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***adeB* and *adeG* Over Expression Increase Antibiotic Resistance in *Acinetobacter baumannii* Clinical Isolates from Burns Infection Patients in Mosul City, Iraq**

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Abstract: The study aims to explore the connection between the efflux systems and antibiotic resistance. A total of 50 pus samples were collected from patients with burn inflammation who were hospitalized in Al-Salam Teaching Hospital and Al-Jumhuri General Hospital, located in Mosul city, Iraq. The sampling period spanned from July 1, 2022, to September 1, 2022. The isolation and diagnosis of the bacteria were conducted based on cultural and morphological characteristics, as well as biochemical properties. Among the isolated samples, four strains of *Acinetobacter baumannii* were identified. One strain was susceptible to antibiotics, while another exhibited resistance to multiple antibiotics. One sensitive and one resistant isolates were chosen for quantification of the gene expression of two specific genes, *adeG* and *adeB*, using the Real-Time qPCR technique. The findings demonstrated variations in gene expression between the antibiotic-resistant bacteria and the susceptible one, indicating the significance of these efflux systems as mechanism for cephalosporin and carbapenem antibiotic resistance.

Keywords: *Acinetobacter baumannii*, Efflux pumps genes, Gene expression, Antibiotic

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Introduction

The emergence of antibiotics was expected to eliminate bacterial infections from the past. However, it resulted in bacteria developing various mechanisms to counteract the effects of antibiotics. One primary mechanism of resistance is through efflux pumps, which are special transporter proteins that enable bacteria to pump

antibiotics out of their cells and into the external environment (Foyssal *et al.*, 2018), Burns are a prevalent global health issue that is highly vulnerable to bacterial infections, making them challenging to treat with conventional wound dressings. Consequently, clinical practice faces significant difficulties in burn wound repair

(Huang, 2022).

In 2017, the World Health Organization published a report prioritizing research and development for novel treatment methods against certain organisms. The top-priority pathogen listed in the report was carbapenem-resistant *Acinetobacter baumannii*. This is due to the increasing prevalence of carbapenem-resistant *A. baumannii* infections, which are extremely challenging to treat as these isolates tend to be resistant to all but last-resort antibiotics like colistin and tigecycline.

Multiple studies have established the ability of *A. baumannii* to persist in hospital environments. For instance, *A. baumannii* was found in approximately 25% of air samples collected from rooms where patients with carbapenem-resistant *A. baumannii* infections were housed. This suggests the possibility of transmission through aerosolization within clinical settings.

While energy-dependent efflux pumps have been implicated in bacterial intrinsic resistance to antibiotics, mounting evidence indicates their involvement in various activities, including nutrient balancing, stress alleviation, pathogenesis, toxin excretion, and heavy metal balancing. Consequently, antibiotic efflux is not their sole primary function. Nevertheless, their role in antibiotic susceptibility remains crucial, as the effectiveness of antibiotics is entirely compromised if bacteria can excrete them before they reach their intended targets. Currently, six different classes of efflux pumps have been identified, and *Acinetobacter spp.* have been shown to possess all six classes of multidrug efflux pumps. These pumps contribute to the reduced susceptibility of *Acinetobacter spp.* to multiple antibiotic classes.

Efflux pumps characterized to date in *Acinetobacter spp.* but, as can be seen in Figure 1, *A. baumannii* contains large numbers of each type of efflux pumps with many remaining to be characterized (Kornelsen and Kumar, 2021).

Figure 1 which provides a schematic

representation of various efflux families and their location within the cell membrane of Gram-negative bacteria. Efflux pumps that span the inner membrane belong to the multidrug and toxic compound extrusion family (MATE), ATP-binding cassette family (ABC), proteobacterial antimicrobial compound efflux family (PACE), major facilitator superfamily (MFS), and small multidrug resistance family (SMR). Some ABC transporters, as depicted, are capable of spanning both the inner and outer membrane. Efflux pumps that span both membranes are classified under the resistance-nodulation-division family (RND). The substrate examples included in the diagram indicate the direction of transport (Kornelsen and Kumar, 2021).

Blocking these pumps is considered a crucial strategy, especially as the availability of new antibiotics diminishes. Efflux pump inhibitors (EPIs), which have the capability to hinder these pumps, are viewed as potential therapeutic agents that can restore the effectiveness of antibiotics against bacterial pathogens that have become resistant. Inhibitors employ various flow-blocking mechanisms and can be derived from natural or synthetic sources (Sharma *et al.*, 2019).

