

PREVALENCE OF EXTENDED-SPECTRUM BETA-LACTAMASE PRODUCING *ENTEROBACTERIACEAE* MEMBERS ISOLATED FROM CLINICALLY SUSPECTED PATIENTS

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ABSTRACT

Objective: Emergence of extended-spectrum beta-lactamases (ESBLs) production poses another clinical problem with Gram-negative bacterial infections. The present study was aimed to evaluate the ESBL producers among various clinical samples of clinically suspected patients.

Methods: A total of 1279 samples (urine [918], pus [207] and stool [154]) were collected and 465 isolates (*Escherichia coli* [320], *Enterobacter aerogenes* [119] and *Klebsiella pneumoniae* [26]) were isolated and screened for the presence of ESBL producers using combination disc method and double disc synergy test.

Results: Of the 465 culture positive isolates, 130 (*E. coli* 93 [29.06%], *E. aerogenes* 35 [29.41%] and *K. pneumoniae* 2 [7.69%]) were identified as ESBL producers. Among the three *Enterobacteriaceae* members, *E. coli* 93 (29.06%) was found to be predominant ESBL producer next in order *E. aerogenes* 35 (29.41%) and *K. pneumoniae* 2 (7.69%). Maximum number of ESBL producers were recovered from urine (n=111) followed by pus (n=14) and stool (n=5). All the ESBL-producing isolates were subjected to antibiotic sensitivity test using 10 different antibiotics. ESBL producers were chiefly resistance to ceftriaxone followed by ceftazidime and cefotaxime. Of 130 ESBL producers, 15 (*E. coli* (8), *E. aerogenes* (6) and *K. pneumoniae* (1)) strains were selected for genotypic identification. Among, only two strains of *E. aerogenes* were positive isolates for CTX-M type ESBL in polymerase chain reaction.

Conclusion: This study concluded that among *Enterobacteriaceae* members, *E. coli* was the predominant ESBL producers and urine was noted as the prime source for the ESBL positive isolates when compared to other source. Genotypic identification was the best method to differentiate ESBL types which were essential to provide proper treatment.

Keywords: Extended-spectrum beta-lactamase, *Enterobacteriaceae*, *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pneumoniae*.

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INTRODUCTION

Infections caused by extended-spectrum beta-lactamase (ESBL)-producing, Gram-negative bacteria are associated with increased morbidity and mortality, which is linked to inappropriate or delayed antimicrobial treatment [1]. Since the introduction to the extended spectrum cephalosporins into clinical use, strains expressing ESBL have been reported from to the world in increasing numbers [2]. There is no consensus on the precise definition of ESBLs. A commonly used working definition is that the ESBLs are β -lactamases capable of hydrolysis of the antibiotics such as penicillins, first-, second- and third-generation cephalosporins and aztreonam (AT) (but not the cefamycins or carbapenems) and which are inhibited by β -lactamase inhibitors such as clavulanic acid [3]. The first report on plasmid-encoded β -lactamases capable of hydrolyzing the extended-spectrum cephalosporins was published in 1983 [4]. Among the family *Enterobacteriaceae*, the production of plasmid-mediated extended-spectrum β -lactamase (ESBLs) has emerged as an important mechanism of resistance to β -lactam drugs [5]. ESBLs have been found mainly in *Klebsiella* spp., and *Escherichia coli*, but have been also reported on other genera worldwide, such as *Citrobacter*, *Enterobacter*, *Morganella*, *Proteus*, *Providencia*, *Salmonella*, *Serratia* and *P.seudomonas* [6,7].

The ESBL genes are mostly plasmid-encoded [8], and most ESBLs can be divided into three genotypes: Temoniera (TEM), sulphydryl

variable (SHV), and CTX-M [3]. The predominant ESBL genotypes were TEM and SHV [9]. Most ESBLs are TEM and SHV enzyme derivatives characterized using a few point mutations at selected loci within the gene [2,10]. This enzyme was found in a blood culture isolate of *E. coli* from a Greek patient named TEM, hence the designation TEM [11]. The SHV-type ESBLs may be more frequently found in clinical isolates than any other type of ESBLs [12]. SHV refers to SHV. In addition, a genotype the CTX-M enzyme emerged worldwide when compared to TEM and SHV [9]. The plasmid-mediated ESBLs, which preferentially hydrolyze cefotaxime (CE) and are better inhibited by tazobactam than by sulbactam and clavulanate so-called as CTX-M enzymes [11]. In the 1990s, a novel type of ESBL, the CTX-M enzyme, emerged worldwide [9]. The CTX-M types, now exceeding 50 different types, can be divided into five groups based on their amino acid identities: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 [13]. These enzymes are not very closely related to TEM and SHV β -lactamases as they show only 40% identity with these enzymes [14].

