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Molecular Characterization of CTX-M β-Lactamase Producing Enterobacteriaceae from Urine Samples in Salem

***Priya R, Sureshkumar BT, Saranya.S and Jasmine M**

Department of Microbiology, Vivekananda College of Arts and Sciences for Women,(Autonomous), Elayampalayam - 637 205, Tiruchengode, Namakkal, Tamilnadu, India

Abstract

The emergence of Extended-spectrum beta lactamase (ESBL) producing bacteria, particularly *Escherichia coli* and *Klebsiella pneumoniae*, is now a critical concern for the development of therapies against bacterial infection. Urinary Tract Infection (UTI) remains the most common bacterial infection in the human population. This observational study was conducted at the SKS Hospital, Salem, from December 2013 to February 2014. Totally 70 urine specimens were used for the study and of which only 30 isolates were found out to be belonging Enterobacteriaceae group of *E. coli, K. Pneumoniae* and *P.aeruginosa*. These 30 isolates were subjected for the antibiotic sensitivity test using Kirby- Bauer method and 22 (73.3%) isolates were found to be ESBL, which were confirmed by using the Double Disc Synergy Test (DDST). ESBL production was detected in 16 (73%) *E. coli,* 2 (9%) *K. Pneumoniae* and 4 (18%) *P.aeruginosa.* ESBL producing organisms showed maximum resistance to Ampicillin, Cloxacillin, Cefuroxime, Lomefloxacin, and Ciprofloxacin while minimum resistance was seen with Imipenem.Proper antibiotic policy and appropriate guidelines to prescribe antibiotics are the routes to prevent dissemination of Multidrug Resistant Organisms. The goal of this study was to detect the ESBL producing Enterobacteriaceae from urine and detect the drug resistance CTX-M gene by PCR method.

Keywords Enterobacteriaceae ; Uropathogens; ESBL; CTX-M, PCR.

INTRODUCTION

Urinary Tract Infections (UTI) are one of the most prevalent extra intestinal infections in the society. Different types of UTIs are a serious health problem that affects millions of people each year. Urinary tract infection includes the infection of urethra, bladder, ureters and kidneys, which comprise the urinary tract [1]. UTI is an important cause of morbidity and mortality in both developing and developed countries of the world, affecting all age groups and both the sexes [2].ESBL organisms produce enzymes that hydrolyze the beta-lactam ring of beta lactam antibiotics like Penicillins and Cephalosporins, rendering them ineffective. Resistant bacteria are emerging the world over as a serious threat in community settings [3]. β-lactamase production is the most common mechanism of resistance in gram negative bacteria. They are of significant concern because they restrict therapeutic options, cause treatment failures and are increasing in occurrence worldwide [4]. The successful use of third-generation Cephalosporins in medicine was considered as a landmark in antimicrobial chemotherapy. Unfortunately, the increased use of these Cephalosporins has led to the emergence of resistance in enterobacterial species, possessing extended spectrum β-lactamases [5].microorganisms producing extended-spectrum beta-lactamases (ESBL) were identified in the early 1980s. The spread of (ESBL) in Enterobacteriaceae has become an over-increasing problem. Gene transfer between bacteria has been shown to play an emerging role in the acquisition of drug resistance and the horizontal genes is now known to be the main cause of resistance transmission [6]. Enterobacteriaceae are the major recipients of extended-spectrum beta-lactamases (ESBLs). Beta lactamase producers are typically gram negative organisms, namely *Escherichia coli, Klebsiella pneumoniae*, and *Klebsiella oxytoca*. However, ESBL production has been observed in *Proteus, Pseudomonas, Serratia*, *Enterobacter, Salmonella, Acinetobacter* and *Citrobacter* species [7]. The most prevalent ESBLs are included in three groups: TEM, SHV and CTX-M types. ESBL were usually plasmid-mediated (or) chromosomal origin β-lactamase inhibitors were Clavulanic acid, Sulbactam and Tazobactam [8]. They are generally inhibiting ESBL producing strains [9]. A new serious of ESBL enzymes, Cefotaximases (CTX-M), resulting in higher MICs of Cefotaxime and Ceftriaxone than of Ceftazidime, has been discovered in several members of the Enterobacteriaceae family and in various countries [10]. The first CTX-M-type β-lactamases were identified as plasmid-encoded enzymes in clinical isolates of Enterobacteriaceae [11]. In contrast with TEM and SHV ESBLs, most of the CTX-M enzymes preferentially hydrolyze and confer resistance to Cefotaxime and Ceftriaxone rather than Ceftazidime [12]. CTX-M types ESBLs show only 40% identify to TEM or SHV ESBLs, but they are closely related to β-lactamases of Kluyvera species [13]. Recent studies on ESBL production in members of Enterobacteriaceae isolated from clinical specimens showed 9-50 percent ESBL producers [14]. Beta-lactams are the first choice for treatment of infections caused by Enterobacteriaceae and might be destroyed by extended spectrum beta-lactamases. ESBLs hydrolyze all beta-Lactam except Cephamycins and Carbapenems, and constitute a large heterogenous group of enzymes with different origins. There are many types of ESBLs among which CTX M is a latest emerged one which carries the bla $_{CTX-M}$ gene. CTX-M

