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MOLECULAR DETECTION OF ANTIBIOTIC RESISTANCE GENES AND SUSCEPTIBILITY IN SERINE AND METALLO-B-LACTAMASE PRODUCING E. COLI

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ABSTRACT

Antimicrobial resistance (AMR) has emerged as one of the most serious global public health threats, significantly increasing morbidity, mortality, and healthcare expenditures worldwide. Multidrug-resistant (MDR) Gram-negative bacteria, particularly Escherichia coli, are of major clinical concern due to their ability to acquire and disseminate β-lactamase enzymes that inactivate β-lactam antibiotics. Carbapenems are often considered last-resort agents for severe infections; however, the emergence of carbapenem-resistant E. coli, especially those producing metallo-β-lactamases (MBLs), severely limits therapeutic options. In addition to MBLs, other β-lactamases such as extended-spectrum β-lactamases (ESBLs) and serine β-lactamases (e.g., SHV-type enzymes) contribute to resistance patterns, complicating treatment strategies. The present study aimed to investigate the molecular detection of antibiotic resistance genes and evaluate antibiotic susceptibility profiles among serine and metallo-β-lactamase-producing E. coli isolates. A total of 20 clinical isolates were collected from diverse patient specimens, including urine, blood cultures, catheter tips, and endotracheal tube secretions, at BJ Micro Lab in collaboration with Abasyn University, Islamabad. Identification of E. coli isolates was performed using standard morphological and biochemical methods. Antimicrobial susceptibility testing was conducted using the Kirby–Bauer disc diffusion method according to established clinical guidelines. Phenotypic detection of MBL production was carried out using the imipenem–EDTA combined disc test (IMP-EDTA CDT), while genotypic confirmation of resistance genes, including MBL and SHV genes, was performed using multiplex polymerase chain reaction (PCR). Antibiotic susceptibility results demonstrated that 10 out of 14 evaluated isolates (71.4%) exhibited resistance to carbapenems, whereas 4 isolates (28.6%) remained susceptible. Among the carbapenem-resistant isolates, one strain (10%) carried the MBL gene, and five strains (50%) harbored the SHV gene. Notably, none of the imipenem-sensitive isolates possessed genes associated with MBL production. A high level of concordance was observed between phenotypic detection using IMP-EDTA CDT and genotypic confirmation by PCR, supporting the reliability of combined diagnostic approaches. The detection of MBL- and SHV-producing E. coli underscores a significant clinical challenge due to limited effective treatment options

and the potential for rapid dissemination of resistance determinants. Early molecular identification of resistance genes, particularly in carbapenem-susceptible isolates, is essential for guiding appropriate antimicrobial therapy, strengthening infection control measures, and reducing the spread of multidrug-resistant organisms in healthcare settings.

Keywords: *Antimicrobial resistance; Escherichia coli; Metallo-β-lactamase (MBL); Serine β-lactamase; SHV gene; Carbapenem resistance; Multiplex PCR; Kirby–Bauer disc diffusion; β-lactam antibiotics; Phenotypic and genotypic detection.*

1- Introduction:

Antimicrobial resistance (AMR) has emerged as one of the most formidable public health challenges of the twenty-first century. The rapid evolution and dissemination of resistant bacterial pathogens threaten the effectiveness of standard antimicrobial therapies, leading to prolonged illness, increased mortality, higher healthcare costs, and limited treatment alternatives. Among the organisms contributing significantly to this global crisis are members of the family Enterobacteriaceae, a diverse group of Gram-negative bacilli responsible for a wide spectrum of community-acquired and hospital-acquired infections. This family includes clinically important genera such as Escherichia, Klebsiella, Enterobacter, Salmonella, Shigella, and Citrobacter, many of which are implicated in urinary tract infections (UTIs), septicemia, pneumonia, intra-abdominal infections, and device-associated infections [1]. Among these, Escherichia coli holds particular clinical and epidemiological importance. Although it commonly exists as a commensal organism within the gastrointestinal tract of humans and animals, pathogenic strains of E. coli are capable of causing severe infections. Furthermore, E. coli functions as a major reservoir for antimicrobial resistance genes due to its remarkable genetic adaptability and ability to acquire resistance determinants via horizontal gene transfer. Consequently, it is widely used as an indicator organism in antimicrobial resistance surveillance programs. β-lactam antibiotics including penicillins, cephalosporins, monobactams, and carbapenems have long been the cornerstone of therapy against Gram-negative bacterial infections. These agents exert their bactericidal action by inhibiting penicillin-binding proteins (PBPs), thereby disrupting peptidoglycan synthesis and compromising bacterial cell wall integrity. Owing to their broad-spectrum activity and favorable safety profile, β-lactams remain the most frequently prescribed class of antibiotics worldwide [2]. However, extensive and often inappropriate use has led to strong selective pressure, promoting the emergence of resistance mechanisms, most notably the production of β-lactamase enzymes. β-lactamases are bacterial enzymes capable of hydrolyzing the β-lactam ring, rendering these antibiotics ineffective. Based on molecular structure and amino acid sequence homology, β-lactamases are classified into four Ambler classes (A, B, C, and D). Classes A, C, and D are serine β-lactamases (SBLs), characterized by the presence of a serine residue at their catalytic site, whereas class B enzymes are metallo-β-lactamases (MBLs), which require zinc ions as essential cofactors for enzymatic activity. These enzyme classes differ significantly in their substrate spectrum, inhibition profile, and clinical impact. A comparative overview of the major β-lactamase classes is presented in Table 1.

Table 1: Molecular classification and characteristics of major β-lactamases

Ambler Class	Enzyme Type	Representative Genes	Catalytic Mechanism	Antibiotics Hydrolyzed	Inhibitor Sensitivity	Clinical Impact
Class A	Serine β-lactamases (ESBLs, KPC)	blaTEM, blaSHV, blaCTX-M, blaKPC	Active-site serine	Penicillins, cephalosporins, some carbapenems	Inhibited by clavulanic acid, tazobactam	Common in hospital settings

Class B	Metallo-β-lactamases (MBLs)	blaNDM, blaVIM, blaIMP	Zinc-dependent hydrolysis	Carbapenems, penicillins, cephalosporins	Inhibited by EDTA	Major global threat
Class C	AmpC β-lactamases	blaAmpC	Serine-mediated hydrolysis	Cephalosporins	Poor inhibition by clavulanate	Often chromosomal
Class D	Oxacillinases (OXA)	blaOXA	Serine-mediated hydrolysis	Oxacillin, carbapenems	Variable inhibition	Increasing prevalence worldwide

Extended-spectrum β-lactamases (ESBLs), particularly TEM, SHV, and CTX-M types, are among the most widely distributed resistance determinants in E. coli. These enzymes confer resistance to third-generation cephalosporins and monobactams, significantly limiting therapeutic options. As a result, carbapenems such as imipenem, meropenem, and ertapenem became the drugs of last resort for severe infections caused by ESBL-producing organisms. Carbapenems possess structural stability against hydrolysis by most ESBLs and AmpC enzymes, making them highly effective in critical infections. However, the emergence of carbapenem-resistant Enterobacteriaceae (CRE) has further complicated treatment strategies. Carbapenem resistance is often mediated by carbapenemases, which include both serine carbapenemases (e.g., KPC and OXA-48) and metallo-β-lactamases (e.g., NDM, VIM, and IMP). A crucial biochemical distinction between serine β-lactamases and MBLs lies in their catalytic mechanism and inhibitor susceptibility. While serine β-lactamases may be inhibited by conventional β-lactamase inhibitors, MBLs are resistant to these inhibitors but can be suppressed by metal chelators such as EDTA. Importantly, MBLs possess the ability to hydrolyze nearly all β-lactam antibiotics, including carbapenems, thereby severely restricting therapeutic options [3]. The global spread of MBL genes is largely driven by their presence on mobile genetic elements such as plasmids, transposons, and integrons, facilitating rapid horizontal gene transfer between bacterial species. The emergence of blaNDM, blaVIM, and blaIMP genes has been particularly alarming in South Asia and other developing regions. The presence of such resistance determinants is associated with higher treatment failure rates, increased hospitalization duration, and elevated mortality. Accurate detection of serine and metallo-β-lactamase-producing *E. coli* is therefore critical for infection control and antimicrobial stewardship. Phenotypic methods such as the Kirby–Bauer disc diffusion test and the imipenem–EDTA combined disc test (IMP-EDTA CDT) are widely used for routine screening. However, molecular techniques such as multiplex polymerase chain reaction (PCR) provide more specific and sensitive identification of resistance genes including blaSHV (serine β-lactamase) and blaIMP (metallo-β-lactamase). Given the increasing burden of carbapenem resistance and the limited availability of molecular epidemiological data from local clinical settings, the present study aims to evaluate antibiotic susceptibility patterns and perform molecular detection of serine (blaSHV) and metallo (blaIMP) β-lactamase genes in clinical E. coli isolates. Understanding the distribution and genetic characteristics of these resistance determinants is essential for guiding empirical therapy, strengthening antimicrobial stewardship programs, and preventing further dissemination of multidrug-resistant pathogens.

2- β-Lactam Antibiotics and Mechanisms of Action:

β-lactam antibiotics constitute the most extensively prescribed class of antimicrobial agents worldwide and remain the primary therapeutic option for treating infections caused by Gram-positive and Gram-negative bacteria. Their widespread clinical use is attributed to their broad-spectrum activity, favorable safety profile, and well-

established efficacy. Structurally, all β-lactam antibiotics share a common four-membered β-lactam ring, which is essential for their antibacterial activity. This ring enables the antibiotic to interact with specific bacterial targets known as penicillin-binding proteins (PBPs), which are enzymes involved in the final stages of peptidoglycan synthesis during bacterial cell wall formation. The mechanism of action of β-lactam antibiotics involves inhibition of transpeptidation, a critical step in cross-linking peptidoglycan chains that provide structural integrity to the bacterial cell wall. By binding to PBPs, β-lactams prevent proper cross-linking, leading to weakening of the cell wall, osmotic instability, and eventual bacterial cell lysis [4]. Because mammalian cells lack peptidoglycan, β-lactam antibiotics exhibit selective toxicity, making them highly effective and relatively safe therapeutic agents. β-lactam antibiotics are categorized into four major subclasses: penicillins, cephalosporins, monobactams, and carbapenems. Each subclass differs in spectrum of activity, structural modifications, resistance to β-lactamase hydrolysis, and clinical application. Carbapenems are considered last-line agents due to their broad activity and relative stability against many β-lactamases. However, the extensive clinical use and misuse of β-lactam antibiotics has exerted strong selective pressure on bacterial populations, resulting in the emergence of resistance mechanisms. The most significant mechanism of resistance is the production of β-lactamase enzymes. These enzymes hydrolyze the β-lactam ring by breaking the amide bond, thereby inactivating the antibiotic before it can bind to PBPs. Over time, β-lactamases have diversified into numerous variants with expanded substrate profiles, including extended-spectrum β-lactamases (ESBLs) and carbapenemases. Additional resistance mechanisms include alterations in PBPs, reduced outer membrane permeability due to porin loss, and overexpression of efflux pumps [5]. Nevertheless, enzymatic degradation via β-lactamase production remains the dominant and clinically most relevant resistance pathway in Escherichia coli and other Enterobacteriaceae. A summary of the major subclasses of β-lactam antibiotics and their characteristics is presented in Table 2.

Table 2: Major subclasses of β-lactam antibiotics and their characteristics

Subclass	Examples	Spectrum of Activity	β-Lactamase Stability	Clinical Importance
Penicillins	Ampicillin, Amoxicillin, Piperacillin	Broad Gram-positive, limited Gram-negative	Low (except β-lactamase inhibitor combinations)	First-line therapy for many infections
Cephalosporins	Ceftriaxone, Ceftazidime, Cefepime	Expanded Gram-negative coverage (3rd/4th gen)	Moderate (variable by generation)	Widely used in hospital settings
Monobactams	Aztreonam	Gram-negative bacteria	Stable against MBLs	Useful in β-lactam allergy cases
Carbapenems	Imipenem, Meropenem, Ertapenem	Very broad spectrum, including ESBL producers	Highly stable (except carbapenemases)	Last-resort antibiotics

The enzymatic hydrolysis of β-lactam antibiotics is illustrated in Figure 1, which provides a detailed representation of the molecular mechanism by which β-lactamase enzymes inactivate these antimicrobial agents. As shown in the figure, β-lactamases recognize and bind to the β-lactam ring structure of the antibiotic molecule. In serine β-lactamases, a nucleophilic serine residue within the active site attacks the carbonyl carbon of the β-lactam ring, forming an acyl-enzyme intermediate

that subsequently leads to cleavage of the amide bond. In metallo-β-lactamases, hydrolysis is facilitated by zinc ions located at the active site, which activate a water molecule to attack the β-lactam ring, resulting in ring opening and structural destabilization. This hydrolytic process disrupts the integrity of the β-lactam ring, which is essential for binding to penicillin-binding proteins (PBPs), thereby rendering the antibiotic biologically inactive. The figure further highlights how this enzymatic degradation prevents inhibition of peptidoglycan cross-linking, allowing bacterial cell wall synthesis to proceed uninterrupted and enabling bacterial survival even in the presence of β-lactam antibiotics.

