CTX-M TYPE EXTENDED SPECTRUM β-LACTAMASES IN ESCHERICHIA COLI ISOLATES FROM CLINICAL SAMPLES FROM KALABURAGI REGION

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ABSTRACT

The β-lactam antibiotics, along with amino glycosides are widely prescribed antibiotics. However resistance to these drugs continues to increase due to their injudicious use. In recent years, resistance in the Indian E. coli population has increased and mostly showing complex mechanisms like extended spectrum β-lactamases (ESBLs) specifically blaCTX-M. The reason may be increased intracontinental movement of the human population. So it should be better to know the prevalence and resistance pattern of these strains, well in advance, to design a systematic policy for empirical therapy. Methods: 150 isolates of Escherichia coli obtained from 170 non-repeat samples of urine, blood, stool, us etc were studied and their resistance rates and patterns were noted. The isolates were screened for prevalent cephalosporin’s resistance (ESBLs) by antibiotic susceptibility test. Minimum inhibitory concentration (MIC) for cephoxatime and ceftazidime was done by HiComb MIC-test strips ESBLs were confirmed by NCCLS-ESBL phenotypic confirmatory test (Combined disc diffusion test) and E-test. Test was carried out using cephoxatime (30μg), ceftazidime (30μg), cephoxatime/clavulanicacid (30/10μg) and ceftazidime/clavulanic acid (30/10 μg) discs. Genotypic conformation for presence of ESBL (CTX-Type) was established by PCR. Results: Resistance to 5 beta-lactam antibiotics tested was varying between 70 to 80 percent i.e. approximately 120 isolates. MICs of ESBL producing isolates ranged from 8 to > 240 μg/ml for both ceftazidime and cephoxatime. 110(92%) of above 120 resistant strains were positive for ESBL production both by the E -test for ESBL and ESBL detection kit (Combination disk method). The PCR band after amplification at 544 bp was visualized.

KEYWORDS: E.coli, ESBLs, CTX-M, Clinical Samples.

INTRODUCTION

The β-lactam antibiotics, especially the cephalosporin’s and β-lactam-b-lactamases inhibitor combinations are major drug classes used to treat infections caused by E. Coli.[1] Among E. coli, β-lactamase production remains the major contributing factor to β-lactam resistance. Extended spectrum b-lactamases are one of the major source of resistance to oxyimino-cephalosporins in Enterobacteriaceae.[2] Most of ESBLs are mutants of TEM and SHV enzymes, but CTX-M enzymes are the newly emerging ESBLs[3] and are increasingly prevalent worldwide among E.coli bacteria. The CTX-M enzymes are wide group with more than 30 alleles categorised into five distinct phylogenetic groups, evolved because of genetic escape and mutation of the chromosomal b-lactamase genes of Kluyvera spp.[2,3] The CTX-M family, first described in 1992[2,3] is known as most dominant non-TEM, non-SHV ESBL among Enterobacteriaceae.

It is identified as a rapidly growing family of ESBLs that selectively prefer to hydrolyze cephoxatime and most of them are active against ceftazidime.[5] Further the incidence of Urinary tract infections (UTI) by ESBL producing E. coli was found to be the highest in India (60%) followed by Hongkong ( 48%) and Singapore (33%).[6] Previous studies from India have reported ESBL production varying from 28% to 84%.[7] On the whole prevalence of ESBL producers was found to vary in different geographical regions and in different institutes. CTX-M enzymes have been the predominant ESBLs in Argentina for >10 years[8] and have prevalence in many parts of the world.[3,9] including Europe.[10,11] Therefore in the present study we are predominantly focussing on prevalence of CTX-M ESBL producing E.coli in our region.
MATERIALS AND METHODS

Isolation and identification of E.coli
For this report, we analysed data for 150 strains of E. Coli from 170 different clinical Samples like urine, stool, blood, pus etc over a period of 3 months in 2013. The clinical samples were collected from hospitals and diagnostic laboratories in Kalburagi region over a period of three months in 2013. Identification was done by culture on EMB agar. Isolation of strains was done by conventional morphological, cultural and biochemical characterisation. Standard strain of E.coli MTCC 443 was obtained from Medical and Phage Therapy Laboratory, department of Biotechnology, Gulbarga University, and Kalaburagi.

