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Molecular characterization of extended spectrum β-lactamase producing bacteria isolated from urinary tract infected patients, Bangladesh

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Abstract. The aims of the study were to examine the presence of extended spectrum β-lactamase (ESBL) producing pathogen in urinary tract infected (UTI) patients and their respected molecular characterization and classification. The isolates collected from UTI patients attending a private hospital during the period between January and June, 2012, were biochemically identified and subjected to double disc synergy method for the detection of ESBL. ESBL genes were detected by multiplex PCR and antibiotic sensitivity test was performed. Thirty two percent of all Gram negative isolates were found as ESBL producer. Among 65 ESBL positive isolates, 77% were *Escherichia coli*, 20% *Klebsiella pneumoniae* and 3% were *Pseudomonas* spp. Around 48% isolates were found carrying at least one of the four ESBL genes, $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV} and bla_{OXA}, and were found in 32%, 23%, 18.5% and 3% of the isolates respectively. In antibiotic sensitivity assay, higher resistance was found in *E. coli* than *K. pneumonia* against ciprofloxacin and nalidixic acid. Interestingly, two *E. coli* and three *K. pneumonia* strains were found resistant to only 3rd generation cephalosporines, but susceptible to all other antibiotics assessed. One *E. coli* strain was found resistant to ciprofloxacin but sensitive to nalidixic acid. *Pseudomonas* spp. was found resistant to most of the antibiotics. The susceptible rate to nitrofurantoin, amikacin, and gentamicin was also not satisfactory. Susceptibility (100%) to meropenem and imipenem render these as good alternatives to treat UTI. The majority of the isolates were positive for $bla_{\text{CTX-M}}$ and adverted to molecular class A. Two strains carrying bla_{OXA} gene along with bla_{SHV}/bla_{TEM}/$bla_{\text{CTX-M}}$, could not be included in any of the established ESBL classification.

INTRODUCTION

Extended spectrum cephalosporins (3rd generation) e.g., ceftriaxone, cefotaxime and ceftazidim acquired widespread clinical use in the early 1980s. These were developed due to the increasing prevalence of ampicillin-hydrolysing β-lactamases (TEM-1, TEM-2 and SHV-1) in Enterobacteriaceae, non-glucose fermenting Gram negative bacilli and some respiratory pathogens such as *Haemophilus influenza* and *Moraxella catarrhalis*. In 1983, a β-lactamase was documented in strains of *Klebsiella pneumonia* from Germany, which was capable to hydrolyze extended-spectrum cephalosporins (Knothe *et al.*, 1983). Their spectrum of activity against oxyimino cephalosporins named these enzymes as extended spectrum β-lactamases (ESBLs); also defined as β-lactamases those are capable to hydrolyze penicillins, broad- and extended-spectrum cephalosporins and monobactams and are inhibited by clavulanic acid (Philippon *et al.*, 2002).

ESBLs carry a number of mutations that permit them to hydrolyze expanded-spectrum β-lactam antibiotics. Most of the ESBLs are derivatives of TEM (named after the patient from whom it was first isolated, Temoniera)

or SHV (Sulfhydryl reagent variable) enzymes (Bush *et al.*, 1995). There are now more than 90 TEM-type and more than 25 SHV-type β-lactamases existing. In both of these groups of enzymes, at selected loci within the gene, a few point mutations result in the extended-spectrum phenotype. TEM- and SHV-type ESBLs are mostly found in *E. coli* and *K. pneumoniae*; however, they have also been reported in *Proteus* spp., *Providencia* spp., and other genera of *Enterobacteriaceae* (Bradford, 2001). CTX-M (first isolated in Munich), a new family of plasmid-mediated ESBLs has also been arisen in recent years that preferentially hydrolyze cefotaxime. They have been found mainly in strains of *Salmonella enteric* serovar Typhimurium and *E. coli*, but also documented in other species of *Enterobacteriaceae* (Bonnet *et al.*, 2000). Another growing family of ESBLs is the OXA-type enzymes. These β-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d (Bush *et al.*, 1995).

A small number of ESBLs have been found which are not closely allied to any of the established families of β-lactamases. In strains of *P. aeruginosa*, the PER-1 (Pseudomonas extended resistant) type β-lactamase was first revealed, which was isolated from patients in Turkey (Nordmann *et al.*, 1993). Another enzyme, somewhat related to PER-1, are VEB-1 (Vietnam extended-spectrum β lactamase) (Poirel *et al.*, 1999); which was initially found in *E. coli* isolated from a patient in Vietnam, but also subsequently found in *P. aeruginosa* reported from a patient in Thailand (Naas *et al.*, 1999); CME-1, was isolated from *Chryseobacterium meningosepticum* (Rossolini *et al.*, 1999); TLA-1, reported in an *E. coli* isolate from a patient in Mexico (Silva *et al.*, 2000).

