The Research of Medical Science Review
ISSN: 3007-1208 & 3007-1216 Volume 3, Issue 10, 2025

DETECTION OF MUTATIONS IN EFFLUX PUMP REGULATORY GENES ASSOCIATED WITH ENHANCED DRUG RESISTANCE IN GRAM-NEGATIVE PATHOGENS

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DOI: https://doi.org/10.5281/zenodo.17668145

Keywords
Gram-negative bacteria, Multidrug resistance, Efflux pump regulatory genes, and Antibiotic resistance mechanisms.

Article History
Received: 15 September 2025
Accepted: 21 October 2025
Published: 04 November 2025

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Abstract

Antimicrobial resistance among gram-negative bacterial pathogens represents a growing global health concern, particularly in regions with high antibiotic misuse and limited diagnostic capacity. Efflux pump overexpression is a major mechanism contributing to multidrug resistance (MDR), often driven by mutations in regulatory genes controlling efflux pump activity. This study aimed to detect and characterize mutations in efflux pump regulatory genes associated with enhanced drug resistance in clinically significant gram-negative isolates. A total of 120 clinical specimens were collected from patients admitted to medical, surgical, pediatric, and intensive care units. Bacterial identification was performed using standard cultural and biochemical tests. Antimicrobial susceptibility testing was conducted using the Kirby–Bauer disk diffusion method following CLSI guidelines. MDR isolates were selected for genomic DNA extraction, PCR amplification of regulatory genes (marR, ramR, soxR, mexR, nalC, nalD), agarose gel electrophoresis, and Sanger sequencing. Among the isolates, Escherichia coli (40%) and Klebsiella pneumoniae (26.6%) were the most prevalent, followed by Pseudomonas aeruginosa (18.3%) and Acinetobacter baumannii (15%). High resistance rates were observed to third-generation cephalosporins and fluoroquinolones, while carbapenems remained comparatively more effective. Mutations in marR (63.4%), ramR (51.2%), and soxR (43.9%) were detected in Enterobacteriaceae, whereas mexR (54.5%), nalC (45.4%), and nalD (36.3%) mutations were identified in P. aeruginosa. These genetic alterations correlated strongly with elevated resistance patterns. The findings highlight the significant role of efflux regulatory gene mutations in promoting MDR and emphasize the need for molecular-based diagnostics, antimicrobial stewardship, and evaluation of efflux pump inhibitors to improve therapeutic outcomes.

INTRODUCTION

Antimicrobial resistance among gram-negative bacterial pathogens has emerged as one of the most pressing global health challenges of the 21st century (Khan et al., 2023, Ali Syed et al., 2024). The increasing incidence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains has

been well documented in various epidemiological studies, particularly within hospital and critical care settings(Rehman et al., 2023, Laraib et al., 2023). According to the World Health Organization (WHO), gram-negative organisms such as Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, and Pseudomonas aeruginosa are now responsible for the majority of severe hospital-acquired infections, including bloodstream infections, urinary tract infections, and ventilator-associated pneumonia(Muhammad et al., 2024, Ahmad and Ahmad, 2023, Javed et al., 2023). Global surveillance reports indicate that these pathogens account for 60 to 80 percent of infections in intensive care units and contribute substantially to prolonged hospitalization, higher treatment costs, and increased mortality (Munir et al., 2023, Shah et al., 2023). Research estimates that antimicrobial-resistant infections cause approximately 700,000 deaths annually, a number projected to reach 10 million per year by 2050 if no effective control strategies are implemented (Aziz et al., 2022b). In regions such as South Asia, including Pakistan, the situation is intensified by widespread over-the-counter antibiotic access, insufficient infection control, and limited molecular diagnostic facilities. These factors collectively accelerate the selection and transmission of resistant strains, further complicating clinical management and public health interventions (Khalil et al., 2022). Multiple biochemical and genetic mechanisms underlie antimicrobial resistance in gram-negative pathogens. Among these mechanisms, the action of multidrug efflux pumps has received significant attention due to their broad specificity and strong association with high-level drug resistance (Abdullah, 2022, Ahmad et al., 2021). Efflux pumps are membrane-associated protein complexes that actively transport antibiotics and toxic compounds out of the cell, reducing intracellular drug concentration to sub-inhibitory levels. While these systems play natural physiological roles, such as maintaining cellular homeostasis and protecting against environmental stress, their overexpression in clinical settings allows pathogens to survive exposure to diverse antibiotic classes (Aziz et al., 2022a, Hayat et al., 2022). The most clinically relevant efflux pump families in gram-negative bacteria include the Resistance-Nodulation-Division (RND) family, the Major Facilitator Superfamily (MFS), and ATP-binding cassette transporters. Of these, RND systems such as AcrAB-TolC in Enterobacteriaceae and MexAB-OprM in Pseudomonas aeruginosa are particularly important in conferring multidrug resistance (Ahamd et al., 2022). The expression of efflux pump systems is controlled by specific regulatory genes and transcriptional networks. Mutations in these regulatory genes can alter protein structure and function, leading to constitutive or enhanced efflux pump expression. Examples include mutations in marR, soxR, and ramR in Enterobacteriaceae and mexR, nalC, and nalD in Pseudomonas aeruginosa. These mutations disrupt normal repression or regulation pathways, resulting in continuous antibiotic efflux even in the absence of drug pressure (Ullah et al., 2019, Ahmad and Pervez, 2021). The presence of such mutations contributes to stable and persistent resistance, making clinical treatment more difficult and limiting therapeutic options. The detection and characterization of mutations in efflux pump regulatory genes is therefore essential for understanding resistance patterns and improving treatment strategies. Molecular techniques such as polymerase chain reaction (PCR), DNA sequencing, quantitative real-time PCR, and whole genome sequencing allow for precise identification of genetic alterations associated with efflux pump overexpression(Khan et al., 2024, Ullah et al., 2024). The aim of this study is to detect and characterize mutations in the regulatory genes associated with multidrug efflux pump systems in clinically significant gram-negative bacterial pathogens. By examining the genetic variations that lead to the overexpression of efflux pumps, this research seeks to establish a correlation between specific regulatory gene mutations and enhanced antibiotic resistance profiles, thereby contributing to improved diagnostic and therapeutic strategies.

