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Study the Role of the Efflux Pump in Multidrug-Resistant *Acinetobacter baumannii*

Nazhad Hassan Mohammed, Sahand Kamaluldeen Arif

Department of Biology, College of Science, University of Sulaimani, Al Sulaymaniyah, Iraq

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Corresponding Author:

Name:

E-mail: Sehand.arif@univsul.edu.iq

Tel:

ABSTRACT

Thirty-one *A. baumannii* isolates were obtained from Shar and Burn hospitals in Sulaimani City, KRG-Iraq. Bacterial identification was confirmed by detecting both *16S rRNA* and bla_{OXA-51} genes. The detection of carbapenem resistant isolates was carried out using Kirby- Bauer, RAPDEC Carba NP and combined disc assays. A high rate of drug resistance was observed, as 74.2% of the isolates were multidrug-resistant (MDR) and 38.7% were extensively drug-resistant (XDR), while carbapenem-resistant *A. baumannii* (CRAB) comprised approximately 58% of the isolates. Among CRAB isolates, carbapenemase production was detected by using the RAPDEC Carba NP and the combined disc assays in 88.8% (16/18) and 55.5% (10/18) of the isolates respectively. Phenotypic activity of efflux pump was detected among 22.5% of all isolates and 38.8% of CRAB isolates. PCR successfully identified the *ade*A, *ade*B, *ade*C, *ade*R, and *ade*S genes in 83.9%, 90.3%, 58%, 80.6%, and 90.3% of the isolates, respectively.

Eight mutations in *ade*R and 15 mutations in *ade*S regulatory genes were recorded. We conclude that carbapenem resistance mechanisms in *A. bumanni* isolates included mutations in *ade*RS regulatory genes and production of carbapenemase.

Introduction

A. baumannii is an opportunistic pathogen which plays a substantial role in the healthcare associated infections particularly in burn, surgical, and intensive care units [1]. *A. baumannii* possess an exceptional ability to get antibiotic resistance traits which finally leads to the appearance of multidrug resistance *A. baumannii* (MDR-AB) [2], to a variety of antibiotic classes, comprising carbapenems , fluoroquinolones, tetracyclines, and aminoglycosides [3]. As a result, eliminating these *A. baumannii* strains is difficult and associated with an increased death rate among infected patients [4]. The formation of various resistance mechanisms in MDR-AB isolates can be attributed to mutations in regulatory and structural genes, as well as the insertion of mobile genetic elements. The endogenous resistance genes identified in *A. baumannii* also include decreased membrane permeability, efflux mechanisms, and genes for carbapenemase that are encoded on the chromosome. Efflux pump overexpression is the major mechanism through which multiple kinds of antibiotics extrusion takes place [5].

In general, bacterial drug efflux pumps belong to one of following five super-families of transport proteins, including the multidrug and toxic compound extrusion (MATE), the ATP binding cassette (ABC), the resistance nodulation division (RND), the small multidrug resistance (SMR) and the major facilitator superfamily (MFS). [6]. The outer membrane (OM, *ade*C), the internal membrane (IM, *ade*B), and the membrane fusion proteins are the three distinct components that make up the efflux system and each have a role in the drug efflux mechanism (MFP, *ade*A) [3].

Previous researches have demonstrated that overexpression of the *ade*ABC, *ade*FGH, and *ade*IJK

as the three main RND pumps, contributes to antibiotic resistance in *A. baumannii isolates*. [7]. A two-component regulatory system (*ade*RS) controls the *ade*ABC efflux pump [8]. *ade*S and *ade*R they work as a sensor kinase and a response regulator respectively. their transcription is in the opposite direction and are placed upstream from *ade*A [9]. This regulatory system is operates as signal transduction pathway in *A. baumannii* that reacts to environmental conditions (pump is dependent on the substrate) [10].

Thus, the goal of this research was to investigate the role of the *ade*ABC efflux pump mechanism in carbapenem resistant *A. baumannii* isolates.

Material and methods

Bacterial isolates

Bacterial isolates were collected between September 2021 and February 2022 from Shar teaching hospital and Burn and Plastic Surgery Hospital in Sulaimani City, KRG-Iraq. The samples were sent to the advanced microbiology laboratory and advanced molecular laboratory in the department of biology at the university of Sulaimani for further investigations.

Identification of bacterial isolates

Bacterial isolates were initially identified by examining morphological characteristics on MacConkey agar, followed by gram staining and biochemical assays such as catalase and oxidase tests.

Molecular confirmation of *A. baumannii*

A. DNA extraction

Bacterial genomic DNA kit (Geneaid, Taiwan) was used to extract the DNA from an overnight culture in tryptic soy broth (TSB), in accordance with the manufacturer's instructions. The quality and quantity of extracted DNA samples were measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

B. 16S rRNA gene detection

For the purpose molecular identification of the bacterial isolates *16S rRNA* primers were used as described by [11]. Briefly, primers described in (Table 1) were used to amplify a 426-bp fragment of the ribosomal RNA gene. The reaction volume was 20µL in total: 5µL OnePCR™ Ultra buffer master mix, 1µL forward and 1µL reverse primers**,** 2µL DNA template and 11µL PCR grade water. And the PCR reaction conditions were as follows: a pre-denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 56.5°C for 1 minute, extension at 72°C for 1 minute, and finally finished with extension at 72°C for 7 minutes.

Table 1: Oligonucleotide primers used in this study

Gene primers	Nucleotide sequence (5' to 3')	Product size (bp)	References
16srRNA- F	GACGTACTCGCAGAATAAGC	426	[11]
16srRNA-R	TTAGTCTTGCGACCGTACTC		
adeA-F	ATCTTCCTGCACGTGTACAT	513	[12]
adeA-R	GGCGTTCATACTCACTAACC		
adeB-F	TTAACGATAGCGTTGTAACC	541	[12]
adeB-R	TGAGCAGACAATGGAATAGT		
adeC-F	AGCCTGCAATTACATCTCAT	560	[13]
adeC-R	TGGCACTTCACTATCAATAC		
adeR-F	ACTACGATATTGGCGACATT	447	[14]
adeR-R	GCGTCAGATTAAGCAAGATT		
adeS-F	TTGGTTAGCCACTGTTATCT	544	[14]
adeS-R	AGTGGACGTTAGGTCAAGTT		
bla_{oxa}-51 -F	TAATGCTTTGATCGGCCTTG	353	[15]
bla_{oxa}-51-R	TGGATTGCACTTCATCTTGG		

C. bla_{OXA-51} gene detection

The amplification of OXA-51 gene was conducted using primers with an amplicon size of 353 base pairs in order to confirm bacterial isolates identity at the species level [15] (Table 1). These primers (1µL each) were added to 5µL OnePCR™ Ultra buffer master mix along with 11µL of PCR grade water and 2µL of DNA template. The reaction conditions consisted of: a pre-denaturation at 95°C for 2 minutes, followed by 45 cycles at 95°C for 10 seconds, 60°C for 34 seconds, 72°C for 30 seconds and a final extension at 72°C for 5 minutes.

