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ORIGINAL ARTICLE

# Molecular Detection of Efflux Pump Genes in Multi-drug-Resistant *Acinetobacter baumannii* Isolated from Clinical Specimens

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***Key words:***
***Efflux pump, Biofilm, Multi-drug Resistance and Acinetobacter baumannii***

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## ABSTRACT

***Background:*** *Acinetobacter baumannii is a critical Gram-negative pathogen whose treatment has become increasingly complicated due to rising multidrug resistance (MDR). A key resistance mechanism involves the expression of multiple efflux pump systems belonging to the Resistance-Nodulation-Cell Division (RND) superfamily.* ***Objectives:*** *This study aimed to correlate the phenotypic resistance of clinical A. baumannii strains with the presence of RND efflux pump genes.* ***Methodology:*** *This study was conducted in the Department of Biotechnology at Al-Nahrain University. Samples were collected from October 2024 to January 2025 from clinical settings, including patients of both sexes and all age groups. A total of 180 clinical samples were obtained. All collected samples were cultured on MacConkey and blood agar. Antibiotic susceptibility testing was performed using the Kirby-Bauer disc diffusion method against 12 antimicrobial agents representing 8 categories, and further confirmed using the AST card on the automated Vitek-2 compact system. Biofilm formation by A. baumannii isolates was quantified using the microtiter plate assay (96-well format). Polymerase chain reaction (PCR) was carried out using primers specific for efflux pump genes (adeB, adeG, and adeJ).* ***Results:*** *A total of 50 A. baumannii isolates were identified. An extremely high rate of multidrug resistance (MDR) was observed. Among these, 82% (41 out of 50) were biofilm producers. PCR analysis revealed a near-ubiquitous presence of RND efflux pump components: adeB (685 bp), adeG (818 bp), and adeJ (417 bp).* ***Conclusion:*** *The present study concluded that effected control necessitates intervention strategies that target both antimicrobial resistance mechanisms and biofilm formation pathways.*

## INTRODUCTION

*Acinetobacter baumannii* is an aerobic, Gram-negative cocci that is a common cause of nosocomial infections, including pneumonia, bloodstream infections, meningitis, and urinary tract infections[1]. For patients in intensive care units (ICUs), particularly those requiring ventilator support, the risk of infection is substantial, contributing to the high mortality rates observed among immunocompromised individuals in these settings[2].

*Acinetobacter baumannii* infections are notoriously difficult to treat due to the bacterium's high epidemic potential, its ability to form biofilms on external body surfaces, and its widespread multidrug resistance (MDR)[3]. Clinical isolates of *A. baumannii* have been widely reported to exhibit resistance to numerous antimicrobial agents, including aminopenicillins, ureidopenicillins, broad-spectrum cephalosporins, most aminoglycosides, quinolones, and chloramphenicol[4].

The underlying molecular mechanisms responsible for multidrug resistance in *A. baumannii* include changes in membrane permeability, alterations of antibiotic target proteins, development of multidrug efflux pumps, and the production of β-lactamase enzymes[5]. Bacterial efflux systems increase the minimum inhibitory concentration (MIC) of antibiotics by reducing their intracellular accumulation[6]. The two major families that constitute the efflux system in *A. baumannii* are the Resistance-Nodulation-Division (RND) superfamily and the Multidrug and Toxic Compound Extrusion (MATE) family[7].

When *A. baumannii* infections become multidrug-resistant, new strategies are required to restore the effectiveness of existing antibiotics. One promising approach is the use of efflux pump inhibitors (EPIs). These inhibitors are compounds that restore bacterial susceptibility to antibiotics by blocking efflux pump activity. Efflux pumps are bacterial mechanisms that actively expel antibiotics, reducing their intracellular concentration and rendering treatment ineffective. EPIs inhibit this activity either by competitively binding to the pump's active site or through non-competitive mechanisms that block its function[8]. This study aimed

to correlate the phenotypic resistance of clinical *A. baumannii* strains with the presence of RND efflux pump genes

## METHODOLOGY

**Bacterial sampling:**

A total of 180 clinical samples were collected from diverse clinical settings at the Baghdad Teaching Laboratories in Medical City, Baghdad/Iraq, for this study at the Biotechnology Department of Al-Nahrain University. Collection occurred from October 2024 to January 2025, and the samples, representing patients of all ages and both sexes, included sputum, wounds, blood, urine, and CSF.

