

CHARACTERIZATION OF ESBLs AND *ISEcp1* INSERTION SEQUENCES FROM *Klebsiella pneumoniae* AND *Escherichia coli*. IN A TERTIARY HOSPITAL IN INDIA

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ABSTRACT

Objective: Many challenging Gram-negative pathogens responsible for infections are having a high resistance rate, mostly because of their production of ESBLs. The development and increase in the rate of resistance in Enterobacteriaceae family are making the treatment of infections difficult. This study focuses on the molecular and phenotypic characterization of Gram negative β -lactamases that include CTX-M, SHV and TEM type enzymes and elaborates on the insertion sequences associated with CTX-M enzymes.

Methods: Total of 141 non-repetitive Gram-negative isolates were subjected to MIC (CLSI) with and without clavulanate to screen for the presence of ESBLs. Plasmid isolation was carried, amplified by PCR with consensus primers recognizing all the known CTX-M variants. The PCR products subjected to digestion with a set of *pst* I and *pvu* II restriction enzymes for RFLP analysis. Plasmid DNA of the isolates was further subjected for amplification of *bla*_{TEM} gene, *bla*_{SHV}, *bla*_{CTX-M-3} and *ISEcp1*.

Results: This study revealed the presence of 49 % CTX-M type ESBLs genotypically, 98 % of which belonged to cluster I by RFLP. Three isolates were co-producers of CTX-M, TEM and SHV and 4 isolates were positive for both SHV and TEM by PCR. This co-production of more than one β -lactamase is quite common among the Enterobacteriaceae.

Conclusion: Our study on ESBLs shows a higher proportion of CTX-M (49.5%), 71 % of which associated with *ISEcp1*. 28 % of the CTX-M positive strains turned out to be positive for CTX-M-3. This high percent of the ESBL prevalence shows the rampant use of antibiotics especially cefotaxime in India.

Keywords: CTX-M, SHV, TEM, PCR-RFLP, sequencing, *ISEcp1*

INTRODUCTION

Most of the organisms belonging to the enterobacteriaceae and the non-fermentative class of Gram-negative bacteria are naturally non-pathogenic in immuno-competent host. However they can become pathogens in an immuno-compromised host. The capacity to prevent and treat infections caused by the Gram negative pathogens are made difficult due to antimicrobial resistance [1]. The major mechanism of resistance seen in the family of Enterobacteriaceae is the production of β -lactamases.

β -lactamases are hydrolytic enzymes targeting the cyclic amide bond, the active group in the β -lactam drug [2]. The most prevalent enzymes (TEM-1 and SHV-1) are able to inactivate penicillins and narrow-spectrum cephalosporins but remained susceptible to β -lactamase inhibitors, such as clavulanic acid [3]. The TEM type Extended Spectrum β -Lactamases (ESBLs) were named after the first patient Temoniera. It gives resistance to penicillins and initial cephalosporins [4]. TEM enzymes, resistant to inhibitors have been noticed as a result of mutations [5].

A Sulphydryl variable (SHV) enzyme confers resistance against ampicillin, ticarcillin, cephalosporins and piperacillin [6]. It may be the most common enzyme found in clinical strains than any other type of ESBLs. These SHV enzymes have been found in many Enterobacteriaceae and occurrence of SHV-harboring *Pseudomonas aeruginosa* and *Acinetobacter* spp. are now reported [7, 8]. Gradual mutations in genes of the TEM and SHV enzymes resulting in amino acid changes in the encoded protein gave rise to the ESBLs [9].

In a study from India by Lal *et al.*, 2007 majority of the isolates from their hospital carried both TEM and SHV in contrast to TEM or SHV alone [10]. When single gene carrying isolates were considered, the ratio of TEM was higher than SHV. Report from other parts of the world showed SHV-5 gene common in *K. pneumoniae* isolates. They suggested that similar studies need to be carried out in other regions of the country to benefit the epidemiology ESBL enzymes.

A new family of ESBLs called CTX-M, known to specifically hydrolyze cefotaxime has come up [11]. CTX-M family is classified in Ambler class A and in group 2be of the Bush, Jacoby and Medeiros classification. They have the ability to hydrolyze broad spectrum cephalosporins and are susceptible to inhibitors such as clavulanic acid, sulbactam and tazobactam [12]. CTX-M-type ESBLs are quickly increasing and are detected in many populated continents. CTX-M-type ESBLs are prevalent in China and India; it could be considered that they are the most common ESBL type enzymes [13].

