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Detection and genetic characterization of extended-spectrum beta-lactamases producers in a tertiary care hospital

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Abstract:

BACKGROUND: Extended-spectrum beta-lactamase (ESBL)-producing organisms inactivate extended beta-lactam antibiotics and monobactams and also exhibit coresistance to many other classes of antibiotics. The present study was carried out to assess the prevalence of the ESBLs and to determine the most prevalent genotype in our hospital.

MATERIALS AND METHODS: All clinically significant Gram-negative isolates were identified, and their antimicrobial susceptibility testing was done by Kirby–Bauers' disc diffusion method. ESBL detection was confirmed by minimal inhibitory concentration method using agar dilution technique for those who screened positive by ceftazidime (30 µg) disc. Further, the established ESBL-positive isolates were subjected to genotyping for bla TEM, bla CTX-M, and bla SHV genes by using conventional polymerase chain reaction.

RESULTS: *Escherichia coli* was the most common (28.84%) Gram-negative bacillus followed by *Klebsiella pneumoniae* (18.07%), while *Pseudomonas* spp. (9.61%) was the most commonly identified nonfermenter. ESBL production was detected in 160 (30.8%) isolates. *Klebsiella oxytoca* (46.7%) followed by *E. coli* (44%) were the common ESBL producers. Most predominant ESBL gene was bla TEM, found in 122 (76.25%) isolates. Combinations of two genes were seen in 109 (68.1%) isolates, the most common (43.12%) combination being bla_{TEM} and bla_{CTX-M}. In this study, 16 (10%) strains had all the three types of genes. Most of the isolated Gram-negative bacilli (GNB) were sensitive to amikacin, imipenem, and colistin.

CONCLUSION: In our study, the 30.8% of GNB were ESBL producers. This is the only study that shows that TEM is the most prevalent ESBL genotypes in our area. Of concern is a good number of isolates showing all three patterns of genes (TEM, SHV, and CTX-M). Amikacin, imipenem, and colistin were the most useful antibiotics in our setup.

Key words:

Bla TEM, extended-spectrum beta-lactamase, genotyping

Introduction

With the broadening front of microorganisms having enzymes capable of destroying beta-lactam drugs, a newer class of drugs known as oxyimino-cephalosporins were developed for the treatment of serious infections due to Gram-negative bacteria to which the microbes responded by creating an

enzyme capable of hydrolyzing them. These enzymes named as extended-spectrum beta-lactamases (ESBLs) have a propensity to inactivate extended-spectrum beta-lactam antibiotics and monobactams except cephamycins and imipenem. In addition, ESBL-producing organisms exhibit coresistance to many other classes of antibiotics resulting in limitation of therapeutic option. These are Ambler Class A β-lactamases, commonly having

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genotypes of such as bla_{SHV}, TEM, and CTX-M types. The present study was undertaken to note the prevalence of ESBL Gram-negative bacilli (GNB) as well as the predominant genotypic pattern.

Materials and Methods

This prospective study was conducted in our department after approval by the institutional ethical committee. During the study period of 2 years, 1509 clinical samples were collected from indoor, outdoor, as well as intensive care unit (ICU) patients. The samples were processed, and the cultures were identified as per the standard protocol. Antibiotic susceptibility testing of all the isolates was carried out by Kirby-Bauer's disc diffusion method and interpreted according to the CLSI guidelines. All the GNB were screened for ESBL production by using ceftazidime (CAZ) (30 µg) discs. The organisms which showed a zone diameter <22 mm were subjected to further confirmatory tests.

Minimum inhibitory concentration (MIC) was carried out by agar dilution technique against antibiotics CAZ and ceftazidime-clavulanic acid (CAC) as per the CLSI breakpoints. About 0.0625 to 32 µg/ml concentrations of CAZ was obtained, and for CAC, 4 µg/ml of clavulanate were added to the respective CAZ dilutions. The lowest concentration of antibiotic that prevented bacterial growth was considered to be the MIC. Organism was considered as ESBL producer if MIC with CAZ is ≥ 2 µg/ml. This was further confirmed when MIC of CAC showed a reduction of ≥ 3 twofold dilution than that of CAZ alone^[1] [Figure 1].

Genomic DNA was isolated from phenotypically confirmed ESBL-producing bacterial cells by lysis method^[2] Extraction has been done by using bacterial genomic DNA extraction kit (HiMedia, Mumbai). The DNA samples were run on 0.8% agarose gel, stained with ethidium bromide, and examined under Gel Doc (Biorad) against uncut Lambda DNA to confirm the presence and note the quantity of DNA.

