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Molecular and Phenotypic Characterization of ESBL- and Carbapenemase-Producing Gram-Negative Bacteria in Clinical Isolates from Peshawar, Pakistan

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ABSTRACT

Introduction: The rising prevalence of extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria and carbapenem-resistant *Enterobacteriaceae* (CRE) poses significant threats to public health. **Methods:** This study investigated the prevalence and antibiotic resistance patterns of ESBL-producing Gram-negative bacteria isolated from various clinical samples in Peshawar, Pakistan. A total of 400 clinical samples were collected, and isolates were identified using biochemical methods. Antibiotic susceptibility testing was performed following CLSI 2023 guidelines. Phenotypic detection of ESBL and carbapenemase production was conducted using the Double-Disk Synergy Test and Modified Hodge Test, respectively. PCR was used to detect β-lactamase genes (*bla*CTX-M, *bla*TEM, *bla*SHV, and *bla*NDM). **Results:** Out of 400 samples, 150 (37.5%) were ESBL-positive, including 50 (33.3%) from urine, 50 (33.3%) from indwelling devices, 25 (16.7%) from pus, and 25 (16.7%) from blood. *Escherichia coli* was the most prevalent species (57.3%), followed by *Klebsiella pneumoniae* (34.0%). Antibiotic susceptibility testing revealed that all ESBL-producing isolates (n=150) were resistant to ampicillin and ceftazidime. High resistance rates were also observed for cefoperazone, though with some species-specific variation. PCR assays characterized β-lactamase genes, with *bla*CTXM detected in 95.3% of ESBL-producing isolates, followed by *bla*TEM (80.7%) and *bla*SHV (70.7%). The Modified Hodge Test showed that 14.7% (22/150) of ESBL isolates were carbapenem-resistant, of which a concerning 86.4% (19/22) carried the *bla*NDM gene. Statistical analysis revealed a significant association between sample type and ESBL positivity (χ^2=16.67, P = 0.0008), with the highest prevalence in urine samples. However, no significant difference in carbapenem resistance was found between *E. coli* and *K. pneumoniae* (χ^2=0.0, P = 1.000). The presence of the *bla*NDM gene was very strongly associated with carbapenem resistance (χ^2=101.42, P < 0.0001; Cramér's V=0.82). **Conclusion:** The high prevalence of ESBL-producing isolates, predominantly carrying the *bla*CTX-M gene, and the emergence of *bla*NDM-mediated carbapenem resistance in Peshawar highlight an urgent need for enhanced surveillance and targeted antimicrobial stewardship to guide effective treatment strategies and public health interventions.

INTRODUCTION

Antibiotics play a crucial role in combating bacterial infections. However, the inappropriate use of these drugs has led to the selection and spread of resistant strains among pathogenic bacteria, resulting in antibiotic resistance. This resistance negatively impacts patient health, leading to prolonged hospitalization, increased mortality rates, and financial burdens. Infections caused by multidrug-resistant (MDR) bacteria pose greater treatment challenges compared to those caused by less resistant or non-resistant strains. Furthermore, antimicrobial resistance (AMR) contributes to economic

hardship by increasing healthcare costs, extending hospital stays, and reducing productivity, disproportionately affecting low-income populations [1]. This threat is increasing globally to dangerously high levels, prompting the World Health Organization (WHO) to identify AMR as one of the top ten global public health threats [2]. A growing number of infections, including tuberculosis, pneumonia, sepsis, gonorrhea, and foodborne illnesses, are becoming increasingly difficult or even impossible to treat due to the diminishing effectiveness of antibiotics [3].

Globally, antibiotic prescription and usage practices need to be modified urgently. Even with the development of new antibiotics, AMR will continue to pose a significant threat unless there are substantial changes in prescribing practices, infection control, and antibiotic stewardship policies [4]. Although AMR is a concern globally, low- and middle-income countries, including Pakistan, bear a disproportionate burden due to unregulated antibiotic use, inadequate infection control, and limited surveillance systems. Recent studies from Peshawar have documented alarming rates of antimicrobial resistance among clinical isolates, including high ESBL prevalence and emerging carbapenem resistance [5]. Carbapenems, a subclass of β-lactam antibiotics, are critical last-line agents for treating severe infections caused by MDR Gram-negative bacteria, particularly those producing extended-spectrum β-lactamases (ESBLs). Their bactericidal activity results from the inhibition of bacterial cell wall synthesis. However, the global emergence of carbapenem-resistant Enterobacteriaceae (CRE) has significantly undermined their clinical utility. CRE infections pose a major public health challenge due to limited treatment alternatives and associated high mortality rates [6]. ESBLs are plasmid-mediated enzymes that are mostly inhibited by clavulanate. ESBL-producing *Enterobacteriaceae* have been responsible for numerous worldwide outbreaks. Because ESBL-encoding plasmids often carry additional resistance determinants, ESBL-producing organisms often exhibit multidrug resistance, complicating the selection of effective antimicrobial therapy. The prevalence of ESBLs is increasing in *E. coli* and *K. pneumoniae* [7]. Currently, carbapenems are considered the most effective antibiotic class against these pathogens. However, due to growing resistance, alternative treatment strategies are increasingly necessary [8].

The rise of carbapenem resistance presents a significant challenge for clinicians, as carbapenems are often the last effective treatment against MDR Gram-negative pathogens [9]. Globally, the occurrence of MDR pathogens has risen, with South Asian countries, including Pakistan, being particularly affected due to factors such as unregulated antibiotic sales, high population density, and limited healthcare infrastructure. Antimicrobial-resistant organisms, such as MDR *Acinetobacter baumannii* and *E. coli*, exhibit high rates of ceftazidime resistance and are frequently associated with septicemia [10]. Carbapenem resistance was first reported in *Enterobacteriaceae* isolates in the early 1990s. Since then, CRE has become widespread, with microbiology laboratories playing a crucial role in its management. Accurate laboratory detection of carbapenemase-producing organisms is therefore critical for guiding appropriate therapy and implementing effective infection control measures [11].