Infections caused by ESBL-producing bacteria often involve immune-compromised patients, making it difficult to eradicate these organisms in high-risk wards, such as intensive care units [15,16]. Drug susceptibility data are of major importance to the clinical management of patients infected by these organisms [10]. Thus, monitoring of the prevalence and the types of extended-spectrum β -Lactamase enzymes

may contribute to defining the degree of the problem with a specific geographical area, and to establish a proper treatment protocol [17]. The aim of this study was to determine the prevalence of ESBL-producing *Enterobacteriaceae* members of the Department of Microbiology, DDC laboratory in Tiruchirappalli, India.

MATERIALS AND METHODS

The study was conducted in the Doctor's Diagnostic Centre, Tiruchirappalli, over a period of month from July to August 2013. A total of 1,279 consecutive, non-repetitive, Gram-negative isolates from various clinical samples such as urine (n=918), pus (n=207), and stool (n=154) were included in the study.

Isolation and identification

The cultures were isolated from selective agar, MacConkey agar, and blood agar (HiMedia, Mumbai) to study their cultural characteristics. A single isolated colony was considered for further studies and identification was done using the standard procedure. Gram's staining, morphological, cultural, and biochemical test were performed [18].

Antibiotic sensitivity test

The resistance to one or more 3rd generation cephalosporin's (ceftazidime [CAZ], ceftriaxone (CTR), cefotaxime, etc.) prompted us to detect ESBL producers, the common mechanism of beta-lactam resistance.

All the isolates were subjected to antimicrobial susceptibility agents and were determined using Disc Diffusion method of Kirby-Bauer [19] on Mueller-Hinton Agar as described by the Clinical and Laboratory Standard Institute (CLSI). The antibiotic discs used (HiMedia, Mumbai) were Cefoxitin (30 mcg), Cefepime (30 mcg), Cefepime+Tazobactam (30/10 mcg), CAZ (30 mcg), ceftazidime+tazobactam (30/10 mcg), CTR (30 mcg), Cephataxime or CE (30 mcg), cephotaxime+clavulanic acid (30/10 mcg), imipenem (10 mcg), and AT (30 mcg) [20].

Combination disc method

The combination disc test using both CE and CAZ, alone and in combination with clavulanic acid, was performed for the detection of ESBL according to the CLSI guidelines. In this test, an overnight culture suspension of the test isolate which was adjusted to 0.5 McFarland standards was inoculated using sterile cotton swab on the surface of a Mueller-Hinton agar plate. The CE (30 µg) and cefotaxime-clavulanic acid (30/10 µg) disks were placed 20 mm apart on the agar. Similarly, CAZ (30 µg) and ceftazidime-clavulanic acid (30 µg/10 µg) disks were placed 20 mm apart. After incubating overnight at 37°C, ≥5 mm increase from the zone diameter for either antimicrobial agent which was tested in combination with clavulanic acid. Its zone, when tested for, was interpreted as positive for ESBL production [21].

Double disc synergy test

The test inoculums (0.5 McFarland tube) was spread as a lawn onto Mueller-Hinton agar (MHA) plate using a sterile cotton swab. A disc of CAZ (30 µg)+clavulanic acid (10 µg) was placed on the surface of MHA, then the disc of CAZ (30 µg) was placed at the distance of 15 mm from the ceftazidime+clavulanic acid disc by the edge to edge. The inoculated plates were incubated at 35°C in the incubator for 18–24 h. The zone of inhibition between the CAZ and ceftazidime+clavulanic acid was compared. The difference in the zone diameter of ≥5 mm was interpreted as positive for ESBL production [22].

