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ORIGINAL ARTICLE

Emergence of CTX-M-15 producing *E. coli* O25b-ST131 clone in a tertiary care hospital of Bangladesh

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Abstract

Background: Extended-spectrum β-lactamase (ESBL) producing uropathogens has become prevalent worldwide. *E. coli* O25b-ST131 clone, associated with *bla*CTX-M-15, has been reported from many parts of the world and is frequently associated with multidrug resistance. Thus far, there are no reports about this clone in Bangladesh. The objective of this study was to investigate ESBL producing uropathogens and to survey the prevalence of *E. coli* O25b-ST131 clone among ESBL positive *E. coli* isolates. *Methods*: From symptomatic urinary tract infection cases, a total of 800 urine samples were collected. Bacterial identification and antimicrobial susceptibility testing was performed using established methods. Screening of ESBL producers was done using the disk diffusion method. Screening positive isolates were phenotypically confirmed by double disk synergy (DDS) test. Genes encoding ESBLs (*bla*CTX-M-15, *bla*OXA-1) were identified both by PCR and DNA sequencing. Phenotypic positive ESBL producers were also studied by PCR for existence of class 1 integron. Subsequently, O25b-ST131 clone was identified by allele specific PCR. *Results:* Of 138 gram-negative uropathogens, 45 (32.6%) were positive for ESBLs. ESBL producers showed high frequency of antimicrobial resistance except imipenem. Among 45 ESBL producers, 36 (80%) produced *bla*CTX-M-15, 18 (40%) produced *bla*OXA-1. Fifteen (33.3%) strains simultaneously produced both *bla*OXA-1 and *bla*CTX-M-15. Class 1 integron was present in 30 (66.7%) isolates. Of the 31 *bla*CTX-M-15 positive *E. coli*, 22 (71%) were positive for *E. coli* O25b-ST131 clone and all (100%) belonged to B2 phylogenetic group. *Conclusion:* Rising antimicrobial resistance among uropathogens, and especially the emergence of *bla*CTX-M-15 positive *E. coli* O25b-ST131 clone in Bangladesh has provided urgency to the development of novel preventive and therapeutic strategies.

Keywords: *E. coli*, *bla*CTX-M-15, O25b-ST131 clone, uropathogens, Bangladesh

INTRODUCTION

Urinary tract infections (UTIs) are frequent bacterial infections worldwide and the most commonly encountered bacterial infection, particularly in women. It is estimated that about 150 million cases of UTI occur worldwide, as many as 50% of women and 12% of men experience at least one symptomatic UTI during their lives and about 25% of affected women have recurrent UTI (RUTI).[1] Although a variety of aetiologies are involved, *Escherichia coli* and *Klebsiella* are the leading causes of UTI.[2] Extended-spectrum β-lactamases (ESBLs) production were reported as a major cause of drug resistance among these bacteria. ESBLs are a heterogroup of enzymes that confer resistance to penicillins, oxyimino-cephalosporins and monobactams.[3] Moreover, ESBL producing organisms are often resistant to several other non β-lactam antibiotics, as the plasmids with the gene encoding ESBLs often carry other resistance determinants.[4] Of all the ESBL variants that have been identified so far, TEM and SHV were the most common during past decade. CTX-M ESBLs have begun to emerge in recent time, and have gained worldwide attention.[5] Currently the most extensively disseminated CTX-M genotype is CTX-M-15 which was first identified in India from *E. coli* isolate in 2001.[6] CTX-M-15 producing *E. coli* with multidrug-resistance is emerged globally as a virulent pathogen causing hospital and community acquired UTIs since 2003.[7]