Carbapenems are considered the last line of defense against MDR bacterial infections [12]. Carbapenemases are enzymes produced by some Gram-negative bacteria that inactivate these antibiotics. *Enterobacteriaceae*, which include both commensal flora and major human pathogens, are increasingly acquiring carbapenemase genes, posing significant therapeutic challenges [13]. Colistin, a polymyxin antibiotic, has re-emerged as a last-resort treatment for MDR Gram-negative pathogens, including CRE, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. [14]. However, colistin resistance is also emerging, further complicating treatment options. Colistin is often administered in combination with other antimicrobials to enhance efficacy and reduce the emergence of resistance [15]. Given the significant burden of AMR in Pakistan, this study aimed to characterize the molecular and phenotypic profiles of ESBL- and carbapenemase-producing Gram-negative bacteria in clinical isolates from Peshawar to inform regional surveillance and treatment guidelines.

MATERIAL AND METHODS

Ethical statement. The study protocol was reviewed and approved by the Institutional Ethics Review Board of the University of the Punjab, Lahore, Pakistan (Approval No. D/119/FIMS). All procedures involving human-derived samples were performed in accordance with the ethical principles of the Declaration of Helsinki and its later amendments.

Sample collection. A total of 400 clinical samples were collected aseptically using sterile swabs, containers, and syringes. Samples were transported immediately to the microbiology laboratory for processing. The sample set comprised 100 specimens from indwelling medical devices, 100 urine specimens, 100 pus specimens, and 100 blood specimens.

Study setting and patient selection. This study was conducted at a tertiary care hospital in Peshawar, Pakistan, and included a total of 400 clinical samples obtained from patients with clinically suspected infections. Patient inclusion was based on clinical criteria assessed by the attending physician. These criteria included systemic signs of infection (fever ≥ 38°C, leukocytosis ≥ 12,000 cells/μL, or leukopenia ≤ 4,000 cells/μL) and/or site-specific findings. Site-specific criteria included dysuria for urine specimens, purulent discharge for pus or surgical site infections, device-site for

inflammation indwelling device specimens, and indicators of sepsis for blood specimens.

Inclusion and exclusion criteria. Inclusion criteria were adult patients (≥18 years) with clinically suspected bacterial infection. All collected clinical samples underwent initial culturing according to standard microbiological procedures. For inclusion in downstream phenotypic and molecular analysis, the primary criterion was the successful isolation of one or more Gram-negative bacterial species. Samples yielding no growth or exclusively Gram-positive growth were excluded from further phenotypic and molecular characterization. This focused approach was designed to specifically investigate the prevalence of ESBL and carbapenemase production among Gram-negative isolates as per the study's objective.

Isolation and identification. Pus swabs, specimens from indwelling devices, and positive blood cultures were inoculated onto blood agar and MacConkey agar. For urine samples, a calibrated loop was used to inoculate cystine-lactose-electrolyte-deficient (CLED) agar. Following incubation at 37°C for 18–24 hours under aerobic conditions, colonies were characterized based on morphology. Isolates identified as Gram-negative rods via Gram staining were subjected to the oxidase test. Species-level identification was performed using the API 20E system (bioMérieux) according to the manufacturer's instructions. Identification was determined by interpreting the numerical profile generated from metabolic reactions against the manufacturer's database. The API 10S system was used for rapid preliminary screening where appropriate.

Biochemical characterization of isolates. Following primary isolation, Gram-negative bacilli were subjected to biochemical testing for species-level identification. The oxidase test was performed to differentiate *Enterobacteriaceae* (oxidase-negative) from non-fermenters. Species identification was performed using the API 20E system (bioMérieux) according to the manufacturer's instructions. The API 10S system was used for rapid preliminary screening where appropriate [16].

Antibiotic susceptibility testing. Antibiotic susceptibility testing (AST) was performed using the Kirby-Bauer disc diffusion method, following the Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. A bacterial suspension was prepared from fresh colonies and adjusted to match a 0.5 McFarland turbidity standard (equivalent to approximately 1.5×10^8 CFU/mL). This inoculum was then evenly swabbed onto Mueller-Hinton agar (MHA) plates (HiMedia). Antibiotic disks (Oxoid, UK) were dispensed onto the agar surface. The panel included: ampicillin (10 µg), amoxicillin-clavulanic acid (20/10 µg), piperacillin-tazobactam (100/10 µg), cefoperazone (75 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), meropenem (10 µg), imipenem (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), nitrofurantoin (300 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), cefoperazone-sulbactam (75/30 µg), and colistin (10 µg). The MHA plates were incubated aerobically at 37°C for 18-24 hours. Following incubation, zones of inhibition were measured, and the isolates were categorized as susceptible, intermediate, or resistant based on CLSI (2023) breakpoint criteria.

Quality control measures. To ensure the reliability and reproducibility of results, quality control (QC) measures were implemented for both AST and PCR analysis. For AST, standard reference strains, including *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853, were tested alongside clinical isolates in accordance with CLSI (2023) guidelines to validate the performance of media and antibiotic discs. All AST assays were performed in duplicate. For PCR assays, each run included previously characterized positive control isolates harboring *bla*TEM, *bla*SHV, *bla*CTX-M, *and bla*NDM to validate primer and reagent efficacy. A no-template control (NTC) was also included in each run to monitor for contamination. Each PCR assay was performed in duplicate, and any discordant results were resolved by repeat testing.