**Cultivation of suspected microbial samples:**

Clinical samples were initially plated on MacConkey and Blood agar and incubated at 37°C for 24 hours. The resulting bacterial isolates were then characterized through morphological and microscopic examination, with final, accurate identification performed using the Vitek-2 system.

**Antibiotics susceptibility test**:

The susceptibility of each pure isolate to the twelve antimicrobial agents listed in Table 1 was determined using a dual-method approach. This included the conventional Kirby-Bauer disc diffusion method and the automated analysis provided by the Vitek-2 compact system using a specific AST card [9].

**Detection of biofilm formation**

Quantification of *A. baumannii* biofilm formation was achieved using the 96-well microtiter plate assay, which assessed the bacterial isolates' ability to produce biofilm, the method adapted according to[10].

**Molecular Study: DNA Extraction**

Genomic DNA was successfully extracted from thirty clinical isolates of *Acinetobacter baumannii* using a Promega/USA commercial purification system according procedure of [11]. The concentration and purity of the isolated DNA were determined via Nanodrop spectrophotometry, and the samples were subsequently quantified and stored at -20 c.

**Primer Design and Molecular screening of Efflux pump Genes:**

Polymerase chain reaction (PCR) was carried out using primer for gene target which their details was listed in (Table 2: A, B). All PCR reactions were done in TECHNE thermo- cyclers, and amplification of target gene followed guide by [12],[13]. 25 microliters of PCR amplification reaction contained 12.5 µl from OneTaq (NEB®) mastermix, 2 µl of DNA sample, 2 µl of (10 pmol/µl) from both primers and 8.5 µl of free-nuclease water. The thermal cycling profile used for all PCR assays was as follows: a 2-minute initial denaturation at 95°C, followed by 30 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C, and 45 seconds of extension at 72°C. The program was completed with a final extension of 5 minutes at 72°C. The annealing time and temperature were uniform 30 sec. at 60C for all primer sets.

**Ethical Approval:**

The Ethics Committee of the Biotechnology Department, College of Science, Al-Nahrain University, approved the study (Reference No.: CSEC/0121/0010).

**Table 1: Antibiotic discs used for the study:**

| No. | Name | Symbol | Cons. | Origin Company |
|---|---|---|---|---|
| **1** | **Trimethoprime-Sulfamethazole** | SXT | 25 µg | Liofilchem / (Italy) |
| **2** | **Levofloxacin** | LE (LVX) | 5 µg | Liofilchem / (Italy) |
| **3** | **Ciprofloxacin** | CIP | 5 µg | Liofilchem / (Italy) |
| **4** | **Tetracycline** | TE (TET) | 30 µg | Liofilchem / (Italy) |
| **5** | **Amikacin** | AK (AN) | 30 µg | Liofilchem / (Italy) |
| **6** | **Gentamicin** | CN (GM) | 10 µg | Liofilchem / (Italy) |
| **7** | **Colistin** | CL (CS) | 10 µg | Liofilchem / (Italy) |
| **8** | **Meropenem** | MRP (MEM) | 10 µg | Liofilchem / (Italy) |
| **9** | **Cefotaxime** | CTX | 30 µg | Liofilchem / (Italy) |
| **10** | **Ceftazidine** | CAZ | 30 µg | Liofilchem / (Italy) |
| **11** | **Piperacillin-Tazobactam** | TZP | 10 µg | Liofilchem / (Italy) |
| **12** | **Piperacillin** | PRL (PIP) | 100 µg | Liofilchem / (Italy) |