Currently two classification schemes for β-lactamases are in use, molecular classification (Ambler, 1980) and functional classification which was initially proposed by Bush in 1989 (Bush, 1989) and expanded in 1995 (Bush *et al.*, 1995). The classification scheme was updated by Bush and Jacoby in 2010 where new functional subgroups have been added as a result of identification and expansion of major β-lactamase families in which variants continue to be identified on a regular basis (Bush & Jacoby, 2010). The molecular classification divides β-lactamases into classes A, B, C, and D on the basis of, amino acid sequence and utilizing serine or zinc ions for substrate hydrolysis. The updated functional classification scheme is based on the substrate and inhibitor profiles and comprises group 1 (class C) cephalosporinases; group 2 (classes A and D) broad-spectrum, inhibitor-resistant, and extended-spectrum β-lactamases and serine carbapenemases; and group 3 metallo β-lactamases (Bush & Jacoby, 2010).

Urinary tract infections cause serious health problems (Warren *et al.*, 1999) and are the second most common type of infection in humans. In suspected UTI patients, antibiotic treatment was usually started before the results of the urine culture were available (Al-Tawfiq & Anani, 2009). Unfortunately, antibiotic resistance has become an alarming problem in many countries.

Since the development in 1960s, fluoroquinolones have been frequently recommended for infectious disease. Infectious Disease Society of America (IDSA) included fluoroquinolones together with trimethoprim-sulfamethoxazole (TMP-SMZ) among the antibiotics for urinary tract infection (Warren *et al.*, 1999). Despite this, TMP-SMZ and fluoroquinolone resistance has been reported in urinary tract infection in many parts of world (Zhanel *et al.*, 2005). As an alternative drug, ampicillin has been frequently used for urinary tract infection as it acts against Gram-negative micro-organisms. Even then, as acquired resistance to β-lactam class antibiotics was found (Neu, 1992), β-lactamase stable cephalosporins were developed. Thus the choice of antibiotics for urinary tract infection was extended (Naber, 1989). But after the report of ESBL-producing organisms in Germany, anxiety over the emergence of cephalosporin resistant bacteria has been rising (Knothe *et al.*, 1983).

The aim of the present study was to examine the presence of extended spectrum β-lactamase (ESBL) producing pathogen in urinary tract infected patients, attended at an urban hospital in Dhaka city, Bangladesh, the presence of ESBL genes in ESBL positive Gram negative bacteria by phenotypic method. Furthermore to classify these isolates according to Bush & Jacob β-lactamase classification scheme. The objective of the study was also to evaluate antibiotics which can be used in patient health care for the treatment of UTIs caused by ESBL producing Gram negative bacteria.

MATERIALS AND METHODS

Sample Collection and Identification of bacterial isolates

During the period of six months between January and June, 2012, a total of 1 006 midstream urine samples were collected form patients having symptoms of UTI attending a private hospital in Dhaka, Bangladesh. Information of the patients e.g., age, sex etc. as well as clinical history were documented properly at the microbiology laboratory of the hospital. Urine samples were collected by mid-stream clean catch urine and were examined under microscope to observe the presence of RBC, yeast, pus cells and were measured if any.

The urine samples were streaked on UTI Chrome agar (Himedia, India) by semi quantitative streaking method for quantification of bacterial load in urine. The inoculated plates were incubated at 37°C and after overnight incubation, plates were examined for growth and cfu was calculated. Pure isolated colonies were picked and subjected to various biochemical test (e.g. motility test, synthesis of oxidase, catalase and urease, utilization of citrate, fermentation of glucose and lactose, production of indole etc.) for further identification purpose.

ESBL detection by Double disc synergy Method

All the Gram negative isolates were tested for detection of ESBL by Double disc synergy test described by Jarlier (Jarlier *et al.*, 1988). The test organism was inoculated onto a Mueller-Hinton Agar plate. Then, two antimicrobial disks were placed 20 mm apart from center to center on the inoculated Mueller-Hinton Agar plate. One disc contained amoxicillin/clavulanic acid (beta lactamase inhibitor) and the other one an expanded-spectrum cephalosporin (e.g., ceftriaxone, cefotaxime or ceftazidime). If after 24 hour of incubation, the zone of inhibition in between the discs was enhanced, the test was taken as positive. The enhancement was the result of inhibition of ESBL by clavulanic acid (provided by the amoxicillin/ clavulanic acid) and the subsequent expanded-spectrum cephalosporin action. For the detection of ESBLs, the test remains a reliable method. Antimicrobial discs (OXOID, UK) used in the study were ceftazidime (CAZ) 30 µg, ceftriaxone (CRO) 30 µg from the group of cephalosporin and amoxicillin/clavulanic acid (AMC) 30 µg as augmentin (20 µg amoxicillin and 10 µg clavulanic acid) and placed 20mm apart center to center, from each other.