Address Correspondence at Department of Microbiology, Vivekananda College of Arts and Sciences for Women,(Autonomous), Elayampalayam - 637 205, Tiruchengode, Namakkal, Tamilnadu, India. Ph:+91-9443316504 Email: btsmicro@gmail.com

lactamase producing Enterobacteriaceae are present in the intestinal flora without harming the host causing infection in extra intestinal sites. The aim of this study was to determine the prevalence and identification of ESBL producing Enterobacteriaceae from urinary tract infections and to characterize the β-lactamase CTX-M gene in multidrug-resistant clinical isolates by molecular techniques.

MATERIALS AND METHODS

Sample Collection

A total of 70 consecutive, non-repetitive, *Enterobacteriaceae* isolates were isolated from urine samples received from SKS Hospital in Salem, Tamilnadu, for a period of three months (December 2013 to February 2014).

Isolation and Identification

All the collected samples were inoculated on Nutrient agar, MacConkey agar, CLED agar and incubated at 37°C for 24 hr. A single isolated colony was considered for further studies and identification was done based on standard bacteriological culture and biochemical characteristics of the isolates. [15].

Antibiotic Sensitivity Test

The resistance to one or more 3rd generation cephalosporin's (Ceftazidime, Ceftriaxone, Cephotaxime, etc.,) prompted use to detect ESBL producers, the common mechanism of Beta-Lactam resistance. All the isolates were subjected to antimicrobial susceptibility agents and were determined by Disc-Diffusion method on Muller–Hinton Agar as described by Clinical and Laboratory Standard Institute (CLSI).The antibiotic discs used (HI-Media) were Cefoxitin (CX) (30mcg), Cefepime (CPM) (30mcg), Ceftazidime (CAZ) (30mcg) ,Ceftriaxone (CTR), (30mcg), Cephotaxime or Cefotaxime (CE) (30mcg), Imipenam (IMP) (10mcg), Aztreonam (AT) (30mcg) [16].

Detection of ESBL by Phenotypic Method

Double Disc Synergy Test (DDST)

In the test, synergy was determined between a disc of Augmentin (20 µg Amoxycillin and 10 µg clavulanic acid) and a 30 mcg disc of third generation Cephalosporin test antibiotic placed at a distance of 30 mm apart on a lawn culture of the resistant isolate under test on Muller-Hinton Agar (MHA, Hi-Media) . The test organism was considered to produce ESBL, if the zone size around the test antibiotic disc increased towards the augmentin disc. This increase occurs because the clavulanic acid present in the augmentin disc inactivates the ESBL produced by the test organism [17].

Detection of CTX-M

Aztreonam, Ceftazidime, Cefotaxime, and Cefotaxime-Clavulanic acid, Ceftriaxone are used to detect CTX-M type ESBL.CTX-M strains are resistant to Cefotaxime and sensitive to Cefotaxime plus Clavulanic acid. The antibiotics were placed on Muller Hinton Agar (MHA) plate. After incubating overnight at 37°C, more than 5-mm increase in the zone diameter for combination with Clavulanic acid, was interpreted as positive for CTX-M production.

