BETA-LACTAMASE-PRODUCING *ESCHERICHIA COLI* IN BANGLADESH: THEIR PHENOTYPIC AND MOLECULAR CHARACTERISTICS

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Key words: Beta-lactamase, *Escherichia coli*, Molecular characteristics

Abstract

The emergence and rapid dissemination of beta-lactamase-producing *E. coli* is now a worldwide problem. A total of 45 *E. coli* obtained from clinical specimens from a medical service centre in Dhaka were selected for this study. Test *E. coli* exhibited variable resistance to 3rd (71.7 - 97.8%, n = 48) and 4th (78%, n = 48) generation beta-lactam antibiotics, with 72% sensitivity to Carbapenem. Analysis of co-resistance indicated that 33.3% of *E. coli* (n = 48) were co-resistant to beta-lactams and ciprofloxacin. ESBL producers were predominant comprising of 84.7% *E. coli*. Among them, 22.7% contained bla_{TEM}, 24.2% contained bla_{CTX-M}, 4.3% contained bla_{SHV} and 9.1% contained bla_{OXA-1} genes. A total of 25.75% isolates were metallo beta-lactamase producers. Of these, 1.5% of *E. coli* strains contained New Delhi metallo beta-lactamase gene and 6% contained AmpC gene. Multiple beta-lactamase genes were detected in some test isolates; 6.7% isolates contained 4, 20% contained 3 and 73.3% contained 2 beta lactamase genes. Fifty per cent of the *E. coli* contained plasmids of variable sizes. In addition, a total of 39% of the *E. coli* contained Class 1 integron. The increasing trend in beta-lactam resistance is of public health concern as it limits treatment regime and indicates to the need of continuous monitoring of resistance pattern.

Introduction

Beta lactamase resistance in Gram-negative bacteria is increasingly reported in different countries of the world. In Bangladesh, beta-lactam antibiotics are readily available over-the-counter and are routinely used for treating infections. This has led to dramatic increase in resistance to beta-lactam agents. Beta-lactamase encoding genes are plasmid-borne and can degrade a range of antibiotics including penicillins, cephalos-porins, and aztreonam and are inhibited by clavulanic acid (CA)[1]. The detection of beta-lactam related genes is challenging[2] and requires nucleic acid amplification and sequence analysis. In earlier studies researchers had investigated the presence of TEM, SHV, OXA and CTX-type enzymes[3,4] and found CTX-M enzymes as the most prevalent types[4,5]. The genes for beta-lactamase production are usually found in mobile genetic elements[6]. Often, ESBL producers are co-resistant to other antibiotics which is of concern

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in deciding treatment strategies[7]. *Escherichia coli* is responsible for causing diarrhoeal disease and UTI. It is also one of the most prevalent ESBL producer known[8,3], thereby making treatment with beta lactam antibiotics difficult. The present study was undertaken to determine the molecular and phenotypic characteristics of ESBL producing clinical *E. coli* in order to characterize the major determinants for ESBL phenotype.

Materials and Methods

A total of 59 *E. coli* isolated from urine were investigated. *E. coli* ATCC 25922 was used as control for antibiotic sensitivity tests and *E. coli* V517 was used as a positive control for plasmid extraction.

All samples were collected by the trained physicians/phlebotomists after having consent from the patients. Bacteria were isolated from the collected samples as part of the diagnostic tests requested by the patients. Isolated and identified bacteria were given code numbers and stored in glycerol broth at –20°C for this study.

Isolates were identified in the laboratory by their cultural characteristics on MacConkey and Eosin Methylene Blue agar. Biochemical characteristics were determined by using Kligler Iron Agar, Citrate utilization agar, Motility Indole Urea agar, Peptone broth for Indole production and Oxidase test.

Isolates were subject to antimicrobial susceptibility testing by disk diffusion method (Bauer and Kirby 1969) using commercial antibiotic disks. The antibiotic disks used in this study were amoxicillin 30 µg (AML), ampicillin 30 µg (AMP), ceftriaxone 30 µg (CRO), cefepime 30 µg (CPM), cefuroxime 30 µg (CXM), cefexime 30 µg (CEF), cefotaxime 30 µg (CTX) and imipenem 10 µg (IPM).