Phenotypic detection of ESBL and carbapenemase production. All Gram-negative isolates were phenotypically tested for carbapenemase production using the Modified Hodge Test (MHT) and for ESBL production using the Double-Disk Synergy Test (DDST).

For the MHT, a 1:10 dilution of a 0.5 McFarland standard suspension of the indicator organism (*E. coli* ATCC 25922) was used to inoculate an MHA plate. A 10 µg meropenem disk was placed in the center. Test isolates, a positive control (*K. pneumoniae* ATCC BAA-1705), and a negative control (*K. pneumoniae* ATCC BAA-1706) were streaked in straight lines from the edge of the disk to the periphery of the plate. Following incubation at 37°C for 16–20 hours, carbapenemase production was indicated by the presence of a cloverleaf-shaped indentation of the indicator lawn along the growth streak of the test organism.

For the DDST, an MHA plate was inoculated as for standard disk diffusion. An amoxicillin-clavulanic acid disk (20/10 µg) was placed at the center of the plate. Disks of ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), and cefepime (30 µg) were then placed 15–20 mm (center to center) from the central disk. After overnight incubation at 37°C, a positive result was identified by a keyhole-shaped enhancement of the inhibition zone of any cephalosporin disk towards the amoxicillin-clavulanic acid disk, confirming phenotypic ESBL production [17, 18].

Molecular detection of resistance genes. Genes encoding ESBLs (*bla*TEM, *bla*SHV, and *bla*CTX-M) and *carbapenemases (bla*NDM) were amplified from all phenotypically resistant isolates using conventional PCR

amplification and gel electrophoresis. Each 25 µL PCR reaction mixture contained: 11.5 µL of nuclease-free water, 2.5 µL of 10x PCR buffer, 2.5 µL of $MgCl_2$ (25 mM), 2.0 µL of dNTPs (2.5 mM), 1.0 µL of each forward and reverse primer (10 µM stock, for a final concentration of 0.4 µM; Table 1), 0.5 µL of *Taq* DNA polymerase, and 5.0 µL of template DNA. Thermal cycling conditions were as follows: an initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. The annealing temperature was adjusted for each gene target: 60°C for *bla*CTX-M, 56°C for *bla*SHV, 55°C for *bla*TEM, and 52°C for *bla*_NDM. Amplified products (5 µL aliquots) were resolved by electrophoresis on a 0.8% (w/v) agarose gel alongside a 1 kb DNA ladder (Thermo Fisher Scientific). The presence of bands of the expected size confirmed gene amplification [19, 20].

Statistical analysis. The Chi-square test was performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA) to compare antibiotic resistance proportions across different bacterial species and clinical sample types.

Table 1. Primer sequences used for the amplification of β-lactamase genes [19, 20].

Gene Target	Primer name	Sequence (5'→3')	Amplicon size (bp)
*bla*CTX-M	CTX-M-F	ATGTGCAGYACCAGTAARGTGATGGC	593
	CTX-M-R	TGGGTRAARTARGTSACCAGAAYCAGCGG	
*bla*SHV	SHV-F	CTTTATCGGCCCTCACTCAA	237
	SHV-R	AGGTGCTCATCATGGGAAAG	
*bla*TEM	TEM-F	CGCCGCATACACTATTCTCAGAATGA	455
	TEM-R	ACGCTCACCGGCTCCAGATTTAT	
*bla*NDM	NDM-F	AACACAGCCTGACTTTCG	699
	NDM-R	GCTCATCACGATCATGCT	

RESULTS

Overall prevalence and key associations. Out of 400 clinical samples, 150 (37.5%) yielded ESBL-producing organisms. Among the 150 ESBL-positive isolates, the distribution by sample type was: urine (n=50, 33.3% of all ESBL-positives; 50% prevalence within urine samples) and indwelling device samples (n=50, 33.3%; 50% prevalence), followed by pus (n=25, 16.7%; 25% prevalence) and blood (n=25, 16.7%; 25% prevalence). A Chi-square test confirmed a statistically significant association between sample type and ESBL positivity ($\chi^2 = 16.67$, $P = 0.0008$). In contrast, no significant difference in carbapenem resistance rates was observed between *E. coli* and *K. pneumoniae* ($\chi^2 = 0.0$, $P = 1.000$). A very strong association was found between the presence of the *bla*NDM gene and phenotypic carbapenem resistance ($\chi^2 = 101.42$, $P < 0.001$). Interestingly, an odds ratio (OR) analysis revealed that ESBL-producing isolates in this cohort were significantly less likely to be carbapenem-resistant compared to non-ESBL isolates (OR = 0.10; 95% CI: 0.05–0.21).

Demographic characteristics of patients with ESBL-producing isolates. Of the 150 patients with ESBL-positive isolates, 82 (54.7%) were male and 68 (45.3%) were female. The age distribution of these patients was as follows: 18–39 years (n=30, 20.0%), 40–59 years (n=65, 43.3%), and ≥ 60 years (n=55, 36.7%), with the highest prevalence observed in the middle-aged group.

Distribution of bacterial species among ESBL-positive isolates. A total of six different bacterial species were identified among the 150 ESBL-positive isolates using API biochemical testing (Table 2; Figure 1). *E. coli* was the most prevalent species, accounting for 86 isolates (57.3%; 95% CI: 49.2–65.1), followed by *K. pneumoniae* with 51 isolates (34.0%; 95% CI: 26.4–41.9). Other species identified included *Enterobacter cloacae* (n=6, 4.0%; 95% CI: 1.5–8.5), *Proteus mirabilis* (n=4, 2.7%; 95% CI: 0.7–6.6), *Serratia marcescens* (n=2, 1.3%; 95% CI: 0.2–4.6), and *Citrobacter freundii* (n=1, 0.7%; 95% CI: 0.02–4.3).