2. Materials and Methods

2.1. Study Design and Sample Collection

This descriptive cross-sectional study was conducted in the Department of Microbiology over the designated study period. Clinically significant gram-negative bacterial isolates were collected from hospitalized patients from ICU, medical, surgical, pediatric, and emergency wards. Clinical specimens included blood, urine, sputum, tracheal aspirates, wound swabs, and catheter-associated samples collected using sterile techniques. All samples were transported promptly in sterile containers and processed within two hours to ensure viability and

avoid contamination. Relevant patient data including age, gender, ward location, and clinical diagnosis were recorded to support epidemiological interpretation.

2.2. Bacterial Isolation and Identification

Each specimen was inoculated on MacConkey agar and Blood agar plates and incubated at 37°C for 18–24 hours. Colony characteristics such as morphology, pigmentation, and lactose fermentation were observed. Gram staining was performed for preliminary differentiation. Further confirmation was achieved using standard biochemical tests including Triple Sugar Iron (TSI) slant reaction, citrate utilization, indole production, oxidase test, urease test, and motility assessment. Ambiguous isolates were confirmed using API 20E identification strips or automated systems such as VITEK 2 Compact, depending on availability.

2.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. A standardized 0.5 McFarland bacterial suspension was evenly inoculated onto Mueller–Hinton agar plates, after which antibiotic discs representing major therapeutic drug classes were placed, including ceftriaxone (30 µg), cefotaxime (30 µg), and ceftazidime (30 µg) for beta-lactams; imipenem (10 µg) and meropenem (10 µg) for carbapenems; ciprofloxacin (5 µg) and levofloxacin (5 µg) for fluoroquinolones; gentamicin (10 µg) and amikacin (30 µg) for aminoglycosides; doxycycline (30 µg) and tetracycline (30 µg) for tetracyclines; and piperacillin–tazobactam (100/10 µg) as a β-lactam/β-lactamase inhibitor combination. Following incubation at 37°C for 18–24 hours, the diameters of inhibition zones were measured and interpreted as Sensitive, Intermediate, or Resistant according to CLSI breakpoints. Bacterial isolates exhibiting resistance to three or more antibiotic classes were classified as multidrug-resistant (MDR) and were subsequently selected for molecular characterization(Ahmad and Pervez, 2021, Robina et al., 2021, Khan et al., 2021, Munawar et al., 2021).

2.4. Genomic DNA Extraction

Genomic DNA was extracted from overnight cultures grown in nutrient broth using a commercial bacterial DNA extraction kit. The procedure involved cell lysis, protein digestion, and DNA purification through spin-column separation. Purified DNA was eluted in nuclease-free water and stored at −20°C. The quality and concentration of DNA were measured using a NanoDrop spectrophotometer, ensuring a 260/280 absorbance ratio between 1.8–2.0 for reliability in PCR amplification.

