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ORIGINAL ARTICLE

Molecular Investigation and Gene Expression of Efflux Pump Genes *adeR* and *adeS* among Extensive Drug Resistance *Acinetobacter baumannii*, Iraq

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A. baumannii, gene expression, efflux pump, adeR, adeS, antibiotic resistance

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ABSTRACT

Background*: The clinical problem of Acinetobacter baumannii has been primarily driven by its remarkable ability to acquire or up-regulate various drug resistances. Antibiotics are ineffective in treating the infections caused by these bacteria, making them one of the major global threats to healthcare settings worldwide. Thus, it becomes one of the most successful multidrug resistance (MDR) and extensive-drug resistant (XDR) organisms.* ***Objective****: to determine the changes in adeR and adeS genes of A. baumannii and their relationship with antibiotic resistance.* ***Methodology****: In the cross-sectional study, from December 2023 to July 2024, 220 samples were collected. Samples, during current study, varied between urine (n=85, 39%), wounds (n=55, 25%), burns (n=45, 20%), sputum (n=20, 9%) and blood (n=15, 7%) from patients at Al-Sadder Medical City, Burn Center Unit, and private laboratories in Al-Najaf city. The isolates were treated for PCR assays with specific primers for (adeR gene, adeS gene) and Realtime-PCR for gene expression.* ***Results****: The recovery rate of A. baumannii isolates was 16 (7.27%) from different clinical samples. The results showed the prevalence of adeR was 15 (93.75%) of 16 A. baumannii isolates, and 15/16 (93.75%) were positive to adeS gene. The results of gene expression demonstrated that patients group displayed a significant increase in the expression levels of all studied genes (adeS=6.210359, and adeR=8.420081) as compared with genes expression of controls samples (adeS=1.00499, and adeR=1.043656).* ***Conclusions****: The genes for efflux pumps (adeR, adeS) may be crucial in A. baumanni's ability to withstand antibiotics*

INTRODUCTION

Acinetobacter baumannii has several characteristics that make it widespread both in natural environments and in various parts of the human body. They are aerobic, Gram-negative, and non-fermentative, thus causing multiple body systemic infections[1,2]. In recent years, it has been reported as a major bacterial cause of morbidity and mortality among health care facilities as an opportunistic bacterium[3].

Since it's a pathogen that has a large probability of acquiring infections, with or without, donating resistance genes to nearby microorganisms. It is currently fixed in the healthcare list, which linked to the (WHO) and (CDC) additional to National Institute of Health (NIH)[4].

Among the high virulence component that *Acinetobacter baumannii* possesses is the ability to form biofilms. The biofilm formation is a strong cause of multi-drug resistance in bacteria and increases their resistance ability against antibiotic by any way, either inherent or acquired, posing a respectable challenge to infection healing especially in virulent strain[5,6].

One of the important bacterial strategies is efflux pumps, which have been demonstrated to be essential to bacterial physiology, pathogenicity, live persistence, and metabolism. Before the use of antibiotics, it seems unlikely that their main function was to extrude drugs. Also, reducing cellular stress, controlling nutritional and heavy metal levels, and extruding toxins are few of these functions[7].

The pump system (Efflux) in *A. baumannii*, contributes to pathogenicity by facilitating biofilm development and quorum sensing scenarios, in addition to exporting antibiotics. By publish quorum-quenching practical and extracellular-polymeric-substances (EPSs) and genes regulation that involved in biofilm formation, efflux pumps may facilitate the production of biofilms[8]. The progression of multidrug and extensive drug resistance is expedite by the efflux pumps function, which can push pharmaceuticals out of bacterial cells, reduce antibiotic level in bacterial cells, and altered the medications' bactericidal effects[9].

The most major efflux mechanism in *A. baumannii* is the AdeABC efflux pump system, and some researchers have proposed that adeABC is a marker for resistance to *A. baumannii*. The chromosome genome of this bacteria contains the gene adeABC, which codes for the proteins AdeA (membrane fusion protein), AdeB (intima efflux protein), and AdeC (outer membrane

channel protein). The AdeABC efflux pump system is managed by the AdeRS two-component regulation system[10].

A research suggested that significance of *adeR* in the development of multidrug resistance, when compared to drug-sensitive strains, multidrug-resistant strains of *A. baumannii* have much increased production of system genes specific for efflux pump expression adeABC; also, *adeR* mutation modifies the chief amino acids[11].

