

Research Article

Detection of Extended Spectrum β -lactamases Producing Genes among Third Generation Cephalosporins Sensitive Bacterial Strains from a Medical College Hospital in Bangladesh

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Abstract

Background: Extended Spectrum β -lactamases (ESBLs) are rapidly evolving group of β -lactamase enzymes produced by the gram-negative bacteria which is very important to detect in clinical laboratory for effective treatment. The aim of the present study was to see the ESBL genes among Third Generation Cephalosporins (3GCs) sensitive bacterial strains.

Methods: This cross sectional study was undertaken in non-repetitive clinical isolates collected from Mymensingh Medical College Hospital, Mymensingh, Bangladesh from both the outpatient and inpatient departments over a period of six months from January 2011 to June 2011. The ESBL status was confirmed by double disc

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diffusion test and minimum inhibitory concentration by agar dilution method as recommended by Clinical and Laboratory Standard Institute 2010 and multiplex polymerase chain reaction for TEM, SHV and CTX-M genes.

Results: A total of 300 Gram negative bacilli were included in the study; among them 236 were resistant and 64 were sensitive to 3GCs by disc diffusion test. Multidrug resistant ESBL production was found 75.8% from resistant isolates and 54.6% from sensitive isolates. Rate of TEM, SHV and CTX-M genes present among sensitive strains were 36.4%, 24.2% and 21.1%, respectively.

Conclusion: Both common and new ESBLs genes were detected from 3GCs sensitive bacteria of which TEM is most common. Routine ESBLs screening for all Gram-negative isolates both from sensitive and resistant to 3GCs strains might be useful for the physicians in selecting effective antibiotics.

Keywords: 3GCs sensitive bacteria; Bangladesh; ESBLs; Genes

Introduction

Increased use of antibiotics, particularly the Third Generation of Cephalosporins (3GCs), has been associated with the emergence of β -Lactamases -a common mechanism of bacterial resistance [1]. These enzymes have serine at their active site and attack the amide bond in the β -lactam ring of antibiotics [2]. Extended Spectrum β -lactamases (ESBLs) are often plasmid mediated, derived from mutations in the classic TEM and SHV genes by one or more amino acid substitutions around the active site [3]. There are also new families of ESBLs, including the CTX-M, OXA, AmpC, KPC -type enzymes as well as novel, unrelated β -lactamases [4], notably the CTX-M types, are becoming increasingly common, evolved via the escape and mutation of chromosomal β -lactamases from *Kluyvera* spp., which is an environmental non pathogenic bacteria [1,4]. ESBLs have been reported worldwide in many different genera of *Enterobacteriaceae* and *Pseudomonas* spp [5,6]. However, they are most common in *Klebsiella pneumoniae* and *Escherichia coli* [7].

Phenotypic methods are not able to distinguish between the specific enzymes responsible for ESBLs production. The determination of whether a specific ESBL present in a clinical isolate is related to TEM and SHV enzymes is a complicated process because point mutations around the active sites of the TEM and SHV sequences have led to amino acid changes that increase the spectrum of activity of the parent enzymes, such as in TEM-1, TEM-2, and SHV-1 [3]. The molecular method commonly used is the PCR amplification of the genes with oligonucleotide primers, followed by sequencing. Sequencing is essential to discriminate between the non-ESBL parent enzymes to different variants of TEM or SHV of ESBLs [8].

Prevalence of ESBL producing from *Escherichia coli* and *Klebsiella pneumoniae* isolates were 41% and 36% in Pakistan [9] and 43.2% and 39.5%, respectively in Bangladesh [10]. Another study conducted in a medical college hospital, Bangladesh found 41.66% ESBL in Gram-negative bacteria [11]. A number of studies in India have reported as ESBL producers were 40.85%, 51.4% and 53.84% in 2004, 2007 and in 2010, respectively [12-14]. However, another two studies in Iran and India documented high prevalence 96% and 97%,

respectively [15,16]. All of these studies were conducted among 3GCs resistant strains. A wide variation of those reports suggests that detection of ESBLs also need to find out among 3GCs sensitive strains. The ESBL genes detected in 3GCs bacterial strains is challenging for physician to treat. Since no previous data is available on the prevalence of genes responsible for ESBLs production in Bangladesh, it is urgent to know whether the bacteria posses the ESBL gene or not and what type of gene they carry. Therefore, the study aimed to detect ESBL genes among the third generation cephalosporin sensitive bacterial strains in Bangladesh.