Molecular detection and identification of ESBL *bla*CTX-M gene by Polymerase Chain Reaction (PCR) amplification

The positive samples were used for Genotype identification. The Genotype identification was carried with following steps;

isolation of Genomic DNA by standard method [18], isolated DNA was amplified by PCR technique [19]. Based on the PCR amplification was predicted that the genes are belongs to CTX-M. To establish the molecular nature of ESBLs involved, PCR was performed for CTX-M-specific genes using specific primers. CTX-M genes were amplified with the following primers. The primers were F: SCS ATG TGC AGY ACC AGT AA and R: CCG CRA TAT GRT TGG TGG TG, where Y S R represents the standard nucleotide combinations. Y is C or T, S is C or G, R is A or G, the ambiguity designed to accommodate sequence variation in the largest number of known CTX-M type enzymes. Polymerase chain reaction technique has been used to amplify genes encoding the CTX-M β-lactamases from genomic DNA of greatest resistant pattern of *E.coli* strain (S-1). Isolates with specific forward and reverse primers (Table.1).DNA amplification was performed in an Eppendorf thermal cycler (Roche Co., Germany) in a final volume of 25µL containing 2.5µL of 10X PCR buffer, 2.5µM of dNTP mix, 10µM of each primer, 0.2 U of Taq DNA polymerase and 0.5µL of the template DNA.PCR conditions for the CTX-M gene comprised an initial denaturation step for 5 minutes at 94ºC, followed by 34 cycles of 94ºC for 45 seconds, 55ºC for 45 seconds, and 72ºC for 1 minute, with extension at 72ºC for 5 minutes. The primers used in the above mentioned reactions can amplify the DNA fragments correspond to 550 base pair (bp) of CTX-M gene. The PCR products were loaded on a 1% (W**/**V) Agarose gel with 0.5µg**/**ml ethidium bromide and were analyzed by gel electrophoresis method.

RESULT AND DISCUSSION

In the present study, out of 70 Urine samples, 30 (42.85%) isolates were found to be Enterobacteriaceae. Out of 30 isolates, 22(73%) isolates of *Escherchia coli* were found to be the most common organisms followed by *Klebsiella pneumonia* 3(10%), *Pseudomonas aeruginosa* 5 (17%) (Table 1,2). All isolates were showed the growth on their respective agar such as EMB, CLED agar, Nutrient agar (Fig.1, 2, 3). Among that 22 isolates were found to be ESBL and CTX-M producers. High prevalence rate of beta- lactamase producing strains have been studied in *E.coli* 15(68.2%), *Pseudomonas aeruginosa* 5(22.7%), *Klebsiella pneumoniae* 2(9.1%) (Fig.4). ESBLs are now problem in hospitalized patients throughout the world. The prevalence of ESBLs among clinical isolates varies greatly worldwide and in geographic areas and are rapidly changing over time [20]. ESBLs are normally encoded by plasmids, which also carry resistant genes for other antibiotics. The ESBL producing Enterobacteriaceae are present in the common intestinal flora without harming the host. Since the source of UTI is mostly the strains from intestine, the urinary tract is the most affected site by the ESBL producing bacteria [21].

The prevalence of Enterobacteriaceae over the age distribution were studied and presented in Table-4. The study subjects were classified into 9 age groups of the 70 urine samples. More number of organisms was found in age group between (0-9) 7 followed by 50-59, 2029, 10-19, 70-79 i.e, (6) and (5) respectively. The age group more than 60-69 shows a growth of 4 and the least growth was shown by the age group 80-89.Infection caused by ESBL-producing organisms may occur in children of all ages. This study revealed that the outbreak of ESBL-producing *K.pneumoniae* isolates in transplant children and found that 80 percent of these children were less than 5 years old. In this study,

Table.1: Sequence of the primers used to detect the ESBL genes

Gene	Primer sequence(5'→3')	PCR condition	Products (bp)
*bla*CTX-M	F: SCSATGTGCAGYACCAGTAA R:CCGCRATATGRTTGGTGGTC	94°C 5 min (94°C 45 s→55°C 45s→72°C 1min) 34 cycles→72° 5min	550

Table.2: Incidence of Enterobacteriaceae isolates from Urine samples

S.No	Type of samples	No. of Clinical samples	No. of isolates	% Incidence of clinical isolates
1	Urine	70	30	42.85%

Table.3: Production of β- Lactamase from clinical isolates of Enterobacteriaceae

S.No	Name of clinical isolates	No. of isolates	β-Lactamase production	% production of β-Lactamasse
1	*Escherichia coli*	22	15	68.2%
2	*Klebsiella pneumonia*	3	2	9.1%
3	*Pseudomonas aeruginosa*	5	5	22.7%
	Total	30	22	100

