found that UPEC strains are resistant to nalidixic acid (49%) and chloramphenicol (44%).

Karisik *et al*. [19] have designed the PCR-based phylogenetic analysis of ESBL producing gene CTX-M of *E. coli* isolates, derived from human commensal phylogenetic type. Similarly, Zarfel *et al*. [20] have reported that 95% of all ESBL producing *E.coli* strains carried genes of the family $bla_{\text{CTX-M}}$. Yasufumi *et al*. [21] have reported that 88% of ESBL producing *E.coli* isolates carried genes $bla_{\text{CTX-M}}$ subgroups. Similarly, the present study found that ESBL producing genes *bla* CTX-M subgroups *bla* CTX-M-14, *bla* CTX-M-15, *bla* CTX-M-24 and *bla* CTX-M-27 are present in all the UPEC strains (100%).

In the present study, 55% of ESBL producing *E. coli* strains were having bla_{TEM} genes. Ahmed *et al*. [22] have reported that 61.1% of ESBL producing *E. coli* were with $bla_{\text{TEM,}}$ Al-Agamy *et al*. [23] and Shahzad *et al*. [24] reported 60.8% and 59 respectively. In the present study, 60% of ESBL producing *E.coli* strains were having bla_{SHV} (60%). Ahmed *et al*. [22] and Bashar *et al*. [25] reported 55.6 and 74% respectively.

In the conclusion, the study confirmed *E. coli* to be a major uropathogen. Further, the bioactive compounds may be determined to understand the mechanism of action of the compounds against all UPEC strains.

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