ISSN Print: 2664-6188
ISSN Online: 2664-6196
Impact Factor: RJIF 5.39
IJCBB 2026; 8(6): 11-19
www.biochemistryjournal.net
Received: xx-02-2026
Accepted: xx-03-2026

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The correlation between the biofilm formation genes and antibiotic resistance in *Acinetobacter baumannii* isolation from hospitalized patients at Basrah, Iraq

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DOI: https://www.doi.org/10.33545/26646188.2026.v8.i6a.143

Abstract
Background: *Acinetobacter baumannii* is a Multidrug-Resistant (MDR) bacterium that has emerged and spread widely, causing major health problems. It is considered an opportunistic bacterium and is commonly associated with hospital-acquired infections in many countries, including Iraq. Recently, an increase in infections in Iraqi hospitals with the highly resistant *A. baumannii* has been observed.
Methods: 160 clinical specimens (burn swabs, wound swabs, sputum, blood, and urine) were collected from patients at five hospitals in Basrah city. Identification of the isolates was performed by standard microbiological methods and confirmed by the VITEK system. The bacterial isolates capacity to produce biofilm was examined using the Micro-titer plate method. DNA was extracted using the Promega kit. PCR technique was used to detect the genes associated with biofilm formation (*bap, ompA, csuE,* and *cpaA*).
Results: A total of 31(19.4%) *A. baumannii* isolates were identified from 160 clinical samples, that obtained from blood samples 11 (30.5%), followed by urine 8 (19.5%), sputum 7 (31.8%), wound samples 4 (16%), and burn samples 1 (2.7%). Statistical analysis showed a significant difference among the different specimen types ($p<0.05$). Of the 31 isolates, 27 were biofilm formers; of them, 13 (41.94%) were strong producers, 9 (29%) were moderate producers, 5 (16.12%) were weak producers, and 4 (13%) were non-producers. The presence of biofilm-related genes (*bap, ompA,* and *csuE*) was detected in *A. baumannii* isolates, where the *bap* gene was present in all isolates at a rate of (100%), followed by the *ompA* gene at a rate of (97%), and the *csuE* gene was (84%), while the *cpaA* gene was not detected in any of the isolates.
Conclusions: The present study demonstrates a marked reduction in the efficacy of commonly used clinical antibiotics against *A. baumannii* isolates, highlighting the alarming rise of multidrug- and extensively drug-resistant phenotypes and the urgent need to re-evaluate current therapeutic strategies in hospital settings and showed a strong association between multidrug-resistant *A. baumannii* isolates and biofilm formation.

Keywords: *Acinetobacter baumannii*, multidrug-resistant, biofilm formation and biofilm-related genes

1. Introduction

Acinetobacter baumannii (*A. baumannii)* is a Gram-negative, nonmotile, obligate, aerobic coccobacillus, oxidase-negative and catalase-positive [1]. It is an opportunistic pathogen associated with nosocomial infections, primarily found in Intensive Care Units (ICUs) and among immunocompromised patients. Additionally, it can be found in various environments such as soil, water, and waste [2].

Bacteria employ a variety of mechanisms to become resistant to various different types of antibiotics. One notable mechanism is the formation of strong biofilms, which are bacterial clusters that provide a strong defense against antibiotics such beta-lactams, fluoroquinolones, and carbapenems [3].Numerous infections, including urinary tract infections, diabetic foot ulcer infections, wounds, otitis media, meningitis, bacteremia, and endocarditis, are thought to be significantly influenced by *A. baumannii* capacity to colonize and form biofilms on abiotic surfaces, especially in patients in intensive care units. [4].

ESKAPE organisms are those that exhibit not only Multiple Drug Resistance (MDR) but also important virulence factors. *A. carbapenem* resistant The WHO has discovered that

baumannii; CRAB was classified as a big problem and specifically as a priority territory for research and development of antibiotics for the year 2018 [7]. There once was. Infections of baumannii often treated with carbapenem Nevertheless, the usage of carbapenems has resulted in an increase in the prevalence of carbapenem resistance [8].