PCR was used for the detection of beta-lactamase genes. PCR primers used in this study are detailed in Table 1.

PCR amplification cycles were carried out by initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 68°C for 1 min/kb of amplicon and final extension at 68°C for 8 min.

Agarose gels of 2.0 % (w/v) were used for resolution of PCR products.

Plasmid DNA was prepared according to the alkaline lysis method of Birnboim and Doly (1979)[17].

Class 1 integron was detected by PCR by amplification of the integrase gene using intI1 primers (Table 1).

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Table 1. Primers used in this study.

Target gene	Primers	Sequence (5′-3′)	Amplicon size (bp)	Annealing temp. (ºC)	References
Bla$_{tem}$	TEM F	5′-ATGAGTATTCAACATTTCCG-3′	858	55	(9)
	TEM R	5′-CCAATGCTTAATCAGTGAGC-3′		(1 min)	
Bla$_{ctx-M}$	CTXU1	5′-ATGCTGCAGYACCAGTAARGT-3′	593	55	(10)
	CTXU2	5′-TGGGTRAARTARGTSACCAGA-3′		(40 sec)	
Bla$_{shv}$	SHV F	5′-CTTTACTCGCTTTATCG-3′	837	55	(11)
	SHV R	5′-TCCCGCAGATAAATCACCA-3′		(30 sec)	
Bla$_{oxa-1}$	OXA 1F	5′-AGCCGTTAATTAAGCCC-3′	882	55	(12)
	OXA 1R	5′-CTTGATTGAAGGGTTGGGCG-3′		(30 sec)	
Bla$_{kpc-1}$	KPC 5F	5′-TGTCACTGTATCGCCGTC-3′	900	48	(13)
	KPC 10R	5′-CTCAGTGCTCTACAGAAAACC-3′		(30 sec)	
Bla$_{ampc}$	AmpC F	5′-CCCCGCTTATAGAGCAACAA-3′	634	55	(14)
	AmpC R	5′-TCAATGGTCGACTTCACACC-3′		(45 sec)	
Bla$_{ndm}$	NDM F	5′-GGTTTGGCGATCTGGTTTTC-3′	621	48	(15)
	NDM R	5′-CGGAATGGCTCATCACGATC-3′		(45 sec)	
IntI	IntI F InH R	5-ACATGTGATGGCGACGCACGA3′	Variable	55	(16)
		5-ATTTCTGTCCTGGCTGGCGA3′			

Results and Discussion

All isolates were found resistant to amoxycillin (100%) and all except one were resistant to amoxy-clav (97.2%). Variable resistance (71.7 - 97.8%) was observed against 3rd generation beta-lactam antibiotics (ceftriaxone, cefixime, cefuroxime, cefotaxime, cefepime and imipenem). About 78% of the test isolates were resistant to the 4th generation Beta-lactam antibiotic, cefepime. In contrast, 72% of the isolates were sensitive to Imipenem. The antibiotic susceptibility pattern of the *E. coli* strains is shown in Fig. 1. Antibiotic resistance is a burning issue in the field of medicine which complicate treatment of infected patients[19]. One of the mechanisms by which Gram-negative bacteria acquire resistance to beta-lactam antibiotics is through the production of beta-lactamases. The present study was conducted to investigate the production of beta-lactamases by amoxycillin resistant clinical isolates collected from a medical diagnostic center in Dhaka city. All isolates showed relatively greater sensitivity to imipenem when compared to other beta-lactams. This could be attributed to treatment of patients with antibiotics which create selective pressure on isolates and help gaining resistance to the antibiotics they are exposed to. Although ESBLs have been described in different Gram-negative bacteria, *Klebsiella pneumoniae* and *Escherichia coli* are the major ESBL-producing microorganisms worldwide [3,20, 21].

Of 27 *E. coli* strains 33.3% exhibited resistance to penicillins-cephalosporins, carbapenems and ciprofloxacin, 55.6% were co-resistant to penicillin-cephalosporin and ciprofloxacin whereas 3.7% was resistant to penicillin-cephalosporin and azithromycin.