Extracted DNA was taken for polymerase chain reaction (PCR) assay to ensure the presence of the bla_{SHV}, bla_{TEM}, and bla_{CTX-M} genes. About 2.5 µl of PCR buffer (without MgCl₂), 2.5 µl of 25 mM MgCl₂, 0.2 µl of 200 mM deoxyribonucleotide triphosphate, 1 µl of each forward and reverse primers^[3,4] (10–12 pmole) [Table 1], and 0.1 µl of 5U Taq polymerase (HiMedia) were used for a single reaction and 1 µl of the extracted genomic DNA was added to the reaction mixture. The volume was made up to 25 µl by using deionized water.

The PCR machine was programmed as per the protocol in Table 2. The amplified PCR products were analyzed

against 100 bp ladder (HiMedia) using 1.5% agarose gel electrophoresis. Gels were stained with ethidium bromide and visualized by ultraviolet transilluminator or Gel Doc instrument [Figure 2].

Results

A total of 1509 clinical specimens were obtained during the study period, of which 712 (47.18%) showed growth of various organisms, 520 (73.03%) yielding single Gram-negative bacteria only. Of these 520 GNBs, 309 (59.42%) were from male patients, while 211 (40.57%) from females with a male-to-female ratio of 1.46:1. Most commonly, 161 (30.96%) of GNBs were isolated from patients in the age group of 16–30 followed by 31–45 years (23.46%). Highest proportion of ESBLs was found in the samples in the age group of 46–60 years (30.62%).

Most of the samples, 418 (80.38%), were collected from the inpatient department, of which 109 (21%) cases were from ICUs. There were only 102 (19.61%) samples from the outpatient department. Most of the ESBL strains (33.9%) were obtained from samples from wards and cabins. ICUs although thought to be the harborer of resistant strains, we had only 30.3% ESBL producers.

Urine was the most predominant (54.07%) sample received during the study period. However, highest amount of ESBL producers were isolated from pus samples (60.34%) followed by those from the blood (58.06%) [Table 3].

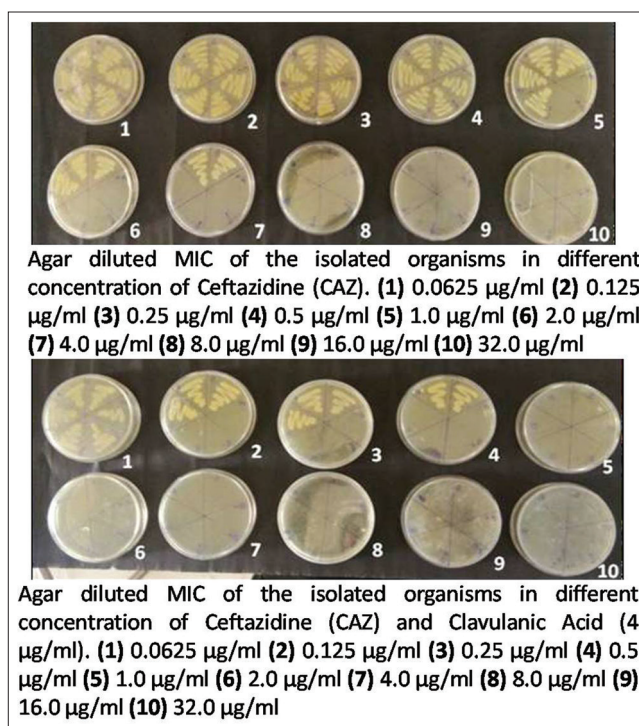


Figure 1: Detection of minimum inhibitory concentration by agar dilution method using ceftazidime alone and ceftazidime with clavulanic acid

Table 1: List of primers for the detection of bla TEM, bla SHV, and bla CTX-M genes (Eurofins)

Primer name	Primer sequence	Size of the PCR product (bp)	Reference
TEM (bla _{TEM})	F 5'- AAGCCATACCAAACGACGAG-3' R 5'- ATTGTTGCCGGAAGCTAGA-3'	108 bp	Yano et al., 1999 ^[3]
SHV (bla _{SHV})	F 5'- TCTCCCTGTTAGCCACCCTG-3' R 5'- CCACTGCAGCAGCTGC (A/C) GTT-3'	593 bp	Yano et al., 1999 ^[3]
CTXM (bla _{CTXM})	F 5'- CGATGTGCAGTACCAGTAA-3' R 5'- TTAGTGACCAGAATCAGCGG-3'	585 bp	Batchelor et al., 2005 ^[4]