Antibiotic Sensitivity Test and MIC Determination
Antibiotic sensitivity test was conducted for E. coli isolates using disk diffusion techniques as described by Bauer-Kirby method by using standard antibiotic disk.[12] Isolates were tested for resistance against commonly used third generation broad spectrum antibiotics like Cefpime (30 µg), Cefotaxime (30 µg), Cefpodoxime (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg). The antibiotic disks were obtained from Hi-Media Laboratories, Mumbai, India. After incubation at 37º C overnight, diameter of the zone of inhibition were measured and results were interpreted according to CLSI guidelines.[13] and recorded as susceptible(S), intermediate susceptible(I) or resistant (R) to the antibiotics. MIC’s of antibiotics were also determined on Mueller–Hinton Agar using Hicomb MIC strips according to CLSI.[14] Following antibiotics were used: cephotaxime (0.0001 to 240 µg), ceftazidime (0.0001 to 240 µg) (Hi-Media, Mumbai, India).

Combination disk method Test for ESBL production
The Combined disk diffusion test (CLSI Recommended) was followed. Test was carried out using cefotaxime (30 µg), ceftazidime (30 µg) and cefotaxime/clavulanic acid30/10g) and ceftazidime/clavulanic acid (30/10 µg) discs. The discs were obtained commercially from Hi-Media Laboratories, Mumbai, India. Standard strain of E.coli MTCC 443 was obtained from Medical and Phage Therapy Laboratory, department of Biotechnology, Gulbarga University, and Kalaburagi. Positive results were taken when there was a ≥/> 5mm increase in ceftazidime/clavulanic acid zone diameter.[14]

Confirmation of ESBL using E- test
The confirmation of ESBL was also performed by E-test ESBL strips (E-test Himedia) and the test was performed in accordance to the manufacturer’s instructions. Double ended strips containing gradient of ceftazidime and ceftazidime +clavulanic acid, cefotaxime and cephoxame+clavulanic acid at the other end were tested. The presence of ESBL was confirmed by the appearance of phantom zone below the formation of TZ inhibition ellipse and clavunate caused a more than or equal to three doubling concentration decrease ratio of > 8 in the MIC values of ceftazidime.[14]

Detection bla CTX-M genes
DNA extraction
DNA was isolated from bacterial cells using DNA purification kit (DNA purification kit, Himedia Mumbia). The purified DNA was stored at -20°C. The samples were run on agarose gel and stained with ethidium bromide. The stained gel was examined for presence of bands under UV light using molecular weight marker.

PCR amplification of bla CTX-M genes
The PCR assay was targeted for the presence of CTX-M genes with specific primers.[15] PCR amplification of bla CTX-M alleles was carried out on 5 resistant isolates with these primers BlaCTX-M 5
TCCGCAGATAATCCAC-3\ for 3'ATGTGCGAYACCAGTAARGT-5'. A single reaction mixture contained 1 µl of DNA extract, 30 pmol of each primer, 100 µM (each) dNTPs, 1.25 U Taq polymerase and buffer with 1.5 mM MgCl2 supplied along with the kit in a total volume of 50 µl. A thermal cycler was used and the following reaction parameters were used: initial denaturation at 94°C for 7 min; denaturation at 94°C for 50 sec, annealing at 50°C for 40 sec and elongation at 72°C for 60 sec, repeated for 35 cycles; and final extension at 72°C for 5 min15. The resulting PCR products were run in 1.6 per cent agarose gels (Himedia, Mumbai) containing 1xtris-acetate EDTA and 0.5 µg of ethidium bromide/ ml and visualized under UV transilluminator and photographed with Gel documentation system.[16]

RESULTS
In the present study 150 isolates of E. coli from 170 non-repeat samples of urine, blood, stools, pus etc were isolated. Out of 150 isolates of E. coli around 120 (70-80%) (Table 1) (Fig 1) isolates were resistant to third generation antibiotic like Cefpime (30 µg), Cefotaxime (30 µg), Cefpodoxime (30 µg), Ceftriaxone (30 µg). The presence of ESBL was confirmed by the appearance of phantom zone below the formation of TZ inhibition ellipse and clavunate caused a more than or equal to three doubling concentration decrease ratio of > 8 in the MIC values of ceftazidime.[14]

According to cephalosporins susceptibility test of E.coli sample in Kalaburagi region 70 to 80 percent of strains were resistant. This was in accordance to others studies in India. In a recent study in north-western India by Kaur and Aggarwal,[17] 45.8% isolates were found to be ESBL producers. In south e Asian region, particularly India, China and Thailand are marked as high-risk countries because of the increased rates of infection caused by ESBL-producing E. coli and K. Pneumoniae.[18] India, with the prevalence >80%, is now said to be the centre of ESBL-producing strain.[19] Another study from south India reported 79.4% of phenotypic positive nosocomial isolates positive for CTX-M genes, of which 63.7% were E. coli and all were positive for the CTX-M-1 group.[20] Suhkla et al.[21] have reported that 72 per cent of isolates were found to be resistant to all 3rd generation cephalosporin’s. These reports are evidence for high
prevalence of CTX-M type ESBL in almost all parts of India. MICs of ESBL producing isolates ranged from 8 to > 240 μg/ml for both ceftazidime and cefotaxime, majority of the isolates (120 i.e. 80%) had MIC > 32 μg/ml and few (40%) >240 μg/ml (fig 3). In the phenotypic confirmatory test for esbl using cephalosporin/clavulanate combination discs, 110(92%) strains showed enhanced susceptibility to ceftazidime and cefotaxime in the presence of clavulanic acid, thus indicating ESBL production in them (fig 2). Five random ESBL positive isolates were tested for genotypic conformation for blaCTX-M gene amplification. Amplification of blaCTX-M produced a band at 544 bp in all five samples.