In the present study, 33.3% of the *E. coli* exhibited co-resistance to penicillins, cephalosporins, carbapanemes and fluoroquinolones, whereas 55.6% were resistant to penicillins, cephalosporins and fluoroquinolones. A similar prevalence was reported earlier[22] that found 61.1% of ciprofloxacin resistant ESBL *E. coli.* The presence of co-resistance in the test isolates which also contained plasmids raises concern over transfer of drug resistance in the environment.
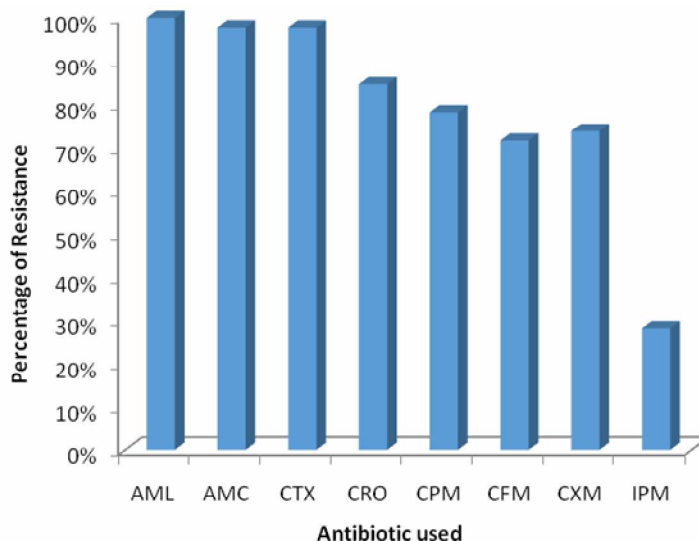

Fig. 1. Resistance of the *E. coli* isolates against different antibiotics. Amoxicillin (AML), ampicillin (AMP), ceftriaxone (CRO), cefepime (CPM), cefuroxime (CXM), cefexime(CEF), cefotaxime (CTX) and imipenem (IPM).

Based on antibiotic resistance pattern, the isolates were classified into four major beta-lactamase producing classes following Ambler's classification[23]. The classification is shown in Table 2. In the present study, 84.7% of the *E. coli* were classified as ESBL producers based on Ambler's classification and subsequent molecular confirmation. In a previous report from Bangladesh 43.2% of *E. coli* were found to be ESBL producers[24]. This was followed by reports of 26.9 and 34.1% ESBL producers by Alim and Mostaqim in 2005 and 2007, respectively[25,26]. Later, in 2014, it was reported 11.8% ESBL producing *E. coli* isolated from clinical sources[27], followed by a report of 31.9% ESBL *E. coli* in 2015[28]. Clearly a prevalence of 84.7% ESBL in the present study indicates an alarming rise of ESBL producers in the community. ESBL prevalence varies in different countries with various prevalence being reported from Iran, India and Pakistan[29-35]. The present study indicated 28.3% *E. coli* that were carbapenem resistant. This finding is a clear contrast to a report by Islam *et al.* (2015)[28] from Bangladesh who indicated 100% sensitivity to carbapenem. Once again, there is a rising trend of resistance to

carbapenems as there was to Beta-lactams. Based on the findings of the present study, carbapenems remain the drug of choice for treatment of *E. coli* infections.

Table 2. Classification of beta-lactamase producers according to Ambler's classification scheme.[23].

Class	Isolates of *E. coli* in the class (n, %)
Class A/D (ESBLs)	19, 20, 22, 26-31, 33-35, 37-39, 42 and 47-48 (18, 39%)
Class B (Metallo beta-lactamases, MBLs)	1, 3, 5, 7-8, 11, 13, 16, 18, 23 and 25 (11, 23.9%)
Class C (AmpC)	-
Untypeable (Isolates showing intermediate susceptibility to carbapenem)	2, 4, 9, 10, 12, 14, 15, 17, 21, 24, 36, 40, 41, 43, 44-46 (n=17, 37%)

Whether an isolate was a metallo beta-lactamase producing strain was determined[36] by Ambler's classification scheme. Out of 66 isolates, 17 (25.75%) were MBL producers. Metallo beta-lactamases belong to class B[37] of Ambler's classification scheme. This class is further divided into subclasses B1, B2, and B3, of which Class-B1 enzymes are the most clinically significant[38-39]. New Delhi metallo beta-lactamase enzymes belong to Class B1 and confer resistance to all beta-lactam antibiotics known[40]. NDMs are no longer confined to India, Bangladesh or Pakistan[41, 42]. In the present study, NDM subclass was prevalent among 2.2% of *E. coli*. NDM encoding genes are usually located on a readily transferable plasmid[43]. It was found that only NDM producing *E. coli* contained a plasmid, which is in concordance with other findings. This indicated that NDM producing genes were present in the chromosome in high frequency in the present study.

