Original Article

Prevalence of CTX-M β lactamases among Gram negative bacteria in a tertiary care hospital in Bangladesh

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Abstract

Extended spectrum beta lactamases (ESBLs) produced by Gram negative bacteria are mainly mediated by three important genes, namely TEM, SHV and CTX-M. In this study, we used a multiplex PCR to determine the prevalence of CTX-M and its subgroups CTX-M-3, CTX-M-14, among the members of *Enterobacteriaceae* family and in *Pseudomonas* spp that were isolated from different clinical samples in a tertiary care hospital in Bangladesh.

A total of 300 culture positive clinical isolates were selected for the study. Out of these, 216 from urine, 45 from wound swab, 39 from pus aspirates. The ESBL status was determined by double disc diffusion test (DDDT) as recommended by Clinical Laboratory Standard Institute 2010 (CLSI) and by multiplex PCR for TEM, SHV and CTX-M, CTX-M-3, CTX-M-14 genes.

Out of 300 isolates tested, 71.3% were positive for ESBL production by DDDT. The rate of positivity for TEM, SHV and CTX-M genes in 107 randomely selected isolates was 83.2%. Among these, 56.2% (50/89) was positive for CTX-M. Among the CTX-M positive isolates, CTX-M-3 and CTX-M-14 were 78.0% (39/50) and 80.0% (40/50) respectively. Our study demonstrated that CTX-M variants were common in *Enterobacteriaceae* and *Pseudomonas* spp prevalent in the hospital of Bangladesh.

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Introduction

Extended spectrum beta-lactamases (ESBLs) are enzymes that mediate resistance to third generation cephalosporins as well as monobactams and are inhibited in vitro by β-lactamase inhibitors such as clavulanic acid and tazobactam.[1] Most ESBLs are mutants of TEM and SHV enzymes, but CTX-M enzymes are also increasingly becoming important. These CTX-M enzymes predominantly hydrolyze cefotaxime.[2] In clinical strains, CTX-M encoding genes have commonly been located on plasmids that vary in size from 7 to 160 kb.[3] ESBLs have been reported worldwide in many different genera of *Enterobactericeae* and *Pseudomonas* spp.[4] ESBL producing organisms have been reported from different parts of the world and ESBLs production rates are now very high in Asia compared to Europe.[5] Epidemiological reports demonstrate that some enzymes are more frequently reported than others. Predominant enzyme type varies with country and that diverse CTX-M types often exist within a single country.[6] CTX-M-3, a variant of CTX-M-5 has also been reported from India.[3] There was no systematic study about ESBL in Bangladesh until 2004. In 2004, it was first reported that 43.2% and 39.5% *Esch. coli* and *K. pneumoniae* isolated from clinical samples were positive for ESBL respectively[7] and in 2010 it increased to 57.89%.[8]

Phenotypic methods cannot distinguish specific enzymes responsible for ESBL properties. Molecular method like PCR amplification followed by sequencing is used to identify the genes responsible for ESBL. Sequencing is essential to discriminate between the non-ESBL

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parent enzymes (e.g. TEM-1, TEM-2, or SHV-1) and different variants of TEM or SHV, CTX-M ESBLs (e.g. TEM-3, SHV-2, etc).[9] In this study, we used a rapid multiplex PCR to identify CTX-M-3, CTX-M-14 a variant of CTX-M-1 and CTX-M-9 family respectively. By using this method, clinical isolates of ESBL-producing members of the family *Enterobacteriaceae,* including *Pseudomonas* spp could be characterized at molecular level.