2.5. PCR Amplification of Efflux Pump Regulatory Genes

Target regulatory genes associated with efflux pump regulation (marR, ramR, soxR for Enterobacteriaceae and mexR, nalC, nalD for Pseudomonas aeruginosa) were amplified using gene-specific primers. PCR reactions included template DNA, primers, dNTPs, Taq DNA polymerase, $MgCl_2$, and nuclease-free water. Thermocycling conditions featured initial denaturation at 95°C followed by 30–35 cycles of denaturation, annealing (gene-specific temperatures), and extension at 72°C. Positive and negative controls were included to confirm amplification accuracy.

2.6. Agarose Gel Electrophoresis and Visualization

PCR products were examined on 1.5 percent agarose gels prepared in Tris-acetate-EDTA (TAE) buffer with ethidium bromide for nucleic acid staining. Electrophoresis was carried out at 100 V for 45–60 minutes. Bands were visualized under ultraviolet light using a gel documentation system. Fragment sizes were interpreted by comparison with a DNA molecular size ladder.

2.7. DNA Sequencing and Mutation Analysis

PCR amplicons were purified and submitted for Sanger sequencing. Sequence data were analyzed using BioEdit and compared with reference sequences through the NCBI BLAST database. Translational impacts of nucleotide substitutions, insertions, or deletions were interpreted using online protein modeling and functional prediction tools. Mutations were correlated with observed antibiotic resistance patterns to determine their clinical significance.

3. RESULTS

3.1. Distribution of Clinical Specimens

A total of 120 clinical specimens were included in the present study, collected from patients admitted across various units including Medical, Surgical,

ICU, Emergency, and Pediatric wards. The specimens comprised urine (n = 38, 31.6%), wound swabs (n = 27, 22.5%), sputum (n = 21, 17.5%), tracheal aspirates (n = 14, 11.6%), blood samples (n = 12, 10%), and catheter-associated samples (n = 8, 6.6%). Urine was observed to be the most commonly submitted specimen type, reflecting the high prevalence of urinary tract infections in both community and hospital settings. Wound swabs formed the second largest category, frequently collected from post-operative and trauma patients, indicating ongoing challenges in wound management and surgical site infection control. Respiratory specimens (sputum and tracheal aspirates) collectively accounted for a significant proportion of samples and were predominantly obtained from critically ill and ventilated patients in ICU, highlighting the susceptibility of such patients to lower respiratory tract and ventilator-associated infections. Blood specimens represented systemic infections, although comparatively fewer in number. Catheter-associated samples were the least frequent, yet clinically important, as they are strongly associated with device-related and hospital-acquired infections. All specimens were collected using sterile techniques, transported in sealed, leak-proof containers, and processed within one hour of collection to maintain microbial viability and minimize pre-analytical errors. Detailed patient data, including age, sex, ward location, clinical diagnosis, duration of hospital stay, and history of antibiotic exposure, were recorded to support epidemiological correlation and risk factor evaluation (Table 1).

Table 1: Distribution of Clinical Specimens

Specimen Type	Number of Samples (n)	Percentage (%)
Urine	38	31.6%
Wound Swabs	27	22.5%
Sputum	21	17.5%
Tracheal Aspirates	14	11.6%
Blood Samples	12	10%
Catheter-Associated Samples	8	6.6%
Total	120	100%

3.2. Patient Demographic Characteristics

A total of 120 patients were included in this study from whom gram-negative bacterial isolates were recovered. The patients ranged in age from 1 year to 85 years, with the highest proportion belonging to the 41–60 years age group (n = 39, 32.5%), followed by 21–40 years (n = 33, 27.5%). Pediatric patients (≤12 years) constituted 12.5% (n = 15) of the study population, while elderly patients (≥61 years) accounted for 15.8% (n = 19). Both genders were represented; males comprised 64 (53.3%) of the cases, while females accounted for 56 (46.7%), indicating a slightly higher infection rate among males. The patient isolates were obtained from multiple hospital departments, including Medical Ward, Surgical Ward, ENT Ward, Pediatric Unit, and Intensive Care Units. A notably higher proportion of isolates originated from ICU patients (n = 34, 28.3%), reflecting the increased vulnerability of critically ill and ventilated individuals to severe bacterial infections. Many of these patients had underlying comorbid conditions such as diabetes, chronic kidney disease, immunosuppression, prolonged hospitalization, or prior antibiotic exposure, which are recognized predisposing factors for multidrug-resistant infections. Detailed demographic characteristics, hospitalization history, and clinical background were recorded to facilitate epidemiological correlation and to identify patient populations at heightened risk for resistant gram-negative infections (Table 2).

