Indian J Microbiol (Jan–Mar 2012) 52(1):35–40
DOI 10.1007/s12088-011-0140-3

ORIGINAL ARTICLE

Study of CTX-M Type of Extended Spectrum β-Lactamase among Nosocomial Isolates of *Escherichia coli* and *Klebsiella pneumoniae* in South India

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Received: 21 December 2009 / Accepted: 1 February 2010 / Published online: 25 January 2011

Abstract Data on CTX-M type extended-spectrum β-lactamase (ESBL) produced by Gram-negative bacteria by molecular methods are limited from India. This study was conducted to investigate the prevalence of CTX-M type ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* from nosocomial isolates in a tertiary care hospital in southern India. A total of 179 clinical isolates of *K. pneumoniae* (n = 72) and *E. coli* (n = 107) were obtained in a period of 3 months and assessed for ESBL production phenotypically. Associated resistance to a panel of antibiotics and Minimum Inhibitory Concentration for 3rd generation cephalosporins was determined. Phenotypically ESBL positive isolates were subjected to PCR for $bla_{\text{CTX-M}}$ gene using two sets of primers for the simultaneous detection of all the five major groups of CTX-M types. All the positive isolates were then subjected to a group specific PCR to detect the prevalent group. Out of 179 isolates, 156 (87.1%) were positive for ESBL phenotypically, which includes 39.2% of *K. pneumoniae* and 60.8% of *E. coli*. All of them were examined by PCR using two primers for the presence of $bla_{\text{CTX-M}}$ genes. Among the 156 phenotypic positive isolates, 124 (79.4%) were positive for $bla_{\text{CTX-M}}$ genes, of which 45 (36.2%) were *K. pneumoniae*, 79 (63.7%) were *E. coli*. When the 124 positive clinical isolates were further tested with CTX-M group-specific primers, all were positive for the CTX-M-1 group. Our findings document evidence of the high prevalence of multidrug resistant CTX-M group 1 type ESBL among nosocomial isolates in this region. High co-resistance to other non-β-lactam antibiotics is a major challenge for management of ESBL infections. This is alarming and calls for the judicious use of carbapenems, especially in developing countries. This has significant implications for patient management, and indicates the need for increased surveillance and for further molecular characterization of these isolates.

Keywords Extended spectrum β-lactamase · CTX-M types · Antibiotic resistance · Nosocomial isolates · South India

β-Lactamases are bacterial enzymes that inactivate β-lactam antibiotics by hydrolysis, which result in ineffective compounds [1]. At least 400 different types of β-lactamases, originating from clinical isolates, have been described [2]. CTX-M is a recently described rapidly growing family of the Extended-Spectrum β-Lactamases (ESBLs). The name CTX reflects the potent hydrolytic activity of these β-lactamases against cefotaxime. They are non-TEM and non-SHV derivatives of molecular class-A ESBLs [3]. They were initially reported in 1989 in Germany, from an *Escherichia coli* isolate, and in 1990 in Argentina, from a *Salmonella* isolate. Approximately 40 CTX-M enzymes have been described to date. Over the last decade they have been identified in various countries of Africa, South America, North America, Asia and Europe [4–8]. CTX-M type β-lactamases may be the most frequent type of ESBLs worldwide. The number of CTX-M-type β-lactamases is rapidly expanding and they have been

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found in different *Enterobacteriaceae* including *Salmonella* spp [9].

CTX-M-β-lactamases can be divided into five groups based on their amino acid sequence identities. Group I includes CTX-M-1, -3, -10 to -12, -15 (UOE-1), -22, -23, -28, -29, and -30. Group II includes CTX-M-2, -4 to -7, and -20 and Toho-1. Group III includes CTX-M-8. Group IV includes CTX-M-9, -13, -14, -16 to -19, -21, and -27 and Toho-2. Finally group V includes CTX-M-25 and -26. The members of these groups exhibit 94% amino acid identity within the group and 90% amino acid identity between groups [3].

The diversity and increasing prevalence of CTX-M-type ESBLs pose a serious threat to the clinical use of third generation cephalosporins for the treatment of severe infections. Currently, the Clinical Laboratory Standards Institute (CLSI) guidelines focus mainly on the detection of ESBLs among *Klebsiella* spp and *E. coli*, while there are currently no definitive recommendations concerning the methods to be used, nor the interpretative criteria to be applied, for other species. In addition, only few PCR based surveys have been performed and reported until now in this country. Hence, we aimed to study the prevalence of CTX-M type ESBL producing *Klebsiella pneumoniae* and *E. coli* from various clinical isolates in this region.