PCR=Polymerase chain reaction

Table 2: Programming of polymerase chain reaction

Stage	Step	TEM	SHV	CTX-M
Initial denaturation	Initial	94°C-2 min	94°C-2 min	94°C- 2 min
	denaturation	1 cycle	1 cycle	1 cycle
Amplification	Denaturation	94° C-1 min	94° C-1 min	94° C-1 min
		30 cycles	30 cycles	30 cycles
	Annealing	58° C-1 min	52° C-30 s	55° C-1 min
		30 cycles	30 cycles	30 cycles
	Extension	72° C-1 min	72° C-45 s	72° C-1 min
		30 cycles	30 cycles	30 cycles
Final extension	Extension	72° C-7 min	72° C-5 min	72° C-5 min
		1 cycle	1 cycle	1 cycle

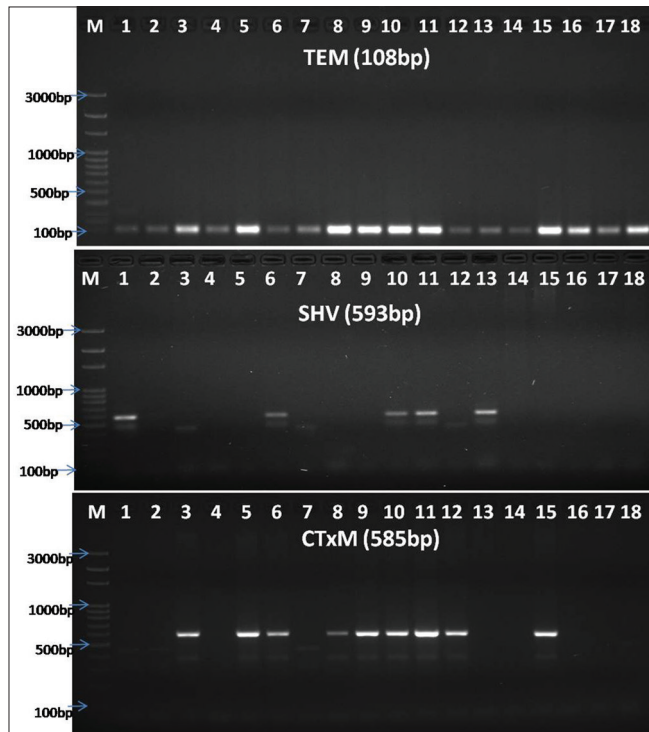


Figure 2: Banding pattern of TEM, SHV, and CTX-M genes through gel electrophoresis (Lane-M: 100 bp ladder, Himedia, India, and Lane-2-18: sample. TEM positive for samples 1-18; CTX-M positive for 1, 6, 10, 11, 13; SHV positive in samples 3, 5, 6, 8, 9, 10, 11, 12, 15)

Escherichia coli were the most common GNB (28.84%) isolated followed by *Klebsiella pneumoniae* (18.07%) and *Citrobacter freundii* (7.7%). *Pseudomonas* spp. (9.61%) followed by *Acinetobacter* spp. (6.9%) were the commonly identified nonfermenters.

Of the 520 GNB, 160 isolates were ESBL producers. *Klebsiella oxytoca* (46.7%) were the most predominant ESBL positive organisms followed by *E. coli* (44%). All the isolates belonging to members of tribe Proteae, *Serratia* spp., *Chromobacterium* spp., and *Shigella* spp. were non-ESBL producers. Of note was *Enterobacter* spp. and *Citrobacter* spp. which were ESBL positive in 48% and 14.7% of cases, respectively. On the other hand, nonfermenters in our study were ESBL producers in 36.1% and 20% for *Acinetobacter* spp. and *Pseudomonas* spp., respectively [Table 4].

In the present study [Table-5], the isolated GNB are mostly sensitive to amikacin (93.7%), imipenem (96.5%), and colistin (98.85%). ESBL producers showed a higher degree of resistance than non-ESBL producers. Amoxiclav, ofloxacin, ceftriaxone, cotrimoxazole, and imipenem activity is reduced in GNBs which show ESBL production. Other antibiotics such as cefoperazone-sulbactam, colistin, and gentamicin are the choicest antibiotics because of their higher or similar efficacy in ESBL producers than their counterparts. Nitrofurantoin showed high sensitivity in urine isolates. It is very effective in ESBL-producing organisms although less than their ESBL-negative counterparts.

