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Research Article

Analysis of Extended Spectrum Beta-Lactamase Producing Gram-Negative Bacilli: Bacteriological Insights from Clinical Samples

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A B S T R A C T

Background: Antimicrobial resistance is a pressing concern in healthcare, driven by new resistance mechanisms, poor infection control, and antibiotic misuse. Proper antibiotic use is crucial for improving patient outcomes. However, inadequate practices foster resistance among bacteria that produce Extended Spectrum Beta-Lactamase (ESBL).

Objective: This study aims to detect and identify ESBL-producing gram-negative bacteria (GNB), offering insights for better management and surveillance.

Methodology: In this study, clinical samples from patients admitted between 2021 and 2024 underwent standard culture procedures. Gram-negative isolates were tested for ESBL production using recommended methods. Genomic DNA was extracted from bacterial isolates, followed by plasmid extraction and gel electrophoresis. Specific primers targeted bla gene sequences for TEM, OXA, SHV, and CTX-M. PCR reactions were conducted Under UV light, visualised with ethidium bromide staining, using agarose gel electrophoresis.

Results: In this analysis, predominant isolates of GNB were from urine (64%), exudates (26%), and respiratory (10%) samples. Out of 144 ESBL-producing GNB obtained, 40% of the isolates were positive for the *blaTEM* gene, 27% for *blaCTX-M*, and 14% for *blaSHV*.

Conclusion: ESBL-producing GNB are widespread in the gut, and highly resistant. Among 2356 isolates, *Escherichia coli* and *Klebsiella* species prevailed. ESBLs, mainly in *E. coli* and *Klebsiella*, pose therapeutic challenges, necessitating updated therapy strategies and prudent cephalosporin use. Routine testing for ESBL detection is crucial in clinical labs for effective control.

Keywords: Antibiotic-Resistant, ESBL Producers, Disc Diffusion Method, Conventional Method

Introduction

Antimicrobial resistance is a serious problem faced in hospitals and communities and may be caused by new resistance mechanisms, poor infection control, and antibiotic misuse. Proper antibiotic use hinges on understanding each drug's action, bacterial resistance, and prevalent local species. Inadequate use, incorrect dosing, and overuse breed resistance.[1] Studies across India have reported varying prevalence rates of ESBL-producing bacteria, with 34.87% of uropathogens in South Mumbai identified as ESBL producers (49.32% *Escherichia coli* and 27.5% *Klebsiella pneumoniae*), 22.76% of Enterobacteriaceae in South India producing ESBLs (31.05% *E. coli* and 16.67% *Klebsiella* species), and 50.81% of Enterobacteriaceae in Gujarat confirmed as ESBL producers (*K. pneumoniae* at 57.4% and *E. coli* at 41.4%).[2–4] International standards for testing antimicrobial susceptibility in clinical labs are crucial to combat resistance. Bacteria that produce ESBL are resistant to common antibiotics and pose significant challenges in hospitals.[5–7] Rapid detection of ESBLs is vital for appropriate treatment. ESBLs, deriving from enzymes like TEM, CTX-M, SHV, and OXA, resist penicillin and cephalosporins. Plasmids transfer ESBL genes between bacteria, complicating treatment. Carbapenems are often the last resort against ESBL-producing bacteria, but resistance is rising. Detecting and characterising ESBL-producing bacteria is crucial for effective treatment and reducing mortality rates—phenotypic and genotypic analyses, following updated guidelines, aid in identifying these strains.[8–10] However, the rapid emergence of ESBLs poses challenges in detection and treatment. Accurate identification of ESBL genes is vital for hospital surveillance and epidemiological studies to understand transmission. Combating antimicrobial resistance requires rigorous testing, judicious antibiotic use, and innovative treatment strategies.[11,12] This study aims to analyse the prevalence, antibiogram, and bacteriological characteristics of Extended Spectrum Beta-Lactamase (ESBL) producing gram-negative bacilli isolated from clinical samples, providing insights into their distribution and resistance patterns to inform effective treatment strategies.

