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Phenotypic and molecular characterisation of bla$_{CTX-M}$ ESBL gene in clinical isolates of Enterobacteriaceae in a tertiary care

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ABSTRACT

Members of the family cause mild to life-threatening infectious diseases in Enterobacteriaceae the recent past all the overworld. ESBL producing Enterobacteriaceae poses a threat to both community and hospital settings, which leads to empirical treatment failure, increasing the rate of morbidity and mortality. A cross-sectional convenient sampling study was done to detect and characterise CTX-M genes among extended spectrum β-lactamases (ESBLs) producing Enterobacteriaceae from clinical isolates in a tertiary care centre A total of 200 isolates of Enterobacteriaceae were collected from various clinical samples. ESBLs production was phenotypically detected by screening with ceftazidime, cefotaxime and cefepime, and then confirmed by disk combination method and multiplex PCR. *Escherichia coli* was the most predominant 87 (43.5%) isolate, followed by Klebsiella pneumonia 72 (36%), Klebsiella oxytoca 15(7.5%), *Proteus species 10* (5%), *Enterobacter species* 5 (2.5%) and *Citrobacter species* 1 (2.5%). ESBLs were detected by phenotypic confirmatory combination method in 144 (72%) isolates 197/200 (98.5%) were ESBLs producers were positive for bla$_{CTX-M}$ genes by PCR. The study highlights the high prevalence of bla$_{CTX-M}$ genes among the Enterobacteriaceae. CTX-M group 1 was the most commonly encountered CTX-M group in our study.

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INTRODUCTION

Resistance to antimicrobials has increased widely in gram-negative bacteria, especially Enterobacteriaceae. They cause a wide range of infections ranging from urinary tract infections, abscesses, meningitis, respiratory infections, diarrhoea and sepsis. The major resistance mechanism among the Enterobacteriaceae is the production of beta-lactamases. There are several types of beta-lactamases like ESBL, AmpC beta-lactamases, KPC.

ESBLs are plasmid mediated enzymes that confer resistance to extended-spectrum penicillins, 1st, 2nd cephalosporins, oxyimino-cephalosporins and monobactam, but sensitive to beta-lactam inhibitors. (Chambers, *et al.,* 2005) There are nine different types of ESBLs like TEM, SHV, CTX-M, TLA, OXA, PER, BES (Paterson DL, Bonomo RA. 2005). Each genotype has variants which differ by few amino acid sequences, and their distribution varies in different geographical regions.

Worldwide ESBL producing Enterobacteriaceae (ESBLP) has been highly prevalent with different genotypes either alone or in combination.

The prevalence rate is high in the Asia-pacific region, (Stephen P. Hawser, 2009).

CTX-M are cefotaximases, which was found in Germany in the year 1989, increased drastically from

the year 2000 worldwide, dominating the other types, with prevalence range from 60-90 %. There are five phylogenetic groups of CTX-M: Group1, 2, 8, 9, 25 (Jemima *et al.,* 2008).

As the antibiotic resistance profile changes in the different geographic regions from time to time, it is necessary to gain the knowledge to change the antibiotic prescription pattern to reduce selection and spread of resistant strains.

The study was undertaken to detect, isolate phenotypic ESBL and to characterise the CTX-M genotype.

MATERIALS AND MATERIALS

A total of 200 Enterobacteriaceae isolated from non-hospitalized and hospitalised patients from January 2015 – November 2016 from a variety of clinical specimens (e.g., urine, pus, sputum, blood catheter tips, endotracheal tube etc.,) were used for this study (Woodford N et al., 2006).

Identification of isolates

Isolates were identified by colony morphology on blood agar, Macconkey agar, Gram staining and Standard biochemical reactions.

Antibiotic susceptibility testing: Antibiotic susceptibility testing was done for cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), Cefepime, Cefepime-tazobactam (30/10 µg), Piperacillin-tazobactam (100/10 µg), Cotrimoxazole (1.25/23.75µg), Amikacin (30 µg), Ciprofloxacin (5 µg), Imipenem (10 µg), Meropenem (10 µg), Gentamicin (10 µg), by Kirby Bauer Disc Diffusion method as per CLSI guidelines 2015. The discs were purchased from Himedia laboratories.

ESBL Screening test: ESBL phenotypic screening was performed for all the isolates by the disk diffusion test using ceftazidime (30 µg) and cefotaxime (30 µg). (The CLSI guidelines for ESBL screen test are Ceftazidime 30 µg $\leqslant$ 22 mm, Cefotaxime 30 µg $\leqslant$ 27 mm, Ceftriaxone 30 µg $\leqslant$ 25 mm). Isolates which were screening test positive for ESBL were subjected to phenotypic confirmatory disc combination test. E. coli ATCC 25922 was used as the control, and the zone diameter was interpreted per the CLSI recommended guidelines.