All the ESBL-producing isolates confirmed by phenotypic methods were analyzed for the presence of bla_{CTXM}, bla_{TEM}, and bla_{SHV} genes [Table-6]. All 160 (100%) isolates showed the presence of bands in any of the three ESBL genes. Most predominant gene was TEM, found in 122 (76.25%) isolates followed by CTX-M in 115 (71.87%) and SHV in 64 (40%). Single type of ESBL gene was seen in 35 (21.9%) isolates; 109 (68.1%) had a combination of 2 genes, while only 16 (10%) strains had all the 3 types of genes. The prevalence of only bla_{TEM} (10.62%) gene was highest followed by bla_{CTX-M} (6.25%) and bla_{SHV} (5%). Coexistence of two genes was observed highest with bla_{TEM} and bla_{CTX-M} gene in 69 (43.12%) isolates followed by bla_{TEM} and bla_{SHV} in 20 (12.5%) isolates and bla_{CTX-M} and bla_{SHV} in 20 (12.5%) isolates only. The most common genotype found in ESBL producers was coexistence of bla_{CTX-M} and bla_{TEM} genes accounting for 43.1% of cases.

Discussion

ESBL-producing GNB have emerged as serious pathogens, as they are capable of hydrolyzing extended-spectrum

Table 3: Sample-wise distribution of isolates

Samples	Total number of samples	Number of samples with GNB isolated	Number of ESBL positive (percentage of ESBL)
Blood	182	31	18 (58.06)
CSF	23	02	0 (0)
High vaginal swab	15	03	0 (0)
Pus	148	58	35 (60.34)
Sputum	71	36	13 (36.1)
Stool	6	2	0 (0)
Tracheal aspirate	104	62	14 (22.58)
Urine	816	235	57 (24.26)
Wound swab	144	91	23 (25.27)
Total	1509	520	160

ESBL=Extended-spectrum beta-lactamases, GNB=Gram-negative bacilli, CSF=Cerebrospinal fluid

Table 4: Prevalence of extended-spectrum beta-lactamases strains among various Gram-negative bacteria isolated

Organisms	Total	ESBL strains (percentage of total)
<i>E. coli</i>	150	66 (44.0)
<i>K. pneumoniae</i>	94	40 (42.6)
<i>A. baumannii</i>	36	13 (36.1)
<i>K. oxytoca</i>	15	7 (46.7)
<i>P. aeruginosa</i>	50	10 (20)
<i>E. cloacae</i>	14	8 (57.14)
<i>C. koseri</i>	35	6 (17.14)
<i>C. freundii</i>	40	5 (12.5)
<i>E. aerogenes</i>	11	4 (36.4)
<i>Salmonella</i> Spp.	8	1 (12.5)
<i>P. mirabilis</i>	24	0 (0)
<i>P. vulgaris</i>	26	0 (0)
<i>S. marcescens</i>	3	0 (0)
<i>Chromobacterium violaceum</i>	1	0 (0)
<i>Providencia</i> Spp.	2	0 (0)
<i>Morganella</i> Spp.	3	0 (0)
<i>Shigella</i> Spp.	8	0 (0)
Total	520	160

E. coli=*Escherichia coli*, *K. pneumoniae*=*Klebsiella pneumoniae*, *A. baumannii*=*Acinetobacter baumannii*, *K. oxytoca*=*Klebsiella oxytoca*, *P. aeruginosa*=*Pseudomonas aeruginosa*, *E. cloacae*=*Enterobacter cloacae*, *C. koseri*=*Citrobacter koseri*, *C. freundii*=*Citrobacter freundii*, *E. aerogenes*=*Enterobacter aerogenes*, *P. mirabilis*=*Proteus mirabilis*, *P. vulgaris*=*Proteus vulgaris*, *S. marcescens*=*Serratia marcescens*, ESBL=Extended-spectrum beta-lactamases

beta-lactam antibiotics and monobactams. In addition, they exhibit coresistance to many other classes of antibiotics, thus limiting the therapeutic options. ESBLs are Ambler Class A β -lactamases commonly having genotypes bla SHV, bla TEM, and bla CTX-M. Other genotypes include bla KPC, VEB, PER, BEL-1, BES-1, SFO-1, TLA, and bla BIC.^[5]

In the present study, 520 Gram-negative bacteria were isolated, of which 160 (30.8%) were ESBL producers.