Table.4: Prevalence of Enterobacteriaceae over the Age Distribution

S.No	Age group	Enterobacteriaceae members			Total No. of organisms
		E.coli	*K.pneumoniae*	*P.aeruginosa*	
1.	0-9	4	2	1	7
2.	10-19	3	1	-	4
3.	20-29	4	-	1	5
4.	30-39	2	-	-	2
5.	40-49	-	-	-	-
6.	50-59	6	-	-	6
7.	60-69	3	-	1	4
8.	70-79	-	-	-	-
9.	80-89	-	-	2	2
Total		**22**	**3**	**5**	**30**

Table.5: Antibiotic Resistance pattern of ESBL producing Enterobacteriaceae

ESBL producers	No of resistant isolates (%)									
	CAZ	CXM	CPM	AT	IPM	CTR	COX	CIP	AMP	LOM
N=22	12 (54.5)	17 (77.3)	14 (63.6)	14 (63.3)	- S	13 (59)	19 (86.4)	16 (72.7)	19 (86.4)	16 (72.7)

CAZ- Ceftazidime, CXM- Cefuroxime, CPM- Cefepime, AT- Aztreonam , IPM- Imipenam , CTR-Ceftriaxone , COX-Cloxacillin , CIP-Ciprofloxacin , AMP- Ampicillin, LOM-Lomefloxacin. S- Sensitive.

Table.6: Phenotypic detection of CTX-M gene in ESBL isolates

Hospital	ESBL positive isolates tested for CTX-M gene	CTX-M gene Positive	CTX-M gene Negative
SKS	*Escherichia coli*	15(68.2%)	-
	Klebsiella pneumoniae	-	2(9.1%)
	Pseudomonas aeruginosa	-	5(22.7%)

almost 90 percent of ESBL-producing *E.coli* and *K.pneumoniae* isolates occurred in children of less than 9 years old, and 70-75 percent occurred in age group of 50-59.The result of this study indicate that younger children tend to acquire ESBL-producing *E.coli* and *K.pneumoniae* isolates more frequently than aged person. The reason for this may be due to the immunological status. The younger children are more vulnerable and get infection easily than older children [22].

Antibiotic resistance pattern of ESBL producing isolates were highly resistant to all tested antibiotics except imipenam (100% sensitive) (Table-5). The antibiotic resistant/sensitive pattern of ESBL isolates to other antibiotics is shown in (Figure 1). All the isolates were sensitive to imipenem.The majority of ESBL producers (.>80%) were resistant to cloxacillin, Ampicillin. Only 70% of ESBL were resistant to Cefuroxime, Ciprofloxacin, Lomefloxacin, and above 50% was found against Ceftazidime,Ceftriaxone. This revealed that previous work of the sensitivity pattern of isolates showed 100 per cent sensitivity to imipenem and 70 per cent to amikacin. Cefoperazone-sulbactam (80%) and piperacillin-tazobactam (63%) showed a good sensitivity *in vitro* but were not prescribed as the isolate was an AmpC producer. Ciprofloxacin gave a sensitivity of 37 per cent, followed by cefepime (32%), cefotaxime (13%), ceftazidime (11%) and amoxicillin-clavulanic acid (9%) [23], [24].

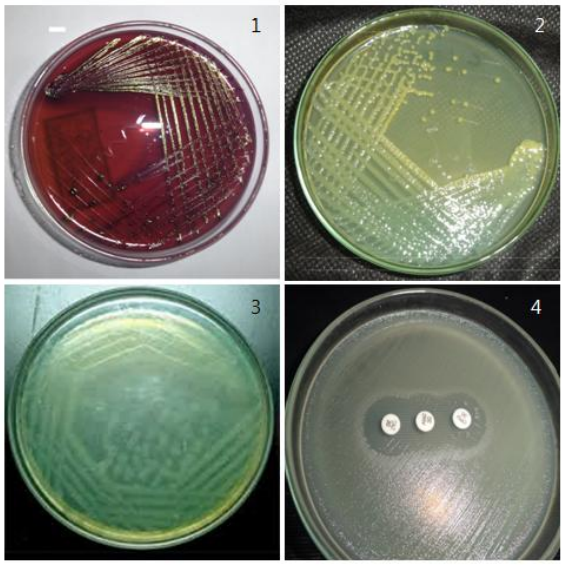


Figure: 1. *E.coli* colonies on EMB agar. **2.** *K.pneumoniae* colonies on C.L.E.D agar. **3.** *P.aeruginosa* on Nutrient agar. **4.** Double Disk Synergy Test(DDST)