**Table 2 A: Primer Sequences of Efflux pump genes**

| Gene | | Sequence of forward and reverse Primer (5´- 3´) | PCR Product Size bp | GC Content% | Ref. |
|---|---|---|---|---|---|
| ***adeB*** | F | AGACCATGGCCGTTTTACCA | **685 bp** | **50** | **NCBI** |
| | R | AGTGCAGCCAAGACAAGGAA | | **50** | |
| ***adeG*** | F | GCGCGTTTACAAGGGGTTG | **818 bp** | **58** | |
| | R | GGTCCACTGACTTCTCGCAT | | **55** | |
| ***adeJ*** | F | CCACCAAATGCAACGCTTGA | **417 bp** | **50** | |
| | R | TGGACGCACACCTACAAGAC | | **55** | |

## RESULTS

**Sampling and Isolation**:

A total of 180 specimens had been collected across five distinct clinical sources (bloodstream, sputum, urine, wound and CSF) from clinical state which involving both sexes with different ages, from Baghdad Teaching laboratories in Medical City in Baghdad/ Iraq. Out of 180 specimens, collected A total of 120 (66.6%) were positive for culturing while 60 (33.3%) negatives for culture, the negative culture cases were attributed to the reasons that some patients were under antimicrobials chemotherapy during culture time. Critically, after final identification of isolates according to colonial morphology (Fig.1), and confirmed by Vitek 2 compact device as a final identification of by using a GN (Gram-Negative) card. The distribution results among specific sources listed in (**Table3**).

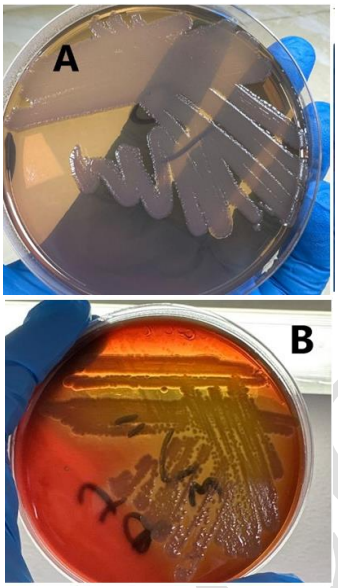


**Fig. 1:** Colonial Morphology of isolates on: A-MacConkey agar. B: Blood agar.

**Table 3: Distribution and Relative Prevalence of *Acinetobacter baumannii* Isolates across Clinical Sample Sources**

| Sample Source | Total Positive Cultures (N=120) | No. of *A. baumannii* Isolates (N=50) | % |
|---|---|---|---|
| **Sputum** | 40 | 20 | 40% |
| **Wound** | 22 | 13 | 26% |
| **Blood** | 36 | 8 | 16% |
| **Urine** | 13 | 7 | 14% |
| **CSF** | 9 | 2 | 4% |
| **TOTAL** | **120** | **50** | **100.0%** |

**Biofilm Formation:**

The analysis of the 50 *A. baumannii* isolates demonstrates that the majority of the strains possess the ability to form biofilms (Table 4).

**Table 4: Biofilm-forming ability of *Acinetobacter baumannii* by using micro-titer plate assay.**

| Biofilm Phenotype | Symbol | No. of Isolates (n=50) | % |
|---|---|---|---|
| **Strong Producer** | S | 8 | 16% |
| **Moderate Producer** | M | 9 | 18% |
| **Weak Producer** | W | 24 | 48% |
| **Non-Producer** | (Blank) | 9 | 18% |
| **Total Producers** | | 50 | 100% |

**Antimicrobial Susceptibility Pattern**

The Kirby-Bauer method, following Clinical and Laboratory Standards Institute (CLSI) recommendations, is a manual technique where antibiotic-containing disks diffuse into agar. The Vitek 2 Compact system is an automated method that uses specialized AST cards. The susceptibility pattern in current study adapted toward **8** categories of antimicrobial agents within 12 types of antibiotics and the results of the susceptibility data for these isolates highlights a critically high level of antimicrobial resistance (**Table 5**).