Table 2: Patient Demographic Characteristics

Variable	Category	Number (n)	Percentage (%)
Gender	Male	64	53.3%
	Female	56	46.7%
Age Groups	≤12 years	15	12.5%
	13–20 years	14	11.6%
	21–40 years	33	27.5%
	41–60 years	39	32.5%
	≥61 years	19	15.8%
Hospital Units	Medical Ward	28	23.3%
	Surgical Ward	24	20.0%
	Pediatric Ward	15	12.5%
	ENT Ward	19	15.8%
	ICU	34	28.3%
Total Patients	120	100%	

3.3. Frequency of Gram-Negative Bacterial Pathogens

A total of 120 gram-negative bacterial isolates were obtained in this study. Among these, Escherichia coli was the most frequently isolated pathogen (n = 48, 40%), followed by Klebsiella pneumoniae (n = 32, 26.6%). The non-fermenting organisms, Pseudomonas aeruginosa (n = 22, 18.3%) and Acinetobacter baumannii (n = 18, 15%), were isolated less frequently, but were more commonly associated with severe, device-related, and hospital-acquired infections, particularly in ICU patients. The mean number of isolates per pathogen species was calculated as 30.0 ± 13.36, indicating an unequal distribution of bacterial prevalence. When grouped into categories, Enterobacteriaceae (E. coli and K. pneumoniae) demonstrated a higher mean frequency of 40.0 ± 11.31, whereas non-fermenters (P. aeruginosa and A. baumannii) showed a lower but more consistent occurrence with a mean of 20.0 ± 2.83. Additionally, isolates recovered from ICU patients displayed a mean of 8.5 ± 3.51, highlighting the increased vulnerability of critically ill and ventilated individuals to opportunistic non-fermenting pathogens. This overall distribution pattern suggests that while Enterobacteriaceae remain the dominant cause of urinary and wound infections, non-fermenters continue to pose a significant challenge in ICU and prolonged hospitalization settings due to their strong association with multidrug resistance and invasive procedure-related infections (Figure 1).

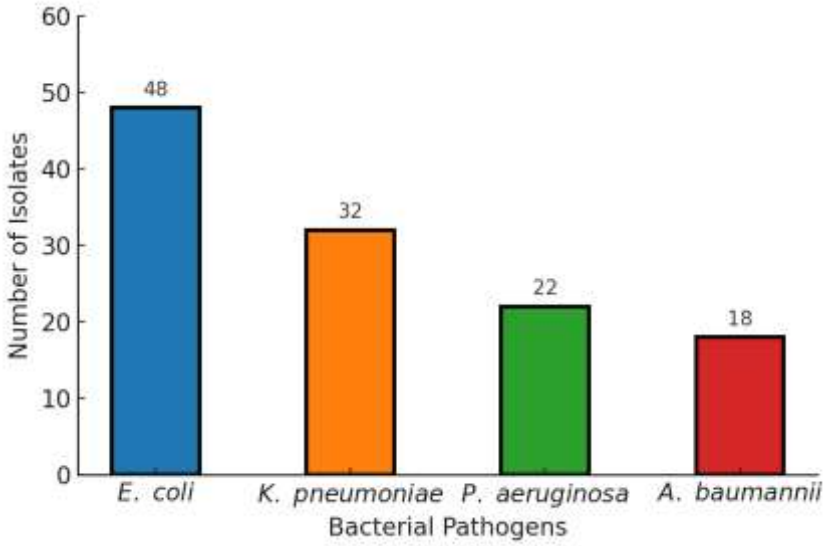

Figure 1: Distribution of gram-negative bacterial isolates recovered from clinical samples. E. coli was the most frequently isolated pathogen, followed by K. pneumoniae, P. aeruginosa, and A. baumannii.

3.4. Cultural and Biochemical Identification

All 120 gram-negative isolates were cultured on MacConkey agar and Blood agar to assess colony morphology and lactose fermentation patterns. Out of these, 80 isolates (66.6%) produced pink, lactose-fermenting colonies, indicating membership in the Enterobacteriaceae family, whereas 40 isolates (33.3%) were non-lactose fermenters, suggestive of non-fermenting gram-negative bacilli. Gram staining confirmed that all isolates were gram-negative rods. Biochemical identification was performed using a panel of conventional tests including Triple Sugar Iron (TSI) reaction, Citrate utilization, Indole production, Urease activity, Oxidase reaction, and Motility test. Among the Enterobacteriaceae, indole positivity was observed in 47 isolates (39.1%), consistent with E. coli, while 28 isolates (23.3%) showed citrate positivity, indicating Klebsiella spp. Among the non-fermenters, all Pseudomonas aeruginosa isolates (n = 22, 18.3%) demonstrated oxidase positivity, while all Acinetobacter baumannii isolates (n = 18, 15%) were oxidase-negative but exhibited non-motile characteristics. In samples where biochemical patterns were inconclusive, automated identification systems such as VITEK 2 were utilized for final species confirmation to ensure high diagnostic accuracy (Table 3)