The CTX-M genes are classified into five clusters: the CTX-M-1, M-2, M-8 M-9 and M-25 clusters [14]. The most common and extensively disseminated subtype of the CTX-M-1 cluster is the CTX-M-3 [15]. In CTX-M-3 it has been observed that a single point mutation (Asp240Gly) has increased the hydrolytic activity of the enzyme against ceftazidime [16].

*bla*_{CTX-M} genes that encode these enzymes are associated with transferable plasmids which are either part of transposons or cassettes in integrons. Three out of the five subtypes of CTX-M genes (M-1, M-2 and M-9) are associated with insertion sequences, *ISEcp1* and hence are very wide spread in dissemination. *ISEcp1* is an insertion sequence that is weakly related to other elements, they belong to the family of IS1380 (IS Database Homepage (<http://www-is.biotoul.fr/page-is.html>)) [14].

Traditionally the phenotypic methods are used for identification of ESBLs. Iso-electric focusing also plays a crucial role in characterizing types but it may not be able to differentiate ESBLs having same iso-electric point [18]. Of late many molecular methods have been employed to detect and distinguish ESBLs; however nucleotide sequencing will remain the standard and accurate method. Among the molecular typing methods, variations of PCR are used to type ESBLs. Arbitrarily primed PCR has been the most used method to study the genetic relatedness of organisms with ESBLs [17].

In India, a multi-centric study has reported 55-61% of ESBL prevalence among clinical isolates of Enterobacteriaceae from various hospitals [19]. Although many studies reported high ratio of ESBL

producers among clinical isolates, information on molecular characterization of these enzymes in India is limited. In this paper we investigated to examine the type of ESBLs belonging to TEM, SHV and CTX-M families in *Klebsiella pneumoniae* and *E. coli* by phenotypic and molecular characterization. We also characterized the CTX-M subtype by Restriction fragment length polymorphism (RFLP), PCR and confirmation by DNA sequence analysis. We have demonstrated the presence of *ISEcp1* insertion sequence in these plasmids.

MATERIALS AND METHODS

Bacterial isolates

A total of 141 consecutive non-duplicate clinical isolates of resistant *Klebsiella pneumoniae* (46), and *Escherichia coli* (95) were obtained from Central Leprosy Teaching & Research Institute (CLT & RI), Chengalpattu, Tamilnadu, India.

ESBL screening by phenotypic method

The isolates were tested by phenotypic method for confirmation of ESBL by determining the minimal inhibitory concentration (MIC) for using CTZ and CTX (Hi-Media) both with and without 4µg/ml concentration of clavulanic acid (Ranbaxy) by agar dilution technique as per National Committee for Clinical Laboratory Standards (NCCLS) now known as CLSI, 2000 [20]. A greater than or equal to 3, two-fold concentration decrease in MIC for CTZ/CTM tested in combination with clavulanic acid versus its MIC when tested alone confirmed the phenotypic presence of ESBL. *E. coli* American Type Culture Collection (ATCC) 25922 and *K. pneumoniae* ATCC 700603 as negative and positive controls respectively were included in this study.

ESBL characterization (TEM, SHV, CTX-M, CTX-M-3, *ISEcp1*)

Plasmid isolation of the isolates was performed by Alkaline lysis method [21]. The primers for detecting TEM, SHV, CTX-M, CTX-M-3 and *ISEcp1* genes are listed in Table 1.

Detection of the *bla*_{TEM} gene

Detection of the *bla*_{TEM} gene was done as reported earlier [17]. Briefly pair of primers matching the conserved sequences specific for TEM gene was used to amplify a specific 1074 bp fragment of *bla*_{TEM} genes (Table 1). PCR was carried out in 50 µl of a reaction mixture, 200 µM of dNTP's, 1U Taq polymerase, 100 pmoles of each set of primers and 10ng of DNA. The samples were subjected to initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1 min (extension), and a final extension at 72°C for 10 min to complete the elongation of the PCR intermediate products.

Detection of *bla*_{SHV} gene

Detection of the *bla*_{SHV} gene was done as reported earlier [17]. A pair of primers matching the conserved sequences of SHV was used to amplify 1016 bp fragment of SHV gene (Table 1). PCR was carried

out in 50 µl of a reaction mixture, 200 µM of dNTP's, 1U Taq polymerase, 100 pmoles of each set of primers and 10ng of DNA. The samples were subjected to initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 1 min (denaturation), 68°C for 1 min (annealing) 72°C for 50 seconds (extension), and a final extension at 72°C for 10 min to complete the elongation of the PCR intermediate products.