Molecular identification

Isolation of DNA

TE buffer was added to overnight grown cells by gentle pipetting. Then, each of the tubes were added with 30 µl of 10% SDS and 3 µl of 20 mg/ml Proteinase K. The tubes were vortexed and then incubated at 37°C for 1 h. A volume of 100 µl of CTAB/NaCl solution was added to 150 µl of 5 M NaCl, mixed well and incubated at 65°C for 10 min and an equal volume

of chloroform:Isoamyl alcohol mixture was added and centrifuged. The aqueous viscous supernatant was transferred to fresh tubes equal volumes of phenol:chloroform:isoamyl alcohol (25:24:4) was added. After centrifugation, to the supernatant ice-cold isopropanol was added and mixed well. The pellets were washed with 70% ethanol, and the nucleic acids were recovered by centrifugation. The pellets were then kept for drying after the complete removal of the supernatant and finally resuspended in 15 µl of distilled water and stored at 4°C [23]. Polymerase chain reaction (PCR) amplification for CTX-M was carried out for all the isolates, based on the producers described by Woodford et al. [24]. After this, the amplified DNA fragments were purified from agarose gels using QIA gel extraction kit manufacturer's protocol and sequenced using forward and reverse about 771 bp were carried out in Xcelris, Ahmedabad.

RESULTS

A total of 1279 various clinical samples were recovered from clinically suspected patients which include, urine 918 (71.77%), pus 207 (16.18%), and stool 154 (12.04%). Based on the standard laboratory procedures, three *Enterobacteriaceae* members (*E. coli* 320 [68.81%], *Enterobacter. aerogenes* 119 [25.59%] and *Klebsiella pneumoniae* 26 [5.59%]) are identified in Table 1.

Further, these 465 isolates were screened for the presence of ESBL producers using combination disc method and double disc synergy test. Of the 465 culture positive isolates, 130 (*E. coli* 93 [29.06%], *E. aerogenes* 35 [29.41%], and *K. pneumoniae* 2 [7.69%]) were identified as ESBL producers, and remaining 335 were nonESBL producers (*E. coli* 93 [70.93%], *E. aerogenes* 35 [70.58%], and *K. pneumoniae* 2 [92.30%]) (Table 2).

A maximum number of ESBL producers were recovered from urine (n=111) followed by pus (n=14) and stool (n=5). Female patients were more prone to infection caused by ESBL producing isolates 78 (urine [38], pus [10], and stool [4]) than male patients 52 (urine [73], pus [4], and stool [1]) (Fig. 1).

The susceptibility profile of ESBL producers is depicted in Fig. 2. Of the 10 antibacterial agents, a maximum number of ESBL producers were resistance to CTR (126/130) followed by CAZ (115/130) and CE (110/130). Interestingly, except one isolate (1/130), all the ESBL producers were susceptible to imipenem.

For the genotypic identification, 15 ESBL positive strains (*E. coli* [8], *E. aerogenes* [6] and *K. pneumoniae* [1]) were selected, among two strains of *E. aerogenes* were positive isolates for CTX-M type ESBL in PCR. The amplified CTX-M gene after gel elution was sequenced using forward and reverse, about 415 and 771 bp were carried out in, Xcelris, Ahmedabad. The sequences obtained were aligned with previously published sequences available in NCBI using BLAST [25]. The gene accession number was KJ131192.1 and KJ131193.1. The sequence of the amplified product was as follows.

KJ131192.1

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GTTCACGCTG ATGGCGACGG CAACCGTCAC GCTGTTGTTA GGAAGTGTGC
CGCTGTATGC
GCAAACGCGG GACGTACAGC AAAAAGTTCG CGAATTAGAG CGGCAGTCCG
GAGGCAGACT
GGGTGTGGCA TTGATTAACA CAGCAGATAA TTCGCAAATA CTTTATCGTG
CTGATGAGCC
CTTTGCGATG TGCAGCACCA GTAAAGTGAT GGCCGCGGCC GCGGTGCTGA
AGAAAAGTGA
AAGCGAACCG AATCTGTTAA ATCAGCGAGT TGAGATCAAA AAATCTGACC
TTGTAACTA
TAATCCGATT GCGGAAAAGC ACGTCAATGG GACGATGTCA CTGGCTGAGC
TTAGCGCGGC
CGCGCTACAG TACAGCGATA ACGTG GCG
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KJ131193.1