Materials and Methods

Total 300 clinical isolates were collected from both the outpatients and inpatients departments of Mymensingh Medical College Hospital (MMCH) over a period of 6 months from January 2011 to June 2011. Urine and pus from skin wound were used as specimen. Specimens were collected aseptically. All samples were routinely cultured on MacConkey and blood agar plates at 37^0C aerobically for 18 hours. Gram negative isolates were identified by standard biochemical tests.[10] The susceptibility to antibiotics was determined by Kirby Bauer method on Muller Hinton agar according to CLSI 2010 protocols for Gram negative panels.[11] ESBL production was determined by double disc diffusion test (DDDT).[12]

Out of 300 isolates, 107 isolates were randomly tested for ESBL specific genes namely TEM, SHV, CTX-M by PCR. CTX- M positive isolates were further analyzed for CTX-M-3 and CTX-M-14 genes by multiplex PCR. For the detection of CTX-M-3 and CTX-M-14 genes by multiplex PCR, plasmid DNA was isolated from bacterial cells by alkaline lyses method.[13] The PCR primers and cycling conditions used were previously described.[14-16] Primers used for CTX-M-3 and CTX-M-14 genes are given in Table-1.

Electrophoresis of the amplified product was done in 1.0% agarose gel and visualized by staining with ethidium bromide (0.5 mg/ml). A 100 bp molecular weight ladder (Roche, USA) was used to measure the molecular weights of the amplified products. The images of ethidium bromide stained DNA bands were digitized using a gel documentation system (AlphaimagerTM 3400, USA). All th laboratory works were carried out in the department of Microbiology at Mymensingh Medical College.

Table-1: *Primers used for the detection of CTX-M-3 and CTX-M-14 in multiplex PCR*

Primer	Sequence	PCR Product
CTX-M-3	F5'-AATCACTGCGCCAGTTCACGCT-3' R5'-GAACGTTTCGTCTCCCAGCTGT-3'	479
CTX-M-14	F5'-TACCGCAGATAATACGCAGGTG-3' R5'-CAGCGTAGGTTCAGTGCGATCC-3'	355

Results

A total of 300 culture positive samples were included in the study of which urine was 216 (72%), wound swab 45 (15%) and pus 39 (13%). Organisms isolated were - *Esch. coli* 156 (52%), followed by *Proteus* spp. 55 (18.3%), *Klebsiella* spp. 45 (15%), *Pseudomonas* spp. 9 (3%) and others (*Enterobacter spp., Citrobacter* spp.) 35 (11.7%). Table-2 shows the rate of ESBL positivity of isolated organisms by DDDT and PCR methods. The overall rate of ESBL positivity by DDDT was 71.3% (214/300) while rate was 83.2% by PCR method (89/107). The rate of ESBL positivity by DDDT ranged from 67% to 80% among *Enterobacteriaceae* while the rate by PCR was 78% to 88%. The rate of ESBL positivity among *Pseudomonas* sp was 88% and 80% respectively by DDDT and PCR methods. Among the genotypic positive ESBLs, CTX-M was positive in 56.2% (50/89) of isolates (Table-3). Among the 50 CTX-M positive isolates, CTX-M-3 and CTX-M-14 genes were present in 78% (39/50) and 80% (40/50) of isolates respectively (Fig-1; Table- 4). About 81%

Table-2: *Rate of ESBL positivity of isolated organisms by DDDT and PCR methods*

Organisms	DDDT method		PCR method	
	No. of isolates tested	Positive for ESBL N (%)	No. of isolates tested	Positive for ESBL* N (%)
Esch. coli	156	105 (67.0)	51	43 (84.3)
Klebsiella spp	45	36 (80.0)	23	18 (78.2)
Proteus spp	55	40 (72.7)	17	15 (88.2)
Pseudomonas spp	09	8 (88.0)	05	4 (80.0)
Others**	35	25 (71.4)	11	9 (81.8)
Total	300	214 (71.3)	107	89 (83.2)

Note: * *Positive for TEM, SHV or CTX-M;*
***Others - Enterobacter* spp, *Citrobacter* spp;

Table-3: *Distribution of CTX-M gene among 89 genotypic positive ESBL organisms*

Name of isolates	Number	Positive for CTX-M N (%)
E.coli	43	29 (67.44)
Klebsiella spp.	18	11 (61.1)
Proteus spp.	15	5 (33.3)
Pseudomonas spp.	4	2 (50)
Others	9	3 (33.3)
Total	89	50 (56.2)