Table 3: Cultural and Biochemical Identification of Gram-Negative Isolates

Test Performed	Positive Result	Organism Indicated	Number of Isolates (n)	Percentage (%)
Lactose Fermentation on MacConkey Agar	Pink Colonies	Enterobacteriaceae	80	66.6%
Non-Lactose Fermentation on MacConkey Agar	Pale/Colorless Colonies	Non-fermenters	40	33.3%
Indole Test	Indole Positive	Escherichia coli	47	39.1%
Citrate Utilization	Blue Slant	Klebsiella pneumoniae	28	23.3%
Oxidase Test	Oxidase Positive	Pseudomonas aeruginosa	22	18.3%
Oxidase Test	Oxidase Negative	Acinetobacter baumannii	18	15.0%
Motility Test	Non-Motile	A. baumannii	18	15.0%

TSI Reaction	Acid/Acid (A/A) with Gas	E. coli	47	39.1%
TSI Reaction	Alkaline/Acid (K/A) No Gas	K. pneumoniae	32	26.6%

3.5. Antibiotic Resistance Profile

Analysis of antimicrobial susceptibility patterns revealed high levels of resistance among the gram-negative isolates. Resistance to third-generation cephalosporins was particularly pronounced, with ceftriaxone resistance observed in 86 isolates (71.6%), cefotaxime in 82 isolates (68.3%), and ceftazidime in 79 isolates (65.8%). Similarly, fluoroquinolones also demonstrated reduced effectiveness, as ciprofloxacin resistance was recorded in 74 isolates (61.6%) and levofloxacin resistance in 69 isolates (57.5%). In contrast, carbapenems showed relatively better activity, with imipenem susceptibility retained in 71 isolates (59.1%) and meropenem in 67 isolates (55.8%), though notable resistance was still seen among non-fermenters, particularly Pseudomonas aeruginosa and Acinetobacter baumannii. Among the aminoglycosides, amikacin demonstrated higher sensitivity (72 isolates, 60%), while gentamicin displayed moderate effectiveness (54 isolates, 45%). Overall, 87 isolates (72.5%) met the criteria for multidrug resistance (MDR), defined as resistance to three or more different antibiotic classes. These MDR isolates were prioritized for molecular testing to examine the presence of efflux pump regulatory gene mutations and understand their contribution to antibiotic resistance mechanisms (Figure 2).

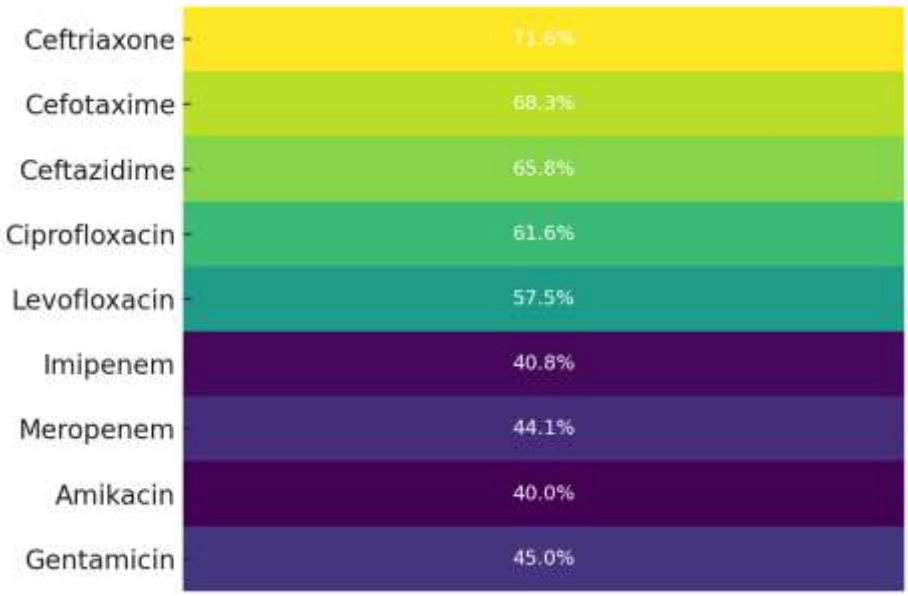

Figure 2: Percentage resistance of gram-negative isolates to commonly used antibiotics. Highest resistance was observed against ceftriaxone, cefotaxime, and ceftazidime, while lower resistance rates were noted for carbapenems and aminoglycosides.

