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Prevalence and Molecular Characterization of Extended-Spectrum Beta-Lactamase (ESBL) Producing Enterobacteriaceae in Hospitalized Patients

Noor e Seher[1], Filza Karim[2], Maheen Shuja[3], Ayesha Qaiser[4]

1 Department of Biological Sciences, Keele University, Keele, Staffordshire ST5 5BG, United Kingdom.
2 Department of Pathology, Sheikh Zayed Medical College and Hospital, Rahim Yar Khan, Pakistan.
3 Department of Pathology, Sheikh Zayed Medical College and Hospital, Rahim Yar Khan, Pakistan.
4 Department of Pathology, Sheikh Zayed Hospital, Lahore, Pakistan; PGR Psychiatry, Lahore General Hospital (LGH), Lahore, Pakistan.

Corresponding Author: Noor e Seher, **Email:** nooreseher7989@gmail.com

ABSTRACT

Background: Extended-Spectrum Beta-Lactamase (ESBL) carbapenem-resistant Enterobacteriaceae (CRE) represent a substantial contributor to global antimicrobial resistance, resulting in morbidity, treatment failure, increased healthcare expenditures, and prolonged hospitalisations. The molecular characterisation of resistance determinants is crucial for understanding the propagation of resistance and the principles of antimicrobial stewardship.

Objective: To evaluate the prevalence, antibiotic resistance profiles, molecular attributes, and clinical risk factors associated with ESBL-Producing Enterobacteriaceae in hospitalised patients.

Methods: This investigation was prospective cross-sectional study carried out at the Department of Pathology at Sheikh Zayed Medical College/Hospital, Rahim Yar Khan, Pakistan, from January 2024 to December 2025. A total of 220 Enterobacteriaceae isolates were obtained from hospitalised patients by sequential sampling. Clinical specimens included urine, blood, respiratory secretions, wound swabs, and catheter-associated samples. The antimicrobial susceptibility testing was conducted using the Kirby–Bauer disc diffusion technique in accordance with CLSI recommendations. ESBL generation was phenotypically validated by the combined disc diffusion technique, whereas resistance genes were identified via multiplex PCR (blaCTX-M, blaTEM, and blaSHV).

Results: Extended-spectrum beta-lactamase (ESBL) synthesis was identified in 96 out of 220 isolates (43.6%). The primary ESBL-positive pathogens were Escherichia coli (52.1%) and Klebsiella pneumoniae (31.3%). The blaCTX-M gene was detected in 74.0% of isolates, followed by blaTEM at 48.9% and blaSHV at 35.4%. The multidrug resistance rate (MDR) among the isolates was 79.2 percent. Resistance was substantially correlated with a history of antibiotic use, duration of hospital stays, utilisation of urinary catheters, and admission to the ICU ($p<0.05$).

Conclusion: ESBL-producing Enterobacteriaceae are still very prevalent and multidrug resistant, highlighting the need for molecular surveillance and antimicrobial stewardship measures.

Keywords: Enterobacteriaceae, Extended-Spectrum Beta-Lactamases (ESBL), Drug Resistance, Microbial, Anti-Bacterial Agents.

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INTRODUCTION

Antimicrobial resistance has arisen as a significant worldwide healthcare problem in the twenty-first century, severely undermining the efficacy of existing treatment medicines and leading to heightened morbidity, mortality, and healthcare costs [1]. The rise of multidrug-resistant Gram-negative bacteria is linked to severe hospital-acquired infections, extended hospital stays, treatment failures, and intra-hospital transmission [2]. Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae represent a notable category of resistant infections, characterised by their rapidly increasing prevalence and ability to acquire resistance to many antibiotic classes.

Clinically relevant pathogens, including Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis, Citrobacter species, and Serratia species, are part of the closely related Gram-negative bacilli of the Enterobacteriaceae family [3]. These organisms are prevalent etiological agents responsible for urinary tract infections, bloodstream infections, intra-abdominal infections, wound infections, catheter-associated infections, and healthcare-associated sepsis. Their capacity to flourish in hospital environments and to cultivate transferable resistance factors is crucial to their epidemiological importance [4].