Materials and Methods

A total of 300 non-repetitive clinical isolates were collected from MMCH both the outpatient and inpatient departments over a period of 6 months from January 2011 to June 2011. Urine and skin wound (pus, wound infection) were used as specimen. Laboratory work was carried out in the department of microbiology in MMC. Specimens were collected aseptically. All samples were routinely cultured on MacConkey and blood agar plates at 37°C aerobically for 18 hours. Gram negative isolates were further characterized by standard biochemical tests [17]. The susceptibility to antibiotics was determined by Kirby Bauer method on Muller Hinton agar (MHA) according to Clinical and Laboratory Standard Institute (CLSI) protocols for Gram negative panels [18].

Isolates were screened for ESBL production by using disc diffusion test on MHA media according to the CLSI recommendations where isolates showing inhibition zone size of ≥ 22 mm with ceftazidime (30 μ g), ≥ 25 mm with ceftriaxone (30 μ g), ≥ 27 mm with cefotaxime (30 μ g) , ≥ 27 mm with aztreonam (30 μ g) were suspected for ESBL production [19] (Figure 1).

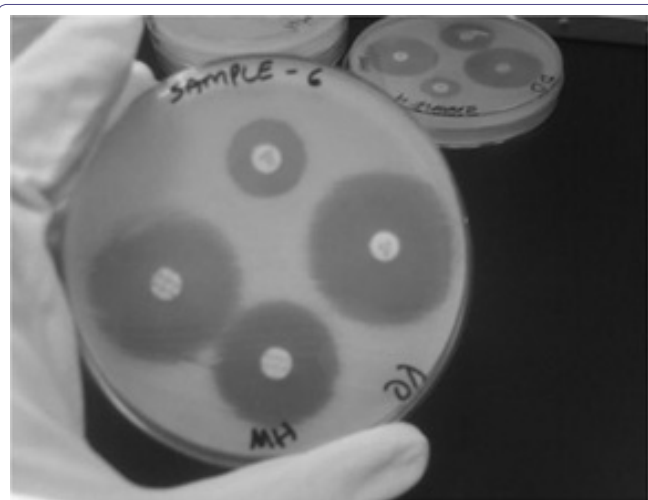


Figure 1: Double disc diffusion test.

Plasmid DNA was isolated from bacterial cells by alkaline lyses method by Medici et al., [20]. A single colony of each organism was inoculated from Mac Conkey agar into 5 ml of Luria-Bertanii broth (LB) and incubated for 20 h at 37°C. Cells from 1.5 ml of the overnight culture was harvested by centrifugation at 12,000 rpm for 5 min. 1.5 ml from LB media containing cells was taken eppendorf tube, than 100 μ l TNE buffer was mixed. The mixture was centrifuged for 1 min at 10000 rpm and supernatant was discarded. Again 100 μ l NaOH (50 mM) was added to pellet. After heating at 40°C in water bath for 1 min, 60 μ l of IM Tris HCl (pH 6.7) was added. Vortex, centrifuge at 10000 rpm for 1 min was done. Then supernatant was used as

template (1 μ l) (Medici et al. 2003 with some modification). Supernatant 1 μ l was used as template. The PCR primers and cycling conditions used were previously described (Table 1) [21-23].

Target gene	Primers	Sequences	Amplicon size
TEM	TEM F	CTTCCTGTTTTTGCTCACCCA	717 bp
	TEM R	TACGATACGGGAGGGCTTAC	
SHV	SHV F	TCAGCGAAAAACACCTTG	471 bp
	SHV R	TCCCGCAGATAAATCACC	
CTX-M-U	CTX-MU1	(5' ATGTGCAGYACCAAGTAAAGT)	593 bp
	CTX-MU2	(5' TGGGTRAARTARGTSACCAGA)	

Table 1: Primer for TEM, SHV, CTX-M-U were used.

