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Molecular Detection of Some Biofilm Genes in *Acinetobacter baumannii* is a Clinically Significant Opportunistic Pathogen in Hospital and Healthcare Environments

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Abstract *Acinetobacter baumannii* is one such opportunistic pathogen in hospital and clinical set up. It can develop strong biofilm on biotic and abiotic surfaces and is therefore tough to unfavorable conditions as well as anti-bacterial drugs. The proteins that are encoded by key genes used in biofilm formation, cilia formation (adhesion), and host immune response suppression include bap, Csu and ompA. This renders the treatment of infections caused by this bacterium especially ventilator-associated pneumonia, septicemia and wound infections to be a major clinical challenge since available treatment has limited efficacy. Biofilm system can form a physical and physiological barrier to prevent penetration of antibiotics, as well as to dampen the host immune response resulting in decreased therapeutic efficacy. Therefore, this time of long-term and chronic microbe exposure becomes extremely difficult to get rid of *A. baumannii* infection. As a result, there has been growing interest in the identification of genetic, molecular and regulatory mechanisms that can interfere with biofilm development or stability. Therefore, a complete perception of the genetic determinants and regulatory circuits involved in biofilm formation, including quorum-sensing systems, two-component control systems and biofilm-associated surface proteins, is an essential requirement to develop novel antibiotics & anti-biofilm strategies against drug-resistant *A. baumannii* isolates.

Keywords: Nosocomial infections, antimicrobial resistance, bacterial adhesion, virulence factors, PCR analysis

Introduction *Acinetobacter baumannii* is a Gram-negative, strict aerobe, opportunistic pathogen, which has become a leading cause of hospital-acquired infections (HAIs) in hospitals around the globe, including intensive care units (ICUs). It is often connected with ventilator-associated pneumonia, bloodstream infections, urinary tract infections, wound infections and sepsis in critically ill and immunocompromised patients. The high survival rates of *A. baumannii* in inanimate surfaces and medical equipment have helped in the survival of this bacterium in

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the health care facility environment hence posing a high risk of nosocomial infection (1). *A. baumannii* is a pathogen with outstanding potential due to its ability to develop multidrug resistance (MDR), extensively drug resistance (XDR) and, in certain instances, pan-drug resistance (PDR). The development of 8-lactamases, overexpression of efflux pumps, modification of the target site, and decreasing the outer membrane permeability are the mechanisms of resistance. Of special interest is resistance to carbapenems that used to be regarded as the final resort against Gram-negative infections. Consequently, treatment possibilities against *A. baumannii* infections are now exceptionally scarce and the World Health Organization has made carbapenem-resistant *A. baumannii* a critical priority pathogen that needs an immediate research and development of a new cure (2). Among the most vital virulence and persistence *A. baumannii* abilities is the formation of biofilms, or complex, surface-fixed aggregates entrenched in an extracellular matrix produced by itself. Biofilms offer protection against host immune defenses which is even more greater and antibiotic penetration is minimal thereby creating persistent infections and treatment failure (3). Various genetic determinants control the formation and evolution of biofilms in *A. baumannii* Biofilm-associated protein (bap) gene encodes a large surface protein that matures the biofilm and provides its structural integrity, and researches have shown that it is associated with increased biofilm formation and multidrug resistance phenotypes (4). The csu operon (e.g., csuE) expresses elements of a chaperone-usher pilus assembly apparatus that facilitates preliminary adhesion to nonbiotic surfaces (e.g. plastics and stainless steel used in clinical environments) and high prevalence of csuE has been shown to be common in very strong biofilm producers. Also, outer membrane protein A (ompA) has multifunctional functions in the pathogenesis of *A. baumannii* through the establishment of the biofilm, immune evasion, environmental stress resistance, and surface attachment (5). The recent reports have reported the prevalence of these genes in clinical and environmental isolates, and the presence of bap, csuE and ompA in the same isolates of multidrug-resistant and biofilm-forming isolates has been frequently reported. These genetic predictors are also related to higher biofilm biomass as well as phenotypes of treatment failure and intractable hospital outbreaks. On a research note, it is of critical importance to investigate the molecular processes involved in the biofilm formation and antimicrobial resistance development in *A. baumannii*, in particular, the functions of bap, csu, and ompA. These studies aid in the formulation of specific anti-biofilm policies, innovative treatment measures and better-infection management procedures to address the world-wide burden of this microbe. Increased knowledge on such pathways has the potential to minimise morbidity and mortality caused by MDR *A. baumannii* infections and the transmission of the pathogen in healthcare settings (6, 7).