Detection and RFLP subtyping of the *bla*_{CTX-M} gene

Detection was performed by the PCR-RFLP method [15]. Briefly pair of primers matching the conserved sequences at positions 205 to 227 and 748 to 727 with respect to the CTX-M translational starting point was used to amplify a specific 544-bp fragment of all the known *bla*_{CTX-M} genes. PCR was carried out in 50 µl of a reaction mixture, 200 µM of dNTP's, 1U Taq polymerase, 100 pmoles of each set of primers and 10ng of DNA. The samples were subjected to initial denaturation at 94°C for 2 min followed by 35 cycles of 95°C for 1 min (denaturation), 51°C for 1 min (annealing) 72°C for 1 min (extension), and a final extension at 72°C for 3 min to complete the elongation of the PCR intermediate products. The RFLP analysis of the PCR products were performed by digesting them with *Pst*I and *Pvu*II restriction enzymes and the digests were analyzed by agarose gel electrophoresis.

Detection of *bla*_{CTX-M-3} subtype

Detection was performed by the PCR and further sequence analysis [22]. PCR was carried out in 50 µl of a reaction mixture, 200 µM of dNTP's, 1U Taq polymerase, 100 pmoles of each set of primers and 10ng of DNA. The samples were subjected to initial denaturation at 94°C for 2 min followed by 35 cycles of 95°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1 min (extension), and a final extension at 72°C for 3 min to complete the elongation of the PCR intermediate products.

Detection of *ISEcp1* gene

Detection was performed by the PCR and further sequence analysis [22]. PCR was carried out in 50 µl of a reaction mixture, 200 µM of dNTP's, 1U Taq polymerase, 100 pmoles of each set of primers and 10ng of DNA. The samples were subjected to initial denaturation at 94°C for 2 min followed by 35 cycles of 95°C for 1 min (denaturation), 51°C for 1 min (annealing) 72°C for 1 min (extension), and a final extension at 72°C for 3 min to complete the elongation of the PCR intermediate products.

DNA sequencing of the *bla*_{CTX-M-3} and *ISEcp1*

DNA sequencing of the CTX-M-3 and *ISEcp1* PCR positive genes were performed by the fluorescent dye based sequencing method using Big dye terminator version 3.1 kit and ABI 3100 AVANT genetic analyzer. This was performed at the Medical Research Foundation, Sankara Nethralaya, Chennai. Bioinformatics sequence analysis was done using BLAST tool from the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Table 1: Details of Primers and PCR conditions used in this study

Amplicon	Primer Sequence(5' to 3')	Size of amplicon(bp)	Annealing Temp(°C)
<i>bla</i> _{CTX-M}	TTT GCG ATG TGC AGT ACC AGT AA CGA TAT CGT TGG TGG TGC ATA	544	51
<i>bla</i> _{TEM}	GAA GAC GAA AGG GCC TCG TG GGT CTG ACA GTT ACC AAT GC	1074	55
<i>bla</i> _{SHV}	CGC CGG GTT ATT CTT ATT TGT CGC TCT TTC CGA TGC CGC CGC CAG TCA	1016	68
<i>bla</i> _{CTX-M}	GGT TAA AAA ATC ACT GCG TC TTG GTG ACG ATT TTA GCC GC	840	55
<i>ISEcp1</i> tnpA1	AAT ACT ACC TTG CTT TCT GA	490	51
<i>ISEcp1</i>	TTC AAA AAG CAT AAT CAA AGC C		
<i>ISEcp1</i> reverse	5'CAA CCA CCT TTC AAT CAT TTT T5'		

RESULTS

Phenotypic and genotypic characterization

A total 141 isolates resistant to third generation cephalosporin (susceptibility towards impenem and meropenem was not studied)

were subjected to phenotypic ESBL screening with both CTZ and CTX both in the presence and absence of the ESBL inhibitor, clavulanic acid. 102 (73%) isolates were positive for ESBL, they showed greater than or equal to 3-fold reduction in MIC of CTZ and CTX in the presence of clavulanic acid when compared to their MIC when tested alone.

Of the plasmid DNA from all the 141 isolates, 70 (49.5%) were positive for *bla*_{CTX-M} gene with a product size of 544bp by genotypic PCR analysis (Table 2). All PCR positive isolates were subjected to RFLP with *Pst*I and *Pvu*II restriction enzymes. All of them, except one of *E. coli* isolate (98 %) belonged to cluster I by RFLP study. The PCR products belonging to cluster I yielded 3 products of size 267bp, 156bp and 121bp.

Of the total 141 isolates, 35 (24.8%) were positive for *bla*_{TEM} gene with a product size of 1074bp and 11 (7.8 %) were found to be positive for *bla*_{SHV} with a product size of 1016bp by PCR (Table 2). However confirmation of the gene by sequencing was not performed in this present study for the *bla*_{TEM} and *bla*_{SHV} genes. Interestingly 18

(24.7 %) of the *bla*_{CTX-M}, 5 (14.3%) of *bla*_{TEM} and 1 (9.1%) of *bla*_{SHV} positive isolates were found to be negative by phenotypic method for ESBL but positive by PCR (Table 2).