Antimicrobial susceptibility testing

The ESBL positive isolates found during the study period, were also subjected to antibiotic sensitivity test using disc diffusion technique by 'Kirby- Bauer method' (Bauer *et al.*, 1966), against different antimicrobial agents : ciprofloxacin (CIP), nitrofurantoin (NF), nalidixic acid (NA), gentamicin (CN), amikacin (AK), imipenem (IPM), meropenem (MEM).

Molecular detection of ESBL genes:

Extraction of DNA

The strains were grown on 5.0 ml of Luria-Bertani (LB) broth. After 18 to 24 hours growth, cells were harvested from the broth and were subjected to alkaline cell lysis. Chromosomal DNA was extracted by phenol: chloroform: isoamylalcohol extraction method (Chowdhury *et al.*, 2000). Dried DNA pellet was dissolved in 30 µL TE buffer and stored at -20°C.

Target genes for ESBL and PCR primers
Detection of ESBL genes of the phenotypically ESBL positive isolates was performed by multiplex PCR using four primer pairs targeting bla_{CTX}, bla_{OXA}, bla_{SHV} and bla_{TEM}. The multiplex PCR reaction was performed choosing primer pairs generating PCR products of different sizes which could easily be separated and distinguished by agarose gel electrophoresis. Primer pair used in the study were: bla_{CTX}-F: 5'-CGC TGTTGTTAGGAAGTGTG-3' bla_{CTX}-R: 5'-GGCTGGGTGAAGTAAGTGAC-3' (GenBank Accession No: DQ303459), bla_{OXA}-F: 5'-ATGGCGATTACTGGATAGATGG-3' bla_{OXA}-R: 5'-AGTCTTGGTCTTGGTTGTGAG-3' (GenBank Accession No: L07945), bla_{SHV}-F: 5'-CGCCTGTGTATTATCTCCCT-3' bla_{SHV}-R: 5'-CGAGTAGTCCACCAGATCCT-3' (GenBank Accession No: EF125011), bla_{TEM}-F: 5'-TTTCGTGTCGCCCTTATTCC-3' bla_{TEM}-R: 5'-ATCGTTGTCAGAAGTAAGTTGG-3' (GenBank Accession No: AB282997) with expected amplicon size 569 bp, 701 bp, 293 bp and 403 bp respectively. PCR was performed in 30 µl reaction mixture containing 1 µl of template DNA, 0.3 µl of 2 U/µl DyNAzyme™ DNA polymerase, 3 µl of 10× buffer for DyNAzyme, 0.6 µl of a mixture of deoxyribonucleoside triphosphates (25 mM of each), and 0.8 µl of 25 µM solution of each primer (Sigma-Aldrich, Germany). The thermocycler conditions with a PTC 200, Peltier thermal cycler (MJ Research, USA) were as follows: a regime of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min for 30 cycles, with an initial denaturation at 96°C for 4 min and a final 7 min extension at 72°C.

RESULTS

Among 1006 urine samples, 182 samples were found UTI positive. In these patients, urinary tract infection was confirmed by the positive urine culture with 10^5cfu/ml. From these 182 UTI positive samples a total of 203 gram negative bacterial isolates was collected and all these were subjected to double disc synergy test (DDST). In DDST, 65 isolates (32%) showed positive result indicating as effective ESBL producer.

These 65 isolates were collected from 63 patients, of which 63.5% were outdoor patients and 36.5% were indoor patients. Out of these 63 patients' 40 were female and 23 were male. According to the biochemical test, out of 65 isolates, 50 isolates (77%) were identified as *E. coli*, 13 isolates (20%) as *K. pneumoniae* and only two isolates (3%) as *Pseudomonas* spp. (Table 1).

In antibiotic sensitivity test, different strains of *E. coli*, *K. pneumonia* showed different resistance pattern against different antibiotics. Strains of *Pseudomonas* spp. were found highly resistant to most of the antibiotic. But all these species were found 100% sensitive to imipenem and meropenem, carbapenem group of antibiotics. Resistance pattern of the isolates has been presented in Figure 1.