Material and methods

Ethical Approval Statement

This study was performed in accordance with ethics and biosafety rules for handling clinical bacterial isolates and molecular diagnosis. The College of Education Ethics Committee, Al-Qadisiyah University, Iraq has authorized this study during the 2025 (Approval number: EDU/BIO/8/7/2025/294).

Samples collection

446 samples were collected from hospitals and health centers in Diwaniyah Governorate, namely (Women's and Children's Hospital, Diwaniyah General Teaching Hospital, and Public Health Laboratory) for the period from Aug. 2024 to Nov.2024, as the study included different clinical samples, collected from different areas of the body for hospitalized patients of all ages and both sexes, as samples were taken from (Burns, Sputum, Urine, and Blood).

Isolation and identification

Swab and sample specimens were promptly transferred to the laboratory, where they were incubated under aerobic conditions at 37°C for 24 hours to assess their cultural, microscopic, and biochemical characteristics. Bacterial isolates were obtained using selective culture media and identified based on their morphological and microscopic features

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observed on the growth plates. The identification of *A. baumannii* isolates was subsequently confirmed using the Vitek 2 Compact automated system.

Different Method to Detection of Biofilm Formation

Congo red agar Method

In this method, brain and heart medium (BHI) was used, along with sucrose and Congo red, in the following formulation: brain and heart agar 52 g/L; sucrose 36 g/L; Congo red 0.8 g/L. The plates were inoculated and incubated for 24–48 hours at 37°C. Dry, crystalline black colonies were considered positive for the assay, while weak or non-biofilm-producing isolates appeared pink (8, 9).

The micro titter plate test

The bacterial suspension was cultured on Tryptic soy broth (TSB) and as per 0.5 McFarland standard. 200 µL of bacterial suspension was added to sterile 96-well flat-bottomed. Only TSB was in negative controls. Subsequently, the plates were covered and allowed to incubate aerobically at 37 o C in 24 hours. The wells were aspirated, rinsed three times with 250 ul of sterile physiological saline, emptied and left to dry after incubation. Next, plate was stained with 2% crystal violet with 5 minutes after which plates were washed severally with distilled water. The plates were air dried later; Bound crystal violet was dissolved using 95% ethanol. At 540 nm, Optic Density (OD) was measured of wells. Weak, strong biofilm producers The isolates with ODs of 0.12> to 0.5 were regarded as weak, and those above 0.5 as strong biofilm producers (10, 11).

Genomic DNA extraction

DNA was extracted from *A. baumannii* using the ready-made Genomeic DNA Mini Kit provided by Geneaid USA and according to the instructions of the company.

Preparation of Agarose Gel

Agarose gel electrophoresis is widely used and efficient technique for the separation and analysis of DNA fragment based on their size and charge (0.5–25 kb). The procedure involves preparing an agarose gel suitable for the fragment size, loading the DNA samples, running the gel under appropriate voltage, and visualizing the DNA either by staining or direct UV illumination if ethidium bromide is used (12).

PCR Master Mix Preparation

Prepare this mixture in PCR tubes according to the instructions of the company that supplied Wizpure PCR FDmix and as shown in the following table (1). After completing the process of preparing the mixture tubes, they were transferred to the Vortex device for 5 seconds, after which the tubes were transferred to the PCR thermocycler device to perform the DNA amplification process according to the ideal condition for thermal cycles. The thermal cycle program required for DNA amplification, The Thermocycler PCR device was used and programmed for the genes under study according

Table (1): Components of the PCR master mix to the reaction.

