IDENTIFICATION AND MOLECULAR CHARACTERISATION OF ESBL PRODUCING UROPATHOGENIC ESCHERICHIA COLI STRAINS ISOLATED FROM A TERTIARY CARE HOSPITAL OF TRIPURA

Arunabha Dasgupta[1], Tapan Majumdar[2], Rajesh Bhowmik[3]

[1]Associate Professor, Department of Medicine, Agartala Government Medical College, Agartala, Tripura, India.
[2]Associate Professor, Department of Microbiology, Agartala Government Medical College, Agartala, Tripura, India.
[3]Junior Research Fellow, Department of Medicine, Agartala Government Medical College, Agartala, Tripura, India.

ABSTRACT

BACKGROUND

Infections caused by Escherichia coli (*E. coli*) have become a significant worldwide public health problem. Furthermore, the situation is worsening due to advent of increased antibiotic resistance due to the evolution of plasmid mediated multi-resistant antibiotic genes, mainly Extended Spectrum B-lactamases (ESBLs). These enzyme producing organisms are also co-resistant to other different classes of antibiotics.

Aims and Objectives- The aim of the study was to determine the antibiotic resistance pattern of Uropathogenic *E. coli* isolated from tertiary care hospital of Tripura with an objective of plasmid profiling of multidrug resistant isolates and to determine the bla$_{TEM}$ and bla$_{CTX-M}$ producers by molecular methods, mainly by PCR using gene specific primer.

MATERIALS AND METHODS

This study was undertaken to identify the presence of ESBLs enzyme by phenotypic method and presence of bla$_{TEM}$ and bla$_{CTX-M}$ gene among the ESBL-producing Uropathogenic Escherichia coli (UPEC) by polymerase chain reaction using gene specific primers at our setup.

RESULTS

Out of 150 clinical isolates from patients of the tertiary care hospital, 85 strains were identified as *E. coli* strains. These isolates were further screened for ESBL production by phenotypic methods using drug-inhibitor combination disks, namely ceftazidime-clavulanate (30 + 10 µg) and cefotaxime-clavulanate (30 + 10 µg) as per CLSI guidelines. Out of 85 isolates, 52 were detected as ESBL producers. Further analysis for plasmid isolation showed that all of them harbour plasmids of size ranging from ~0.5 to 20 kb. Molecular analysis of ESBL gene indicated predominance of bla$_{TEM}$ 42% followed by bla$_{CTX-M}$ 35%, bla$_{OXA}$ + bla$_{TEM}$ 15% and bla$_{CTX-M}$ +bla$_{TEM}$ 8% respectively.

CONCLUSION

This study highlights increased proportion of ESBL producers among uropathogenic *E. coli* isolated from the tertiary care setup of Tripura. These ESBL producers were simultaneously resistant to three or more than three groups of other antibiotics and were assigned as multidrug resistant (MDR) *E. coli*. The isolates harboured plasmids of varied size and numbers. Molecular analysis of ESBL gene indicated predominance of bla$_{TEM}$ and bla$_{CTX-M}$, which can be successfully detected by PCR. Detection of ESBL by phenotypic tests if supplemented with molecular methods may provide a valuable diagnostic and research tool. These will strengthen the antibiotic stewardship programme of the hospital and aid in preserving the efficacy of life saving antibiotics.

KEY WORDS

Escherichia coli, Anti-Bacterial Agents, Tertiary Care Centre, Drug Resistance, Urinary Tract Infections, Plasmids, DNA, Polymerase Chain Reaction.

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BACKGROUND

Urinary tract infection (UTI) is a common bacterial disease.[1] *E. coli* is reported to be the major cause (85% - 95%) of urinary tract infection.[2] The treatment of choice for urinary tract infections are uses of specific antibiotics.

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Corresponding Author:
Dr. Tapan Majumdar,
Associate Professor,
Department of Microbiology,
Agartala Government Medical College,
PO. Kunjaban-799006, Tripura, India.
E-mail: drtapan1@rediffmail.com, drtapan1960@gmail.com
DOI: 10.14260/jemds/2018/638

However, injudicious uses of antibiotics exert selective pressure on the bacteria causing emergence of resistant strains to multiply and spread. Therefore, multidrug resistant (MDR) organisms are frequently found in urinary tract infection.[3] Successful antibiotic therapy against Uropathogenic *E. coli* is often impaired due to the resistance to commonly used antimicrobial agents.[4]

β-lactams are the most commonly used antibiotic in hospitals and community setup resulting in increased morbidity, mortality and health-care costs because of emergence of resistant strain.[5] This is due to the production of β-lactamases followed subsequently by the evolution of extended spectrum β-lactamases [ESBLs]. Extended spectrum β-lactamases are a large, rapidly evolving group of plasmid-mediated enzymes capable of hydrolysing and inactivating penicillins, cephalosporins and monobactams and are inhibited by β-lactamase inhibitors such as clavulanate,

sulbactam and tazobactam.[6,7,8] Detection of ESBLs are not routinely done in resource poor setup.