Recently, a very successful pandemic

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uropathogenic CTX-M-15 positive *E. coli* clonal group named ST131 has been reported worldwide as an important cause of community acquired UTI. Serogroup O25b is associated with it. This clonal group belongs to phylogenetic group B2 which is highly virulent, and harbors multidrug-resistant incompatibility group FII plasmids.[8] This clone is commonly associated with the worldwide emergence of fluoroquinolone-resistant and *bla*CTX-M producing *E. coli*.[7] Current surveillance studies have demonstrated that ST131 accounts for 30% of total *E. coli* isolates, 60% of ESBL producing isolates, and 80% fluoroquinolone-resistant isolates.[9] Alarmingly, ST131 showed resistance to carbapenems further limiting treatment options for this clone.[10]

Many of the ESBL genes are frequently associated with integron-like elements carried on plasmids, which facilitate their rapid dissemination and often associated with non β-lactam antimicrobial resistance genes.[11] Until now, this *E. coli* O25b-ST131 clone has not yet been reported in Bangladesh. This study was designed to observe the prevalence of CTX-M-15 type of ESBL among uropathogens and concurrently the occurrence of O25b-ST131 clone among *bla*CTX-M-15 producing *E. coli* isolates.

MATERIALS AND METHODS

Samples

This cross sectional study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Dhaka Medical College, Dhaka, Bangladesh. A total of 800 consecutive urine samples were collected from urinary tract infected patients of different age and sex attending either at the outpatient departments or admitted in the Dhaka Medical College Hospital, Dhaka, Bangladesh from January 2014 to December 2014. Informed written consent was taken from each participant prior to collecting samples.

Bacterial isolation

Urine samples were collected following clean catch mid-stream urine collection technique. Two ml of urine sample was transferred aseptically from each sample into a sterile test tube for culture. A 10 ml aliquot of urine was centrifuged at 3000 RPM for five minutes. The supernatant was discarded and the sediment was remixed by tapping the base of the tube. One drop of the well mixed sediment was placed on a clean labeled glass slide, covered with a clean cover slip and then examined under light microscope. All those urine samples having a pus cell count ≥5 pus cells/HPF were inoculated onto the Blood agar and MacConkey agar media by the semi-quantitative culture technique using the calibrated wire loop (0.001 ml). These plates were routinely incubated at 37°C aerobically and after overnight incubation, they were checked for bacterial growth. The sample plate which yielded a colony count of $\geq 10^5$ colony-forming units (cfu)/ml of urine was considered as suggestive of significant bacteriuria. The uropathogens were further identified by observing colony morphology, hemolytic criteria, staining characters, pigment production, motility and other relevant biochemical characteristics as per standard microbiological techniques.[12]

Antimicrobial susceptibility testing and screening of ESBL producers

All bacterial isolates were tested for antimicrobial susceptibility by the Kirby Bauer modified disc diffusion technique on Mueller-Hinton agar media as described by the Clinical and Laboratory Standard Institute.[13] *E. coli* ATCC 25922 was used as a control strain to assess the performance of the method. The antibiotics tested were ceftazidime, ceftriaxone, cefotaxime, amoxicillin-clavulanic acid, ciprofloxacin, gentamicin, co-trimoxazole, azithromycin, nitrofurantoin, and imipenem. All the isolates showing resistance to 3rd generation cephalosporins, particularly ceftazidime, ceftriaxone and cefotaxime, were further tested by phenotypic techniques to confirm β-lactamase production.[13]

Detection of ESBL producers by double disk synergy (DDS) test

ESBL producers were further confirmed for ESBL production by DDS test as described previously.[14] Amoxicilin-clavulanic acid disk was placed at the center of the inoculated Mueller-Hinton agar plate and third generation cephalosporins (ceftriaxone, ceftazidime and cefotaxime) were placed 15 mm apart from center of the amoxicilin-clavulanic acid disk. After incubation at 37ºC for 24 hours, if there was clear augmentation of any of the cephalosporin inhibition zone towards amoxicilin-clavulanic acid disk, an organism was classified as having an ESBL producing phenotype.

