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Original Article

Phenotypic Screening and Genotypic Characterization of Extended-spectrum-beta-lactamase Producing Uropathogenic *Escherichia coli* (UPEC) isolated from a Tertiary Care Hospital in Bangladesh

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Abstract

Beta-lactamases are the most important factors in the resistance to beta-lactam antibiotics among gram-negative bacteria, especially *Escherichia coli*. The prevalence of infections caused by extended-spectrum β-lactamases (ESBLs)-producing *Esch. coli* is increasing, as one of the emerging health problems worldwide. This study aimed to investigate the prevalence of ESBL producing *Esch. coli* isolated from urinary tract infections (UTIs) and to observe the antibiotic resistance gene among them. This cross-sectional study was conducted in the Department of Microbiology, Dhaka Medical College from July 2015 to June 2016. Out of 280 urine samples with pus cells ≥ 5/HPF, 60 (72.29%) isolated bacteria was *Escherechia coli* identified by standard laboratory methods. Antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method. ESBL producers were detected phenotypically by Double-disk-synergy (DDS) test. Genotypically ESBL genes (blaCTX-M-15 and blaOXA-1) among the ESBL producers were detected by PCR. Among 60 isolated *Escherichia coli*, 22 (36.67%) ESBL producers were detected by DDS test. Ten (45.45%) ESBL producers were positive for blaCTX-M-15, 5(22.73%) were positive for blaOXA-1 and 4(18.18%) isolates were harbouring the both ESBL genes. The results of this study showed high proportion of ESBL producing *Esch. coli* in Bangladesh.

Keywords: Beta-lactamases, Uropathogenic Esch. Coli, Phenotypic Screening, Genotypic,Characterization, Urinary tract infection

Introduction

Urinary tract infection is the common bacterial infection for both female and male, especially *Escherichia coli* is a common UTI causing gram negative bacteria.[1,2] The varitype of *Esch. coli* causing UTI is called Uropathogenic *Escherichia coli* (UPEC) which accounts for up to 80% of UTI.[3] Uropathogenic *Esch. coli* possess various virulence factors, with increased prevalence of ESBL accounting up to 17% of community acquired UTI and 58% of nosocomial UTI infection.[4] Extended spectrum β–lactamases(ESBLs) are plasmid mediated enzymes that confer resistance to the penicillins, first, second, and third-generation cephalosporins and monobactams (i.e. aztreonam) but do not affect cephamycins (i.e. cefoxitin and cefotetan) or carbapenems and inhibited by β-lactamase inhibitors such as clavulanic acid.[5,6] Genotypic characterization of ESBL in UPEC has become important objective in antibiotic resistance of infectious agents. The common genes responsible for resistance against β-lactam groups are TEM, SHV, CTX-M.[7] CTX-M gene possessing 291 aminoacid and any change in it results in emerge of a new variant CTX-M gene.[8] Currently the most widely distributed CTX-M enzyme is CTX-M-15.[9] OXA-1 has been described as the most

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common OXA-type beta-lactamase.[10] Genotypic and phenotypic expression may differ in wild and laboratory conditions based on gene regulation. Patients with increased threat of colonization and infection with ESBL producing microorganism makes the patients fatally sick with prolong hospital stay.[6] Therefore the present study has been carried out to detect ESBL by double disc method and ESBL gene by using designed primers through PCR assay in clinical isolates of *Esch.coli* obtained from patients with UTIs from Dhaka Medical College Hospital in Bangladesh.

Materials and methods

A cross sectional study was conducted in the Department of Microbiology, Dhaka Medical College, Dhaka, from July, 2015 to June, 2016. This research protocol was approved by Research Review Committee and Ethical Review Committee of Dhaka Medical College. All the patients presenting with the clinical features of UTI irrespective of age & sex who attended in the outdoor patient Department (OPD) or admitted in the indoor patient department (IPD) of the hospital were included as the study population. A total of 280 non-repetitive urine specimens were included in the study.

Sample collection:

A mid-stream urine were collected aseptically in a sterile, dry, wide necked, leak proof container and catheterized urine samples received from inpatients of the hospital and were labelled appropriately and transported immediately to the Microbiology laboratory for further processing.

