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Effect of Tryptanthrin on the Expression of the *Bap Gene* in Biofilm-Forming *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is an opportunistic pathogen that mainly causes disease in individuals with immunocompromising conditions, burn and wound infections, and individuals who are hospitalized in an intensive care unit (ICU). The aim of this study was to investigate the prevalence of biofilm-forming *A.baumannii* isolated from burn and wound infections in Baghdad hospitals and to evaluate the effect of Tryptanthrin on the expression of the biofilm-associated *bap* gene using real-time PCR analysis. A total of 100 clinical samples were collected from patients with burn and wound infections admitted to multiple hospitals in Baghdad City. Thirty *A. baumannii* isolates were obtained from clinical samples, including 17 isolates from burn infections (56.7%) and 13 isolates from wound infections (43.3%). Antibiotic susceptibility testing revealed high resistance rates among the isolates. Resistance was observed to β-lactam antibiotics (58.5%), cephalosporins (77.2%), carbapenems (75%), aminoglycosides (43.5%), and fluoroquinolones (60%). The biofilm-forming ability of the isolates was also evaluated using the microtiter plate assay, and all isolates demonstrated the ability to produce biofilm with varying levels of intensity. PCR analysis revealed that the bap gene was detected in 19 (62%) of the isolates, suggesting its role in biofilm formation. Real-time PCR analysis demonstrated a reduction in bap gene expression after treatment with tryptanthrin, suggesting its potential role as an antibiofilm agent against *A. baumannii*. These results suggest that Tryptanthrin may function as an anti-virulence agent because it inhibited the biofilm-associated *bap* gene expression and could be utilized as a method in controlling the virulence of *A. baumannii*.

Keywords: Tryptanthrin, *bap* gene, *Acinetobacter baumannii*, Biofilm formation, Gene expression, Antibacterial.

1. Introduction

Although previously classified as a low-virulence opportunistic pathogen, *A. baumannii* has increased in clinical significance over the past thirty years as an opportunistic pathogen that primarily affects immunocompromised patients, burn and wound patients, and patients in the intensive care unit (ICU)[1]. During the wars in Iraq and Afghanistan, *A. baumannii* was isolated from American soldiers suffering from ulcerations and wound infections[2]. Members of the genus *Acinetobacter* are described as Gram-negative, non-lactose fermenting, aerobic, non-spore-forming, coccobacilli, oxidase-negative, and catalase-positive[3].

A. baumannii is an opportunistic pathogen commonly associated with hospital-acquired infections, particularly ventilator-associated pneumonia and bloodstream infections. The pathogenicity of this organism is strongly linked to its ability to form biofilms on biotic and abiotic surfaces, which contributes to persistence in hospital environments and increased resistance to antimicrobial agents[4,5]. Many factors contribute to the risk of infection with *A. baumannii*, including patient age, prolonged hospitalization, and a weakened immune system[6]. In

2017, the World Health Organization (WHO) classified carbapenem-resistant *A. baumannii* as a critical priority pathogen requiring the development of new antibiotics due to its high mortality rate and multidrug resistance. In relation to its pathogenicity, *A. baumannii* is associated with virulence factors that include biofilm, biofilm-associated protein (Bap), capsule, outer membrane vacuoles, enzymes (such as proteases and lipases), and type 1 fimbriae, which facilitate adhesion to surfaces and antibiotic resistance, including penicillins, cephalosporins, and aminoglycosides[7]. Although the *bap* gene contributes to biofilm formation, it is not the sole determinant. Other factors such as *ompA*, csu pili, the pgaABCD operon, and the bfmRS regulatory system have also been reported to play important roles in biofilm development[8]. Accordingly, Bap is a large surface protein that contributes to bacterial adhesion and biofilm stability in *A. baumannii*[9].
Tryptanthrin is a naturally occurring indoloquinazoline alkaloid that has been reported to exhibit antimicrobial and antibiofilm activities against several bacterial pathogens, including *A. baumannii*[10].
The aim of this study was to investigate the prevalence of biofilm-forming *A. baumannii* isolated from burn and wound infections in Baghdad hospitals and to evaluate the effect of Tryptanthrin on the expression of the biofilm-associated *bap* gene using real-time PCR analysis.