CTX-M subtyping and insertion sequence

Of the 70 *bla*_{CTX-M} gene positive isolates, 20 [28.57 %] isolates were found to be positive for the *bla*_{CTX-M-3} variant with a product size of 840bp by PCR using specific primers and 50 [71.42 %] isolates were found to be negative (Table 3). The negative isolates may belong to some other variants in CTX-M cluster I other than CTX-M-3. Interestingly, 50 [73.53 %] of the 70 CTX-M gene positive isolates were found to harbor the *ISEcp1* type insertion sequence with a product size of 490bp by PCR using specific primers (Table 3).

Table 2: Correlation between phenotypic and molecular methods for CTX-M, TEM & SHV ESBLs

Organisms	<i>Escherichia coli</i>		<i>Klebsiella pneumoniae</i>		Total (in numbers)
	Phenotypic method (in numbers)		Phenotypic method (in numbers)		
	Positive	Negative	Positive	Negative	
<i>bla</i> _{CTX-M} PCR Positive	38	09	14	09	70
<i>bla</i> _{CTX-M} PCR Negative	32	16	18	05	71
Total	70	25	32	14	141
<i>bla</i> _{TEM} PCR Positive	22	04	8	01	35
<i>bla</i> _{TEM} PCR Negative	48	21	24	13	106
Total	70	25	32	14	141
<i>bla</i> _{SHV} PCR Positive	06	0	04	01	11
<i>bla</i> _{SHV} PCR Negative	64	25	28	13	130
Total	70	25	32	14	141

Table 3: CTX-M subtyping for CTX-M-3 and *ISEcp1*

Organisms	<i>bla</i> _{CTX-M-3}		<i>ISEcp1</i>	
	Positive	Negative	Positive	negative
<i>Escherichia coli</i>	15	32	38	9
<i>Klebsiella pneumoniae</i>	5	18	12	11
Total	20	50	50	20

The CTX-M-3 PCR and *ISEcp1* positive plasmids were subjected to gene sequencing and subsequent BLAST analysis to confirm whether the variant is CTX-M-3 or CTX-M-15. The NCBI-BLAST analysis of the sequenced CTX-M-3 gene amplicon showed most number of hits against the known *Escherichia coli* CTX-M-15 gene [Accession Number AM040706]. The BLAST analysis of the *ISEcp1* gene was also found to have more number of hits against *Escherichia coli* CTX-M-15 [Accession Number AM040706]. This insertion sequence gene was found to be aligned at position between 3056-3490 bp of the CTX-M-15 gene.

Co-production of more than one enzyme type

Among the ESBL-positive isolates detected in this study, three tested positive for CTX-M β-lactamases and also co-produced TEM and SHV enzymes. In four isolates co-production of SHV and TEM enzymes were observed. Our finding showed a higher percentage, 49.5 % of CTX-M positive with CTX-M-3 specific primer positive being 28.57 %. 71.42 % of the CTX-M positive isolates were found to harbor *ISEcp1* insertion sequence.

DISCUSSIONS

Antibiotic resistance has been first observed with *Staphylococcus aureus*, which showed resistance against the first antibiotic penicillin [22]. Bacterial resistance pattern has become worldwide making treatment difficult resulting in high death rates [23]. This study is a report on the phenotypic and genotypic characterization of ESBL producers in Chennai, India, of particular reference to CTX-M, TEM and SHV type enzymes. ESBLs are resistant to oxyimino-cephalosporins but the MIC of the antibiotics are not always increase in their presence. A combination method would hence be a better way to detect their presence. The phenotypic detection method involving the known ESBL inhibitor clavulanic acid suggested positive for ESBL with CTZ and CTX antibiotics in 73% of the isolates. The phenotype method can only detect the presence of the

ESBL but not the subtype. The screening for ESBL alone is also not sufficient enough to prove that the organism produces ESBL; this is very well shown by some of the isolates in this study with phenotypic negative result yet positive for PCR.

