

ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF EXTENDED SPECTRUM β -LACTAMASE PRODUCING GRAM-NEGATIVE BACTERIA ISOLATED FROM WOUND AND URINE IN A TERTIARY CARE HOSPITAL, DHAKA CITY, BANGLADESH

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Abstract. From a total of 320 bacterial samples from wound swab and urine 169 (53%) gram-negative bacteria were isolated, of which 42 (25%) extended-spectrum beta-lactamase (ESBL) producers were detected by double-disk synergy test. ESBL producers were significantly more resistant against amoxiclav, Co-trimoxazole, ciprofloxacin, amikacin and gentamicin than non-ESBL producers. Among the 42 ESBL producers, 76% were positive for *bla*_{CTX-M} and 43% were positive for *bla*_{OXA}, with *bla*_{CTX-M} predominantly (97%) observed in *E. coli* and *bla*_{OXA} predominantly (80%) in *Pseudomonas* spp. Class 1 integron was found in 75% of *bla*_{CTX-M} positive and 56% of *bla*_{OXA} positive strains. Combinations of ESBL genes and class 1 integron were observed in 29 (69%) of the ESBL producers. The findings of this study infer that CTX-M and OXA producers are emerging in Bangladesh and we report the presence of *bla*_{OXA} for the first time in Bangladesh.

Keywords: ESBL, antimicrobial resistance, gram-negative bacteria, Bangladesh

INTRODUCTION

The emergence of extended-spectrum β -lactamases (ESBLs) in gram-negative bacteria has increased in recent years, which has led to global concern regarding the management of bacterial infections (Paterson, 2006; Bourjilat *et al*, 2011). Though the prevalence of ESBL producers varies with geographical areas and time, a relatively high prevalence rate of ESBL producers in the Asia-Pacific area were

documented by several surveillance studies (Bell *et al*, 2002; Hirakata *et al*, 2005). Previous studies in Bangladesh reported the prevalence of ESBL producers ranging from 23.2% to 80% (Islam, 2008; Biswas, 2009). ESBLs compromise the effects of penicillin, first-, second-, third-generation cephalosporins and monobactams but do not affect cephamycin or carbapenem (CDC, 2012). Currently, over 300 different ESBLs have been identified in gram-negative bacilli (Rawat and Nair, 2010). At present, *bla*_{CTX-M} is considered as the most frequent type of ESBLs worldwide. Another growing family of ESBLs, oxacillin hydrolyzing (OXA) types have been reported, which are not usually inhibited

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by clavulanic acid except OXA-18 and OXA-45 (Philippon *et al*, 1997; Toleman *et al*, 2003; Rawat and Nair, 2010). Many of the ESBL genes are frequently found within integron-like elements carried on plasmids, which facilitate their rapid dissemination and often associated with non- β -lactam antimicrobial resistance genes (Villa *et al*, 2000; Yao *et al*, 2007).

The present study was designed to investigate the prevalence of ESBL producers from wound and urine samples in Dhaka Medical College hospital, Bangladesh and to identify the distribution of *bla*_{CTX-M} and *bla*_{OXA} among them. Our study also determined the antimicrobial resistance patterns among the ESBL and non-ESBL producers.

MATERIALS AND METHODS

Bacterial isolates

A total of 320 samples were collected during July 1, 2010 to June 30, 2011 from the Department of Microbiology, Dhaka Medical College, Bangladesh. Approval was obtained from research review committee (RRC) and ethical review committee (ERC) of Dhaka Medical College according to the Declaration of Helsinki and national and institutional standards. Written consent was obtained from all participants. One hundred and seventy wound and 150 urine samples were studied. Gram-negative bacteria were examined phenotypically for ESBLs production and then for the presence of *bla*_{CTX-M} and *bla*_{OXA} by PCR among the phenotypic-positive ESBL producers.

Isolation of gram-negative bacteria

All the wound swabs and urine samples were inoculated on blood agar and MacConkey agar and incubated at 37°C aerobically for 24 hours. Incubated

plates were then examined for the presence of bacterial growth. Organisms were identified by colony morphology, hemolytic criteria, staining character, pigment production and biochemical tests (Baron *et al*, 1994).

Antimicrobial susceptibility testing and screening of ESBL producers

According to CLSI guidelines, the antimicrobial susceptibility pattern was determined by disk-diffusion technique using commercially available antibiotic disks (Oxoid, Hampshire, UK) (CLSI, 2009). *Escherichia coli* ATCC 25922 was used for quality control. ESBL producers were screened by disk-diffusion method using ceftazidime, cefotaxime, ceftriaxone and aztreonam. If the isolates are resistant to any of these drugs, they are considered as suspected ESBL producers (CDC, 2012).