The study aimed to investigate the genetic characterization of some efflux pump genes (*adeR*, and *adeS*) and the relation between local multidrug-resistant *A.baumannii* isolates (from burns, wounds, sputum, blood and urine infections) and gene expression by RT-PCR.

METHODOLOGY

Study design: cross sectional study

Sample selection

220 samples varied between urine 85 (39%), wound 55 (25%), burns 45 (20%), sputum 20 (9%), and blood 15 (7%) were collected from patients attended at Al-Sadder Medical City, Burn Centre Unit, and private laboratories during the period from December 2023 to July 2024. *A. baumannii* was isolated from urine 2 (0.9%), wound 7 (3.18%), burns 5 (2.27%), sputum 2 (0.9%), and 0 (0%) blood samples.

Under complete sterile conditions, all patients specimens were obtained by sterile swabs and the patients' information were recorded. Then all samples were transported to the laboratory. All swabs were streaked on differential and selective media (MacConkey Agar, Blood Agar and chromogenic agar) for bacterial isolate and incubated at 37ºC for 24 hours aerobically[12]. Biochemical tests and the Vitek2 system with antibiotics susceptibility test were used to confirm the diagnosis of the isolated bacterial colonies as *Acinetobacter baumannii*.

Six antibiotic classes were used, and then all *A. baumannii* isolates were classified into three groups: sensitive, intermediate, or resistant for each antibiotic according to standard Clinical and Laboratory list 2021[13]

Polymerase Chain Reaction:

The guidelines were provided by the manufacturer of the Genomic DNA Extraction Kit (FavorPrep, Austria), bacterial DNA was extracted from the isolates of *A. baumannii*. Each DNA extract's purity was assessed using the NanoDrop device (THERMO, USA).

The isolates were treated for PCR assays with specific primers for detection of efflux pump gene (*adeR* , *adeS* gene) and Realtime-PCR for gene expression. Table (1) explain all primers details.

Table 1: Sequencing Primers

N.	Gene	Primer Sequence		Amplicon size (bp)	Annealing Temp./time	Reference
1	*AdeR*	F	GTTAAGGCAATAAAAAGTTGCTT	800bp	55 ºC/45 sec	[14]
		R	TGGAGTAAGTGTGGAGAAATACG			
2	*AdeS*	F	CTTGGTTAGGTTAGATATGGCATT	1200bp	55 ºC /45sec	
		R	GGCGTGGGATATAGGCTAGATAA			

Gene expression

The quantitative Real-Time PCR technique was used to analyse the gene expression of the efflux pump genes (*adeR* and *adeS*) in *A. baumannii* strains. Briefly, 96-well plates were filled with 100 µl of each bacterial solution, and they were then aerobically incubated for 18 hours at 37°C. Then, adhering cells were scraped off using LB broth after each well was cleaned with nuclease-free water. Following instructions, an RNA extraction kit (TransZolUp plus RNA kit) was used to extract the total RNA of the strains that had been collected. The purified RNA was transformed into complementary DNA (cDNA) using a cDNA synthesis kit (TransZolUp plus RNA kit). Using Go Taq® qPCR Master Mix Kit RT-PCR equipment (Korea), each cDNA was employed as a template in a 20 µl final volume that contained 2 µl of cDNA, 10 pmol of each primer (Table 1), and 10 µl of CXR dye PCR Master Mix (Go Taq® qPCR Master Mix Kit). The relative expression of the *adeR* and *adeS* genes was determined using the ΔΔCT technique, and the 16SrRNA was employed as a housekeeping gene to standardize the levels of mRNA expression[15,16].

Statistical analysis: Chi-square, P value ≤ 0.05.

RESULTS

Isolation and Identification of *A. baumannii*

Only 144 (65%) of the 220 samples that were collected gave positive growth culture, while 76 (35%) showed negative culture results. Based on colony morphology and biochemical assays, only 16 isolates (7.3%) were identified to be *A. baumannii*, as shown in Table 2.