In burn patients, inflammation represents a significant cause of morbidity and mortality. It encompasses factors that increase the patient's susceptibility to infections and complications resulting from burns, such as prolonged hospital stays and immune effects (El Hamzaoui *et al.*, 2021). Burn injuries provide an ideal environment for bacterial growth due to the extensive affected area and the extended duration of hospitalization. Severe burns also contribute to immunosuppression, rendering these patients susceptible to invasive bacterial infections. Mortality among burn patients who survive the initial shock and trauma primarily stems from infections, depending on the percentage of the body surface area affected. Although systemic prophylactic antibiotics are commonly administered to outpatients with burns, their general use in inpatients lacks support from

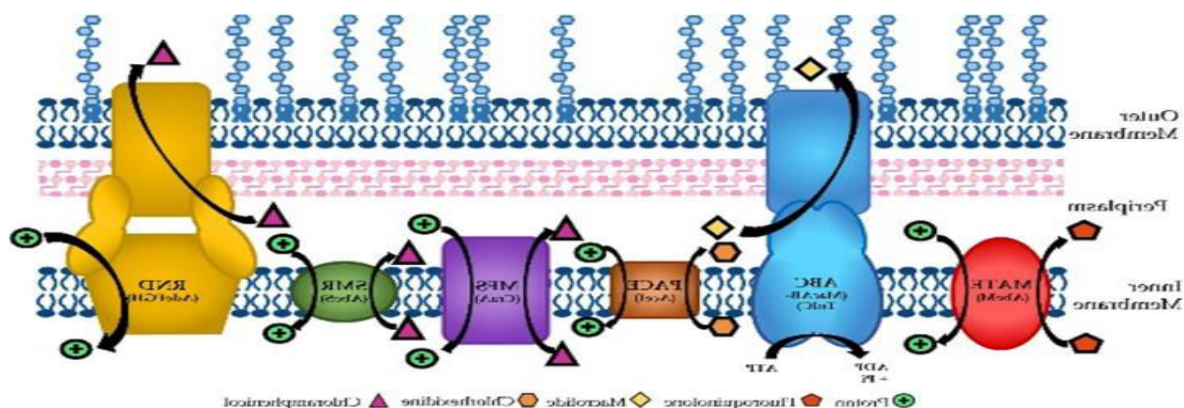


Fig. 1: Schematic representation of various efflux families and their location within the cell membrane of Gram-negative bacteria.

current data.

Regular assessment of wounds is crucial to ensure prompt and appropriate treatment of burn patients while minimizing unnecessary antibiotic exposure. Continuous monitoring of microorganisms and regular updates on their antibiotic resistance patterns are essential for effective infection control programs in the hospital burn unit (Agnihotri *et al.*, 2004). The present study aims to explore the connection between the efflux systems and antibiotic resistance.

Materials and Methods

Sample collection:

Fifty samples of burn pus smears were collected from patients hospitalized in Al-Salam Teaching Hospital and Al-Jumhuri General Hospital in Mosul city, Iraq for the period from July 1, 2022 to September 1, 2022 to isolate *Acinetobacter baumannii*.

Acinetobacter Isolation:

Acinetobacter baumannii isolated from cases of burn inflammation that had been previously collected as a swab was taken and placed in sterile tubes containing 5 ml of brain heart infusion broth. The tubes were incubated at 37 °C for 24 h. After the end of the incubation period and noticing the presence of bacterial growth, the bacterial suspension was cultured on MacConkey agar,

Nutrient Agar medium, and Blood Agar medium by streaking method, then incubated for 24 h at 37 °C in order to observe the characteristics and shapes of the colonies. The growing isolates were diagnosed by API20E system.

Antibiotic resistance test:

The Kirby-Bauer method was employed for the antibiotic resistance test. 3-5 colonies of bacterial isolates grown on MacConkey agar for 24 h were transferred to tubes containing 5 ml of normal physiological saline. The turbidity of the solution was adjusted to match the turbidity of a previously prepared MacFarland solution, equivalent to 1.5×10^8 cells/ml. Sterile cotton swabs were then dipped into the bacterial suspension for each bacterial type, excess liquid was removed by pressing the swabs against the inner wall of the tube. The swabs were streaked several times and in different directions on plates containing Mueller-Hinton Agar to achieve uniform growth. Antibiotic tablets, comprising 10 different types of antibiotics, were placed equidistantly on the surface of the culture medium. The tablets were gently pressed using sterile forceps. The plates were incubated at 37°C for 24 h, and the diameters of the inhibition zones around the discs were measured in millimeters using a ruler. The results were then compared with standard tables (Vandepitte *et al.*, 2003).