This study was undertaken to identify the presence of ESBLs enzyme by phenotypic method and presence of bla*TEM* and bla*CTX-M* gene among the ESBL-producing Uropathogenic Escherichia coli (UPEC) by polymerase chain reaction using gene specific primers at our setup.

MATERIALS AND METHODS

Identification of Organisms

Midstream urine samples received in the department were inoculated CLED agar plates. These plates were incubated at 37°C aerobically and after overnight incubation they were checked for bacterial growth. The organisms were identified by their colony morphology, staining characters, pigment production, motility and other relevant biochemical tests as per standard laboratory protocol.[9,10]

Antimicrobial Susceptibility Test done by Modified Kirby-Bauer Sensitivity Testing/ Method

As per CLSI guidelines, modified Kirby-Bauer sensitivity testing method was used for this purpose.[11] Bacterial suspension having visually equivalent turbidity to 0.5 McFarland standards were inoculated in Mueller-Hinton agar media using swab stick. Inoculated plates were incubated at 37°C for 24 hours. The next day plates were read by taking measurement of zone of inhibition. Antimicrobial discs were used for Amikacin (30 µg), Amoxyclav (30 µg), cephotaxime (30 µg), Ceftazidime (30 µg), Ciprofloxacin (5 µg), nitrofurantoin (300 µg), ofloxacin (5 µg) Piperacillin (75 µg), Cefotaxime/ clavulanic acid (30/10 µg), Ceftazidime/clavulanic acid (30/10 µg), Piperacillin/Tazobactam (100/10 µg).

Confirmatory Test for ESBLs

Double Disk Diffusion Method (DDDT)

For this test as per CLSI document M-100 S-25, lawn culture of the test isolates inoculated on Mueller-Hinton agar plate followed by putting up of disc of ceftazidime (30 µg), cefotaxime (30 µg) alone and a disc of ceftazidime and cefotaxime in combination with clavulanic acid (30/10 µg), the discs were placed 25 mm apart, Center to Center and incubated overnight at 37°C. A ≥ 5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was designated as ESBL positive.[12]

Determination of Minimum Inhibitory Concentration (MIC) by Macro Broth Dilution Method

Preparation of Inoculum

- Broth suspension of isolated colonies selected from an 18- to 24-hour agar plate were used to prepare the inoculum by making turbidity equivalent to a 0.5 McFarland, which is approximately equivalent to 1 to 2 x 10^8 colony forming units (CFU)/mL.
- This suspension was further diluted to adjust the inoculum to contain approximately 5 x 10^5 CFU/mL. This was accomplished by diluting the 0.5 McFarland suspensions 1: 150, resulting in a tube containing approximately 1 x 10^6 CFU/mL. The subsequent 1: 2 dilution in step 3 brings the final inoculum to 5 x 10^5 CFU/mL.[13]

Inoculation

1 mL of the adjusted inoculum added to each tube containing 1 mL of antimicrobial agent in the dilution series (and a positive control tube containing only broth) and mixed. This results in a 1: 2 dilution of each antimicrobial concentration and a 1: 2 dilution of the inoculums.

Incubation

Tubes were incubated at 35ºC for 16 to 20 hours in an ambient air incubator.

Interpretation

The lowest concentration at which the isolate was completely inhibited (as evidenced by the absence of visible bacterial growth) was recorded as the minimal inhibitory concentration or MIC.

Genotypic Detection

A) Preparation of Plasmid DNA

A pure colony of each isolate was inoculated from CLED agar into 5 mL of Luria-Bertani broth and incubated for 20 h at 37ºC. Cells from the overnight culture were harvested by centrifugation at 12,000 rpm for 5 mins. Plasmid DNA were isolated by alkaline lysis method. The DNA samples were stored at -20ºC until used.[14]

B) Profiling of Plasmid

1) Preparation of Agarose Gel

0.8 gm agarose in 100 mL of diluted TAE buffer was prepared by using a microwave oven. The melted agarose was allowed to cool at 50ºC and 20 µL ethidium bromide was mixed and poured into gel tray after shaking and combs were placed. After solidification of the gel, the comb was removed. During electrophoresis, the gel was placed in a horizontal electrophoresis apparatus containing TAE buffer and ethidium bromide.

2) Gel Electrophoresis of the Sample

The mixture containing 5 µL of sample and 2.0 µL of loading buffer was slowly loaded into the well using disposable micropipette tips. 100 bp molecular weight marker was loaded in one well to determine the size of the amplified PCR products. Electrophoresis was carried out at 100 volts for 35 minutes.