Phenotypic resistance profile summary. All 150 isolates included in the study cohort were confirmed as ESBL-producers by the Double-Disk Synergy Test (DDST; Figure 2). Subsequent screening for carbapenemase production revealed that 22 of these 150 ESBL-positive isolates (14.7%) were positive by the Modified Hodge Test (MHT; Figure 3).

Fig. 1. Representative examples of biochemical identification of *Enterobacteriaceae* isolates using API systems. (A) An API 20E strip showing the biochemical profile for *E. coli*. The resulting numerical profile corresponded to an identification confidence of >99% according to the manufacturer's database. (B) An API 10S strip showing the characteristic biochemical profile for *K. pneumoniae*, allowing for rapid presumptive identification.

Table 2. Summary of key phenotypic and biochemical characteristics of the identified ESBL-producing isolates.

Species identified	Culture characteristics on MacConkey/blood agar	Gram stain morphology	Representative API 10S codes
E. coli	• **LF:** Dry, flat, pink colonies • May exhibit spreading growth	Gram-negative bacilli	7105, 6105, 6305
K. pneumoniae	• **LF:** Large, mucoid, dark pink colonies • Positive string test	Gram-negative bacilli	7504, 7524, 7124
E. cloacae	• **LF:** Large, mucoid, light pink colonies	Gram-negative bacilli	7604
S. marcescens	• **NLF:** Convex, mucoid, non-pigmented colonies •Some strains may produce red pigment (prodigiosin)	Gram-negative bacilli	3720, 3724
P. mirabilis	• **NLF:** Pale/colorless colonies • Exhibits swarming motility on blood agar • Characteristic odor	Gram-negative bacilli	2634, 2664
C. freundii	• **LF:** Small, moist, convex, pink/shiny colonies	Gram-negative bacilli	7410

LF = Lactose-fermenting; NLF = Non-lactose-fermenting.

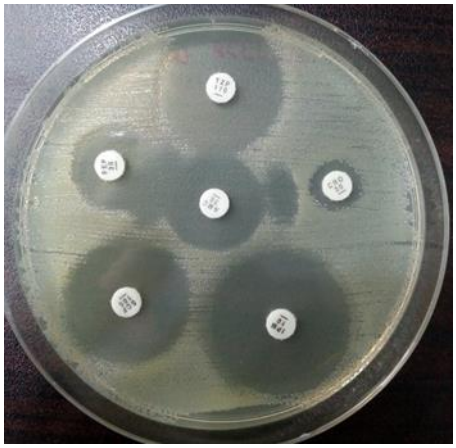


Fig. 2. Phenotypic detection of ESBL production using the DDST. The image displays the characteristic synergistic enhancement (keyhole effect), where the zone of inhibition around the cephalosporin disk extends towards the central amoxicillin-clavulanic acid disk, confirming ESBL production.

Prevalence and distribution of resistance genes. Molecular analysis of the 150 ESBL-producing isolates revealed a high prevalence and co-occurrence of β-lactamase genes. The most widespread ESBL genotype was *bla*CTX-M, detected in 143 isolates (95.3%), followed *by bla*TEM in 121 isolates (80.7%) and *bla*SHV *in* 106 isolates (70.7%). Among the 22 phenotypically carbapenem-resistant isolates, a concerning 19 (86.4%) were positive for the carbapenemase gene *bla*NDM (Figure 4).

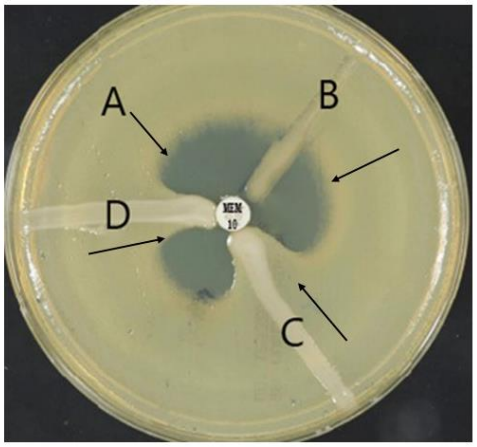


Fig. 3. Modified Hodge Test (MHT) for the detection of carbapenemase production. The plate shows the indicator lawn of *E. coli* ATCC 25922 with streaks of: (A) the negative control strain (*K. pneumoniae* ATCC BAA-1706), (B) the positive control strain (*K. pneumoniae* ATCC BAA-1705), and (C) a representative positive clinical isolate.

