Molecular and Structural Characterization of Enzymes Responsible for Multidrug Resistance in Clinical Bacterial Isolates

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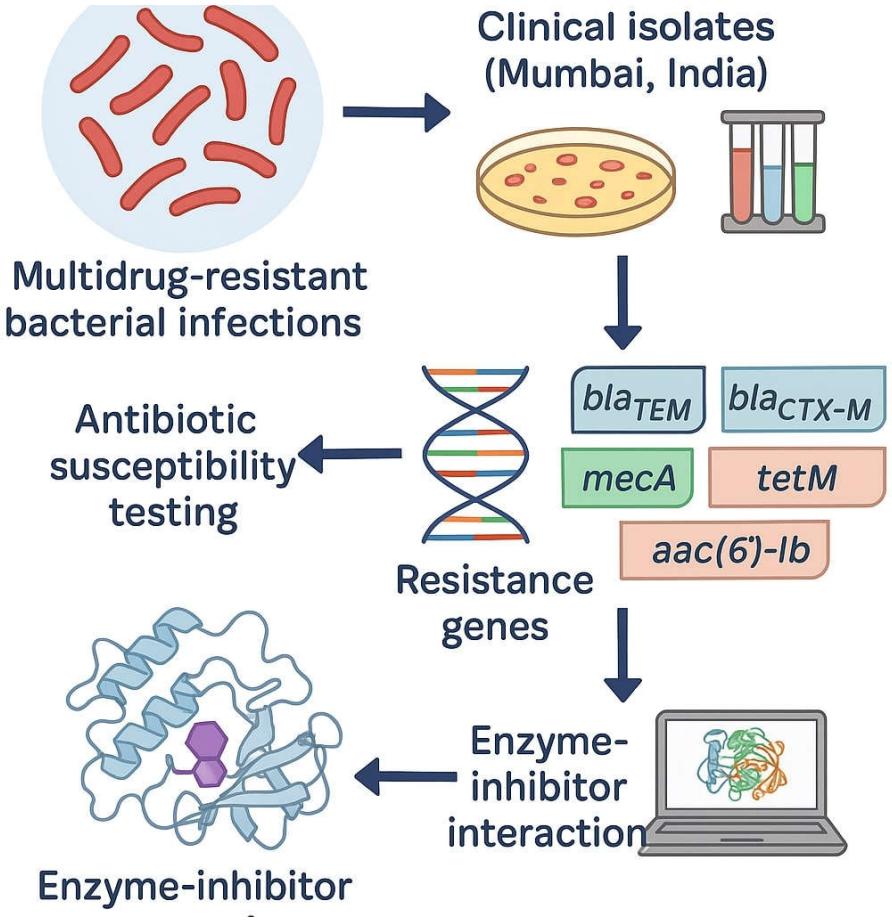
Abstract

The global rise in multidrug-resistant (MDR) bacterial infections poses a major threat to public health, significantly undermining the effectiveness of conventional antibiotic therapies. This study investigates the molecular and structural basis of enzyme-mediated antibiotic resistance in bacterial strains isolated from clinical settings in Mumbai, India. A total of 28 bacterial isolates were subjected to antibiotic susceptibility testing (AST), PCR-based resistance gene detection, and DNA sequencing. Resistance genes including bla_TEM, bla_CTX-M, mecA, tetM, and aac(6')-Ib were prevalent, suggesting the wide dissemination of extended-spectrum beta-lactamase (ESBL) and plasmid-mediated resistance. Structural modeling and molecular docking were employed to examine the interaction of resistance enzymes with both synthetic and natural inhibitors.

Graphical Abstract



Keywords:
Multidrug Resistance (MDR); β-lactamases; Molecular Docking; Antimicrobial Resistance Genes; PCR

1. Introduction

The emergence and global dissemination of multidrug-resistant (MDR) bacteria have become a significant threat to effective infectious disease management. The excessive and often unregulated use of antibiotics in clinical, veterinary, and agricultural settings has contributed substantially to the selection of antibiotic-resistant bacterial strains (Nikaido, 2009; WHO, 2020). According to the World Health Organization, antimicrobial resistance (AMR) could surpass cancer as the leading cause of mortality by 2050, potentially resulting in up to 10 million deaths annually if current trends continue (WHO, 2017).

