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Screening of CTX-M Type Extended Spectrum BETA-Lactamases Producing Clinical Isolates from Tamil Nadu, India.

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ABSTRACT

The world is facing a major problem of antimicrobial resistance that is more severe in developing countries where the infectious diseases get spread more easily and the cost of treatment and prevention of them is very high. Multidrug resistance and extended spectrum β-lactamases (ESBL) production is a common problem, which needs empirical therapy to overcome and to reduce this drug resistance. In this study 88 clinical isolates were studied for ESBL production and presence of CTX-M genotypically. Out of 88 isolates, 32 were *Escherichia coli* (36%), 20 *Klebsiella pneumoniae* (23%), 14 *Pseudomonas aeruginosa* (16%), 12 *Enterobacter cloacae* (14%), 3 *Proteus vulgaricus* (3%), 5 *Proteus mirabilis* (7%) and 2 *Acinetobacter baumannii* (2%). Antibiotic susceptibility pattern showed 92% of the isolates were resistant to most beta-lactam antibiotics and plasmid was isolated from 72 isolates irrespective of its susceptibility pattern. 29 isolates were found to have CTX-M group I genes that includes 17 of 32 *E. coli* (53%), 8 of 20 *K. pneumoniae* (40%), 1 of 14 *P. aeruginosa* (7%) and 3 of 12 *Enterobacter cloacae* (25%). This study describes the genetic characteristics and molecular epidemiology of ESBLs among *Enterobacteriaceae* isolates and provides insight emergence of bacterial strains harbouring ESBL genes.

Keywords: Gram negative bacteria, beta-lactamase, multi-drug resistant, plasmid profile, phenotypic variants.

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INTRODUCTION

Extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae*, especially *Klebsiella pneumoniae* and *Escherichia coli*, were shown to have a significant impact on treatment options and clinical outcome in patients [1]. Further, ESBL- producing bacteria have shown to cause higher morbidity, mortality and fiscal burden [2]. *Enterobacteriaceae* producing β-lactamase displaying multi-drug resistance and causing therapeutic problems have been found in human and veterinary practice [3]. ESBLs are dangerous, as they are plasmid associated and there can be a cross species- dissemination of these plasmids. Moreover, these plasmids can carry co-resistance of antibiotics such as aminoglycosides, fluroquinolones, tetracycline, chloramphenicol and sulfamethoxazole-trimethoprim [4]. As ESBLs are the main reason of multi-drug resistance (MDR) in Gram negative clinical pathogens, their early detection and identification of the resistance enzymes will help us to optimize antimicrobial therapy and to prevent the spread of these organisms in hospitals and outbreak of any epidemic symptoms [5]. The most important mechanism of resistance in clinically significant bacteria is the production of one or more β-lactamase enzymes, which hydrolyze the β-lactam ring of β- lactam antibiotics. ESBL, a plasmid-mediated trait typically found in *Enterobacteriaceae*, is an emerging public health problem. An excessive use of antibiotics to prevent and reduce the frequency of infections has led to the selection and emergence of resistant bacteria, and they are involved in variety of infections like urinary tract infection (UTI) [6], septicaemia, hospital acquired pneumonia, intra-abdominal abscess, brain abscess, surgery device related infections and also from environments such as touch screens of ATM machines [7].

METHODOLOGY

Identification of clinical isolates

A total of 88 clinical isolates were isolated from 300 samples, recovered from patients infected with *Enterobacteriaceae* within the ages of 3 to 58 among both genders during August 2013 to November 2014 of tertiary care hospitals in North Eastern part of Tamil Nadu. Clinically significant bacteria were isolated by using standard microbiological procedures and identified at species level by automated methods with mini Api identification system. The study was carried out in Antibiotic resistance laboratory, VIT University, Vellore.

