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ORIGINAL ARTICLE

Extended-Spectrum β-Lactamases in tribal area: A Clinical Update

Dinal B. Prajapati, Jeni R Patel
Biology Department, The Patidar Gene Science College, Bardoli, Gujarat.
Email: dinalprajapati12@gmail.com

ABSTRACT

Extended-spectrum β-lactamases (ESBLs) are a rapidly evolving group of β-lactamases that share the ability to hydrolyse third-generation cephalosporins and aztreonam yet are inhibited by clavulanic acid. The impact of these enzymes has posed a major threat to the health sectors and has challenged the available treatment options for both community and hospital-acquired infections. It is an alarming situation since there is a steep rise in MDR-Beta lactamase pathogens mainly in Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, and Pseudomonas aeruginosa. A total of 519, non-duplicate third-generation cephalosporin (3GC) resistance pathogens were collected from District Hospital, vyara from November 2020 to July 2023. The prevalence of ESBL-producing gram-negative bacteria was 84.7% detected by the modified double disk synergy test (MDDST). Further genotypic characterization was performed for only selected samples using RT PCR (Real-time polymerase chain reaction) assays for the molecular identification of ESBL genes. Most ESBL-producing GNB showed a multi-drug-resistant phenotype, and blaCTX-M was the predominant ESBL genotype in this study. This study provides an overview of the background, associated with AMR and ESBL producers in GNB with a focus on the current situation and future threat in the development of definite control strategies to limit the abuse of antimicrobial agents in the developing countries of the western region of Gujarat state.

Keywords: *Multidrug resistance, Extended-spectrum β-lactamases, modified double disk synergy test, Real-time polymerase chain reaction.*

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INTRODUCTION

In 1983, SHAH, P. M., & STILLE, gave the first report of the strain *K. pneumonia* and *E. coli* were resistant to third-generation cephalosporins [1] So, according to this report the Enterobacteriaceae producing extended-spectrum b-lactamases (ESBLs) has become a concern in the field of medical bacteriology and the infection due to ESBL producing organisms have been described all over the world [2]. β-lactam antibiotics are used for the treatment of Gram- negative bacteria but the continuous exposure of these antibiotics and misuse has led to the development of β-lactamase-producing bacterial strains, expanding their activity even against the newly developed β-lactam antibiotics. These enzymes are some of the most prevalent and well-studied families of enzymes, especially in the area of antibiotic resistance known as extended-spectrum β-lactamases (ESBLs). Detection of ESβL producing MDR strain is a key challenge in addressing this issue [3,4,5,6]. ESBL-producing bacteria that carry genetic mutations in the enzyme capable of hydrolysing a β-lactam bond in commonly prescribed β- lactam antibiotics, such as penicillin's, oxyimino-cephalosporin (third- and fourth-generation) and monobactam (aztreonam) but not cephamycin (cefoxitin) or carbapenem (meropenem, imipenem, ertapenem, and doripenem) and this enzyme is one of the most common types of antibiotic resistance found in *Enterobacteriaceae* and based on their amino acid sequence these enzymes have been gathered into nine different structural and evolutionary families [2,7]. Generally, these enzymes are inhibited by β-lactamase inhibitors (BLIs) such as clavulanic acid, sulbactam, and tazobactam but in case of high level of enzyme production, these enzymes are resistant to β-lactamase inhibitors [8]. The ESBL genes are widely transmitted horizontally or by conjugation between bacteria through plasmid which is double-stranded, the extra chromosomal genetic

material that replicates independently, leading to the spread of community-acquired and nosocomial infections [9]. The genotypes of ESBL-producing bacteria include TEM (Temorina Escherichia coli mutant), SHV (Sulfhydryl variant), and CTX-M (Cefotaximase-munich) encoded by the blaTEM, blaSHV, and blaCTX-M genes respectively [10, 11], from this genotype CTX-M belong to type class A are serine β-lactamase and OXA- type enzymes belongs to class D serine β-lactamase. From this gene, the CTX-M ESBL emerged worldwide and in 2006 Pandemic was declared due to the CTX-M gene [12, 13].