Amplification of ESBL genes

Isolates positive for ESBL production were

screened by PCR using primers specific for the detection of *bla*CTX-M-15 and *bla*OXA-1 genes.[15,16] Presence of class 1 integron among the phenotypically confirmed ESBL producers was also determined by PCR.[17] For class-1 integron, the primers used were IntI1F/IntI1R for amplifying the integrase gene *intI1*. Template DNA for PCR was prepared by boiling method. Details of primer sequences and expected PCR product sizes are shown in Table 1.

PCR was done in a 25 µl reaction mixture containing 12.5 µl of master mix (mixture of dNTP, *taq* polymerase $MgCl_2$ and PCR buffer), 2 µl of each forward and reverse primers (Promega Corporation, USA), 2 µl of DNA template and 6.5 µl of nuclease free water in a PCR tube. Amplification of DNA was performed using Mastercycler Personal Thermal Cycler (Mastercycler gradient, Eppendorf AG, Germany). The thermal cycling conditions were: initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 1 min, primer annealing at 55°C (for both *bla*CTX-M-15 and *bla*OXA-1), or 50°C (for class 1 integron) for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. The amplified DNA was analyzed by 1.5% agarose gel-electrophoresis at 100 volts for 35 mins, stained with 1% ethidium bromide and visualized under UV light.

Detection of O25b-ST131 clone by PCR

E. coli isolates that produce *bla*CTX-M-15 were tested by PCR specific to the detection of the pabB gene developed by Clermont *et al*[18] to determine whether they belonged to the O25b-ST131 clone. Primers 025pabBspe.F and O25bpabBspe.R were used to amplify a 347 bp fragment of the pabB gene. Details of primer sequences are shown in Table 1. Amplification was carried out according to the following thermal cycling conditions: initial denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 50 sec, annealing at 65°C for 60 sec, extension at 72°C for 2 min, and final extension at 72°C for 5 min.

Phylogenetic analysis

For the detection of phylogenetic group, all O25b-ST131 positive isolates were screened by an established triplex PCR-based method[19] that uses a combination of three DNA markers (*chuA*, *yjaA* and TspE4.C2). The primers and sizes of the expected amplification product are listed in Table 1. The PCR steps were: initial denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 30 sec, annealing at 59°C for 30 sec, extension at 72°C for 30 sec, and final extension at 72°C for 7 min. Band patterns on agarose gels were then analyzed to determine the phylogenetic group (A, B1, B2, and D) according to the dichotomous decision tree (Fig. 1).

*DNA sequencing of bla*CTX-M-15 *and bla*OXA-1

PCR products were purified using kit (FAVOGEN, Biotech. Corp) and sequencing was done by capillary method (ABI 3500).

Data analysis

Data were analyzed by using Microsoft Excel

TABLE 1: Selected primers used in this study

Primer name	Primer sequence (5′-3′)	Size of product (bp)	References
*bla*CTX-M-15	F-CACACGTGGAATTTAGGGACT R-GCCGTCTAAGGCGATAAACA	996	15
*bla*OXA-1	F-ACCAGATTCCAACTTTCAA R-TCTTGGCTTTTATGCTTG	598	16
IntI1	F-GGCATCCAAGCAGCAAGC R-AAGCAGACTTGACCTGAT	variable	17
025pabBspe	F-TCCAGCAGGTGCTGGATCGT R-GCGAAATTTTTCGCCGTACTGT	347	18
ChuA	F-GACGAACCAACGGTCAGGAT R-TGCCGCCAGTACCAAAGACA	279	19
YjaA	F-TGAAGTGTCAGGAGACGCTG R-ATGGAGAATGCGTTCCTCAAC	211	19
TspE4.C2	F-GAGTAATGTCGGGGCATTCA R-CGCGCCAACAAAGTATTACG	152	19