D. Gel electrophoresis of PCR products

Ethidium bromide was used to stain the PCR products and gel electrophoresis carried out using 1% agarose gel. Using a Bio-Rad Gel Doc XR+ imaging technique, images were captured. Agarose gel electrophoresis (Cleaver, Scientific Ltd., U.K.) was used to separate the PCR products in 1x TBE buffer at 80V for 60 minutes [16].

Antimicrobial susceptibility testing

Antibiotic susceptibility was determined by Kirby-Bauer disc diffusion for 11 antibiotics (Himedia, India). The antibiotics used were ciprofloxacin (10µg), colistin (25µg), meropenem (10µg), imipenem (10µg), tigecycline (15µg), ceftazidime (30µg), piperacillin (100µg), tobramycin (10µg), tetracycline (30µg), piperacillin-tazobactam (100/10µg), and trimethoprim-sulfamethoxazole (1.25/23.75µg) results were interpreted according to

the guidelines of Clinical and Laboratory Standards Institute [17]. *A. baumannii* ATCC 19606 was used as control strain.

Phenotypic detection of carbapenem resistant isolates

A. CarbaNP test

RAPIDEC CARBA NP (BioMérieux, France) was used to detect carbapenemase-producers according to the manufacturer's protocol. After the isolates were grown overnight on Mueller-Hinton Agar (MHA) plates, they were inoculated into the test strip wells and incubated at 37°C for 30 minutes to 2 hours. The results were visualized by comparing the color in the test strips to those in the control. Color changes from red to yellow or orange within 30 minutes were reported as positive results; otherwise, the incubation time was extended to 2 hours before a negative result was issued [18, 19].

B. Meropenem+EDTA combination disc test (EDTA-CDT)

In order to prepare a 0.5M EDTA solution, 18.6 gm of EDTA (Sigma-Aldrich, Germany) was dissolved in 100 mL of distilled water and mixed well. To bring the pH of this solution to 8.0, NaOH was gradually added. Tested isolates were adjusted to the 0.5 MacFarland standard and streaked on MHA plates, to a MHA plate, two discs of meropenem (MEM) were placed, and 10µL of 0.5 M EDTA solution containing 750µg of EDTA was dropped on one of the two discs, after an overnight incubation at 37^{o}C. Isolates showing ≥7 mm increase in the inhibition zone size of MEM-EDTA disc than the MEM disc alone were considered as a positive test [20].

C. Detection of efflux pumps

1. Phenotypic detection

All *A. baumannii* isolates were assessed to determine phenotypic expression of the efflux pump using MHA plates with meropenem double serial dilutions (0.125-256 µg/mL) in the presence and absence of 25µg/L CCCP. A reduction by at least fourfold in the MIC of meropenem in the presence of CCCP was defined as positive phenotypic detection of an efflux system [21].

2. Genotypic detection using PCR

Efflux pump-encoding genes *ade*A, *ade*B, and *ade*C, as well as their regulatory (*ade*R and *ade*S) genes were targeted using specific primers (Table 1), The reaction volume was 20µL in total: 5µL OnePCR™ Ultra buffer master mix, 1µL forward and 1µL reverse primers**,** 2µL DNA template and 11µL PCR grade water. And the following PCR conditions were applied: initial denaturation at 94°C for 5 minutes, then followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 56.5°C for 1 minute, extension at 72°C for 1 minute, and finally finished with extension at 72°C for 7 minutes [12-14].

DNA sequence analysis

Twelve Carbapenem resistant isolates harboring *ade*ABC and *ade*RS genes were selected for DNA sequencing, 20µl of PCR products and 2µl of each primer were sent to (Macrogen, South Korea) for sequencing, the sequenced genes were recorded in the database of NCBI under these GenBank accession numbers: *ade*R, OP756503- OP756509 and *ade*S, OP756510- OP756521.

Results

A. baumannii was identified preliminarily by observing morphological aspects such as growth on MacConkey agar and gram staining, in addition to performing two biochemical tests such as oxidase and catalase test, and finally confirmation was conducted by PCR targeting of *16S rRNA* and $bla_{\text{OXA-51}}$ genes.

Molecular detection of *A. baumannii*

PCR identification of *A. baumannii* has taken place by detecting *16S rRNA* and $bla_{\text{OXA-51}}$ genes by two distinct PCR reactions (Figure 1: A and B). *A baumannii* in both clinical isolates and the reference strain were successfully detected by the primers targeting *16S rRNA* and $bla_{\text{OXA-51}}$ genes.

Antibiotic susceptibility assay

Zone diameter around the antibiotic discs were measured to determine the resistance profile of the tested bacteria by using Kirby-Bauer disc diffusion method, the current study exhibited remarkable resistance-patterns, according to the findings, *A. baumannii* showed resistance to penicillins; piperacillin (74%), carbapenems; meropenem (45%), imipenem (58%) aminoglycosides; tobramycin (61%), fluoroquinolones; ciprofloxacin (65%), cephalosporin; ceftazidime (80%), folate pathway antagonists; trimethoprim/sulfamethoxazole (61%), tetracyclines; tetracycline (42%), glycylcyclines; tigecycline (35%) while it showed an intermediate resistance to β-Lactam-β-lactamase-inhibitor combination; piperacillin-tazobactam (42%), however all the isolates were fully susceptible to lipopeptides; colistin (100%) (Table2).

In addition, the isolates categorized into multi-drug resistant (MDR: resistant to ≥ one agent in ≥ 3 antimicrobial classes) and extensively-drug resistant (XDR: resistant to ≥ one agent in all but ≤ two antimicrobial classes) based on the phenotypic resistance patterns. About 74.2% (23/31) of the *A. baumannii* isolates were MDR which exhibited 8 different patterns, pattern 1 was the most common and showed resistance to penicillins, carbapenems, cephalosporin, fluoroquinolones, aminoglycosides, folate pathway antagonists.

