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Original Article

Phenotypic and Genotypic Characterization of Extended-spectrum beta-lactamase Producing Escherichia coli and Klebsiella species Isolated from a Tertiary Care Hospital in Bangladesh

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Key word: ESBL Producing *E. coli* and *Klebsiella* spp.

Abstract:
This study was conducted to determine the presence of ESBL producing Escherichia coli and Klebsiella species in a tertiary care hospital of Bangladesh, as well as to observe the patterns of antibiotic resistance and antibiotic resistance genes among them. A total of 166 Escherichia coli,Klebsiella pneumoniae and Klebsiella oxytoca were isolated from urine, wound swab, pus, sputum and blood samples of patients of Dhaka Medical College Hospital. Antibiotic susceptibility test wasperformed by disk-diffusion technique. ESBL producers were detected phenotypically by Double-disk synergy (DDS) test. Genotypically ESBL genes (blaCTX-M-15, blaOXA-1) among the ESBL producers with presence of class 1 integron among them were detected by PCR. Eighty seven (52.41%) ESBL producers were detected by DDS test. CTX-M-15 (80.46%) was the dominant genotype in ESBL producing strains detected by PCR. Class 1 integron was found in 58 (66.67%) of the phenotypic positive ESBL producers. The results of this study showed high proportion of ESBL producing Escherichia coli and Klebsiella species in Bangladesh.

Key word: Extended-spectrum beta-lactamase; *Escherichia coli; Klebsiella* species;

Introduction:

Gram negative bacteria intrinsically can produce both Various resistance mechanisms facilitate the emergence and spread of multidrug-resistant phenotypes of *Escherichia coli* and *Klebsiella* species[1]. Among gram negative organisms, beta-lactamase production represents the single greatest contributor to beta-lactam resistance, including resistance to the oxyimino-cephalosporins and carbapenems[2]. ESBLs confer resistance to penicillins, first, second and third generation cephalosporins as well as aztreonam (but not cephamycins or carbapenems) and inhibited by beta-lactamase inhibitors such as clavulanic acid[3]. The CTX-M family is known to be the most dominant non-TEM, non-SHV ESBL among *Enterobacteriaceae* and is recognized as a rapidly growing family of ESBLs that selectively prefer to hydrolyze cefotaxime rather than ceftazidime[4]. Currently the most widely distributed CTX-M enzyme is CTX-M-15[5]. OXA-1 has been described as themost common OXA-type beta-lactamse[6]. The continuous monitoring and rapid detection of these resistant determinants may reduce their spread and play a vital role in infection control. Therefore, the present study has been carried out to detect ESBL genes in *Escherichia coli* and *Klebsiella* species isolated from various clinical samples of patients of Dhaka Medical College Hospital in Bangladesh.

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Materials and Methods

Bacterial isolates

A cross-sectional study was conducted in the Department of Microbiology of Dhaka Medical College, Dhaka, Bangladesh, during January 2015 to December 2015. This research protocol was approved by the research review committee and ethical review committee of Dhaka Medical College. Written informed consent was taken from each patient. Escherichia coli, Klebsiella pneumoniae and Klebsiella oxytoca were isolated from 340 urine, wound swab, pus, sputum and blood samples of clinically suspected infected patients of in-patient and out-patient departments of Dhaka Medical College Hospital, irrespective of age and sex. All samples were inoculated in blood agar and MacConkey agar media and incubated at 37oC aerobically for 24 hours. Incubated plates

were then examined for the presence of colonies of bacteria. Primary blood culture was done in Trypticase Soy Broth then subculture was done on blood agar and MacConkey agar media. Escherichia coli, Klebsiella pneumoniae and Klebsiella oxytoca were identified by colony morphology, hemolytic criteria, staining character, pigment production and biochemical tests as per standard techniques[7].

Antimicrobial susceptibility testing

Isolated Escherichia coli, Klebsiella pneumoniae and Klebsiella oxytoca were tested for antimicrobial susceptibility by disk diffusion method following the guidelines of Clinical and Laboratory Standards Institute[8], using commercially available antibiotic disks (Oxoid Ltd, Basingstoke, United Kingdom). Antibiotic disks such as ceftazidime (30µg), cefuroxime (30 µg),ceftriaxone (30µg), cefoxitin (30 µg), cefepime (30 µg), imipenem (10µg), amoxiclav (amoxicillin and clavulanic acid) (20/10µg), ciprofloxacin (5µg), amikacin (30 µg), gentamicin (10 µg), colistin (10µg), sulfamethoxazole-trimethoprim (1.25/23.75µg), tigecyline (15µg) were used. Mueller-Hinton agar media was used for antimicrobial susceptibility test. Criteria of the United States Food and Drug Administration was used for interpretation of zone of inhibition of tigecycline[9]. Escherichia coli ATCC 25922was used as control strain for susceptibility test. 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-disk synergy (DDS) test for detection of ESBL producing organism[10]

Using sterile cotton swab, test inoculums (compared with McFarland standard) were inoculated in Mueller-Hinton agar plate. Third generation cephalosporins (ceftriaxone, ceftazidime and cefotaxime) were placed 20 mm apart from center of the amoxiclav disk. Inoculated plate was incubated at 37°C for 24 hours. A clear extension of the edge of the inhibition zone of cephalosporin disks towards amoxiclav disk was interpreted as positive for ESBLs production.