Table 1. Details of ESBL positive strains

ID of isolates	Age	sex	Ip/op	Identified Organisms	ESBL genotype	Antibiotic resistance pattern
apmb123du	61	M	OP	*E. coli*	$bla_{CTX\text{-}M}$	CIP, NA, CN, CRO, AMC, CAZ
apmb124du	82	F	IP	*E. coli*	$bla_{CTX\text{-}M}$	CIP, NA, AK,CRO, CAZ
apmb126du	62	F	OP	*E. coli*	bla_{TEM}/$bla_{CTX\text{-}M}$	CIP, NA, AK, NA, CRO, AMC, CAZ
apmb128Edu	93	F	IP	*E. coli*	–	CIP, NA, AK, CRO, AMC, CAZ
apmb128Kdu				*Klebsiella pneumoniae*	bla_{SHV}	NF, NA, CRO, AMC, CAZ

apmb129du	67	F	IP	*Klebsiella pneumoniae*	bla_{SHV}/bla_{TEM}	NA, CRO, CAZ
apmb1210du	2	M	IP	*Klebsiella pneumoniae*	$bla_{SHV}/bla_{TEM}/bla_{CTX\text{-}M}$	NF, CIP, NA, AK, CN, CRO, AMC, CAZ
apmb1212Kdu	56	F	IP	*Klebsiella pneumoniae*	$bla_{SHV}/bla_{TEM}/bla_{CTX\text{-}M}$	NF, CIP, NA, AK, CN, CRO, AMC, CAZ
apmb1215du	8	M	OP	*E. coli*	–	CIP, NA, CRO, CAZ
apmb1218du	65	F	OP	*E. coli*	–	CIP, NA, CRO,
apmb1219du	56	F	IP	*E. coli*	$bla_{CTX\text{-}M}$	CIP, NA, CRO, CAZ
apmb1220du	17	F	IP	*E. coli*	$bla_{CTX\text{-}M}$	NF, CIP, NA, CN, CRO, AMC, CAZ
apmb1221du	59	F	OP	*E. coli*	$bla_{CTX\text{-}M}$	CIP, NA, CRO, AMC, CAZ
apmb1222du	62	F	OP	*E. coli*	$bla_{TEM}/bla_{CTX\text{-}M}$	NF, CIP, NA, CN, CRO, AMC, CAZ
apmb1223du	39	F	OP	*E. coli*	$bla_{CTX\text{-}M}$	CIP, NA, CRO, AMC, CAZ
apmb1224du	11 m	M	OP	*Klebsiella pneumoniae*	bla_{SHV}/bla_{TEM}	NF, NA, CN, CRO, AMC, CAZ
apmb1226du	75	M	IP	*E. coli*	–	CIP, NA, CRO
apmb1227du	76	M	OP	*Klebsiella pneumoniae*	bla_{SHV}/bla_{TEM}	CIP, NA, CRO, AMC, CAZ
apmb1228du	1	M	OP	*E. coli*	$bla_{CTX\text{-}M}$	CIP, NA, CRO, CAZ
apmb1229du	51	F	OP	*E. coli*	$bla_{CTX\text{-}M}$	CIP, CN, CRO, AMC, CAZ
apmb1230du	37	F	OP	*E. coli*	$bla_{SHV}/bla_{TEM}/bla_{CTX\text{-}M}$	NA, CRO, CAZ
apmb1232du	62	F	OP	*E. coli*	–	CIP, NA, CN, CRO, AMC, CAZ
apmb1233du	27	F	OP	*E. coli*	–	CIP, NA, CRO, AMC, CAZ
apmb1234du	1	F	OP	*E. coli*	–	CIP, NA, CN, CRO, AMC, CAZ
apmb1236du	30	M	OP	*E. coli*	–	CIP, NA, CN, CRO, AMC,
apmb1238Edu	10 month	F	OP	*E. coli*	$bla_{SHV}/bla_{TEM}/bla_{CTX\text{-}M}$	CRO
apmb1238Kdu				*Klebsiella pneumoniae*	bla_{SHV}/bla_{OXA}	CRO
apmb1241du	66	M	OP	*E. coli*	$bla_{SHV}/bla_{TEM}/bla_{CTX\text{-}M}$	CIP, NA, CRO, AMC, CAZ
apmb1242du	7 month	M	IP	*Klebsiella pneumoniae*	–	CRO, CAZ
apmb1243du	40	F	IP	*E. coli*	–	CIP, NA, CRO, CAZ
apmb1245du	60	F	IP	*E. coli*	–	CIP, NA, CRO, AMC, CAZ
apmb1246du	65	F	IP	*E. coli*	–	CIP, NA, CRO
apmb1248du	40	M	OP	*E. coli*	–	CIP, NA, CRO, AMC, CAZ
apmb1249du	90	M	IP	*E. coli*	–	NF, CIP, NA, CRO
apmb1250du	69	F	OP	*E. coli*	–	CIP, NA, CRO
apmb1252du	29	M	OP	*Klebsiella pneumoniae*	–	NF, CIP, NA, CRO, CAZ