MATERIAL AND METHODS

Study design

A total of 519 non-duplicate third-generation cephalosporin (3GC)- resistant clinical isolates of Gram-negative bacilli were investigated in this study for the screening of Extended-spectrum β- lactamase and β-lactamase genes (bla CTX-M, bla TEM, bla SHV, blaOXA). The present study was performed in the Department of Microbiology at District Hospital, Vyara, Gujarat over a period from November 2021 to July 2023. The sample sources of these isolates were Blood, urine, pus, sputum, endotracheal secretions, stool, and various body fluids from OPD and IPD. Work permission for this study was approved by the hospital authority and the study was approved by the institutional local ethical committee of The Patidar Gene Science College, Bardoli, Gujarat.

Culture of clinical specimens

All collected clinical specimens were inoculated on Nutrient agar, MacConkey agar, Blood agar, chocolate agar, and CLED (cysteine–lactose–electrolyte-deficient agar or medium) agar and incubated overnight at 37°C aerobically. Bacterial isolates were subjected for identification upon microscopy, cultural characteristics, and other relevant biochemical reactions. Moreover, antibiotic susceptibility testing, DNA extraction, and RT-PCR were performed.

Antibiotic susceptibility test and screening of extended-spectrum β-lactamase

Commercially available antibiotic disks (HiMedia, Mumbai) of different classes, Dodeca Enterobacteriaceae-1-DE053 and Dodeca Enterobacteriaceae-2-DE054 were used for antimicrobial susceptibility test. Antimicrobial susceptibility tests and ESBL screening were assessed by Kirby Bauer disc diffusion method as per the Clinical Laboratory Standards Institute (CLSI) guidelines [14]. The antibiotic used for this study were gentamicin (10µg), amikacin (30µg), Ciprofloxacin (5 µg), Ofloxacin (5µg), co-trimoxazole (sulpha/Trimithoprim) (25 µg), Amoxyclav (30 µg), Cefuroxime (30 µg), Ceftazidime (30 µg), ceftazidime/clavulanic acid (30 µg), cefepime (30µg), Impenem (10µg), cefotaxime (30 µg), ceftriaxone (30 µg), Cefoxitin (30 µg), Meropenem (10µg), Levofloxacin (5µg), Ticarcillin- clavulanic acid (85 µg), piperacillin-tazobactam (110µg), aztreonam (30 µg), Gatifloxacin (5 µg), ampicillin/sulbactam (10/10 µg), cefoperazone (75 µg), ceftrizoxime (30 µg) and polymyxin

B. After incubation at 37 °C for 18–24 h., the plates were examined for confluent growth, and a circular Zone of inhibition for each antimicrobial agent was interpreted and organisms were classified as resistant, intermediate, or susceptible. After that data management and analysis was done by WHONET software.

Phenotypic detection of ESBLs using modified double disk synergy test

In this study, the isolates were further confirmed for ESBL production by the Modified Double Disc Synergy Test (MDDST). The organism to be tested was spread onto a Mueller–Hinton agar plate. All of the isolates that were resistant to third-generation cephalosporin and showed sensitivity to β-lactamase inhibitors (BLIs) such as clavulanic acid, sulbactam, and tazobactam in combination disk were subjected to the MDDS test. The antibiotic Ceftriaxone (30µg), Cefotaxime (30µg), Ceftazidime (30µg)(three third-generation cephalosporins discs) and one fourth-generation cephalosporin disc (cefepime 30 µg) along with Amoxicillin/Clavulanic acid (20/10 µg) were placed at distances of 15 mm 20 mm (edge to edge) respectively, from the Amoxicillin/Clavulanic acid disc that was placed in the middle of the plate. After incubation positive results for ESBL production are considered by any increase in the inhibition zone around any of these cephalosporin discs towards the disc of amoxicillin-clavulanate[15, 16, 17].

Genotypic characterization of extended-spectrum β-lactamases

DNA extraction and genotypic identification of SHV, CTX-M, TEM, and OXA - 10/11 genes was done at the Microbiology Department (PCR laboratory) of District Hospital, vyara, Gujarat.

DNA Extraction

The genomic DNA of the isolates was extracted using a Multi-Sample DNA Purification Kit (HiPurA® - Himedia – Mumbai - MB554) and the extraction protocol was followed according to the manufacturer's instructions.

Detection of TEM, SHV, OXA – 10/11 and CTX-M genes by PCR

ESBL detection was performed by the instruction manual provided with the commercial kits procedure from HiMedia Laboratories Pvt. Ltd., Mumbai: MBPCR131 (ESBL detection kit – Master mix Reaction: SHV, CTX-M, TEM, OXA-10/11).

