

Original Article

Transfer of antibiotic resistant gene CTX-M -An alarm for the clinician.

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Abstract

A total of 20 known CTX-M gene positive Extended spectrum lactamase (ESBL) producing strains (18 *Escherichia coli* and 2 *Klebsiella spp.*) from three tertiary care hospitals were detected using screening test, double disc synergy test & PCR. All isolates were screened for detection of horizontal transfer of CTX-M gene by conjugation assay. Among them 1 positive conjugal transfer of CTX-M gene has occurred between CTX-M gene positive ESBL producing *Esch.coli* as donor & DH5 - *Esch.coli* (CTX-M gene negative, non ESBL strain) as recipient. This is the first report of identifying horizontal transfer of CTX-M gene by conjugation assay in our country. This finding signifies the reason of gradual increase of ESBL producing organism. This study provides further evidence of the global dissemination of CTX-M type ESBLs and emphasizes the need for their epidemiological monitoring.

Keywords: ESBL, CTX-M gene, DH5 - *Esch.coli*, PCR.

Introduction

Resistance to extended spectrum cephalosporins can occur in *Enterobacteriaceae* via the production of extended spectrum β -lactamases (ESBLs) that are capable of hydrolyzing the oxyiminocephalosporins and monobactams¹. In recent years a new family of plasmid mediated CTX-M (Cephotoximase) extended spectrum β -lactamases (ESBLs) called CTX-M has arisen and reported in the literature with increasing frequency from Europe, Africa, Asia, South America and North America.² These ESBLs were named CTX-M type β -actamases, owing to their high activity against cefotaxime². Now a days gradual increase of ESBL producing organism is a global concern. In a study at Indian hospitals 73% (72% of total *K.pneumoniae* and 73% of total *E.coli*) 3rd generation cephalosporin-resistant isolates were found to carry CTX-M gene³. In a earlier study in our country 66.5% ESBL producing strains were found positive for CTX-M gene⁴. Although molecular type of ESBLs has been reported from

Bangladesh earlier, there is no information of their spreading mechanism. Hence the present study was undertaken to see whether horizontal transfer of antibiotic resistant gene CTX-M was possible or not?

Materials and Methods

This cross-sectional study was carried out in the department of Microbiology, Sir Salimullah Medical College for a period of one year from January, 2009 to December, 2009. A total of 20 known CTX-M gene positive ESBL producing strains (18 *Escherichia coli* and 2 *Klebsiella spp.*) from three tertiary care hospitals (Sir Salimullah Medical College, BSMMU and BIRDEM) were taken as study strain which were previously detected using screening test, double disc synergy test & PCR. All this isolates were screened for detection of horizontal transfer of CTX-M gene by conjugation assay.

Study of horizontal gene transfer

All the 20 known CTX-M gene positive ESBL producing strains were screened for detection of horizontal transfer of CTX-M gene by conjugation assay.

Steps involved in Conjugational gene transfer

1. A freshly grown isolated colony of each donor strain (CTX-M gene positive ESBL isolates both *Esch.coli* &

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Klebsiella spp.) was inoculated into 5ml of Luria-Bertani (LB) broth and incubated overnight at 37°C. (Figure-1)

- In another test tube a single isolated colony of recipient strain (CTX-M gene negative & non ESBL producer, also treated & proved in our laboratory) DH5 *Esch.coli* (Kindly donated by Department of Microbiology, Dhaka University). was inoculated which contained 5ml of LB broth and incubated overnight at 37°C. (Figure- 2)
- After overnight incubation, 20 µl broth was taken from each donor and recipient strains containing test tubes and they were placed in the same tube containing 5ml fresh LB broth for conjugation and incubated overnight at 37°C. (Figure- 3)
- After overnight incubation, inoculum was taken and inoculated on cefotaxime (30 µl/ml) containing Muller-Hinton agar plate.
- After overnight incubation both pale colonies of ESBL isolates (donor strain) and green fluorescent colonies of DH5 *Esch.coli* (recipient strain) were found in this antibiotic containing plate when visualized under UV transilluminator (Figure-4) Previously this DH5 *Esch.coli* was susceptible to this antibiotic at the same concentration and no colony was formed (Figure-5) but now it was resistant to the antibiotic and colonies were formed.
- For further phenotypic and genotypic confirmation of DH5 *Esch.coli*, Double disc synergy test and PCR were done respectively to prove that there has been transfer of resistant gene CTX-M from donor to recipient strain.⁵

Double disc synergy test

ESBLs production was considered positive when the inhibition zone around the test antibiotic disc (cefazidime, ceftriaxone, cefotaxime and aztreonam disc) was increased towards the augmentin disc (20 µg amoxicillin and 10 µg clavulanic acid) which was placed in the centre of the plate and 20 mm apart from other discs.⁶

Polymerase Chain Reaction (PCR):