2. Materials and Methods

2.1. Collection and Identification

A total of 100 clinical samples were collected from patients with burn and wound infections admitted to multiple hospitals in Baghdad between August 2025 and November 2025. Ethical approval for the study was obtained from the Scientific Research Ethics Committee of the College of Education for Pure Sciences (Ibn Al-Haitham), University of Baghdad (Approval No. EC-87). Verbal consent was obtained from patients prior to sample collection.
Isolation of bacterial strains was performed by culturing clinical samples on MacConkey agar and blood agar. Microscopic diagnostic analysis was conducted by examining all of the bacterial isolates using Gram staining. Biochemical tests including oxidase, catalase, and IMViC reactions were performed mainly to exclude Enterobacteriaceae and support the identification of non-fermenting Gram-negative bacteria.
Final identification of *A. baumannii* isolates was confirmed using the VITEK 2 Compact system (bioMérieux, France). Antibiotic susceptibility testing and minimum inhibitory concentration (MIC) determination were performed using the VITEK 2 system, and results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [12].

2.2. Biofilm formation assay

Biofilm formation was evaluated using the microtiter plate (MTP) assay. Briefly, overnight bacterial cultures were diluted in tryptic soy broth and inoculated into 96-well microtiter plates. After incubation at 37°C for 24 hours, the wells were washed, fixed with methanol, and stained with crystal violet, and the absorbance was measured at 570 nm using a microplate reader [13].

2.3. DNA Extraction

Genomic DNA was extracted using the Geneaid Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's instructions. Extracted DNA was stored at −20°C until PCR analysis. One microliter of extracted DNA was put into the instrument to estimate concentration (ng/µl). The purity was quantified as the ratio of absorbance at 260–280 nm to determine contamination with protein or RNA. DNA samples with a purity ratio of 1.8-2.0 were classified as highly pure for PCR gene amplification.

2.4. Polymerase Chain Reaction (PCR)

Molecular identification of the bacterial isolates used in this study was performed by polymerase chain reaction (PCR) followed by detection of the presence of the *bap* gene (**Table 1**). The primer sequences used for amplification of the *bap* gene were adopted from the study by[14]. PCR master mix was prepared according to the manufacturer's instructions (Bioneer, Korea).

Primers targeting the bap gene were synthesized by Macrogen Company (South Korea). Primers for the *bap* gene of *A. baumannii* were prepared using sterile, deionized distilled water (ddH_2O), making a final concentration of 100 pmol/µL by adding 10 µL of the primer solution to 90 µL of ddH_2O; the solution was swirled to ensure homogeneity, which resulted in a final concentration of 1 pmol/µL, and subsequently stored at –20°C. The solution was mixed again using a vortex mixer prior to use.

The PCR master mix was thawed to room temperature, and to facilitate its use, the entire contents were mixed gently with a mechanical mixing device. The reaction mixture was prepared as per **Table 2**, using the necessary volumes and concentrations of the primers and template DNA. Polymerase chain reaction (PCR) was performed to detect the *bap gene* in *A. baumannii* isolates. The amplification reaction was carried out in a final volume of 25 µL containing 5 µL of PCR master mix, 1 µL of forward primer, 1 µL of reverse primer, 5 µL of template DNA, and 13 µL of nuclease-free water. Amplification was conducted in a thermal cycler according to the conditions shown in **Table 3**.

Table 1. Primer sequences used in the study.

Gene	Primer Sequence (5'→3')	Gene Size (bp)
Bap	F: AATGCACCGGTACTTGATCC	205
	R: TATTGCCTGCAGGGTCAGTT	

Table 2. Components required for PCR amplification of the *Bap* Gen.

