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The Role of Antimicrobial Peptide Buforin2 in the Activity of *AdeIJK* Efflux Pump among Carbapenem-Resistant*Acinetobacter baumannii* Clinical Isolates

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Abstract: *Acinetobacterbaumannii* is a multidrug-resistant (MDR) nosocomial pathogen that can cause severe infections. The efflux pumps especially the *adeIJK* system they developed are particularly important with respect. In this paper, we explore the impact of the antibacterial peptide Buforin2 on the activity of *adeIJK* efflux pump in carbapenem-resistant clinical isolates of *A. baumannii*. In this study, a total of 150 different clinical specimens (burns, urine, blood and sputum) were collected from two hospitals in Baghdad. Identification of *A. baumannii* isolates was performed using CHROMagar Acinetobacter medium, biochemical tests, and VITEK 2 system. Antibiotic susceptibility testing was carried out by the disc diffusion method. The role of efflux pumps was assessed by measuringthe minimum inhibitory concentrations (MICs) of antimicrobial agents (Meropenem, Amikacin, Ciprofloxacin, and Tigecycline) and Buforin2 in the presence and absence ofthe efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Quantitative RT-qPCR was usedto evaluate the expression levels of efflux pump genes *(adeJ* and *adeK*). Among 150 clinical specimens, 65 (43.3%) were identified as *A. baumannii*, with the highest prevalence in burn infections (47.7%). Antibiotic susceptibility testing revealed high resistance rates, with 64.6% of isolates classified as MDR.Carbonyl cyanide 3-chlorophenylhydrazone significantly reduced MICs of Meropenem (8–64 fold) and Amikacin (8–32 fold), while it had a minimal effect on Tigecycline (1–2 fold) and ciprofloxacin (4-8 fold). Buforin2 exhibited a (2–4-fold) decrease in MICs with CCCP, suggesting a limited role of efflux pumps in its activity. Gene expression analysis showed significant upregulation of *adeJ* and *adeK* in response to Meropenem (4.62 and 3.51 fold, respectively), whereas Buforin2 led to downregulation of *adeJ* (0.41 fold) and a minimal change in *adeK* (1.24 fold). The *adeIJK* efflux pump contributes to antibiotic resistance in *A. baumannii*, particularly against carbapenems and aminoglycosides. Buforin2 exhibited limited susceptibility to efflux pump inhibition, suggesting a different mechanism of action. These findings highlight the potential of antimicrobial peptides as alternative therapeutics against MDR *A. baumannii* infections.

Keywords: *AcinetobacterBaumannii*, Bufforin 2 peptide, *adeIJK* efflux pump.

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Introduction

Multidrug-resistant (MDR) *Acinetobacter baumannii* (*A. baumannii*) is a major global health challenge, being an important nosocomial pathogen responsible for serious infections, including pneumonia, bloodstream infections, and wound infections (1,2). Treatment options are also hampered by the rapid emergence of carbapenem-resistant *A. baumannii* (CRAB) strains which show resistance to nearly all available antibiotics including last-resort

carbapenems (3). A main mechanism of resistance is the overexpression of efflux pumps, especially the Resistance-Nodulation-Division (RND) family pumps, like *adeIJK*(4,5).

For understanding the efflux pumps role in bacterial resistance against antibiotics the basic principle should be considered, which is the efflux pump actively export the antimicrobial agents out of the cell, therefore decreasing the effectiveness of the drug as it intracellular concentrations (6). In particular, the *adeIJK* efflux pump has been previously reported to play a key role in *A. baumannii* resistance to different antibiotics, including carbapenems, aminoglycosides, and fluoroquinolones (7,8). Moreover, biofilm assistance was related to efflux pumps to support the bacteria in clinics(9).

Recently, amphipathic antimicrobial peptides (AMPs) have attracted interest as potential candidates for non-traditional antibiotics because they generally exert their effects by disrupting the bacterial membrane or by interfering with an essential intracellular function (10). Histone-derived antimicrobial peptides (AMPs) such as buforin2 have demonstrated potent anti-Gram-negative pathogenic activity against a variety of pathogens, targeting *A. baumannii*(11). Although efflux pump activity does not seem to affect the activity of Buforin2, experience with other AMPs suggests that efflux pump activity could have a major influence on activity (12).