Antibiotic Susceptibility Pattern

Antibiotic susceptibility testing of the isolates was performed by the disk diffusion method, as per Clinical Laboratory Standard Institute [8] guidelines. Briefly, the inoculum was adjusted to the turbidity of 0.5McFarland was swabbed on to MH agar plates and commercial antibiotic disks were used (Hi-Media, Mumbai, India). The concentrations of used antibiotics were ampicillin (10µcg), amikacin (30µcg), gentamycin (10µcg), ciprofloxacin (5µcg), norfloxacin (10µcg), levofloxacin (5µcg), nitrofurontoin (30µcg), tobramycin (10µcg), ceftriaxone (30µcg), cefotaxime (30µcg), cefoxitin (30µcg), ceftazidime (30µcg), cefuroxime (30µcg), cefepime (30µcg), co-trimoxazole (25µcg), amoxicillin clavulanic acid (30µcg), imipenem (10µcg), meropenem (10µcg), piperacillin tazobactum (100/10µcg), ticarcillin clavulanic acid (75/10µcg), and Nitrofurantoin. The antibiotic potency of the disks was standardised against the reference strains.

Molecular studies

Plasmid profiling was done by using the plasmid DNA obtained by modified alkaline lysis method. PCR amplification for $bla_{CTX\text{-}M}$ group genes was carried out for all the isolates using PCR conditions and primers (Table-1) as previously described [9]. The selected PCR product sequence analysis was done using the Big Dye terminator cycle sequencing method (Applied Biosystems3730 and 3730*xl*) in (Macrogen) and compared to sequences within the NCBI database (http://www/ncbi.nlm.nih.gov/) by using Basic Local Alignment Search Tool (BLAST) [10].

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Table 1: PCR primer sequences for CTX-M group genes.

Primer Name	Primer Sequence (5'-3')	Product Size (bp)
CTX-M group 1 – f	AAAAATCACTGCGCCAGTTC	415
CTX-M group 1 – r	AGCTTATTCATCGCCACGTT	415
CTX-M group 2 – f	CGACGCTACCCCTGCTATT	552
CTX-M group 2 – r	CCAGCGTCAGATTTTTCAGG	552
CTX-M group 9 – f	CAAAGAGAGTGCAACGGATG	205
CTX-M group 9 – r	ATTGGAAAGCGTTCATCACC	205
CTX-M group 26 – f	GCACGATGACATTCGGG	327
CTX-M group 8/26 – r	AACCCACGATGTGGGTAGC	327 or 666
CTXM group 8 fwdII	TCGCGTTAAGCGGATGATGC	666

RESULTS

Identification of isolates

Out of 88 isolates, the most encountered pathogenic organisms were *Escherichia coli* (36%) followed by *Klebsiella pneumoniae* (23%), *Pseudomonas aeruginosa* (16%), *Enterobacter cloacae* (14%), *Proteus vulgaricus* (3%), *Proteus mirabilis* (7%) and *Acinetobacter baumannii* (2%) (Fig.1.).

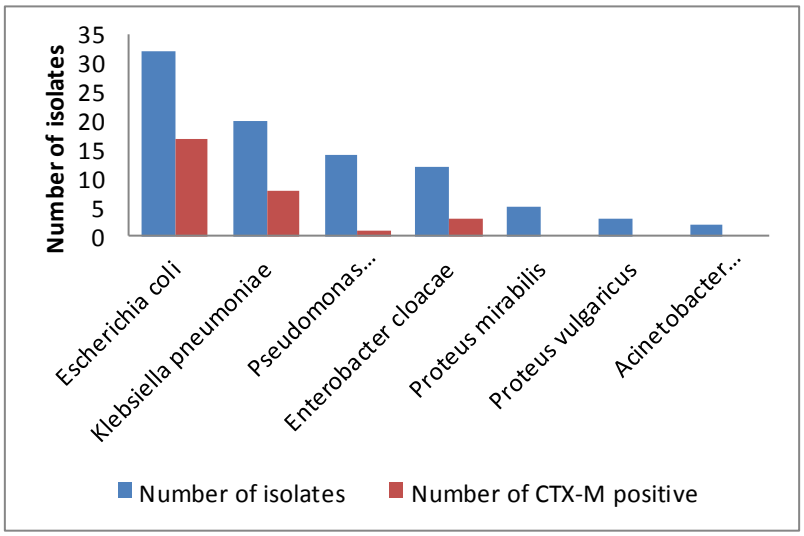

Figure 1: Comparison of CTX-M group gene positive isolates with total number of isolates.