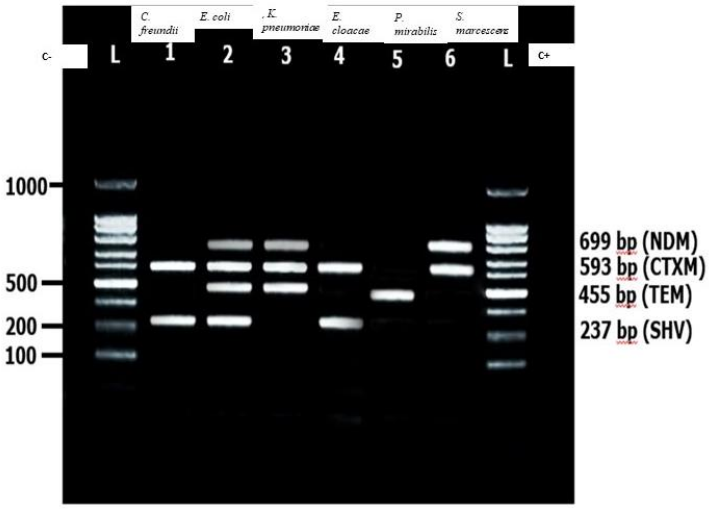


Fig. 4. Representative agarose gel electrophoresis of PCR products for the detection of β-lactamase genes in clinical isolates. Lane L: 100 bp DNA ladder. Lanes 1–6 contain amplicons from the following representative isolates: (1) *C. freundii* (*bla*CTX-M, *bla*SHV); (2) *E. coli* (*bla*NDM, *bla*CTX-M, *bla*TEM, *bla*SHV); (3) *K. pneumoniae* (*bla*NDM, *bla*CTX-M, *bla*TEM, *bla*SHV); (4) *E. cloacae* (*bla*CTX-M, *bla*SHV); (5) *P. mirabilis* (*bla*TEM); and (6) *S. marcescens* (*bla*NDM, *bla*TEM). **Lane C-**: Negative control (nuclease-free water). **Lane C+**: Positive control. The expected amplicon sizes are indicated on the right: *bla*NDM (699 bp), *bla*CTX-M (593 bp), *bla*TEM (455 bp), and *bla*SHV (237 bp).

Antimicrobial susceptibility profiles of clinical isolates. Antimicrobial susceptibility testing revealed high rates of resistance among the 150 ESBL-producing isolates with detailed species-specific and overall patterns presented in Figure 5. Colistin demonstrated excellent activity, with 100% susceptibility among *K. pneumoniae*, *E. cloacae*, *C. freundii*, and *P. mirabilis* isolates, and 98.8% (85/86) susceptibility among *E. coli* isolates. As expected, both *S. marcescens* isolates exhibited intrinsic resistance to colistin. Meropenem also retained high activity against most isolates; however, 22/150 (14.7%) of all ESBL-producers were phenotypically resistant to carbapenems. This resistance was strongly correlated with the presence of the *bla*NDM gene, which was detected in 19/22 (86.4%) of these resistant isolates.

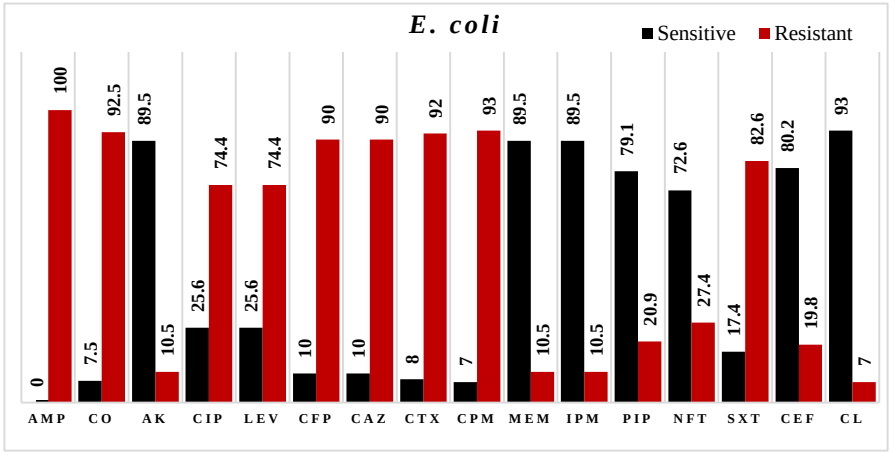
Conversely, widespread resistance was observed for several antibiotic classes. All ESBL-producing isolates were, by definition of the screening criteria, resistant to third-generation cephalosporins like ceftazidime and ceftriaxone. Ampicillin was the least effective antibiotic, with near-total resistance across all species. For the most prevalent species, *E. coli*, high resistance rates were observed for ampicillin (92.4%), cefoperazone (78.6%), and ceftriaxone (74.1%).

Distinct resistance patterns were observed for the most prevalent species. For the 51 *K. pneumoniae* isolates,

complete resistance (100%) was noted for ampicillin. Resistance rates against other key antibiotics were 27.5% for amikacin and 8.4% for meropenem. Similarly, for the 6 *E. cloacae* isolates, 100% resistance was observed against ampicillin, ciprofloxacin, and levofloxacin, with lower resistance to meropenem (6.7%) but higher resistance to amikacin (50%). Among the 4 *P. mirabilis* isolates, all were resistant to ampicillin and cefoperazone (100%), and 75% were resistant to trimethoprim-sulfamethoxazole.

For the species represented by very few isolates, specific susceptibility patterns were noted. The two *S. marcescens* isolates tested were susceptible to meropenem, imipenem, and amikacin. Both isolates were resistant to ampicillin (100%), as well as to amoxicillin-clavulanic acid and ceftriaxone. The single isolate of *C. freundii* was resistant to all tested antibiotics except colistin, to which it was susceptible.

Overall susceptibility rates across all 150 ESBL-producing isolates highlighted significant resistance. Colistin was the most active agent, with an overall susceptibility rate of 98.7%. This was followed by meropenem (85.3%), amikacin (80.7%), cefoperazone-sulbactam (74.0%), and piperacillin-tazobactam (72.7%). Conversely, near-complete resistance was observed against several β-lactams, with overall susceptibility rates of 0% for ampicillin, cefoperazone, and ceftazidime.