This study is designed to determine the influence of the *adeIJK* efflux pump on the sensitivity of Buforin2 in carbapenem-resistant *A. baumannii* clinical isolates. The effects of efflux pump inhibition along with corresponding gene expression of both *adeJ*and *adeK*, will be analysed in order to elucidate the interplay between efflux mediated resistance, and antimicrobial peptide function. Characterization of these mechanisms provides key knowledge for the development of new treatment options for MDR *A. baumannii* infections and their successful treatment.

Materials and Methods

Isolation and identification of *Acinetobacter baumannii*

The study is a cross sectional study conducted from January 2025 to March 2025 in some hospitals (Al-Shaheed Ghazi Al-Hariri Hospital for Specialized Surgeries, Specialized Burns Hospital) in medicalcity, Baghdad, Iraq. *AcinetobacterBaumannii* isolation using CHROMagar Acinetobacter, McConkey agar and Blood agar. Identification of the isolates were conducted by biochemical tests then by using the VITEK 2 system (bioMerieux, France) according to the manufacturer's recommendation. Demonstration of 150 different clinical samples (Burn swabs /72, urine /20, blood /35 and sputum /23).

Antibiotic Susceptibility Test

Antimicrobial susceptibility test was assessed by the disc diffusion method. *AcinetobacterBaumannii* was grown overnight on CHROMagar before being suspended in normal saline. The turbidity used in the suspension was adjusted at 0.5 McFarland and the suspension was inoculated on Mueller-Hinton agar (Oxoid) plates. The following antibiotic discs were used in this study: Aztreonam (AZM) 30µg/mL1, Amikacin (AK)30 µg/mL1, Gentamicin (GEN)10 µg/mL1, Imipenem (IPM)10 µg/mL1, Meropenem (MEM)10 µg/mL1, Levofloxacin (LEV)5 µg/mL1, Ciprofloxacin (CIP)10 µg/mL1, Tigecycline (TCG)5 µg/mL1, Cefotaxime (CTX)30 µg/mL1,

Ampicillin (AMP)10 µg/mL1, Colistin (CL)10µg/mL1, Piperacillin (PI)30 µg/mL1, Cefipime (FEP)30 µg/mL1, Trimethoprim / Sulfamethoxazole (SXT)5 µg/mL1 and Ceftazidime (CAZ)10 µg/mL1. After 24 hrs of incubation of the agar plates at 37 °C, the inhibition zone was detected and interpreted according to CLSI breakpoint interpretation criteria (CLSI, 2021) as relevant percentage of susceptible intermediate or resistant isolates.

Effect of efflux pump inhibitor: Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) inclusion

The resistance to antibiotics (Meropenem and Amikacin) and the resistance to antimicrobial peptide (Buforin2) were evaluated with or without carbonyl cyanide 3-chlorophenylhydrazone (CCCP) inhibition. Test plates were prepared in two distinct ways. Then, 100 µl CCCP with concentration 20 Mg /ml was added to the corresponding plate. We determined the minimal inhibitory concentration (MIC) of each antibiotic with and without CCCP (an inhibitor). A 4-fold increase or greater in the MIC following the addition of the CCCP as compared to the MIC an established criterion (13) was defined as a significant increase in MIC values in the presence of the CCCP. Microtiter plate dilution assay (14) was used to determine the minimum inhibitory concentration (MIC) of each antimicrobial agent.

Gene expressionof*adeJ*and *adeK* genes using RT- qPCR assay

The design of this experiment utilized four isolates of carbapenem-resistant *A. baumannii* each with two efflux pump genes (*adeJ* and *adeK*) and different MIC values. Gene expression for the two resistant isolates was analyzed before and after the treatments. These agents were utilized during the treatment at sub-MIC levels for the induction of bacterial growth and resistance. After 24 hrs measurement was done. Each well was repeated, and each plate was replicated. The copy was used for a gene expression study.

Total RNA extraction

The isolation of RNA from samples was carried out according to the TRIzol^{TM} Reagent (Promega, USA) protocol. The extracted RNA was quantified using a Quantus Fluorometer to determine the quality of samples for downstream applications.

Preparation of primers

The primers were lyophilized and provided by the company of Macrogen. To generate a stock solution, lyophilized primers were first re-suspended in nuclease-free water to a concentration of 100 pmol/µl. Ten microliters of primer from the stock solution that was stored at - 20 °C were diluted with ninety microliters of nuclease free water to provide a working solution of primer at ten pmol/µl and gene expression specific primers were used previously as mentioned in (Table 1).