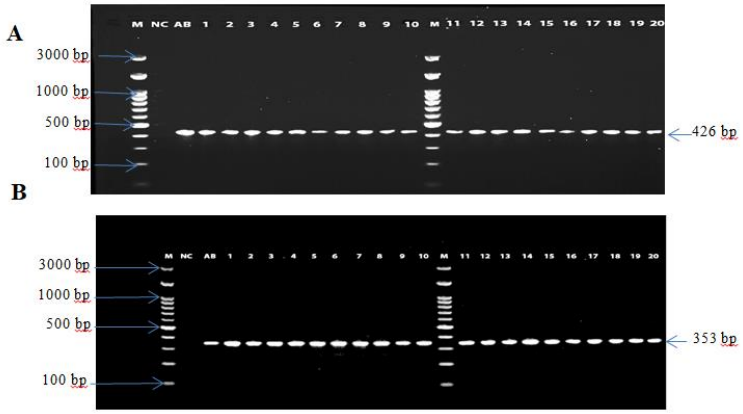

Fig. 1: Agarose gel electrophoresis image of PCR products for *16S RNA* and bla_{OXA-51} gene

A: Amplified fragments of *16S RNA* gene (426 bp) in *A. baumannii* (1-4), Negative control (NC), *A. baumannii* ATCC 19606 (AB), Lane M: DNA ladder 100bp. **B**: PCR amplification of bla_{OXA-51} gene (353 bp) of *A. baumannii* (1-4), Negative control (NC), *A. baumannii* ATCC19606 (AB)

Furthermore, based on the findings about 38.7% (12/31) were XDR and exhibited 4 different patterns, pattern 1 was the most common and showed resistance to all antibiotics except for β-Lactam-β-lactamase-inhibitor combination and lipopeptides (Table 3).

Phenotypic detection of carbapenem resistant isolates

In the present study, 18 (58%) of *A. baumannii* isolates exhibited carbapenem resistance (CRAB) as determined by the disc diffusion method were selected for carbapemenase detection using RAPIDEC CARBA NP and meropenem-EDTA

A. CarbaNP test

According to the RAPIDEC CARBA NP test, carbapenemase was produced by 88.8% (16/18) of the isolates (Figure 2).

Table 2: Antimicrobial susceptibility patterns of *A. baumannii* to various antimicrobial agents

Antimicrobial Classes	Antimicrobial agents	*A. baumannii* (N=31)					
		S		**I**		**R**	
		N	%	n	%	N	%
Penicillins	Piperacillin	6	20	2	6	23	74
β - lactam- β-lactamase inhibitor	piperacillin-tazobactam	11	35	13	42	7	23
Carbapenem	Meropenem	12	39	5	16	14	45
	Imepenem	12	39	1	3	18	58
Lipopeptides	Colistin	31	100				
Cephalosporin	Ceftazidime	3	10	3	10	25	80
Folate pathway antagonists	Trimethoprim/ Sulfamethoxazole	12	39			19	61
Aminoglycosides	Tobramycin	12	39			19	61
Fluoroquinolones	Ciprofloxacin	11	35			20	65
Glycylcycline	Tigecycline	15	49	5	16	11	35
Tetracyclines	Tetracycline	14	45	4	13	13	42

Table 3: Resistant Patterns of *A. baumannii*

Antimicrobial classes	Resistant Patterns of *A. baumannii* (N=31)											
	(MDR 23/31)								XDR (12/31)			
	Patterns (P)								Patterns (P)			
	P 1	P 2	P 3	P 4	P 5	P 6	P 7	P 8	P 1	P 2	P 3	P 4
Penicillins	R	R	R	R	R	R	R	R	R	R	R	R
β - lactam- β-lactamase inhibitor	S	S	R	S	S	R	S	S	S	R	R	R
Carbapenem	R	S	R	S	S	S	R	R	R	R	R	S
Cephalosporin	R	R	S	R	R	R	R	R	R	R	R	R
Folate pathway antagonists	R	R	S	R	S	S	S	R	R	R	R	R
Aminoglycosides	R	R	R	R	S	S	S	S	R	R	R	R
Fluoroquinolones	R	S	R	S	R	S	R	R	R	R	R	R
Lipopeptides	S	S	S	S	S	S	S	S	S	S	S	S
Tetracyclines	S	R	S	S	S	R	R	S	R	R	S	R
Samples N (%)	4 (36%)	1 (9%)	1 (9%)	1 (9%)	1 (9%)	1 (9%)	1 (9%)	1 (9%)	6 (50%)	3 (25%)	2(17%)	1 (8%)

B. Meropenem+EDTA combined disc test (EDTA-CDT)

Using the combined Meropenem/EDTA test, all CRA B isolates were examined for metallo-β-lactamase (MBL) production,
the results indicated that MBL activity was present in 10 (55.5%) of the isolates (Figure 3).

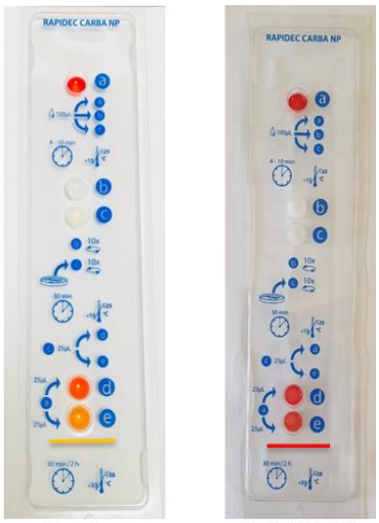

Fig. 2: phenotypic detection of carbapenemase producing *A. baumannii* using the RAPIDEC CARBA NP.

Isolate no.20 shows positive carbapenemase production (positive: yellow color), *A. baumannii* (ATCC 19606) reference strain control show negative for carbapenemase production (negative: red color).

C. Efflux pump mechanism

1. Phenotypic detection

The phenotypic expression of the efflux pumps in all *A. baumannii* including 18
CRAB isolates with and without addition of 25µg/mL of CCCP was analyzed. 4 to 12- fold decrease in MICs to meropenem were detected in 22.5%, (7/31) among CRAB isolates (Figure 4).