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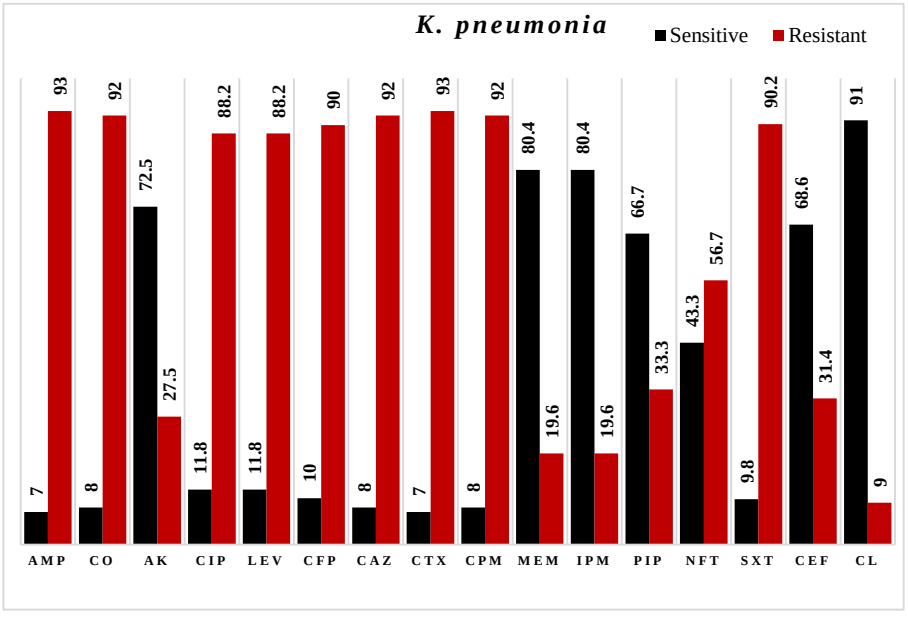

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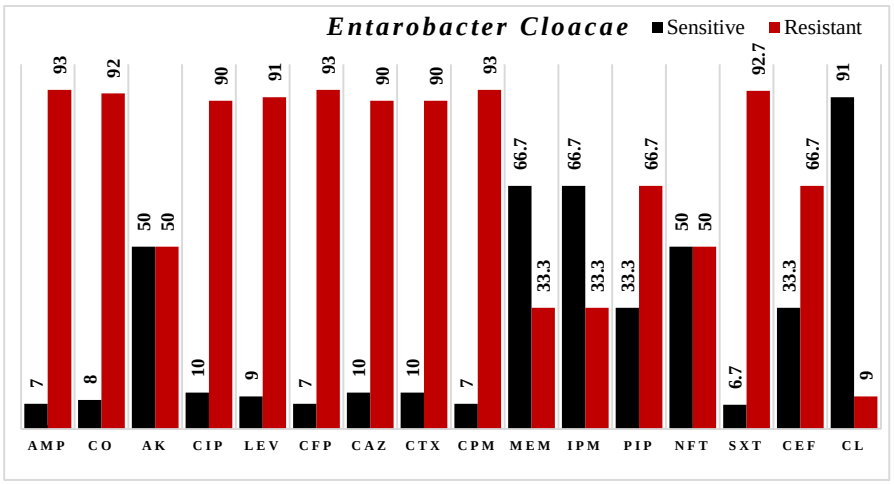

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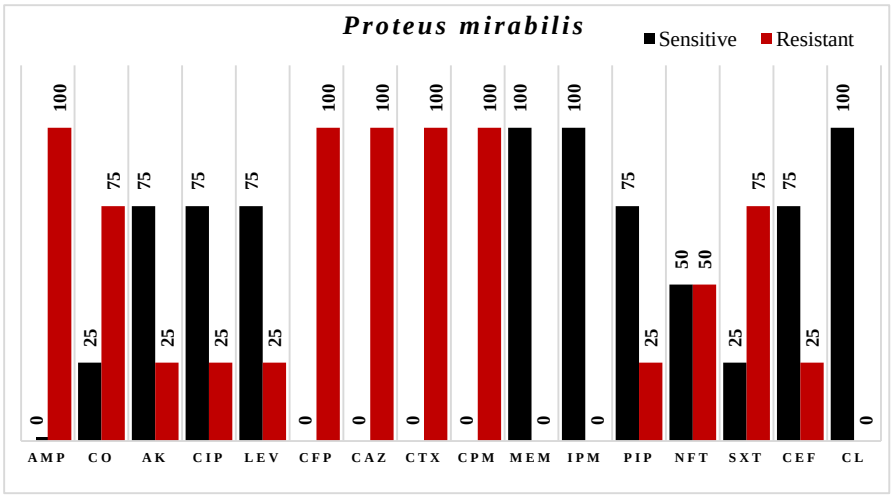

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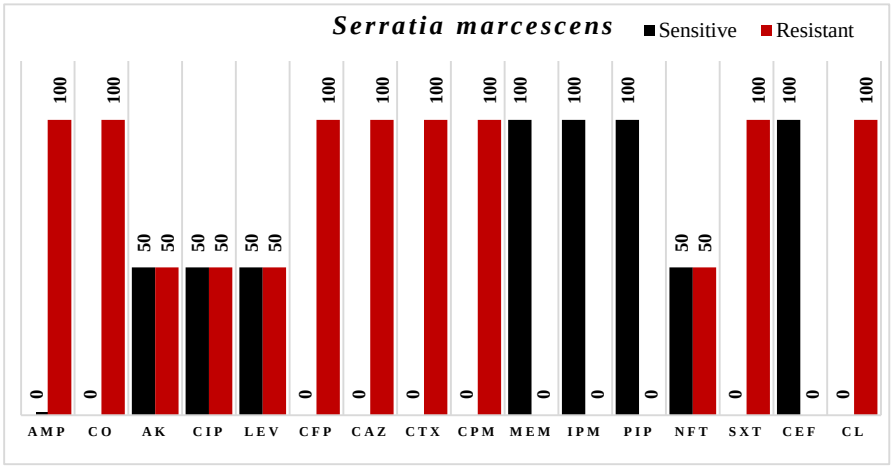

