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CONCURRENT ESBL PRODUCTION AND BIOFILM-FORMING POTENTIAL IN GRAM-NEGATIVE CLINICAL ISOLATES FROM A TERTIARY CARE HOSPITAL IN KARNATAKA WITH IMPLICATIONS FOR ANTIMICROBIAL STEWARDSHIP

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ABSTRACT
Extended-spectrum beta-lactamase (ESBL)-producing Gram-negative bacteria complicate empirical treatment and elevate healthcare costs in tertiary care settings. This prospective, single-center laboratory-based study aimed to evaluate eight classified culture media for Gram-negative recovery, confirm ESBL production using a comprehensive six-method phenotypic panel, quantify biofilm formation by three validated methods, analyze the statistical association between ESBL production and biofilm-forming capacity, and determine the antibiotic susceptibility and multidrug resistance (MDR/XDR) profile of ESBL-confirmed isolates. Of 125 consecutive clinical specimens, 100 (80.0%) yielded Gram-negative growth across seven species; the remaining 25 yielded no growth or non-qualifying organisms. Escherichia coli predominated (46.0%). MacConkey agar achieved the highest recovery (96.0%). ESBL production was phenotypically confirmed in 77 of 100 isolates (77.0%) using a comprehensive six-method panel comprising double disc synergy test (DDST), combination disc test (CDT), E-test for ESBL, MIC by agar dilution, inhibitor potentiated disc diffusion test (IPDD), and modified CLSI phenotypic confirmatory method using phenylboronic acid and EDTA (Boronic Acid-EDTA method). Biofilm was detected in 86 isolates (86.0%) by the microtiter plate reference method; ESBL-positive isolates showed significantly greater biofilm-forming capacity than ESBL-negative isolates (93.5% vs. 60.9%; OR = 9.26; 95% CI: 2.81-30.50; $p < .001$). Colistin susceptibility was universal (100%), and tigecycline retained high activity (94.8%). MDR was identified in 80.5% and XDR in 13.0% of ESBL-confirmed isolates. Consequently, tracking concurrent ESBL and biofilm profiles is crucial for effective antibiotic stewardship in Karnataka.

KEYWORDS: ESBL; Biofilm; Multidrug resistance; Gram-negative bacteria; DDST; Karnataka

1. INTRODUCTION

Healthcare-associated infections caused by multidrug-resistant Gram-negative pathogens are a major challenge to modern clinical therapeutics (Weiner-Lastinger et al., 2020; Antimicrobial Resistance Collaborators, 2022). Among these, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter baumannii pose particular risks in tertiary care environments due to their capacity to acquire and express complex resistance determinants. The proliferation of extended-spectrum beta-lactamases (ESBLs) — plasmid-mediated enzymes capable of degrading third- and fourth-generation cephalosporins as well as monobactams — has compromised empirical treatment algorithms, increasing reliance on carbapenems and restricted reserve agents (Pitout and Laupland, 2008; Bush and Bradford, 2016). Consequently, the World Health Organization has classified ESBL-producing Enterobacteriaceae as critical priority pathogens requiring intensive epidemiological surveillance and novel containment strategies (Tacconelli et al., 2018).

In India, the baseline prevalence of ESBL expression among clinical Gram-negative isolates is markedly higher than global averages. National surveillance data published by the Indian Council of Medical Research indicated that ESBL phenotypic positivity frequently reaches 76-81% in E. coli isolates recovered from bacteraemia cases in tertiary settings (NARS-Net, 2023). Within this landscape, Karnataka represents a high-burden region; baseline surveillance from tertiary hospitals in Chamarajanagar and Hubballi has documented ESBL rates spanning 67-80% among general clinical specimens (Metri et al., 2011; Wadekar et al., 2020). Localized studies from Davangere and Shimoga further indicate that a substantial proportion of these resistant phenotypes — often exceeding 50-72% — concurrently show biofilm-forming capability, consistent with a regional trend toward dual-phenotype resistance (Zubair and Mohammad, 2023; Li et al., 2024).

The co-occurrence of ESBL production and biofilm formation compounds the clinical challenge through two converging mechanisms, structural and genetic. Structurally, the extracellular polymeric substance matrix of a mature biofilm acts as a diffusion barrier, retarding the penetration of hydrophilic beta-lactams and granting cell-bound ESBL enzymes sufficient time to hydrolyze the antibiotic before it reaches its lethal targets on the bacterial cell wall (Laure and Ahn, 2022; Zubair and Mohammad, 2023). Genetically, the horizontal dissemination of ESBL-encoding genes is frequently mediated by high-molecular-weight, conjugative plasmids belonging to IncF, IncI, or IncHI incompatibility groups. These specific

plasmid backbones often co-carry regulatory clusters for surface adhesins, such as type I fimbriae operons and csgD regulons, which actively upregulate biofilm initiation upon plasmid acquisition (Schembri et al., 2003; Bae et al., 2008). Furthermore, structural investigations reveal that specific Class A and Class D beta-lactamases modulate cell-surface hydrophobicity and interact with low-molecular-weight penicillin-binding proteins to facilitate early bacterial attachment (Gallant et al., 2005). This results in biofilm-dwelling populations demonstrating up to a 1,000-fold increase in phenotypic antibiotic tolerance compared to their planktonic counterparts (Li et al., 2024).

Despite the clinical importance of this association, data quantifying the statistical and phenotypic correlation between ESBL production and biofilm metrics remain limited within Karnataka's tertiary healthcare networks. Most existing regional literature relies on limited dual-disc screening without assessing co-existing metallo-beta-lactamase (MBL) or AmpC interference, leaving an incomplete picture of the local resistome (Dumaru et al., 2019); molecular studies linking MBL and biofilm-associated genes in Gram-negative isolates remain similarly limited (Yadav et al., 2024). To address this gap, the present prospective, laboratory-based study was designed to evaluate eight classified isolation media, confirm ESBL expression using a six-method phenotypic panel, quantify biofilm production using three validated techniques, and characterize the multidrug and extensive drug resistance (MDR/XDR) profiles of these isolates. These findings are intended to inform empirical antibiotic stewardship protocols within secondary and tertiary care centers across Karnataka.

2. MATERIAL AND METHODS

Study design and ethical considerations

This was a prospective, observational, single-center laboratory-based study conducted over a six-month period in the Department of Microbiology, Davangere University, Karnataka, India. Bacterial isolates recovered from routine diagnostic clinical specimens were studied. All isolates were handled in de-identified form; no patient-linked personal or clinical data were accessed at any stage. The study was confined to routine microbiological processing without patient contact or experimental intervention. All procedures complied with the Declaration of Helsinki (2013 revision).