Genomic DNA extraction:

The kit prepared by Geneaid Company was used for the purpose of extracting genomic DNA. The extraction was carried out according to the attached protocol, and a nano drop DNA concentration meter was used to measure DNA purity and concentration.

Extracted DNA electrophoresis:

5 µl of DNA sample were mixed with 0.5 µl of dye loading solution. Then the mixture was transferred to the pits in to the electrophoresis tank containing 1x TBE buffer, and the DNA samples were migrated by passing them over a potential difference of 100 volts for 2 h. The extracted DNA bundles were located using an ultraviolet light source at wavelength 320 nm, images of the gel were taken for the purpose of confirming the presence of DNA (Sambrook and Russell, 2001).

A volume of 2% (v/v) of preculture inoculum containing 17 h old bacterial inoculums at a concentration of 1×10^9 cells/ml was inoculated into a 250 ml Erlenmeyer flask containing 0.3% of agar in 100.0 ml of marine semi-solid medium (Difco, United Kingdom), initial pH of 7.5 and incubated at 25 °C in an orbital shaker at 120 rpm for 72 h (Giri *et al.*, 2004). The samples were harvested at every 8 h intervals and analyzed for prodigiosin yield and anti- MRSA activity. The best cultivation time to produce the highest amount of prodigiosin was determined.

Quantitative Reverse Transcription Real-Time PCR of adeG and adeB:

Performed quantification detection and gene expression analysis of antibiotics resistance efflux pumps genes and normalized by housekeeping (Real-Time PCR) gene in extensive multidrug resistance *Acinetobacter baumannii* isolate as well as the sensitive isolation was carried out according to method which include the following steps:

Total RNA extraction:

RNA was isolated using an extraction kit (Genezol TM TRI RNA Pure kit, Geneaid company, Taiwan) for total RNA isolation by mechanical disruption, according to the manufacturer's instruction.

Convert RNA to cDNA:

The RNA was converted to the complementary nucleic acid strand by using synthesis kit (WizScript™ cDNA Synthesis kit, Wizibio Company, South Korea) under the following condition 25 °C for 10 min, 37°C for the 120 min, 85°C for 5 min and a cooling step to 4 °C for 5 min.

Estimateion of cDNA concentration:

The concentration of the cDNA strand was measured from the extracted RNA using TheQuantus Fluorometer, which is based on the principle of fluorescence of qualified template DNA (cDNA) in the real-time quantitative amplification process (Real-Time qPCR) through fluorescence detection of nucleic acids using programming settings. Quantifluor Biosciences (Canadian origin) pre-detection kit was used for DNA Broad range including ssDNA and dsDNA to estimate the concentration of nucleic acids. 1µl of cDNA was taken with 199 µl of Dye Quanty Flour diluted and mixed well using a micropipette after which it was incubated at room temperature in a dark place for 5 min. The DNA concentration of the complementary cDNA strand, which is eligible for work, was measured, after which it was kept at - 20 °C.

Designing of primer:

The primers (Table 1) were obtained in a lyophilized form from the Korean manufacturer Oligomer. Each primer was dissolved by adding anionic distilled water (nuclease free) in order to obtain concentration of 100 pmol/ml, and then preserved by freezing until use.

Calculated the amount of change in the level of gene expression as shown by the following equations:

$$\Delta ct = ct \text{ of tested gene} - ct \text{ of house keeping gene (16 SrRNA)}$$

$$\Delta\Delta CT = \Delta ct(\text{sample}) - \Delta ct(\text{control})$$

Table 1: Primers and their sequences used in the Real-Time PCR quantification

S. No.	Primer name	Sequence (5'-3')		Product Size	Origin
1	<i>adeG</i>	TCATCTAGCCAAGCAGAAG	F	160	This study
		GTGTAGTGCCACTGGTTACT	R		
2	<i>adeB</i>	ATTTGGATTGCTGAGCATTTC	F	160	This study
		GTAAACCTTGCTGACGTACA	R		
3	Housekeeping gene	GACGATGCAGGTGCTGAGTA	F	145	
		GACACCCATCACAAACATGG	R		