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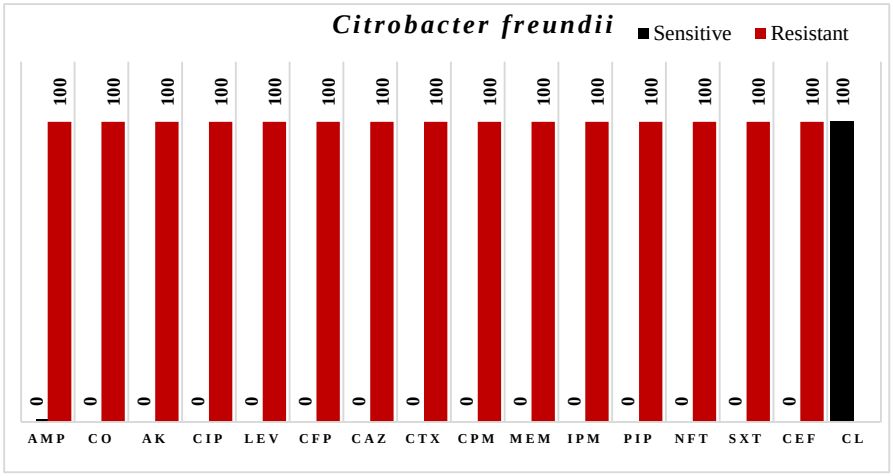

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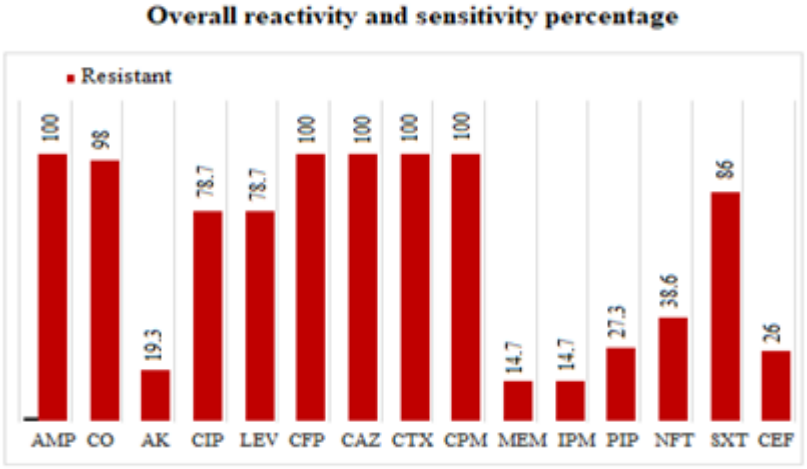

(G)

Fig. 5. Antimicrobial susceptibility patterns of ESBL-producing isolates. The graphs show the percentage of isolates (vertical axis) that were sensitive (black bars) or resistant (red bars) to a panel of antimicrobial agents (horizontal axis). Panels display data for: (A) *E. coli* (n=86), (B) *K. pneumoniae* (n=51), (C) *E. cloacae* (n=6), (D) *P. mirabilis* (n=4), (E) *S. marcescens* (n=2), (F) *C. freundii* (n=1), and (G) the overall susceptibility percentages for all 150 isolates combined. **Abbreviations:** AMP, ampicillin; CO, ceftriaxone; AK, amikacin; CIP, ciprofloxacin; LEV, levofloxacin; CFP, cefoperazone; CAZ, ceftazidime; CTX, cefotaxime; CPM, cefepime; MEM, meropenem; IPM, imipenem; PIP, piperacillin-tazobactam; NFT, nitrofurantoin; SXT, trimethoprim-sulfamethoxazole; CEF, cefoperazone-sulbactam; CL, colistin

DISCUSSION

The high prevalence (37.5%) of ESBL-producing bacteria found in this study underscores their significant and ongoing threat to public health in Peshawar. Infections caused by these MDR organisms are known to compromise patient outcomes, as they are often difficult to treat and are associated with prolonged hospitalizations and higher mortality rates [3, 21].

Our finding of a 37.5% prevalence rate for ESBL-producing organisms, predominantly *E. coli* (57.3%) and *K. pneumoniae* (34.0%), in a Peshawar tertiary care hospital aligns with the growing body of evidence identifying these pathogens as primary drivers of AMR in the region. Descriptive data indicated a slightly higher prevalence among male patients (54.7%) and those aged 40–59 years. Although these demographic trends did not reach statistical significance, the higher prevalence in older patients likely reflects immune senescence and increased healthcare exposure. Critically, the high proportion of ESBL-positive isolates from hospitalized patients strongly suggests a significant burden of nosocomial transmission, highlighting the need for reinforced infection prevention and control measures. The wide distribution of these isolates across diverse clinical samples—including urine, pus, blood, and indwelling devices—further underscores the broad clinical impact and varied manifestations of these challenging infections.

The predominance of *E. coli* and *K. pneumoniae* among our ESBL-producing isolates is consistent with global epidemiological data, which identifies these two species as the primary reservoirs and vectors of ESBL-mediated resistance worldwide [21]. The identification of other opportunistic pathogens, such as *E. cloacae* and *P. mirabilis*, though at lower frequencies, highlights the diverse range of species contributing to the ESBL burden in this clinical setting [22].

Our finding that *E. coli* and *K. pneumoniae* were the most prevalent ESBL-producing bacteria is consistent with previous reports that have identified these species as the major etiologic agents of ESBL-associated infections [23].

The high prevalence of ESBL-producing bacteria observed in our hospital (37.5%) contributes to the body of evidence showing their alarming proliferation in both healthcare and community settings globally [24, 25]. This escalating trend underscores the urgent need for robust infection prevention and control (IPC) measures and comprehensive antibiotic stewardship programs to mitigate the further spread of these multidrug-resistant pathogens.