Component	Volume (µL)
PCR Master Mix	5
Forward primer	1
Reverse primer	1
DNA Solution	5
Deionized Sterile Water (ddH_2O)	13
Total Volume	25

Table 3. Optimal **PCR conditions for the *bap gene*.**

Step	Temperature (°C)	Time	Cycle
Initial Denaturation	95	3 min	1
Denaturation	95	30 sec	35
Annealing	60	30 sec	
Extension	72	30 sec	
Final Extension	72	7 min	1

2.5. Gel Electrophoresis

Agarose solution was prepared by adding 1 g of agarose powder into 100 mL of 1× TBE buffer (1.5% agarose) and heated until it melted. The solution was allowed to cool to ~45–50°C while gently swirling the flask during cooling. The agarose powder was added to ethidium bromide (TBE) buffer and heated until completely melted. The solution was then poured into the casting tray and allowed to solidify before electrophoresis. Five µL of the DNA sample was mixed with 3 µL of DNA loading dye and was subsequently loaded onto the agarose gel. The electrophoresis voltage and running time improved reproducibility. The gel electrophoresis was performed at 100 V for approximately 80 minutes, and the amplified products were visualized under a UV transilluminator.

2.6. Real-Time PCR and gene expression

Total RNA was extracted from *Acinetobacter baumannii* isolates before and after treatment with Tryptanthrin using a commercial RNA extraction kit (Geneaid, Taiwan) according to the manufacturer's instructions. The extracted RNA concentration and purity were measured using a NanoDrop spectrophotometer and stored at −20 °C until further use.

Complementary DNA (cDNA) was synthesized from the extracted RNA using a cDNA synthesis kit (Bioneer, Korea) following the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix (Bioneer, Korea) to evaluate the expression level of the bap gene. The reaction mixture (25 µL) consisted of 5 µL of qPCR Master Mix, 1 µL of forward primer, 1 µL of reverse primer, 5 µL of cDNA template, and 13 µL of nuclease-free water (**Table 4**).

The amplification protocol included an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 5 s. A melting curve analysis was performed at the end of the amplification process to confirm the specificity of the PCR products.

Gene expression levels were normalized using the 16S rRNA gene as an internal housekeeping control. Relative gene expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method, and expression values were expressed as fold change relative to untreated control samples. A no-template control (NTC) was included in each run to ensure the absence of contamination[15].

Table 4. Optimal Real-Time PCR Conditions for the *bap gene*.

Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	95	5 min	1
Denaturation	95	5 sec	40
Annealing & Extension	60	5 sec	40
Hold	25	1 min	1

2.7. Tryptanthrin Preparation

The compound Indolo[2,1-b]quinazoline-6,12-dione (Tryptanthrin) with 97% purity and a molecular weight of 248.24 g/mol was prepared by dissolving 20 mg of the powder in 1 mL of dimethyl sulfoxide (DMSO) to obtain a stock solution. The solution was mixed using a vortex mixer and sterilized through a 0.22 µm membrane filter. Serial dilutions were subsequently prepared to obtain the required concentrations for gene expression experiments [16].

The compound Indolo[2,1-b]quinazoline-6,12-dione (Tryptanthrin) was made with 97% purity and a molecular weight of 248.24 g/mol by dissolving 20 mg of the powder in 1 mL of dimethyl sulfoxide (DMSO) to compound a stock solution, which was later diluted to obtain the desired working concentrations used in the experiments. The solution was well mixed with a vortex mixer and filtered for sterilization through a 0.22 µm filter from which serial dilutions were prepared to obtain lower working concentrations used in the gene expression experiment[17].

2.8. Statistical analysis

Statistical analysis was performed using the Statistical Analysis System (SAS) software. Differences between gene expression levels before and after treatment with Tryptanthrin were analyzed using the paired t-test. Values of $P \leq 0.05$ were considered statistically significant[18].

3. Results

Out of 100 clinical specimens collected from patients with burn and wound infections admitted to several hospitals in Baghdad, bacterial growth was observed in 30 samples, while 70 samples showed no bacterial growth. After performing biochemical tests and confirmation using the VITEK 2 Compact System, 30 isolates were identified as *A. baumannii* from the total bacterial growth samples **(Table 5**).