In Phenotypic detection of CTX-M gene test, totally 22 isolates were subjected to detect the CTX-M gene. Among that, 15(68.2%) isolates of *E.coli* showed positive for CTX-M gene, and 7 (31.8%) isolates of both (*Klebsiella* and *Pseudomonas* sp.) showed negative (Table 6). Among the 22 ESBL producing clinical isolates, the gene CTX-M type of β-lactamase were identified by PCR. The amplified product was visualized at 550 bp by agarose gel electrophoresis (Figure 6). The results of PCR method showed that the prevalence of CTX-M genes among ESBLs-positive isolates of greatest resistant pattern of *Escherichia coli* (S-1).

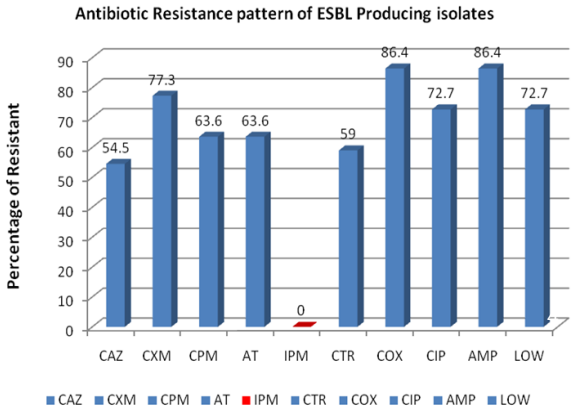
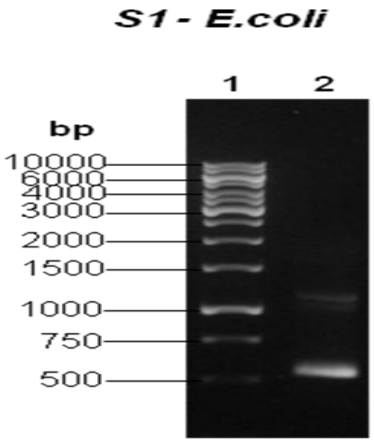


Figure.5: Resistant of the ESBL isolates to various antibiotics



Lane-1: 1Kb DNA ladder ; Lane-2: PCR product of S1 E.coli

Figure.6: Amplification of CTX-M gene by Gel Electrophoresis

Molecular characterization by PCR revealed that ^{bla} CTX-M gene was prevalent in 66.5% of these ESBL producers in Enterobacteriaceae. A report on ESBL types in *Enterobacteriaceae* in Argentinean public hospitals found that CTX-M accounted for roughly 70% of all ESBLs found, with similar findings being reported in studies conducted in Japan, China, United Kingdom and Spain [25-29]. The prevalence of ^{bla} CTX-M was also reported by Shahid *et al.*, with 72 (77.4%) of the 93 *E.coli* isolates being found to be CTX-M group -1 positive by PCR in the north Indian isolates [30]. In a study at Indian hospitals 73% (72% of total *K.pneumoniae* and 73% of total *E.coli*) 3rd generation cephalosporin-resistant isolates were found to carry CTX-M gene [31]. In contrast to the findings of above studies the prevalence of CTX-M type ESBL was less in the present study. In India, Sekar *et al.* reported that 44.4% of *E. coli* and 35.29% of *K.pneumoniae* strains were found to be positive for ^{bla} CTX-M gene by PCR[32].16 In this study 68.2% of *E.coli* were found to be positive for ^{bla} CTX-M gene by CTX-M detection method. Among that only one E.coli isolates were found to be positive for ^{bla}CTX-M gene by PCR [32].

CONCLUSION

During the past 20 years, the increase of antibiotic resistant bacteria especially ESBLs is becoming the major public health problem. Treatment with the 3rd generation cephalosporins often fails when the ESBLs producing bacteria are present. The prevalence of ESBL among clinical isolates varies greatly

worldwide and the patterns are changing overtime. The spread of ESBL positive strains in hospitals, there is a need to formulate a policy of empirical therapy in high risk unit were infection due to resistant organism is much higher. Equally important is the information on an isolate from a patient to avoid the misuse of extended spectrum Cephalosporins, which still remain as an important component of antimicrobial therapy in high risk wards. Imipenam and Carbapenem are the most active and reliable treatment options for infections which are caused by the ESBL producing isolates.

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