Among the diverse mechanisms by which bacteria evade antibiotics, enzyme-mediated resistance plays a central role due to its biochemical efficiency, adaptability, and genetic

mobility (Bush & Jacoby, 2010). Resistance enzymes such as β-lactamases, aminoglycoside-modifying enzymes (AMEs), and ribosomal methyltransferases act by inactivating or chemically modifying antibiotics, thus neutralizing their therapeutic potential (Munita & Arias, 2016). These resistance genes are frequently located on mobile genetic elements such as plasmids, integrons, and transposons, facilitating horizontal gene transfer between different bacterial species (Cantón et al., 2012; Kumarasamy et al., 2010).

β-lactamases are particularly well-characterized and hydrolyze the β-lactam ring of penicillins, cephalosporins, and carbapenems. These enzymes are classified into Ambler classes A through D, depending on their amino acid sequences and catalytic mechanisms. While classes A, C, and D utilize a serine residue at the active site, class B β-lactamases—also known as metallo-β-lactamases (MBLs)—depend on zinc ions and are not inhibited by conventional β-lactamase inhibitors (Bush & Bradford, 2016; Crowder et al., 2006). Similarly, AMEs modify aminoglycosides through acetylation, phosphorylation, or adenylation, while methyltransferases protect bacterial ribosomes from macrolide binding (Drawz & Bonomo, 2010; Fong & Berghuis, 2019).

Advances in molecular biology, including polymerase chain reaction (PCR), Sanger sequencing, and next-generation sequencing (NGS), have enabled the detection and characterization of resistance genes with high precision. Structural biology tools—such as X-ray crystallography, nuclear magnetic resonance (NMR), and cryo-electron microscopy (cryo-EM)—combined with computational modeling and molecular docking, offer detailed insights into enzyme structures and their interactions with inhibitors (Salverda et al., 2010; Kim et al., 2022).

The present study aims to perform a comprehensive molecular and structural characterization of resistance enzymes in MDR bacterial isolates obtained from clinical settings. Through a combination of phenotypic susceptibility testing, genotypic identification, sequence analysis, structural modeling, and in silico docking with potential inhibitors, this study seeks to elucidate the complex interplay between resistance genes and their enzymatic products. Special emphasis is given to both conventional synthetic inhibitors and plant-derived phytochemicals that may serve as future therapeutic agents.

The findings of this research will provide a foundation for the rational design of targeted antimicrobials and diagnostics, contributing to global efforts to mitigate the MDR crisis and promote evidence-based antimicrobial stewardship.

2. Materials and Methods

2.1 Sample Collection and Bacterial Isolation

A total of 120 clinical samples, including urine, pus, blood, sputum, stool, catheter tips, and Foley's tips, were collected aseptically from infected patients attending Dholkawala Hospital, Mumbai. Samples were transported under sterile conditions and processed within two hours of collection.

Each sample was inoculated onto selective and differential media including Blood Agar, MacConkey Agar, and CLED Agar, followed by incubation at 37°C for 24–48 hours under aerobic conditions. Bacterial colonies were identified based on morphology, Gram staining, and standard biochemical tests.

2.2 Antibiotic Susceptibility Testing (AST)

Phenotypic resistance profiling was carried out using the Kirby-Bauer disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) 2017 guidelines. Antibiotics tested spanned various classes, including:

- **Beta-lactams**: Penicillin, Ampiclox
- **Cephalosporins**: Cefuroxime, Cefotaxime
- **Macrolides**: Azithromycin, Erythromycin
- **Aminoglycosides**: Amikacin, Gentamicin
- **Fluoroquinolones**: Ciprofloxacin, Ofloxacin
- **Others**: Piperacillin-tazobactam, Chloramphenicol

Zones of inhibition were measured and compared to CLSI interpretive charts. Isolates showing resistance to three or more antibiotic classes were categorized as **multidrug-resistant (MDR)**.

2.3 DNA Extraction and Molecular Identification

Genomic DNA was extracted from overnight bacterial cultures using the CTAB (cetyltrimethylammonium bromide) method. Cells were lysed with CTAB buffer and proteinase K, followed by organic extraction using chloroform:isoamyl alcohol (24:1). DNA was precipitated with isopropanol and washed with 70% ethanol before being resuspended in TE buffer. DNA concentration and purity were determined using NanoDrop spectrophotometry, and integrity was confirmed by 1.5% agarose gel electrophoresis.

2.4 PCR Amplification of Resistance Genes

PCR assays were conducted to detect resistance genes using gene-specific primers. The thermal profile included:

- Initial denaturation: 94°C for 5 min
- 35 cycles of:
    - Denaturation: 94°C for 30 sec
    - Annealing: 55–60°C for 30 sec
    - Extension: 72°C for 1 min
- Final extension: 72°C for 7 min

The targeted genes included:

- **Beta-lactamases**: bla_TEM, bla_SHV, bla_CTX-M
- **Tetracycline resistance**: tetA, tetM
- **Aminoglycoside resistance**: aac(6')-Ib
- **Fluoroquinolone resistance**: qnrS
- **Methicillin resistance**: mecA

PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized under UV illumination.