Detection of ESBL encoding genes

ESBL genes (blaCTX-M-15 and blaOXA-1) among the ESBL producing Escherichia coli, Klebsiella pneumoniae and Klebsiella oxytoca with presence of class 1 integron among them were detected by PCR. To prepare bacterial pellets, a loop full of bacterial colonies was inoculated into a Falcon tube containing Trypticase Soy Broth. After incubation overnight at 37°C, the Falcon tubes were centrifuged at 4,000 rpm for 10 minutes, after which the supernatant was discarded. A small amount of sterile Trypticase SoyBroth was added into the Falcon tubes with pellets and mixed evenly. Then an equal amount of bacterial suspension was placed into 2 to 3 to micro centrifuge tubes. The micro centrifuge tubes were then centrifuged at 4,000 rpm for 10 minutes and the supernatant was discarded. The micro centrifuge tubes containing bacterial pellets were kept at -20°C until DNA extraction. Bacterial DNA was extracted by the boiling method[11]. PCR screening for presence of different genes were performed using primers and conditions described previously[12, 13]. The amplified DNA were loaded into a 1.5% agarosegel, electrophoresed at 100 volts for 35 minutes, stained with 1% ethidium bromide, and visualized under UV light.

Statistical analysis

Data were analyzed by using Microsoft Office Excel (2013) software (Microsoft, Redmond, WA, USA).

Results

One hundred and sixty six Escherichia coli, Klebsiellapneumoniae and Klebsiella oxytoca were isolated from 340 samples. Of them, 57.35% (78/136) isolates were recovered from urine, whilst other sources included, wound swab, pus, sputum and blood. Out of 166 isolates, 106 (40.77%) wereEscherichia coli, 42 (16.15%) were Klebsiellapneumoniae and 18 (6.92%) were Klebsiella oxytoca. Most of the isolates showed high resistance rate to several antimicrobial classes whereas imipenem, colistin and tigecycline were found to be the most effective drugs (Table I).

Table I. Antibiotic resistance pattern of isolated strains (N=166).

Antimicrobial Drugs	*Escherichia coli* (N=106) n (%)	*Klebsiella pneumoniae* (N=42) n (%)	*Klebsiella oxytoca* (N=18) n (%)
Amoxiclav	82 (77.36)	42 (100.00)	18 (100.00)
Cefuroxime	73 (68.87)	38 (90.48)	15 (83.33)
Cefoxitin	40 (37.74)	21 (50.00)	6 (33.33)
Ceftriaxone	86 (81.13)	42 (100.00)	18 (100.00)
Ceftazidime	78 (73.58)	42 (100.00)	18 (100.00)
Cefepime	71 (66.98)	38 (90.48)	17 (94.44)
Gentamicin	88 (83.02)	32 (76.19)	14 (77.78)
Amikacin	75 (70.75)	31 (73.81)	11 (61.11)
Ciprofloxacin	89 (83.96)	35 (83.33)	14 (77.78)
Sulfamethoxazozole-trimethoprim	96 (90.57)	40 (95.24)	17 (94.44)
Imipenem	21 (19.81)	12 (28.57)	4 (22.22)
Colistin	4 (3.77)	0 (0.00)	0 (0.00)
Tigecycline	0 (0.00)	2 (4.76)	0 (0.00)

Note: N =Total number of bacteria; n = Number of resistant bacteria.

Of the 166 isolates, 52.41% (n=87) were suspected to be ESBL producers using DDS test, of which 21.69% (36/166) were isolated from urine, 13.25% (22/166) from wound swab, 8.43% (14/166) from pus, 5.42% (9/166) from sputum and 3.61% (6/166) from blood samples.Sixty one (57.55%) of the 106Escherichia coli, 20 (47.62%) of the 42Klebsiella pneumoniae and 6 (33.33%) of the 18 Klebsiella oxytoca, were ESBL producers.All (100%) the ESBL producing isolateswere resistant to ciprofloxacin and sulfamethoxazole-trimethoprim. Among the isolated ESBL producing Escherichia coli, 88.52% were resistant to cefepime, 86.89% to amikacin, 88.52% to gentamicin and 6.56% to colistin. All (100%) were sensitive to cefoxitin, imipenem and tigecycline. All (100%) the ESBL producing Klebsiella pneumoniae were resistant to gentamicin, 85% to cefepime, 90% to amikacin and 10% to tigecycline. All (100%) were sensitive to cefoxitin, imipenem and colistin. All (100%) the ESBL producing Klebsiella oxytoca were resistant to gentamicin and 66.67% to cefepime, 83.33% to amikacin. All (100%) were sensitive to cefoxitin, imipenem, colistin and tigecycline.