Concentration	Component
1 U	*Top* DNA Polymerase
10Mm	Tris-HCl (pH9.0)
30mM	KCl
1.5mM	$MgCl_2$
250µM	dNTPs (dATP, dCTP, dGTP, dTTP)

Gene Expression

Total RNA (RNATotal) was extracted from these bacterial isolates using the TriRNA GENEzolTM extraction kit. The concentration of the extracted RNA ranged between 10.7 and 13.4 ng/µL, the RNA purity ranged between 1.8 and 2.0. The RNA was then reverse into complementary DNA(cDNA) and all synthezied cDNAs were stored at −20°C untile further use.the Real-time PCR amplification was carried out in a total reaction volum of 20 µL, consisting 400 ng cDNA template, 10 µL RealQ Plus Master Mix Green 0.5 µL each of forward and reverse primers, and RNase- and DNase-free

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water add to reach final volume 20 µL The Primer paris employed in this study targeted genes associated with biofilm formation, including (bap,Csu,ompA) are shown in table (2), and then the gene expression was analysis using the real-time polymerase chain reaction (RT-PCR) technique. the 16S rRNA gene used to measure the gene expression, the biofilm genes (OmpA, Csu, Bab) were investigated in the bacterial isolates after growing them aerobically in nutrient broth and incubating them for 24 hours.

Table (2): Primers used in this study with their nucleotide sequence and amplification size

Oligo Name	Sequence (5' → 3')
bap-F	ACCCAGGTTTGAATGATGGC
bap-R	TTGCTGGGCCTGATGTATTG
Csu-F	ATTGACGGTGGTGAACGTAC
Csu-R	TTTGTACGAGCAGCATCACG
ompA-F	TGCTTTCTGGCGCTTAAACG
ompA-R	ACGTTTAAGCCAGCAAGAGC
16SrRNA-F	CAGCTCGTGTCGTGAGATGT
16SrRNA-R	CGTAAGGGCCATGATGACTT

Result

Numbers and percentages of *A. baumannii* isolation

446 clinical samples were obtained after burns, sputum, urine and blood. Out of these samples, 25 isolates were found to be A. baumannii, and the total isolation rate was 5.6% Burn samples recorded the highest isolation rate of 8.3 % and then sputum samples (6.7 %). The isolation rates were found to be lower in urine (3.3%) and blood samples (4.7%). The occurrence and the percentages of the isolates are summarized in Table (3).

Table (3): Percentages and numbers of *A.baumannii* isolates isolated from clinical samples

Percentage rate	Total number of samples	Number of bacterial isolates	Sample source
8.3%	120	10	Burns
6.7%	90	6	Sputum
3.3%	150	5	Urine
4.7%	86	4	Blood
5.6%	446	25	Total

Detection of Biofilm Formation

When tested by the microtiter plate assay, all the A. baumannii isolates (25/25; 100%) were able to form biofilms. Biofilm production was categorized into three based on optical density at 570 nm. Seven isolates (28) were found strong biofilm producers, thirteen isolates (52) were moderate biofilm producers, and five isolates (20) were weak biofilm producers as indicated in Table (4). Figure (1) depicts the difference in biofilm forming capacities among the isolates and it shows that there is a variation in the optical density value that relates to the degree of biofilm production.

Table (4): Levels of biofilm formation in *A. baumannii* bacteria.