Table (1): Primer sequences were used for the gene expression study of efflux pump genes.

No.	*Target gene*	Primer name	Sequence (from Macrogen) 3-5	Product sizebp	Reference
1	*adeJ*	*adeJ* (F)	CATCGGCTGAAACAGTTGAA	109	(15)
		adeJ (R)	GCCTGACCATTACCAGCACT		
2	*adeK*	*adeK* (F)	TTGATAGTTACTTGACTGTTC	163	(16)
		adeK (R)	GGTTGGTGAACCACTGTATC		
3	*16S rRNA*	*16S* (F)	CAGCTCGTGTCGTGAGATGT	150	(17)
		16S (R)	CGTAAGGGCCATGATGACTT		

One step Quantitative Real-time PCR Assay (RT- qPCR)

The extracted RNA, primers and RT-qPCR master mix were thawed at 4 ºC and mixed well by vortex. RT-qPCR reaction tubes were placed into the thermocycler q- PCR. The reaction mix for one-step quantitative RT-qPCR included 5 µl of SYBER Green MasterMix, 0.5 µl of T mix and $MgCl_2$, 0.5 µl of Forwardand reverse primers, 2.5 of NucleaseFreeAquatic, and 1 µl of RNA.The total volume was 10 µl. (Table 2), presents the reaction conditions of one-step quantitative RT-qPCR.

Table (2): A Detailed Protocol for Thermocycler in RT-qPCR (one-step RT-qPCR).

Step	Temperature	Time	Cycles
Reverse Transcription	37 °C	15 minutes	1
a. intial Denaturation	95	05:00	1
b. Denaturation	95 °C	00:20	40
c. Annealing	55,60 or 53°C	00:20	40
d. Extention	72 °C	00:20	40

***16S rRNA*:55°C; *adeJ*: 60°C; *adeK*: 53°C.**

Delta delta Ct (ΔΔCt) method

The signal was measured as a cycle number (Ct) at the logarithmic phase threshold crossed by the signal. Fold changes and cycle threshold (Ct) were compared for the treatment groups and each gene's calibrator. This was the way these values were calibrated to the expression of the housekeeping gene (*16S rRNA*; as shown in the following figure.

• Delta delta Ct method (ΔΔCt)

Where ΔCT = House Keeping gene (HKG) — CT gene

Note: • ΔΔCT = (ΔCT Control − ΔCT Treated)

• Folding = 2-ΔΔCT

Step 1: the ΔCt between the target gene and the HKG gene for each sample (calibrator sample and unknown samples) were calculated

How we equations • ΔCt = Ct reference gene − Ct target gene

ΔΔCt is next calculated by determining the difference between the ΔCt of the calibrator and the ΔCt of the unknown:

• ΔΔCt = sample − (Ct target − Ct reference) calibrator (Ct target − Ct reference)

Thus the amount of target in the normalized sample = 2-ΔΔCt and this value can be used to compare expression levels in samples (18). Samples were analysedin duplicates and compared against the expression levels of the *adeJ* and the *adeK* gene. When assessing the differences in mRNA expression levels between antibiotic-exposed and non-exposed *A. baumannii*, the comparative threshold cycle (CT) method and 2−ΔΔCt were used.

Statistical Analysis

The Statistical Packages of Social Sciences-SPSS (2019) program was used to detect the effect of difference factors in study parameters. The chi-square test was used to significant compare proportions with significance set at$P\leq0.05$ and$P\leq0.01$ in this study (19).