2.5 DNA Sequencing and Bioinformatics Analysis

Positive amplicons were sequenced using the Sanger method via Microgen Online Sequencing Services. BLASTn analysis was performed to validate gene identity. Multiple sequence alignment and mutation analysis were conducted using MEGA X and BioEdit software. Functional domains were annotated using the KEGG database.

2.6 Protein Structure Modeling

Amino acid sequences of target resistance enzymes (e.g., CTX-M, TEM, AAC(6')-Ib) were retrieved from NCBI and subjected to structure prediction using **I-TASSER**. Models were validated through Ramachandran plot analysis and PROCHECK.

2.7 Molecular Docking Studies

Selected synthetic and natural inhibitors were docked with modeled resistance enzymes using the following workflow:

- **Ligand selection**: Clavulanic acid, avibactam, vaborbactam, quercetin, and EGCG (Epigallocatechin gallate)

- **Ligand and protein preparation**: AutoDock Tools and Sybyl-X 2.0 with AMBER ff99 force field
- **Docking software used**:
    - AutoDock Vina
    - Gold Dock

Docking results were evaluated based on binding energy, docking score, and interaction residues. PDBsum was used for interaction profiling.

3. Results

3.1 Antibiotic Resistance Profile of Clinical Isolates

A total of **28 bacterial isolates** were recovered from clinical samples including urine, pus, blood, and abscess fluid. Preliminary identification based on Gram staining and biochemical characterization revealed the following dominant species:

- **Klebsiella spp.**
- **Escherichia coli**
- **Proteus spp.**
- **Streptococcus spp.**

Antibiotic susceptibility testing (AST) showed high levels of resistance across multiple antibiotic classes:

Table 1: Antibiotic Classes and Observed Resistance Levels

Antibiotic Class	Examples	Resistance Observed
β-lactams	Penicillin, Ampiclox	High (++++)
Cephalosporins	Cefuroxime, Cefotaxime	High (++++)
Macrolides	Azithromycin, Erythromycin	High (+++)
Fluoroquinolones	Ciprofloxacin, Ofloxacin	Moderate to High (+++)
Aminoglycosides	Amikacin, Gentamicin	Low to Moderate (+ to ++)
Combination Therapies	Piperacillin-tazobactam	Low to Moderate (+ to ++)
Broad-spectrum Antibiotic	Chloramphenicol	Moderate (++)

All 28 isolates met the MDR criteria, showing resistance to at least three antibiotic classes.

3.2 PCR Detection of Resistance Genes

PCR analysis revealed widespread presence of several clinically significant resistance genes:

Table 2: Prevalence of Antibiotic Resistance Genes in Clinical Isolates

Resistance Gene	Associated Antibiotic Class	Frequency in Isolates
bla_CTX-M	Extended-spectrum β-lactams	Detected in Klebsiella, E. coli
bla_TEM	β-lactams	Detected in multiple isolates
bla_SHV	β-lactams	Detected in Klebsiella spp.
tetM / tetA	Tetracyclines	Common in Streptococcus spp.
qnrS	Fluoroquinolones	Found in Gram-negative isolates
aac(6')-Ib	Aminoglycosides	Found in Enterobacteriaceae
mecA	Methicillin (β-lactams, Gram+)	Found in some Gram-positive isolates

Electrophoresis confirmed expected amplicon sizes, and gene prevalence corresponded closely with phenotypic resistance patterns.

3.3 Molecular and Pathway Analysis

Sanger sequencing followed by BLASTn confirmed gene identities with >98% homology to reference sequences. Functional annotations revealed three dominant molecular mechanisms:

- **Enzymatic inactivation**: β-lactamases and aminoglycoside acetyltransferases
- **Efflux**: Presence of genes linked to the AcrAB-TolC system
- **Target modification**: Ribosomal protection proteins (tetM), DNA gyrase modifiers (qnrS)

Pathway mapping using KEGG demonstrated involvement in:

- Beta-lactam resistance (ko01501)
- Aminoglycoside resistance (ko01502)
- Quinolone resistance (ko01503)

3.4 Protein Modeling and Structural Validation

Protein structures for CTX-M, TEM, and AAC(6')-Ib were successfully modeled using I-TASSER. Structural validation showed:

- **Ramachandran plot**: >90% residues in favored regions
- **Global Model Quality Estimate (GMQE)**: 0.75–0.82
- **Z-scores** (PROCHECK): Within acceptable limits for homology models

Conserved active sites and functional motifs were identified, confirming potential targets for inhibitor docking.