The reaction system and PCR cycle used are as follows.

component	Volume to be added for 1R (for a 25 µL reaction)
2X Super Master mix	12.5 µL
ESBL Primer-Probe Mix	3 µL
Internal Control Primer-Probe Mix	1 µL
Internal Control DNA	1 µL
Molecular Biology Grade Water for PCR	2.5 µL
Sensitive Control / Negative Control / Template DN	5 µL
Total volume	25 µL

Centrifuge the tube briefly at 6000 rpm for about 10 seconds. Place the tubes in Real-time PCR machine and set the recommended PCR program (mentioned below). Interpret the data from the amplification plot.

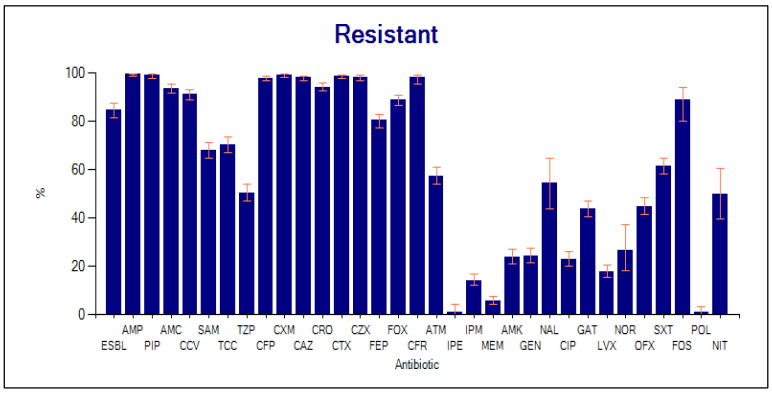
PCR cycle

Initial denaturation : 95°C for 10 minutes
Denaturation : 95°C for 15 seconds
Annealing and Extension : 60°C for 30 seconds (Plate Read)
No. of cycles : 40 Plate Read FAM/HEX//Cy5/Cy5.
Hold : 4°C for ∞

After completion of the run, a cycle threshold (Ct) was calculated by determining the signal strength at which the fluorescence exceeded a threshold limit. Samples possessing a fluorescence signal above this value were assessed as positive.

RESULTS

A total of 828 consecutive gram-negative isolates were obtained from various clinical samples during the study period. Out of 828 MDR strains, 439 (53.0 %) strains were investigated as ESBL producers and 80 strains as non-ESBL producers using the disc diffusion test and combined disc test method. According to the breakpoints that were indicated in CLSI guidelines [14], the antibiotic susceptibility patterns for all isolates were analysed. The resistance profile for all gram-negative bacteria was analysed by WHONET 2021 software (shown in Fig 1). AST results recorded 100% resistance to ampicillin, Piperacillin, and Cefuroxime. All GNB showed a higher percentage of non - non-susceptibility against ceftazidime (98%), ceftriaxone (94.32), cefotaxime (98.79), ceftrizoxime (98.3), cefoperazone (97.94%) and cefepime (80.43%) and proportion of non - susceptibility for beta-lactam/beta-lactamase inhibitor combinations were significantly higher in amoxicillin/clavulanic acid (93.8) than ceftazidime/ clavulanic acid (91.3%), ticarcillin/clavulanic acid (70.41%), ampicillin/sulbactam (68.23%) and pipracillin/ tazobactam (50.42%) (Fig 1A). The most effective antimicrobial classes in ESBL producers were aminoglycosides, fluoroquinolones, and carbapenem drugs. (Fig 1C).