Specimen collection and inclusion criteria

One hundred and twenty-five consecutive, non-duplicate clinical specimens submitted for routine bacterial culture and sensitivity testing were enrolled: urine (n = 48), pus and wound swabs (n = 32), blood cultures (n = 18), sputum (n = 16), and miscellaneous body fluids including tracheostomy secretions and pleural fluid (n = 11). Of the 125 specimens, 100 (80.0%) yielded Gram-negative growth; the remaining 25 were culture-negative (n = 14), yielded Gram-positive organisms (n = 8), or yielded polymicrobial growth with Gram-positive predominance (n = 3) and were excluded from further analysis. Each specimen was inoculated directly onto all general and specialized media (MacConkey agar, blood agar, EMB agar, chocolate agar, CLED agar, and XLD agar) simultaneously within one hour of receipt. Selective media (cetrimide agar for P. aeruginosa and Leeds Acinetobacter Medium for A. baumannii) were inoculated from primary growth on general media upon detection of Gram-negative non-fermenting morphology after 18-24 hours of incubation.

Culture media and bacterial identification

Eight classified media were employed. General isolation media comprised MacConkey agar, Eosin Methylene Blue (EMB) agar, blood agar, and chocolate agar. Specialized media included Cystine-Lactose-Electrolyte-Deficient (CLED) agar and Xylose Lysine Deoxycholate (XLD) agar. Selective media comprised cetrimide agar for Pseudomonas aeruginosa and Leeds Acinetobacter Medium for Acinetobacter baumannii. All media were sourced from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Recovered isolates were identified to species level using standard conventional biochemical characterization per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Media performance is reported as descriptive recovery rate — the proportion of total isolates recovered on each medium — rather than formal diagnostic accuracy, as all specimens were concurrently processed across all eight media without an independent reference standard.

Phenotypic detection and characterization of ESBL and beta-lactamase resistance

All 100 Gram-negative isolates were subjected to disc susceptibility testing (DST) for general antimicrobial susceptibility, followed by a comprehensive six-method phenotypic panel to detect ESBL production, characterize co-existing beta-lactamase resistance mechanisms, and determine minimum inhibitory concentrations. Methods are organized below by clinical purpose.

Phenotypic screening and disc susceptibility testing

Disc susceptibility testing (DST) was performed by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA; HiMedia, India), following CLSI M100-2023 (33rd edition) guidelines (CLSI, 2023). Bacterial suspensions were prepared in sterile normal saline and adjusted to 0.5 McFarland turbidity. Inoculated plates were incubated at 35 ± 2°C for 16-18 hours. Zone diameters were measured with a ruler under reflected light and interpreted as susceptible, intermediate, or resistant per CLSI M100-2023 breakpoints. Disc concentrations: piperacillin-tazobactam (100/10 µg), cefepime (30 µg), ertapenem (10 µg), meropenem (10 µg), imipenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), and tigecycline (15 µg; interpreted per EUCAST v14.0 breakpoints). Colistin was not evaluated by disc diffusion, as this method is not validated by CLSI or EUCAST for polymyxins. Quality control strains E. coli ATCC 25922 and P. aeruginosa ATCC 27853 were run on every testing day.

Phenotypic detection and confirmation of ESBLs

ESBL detection was performed using three sequential methods applied to all 100 isolates. (a) Double Disc Synergy Test (DDST): Ceftazidime (30 µg), cefotaxime (30 µg), and ceftriaxone (30 µg) discs were positioned exactly 20 mm center-to-center from an amoxicillin-clavulanate disc (20/10 µg) on MHA. After incubation at 35 ± 2°C for 16-18 hours, a positive result was indicated by enhancement or extension of the inhibition zone toward the clavulanate disc — the characteristic keyhole or egg-shape synergy pattern. DDST was applied to all Enterobacteriaceae isolates. For non-fermenters (P. aeruginosa and A. baumannii), DDST was also performed. However, due to the intrinsically unreliable

DDST synergy pattern in non-fermenting species, final ESBL classification for these organisms was based on CDT alone as the reference, consistent with CLSI M100-2023 guidance. (b) Combination Disc Test (CDT): Paired disc sets of ceftazidime ± clavulanate (30/10 µg) and cefotaxime ± clavulanate (30/10 µg) were placed on MHA inoculated to 0.5 McFarland turbidity. A zone diameter increase of ≥ 5 mm for the clavulanate-containing disc versus the disc without clavulanate confirmed ESBL production per CLSI M100-2023, Table 3A (CLSI, 2023). For Enterobacteriaceae, an isolate was classified ESBL-positive only when both DDST and CDT were concordantly positive. For non-fermenters, CDT positivity alone was the classification criterion. Quality controls: K. pneumoniae ATCC 700603 (ESBL-positive) and E. coli ATCC 25922 (ESBL-negative) were tested concurrently on every day of testing. (c) E-test for ESBL: ESBL E-test strips (HiMedia/bioMerieux) incorporating cefotaxime-clavulanate (CT/CTL) and ceftazidime-clavulanate (TZ/TZL) gradients were applied to MHA inoculated to 0.5 McFarland turbidity. After incubation at 35 ± 2°C for 18 hours, ESBL production was confirmed when the MIC ratio (MIC of cephalosporin alone divided by MIC with clavulanate) was ≥ 8, or when a phantom zone or ellipse deformity was observed. The E-test provided quantitative MIC endpoints for cefotaxime and ceftazidime.

Determination of minimum inhibitory concentrations (MIC)

MIC by agar dilution was performed for a representative subset of 30 isolates (selected to include all seven species) for cefotaxime, ceftazidime, and ceftriaxone per CLSI M07-2018 guidelines (CLSI, 2018). Serial two-fold dilutions of each antibiotic (range 0.25-512 µg/mL) were incorporated directly into Mueller-Hinton agar. Plates were inoculated using a multipoint inoculator device at a final inoculum of approximately 10^4 CFU/spot, prepared from bacterial suspensions adjusted to 0.5 McFarland turbidity and diluted 1:10. Plates were incubated at 35 ± 2°C for 18 hours; the MIC endpoint was the lowest antibiotic concentration at which no visible growth was observed at the inoculation spot. MIC values for meropenem, imipenem, and colistin were also determined in this subset. For colistin, broth microdilution MIC was performed using cation-adjusted Mueller-Hinton broth (CAMHB) in untreated polystyrene microtiter plates per EUCAST guidelines, as colistin binds to certain plastics and disc diffusion is not validated for polymyxins (EUCAST, 2024).

Supplementary ESBL Confirmation — Boronic Acid-EDTA Method and IPDD

(a) Boronic Acid-EDTA Method (Modified CLSI): A phenylboronic acid (PBA; 40 mg/mL) and EDTA (0.1 M) enhanced double-disc synergy approach was used on MHA at 0.5 McFarland turbidity to distinguish true ESBL producers from AmpC/MBL co-producers (Tsakris et al., 2009; Poulou et al., 2014). Cefotaxime and ceftazidime discs (30 µg each), their clavulanate combinations (30/10 µg), and duplicate cephalosporin discs with 10 µL PBA or EDTA were incubated at 35-37°C for 16-18 hours. Zone enhancement ≥ 5 mm with clavulanate, or synergy toward the clavulanate disc, confirmed ESBL production when reproduced in PBA/EDTA duplicates. Meropenem was not employed.

