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Original Article

Microbiology Section

The Occurrence of CTX-M3 Type Extended Spectrum Beta Lactamases among *Escherichia Coli* Causing Urinary Tract Infections in a Tertiary Care Hospital in Puducherry

MALINI A. BHAT, SAGEERABANOO S., KOWSALYA R., GAUTAM SARKAR

ABSTRACT

Introduction: The Extended Spectrum Beta Lactamases (ESBLs) are the class A plasmid mediated enzymes that hydrolyze the oxyimino-cephalosporins and the monobactams but not the cephamycins and they are inhibited by clavulanic acid. In the recent years, the CTX-M type are the most common ESBLs which have been isolated. The present study was undertaken to know the prevalence of the ESBL producing *E. coli* in urinary tract infections (UTIs) and also to know the occurrence of the CTX-M3 genotype among these *E. coli* which were isolated in our hospital.

Materials and Methods: The routine antimicrobial susceptibility testing was done for the *E. coli* which were isolated from the urine samples. The strains of *E. coli* that were resistant to cefotaxime and ceftriaxone were selected for the ESBL testing. The strains were tested for ESBL production by the Double Disc Synergy Test (DDST) and the phenotypic confirmatory double disc test (PCDDT) as per the CLSI guidelines. Fifty four isolates of *E. coli* were found to be positive for ESBL by the phenotypic methods, out of which fifty isolates were tested for the presence of CTX-M3 by PCR and also their minimum inhibitory concentrations (MICs) for ceftazidime were determined.

Results: Out of a total of 157 *E. coli* isolates, 54 isolates (34.4%) were found to be positive for ESBL by the phenotypic methods, DDST and PCDDT. Nearly 21(38.8%) ESBL positive *E. coli* isolates had an MIC of ≥ 128µg/ml for ceftazidime. Out of the 50 strains of ESBL positive *E. coli* that were run for PCR, 42(84%) were positive for the CTX-M3 gene.

Conclusion: Our study showed that the ESBL producing urinary isolates of *E. coli* were not only resistant to the third generation cephalosporins but also to ciprofloxacin, cotrimoxazole and gentamicin. The occurrence of the CTX-M3 genotype was high among the *E. coli* isolates in our study.

Key Words: CTX-M3 genotype, ESBL, *Escherichia coli* , UTI

INTRODUCTION

The Extended Spectrum Beta Lactamases (ESBL) are the class A plasmid mediated enzymes that hydrolyze the oxyimino-cephalosporins and the monobactams but not the cephamycins and they are inhibited by clavulanic acid [1].

ESBLs are originally the mutants of the TEM and the SHV enzymes and they were first described in *Klebsiella pneumoniae* in Europe [2,3]. Recently, the CTX-M type ESBLs are being reported worldwide commonly. The CTX-M type ESBLs predominantly hydrolyze cefotaxime and they are less active against ceftazidime [2,4]. The CTX-M type of ESBLs are a diverse group which consists of >70 types presently and they are grouped into five clusters based on their amino acid sequences [5]. CTX-M14, CTX-M3 and CTX-M2 are the most widespread types which have been seen [6]. The presence of the CTX-M gene is also associated with the resistance to the fluoroquinolones, the aminoglycosides and cotrimoxazole [5]. In the recent years, the CTX-M type are the most common ESBLs which are being isolated and *Escherichia coli* is the most common ESBL carrying organism [5].

The present study was undertaken to know the prevalence of the ESBL producing *E. coli* in urinary tract infections (UTIs), to know their resistance pattern and also to know the occurrence of the CTX-M3 genotype among these *E. coli* which were isolated in our hospital.

MATERIALS AND METHODS

This study was done on all the isolates of *E. coli* which were obtained from the inpatients and the outpatients with UTI, during February 2009 to March 2010.The urine samples were inoculated on Mac Conkey's agar and on 5% sheep blood agar. The urine culture was done by the standard loop method, which is a semi-quantitative method. The organisms were identified by using standard methods [7].

The antibiotic susceptibility testing was done on Mueller Hinton agar by the Kirby-Bauer disc diffusion test and the results were interpreted as per the Clinical Laboratory Standards Institute (CLSI) guidelines[8] for the following antibiotics, ampicillin 10µg, cefotaxime 30µg, ceftriaxone 30µg , gentamicin 10 µg, cotrimoxazole 1.25/23.75 µg, ciprofloxacin 5 µg, amikacin 30 µg, piperacillin-tazobactam 100/10 µg and imipenem 10µg. (Hi-media, Mumbai)

The strains of *E. coli* that were resistant to cefotaxime and ceftriaxone or those which showed an inhibition zone of ≤ 27mm for

cefotaxime and of ≤ 25mm for ceftriaxone were tested for ESBL production by the phenotypic methods. The MIC for ceftazidime was also determined for 50 strains of *E. coli* that were ESBL positive. The presence of the CTX-M3 genotype was also determined by PCR, among the 50 ESBL positive strains of *E. coli* for which the MIC was also determined.