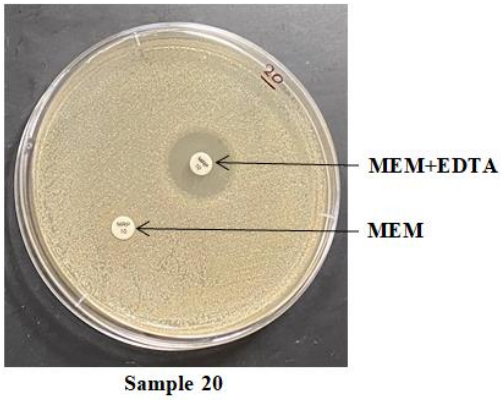

Fig. 3: Combined disc test (CDT) revealing presence of Metalo Beta Lactamase (MBL) class B.

2. Genotypic detection

Our PCR assay revealed that 83.9% (26/31) of the tested isolates carry *ade*A gene, 90.3% (28/31) carry *ade*B gene and 58.0% (18/31) carry *ade*C (Figure 4). While 80.6% (25/31) of the tested isolates carry *ade*R gene and 90.3% (28/31) carry *ade*S gene on their DNA (Figure 5).

Sequence analysis

Twelve CRAB isolates were sent for sequence analysis, 7 of them were efflux pump positive (≥ 4 fold reduction of MIC) and 5 of them were efflux pump negative (< 4 fold reduction of MIC). Some results were excluded from the study because of incomplete sequence data. Sequence analysis of the regulatory genes revealed several nucleotide mutations in the *ade*R and *ade*S genes. The genes were translated to proteins, in *ade*R the most common mutation was D698G (6 isolates), followed by, V747I and A763V (1 isolates each), while more mutations detected in *ade*S protein, A910V, G1002V and F1030L found in (5 isolates each) (Table 4 and figure 6). All three efflux pump-negative samples revealed a single mutation in the *ade*S protein at position G1002V.

Discussion

Drug-resistant *A. baumannii* is considered to be the "top priority" pathogen by WHO in 2018 in the

summit of critical bacteria resistant, Recently, the urgent need for study search for new therapy choices has been pointed out. [22, 23]. There are few treatments available for this tenacious microorganism as a result of the participation of various mechanisms of resistance in the survival of *A. baumannii* [24]. The largest portion of the Ambler Class D oxacillinase group is an OXA-51-like gene (OXA-type). So far, numerous variants of this group have been discovered [25].

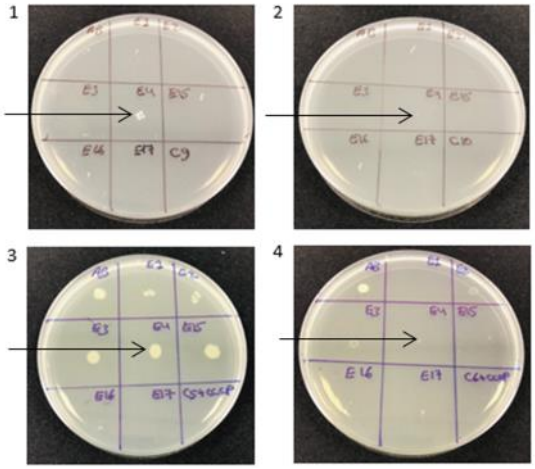

Fig. 4: MIC decrease of meropenem with and without CCCP (25 µg/mL)

1- Bacterial growth not suppressed by meropenem (32µg/mL) in the absence of CCCP.
2- Bacterial growth suppressed by meropenem (64µg/mL) in the absence of CCCP.
3- Bacterial growth not suppressed by meropenem (2µg/mL) in the presence of CCCP.
4- Bacterial growth suppressed by meropenem (4µg/mL) in the presence of CCC.

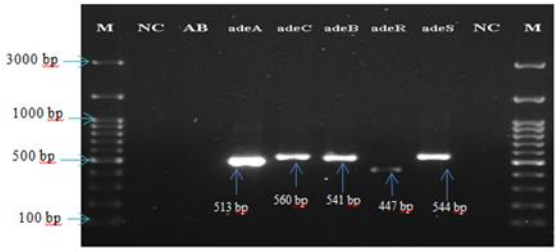

Fig. 5: gel electrophoresis image of PCR product of *ade*ABC-*ade*RS system

Amplified fragments of *ade*ABC genes of *A. baumannii*; *ade*A gene (513 bp), *ade*B (541 bp) and *ade*C (560 bp), and regulatory genes; *adeR* (447 bp) and *ade*S (544 bp). Negative control (NC), *A. baumannii* ATCC 19606 (AB), Lane M: DNA ladder 100bp.

Table 4: Amino acid substitution in *ade*R and *ade*S

Isolate no.	Fold reduction in MEM MIC	Changes in *ade*R	Changes in *ade*S	No. of mutations
2	6	D698G	A910V, F1030L	3
3*	1	-	G1002V	1
4	4	D698G	A910V, F1030L	3
6	6	D698G	A910V, F1030L	3
8	5	-	G1002V	1
10*	1	-	G1002V	1
11*	2	-	G1002V	1
13	4	D698G	A910V, F1030L	3
18	4	D698G	A910V, F1030L	3
19	12	D698G, V747I, A763V	G1002V	4