DH5 *Esch.coli* isolate was screened for the resistance gene CTX-M by PCR assay using universal primers.⁷ Bacterial DNA extraction was performed by simple boiling method.⁸ PCR amplification reactions were performed in a volume of 375 µl for 15 PCR reaction containing 300 µl PCR Master mix (Invitrogen, USA), 15 µl of each primer, and 3 µl of DNA template. The cycling parameters were as follows: an initial denaturation at 94°C for 7 min; followed by 35 cycles of 94°C for 50s, 54°C for 40s, and 72°C for 60s; and with a final extension at 72°C for 5 min.⁷ The amplified PCR

products were subjected to electrophoresis at a 1.5% agarose gel in 1x TBE buffer. Strain with known β -lactamase type was included as reference. Here *E.coli* j53 (met pro) pMG 298 used as a positive control for CTX-M gene detection (Kindly donated by George Jacoby, USA).

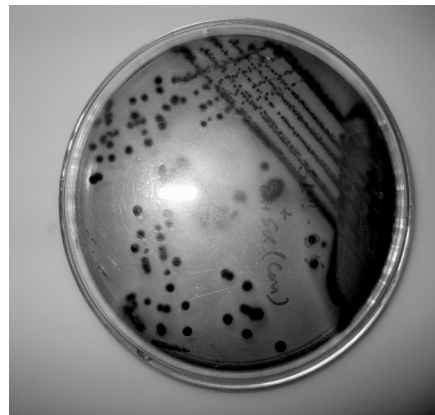


Figure 1. CTX-M gene positive ESBL producing *Esch.coli* in MacConkey agar. This strain used as donor strain in conjugation assay.

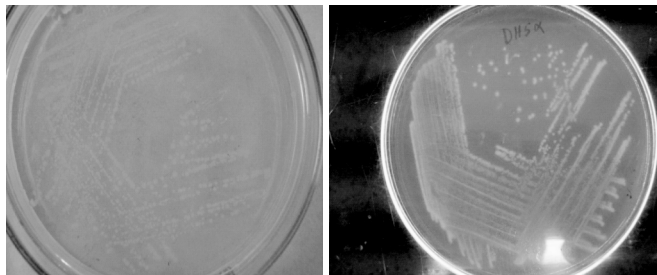


Figure 2. DH5- *Esch.coli* (Non-ESBL producer) in Mueller-Hinton agar on normal light (left) and on UV-light (right). This strain used as recipient strain in conjugation assay.

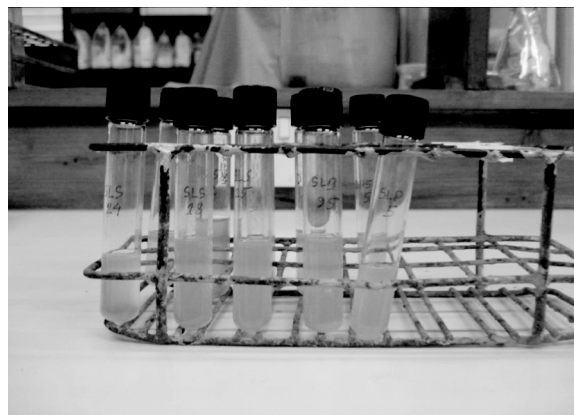


Figure 3. These tubes contain both donor strain (ESBL producer) & recipient strain (Non-ESBL producer DH5 *Esch.coli*). They are placed in the same tube for conjugation.

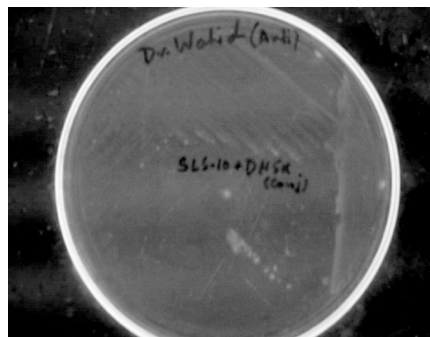


Fig 4. Single green fluorescent colonies of DH5- *Esch. coli* on antibiotic (cefotaxime) containing Mueller-Hinton agar plate under UV-light after conjugation.



Figure 5. Antibiotic (cefotaxime) containing Mueller- Hinton agar plate showed no growth of DH5- *Esch. coli* .

Result:

A total of 20 known CTX-M gene positive ESBL producing strains were screened for detection of horizontal transfer of CTX-M gene by Conjugation assay, which include 18 *Escherichia coli* and 2 *Klebsiella spp.* All this strains were previously identified both phenotypically and molecularly.

Among the 20 strains 1 positive conjugal transfer of CTX-M gene was found between CTX-M positive ESBL producing *Esch. coli* as donor & DH5 - *Esch. coli* (CTX-M negative, non ESBL strain) as recipient.