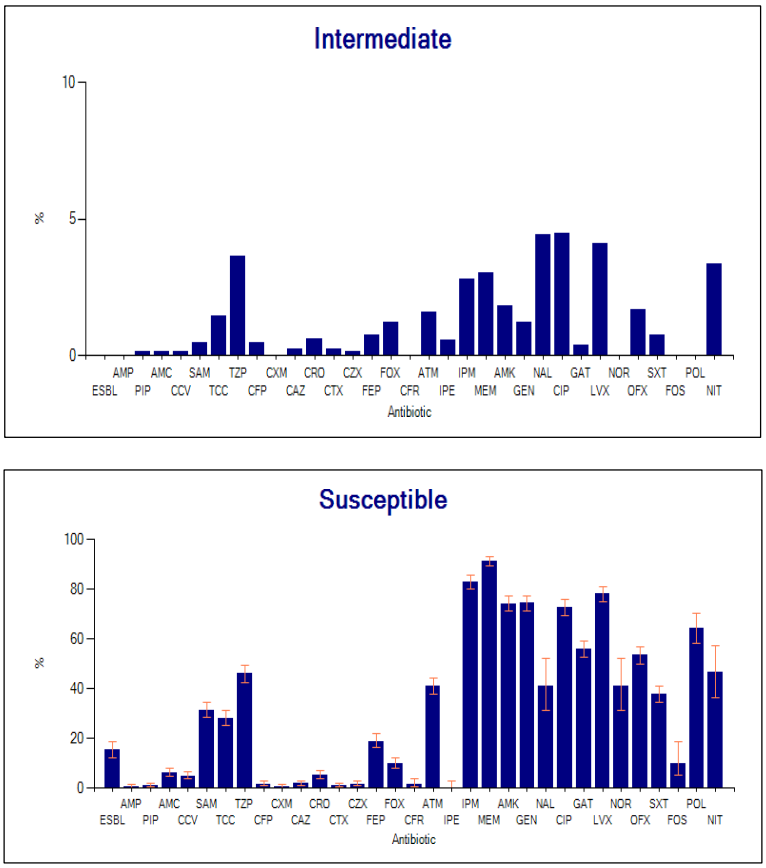


Figure 1. Overall Antibiogram of Gram negative pathogen

In this study, 3 GC resistance isolates and isolates showed resistance or sensitivity to beta- lactamase inhibitors (BLIs) such as clavulanic acid, sulbactam, and tazobactam in Augmentin disk were further phenotypically (fig 2) and genetically detected (fig 4) for ESBL enzyme. The Determination of ESBL production by the MDDST method showed that 177 isolates of *K. pneumonia,* 136 isolates of *E. Coli,* 97 isolates of *pseudomonas spp.,* 79 isolates of *Acinetobacter spp.,* 23 isolates of *proteous spp.* And 3 *Citrobacter spp.* were considered positive for ESBL production. At the same time selected samples were tested by VITEK 2 compact system for ESBL and the same results were obtained.

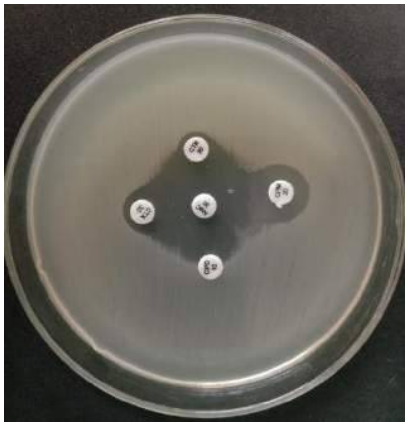


Figure 2. Modified double disk synergy test for determination of ESBL production

Confirmations of phenotypes of selected MDDST positive samples were processed using RT PCR (Real time polymerase chain reaction). Figure 4 represents a comprehensive quantification report of RT-PCR that

showed amplification plot of CTX-M, SHV, TEM and OXA-10/11 gene. The analysis showed the presence of a maximum of two ESBL genes (CTX-M and SHV) and the most frequently isolated ESBL gene was CTX-M.