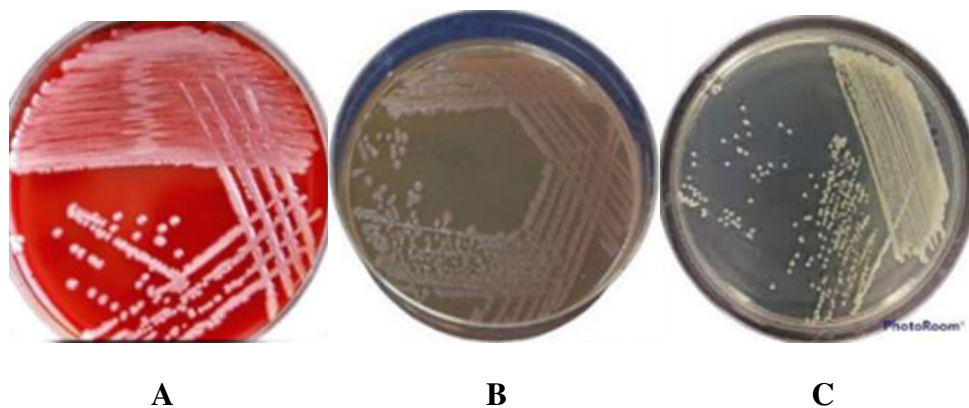


Fig. 2: *Acinetobacter baumannii* on Blood agar (A), on MacConkey agar (B), on nutrient agar (C).



Fig. 3: Microscopic examination of bacteria *Acinetobacter baumannii* under a light microscope with 1000X magnification.

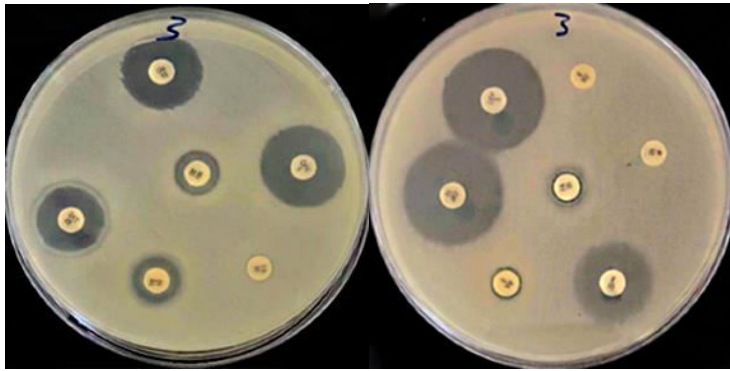


Fig. 4: Antibiotic susceptibility test of *A. baumannii*.

Table 2: Gene expression level of the *adeB* gene in *Acinetobacter baumannii* sensitive and resistant bacteria

Transactions	<i>H.K Ct</i>	<i>adeB Ct</i>	ΔCt	$\Delta\Delta Ct$	Folding
sensitive <i>A.baumannii</i> isolate (S1)	24.39	24.26	-0.13	-0.13	1.10
resistant <i>A.baumannii</i>	24.58	20.16	-4.42	-4.42	21.39

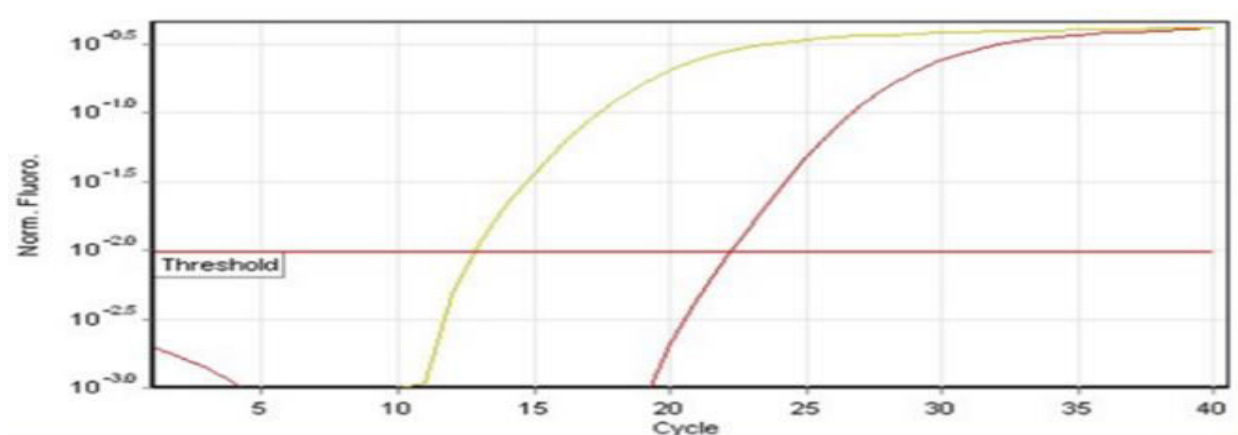


Fig. 5: An increase in gene activity.