Materials And Methods

This descriptive study was conducted in the Microbiology Department at Chettinad Hospital and Research Institute in Kelambakkam, Chennai. Before the study commenced, clearance was obtained from the Institutional Human Ethics Committee (IHEC-I/0147/21). Informed consent was obtained from all participants before sample collection. Clinical samples collected from patients admitted to various departments, including medicine, surgery, obstetrics, and gynaecology, as well as the outpatient department, over a period of 4 years (2021-2024), were processed as per standard guidelines. Out of the total 6672 samples processed, the distribution was as follows: 4272 urine samples, 1759 exudate samples, and 641 sputum samples. From these, a total of 1176 non-duplicate GNB isolates were obtained. Statistical analysis was conducted utilizing MS Excel (Microsoft) spreadsheets to organize the collected data, while categorical data were presented as numbers or percentages. SPSS Statistics version 20.0 was employed for the statistical computations.

Inclusion Criteria

The collected ESBL-producing GNB from clinical samples including urine, sputum, central venous catheter tip, exudates, throat swab, bronchoalveolar lavage, pleural fluids, and other body fluids, with no duplicates included.

Exclusion Criteria

Gram-negative bacilli which were not ESBL producers, gram-positive cocci, and gram-negative cocci isolated from various clinical samples were excluded from this study.

Analytical Approaches

Sample Processing and Identification

Clinical samples underwent standard culturing procedures on MacConkey agar and Blood agar plates, incubated at 37 °C for 18 hours to promote bacterial growth. Gram-negative isolates underwent additional characterisation using established biochemical assays. These tests aimed to identify and differentiate various bacterial species based on their metabolic properties, aiding in accurate classification. This systematic approach ensured a comprehensive assessment of microbial presence and characteristics within the clinical samples.

Detection of ESBL

Antibiotic Susceptibility Test

- **ESBL Detection Method:** ESBL detection was performed using Muller Hinton Agar plates and the Double Disc Diffusion Method (DDDM), as per CLSI guidelines. The DDDM detects ESBL production by comparing the inhibition zones of a cephalosporin disc (e.g., cefotaxime or ceftazidime) and a combination disc containing the same cephalosporin plus clavulanic acid. After incubation at 37 °C for 18 hours, a ≥ 5 mm increase in the zone diameter around the combination disc indicates ESBL production, as clavulanic acid inhibits the ESBL enzymes, restoring antibiotic activity. Standardised interpretation guides the efficient identification of ESBL-producing bacteria, informing subsequent analyses and treatments.

Genotypic Characterisation of Genes Responsible for ESBL Producers

To extract genomic DNA from bacterial isolates, nutrient agar culture plates were incubated overnight with a pure culture of the isolated bacteria.

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- **Cell Lysis and Extraction of DNA:** Plasmid extraction from ESBL producers was conducted via alkaline lysis, starting with colony inoculation into Luria broth, followed by overnight incubation at 37 ˚C. After centrifugation and resuspension in solutions, plasmid DNA was collected, precipitated with ethanol, and resuspended in TE buffer. Gel electrophoresis with molecular weight markers was performed for analysis, running for 2.5 hours at 50–70 V, followed by UV light examination for DNA bands.
- **Primers Used for Amplification:** The primers (Forward-F, Reverse-R) previously designed were employed specifically for the amplification of the bla gene sequences corresponding to TEM, OXA, SHV, and CTX-M (Table 1).[13] These genes encode important beta-lactamases that confer resistance to various classes of antibiotics. By targeting these specific bla gene variants, the study aimed to elucidate the presence and distribution of ESBL-producing bacteria harbouring these resistance determinants.

PCR Procedure

A 25 µL Master Mix was prepared, comprising a blend of 0.4 mM dNTPs, 2 units of Taq DNA polymerase proofreading, 10X Taq buffer, and 2 mM $MgCl_2$ combined for the reaction mixture.