apmb1254du	61	F	OP	*E. coli*	–	NF, CIP, NA, CN, CRO, AMC, CAZ
apmb1256du	60	F	OP	*E. coli*	$bla_{\text{CTX-M}}$	NF, CIP, NA, CRO, AMC, CAZ
apmb1260du	29	F	IP	*E. coli*	$bla_{\text{CTX-M}}$	CIP, NA, CN, CRO, AMC, CAZ
apmb1261du	48	F	IP	*E. coli*	–	CIP, NA, CN, CRO, AMC, CAZ
apmb1262du	26	M	OP	*E. coli*	$bla_{\text{CTX-M}}$	NF, CIP, NA, CN, CRO, AMC, CAZ
apmb1263du	46	F	IP	*E. coli*	bla_{TEM}	NF, NA, CRO, AMC, CAZ
apmb1265du	42	F	IP	*E. coli*	–	CIP, NA, CRO, AMC, CAZ
apmb1266du	51	F	OP	*E. coli*	–	CIP, NA, CRO, AMC, CAZ
apmb1267du	62	M	OP	*Pseudomonas* spp.	–	NF, CIP, NA, CN, CRO, AMC, CAZ
apmb1268du	23	F	OP	*E. coli*	–	CIP, NA, CRO, CAZ
apmb1269du	56	M	OP	*Klebsiella pneumoniae*	–	NF, CN, CRO, AMC
apmb1270du	45	F	IP	*E. coli*	$bla_{\text{CTX-M}}$	CIP, NA, NF, CRO
apmb1271du	6	M	OP	*E. coli*	–	CIP, NA, CRO, CAZ
apmb1272du	37	F	OP	*E. coli*	–	CIP, NA, CN, CRO, AMC, CAZ
apmb1273du	74	F	OP	*E. coli*	–	CIP, NA, CRO, AMC, CAZ
apmb1274du	71	F	OP	*E. coli*	bla_{TEM}/$bla_{\text{CTX-M}}$/ bla_{OXA}	CIP, NA, CRO
apmb1275du	43	F	IP	*E. coli*	–	CIP, NA, CRO, AMC
apmb1276du	72	F	OP	*E. coli*	–	CIP, NA, CRO
apmb1277du	9 m	M	OP	*Klebsiella pneumoniae*	bla_{SHV}	CAZ
apmb1278du	43	M	OP	*E. coli*	–	CRO
apmb1279du	2	M	OP	*Klebsiella pneumoniae*	bla_{SHV}/bla_{TEM}	NF, CIP, NA, AK, CN, CRO, CAZ
apmb1282du	60	F	OP	*E. coli*	–	CIP, NA, CRO, AMC, CAZ
apmb1283du	61	F	IP	*Pseudomonas* spp.	–	NF, CIP, NA, CN, CRO, AMC
apmb1284du	104	M	IP	*E. coli*	bla_{TEM}	CIP, NA, CRO, AMC, CAZ
apmb1285du	52	M	OP	*E. coli*	–	CIP, NA, CN, CRO
apmb1286du	13	F	IP	*E. coli*	bla_{TEM}	CIP, NA, CRO, AMC, CAZ
apmb1287du	79	F	OP	*E. coli*	–	CIP, NA, CRO
apmb1288du	31	F	OP	*E. coli*	$bla_{\text{CTX-M}}$	CIP, NA, CRO, CAZ
apmb1290du	79	M	IP	*Klebsiella pneumoniae*	–	CIP, NA, CRO, AMC, CAZ

Abbreviations: ciprofloxacin (CIP), nitrofurantoin (NF), nalidixic acid (NA), gentamicin (CN), amikacin (AK), imipenem (IPM), meropenem (MEM).ceftazidime (CAZ), ceftriaxone (CRO) and amoxicillin/clavulanic acid (AMC).