(b) Inhibitor Potentiated Disc Diffusion Test (IPDD): Bacterial suspensions at 0.5 McFarland turbidity were plated on MHA with and without 0.004 mg/L potassium clavulanate (Sigma Aldrich, Bangalore) (Harwalkar et al., 2013). Ceftazidime, cefotaxime, and cefpodoxime discs (30 µg each) were placed on both plates; after overnight incubation at 37°C, a zone difference of ≥ 10 mm confirmed ESBL production.

Biofilm detection

Three validated phenotypic methods were employed. (i) Microtiter plate (MTP) method, reference standard: overnight broth cultures adjusted to 0.5 McFarland turbidity were inoculated in quadruplicate wells of 96-well flat-bottomed polystyrene microplates (HiMedia, India) and incubated at 37°C for 24 hours under static conditions. Wells were emptied, washed three times with phosphate-buffered saline (PBS), air-dried, fixed with 99% methanol, and stained with 0.1% crystal violet for 15 minutes. After washing and solubilization with 33% glacial acetic acid, absorbance was read at OD570 nm. Optical density cut-off (ODc) was defined as mean negative control OD + 3 standard deviations (SD). Biofilm was graded: strong (OD > 4 × ODc), moderate (2 × ODc < OD ≤ 4 × ODc), weak (ODc < OD ≤ 2 × ODc), and non-producer (OD ≤ ODc) per Stepanovic et al., 2000, 2007. (ii) Tube method (TM): Trypticase soy broth supplemented with 1% glucose was inoculated and incubated statically at 37°C for 24 hours; biofilm adherent to tube walls was stained with crystal violet (Kırmusaoglu, 2019). (iii) Congo Red Agar (CRA) method: brain heart infusion agar supplemented with Congo red dye (0.8 g/L) and sucrose (50 g/L); black colony morphology indicated slime production (Freeman et al., 1989). Inter-method agreement between TM and CRA against the MTP reference was assessed by Cohen's kappa (κ) with 95% confidence intervals.

MDR and XDR classification

MDR and XDR classification followed the Magiorakos et al., 2012 international expert consensus definitions: MDR = non-susceptibility to ≥ 1 agent in ≥ 3 antimicrobial categories; XDR = non-susceptibility to ≥ 1 agent in all but ≤ 2 categories. XDR is a stricter, higher category than MDR; isolates meeting XDR criteria are reported exclusively as XDR and are not double-counted as MDR. Six antimicrobial categories were assessed: penicillin/beta-lactamase inhibitor combinations, advanced cephalosporins, carbapenems, aminoglycosides, fluoroquinolones, and polymyxins.

Statistical analysis

Data entry and statistical analysis were performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Categorical variables were compared by the Chi-square test or Fisher's exact test, as appropriate, using standard formula-based calculations. Odds ratios (OR) with 95% confidence intervals (CI) were calculated for the ESBL-biofilm association. Mean OD570 biofilm values were compared between ESBL-positive and ESBL-negative isolates by independent samples t-test. Inter-method agreement for biofilm detection was quantified by Cohen's kappa (κ) with 95% CI, interpreted using Landis and Koch, 1977 benchmarks. All tests were two-tailed; $p < .05$ was considered statistically significant.

RESULTS

Specimen yield and species distribution

Of 125 specimens processed, 100 (80.0%) yielded Gram-negative growth. Seven species were recovered: Escherichia coli (n = 46; 46.0%), Klebsiella pneumoniae (n = 28; 28.0%), Klebsiella oxytoca (n = 8; 8.0%), Pseudomonas aeruginosa (n = 7; 7.0%), Acinetobacter baumannii (n = 5; 5.0%), Proteus vulgaris (n = 4; 4.0%), and Klebsiella aerogenes (n = 2; 2.0%). Klebsiella species together accounted for 38.0% of all isolates. Species distribution by specimen type is detailed in Table 1 and Figure 1.

Table 1. Distribution of Gram-negative clinical isolates by bacterial species and specimen type (n = 100)

Bacterial species	Urine (n=48)	Pus/wound (n=32)	Blood (n=18)	Sputum (n=16)	Other (n=11)	Total n (%)
E. coli	28	8	6	3	1	46 (46.0)
K. pneumoniae	8	7	6	4	3	28 (28.0)
K. oxytoca	4	0	0	3	1	8 (8.0)
P. aeruginosa	0	5	0	2	0	7 (7.0)
A. baumannii	0	2	2	0	1	5 (5.0)
P. vulgaris	1	2	0	0	1	4 (4.0)
K. aerogenes	0	0	2	0	0	2 (2.0)
Total	48	32	18	16	11	100 (100.0)

Other specimens: tracheostomy secretions and pleural fluids.

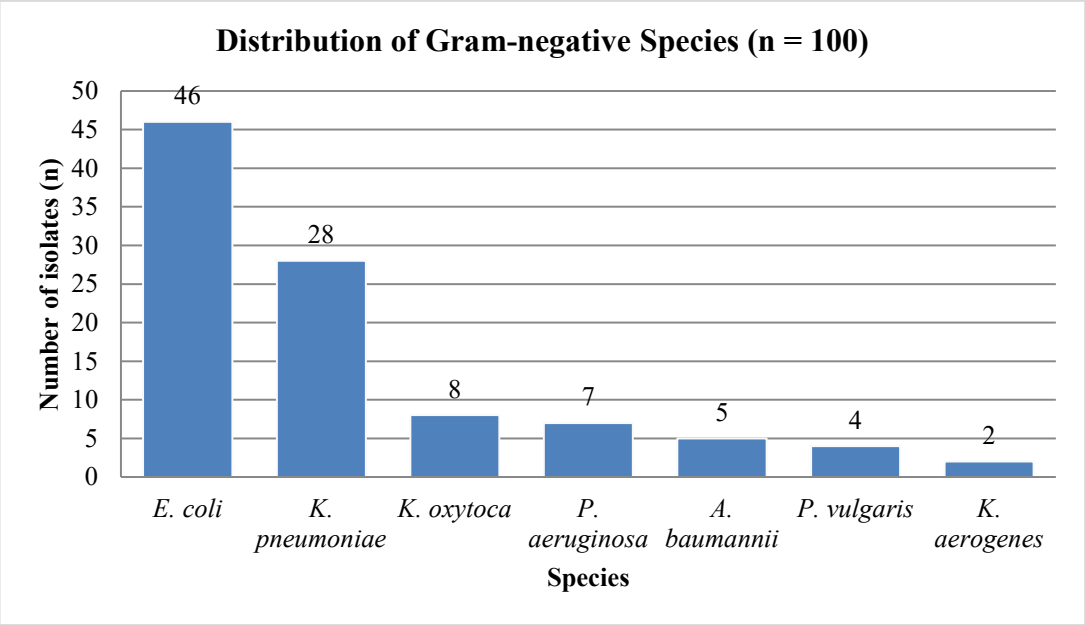

Figure 1. Distribution of Gram-negative bacterial species recovered from clinical specimens (n = 100). Bar chart showing isolate frequency by species, corresponding to the data in Table 1.