The reaction setup was as follows:

In a PCR vial, 25 µL of Master Mix was combined with 1 µL each of F and R primers (sequence mentioned in Table 1) (10 pmoles/µL), genomic DNA (1 µL), and 22 µL of nuclease-free water (22 µL), resulting in 50 µL total volume. The mixture was gently mixed to ensure thorough blending and briefly centrifuged to collect any droplets. The vials were then placed into the PCR machine for amplification.

Conventional PCR Method

For this study, we employed the conventional PCR method. Initially, the conditions were set at 95 °C for 15 minutes, followed by 30 cycles of amplification at 94 °C for 30 seconds, 59 °C for 30 seconds, and 72 °C for 30 seconds each. The final step of PCR amplification involved raising the temperature to 72 °C for 10 minutes.

Agarose Gel Electrophoresis Procedure

Agarose 2% solution was prepared and melted in a microwave oven. Once the temperature of the agarose gel reached approximately 60 °C, ethidium bromide (5 µL) was added. The warm agarose solution was slowly poured into the gel platform. The gel set was left undisturbed until solidifying the agarose. The submarine gel tank was filled with 1X TAE buffer. The gel platform carefully, gel platform was placed into the tank, ensuring that the level of buffer remained approximately 0.5cm above the gel surface throughout the procedure. PCR samples, mixed with gel loading dye, were loaded onto the gel along with a 100 bp DNA ladder (10 µL). Gel electrophoresis was conducted at 50 V until the dye migrated approximately three-fourths of the distance across the gel. The gel was then seen under a UV transilluminator to observe the band patterns.

Results

Sample Distribution

This study encompassed a total number of 6672 clinical isolates which included 4272 urine samples, 1759 exudates, and 641 respiratory samples (Table 2).

Table 1.Sequence of Primers Utilised in This Study[13]

S. No.	Gene	Primers		Product (bp)
1	*blaSHV*	F	5’ CTTTCCCATGATGAGCACCT-3’	580
		R	5’ CAATGCGCTCTGCTTTGTTA-3’	
2	*blaCTX-M*	F	5’ CGCAGATAATACGCAGGTGCTTT-3’	296
		R	5’ GGCCGCCATAACTTTACTGGT-3’	
3	*blaTEM*	F	5’ AAAATTCTTGAAGACG -3’	250
		R	5’ TTACCAATGCTTAATCA-3’	
4	*blaOXA*	F	5’ TCAACAAATCGCCAGAGAAG-3’	276
		R	5’ TCCCACACCAGAAAAACCAG-3’	

Table 2.Distribution of Samples

Type of Clinical Sample	No. of Patients	Percentage
Urine	4272	64
Exudate	1759	26
Respiratory	641	10
Total	6672	100

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Distribution of Gram-Negative Bacteria

Out of the 6672 clinical isolates obtained, 4272 were from urine, 1759 were exudate samples and 641 were respiratory samples. 2356 isolates were GNB (35.3%), of which, 1176 (49.91%) were from urine, 846 (35.9%) from exudates and 334 (14.17%) were from respiratory samples. The highest proportion of GNB isolates was found in urine samples, accounting for 64% of the total isolates. Pus and exudates samples followed with 26%, while respiratory samples yielded 10% of the isolates among the various clinical samples obtained (Figure 1).

Isolation of Gram-Negative Bacteria from Urine Samples

Of the 4272 urine isolates, 1176 showed significant growth of GNB. Among these isolates, 735 (63%) were *Escherichia coli*, 111 (9%) were *Klebsiella* species, 76 (6%) were *Acinetobacter* species, 80 (8%) were *Pseudomonas* species, 64 (5%) were *Citrobacter* species, 69 (6%) were *Proteus* species, and 41 (3%) were *Enterobacter* species. The details are shown in Table 3.