*: Efflux pump negative sample

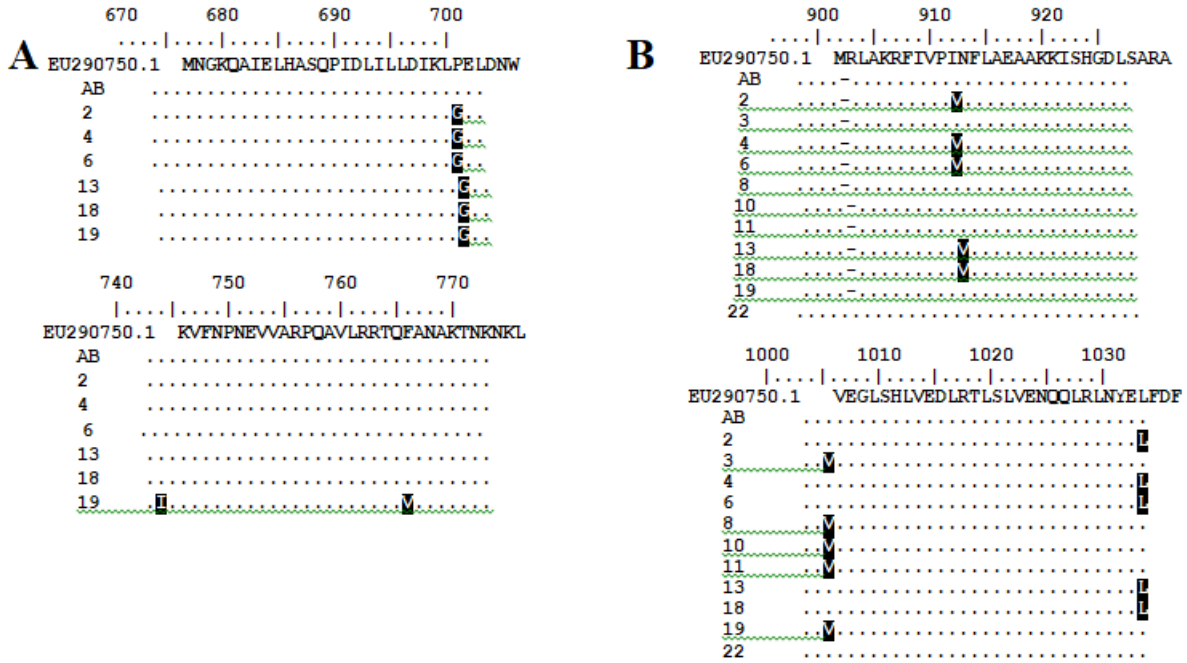

Fig. 6: Multiple-sequence alignment of *ade*R (A) and *ade*S (B) and comparing the amino acid sequence with that of the sensitive strain (*A. baumannii* EU290750.1).

Substitution of the amino acids was highlighted in black. In Some instances, mutations leading to successive expression of *ade*ABC efflux pump in the isolates such as D698G, V741I and A763V in *ade*R and A910V, G1002V and F1030L in *ade*S.

These enzymes are intrinsic and naturally exist in the chromosomal DNA of *A. baumannii* inherited into all strains. This fact makes it a wonderful marker successfully applied for the detection of *A. baumannii* at the species level [16].

Our results revealed high resistance for cephalosporins, carbapenems, fluoroquinolones, piperacillin, piperacillin-tazobactam, aminoglycosides and trimethoprim/sulfamethoxazole; moderate resistance for tetracyclines, and interestingly, all isolates were susceptible to colistin, Antibiotic susceptibility patterns were in consistent with [26-30] .

In the present study about 74.2% (23/31) of the *A. baumannii* isolates were MDR; several studies have investigated imipenem/meropenem resistance in Iraq. In 2012, a local study in Sulaimani, found that 57% of *A. baumannii* found in burn patients were resistant to imipenem [31]. Researchers in Baghdad, conducted a study during 2014 that included clinical and environmental samples and found that 58.26% of the isolates were resistant to carbapenem antibiotics [32]. In addition to this, alarmingly high levels of resistance (92%) to imipenem and meropenem among *A. baumannii* calcoaceticus complex was recorded in 2016 among isolates obtained from a number of different hospitals located in two cities within the Kurdistan region of Iraq [33]. Recently, researchers in 2019 recorded MDR-AB in 73.3% of their isolates in Hilla [34], other studies which were conducted during 2020 in Ninawa reported 100% [35] and in Duhok during 2022 recorded 96% [36].

About 38.7% of our isolates were XDR, similar data (30%) was recorded by Radhi and Al-Charrakh in 2019 [34], while other researchers found much higher XDR result (84.2%) in Ninawa during 2020 [35], The Taiwan Surveillance of Antimicrobial Resistance program found that from 2002 to 2010, the frequency of the XDR A. baumannii jumped from 1.3% in 2002 to 41.0% in 2012. This indicates that XDR *A. baumannii* is emerging worldwide [37].

A baumanii produce carbapenemase, a beta lactamase that hydrolyzes carbapenem which is a last resort for treating multidrug-resistant infections, consequently, therapeutic options is reduced [19]. These bacteria develop resistance to carbapenem primarily as a result of three distinct or combined mechanisms: carbapenemase production (class D OXA-type and class B metallo-β-lactamases), permeability loss, and efflux pumps [38].

The global distribution of carbapenemases emphasizes the significance of their rapid identification. Even though PCR is regarded as the gold standard for detection of carbapenemase, most laboratories can't use this method because it's expensive and necessitates special equipment. Biochemical assays recommended by Clinical laboratory Standard institute (CLSI) such as the Carba NP method in which the hydrolysis of the B -lactam ring is seen by pH indicator's color change, can identify carbapenemase activity [38].

In the present work, 58% of the isolates showed resistance to carbapenem (CRAB) by the Kirby-

Bauer disc diffusion susceptibility testing, and among these CRAB isolates, carbapenemase activity was detected by the RAPDEC Carba NP and Meropenem+EDTA CDT tests in 88.8% and 55%, respectively. which was inconsistence with studies conducted recently in Sulaimani city [15] and in Duhok city [16], furthermore, high resistance to carbapenems reported in Iran 94% in 2020 [12], 99% in 2021 [24] and in Jordan 75.1% in 2022 [39].

To determine the role of efflux pump activity, we used EP inhibitor (CCCP) to observe the reduce in the sensitivity of the tested isolates toward meropenem, the MIC was reduced by at least fourfold in the presence of 25µg/mL CCCP in 22.5% (7/31) of all isolates and 38.8% (7/18) among CRAB isolates. in a study which was conducted by Alcántar-Curiel, et al. in 2014 only 14.5% of isolates exhibited MIC reduction to meropenem [21], whereas Ju, et al. in 2021 found that the MIC reduced to meropenem in 58.8% of the isolates [40]. About 80% of the *A. baumannii* isolates harbor *ade*ABC, which is chromosomally encoded and considered to be the major efflux mechanism linked to carbapenem resistance [41]. Our study indicated that *ade*A, *ade*B, and *ade*C genes were present in 83.9%, 90.3%, and 58.0% of the samples, respectively. Japooni et al. revealed that the frequency of *ade*A, *ade*B, and *ade*C genes was 100, 100, and 96.5% in *A. baumannii* isolates respectively, another study found *ade*ABC genes in all *A. baumannii* isolates [42, 43], while others researchers have found that their prevalence is between 53% and 97% [44, 45].