Screening of ESBL-producers by plate-based assay is challenging[2,44]. Detection of specific genes by PCR and sequencing are usually conducted for the final confirmation of ESBL production. Molecular characterization of isolates by identifying specific genes with the help of PCR was needed for final confirmation of beta-lactamase production. The association of ESBLs and the presence of TEM, SHV, OXA and CTX-M-type enzymes have been investigated in many studies[3,4]. As reported by other researchers in earlier studies[4,5], this study was also found predominance of CTX-M gene (34.8%) closely followed by TEM genes (32.6%). The CTX-M type of beta-lactamase represent a rapidly emerging group worldwide and have been found predominantly in *Enterobacteriaceae*, particularly in *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Salmonella typhimurium*[5]. In contrast to a previous report from Bangladesh[27], our study detected a much lower frequency of OXA-1 (13%) and SHV (4.3%) producers. CTX-M and SHV-type genes were reported in *E. coli* isolated from surface water in Bangladesh[45]. PCR based methods were used to identify specific genes for various classes of beta-lactamases. Gene targeted for PCR and number of positive isolates for each gene are shown in Table 3.

In this study, ESBL producers were the most prevalent comprising of 84.7%, followed by AmpC beta-lactamase producers which contained 6% and metallo beta-lactamase producers consisting of 1.5% *E. coli.* . Occurrence of ESBL production among *E. coli* strains is important as they form a major part of the commensal intestinal flora and serve as a reservoir of infection in the environment. Problem with emergence of ESBL is enhanced further by the fact that the ESBL trait can be transferable via plasmids to other organisms. Resistance genes to other agents like fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole may also be transferred by conjugation. Of all isolates in this study, 31.34% produced CTX-M, 25.7% produced TEM, 10.6% produced OXA-1and 5.8% produced SHV. A total of 15 of the test isolates produced multiple beta-lactamases as shown in Table 4. Of these 6.7% contained 4, 20% contained 3 and 73.3% contained 2 beta-lactamase genes (Table 4).

Table 3. Targeted gene and number of positive isolates for each gene of *Escherichia coli*.

Class	Target gene	Amplicon size (bp)	Isolates of *Escherichia coli* (n, %)
B	Bla_{NDM}	621	2 (n = 66, 1.5%)
C	Bla_{AmpC}	634	2, 12, 15, 17 (n = 66, 6%)
A/ D	Bla_{TEM}	858	4, 10, 14, 15, 17, 20, 21, 24, 25, 27, 28, 36, 41, 47, 48 (n = 66, 22.7%)
	Bla_{CTX-M}	593	2, 19, 20, 26, 27, 30, 31, 33, 34, 35, 38, 41, 42, 46, 47, 48 (n = 66, 24.2%)
	Bla_{SHV}	827	19, 20 (n = 2, 4.3%)
	Bla_{OXA-1}	882	20, 25, 26, 27, 28, 31 (n = 6, 9.1%)

Table 4. Presence of multiple beta-lactamase genes in tested isolates.

	bla_{CTX-M}	bla_{TEM}	bla_{OXA-1}	bla_{SHV}	NDM	AmpC
E. coli						
2	+	−	−	−	+	+
12	−	−	−	−	+	+
15	−	−	−	−	+	+
17	−	−	−	−	+	+
19	+	−	−	+	−	−
20	+	+	+	+	−	−
25	−	+	+	−	−	−
26	+	−	+	−	−	−
27	+	+	+	−	−	−
28	−	+	+	−	−	−
31	+	−	+	−	−	−
41	+	+	−	−	−	−
47	+	+	−	−	−	−
48	+	+	−	−	−	−