Isolation of Gram-Negative Bacteria from Exudate Samples

Out of the 1759 exudate samples that were processed, 846 samples yielded gram-negative bacilli. Among these, the bacterial distribution was as follows: *Escherichia coli*: 164 (19%), *Klebsiella* species: 129 (15%), *Acinetobacter* species: 106 (13%), *Pseudomonas* species: 245 (29%), *Citrobacter* species: 108 (13%), *Proteus* species: 68 (8%), and *Enterobacter* species: 26 (3%) (Table 3).

Isolation of Gram-Negative Bacteria from Respiratory Samples

Out of the 641 respiratory samples processed, 334 samples yielded gram-negative bacilli. The bacterial distribution in these samples was as follows: *Escherichia coli*: 34 (10%), *Klebsiella* species: 79 (24%), *Acinetobacter* species: 84 (25%), *Pseudomonas* species: 105 (31%), and *Citrobacter* species: 32 (10%). The details of the isolates are shown in Table 3.

Percentage of ESBL Producers among Various Isolates Obtained from All Clinical Samples

By phenotypic methods, the total number of ESBL-producing GNB was found to be 144 out of 2356, which were distributed as 80 which was 56% of isolates from urine, 43 (30%) from exudates and 21 (14%) from respiratory samples. Among the 144 ESBL producers, the highest percentage of ESBL producers were *Escherichia* coli: 60 isolates (42%), followed by *Klebsiella* species: 35 isolates (24%), *Acinetobacter* species: 15 (10%), *Pseudomonas* species: 16 (11%), *Citrobacter* species: 8 (6%), *Proteus* species: 6 (4%) and *Enterobacter* species: 4 (3%). Table 4 shows the details of ESBL distributions among samples.

Detection of ESBL Producers among Different Isolates by the Genotypic Method

The presence of ESBL resistance genes in 144 isolated strains of *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., and *Acinetobacter* spp. was detected using a conventional PCR method, with specific targeting of the *blaCTX-M*, *blaSHV*, *blaOXA*, and *blaTEM* genes. Among the ESBL isolates, a predominant proportion of 58 (40%) harboured the *blaTEM* gene within their plasmid DNA (Figure 2), with the *blaCTX-M* gene present in 39 (27%) isolates (Figure 3). Conversely, only a minority of 20 (14%) exhibited the *blaSHV* gene within their plasmid DNA (Figure 4). None of the isolated strains showed a positive presence of the *blaOXA* gene. The details of the ESBL-resistant genes are illustrated in Table 5.

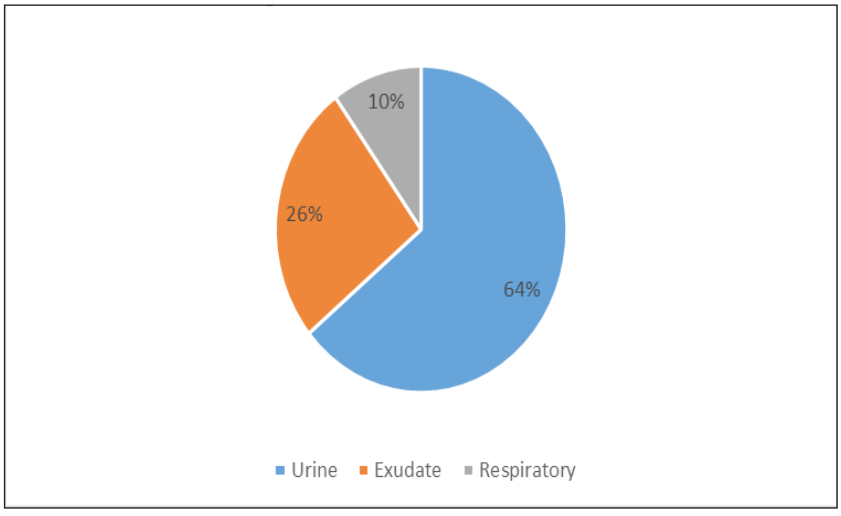

Figure 1.Distribution of Isolated Gram-Negative Bacteria among Different Types of Samples