3) Gel Documentation

The products electrophoresis was visualised by trans-illuminator followed by photographed transferring of data to a computer for further analysis.

C) Detection of bla*TEM* and bla*CTX-M* by PCR

PCR amplification of bla*TEM* and bla*CTX-M* genes was done following the published protocol of Mukherjee et al.[15] Briefly, Primers used to detect *bla*TEM specific gene were 5'-ATGAGTATTCAACATTTCCGTG-3' (forward primer) and 5'-TTACCAATGCTTAATCA GTGAG-3' (reverse primer) and for *bla*CTX-M gene, a degenerate primer set comprising of forward primer: 5' ATGYGCAGYACCAGTAAG 3' (Y represents T/C) and reverse primer: 5' ATATCRTTGGTGGTGCCRT 3' (R represents A/G) procured from Integrated DNA Technologies Pte. Ltd. (IDT), USA through BioApps. The extracted plasmid DNA was used as the template in the PCR reaction. PCR mix

was prepared for a final reaction volume of 20 µL using H_2O (Milli-Q grade), 50 pmol and 20 pmol of *bla*CTX-M and *bla*TEM gene specific primers respectively, 250 µM of each dNTP, 1.5 units of Taq polymerase, 2.0 µL of 10X PCR buffer containing 1.5 mM $MgCl_2$ and 100 ng DNA template. Initial denaturation at 94ºC for 3 mins followed by 30 cycles of denaturation at 94ºC for 30 secs, annealing at 52ºC for 30 secs and elongation at 72ºC for 1 min was carried out in a thermocycler. The final elongation step was extended to 10 mins at 72ºC. The amplified products were separated in agarose gel containing ethidium bromide (0.5 mg/mL). To measure the molecular weights of amplified products, 100 bp DNA ladder was used. The images of ethidium bromide stained DNA bands were visualised and documented using a gel documentation system.[15,16,17]

RESULTS

150 clinical isolates from patients of the tertiary care hospital, Agartala, Tripura were included in the study. Out of these 85 strains were identified as Escherichia coli, 40 strains as Klebsiella pneumoniae, 14 strains as Enterobacter species, 8 strains as Pseudomonas species and 3 strains as Acinetobacter species.

Antibiogram of the *E. coli* isolates shows 79.36% sensitive to Nitrofurantoin followed by Amikacin 74.36%, Ofloxacin 60.31%, Ceftazidime 52.38%, Cefotaxime 44.44%, Amoxyclav-54%, Ciprofloxacin 28.57% Piperacillin 38.01%, Piperacillin-Tazobactam 81.71% percent respectively which is shown in Fig. 1.

Phenotypic analysis by DDDT (Fig. 2) shows 61% (52/85) are confirmed ESBL producer and 39% (33/85) are β-lactamase inhibitor resistant (BLIR). Minimal inhibitory concentration (MIC) studies using macro broth dilution method against ceftazidime and cefotaxime on the ESBL and BLIR isolates indicated that the average values of the ESBL producers were < 512 µg/mL and BLIR were > 512 µg/mL respectively as shown in Table 1.

Plasmids were extracted from all 52 clinical isolates of *E.coli* and used as template in PCR to identify the β-lactamase genes. All the isolates were found to harbour plasmids, size ranging from ~0.5 to 20 kb. Molecular analysis of ESBL gene shown in Fig. 3 indicated predominance of bla_{TEM} 42% followed by $bla_{CTX\text{-}M}$ 35%, bla_{OXA} + bla_{TEM} 15% and $bla_{CTX\text{-}M}$ +bla_{TEM} 8% respectively, which is shown in Table 2.

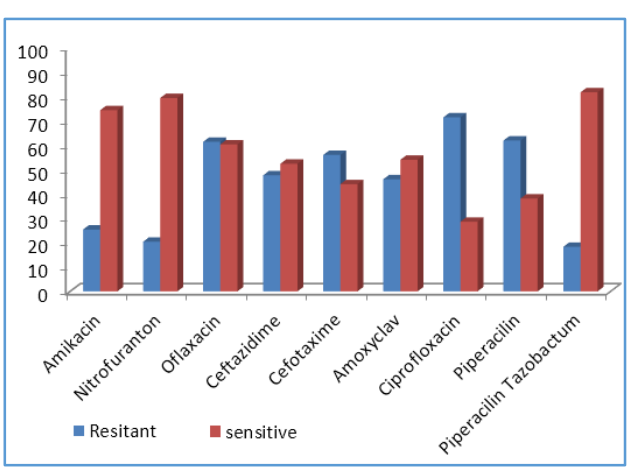

Figure 1. Antibiogram of Escherichia coli Isolates (n= 85) recovered from UTI Patients

Figure 2. Showing Double Disc Diffusion Test (DDDT)

***Interpretation**

An increase of ≥ 5 mm in inhibition zone diameter around combination disk of Ceftazidime + Clavulanic acid against the inhibition zone diameter around Ceftazidime disk alone confirms ESBL production.