Phenotypic Tests for the Detection of ESBL

The resistant strains of *E. coli* were tested for ESBL production by the Double Disc Synergy Test (DDST) and the Phenotypic Confirmatory Double Disc Test (PCDDT) as per the CLSI guidelines [8,9]. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as the positive and the negative controls respectively for the testing.

DDST

A Mueller Hinton agar plate was inoculated with the standard inoculum (0.5 McFarland's standard) of the isolate. A ceftazidime disc (30µg) and a cefpodoxime disc (30µg) were placed on the agar, 15mm away from the centre of the augmentin disc (amoxycillin 20µg and clavulanic acid 10µg).When the inhibition zone around the test disc increased towards the augumentin disc or when neither disc were inhibitory alone by the bacterial growth, but were inhibited where the two antibiotics diffused together, then the organisms were considered as ESBL producers [9,10] [Table/Fig-1]

PCDDT

The Mueller Hinton agar (MHA) was inoculated with the standard inoculum (0.5McFarland's standard) of the isolate. A ceftazidime 30µg disc was tested alone and also along with a combination of 10µg of clavulanic acid. An increase in the zone diameter of ≥ 5mm for the ceftazidime and the clavulanic acid combination was considered to be produced by an ESBL producer [9,11] [Table/Fig-2].

MIC for ceftazidime: The MIC for ceftazidime was determined by the agar dilution method for the 50 strains of *E. coli* that were ESBL positive. Mueller Hinton agar plates were prepared with ceftazidime which was incorporated in two fold dilutions in the concentration range from 0.5µg/ml to 256 µg/ml. An inoculum size of 10^5cfu/ml was used for the test isolate and 10µl of the suspension was spot inoculated on the plates [9].

The Detection of the CTX-M3 Gene by PCR

Fifty strains of *E. coli* that were positive for ESBL by the phenotypic methods were tested for the presence of CTX-M3 by PCR. The *E. coli* which was grown on Mac-Conkey's agar was inoculated onto 5ml of the Luria- Bertani broth (Himedia, Mumbai) and the broth was incubated for 20hrs at 37ºC. It was centrifuged at 12,000 rpm for 5 minutes.1.5ml of the sediment was transferred to sterile microcentrifuge tubes. Plasmid DNA was extracted by the alkaline lysis method as per the manufacturer's instructions (Medox Biotech India Pvt Ltd, Chennai) [12]. The plasmid DNA which was extracted was subjected to PCR. The forward primer for the CTX-M3 gene was 5^1AATCACTGCGCCAGTTCACGCT 3^1 and the reverse primer was 5^1 GAACGTTTCGTCTCCCAGCTGT 3^1 .The primers were supplied by Medox Biotech India Pvt Ltd, Chennai and the product size was 540-600bp [13,14]. A single reaction mixture of 25 µl consisted of 1 µl plasmid DNA, 10 pmol(1 µl) of each primer and 5 µl of the 5X master mix which consisted of 1U of Taq DNA Polymerase, 7.5mM $MgCl_2$ and 1mM of deoxynucleoside triphosphates (Medox, Chennai).The volume was made up by adding 17 µl of RNAase and DNAase free water. The reactions were run in a thermal cycler under the following conditions: initial denaturation at 94ºC for 5 minutes, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 1minute and 20seconds, extension at 72ºC for 1minute and 20seconds and the final extension at 72ºC for 5 minutes. The PCR products were subjected to electrophoresis in a 1.2% agarose gel with ethidium bromide in the concentration of 0.5 µg/ml. A molecular weight standard (100bp ladder) was also included on each gel. The gel was later observed with a UV transilluminator. A molecular band between 500-600bp was taken as suggestive for the presence of the bla_{ctxm3} gene [Table/Fig-3].