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Table 3.Isolated Organisms from the Clinical Samples

Type of GNB	Urine Samples		Exudate Samples		Respiratory Samples	
	Number	Percentage of Isolates	Number	Percentage of Isolates	Number	Percentage of Isolates
E. coli	735	63	164	19	34	10
Klebsiella spp.	111	9	129	15	79	24
Acinetobacter spp.	76	6	106	13	84	25
Pseudomonas spp.	80	8	245	29	105	31
Citrobacter spp.	64	5	108	13	32	10
Proteus spp.	69	6	68	8	0	0
Enterobacter spp.	41	3	26	3	0	0
Total	1176	100	846	100	334	100

GNB: Gram-Negative Bacteria

Table 4.Percentage of ESBL Producers

Type of Organisms	Total Isolates	ESBL Producer in Urine n=80 (56%)	ESBL Producer in Exudates n=43 (30%)	ESBL Producer in Respiratory n=21 (14%)	Total ESBL Isolates n=144 (100%)	ESBL Percentage (%)
Escherichia coli	933	39	17	4	60	42
Klebsiella spp.	319	14	13	8	35	24
Acinetobacter spp.	266	7	5	3	15	10
Pseudomonas spp.	430	6	5	5	16	11
Citrobacter spp.	204	6	1	1	8	6
Proteus spp.	137	5	1	0	6	4
Enterobacter spp.	67	3	1	0	4	3

ESBL: Extended Spectrum Beta-Lactamase

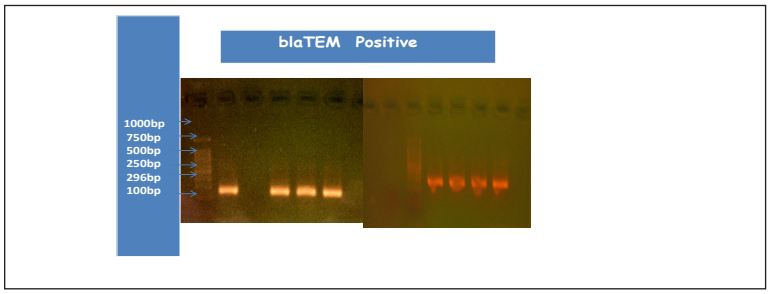

Figure 2.*blaTEM* Gene Detection in Isolated ESBL Strains

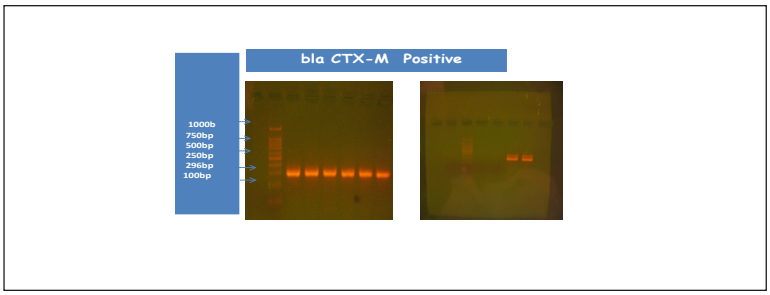

Figure 3.*blaCTX-M* Gene Detection in Isolated ESBL Strains

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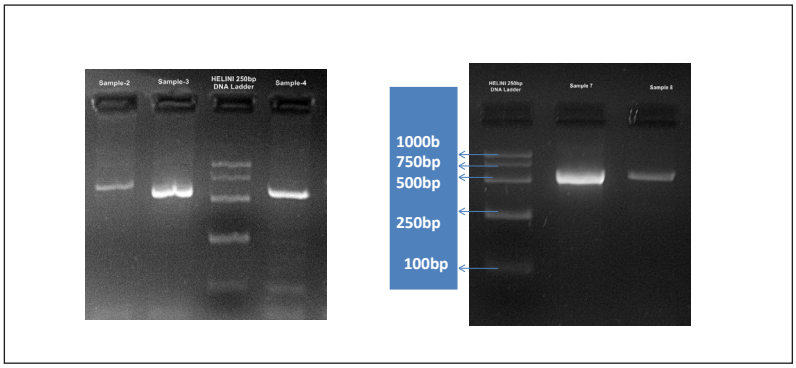