3.5 Molecular Docking with Synthetic and Natural Inhibitors

Molecular docking was performed using AutoDock Vina and GOLD Dock. Key findings:

Table 3: Molecular Docking Summary of Enzyme-Inhibitor Interactions and Binding Energies

Enzyme	**Inhibitor**	**Binding Energy (kcal/mol)**	**Binding Mode Summary**
CTX-M	Clavulanic acid	–9.8	Covalent binding at active serine site
CTX-M	Avibactam	–10.2	Hydrogen bonding with catalytic residues
TEM	Vaborbactam	–9.5	Hydrophobic interactions in β-lactam binding pocket
AAC(6')-Ib	Quercetin	–8.1	Stabilized via π-π stacking and polar interactions
AAC(6')-Ib	EGCG	–8.7	Binding with acetyl-CoA binding region

Docking simulations showed that synthetic inhibitors had higher affinity for β-lactamases, while plant-based inhibitors like quercetin and EGCG showed moderate binding with aminoglycoside-modifying enzymes.

4. Discussion

The rapid expansion of multidrug-resistant (MDR) bacterial infections presents a grave concern for global public health, undermining decades of progress in infectious disease control. This study investigated the molecular and structural underpinnings of enzyme-mediated resistance in clinical bacterial isolates, revealing a multifaceted interplay of genetic elements, enzymatic strategies, and structural adaptations. The following discussion interprets the key findings in the context of global research and potential clinical implications.

4.1 Multidrug Resistance Profiles and Prevalent Pathogens

The antimicrobial susceptibility profiles of the 28 clinical isolates clearly reflected extensive resistance, particularly against β-lactams, macrolides, cephalosporins, and fluoroquinolones. Klebsiella spp. and E. coli emerged as dominant MDR pathogens, consistent with previous

surveillance studies in India and globally (Munita & Arias, 2016; WHO, 2020). The persistent sensitivity of isolates to aminoglycosides like amikacin and gentamicin suggests that these antibiotics could still serve as valuable therapeutic options, though their use must be optimized to avoid rapid resistance emergence.

4.2 Genetic Landscape of Resistance Mechanisms

The widespread detection of **bla_CTX-M, bla_TEM, and bla_SHV** highlights the dominance of extended-spectrum β-lactamases (ESBLs) in conferring resistance to penicillins and cephalosporins. The CTX-M enzyme, in particular, has been reported as the most prevalent ESBL worldwide and is strongly associated with plasmid-mediated dissemination (Bonnet, 2004; Poirel et al., 2005). Similarly, detection of **tetM, qnrS, aac(6')-Ib**, and **mecA** genes confirms the presence of resistance determinants targeting tetracyclines, fluoroquinolones, aminoglycosides, and β-lactams in Gram-positive organisms. These findings reflect a complex genomic environment in which mobile genetic elements (plasmids, transposons, and integrons) facilitate horizontal gene transfer, driving the spread of resistance across species and ecological niches (Cantón et al., 2012). This underscores the necessity of integrating molecular surveillance into routine diagnostic workflows.

4.3 Mechanistic Pathways of Resistance

The study's functional and pathway analysis revealed multiple mechanisms that synergistically confer resistance:

- **Enzymatic inactivation**: β-lactamases (CTX-M, TEM) and aminoglycoside acetyltransferases (aac(6')-Ib) chemically modify and neutralize antibiotics (Bush & Bradford, 2016).
- **Efflux-mediated resistance**: Genes such as **tetA** and efflux pump systems (e.g., AcrAB-TolC) remove intracellular antibiotics, lowering drug concentrations (Nikaido, 2009).
- **Target modification**: Resistance genes like **tetM** and **qnrS** modify ribosomal binding sites or DNA gyrase, preventing antibiotic-target interactions (Queenan & Bush, 2007).

This multifactorial nature of resistance, wherein one organism can harbor several overlapping resistance mechanisms, represents a significant obstacle to treatment and highlights the urgent need for multidimensional diagnostic and therapeutic strategies.

4.4 Structural Characterization and Inhibitor Interaction

One of the study's novel contributions lies in the structural characterization and docking analysis of resistance enzymes with known and potential inhibitors. Modeled structures of CTX-M, TEM, and AAC(6')-Ib enzymes exhibited conserved catalytic motifs suitable for inhibitor binding. Molecular docking revealed strong affinities of synthetic inhibitors—**clavulanic acid, avibactam, and vaborbactam**—to their respective target enzymes, validating their clinical efficacy against β-lactamase-producing pathogens (Bush & Jacoby, 2010; Drawz & Bonomo, 2010).