Biofilm production levels	**Isolates number**	**Percentage rate**
Strong production	**7**	**28%**
Medium production	**13**	**52%**
Weak production	**5**	**20%**
%Total	**25**	**100%**

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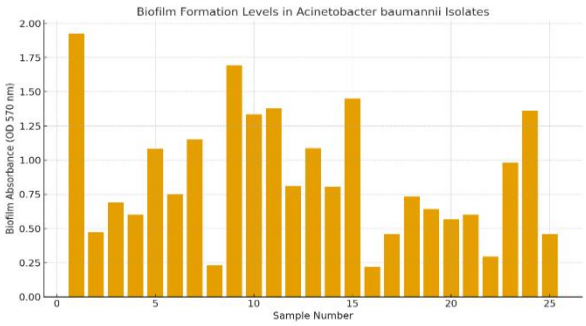

Figure (1): The figure shows the biofilm-forming levels of *A. baumannii* isolates based on light absorption at 570 nm.

Gene Expression

Gene expression was determined to be expressing biofilm-related genes, bap, csu, and ompA, in all the *A. baumannii* isolates. In quantitative real-time PCR experiments, it was shown that the levels of expression of these genes vary among strong, moderate and weak biofilm-producing isolates. Bap, csu and ompA were found to be more expressed in strong biofilm producers than moderate and weak ones. Figure (2) shows the relative level of expression (fold change) of the studied genes.

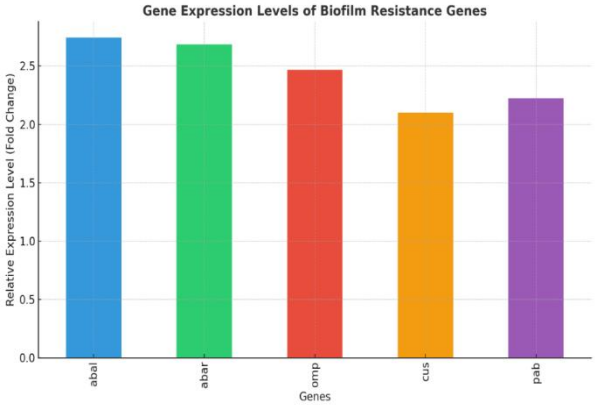

Figure (2): The level of expression of genes associated with biofilm resistance (abal, abar, omp, cus, pab).

Discussion

Isolation rate of *A. baumannii* (5.6%) in the current research indicates its persistence as a nosocomial pathogen of clinical importance in hospitals. The greater occurrence of the organism in burn and sputum samples is indicative of the close relationship of the organism with wound and respiratory infection especially in settings where there is a history of extended hospital stay and invasive procedures (13). The same trends have been observed in earlier regional and international surveys where burn units and intensive care units were found to be the significant sources of *A. baumannii* infections (14).

The capacity of the isolates to form biofilm confirms the critical role played by biofilm formation in *A. baumannii* persistence and pathogenicity. The fact that moderate and powerful producers of biofilm are dominant implies greater ability of adhering to the surface and resistance to environmental stresses, which can lead to failure in treatment and repeated infections (15, 16). These results confirm previous accounts that biofilm formation is a frequent (17) , and The expression of bap, csu, and ompA genes and their differential expression further confirm that they are involved in biofilm formation (18, 19). The increased level of expression of these genes in good biofilm producing isolates suggests that gene expression and not gene presence is a determining factor in biofilm strength. This finding is consistent with the prior research that has shown positive relationship between the expression of biofilm-related genes and the strength of biofilm formation (20, 21), conserved feature of clinical *A. baumannii* isolates (22, 23). All in all, the findings highlight the multidimensionality of biofilm formation in *A. baumannii* and the significance of focusing on biofilm-related processes in designing interventions on infection control and treating biofilms.

Conclusion

The current research showed that *A. baumannii* was detected in 5.6 % of clinical samples, and the burn (8.3 %) and sputum (6.7 %) samples had the highest rates. All Isolates exhibited the ability to form biofilms, and high proportions of biofilm-related genes, suggesting their virulence and role in persistent infections and these findings highlight the urgency of continuous local epidemiological surveillance and implementation of specific measures aimed at controlling the spread of this multidrug resistant pathogen, especially in high-risk units, such as burns wards and in intensive care settings.

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