A is shaped by a multitude of factors. Biofilms of baumannii. Among these proteins are the outer membrane protein A (OmpA), the biofilm-associated protein (Bap) and chaperone usher (Csu) pathway for pilus formation, which may provide structural basis for A. Formation of biofilms by baumannii species As the significant porin of A. In case of *A. baumannii*, the outer membrane protein A (ompA) is essential for the adhesion and invasion of epithelial cells through interaction with fibronectins, biofilm formation and persistence, induction of apoptosis, and resistance towards antibiotics [9].

Biofilm associated protein (*Bap*) is a large cell surface protein that plays a critical role in cell-to-cell interactions and contributes to biofilm formation, maturation, and maintenance [10, 11]. The *csuE* protein is important, which works with other proteins to form pili and participate in adhesion to surfaces and has a major role in the formation of biofilms in *A. baumannii* bacteria and participates in the colonization of various surfaces [12].

Although many studies have investigated antimicrobial resistance and virulence characteristics of *A. baumannii*, but there are limited studies examined the combined relationship between biofilm-related genes (*bap, ompA, csuE*, and *cpaA*) and antimicrobial resistance patterns among clinical isolates, especially in clinical isolates obtained from hospitalized patients.

Therefore, the current study was conducted to investigate the association between biofilm-related genes and antibiotic resistance in Basrah city

2. Material and Methods

2.1 Study design and setting

This observational research was conducted in the province of Al-Basrah of Iraq. Five Basrah hospitals which were used for sampling were Al-Fayhaa Hospital, Al-Sadr Teaching Hospital, Al-Mawani Teaching Hospital, Al-Basrah Teaching Hospital and Basrah Hospital for Women and Children. All molecular and microbiological analysis were performed at the Department of Biology, College of Science, University of Basrah.

2.2 Sample collection and bacterial identification

A total of 160 clinical samples were collected during the period from October 2024 to March 2025. The samples included burn swabs, wound swabs, sputum, blood, and urine samples from patients suffering from urinary tract infections under aseptic conditions. Samples were cultured on standard media, including Blood Agar, MacConkey Agar and HiCrome *Acinetobacter* Agar (HiMedia, India), and incubated at 37 °C for 24 h.

Using standard morphological characteristics, Gramme staining, and biochemical test reactions – the oxidase and catalase tests are commonly used to determine suspected colonies. We picked a single colony from a bacterial culture of suspected A. in the lab for identification. isolates of baumannii The GN ID card and VITEK-2 automated system (bioMérieux, Marcyl'Étoile, France) manufacturer's recommendations served as a basis for final recognition.

2.3 Antimicrobial susceptibility testing

Antibiotic susceptibility of A was determined using the VITEK-2 system (bioMérieux, France). The ASTN419 card was used to detect the sensitivity of *A. baumannii* isolates to different antibiotics including Ampicillin with sulbactam (AMS), Piperacillin/Tazobactam (PRL). Cefepime, Meropenem, Imipenem, Ciprofloxacin, Trimethoprim/sulfamethoxazole, Tobramycin, Gentamicin, and Colistin. In line with the manufacturer's instructions, antibiotic quantities were added to the card wells as preset minimum inhibitory concentration (MIC) dilution ranges. Test results were interpreted in accordance with Clinical and Laboratory Standards Institute (CLSI) [13] recommendations.

2.4 Biofilm formation (quantitative biofilm production assay)

The biofilm development experiment was carried out using the method outlined by Babapour [14].

2.5 Genetic work

2.5.1 Extraction of genomic DNA

Genomic DNA was extracted from the A. The baumannii isolates were taken for the Wizard® Genomic DNA Isolation Kit for Bacteria (Promega, USA) as per the manufacturer instructions. The isolates were stored at -20 °C.

2.5.2 Detection of biofilm-related genes by PCR

The sequencing of bap, csuE, ompA, and cpaA genes was carried out using PCR assays (Table 1) using specific primers. The total volume of green master mix for the PCR was 50 ul provided by the Promega Company (USA). The PCR mix comprised of a total of 20 µL containing PCR Master Mix, 1.0 µL of 10 pmole forward primer, an and 1.0 µL of 10 pmole reverse primer. Genomic DNA totaling 5 µL as well as 23 µL of water without any nuclease. The thermal cycler was used for amplifying the genes. Table 2 illustrates the setup parameters for the PCR assay. The PCR results were visualised by agarose gel electrophoresis (1.5%) stained with ethydium bromide and viewed under UV-light.