Descriptive recovery performance of culture media

MacConkey agar achieved the broadest recovery (96/100; 96.0%), capturing 74 of 77 ESBL-producing isolates (96.1%), and serves as the reference for general Gram-negative bacteriology. Blood agar and EMB agar followed at 94.0% and 92.0%, respectively. CLED agar yielded 82.0% and chocolate agar 78.0%. XLD agar demonstrated poor recovery (21.0%), reflecting its design specificity for enteric pathogens absent from this cohort. Cetrimide agar achieved complete recovery of all P. aeruginosa isolates (100%), and Leeds Acinetobacter Medium recovered all A. baumannii isolates (100%). Full recovery data are presented in Table 2 and Figure 2.

Table 2. Descriptive recovery performance of eight classified culture media (n = 100)

Culture medium	Category	GNB recovered n (%)	Recovery rate (%)	ESBL isolates recovered n (%)	Remarks
MacConkey agar	General	96 (96.0)	96.0	74/77 (96.1)	Reference; direct specimen plating
Blood agar	General	94 (94.0)	94.0	72/77 (93.5)	Direct specimen plating
EMB agar	General	92 (92.0)	92.0	71/77 (92.2)	Direct specimen plating
CLED agar	Specialized	82 (82.0)	82.0	63/77 (81.8)	Direct plating; optimized for urine
Chocolate agar	General	78 (78.0)	78.0	60/77 (77.9)	Direct specimen plating
XLD agar	Specialized	21 (21.0)	21.0	16/77 (20.8)	Enteric-specific; low yield here
Cetrimide agar	Selective	7/7 (100.0)a	100.0a	5/5 (100.0)	P. aeruginosa only; subculture from primary GNB growth
Leeds Acinetobacter Medium	Selective	5/5 (100.0)b	100.0b	4/4 (100.0)	A. baumannii only; subculture from primary GNB growth

Remarks: Recovery rates for selective media are calculated against confirmed target organisms only. a Cetrimide agar inoculated from primary Gram-negative non-fermenter growth; 100% recovery of P. aeruginosa (n = 7). b Leeds Acinetobacter Medium inoculated from primary Gram-negative growth; 100% recovery of A. baumannii (n = 5).

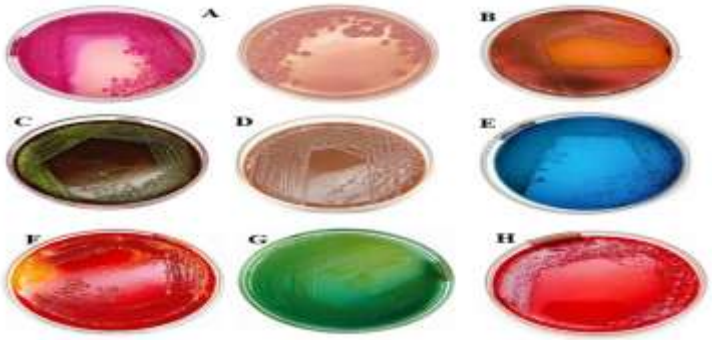

Figure 2. Representative growth of Gram-negative isolates on the eight culture media used for recovery. (a) MacConkey agar — lactose-fermenting (pink) colonies of Escherichia coli and non-fermenting (pale) colonies of Klebsiella pneumoniae; (b) blood agar showing growth of Pseudomonas aeruginosa; (c) Eosin Methylene Blue (EMB) agar showing the characteristic green metallic sheen of Escherichia coli; (d) chocolate agar showing growth of Klebsiella pneumoniae; (e) Cystine-Lactose-Electrolyte-Deficient (CLED) agar showing growth of Proteus vulgaris; (f) Xylose Lysine Deoxycholate (XLD) agar showing growth of Klebsiella pneumoniae; (g) cetrimide agar showing pyocyanin-pigmented growth of Pseudomonas aeruginosa; (h) Leeds Acinetobacter Medium showing colonies of Acinetobacter baumannii. Scale bar = 1 cm.

ESBL production — six-method panel results

ESBL production was confirmed in 77 of 100 isolates (77.0%) by the composite reference standard. For Enterobacteriaceae (n = 88), DDST and CDT were both required to be concordantly positive; for non-fermenters (P. aeruginosa n = 7, A. baumannii n = 5), CDT alone served as the classification criterion per CLSI M100-2023 guidance. Rates ranged from 50.0% (K. aerogenes and P. vulgaris) to 80.4% (E. coli). The E-test confirmed ESBL in 74 of 77 reference-positive isolates (96.1% concordance). MIC by agar dilution (n = 30 subset) showed cefotaxime MIC > 2 µg/mL in all 23 ESBL-positive isolates tested (range 8 to > 256 µg/mL), with ≥ 8-fold MIC reduction in the presence of clavulanic acid. The Boronic Acid-EDTA method (Tsakris et al., 2009; Poulou et al., 2014) confirmed genuine ESBL production by excluding AmpC and carbapenemase co-production in all 77 ESBL-positive isolates; the PBA-supplemented duplicate discs identified two K. pneumoniae isolates where AmpC co-production was masking the ESBL phenotype, and the EDTA-supplemented duplicates excluded MBL co-production in five P. aeruginosa and three A. baumannii isolates. The IPDD (Harwalkar et al., 2013) independently confirmed ESBL production in 75 of 77 reference-positive isolates (≥ 10 mm zone increase with clavulanate-supplemented plates). Species-wise ESBL data are shown in Table 3 and Figure 3

Table 3. ESBL production by bacterial species with detection methods applied (n = 100)