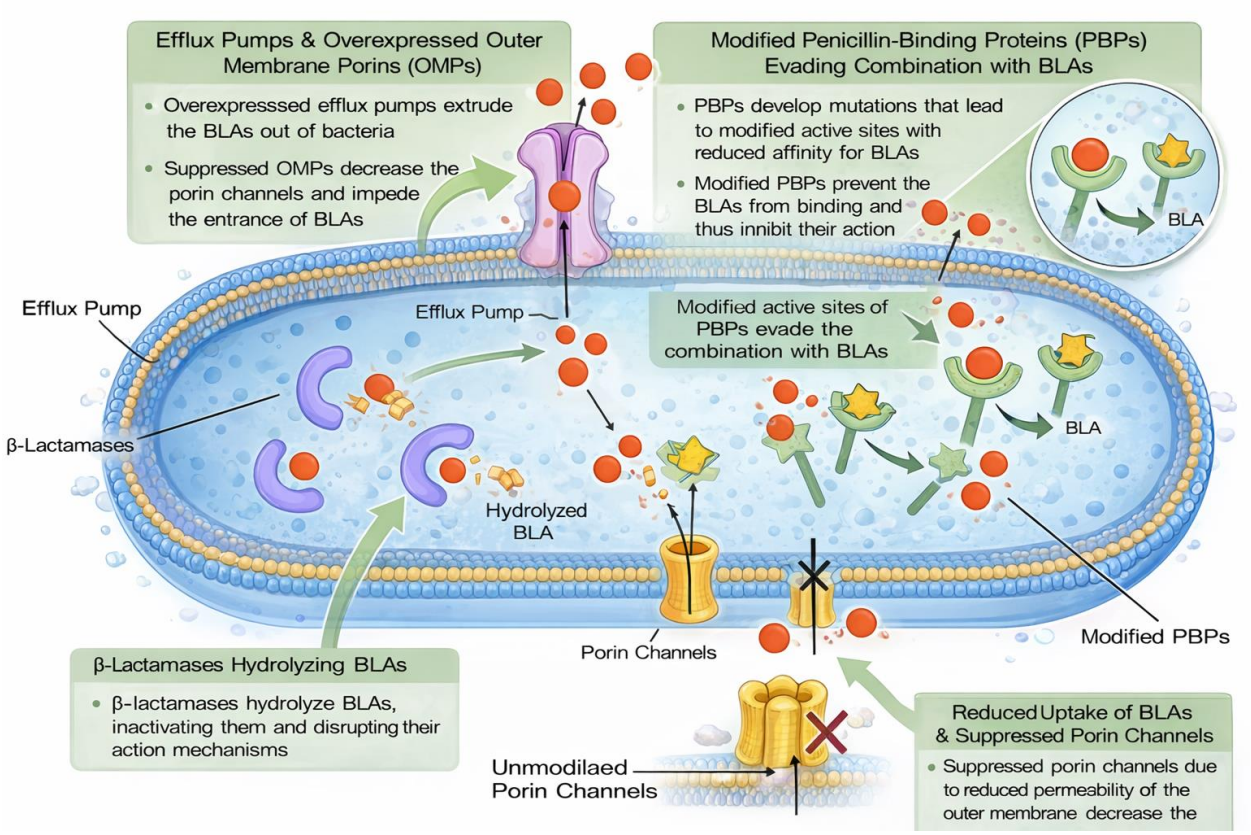


Figure 1: Mechanism of β-lactam antibiotic action and β-lactamase-mediated resistance

The progressive diversification of β-lactamases, particularly extended-spectrum β-lactamases and carbapenemases, has dramatically reduced the clinical utility of many β-lactam agents. The continued emergence of serine β-lactamases and metallo-β-lactamases underscores the urgent need for improved detection strategies and molecular surveillance to guide effective antimicrobial therapy.

3- Emergence of Carbapenem Resistance in Escherichia coli:

Carbapenems have long been considered the cornerstone of therapy for severe infections caused by multidrug-resistant (MDR) Gram-negative bacteria, particularly extended-spectrum β-lactamase (ESBL)-producing Escherichia coli. Their unique molecular structure characterized by a carbapenem nucleus with a trans-6-hydroxyethyl group confers enhanced stability against hydrolysis by most ESBLs and AmpC β-lactamases. Due to this stability, carbapenems such as imipenem, meropenem, doripenem, and ertapenem have historically been reserved as last-resort antibiotics for life-threatening infections, including septicemia, complicated urinary tract infections, intra-abdominal infections, and ventilator-associated pneumonia. However, over the past two decades, the global emergence of carbapenem-resistant E. coli (CREC) has significantly

undermined the effectiveness of this critical antibiotic class. Carbapenem resistance represents a major public health concern because it is frequently associated with limited therapeutic alternatives, higher mortality rates, increased hospital stays, and elevated healthcare costs [6]. The dissemination of carbapenem resistance is particularly alarming in healthcare settings where selective antibiotic pressure facilitates the survival and spread of resistant strains. Carbapenem resistance in E. coli is complex and multifactorial, involving enzymatic and non-enzymatic mechanisms that may act independently or synergistically. The most clinically significant mechanism is the production of carbapenemase enzymes, which directly hydrolyze carbapenem antibiotics. Carbapenemases are classified according to the Ambler system into serine carbapenemases (Classes A and D) and metallo-β-lactamases (Class B). Class A carbapenemases include Klebsiella pneumoniae carbapenemase (KPC), while Class D includes OXA-48-like enzymes. Metallo-β-lactamases (MBLs), such as NDM (New Delhi metallo-β-lactamase), VIM (Verona integron-encoded metallo-β-lactamase), and IMP (imipenemase), require zinc ions for catalytic activity and exhibit an exceptionally broad substrate spectrum. Among these enzymes, MBLs are considered the most threatening due to their ability to hydrolyze nearly all β-lactam antibiotics, including carbapenems, penicillins, and cephalosporins, while remaining unaffected by conventional β-lactamase inhibitors such as clavulanic acid, sulbactam, tazobactam, and avibactam. Their genes are commonly located on conjugative plasmids, integrons, and transposons, facilitating horizontal gene transfer across bacterial species. This genetic mobility accelerates the dissemination of resistance within hospitals and across geographic regions. In addition to carbapenemase production, alterations in outer membrane permeability contribute substantially to carbapenem resistance. Mutations or downregulation of porin proteins such as OmpF and OmpC reduce antibiotic entry into the periplasmic space [7]. When porin loss occurs in conjunction with ESBL or AmpC production, even low-level carbapenem hydrolysis can result in clinically significant resistance. Efflux pump overexpression, particularly the AcrAB-TolC system, further enhances resistance by actively exporting antibiotics from the bacterial cell. These mechanisms may operate synergistically, resulting in high-level resistance phenotypes that are difficult to treat. A comprehensive overview of these mechanisms is presented in Table 3.

Table 3: Advanced molecular mechanisms of carbapenem resistance in Escherichia coli

Mechanism	Molecular Basis	Representative Genes/Proteins	Functional Effect	Clinical Consequence
Serine carbapenemases	Enzymatic β-lactam ring hydrolysis via serine residue	blaKPC, blaOXA-48	Direct carbapenem inactivation	Rapid hospital outbreaks
Metallo-β-lactamases (MBLs)	Zinc-dependent hydrolysis	blaNDM, blaVIM, blaIMP	Broad-spectrum β-lactam degradation	Extremely limited treatment options
Porin loss	Mutation/downregulation of membrane channels	OmpF, OmpC	Reduced antibiotic influx	Synergistic resistance with ESBL
Efflux pump overexpression	Increased active extrusion	AcrAB-TolC	Decreased intracellular drug levels	Multidrug resistance phenotype
ESBL/AmpC + porin alteration	Combined enzymatic + permeability defect	blaTEM, blaSHV, blaAmpC	Reduced carbapenem susceptibility	Therapeutic failure risk

The integrated molecular pathways contributing to carbapenem resistance are illustrated in Figure 2, which provides a comprehensive schematic representation of the multifactorial mechanisms operating within Escherichia coli. As depicted, carbapenem resistance is not typically the result of a single genetic alteration but rather the outcome of complex interactions between enzymatic and non-enzymatic mechanisms. The figure demonstrates the direct hydrolysis of carbapenem molecules by carbapenemase enzymes, including both serine carbapenemases and metallo-β-lactamases, which cleave the β-lactam ring and render the antibiotic inactive before it can bind to penicillin-binding proteins. In parallel, alterations in outer membrane permeability primarily through mutation, downregulation, or loss of porin proteins such as OmpF and OmpC restrict antibiotic entry into the periplasmic space, thereby reducing effective intracellular drug concentration. Additionally, the overexpression of efflux pump systems, particularly the AcrAB–TolC complex, actively expels carbapenem molecules from the bacterial cell, further diminishing antimicrobial activity [8]. The figure also highlights the synergistic interplay between these mechanisms, where carbapenemase production combined with porin loss or efflux pump activation results in significantly elevated resistance levels. This coordinated resistance strategy enhances bacterial survival under intense antibiotic selective pressure and contributes to the persistence and dissemination of carbapenem-resistant strains within clinical environments. Understanding these integrated molecular pathways is essential for accurate diagnostic interpretation, development of targeted therapeutic strategies, and implementation of effective infection control measures.

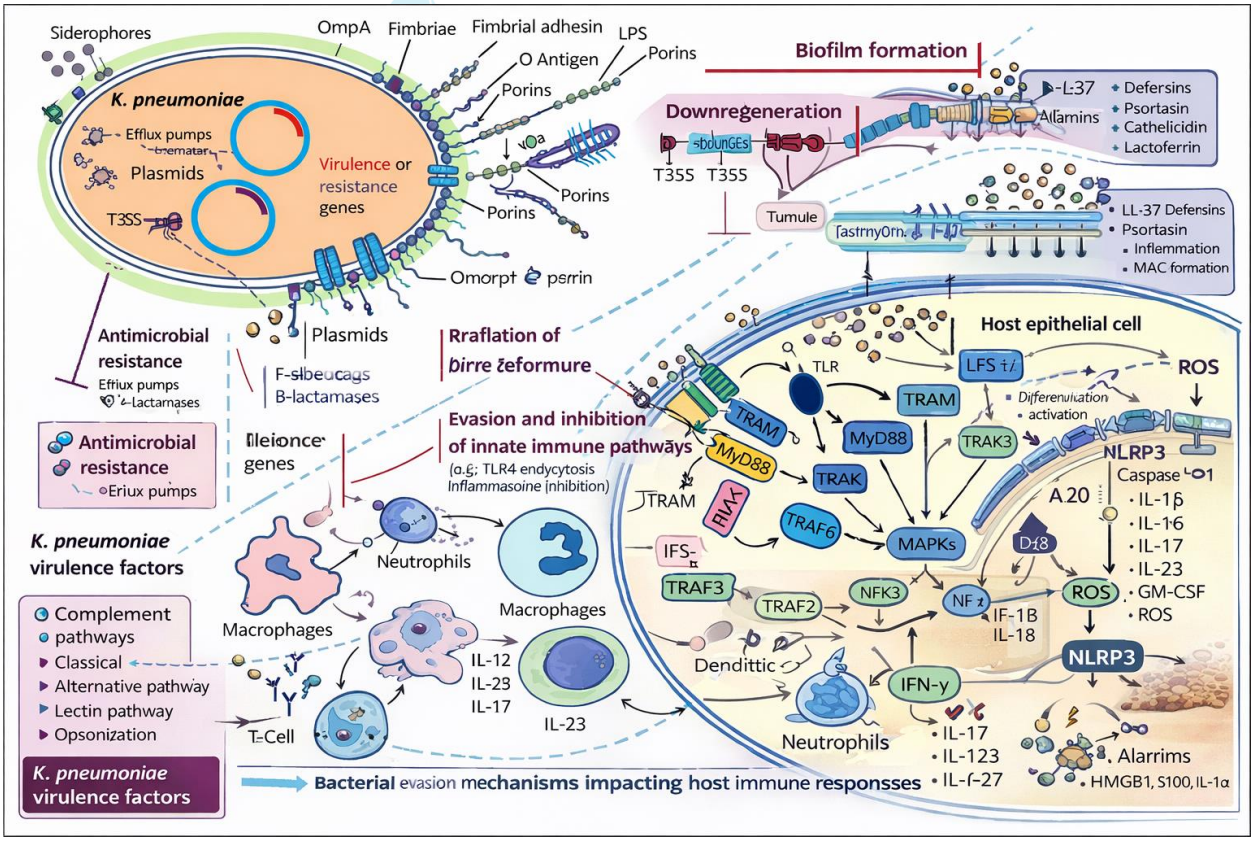


Figure 2: Integrated mechanisms of carbapenem resistance in Escherichia coli

The rapid emergence of carbapenem-resistant E. coli underscores the urgent need for early detection, molecular surveillance, and strict antimicrobial stewardship. In particular, the

plasmid-mediated spread of MBL genes poses a severe epidemiological threat due to their capacity for interspecies transmission and rapid global dissemination. Understanding these resistance mechanisms at both molecular and clinical levels is essential for developing effective diagnostic strategies, optimizing therapeutic interventions, and limiting the spread of multidrug-resistant organisms in healthcare environments.

4- Methodology:

This laboratory-based cross-sectional study was undertaken to comprehensively evaluate antimicrobial susceptibility patterns and to detect serine and metallo-β-lactamase-producing Escherichia coli isolates using both phenotypic and genotypic diagnostic approaches. The study was designed to provide an integrated assessment of resistance mechanisms by combining conventional microbiological methods with advanced molecular techniques. Clinical specimens were collected from patients admitted to a tertiary care hospital, ensuring representation of diverse infection sources including urinary, bloodstream, respiratory, and device-associated infections. All samples were processed using standard microbiological protocols for isolation, culture, and identification of E. coli, including selective media growth, Gram staining, and biochemical confirmation. Antibiotic susceptibility testing was performed using the Kirby–Bauer disc diffusion method in strict accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines to ensure reproducibility and standardized interpretation of results. Carbapenem-resistant isolates were further subjected to phenotypic screening for β-lactamase production using EDTA-based synergy assays, enabling differentiation between serine β-lactamase and metallo-β-lactamase activity based on inhibitor response patterns [9]. To achieve precise molecular confirmation, genomic DNA was extracted from confirmed isolates and polymerase chain reaction (PCR) amplification was performed targeting blaSHV (serine β-lactamase) and blaIMP (metallo-β-lactamase) genes. Agarose gel electrophoresis was used to verify gene-specific amplicons. Finally, statistical analysis was conducted to determine the prevalence of resistance phenotypes and genotypes, assess the distribution of β-lactamase genes, and evaluate the agreement between phenotypic and molecular detection methods. This comprehensive methodological framework ensured accurate characterization of antimicrobial resistance mechanisms in clinical E. coli isolates.

4.1- Study Population and Sampling Strategy:

The present investigation was conducted at Pakistan Institute of Medical Sciences (PIMS), Islamabad, a major tertiary care referral hospital that caters to a diverse patient population from both urban and rural regions. The hospital's multidisciplinary clinical departments including internal medicine, surgery, intensive care units (ICUs), nephrology, pulmonology, and emergency medicine provided access to a wide range of infection types. This diversity ensured that the study captured isolates from multiple anatomical sites and varying clinical severities, thereby improving the representativeness of the findings. A total of 300 clinical specimens were collected during the study period for microbiological evaluation. These specimens comprised urine samples, blood cultures, catheter tips, indwelling catheter samples, and endotracheal/tracheostomy tube secretions. Inclusion of these specimen types enabled assessment of infections associated with urinary, bloodstream, respiratory, and device-related sources, which are commonly implicated in Escherichia coli infections. All samples were collected aseptically by trained healthcare personnel following hospital infection control protocols and were transported to the microbiology laboratory without delay to preserve sample integrity [10]. A convenience sampling strategy was employed due to the reliance on routine clinical specimen submission during the defined timeframe. While probability-based sampling enhances generalizability, convenience sampling is frequently utilized in hospital-based microbiological research where sample acquisition depends on diagnostic workflow. This approach facilitated efficient collection of clinically relevant isolates within logistical and temporal constraints.

To ensure ethical compliance, patient identifiers were removed during data recording, and confidentiality was strictly maintained. Informed consent was obtained in accordance with institutional guidelines and hospital policy. Eligibility criteria were established to maintain consistency and scientific rigor. Only adult patients aged 18 years and above admitted to PIMS Islamabad with suspected or confirmed bacterial infections were included. Samples demonstrating confirmed growth of E. coli on culture media were selected for further phenotypic and genotypic analysis. Patients younger than 18 years, patients not admitted to PIMS, and specimens with no bacterial growth or growth of organisms other than E. coli were excluded. The distribution of collected specimens is summarized in Table 4.