Table 1: The primers used in this study for the detection of biofilm-related genes

Type of gene	Sequence type	Sequence (5'-3')	Size of gene	References
bap	Forward primer	TGCTGACAGTGACGTAGAACCACA	184 bp	[44]
	Reverse primer	TGCAACTAGTGGAATAGCAGCCCA		
csuE	Forward primer	ATGCATGTTCTCTGGACTGATGTTGAC	976 bp	[45]
	Reverse primer	CGACTTGTACCGTGACCGTATCTTGATAAG		
ompA	Forward primer	ATTTACCAGGATGGGCCGTG	182 bp	[46]
	Reverse primer	GCGCCACAACCAAGCAATTA		
cpaA	Forward primer	CTG CTT TAG GAA AAT GGG	669 bp	[47]
	Reverse primer	CGCCTTCAATCATTCTAAG		

Temperature (°C) / time Cycling conditions						
Genes	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
bap	95°C / 5 min.	95°C / 1 min	54°C / 30 sec	72°C / 1 min.	72°C / 5 min.	35
csuE	94°C / 5 min.	94°C / 1 min	60°C / 1 min.	72°C / 1 min.	72°C / 10 min.	30
ompA	95°C / 10 min.	95°C / 15 sec	60°C / 1 min.	72°C / 1 min.	72°C / 5 min.	40
cpaA	95°C / 3 min.	95°C / 30 sec	63°C / 30 sec	72°C / 1 min.	68°C / 5 min.	35

Fig 1: PCR conditions for *bap, csuE, ompA,* and *cpaA* gene amplification

2.5.3 Gradient PCR amplification procedure

Gradient PCR enables optimization of a PCR protocol to find which annealing temperature works best. Conditions of PCR were optimised by varying the annealing temperatures (58 °C to 65 °C) based on capA gene primer and number of cycles (35). This technique saves time and uses fewer reagents when compared to a normal PCR optimisation technique.

3. Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 27 was used to analyze the data, and a chi-square test was performed for the sample, with the significance level set at p-vale <0.05 for all statistical tests [15].

4. Results and Discussion

4.1 Isolation of *A. baumannii*

The results in the current study showed that 123 (77%) bacterial growth, while 37 (23%) showed no growth. Among the positive growth, 31 (19.4%) were of *A. baumannii* isolates. As appeared in Figure 2.

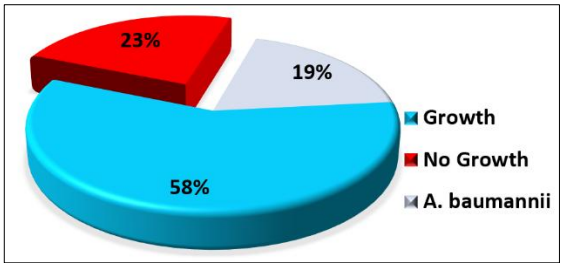
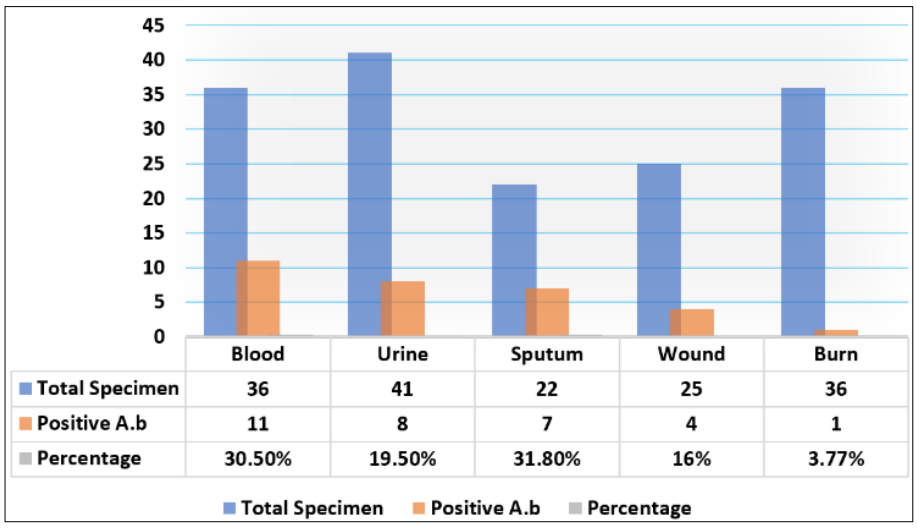