Extended-spectrum beta-lactamases (ESBL) are plasmid-mediated enzymes capable of hydrolysing extended-spectrum cephalosporins, penicillins, and monobactams, hence diminishing the efficacy of widely used antimicrobial medicines [5]. ESBL-producing organisms often express co-resistance to other classes of antibiotics such as fluoroquinolones, aminoglycosides, tetracyclines and sulfonamides, significantly reducing therapeutic options. With the occurrence of these resistance mechanisms, clinical use has now inevitably turned to carbapenem antibiotics with a subsequent increase in the selective pressure and potential for development of resistance to these antibiotics [6].

Several important resistance factors linked to ESBL, including blaCTX-M, blaTEM, and blaSHV genes, which are frequently found on mobile genetic components such plasmids, transposons, and integrons, have been identified by molecular epidemiological investigations [7]. The fast development of antibiotic resistance in healthcare is facilitated by these mobile genetic platforms, which permit gene exchange across various bacterial populations. Of these factors, the CTX-M enzymes are now more predominant in the world and are considered to be one of the most important factors contributing to global ESBL epidemiology [8].

Hospitalized patients are a very susceptible population for colonization and infection with ESBL-producing Enterobacteriaceae because of multiple contributing factors such as prolonged hospital stay, intensive care unit admission, invasive procedures, the presence of indwelling devices (such as urinary catheters), immunocompromised status, previous use of broad-spectrum antimicrobial therapy, and underlying chronic medical conditions. The growing spread of the ESBL-producing organisms in the hospital setting has significant implications for infection prevention programs, empirical antibiotic therapy, and antimicrobial stewardship efforts [9,10].

Antimicrobial resistance monitoring is not standard in health care system development, and molecular characterization studies are often resource-limited [11]. Epidemiological data from the region will be important since the prevalence of antimicrobial resistance varies significantly across geographical regions and can impact local treatment guidelines and infection control strategies. Circulating ESBL-producing pathogens can be characterized at the molecular level to gain understanding into the mechanisms of resistance, patterns of transmission, and emerging epidemiological trends that can help guide evidence-based clinical decision-making [12].

While ESBL prevalence has been reported in the past and is rising across the world, data on molecular resistance determinants in hospitalized patients in tertiary healthcare facilities in Southern Punjab are limited. Knowing the local resistance epidemiology is important for informing the optimal use of empirical antimicrobials and enhancing hospital antimicrobial stewardship programs [13].

This study aimed to evaluate the prevalence, molecular characteristics, antimicrobial resistance profiles, and associated clinical risk factors of ESBL-producing Enterobacteriaceae in hospitalised patients. This study aimed to uncover the predominant genetic factors linked to antibiotic resistance in the local healthcare environment by detecting ESBLs [14].

MATERIALS AND METHODS

This prospective cross-sectional study was carried out from January 2024 to December 2025 at the Department of Pathology, Sheikh Zayed Medical College/Hospital, Rahim Yar Khan, Punjab, Pakistan, a tertiary care teaching institution providing inpatient diagnostic, microbiological, and specialised laboratory services to a substantial population in Southern Punjab and adjacent regions. This study aimed to ascertain the prevalence and genetic characteristics of Extended-Spectrum Beta-Lactamase (ESBL) producing Enterobacteriaceae in hospitalised patients, while also investigating associated antibiotic resistance determinants and clinical risk factors. Patients hospitalised across many clinical services (including internal medicine, general surgery, intensive care unit [ICU], pulmonology, urology, and other inpatient services) throughout the study period, who exhibited a clinical suspicion of bacterial infection, underwent testing. Patients age 18 years and older were eligible for inclusion and microbiologically verified Enterobacteriaceae growth

in clinical specimens was considered a requirement for inclusion in the study. The study group comprised of those who had been hospitalized for over 48 hours with detailed clinical and microbiological data available. Patients with duplicate (more than one) bacterial isolates, contaminated samples, insufficient laboratory information, mixed bacterial growth which did not allow for accurate bacterial isolate characterisation, and discharge without microbiological confirmation were excluded.