Table 4: Distribution of Clinical Specimens Collected (n = 300)

Specimen Type	Number of Samples	Percentage (%)	Associated Clinical Condition
Urine samples	140	46.7%	Urinary tract infections
Blood cultures	60	20.0%	Septicemia / bloodstream infections
Catheter tips	40	13.3%	Device-associated infections
Indwelling catheter samples	30	10.0%	Nosocomial infections
Endotracheal/Tracheostomy secretions	30	10.0%	Respiratory infections
Total	**300**	**100%**	–

The distribution of specimen types demonstrates that urinary and device-associated infections constituted a substantial proportion of clinical submissions, reflecting the epidemiological pattern of E. coli-related infections in tertiary care settings. Bloodstream and respiratory samples further represent severe or hospital-acquired infections, underscoring the clinical relevance of investigating antimicrobial resistance in these isolates. This heterogeneous sample pool strengthened the robustness of downstream phenotypic and molecular analyses by ensuring inclusion of isolates from multiple infection niches. To provide a clearer understanding of the sample selection process from initial collection to final inclusion of confirmed E. coli isolates a systematic workflow is presented in Figure 3.

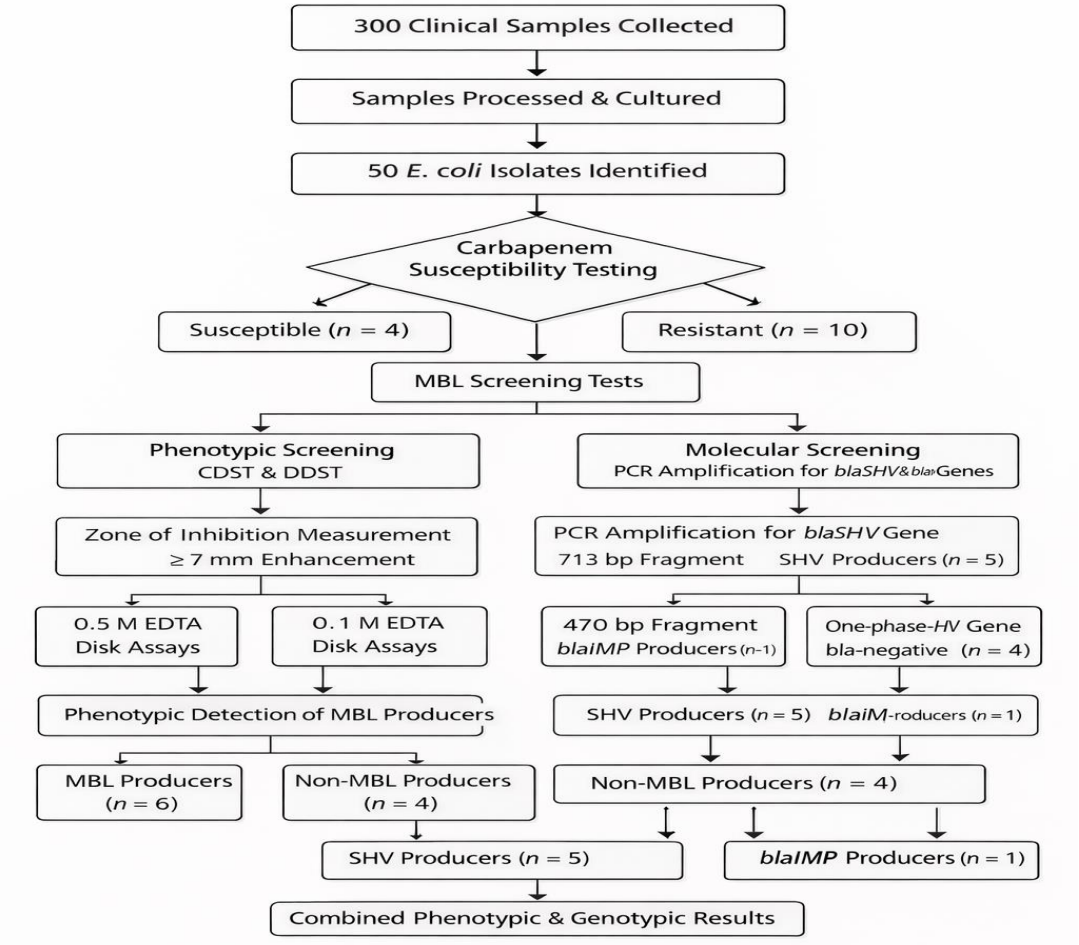


Figure 3: Flow diagram of sampling and isolate selection process

Figure 3 illustrates the sequential process beginning with collection of 300 clinical specimens, followed by culture screening, exclusion of non-E. coli isolates, confirmation through biochemical testing, and final selection of eligible isolates for antimicrobial susceptibility testing and molecular analysis. This structured sampling and selection framework ensured methodological transparency, reproducibility, and appropriate representation of clinically significant E. coli isolates.

4.2- Isolation and Identification of Escherichia coli:

All clinical specimens received in the microbiology laboratory were processed according to standard bacteriological procedures to ensure accurate isolation and identification of Escherichia coli. Upon receipt, samples were examined for labeling accuracy and processed under aseptic conditions within a biosafety cabinet to prevent contamination. Each specimen type was inoculated onto appropriate selective and differential media, including MacConkey agar and Blood agar plates. MacConkey agar, a selective and differential medium for Gram-negative enteric bacteria, was primarily used to differentiate lactose-fermenting organisms from non-lactose fermenters. Blood agar served as a general-purpose enriched medium to support the growth of fastidious organisms and to observe hemolytic activity. Inoculated plates were incubated aerobically at 37°C for 18–24 hours. After incubation, colonies were examined for morphological characteristics such as size, shape, margin, elevation, color, and lactose fermentation [11]. Typical E. coli colonies appeared as pink to reddish colonies on MacConkey agar due to

lactose fermentation, often surrounded by a zone of bile salt precipitation. On Blood agar, colonies appeared smooth, circular, moist, and grayish-white without significant hemolysis. Suspected lactose-fermenting colonies were sub-cultured to obtain pure isolates for further confirmation. Preliminary identification was performed using Gram staining, where smears were prepared from isolated colonies. Gram-negative, short rod-shaped bacilli were selected for biochemical characterization. Only Gram-negative bacilli consistent with Enterobacteriaceae morphology were subjected to further confirmatory testing. Biochemical identification involved a series of conventional tests including oxidase and catalase tests, followed by confirmation using the Analytical Profile Index (API) identification system. The oxidase test was performed to differentiate Enterobacteriaceae (oxidase-negative) from other Gram-negative organisms such as Pseudomonas spp. Catalase testing confirmed the presence of catalase enzyme activity. For API testing, a standardized bacterial suspension equivalent to 0.5 McFarland turbidity standard was prepared in sterile saline solution. The suspension was inoculated into API microtubes containing dehydrated substrates, following the manufacturer's protocol. The API strips were incubated at 37°C for 18–24 hours, and color reactions were recorded. Results were interpreted using the API identification database, enabling species-level confirmation of E. coli. The key morphological and biochemical characteristics used for identification are summarized in Table 5.

Table 5: Morphological and Biochemical Characteristics of Escherichia coli

Test / Characteristic	Expected Result for E. coli	Interpretation
Gram stain	Gram-negative rods	Member of Enterobacteriaceae
MacConkey agar	Pink lactose-fermenting colonies	Lactose positive
Blood agar	Smooth, moist colonies	Non-hemolytic (usually)
Oxidase test	Negative	Differentiates from non-Enterobacteriaceae
Catalase test	Positive	Presence of catalase enzyme
API profile	Species-specific numerical code	Confirmatory identification

The sequential workflow from initial specimen processing to the confirmed identification of Escherichia coli isolates is presented in Figure 4, which provides a comprehensive overview of the structured laboratory protocol implemented in this study. As depicted, the process begins with aseptic specimen receipt and primary inoculation onto selective and differential culture media, followed by incubation under standardized conditions to promote optimal bacterial growth. Subsequent evaluation of colony morphology particularly lactose fermentation characteristics on MacConkey agar allows preliminary differentiation of potential E. coli isolates. Gram staining is then performed to confirm the presence of Gram-negative bacilli, ensuring that only appropriate isolates proceed to further biochemical characterization. The workflow continues with confirmatory biochemical testing, including oxidase and catalase assays, to exclude non-Enterobacteriaceae organisms and validate enzymatic activity profiles consistent with E. coli. Final species-level identification is achieved using the Analytical Profile Index (API) system, where metabolic substrate utilization patterns generate a numerical profile that is interpreted against standardized databases [12]. By outlining each step in a systematic sequence, Figure 4 highlights the methodological rigor and quality control measures applied to ensure accurate and reproducible identification of clinical isolates prior to antimicrobial susceptibility testing and molecular analysis.

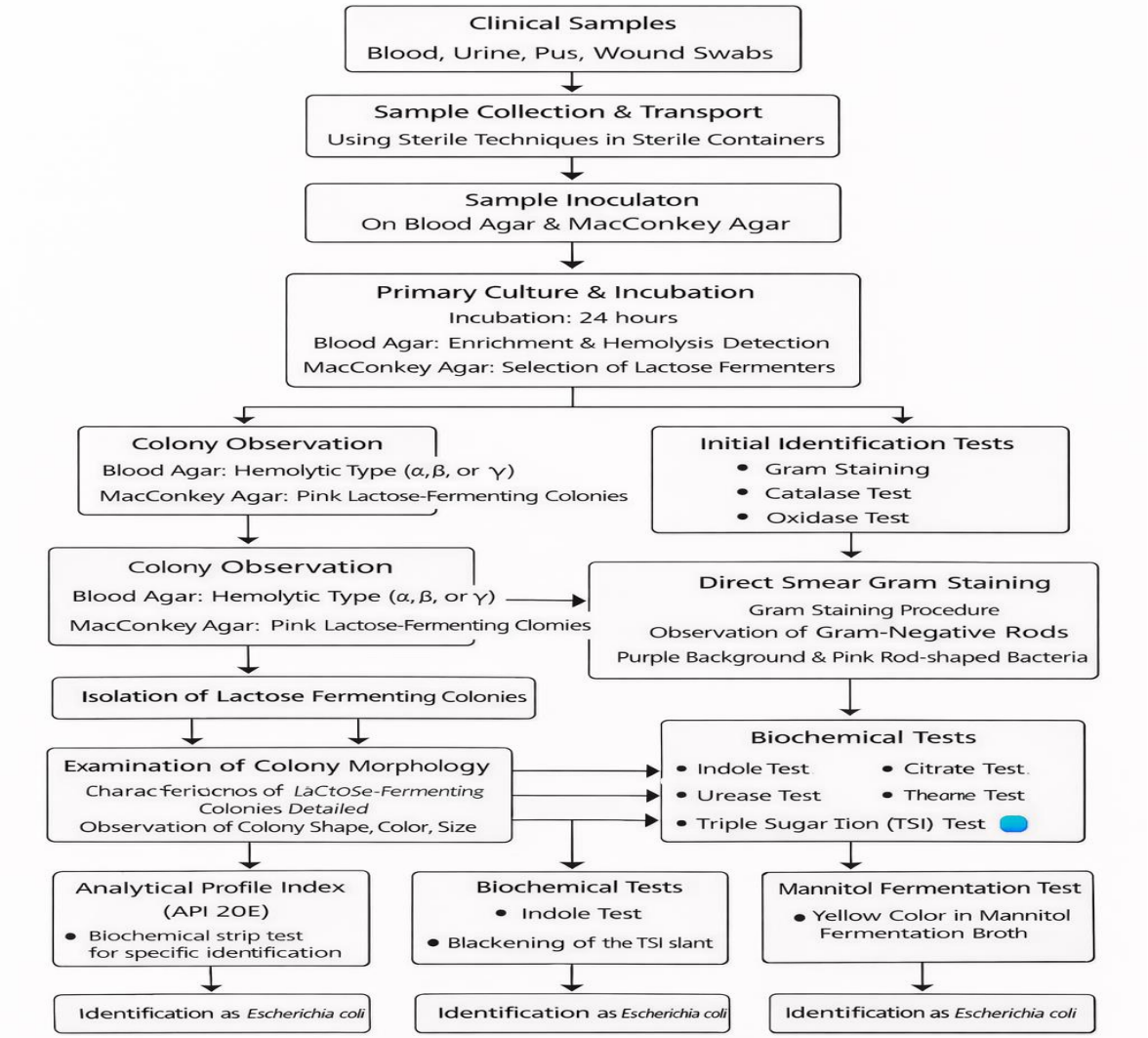


Figure 4: Workflow for isolation and identification of Escherichia coli

This comprehensive identification strategy ensured precise and reliable isolation of Escherichia coli from diverse clinical specimens before proceeding to antimicrobial susceptibility testing and molecular characterization. By integrating selective culture techniques, morphological evaluation, Gram staining, and confirmatory biochemical profiling, the study minimized the risk of misidentification and cross-contamination. The stepwise approach allowed differentiation of E. coli from other Gram-negative bacilli commonly encountered in clinical settings, thereby enhancing diagnostic specificity. Accurate species-level identification was critical for ensuring that subsequent phenotypic assays and genotypic analyses were performed exclusively on confirmed isolates, thereby preserving the validity of resistance profiling. Furthermore, implementation of standardized protocols and quality control measures improved reproducibility and reduced analytical variability. This rigorous identification framework established a robust foundation for downstream investigations into antimicrobial resistance patterns and β-lactamase gene detection, ultimately strengthening the overall reliability and scientific integrity of the study findings.

4.3- Antimicrobial Susceptibility Testing:

Antimicrobial susceptibility testing (AST) was conducted to determine the resistance profile of confirmed Escherichia coli isolates against

commonly prescribed antibiotics. The Kirby–Bauer disc diffusion method was employed using Mueller–Hinton (MH) agar, following the standardized procedures outlined in the Clinical and Laboratory Standards Institute (CLSI) M100, 31st Edition (2022) guidelines. The use of CLSI-recommended protocols ensured consistency, reproducibility, and international comparability of susceptibility results. Prior to inoculation, a standardized bacterial suspension equivalent to 0.5 McFarland turbidity standard (approximately 1.5×10^8 CFU/mL) was prepared by emulsifying 4–5 well-isolated colonies in sterile saline. The turbidity was adjusted visually and, when necessary, confirmed using a turbidity meter. Within 15 minutes of preparation, the standardized suspension was uniformly swabbed over the entire surface of MH agar plates to create a confluent bacterial lawn. Commercially prepared antibiotic discs were placed on the inoculated agar surface using sterile forceps or a disc dispenser, maintaining a minimum distance of 24 mm (center-to-center) to prevent overlapping zones of inhibition [13]. The selected antibiotics included representatives from different antimicrobial classes, with special emphasis on carbapenems due to their clinical importance in treating multidrug-resistant infections. Plates were incubated aerobically at 37°C for 18–24 hours. After incubation, the diameter of inhibition zones around each antibiotic disc was measured in millimeters using a calibrated ruler. Results were interpreted as Susceptible (S), Intermediate (I), or Resistant (R) according to CLSI breakpoint criteria. Carbapenem-resistant isolates identified through this screening were subsequently subjected to phenotypic and genotypic analyses for detection of β-lactamase mechanisms. The antibiotics tested and their respective CLSI breakpoint interpretations are summarized in Table 6.