Fig 2: The percentages of clinical specimens

4.2 Distribution of *A. baumannii* isolates according to the sources

The results shown in Figure 3 indicate that the highest percentage of *A. baumannii* isolates was obtained from blood samples 11 (30.5%), followed by urine 8 (19.5%), sputum 7 (31.8%), wound samples 4 (16%), and burn samples 1 (2.7%). The isolation rate of *A. baumannii* changes according to the source of the clinical sample, according to statistical analysis, that showed a significant difference between the various specimen types (p<0.05).



Significant difference (p<0.05) between all sample types (done by Chi-square)

Fig 3. Distribution of *A. baumannii* isolates according to the sources

The predominance of isolates from blood samples can be attributed to *A. baumannii* ability to cause hospital-acquired bloodstream infections, especially among critically ill patients and those exposed to surgical procedures such as intravenous catheters and prolonged hospitalization. [16]

4.3 Identification of *A. baumannii*

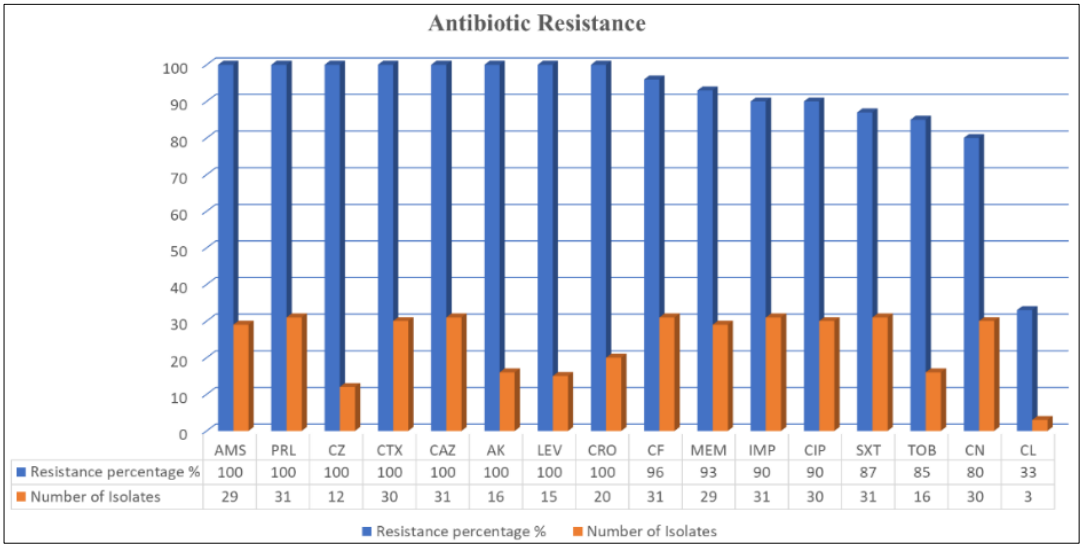
A. baumannii isolates on the MacConkey agar appeared as a pale pinkish tint or smooth transparent colonies, due to the non-lactose fermenting colonies [17]. While on blood agar, *A.baumannii* colonies appear round, gray in color and

nonhemolytic [18] and on the HiCrome *Acinetobacter* agar, appeared as light purple colonies after 24 hr. and incubation at 37 °C [19]. Then characteristics of isolates were subjected identified by biochemical test as shown a negative oxidase test, catalase-positive, Citrate utilization positive, Indol production negative and growth at 44 °C.

Also, the Vitek 2 System was used to validate the identification of the bacterial isolates. A GN ID card was used to identify the Gram-negative bacteria. This process is very accurate, with an approximate success rate of (98-99%) and by very little mistake [20].