Out of 100 clinical specimens obtained from patients with burn and wound infections, 30 (30%) were isolated bacteria such as *A. baumannii* from various hospitals across Baghdad. Among the confirmed isolates, 17 isolates (56.7%) were obtained from burn infections, whereas 13 isolates (43.3%) were isolated from wound infections (**Table 5**).

Table 5: Percentage and number of bacterial isolates recovered from clinical samples.

Sample Source	Total Samples (%)	Samples with Bacterial Growth (%)
Burns	53 (53%)	17 (56.7%)
Wounds	47 (27%)	13 (43.3%)
Total	100	30 (100%)

3.1. Cultural and microscopic identification

The isolates were characterized using morphological, cultural, and microscopic analyses. Colonies grown on MacConkey agar appeared pale and colorless, indicating that *A. baumannii* is a non-lactose-fermenting bacterium, and the colonies that were grown on blood agar developed a gray color. After gram staining, all isolates were identified as being gram negative and coccobacillary in shape (**Figure 1**).

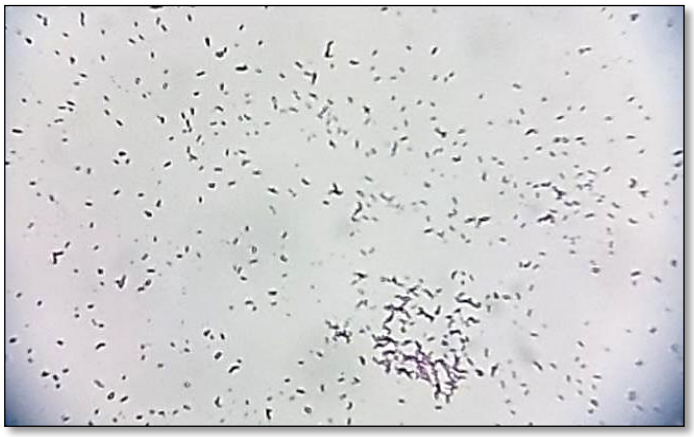

Figure 1. Gram-negative coccobacilli of *A. baumannii* observed under light microscope.

3.2. Biochemical tests

The biochemical tests were negative for Oxidase, Indole, Methyl Red, and Voges-Proskauer but positive for Catalase and Citrate utilization Tests. The final identification of all isolates as *A. baumannii* was provided by the VITEK 2 Compact System.

3.3. Final Identification

The isolates showed high resistance rates to several antibiotic classes. Resistance was observed to β-lactam antibiotics (58.5%), cephalosporins (77.2%), carbapenems (75%), aminoglycosides (43.5%), and fluoroquinolones (60%) (**Figure 2**). Based on the resistance patterns observed, the majority of the isolates were classified as multidrug-resistant (MDR) because they exhibited resistance to multiple classes of antibiotics.