ResultsandDiscussion

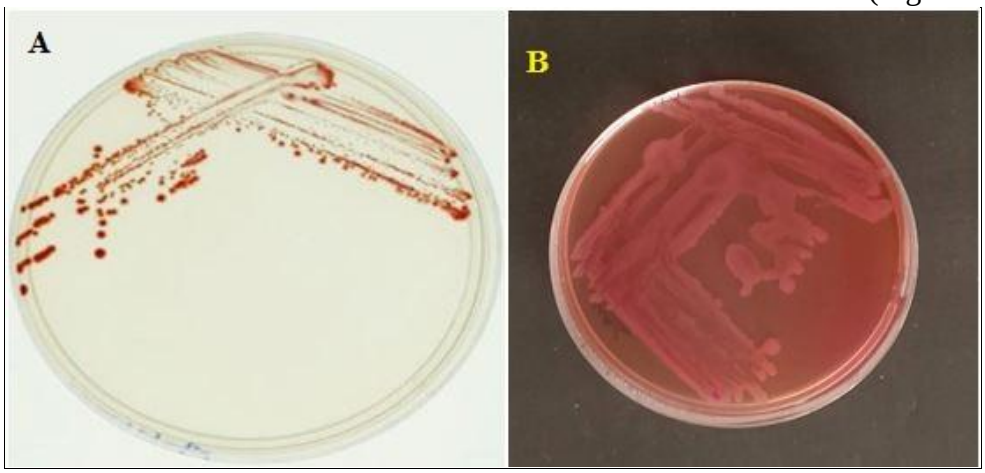
***Acinetobacter Baumannii* isolation and identification**

The specimens were isolated and identified based on their morphology identification by Gram's staining, cultural properties and biochemical profiles in addition to VITEK 2 system . The 150 various types of clinical

specimens were cultured onto CHROMagar Acinetobacter, MacConkey agar and Blood agar plates for 24hrs and at 37° C. The identified isolates obtained from these media and were identified by the following characteristics observed:

MacConkey agar and Acinetobacter CHROMagar colonies

The specific isolation of Acinetobacter species was performed on CHROMagar Acinetobacter medium. Isolates were seen as colonies bright salmon red grown on the CHROMagar at 37 °C for 24 hrs as shown in (figure 1, A). MacConkey agar appears to have mucoid structure and bright pink colonies, which are characteristic features of these bacteria (Figure 1, B).



Figure(1) : (A, B): (A) Acinetobactermucoid colonies onCHROMagarAcinetobactermedium agar (B) ColoniesofA. *baumannii*onMacConkey agarmedium at 37˚C for 24 hrs.

The *A. baumannii* pathogen species was specifically isolated with CHROM agar Acinetobacter medium, with bright salmon red colonies after 24 hours of incubation at 37 °C figure1 (A).This medium is selective for some Gram negative pathogens which appear in specified color for each bacterial genus such as colonies of *Escherichia coli* appear as blue colonies if not inhibited.

Due to its reliable identification, with extremely low false positive rates, CHROMagar Acinetobacter medium is the standard preferred medium, which also reduces the requirement for use of different reagents and further confirmatory tests indicating that CO medium is cost-effective compared to standard urine culture methods and significantly less work in microbiology lab when compared to Blood agar and MacConkey agar and should be considered for adaptation as an alternative method of culture for detection and reporting of *Acinetobacter baumannii*(20,21).

Identification of *AcinetobacterBaumannii* bacteria by VITEK 2

The identification of *A. baumannii* was performed accurately and reliably by the VITEK 2 system (BioMerieux, France) for bacteria identification. This device diagnoses bacteria accurately; it is 99% accurate. The initial results of identification showed that the number of *A. baumannii* isolates was 65 (43.3%), In contrasty after being identified via the biochemical tests at the Identification step it was 69 isolates were found.

Distribution of *AcinetobacterBaumannii* among Patients

A total of 65 *A. baumannii*isolates among 150 clinical specimens from 2 hospitals in Baghdad (Al-Shaheed

Ghazi Al-Hariri Hospital for Specialized Surgeries and Specialized Burns Hospital) were characterized. Burn infections specimens were the most frequent specimens from which *A. baumannii*were isolated 31 out of 65(47.7%). From blood, *A. baumannii* tended to be the second level, with 23.1% (15/65). Also, thisspecies was found in 10 (15.4%) sputum,while the urine samples contained nine isolates (13.8%).

The prominence of burn infections as a source of *A. baumannii* isolates in the Baghdad study is noteworthy.Burn patients are particularly susceptible to infections due to the loss of skin integrity and the immunocompromised state associated with severe burns.A study focusing on isolates from burns and wounds in Baghdad hospitals found that 36.5% of *A. baumannii* isolates were from burn samples, underscoring the vulnerability of burn patients to such infections(22). Urinary tract infections (UTIs) due to *A. baumannii*, while less common, remain clinically relevant.The 13.8% isolation rate from urine samples in the Baghdad study is higher than some reports but indicates the bacterium's potential to cause UTIs.A study found that 9.4% of *A. baumannii* isolates were from urine samples, highlighting the need for vigilance in catheterized or otherwise susceptible patients(23).