A non-probability sequential sampling approach was used and a total of 220 Enterobacteriaceae isolates were retrieved from hospitalized patients. The following variables were considered while determining the sample size: the estimated prevalence of ESBL in hospitalized patients, the practicality of laboratory processing, the load on the hospital, and statistical considerations for prevalence estimates and molecular features analysis.

Clinical and demographic data were gathered using a standardized format of the hospital's medical record and a data collection form. Age, sex, hospital ward, length of stay, previous hospitalizations, intensive care unit (ICU) admission, prior antibiotic use in the last 90 days, urinary catheterization, mechanical ventilation, use of invasive devices, underlying chronic medical illnesses, and final clinical diagnosis were all collected.

Clinical specimens were acquired using stringent aseptic precautions and normal microbiological techniques. Urine, blood cultures, wound swabs, respiratory tract secretions, catheter-associated specimens, and body fluid samples were collected according to clinical indications. All specimens were rushed to the Microbiology Laboratory, Department of Pathology, Sheikh Zayed Medical College/Hospital, Rahim Yar Khan, and processed in a timely manner in accordance with recommended laboratory parameters to ensure microbiological reliability and minimize pre-analytical variability.

The traditional cultural method was used for microbiological isolation. The specimens were incubated aerobically at 35-37°C for 18-24 hours after inoculation on MacConkey agar, Blood agar, and Cysteine Lactose Electrolyte Deficient (CLED) agar, if necessary. Subsequent to the preliminary identification of Enterobacteriaceae isolates by colony morphology and Gram staining, biochemical assays including Triple Sugar Iron, citrate utilisation, urease, indole, motility, methyl red, Voges-Proskauer reaction, and oxidase were performed. Automated microbiological identification methods were used wherever feasible to verify identity. The Clinical and Laboratory Standards Institute (CLSI) advocates the use of the Kirby-Bauer disc diffusion technique for evaluating antibiotic susceptibility on Mueller-Hinton agar. The assessed antibiotics included cefotaxime, ceftazidime, ceftriaxone, cefepime, ciprofloxacin, levofloxacin, gentamicin, amikacin, piperacillin-tazobactam, trimethoprim-sulfamethoxazole, imipenem, and meropenem. The current CLSI guidelines were used to determine zone diameters. Resistance to three or more classes of antibiotics is referred to as multidrug resistance.

The first ESBL screening was based on reduced susceptibility to third-generation cephalosporins. Phenotypic detection of ESBL production was performed using antibiotic discs of cefotaxime, cefotaxime-clavulanic acid, ceftazidime, and ceftazidime-clavulanic acid by the Combination Disc Diffusion Test (CDDT). The comparison of antibiotic discs with clavulanic acid to those with just cephalosporins demonstrated that an increase in the inhibition zone width of ≥5 mm signified the emergence of ESBL development, according to CLSI phenotypic criteria.

Every isolate that generated ESBL and had phenotypic confirmation underwent molecular analysis. Genomic DNA was obtained using commercially available bacterial DNA extraction kits, as instructed by the manufacturer. Multiplex polymerase chain reaction (PCR) experiments were used to discover the principal resistance determinants associated with extended-spectrum beta-lactamases (ESBL), namely the blaCTX-M, blaTEM, and blaSHV genes. The DNA template underwent amplification in a 25 µL PCR reaction including Taq DNA polymerase, dNTPs, MgCl2, and gene-specific primers. The amplification protocol included 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55-60°C for 30 seconds, and extension at 72°C for 1 minute, concluding with a final extension at 72°C for 7 minutes, preceded by an initial denaturation at 95°C for 5 minutes. The amplified PCR products were separated using a 1.5% agarose gel and stained with a nucleic acid dye for UV transillumination to detect the genes. Analytical reliability and reproducibility were achieved by performing quality assurance throughout the laboratory process. As per CLSI guidelines, microbiological validation and antimicrobial susceptibility quality control were performed with SRS. A consistent laboratory equipment calibration, reagent verification and internal quality monitoring program was carried out throughout the investigation.