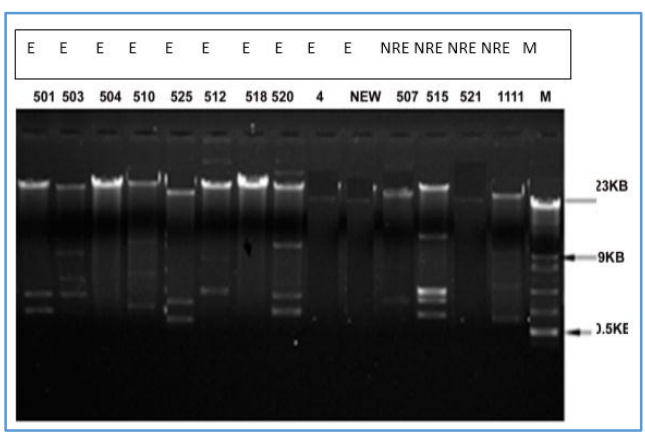

Figure 3. PCR Results of Extracted Plasmid DNA

*E- ESBL producer, NRE- β-lactamase inhibitor resistant, NE- susceptible to cephalosporins.

Dilution Range (µg/mL)	Cefotaxime		Ceftazidime	
	ESBL Positive (*n*=52)	ESBL Negative (*n*=33)	ESBL Positive (*n*=52)	ESBL Negative (*n*=33)
≤0.25	-	-	-	-
0.5	-	-	-	-
1	-	-	-	-
2	-	-	-	
4	-	-	-	-
8	-	-	-	-
16	-	-	-	-
32	-	-	-	-
64	08	-	07	-
128	21	-	27	-
256	23	-	18	-
512	-	17	-	23
1024	-	19	-	10

Table 1. Detection of MIC Values in ESBL producers and Non-ESBL producer by Macro Broth Dilution Method

β-Lactamase Genes	ESBL producing *E. coli*
bla*TEM*	42%(22/52)
bla*CTX-M*	35%(18/52)
bla*OXA* + bla*TEM*	15%(8/52)
bla*TEM* + bla*CTX-M*	8%(4/52)

Table 2. β-Lactamase Genotypes in ESBL (n= 52) producing Uropathogenic E. coli Isolates

DISCUSSION

This study highlights increased proportion of ESBL producers among uropathogenic *E. coli* isolated from the tertiary care setup of Tripura. These ESBL producers were simultaneously resistant to three or more than three groups of other antibiotics and were assigned as MDR.

E. coli- These MDR *E. coli* exhibited simultaneous resistance to large number of antibiotics like Ciprofloxacin, Amoxyclav, Ciprofloxacin, Ceftazidime, Ofloxacin and Cefotaxime. Whereas Amikacin, Piperacillin/Tazobactam and Nitrofurantoin were found to be the most active antimicrobials against these organisms. The isolates harboured plasmids of varied size and numbers. Molecular analysis of ESBL gene indicated predominance of bla*TEM* and bla*CTX-M*, which could be successfully detected by PCR.

Clinical Microbiology laboratories should report about the ESBLs phenotypes, which may help clinician to be aware and alert about choosing both empirical and targeted antibiotic therapy against uropathogenic *E. coli*. At the same time, molecular characterisation of these ESBL producers will assist in understanding the pathogenesis as well as management of UTI. Similar study by Grover et al emphasised about molecular detection, where facilities are available.[18] Molecular characterisation also help in detecting exact ESBLs subtypes that cannot be detected by phenotypic analysis.[19,20] Detection of ESBLs by phenotypic tests if supplemented with molecular methods may provide a valuable diagnostic and research tool. These will strengthen the antibiotic stewardship programme of the hospital and aid in preserving the efficacy of life saving antibiotics.

CONCLUSION

ESBL producers among uropathogenic *E. coli* isolated from the tertiary care set-up of Tripura are on the rise. These ESBL producers were simultaneously resistant to three or more than three categories of other antibiotics and were designated as MDR *E. coli*. Plasmids of varied size and numbers were responsible for carrying the genes of drug resistance. Molecular analysis of ESBL gene indicated predominance of bla*TEM* 42% followed by bla*CTX-M* 35%, blaOXA + bla*TEM* 15% and bla*CTX-M* + bla*TEM* 8% respectively. Detection of ESBLs by phenotypic tests if supplemented with molecular methods may provide a valuable diagnostic and research tool. These will strengthen the antibiotic stewardship programme of the hospital and aid in preserving the efficacy of life saving antibiotics.

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