Species	Total n	ESBL-positive n (%)	ESBL-negative n (%)	CDT confirmed n (%)	Detection methods applied
Enterobacteriaceae (classified by DDST + CDT concordance)					
E. coli	46	37 (80.4)	9 (19.6)	37 (80.4)	DDST + CDT + E-test + MIC
K. pneumoniae	28	22 (78.6)	6 (21.4)	22 (78.6)	DDST + CDT + E-test + MIC + Boronic Acid-EDTA + IPDD
K. oxytoca	8	6 (75.0)	2 (25.0)	6 (75.0)	DDST + CDT + E-test
P. vulgaris	4	2 (50.0)	2 (50.0)	2 (50.0)	DDST + CDT
K. aerogenes	2	1 (50.0)	1 (50.0)	1 (50.0)	DDST + CDT
Non-fermenters (DDST performed; CDT alone used for classification — see note)					
P. aeruginosa	7	5 (71.4)	2 (28.6)	5 (71.4)	DDST (performed) + CDT + Boronic Acid-EDTA + IPDD
A. baumannii	5	4 (80.0)	1 (20.0)	4 (80.0)	DDST (performed) + CDT + Boronic Acid-EDTA + IPDD
Total	**100**	**77 (77.0)**	**23 (23.0)**	**77 (77.0)**	**All 6 methods applied**

DDST was performed on all 100 isolates. For Enterobacteriaceae, concordant DDST + CDT positivity was required for ESBL classification. For P. aeruginosa and A. baumannii, CDT alone served as the ESBL classification criterion (CLSI M100-2023; DDST synergy unreliable in non-fermenters). DDST, double disc synergy test; CDT, combination disc test (CLSI M100-2023, Table 3A); E-test, Epsilometer test for ESBL; MIC, minimum inhibitory concentration by agar dilution; Boronic Acid-EDTA, modified CLSI phenotypic confirmatory method using phenylboronic acid and EDTA (Tsakris et al., 2009; Poulou et al., 2014); IPDD, inhibitor potentiated disc diffusion test using clavulanate-supplemented Mueller-Hinton agar (Harwalkar et al., 2013). The imipenem-EDTA combined disc test was not employed; all supplementary ESBL confirmation used the Boronic Acid-EDTA method and IPDD exclusively.

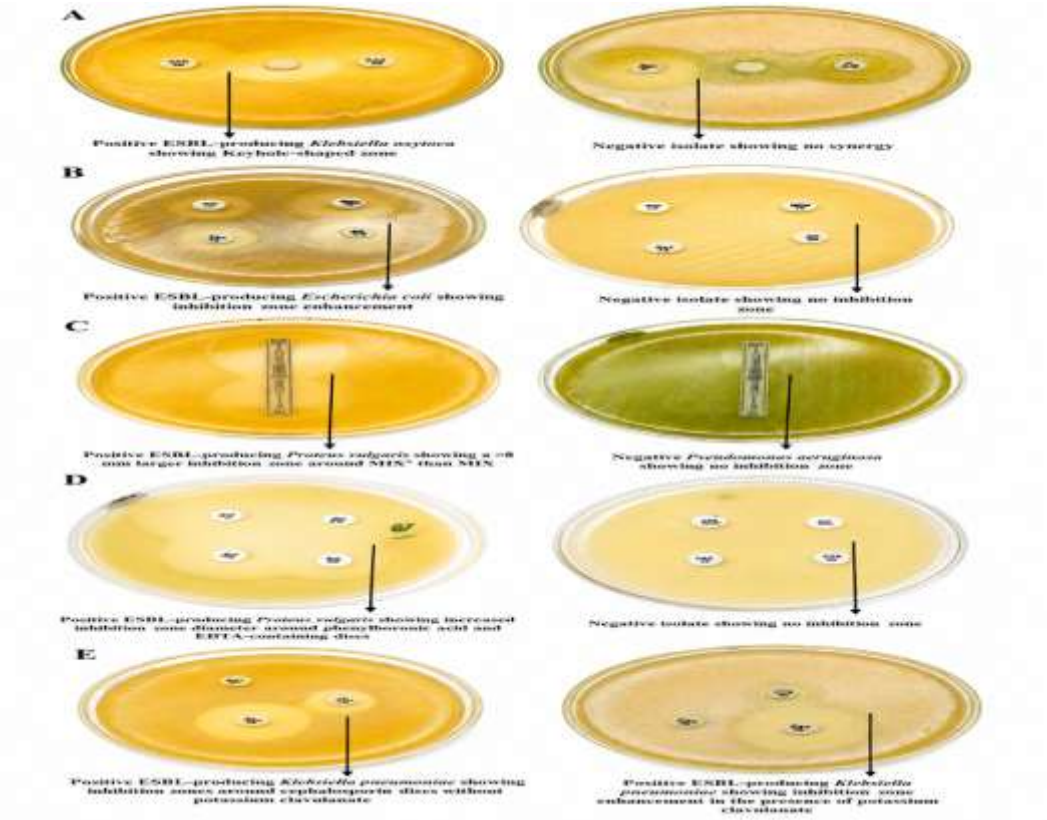

Figure 3. Phenotypic ESBL detection methods. (a) Double Disc Synergy Test (DDST) showing the characteristic keyhole-shaped zone of enhancement between a cephalosporin disc and the amoxicillin-clavulanate disc; (b) Combination Disc Test (CDT) showing paired cephalosporin ± clavulanate discs with a ≥ 5 mm zone increase per CLSI M100-2023, Table 3A; (c) E-test strip showing the cefotaxime-clavulanate (CT/CTL) MIC gradient with a

phantom zone; (d) Boronic Acid-EDTA method showing zone enhancement with phenylboronic acid and EDTA discs distinguishing AmpC/MBL co-production; (e) Inhibitor Potentiated Disc Diffusion Test (IPDD) showing paired plates with and without potassium clavulanate.

Biofilm formation

The MTP method detected biofilm formation in 86 of 100 isolates (86.0%): strong producers 44 (44.0%), moderate 28 (28.0%), and weak 14 (14.0%); 14 isolates (14.0%) were non-producers. All P. aeruginosa, A. baumannii, and K. aerogenes isolates were biofilm-positive (100% each). The tube method detected 80 biofilm-positive isolates, with substantial agreement against the MTP reference (κ = 0.72; 95% CI: 0.56-0.88; sensitivity 91.9%; specificity 92.9%). Congo Red agar detected 75 biofilm-positive isolates, achieving moderate agreement (κ = 0.47; 95% CI: 0.28-0.66; sensitivity 83.7%; specificity 78.6%). Species-wise biofilm grading and method comparison are shown in Tables 4 and 5, with representative images of all three methods in Figure 4.