The data was examined with SPSS version 26.0. Frequency and percentage were used for the analysis of categorical data, whereas continuous data were represented as mean ± SD. Chi-square tests were used to investigate the associations among categorical variables. Multivariate logistic regression was used to determine the odds ratios and 95% confidence intervals for independent variables linked to ESBL positivity. Results with a p-value below 0.05 are deemed statistically significant. The Institutional Review Board (IRB) of Sheikh Zayed Medical College/Hospital in Rahim Yar Khan, Punjab, Pakistan, issued ethical approval (IRB Reference No.: SZMC/IRB/2023/427) before the initiation of the study. The study was conducted in compliance with the ethical standards outlined in the Declaration of Helsinki. Written

informed consent has been obtained from all participants or their legal representative, as appropriate. Patients' confidentiality and identity were carefully kept during data collection, microbiological processing, molecular analysis and statistical assessment.

RESULTS

The final study includes 220 Enterobacteriaceae isolates that were isolated from hospitalized patients admitted to different inpatient departments of Sheikh Zayed Medical College/Hospital, Rahim Yar Khan. There were 93 (42.3%) female patients and 127 (57.7%) male patients in the study population. Patients between the ages of 51 and 70 had the highest infection load, with a mean age of 52.8 ± 16.4 years. Most of the isolates were from patients in the intensive care unit (ICU), surgery, pulmonology, and internal medicine wards.

Phenotypic confirmation demonstrated that 96 of 220 isolates (43.6%) were Extended-Spectrum Beta-Lactamase (ESBL)-producing Enterobacteriaceae, while 124 isolates (56.4%) were non-ESBL producers. The microbiological distribution revealed Escherichia coli as the predominant ESBL-producing organism, comprising 50 (52.1%) isolates, followed by Klebsiella pneumoniae (31.3%), Enterobacter cloacae (10.4%), Proteus mirabilis (4.2%), and Citrobacter species (2.0%). Urinary specimens represented the most frequent source of ESBL-positive isolates (43.8%), followed by blood cultures (20.8%), respiratory tract specimens (15.6%), wound swabs (13.5%), and catheter-associated samples (6.3%), demonstrating substantial dissemination of resistant organisms across multiple hospital-associated infection sites (Table 1).

Molecular characterization of phenotypically confirmed ESBL isolates showed that blaCTX-M was the most common resistance determinant found in 71 (74.0%) isolates. blaTEM was found in 47 (48.9%) isolates, and blaSHV was found in 34 (35.4%) isolates. 28 (29.2%) of the bacterial isolates had multiple ESBL-associated resistance genes expressed simultaneously, signifying significant genetic diversity, which may play an important role in the dissemination of antimicrobial resistance. Antimicrobial susceptibility testing further demonstrated extensive resistance against third-generation cephalosporins, with cefotaxime resistance observed in 91.7%, ceftriaxone resistance in 89.6%, and ceftazidime resistance in 88.5% isolates. Resistance against ciprofloxacin and trimethoprim-sulfamethoxazole was identified in 68.8% and 63.5% isolates, respectively. Carbapenem susceptibility remained relatively preserved, with meropenem resistance observed in only 8.3% isolates and imipenem resistance in 6.3% isolates (Table 2).