Prevalence of ESBL varies worldwide. A strikingly high frequency rate of ESBLs (44.9%) has been reported from Latin America,^[6] whereas frequency rates in Greece, Portugal, Germany, and the Netherlands is 27.4%, 15.5%, 2.6%, and 2%, respectively.^[7] ESBL prevalence in pathogens tested as part of the Surveillance of Antimicrobial Resistance (SARI) in (2004–2006) study in India was 88%.^[8] Such high prevalence is probably due to rampant and inadvertent use of the third-generation cephalosporins. Over-the-counter availability could be another cause when patients resort to self-medication.^[9] A previous study from our institute reported 51.78% of uropathogens as ESBL producers.^[10] A reduction in the percentage of ESBL producers in our hospital is probably due to a leap in training and adherence to infection control procedures in recent past.

Maximum GNB samples in this study were isolated from wards and cabins 221 (42.5%), followed by ICUs 109 (20.96%) and Outpatient department (OPD) 102 (19.61%). Wards had the highest prevalence of ESBL-producing GNBs followed by ICUs. OPD samples were no less behind in harboring ESBL-producing GNBs. Vaguely, this suggests that in our area, the prevalence of ESBL in community is high. Although the ESBL-related prevalence studies are traditionally associated with health-care settings, there is an increasing number of reports documenting the isolation of ESBLs in community settings as well.^[11]

E. coli (150) and *Klebsiella* spp. (109) were the two most common isolates in the present study. *E. coli* (44%) was the most predominant ESBL producer followed by *Klebsiella* spp. (43.1%). A previous study^[12] ESBL production was observed in 41% of *E. coli* and 40% of *K. pneumoniae*.

Other members of *Enterobacteriaceae* when pondered into *Enterobacter* spp. showed a very high degree (48%) of ESBL production. None of the strains of *Proteus* spp. and *Shigella* spp. produced any ESBL. Nonfermenters such as *Pseudomonas* spp. and *Acinetobacter* spp. were positive for ESBL in 20% and 36% cases, respectively. Although the data for GNBs other than *E. coli* and *Klebsiella* spp. are limited, a study^[13] has found 15% of *Pseudomonas* spp. to be ESBL producers. In an Indian study,^[14] 28% of the *Acinetobacter* spp. isolates were ESBL producing. However, these are notorious because of the transferable nature of the ESBL plasmids in *Acinetobacter*^[15] which is further facilitated by the ability of this bacteria to survive for a long time in the hospital environment.^[14] Routine detection of ESBL-producing strains may be difficult because the synergy between cephalosporin and clavulanic acid, typically observed with ESBL-producing members of *Enterobacteriaceae*, tends to be minimal with *Acinetobacter* spp.^[16] Thus, our study, due to the inherent

Table 5: Comparison of resistant pattern of extended-spectrum beta-lactamases and nonextended-spectrum beta-lactamases isolates

Drugs	Number of resistant organism among ESBL-producing isolates (percentage resistance) (n=160), n (%)	Number of resistant organism among non-ESBL-producing isolates (percentage resistance) (n=360), n (%)	Overall percentage of resistance (n=520), n (%)
Ampicillin (10 µg)	157 (98.12)	358 (99.44)	99.03
Amoxiclav (20/10 µg)	48 (30)	74 (20.55)	23.46
Amikacin (10 µg)	7 (4.37)	26 (7.22)	6.34
Ceftriaxone (30 µg)	160 (100)	159 (44.16)	61.34
Cefoperazone-sulbactam (75/10 µg)	0	263 (73.05)	50.57
Cotrimoxazole (25 µg)	122 (76.25)	238 (66.11)	69.23
Cefotaxime (30 µg)	157 (98.12)	143 (67.50)	57.69
Gentamicin (10 µg)	60 (37.5)	242 (67.22)	58.07
Ofloxacin (5 µg)	160 (100)	285 (79.16)	85.57
Nitrofurantoin (300 µg)*	6 (10.62)	11 (6.18)	7.6
Imipenem (10 µg)	15 (9.37)	3 (0.83)	3.46
Tigecycline (15 µg)	10 (6.25)	40 (11.11)	9.61
Colistin (10 µg)	1 (0.62)	5 (1.39)	1.15

*Nitrofurantoin used only in urine samples. ESBL=Extended-spectrum beta-lactamases

Table 6: Genotypic pattern of extended-spectrum beta-lactamases-producing GNB isolates