CAGTTCACGC TGATGGCGAC GGCAACCGTC ACGCTGTTGT TAGGAAGTGT
 GCCGCTGTAT
 GCGCAAACGG CGGACGTACA GCAAAAACCTT GCCGAATTAG AGCGGCAGTC
 GGGAGGCAGA
 CTGGGTGTGG CATTGATTAA CACAGCAGAT AATTCGCAAA TACTTTATCG
 TGCTGATGAG
 CGCTTTGCGA TGTGCAGCAC CAGTAAAGTG ATGGCCGCGG CCGCGGTGCT
 GAAGAAAAGT
 GAAAGCGAAC CGAATCTGTT AAATCAGCGA GTTGAGATCA
 AAAAATCTGA CCTTGTTAAC

TATAATCCGA TTGCGGAAAA GCACGTCAAT GGGACGATGT CACTGGCTGA
 GCTTAGCGCG
 GCCGCGCTAC AGTACAGCGA TAACGTGG

DISCUSSION

In hospital environment, resistance of bacterial pathogens to common antibiotic therapies was increasing at an alarming rate [26]. The use of antibiotics for any infection causes a “selective pressure” on bacterial populations, which emerge the resistant mutants and it can flourish [27]. The incidence of ESBL producing strains of clinical isolates has been steadily increasing from the past years resulting in limitation of therapeutic options [28]. These bacteria are showing rising rates of resistance to current therapies [29]. The problems which are associated with ESBLs include multidrug resistance, difficulty in detection and treatment, and increased mortality. ESBL-producing organisms, being the most common nosocomial pathogens, it is essential to detect and treat them as early as possible [30].

Nosocomial infections with Gram-negative bacilli are not uncommon for the local setting and can be perceived as a growing threat to public health [31]. In this study, out of 1279 different clinical samples, 465 Gram-negative isolates were recovered among that *E. coli* 320 (68.81%), *E. aerogenes* 119 (25.59%), and *K. pneumoniae* 26 (5.59%). Similarly, a previous study recorded that, *E. coli* (42.4%) was the most predominant isolate followed by *K. pneumoniae* (28.5%) [32]. However, the study conducted by Nazneen et al. (47%) [31], Menon et al. (47.14%) [33], Shobha et al. (45.62%) [34], and Nevine et al. (41.17%) [35] recorded that *K. pneumoniae* was the predominant isolate.

The important reason for its detection is a failure to treat ESBL-producing organisms due to limited therapeutic choices [36]. The isolates resistant to cefotaxime were tested for ESBL production by double disc synergy test method [37-39]. Similarly, in the present study, ESBL production was detected using combination disc method and double disk synergy test. Similarly, a study conducted by Umadevi et al. [30] used the combination disc method, and some other studies used the double disk synergy test of the detection of ESBL producer [40-43].

ESBL-producing organisms predominantly isolated from urine sample [31,44-47]. Similarly, in our study, urine sample (85.38%) was the major source of ESBL producing strains followed by pus (10.76%) and stool (3.84%). However, a study by Sharma et al. [41] recorded that high prevalence of ESBL producers we isolated from respiratory tract samples (63.83%) was the major source of ESBL-producing strains followed by stool samples, urine, body fluid, pus, and blood.

The sex plays an important role in the infection which was affected by Gram-negative ESBL-producing organisms. A study by Shah et al. [48] was documented that male patients 10 (62.50%) were predominant than female 6 (37.50%) for the ESBL producers. Conversely, in our study, ESBL-producing isolates were predominantly recorded in female (n=78) than male (n=52). ESBL-producing isolates from urine were more common to males than females, in agreement with a previous report [49]. Contrary, in our study, male patients were more prevalent to ESBL in pus (10) and stool (5) samples than the female patients. On the other hand, ESBL prevalence in female patients was found to be the highest in urine samples (73).