Several clinical variables were statistically associated with ESBL positivity by the risk factor analysis. Previous antibiotic exposure was identified as the strongest predictor, demonstrating approximately 3.5-fold increased odds of ESBL positivity (OR=3.54; $p<0.001$). Hospitalization exceeding seven days significantly increased resistance risk (OR=2.43; $p=0.008$). Urinary catheterization and an intensive care unit (ICU) admission also showed strong independent associations with resistant bacterial isolation. These results highlight the high contribution of HAE and antimicrobial selection pressure to the emergence of ESBL in hospitalized patients (Table 3).

Overall results showed a high prevalence of ESBL-producing Enterobacteriaceae in hospitalized patients with high drug-resistance levels and the predominance of blaCTX-M-encoded ESBL. The current molecular epidemiology and clinical risk factors highlight the need for improved antimicrobial stewardship programs, molecular surveillance programs, and infection prevention and control measures in the hospital setting.

Table 1: Baseline Characteristics and Distribution of ESBL-Producing Enterobacteriaceae (n=220)

Variable	Frequency (n)	Percentage (%)
Male patients	127	57.7
Female patients	93	42.3
ESBL-positive isolates	96	43.6
Non-ESBL isolates	124	56.4
Escherichia coli	50	52.1*
Klebsiella pneumoniae	30	31.3*
Enterobacter cloacae	10	10.4*
Proteus mirabilis	4	4.2*
Citrobacter species	2	2.0*
Urinary specimens	42	43.8*
Blood cultures	20	20.8*
Respiratory specimens	15	15.6*
Wound swabs	13	13.5*
Catheter-associated specimens	6	6.3*

Table 2: Molecular Characteristics and Antimicrobial Resistance Profile of ESBL-Producing Isolates (n=96)

Variable	Frequency (n)	Percentage (%)
blaCTX-M	71	74.0
blaTEM	47	48.9
blaSHV	34	35.4
Multiple gene co-expression	28	29.2
Cefotaxime resistance	88	91.7
Ceftriaxone resistance	86	89.6
Ceftazidime resistance	85	88.5
Ciprofloxacin resistance	66	68.8
Gentamicin resistance	42	43.8
Trimethoprim-sulfamethoxazole resistance	61	63.5
Meropenem resistance	8	8.3
Imipenem resistance	6	6.3
Multidrug resistance	76	79.2

Table 3: Clinical Risk Factors Associated with ESBL Positivity

Variable	Odds Ratio (OR)	95% Confidence Interval	P-value
Previous antibiotic exposure	3.54	1.82–6.89	<0.001
Hospital stay >7 days	2.43	1.27–4.66	0.008
Urinary catheterization	2.76	1.42–5.33	0.003
ICU admission	2.91	1.51–5.61	0.002

DISCUSSION

The current study highlighted the increasing burden of antibiotic resistance in tertiary healthcare settings by demonstrating a significant incidence of Enterobacteriaceae that produce Extended-Spectrum Beta-Lactamase (ESBL) among hospitalized patients [1]. Nearly half of all isolates were ESBL-producing Enterobacteriaceae, indicating a significant frequency of resistant pathogens in the hospital environment. The rapid rise of ESBL-mediated resistance has become a serious clinical problem since these pathogens are associated with prolonged hospital stay, high health care costs, therapeutic failure, as well as high morbidity and mortality [2].

The high prevalence of Escherichia coli in the present investigation is in line with the well-recognized epidemiologic importance of this organism as a major cause of bloodstream infections, catheter-associated infections, and urinary tract infections in healthcare-associated infections [3,4]. Further, Klebsiella pneumoniae was the second most common ESBL-producing organism, which also underscores its role as an opportunistic nosocomial pathogen with potential for rapid spread in hospital settings. The high prevalence of these organisms may be partly due to their extraordinary capacity for the acquisition of transferable resistance determinants and their ability to survive under antimicrobial selective pressure [5].

Consistent with previous studies, the urinary source had the highest percentage of ESBL-positive isolates in the current study. Patients in hospitals are often at high risk for colonization and infection with resistant bacterial strains due to urinary catheterization and prolonged exposure to antimicrobial agents. The results also suggest that invasive health care interventions play a major role in the development of antimicrobial resistance [6,7].