Antibiotic susceptibility test

There is high antibiotic resistance against most of the tested antibiotics was demonstrated in this study (Table3). The results demonstrate that isolates were defined as extensive-drug resistant (XDR) (100%)

Table 2: Percentage of *A. baumannii* depending on sample source

Sample source	Total number	Bacterial culture results No. (%)		
		No growth	Bacterial growth	
			A.baumannii isolate	Other bacteria
Urine	85 (39%)	44 (51.7%)	2 (2.3%)	39 (45.8%)
Wound	55(25%)	4 (7.3%)	7 (12.7%)	44 (80%)
Burn	45(20%)	6 (13.3%)	5 (11.1%)	34 (75.5%)
Sputum	20(9%)	8 (40%)	2 (10%)	10 (50%)
Blood	15(7%)	14 (93.3%)	0 (0%)	1 (6.7%)
Total	220(100%)	76 (34.5%)	16 (7.3%)	128 (58.1%)
P-value (P ≤0.05)		< 0.0001*	0.0014*	< 0.0001*
*Significant differences at (P ≤0.05) by chi Squair test				

Table 3: Antibiotics susceptibility patterns of *A. baumannii* isolates

Antibiotics classes	Antibiotics	Resistant (R)		Sensitive (S)		P-value (P ≤0.05)
		Isolates No.	%	Isolates No.	%	
Beta-lactam combination agents	Piperacillin/Tazobactam	16	100%	0	0%	
Cephalosporins	Cefazolin (3rd G)	12	75%	4	25%	< 0.0001*
	Ceftriaxone (3rd G)	16	100 %	0	0%	--------
	Ceftazidime (3rd G)	16	100 %	0	0%	---------
	Cefepime (4th G)	16	100 %	0	0%	---------
Carbapenem	Meropenem	16	100%	0	0%	-------
Aminoglycoside	Amikacin	16	100%	0	0%	--------
	Gentamicin	16	100%	0	0%	--------
Folate pathway antagonsits	Trimethoprim/ Sulfamethoxazole	13	81.25%	3	18.75%	< 0.0001*
Quinolone	Ciprofloxacin	16	100%	0	0%	--------
P-value (P ≤0.05)		0.5053*		< 0.0001*		
*Significant differences at (P ≤0.05) by chi Squair test						

Molecular screening of *adeR* and *adeS* genes

Conventional PCR technique was carried out to detect *adeR* and *adeS* genes. The findings showed that 15 (93.75%) of 16 isolates of *A. baumannii* produced a single amplicon (800 bp) bands on gel electrophoresis to the *adeR* gene, which were verified by comparing their molecular weight with the 100–1500 bp DNA ladder as pointed out in Figure (1), which displays the efflux pump gene distribution rate among 16 XDR *A. baumannii* isolates.

However, *adeS* gene were studied in all 16 *A. baumannii* isolates. Figure (2) showed that 15/16 positive for *adeS* gene, about 93.75%, when PCR product size was approximately 1200 bp.

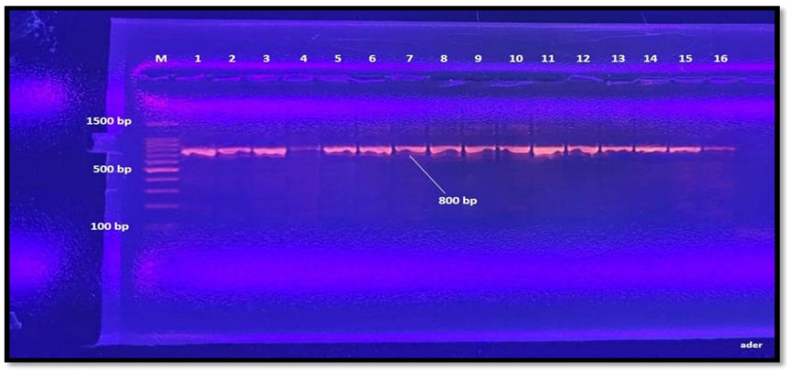

Fig. 1: Agarose gel electrophoresis (1.5%) of RCR amplified of *adeS* gene (800bp) of *A. baumannii* for (55) min at (70) volt M: ladder (DNA marker). Number (1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16) positive *A. baumannii* isolates, while number (2) was negative.

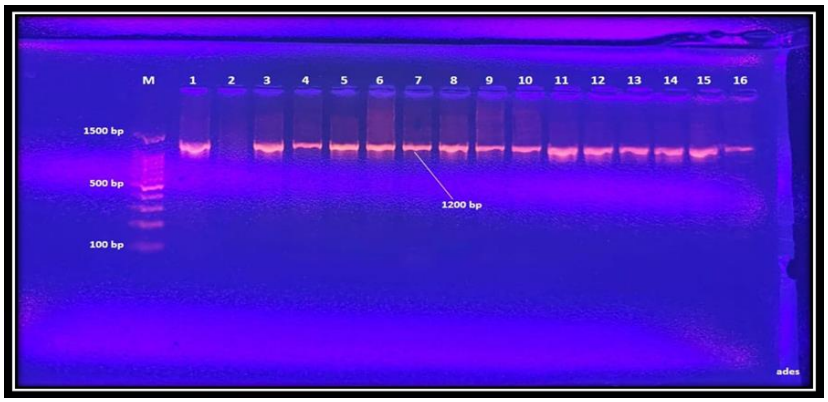

Fig. (2): Agarose gel electrophoresis (1.5%) of RCR amplified of *adeR* gene (800bp) of *Acinetobacter baumannii* for (55) min at (70) volt M: ladder (DNA marker). Number (1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16) positive *A. baumannii* isolates, while number (4) was negative.