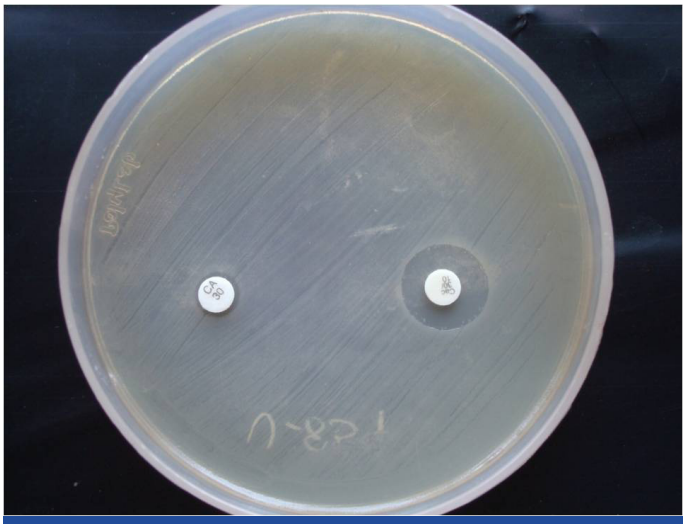
RESULTS

A total number of 982 urine samples were received during the study period, out of which 312 (31.7%) samples yielded significant bacteriuria. Out of 312 isolates, 220(70.5%) were gram negative organisms. The various gram negative organisms which were isolated from the urine were as shown in the [Table/Fig-4].

A total of 62 (28.2%) isolates were ESBL producing, which included *E. coli* (54 no) and *Klebsiella* species (8 nos).

Out of a total of 157 *E. coli* strains which were isolated from urine samples, 54 isolates (34.4%) were resistant to the third generation

[Table/Fig-1]: DDST showing an enhancement of inhibition zone of ceftazidime and cefpodoxime towards amoxycillin-clavulanic acid



[Table/Fig-2]: PCDDT showing an increase in zone diameter for ceftazidime-clavulanic acid

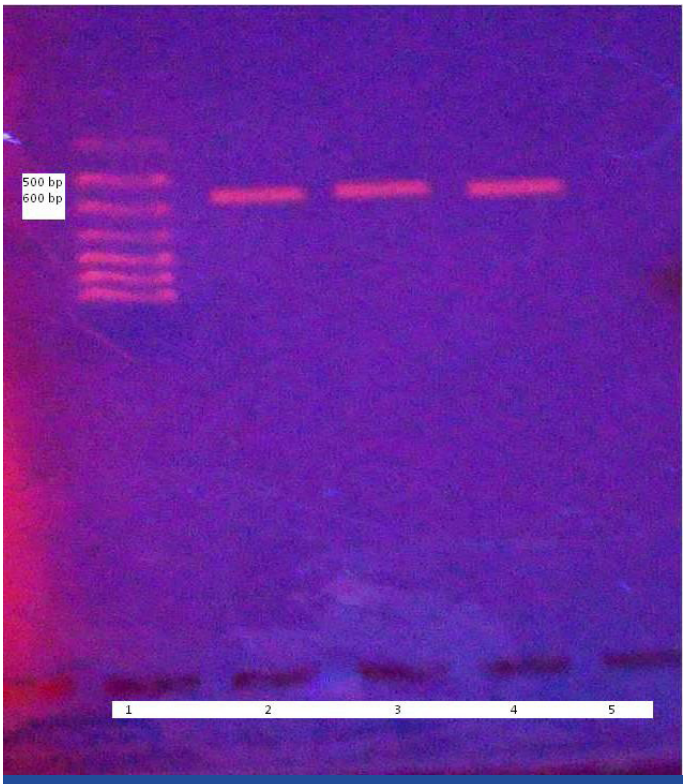
cephalosporins. These 54 isolates were found to be positive for ESBL by the phenotypic methods, DDST and PCDDT.

The ESBL producing strains of *E. coli* not only showed a high resistance to ceftriaxone (86.5%) and cefotaxime (84.2%), but they also showed resistance to gentamicin (65%), cotrimoxazole (74%) and ciprofloxacin (80.5%).They showed a good sensitivity to amikacin (81.8%), piperacillin-tazobactam (97.5%) and imipenem (100%).

Among the 54 ESBL producing strains of *E. coli* , a majority were resistant to both cefotaxime and ceftriaxone. Seven strains showed resistance to only cefotaxime and two strains showed resistance to only ceftriaxone.

The MIC value of the 50 strains of *E. coli* for ceftazidime were as shown in [Table/Fig-5]. A majority of the isolates had an MIC value of ≥ 128µg/ml.

Out of the 50 strains of ESBL positive *E. coli* that were run for PCR, 42(84%) were positive for the CTX-M3 gene.