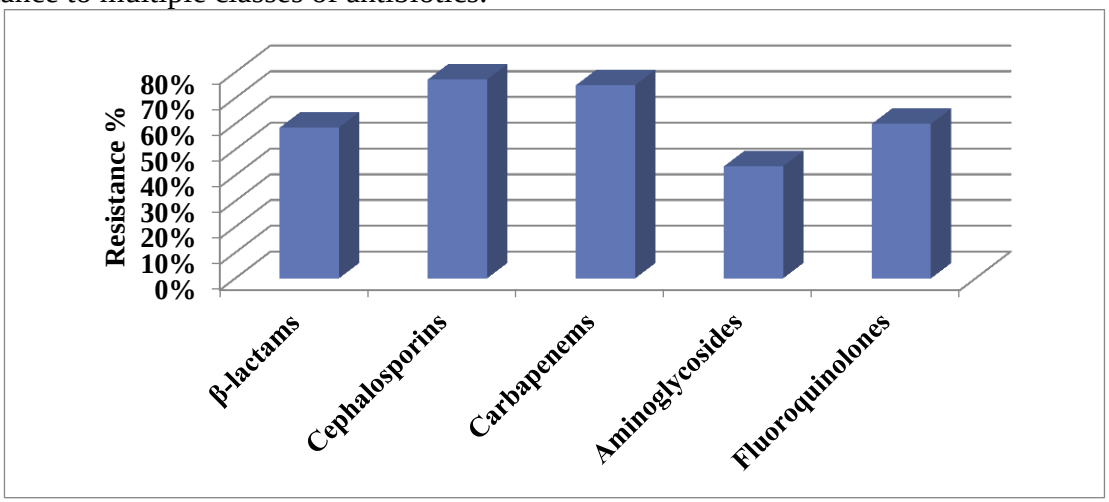

Figure 2 . Antibiotics groups' susceptibility

3.4. Biofilm Formation Assay

The isolates' ability to produce biofilm was tested through the Microtiter Plate (MTP) method. All isolates (30/30) were able to produce biofilm, and no non-biofilm-forming isolates were detected (**Table 6**). The isolates were categorized into weak, moderate, and strong biofilm

producers according to the optical density measurements obtained from the microtiter plate assay.

Table 6: Biofilm Production Levels in *A. baumannii*.

Biofilm Production Level	Number of Isolates	Percentage (%)
Strong	21	70%
Moderate	3	10%
Weak	6	20%
Total	30	100%

3.5. Detection of the *Bap gene* by PCR

The presence of the *bap* gene was investigated using polymerase chain reaction (PCR). The results showed that 19 out of 30 isolates (62%) were positive for the bap gene. The amplified PCR product showed the expected size of 205 bp as confirmed by agarose gel electrophoresis (**Figure 3**).

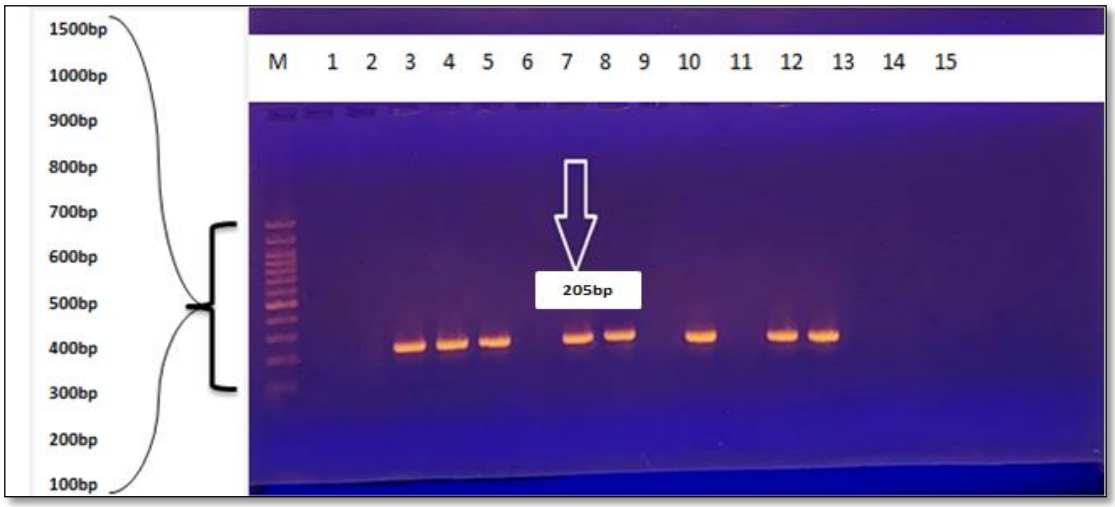

Figure 3. Agarose gel electrophoresis of PCR products showing amplification of the bap gene (205 bp) in *A. baumannii* isolates. M: DNA marker; lanes 1–10 represent clinical isolates showing positive amplification.