Bacterial isolates

All samples were inoculated on blood agar and MacConkey agar media. The inoculated culture plates were aerobically incubated at 37°C for 24 hours. Incubated plates were observed for the presence of any bacterial growth after 24 hours. If growth occurred, colony count was done to calculate the number of colony forming unit per ml of urine. A count of 1 x 10^5 or more bacteria per ml of urine was considered as clinically significant. *Esch. coli* was identified by colony morphology, staining character and biochemical tests including: fermentation of glucose, lactose, sucrose, gas and H_2S production into TSI agar, citrate utilization, indole production and urease test into MIU media as per standard laboratory techniques.[11]

Antimicrobial susceptibility testing

Susceptibility test to antimicrobial agents of all isolated *Escherichia coli* were done by Kirby-Bauer modified disc diffusion technique as described by CLSI guideline (CLSI, 2015).[12] Antibiotic discs used were ceftazidime (30 µg /disc), ceftriaxone (30 µg/disc), cefixime (30 µg /disc), cefuroxime (30 µg/disc), imipenem (10 µg/disc), amoxiclav (amoxicillin and clavulanic acid) (20/10 µg/disc), ciprofloxacin (5 µg/disc), gentamicin (10 µg/disc), amikacin (30 µg/disc), co-trimoxazole(1.25/23.75 µg/disc), azithromycin (15 µg/disc), nitrofurantoin (300 µg/disc), colistin sulphate (10 µg/disc), piperacillin-tazobactum (100/10 µg/disc), tigecycline (15 µg/disc) (Oxoid Ltd. UK). Mueller Hinton agar media was used for antimicrobial susceptibility test. Susceptibility of the Enterobacteriaceae to tigecycline was determined using 15 µg tigecycline disc and the criteria of the United States Food and Drug Administration was used for interpretation.[13] *Escherichia coli* ATCC 25922 was used as the quality control strain. Study isolates were phenotypically characterized for the production of ESBL by using DDS test. Antimicrobial susceptibility testing of all ESBL producers were also performed.

Double disc synergy (DDS) test for detection of ESBL producing organism[14]

This test was performed on Mueller-Hinton agar plate. Amoxiclav (amoxicillin 20 µg + clavulanic acid 10 µg) disc was placed at the centre of the plate. Third generation cephalosporins (ceftriaxone, ceftazidime and cefotaxime) were placed 20 mm apart from center of the amoxiclav disc. Inoculated plate was incubated at 37ºC for 24 hours. A clear extension of the edge of the inhibition zone of cephalosporin disc towards amoxiclav disc was interpreted as ESBLs production.

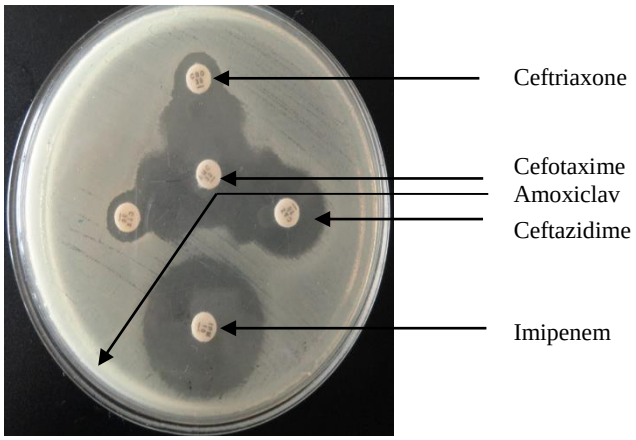

Figure I: Double-Disk Synergy test for detection of ESBL producers.

Detection of ESBL encoding Genes by PCR[15]

Bacterial pellet formation

PCR was used for identification of ESBLs encoding genes (blaCTX-M-15 and blaOXA-1) among phenotypically confirmed ESBLs producing uropathogenic *Escherichia coli.* To prepare bacterial pellet, a loop full of bacterial colonies was inoculated into a falcon tube containing Trypticase Soy Broth. After incubation overnight at37^0C, the Falcon tubes were centrifuged at 4,000 rpm for 10

minutes, after which the supernatant was discarded.A small amount of sterile trypticase soy broth was added into the Falcon tubes with pellets and mixed evenly. Then an equal amount of bacterial suspension was placed into 2 to 3 microcentrifuge tubes. The microcentrifuge tubes were then centrifuged at 4,000 rpm for 10 minutes and the supernatant was discarded. The microcentrifuge tubes containing bacterial pellets were kept at -20^{0}C until DNA extraction.

DNA extraction

Three hundred µl of sterile distilled water was added into microcentrifuge tubes having bacterial pellet and vortexed until mixed. Mixture was heated at 100°C for 10 minutes in a heat block. After heating, immediately the microcentrifuge tubes were placed on ice for 5 minutes and then centrifuged at 14,000Xg at 4°C for 6 minutes. Supernatant was taken into another microcentrifuge tube by micropipette and was used for PCR. Extracted DNA was preserved at 4°C for 7-10 days and - 20°C for long time.