Twelve (CRAB, *ade*ABC and *ade*RS genes positive) isolates were selected for studying the possible mutation in *ade*R and *ade*R genes by DNA sequencing analysis. Sequencing analysis revealed mutations in different positions of both genes which lead to changes in 6 different amino acids. *ade*R protein sequences showed following changes: D698G in 6 isolates, V747I in one isolate and A763V in 1 isolate, while *ade*S protein showed the following changes: A910V, G1002V , F1030L in (5 isolates each). Numerous studies demonstrated the association between antibiotic resistance and RND efflux pump overexpression [24, 46-48]. Overexpression of this mechanism is strictly controlled by *ade*RS genes, mutation in *ade*RS genes provides resistance or reduced susceptibility to aminoglycosides, chloramphenicol, trimethoprim, tetracycline, erythromycin, fluoroquinolones, and carbapenems [41, 49-51]. The present study demonstrated that 7 of the EP-positive isolates also exhibited carbapenemase activity, which may suggest a combined action of enzymatic (carbapenemase) and non-enzymatic (efflux pump) mechanism in carbapenem resistance among our CRAB isolates, as production of carbapenemase were documented by two different assays: the RAPDEC Carba NP test which was used to detect carbapenem-hydrolysing oxacillinases and the combined disc test, which was used to detect metallo-β-lactamases, furthermore, non-enzymatic mechanism confirmed by detecting efflux pump activity with and without efflux pump inhibiter and recording mutations in *ade*RS regulatory genes which leads to overexpression of the *ade*ABC efflux pump.

A study conducted in UAE in 2019 concluded that carbapenemases, in addition to the downregulation of porin and the overexpression of efflux, were the major mechanisms of carbapenem resistance in *P. aeruginosa*. Prior studies established that the existence of three genetic markers of carbapenem resistance in a particular isolate leads to an elevated MIC of imipenem Moubareck, et al. [52] and Al-Agamy, et al. [53]

Conclusions

In the current investigation, mutations in the efflux pump regulatory system (*ade*RS) and carbapenemase production were documented as carbapenem resistance mechanisms in *A. baumannii* isolates.

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References

[1] M. Amin, T. Navidifar, F. S. Shooshtari, M. Rashno, M. Savari, F. Jahangirmehr, *et al.*, "Association between biofilm formation, structure, and the expression levels of genes related to biofilm formation and biofilm-specific resistance of Acinetobacter baumannii strains isolated from burn infection in Ahvaz, Iran," *Infection and Drug Resistance*, vol. 12, p. 3867, 2019.

[2] G. S. GIEBINK, "Otitis media: the Chinchilla model1," *Microbial drug resistance*, vol. 5, pp. 57-72, 1999.

[3] H. Li, X. Wang, Y. Zhang, C. Zhao, H. Chen, S. Jiang, *et al.*, "The role of RND efflux pump and global regulators in tigecycline resistance in clinical Acinetobacter baumannii isolates," *Future microbiology*, vol. 10, pp. 337-346, 2015.

[4] L. Azimi, A. E. Namvar, A. R. Lari, and S. Jamali, "Comparison of efflux pump involvement in antibiotic resistance among Pseudomonas aeruginosa isolates of burn and non-burn patients," *Archives of Pediatric Infectious Diseases*, vol. 4, 2016.

[5] É. Ruppé, P.-L. Woerther, and F. Barbier, "Mechanisms of antimicrobial resistance in Gram-negative bacilli," *Annals of intensive care*, vol. 5, pp. 1-15, 2015.

[6] E.-J. Yoon, P. Courvalin, and C. Grillot-Courvalin, "RND-type efflux pumps in multidrug-resistant clinical isolates of Acinetobacter baumannii:

major role for AdeABC overexpression and AdeRS mutations," *Antimicrobial agents and chemotherapy*, vol. 57, pp. 2989-2995, 2013.
[7] A. Y. Peleg, H. Seifert, and D. L. Paterson, "Acinetobacter baumannii: emergence of a successful pathogen," *Clinical microbiology reviews*, vol. 21, pp. 538-582, 2008.
[8] D. B. Knight, S. D. Rudin, R. A. Bonomo, and P. N. Rather, "Acinetobacter nosocomialis: defining the role of efflux pumps in resistance to antimicrobial therapy, surface motility, and biofilm formation," *Frontiers in microbiology*, vol. 9, p. 1902, 2018.
[9] A. R. Lari, A. Ardebili, and A. Hashemi, "AdeR-AdeS mutations & overexpression of the AdeABC efflux system in ciprofloxacin-resistant Acinetobacter baumannii clinical isolates," *The Indian journal of medical research*, vol. 147, p. 413, 2018.
[10] R. Krasauskas, J. Skerniškytė, J. Armalytė, and E. Sužiedėlienė, "The role of Acinetobacter baumannii response regulator BfmR in pellicle formation and competitiveness via contact-dependent inhibition system," *BMC microbiology*, vol. 19, pp. 1-12, 2019.
[11] L. Chen, H. Li, H. Wen, B. Zhao, Y. Niu, Q. Mo, *et al.*, "Biofilm formation in Acinetobacter baumannii was inhibited by PAβN while it had no association with antibiotic resistance," *MicrobiologyOpen*, vol. 9, p. e1063, 2020.
[12] H. Mahmoudi, L. Shokoohizadeh, N. Zare Fahim, A. Mohamadi Bardebari, S. Moradkhani, and M. Y. Alikhani, "Detection of adeABC efflux pump encoding genes and antimicrobial effect of Mentha longifolia and Menthol on MICs of imipenem and ciprofloxacin in clinical isolates of Acinetobacter baumannii," *BMC complementary medicine and therapies*, vol. 20, pp. 1-7, 2020.
[13] S. S. Gurpinar, D. Kart, and M. Eryilmaz, "The effects of antidepressants fluoxetine, sertraline, and amitriptyline on the development of antibiotic resistance in Acinetobacter baumannii," *Archives of Microbiology*, vol. 204, pp. 1-10, 2022.
[14] Y. Chen, L. Ai, P. Guo, H. Huang, Z. Wu, X. Liang, *et al.*, "Molecular characterization of multidrug resistant strains of Acinetobacter baumannii isolated from pediatric intensive care unit in a Chinese tertiary hospital," *BMC Infectious Diseases*, vol. 18, pp. 1-7, 2018.
[15] A. A. Shali, P. J. Jalal, and S. K. Arif, "Dissemination and Genetic Relatedness of Multidrug-Resistant and Extensively Drug-Resistant Acinetobacter baumannii Isolates from a Burn Hospital in Iraq," *Canadian Journal of Infectious Diseases and Medical Microbiology*, vol. 2022, 2022.
[16] M. K. Qader, "Molecular study of Acinetobacter baumannii using 16S rRNA and blaOXA-51 gene isolated from hospitals in Duhok-Kurdistan region, Iraq," *Journal of Duhok University*, vol. 24, pp. 19-25, 2021.
[17] M. P. Weinstein and J. S. Lewis, "The clinical and laboratory standards institute subcommittee on antimicrobial susceptibility testing: background, organization, functions, and processes," *Journal of clinical microbiology*, vol. 58, pp. e01864-19, 2020.
[18] L. Dortet, L. Poirel, C. Errera, and P. Nordmann, "CarbAcineto NP test for rapid detection of carbapenemase-producing Acinetobacter spp," *Journal of clinical microbiology*, vol. 52, pp. 2359-2364, 2014.
[19] K. T. Suryavanshi and K. K. Lahiri, "Assessment of RapidecCarba NP test, as screening test to detect carbapenemase, specially metallo-[beta]-lactamase producing Gram negative bacteria at tertiary care centre," *IJHSR*, vol. 8, pp. 56-61, 2018.
[20] B. Bedenić, R. Ladavac, M. Vranić-Ladavac, N. Barišić, N. Karčić, K. B. Sreter, *et al.*, "False positive phenotypic detection of metallo-beta-lactamases in Acinetobacter baumannii," *Acta clinica Croatica*, vol. 58, p. 113, 2019.
[21] M. D. Alcántar-Curiel, L. F. García-Torres, M. I. González-Chávez, R. Morfín-Otero, C. Gayosso-Vázquez, M. D. Jarillo-Quijada, *et al.*, "Molecular mechanisms associated with nosocomial carbapenem-resistant Acinetobacter baumannii in Mexico," *Archives of medical research*, vol. 45, pp. 553-560, 2014.
[22] J. A. Viehman and M. H. Nguyen, "Treatment options for carbapenem-resistant and extensively drug-resistant Acinetobacter baumannii infections," *Drugs*, vol. 74, pp. 1315-1333, 2014.
[23] E. Tacconelli, E. Carrara, A. Savoldi, S. Harbarth, M. Mendelson, D. L. Monnet, *et al.*, "Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis," *The Lancet Infectious Diseases*, vol. 18, pp. 318-327, 2018.
[24] B. Salehi, Z. Ghalavand, A. Yadegar, and G. Eslami, "Characteristics and diversity of mutations in regulatory genes of resistance-nodulation-cell division efflux pumps in association with drug-resistant clinical isolates of Acinetobacter baumannii," *Antimicrobial Resistance & Infection Control*, vol. 10, pp. 1-12, 2021.
[25] B. A. Evans and S. G. Amyes, "OXA β-lactamases," *Clinical microbiology reviews*, vol. 27, pp. 241-263, 2014.
[26] N. D. Ghulam and H. Hamimah, "Emergence of extensive drug-resistant Acinetobacter baumannii in North of Jordan," *African Journal of Microbiology Research*, vol. 5, pp. 1070-1074, 2011.
[27] H. Hussein, H. J. F. Al-Mathkhury, and A. Sabbah, "Imipenem-Resistant Acinetobacter baumannii isolated from patients and hospitals environment in Baghdad," *Iraqi Journal of Science*, vol. 54, pp. 803-812, 2013.
[28] N. Obeidat, F. Jawdat, A. G. Al-Bakri, and A. A. Shehabi, "Major biologic characteristics of Acinetobacter baumannii isolates from hospital environmental and patients' respiratory tract sources,"