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

ESBL producers were further confirmed for ESBL production by DDS test as described previously (Jarlier *et al*, 1988). Amoxiclav disk was placed at the center of the inoculated Mueller-Hinton agar plate. Third generation cephalosporins (ceftriaxone, ceftazidime and cefotaxime) were placed 15 mm apart from center of the amoxiclav disk. After incubation at 37°C for 24 hours, a clear extension of the edge of the inhibition zone of cephalosporins disks towards amoxiclav disk is interpreted as ESBL producer.

Amplification of ESBL genes

Phenotypic-positive ESBL producing isolates were examined for the presence of CTX-M and OXA genes by PCR. Presence of class 1 integron among the phenotypically confirmed ESBL producers also was determined by PCR. Genomic DNA was extracted by boiling method.

Table 1
Antimicrobial resistance patterns of ESBL ($n=42$) and non-ESBL ($n=127$) producing gram-negative bacilli.

Antibiotic	% ESBL resistant	% non-ESBL resistant	Chi-square value	Difference (p)
Amoxiclav	93	62	14.1	$p<0.001$
Co-trimoxazole	95	53	23.8	$p<0.001$
Ciprofloxacin	93	69	9.4	$p<0.01$
Gentamicin	81	67	3	$p<0.1$
Amikacin	64	39	8.4	$p<0.01$

The following pairs of previously used primers were employed: for *bla*_{CTX-M} SC-SATGTGCAGYACCAGTAA (forward) and CGCCRATATGRTTGGTGGTG (reverse) (S=G or C, Y=C or T, R=A or G); for *bla*_{OXA} ACACAATACATATCAACTTCGC (forward) and AGTGTGTTAGAATG-GTGATC (reverse) and for class 1 integron GGCATC CAAGCAGCAAGC (forward), and AAGCAGACTTGACCTGAT (reverse) (Khan *et al*, 2009; Vranic-Ladavac *et al*, 2010). Primers that were used for amplifying *bla*_{CTX-M} could not amplify the coding sequence of CTX-15 and CTX-28 (Vranic-Ladavac *et al*, 2010). Amplification was performed at 95°C for 10 minutes followed by 30 cycles of 95°C for one minute, at 61°C for *bla*_{CTX-M} 57°C for *bla*_{OXA} and 58°C for class 1 integron for 45 seconds, and at 72°C for 90 seconds, and final heating at 72°C for 10 minutes. The amplified DNA was analyzed by 1.5% agarose gel-electrophoresis at 100 volts for 35 minutes, stained with 1% ethidium bromide and visualized under UV light (Vranic-Ladavac *et al*, 2010).

Data analysis

Data were analyzed using Microsoft Excel (2007) and comparisons were performed using chi-square test.

RESULTS

Out of the 320 samples, 169 (53%) gram-negative bacteria were isolated, comprising 90 (53%) *E. coli*, 25 (15%) *K. pneumoniae*, 15 (9%) *Proteus* spp and 39 (23%) *Pseudomonas* spp.

From the isolated gram-negative bacteria, 42 (25%) were ESBL producers as detected by DDS test. Twenty nine (32%) ESBL producing *E. coli*, 5 (20%) *K. pneumoniae*, 3 (20%) *Proteus* spp and 5 (13%) *Pseudomonas* spp were identified among the isolated organisms.

All the ESBL producers were resistant to amoxicillin, aztreonam, cephadrine, cefuroxime, cefotaxime, ceftriaxone, and ceftazidime. The proportion resistant to the other antimicrobials tested was 95% for Co-trimoxazole, 93% amoxiclav, 93% ciprofloxacin, 81% gentamicin and 64% amikacin. However, all strains were sensitive to imipenem. Resistance of ESBL producers against Co-trimoxazole ($p<0.001$), amoxiclav ($p<0.001$), ciprofloxacin ($p<0.01$), gentamicin ($p<0.1$) and amikacin ($p<0.01$) is statistically different when compared to non-ESBL producing gram-negative bacteria (Table 1).

In this study, 32 (76%) ESBL producers

Table 2
Distribution of ESBL encoding genes among ESBL producers isolated from wound and urine.