Note: Others - *Enterobacter* spp, *Citrobacter* spp

Table-4: *Pattern of CTX-M-3, CTX-M-14 distribution in CTX-M positive isolates*

Name of isolates	Number	CTX-M-3 N (%)	CTX-M-14 N (%)
Esch. coli	29	27 (93.1)	28 (96.6)
Klebsiella spp	11	9 (81.8)	9 (81.8)
Proteus spp	5	01(20)	01 (20)
Pseudomonas spp	2	01(50)	01 (50)
Others	3	01(33.3)	01 (33.3)
Total	50	39 (78.0)	40 (80.0)

Note: *Others - *Enterobacter* spp, *Citrobacter* spp

to 96% *Esch. coli* and *Klebsiella* spp was positive for CTX-M-3 and CTX-M-14. Both the genes were overlapping.

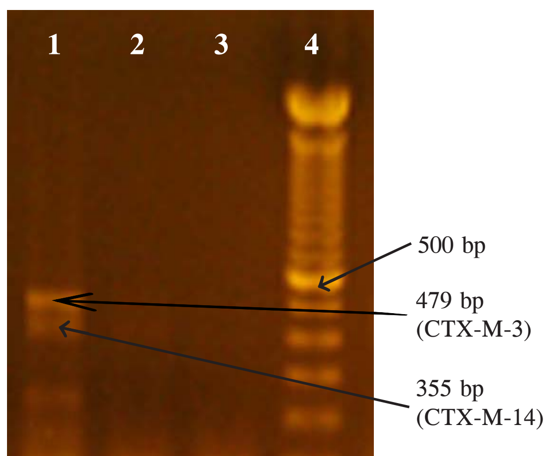

Fig-1. *Multiplex PCR of CTX-M-3 and CTX-M-14 genes. Lane1: sample E.coli, Lane2&3: Negative control, Lane4: DNA Ladder*

Discussion

The CTX-M-type enzymes are a group of molecular class A extended-spectrum β-lactamases (ESBLs) that exhibit an overall preference for cefotaxime (CTX; hence the name CTX-M) and ceftriaxone and a higher susceptibility to tazobactam than to clavulanate.[17] CTX-M gene is now the most common in *Esch.coli* in community and it may be due to overuse of ceftriaxone or due to faecal carriage and the spread of gene by horizontal transmission.[18] These enzymes are emerging among the members of the family *Enterobacteriaceae*, where they can cause resistance to Cefotaxime and other expanded-spectrum β-lactams.[19] The family of CTX-M type ESBL genes consists of five phylogenetic branches on the basis of their amino acid sequence similarities. They are CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Cluster 1 includes CTX-M-1, CTX-M-3, CTX-M-15, CTX-M-22, CTX-M-25 and others. Cluster 9 includes CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-18 and others.[4] In the present study the prevalence of CTX-M gene for ESBL among the Gram negative bacterial isolates from a tertiary care hospital was 56%. The CTX-M gene was present in higher frequency in *Esch. coli* and *Klebsiella* spp compared to other enerobacteriacae and *Pseudomonas* spp. Majority of the *Esch. coli* and *Klebsiella* isolates contained CTX-M-3 and CTX-M-14. But its frequency was much less among the other enterobacteriacae and *Pseudomonas* spp.

In Europe, CTX-M is also predominant and among them CTX-M-3 and CTX-M-14 are most frequently detected.[21] In 2002, CTX-M-1, CTX-M-3 and CTX-M 14 were isolated from *Enterobacteriaceae* in France and China.[20,22] Similar predominance of CTX-M-3 and CTX-M-14 has been reported from other countries of Asia and America.[15,23-26] In our isolates the CTX-M-3 and CTX-M-14 were found to co-exist in both *Esch. coli*, *Klebsiella* and other *Enterobacteriaceae*. The co-existence of two or more kinds of ESBLs in a single isolates also common in study done by Lin et al.[27] Other variants of CTX-M genes may exist elsewhere in Bangladesh. Regular screening and national surveillance characterizing the CTX-M genes needs to be instituted at different geographical locations and healthcare settings to monitor the transmission and spread of ESBL mediated resistance.

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