Antibiotic susceptibility pattern

Among 88 bacterial pathogens isolated from the clinical specimens, 81 (92%) strains were phenotypically resistant to ESBLs (Fig.2.). The antimicrobial sensitivity patterns showed the overall resistance pattern of ceftriaxone was 88.8% followed by norfloxacin (95%), ciprofloxacin (88.8%), nitrofurantoin (45%), co-trimoxazole (77.5%), cefoxitin (55.5%) and levofloxacin (77.5%) though *Pseudomonas* showed resistance against norfloxacin (40%), levofloxacin (45%), tobramycin (60%), amikacin (60%), cefepime (40%) and ceftazidime (40%) but 80% of *klebsiella* spp. showed resistance against cefuroxime axetil, ceftriaxone, co-trimoxazole, ciprofloxacin and levofloxacin. The strains of *Proteus mirabilis* and *Proteus vulgaricus* showed maximum susceptibility against norfloxacin, levofloxacin, cefuroxime axetil, nitrofurantoin and ceftriazone. All the isolates were found to have resistance against cephalosporins.

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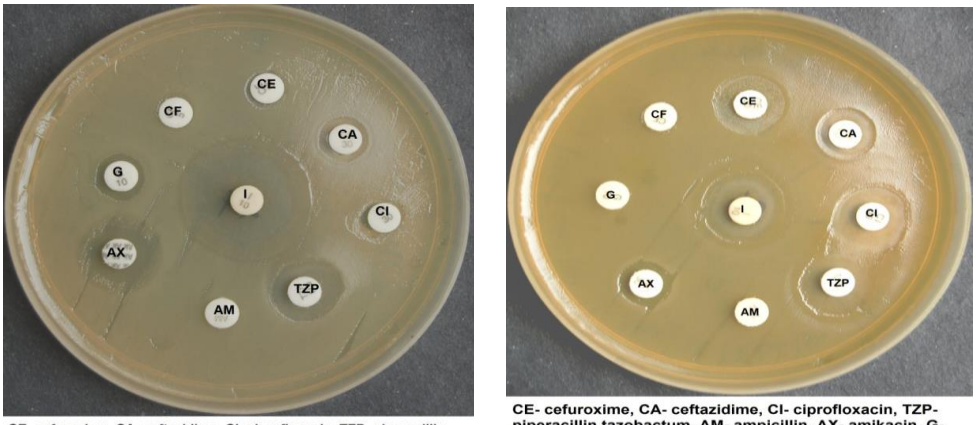

Figure 2: Antibiotic susceptibility test results with nine different beta-lactam antibiotic discs placed over MH agar swabbed with *E. coli* (left) and *K. pneumoniae* (right).

Molecular studies

Out of 88 selected strains, 72 (82%) strains yielded plasmids, while the remaining 16 (18%) were plasmid free. On agarose gel, multiple plasmids were observed for *E. coli, Klebsiella* spp. and *Proteus* spp. Plasmid copy number in these isolates ranged from 5 to 7, while the remaining had a plasmid copy number of 1 to 4. Plasmid DNA was isolated from the ESBL producers and they were subjected to multiplex PCR for the identification of $bla_{CTX\text{-}M}$ genes (Fig.3.). Of these 88 isolates, 29 strains (33%) were found positive for group I CTX-M genes (CTX-M-15), that includes 17 of 32 *E. coli* (53%), 8 of 20 *K. pneumoniae* (40%), 1 of 14 *P. aeruginosa* (7%) and 3 of 12 *Enterobacter cloacae* (25%) which is around 415bp in size. The sequenced gene was submitted to NCBI (accession no - KJ131190.1) and the ESBL was identified as CTX-M-15 type.

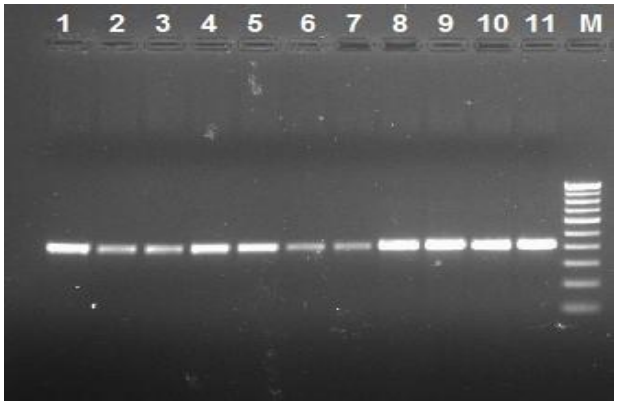

Figure 3: Multiplex PCR amplification of CTX-M group genes. Lane 1-11 with clinical isolates positive for CTX-M group 1 gene and M- DNA molecular ladder.