PCR for ESBL specific genes showed that blaCTX-M-15 type and blaOXA-1 type genes were present in 70 (80.46%) and 36 (41.38%) isolates, respectively (Table II). Out of 87 ESBL producing isolates,58 (66.67%) were positive for class 1 integron.Class 1 integron was found in 46 (65.71%) of blaCTX-M-15 positive strains and 24 (66.67%) of blaOXA-1 positive strains.

Table II. Genetic study of ESBL producing isolates.

ESBL producing organisms	ESBL encoding genes	
	CTX-M-15 n (%)	OXA-1 n (%)
Escherichia coli (N=61)	56 (91.80)	35 (57.38)
Klebsiella pneumoniae (N=20)	13 (65.00)	1 (5.00)
Klebsiella oxytoca (N=6)	1 (16.67)	0 (0.00)
Total (N=87)	70 (80.46)	36 (41.38)

Note: N= Total number of bacteria;
n= Number of ESBL genes carrying bacteria;

Discussion

The emergence of ESBL producing Escherichia coli,Klebsiella species has been reported in many parts of the world[14, 15]. The present study showed that around 57.55% Escherichia coli, 47.62% Klebsiella pneumoniae and 33.33% Klebsiella oxytoca were ESBL producers which is higher than a previous study conducted in Bangladesh[16]. ESBL producers are increasing with time in Bangladesh which might be due to indiscriminate and inappropriate use of antibiotics.In the present study, ESBLproducers showed high resistance rate to ciprofloxacin, sulfamethoxazole-trimethoprim, cefepime, amikacin and gentamicin.ESBL producing bacteria which were resistant to non-beta-lactam antibiotics may carry genes encoding resistance to aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole[17]. All ESBL-producing strains were susceptible to carbapenems, the current drug of choice for the treatment of patients infected with multidrug-resistant ESBL-producing bacteria. In the present study among 87 ESBL producing strains,blaCTX-M-15 was identified as the predominant genotype (n= 70, 80.46%). Fifty six (91.80%) of the 61 ESBL producing Escherichia coli, were positive forblaCTX-M-15 gene.Previous reports within or outside of Bangladesh showed a high prevalence of CTX-M-15 groupin ESBL producingEscherichia coli[18, 19]. The high prevalence of CTX-M-15 type ESBL among Escherichia coli identified in these studies reflect the global spread of blaCTX-M-15 type ESBL in Escherichia coli. A previous study determined the complete sequence of three plasmids that encode CTX-M ESBLs within three different lineage of clone ST131 and showed that IncFII plasmids harboring blaCTX-M-15, blaOXA-1, blaTEM, aac (6i)-Ib-cr and aac (3)-II have played a crucial role in the rapid global spread of CTX-M-15 beta-lactamases in Escherichia coli[20]. Further analysis is needed in Bangladesh to investigate the dissemination of this clone in future.Present study observed 36 (41.38%) OXA-1 producers among the 87 ESBL producing Escherichia coli, Klebsiella pneumoniae and Klebsiella oxytoca. A recent study in Bangladesh reported that 40% OXA-1 producers isolated from urine, which was close to the present findings[21]. Using specific primers for blaCTX-M-15 and blaOXA-1, present study could not detect any ESBL genes in 17 (19.54%) 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[17]. The reason for the absence of blaCTX-M-15 and blaOXA-1 in those 17 phenotypic positive ESBL producers might be due to the presence of other variants of ESBL genes. Hence, we examined the presence of class 1 integron. In our study, out of 87 ESBL producingstrains, 66.67% (n=58) isolates harbored class 1 integron. Concurrent presence of integrons and ESBL genes in the same isolates probably due to genetic linkage between them[22].

In conclusion, the present study reflected that the proportion of blaCTX-M-15among Escherichia coli and Klebsiella species are increasing in Bangladesh. CTX-M-15was mainly found in Escherichia coli. Imipenem was found to be most effective drug for the treatment of infection by ESBL

producers.High prevalence of ESBL encoding genes in Escherichia coli and Klebsiella species possibly reflects the overuse and misuse of antibiotics in Bangladesh and severely limits the therapeutic options in Bangladesh. Prompt and accurate detection of drug resistant bacterial strains will prevent their spread and in vitro resistance patterns of these strains will guide the clinicians to the use of appropriate antibiotics.

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