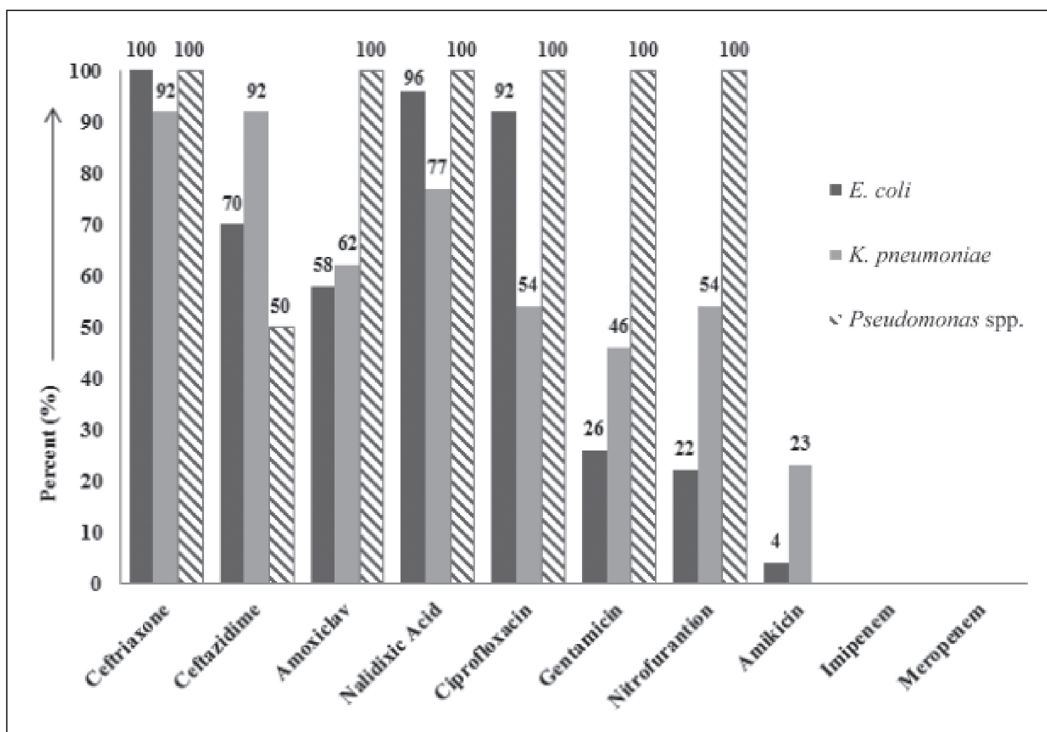

Figure 1. The antibiotic resistance pattern of ESBL producing isolates collected from urine culture to different antibiotics.

Among 65 isolates, 31 were (around 48%) found carrying at least one of the four genes assessed.

Twenty one isolates (32%) were found positive for $bla_{\text{CTX-M}}$ gene, of which 18 were *E. coli* and three were *K. pneumonia*. Fifteen (23%) isolates (eight *E. coli* and seven *K. pneumonia*), were found positive for bla_{TEM}; and 12 (18.5%) isolates (two *E. coli* and ten *K. pneumonia*) were found carrying bla_{SHV}. Only two (3%) isolates (one *E. coli* strain and one *K. pneumonia*) were found bla_{OXA} positive (Figure 2). Several isolates were found to harbour more than one among four genes assessed (Table 2). No *E. coli* strains were found to carry bla_{SHV} alone; rather the gene was present along with other ESBL genes. But in the case of *K. pneumonia*, bla_{SHV} was seen alone and also in combination with other genes. Thirteen *E. coli* strains and three *E. coli* strains were found to carry $bla_{\text{CTX-M}}$ and $bla_{\text{TEM-}}$ alone, but these two genes were seen only in combination with other genes in *K. pneumonia*. Three *E. coli* isolates and three *K. pneumonia* isolates were found to habour three ESBL genes simultaneously, indicating highly resistant bacterial strains. No *Pseudomonas* spp. was found possessing any of the ESBL genes assessed in the study.

DISCUSSION

The prevalence of ESBL-producing organisms is increasing throughout the world. In Asia, the prevalence of ESBL-producing strains in *Enterobacteriaceae* differs from country to country and from species to species (Bell *et al.*, 2002).

There has been limited study on ESBL producing organisms in Bangladesh. The first reported study was conducted by Rahman *et al.* (2004) in urine, sputum, pus, throat swab, etc. and reported 43.2% of *E. coli* and 39.5% of *K. pneumonia* were found to be ESBL producer. Studies in Dhaka city found that 31.9% of uropathogenic *E. coli* isolates (Islam *et al.*, 2015), 25% of the Gram negative isolates (Farzana *et al.*, 2013) and 40.9% of