ESBL Phenotypic confirmatory double disc combination test

Lawn culture of the isolates adjusted to 0.5 Mc Farland was done in Muller Hinton agar (MHA). 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), (Hi-media, Mumbai, India) was placed on the MHA plate. The plates were incubated at 37ºC for 18 hrs. E. coli ATCC 25922 was used as negative control, and K. pneumoniae ATCC 700603 was used as positive control. The tests were interpreted according to CLSI guidelines, 2015. A 5 mm or more, increase in the zone of inhibition for CTX/CLA, CAZ/CLA containing disks compared the corresponding CTX, CAZ alone was considered as ESBL positive.

Molecular characterisation of ESBL isolates

All the 200 isolates were subjected to detection of five groups of CTX-M as described by Woodford N *et al.,* 2006. Few colonies from overnight culture on Nutrient agar plate was suspended in 500µl of distilled water in a microcentrifuge tube, and the cells were lysed by heating at 95°C for 10 minutes. Cellular debris was removed by microcentrifugation at 8,500 rpm for 10 minutes, and the supernatant was used as a source of template DNA. Forward and reverse set primers was used for simultaneous detection of all five groups of CTX-M type were used.

The primers used (Woodford N et al., 2006) table 1 with two specific forward primers and a shared reverse primer. Amplification was done in Eppendorf thermocycler. Each reaction mix of 25µl was used. The Initial denaturation was done for 5 minutes at 94°C, 30 cycles of 94°C for 25s, 52°C for the 40s and 72°C for 50s and a final elongation of 72°C for 6 minutes.

Table 1: The primers used

Group	Sequence	Amplicon
Group 1	5'-AAA AAT CAC TGC GCC AGT TC and 5'-AGC TTA TTC ATC GCC ACG TT	415 bp
Group 2	5'-CGACGCTAC CCCTGC TAT T and 5'-CCAGCGTCAGAT TTT TCA GG	552 bp
Group 9	5'-CAA AGA GAG TGC AAC GGA TG and 5'-ATT GGA AAG CGT TCA TCA CC.	205 bp
Group 8	5'-TCG CGT TAA GCG GAT GAT GC and 5'-AAC CCA CGA TGT GGG TAG C	666 bp
Group 25	5'-GCA CGA TGA CAT TCG GG and and 5'-AAC CCA CGA TGT GGG TAG C	327 bp

The PCR products were analyzed by 1.5% w/v agarose gel electrophoresis, the gels were stained with ethidium bromide and visualized under UV light. A100 bp ladder was used.

RESULTS

Among the 200 isolates, all the isolates were 100% resistant to cefotaxime and ceftazidime, 69.5% to cefepime, 27.5% to cefepime-tazobactam, 29.5%

to piperacillin-tazobactam, 32.5% to imipenem, 26.5% to meropenem, 48% to Amikacin ,65.5%% to gentamycin, 70.5% to cotrimoxazole, and 68.5% to ciprofloxacin (Figure 1). 25-30% of the isolates were resistant to ceftazidime-clavulanic acid, cefotaxime-clavulanic acid and cefepime-tazobactam, piperacillin-tazobactam. About 50-70% were resistant to amikacin, gentamicin, cotrimoxazole, ciprofloxacin. Only 5.5% were sensitive to ceftriaxone.

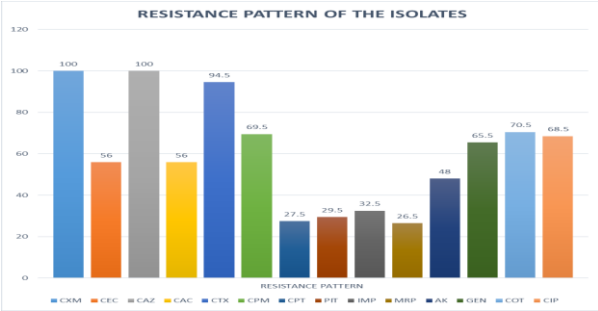

Figure 1: Resistance pattern of the isolates

CXM –cefotaxime, CEC – cefotaxime-clavulanic acid, CAZ- ceftazidime, CTX- ceftriaxone, CPM – cefepime, CPT – cefepime-tazobactam, PIT- piperacillin-tazobactam, IMP – imipenem, MRP – Meropenam, AK – Amikacin, GEN – gentamycin, COT – cotrimoxazole, CIP – ciprofloxacin.

In *Escherichia coli* was the most predominant 87 (43.5%) isolate, followed by Klebsiella pneumoniae 72 (36%), Klebsiella oxytoca 15(7.5%), *Proteus species 10* (5%), *Enterobacter species* 5 (2.5%) and *Citrobacter species* 1 (2.5%). The prevalence of ESBL has increased drastically over the years in the two genus of the family Enterobacteriaceae, Escherichia coli and Klebsiella pneumoniae.