Gene expression of *adeR* and *adeS* genes

The present study demonstrated that, the patients group showed significant differential gene expression of the target genes (*adeS*, and *adeR*), which representing a significant difference with a p-value of ≤ 0.05. Patients group displayed an increase in the expression levels of all studied genes (*adeS*=6.210359, and *adeR*=8.420081) significantly as compared with genes expression of controls samples (*adeS*=1.00499, and *adeR*=1.043656) as explained in Tables (4 and 5), and shown in Figures (3 and 4).

Table 4: *adeS* Fold Gene Expression between Control and Patients versus the reference gene (16 SRNA)

Groups	N	*adeS* Expression levels (2^-(ΔΔCt))		
		Mean	SD	SE
Controls	5	1.00499	0.114067	0.051012
Patients	5	6.210359	1.147402	0.573701
P value	0.000518*			
* represent a significant difference under p-value ≤ 0.05. SD, SE: Standard Deviation and Error				

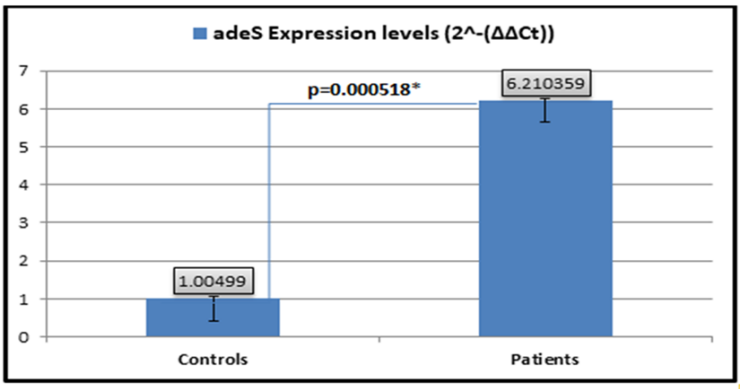

Fig. 3: *adeS* Fold Gene Expression between Control and Patients versus the reference gene (16 SRNA)

Table 5: *adeR* Fold Gene Expression between Control and Patients versus the reference gene (16 SRNA)

Groups	N	*adeR* Expression levels (2^-(ΔΔCt))		
		Mean	SD	SE
Controls	5	1.043656	0.409519	0.204759
Patients	5	8.420081	1.633758	0.816879
P value	0.000498*			
* represent a significant difference under p-value ≤ 0.05. SD, SE: Standard Deviation and Error				

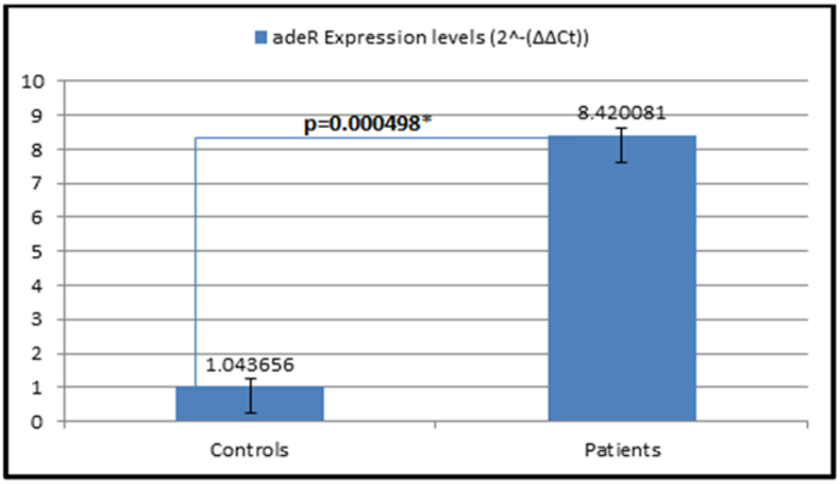

Fig. 4: *adeS* Fold Gene Expression between Control and Patients versus the reference gene (16 SRNA)

DISCUSSION

The current results revealed that the percentage of *A. baumannii* among clinical isolates was 16 (7.3%) and this is similar to other local studies in Baghdad by Al-Dulaimi *et al*[17] as the percentage of isolation of *A. baumannii* was (8.2%), while in Al-Hussein Medical City in Karbala by AL-Baroody etal[18], who established that the isolation rate of *A. baumannii* was (11.11%).