AntibioticSusceptibilityof*Acinetobacter Baumannii*

Antimicrobial susceptibility was performed on all 65*A. baumannii*isolates to 15 antibiotics represented by disc diffusion method towardsAztreonam (AZM)30 µg/mL1, Amkacin (AK)30µg/mL1, Gentamicin (GEN)10 µg/mL1, Imipenem (IPM)10 µg/mL1, Meropenem (MEM)10 µg/mL1, Levofloxacin (LEV)5µg/mL1, Ciprofloxacin (CIP)10 µg/mL1, Tigecycline (TCG)5 µg/mL1, Cefotaxime (CTX)30 µg/mL1, Ampicillin (AMP)10µg/mL1, Colistin (CL)10µg/mL1, Piperacillin (PI)30 µg/mL1, Cefipime (FEP)30 µg/mL1, Trimethoprim / Sulfamethoxazole (SXT)5µg/mL1 and Ceftazidime (CAZ)10µg/mL1.by disc diffusion method (Table 3).

Table (3): Shows the percentages of antimicrobial susceptibility rate of 65*A. baumannii*isolates against 15 antimicrobial agents.

Antibiotics (µg/mL)	Resistance (%)	Intermediate (%)	Sensitive (%)	P-value
AZM (30 µg/mL)	61(93.8%)	0 (0.0%)	4(6.2%)	0.0001 **
LE (5 µg/mL)	40(61.5%)	1(1.5%)	24(36.9%)	0.0001 **
TS (5 µg/mL)	56(86.2%)	0(0.0%)	9(13.8%)	0.0001 **
CO (10 µg/mL)	8(12.3%)	20(30.8%)	37(56.9%)	0.0001 **
TGC (5 µg/mL)	3(4.6%)	5(7.7%)	56(86.2%)	0.0001 **
CIP (10 µg/mL)	56(86.2%)	5(7.7%)	3(4.6%)	0.0001 **
GM (10 µg/mL)	39(60.0%)	4(6.2%)	20(30.8%)	0.0001 **
AK (30 µg/mL)	59(90.8%)	1(1.5%)	5(7.7%)	0.0001 **
MEM (10 µg/mL)	36(55.4%)	1(1.5%)	28(43.1%)	0.0001 **

Antibiotics (μg/mL)	Resistance (%)	Intermediate (%)	Sensitive (%)	P-value
IPM (10 μg/mL)	29(44.6%)	0(0.0%)	35(53.8%)	0.0001 **
FEP (30 μg/mL)	49(75.4%)	8(12.3%)	8(12.3%)	0.0001 **
CAZ (10 μg/mL)	56(86.2%)	1(1.5%)	5(7.7%)	0.0001 **
CTX (30 μg/mL)	60(92.3%)	0(0.0%)	5(7.7%)	0.0001 **
PI (30 μg/mL)	58(89.2%)	2(3.0%)	5(7.7%)	0.0001 **
AMP (10 μg/mL)	60(92.3%)	1(1.5%)	4(6.2%)	0.0001 **
P-value	0.0001 **	0.0001 **	0.0001 **	---
**** (P≤0.01)**				

Aztreonam (AZM), Amikacin (AK), Gentamicin (GEN), Imipenem (IPM), Meropenem (MEM), Levofloxacin (LEV), Ciprofloxacin (CIP), Tigecycline (TCG), Cefotaxime (CTX), Ampicillin (AMP), Colistin (CL), Piperacillin (PI), Cefipime (FEP), Trimethoprim / Sulfamethoxazole (SXT) and Ceftazidime (CAZ).

The results of the study showed that the percentage of antibiotic sensitivity against *A. baumannii* was the highest for Tigecyclin (86.2%), moderate sensitivity to Colistin (56.9%), Meropenem (43.1%), and Imipenem (53.8%). In contrast the percentage of antibiotic resistance was high by *A. baumannii* for Aztreonam (94.44%) and moderate by Ampicillin, Cefotaxime, Amikacin, Trimethoprim / Sulfamethoxazole, Piperacillin, Ceftazidime and Ciprofloxacin. From the 65 isolates of *A. baumannii*, 42(64.6 %) were MDR since they were resistant to more than three classes of selected antibiotics and, as illustrated in the antibiogram in this study, most of the antibiotics used are enormously resistant.