Table 4. Biofilm formation grades by bacterial species — Microtiter plate reference method (n = 100)

Species	n	Strong n (%)	Moderate n (%)	Weak n (%)	Non-producer n (%)	Biofilm+ n (%)
E. coli	46	15 (32.6)	14 (30.4)	8 (17.4)	9 (19.6)	37 (80.4)
K. pneumoniae	28	14 (50.0)	8 (28.6)	3 (10.7)	3 (10.7)	25 (89.3)
K. oxytoca	8	4 (50.0)	2 (25.0)	1 (12.5)	1 (12.5)	7 (87.5)
P. aeruginosa	7	6 (85.7)	1 (14.3)	0 (0.0)	0 (0.0)	7 (100.0)
A. baumannii	5	4 (80.0)	1 (20.0)	0 (0.0)	0 (0.0)	5 (100.0)
P. vulgaris	4	1 (25.0)	1 (25.0)	1 (25.0)	1 (25.0)	3 (75.0)
K. aerogenes	2	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	2 (100.0)
Total	**100**	**44 (44.0)**	**28 (28.0)**	**14 (14.0)**	**14 (14.0)**	**86 (86.0)**

Classification per Stepanovic et al., 2000, 2007: Strong (OD > 4 × ODc), Moderate (2 × ODc < OD ≤ 4 × ODc), Weak (ODc < OD ≤ 2 × ODc), Non-producer (OD ≤ ODc). ODc: optical density cut-off = mean negative control OD + 3 SD. OD measured at 570 nm.

Table 5. Comparison of three biofilm detection methods against the Microtiter plate reference standard (n = 100)

Detection method	Biofilm+ n (%)	Sensitivity (%)	Specificity (%)	PPV (%)	Cohen's kappa (95% CI)
Microtiter plate (reference)	86 (86.0)	—	—	—	Reference standard
Tube method	80 (80.0)	91.9	92.9	98.8	κ = 0.72 [0.56-0.88] (substantial)
Congo Red agar	75 (75.0)	83.7	78.6	96.0	κ = 0.47 [0.28-0.66] (moderate)

K interpretation per Landis and Koch, 1977: 0.61-0.80 = substantial; 0.41-0.60 = moderate. PPV: positive predictive value.

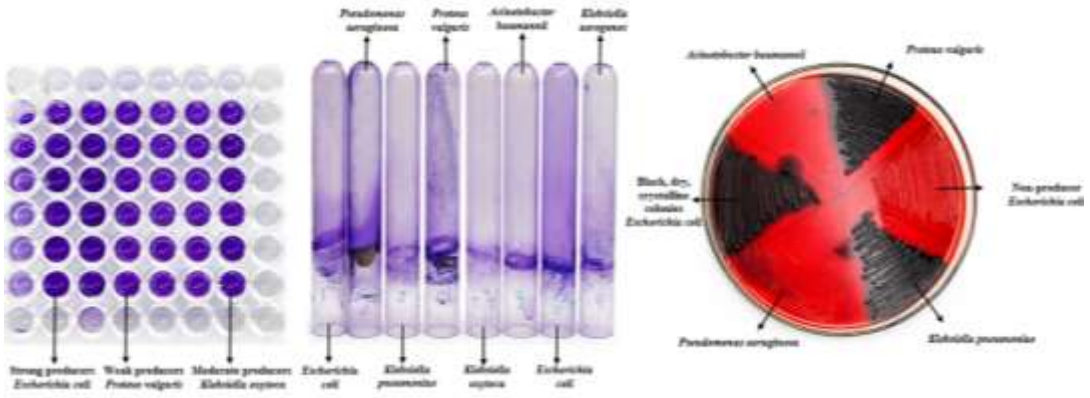

Figure 4. Phenotypic biofilm detection methods. (a) Microtiter plate (MTP) method showing crystal violet-stained wells in quadruplicate; deep purple wells indicate strong producers (E. coli), pale wells indicate weak producers (P. vulgaris), and intermediate wells indicate moderate producers (K. oxytoca), with optical density read at 570

nm. (b) Tube method (TM) showing adherent crystal violet-stained biofilm as dark sediment and wall deposits in the lower portion of the tube in positive producers (from left: E. coli, K. pneumoniae, K. oxytoca, E. coli, P. aeruginosa, A. baumannii, K. aerogenes), compared with faint or no staining in negative controls. (c) Congo Red Agar (CRA) method showing black, dry, crystalline colonies indicating slime production (E. coli, K. pneumoniae, P. aeruginosa, A. baumannii) compared with smooth red colonies in non-producers (E. coli & P. vulgaris).

Association between ESBL production and biofilm formation

Biofilm production was detected in 72 of 77 ESBL-positive isolates (93.5%) compared with 14 of 23 ESBL-negative isolates (60.9%; OR = 9.26; 95% CI: 2.81-30.50; p < .001). This finding represents the primary result of this study. The disparity was most pronounced for strong biofilm formation: 40 of 77 ESBL-positive isolates (51.9%) versus 4 of 23 ESBL-negative isolates (17.4%; p = .003). Mean OD570 was significantly higher among ESBL-positive isolates (1.18 ± 0.52) than ESBL-negative isolates (0.57 ± 0.40; p < .001). Full association data are presented in Table 6 and Figure 5.

Table 6. Association between ESBL production and biofilm formation — Microtiter plate method (n = 100)

Parameter	ESBL-positive (n=77) n (%)	ESBL-negative (n=23) n (%)	Total (n=100) n (%)	p-value / OR (95% CI)
Total biofilm producers	72 (93.5)	14 (60.9)	86 (86.0)	p < .001; OR = 9.26 (2.81-30.50)
Non-biofilm producers	5 (6.5)	9 (39.1)	14 (14.0)	—
Strong biofilm formers	40 (51.9)	4 (17.4)	44 (44.0)	p = .003
Moderate biofilm formers	22 (28.6)	6 (26.1)	28 (28.0)	p = .824
Weak biofilm formers	10 (13.0)	4 (17.4)	14 (14.0)	p = .602
Mean OD570 ± SD	1.18 ± 0.52	0.57 ± 0.40	0.98 ± 0.61	p < .001 (t-test)

Overall biofilm: Chi-square test. Strong/moderate/weak: Fisher's exact test. Mean OD570: independent samples t-test. Strong: OD > 4 × ODc; Moderate: 2 × ODc < OD ≤ 4 × ODc; Weak: ODc < OD ≤ 2 × ODc. OD: optical density at 570 nm; SD: standard deviation; OR: odds ratio; CI: confidence interval.