ESBLs were detected by phenotypic confirmatory combination method in 144 (72%) isolates. 197/200 (98.5%) were ESBLs producers were positive for bla_{CTX-M} genes by PCR. All 197 isolates were CTX-M group 1 positive (Figure 2).

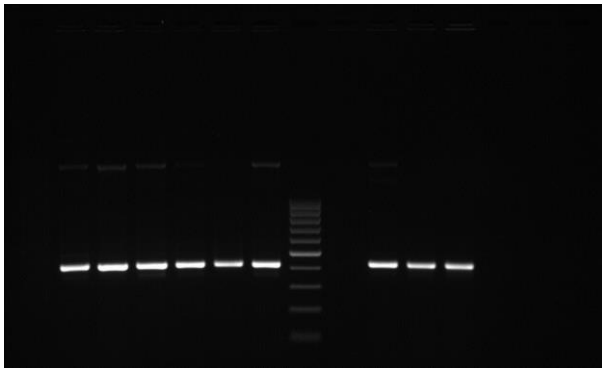

Figure 2: Gel electrophoresis of PCR amplification product of blactx-m group 1(415bp).
Only lane 7 negative for CTX-M gene. Lane 1, 2, 3, 4, 5, 8, 9, 10 are CTX-M group. Lane 6 DNA molecular ladder

DISCUSSION

Resistance to beta-lactam antibiotics due to beta-lactamases is a major problem in developing and developed countries. The ESBL producing Enterobacteriaceae has emerged as a challenge for both communities acquired and hospital-acquired infections in *India* (Varsha Gupta, 2007). The prevalence rate of ESBL in India is around 62-100% (Mathai *et al.,* 2009) These organisms are widely distributed in *E. coli* and *Klebsiella* species of the Enterobacteriaceae, which is also evident in our study. The ESBL prevalence rate in *E. coli* was 43.5%, and Klebsiella pneumoniae was 36% which was similar to (Sekar *et al.,* 2006) study.

Resistance to gentamycin, cotrimoxazole and ciprofloxacin have been shown in the isolates. Such Multidrug resistance has been attributed to the CTX-M gene because of its association with sul 1 type integrons. Genetically the structure is linked to class 1 integrons that combine with antibiotic-resistant gene cassettes responsible for resistance to aminoglycosides, β-lactams, chloramphenicol, sulphonamides, and to a lesser extent rifampicin (Pitout JD *et al.,* 2005).

In our study resistance to carbapenems like meropenem and imipenem has been reported. But in Manoharan *et al.,*2011, the study showed 100% susceptibility. This may be due to carbapenemases or porin loss in these isolates. Majority of isolates were from urine, followed by pus, wound.

The prevalence rate of CTX-M was 98.5% which was almost similar to the recent study by *Khosrow Zamani et al., 2015* showing 91.5%**.** The CTX-M is the predominant type of ESBL that is prevalent Worldwide for the past two decades. This may be due to the widespread use of cefotaxime for treating infections. First, it was reported from Europe, South America and Asia (Hayley Wilson and M. Estee Torok 2018). The high prevalence of CTX-M among the Enterobacteriaceae may be attributed to following reasons: colonization, irrational use of antibiotics, animal source, dissemination through the medical personnel, improper infection control measures, spread through transposons (Chong Y *et al.,* 2011).

CTX-M genes are found mainly in the family Enterobacteriaceae and are described as “epidemic resistance plasmids” because of the ability to acquire resistance genes and transfer among bacteria (Carattoli, 2011).

CTX-M group 1 is the most commonly identified group in Europe, Asia, Africa and the USA. All the CTX-M belong to group 1 in our study which is similar to the study done by R.Mohamudhaparveena, *et al., 2012,* and also in the various parts of India.

About 28% isolates were negative for PCDDT, but positive for CTX-M type this indicates that even if phenotypic confirmatory test is negative, it does not indicate that the isolate is negative for ESBL. This is due to simultaneous production of other beta-lactamases like AmpC beta-lactamases, carbapenemases, and porin loss.

Molecular detection methods are preferable than phenotypic tests which can detect the resistant genes and provides information on the epidemiology of resistance mechanism on a genetic basis, which suggests valuable information for infection control practices (Sundsfjord A *et al.,* 2004).

CONCLUSION

ESBLPE are increasing drastically, as all our isolates were positive for ESBL. About 60-70% are MDR, which warrants the need for strategies to be planned and implemented to the rational use of antibiotics. CTX-M prevalence is alarming. However, the other ESBLs genes can also remain in the isolates. Thus, study on ESBL must be done regularly to formulate the empirical therapy.

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