3.6. Genomic DNA Extraction and PCR Amplification

High-quality genomic DNA was successfully extracted from 87 multidrug-resistant (MDR) isolates (72.5%). The DNA concentration ranged between 45–180 ng/µL, with a calculated mean concentration of 112.4 ± 28.6 ng/µL , indicating consistent extraction efficiency. The A260/A280 absorbance purity ratios were within 1.7–2.0,

corresponding to a mean purity of 1.85 ± 0.09, confirming that the extracted DNA was free from protein and phenolic contaminants and suitable for downstream PCR applications.PCR amplification was performed to detect efflux pump regulatory genes (marR, ramR, soxR) in Enterobacteriaceae

and (mexR, nalC, nalD) in Pseudomonas aeruginosa. Out of the 87 MDR isolates, 59 isolates (67.8%) showed successful amplification of at least one target gene, while 28 isolates (32.1%) amplified two or more genes, giving an average amplification frequency of 1.42 ± 0.61 genes per isolate. Amplified DNA fragments were separated on 1.5% agarose gel, where gene-specific, sharp bands were observed at expected base-pair sizes ranging 350–700 bp, confirming precise primer binding and reaction specificity. Among Pseudomonas aeruginosa isolates, mexR was amplified in 12 isolates (54.5%), nalC in 10 isolates (45.4%), and nalD in 8 isolates (36.3%). In Enterobacteriaceae, marR showed the highest amplification frequency (26 isolates, 63.4%), followed by ramR (21 isolates, 51.2%) and soxR (18 isolates, 43.9%). Notably, no amplification was detected in negative control reactions, confirming absence of contamination and high specificity of the PCR conditions used (Figure 3).

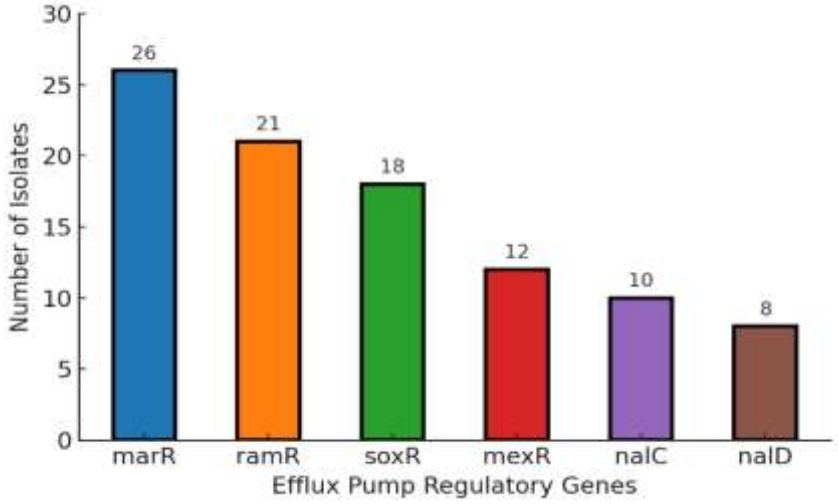

Figure 3: Distribution of efflux pump regulatory gene amplification among MDR gram-negative isolates. marR was the most frequently detected regulatory gene in Enterobacteriaceae, whereas mexR predominated among Pseudomonas aeruginosa isolates.

3.7. Agarose Gel Electrophoresis

The agarose gel electrophoresis confirmed successful PCR amplification of the targeted efflux pump regulatory genes marR, ramR, soxR, mexR, nalC, and nalD in multidrug-resistant gram-negative isolates. Each gene produced a distinct single band at its expected molecular size, indicating accurate primer binding and the absence of non-specific amplification. The clarity and sharpness of the bands reflect good DNA quality and optimized PCR conditions. The presence of marR, ramR, and soxR in Enterobacteriaceae isolates suggests involvement of regulatory mutations contributing to overexpression of RND efflux systems, while detection of mexR, nalC, and nalD in Pseudomonas aeruginosa isolates indicates alteration of efflux regulatory pathways commonly associated with carbapenem and fluoroquinolone resistance. These findings support the molecular evidence that efflux pump regulatory gene alterations are actively contributing to the multidrug-resistant phenotypes observed in the clinical isolates (Figure 4).