Organisms	ESBL number	TEM	CTxM	SHV	TEM+CTxM+SHV	TEM+ CTxM	TEM+ SHV	CTxM+ SHV
<i>E. coli</i>	66	6	2	1	3	39	8	7
<i>K. pneumoniae</i>	40	4	2	2	8	15	4	5
<i>Acinetobacter</i> spp.	13	1	2	1	2	1	3	3
<i>K. oxytoca</i>	7	1	1	1	0	2	1	1
<i>Pseudomonas</i> spp.	10	0	0	1	2	2	2	3
<i>E. cloacae</i>	8	0	1	1	1	4	1	0
<i>C. freundii</i>	5	1	1	0	0	2	0	1
<i>C. koseri</i>	6	2	0	0	0	3	1	0
<i>E. aerogenes</i>	4	1	1	1	0	1	0	0
<i>Salmonella</i> spp.	1	1	0	0	0	0	0	0
Total	160	17	10	8	16	69	20	20

GNB=Gram-negative bacilli, ESBL=Extended-spectrum beta-lactamases, *E. coli*=*Escherichia coli*, *K. pneumoniae*=*Klebsiella pneumoniae*, *K. oxytoca*=*Klebsiella oxytoca*, *E. cloacae*=*Enterobacter cloacae*, *C. freundii*=*Citrobacter freundii*, *C. koseri*=*Citrobacter koseri*, *E. aerogenes*=*Enterobacter aerogenes*

limitations, may have underestimated the prevalence of ESBL in *Acinetobacter* spp.

The antibiotic susceptibility testing results of all 520 isolates showed most of the isolates (72.69%) in the present study were multidrug-resistant (MDR) (resistance to three or more group of antibiotics). Similarly, higher rate of MDR was also reported by Ansari *et al.*^[17] (78%). Amikacin, colistin, tigecycline, and imipenem were the most effective antibiotics in ESBL-producing organisms in the present study. Other studies^[18,19] denoted amikacin and imipenem are the choicest drugs for ESBL-positive strains. In our study, quinolones and beta-lactams showed the lowest efficacy similar to other studies.^[18,19] Among aminoglycosides, amikacin was a more effective antibiotic than gentamicin. This has also been noted in a similar to study by Rahman *et al.*^[20] In our study, ESBL-positive isolates showed higher degree of resistance to amoxiclav, cotrimoxazole, ofloxacin, nitrofurantoin, beta-lactam drugs, and imipenem than the non-ESBL strains. A similar study^[21] showed gentamicin, amikacin, norfloxacin, levofloxacin, ciprofloxacin, and netilmicin

having a significant reduced activity in ESBL producers than the non-ESBL producers. The non-ESBL strains were more resistant to aminoglycosides than ESBL strains unlike the above study findings, thus proposing other mechanisms of aminoglycoside resistance.

There is a paucity of literature as to the genotypic pattern of ESBL producers in our area. Genotyping of 160 ESBL isolates showed that TEM 122 (76.25%) is the predominant ESBL gene in GNB isolates. Few of the recent studies corroborate this data.^[9,22] Until the year 2000, TEM was the most prevalent ESBL gene in the Indian bacterial population but was replaced by CTX-M in the following decade.^[23] Reports from South America, Israel, Spain, New York, the United Kingdom, and several parts of the Indian subcontinent have revealed CTX-M as the predominant gene.^[23] In this study, CTX-M was seen in 115 (71.87%) cases either singly or in combination with other genes. CTX-M gene constitutes a distinct phylogenetic lineage of molecular class A beta-lactamases that exhibit a higher preference for cefotaxime and ceftriaxone, than CAZ, and a higher susceptibility to

tazobactam than to clavulanate. They still exist among the Indian bacterial population, especially in our settings and hence are a matter of concern for clinicians.^[24]

Coexistence of all the three bla genes was observed in 16 (10%) isolates in our study, which was higher than the report from North India where they observed only in 6.45% isolates.^[22] The occurrence of TEM, SHV, and CTX-M together along with impermeability can cause resistance to carbapenems^[25] which is a matter of concern in India which has high ESBL prevalence.

Conclusion

In our study, the 30.8% of GNB were ESBL producers. *K. oxytoca* was the most predominant ESBL organism followed by *E. coli*. Amikacin, colistin, tigecycline, and imipenem were the most effective antibiotics in ESBL-producing organisms in the present study. The rampant use of carbapenems for treating ESBL-producing organisms may lead to widespread carbapenem resistance. Bla TEM is the most predominant genotype in our locality in contrast to CTX-M which is prevalent in most parts of India. Ten percent cases in the present study had coexistence of all the genotypes which is a matter of concern because such isolates carry higher propensity for carbapenemase production as well.

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Conflicts of interest

There are no conflicts of interest.

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