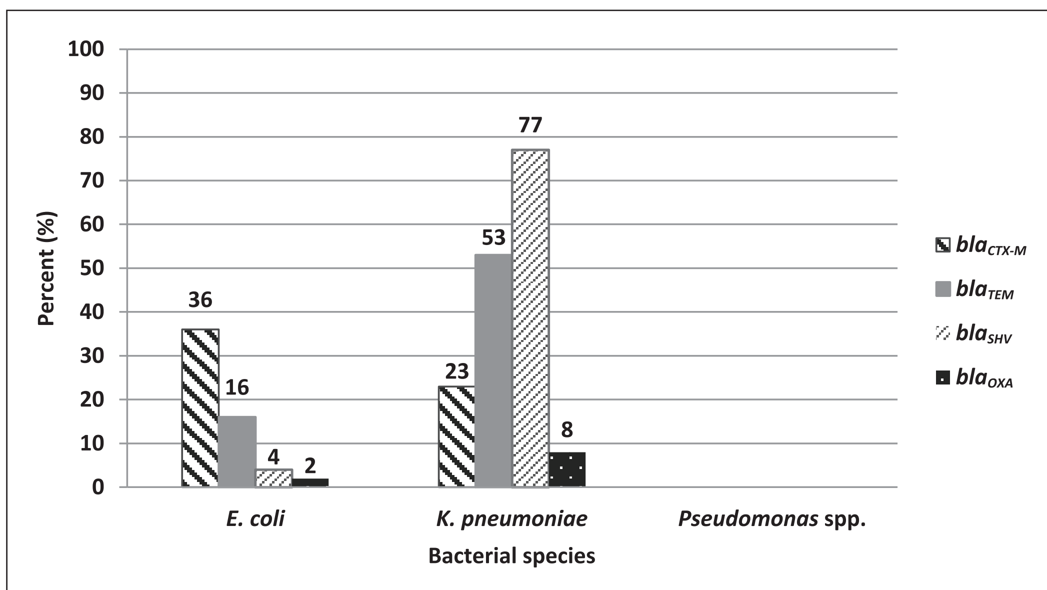

Figure 2. Presence of different ESBL genes in different bacterial species.

Table 2. Presence of different β-lactamase genes in the UTI isolates

Name of the genes	Number of isolates harboring β-lactamase genes			Molecular classification (Bush & Jacoby, 2010)
	E. coli	*K. pneumoniae*	*Pseudomonas* spp.	
bla_{SHV}	–	2	–	Molecular Class A
bla_{TEM}	3	–	–	Molecular Class A
$bla_{CTX\text{-}M}$	13	–	–	Molecular Class A
bla_{SHV}+bla_{OXA}	–	1	–	–
bla_{SHV}+bla_{TEM}	–	4	–	Molecular Class A
bla_{TEM}+$bla_{CTX\text{-}M}$	2	–	–	Molecular Class A
bla_{SHV}+bla_{TEM}+$bla_{CTX\text{-}M}$	2	3	–	Molecular Class A
bla_{TEM}+bla_{OXA}+$bla_{CTX\text{-}M}$	1	–	–	–

E. coli strains (Masud *et al.*, 2014) were ESBL producers. The present study found 32% of the isolated gram negative strains as ESBL producer. In Mymensingh and Chittagong, other cities of Bangladesh, studies found 71.3% of the clinical isolates (Yasmin, 2012) and 55.56% Gram-negative bacteria (Karim Chowdhury *et al.*, 2015) were ESBL producer respectively. Another study from Rajshahi city, revealed ESBL characteristics in 57.89% *Klebsiella* spp., 50.0% *Proteus* spp., 47.83% *E. coli* and 31.35% *Pseudomonas* spp. (Haque & Salam, 2010). This study has revealed that the percentage of ESBL producer was less than 50% in Dhaka city, whereas in most of the other studies outside Dhaka city, e.g. Mymensingh, Rajshahi, Chittagong, percentage was higher than 50%.

In this study, among 63 patients, 64% was female and 36% was male. All these patients were infected with ESBL producing bacterial species. It is already known that UTI is more common in female than in male (Yamamoto *et al.*, 1997), so in this study, higher percent of female UTI patients and infection caused by ESBL producer could not be correlated. The reasons behind higher percentage of UTI in female are, female have shorter

urethra than men, menstrual bleeding, fecal contamination from the perineum (Kumar *et al.*, 2006). The enteric bacteria are mostly found as the causative agent of UTIs, accounting uropathogenic *E.coli* (UPEC) for about 80% of the cases. Other bacterial species are *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., *Pseudomonas* spp., *Staphylococcus saprophyticus* and *Enterococcus* spp. (Ronald, 2003). In this study, *E. coli* was responsible for 77% of the cases, *Klebsiella* spp. and *Pseudomonas* spp. were for 20% and 3% cases respectively.

In multiplex PCR assay, $bla_{\text{CTX-M}}$ type was found in isolates with highest percent followed by bla_{TEM}, bla_{SHV} and bla_{OXA}. Around 77% of *Klebsiella* spp. and 42% of *E. coli* isolates were found to carry at least one of the ESBL genes assessed, whereas *Pseuodomonas* spp. was found to carry none of the four ESBL genes (Table 2, Figure 2). Many isolates were found to contain multiple genes. According to many previously published studies performed on ESBL genotype at various regions, most of the ESBL genes have been found in *E. coli*, *K. pneumoniae*, and other *Enterobacteriaceae*, but the OXA-type ESBLs have mostly been found in *P. aeruginosa* (Hall *et al.*, 1993, Mugnier *et al.*, 1998, Danel *et al.*, 1999). In the present study bla_{OXA} was detected either in *E. coli* or in *K. pneumoniae*, moreover *Pseuodomonas* spp. was found negative for any of the ESBL genes as observed by PCR.