Mixing of mastermix and specific primers (Table-I) with DNA template and Amplification in thermal cycler were done. The amplified DNA were loaded into a 1.5% agarosegel, electrophoresed at 100 volts for minutes, stained with 1% ethidium bromide, and visualized under UV light.

Table-I: The primers used in this study

Genes	Sequence (5′-3′)	Bp
CTX-M-15-SF	CACACGTGGAATTTAGGGACT	996
CTX-M-15 SR	GCCGTCTAAGGCGATAAACA	
OXA-1-F	ACCAGATTCCAACTTTCAA	598
OXA-1-R	TCTTGGCTTTTATGCTTG	

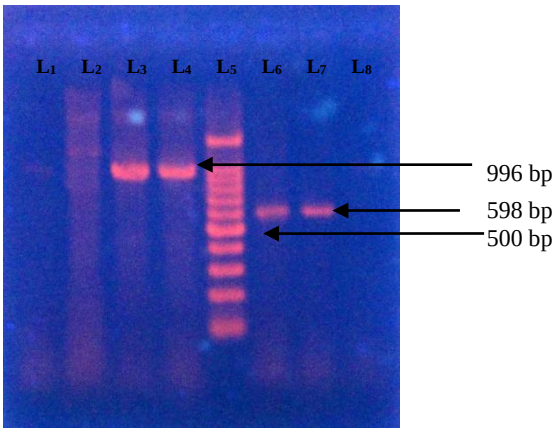

Figure II: Photograph of gel electrophoresis of amplified DNA of 996 bp for *bla*CTX-M-15 gene (Lane 3 and 4). Amplified DNA of 598 bp for *bla*OXA-1-group gene (Lane 6 and 7). Hundred bp DNA ladder (Lane 5). Negative control without DNA (Lane 2).

Statistical analysis

The results of the study were recorded systematically. Data analysis was done by using 'Microsoft Office Excel 2013' program.

Results

Among the isolated 60 uropathogenic *Esch. coli*, 22(36.67%) ESBL producers were detected by DDS test. Among the ESBL producers 95.45% were resistant to ceftriaxone and cefixim, 90.91% were resistant to ceftazidime, cefuroxime and co-trimoxazole, 77.27% were resistant to amoxiclave and azithromycin, 72.73% were resistant to ciprofloxacin, 45.45% were resistant to gentamycin and pipercillin-tazobactam, 22.73% were resistant to nitrofurantoin and low level of resistance to imipenem 4.54%. All (100%) the ESBL producers were sensitive to colistin and tigacycline. (Table-II)

Table-II: Antibiotic resistance rates of *Esch. coli* isolates tested in this study.

Antibiotic	ESBL producer (N=22)-n(%)	Non-ESBL producer (N=48)-n(%)	Total (N=60)n(%)
Amoxiclave	17(77.27)	26(54.17)	43(71.67)
Ceftriaxone	21(95.45)	25(52.08)	46(76.67)
Ceftazidime	20(90.91)	29(60.41)	49(81.67)
Cefixime	21(95.45)	25(52.08)	46(76.67)
Cefuroxime	20(90.91)	29(60.42)	49(81.67)
Azithromycin	17(77.27)	31(64.58)	48(80.00)
Ciprofloxacin	16(72.73)	34(70.83)	50(83.33)
Cotrimoxazole	20(90.91)	34(70.83)	54(90.00)
Gentamicin	10(45.45)	17(35.42)	27(45.00)
Amikacin	4(18.18)	5(10.42)	9(15.00)
Nitrofurantoin	5(22.73)	9(18.75)	14(23.33)
Piperacillin-tazobactam	10(45.45)	4(8.33)	14(23.33)
Imipenem	1(4.54)	8(16.67)	9(15.00)
Colistin	0(00)	1(2.08)	1(1.67)
Tigecycline	0(00)	1(2.08)	1(1.67)

Note: N = Total number of isolates; n = number of positive cases

PCR for ESBL specific genes was performed for all the 22 ESBL isolates that were detected by DDS test. Ten (45.45%) ESBL producers were positive for blaCTX-M-15, 5(22.73%) were positive for blaOXA-1 and 4(18.18%) isolates were harbouring the both ESBL genes. (Table-III)

ESBL encoding genes	Positive n (%)
CTX-M-15 alone	10 (45.45)
OXA-1 alone	5 (22.73)
CTX-M-15 + OXA-1	4 (18.18)
Total	**19 (86.36)**

Discussion:

The emergence and rapid dissemination of multidrug-resistant Enterobacteriaceae worries the whole world and, in particular, ESBL-producing enterobacteriaceae. Since 2000s, ESBL producing *Escherichia coli* have been considered as serious pathogens both in nosocomial and community infections around the world, and their virulence varies by region.[16] ESBL producing *Escherichia coli* are more common in females and in hospital settings and are for more than half of nosocomial origin, which is often very complicated to treat.[17] In this study, among the 60 isolated *Esch. coli*, 22 (36.67%) ESBL producers were detected by DDS test. In agreement with this findings, Rajabnia *et al*,. (2019) reported 37.11% ESBL producing *Esch. coli* isolated from UTI.[18] A few other studies Motaqim (2007) and Kulkarni *et al*,.(2016) also reported 34.1% and 46.87% ESBL producers respectively.[19,20] In Bangladesh Rahman *et al*,. (2004) found that 43.20% *Esch. coli* were ESBL producer which is higher than the present study.[21] Shigh *et al*,. (2016) reported a high proportion of ESBL which was 82.6%.[22] However, 80% ESBL producers was also reported in Bangladesh in another study.[23] Canton *et al*,. (2008) reported that the higher prevalence of ESBL producers in Asia than in Europe and America was observed because of spread of mobile genetic elements, mainly 148 epidemic plasmids, and the dispersion of specific clones have been responsible. Moreover, the prevalence of ESBL producers varies with time as well as from country to country, city to city and even hospital to hospital of one city.[24]

In this study, sensitivity pattern of ESBL producing *Esch. coli* was done against different antibiotics and has shown that, the highest antibiotic resistance among ESBL was observed with ceftriaxone and cefixim (95.45%), ceftazidime, cefuroxime and cotrimoxazole (90.91%), amoxiclave and azithromycin (77.27%), ciprofloxacin (72.73%), gentamycin and pipercillin-tazobactum (45.45%). The highest antibiotic sensitivity was observed with colistin and tigecycline (100%), imipenem (95.46%). Islam *et al*., from Bangladesh reported that ESBL producing *Esch. coli* strains have 100% resistant to amoxicillin, ceftriaxone, ceftazidime and cefotaxim, 86.4% resistant to cotrimoxazole, 54.5% resistant to nitrofurantoin and all strains were sensitive to imipenem.[25] Another study by Halabi *et al*., reported that highest antibiotic resistance was observed with amoxicillin (100%), cefixim (96%), cefotaxime (91%), ceftazidime (90%) and the highest sensitivity was observed with colistin (100%) and imipenem (96%).[26] This findings are very much similar to the present study. Similar findings were also observed by Shamsuzzaman who reported that all the ESBL producers were sensitive to colistin.[27] Canigia and Dowzicky reported that more than 95.0% of the ESBL producing *Esch. coli* were sensitive to tigecycline and imipenem which is almost similar to present findings.[28]

In the present study among 22 ESBL producing strains, 14 (63.63%) were positive for blaCTXM-15 gene. In agreement with the present findings Yeasmin (2016) from DMCH and Sedighi *et al*., (2015) from Iran, reported that the detection rate of the blaCTX-M-15 gene in patient with UTI were 62.16% and 66.67% respectively.[29,30] Previous reports within or outside of Bangladesh showed a high prevalence of CTX-M-15 group in ESBL producing *E. coli*.[31,32] Present study observed 9 (40.91%) OXA-1 producers among the 22 ESBL producing *Esch. coli.* A recent study in Bangladesh reported that 40% OXA-1 producers isolated from urine, which was very close to the present findings.[33] Using specific primer for blaCTX-M-15 and blaOXA-1, present study could not detect any ESBL gene in 3 (13.64%) of the ESBL producers. Other than blaCTX-M-15 and blaOXA-1, till now there are many variants of ESBL genes, of them TEM and SHV genes are commonly present worldwide.[28] The reason for the absence of blaCTX-M-15 and blaOXA-1in those 3 phenotypic positive ESBL producers might be due to the presence of other variants of ESBL genes.

Conclusion

The present study reflected that the high prevalence of ESBL uropathogenic *Esch. coli*, especially blaCTX-M-15 (63.63%) are increasing in Bangladesh. Imipenem, colistin and tigecycline were found to be most effective drug

for the treatment of ESBL producers. It is thought that ESBLs should be detected by phenotypic or genotypic methods for selecting the appropriate antibiotics regarding treating the patients with UTIs. Surveys and monitoring should be done routinely for recognizing the antimicrobial resistance and to setup the antibiotic policy.

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