**Table 5: Antibiotic Resistance Count and Percentage.**

| Antibiotic (7 Class) | Antibiotic (12 Type) | No. of Resistance Isolates | (%) |
|---|---|---|---|
| **Penicillins/Cephalosporins** | Piperacillin (PRL) | 40 | 97.6% |
| | Cefotaxime (CTX) | 40 | 97.6% |
| | Ceftazidine (CAZ) | 35 | 85.4% |
| | Piperacillin/Tazobactam (TZP)* | 30 | 73.2% |
| **Carbapenems** | Meropenem (MRP) | 34 | 82.9% |
| **Polymyxins** | Colistin (CL) | 35 | 85.4% |
| **Aminoglycosides** | Gentamicin (CN) | 38 | 92.7% |
| | Amikacin (AK) | 31 | 75.6% |
| **Fluoroquinolones** | Ciprofloxacin (CIP) | 35 | 85.4% |
| | Levofloxacin (LE) | 33 | 80.5% |
| **Folate Inhibitors** | Trimethoprime-Sulfamethazole (SXT) | 35 | 85.4% |
| **Tetracyclines** | Tetracycline (TE)** | 26 | 63.4% |

**Molecular Detection**

The gel electrophoresis results show a clear and strong band at the expected 685 bp size for virtually all tested isolates (Figure 2).

The PCR results for the detection of *adeG* gene, showed a high frequency of detection, with the expected 818 bp amplicon band visible across the majority of the isolates (Figure 3).

On the other hand, the PCR screening for *adeJ* gene indicate all isolates adapted gave a true amplification where the presence of a strong amplicon band at **417 bp** in all tested lanes confirms the widespread existence of this regulatory element among the highly resistant isolates (Figure 4).

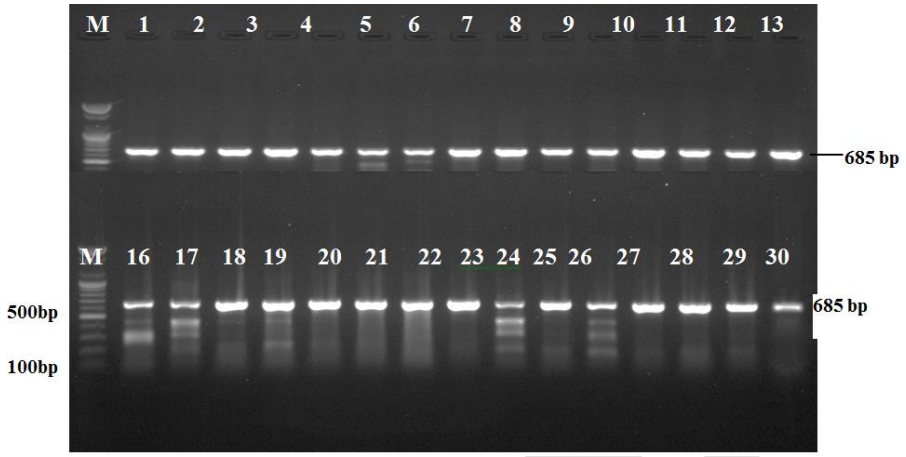


**Fig. 2: Gel** electrophoresis of the amplified *adeB* gene of *A. baumannii* isolates. Electrophoresis was done on 1% agarose at 75 V for 1 h, Lane M: DNA ladder marker (100 bp), Lanes 1-30: amplified *adeB* gene (685 bp) of *A. baumannii* isolates.