Materials and Methods

Clinical Isolates

A total of 179 clinical isolates were obtained in a period of 3 months (December 2008–February 2009) from patients admitted in our hospital, which is a 750 bedded tertiary care hospital in South India. They included 72 (40.2%) *K. pneumoniae* and 107(59.7%) *E. coli*. The distributions of the sources of the isolates were: urine (n = 75), blood (n = 41), wound discharge (n = 23), tracheal aspirate (n = 11), peritoneal fluid (n = 14), ascitic fluid (n = 6), sputum (n = 5), and CSF (n = 4).

Only one positive culture per patient was included. Infections caused by more than one organism and the isolates for which it was impossible to discriminate between contamination and infection were excluded. Patients' demographic data, clinical diagnoses, and specimen types were recorded. Hospital associated infection was defined as occurrence of infection 48 h or more after hospital admission, without evidence that the infection was present or incubating on admission, in patients without prior history of stay in a healthcare facility [10]. All the isolates were identified as per the standard biochemical methods [11] and stocked in 0.2% semi-solid agar until analyzed.

Phenotypic ESBL Detection

ESBL phenotypic screening was performed for all the isolates by the disk diffusion test using ceftazidime (30 µg) and cefotaxime (30 µg), cefpodoxime (30 µg) and aztreonam (30 µg) disks. *E. coli* ATCC 25922 was used as the control and the zone diameter was interpreted per the CLSI recommended guidelines [12]. ESBL phenotypic confirmatory test was performed by the double disk diffusion method using antibiotic disks containing a combination of cephalosporin plus clavulanic acid in combination with a corresponding cephalosporin disk alone. The following antibiotic disks were used: ceftazidime (CAZ 30 µg), ceftazidime plus clavulanic acid (CAZ/CA 30/10 µg), cefotaxime (CTX 30 µg) and cefotaxime plus clavulanic acid (CTX/CA 30/10 µg), cefpodoxime (CPD 30 µg) and cefpodoxime plus clavulanic acid (CPD/CA 30/10 µg) (Hi media, Mumbai, India). *E. coli* ATCC 25922 was used as the negative control and *K. pneumoniae* ATCC 700603 was used as the positive control. The tests were interpreted according to CLSI guidelines [12] and $\geq$5 mm increase in the zone of inhibition for CTX/CLA, CAZ/CLA or CPD/CLA containing disks versus the corresponding CTX,CAZ or CPD disk alone was considered as ESBL positive.

Antibiotic Susceptibility Testing

The antibiotic susceptibility pattern of the ESBL-producing isolates to a panel of antibiotics was done by the standard Kirby Bauer's Method. The following antibiotics were used; amikacin (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg), piperacillin/tazobactam (100/10 µg), cefepime (30 µg), cefoxitin (30 µg), and trimethoprim/sulfmethoxazole (1.25/23.75 µg) (Himedia, Mumbai, India). Approved CLSI breakpoints were used [12]. Each ESBL producing isolate was subjected to MIC by the agar dilution test according to CLSI [12]. Antibiotic powders included cefotaxime, ceftriaxone, ceftazidime (Hi Media, Mumbai, India). Control strain *E. coli* ATCC 25922 was included in each series.

Molecular Characterization

PCR for bla_{CTX-M} Gene

The isolates which were ESBL positive by the phenotypic confirmation test were subjected to molecular characterization for the simultaneous detection of bla_{CTX-M} gene by a PCR, with some modifications [13]. Template DNA was prepared from an overnight culture (18–24 h) on a Mueller–Hinton agar plate. Two colonies were suspended in 100 µl of distilled water and the cells were lysed by heating at 95°C for 10 min. Cellular debris was removed by

centrifugation at 15,000 rpm for 2 min, and the supernatant was used as a source of template DNA for amplification. Two sets of primers for the simultaneous detection of all the five groups of CTX-M types were used. The primers used for the PCR assay were given in the Table 1.