[Table/Fig-3]: Agarose gel electrophoresis of PCR amplified products with *ctxM3* specific primers (540-600 bp). Lanes: 1 DNA Size Marker (100 bp ladder) ; 2-4, clinical isolates; 5, negative control (ATCC *E. coli* 25922)

Organism	No. of isolates (%)	No of ESBL producers
1. *Escherichia coli*	157 (71.36)	54
2. *Klebsiella species*	27 (12.27)	8
3. *Proteus species*	14 (6.36)	-
4. *Pseudomonas species*	08 (3.63)	-
5. *Citrobacter species*	06 (2.72)	-
6. *Acinetobacter species.*	05 (2.27)	-
7. *Enterobacter species*	03 (1.36)	-
Total no.	220	

[Table/Fig-4]: Gram negative bacilli isolated from urine and the ESBL producers among them

MIC (µg/ml)	No. of isolates
16	7
32	13
64	09
128	10
256	10
>256	01

[Table/Fig-5]: MIC of 50 isolates of *E. coli* for ceftazidime by agar dilution method.

DISCUSSION

The CTX-M type of ESBLs have become the most frequently isolated variants globally [15]. The CTX-M type shows a higher resistance against cefotaxime than ceftazidime. However, the variants of CTX-M, like CTX-M15,CTX-M16 and CTX-M19 hydrolyze ceftazidime also [16]. Currently, there are about 5 clusters of the CTX-M genotype: CTX-M1, CTX-M2, CTX-M8, CTX-M9 and CTX-M25 [6]. The CTX-M3 type belongs to the CTX-M9 cluster.

The prevalence of the ESBL producing organisms ranges from 6% to 88% in various hospitals in India [2]. In our study, it was 34% among the urinary isolates of *E. coli*. The ESBL producing *E. coli* in our study showed high resistance to ciprofloxacin, co-trimoxazole and gentamicin. A study from south India also reported a high resistance to cefotaxime, ceftazidime, co-trimoxazole (82.6%), gentamicin (91%) and ciprofloxacin (82.6%) [16]. Similar observations were made among the *E. coli* which were isolated from UTIs in a study from Cambodia [5]. The CTX-M carriage is associated with a resistance to fluoroquinolones, aminoglycosides and cotrimoxazole [5].

In our study, 42 strains (84%) of *E. coli* which were isolated from UTIs showed the presence of the CTX-M3 gene. There are few reports on the molecular types which prevail in India. CTX-M3 was reported from India in a study [15]. A study which was done in a tertiary care hospital in south India showed a high prevalence of the CTXM1 variant of ESBL [17].

Another study from north India showed an ESBL prevalence rate of 63.6% among *E. coli*. CTX-M was the commonest genotype (54.3%) in their study, where 97% of them belonged to the CTX-M1 cluster [18].

A study in Korea revealed that the CTX-M3 and the CTX-M15 genotypes were the most common types of ESBLs in *E. coli* [4].

The epidemiology of the ESBL carrying organisms has been changing in the recent years, with the CTX-M type ESBL having emerged within the community, especially among the *E. coli* which were isolated from UTIs [5].

The CTX-M type may have emerged as a dominant type, probably because it had a high capacity to disseminate or it may have had an ecological advantage of persisting in the community [5].

Studies on the different genotypes which prevail in an area is necessary for epidemiological purposes and for the surveillance in hospital infections. It also throws light on the pattern of the strains which changes over a period of time. More of such studies on the genotypes which prevail in different parts of India are needed.

CONCLUSION

Our study showed an ESBL prevalence rate of 34% among the *E. coli* which were isolated from urine and a majority of these strains

showed an MIC of ≥ 128µg/ml for ceftazidime. The isolates also showed a high rate of resistance to cotrimoxazole, ciprofloxacin and gentamicin and a high occurrence of the CTX-M3 genotype (84%) among them.

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AUTHOR(S):

1. Dr. Malini A. Bhat
2. Dr. Sageerabanoo S.
3. Dr. Kowsalya R.
4. Dr. Gautam Sarkar

PARTICULARS OF CONTRIBUTORS:

1. Associate Professor, Department of Microbiology, Indira Gandhi Medical College & Research Institute, Vazhudavur Road, Kadirkammam, Puducherry-605009, India.
2. Assistant Professor, Department of Microbiology, Aarupadai Veedu Medical College, Puducherry, India.
3. Assistant Professor, Department of Biochemistry, Institute of Nephrourology, Bangalore, India.
4. Professor & HOD, DepARTMENT of Biochemistry, Subharathi Medical College, Meerut, UP, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Malini A. Bhat
Associate Professor, Dept of Microbiology,
Indira Gandhi Medical College & Research Institute,
Vazhudavur Road, Kadirkammam, Puducherry-605009 (India).
Phone: 9944758049
E-mail: drmalinirb@yahoo.co.in

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