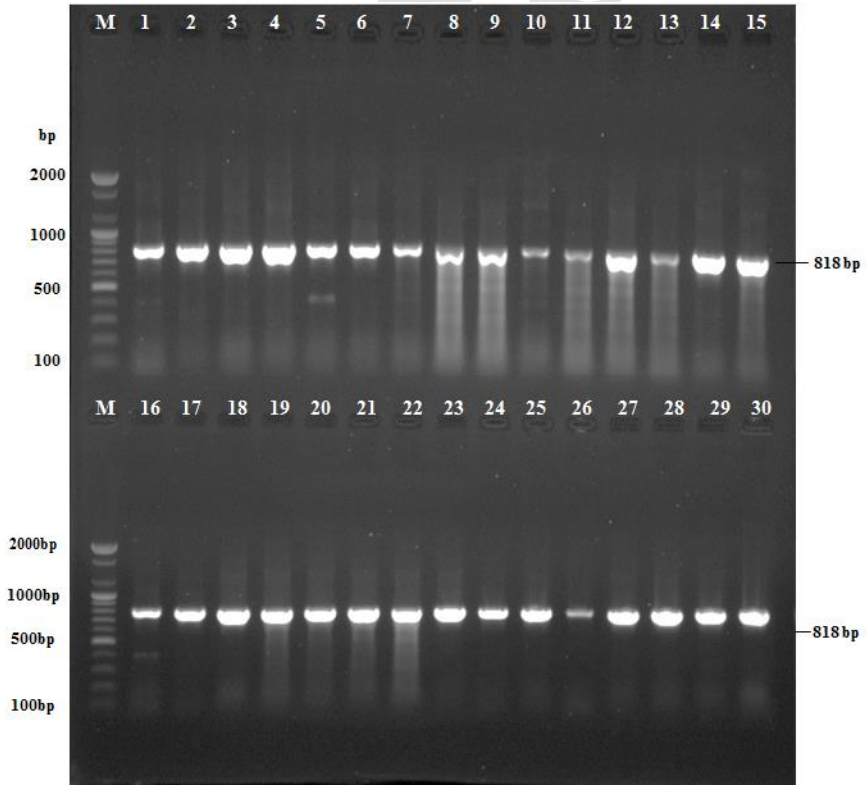


**Fig. 3:** Gel electrophoresis of the amplified *adeG* gene of *A. baumannii* isolates. Electrophoresis was done on 1% agarose at 75 V for 1 h, Lane M: DNA ladder marker (100 bp), Lanes 1-30: amplified *adeG* gene (818 bp) of *A. baumannii* isolates.

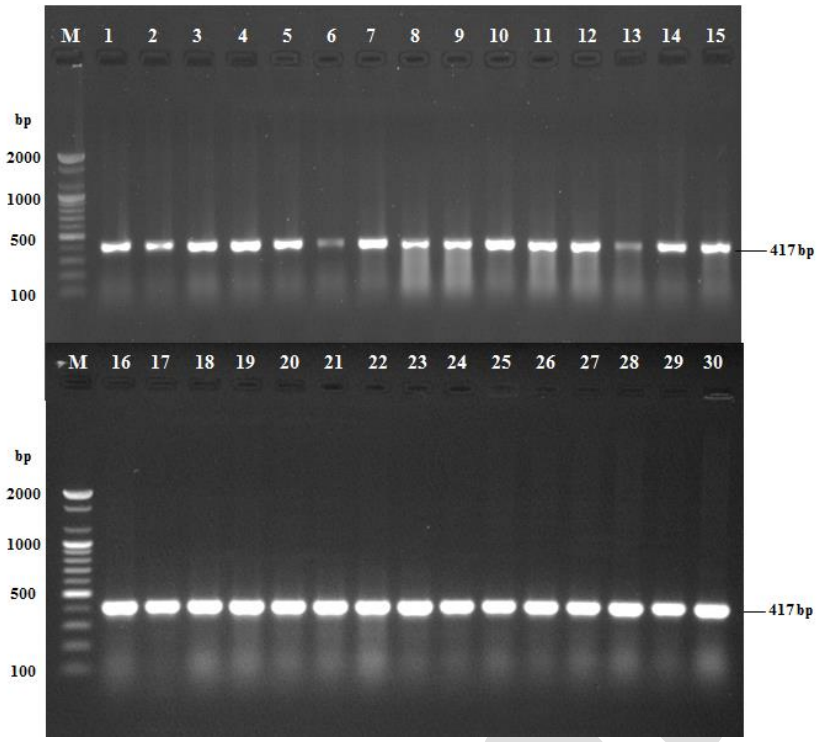


**Fig. 4:** Gel electrophoresis of the amplified *adeJ* gene of *A. baumannii* isolates. Electrophoresis was done on 1% agarose at 75 V for 1 h, Lane M: DNA ladder marker (100 bp), Lanes 1-30: amplified *adeJ* gene (417 bp) of *A. baumannii* isolates.