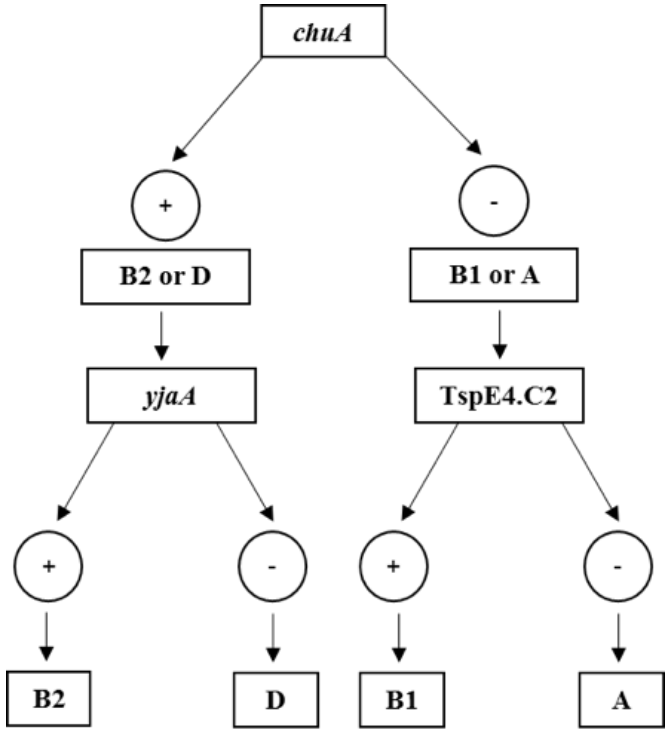

FIG. 1: Dichotomous decision tree to determine the phylogenetic group of an *E. coli* strain by using the results of PCR amplification of the *chuA*, *yjaA* genes, and DNA fragment TspE4.C2

(2007) software (Microsoft, Redmond, WA, USA).

RESULTS

Out of the 800 urine samples, 320 samples were positive for significant pus cells (pus cells ≥5/HPF), which were selected for culture. A total of 150 (46.8%) of the 320 samples were culture positive. Among the 150 culture positive cases, 67% were from female patients and 33% were from male patients. The frequency of UTI in the case of female patients was found highest (45%) in the age group of 16-30 years followed by 38% in the age group of 31-45 years. In the case of males, the majority (32%) of UTI occurred in the age group of >60 years. Of the 150 isolates, 138 (92%) were gram negative bacilli and 12 (8%) were gram positive cocci (Table 2). Among the isolated 138 gram negative bacilli, 45 (32.6%) ESBL producers were detected by DDS test (Table 3). All (100%) the ESBL producers were found resistant to ceftriaxone, ceftazidime, and cefotaxime but all were sensitive to imipenem (Table 4). Thirty-six (80%) ESBL producing bacteria were positive for *bla*CTX-M-15 and 18 (40%) were positive for *bla*OXA-1 with consensus primers by PCR amplification and sequencing (Table 5) (Fig. 2). Fifteen isolates (33.3%) contained both *bla*OXA-1 and *bla*CTX-M-15, 24 (53.4%) contained single ESBL gene, either *bla*OXA-1 or *bla*CTX-M-15, and 6 (13.3%) had neither *bla*OXA-1 nor *bla*CTX-M-15. Among the phenotypic-positive ESBL producing organisms, 30 (66.7%) isolates were positive for class 1 integron. Class 1 integron was found in 28 (77.8%) of the 36 *bla*CTX-M-15 positive strains and 10 (55.6%) of the 18 *bla*OXA-1 positive strains. Combination of ESBL encoding genes and class 1 integron was found in 28 (62.2%) of the ESBL producers, with a combination of *bla*CTX-M-15 and class 1 integron being predominant (40%) (Table 6). Of the 31 *bla*CTX-M-15 positive *E. coli* isolates, 22 (71%) were positive for O25b-ST131 clone (Fig. 3). All (100%) clone positive isolates belonged to phylogenetic group B2.