Table 6: Antibiotics Used for Susceptibility Testing and CLSI Interpretation Criteria

Antibiotic	Class	Disc Potency	CLSI Interpretation Basis	Clinical Significance
Imipenem	Carbapenem	10 µg	Zone diameter (mm)	Detection of carbapenem resistance
Meropenem	Carbapenem	10 µg	Zone diameter (mm)	Last-line therapy indicator
Ceftriaxone	3rd Gen Cephalosporin	30 µg	Zone diameter (mm)	ESBL screening
Ceftazidime	3rd Gen Cephalosporin	30 µg	Zone diameter (mm)	Gram-negative coverage
Amoxicillin-Clavulanate	Penicillin + inhibitor	20/10 µg	Zone diameter (mm)	β-lactamase activity assessment
Ciprofloxacin	Fluoroquinolone	5 µg	Zone diameter (mm)	Broad-spectrum therapy
Gentamicin	Aminoglycoside	10 µg	Zone diameter (mm)	Combination therapy indicator

The procedural workflow for antimicrobial susceptibility testing is illustrated in Figure 5, which provides a systematic overview of the standardized steps followed in the Kirby–Bauer disc diffusion method. As depicted, the process begins with the preparation of a standardized bacterial inoculum adjusted to a 0.5 McFarland turbidity standard to ensure uniform cell density and reproducibility of results. This is followed by the even inoculation of Mueller–Hinton agar plates to create a confluent bacterial lawn, thereby allowing consistent diffusion of antibiotics from the discs into the surrounding medium. Subsequently, selected antibiotic discs are carefully

placed at appropriate distances to prevent overlapping zones of inhibition. After incubation under controlled temperature and time conditions, clear zones around each disc are measured in millimeters using a calibrated ruler or digital caliper. These measurements are then interpreted according to CLSI breakpoint criteria to classify isolates as susceptible, intermediate, or resistant [14]. The figure emphasizes the importance of maintaining standardized conditions at each stage including inoculum density, agar depth, disc spacing, and incubation parameters to minimize variability and ensure reliable antimicrobial susceptibility profiling. This standardized antimicrobial susceptibility testing protocol ensured accurate detection of resistant phenotypes, particularly carbapenem-resistant E. coli isolates, which were subsequently analyzed for β-lactamase production through phenotypic and molecular techniques. The integration of CLSI-based interpretation strengthened the validity and clinical relevance of the resistance data generated in this study.

4.4- Phenotypic Differentiation of Serine and Metallo-β-Lactamase:

Phenotypic differentiation of serine β-lactamase and metallo-β-lactamase (MBL) production among carbapenem-resistant *Escherichia coli* isolates was carried out using EDTA-based synergy assays. The rationale for employing EDTA in these assays is based on the biochemical mechanism of metallo-β-lactamases, which require divalent zinc ions at their active site for enzymatic hydrolysis of β-lactam antibiotics. Ethylenediaminetetraacetic acid (EDTA), a metal chelating agent, binds zinc ions and disrupts the catalytic activity of MBL enzymes, thereby restoring the inhibitory effect of carbapenem antibiotics in vitro. In contrast, serine β-lactamases do not rely on metal ions for activity and therefore remain unaffected by EDTA. This fundamental mechanistic difference allows phenotypic discrimination between these two major classes of carbapenemases. In the present study, carbapenem-resistant isolates identified through antimicrobial susceptibility testing were subjected to two principal EDTA-based approaches: the Combined Disc Test (CDT) and the Double Disc Synergy Test (DDST), each performed using two concentrations of EDTA (0.1 M and 0.5 M). For all assays, a standardized bacterial suspension equivalent to 0.5 McFarland turbidity was prepared and uniformly inoculated onto Mueller–Hinton agar plates to ensure consistent growth density. Imipenem (10 µg) discs were selected as the indicator antibiotic because carbapenems are primary substrates for MBL-mediated hydrolysis [15]. In the combined disc test, two imipenem discs were placed approximately 30 mm apart (center-to-center). A defined volume (10 µL) of EDTA solution at either 0.1 M or 0.5 M concentration was added to one of the discs to achieve the desired chelating effect. Following incubation at 37°C for 18–24 hours, inhibition zones around both discs were measured in millimeters. An increase in zone diameter of ≥7 mm around the imipenem–EDTA disc compared to imipenem alone was interpreted as indicative of MBL production. In the double disc synergy test, an EDTA-impregnated blank disc was placed approximately 20 mm away from an imipenem disc on the inoculated plate. After incubation, enhancement or distortion of the inhibition zone toward the EDTA disc was considered evidence of synergy, supporting MBL activity. The application of two EDTA concentrations allowed comparative evaluation of assay sensitivity and minimized the likelihood of false-negative results due to suboptimal chelation. Isolates that did not demonstrate significant zone enhancement in the presence of EDTA were considered negative for MBL production and were suggestive of alternative resistance mechanisms, including serine β-lactamase activity. A summary of the phenotypic tests employed in this study is presented in Table 7.

Table 7: Phenotypic assays used for differentiation of serine and metallo-β-lactamase

Assay Method	EDTA Concentration	Indicator Antibiotic	Interpretation Criterion	Enzyme Type Detected
Combined Disc Test (CDT)	0.5 M	Imipenem (10 µg)	≥7 mm zone increase	MBL
Combined Disc Test (CDT)	0.1 M	Imipenem (10 µg)	≥7 mm zone increase	MBL
Double Disc Synergy Test (DDST)	0.5 M	Imipenem (10 µg)	Zone enhancement toward EDTA	MBL
Double Disc Synergy Test (DDST)	0.1 M	Imipenem (10 µg)	Zone enhancement toward EDTA	MBL

The overall laboratory configuration and interpretative framework of the EDTA-based phenotypic assays are illustrated in Figure 6, which provides a comprehensive visual representation of the methodological setup and analytical criteria employed for the detection of metallo-β-lactamase (MBL) activity. As shown in the figure, the assay involves the strategic placement of imipenem discs alone and in combination with EDTA-impregnated discs on a uniformly inoculated Mueller–Hinton agar plate. The spatial arrangement of discs, including standardized center-to-center distances, ensures optimal diffusion gradients and reliable comparison of inhibition zones. Following incubation, the inhibition zones surrounding each disc are carefully measured and evaluated for evidence of zone enhancement or distortion toward the EDTA-containing disc [16]. The figure further demonstrates how chelation of zinc ions by EDTA disrupts the catalytic activity of MBL enzymes, leading to restoration or expansion of the carbapenem inhibition zone in MBL-producing isolates. In contrast, isolates harboring serine β-lactamases typically do not exhibit significant changes in zone diameter upon EDTA exposure, thereby allowing differentiation based on inhibitor response patterns. By visually summarizing disc placement strategy, diffusion dynamics, and synergistic enhancement patterns, Figure 5 reinforces the interpretative criteria used to classify isolates as MBL-positive or MBL-negative. This structured representation supports methodological transparency and ensures reproducibility of phenotypic detection procedures in routine clinical microbiology laboratories.

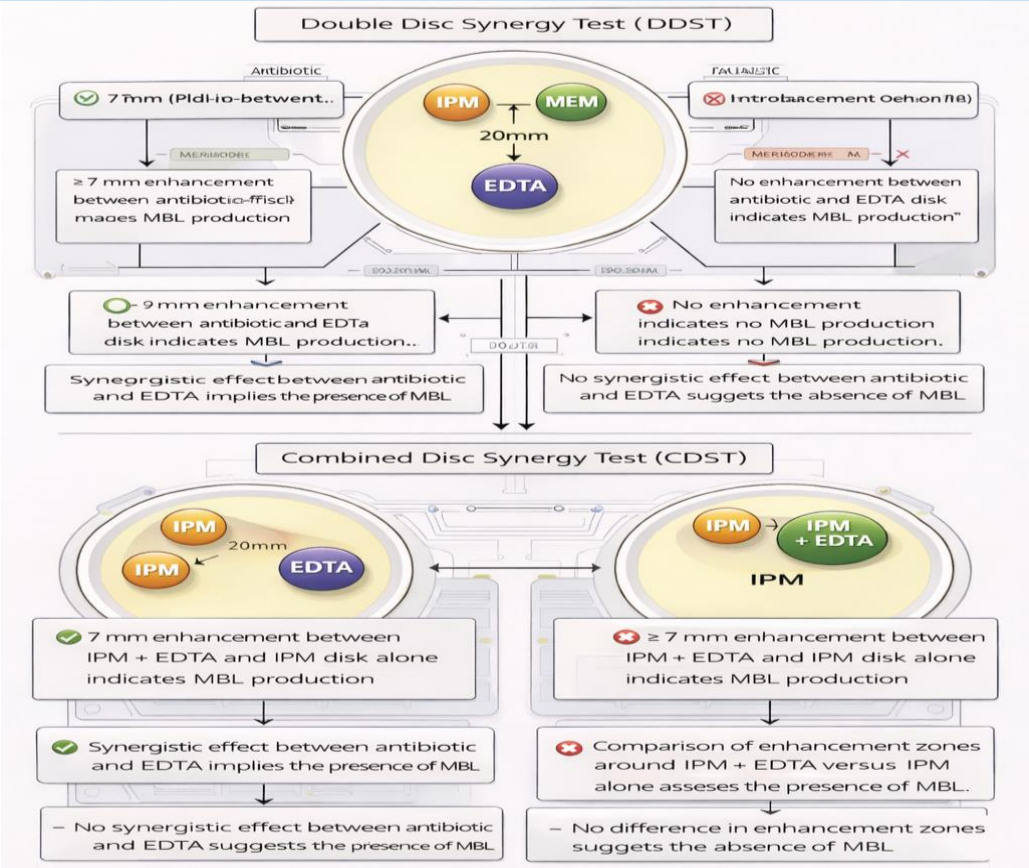


Figure 5: EDTA-based phenotypic detection of metallo-β-lactamase activity

The integration of multiple phenotypic assays increased diagnostic reliability and provided preliminary classification of resistance mechanisms prior to molecular confirmation. Although EDTA-based methods are cost-effective and suitable for routine clinical laboratories, their interpretation may be influenced by borderline zone differences and variable enzyme expression. Therefore, phenotypic findings were subsequently validated using polymerase chain reaction (PCR) targeting specific β-lactamase genes to ensure accurate classification of resistance determinants.

4.5- Genotypic Detection of β-Lactamase Genes:

Molecular detection of β-lactamase genes was performed to accurately characterize the genetic determinants responsible for serine and metallo-β-lactamase production in phenotypically identified Escherichia coli isolates. While phenotypic assays provide preliminary evidence of enzyme activity, molecular analysis offers definitive confirmation through direct detection of resistance genes. Therefore, polymerase chain reaction (PCR)-based amplification of specific β-lactamase genes was employed to validate the presence of blaSHV (serine β-lactamase) and blaIMP (metallo-β-lactamase) genes [17]. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method, a reliable and cost-effective protocol for purification of high-quality bacterial DNA. Fresh overnight cultures of confirmed *E. coli* isolates were used as the starting material. A loopful of bacterial colonies was suspended in CTAB extraction buffer containing CTAB, Tris-HCl (pH stabilization), EDTA (metal chelator), and NaCl (ionic strength regulator). The mixture was incubated at 65°C to promote cell wall disruption and release of intracellular contents. Protein denaturation and removal were achieved through chloroform-isoamyl alcohol extraction, followed by centrifugation at high speed to separate the aqueous DNA-containing phase from organic contaminants. DNA

precipitation was performed using chilled reagents, and the resulting pellet was washed, air-dried, and resuspended in nuclease-free water. The purified genomic DNA was stored at −20°C until amplification. DNA integrity and purity were evaluated by agarose gel visualization and, where applicable, spectrophotometric assessment. PCR amplification was carried out using gene-specific primers targeting conserved regions of blaSHV and blaIMP genes. Each reaction was prepared in a final volume of 25 µL containing 2× PCR master mix (including Taq DNA polymerase, dNTPs, $MgCl_2$, and buffer), forward and reverse primers (10 pmol each), template DNA, and nuclease-free water. Amplification reactions were performed using a BIO-RAD T100™ thermal cycler under optimized cycling conditions. The thermal profile consisted of an initial denaturation step at 95°C for 5 minutes to ensure complete DNA strand separation, followed by 35 amplification cycles comprising denaturation at 95°C, annealing at 50°C to allow primer binding, and extension at 72°C for DNA synthesis [18]. A final extension step at 72°C ensured complete elongation of amplified fragments. PCR products were analyzed using 1.5% agarose gel electrophoresis prepared in 1× TBE buffer. Ethidium bromide was incorporated into the gel matrix for DNA staining, enabling visualization under ultraviolet (UV) illumination. A molecular weight DNA ladder was loaded alongside samples to confirm amplicon size. The expected product sizes were 713 base pairs (bp) for blaSHV and 470 bp for blaIMP. Clear, distinct bands corresponding to these sizes were interpreted as positive gene amplification, confirming the presence of respective β-lactamase genes. The primer sequences, amplicon sizes, and optimized PCR conditions used in this study are summarized in Table 8.

Table 8: Molecular parameters for detection of blaSHV and blaIMP genes

Target Gene	Primer Sequence (5′–3′)	Amplicon Size	Annealing Temp.	Final Extension	Total Cycles
blaSHV	F: AGCCGCTTGAGCAAATTAAAC	713 bp	50°C	72°C, 10 min	35
	R: ATCCGCAGATAAATCACCAC				
blaIMP	F: TTGACACTCCATTTACGGCT	470 bp	50°C	72°C, 10 min	35
	R: TGCGTCTCCAACTTCACTGT				

The comprehensive molecular workflow from genomic DNA extraction to PCR amplification and subsequent agarose gel electrophoresis analysis is depicted in Figure 7, which provides a systematic representation of the stepwise procedures undertaken for genotypic confirmation of β-lactamase genes. As illustrated, the process begins with careful extraction of high-quality bacterial DNA using the CTAB method, ensuring effective cell lysis, removal of protein contaminants, and purification of nucleic acids suitable for downstream amplification [19]. The integrity and purity of the extracted DNA are critical, as they directly influence the efficiency and specificity of PCR amplification. Following DNA isolation, the workflow proceeds to preparation of the PCR reaction mixture, including optimized concentrations of primers, master mix components, and template DNA. The figure further outlines the thermal cycling program applied in the BIO-RAD T100™ thermal cycler, highlighting the sequential phases of denaturation, annealing, and extension that enable exponential amplification of the target blaSHV and blaIMP gene fragments. The importance of precise annealing temperature and cycle optimization is emphasized to minimize non-specific amplification and ensure accurate detection of resistance determinants. The final stage illustrated in Figure 7 involves separation of amplified products on a 1.5% agarose gel, staining with ethidium bromide, and visualization under

ultraviolet illumination. Distinct DNA bands corresponding to the expected amplicon sizes confirm the presence of specific β-lactamase genes. By presenting the entire molecular detection pathway in a structured and sequential format, Figure 6 enhances methodological transparency, demonstrates procedural rigor, and supports reproducibility of genotypic analysis in clinical microbiology laboratories.