Organism	Wound		Urine		Total	
	CTX-M No. (%)	OXA No. (%)	CTX-M No. (%)	OXA No. (%)	CTX-M No. (%)	OXA No. (%)
<i>E. coli</i> (n=29)	16 (55)	7 (24)	12 (41)	5 (17)	28 (96)	12 (41)
<i>K. pneumoniae</i> (n=5)	2 (40)	1 (20)	1 (20)	0 (0)	3 (60)	1 (20)
<i>Proteus</i> spp (n=3)	0 (0)	1 (33)	0 (0)	0 (0)	0 (0)	1 (33)
<i>Pseudomonas</i> spp (n=5)	1 (20)	4 (80)	-	-	1 (20)	4 (80)
Total (n=42)	19 (45)	13 (31)	13 (31)	5 (12)	32 (76)	18 (43)

(-) indicates not applicable

Table 3
Distribution of ESBL encoding genes and class 1 integron among phenotypic-positive ESBL producers.

Genotype	Number (%)
CTX-M + OXA + class 1 integron	9 (21)
CTX-M + OXA	4 (9)
CTX-M + class 1 integron	15 (36)
OXA + class 1 integron	1 (2)
CTX-M	4 (9)
OXA	4 (9)
Class 1 integron	1 (2)
Absence of examined genes	4 (9)

were positive for *bla*_{CTX-M}. Twenty eight (96%) of the 29 ESBL producing *E. coli*, 3 (60%) of the 5 *K. pneumoniae* and 1 (20%) of the 5 *Pseudomonas* spp were CTX-M producers. No ESBL producing *Proteus* spp carried *bla*_{CTX-M}. Eighteen (43%) *bla*_{OXA} ESBLs were identified. Four (80%) of the 5 *Pseudomonas* spp, 12 (41%) of the 29 *E. coli*, 1 (33%) of the 3 *Proteus* spp and 1 (20%) of the 5 *K. pneumoniae* were positive for *bla*_{OXA} (Table 2). Thirteen isolates (31%) contained both *bla*_{CTX-M} and *bla*_{OXA}, 24 (57%) a single ESBL gene, either *bla*_{CTX-M}

or *bla*_{OXA}, and 5 (12%) had neither *bla*_{CTX-M} nor *bla*_{OXA}.

Among the phenotypic-positive ESBL producing organisms, class 1 integron was present in 26 (62%) of the isolates. Class 1 integron was found in 24 (75%) of the 32 *bla*_{CTX-M} positive strains and in 10 (55.6%) of the 18 *bla*_{OXA} positive strains. Combination of ESBL genes and class 1 integron was observed in 29 (69%) of ESBL producers, with a combination of *bla*_{CTX-M} and class 1 integron being predominant (36%) (Table 3).

DISCUSSION

Forty-two (25%) ESBL producing gram-negative bacteria were detected in this study. The higher prevalence of ESBL producers in Asia than in Europe and America was observed in a previous study (Cantón *et al*, 2008). Previous studies in Bangladesh revealed 23% to 31% ESBL producers from gram-negative bacteria (Islam, 2008), which is similar to the present study. However, the proportion of ESBL producers was reported as 80% in another study in Bangladesh (Biswas, 2009). The discrepancy of the findings between the latter and the present study

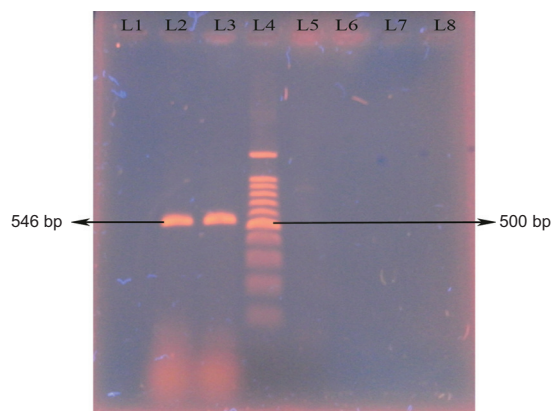


Fig 1—Amplification of $bla_{\text{CTX-M}}$. Hundred bp DNA ladder (lane 4), 546 bp amplicon of $bla_{\text{CTX-M}}$ (lanes 2 and 3), amplicon of *Escherichia coli* ATCC 25922 as negative control (lane 1).