American journal of infection control, vol. 42, pp. 401-404, 2014.
[29] B. A. Almomani, A. McCullough, R. Gharaibeh, S. Samrah, and F. Mahasneh, "Incidence and predictors of 14-day mortality in multidrug-resistant Acinetobacter baumannii in ventilator-associated pneumonia," *The Journal of Infection in Developing Countries*, vol. 9, pp. 1323-1330, 2015.
[30] A. Batarseh, A. Al-Sarhan, M. Maayteh, S. Al-Khatirei, and M. Alarmouti, "Antibiogram of multidrug resistant Acinetobacter baumannii isolated from clinical specimens at King Hussein Medical Centre, Jordan: a retrospective analysis," *East Mediterr Health J*, vol. 21, p. 11, 2015.
[31] A. A. Shali, "Identification of Multidrug-Resistant Genes in" Acinetobacter baumannii" in Sulaimani City-Kurdistan Regional Government of Iraq," *Asian Journal of Medical Sciences*, vol. 4, pp. 179-183, 2012.
[32] N. H. Hussein, H. J. F. Al-Mathkhury, and M. A. Sabbah, "Identification of imipenem-resistant genes in Acinetobacter baumannii isolated from Baghdad Hospitals," *J Med Microb Diagn*, vol. 3, p. 170, 2014.
[33] A. R. Ganjo, D. M. Maghdid, I. Y. Mansoor, D. J. Kok, J. A. Severin, H. A. Verbrugh, *et al.*, "OXA-carbapenemases present in clinical Acinetobacter baumannii-calcoaceticus complex isolates from patients in Kurdistan region, Iraq," *Microbial drug resistance*, vol. 22, pp. 627-637, 2016.
[34] S. H. Radhi and A. H. Al-Charrakh, "Occurrence of MBLs and Carbapenemases among MDR and XDR Acinetobacter baumannii Isolated from Hospitals in Iraq," *SCOPUS IJPHRD CITATION SCORE*, vol. 10, p. 668, 2019.
[35] N. H. Ahmad and G. A. Mohammad, "Identification of Acinetobacter baumannii and Determination of MDR and XDR Strains," *Baghdad Sci J*, vol. 17, 2020.
[36] N. H. Mahmood and A. Al-Brefkani, "Molecular Characterisation of Carbapenemase-Producing Acinetobacter baumannii isolates from Hospitalised Patients in Iraq," *Journal of Life and Bio Sciences Research*, vol. 3, pp. 27-32, 2022.
[37] S.-C. Kuo, S.-C. Chang, H.-Y. Wang, J.-F. Lai, P.-C. Chen, Y.-R. Shiau, *et al.*, "Emergence of extensively drug-resistant Acinetobacter baumannii complex over 10 years: nationwide data from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) program," *BMC Infectious Diseases*, vol. 12, pp. 1-9, 2012.
[38] L. R. Pancotto, C. S. Nodari, F. P. Rozales, T. Soldi, C. G. Siqueira, A. L. Freitas, *et al.*, "Performance of rapid tests for carbapenemase detection among Brazilian Enterobacteriaceae isolates," *brazilian journal of microbiology*, vol. 49, pp. 914-918, 2018.
[39] M. Al-Tamimi, H. Albalawi, M. Alkhawaldeh, A. Alazzam, H. Ramadan, M. Altalalwah, *et al.*, "Multidrug-Resistant Acinetobacter baumannii in Jordan," *Microorganisms*, vol. 10, p. 849, 2022.
[40] Y. Ju, Y.-J. Kim, C. L. Chang, G.-E. Choi, and K.-Y. Hyun, "Relationship between AdeABC Efflux Pump Genes and Carbapenem in Multidrug-resistant Acinetobacter baumannii," *Biomedical Science Letters*, vol. 27, pp. 59-68, 2021.
[41] I. Abbott, G. M. Cerqueira, S. Bhuiyan, and A. Y. Peleg, "Carbapenem resistance in Acinetobacter baumannii: laboratory challenges, mechanistic insights and therapeutic strategies," *Expert review of anti-infective therapy*, vol. 11, pp. 395-409, 2013.
[42] A. R. JaponiNejad, M. Sofian, and E. Ghaznavi-Rad, "Molecular detection of AdeABC efflux pump genes in clinical isolates of Acinetobacter baumannii and their contribution in imipenem resistance," *ISMJ*, vol. 17, pp. 815-823, 2014.
[43] H. Khayat, N. Sadeghifard, I. Pakzad, L. Azimi, S. Delfani, K. Sayehmiri, *et al.*, "Determination of different fluoroquinolone mechanisms among clinical isolates of Acinetobacter baumannii in Tehran, Iran," *Iranian Red Crescent Medical Journal*, vol. 19, 2017.
[44] S. Coyne, P. Courvalin, and B. Périchon, "Efflux-mediated antibiotic resistance in Acinetobacter spp," *Antimicrobial agents and chemotherapy*, vol. 55, pp. 947-953, 2011.
[45] S. Coyne, G. Guigon, P. Courvalin, and B. Périchon, "Screening and quantification of the expression of antibiotic resistance genes in Acinetobacter baumannii with a microarray," *Antimicrobial agents and chemotherapy*, vol. 54, pp. 333-340, 2010.
[46] M. Choquet, E. Lohou, E. Pair, P. Sonnet, and C. Mullié, "Efflux Pump Overexpression Profiling in Acinetobacter baumannii and Study of New 1-(1-Naphthylmethyl)-Piperazine Analogs as Potential Efflux Inhibitors," *Antimicrobial agents and chemotherapy*, vol. 65, pp. e00710-21, 2021.
[47] C.-M. Kim, G. Park, Y. J. Ko, S.-H. Kang, and S. J. Jang, "Relationships between relative expression of RND efflux pump genes, H33342 efflux activity, biofilm-forming activity, and antimicrobial resistance in Acinetobacter baumannii clinical isolates," *Japanese Journal of Infectious Diseases*, vol. 74, pp. 499-506, 2021.
[48] Y. Shi, X. Hua, Q. Xu, Y. Yang, L. Zhang, J. He, *et al.*, "Mechanism of eravacycline resistance in Acinetobacter baumannii mediated by a deletion mutation in the sensor kinase adeS, leading to elevated expression of the efflux pump AdeABC," *Infection, Genetics and Evolution*, vol. 80, p. 104185, 2020.
[49] F. Perez, R. Ponce-Terashima, M. D. Adams, and R. A. Bonomo, "Are we closing in on an" elusive enemy"? The current status of our battle with Acinetobacter baumannii," vol. 2, ed: Taylor & Francis, 2011, pp. 86-90.
[50] L. Poirel, B. Berçot, Y. Millemann, R. A. Bonnin, G. Pannaux, and P. Nordmann, "Carbapenemase-producing Acinetobacter spp. in