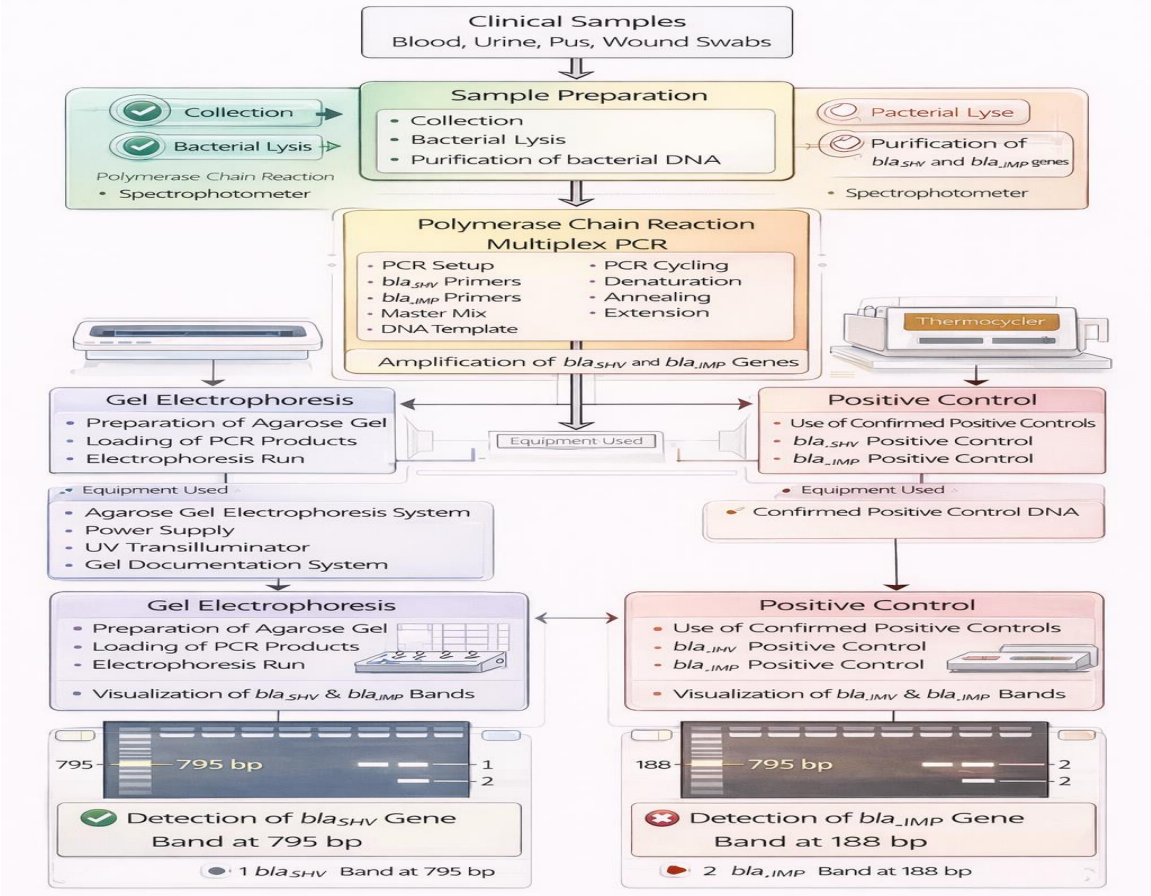


Figure 6: Workflow of molecular detection of blaSHV and blaIMP genes

Statistical analysis of genotypic data was performed using SPSS version 20.0. The prevalence of blaSHV and blaIMP genes was calculated as percentages of total isolates tested. Associations between phenotypic resistance patterns and genotypic findings were evaluated using appropriate statistical tests. A p-value less than 0.05 was considered statistically significant at a 95% confidence interval. Comparative analysis between phenotypic EDTA-based assays and PCR results was conducted to assess diagnostic agreement and validate molecular confirmation.

5- Results and Discussion:

The present study was carried out at BJ Micro Lab (Private) Limited in collaboration with Abasyn University, Islamabad, with the objective of investigating antimicrobial resistance patterns and β-lactamase gene distribution among clinical Escherichia coli isolates. The laboratory setting provided access to a diverse range of clinical specimens submitted from a tertiary care hospital environment, thereby ensuring representation of multiple infection types and patient demographics. During the defined study period, a total of 300 clinical specimens were systematically collected, processed, and analyzed using

standardized microbiological procedures. Each specimen underwent culture, isolation, and preliminary identification using conventional morphological techniques, followed by biochemical confirmation and subsequent molecular validation where required [20]. Out of the total 300 specimens processed, 50 isolates (16.66%) were confirmed as Escherichia coli. Confirmation was achieved through a combination of colony morphology assessment on selective media, Gram staining, biochemical profiling including API testing, and molecular characterization to ensure diagnostic accuracy and eliminate misidentification. The integration of phenotypic and genotypic approaches strengthened the reliability of species confirmation. The confirmed E. coli isolates were recovered from patients representing various age groups and both genders, reflecting the broad epidemiological distribution of E. coli-associated infections within the hospital population. This demographic diversity enhanced the representativeness of the sample set and provided a robust foundation for subsequent antimicrobial susceptibility testing and β-lactamase gene detection analyses. The demographic distribution of the 300 patients included in this study is summarized in Table 9. The majority of patients belonged to the 41–50 years age group (30.0%), followed by >50 years (26.7%). Female patients constituted a higher proportion (67.0%) compared to male patients (33.0%).

Table 9: Age and Gender-wise Distribution of Patients (n = 300)

Variable	Groups	Frequency (%)
Age	20–30 years	60 (20.0%)
	31–40 years	70 (23.3%)
	41–50 years	90 (30.0%)
	>50 years	80 (26.7%)
Gender	Male	99 (33.0%)
	Female	201 (67.0%)

Out of the total 300 clinical specimens collected and processed during the study period, 50 samples (16.66%) yielded confirmed Escherichia coli isolates following culture, morphological examination, and biochemical verification. This isolation rate reflects the proportion of clinically significant E. coli infections detected among the submitted specimens and underscores the role of this organism as a common etiological agent in both community- and hospital-acquired infections. The confirmed isolates were recovered from a range of specimen types, representing different anatomical sites and infection categories, including bloodstream infections, urinary tract infections, respiratory tract infections, and device-associated infections. The variability in isolation frequency across specimen categories provides insight into the clinical distribution of E. coli within the hospital setting and may reflect differences in infection prevalence, patient comorbidities, and exposure to invasive medical devices [21]. A detailed breakdown of isolate distribution according to specimen type, including the total number of samples processed and the corresponding number and percentage of E. coli isolates recovered from each category, is presented in Table 10. This tabulated data facilitates comparison of isolation rates across clinical sources and supports further interpretation of infection patterns observed in the study population.

Table 10: Distribution of E. coli Isolates from Clinical Specimens

Sample Type	Total Samples (N)	Number of Isolates (%)
Blood	142	22 (15.49%)
Catheter tip	83	16 (19.27%)
Endotracheal tube secretions	39	08 (20.51%)

Urine	36	04 (11.11%)
Total	**300**	**50 (16.66%)**

Endotracheal tube secretions showed the highest proportion of E. coli isolation (20.51%), followed by catheter tips (19.27%). Urine samples showed the lowest isolation rate (11.11%). On MacConkey agar, lactose-fermenting colonies appeared as pink colonies, confirming lactose fermentation ability. Among the 50 isolates, 14 lactose-fermenting isolates were selected for further detailed analysis (Figure 7).

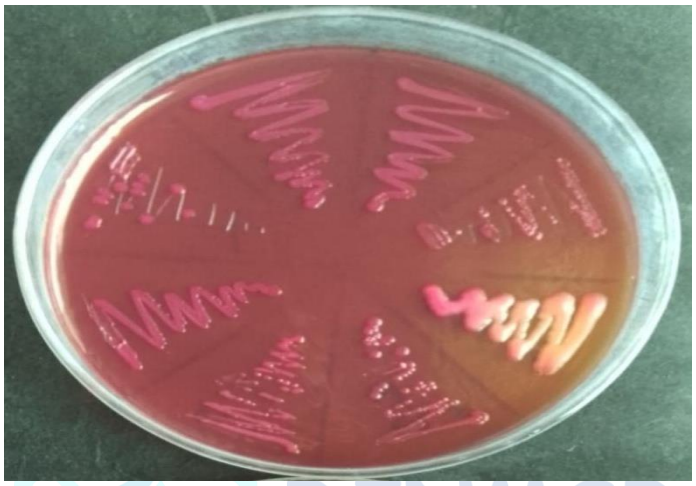


Figure 7: Lactose fermenting Gram Negative bacteria

Microscopic examination of the bacterial smears following Gram staining demonstrated the presence of Gram-negative, short rod-shaped bacilli, consistent with the morphological characteristics of Escherichia coli. The organisms appeared as pink to reddish cells under oil immersion microscopy due to retention of the counterstain (safranin), confirming their Gram-negative cell wall structure. The bacilli were observed predominantly as single cells, although occasional short chains and paired arrangements were also noted. The uniformity in cell size, smooth cellular outline, and absence of spore formation further supported the identification of these isolates as members of the Enterobacteriaceae family. The Gram-staining results served as a critical preliminary diagnostic step, allowing differentiation from Gram-positive cocci and other morphologically distinct bacteria that may be present in clinical specimens. Only isolates exhibiting typical Gram-negative bacillary morphology were subjected to further biochemical and molecular confirmation. The representative microscopic morphology of the isolates is illustrated in Figure 8, which depicts the characteristic appearance of Gram-negative rods observed during the study.

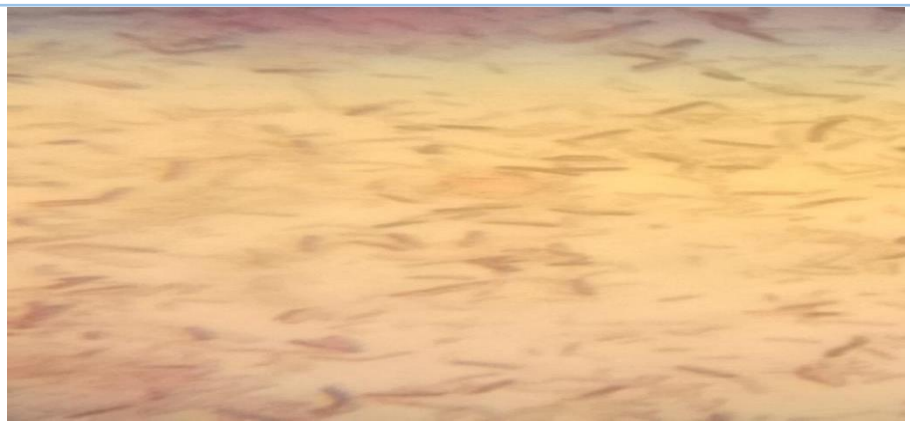


Figure 8: Gram-negative rods

All confirmed isolates exhibited a consistent biochemical profile characteristic of Escherichia coli, demonstrating oxidase-negative and catalase-positive reactions. The oxidase test yielded no color change upon application of oxidase reagent, confirming the absence of cytochrome c oxidase activity and thereby supporting classification within the Enterobacteriaceae family. This result effectively differentiated the isolates from non-fermentative Gram-negative organisms such as Pseudomonas species, which are oxidase-positive. In contrast, the catalase test produced immediate bubble formation upon exposure to 3% hydrogen peroxide, indicating the presence of catalase enzyme activity and the organism's ability to decompose hydrogen peroxide into water and oxygen. To further ensure species-level confirmation and eliminate the possibility of misidentification based solely on conventional biochemical reactions, the isolates were subjected to Analytical Profile Index (API) testing [22]. The API system evaluates multiple metabolic and enzymatic reactions simultaneously, generating a numerical biochemical profile that is interpreted against an established database. The resulting API codes corresponded with high probability to E. coli, thereby confirming the identity of the isolates with greater diagnostic accuracy. The representative biochemical reactions and API test results are illustrated in Figure 9, which demonstrates the standardized identification approach employed in this study.

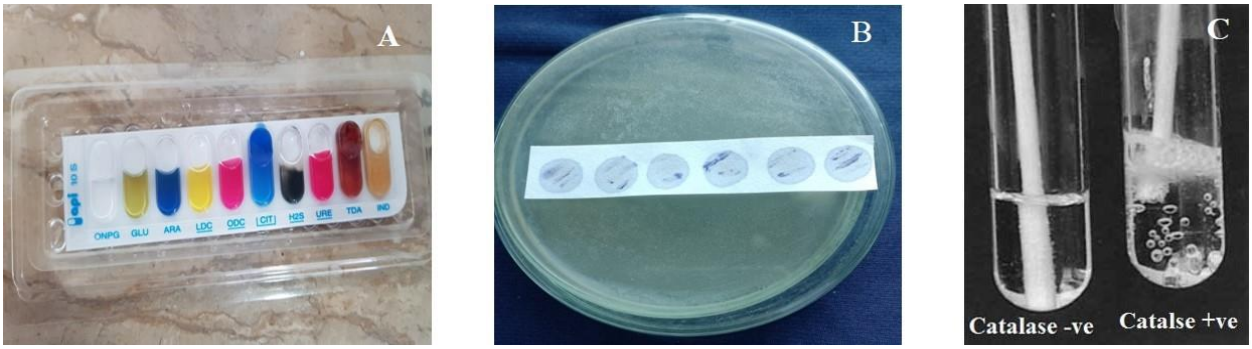


Figure 9: (A) API testing, (B) Oxidase test, (C) Catalase test

A total of 14 isolates were tested for antibiotic sensitivity pattern. Out of which 10 samples of E. coli isolates showed resistance to carbapenem, a small number of isolates showed perfect susceptibility to carbapenem. 10 bacterial isolates which were resistant to carbapenem, were selected for further evaluation.

Table 11: Resistance and Sensitivity pattern of E. coli against carbapenem drug

Total No. of Samples	Resistant	Sensitive
14	10	4

In 10 of the 14 clinical isolates of E. coli that tested positive for MBL screening were resistant to IPM. The 10 IPM-resistant isolates underwent additional processing to validate their identity using several techniques for phenotypic MBL producer recognition. 10 (71.42%) of the 14 *E. coli* isolates in this investigation tested positive for MBL production. Using the CDST 0.1 M EDTA method, 06 (60%) of the 10 isolates were MBL positive; the DDST 0.1 M EDTA method produced 5 (50%) and the DDST 0.5 M EDTA method produced 4 (40%) MBL positive isolates (Figure 10).