Preparation of reaction mixture for PCR amplification, 1 μ l of template DNA was added to 50 μ l of master mixture containing 4 μ l of dNTP mixture (2.5 mM of each), 10X PCR buffer 5 μ l (Ex *Taq*), 0.5 μ l of *Taq* polymerase (250 U), 1 μ l of each primer stock solution (50 pmol/ μ l), and remaining 38.5 μ l volume was fulfilled by nuclease free water (Takara Japan).

For TEM, SHV gene

Initial denaturation at 94°C for 3 minute
 Denaturation at 94°C for 30 sec
 Annealing at 50°C for 30 sec
 Extension at 72°C for 2 min
 Final extension at 72°C for 10 minutes

} 35 cycles

For CTX-M gene

Initial denaturation at 94°C for 7 minute
 Denaturation at 94°C for 50 sec
 Annealing at 50°C for 40 sec
 Extension at 72°C for 1 min
 Final extension at 72°C for 5 minutes

} 30 cycles

Amplification was performed in an Eppendorf Thermocycler (Takara, Japan) in 1.0% agarose gel and visualized by staining with ethidium bromide (0.5 mg/ml), running through horizontal electrophoresis. A 100 bp ladder molecular weight marker (Roche, USA) was used to measure the molecular weights of amplified products. The images of ethidium bromide stained DNA bands were digitized using a gel documentation system (AlphaimagerTM 3400, USA) by UV light (Figure 2).

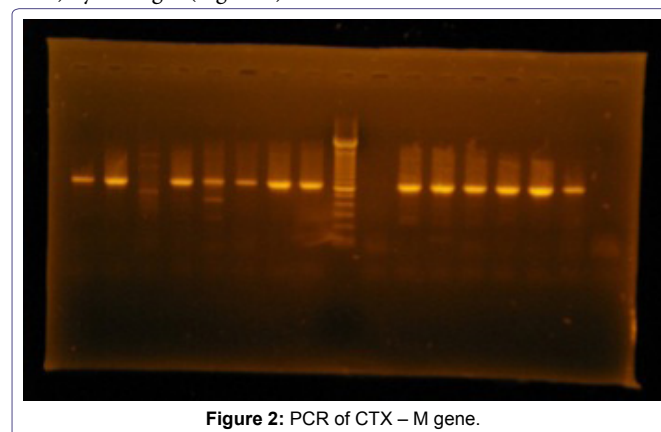


Figure 2: PCR of CTX - M gene.

Results

During the 6 months study period a total of 300 various bacterial isolates were yielded. The specimens were urine 216 (72%), wound

swab 45 (15%), pus 39 (13%). Out of 300 Gram negative isolates in this study more than half (52%) were *Escherichia coli* followed by *Proteus* spp. (18.3%), *Klebsiella* spp. (15%), *Pseudomonas* spp. (3%) and others for example *Enterobacter* spp., *Citrobacter* spp. were 11.7%. Among the Gram negative isolates ESBLs producers were 179/236 (75.8%) from resistant isolates and 35/64 (54.6%) from sensitive (Table 2). Rate of TEM, SHV and CTX-M genes present in 3GCs sensitive strains were 36.4%, 24.2% and 21.1%, respectively. Among them single strain contain three genes and five strains contain two combine genes (Table 3).

3 GCs	ESBL Positive n (%)	ESBL Negative n (%)	Total n (%)
Sensitive	35 (54.6)	29 (45.3)	64 (100.0)
Resistance	179 (75.8)	57 (24.1)	236 (100.0)
Total	214 (71.3)	86 (28.7)	300 (100.0)

Table 2: Distribution of ESBL producing Bacteria according to Third Generation Cephalosporin Sensitivity (n=300).

* χ^2 value 11.02 at df 3; p<0.001: Highly Significant; 3GCs: Third Generation Cephalosporins

Name of Gene	Frequency	Percentage
TEM gene	12	36.4
SHV gene	8	24.2
CTX-M gene	7	21.2
Combined gene	6	18.2
Total	33	100.0

Table 3: Distribution of TEM, SHV and CTX-M genes among 3GCs sensitive but phenotypic confirmed ESBL producers.