Amplification was performed on a Corbett Research thermal cycler (HP, USA). The reaction mixtures comprised of 12.5 µl of red eye PCR master mix (Amplicon III), 2 µl of lM CTX-MA1, CTX-MA2, CTX825-F and CTX-825R primers each, 2.5 µl of nano pure water and 2.0 µl of template DNA. The final volume was 25 µl. Amplification condition were initial denaturation for 6 min at 94°C, followed by 30 cycles of 92°C for 1 min, 55°C for 1 min and 72°C for 1 min, and final extension of 72°C for 7 min. Known CTX-M 15 producing *K. pneumoniae* was used as the positive control.

The PCR products analyzed by electrophoresis in 1.5% w/v agarose gels in 0.5× TBE buffer (1× TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA) 100 bp DNA ladder (Fermentas) was used, the gels were stained with ethidium bromide and were visualized under UV light.

CTX-M Group Specific PCR

The entire isolates positive for $bla_{\text{CTX-M}}$ gene by PCR were subjected to the CTX-M group specific PCR [14]. Template DNA, positive control and visualization of the PCR products were same as above. Primers were procured from Biobasic, Canada. The primers and annealing temperature used were given in the Table 2.

Results and Discussion

Phenotypic ESBL Detection

Out of the total 179 clinical isolates, 87.1% (156/179) showed resistance to at least one of the third generation cephalosporins used by the disk diffusion susceptibility test. Among them, 60.8% (95/156) were *E. coli* and 39.2% (61/156) were *K. pneumoniae.* All the 156 isolates showed resistance to cefpodoxime. In the CLSI phenotypic confirmatory test using cephalosporin/clavulanate combination discs, all the 156 strains showed enhanced susceptibility to ceftazidime and/or cefotaxime in the presence of clavulanic acid, thus indicating ESBL production in them. The strains were positive for at least one of the confirmations tests, but by using both CTX/CLA and CAZ/CLA disk; all strains producing an ESBL were detected. Table 3 shows the comparison of screening and confirmatory tests in the phenotypic detection of ESBL.

The problem is, the under detection of ESBL production in clinical isolates, as it is common practice to screen for ceftazidime resistance as an indicator of ESBLs, ceftazidime is usually the best substrate for TEM and SHV ESBLs [15]. The CLSI guidelines for ESBL detection in *E. coli* and *Klebsiella* spp. include an initial screening with CPD, CTX, CAZ, ceftriaxone, or aztreonam, followed by a confirmation test using both CTX and CAZ in combination with clavulanate [12]. When used alone this practice could fail to recognize CTX-M-producing isolates susceptible to ceftazidime as ESBL producers and therefore greatly

Table 1 Primers used for simultaneous detection of $bla_{\text{CTX-M}}$ groups

Primer	Sequence (5′–>3′)	Amplicon size (bp)	Interpretation
CTXMA-1	**S**CS ATG TGC AG**Y** ACC AGT AA	450	Detection of Groups 1,2 and 9
CTXMA-2	CCG C**R**A TAT G**R**T TGG TGG TG		
CTXM825F	CGC TTT GCC ATG TGC AGC ACC	307	Detection of Groups 8 or 25
CTXM825R	GCT CAG TAC GAT CGA GCC		

S = C/G; **Y** = C/T; **R** = A/G

Table 2 Primers and its annealing temperature used for $bla_{\text{CTX-M}}$ Group specific PCR

Primer	Sequence (5′–3′)	Annealing temp (°C)	Product size (bp)	CTX-M target
CTXM1-F3	GACGATGTCACTGGCTGAGC	55	499	Group I
CTXM1-R2	AGCCGCCGACGCTAATACA			
TOHO1-2F	GCGACCTGGTTAACTACAATCC	55	351	Group II
TOHO1-1R	CGGTAGTATTGCCCTTAAGCC			
CTXM825F	CGCTTTGCCATGTGCAGCACC	55	307	Group III
CTXM825R	GCTCAGTACGATCGAGCC			
CTXM914F	GCTGGAGAAAAGCAGCGGAG	62	474	Group IV
CTXM914R	GTAAGCTGACGCAACGTCTG			

Table 3 Comparison of screening and confirmatory tests in the phenotypic detection of ESBL

Strains (n)	Number (%) of strains positive						
	Screening test			Disk confirmatory test			CPD/CLA
	CPD	CTX	CAZ	CTX/CLA	CAZ/CLA	Both CTX/CLA and CAZ/CLA	
E. coli (95)	95 (100)	93 (98)	87 (91)	94 (99)	83 (87)	95 (100)	95 (100)
K. pneumoniae (61)	61 (100)	60 (99)	59 (97)	61 (100)	56 (91)	61 (100)	61 (100)
Total (156)	156 (100)	153 (98)	146 (94)	155 (99)	139 (89)	156 (100)	156 (100)