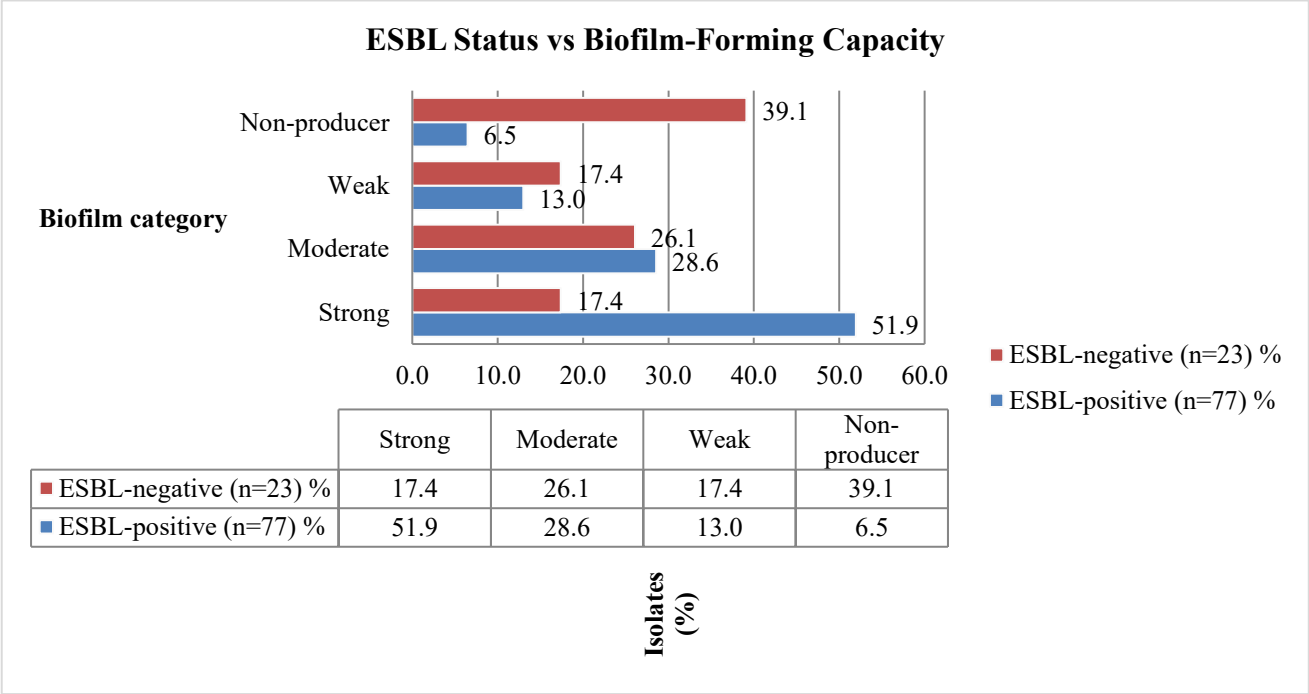

Figure 5. Association between ESBL production and biofilm-forming capacity. Grouped bar chart comparing the proportion of strong, moderate, weak, and non-biofilm producers among ESBL-positive (n = 77) and ESBL-negative (n = 23) isolates, corresponding to the data in Table 6.

Antibiotic susceptibility profile

Colistin, assessed exclusively by broth microdilution MIC in cation-adjusted Mueller-Hinton broth in untreated polystyrene plates per EUCAST guidelines, demonstrated universal susceptibility across all species (77/77; 100%) (EUCAST, 2024). Tigecycline was the second-most reliably active agent (73/77; 94.8%). Carbapenem susceptibility was preserved among Enterobacterales: meropenem and imipenem each 83.1% (64/77). A. baumannii showed meropenem/imipenem susceptibility of only 25% (1/4), consistent with carbapenem-resistant A. baumannii (CRAB) emergence. Amikacin substantially outperformed gentamicin (79.2% vs. 46.8%). Fluoroquinolones showed poor activity

(ciprofloxacin 26.0%; levofloxacin 29.9%). The low susceptibility observed for cefepime (15.6%) and piperacillin-tazobactam (32.5%) suggests limited utility of these agents for empirical therapy in our setting. Complete results are in Table 7.

Table 7. Antimicrobial susceptibility profile of 77 ESBL-producing Gram-negative clinical isolates (susceptible n/total and %)

Antimicrobial agent	***E. coli* (n=37)**	***K. pneumoniae* (n=22)**	***K. oxytoca* (n=6)**	***P. aeruginosa* (n=5)**	***A. baumannii* (n=4)**	***P. vulgaris* (n=2)**	***K. aerogenes* (n=1)**	**Total (n=77)***
Piperacillin-tazobactam	12/37 (32%)	7/22 (32%)	2/6 (33%)	1/5 (20%)	2/4 (50%)	1/2 (50%)	0/1 (0%)	25/77 (32.5%)
Cefepime	6/37 (16%)	3/22 (14%)	1/6 (17%)	2/5 (40%)	0/4 (0%)	0/2 (0%)	0/1 (0%)	12/77 (15.6%)
Ertapenem	33/37 (89%)	18/22 (82%)	5/6 (83%)	N/Aa	1/4 (25%)	2/2 (100%)	1/1 (100%)	60/72 (83.3%)
Meropenem	34/37 (92%)	19/22 (86%)	5/6 (83%)	2/5 (40%)	1/4 (25%)	2/2 (100%)	1/1 (100%)	64/77 (83.1%)
Imipenem	34/37 (92%)	19/22 (86%)	5/6 (83%)	2/5 (40%)	1/4 (25%)	2/2 (100%)	1/1 (100%)	64/77 (83.1%)
Amikacin	30/37 (81%)	17/22 (77%)	5/6 (83%)	3/5 (60%)	3/4 (75%)	2/2 (100%)	1/1 (100%)	61/77 (79.2%)
Gentamicin	18/37 (49%)	10/22 (45%)	3/6 (50%)	2/5 (40%)	1/4 (25%)	1/2 (50%)	1/1 (100%)	36/77 (46.8%)
Ciprofloxacin	9/37 (24%)	6/22 (27%)	2/6 (33%)	1/5 (20%)	1/4 (25%)	1/2 (50%)	0/1 (0%)	20/77 (26.0%)
Levofloxacin	10/37 (27%)	7/22 (32%)	2/6 (33%)	2/5 (40%)	1/4 (25%)	1/2 (50%)	0/1 (0%)	23/77 (29.9%)
Tigecycline (EUCAST v14.0)	36/37 (97%)	21/22 (95%)	6/6 (100%)	4/5 (80%)	3/4 (75%)	2/2 (100%)	1/1 (100%)	73/77 (94.8%)
Colistin (BMD, EUCAST)	37/37 (100%)	22/22 (100%)	6/6 (100%)	5/5 (100%)	4/4 (100%)	2/2 (100%)	1/1 (100%)	77/77 (100%)

Disc diffusion per CLSI M100-2023 (CLSI, 2023). Tigecycline: EUCAST v14.0 breakpoints. Colistin: broth microdilution MIC in cation-adjusted Mueller-Hinton broth in untreated polystyrene plates per EUCAST (EUCAST, 2024); disc diffusion not validated for polymyxins. * Total column denominator = 77 for all agents except ertapenem. a Ertapenem not tested for P. aeruginosa (intrinsic resistance); ertapenem denominator = 72.