DISCUSSION

Multidrug resistance is a major problem in the management of uropathogens.[20] Microorganisms responsible for UTIs, specifically *E. coli* and *Klebsiella* species are often associated with

TABLE 2: Isolated bacteria from urine culture

Bacteria	Number (%)
E. coli	90 (60.00)
Klebsiella spp.	20 (13.33)
Citrobacter spp.	10 (6.67)
Pseudomonas spp.	8 (5.33)
Proteus spp.	5 (3.33)
E. fergusonii	2 (1.33)
Acinetobacter spp.	1 (0.67)
Enterobacter spp.	2 (1.33)
Staphylococcus aureus	8 (5.33)
CONS*	4 (2.67)
Total	150 (100)

*CONS= Coagulase negative *Staphylococcus*

production of ESBLs. Therapeutic options for ESBL producers have become drastically limited.[21] Recently, *bla*CTX-M-15 positive *E. coli* O25b-ST131 clone has been reported from all over the world which is frequently associated with UTIs. The continuous monitoring and rapid detection of these increasingly prevalent microorganisms is essential to limit their spread and also to establish antimicrobial stewardship guidelines.

In concordance with other studies[22-24] *E. coli* was found to be the most frequently identified uropathogen followed by *Klebsiella* in this study. UTI was found more common in females and highest frequency was observed in 16-45 years in comparison to other studies.[24,25] The reasons for women to be more susceptible to UTI are due to shortness of urethrae, close proximity of urethrae with vagina and anus which makes them more prone to get infection from perineal colonization and after sexual intercourse.[26] In males, the incidence of UTI was more (32%) in the age group of >60 years. The chances of UTI increases in men with advanced age may

TABLE 3: Distribution of ESBL producing gram negative bacilli identified by DDS test

Gram negative bacilli	ESBL producers by DDS test	
	Positive (%)	Negative (%)
E. coli (n=90)	34 (37.8)	56 (62.2)
Klebsiella spp. (n=20)	6 (30.0)	14 (70.0)
Citrobacter spp. (n=10)	3 (30.0)	7 (70.0)
Pseudomonas spp. (n=8)	1 (12.5)	7 (87.5)
Proteus spp. (n=5)	1 (20.0)	4 (80.0)
E. fergusonii (n=2)	0 (0)	2 (100)
Acinetobacter spp. (n=1)	0 (0)	1 (100)
Enterobacter spp. (n=2)	0 (0)	2 (100)
Total (n=138)	45 (32.6)	93 (67.4)

TABLE 4: Antimicrobial drug resistance among different species of ESBL producers

Antibiotics	*E. coli* (n=34)	*Klebsiella* (n=6)	*Citrobacter* (n=3)	*Pseudomonas* (n=1)	*Proteus* (n=1)	Total (n=45)
Amoxicilin-clavulanic acid	32 (94.1)	6 (100)	3 (100)	1 (100)	1 (100)	43 (95.5)
Ceftriaxone	34 (100)	6 (100)	3 (100)	1 (100)	1 (100)	45 (100)
Ceftazidime	34 (100)	6 (100)	3 (100)	1 (100)	1 (100)	45 (100)
Cefotaxime	34 (100)	6 (100)	3 (100)	1 (100)	1 (100)	45 (100)
Imipenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ciprofloxacin	32 (94.1)	6 (100)	3 (100)	1 (100)	1 (100)	43 (95)
Cotrimoxazole	34 (100)	6 (100)	3 (100)	1 (100)	1 (100)	45 (100)
Gentamicin	30 (88.2)	6 (100)	2 (66.6)	0 (0)	0 (0)	38 (84.4)
Azithromycin	31 (91.1)	4 (66.6)	1 (33.3)	0 (0)	1 (100)	37 (82.2)

TABLE 5: ESBLs encoding genes among various species of phenotypically positive ESBL producers

Gram negative bacteria	*bla*CTX-M-15	*bla*OXA-1
E. coli (n=34)	31 (91.2)	16 (47.1)
Klebsiella spp. (n=6)	4 (66.7)	2 (33.3)
Citrobacter spp. (n=3)	1 (33.3)	0 (0)
Pseudomonas spp. (n=1)	0 (0)	0 (0)
Proteus spp. (n=1)	0 (0)	0 (0)
Total (n= 45)	36 (80)	18 (40)