3.6. Expression analysis of the *bap* gene

Gene expression analysis of the *bap gene* was performed using real-time PCR to evaluate the effect of Tryptanthrin treatment. The 16S rRNA gene was used as a housekeeping gene for normalization in the real-time PCR analysis. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The results indicated a decrease in *the bap gene* expression after treatment with Tryptanthrin, suggesting a down-regulation effect on the biofilm-associated gene.

Real-time PCR analysis showed a decrease in bap gene expression after treatment with tryptanthrin, with the mean expression level decreasing from 1.14 before treatment to 0.50 after treatment. However, this reduction was not statistically significant ($p = 0.11$). The experiment was performed in triplicate, and the data are presented as mean ± standard deviation (SD) (**Table 7**). The decreased expression of bap as a result of Tryptanthrin could represent a potential anti-virulence mechanism to reduce biofilm formation in clinical strains of *A. baumannii*. (**Figures 4 and 5**).

Table 7. Effect of Tryptanthrin on *bap gene* expression

Before Folding (2^–ΔΔCt)	After Folding (2^–ΔΔCt)	Mean Before Folding	Mean After Folding	95% CI of Difference	T-Test	Sig	Mean (D)	Std. Deviation	Correlation	df	N
2.14355	0.57435	1.14169	0.50233	1.50481 – 0.50233	-0.205	0.11	0.63936	0.69701	-0.22610	4	5
1.07177	0.43528										
0.70711	0.65975										
1.31951	0.26794										
0.46652	0.57435										
2.14355	0.57435										
1.07177	0.43528										

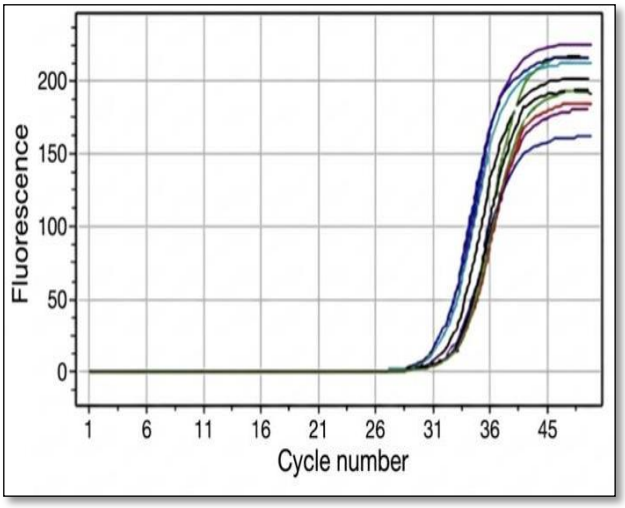

Figure 4. Quantitative PCR Curve for the *bap gene*.

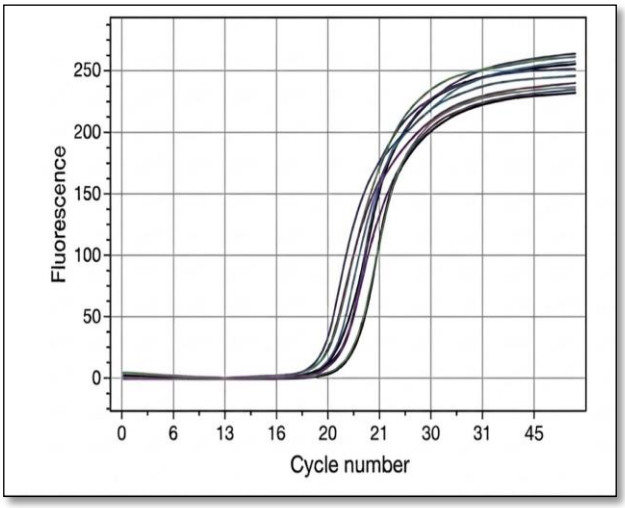

Figure 5. Quantitative PCR Curve for the Reference Gene

4. Discussion

The results of this study highlight the clinical relevance of *A. baumannii* in burn and wound infections. In the present study, 30% of the clinical samples yielded *A. baumannii* isolates. In addition to its presence in these infections, the isolates demonstrated multidrug resistance and strong biofilm-forming ability, which are important factors contributing to persistence in hospital environments[19]. The isolation rate observed in the present study is comparable to findings reported in several regional and international studies, where *A. baumannii* has been frequently isolated from burn and wound infections. Similar studies have also reported high levels of multidrug resistance among clinical isolates[20].