Discussion

Identify that ESBLs are a major challenge for the clinical microbiology laboratory. This study detected a significant rate of ESBL gene in the 3GCs sensitive bacterial strains. Sensitivity breakpoints designated in the CLSI guidelines for *Klebsiella* spp. and *Escherichia coli* against cefotaxime, ceftriaxone, and ceftazidime are [17] inefficient at detecting ESBLs [24,25]. Failure to detect ESBL production by routine disc-diffusion tests has been well documented [3,24]. Highly revealing studies performed in the United States and Europe by Tenover et al., and Livermore et al., respectively, reported that errors in the detection of ESBL mediated resistance are frequently encountered with both automated and disk diffusion methods [8,24]. It might be due to the variable affinity of enzymes for different substrates and inoculum effect [26]. It is also documented that ESBL-producing organism with third-generation cephalosporins may result in clinical failure if the infection is outside the urinary tract [25]. Despite *in vitro* susceptibilities, reports of failures in both animal models and clinical settings are well documented when third-generation cephalosporins are used to treat ESBL infections [27-29]. The sensitivity of using any particular extended-spectrum cephalosporin as a screening test can be dependent on the geographic variation and the resistance patterns of these organisms.

Our study documented that among the 3GCs sensitive strains more than half (54.6%) were phenotypic positive for ESBLs production (Table 2). Most of the strains were sensitive to ceftazidime, ceftriaxone, and cefotaxime disc but some were only to cefotaxime. They showed more resistant to ceftazidime, which is supported by a study of SHV-3 like ESBLs found 100.0% resistant to ceftriaxone where as only 50.0% were resistant to ceftazidime *in vitro* [30]. Moland et al., found the sensitivity of using ceftazidime resistance as marker for ESBL production to be 78.0% in a broth dilution format

[31]. Similarly we found statistically more significant by these combination. Though ceftazidime plus clavulanic acid (CAZ/CAZC) was the best single disc diffusion test was recommended by George et al., [32] but it cannot cover all the strain. Use of only one combination may fail to detect ESBL positive strains and thus might cause low prevalence [10]. The use of cefotaxime, ceftazidime, and ceftriaxone as the only indicator of ESBLs screening can no longer be recommended. If only one indicator antibiotic would be used for screening, cefpodoxime has proven to be the best molecule for screening all types ESBLs producers in clinical sample, that also produce AmpC β -lactamase [33]. In this study we used two combinations with clavulanic acid (CAZ/CAZC and CTX/CTXC) and found that *Escherichia coli* and *Klebsiella* spp. showed maximum ESBLs production in CAZ/CAZC combination, which correlates with other studies [32,34]. But the combination of ceftazidime and cefotaxime still missed two strains producing the SHV-5 and SHV-7 ESBLs [31].

Occurrence and distribution of ESBLs differs from country to country and from hospital to hospital [35]. Since no previous data is available about the prevalence of genes responsible for ESBLs production in Bangladesh, it is assumed that this high rate of ESBLs by phenotypic method may be due to mutation of first two parent gene TEM-1, SHV-1 and newer most prevalent gene CTX-M in the world and our neighbor country India [28]. CTX-M gene is now the most common in *Escherichia coli* in community and it may be due to overuse of ceftriaxone or due to fecal carriage and transfer gene by horizontal transmission [3,6,27,36]. The present study detected TEM, SHV and CTX-M genes in 36.4%, 24.2%, and 21.1% from phenotypically confirmed ESBLs producers, respectively. Moreover, presence of multiple genes is also found in these bacteria.

Our study should be viewed in the context of several limitations. Firstly, because of constraints of time and budget, this was a single centered study with relatively a small sample; therefore the results cannot be generalized to the whole country. Finally, we did not take into consideration of antibiotic history of the patients before and after *in vitro* sensitivity test, and we checked only *in vitro* drug response. However, the study represents the first study to determine the presence of ESBL genes from the 3GCs sensitive gram-negative bacterial strains in Bangladesh. Further study need to be conducted to correlate between *in vitro* and *in vivo* drug response among such a patients, and to compare antibiotic usage with less or no antibiotic usage.

In conclusion, it can be said that the 3GCs sensitive bacteria possessed both common and newer ESBL genes of which TEM is most common. By considering the fact that Bangladesh is a common place of irrational use and abuse of antibiotics, routine ESBLs screening for all Gram-negative isolates both from sensitive and resistant to 3GCs sensitive bacterial strains might be helpful for the physicians in selecting effective antibiotic therapy.

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