In this study, 50% of the *E. coli* strains contained plasmids of variable sizes (Fig. 2). However, there was no correlation between beta-lactam resistance and presence of plasmids. Production of ESBL is frequently reported to be plasmid encoded and bears clinical significance. Plasmids carrying ESBL genes may also carry genes for resistance to other antibiotics. Determining the presence of plasmids in ESBL bacteria and establishing co-relation between presence of plasmid and ESBL production is important because spread of resistance via plasmids can lead to outbreaks or endemic occurrence[46]. Plasmid profiles of all isolates were studied to demonstrate variation and epidemiological linkage among the ESBL producers. Plasmids were found in 50% of *E. coli*. However, no correlation was found between presence of plasmids and pattern of resistance to beta-lactam antibiotics. About 56% of the plasmid-containing *E. coli* did not exhibit resistance to the beta-lactams. Thus it can be inferred that mechanisms of antibiotic resistance was mediated by chromosome-borne factors in addition to plasmid-mediated genes, if any. Further analyses are needed to determine the exact location of beta-lactam resistance genes. We classified plasmids based on a boundary of 1.33 MDa, since the smallest plasmid clearly discernible in our gel from *E. coli* V157 (positive control) was of this size. Plasmids in the present study ranged in size between less than one kb to about 7 kb. Prevalence of plasmids found in the present study was found to be different from previous findings[27, 47].
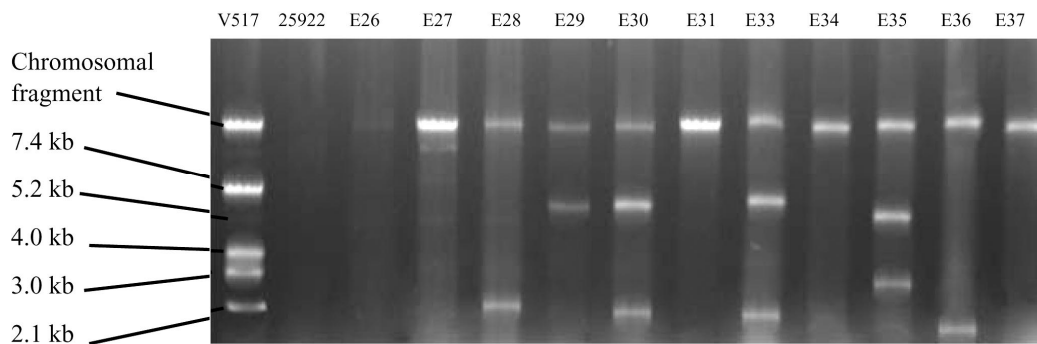

Fig. 2. Plasmid profile of test isolates. From left, lane 1: *E. coli* V517, lane 2: *E. coli* 25922, lanes 3-13: Clinical *E. coli* investigated.

Integrons are mobile genetic elements which can carry beta-lactamase gene. A total of 39% of the *E. coli* isolates contained Class 1 integron. However, no correlation could be found between the occurrence of integron and beta-lactam resistance/ plasmid. In our study, 39% of the *E. coli* isolates contained Class 1 integrons. A much higher prevalence of Class 1 integrons (50%) was reported earlier[27], who reported 50% Class 1 integron containing ESBL producers.

Detection of ESBL producers is of significance because this facilitates adoption of treatment options. Spread of ESBL positive bacteria in the community and within

hospitals may lead to endemic outbreaks. Moreover failure to treat ESBL infections limits therapeutic choices. It is imperative to investigate the prevalence of ESBL positive strains to formulate a policy to treat infections due to resistant organisms. The present study indicated a rising pattern of resistance to beta-lactam antibiotics when compared to earlier reports. Prevalence of beta-lactamase producing *E. coli* isolates reflects the overuse and misuse of antibiotics in Bangladesh. This limits therapeutic options for treatment. It is necessary to continuously monitor multi-drug resistant bacteria causing various infections to aid appropriate treatment strategies. Based on the findings of the present study, carbapenems still remain the drug of choice for treatment when choosing beta lactams. However, future studies need to be performed to determine the prevalence of other metallo beta-lactamases *viz.* IMP, VIM and SPM. The lack of correlation between mobile genetic determinants and antibiotic resistance emphasizes the need to actually locate the position of resistance genes for epidemiological significance.

Acknowledgements

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