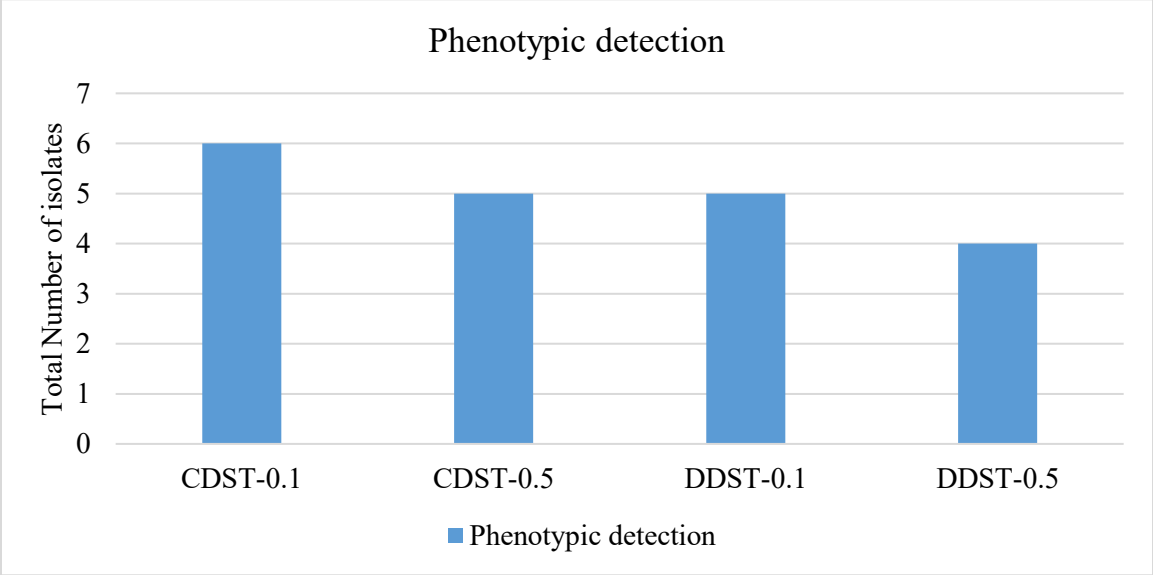


Figure 10: Phenotypic analysis for the detection of serine and metallo β lactamase.

Table 12 presents the detailed measurements of inhibition zones obtained from the Combined Disc Synergy Test (CDST) performed using imipenem discs alone and in combination with two different concentrations of ethylenediaminetetraacetic acid (EDTA), specifically 0.5 M and 0.1 M. The zone of inhibition (ZOI) values, recorded in millimeters, were measured following incubation on Mueller–Hinton agar plates inoculated with carbapenem-resistant Escherichia coli isolates. Comparison of inhibition zones between imipenem alone and imipenem supplemented with EDTA allowed phenotypic identification of metallo-β-lactamase (MBL) activity based on the predefined criterion of a ≥7 mm increase in zone diameter. The table provides a quantitative assessment of EDTA-mediated enhancement effects for each isolate, facilitating evaluation of MBL production patterns and comparison between the two EDTA concentrations.

Table 12: Combined disk synergy test with 0.5 M and 0.1 M ethylene diamine tetra acetic acid zone of inhibition (ZOI)

Sample No.	IPM	IPM+0.5 M EDTA	IPM+0.1 M EDTA
E1 A	21mm	23mm	23mm
E2 A	26mm	29mm	25mm
E4 A	24mm	29mm	24mm

E5 A	28mm	32mm	30mm
E1 P	30mm	31mm	30mm
E2 P	21mm	23mm	22mm
E 5	25mm	30mm	28mm
E 6	22mm	25mm	22mm
E 7	No Activity	25mm	10mm
E 11	25mm	22mm	21mm

Table 13 summarizes the zone of inhibition (ZOI) measurements obtained from the Double Disc Synergy Test (DDST) performed using imipenem discs in conjunction with EDTA at concentrations of 0.5 M and 0.1 M. The inhibition zones, measured in millimeters after incubation on Mueller–Hinton agar plates inoculated with carbapenem-resistant Escherichia coli isolates, were evaluated to determine the presence of metallo-β-lactamase (MBL) activity. In this assay, enhancement or distortion of the inhibition zone toward the EDTA-impregnated disc was interpreted as indicative of zinc chelation–mediated inhibition of MBL enzymes. The table provides isolate-specific comparative data, enabling assessment of synergistic effects between imipenem and EDTA at varying concentrations. These quantitative findings contribute to the phenotypic differentiation of MBL-producing isolates from those harboring serine β-lactamases or other resistance mechanisms.

Table 13: Double-disk synergy test with 0.5 M and 0.1M ethylene diamine tetra acetic acid zone of inhibition (ZOI)

Sample No.	IPM	0.5 M EDTA	0.1 M EDTA
E1 A	23mm	21mm	11mm
E2 A	26mm	20mm	8mm
E4 A	23mm	20mm	15mm
E5 A	28mm	23mm	11mm
E1 P	30mm	33mm	24mm
E2 P	20mm	12mm	10mm
E 5	28mm	18mm	10mm
E 6	20mm	18mm	9mm
E 7	No Activity	20mm	11mm
E 11	20mm	19mm	10mm
E1 A	22mm	28mm	18mm

In-silico PCR was used to simulate the polymerase chain reaction (PCR) process computationally. This technique helped design primers, optimize PCR conditions, and analyze DNA sequences. Table 14 shows the Sequence of blaSHV primers along with their annealing temperature and product size

Table 14: Sequence of blaSHV primers along with their annealing temperature and product size

Primer	Sequence	Ann. Temp	Product size
Bla_{SHV}	F' AGCCGCTTGAGCAAATTAAAC R' ATCCCGCAGATAAATCACCAC	50-51°C	713bp

F' AGCCGCTTGAGCAAATTAAAC

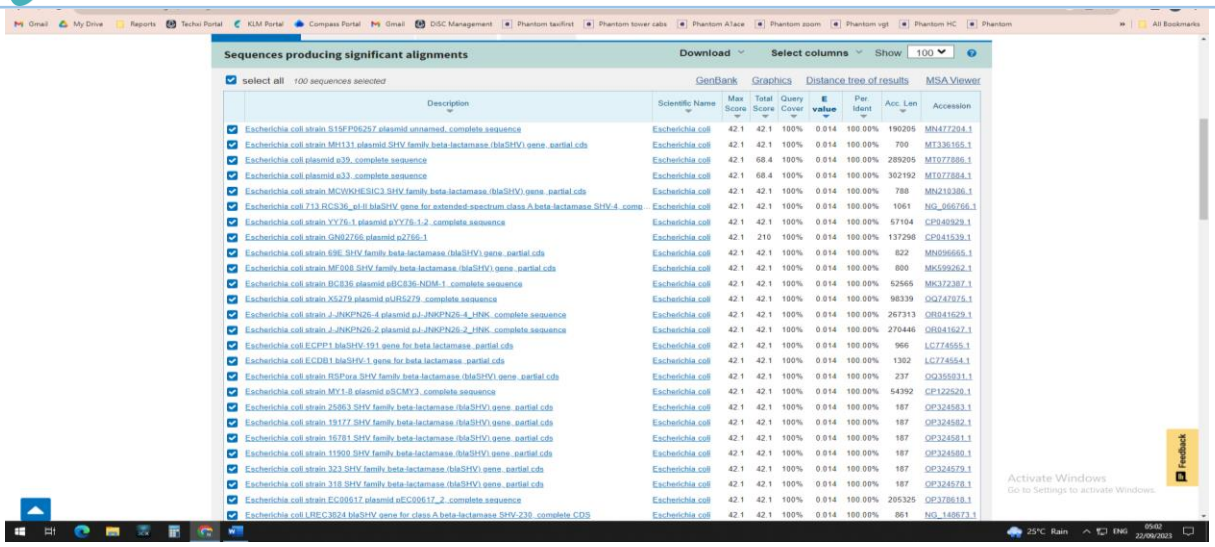


Figure 11: blaSHV forward sequence

R' ATCCCGCAGATAAATCACCAC

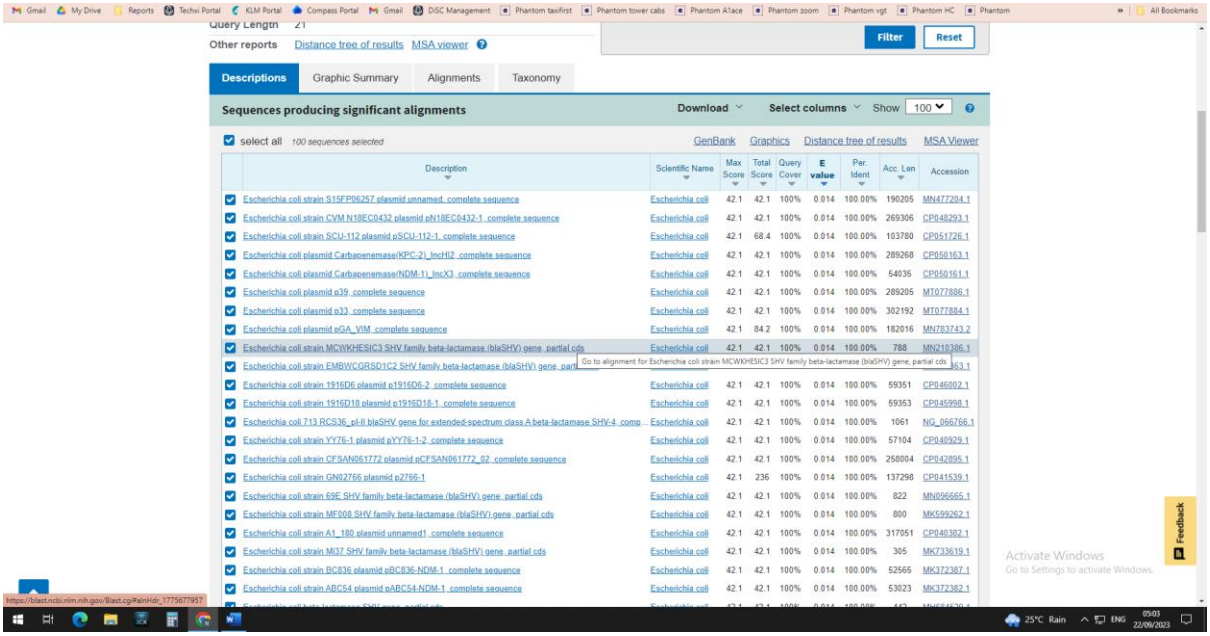
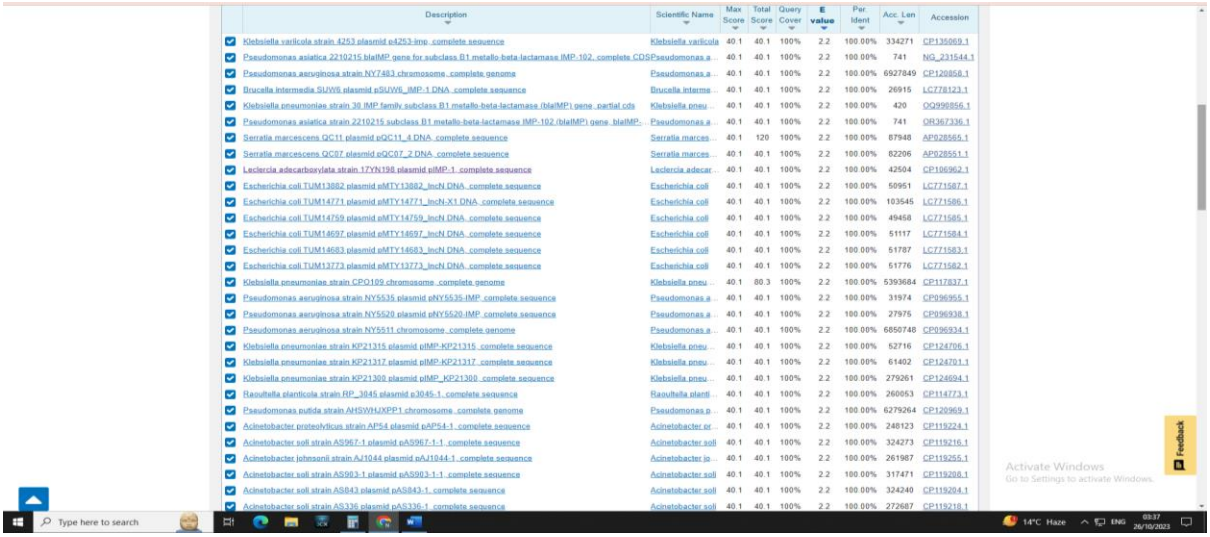