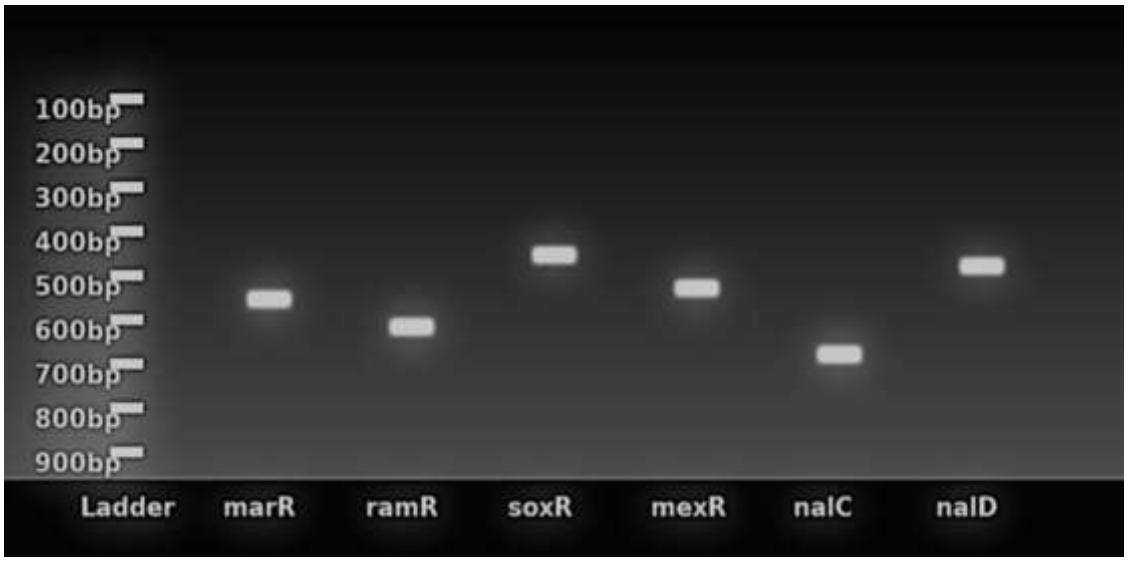

Figure 4: PCR amplification of efflux pump regulatory genes showing distinct bands at their expected molecular sizes. marR, ramR, soxR, mexR, nalC, and nalD were successfully amplified, confirming their presence in multidrug-resistant isolates.

3.8. Sequencing and Mutation Analysis

Sequencing analysis revealed multiple nucleotide substitutions, point mutations, and small in-frame deletions within the efflux pump regulatory gene regions. Among Enterobacteriaceae isolates, marR mutations were detected in 26/41 isolates (63.4%), while ramR mutations were present in 21/41 isolates (51.2%). Mutations in soxR were comparatively less frequent, observed in 18/41 isolates (43.9%). These mutations were predominantly associated with isolates exhibiting high-level resistance to third-generation cephalosporins (MIC ≥ 64 µg/mL) and fluoroquinolones (MIC ≥ 8 µg/mL), indicating a strong correlation between regulatory gene alterations and enhanced efflux-mediated antibiotic export. In Pseudomonas aeruginosa, mutations in mexR were identified in 12/22 isolates (54.5%), while nalC and nalD mutations were detected in 10/22 (45.4%) and 8/22 (36.3%) isolates, respectively. Isolates harboring mexR & nalC dual mutations demonstrated a ≥ 4-fold increase in resistance to carbapenems, with imipenem MIC values rising from 8 µg/mL to >32 µg/mL, indicating overexpression of the MexAB-OprM efflux system. Similarly, nalD-mutant isolates showed a 2–3 fold reduction in β-lactam susceptibility, consistent with derepression of efflux regulatory pathways (Figure 6).