In PCR based identification 49.5% positive strains for *bla*_{CTX-M} were identified which is quite higher percentage. Most prevalent cluster among these isolates was found out by RFLP study that is 98 % belonged to cluster I, which may include any one of the variants (CTX-M-1, -3, -10, -11, -12, -15, -22, -23, -27, -28, -29, -30, -32, -33, -34, -36, -37 and -42). The second and third higher genes found prevalent in this study was TEM type β-lactamases with 24.8% isolates positive in PCR and SHV type β-lactamases with 7.8 % positive. A recent study from India on molecular characterization of *bla*_{TEM} and *bla*_{SHV} revealed that TEM gene was present in 19(20%), SHV in 8(8.4%) and both TEM and SHV genes in 67.3 percent (64) of the isolates studied [10]. Our study on molecular characterization of CTX-M, TEM and SHV ESBLs revealed a higher proportion of CTX-M (49.5%) than TEM (24.8%) and SHV (7.8%). This could be due to rampant use of cefotaxime in Indian hospitals.

The combined detection gave way to the presence of strains with no ESBL activity phenotypically but positive with PCR for their corresponding genes. This may be due to two possibilities, one it could be due to the presence of "silent" genes, which are not expressed at that particular time of study, thus not showing phenotypic positivity. Second, the inhibitor used in the phenotypic test was clavulanic acid and like inhibitor resistant TEM (IRT) some β-lactamases have become resistant to inhibitors.

Many of the CTX-M strains were positive to *bla*_{CTX-M-3} variant, which is a member of the CTX-M cluster I. CTX-M-15 gene differs from CTX-M-3, which is one of the most prevalent ESBLs, by only a single amino acid change (Asp-240 Gly) [1]. Hence the use of specific primer to identify CTX-M-3 gene is not sufficient enough to distinguish the two variants. Sequence analysis of the amplified CTX-

M-3 gene was done in an effort to identify the single amino acid mutation. The sequenced gene was confirmed using BLAST analysis, which showed that these isolates shared maximum to identify *Escherichia coli* CTX-M-15 gene. The first CTX-M in Enterobacteriaceae in India was reported from New Delhi, all isolates tested produced CTX-M-15. Many Indian surveys since have shown the presence of ESBLs in isolates based on phenotypic tests [27]. In one study from South India it was seen that out of 23 isolates screened for *bla*_{CTX-M} genes, 19 yielded positive amplicons and sequence analysis from three transconjugants and whole gene amplified from a transconjugant matched the sequence of CTX-M type enzyme in the GenBank Database (AYO 13478) called UOE-1 or CTX-M-15 [28].

Most of the CTX-M strains were found to be associated with *ISEcp1* type insertion sequence. *ISEcp1* is a transposable element which can disseminate genes responsible for drug resistance among bacterial population. The presence of these insertion sequences stress the epidemiological importance of these CTX-M type ESBLs and their widespread prevalence. *ISEcp1* is always found upstream of *bla*_{CTX-M-15} and is strongly implicated in the mobilization of this antibiotic resistance gene [25]. IS26 has also been reported in association with *bla*_{CTX-M} including *bla*_{CTX-M-15} and more specifically, has been found to insert within *ISEcp1*, although interestingly, the insertion of IS26 differs from strain to strain [24, 26].

The molecular detection methods revealed 92.6% (116 of 141) of the isolates to be positive for any one of the ESBL genes (*bla*_{CTX-M}/*bla*_{TEM}/*bla*_{SHV}) screened, where as phenotypic method identified only 73% (102 of 141) of the isolates as ESBL producers. Co-production of more than one type of β -lactamase was also observed in our study, which is quite common among the Enterobacteriaceae. A report on ESBL types in Enterobacteriaceae in Argentinean public hospitals found that CTX-M accounted for roughly 70% of all ESBLs found, with similar findings reported in studies conducted in Japan, China, and United Kingdom [29, 30, 31, 32]. Recently in India, Jemima *et al.*, reported 15.8 % (95/600) of *bla*_{CTX-M} among Enterobacteriaceae [33]. All these strains belonged to CTX-M-1 cluster by RFLP study. In another study from India, Padmini *et al.*, reported 80.2% (19/23) *bla*_{CTX-M} among cefotaxime and ceftazidime resistant *E. coli* and *Klebsiella* strains [28].

CONCLUSIONS

Latest reports of treatment failure due to ESBL producing bacteria are of great concern in clinical practice. High incidence of infections caused by ESBL producing bacteria in Intensive care units were reported in different countries. If the molecular detection assays were available at the time of an outbreak and performed by a reference laboratory, early recognition and the possible mechanism by which resistance is spread can be identified in a timely manner. The key to effective surveillance is the use of both phenotypic and genotypic analysis. In our study also 24% of isolates found negative by phenotypic method were positive for *bla*_{CTX-M} or *bla*_{TEM} or *bla*_{SHV} by PCR. This emphasise that both genotypic and phenotypic methods must be studied together to determine the presence of ESBL producers.

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