In Bangladesh, a study reported, $bla_{\text{CTX-M-1}}$ was present in 100% ESBL producing clinical isolates, bla_{TEM} and $bla_{\text{OXA-1}}$ in 82.5% and 47.5% isolates respectively (Lina *et al.*, 2014). Farzana R *et al.* (2013) found that 76% and 43% of the ESBL producer were $bla_{\text{CTX-M}}$ and bla_{OXA} positive respectively, where $bla_{\text{CTX-M}}$ was predominantly found in *E. coli* and bla_{OXA} in *Pseudomonas* spp. (Farzana *et al.*, 2013). Yasmin T *et al.* (2012) observed bla_{TEM}, $bla_{\text{CTX-M}}$ and bla_{SHV} β-lactamases genes in 50.5%, 46.7% and 18.7% isolates respectively. In these three studies, $bla_{\text{CTX-M}}$ was found in significantly higher percent than the rest of the genes, similar to the present study. However, bla_{OXA} was also found in *E. coli* and *K. pneumoniae* instead of *Pseudimonas* spp. unlike other studies previously mentioned.

According to the Ambler molecular and Bush *et al.*, 1995 (Bush & Jacoby, 2010) classification scheme for bacterial β-lactamases, the $bla_{\text{CTX-M}}$, bla_{SHV} and/or bla_{TEM} positive isolates in this study, have been included in the molecular class A and functional group 2be as these strains were resistant to extended spectrum β-lactamase. The classification method placed the bla_{OXA} positive isolates into molecular class D. But in this study two strains have been found to carry bla_{OXA}, among these one was also positive for bla_{SHV} and another one was positive for bla_{TEM} and $bla_{\text{CTX-M}}$. These findings made us difficult to assign them in any of the established classification. Among these two, one was *E. coli* and another was *K. pneumonia* strain (Table 2).

The present study shows about 52% of the 65 phenotypically ESBL positive isolates was found carrying none of the ESBL genes assessed. The phenotypic ESBL positive isolate devoid of relevant genes assessed, may possess some other genes (e.g. PER-1, VEB-1, CME-1, TLA-1) not included in this study or may possess other mechanism involved in their resistance yet uncharacterized.

It is plenary that patients having infections caused by an ESBL-producing organism are at an enraged risk of treatment failure with an expanded-spectrum β-lactam antibiotic. *E. coli* isolates showed higher resistant rate against ceftriaxone, ceftazidime, amoxicillin-clavulanic acid, nalidixic acid and ciprofloxacin than *K. pneumonia*. On the other hand, *K. pneumonia* strains were found to be more resistant against gentamicin, nitrofurantoin and amikicin. Interestingly, two *E. coli* strains and three *K. pneumonia* strains were found resistant to only 3rd generation antibiotics, e.g. ceftriaxone and/or ceftazidime, but susceptible to all other antibiotics assessed. One *E. coli* isolates was also found to be resistant to ciprofloxacin but sensitive to nalidixic acid (Table 1). Ciprofloxacin is an antibiotic of 2nd generation quinolone group and nalidixic

acid is a 1st generation antibiotic of quinolone group. This *E. coli* strain was possibly resistant to nalidixic acid previously and reverted the resistant mode and became sensitive as now-a-days. In current days, Nalidixic acid is not commonly used to treat UTI or maybe it was never resistant to nalidixic acid and only inherited resistant mode against ciprofloxacin. *Pseudomonas* spp. was found highly resistant to all the antibiotics except amikicin, meropenem and imipenem. Antibiotic susceptibility test of the ESBL isolates revealing their high resistance rate to ciprofloxacin and nalidixic acid, render these antibiotic no more appropriate for the treatment of urinary tract infection. The susceptibility rate to nitrofurantoin and gentamicin was also not satisfactory. Though 96% *E. coli* and 100% *Pseudomonas* spp. were susceptible to amikacin but susceptible rate of *K. pneumonia* (77%) was not acceptable. However, all the strains of *E. coli*, *K. pneumoniae* and *Pseudomonas* spp. were found susceptible to meropenem and imipenem and hence render the carbapenem group of antibiotic as a good alternate choice for the treatment for UTI. But to ensure the use of a specific group of antibiotic for a certain type of infection, large-scale study on a huge population encompassing wide and broad area as much as possible, is needed. It is also necessary to keep continuous monitoring for the sensitivity pattern against first line antibiotics, as the bacterial strains may acquire resistant gene from the surrounding environment and become resistant to that particular antibiotic against which it was sensitive previously.

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