## DISCUSSION

*A. baumannii* accounted for the majority of these positive cultures, representing 50 out of 120 isolates, of all recovered bacteria, while other 70 indicate to other type of bacteria. The distribution among specific sources highlights its predilection for certain anatomical sites where *A. baumannii* was most frequently isolated from sputum (40%), a finding consistent with its role as a leading cause of hospital-acquired pneumonia. The organism also showed a significant presence in wound samples (26%) and bloodstream (16%), emphasizing its capacity to cause severe, invasive infections like sepsis and complicated soft-tissue infections. The presence in CSF (4%) also points to its ability to infect the central nervous system, albeit less frequently.

After the final diagnosis of the isolates and obtaining 50 isolates, these isolates were subjected to the biofilm formation test, where the results showed that 9 isolates were unable to form a biofilm, and the result was negative when comparing the absorbance of these isolates with the absorbance of the control. Overall, 82.0% (41 out of 50 isolates) were classified as biofilm producers (Weak, Moderate, or Strong). The largest proportion of isolates (48.0%) were classified as weak producers. While these strains form a less robust structure, their high prevalence suggests that biofilm production is a common, baseline characteristic among these clinical isolates.

Critically, 34.0% (17 isolates) demonstrated a capacity for moderate biofilm formation and (6 isolate) (16.0%) strong biofilm formation. These strains represent the highest clinical risk, as robust biofilm production provides the bacteria with increased protection from host immune responses and antibiotic penetration, contributing significantly to chronic colonization and persistent infections. 48% of isolates demonstrate a weak biofilm formation. Only a small minority of isolates (18.0%) were classified as non-producers, further emphasizing that biofilm formation is a highly conserved virulence mechanism in this *A. baumannii* population.

This high frequency of biofilm-competent strains is highly relevant in the clinical context, as the extracellular matrix provides a substantial barrier against both host immune defenses and antimicrobial agents [11], thereby enhancing the recalcitrance of infections a threat amplified when combined with the extreme drug-resistant phenotypes observed in this study.

Biofilm formation is a major virulence factor in *A. baumannii* infections, particularly those associated with foreign materials like catheters, ventilators, and surgical implants. The presence of a biofilm makes bacteria encased in a biofilm matrix are notoriously difficult to

eradicate where the matrix acts as a physical barrier, slowing the penetration of antibiotics [12]. Furthermore, metabolic changes within the biofilm (decreased growth rate) make the bacteria less susceptible to agents like β-lactams and aminoglycosides, which primarily target actively growing cells[13].

The non-susceptibility to β-lactam agents, which are the backbone of therapy for most Gram-negative bacteria, is near-universal. The rates of non-susceptibility to Piperacillin (97.6%) and Cefotaxime (97.6%). Even with the β-lactamase inhibitor combination, Piperacillin/Tazobactam is ineffective against 73.2% of the isolates. The high rate of non-susceptibility to Meropenem (82.9%) is the most critical feature defining this MDR population. Loss of carbapenems eliminates the primary empirical treatment option for severe Acinetobacter infections and immediately shifts treatment protocols toward more toxic or less reliable agents, reinforcing the severity of the MDR status.