*figures in parentheses represent percentage

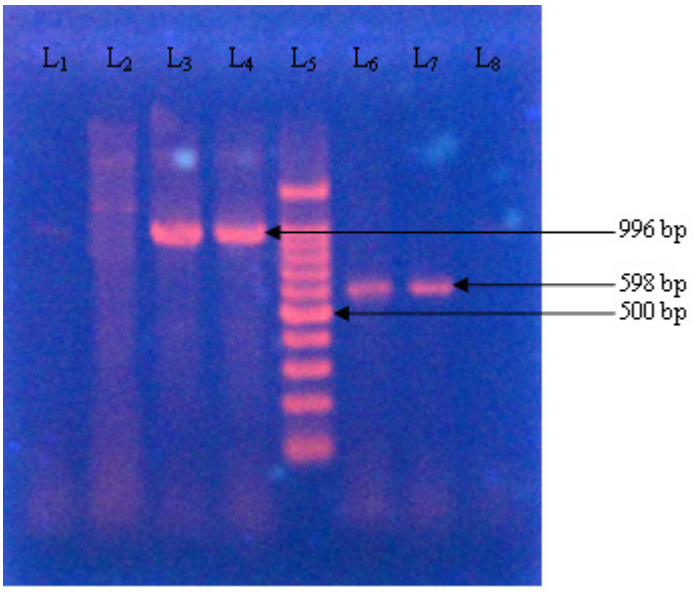

FIG. 2: Photograph of gel electrophoresis of amplified DNA of 996 bp for *bla*CTX-M-15 gene (Lane 3 and 4). Amplified DNA of 598 bp for *bla*OXA-1 gene (Lane 6 and 7). Hundred bp DNA ladder (Lane 5). Negative control *Escherichia coli* ATCC 25922 (Lane 2)

TABLE 6: Distribution of *bla*CTX-M-15, *bla*OXA-1, and class 1 integron among phenotypic-positive ESBL producers

Genotype	Number (%)
*bla*CTX-M-15 + *bla*OXA-1 + class 1 integron	10 (22.2)
*bla*CTX-M-15 + *bla*OXA-1	5 (11.1)
*bla*CTX-M-15 + class 1 integron	18 (40.0)
*bla*CTX-M-15	3 (6.7)
*bla*OXA-1	3 (6.7)
Class 1 integron	2 (4.4)
Absence of examined genes	4 (8.9)

be due to prostate enlargement and decrease of bacteriostatic prostatic secretions.[27]

Our study reported 32.6% ESBL producers resembling those reported by Farzana R *et al* in Bangladesh (32%)[28] but unlike those reported in another study in Bangladesh (40.9%)[29] and in India (66.9%).[30] The possible explanation behind the variation of results from different studies may be that the overall prevalence of ESBLs production varied considerably in different geographical areas and in different institutes.[31]

Most of the ESBL producing organisms were found to be co-resistant to ciprofloxacin, co-trimoxazole, and gentamicin, which correlate with previous studies.[28,32] This was due to the genes encoding these β-lactamases were often located on large plasmids that also encode genes for resistance to other antibiotics, including aminoglycosides, tetracycline, sulfonamides, trimethoprim, quinolones, and chloramphenicol.[33] Like other studies[28,34] all the ESBL producers were found sensitive to imipenem.

Recent reports have shown a rapid and alarming dissemination of CTX-M-15 type ESBL in certain countries including India and have become the most prevalent ESBL-type worldwide. The majority of them are now recovered from *E. coli* isolates causing UTIs.[35,36] We also found *bla*CTX-M-15 to be the most prevalent ESBL, as it was detected in 91.2% of the phenotypic positive ESBL producing urinary *E. coli* isolates (Table 5) which corroborates with contemporary findings from around the world.