The highly resistant levels of antibiotic resistance that were found in this study, especially against β-lactams, cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones, are highly relevant given the world-wide anxiety about multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains[21,22]. Previous studies have reported similar findings, attributing them to β-lactamase production, decreased membrane permeability, efflux pump presence, and the inappropriate use of antibiotics, all intrinsic to greater virulence in bacteria[20].

The capacity of bacteria to form biofilms represents a significant virulence factor that allows bacteria to remain viable on biotic and abiotic surfaces, including on medical devices and catheters, and it is associated with increased antimicrobial resistance[23]. In the current study it was demonstrated that all isolates were capable of biofilm production, supporting previous findings that found 80-100% of clinical *A. baumannii* isolates produce biofilms[27]. Biofilm formation in *A. baumannii* is a multifactorial process involving several genetic determinants. In the present study, all isolates demonstrated the ability to form biofilms, while 62% of the isolates carried the bap gene. This indicates that although bap contributes to biofilm formation, it is not the only determinant responsible for this phenotype. Previous studies have demonstrated that other genes, including *ompA*, csu pili operon, and pgaABCD, also play important roles in biofilm development in bap-negative isolates[9]. In the present study, approximately 62% of biofilm-forming isolates possessed the *bap* gene, supporting a role for this gene in contributing to virulence and persistence in clinical isolates. This prevalence is similar to what has been reported in the literature from previous studies, including[24], who reported a 66.2% prevalence, [25] at 84%; and[11] at 92%, highlighting the prevalence of *bap* gene-positive multidrug resistant *A. baumannii*. The strong association of biofilm formation and the presence of the *bap* gene supports the hypothesis that Bap contributes to persistence and pathogenicity in the hospital.

The real-time PCR analysis showed a reduction in the bap gene expression after treatment with Tryptanthrin. However, this reduction did not reach statistical significance ($P = 0.11$). Therefore, the observed decrease may indicate a potential trend toward down-regulation, but additional studies with larger sample sizes are required to confirm this effect. Furthermore, since 38% of the biofilm-forming isolates lacked the bap gene, the inhibition of biofilm formation cannot be explained solely by the reduction of bap gene expression. Other biofilm-associated genes and regulatory systems may contribute to biofilm formation in these isolates.

The bap protein is localized to the bacterial surface, where it forms a network that acts as a basic structural matrix to promote adhesion to human epithelial cells and to assist with adherence to biotic and abiotic surfaces, including medical devices and catheters, further promoting persistence in hospital settings[26-28]. While the present study did not observe a statistically significant down-regulation, this data is in line with the research of[29,30], which showed Tryptanthrin inhibits biofilm formation in *A. baumannii* by down-regulating biofilm-related gene expression. Differences between studies may be attributed to geographical variation, sample size, bacterial strain diversity, and differences in laboratory methods. Tryptanthrin may act as an anti-virulence agent by suppressing biofilm-associated gene expression rather than directly killing bacterial cells[31].

5. Conclusion

In conclusion, *Acinetobacter baumannii* isolates demonstrated high levels of antibiotic resistance and strong biofilm-forming ability. The bap gene was detected in a considerable proportion of isolates and was associated with biofilm formation. Treatment with Tryptanthrin resulted in reduced bap gene expression, suggesting its potential as an anti-virulence agent for controlling biofilm-related infections caused by *A. baumannii*.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Funding

No founding.

Ethical Clearance

The samples were obtained according to the approval of the Scientific Research Ethics Committee at the College of Education for Pure Sciences Ibn Al-Haitham, University of Baghdad (EC-87) on 19 /11 /2025.

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