Figure 4.*blaSHV* Gene Detection in Isolated ESBL Strains

Table 5.Distribution of ESBL-Responsible Genes among Isolated Strains

Type of isolate	*blaTEM ESBL* Type	*blaCTX-M ESBL* Type	*blaSHV ESBL* Type	*blaOXA ESBL* Type
E. coli	35	09	12	Nil
Klebsiella spp.	11	17	05	Nil
Acinetobacter spp.	06	05	02	Nil
Pseudomonas spp.	06	08	01	Nil

ESBL: Extended Spectrum Beta-Lactamase

Discussion

The study included a total of 4,272 urine samples, 1,759 exudate samples, and 641 respiratory samples. Among these clinical samples, the highest proportion of GNB isolates was observed in urine samples, accounting for 58%, followed by exudates at 29%, and Broncho alveolar lavage (BAL) and sputum samples at 13%. Notably, the GNB prevalence in urine samples was significantly higher than the findings of a study conducted by Shobha et al. in 2009.[14] In our present study, we observed an ESBL prevalence rate of 6% (144 out of 2,356 isolates). This rate is notably lower compared to findings from other studies. For instance, Osman in 2019[15] reported an ESBL prevalence rate of 66.61% (804 out of 1,207 isolates), while Li et al. in 2009 found a prevalence rate of 40.0% (56 out of 140 isolates).[16] In contrast to our study, Duguid et al. in 1989 reported an exceptionally high ESBL prevalence rate of 88% (105 out of 119 isolates). This rate stands out as significantly higher compared to our findings.[17]

The percentage of ESBL producers detected by phenotypic identification in our study includes *Escherichia coli*: 60 (42%), *Klebsiella* species: 35 (24%), *Acinetobacter* species: 15 (10%), *Pseudomonas* species: 16 (11%), *Citrobacter* species: 8 (6%), *Proteus* species: 6 (4%), and *Enterobacter* species: 4 (3%). Notably, among these ESBL-producing isolates, *Escherichia coli* exhibited intense ESBL production. However, compared to the findings of Dalela in 2012, our prevalence rates differ. Their study reported a prevalence rate of *Klebsiella* species at 51% (22 out of 43 isolates), *E. coli* at 70% (82 out of 117 isolates), *Pseudomonas* species at 31% (11 out of 35 isolates), *Proteus* species at 50% (2 out of 4 isolates), *Acinetobacter* species at 57% (4 out of 7 isolates), and *Citrobacter* species at 50% (2 out of 4 isolates).[18]

In several studies, the predominant genotypes of ESBLs are diverse. In this study, we found a relatively high presence of *blaTEM* (58, 40%), followed by *blaCTX-M* (39, 27%), and *blaSHV* (20, 14%). The most common ESBL genotype among our isolates was the combination of *blaTEM* and *blaCTX-M*. However, another study conducted by Bora et al. in 2014 reported different findings. They found that the most prevalent genotype was *blaCTX-M* (89%), followed by *blaTEM* (78%). In their study, the *blaCTX-M* ESBL type was highly common among *E. coli* isolates, while the *blaTEM* ESBL type was predominant among *Klebsiella* species. These results were consistent with our findings.[19]

Conclusion

ESBL-producing GNB are prevalent in the human gut and are resistant to various antibiotics. Among 2356 GNB isolates, *Escherichia coli* and *Klebsiella* species were predominant. ESBLs, primarily *blaTEM* and *blaCTX-M*, were mainly detected in these species, posing therapeutic challenges. Revised therapy strategies and cautious cephalosporin use are crucial, with routine phenotypic disc diffusion testing recommended for ESBL detection in clinical microbiology laboratories.

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Conflict of Interest: None

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