In a study performed in hospitals in Baghdad, the antibiotic susceptibility test results revealed that 100% of *A. baumannii* isolates were multidrug-resistant with 100% resistance to gentamycin and ciprofloxacin.Tigecycline and colistin were the most effective antibiotics (100% susceptibility). All *A. baumannii* isolates had ceftriaxone MIC ≥32 mg/L. Multiple virulence factors and antibiotic resistance were exhibited by each hospital strain of *A. baumannii*(24).

Carbapenem-resistant *A. baumannii* (CR-Ab) is a global issue due to the fact that these germs frequently exhibit resistance to every other routinely used antibiotic. As a result, infections linked to pathogenic multidrug resistance *A. baumannii* (MDR-Ab) become challenging to remove. Outbreaks of widespread drug resistance are caused by plasmid-mediated resistance, XDR-Ab *A. baumannii*. In order to control (XDR-Ab) infections, appropriate monitoring, prophylaxis, and therapy are necessary. The pathogenic strain of *A. baumannii* uses attachment, biofilm formation and toxin production, and mild stimulation of inflammatory responses as mechanisms**(**25).

Efflux pumps and their role in resistance mechanisms against peptides and antibiotics

This step aimed to compare minimal inhibitory concentrations of the antimicrobial peptide buforin2 and the antibiotics (Meropenem, Amikacin, Tigecycline, and Ciprofloxacin) in the presence and absence of this efflux

inhibitor (20 µg/ml) in four multidrug resistant isolates (Table 4) to improve the role of this inhibitor on efflux pumps.

It was discovered that the MICs of Meropenem and Amikacin were significantly lowered from 8 to 64 fold and 8 to 32 fold, respectively, upon the addition of the CCCP at a final concentration of 20 µg/mL. Additionally, the impact of CCCP on ciprofloxacin MICs, decreased from 4 to 8 fold. (Table 4) shows that the efflux pump inhibitor caused the minimum effect on Tigecycline with 1–2fold decrease in the minimum inhibitory concentrations (MICs). The inhibitor's greatest impact on MIC reduction was observed in relation to Meropenem and Amikacin. Also, there was no significant effect of the efflux pump inhibitor on the activity of the antimicrobial peptide buforin2 with a 2–4-fold decrease in the minimum inhibitory concentrations (MICs).

Table (4): Antimicrobial susceptibility of clinical *A. baumannii* isolates in the presence (20 µg/mL-1 CCCP) and absence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), an efflux pump inhibitor.

Isolate No.		TGC	MEM	AK	CIP	Peptide (Buforin2)	** (P≤0.01).
A10	**alone**	256	64	128	16	64	0.0001 **
	+EPI	128	8	16	4	32	0.0001 **
A11	**alone**	256	32	64	8	128	0.0001 **
	+EPI	128	2	4	1	64	0.0001 **
A36	**alone**	128	64	64	16	128	0.0001 **
	+EPI	128	8	4	2	64	0.0001 **
A44	**alone**	256	128	64	8	64	0.0001 **
	+EPI	128	2	2	2	16	0.0001 **
Fold of reductionIn MIC+ EPI		1-2	8-64	8-32	4-8	2-4	---
** (P≤0.01).							

MIC, Minimum inhibitory concentration; EPI=Efflux Pump Inhibitor carbonyl cyanide 3-chlorophenylhydrazone *(CCCP)*. MEM (Meropenem), TGC (Tigecycline), AK (Amikacin), CIP (Ciprofloxacin)

Several studies have examined the effect of efflux pump inhibitors, such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (26,27) on antimicrobial susceptibility.