The 14.7% carbapenem resistance rate observed in our study is comparable to a previous report from Khyber Pakhtunkhwa documenting 15% resistance, suggesting a consistent and alarming regional trend [5]. While this rate is lower than those reported in specialized patient populations—for example, 52.2% carbapenem resistance among renal patients in Pakistan [26]—the difference likely reflects variations in patient populations and sample sources. Discrepancies in clinical outcomes and resistance patterns across different regions can often be attributed to differing local antibiotic prescribing practices and stewardship policies [27].

Furthermore, the 37.5% ESBL prevalence in our hospitalized patient cohort is substantially higher than rates reported from community surveillance in Europe, where the intestinal carriage of ESBL-producing organisms is estimated to be between 6–8% [19]. For example, environmental surveillance in the UK has detected ESBL-producing bacteria at a prevalence of 9.2%, and in Switzerland, wastewater studies have shown stable circulation of ESBL-producing *E. coli* at approximately 10–11% since 2015 [28]. While these data reflect indirect indicators of human carriage rather than direct clinical isolates, they highlight the ongoing presence and spread of ESBL-producing organisms in the community. Regional surveillance of fecal samples in the UK also shows community prevalence ranging from 8.5% to 17.0% [29]. This significant difference between the rates in our clinical isolates and those in European community settings highlights the critical role of the hospital environment in amplifying resistance. This gap is likely exacerbated by challenges in Pakistan's healthcare system compared to the robust infection control infrastructure and regulated antibiotic use in countries such as Switzerland and the UK. The continued rise in resistance to last-line agents like carbapenems underscores the urgent need for enhanced surveillance and intervention.

The high rate of fluoroquinolone resistance observed in our isolates (72.7%) is likely a consequence of their widespread empirical use, a trend that has been documented globally [30]. Our finding of near-universal resistance to ampicillin and third-generation cephalosporins aligns with the defining characteristic of ESBL-producing pathogens and confirms the clinical obsolescence of these antibiotics for treating such infections [31].

The molecular findings of this study characterize the genetic drivers of resistance in Peshawar. The high prevalence of the *bla*CTX-M gene (95.3%) among ESBL-producing isolates confirms its status as the most widespread ESBL genotype, both globally and within this region. This finding has direct clinical implications, as CTX-M enzymes confer high-level resistance to third-generation cephalosporins, which remain commonly used for empirical treatment of community-acquired infections in many healthcare settings. Even more concerning is the high prevalence of the *bla*NDM gene, detected in 86.4% of our carbapenem-resistant isolates. The emergence and dissemination of NDM-producing organisms severely threaten the efficacy of carbapenems, which are often the last resort for treating infections caused by ESBL-producers. The co-existence of both potent ESBL and carbapenemase genes in pathogens like *E. coli* and *K. pneumoniae* creates a scenario of extensive drug resistance, posing significant challenges for clinical management and highlighting the urgent need for continuous molecular surveillance to track their spread. Our results demonstrated a strong negative association between ESBL production and carbapenem resistance (OR = 0.10; 95% CI: 0.05–0.21). This indicates that ESBL-producing isolates were approximately 10-fold less likely to exhibit carbapenem resistance compared to non-ESBL-producing isolates. Although this finding appears counterintuitive compared with much of the literature, it may reflect the independent acquisition and spread of carbapenemase genes, particularly *bla*NDM, rather than a sequential evolution from ESBL activity. This highlights the complexity of resistance dynamics in our setting and emphasizes that ESBL production does not always predict carbapenem resistance.

This study has several limitations. First, it was conducted at a single tertiary care hospital in Peshawar, which may introduce selection bias and limit the generalizability of findings. Consequently, our findings primarily reflect hospital-acquired infections and may not be generalizable to community-acquired infections or other healthcare settings. Second, the study's cross-sectional nature prevents the evaluation of temporal trends and restricts insights into the longitudinal dynamics of resistance development and spread. Therefore, future

multicenter, longitudinal studies are necessary to provide a more comprehensive understanding of the epidemiology and evolution of ESBL and carbapenem resistance in this region.

ESBL-producing Gram-negative bacteria, predominantly *E. coli* and *K. pneumoniae* harboring the *bla*CTX-M gene, are highly prevalent in clinical isolates from Peshawar. Crucially, the emergence of carbapenem resistance, mediated by the alarming prevalence of the *bla*NDM gene in these isolates, signals a significant threat to the efficacy of last-line antibiotics in this region. While phenotypic testing confirmed that colistin remains a highly active agent against most isolates, widespread resistance to older β-lactams and cephalosporins severely limits empirical treatment options. These findings underscore the urgent need for continuous, integrated surveillance of both phenotypic and genotypic resistance patterns. These data are essential for developing targeted antimicrobial stewardship initiatives and effective infection control strategies to combat the spread of these multidrug-resistant pathogens.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest associated with this manuscript.

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AI DISCLOSURE

AI-based tools were used for assistance with language editing only. All scientific content, analysis, and conclusions are the authors' own work.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. No publicly archived datasets were used or generated.

AUTHORS' CONTRIBUTIONS

HAA: Conceptualization; Methodology; Investigation; Data Curation; Formal Analysis; Writing – Original Draft Preparation; Visualization. JA: Methodology; Validation; Resources; Writing – Review & Editing; Investigation. FK: Formal Analysis; Data Curation; Visualization; Software; Writing – Review & Editing. MA: Data Analysis; Software; Supervision; Project Administration; Writing – Review & Editing.

ETHICS STATEMENT

The study protocol was approved by the Institutional Ethics Review Board of the University of the Punjab, Lahore (Approval No. D/119/FIMS), which served as the primary research institution, and the study was conducted in accordance with local institutional guidelines at the participating tertiary care hospital in Peshawar. All procedures involving human-derived samples were performed in accordance with the ethical principles of the Declaration of Helsinki and its later amendments.

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