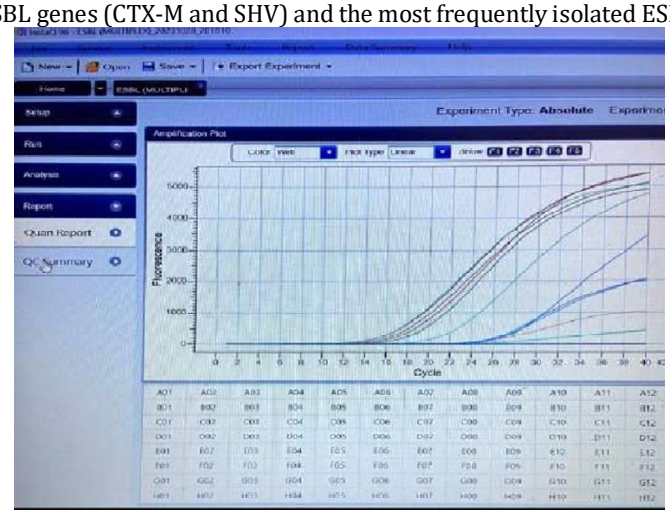


Figure 3 Quantitative report of RT PCR Multiplex

DISCUSSION

β-lactam antibiotics are the most widely prescribed antibiotics for bacterial infection treatment and this class of antibiotics works by inhibiting the synthesis of bacterial cell walls, ultimately leading to the death of the bacteria. Bacteria have evolved various mechanisms to resist the action of β-lactam antibiotics. So, resistance to them has become an increasing problem in recent years [18, 19]. The results of the present study showed that a greater prevalence of ESBL production was noted in K. pneumonia followed by E. Coli, pseudomonas spp., and Acinetobacter spp., Similar to our study, In India, a multi-centric study reported that ESBL production was seen 42% in K. pneumonia and 33% in *E. coli* [20]. The reports of Roopashree, S., & Kaup, S. showed that the predominant ESBL producer isolates obtained from the various clinical samples were *Escherichia coli*, constituting 70.4% of the isolates followed by Klebsiella species accounting for 22.53% [21]. In India, the prevalence rate of ESBLs ranges from 60 to 80%[22], and in our study, the magnitude of ESBL-producing GNB isolates was found to be 84.7 %. The majority of ESBLs producing strains were E. coli (96, 45.71%) followed by *K. pneumoniae* (45, 21.43%) highlighted by Salvia, T., *et al* in 2024 [23].

Our results revealed a high resistance rate (Greater than 80%) which was recorded with ampicillin, Piperacillin, Cefuroxime, ceftazidime, ceftriaxone, cefotaxime, cefoperazone, cefepime, ceftazidime /clavulanic acid, ampicillin/sulbactam, piperacillin/tazobactam and ticarcillin/clavulanic acid amoxicillin/ clavulanic acid, these results are in agreement with the result of studies done by Elsafi, S. H. (2020) which stated that the carbapenem and the aminoglycosides class of drug were most effective[24]. A similar study was conducted in India, with more than 70% of Acinetobacter baumannii, E. coli, and Klebsiella pneumoniae while more than 50% of P. aeruginosa resistant to third-generation cephalosporins and fluoroquinolones reported by Jani *et al*. in 2021[25].

The current study also included the molecular outlook of resistance through the identification of genes carried by these resistant isolates. The ESBL genes determined in these isolates belonged to the CTX-M and SHV types, while the TEM and OXA type was not detected in any of the tested isolates. The most prevalent genes among the isolates were CTX-M in this study. The first recognition of the emergence of CTX-M β-lactamases occurred nearly simultaneously in Europe and South America at the beginning of 1989 [26]Ensor, V. M., et al. reported in their study that the first report of CTX-M producing Enterobacteriaceae came from New Delhi in a study carried out by Karim, A., *et al* in 2001. Out of 2000 isolates Six isolates were investigated [27,28]. A previous report from North India and a study from Central India (Rajasthan) showed commonest genotype was blaCTX-M followed by blaTEM and blaSHV [29,30]. In addition, Verma et al. and Salvia, T. et al highlighted that blaTEM was the most predominant ESBL gene followed by blaCTX-M and blaSHV[23,31]. Among the different ESBL families, CTX-M has become the most prevalent worldwide; both in nosocomial and community-acquired infections [32]. The limitation of our study is that molecular methods were employed for only selected samples.

CONCLUSION

Our results highlighted the higher prevalence and rapid emergence of ESBL production in clinical MDR strains isolated from the Western region of India in Gujarat state. A substantial level of resistance to first-,

second-, and third-generation cephalosporin was also observed. This poses a significant problem for hospitalized patient's treatment. There are very limited cases of prevalence studies about drug resistance investigated in the Tribal area of Tapi district, Gujarat, which is still underdeveloped in healthcare sectors compared to other parts of India. The prevalence and spread of ESBL-producing Gram-negative bacteria is worrisome and E. coli and K. pneumoniae were the most frequent isolates among different clinical samples. TEM, SHV, OXA – 10/11, and CTX-M ESBL genes were tested among GNB with prevalent of blaCTX-M over blaSHV gene. This work adds to the evidence of blaCTX-M and blaSHV genes in the tribal areas. The knowledge of the resistance pattern of bacterial strains in a geographical area will help to guide the appropriate antibiotic use and such institutional studies will help to formulate an empirical antibiotic policy to treat Gram-negative infections. In this regard, the present study may need more focus on rural areas, which often lack access to advanced healthcare facilities and may have limited resources. Investing in research and development in these areas can help address the unique challenges faced by rural communities and improve their healthcare outcomes.

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CONFLICT OF INTEREST
Author declares that the patient was not personally involved in this study. All the data obtained is solely for research purposes and was not used for any therapeutic or treatment decisions.

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