DISCUSSION

The prevalence of CTX-M producing organisms has progressively increased and become a major clinical problem worldwide. Infections by the ESBL producers are infamous for unfavourable outcomes and high mortality rates. Our study showed *Escherichia coli* was the most prevalent strain with 36%, followed by *Klebsiella pneumoniae* (23%) and *Pseudomonas aeruginosa* (16%). The isolates of *E.coli* showed maximum susceptibility against ceftriaxone (88.8%), norfloxacin (95%), ciprofloxacin (88.8%), nitrofurantoin (45%), co-trimoxazole (77.5%), cefoxitin (55.5%) and levofloxacin (77.5%). High amount of ESBL-producing bacteria from inpatients and outpatients indicates the continent wide spread of these species and particularly in *E. coli* with great variations in distribution and occurrence among different countries. Haque *et al*., (2012) had showed that the most prevalent isolates of *Escherichia coli* 58.6% and *Pseudomonas* 8.6% were resistance against most of the antibiotics including cefazolin, ceftriaxone, cefuroxime, ampicillin and co-trimoxazole [4]. The

Pseudomonas showed resistance against norfloxacin (40%), levofloxacin (45%), tobramycin (60%), amikacin (60%), cefepime (40%) and ceftazidime (40%). The isolates of *Klebsiella pneumoniae* showed resistance against cefuroxime axetil (80%), cefepime (40%), co-trimoxazole (45%), amikacin (35%) and levofloxacin (50%). This compared with the study by An *et al*.,(2012) reported that the resistance rated to drugs with lower overall resistance to amikacin, cefeoime, piperacillin/tazobactum, cefotetan and imipenem were 26.6%, 22.2%, 10.1%, 8.2% and 3.8% respectively [11]. A study by Oleson *et al*., (2013) showed that out of 115 ESBL isolates, 92% isolates produced CTX-M enzymes, most commonly 52% of CTX-M-15 [12]. A study by Achour *et al*., (2009) in *Klebsiella pneumoniae* reported the first evidence of presence of CTX-M-28 gene by sequencing its PCR products [13]. Another study reported that out of 140 *E. coli* strains screened for $bla_{\text{CTX-M}}$ group genes 50 showed (35.7%) presence of CTX-M group-1 genes [14]. In our study, all the $bla_{\text{CTX-M}}$ positive isolates (35%) were found to have CTX-M group 1 gene that was already reported in Iran [14], France [15], Switzerland [16] and Austria [17]. The remaining 52 isolates were found to be negative for $bla_{\text{CTX-M}}$ genes but they were phenotypically positive indicating the possibility for presence of ESBLs other than CTX-M and this result do not negate the possibility for other modified $bla_{\text{CTX-M}}$ in these isolates. As Gram negative organisms show increased resistance towards beta-lactam antibiotics future surveillance studies should use both phenotypic and genotypic tests for effective analysis. The high rate of ESBL production is due to the excessive use of broad-spectrum antibiotics in clinical sectors with lack of attention towards ESBL production in clinical pathogens [14]. May be spread of one single clone and/or plasmid is a reason for ESBL production with one common CTX-M gene at higher rate within these clinical isolates and owing to a number of limitations we could sequence only one CTX-M gene and could not determine the plasmid profiling for these isolates.

CONCLUSION

In the present study, *Escherichia coli* and *Klebsiella pneumoniae* were the species with highest ESBL production and they showed the highest prevalence of $bla_{\text{CTX-M}}$ group 1 (CTX-M-15) gene. It was also to be noted that not all phenotypically ESBL resistant isolates were found to have CTX-M group genes detected in this study. The results of this study describe the genetic characteristics and molecular epidemiology of ESBLs among *Enterobacteriaceae* isolates and provides insight emergence of bacterial strains harbouring ESBL genes. There is an urgent need to monitor the spread of these ESBL producers throughout the globe. If we don't give proper importance to monitor this MDR, in future we may lose all available antibiotics.

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