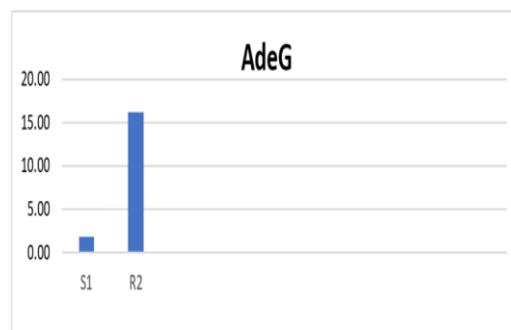


Fig. 6: Schematic showing gene activity *adeG*.

$Folding = 2^{-\Delta\Delta ct}$

$ct \rightarrow$ cycle number

$\Delta ct \rightarrow$ cycle threshold

Results and Discussion

Isolation and Identification:

Figure 2 shows the colonies of *Acinetobacter baumannii* on blood agar, appearing as smooth, convex, shiny, circular colonies with 1-2 mm diameter. On MacConkey agar, the colonies appeared as small, pale yellow to pink non-fermenting colonies, partially Lactose and oxidase-negative, but turning red after a short incubation period. The isolates exhibited colony growth in the nutrient broth medium, sometimes with a mucous appearance, and pale yellow to grayish-white color (Al-Ahmer and Al-Asady, 2021).

The bacteria appeared under light microscope (1000x) as gram negative coccobacilli or bicocci, as shown in the Figures 3.

The studied isolate exhibited resistance to all antibiotics utilized in the study, indicating the presence of multi-drug resistance in this particular isolate (Fig. 4). The term "multi-drug resistance" refers to bacteria that demonstrate resistance to antibiotics belonging to various classes (Sweeney *et al.*, 2018; Santos *et al.*, 2020).

The acquisition of multiple resistance traits by bacteria can be attributed to two main factors. Firstly, the presence of antibiotics in the natural environment of bacteria leads to the natural selection of mutant strains that are resistant to these antibiotics. Secondly, bacteria can acquire multiple resistance genes through horizontal gene transfer facilitated by resistance plasmids. Additionally, bacteria may possess inherent structural properties that confer resistance to certain antigens. For instance, Gram-negative bacteria's outer envelope acts as a barrier, impeding the permeability of many antigens and thereby conferring resistance against them (Hasan and Al-Harmoosh, 2020).

Measurement of gene expression of the *adeB* gene of sensitive (S1) and resistant (R2) *Acinetobacter baumannii*:

There was a difference in the gene expression value of the aforementioned gene (21-39) between the sensitive (S1) and the resistant (R2) *A.baumannii* compared to the gene expression value of the standard gene, meaning that the resistance increased in these bacteria as a result of the activity of the aforementioned gene (Table 2, Fig. 5).

The result of the current study demonstrate that the over-expression of the efflux pump gene has an additional impact on antibiotic resistance in isolates with a specific resistance mechanism. This suggests that suppressing the function of these genes is a viable approach, which can be further utilized to manage multidrug-resistant isolates. The study's results align with the researchers' previous findings (Abdi *et al.*, 2020). This suggests that PNA could be a potential strategy for combating antibiotic resistance associated with drug efflux pumps. It is also consistent with the findings of Lin *et al.* (2017), who found *Acinetobacter baumannii*, which had diverse pump gene express.

The results of the study also corroborate the findings of Salehi *et al.* (2021), who emphasized the involvement of RND (Resistance-Nodulation-Division) efflux pumps in the resistance of *A. baumannii* to multiple antibiotics, especially tigecycline. They highlighted the significance of various individual mutations in the regulatory systems associated with these efflux pumps. Further research is needed to fully understand the specific role of RND efflux pumps in multidrug-resistant clinical isolates of *A. baumannii*.

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