MDR and XDR classification

MDR/XDR classification was applied to all 77 ESBL-confirmed isolates using the Magiorakos et al., 2012 criteria. MDR only was identified in 52 of 77 isolates (67.5%), and XDR in 10 of 77 isolates (13.0%); combined, 62 of 77 isolates (80.5%) met criteria for either MDR or XDR. The highest combined MDR/XDR rates were in A. baumannii (4/4; 100%), P. vulgaris (2/2; 100%), and K. aerogenes (1/1; 100%). No pandrug-resistant (PDR) isolates were identified. Seven isolates (9.1%) did not meet MDR criteria. Of the four A. baumannii isolates, two met MDR only criteria and two met XDR criteria. Data are in Table 8.

Table 8. MDR and XDR classification of 77 ESBL-producing Gram-negative clinical isolates by species (Magiorakos et al., 2012)

Classification	***E. coli* (n=37)**	***K. pneumoniae* (n=22)**	***K. oxytoca* (n=6)**	***P. aeruginosa* (n=5)**	***A. baumannii* (n=4)**	***P. vulgaris* (n=2)**	***K. aerogenes* (n=1)**	**Total n (%) (n=77)**
MDR only n (%)	25 (67.6)	15 (68.2)	4 (66.7)	3 (60.0)	2 (50.0)	2 (100.0)	1 (100.0)	52 (67.5)
XDR n (%)	3 (8.1)	3 (13.6)	1 (16.7)	1 (20.0)	2 (50.0)	0 (0.0)	0 (0.0)	10 (13.0)
MDR + XDR combined n (%)	28 (75.7)	18 (81.8)	5 (83.3)	4 (80.0)	4 (100.0)	2 (100.0)	1 (100.0)	62 (80.5)
Non-MDR n (%)	**6 (16.2)**	**1 (4.6)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**7 (9.1)**

MDR: non-susceptibility to ≥ 1 agent in ≥ 3 antimicrobial categories. XDR: non-susceptibility to ≥ 1 agent in all but ≤ 2 categories. Per Magiorakos et al., 2012. XDR isolates are not double-counted as MDR; the 'MDR only' row excludes all XDR isolates. No PDR isolates identified.

DISCUSSION

This study characterizes ESBL production, biofilm-forming capacity, and antimicrobial resistance phenotypes in Gram-negative clinical isolates from a Karnataka tertiary care center, applying a six-method phenotypic panel for ESBL and beta-lactamase characterization to all 100 isolates.

The overall ESBL prevalence of 77.0% is consistent with the upper range of Karnataka tertiary care data. Comparable rates of 67.4% and 67-80% have been reported from Chamarajanagar and north Karnataka tertiary hospitals (Wadekar et al., 2020; Metri et al., 2011). The ICMR AMRSN report documented ESBL E. coli rates of 76-81% from Indian tertiary blood cultures (NARS-Net, 2023), closely matching the 80.4% observed for E. coli in this cohort.

The strong ESBL-biofilm association observed here (OR = 9.26; 95% CI: 2.81-30.50; $p < .001$) is biologically plausible through two converging mechanisms. At the structural level, the extracellular polymeric substance matrix physically impedes beta-lactam diffusion, giving ESBL enzymes additional time to hydrolyze antibiotics before they can reach the bacterial cell wall (Laure and Ahn, 2022; Zubair and Mohammad, 2023). At the genetic level, ESBL-encoding IncF and IncI plasmids frequently co-carry biofilm-regulatory determinants including type I fimbriae and csgD regulon components, so that a single horizontal transfer event can confer both phenotypes simultaneously (Bae et al., 2008; Schembri et al., 2003). Structural investigations have further demonstrated that Class A and Class D beta-lactamases interact with low-molecular-weight penicillin-binding proteins, altering peptidoglycan remodeling and influencing cell-surface adhesins required for biofilm initiation (Gallant et al., 2005). At the clinical level, biofilm-dwelling Gram-negative bacteria display up to 1,000-fold greater antibiotic tolerance compared with planktonic counterparts, a phenomenon well-documented for ESBL- and carbapenemase-producing K. pneumoniae (Li et al., 2024). Our findings align closely with regional data: studies from Karnataka have similarly reported that over 50-72% of ESBL-producing Gram-negative isolates exhibit strong biofilm phenotypes (Zubair and Mohammad, 2023), and studies on Klebsiella pneumoniae clinical isolates have similarly reported higher biofilm-forming capacity among ESBL-positive strains compared with non-producers. Notably, some literature reports a more variable relationship for specific ESBL enzyme classes depending on specimen type and region (Dumaru et al., 2019); the magnitude of the association in our cohort (OR = 9.26) is consistent with this pattern and contributes additional quantitative data to the Karnataka and South Indian surveillance record.

The antibiotic susceptibility findings have direct clinical implications for empiric prescribing. Colistin susceptibility was confirmed exclusively by broth microdilution in cation-adjusted Mueller-Hinton broth per EUCAST recommendations, yielding 100% susceptibility across all species (EUCAST, 2024). High tigecycline activity (94.8%) positions these two agents as reliable last-resort options for MDR ESBL infections (Agarwal et al., 2022; NARS-Net, 2023). Carbapenem susceptibility, while preserved for E. coli (92%) and K. pneumoniae (86%), was only 25% for A. baumannii, paralleling nationally documented CRAB emergence (NAMS Task Force, 2025). The low susceptibility observed for cefepime (15.6%) and piperacillin-tazobactam (32.5%) suggests limited utility of these agents for empirical therapy in our setting, consistent with CLSI and EUCAST guidance (CLSI, 2023; EUCAST, 2024). An MDR prevalence of 80.5% and XDR rate of 13.0% indicate a substantial resistance burden that warrants institutional antimicrobial stewardship intervention.

Limitations of this study include the single-center design, the 100-isolate sample size, and the exclusive reliance on phenotypic ESBL confirmation without molecular genotyping of bla gene variants (blaCTX-M, blaTEM, blaSHV) — an important gap that should be addressed in future investigations. The absence of molecular data represents an inherent constraint on mechanistic conclusions. Plasmid replicon typing and clinical outcome correlation are planned in a companion molecular study from this cohort.

CONCLUSION

ESBL production and biofilm formation co-occur at high frequency in Gram-negative clinical isolates from this Karnataka tertiary care center, with ESBL-positive isolates being nine times more likely to form biofilm than ESBL-negative counterparts. A six-method phenotypic panel characterized both ESBL production and co-existing resistance mechanisms in all isolates. MDR was identified in 80.5% and XDR in 13.0% of ESBL-confirmed isolates. Colistin and tigecycline retained near-universal activity, while cefepime, piperacillin-tazobactam, and fluoroquinolones showed limited utility for empirical therapy in this setting. Consequently, tracking concurrent ESBL and biofilm profiles is crucial for effective antibiotic stewardship in Karnataka, and these findings support integrating routine ESBL detection, quantitative biofilm assessment, comprehensive antibiotic susceptibility testing, and standardized MDR/XDR classification into tertiary care microbiology practice across India.

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