CPD cefpodoxime (30 μg) disk, *CPD/CLA* cefpodoxime/clavulanate (30/10 μg) disk, *CTX* cefotaxime (30 μg) disk, *CTX/CLA* cefotaxime/clavulanate (30/10 μg) disk, *CAZ* ceftazidime (30 μg) disk, *CAZ/CLA* ceftazidime/clavulanate (30/10 μg) disk

hamper the control of the spread of the isolates harbouring the CTX-M enzyme. The recent emergence of isolates producing CTX-M variants capable of hydrolyzing ceftazidime, as well as cefotaxime, with higher efficiency has rendered their phenotypic recognition extremely difficult.

Data from this study indicate that 6% of ESBL-producing strains will not be detected if CAZ is used alone as the initial screen. However, in this study, CPD detected all of the ESBL-positive strains. Only 89% of ESBL producing strains were reported as ESBL positive when CAZ/CLA was only used and 99% of the ESBLs were detected when CTX/CLA was used as confirmation test (Table 3). The confirmatory test using CPD/CLA may give false positive results which are influenced by the TEM and AmpC production [16]. Therefore, a more proper is to screen the organisms using CPD followed by disk tests using CTX/CLA and CAZ/CLA.

Antibiotic Susceptibility Testing

The overall resistance of ESBL producers to various antibiotics was as follows: cefepime (100%), piperacillin/tazobactam (100%), gentamicin (89%), ciprofloxacin (82.7%), cefoxitin (100%), and Trimethoprim/sulfmethoxazole (84%) respectively, thus revealing multi drug resistance. A total of 18.1% isolates were resistant to amikacin, 3.4% to meropenem and all were sensitive to imipenem. MIC determination of the ESBL positive isolates to cefotaxime showed that all of them were in the range of 256 to >1,024 μg/ml and to ceftazidime and ceftriaxone were in the range of 16 to >256 μg/ml, thus exhibiting a higher range of resistance against the third generation cephalosporins tested, particularly to cefotaxime.

The multidrug resistance of $bla_{\text{CTX-M}}$ is due to the association of Class I integrons responsible for resistance to β-lactams, aminoglycosides, cholarmphenicol, sulphonamides [17]. All of the isolates were 100% resistant to cefepime, the fourth generation cephalosporin, which correlates with the finding that cefipime resistance may be more frequent in strains which produce the CTX-M-type ESBLs [18].

The susceptibility to amikacin was (81.9%) followed by meropenem (96.6%), and imipenem (100%) which suggests that these are the drugs of choice for serious life-threatening infections due to ESBL-producing organisms in our setting. By MIC, majority of the isolates conferred high resistance to cefotaxime (>256 to >1,024 μg/ml) as well as to ceftazidime (16 to >256 μg/ml). Thus, MIC determination to cefotaxime and ceftazidime is not a reliable approach for CTX-M, this correlates with other studies [19].

Molecular Analysis by PCR

Molecular characterization showed that, out of the 156 phenotypically ESBL positive isolates, 79.4% (124/156) were positive for the $bla_{\text{CTX-M}}$ gene. All were found to be of CTX-M group1, 2 or 9. It included 36.2% (45/124) of *K. pneumoniae* and 63.7% (79/124) of *E. coli*. Agarose gel electrophoresis of the amplified product of CTX-M PCR is shown in the Fig. 1. The 124 isolates positive for $bla_{\text{CTX-M}}$ gene PCR were subjected to CTX-M group specific PCR and it showed that all were of CTX-M group 1.

In the past 15 years, CTX-M-type ESBLs have become more prevalent worldwide and several aspects of them are worrying. These enzymes are becoming increasingly expressed by many strains of pathogenic bacteria with a potential for dissemination. They compromise the activity of wide-spectrum antibiotics creating major therapeutic difficulties with a significant impact on the outcome of patients [20, 21].