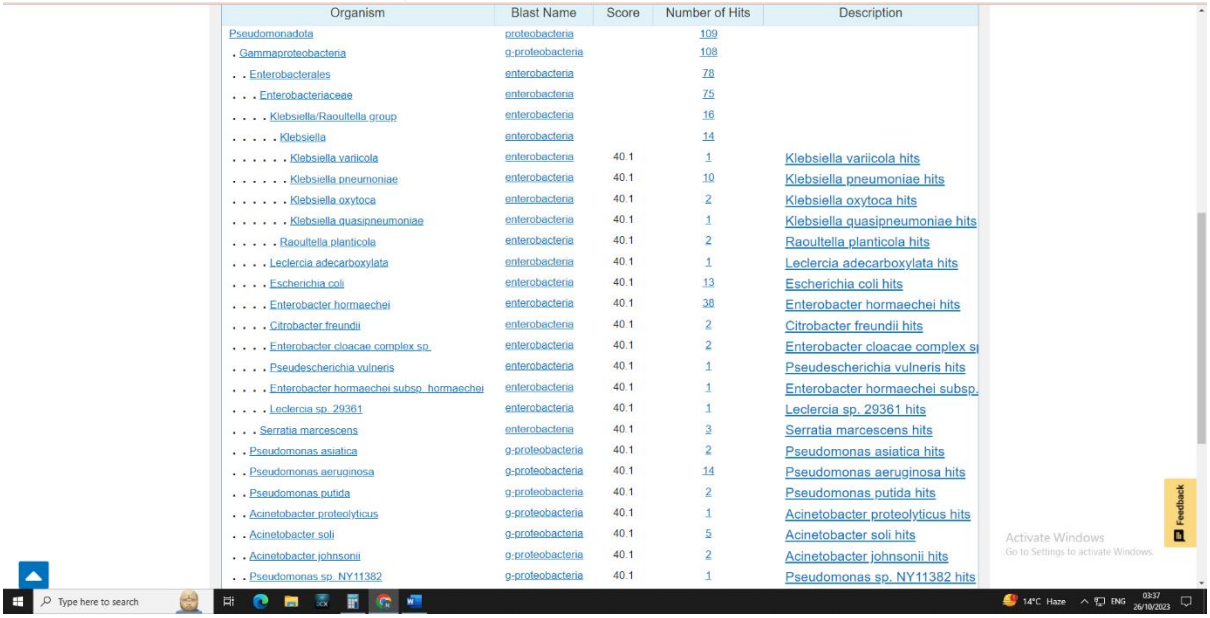
Figure 12: blaSHV reverse sequence

F' TTGACACTCCATTTACGGCT



Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc. Len	Accession
Klebsiella variicola strain 4253 plasmid p4253-imp, complete sequence	Klebsiella variicola	40.1	40.1	100%	2.2	100.00%	334271	CP135069.1
Pseudomonas asiatica 2210215 blaIMP gene for subclass B1 metallo-beta-lactamase IMP-102, complete CDS	Pseudomonas a...	40.1	40.1	100%	2.2	100.00%	741	NG_231544.1
Pseudomonas aeruginosa strain NY7483 chromosome, complete genome	Pseudomonas a...	40.1	40.1	100%	2.2	100.00%	6927849	CP120858.1
Brucella intermedia SUW6 plasmid pSUW6_IMP-1 DNA, complete sequence	Brucella interme...	40.1	40.1	100%	2.2	100.00%	26915	LC778123.1
Klebsiella pneumoniae strain 30 IMP family subclass B1 metallo-beta-lactamase (blaIMP1 gene, partial cds	Klebsiella pneu...	40.1	40.1	100%	2.2	100.00%	420	OQ996856.1
Pseudomonas asiatica strain 2210215 subclass B1 metallo-beta-lactamase IMP-102 (blaIMP) gene, blaIMP-...	Pseudomonas a...	40.1	40.1	100%	2.2	100.00%	741	OR367336.1
Serratia marcescens QC11 plasmid pQC11_4 DNA, complete sequence	Serratia marces...	40.1	120	100%	2.2	100.00%	87948	AP028565.1
Serratia marcescens QC07 plasmid pQC07_2 DNA, complete sequence	Serratia marces...	40.1	40.1	100%	2.2	100.00%	82206	AP028551.1
Leclercia adecarboxylata strain 17YN198 plasmid pIMP-1, complete sequence	Leclercia adecar...	40.1	40.1	100%	2.2	100.00%	42504	CP106962.1
Escherichia coli TUM13882 plasmid pMTY13882_IncN DNA, complete sequence	Escherichia coli	40.1	40.1	100%	2.2	100.00%	50951	LC771587.1
Escherichia coli TUM14771 plasmid pMTY14771_IncN-X1 DNA, complete sequence	Escherichia coli	40.1	40.1	100%	2.2	100.00%	103545	LC771586.1
Escherichia coli TUM14759 plasmid pMTY14759_IncN DNA, complete sequence	Escherichia coli	40.1	40.1	100%	2.2	100.00%	49458	LC771585.1
Escherichia coli TUM14697 plasmid pMTY14697_IncN DNA, complete sequence	Escherichia coli	40.1	40.1	100%	2.2	100.00%	51117	LC771584.1
Escherichia coli TUM14683 plasmid pMTY14683_IncN DNA, complete sequence	Escherichia coli	40.1	40.1	100%	2.2	100.00%	51787	LC771583.1
Escherichia coli TUM13773 plasmid pMTY13773_IncN DNA, complete sequence	Escherichia coli	40.1	40.1	100%	2.2	100.00%	51776	LC771582.1
Klebsiella pneumoniae strain CPO109 chromosome, complete genome	Klebsiella pneu...	40.1	80.3	100%	2.2	100.00%	5393684	CP117837.1
Pseudomonas aeruginosa strain NY5535 plasmid pNY5535-IMP, complete sequence	Pseudomonas a...	40.1	40.1	100%	2.2	100.00%	31974	CP096955.1
Pseudomonas aeruginosa strain NY5520 plasmid pNY5520-IMP, complete sequence	Pseudomonas a...	40.1	40.1	100%	2.2	100.00%	27975	CP096938.1
Pseudomonas aeruginosa strain NY5511 chromosome, complete genome	Pseudomonas a...	40.1	40.1	100%	2.2	100.00%	6850748	CP096934.1
Klebsiella pneumoniae strain KP21315 plasmid pIMP-KP21315, complete sequence	Klebsiella pneu...	40.1	40.1	100%	2.2	100.00%	52716	CP124706.1
Klebsiella pneumoniae strain KP21317 plasmid pIMP-KP21317, complete sequence	Klebsiella pneu...	40.1	40.1	100%	2.2	100.00%	61402	CP124701.1
Klebsiella pneumoniae strain KP21300 plasmid pIMP_KP21300, complete sequence	Klebsiella pneu...	40.1	40.1	100%	2.2	100.00%	279261	CP124694.1
Raoultella planticola strain RP_3045 plasmid p3045-1, complete sequence	Raoultella planti...	40.1	40.1	100%	2.2	100.00%	260053	CP114773.1
Pseudomonas putida strain AHSWHJXPP1 chromosome, complete genome	Pseudomonas p...	40.1	40.1	100%	2.2	100.00%	6279264	CP120969.1
Acinetobacter proteolyticus strain AP54 plasmid pAP54-1, complete sequence	Acinetobacter pr...	40.1	40.1	100%	2.2	100.00%	248123	CP119224.1
Acinetobacter soli strain AS967-1 plasmid pAS967-1-1, complete sequence	Acinetobacter soli	40.1	40.1	100%	2.2	100.00%	324273	CP119216.1
Acinetobacter johnsonii strain AJ1044 plasmid pAJ1044-1, complete sequence	Acinetobacter jo...	40.1	40.1	100%	2.2	100.00%	261987	CP119255.1
Acinetobacter soli strain AS903-1 plasmid pAS903-1-1, complete sequence	Acinetobacter soli	40.1	40.1	100%	2.2	100.00%	317471	CP119208.1
Acinetobacter soli strain AS843 plasmid pAS843-1, complete sequence	Acinetobacter soli	40.1	40.1	100%	2.2	100.00%	324240	CP119204.1
Acinetobacter soli strain AS336 plasmid pAS336-1, complete sequence	Acinetobacter soli	40.1	40.1	100%	2.2	100.00%	272687	CP119218.1

Figure 13: (a) blaIMP forward reaction



Organism	Blast Name	Score	Number of Hits	Description
Pseudomonadota	proteobacteria		109	
. Gammaproteobacteria	g-proteobacteria		108	
. . Enterobacterales	enterobacteria		78	
. . . Enterobacteriaceae	enterobacteria		75	
. . . . Klebsiella/Raoultella group	enterobacteria		16	
. . . . . Klebsiella	enterobacteria		14	
. . . . . Klebsiella variicola	enterobacteria	40.1	1	Klebsiella variicola hits
. . . . . Klebsiella pneumoniae	enterobacteria	40.1	10	Klebsiella pneumoniae hits
. . . . . Klebsiella oxytoca	enterobacteria	40.1	2	Klebsiella oxytoca hits
. . . . . Klebsiella quasipneumoniae	enterobacteria	40.1	1	Klebsiella quasipneumoniae hits
. . . . . Raoultella planticola	enterobacteria	40.1	2	Raoultella planticola hits
. . . . Leclercia adecarboxylata	enterobacteria	40.1	1	Leclercia adecarboxylata hits
. . . . Escherichia coli	enterobacteria	40.1	13	Escherichia coli hits
. . . . Enterobacter hormaechei	enterobacteria	40.1	38	Enterobacter hormaechei hits
. . . . Citrobacter freundii	enterobacteria	40.1	2	Citrobacter freundii hits
. . . . Enterobacter cloacae complex sp.	enterobacteria	40.1	2	Enterobacter cloacae complex sp
. . . . Pseudescherichia vulneris	enterobacteria	40.1	1	Pseudescherichia vulneris hits
. . . . Enterobacter hormaechei subsp. hormaechei	enterobacteria	40.1	1	Enterobacter hormaechei subsp.
. . . . Leclercia sp. 29361	enterobacteria	40.1	1	Leclercia sp. 29361 hits
. . . Serratia marcescens	enterobacteria	40.1	3	Serratia marcescens hits
. . Pseudomonas asiatica	g-proteobacteria	40.1	2	Pseudomonas asiatica hits
. . Pseudomonas aeruginosa	g-proteobacteria	40.1	14	Pseudomonas aeruginosa hits
. . Pseudomonas putida	g-proteobacteria	40.1	2	Pseudomonas putida hits
. . Acinetobacter proteolyticus	g-proteobacteria	40.1	1	Acinetobacter proteolyticus hits
. . Acinetobacter soli	g-proteobacteria	40.1	5	Acinetobacter soli hits
. . Acinetobacter johnsonii	g-proteobacteria	40.1	2	Acinetobacter johnsonii hits
. . Pseudomonas sp. NY11382	g-proteobacteria	40.1	1	Pseudomonas sp. NY11382 hits

Figure 13: (b) blaIMP forward reaction

R' TGCGTCTCCAACTTCACTGT

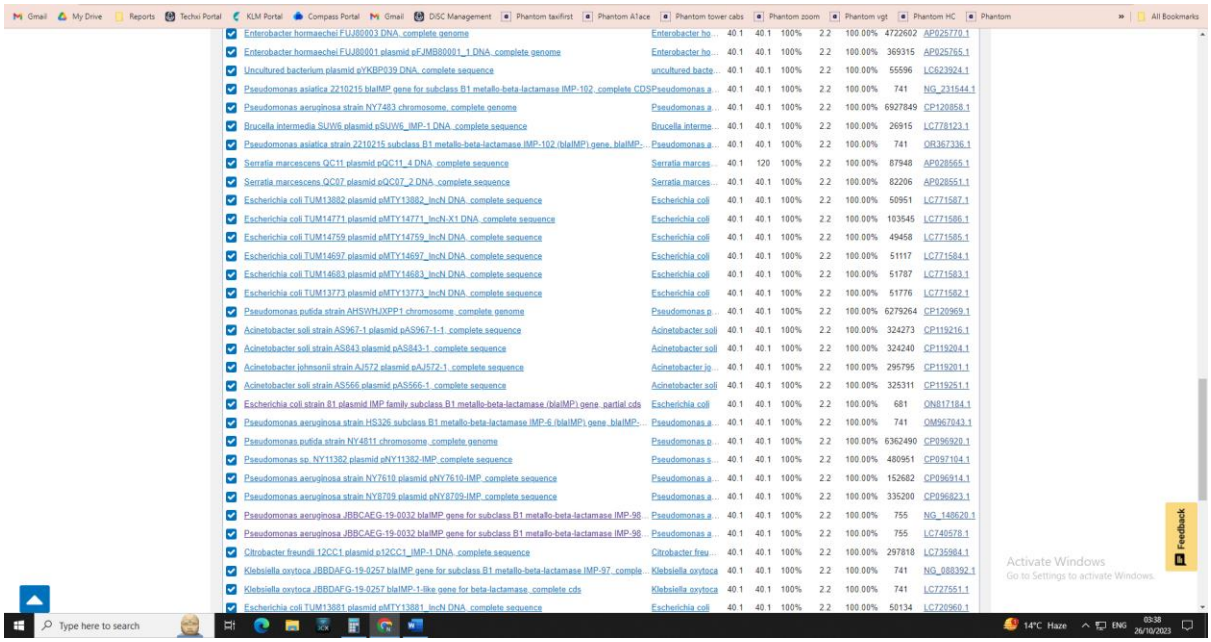


Figure 14: (a) blaIMP reverse reaction

Organism	Blast Name	Score	Number of Hits	Description
Bacteria	bacteria		111	
. Pseudomonadota	proteobacteria		110	
. . Gammaproteobacteria	g-proteobacteria		108	
. . . Enterobacterales	enterobacteria		78	
. . . . Enterobacteriaceae	enterobacteria		74	
. . . . . Leclercia adecarboxylata	enterobacteria	40.1	1	Leclercia adecarboxylata hits
. . . . . Enterobacter hormaechei subsp. hormaechei	enterobacteria	40.1	1	Enterobacter hormaechei subsp.
. . . . . Enterobacter hormaechei	enterobacteria	40.1	52	Enterobacter hormaechei hits
. . . . . Escherichia coli	enterobacteria	40.1	17	Escherichia coli hits
. . . . . Citrobacter freundii	enterobacteria	40.1	1	Citrobacter freundii hits
. . . . . Klebsiella oxytoca	enterobacteria	40.1	2	Klebsiella oxytoca hits
. . . . Serratia marcescens	enterobacteria	40.1	3	Serratia marcescens hits
. . . . Providencia rettgeri	enterobacteria	40.1	1	Providencia rettgeri hits
. . . Pseudomonas aeruginosa	g-proteobacteria	40.1	17	Pseudomonas aeruginosa hits
. . . Acinetobacter proteolyticus	g-proteobacteria	40.1	1	Acinetobacter proteolyticus hits
. . . Acinetobacter johnsonii	g-proteobacteria	40.1	2	Acinetobacter johnsonii hits
. . . Acinetobacter soli	g-proteobacteria	40.1	5	Acinetobacter soli hits
. . . Pseudomonas asiatica	g-proteobacteria	40.1	2	Pseudomonas asiatica hits
. . . Pseudomonas putida	g-proteobacteria	40.1	2	Pseudomonas putida hits
. . . Pseudomonas sp. NY11382	g-proteobacteria	40.1	1	Pseudomonas sp. NY11382 hits
. . Brucella intermedia	a-proteobacteria	40.1	1	Brucella intermedia hits
. . Achromobacter xylosoxidans	b-proteobacteria	40.1	1	Achromobacter xylosoxidans hits
. uncultured bacterium	bacteria	40.1	1	uncultured bacterium hits

Figure 14: (b) blaIMP reverse reaction

The gradient PCR technique was employed to optimize the amplification conditions for the targeted β-lactamase genes, specifically the serine β-lactamase gene (blaSHV) and the metallo-β-lactamase gene (blaIMP). Optimization of annealing temperature is a critical step in PCR, as it directly influences primer specificity, amplification efficiency, and reduction of non-specific binding. By applying a temperature gradient across multiple reaction lanes, a range of annealing temperatures was systematically evaluated to determine the most suitable condition that produced clear, sharp, and specific amplicon bands corresponding to the expected product sizes. This approach ensured reliable amplification while minimizing primer-dimer formation and non-specific products [23]. The results of the gradient PCR optimization are illustrated in Figure 4.17, which displays agarose gel electrophoresis patterns obtained at varying

annealing temperatures. Distinct DNA bands corresponding to the expected amplicon sizes 713 bp for blaSHV and 470 bp for blaIMP were observed at optimal temperatures, indicating successful and specific amplification. The gel image demonstrates comparison across multiple temperature settings, including both positive amplification lanes and a negative control to confirm absence of contamination. The temperature showing the most intense and well-defined bands without background smearing was selected as the optimal annealing temperature for subsequent PCR assays. This optimization step enhanced the accuracy and reproducibility of molecular detection for both serine and metallo β-lactamase genes in the study.