cattle, France," *Emerging infectious diseases*, vol. 18, p. 523, 2012.
[51] J. Vila, S. Martí, and J. Sanchez-Céspedes, "Porins, efflux pumps and multidrug resistance in Acinetobacter baumannii," *Journal of antimicrobial chemotherapy*, vol. 59, pp. 1210-1215, 2007.
[52] C. A. Moubareck, D. H. Halat, C. Akkawi, A. Nabi, M. A. AlSharhan, Z. O. AlDeesi, *et al.*, "Role of outer membrane permeability, efflux mechanism, and carbapenemases in carbapenem-nonsusceptible Pseudomonas aeruginosa from Dubai hospitals: Results of the first cross-sectional survey," *International Journal of Infectious Diseases*, vol. 84, pp. 143-150, 2019.
[53] M. H. Al-Agamy, K. Jeannot, T. S. El-Mahdy, H. A. Samaha, A. M. Shibl, P. Plésiat, *et al.*, "Diversity of molecular mechanisms conferring carbapenem resistance to Pseudomonas aeruginosa isolates from Saudi Arabia," *Canadian Journal of Infectious Diseases and Medical Microbiology*, vol. 2016, 2016.

دراسة دور مضخة التدفق في مقاومة بكتيريا *A. baumannii* المقاومة للأدوية المتعددة

سهند كمال الدين عارف ، نژاد حسن محمد

قسم علوم الحياة ، كلية العلوم ، جامعة السليمانية ، السليمانية ، العراق

الملخص

تم عزل 31 عزلة من *A. baumannii* من المرضى الراقدين في مستشفى شار و مستشفى الحروق في مدينة السليمانية إقليم كردستان العراق. اكد تشخيص البكتيريا من خلال الكشف عن الجينين *16S rRNA* و bla_{OXA-51}. لقد لوحظ وجود معدل مرتفع للمقاومة ضد الأدوية ، حيث أن 74.2٪ من العزلات كانت مقاومة للأدوية المتعددة (MDR) و 38.7٪ كانت مقاومة للأدوية على نطاق واسع (XDR) ، بينما شكلت *A. baumannii* المقاومة للكاربابينيم (CRAB) حوالي 58٪ من مجموع العزلات.

لوحظ الفعالية المظهرية لمضخة التدفق(Efflux Pump) في 22.5٪ من المجموع الكلي للعزلات و 38.8٪ من عزلات CRAB. استخدمت تقنية PCR في الكشف عن الجينات *adeA* و *adeB* و *adeC* و *adeR* و *adeS* وشكلت 83.9٪ و 90.3٪ و 58٪ و 80.6٪ و 90.3٪ من العزلات على التوالي, كما سجلت 8 طفرات في الجين *adeR* و 15 طفرة في الجين *adeS*. استنتج من الد راسة وجد آليات مختلفة لمقاومة البكتريا ضد Carbapenem, من ضمنها طفرات في الجينات التنظيمية لمضخة التدفق (*adeRS*) بالاضافة الى إنتاج انزيم carbapenemase.