Due to the overlapping phenotypes resulting in interference from other β-lactamases produced by the organism hydrolyzing ceftazidime, phenotypic differentiation of organisms producing CTX-M β-lactamases from other types of ESBLs are difficult. Hence, molecular methods have advantages over phenotypic tests in that they accurately and rapidly detect resistant genes and by defining the precise genetic basis of the resistance mechanism, provide information valuable for reaching decisions concerning the early introduction of the targeted infection. This will ensure the early recognition of an outbreak involving

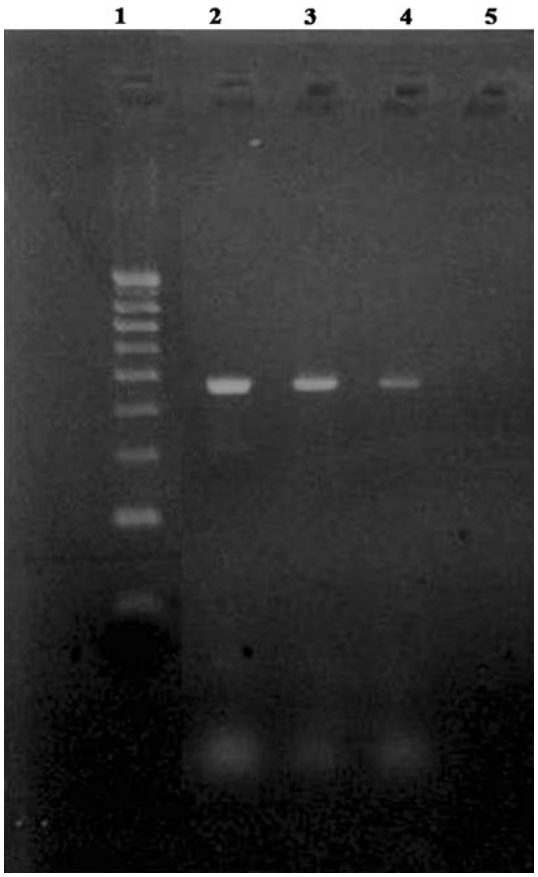

Fig. 1 PCR amplification products of $bla_{\text{CTX-M}}$ gene: *Lane 1* 100 bp DNA ladder, *lane 2* $bla_{\text{CTX-M group-1}}$ gene positive control strain, *lane 3* positive test *K. pneumoniae*, *lane 4* positive test *E. coli*, *lane 5* negative water control

organism producing CTX-M enzymes and the timely introduction of appropriate infection control procedures.

Most investigators in India have used phenotypic methods, and have reported prevalence ranging from 6.6 to 88.8% [22]. There are few reports of molecular identification of these β-lactamases. In India, the very first report of the presence of CTX-M-producing *Enterobacteriaceae* came from New Delhi, all produced CTX-M-15 [23]. A variant of the CTX-M-3 enzyme, designated CTX-M-15 was reported in May 2000 [3]. CTX-M-15 appears to be the predominant ESBL in northern India [19, 24, 25]. Jemima et al. [22] reported prevalence of CTX-M-1 type gene in 58.3% *Klebsiella* spp. and in 36% of *E. coli* from South India. These studies used the universal CTX-M primers and then CTX-M-1 group targeted primers. Unlike them, we attempted to look for the presence of all the 5 types of CTX-M ESBLs in our isolates.

This is a preliminary study which documents the emergence of $bla_{\text{CTX-M}}$ gene in clinical isolates from Pondicherry for the first time. Among the 179 clinical isolates studied, 87.1% showed ESBL phenotypically among them. Around, 79.4% of the isolates were positive for the CTX-M group 1 gene, thus showing the prevalence of CTX-M group 1 enzymes in this region, as reported from other parts of the country.

Conclusion

ESBLs are now a problem in hospitalized patients throughout the world. The prevalence of ESBLs among clinical isolates vary greatly world wide and in geographic areas and are rapidly changing over time. We have found a high prevalence of CTX-M-1 group of β-lactamase and the high rates of antimicrobial resistance encountered among them. Thus, it indicates the need for a more detailed surveillance to adopt appropriate control measures to reduce the ESBL burden in this region.

Though the molecular methods are not possible routinely in the laboratories in the developing countries, some extra efforts such as PCR should be carried out in the correct identification of the genes involved in ESBL mediated resistance for the surveillance, epidemiological studies and control of their transmission in hospitals.

Acknowledgment The corresponding author (B. N. Harish) acknowledges Univesity Grants Commission, New Delhi for providing funds for this project.

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