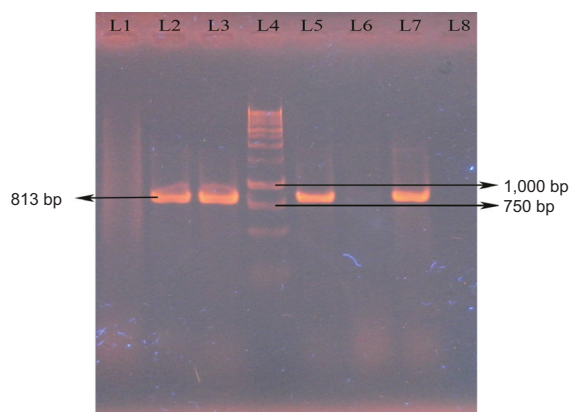


Fig 2—Amplification of bla_{OXA} . One kb DNA ladder (lane 4), 813 bp amplicon of bla_{OXA} (lanes 2, 3, 5 and 7), amplicon of *Escherichia coli* ATCC 25922 as negative control (lane 1).

may be due to the varying prevalence of ESBL producers with time as well as from country to country, city to city and even hospital to hospital in one city.

E. coli (32%) was the predominant ESBL producers in this study, in agreement with a previous report (Islam, 2008). However, differences in ESBL production

among other bacteria species of ESBL producers are not statistically significant.

Significant resistance against amoxicillin, ceftriaxone, aztreonam, Co-trimoxazole, amoxiclav, ciprofloxacin, gentamicin and amikacin was observed compared to non-ESBL producers. In Bangladesh, detection of ESBL producers is not routinely conducted because of deficiency of resources and facilities in most of the laboratories. Significant antimicrobial resistance among the ESBL producers suggests the urgent necessity for inclusion of detection of ESBL producers in routine laboratories. Simultaneous resistant to Co-trimoxazole, ciprofloxacin, gentamicin and amikacin was frequent and the reason might be due to concomitant presence of other drug resistance genes, as described previously (Villa *et al*, 2000; Yao *et al*, 2007).

The present study detected 32 (76%) $bla_{\text{CTX-M}}$ among the phenotypic-positive ESBL producers. Increasing frequency of CTX-M producers among ESBL producers has been reported from America, Africa, Europe and Asia (Radice *et al*, 2002; Jouini *et al*, 2007; Coque *et al*, 2008). The presence of $bla_{\text{CTX-M}}$ in the *E. coli* and *K. pneumoniae* have been described in earlier several studies (Kiratisin *et al*, 2008; Jemima and Verghese, 2008; Goyal *et al*, 2009; Sun *et al*, 2010). The finding of this study indicated that high proportion of CTX-M ESBLs in Bangladesh was not only in *E. coli* (96%) and *K. pneumoniae* (60%) but also in *Pseudomonas* spp (20%).

Eighteen (43%) of the 42 ESBL producers carried bla_{OXA} . Many variants of this type of ESBLs have been identified from Turkey, France and China, predominantly in *P. aeruginosa* and *Acinetobacter* spp, and also in other gram-negative bacteria (Bradford, 2001; Rawat and Nair, 2010). Our study demonstrated that OXA

ESBLs in Bangladesh is predominantly (80%) found in *Pseudomonas* spp. To our knowledge, it is the first report on *bla*_{OXA} in Bangladesh. The absence of ESBL genes (12%) in phenotypic-positive ESBL producer might be due to other genes than *bla*_{CTX-M} and *bla*_{OXA} that were not studied.

The concomitant presence of class 1 integron (62%) among the ESBL producers was found. Many ESBL genes are frequently found within integron-like elements (Yao *et al*, 2007). The existence of ESBL genes in plasmids is associated with nosocomial outbreaks and is often associated with class 1 integron (Villa *et al*, 2000). However, the presence of class 1 integron and ESBL genes in same bacteria could not be established in this study due to lack of laboratory facility. ESBL genes and class 1 integron were shown in various combinations in the present study. Multiple transferable antimicrobial resistance determinants among enteric flora have been demonstrated in Bangladesh, which serve as reservoirs of antimicrobial resistances (Mamun *et al*, 1993). The reason for the presence of more than one antimicrobial resistance determinants might be due to transfer of ESBLs genes via plasmids (Villa *et al*, 2000).

Emergence of ESBLs in gram-negative bacteria reduces therapeutic options. A high prevalence (25%) of ESBL producers creates a need to report ESBL producers routinely in the laboratory, because ESBL producers are significantly resistant to both β -lactams and non- β lactams than non-ESBL producers. Regarding antimicrobial susceptibility, imipenem is the most effective antibiotic for the management of infection by ESBL producers. However, an evaluation of combined therapy is required in order to determine the efficacy of treating infections by ESBL producers.

The finding of this study indicates an urgent surveillance of ESBL producers in larger number of cohorts in order to obtain an accurate prevalence of ESBL producers in Bangladesh. In addition, increasing reports of carbapenem resistant isolates provide further necessity for monitoring and controlling infections caused by ESBL producing bacteria (Meyer *et al*, 2003; Cisneros *et al*, 2005).

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