A study done in Al-Diwaniya city by Ghadeer[19] revealed that the incidence of *A. baumannii* was (11.7%). Also, another study in Al-Najaf city showed a relatively low incidence (5.36%) of *A. baumannii*[20].

Additionally, a study in India reported that from 138 strains isolates about 11.49% *Acinetobacter*, were obtained from various specimens[21]. Sometimes the differences in the study design, methodology, and study duration may be the cause of the observed variations in prevalence rates.

Our study observed that the highest percentage was detected from wound 7 (12.7%), followed by burn 5 (11.1%), sputum 2 (10%), and urine 2 (2.3%). Different works were concerning with the study of *A. baumannii* from various clinical sites and revealed varying frequencies; a study was done in Babylon/Iraq resulting in (20%) overall isolation rate divided into (35%) from burn swabs, (40%) from wound swabs, while (25%) from urine[22]. Another study at Babylon City/Iraq showed that *A. baumannii* was mostly isolated from respiratory tract infection with (27%) followed by (14%) and (10%) from burns and urine respectively, while the lowest was from wound (8%)[23].

The body's first line of defence against foreign infections is the skin due to its feature as the biggest organ. When there is injury, opportunistic microorganisms can interfere with the natural healing process, which can prolong wound care and raise the risk of serious infections[24,25]. Chronic wound infections are complicated, with factors such as biofilm formation, antibiotic resistance and hypoxia contributing to the limited effectiveness of many medications and encouraging the persistence of bacteria[26,27,28].

The present study, demonstrated that *A. baumanii* isolates had *adeR* and *adeS* genes (93.75%). Another study, revealed that prevalence of *adeS*, *adeR* genes of *A. baumannii* strains was (68%) and (96%), respectively[29].

Another study done by Owaid and Abood[30], that employed the *adeR* gene as molecular marker for the purpose of identifying efflux pump activity by PCR technique. The obtained results indicated a 100% presence of this gene across all isolates.

One of the well-known bywords of a pathogen with several efflux pump systems is *Acinetobacter baumannii*, which makes it MDR-to-pan-drug resistant and a current public-health-threat[31].

In the clinical field, imipenem and meropenem are used in wide path as broad-spectrum antibiotics in healing nosocomial infections[32]. The difference of efflux pumps found in *A. baumannii* can use these antibiotics as substrates and carry out them from the cell[33]. The overexpression of efflux pumps plays an essential role in drug resistance in *A. baumannii*[34]. *adeS* is considered the first gene to regulate the AdeABC system and some point mutations in this gene may lead to induced efflux pump production at a high rate, according to Lin *et al*.[35]

The high expressions of efflux pumps may have an effect on the multidrug resistance ability, and are capable of expelling a diverse range of dissimilar drugs in their classes[36].

Between the abundant bacterial pumps, the RND pumps are the most clinically significant efflux pump family in relation to AMR infections brought on by Acinetobacter species[37,38]. Technical ways, that targeting these regulatory pathways, may be essential to prevent efflux pump gene overexpression and as a result compromising the removal of harmful chemicals, as the expression of this pump is usually closely regulated[39].

Efflux pumps have been connected to multiple drug resistance (MDR); they can carry a wide range of different compounds, including antibiotics of several classes, or they can be specialized for a single substrate[40,41].

CONCLUSION

The genes for efflux pumps (*adeR*, *adeS*) may be crucial in *A. baumanni*'s ability to withstand antibiotics.

Study limitations: short time of study, high cost and difficult diagnosis.

Recommendations

Explore other mechanisms employed by *A. baumannii* to resist antibiotics to gain a deeper understanding of its resistance strategies.

Assignment

All the participants provided informed consents for inclusion in the study and were assured that all the information provided would be used solely for the purposes of this study and treated confidentially.

Ethical Approval Declaration

The research was in proper accordance to the ethical standards established in the Declaration of Helsinki. Prior to sampling, consent was obtained from the patient or their partner. The research methodology, subject information, and permission form, received approval from the College of Medicine, Al-Qadisiyah University.

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Competing interests

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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