Table I : Result of Conjugation between CTX-M positive ESBL strains as donor & DH5 - *Esch. coli* (CTX-M negative, non ESBL strain) as recipient.

Strain no (n=20)	CTX-M positive ESBL strain	Conjugation with DH5 <i>Esch. coli</i> (CTX-M negative, non ESBL strain	Single green fluorescence colony isolated on antibiotic-agar plate	Conjugal transfer of resistance determinant (CTX-M gene)
SLS 1	<i>Klebsiella spp.</i>	Done	-	-
SLS 2	<i>Esch. coli</i>	Done	-	-
SLS 3	<i>Esch. coli</i>	Done	-	-
SLS 4	<i>Esch. coli</i>	Done	-	-
SLS 5	<i>Esch. coli</i>	Done	-	-
SLS 6	<i>Esch. coli</i>	Done	-	-
SLS 7	<i>Esch. coli</i>	Done	-	-
SLS 8	<i>Esch. coli</i>	Done	-	-
SLS 9	<i>Esch. coli</i>	Done	-	-
SLS 10	<i>Esch. coli</i>	Done	+	+
SLS 18	<i>Esch. coli</i>	Done	-	-
SLS 24	<i>Klebsiella</i>	Done	-	-
SLS 25	<i>Esch. coli</i>	Done	-	-
SLP 5	<i>Esch. coli</i>	Done	-	-
SLP 6	<i>Esch. coli</i>	Done	-	-
SLP 7	<i>Esch. coli</i>	Done	-	-
SLB 33	<i>Esch. coli</i>	Done	-	-
SLB 34	<i>Esch. coli</i>	Done	-	-
SLB 35	<i>Esch. coli</i>	Done	-	-
SLB 95	<i>Esch. coli</i>	Done	-	-

SLS- Strain collected from SSMC&MH
 SLP- Strain collected from BSMMU
 SLB- Strain collected from BIRDEM

Discussion

Bacterial antibiotic resistance has become a major clinical concern worldwide including Bangladesh⁹.

Although several studies addressed the issue of the emergence of ESBL producing *Enterobacteriaceae* worldwide, no reports of their spreading mechanism have been published from Bangladesh till now. It has been mostly reported that resistance to β -lactam antibiotics is on the rise among clinical isolates in different hospitals of Bangladesh, expressing the need for an exhaustive research. The present report is the first report of identifying horizontal transfer of CTX-M gene by conjugation assay in our country.

A total of 20 known CTX-M gene positive ESBL producing strains (18 *Escherichia coli* and 2 *Klebsiella spp.*) from three tertiary care hospitals (Sir Salimullah Medical College, BSMMU and BIRDEM) were taken as study strain which were previously detected using screening test, double disc

synergy test & PCR. All this isolates were screened for detection of horizontal transfer of CTX-M gene by conjugation assay.

Among the 20 strains, 1 positive conjugal transfer of CTX-M gene was found between CTX-M positive ESBL producing *Esch.coli* as donor & DH5 - *Esch.coli* (CTX-M negative, non ESBL strain) as recipient. Successful transfer of CTX-M gene from a donor strain to a recipient strain by conjugation assay used here in the table is sufficient to prove that horizontal transmission is possible of this resistant determinant. In a study at Indian hospital conjugation assay were carried out & plasmid analysis of the transconjugants showed that the CTX-M beta lactamase gene was associated with the plasmid. Oxyimino- β -lactam antibiotic resistance was transferred to recipient strain¹⁰. In contrast to the findings of Jemima (2008), plasmid analysis of the transconjugants was not done in this study due to lack of facilities rather a search for CTX-M gene was made from the recipient strain (DH5 - *Esch.coli*) having CTX-M gene transferred from donor strain (ESBL producing *Esch.coli*) after DNA extraction by PCR. In another study in Northern Italy conjugational transfer could not be detected for the CTX-M-15 determinant carried by the *K.pneumoniae* isolate, while both the CTX-M-1 determinant found in *Esch.coli* and the CTX-M-2 determinant found in *P.vulgaris* were readily transferable by conjugation⁷. In contrast to the finding of Laura Pagani (2003), genotyping characterization was not done due to lack of facilities. Here one CTX-M determinant found in *Esch.coli* was readily transferable by conjugation.

Our finding emphasizes the increasing role of the CTX-M gene in β -lactam antibiotic resistance worldwide and leads to consideration of empirical treatment for infections caused by coliforms, especially in patients compromised by underlying disease or immunological status. Identification of horizontal transfer of CTX-M gene is uncommon; and this is the first report from Bangladesh. In conclusion, the data presented here illustrates the complexity and extent of the spread of ESBL producing *Enterobacteriaceae* in Bangladesh. This finding signifies the reason of gradual increase of ESBL producing organism. This study provides further evidence of the global dissemination of CTX-M type ESBLs and emphasizes the need for their epidemiological monitoring.

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