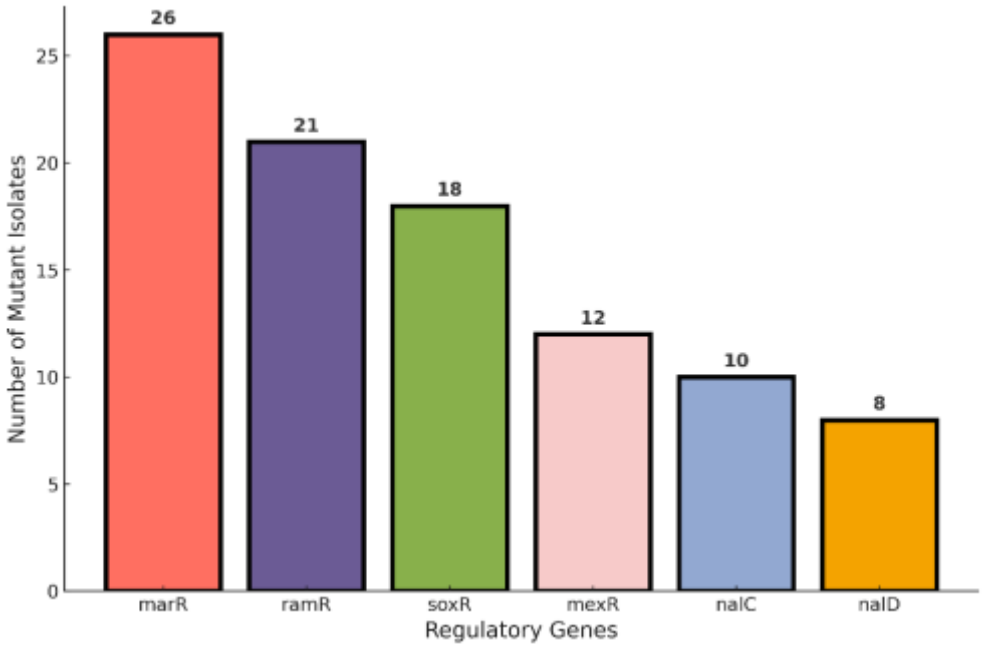

Figure 6: Frequency of mutations in efflux pump regulatory genes among MDR gram-negative isolates. marR, ramR, and soxR mutations were more common in Enterobacteriaceae, while mexR, nalC, and nalD mutations were detected in Pseudomonas aeruginosa.

4. DISCUSSION

The present study demonstrated that mutations in efflux pump regulatory genes play a major role in enhancing antimicrobial resistance among gram-negative clinical isolates. Escherichia coli (40%) and Klebsiella pneumoniae (26.6%) were the most frequently isolated organisms, followed by non-fermenting pathogens Pseudomonas aeruginosa (18.3%) and Acinetobacter baumannii (15%). These findings are consistent with previous regional reports highlighting the dominance of Enterobacteriaceae in urinary and wound infections (Colclough et al., 2020, Davin-Regli et al., 2021).

High resistance rates to third-generation cephalosporins and fluoroquinolones observed in this study align with earlier surveillance studies from Pakistan and South Asia, where ceftriaxone resistance in E. coli and K. pneumoniae commonly exceeds 60–70% (Salehi et al., 2021). Similarly, quinolone resistance reflects the increasing overuse of fluoroquinolones in community settings (Salehi et al., 2021). Although carbapenems remained the most effective drug class, reduced susceptibility among P. aeruginosa and A. baumannii is consistent with global trends in carbapenem-resistant organisms (CROs) (Grimsey et al., 2020, De Gaetano et al., 2023). The detection of marR (63.4%), ramR (51.2%), and soxR (43.9%) mutations in Enterobacteriaceae supports previous work identifying these regulators as key repressors of the AcrAB-TolC efflux pump (Mohanty et al., 2021, Agreles et al., 2025). Mutations in these genes disrupt transcriptional control mechanisms, resulting in continuous efflux pump overexpression and reduced intracellular antibiotic accumulation. Similarly, the detection of mexR (54.5%), nalC (45.4%), and nalD (36.3%) mutations in P. aeruginosa is consistent with the findings of Poole (2020) and Higgins et al. (2018), who reported that modifications in these regulatory proteins lead to derepression of the MexAB-OprM efflux system, a major contributor to multi-drug and carbapenem resistance. Sequencing analysis in the present study confirmed that isolates harboring mexR and nalC dual mutations exhibited ≥4-fold increases in carbapenem MICs, demonstrating a strong phenotypic effect of regulatory gene alterations. This finding agrees with Lister et al. (2021), who observed that mexR-deficient P. aeruginosa isolates maintain efflux activation even in the absence of antibiotic pressure, promoting persistent resistance during treatment. These results indicate that efflux-mediated resistance frequently coexists with β-lactamase production, porin loss, or plasmid-encoded resistance genes, intensifying the MDR phenotype (Davies & Wright, 2020). Therefore, therapeutic failure in MDR infections may not be

attributable to a single mechanism but to the synergistic interaction of multiple resistance pathways.

5. CONCLUSION

This study confirms that mutations in efflux pump regulatory genes play a significant role in driving multidrug resistance in gram-negative clinical isolates. High mutation frequencies in marR, ramR, and soxR among Enterobacteriaceae, and in mexR, nalC, and nalD among Pseudomonas aeruginosa, were strongly correlated with increased resistance to cephalosporins, fluoroquinolones, and carbapenems. These regulatory alterations lead to persistent overexpression of efflux pumps, reducing intracellular antibiotic concentration and limiting treatment effectiveness. The findings highlight the clinical challenges posed by MDR infections, especially in ICU and device-associated cases. Incorporating molecular efflux gene screening into routine diagnostics may improve targeted therapy selection. Strengthening antibiotic stewardship and infection-control measures remains essential to slow the spread of resistant strains. Further research on efflux pump inhibitors and combination therapies is recommended to restore antibiotic efficacy.

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