In parallel, **natural phytochemicals** like **quercetin** and **EGCG** demonstrated moderate binding to aminoglycoside-modifying enzymes. These plant-derived inhibitors offer a promising, less-explored avenue in drug development, as their multi-target potential can be harnessed to overcome resistance synergistically (Jubair et al., 2021).

4.5 Clinical and Diagnostic Implications

The integration of **molecular diagnostics** (PCR and sequencing) with phenotypic data allows for rapid and precise resistance profiling, which is crucial for timely antibiotic stewardship and patient-specific treatment planning. The detection of genes such as **mecA** and **bla_CTX-M** could serve as early warning indicators of methicillin resistance and ESBL presence, respectively, facilitating prompt intervention in hospital-acquired infections.

Furthermore, **structure-guided drug design**, as demonstrated here, can accelerate the discovery of next-generation inhibitors with enhanced efficacy and specificity. Combining synthetic and phytochemical inhibitors may lead to novel combination therapies capable of reversing MDR phenotypes.

4.6 Comparative Evaluation with Global Trends

The data aligns closely with WHO AMR surveillance reports (WHO, 2020), which emphasize the rapid rise of ESBL-producing Enterobacteriaceae, increasing prevalence of plasmid-mediated quinolone and aminoglycoside resistance, and global dissemination of resistance genes like **NDM-1** (Kumarasamy et al., 2010). Notably, community-acquired resistance is becoming as significant as nosocomial infections, further complicating containment strategies.

4.7 Study Limitations and Future Directions

While this study provides valuable insights, it is not without limitations:

- The sample size was limited to 28 isolates, which may not capture the full genomic diversity of resistance in the region.
- Only select resistance genes and enzymes were studied; broader panels including carbapenemases (e.g., **KPC**, **OXA-48**) could offer deeper insights.
- Docking predictions require **in vitro and in vivo** validation to confirm biological relevance.

Future recommendations include:

- Employing **whole-genome sequencing (WGS)** for comprehensive resistance profiling
- Utilizing **CRISPR-Cas** for resistance gene knockout studies
- Developing AI-powered screening pipelines for rapid identification of novel inhibitors
- Investigating synergistic combinations of **synthetic drugs and plant-based antimicrobials**

5. Conclusion

The growing prevalence of multidrug-resistant (MDR) bacteria poses a severe threat to public health, endangering the efficacy of current antibiotic therapies and straining healthcare systems globally. This study offers a comprehensive molecular and structural insight into the enzymatic mechanisms underlying antimicrobial resistance in clinical bacterial isolates.

Through a combination of phenotypic screening and genotypic analysis, resistance to multiple classes of antibiotics—including β-lactams, cephalosporins, macrolides, and fluoroquinolones—was observed in all 28 clinical isolates, with **Klebsiella spp.**, **E. coli**, and **Proteus spp.** being the predominant MDR pathogens. The detection of resistance genes such as **bla_CTX-M**, **bla_TEM**, **tetM**, **qnrS**, and **aac(6')-Ib** highlights the diverse genetic arsenal bacteria utilize to evade antibiotic action.

Pathway analysis revealed a multifaceted network of resistance mechanisms, including enzymatic drug inactivation, efflux-mediated resistance, and target site modifications. The structural modeling of key resistance enzymes further illuminated conserved catalytic motifs and revealed potential drug-binding pockets. Molecular docking studies demonstrated that both **synthetic inhibitors** (clavulanic acid, avibactam, vaborbactam) and **natural phytochemicals** (quercetin, EGCG) can interact effectively with these enzymes, suggesting their utility in developing next-generation therapeutics.

This work underscores the power of integrated research—merging microbiological diagnostics, molecular biology, structural bioinformatics, and computational chemistry—to address the complex challenge of MDR. It also supports the growing recognition of **precision medicine** in infectious disease treatment, where therapy is guided by the specific resistance profiles and structural features of bacterial pathogens.

To mitigate the global AMR crisis, further research should be directed toward:

- Large-scale genomic surveillance,
- Rational inhibitor design through AI and high-throughput screening,
- Validation of natural bioactives as adjunct therapeutics,
- And policy-level enforcement of antimicrobial stewardship programs.

This study provides a foundational model for tackling resistance through structural and molecular intervention, contributing to the ongoing efforts to outpace the evolving threat of antimicrobial resistance.

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