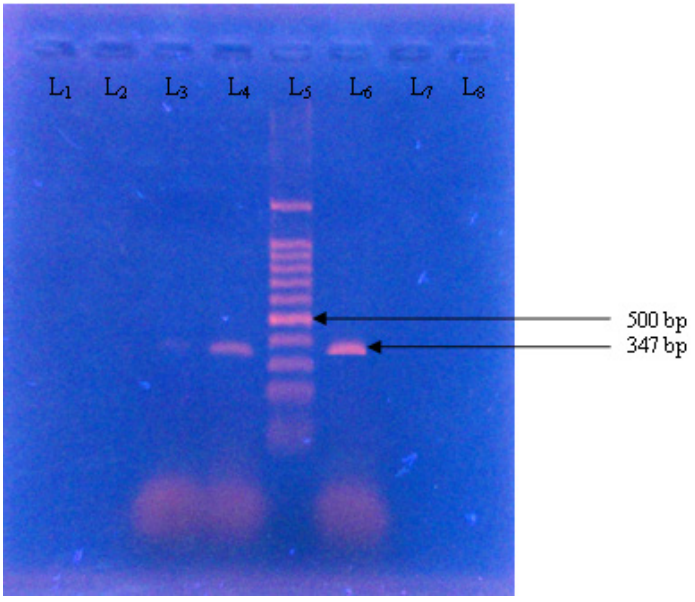

FIG. 3: Photograph of amplified gene for *E. coli* O25b-ST131 clone. Hundred bp DNA ladder (Lane 5), amplified DNA of 347 bp for *E. coli* O25b-ST131 clone (Lane 4 and 6), negative control *Escherichia coli* ATCC 25922 (Lane 3)

The concomitant presence of class 1 integron (66.7%) among the ESBL producers was found. Existence of ESBL genes in plasmids from bacteria are responsible for hospital outbreaks and often associated with class 1 integron.[37] ESBL genes and class 1 integron were shown in various combinations in the present study.

Among 45 ESBL producers, 15 (33.3%) strains simultaneously produced both *bla*CTX-M-15 and *bla*OXA-1. Bacteria producing CTX-M-15 often associated with carriage of other β-lactamase such as TEM-1 and OXA-1.[7] Previous studies in Bangladesh revealed 31% to 80% ESBL producers contained both *bla*CTX-M-15 and *bla*OXA-1[28,38] which is in agreement with present finding. In this study, 13.3% phenotypically positive ESBL strains lacked *bla*CTX-M-15 and *bla*OXA-1, which might be due to the presence of other variants of ESBL genes in the studied isolates.

In the current study, out of 31 *bla*CTX-M-15 positive *E. coli* isolates, 22 (71%) of them were identified as O25b-ST131 clone of B2 phylogroup by PCR. These findings strongly coincide with recent studies done in Riyadh (66.7%) and India (70%).[36,39] It was reported that CTX-M-15 producing *E. coli* of O25b-ST131 lineage, were positive among 88% CTX-M-15 positive *E. coli* isolates.[40] Returning travelers from Indian subcontinent, Africa and the Middle East carry the high risk of being affected by UTI which is primarily due to *bla*CTX-M-15 producing *E. coli* ST131 clone found in a Canadian research.[41,42] Our data confirm other reports of global dissemination of *bla*CTX-M-15 carrying *E. coli* O25b-ST131 clone. Our study also demonstrates the striking predominance of this clonal group in Bangladesh.

Conclusion

Our study demonstrates a high (32.6%) prevalence of ESBL producing urinary isolates with *bla*CTX-M-15 enzyme being the predominant ESBL. In addition, our preliminary study showed that the O25b-ST131 clone is widely distributed among *E. coli* isolates causing UTIs in Bangladesh. These findings indicate a significant public health threat and therefore a need for additional studies to determine the distribution and risk factors for acquisition of this clone so that effective control measures can be devised.

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