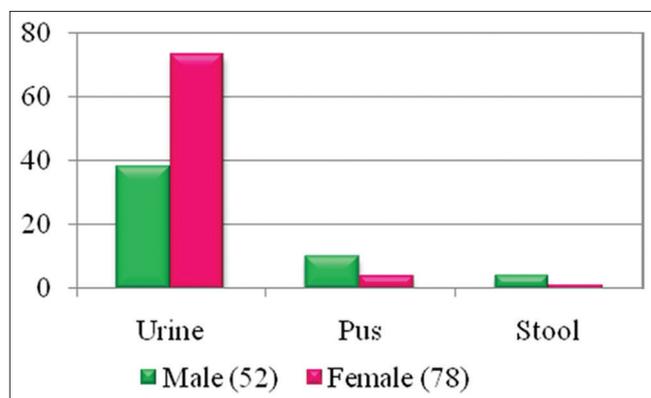


Fig. 1: Prevalence of extended-spectrum beta-lactamase over the sex distribution

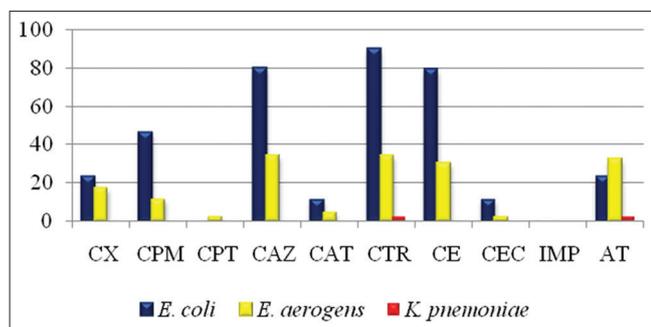


Fig. 2: Resistant patterns of extended-spectrum beta-lactamase producers from different sample

Table 1: Frequency of Gram-negative bacteria among various clinical samples

| Sample | Culture positive | | | Total |
|---------------|------------------|---------------------|----------------------|-------|
| | <i>E. coli</i> | <i>E. aerogenes</i> | <i>K. pneumoniae</i> | |
| Urine (n=918) | 253 | 101 | 18 | 372 |
| Pus (n=207) | 34 | 18 | 8 | 60 |
| Stool (n=154) | 33 | - | - | 33 |
| Total | 320 | 119 | 26 | 465 |

Escherichia coli: *E. coli*, *Enterobacter aerogenes*: *E. aerogenes*, *Klebsiella pneumoniae*: *K. pneumoniae*, ESBL: Extended-spectrum beta-lactamase

Table 2: Detection of ESBL production in *E. coli*, *E. aerogenes* and *K. pneumoniae*

| Sample | ESBL producers | | | | NonESBL producers | | | |
|--------|----------------|---------------------|----------------------|-------|-------------------|---------------------|----------------------|-------|
| | <i>E. coli</i> | <i>E. aerogenes</i> | <i>K. pneumoniae</i> | Total | <i>E. coli</i> | <i>E. aerogenes</i> | <i>K. pneumoniae</i> | Total |
| Urine | 80 | 30 | 1 | 111 | 173 | 71 | 17 | 261 |
| Pus | 8 | 5 | 1 | 14 | 26 | 13 | 7 | 46 |
| Stool | 5 | - | - | 5 | 28 | - | - | 28 |
| Total | 93 | 35 | 2 | 130 | 227 | 84 | 24 | 335 |

Escherichia coli: *E. coli*, *Enterobacter aerogenes*: *E. aerogenes*, *Klebsiella pneumoniae*: *K. pneumoniae*, ESBL: Extended-spectrum beta-lactamase