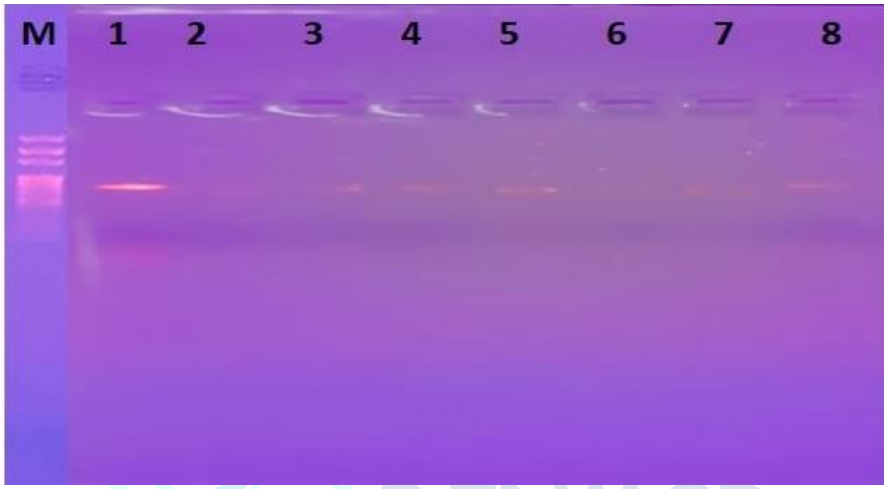


Figure 15: Temperature gradient for MBL and SBL genes shown by agarose gel electrophoresis using certain primers.

All genomic DNA samples extracted from the confirmed clinical isolates of Escherichia coli were subjected to polymerase chain reaction (PCR) amplification to detect the presence of β-lactamase genes, with particular emphasis on the metallo-β-lactamase gene blaIMP. Gene-specific primers targeting conserved regions of blaIMP were used to ensure selective and accurate amplification of the target sequence. The PCR reactions were carried out under optimized thermal cycling conditions, and the amplified products were subsequently analyzed by electrophoresis on a 1.5% agarose gel. Visualization under ultraviolet illumination allowed confirmation of the expected 470 base pair (bp) amplicon corresponding to the blaIMP gene. The inclusion of a molecular weight marker enabled precise determination of fragment size, while negative controls ensured the absence of contamination [24]. In addition to blaIMP screening, amplification of the blaSHV gene was performed to evaluate the presence of serine β-lactamase determinants among the isolates. Analysis of the PCR results revealed that, among the ten carbapenem-resistant isolates tested, five isolates (50%) carried the blaSHV gene, indicating a substantial prevalence of serine β-lactamase production within the studied population. In contrast, only one isolate (10%) demonstrated the presence of the blaIMP gene, confirming metallo-β-lactamase production at the molecular level. The remaining isolates did not show amplification of the targeted genes, suggesting either the presence of alternative resistance mechanisms or other carbapenemase genes not included in the present investigation. These findings indicate that serine β-lactamase–mediated resistance was more prevalent than metallo-β-lactamase production among the analyzed E. coli isolates, highlighting the importance of comprehensive molecular screening for accurate resistance profiling.

Table 15: Extracted DNA was quantified by using nanodrop.

Sample ID	ng/µL	260/280
E1 A	235	1.66
E2 A	128	1.89
E4 A	208	1.51
E5 A	161	1.60
E1 P	347	1.67
E2 P	244	1.63
E 5	194	1.60
E 6	57	1.47
E7	557	1.81
E 11	1511	1.75

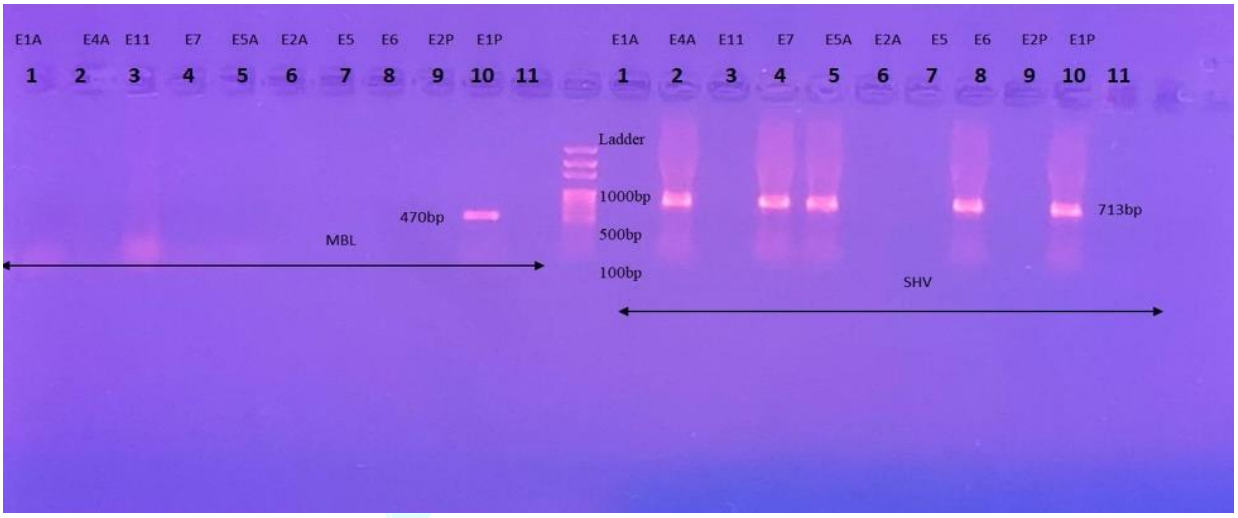


Figure 16: Agarose gel electrophoresis showing the presence MBL (470bp) and SHV (713bp) target gene by PCR with specific primers.

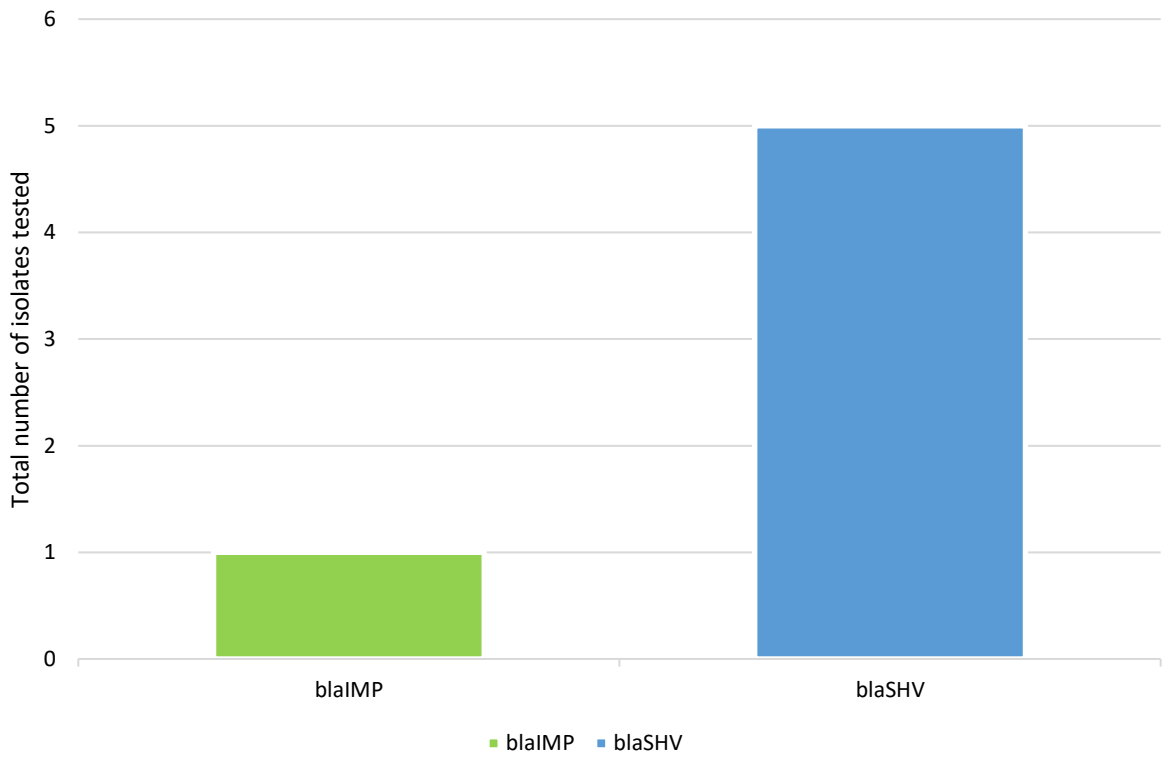


Figure 17: Among the total 10 Isolates 50% (05/10) bearing the blaSHV while 10% (01/10) showed the presence of blaIMP

The findings of the present study highlight a concerning burden of carbapenem resistance among clinical Escherichia coli isolates, with a notable predominance of serine β-lactamase (blaSHV) compared to metallo-β-lactamase (blaIMP) at the molecular level. Although phenotypic assays suggested a higher frequency of MBL activity, genotypic confirmation revealed that only a limited proportion of isolates carried the blaIMP gene, underscoring the importance of combining phenotypic and molecular approaches for accurate characterization of resistance mechanisms [25]. The discrepancy between phenotypic and genotypic detection also suggests the possible presence of additional carbapenemase genes not investigated in this study, such as blaNDM or blaVIM. The emergence and dissemination of β-lactamase–producing E. coli in a tertiary care setting represent a significant therapeutic and infection control challenge. These findings emphasize the urgent need for continuous molecular surveillance, rational antibiotic stewardship, and strict infection prevention strategies to limit the spread of multidrug-resistant strains and preserve the effectiveness of last-resort antibiotics.

6- Future Work:

While the present study successfully demonstrates the effectiveness of combining phenotypic (IMP–EDTA CDT) and genotypic (multiplex PCR) methods for detecting metallo-β-lactamase (MBL) production in Escherichia coli, several important avenues remain for future investigation. Expanding the scope of research is essential to better understand the evolving dynamics of carbapenem resistance and to strengthen infection control strategies at both local and global levels [26]. First, future studies should involve a substantially larger and more diverse sample size collected from multiple healthcare institutions and geographic regions. A broader dataset would allow for more accurate estimation of MBL prevalence and provide insight into regional variations in resistance patterns. Longitudinal surveillance studies are also recommended to

monitor temporal trends in carbapenem resistance and to evaluate whether the frequency of MBL-producing isolates increases over time. Such epidemiological monitoring would help inform antibiotic stewardship programs and guide policy-level interventions. Second, further molecular characterization of resistant isolates is necessary. In addition to screening for MBL genes and SHV genes, future work should include the detection of other clinically significant carbapenemase genes such as NDM, VIM, IMP, OXA-48, and KPC [27]. Whole genome sequencing (WGS) could provide a deeper understanding of resistance determinants, plasmid-mediated gene transfer, clonal relationships, and horizontal gene dissemination. Investigating mobile genetic elements such as integrons, transposons, and plasmids would clarify how resistance genes spread within and between bacterial populations. Third, functional and expression-based studies should be conducted to determine whether detected resistance genes are actively expressed or remain silent under certain conditions. Quantitative real-time PCR (qRT-PCR) and proteomic analyses may help evaluate gene expression levels and correlate them with phenotypic resistance patterns. This would address the hypothesis of "concealed" or low-expression MBL genes that may not always manifest strongly in routine susceptibility testing but could be activated under antibiotic pressure [28]. Fourth, future research should evaluate clinical outcomes associated with MBL-producing infections. Correlating microbiological findings with patient data such as treatment regimens, duration of hospitalization, morbidity, and mortality would provide clinically meaningful insights into the real-world impact of carbapenem resistance. Such data could support the development of targeted therapeutic guidelines and optimize empirical treatment strategies for severe infections. In addition, alternative therapeutic options and combination therapies should be explored in vitro and in vivo against MBL-producing isolates. The efficacy of newer β-lactam/β-lactamase inhibitor combinations, non-β-lactam antibiotics, and synergistic drug regimens should be systematically evaluated. Investigating novel antimicrobial agents, bacteriophage therapy, antimicrobial peptides, and anti-plasmid strategies could also provide promising directions for combating multidrug-resistant organisms [29]. Finally, future work should focus on strengthening diagnostic capacity in routine clinical laboratories. The development of rapid, cost-effective, and point-of-care molecular diagnostic tools would significantly enhance early detection of MBL producers. Implementing automated molecular platforms and integrating artificial intelligence–based surveillance systems may further improve real-time resistance monitoring and outbreak prediction.

Conclusion:

The present study provides compelling evidence that a combined phenotypic and genotypic strategy offers a reliable, highly sensitive, and clinically practical approach for the detection of metallo-β-lactamase (MBL) production among Escherichia coli isolates. By integrating the disc diffusion method for antibiotic susceptibility testing with multiplex PCR for gene detection and the imipenem–EDTA combined disc test (IMP–EDTA CDT) for phenotypic confirmation, our research establishes a comprehensive diagnostic framework capable of identifying both overt and concealed resistance mechanisms. This dual approach strengthens diagnostic accuracy and supports timely clinical decision-making, particularly in the context of carbapenem therapy. The findings reveal a concerning prevalence of carbapenem resistance, with 10 out of 14 E. coli isolates demonstrating resistance, while only four remained susceptible. Resistant isolates were subjected to further evaluation for MBL production, highlighting the importance of targeted molecular investigation following phenotypic screening. Notably, only one resistant strain (10%) harbored an MBL gene, whereas five isolates (50%) carried the SHV gene, indicating the co-existence of multiple β-lactam resistance determinants within the study population. Importantly, none of the imipenem-sensitive isolates carried genes associated with MBL production, reinforcing the correlation between phenotypic resistance and genotypic expression of carbapenemase-related mechanisms. Although the

proportion of confirmed MBL gene carriers was relatively low, the presence of even a single MBL-producing strain is epidemiologically significant. MBL-producing organisms represent a major public health threat due to their ability to hydrolyze a broad range of β-lactam antibiotics, including carbapenems, which are often reserved as last-line agents for severe infections. The detection of hidden or silent MBL genes in resistant isolates underscores the complexity of antimicrobial resistance patterns and suggests that phenotypic resistance may sometimes precede or mask genotypic confirmation. This observation warrants further large-scale investigations to explore the genetic diversity, transmission dynamics, and clinical impact of MBL-producing strains. The study highlights the urgent need for continuous surveillance and routine implementation of both phenotypic and molecular diagnostic techniques in clinical microbiology laboratories. Early identification of MBL-producing organisms is essential to guide appropriate antimicrobial therapy, prevent treatment failure, and limit the spread of resistant strains within healthcare settings. Without vigilant detection and monitoring, the increasing emergence of carbapenem-resistant E. coli could significantly compromise infection control strategies and lead to adverse clinical outcomes. Overall, our research reinforces the critical importance of integrated diagnostic approaches in combating antimicrobial resistance and emphasizes that proactive laboratory surveillance remains a cornerstone in preventing the escalation of carbapenem-resistant infections.

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