Extensive research has been don on how efflux pumps contribute to Gram negative bacteria's biofilm formation and antibiotic resistance. These pathogens are the most common cause of nosocomial infections because of their innate resistance, and they are fast becoming one of the main issues facing modern medicine. The existence of efflux pumps has rendered many bactericides that were once effective against Gram-negative bacteria ineffective. Several families of antibiotics, including biocides, beta-lactams, carbapenems, tetracyclines, and macrolides.according to research (28,29)has been demonstrated to be resistant to the presence of efflux pumps linked to the resistance-nodulation-cell division (RND) family. In one local study published on the efflux pump genes (*adeABC*) in multidrug-resistant *A. baumannii*, the isolates were highly susceptible to amikacin, gentamicin, ciprofloxacin, levofloxacin, tigecycline, and likely tigecycline when an efflux pump inhibitor was present. Hence, the use of Phe-arg-beta-naphthylamide (PAβN) reduced the minimum inhibitory concentrations (MICs) up to 4-32 fold. In addition, it reduced the carbapenem MICs two to eightfold(30).

Evaluate the gene expression of efflux pump genes

The minimum inhibitory concentration of each sample was determined, and bacterial growth with antibiotics (Meropenem and Amikacin), and the antimicrobial peptide (Buforin2) was compared by this technique, where the treated and untreated samples were analysed by RT-qPCR in a quantitative manner. The analysis focused on determining the mRNA expression of the genes for the efflux pumps (*adeJ* and *adeK*). Quantitative RT-qPCR software was used to detect the Ct values of amplified genes. Gene expression fold changes were calculated by delta delta Ct value using the relative quantification (RQ) method. Our results showed that the efflux pump genes (*adeJ* and *adeK*) were significantly up-regulated in the local isolates (figures 2 and 3). The results of this experiment were the average gene expression of four isolates in comparison with the fold change of the control (the fold change is 1). Meropenem had the highest fold values at 4.62 with the gene *adeJ* (figure 2) and 3.51 with the gene *adeK* (figure 3). Additionally, the four isolates' gene expression folds ranged from 2.43 to 3.84 for the two genes, these resultswere greater than the fold of the control (the fold change is 1).

The results of the peptide Buforin2 recorded the down-regulationof the fold value (0.41) with the genes *adeJ*, while the fold change of the gene *adeK* exhibited the value similar to the control (1,24).

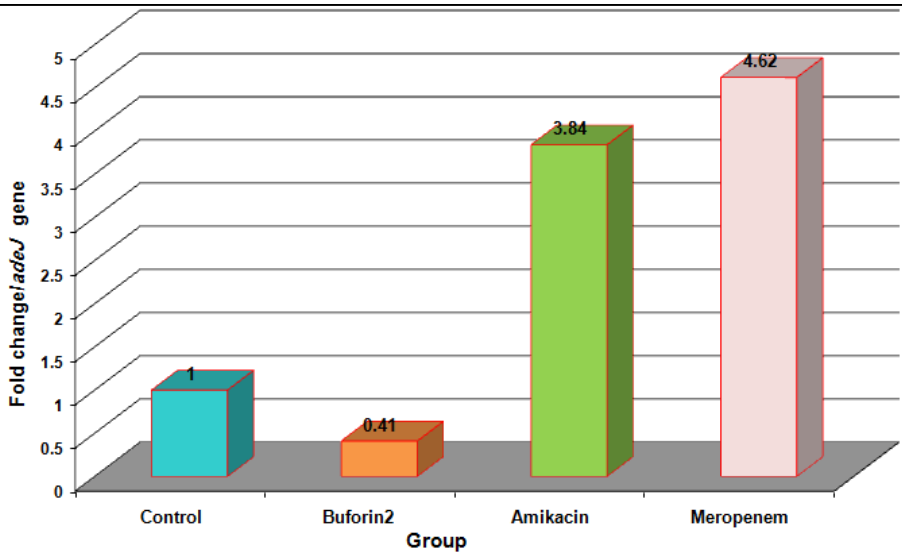


Figure (2): Fold of gene expression of *adeJ* gene for different *A. baumannii* isolates at the sub-inhibitory concentrations of Buforin2 and antibiotics depending on ΔΔCt method.