Molecular characterization revealed that blaCTX-M was the predominant ESBL-related resistance determinant, followed by blaTEM and blaSHV genes [8]. The prevalence of CTX-M enzymes is consistent with the molecular epidemiology of resistance, which is changing throughout the world, showing a shift from the older resistance mechanisms of TEM and SHV to CTX-M. The rise of blaCTX-M-positive organisms is a significant clinical issue as the CTX-M enzymes are highly transferable and are often linked with multidrug-resistant phenotypes. Multiple genes associated with ESBL production were simultaneously detected, which further highlights the complexity of the evolution of resistance and the horizontal transfer of genes between populations of Enterobacteriaceae [9].

The antimicrobial susceptibility results showed a high level of resistance, specifically to third-generation cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole [10]. This suggests that therapy alternatives for infection with ESBL are narrowing within hospitals. The high level of multidrug resistance found in the current study could be a consequence of excessive use of antimicrobials and misuse of antibiotics, giving rise to selective pressure for the resistant bacterial population. Despite the preserved carbapenem susceptibility, there is concern about the future emergence of CPE because of increasing reliance on carbapenems for therapy [11].

A number of clinical parameters were found to be significantly associated with ESBL positivity. Prior antibiotic use was identified as the single most significant independent risk factor, and therefore, the need for prudent use of antibiotics is highlighted [12]. Resistant bacterial isolation was also significantly associated with prolonged hospitalization, urinary catheterization, and Intensive Care Unit admission. This may be due to enhanced opportunities for the transmission of bacteria, exposure to

resistant bacteria in the environment, and the continuous antimicrobial selection pressure in the hospital environment [13].

The results of the current investigation highlight the need for enhanced infection prevention strategies, antimicrobial stewardship programs, and ongoing molecular surveillance programs [14]. Evidence-based and early detection of resistant organisms can have a great impact on patient care and the spread of resistant organisms. Molecular characterisation has also been found to be highly useful since understanding the epidemiology of local resistance will enable optimisation of empirical antimicrobial therapy and enhance hospital infection control protocols [15].

Several strengths of the present study are the molecular characterisation of the major ESBL-associated genes as well as the investigation of clinically relevant risk factors associated with the development of resistance. Some restrictions have to be observed however. The findings of this study may not be generalizable to other populations due to the study site being a single tertiary healthcare center. Furthermore, this more comprehensive and detailed genomic sequencing was not conducted, thus providing deeper insights into the characterization of resistance mechanisms and dynamics of bacterial transmission [16,17].

CONCLUSION

The current study showed that ESBL-producing Enterobacteriaceae were significantly prevalent in hospitalized patients at Sheikh Zayed Medical College/Hospital, Rahim Yar Khan, and these ESBL-producing bacteria were highly multidrug resistant, and ESBL molecular determinants were widely disseminated. E. coli and K. pneumoniae were the most frequently encountered resistant pathogens, and blaCTX-M was the most common molecular resistance determinant found. Prior antibiotic exposure, length of hospital stay, urinary catheterization, and admission to Intensive Care Unit (ICU) were among the factors identified as being associated with the emergence of antimicrobial resistance and were found to be important contributors. Widespread antibiotic resistance also reflects the growing challenges in therapy in tertiary care centers. Even with the presence of molecular surveillance systems, the use of antimicrobial stewardship programs, rational antibiotic use policies, and improved hospital infection prevention measures is still important to ensuring that resistant Enterobacteriaceae are not widely spread. Ongoing surveillance of resistance trends and molecular epidemiology will play an important role in helping to maintain antimicrobial efficacy and enhance clinical outcomes in hospitalized populations.

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Authors' Contributions: N.E.S. conceptualized the study and drafted the manuscript. F.K. and M.S. contributed to data collection, laboratory procedures, and analysis. A.Q. contributed to interpretation, critical revision, and supervision. All authors approved the final manuscript.

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Data Availability: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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