According to the geographical region, the fraction of putative ESBL-producing isolates can vary. These variations could be due to the differences in selecting a type of antibiotic, antibiotic selection pressure, local antibiotic, and prescribing habits, which differ from state to state, country to country, and from institution to institution. The prevalence of ESBL-producing bacteria has been on the rise, particularly in Asia compared to other regions [50]. A study from China, the figures of ESBL producers vary between 25 and 40% [51]. In India, the prevalence rate varies in different institutions from 28% to 84% [52], but Ali *et al.* [53] reported that ESBL producers making a frequency of 45%. Another study recorded that ESBL production was found to be 52.49% [41]. Similarly, a study from Malaysia in 2001 by Nurul *et al.* [54] depicted a prevalence of ESBL as 58.6%. A recent study in 2005, from New Delhi, showed 68.78% of the strains of Gram-negative bacteria to be ESBL producers [55]. A study by Nazneen *et al.* [31], Mathur *et al.* [56], and Nevine *et al.* [35] noted 61%, 68%, and 65.8% of ESBL producer correspondingly, these prevalence were quite high when compared to our studies. Differently, in our study, the prevalence of ESBL producers was 27.95%. A study by Basavaraj *et al.* [57] from Karnataka and Sharma and Grover [58] from Himachal-Pradesh reported an incidence of 32.1% and 38.5% which is slightly accordance with our results. While studies conducted by Rodrigues *et al.* [59], Kumar *et al.* [60], and Menon *et al.* [33] reported 6.9%, 19.8%, and 20% respectively, which were quite low when compared to our study.

ESBLs in Gram-negative bacteria have emerged as a major problem of hospitalized as well as community-based patients [31]. Important ESBL-producing Gram-negative bacilli includes *K. pneumoniae*, *E. coli*, and *P. mirabilis*, *Enterobacter* spp., *Citrobacter freundii*, *P. aeruginosa*, *Acinetobacter* and *Stenotrophomonas maltophilia* [61]. A study from Egypt reported that 46% of ESBL-producing isolates of *K. pneumoniae* were from the clinical isolates [62]. ESBL are more prevalent in *Klebsiella* spp. followed by *E. coli* [63]. Similarly, a study by Nazneen *et al.* [31], Mathur *et al.* [56], Gupta *et al.* [64], Sharma *et al.* [41], and Ali *et al.* [53], were reported that, *K. pneumoniae* 74%, 73%, 71%, 67%, and 57% was more prevalent than *E. coli* 62%, 62%, 64%, 57%, and 39.1%, respectively. Conversely, in the present study, the highest incidence of ESBL was noted in *E. coli* (29.06%), *E. aerogenes* (29.41%), and *K. pneumoniae* (7.69%).

Klebsiella is the genus which frequently harbors ESBL [65]. However, in other studies, *E. coli* was the major ESBL producer [30,44,66,67]. Similarly, in the present study, the highest incidence of ESBL was noted in *E. coli* (29.06%), *E. aerogenes* (29.41%) and *K. pneumoniae* (7.69%). Following studies were also reported that *E. coli* was the predominant ESBL producers. A study reported that 81% of the *E. coli* and 74% of the *K. pneumoniae* isolates were ESBL producers [30]. In the same way, a study noted that the highest ESBL producer was *E. coli* (64.2%) followed by *K. pneumoniae* (60.1%) [32]. Another study from southern India reported an incidence of 58.06% for ESBL producing *E. coli* and 57.14% for ESBL-producing *Enterobacter* spp. [68]. A similar prevalence was observed that 55.69% (n=93) were *E. coli* and 44.31% (n=74) were *K. pneumoniae* isolates were ESBL producer [69]. Shrestha *et al.* [70] reported that the prevalence of ESBL-producing organisms was found to be 54 (18%); among which *E. coli* was 29 (53.7%), *K. pneumoniae* (14.8%). A study by Jain *et al.* reported that maximum ESBL producers were found among *E. coli* isolates (80.9%) followed by *K. pneumoniae* (75%) [71]. A report from Coimbatore (India) showed that ESBL production was 41% in *E. coli* and 40% in *K. pneumoniae* [72]. Kulkarni *et al.* [47] in their study reported that *E. coli* (40.7%) was the most frequent and *K. pneumoniae* (15.9%).

CONCLUSION

The study results suggested that, among *Enterobacteriaceae* members, *E. coli* was the predominant ESBL producers and urine was noted as the prime source for the ESBL positive isolates when compared to another source. Although many phenotypic methods were available, genotypic identification was the best method to differentiate ESBL types which were essential to provide proper treatment. Constant and careful

surveillance, proper detection methods, and proper management are recommended to control the spread of these organisms as the infections by ESBL-producing organisms.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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