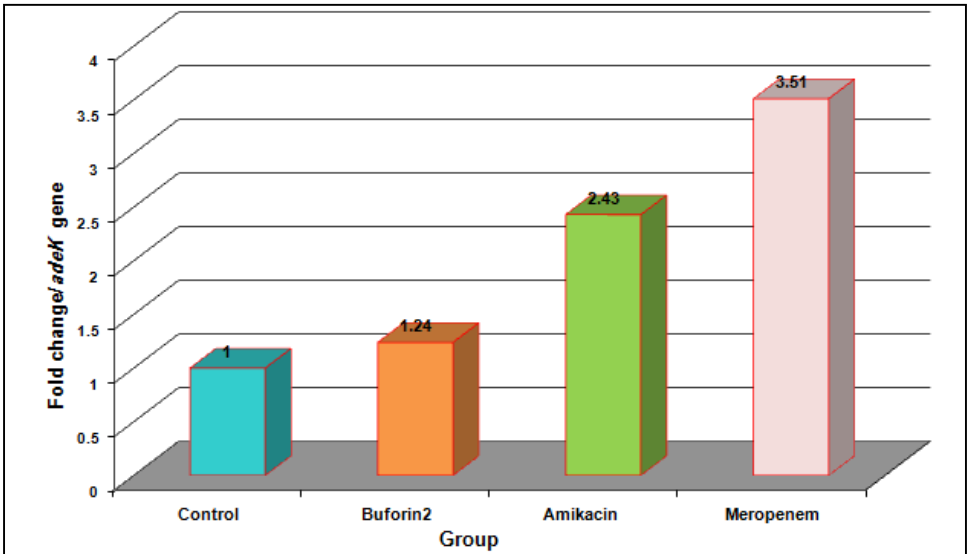


Figure (3): Fold of gene expression of *adeK* gene for different *A. baumannii* isolates at the sub-inhibitory concentrations of Buforin2 and antibiotics depending on ΔΔCt method.

Previous studies that use relative quantification usually compare the expression levels of a given gene across several samples. Because of this RT-qPCR differs from other gene expression methods. The amplification was recorded using the cycle threshold, also known as the Ct value. The housekeeping gene used in this study was the *16S rRNA* gene. The gene's utility in molecular studies is predicated on its steady expression in the cells or tissues being examined under various circumstances (31).

The results of previous study demonstrate the correlation between *adeABC* and carbapenem resistance in *A. baumannii*, as well as the overexpression of *adeJKL*, which was reversed by the addition of efflux pump inhibitors. Imipenem and trimethoprim worked well together against every strain that was tested when imipenem and cinnamon oil were mixed (32).

Many researchers indicated the basic RND system for bacteria in the genus Acinetobacter is *adeIJK*. Since *adeIJK* may export a wide variety of antibiotics and performs essential cellular processes including lipid regulation of the cell membrane, it is probable that all Acinetobacter depend on *adeIJK* for survival and homeostasis. Other RND systems, such as*adeABC* and *adeFGH*, on the other hand, were only discovered in a fraction of infection-associated Acinetobacter. An understanding of the roles and mechanisms of Acinetobacter's RND efflux systems could guide infection treatment in such a way that development of efflux-mediated resistance is prevented, and patient outcomes improved (32).

In contrast, CCCP was reported to decrease the minimum inhibitory concentration (MIC) values of tigecycline (opposite to our data), and all tested isolates were treated as resistant to tigecycline. In addition, up-regulation of the RND efflux pumps plays a role in tigecycline resistance in clinical *A. baumannii* isolates from the Western Balkans (33), respectively, (1.35 to 2.82 fold) and (1.62 to 4 fold) when compared to ATCC19606 though the expression of *adeB*, *adeG* and *adeJ* genes in five, sixteen, and twelve isolates, respectively overexpression (1.16 to 3 fold), (1.35 to 2.82 fold), and (1.62 to 4 fold) when compared to ATCC19606 (33), respectively (1.16 to 3 fold), (1.35 to 2.82 fold), and (1.62 to 4 fold) compared to ATCC19606 (33).

Conclusion

The present study confirms that the *adeIJK* efflux pump represents an important team player in antibiotic resistance in clinical isolates of carbapenem-resistant *A. baumannii*. The MIC of Meropenem and Amikacin were significantly decreased with the addition of the efflux pump inhibitor CCCP, further indicating the effect of efflux pumps on the resistance. In contrast, the antimicrobial peptide Buforin2 showed relatively few changes in susceptibility, suggesting a different mode of action (Fig. 3). This result was confirmed with analysis of the expression levels of genes targeting the *adeABC* efflux pump using Gene expression analysis: recognition of *adeJ* and *adeK* being upregulated with antibiotics and downregulated (*adeJ*) and no significant change (*adeK*) with Buforin2 ($p<0.01$) in comparison to the control.

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