Resistance in last-resort and alternative therapies where the MDR status is compounded by significant non-susceptibility in critical last-line classes, where the current results indicate to non-susceptibility to Colistin stands at 85.4%. As Colistin is often the single remaining effective agent against carbapenem-resistant strains, this concurrent resistance is a key factor driving many isolates into the PDR category. Its loss leaves clinicians with no available options in the tested classes. High non-susceptibility to Gentamicin (92.7%) and Amikacin (75.6%) removes another critical group of compounds often used in combination therapy to combat MDR pathogens. While the non-susceptibility to Tetracycline (63.4%) is the lowest among the tested classes, it still represents a substantial loss of activity.

The co-occurrence of high multidrug resistance (MDR) and robust biofilm formation in *A. baumannii* is a critical finding. The fact that the 41 isolates tested for antibiotic resistance were nominated as biofilm producers establishes a direct, causative link between this virulence factor and the observed severe MDR profile. The extremely high rate of Multidrug Resistance (MDR) observed in this study (90.2%) of isolates classified as MDR can be directly linked to the isolates' capacity for biofilm formation.

The finding that 82.0% (41 out of 50) of the overall isolates are biofilm producers (Weak, Moderate, or Strong) provides the underlying mechanism for the high antibiotic failure rates. Biofilms form a protective, matrix-encased community (composed of exopolysaccharides, proteins, and DNA) that acts as a physical barrier. This matrix effectively hinders the diffusion and penetration of antibiotics, especially large molecules [14],s such aminoglycosides (Gentamicin 92.7% non-susceptible) and polymyxins (Colistin 85.4% non-susceptible), preventing them from reaching the deeper bacterial cells. Cells embedded deep within the biofilm often exist in a state of nutrient limitation and low oxygen, leading to a slowed metabolic rate. Antibiotics, particularly those that target active cell growth and replication such as (β-lactams (Meropenem) 82.9% non-susceptible), become significantly less effective against these slow-growing cells (often referred to as persisters). This phenotypic change alone can increase antibiotic tolerance by up to 1,000 times compared to planktonic (free-floating) cells [15].

The close proximity of bacterial cells within the biofilm matrix facilitates the horizontal gene transfer (HGT) of resistance genes, particularly those carried on plasmids or transposons [16], (such genes for carbapenemases). The collective environment of the biofilm serves as a "hotspot" for the rapid acquisition and dissemination of the resistance determinants responsible for the observed MDR profile [17].

The molecular screening for the *adeB* gene, which serves as the multidrug transporter component of the crucial *AdeABC* efflux pump system, demonstrated a near-ubiquitous presence among the highly resistant isolates. The finding is highly significant as the *AdeABC* efflux system is one of the most important mechanisms conferring resistance to multiple, structurally unrelated classes of antibiotics in *A. baumannii*, including aminoglycosides, macrolides, fluoroquinolones, tetracyclines, and chloramphenicol [18].

The *adeG* gene, which encodes the multidrug transporter of the *AdeFGH* efflux pump system, similarly showed a high frequency of detection, with the expected 818 bp amplicon band visible across the majority of the isolates (Figure 3). The *AdeFGH* pump is an RND-type system that is regulated by the *LysR*-type transcriptional regulator *AdeL*, and its overexpression confers resistance to a broad range of drugs including fluoroquinolones, trimethoprim, sulfamethoxazole, tetracyclines, and importantly, the last-resort drug tigecycline[19]. The simultaneous presence of both *adeB* and *adeG* efflux system components in the same isolate confirms that these clinical strains possess multiple, overlapping efflux systems for antibiotic expulsion. This redundancy is crucial, as it allows the bacteria to maintain high-level resistance even if one pump system is inhibited or unable to completely clear a specific antibiotic.

## CONCLUSION

The study conclude that effective control necessitates intervention strategies that target both antimicrobial resistance mechanisms and biofilm formation pathways. The extremely high rate of Multidrug Resistance (MDR) observed in this study (90.2%) of isolates classified as MDR. The finding that 82.0% (41 out of 50) of the overall isolates are biofilm producers which provides the underlying mechanism for the high antibiotic failure rates. Also the high-level

resistance is directly correlated with the near-ubiquitous presence of Resistance-Nodulation-Cell Division (RND) efflux pump genes in the screened isolates.

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