Table 1: Percentage of resistance among E.coli isolates.

<table>
<thead>
<tr>
<th>Sl no</th>
<th>Antibiotic Used</th>
<th>% of resistance</th>
<th>No of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cefipime(30 μg)</td>
<td>80</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>Cefotaxime(30 μg)</td>
<td>76</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
<td>Cefpodoxime(30 μg)</td>
<td>76</td>
<td>63</td>
</tr>
<tr>
<td>4</td>
<td>Ceftriaxone (30 μg)</td>
<td>70</td>
<td>63</td>
</tr>
<tr>
<td>5</td>
<td>Ceftazidime(30)</td>
<td>74</td>
<td>66</td>
</tr>
</tbody>
</table>

Figure 1: Antibiotic Resistance to cephalosporins antibiotic.

Figure 2: MIC for Resistant Strains.

Figure 3: PCR Amplification of bla CTX-M gene.

Lane M: 1Kb DNA ladder; Lane 1-5: ESBL positive strains.

DISCUSSION

The present study clearly highlight that most of the isolated E.coli were resistant to third generation cephalosporin’s like Cefipime, ceftazidime, cefpodoxime, cefotaxime and cefuroxime. E. coli possessing CTX-M gene causing UTI’s began to be reported from earlier this decade from USA, Saudi Arabia, India, Japan, Nepal, China and Brazil.[36] Sundram Medical Foundation India has reported an ESBL positivity rate between 26.9% and 48.3%.[22-25] The present study clearly shows that there is high prevalence of ESBL E. coli in clinical isolates and its resistance to commonly used antibiotics in this region. ESBL producers do not respond well to the usually prescribed empirical therapy. Also, there is an rising risk of associated mortality and high cost of therapy when the patients are put on the standard empirical therapy.[26] Recent surveys have shown that ESBLs in Enterobacteriaceae in India range from 70–90%.[27] consequently making the use of reserved antibiotics such as carbapenems necessary.[28,29] Though the rates of cephalosporin resistance are lower in other countries in comparison to India but the growing prevalence of ESBL producers is sufficient to drive a greater dependence on carbapenems. Along with time the selection pressure for
carbapenem resistance in Enterobacteriaceae will increase leading to its widespread prevalence. This will be a worldwide public health concern, since there are very few antibiotics beyond carbapenems.\(^{30}\) Already *Klebsiella pneumonia* clones with KPC carbapenemase are a major problem in the USA, Greece, and Israel, and plasmids encoding the VIM metallo-carbapenemase have disseminated among *K pneumoniae* in Greece.\(^{30}\) Most investigators in India have used phenotypic methods, and have reported prevalence ranging from 6.6 to 88.8 per cent prevalence of ESBLs. There are a quite a few reports of molecular identification of beta lactamases in India. In south India Sekar et al.\(^{31}\) reported the prevalence of blaCTX-M gene in the 39 selected clinical isolates. The prevalence of blaCTX-M was reported by Shahid et al., 2006\(^{32}\) and 72 (77.4%) of the 93 *E.coli* isolates were found to be CTX-M group -1 positive by PCR in north Indian isolates.

In conclusion, Non-prescription sale and use of antibiotics in India is leading to selection pressure on ESBL producing *E.coli*.\(^{33}\) This increase in resistance is of great public health concern because there only few antibiotic in reserve.\(^{34}\) More importantly, failure to initiate appropriate antibiotic therapy from the start appears to be responsible for higher patient mortality (35) to avoid this indiscriminate use of antibiotic and to control the selection pressure over ESBL producing *E.coli* population, knowledge of local and recently susceptibility trends is useful. There should be constant vigilance over the local ESBL prevalence as there is variation in report of ESBL positive samples in different parts of India. Our report gives brief insight of susceptibility pattern of *E.coli* in Kalaburagi urban areas. A proper empirical therapy can be designed using this data.

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**Conflict of interest statement**

We declare that no conflict of interest.

**REFERENCE**