4.4 Antibiotics susceptibility test

Following the Clinical and Laboratory Standards Institute CLSI (2024) requirements, Vitek®2 was applied for the identification and validation of antibiotic susceptibility testing and their research. The findings showed that *A. baumannii* clinical isolates are highly resistant to most of antibiotics. The results shown in Figure 4 that resistance rates were (100%) for Ampicillin/sulbactam (AMS), Piperacillin/Tazobactam (PRL), Cefazolin (CZ), Cefotaxime (CTX), Ceftazidime (CAZ), Amikacin (AK), Levofloxacin (LEV), and Ceftriaxone (CRO). While the resistance rates were Cefepime (CF) (96%), Meropenem (MEM) (93%) and (90%) for both Imipenem (IMP) and Ciprofloxacin (CIP), followed by (87%), (85%), and (80%) respectively for each of Trimethoprim/sulfamethoxazole (SXT), Tobramycin (TOB) and Gentamicin (GN). The lowest resistance rate was observed with Colistin (CL), reaching 33%.



No of *A. baumannii* isolates =31

Fig 4: The proportion of *A. baumannii* isolates that are susceptible to antibiotics

These results were consistent with several studies, including a local study conducted in Babil Governorate, which confirmed a 100% resistance rate to both the antibiotics Ampicillin /Sulbactam, Piperacillin/tazobactam, and Cephalosporins specifically Cefazolin, Cefoxitin, Ceftazidime and Ceftriaxone [21]. Also, a study in Iran showed a 100% resistance rate for Ceftriaxone, Ceftazidime, and Cefazolin [22].

A's results. The current study's baumannii isolates were found to have an Amikacin resistance rate of 100% and a Gentamicin and Tobramycin resistance rate of 80% and 85%, respectively. These results are almost consistent with a study conducted in Morocco, where the resistance rate of isolates reached 87% and 81%, respectively, for both Gentamicin and Tobramycin, but they did not agree with them in the percentage of Amikacin, as the percentage was only 43%[23]].

The current study which Fluoroquinolone antibiotics involving Levofloxacin and Ciprofloxacin, the *A. baumannii* isolates displayed 100% resistance to Levofloxacin and 90% to Ciprofloxacin. A study conducted in Erbil revealed 100% resistance to these antibiotics [24].

The results showed that 93% and 90% of the isolates in the current study were resistant to the antagonists Imipenem and Meropenem, respectively. and this result is consistent with a study conducted in Erbil, where resistance to Meropenem antagonists was 96% [25] and disagree with Hussain that was 64.4% Imipenem resistance [26]. Carbapenems antibiotics resistant mechanism in gram negative pathogens bacteria in first place, is the efflux pumps that push the drug away from where it attaches to the cell, it also changes the link location in the penicillin-binding proteins (DD-transpeptidases) [27].

The Polymyxin antibiotic involving Colistin shows 33% resistance *A. baumannii* isolates against this antibiotic were the least resistant compared to other antimicrobial agents tested in this study. The current study showed Colistin is still effective for the treatment of *A. baumannii* infection. In recent study conducted in Karbala, the resistance rate to Colistin was 10% [28].

The resistance of *A. baumannii* bacteria to many antibiotics is linked to its ability to produce beta-lactamase enzymes and possess efflux pumps that expel the antibiotic from the cell or reduce the permeability of the outer membrane, thus preventing the antibiotic from reaching the target site, as well as al in Jordan tering metabolic pathways and changing the target site of the antibiotic through genetic mutations [30].

The results of the current study showed that all *A. baumannii* isolates are resistant to at least one antibiotic for three groups of antibiotics. Where all 31 *A. baumannii* isolates were found to have MDR (100%) and 29/31 isolates were found with XDR (93.55%). Therefore, all 31 isolates from all clinical sources are 100% multi-drug resistance (MDR). The results of this study are consistent with a study conducted in Italy showed that MDR isolates 95.8% [31]. Also, another study conducted in Italy indicate to MDR rate was 89.5% [32]. The emergence and spread of Multidrug

Resistance in the hospital environment by *A. baumannii* is a matter of concern and is among the most important problems facing healthcare., because it limits treatment options for infected patients, especially if the isolates are also resistant to Carbapenems.

4.5 Molecular detection of biofilm-related genes by PCR

In the current study, the biofilm related genes in *A. baumannii* isolates were investigated. These genes included: Bap, ompA, csuE, and capA, to determine their relationship to biofilm formation and their role in antibiotic resistance.

As apparent in Figure 4, the most common gene found among biofilm-related genes was bap (100%), followed by ompA (97%), csuE (84%), and cpaA (0%).

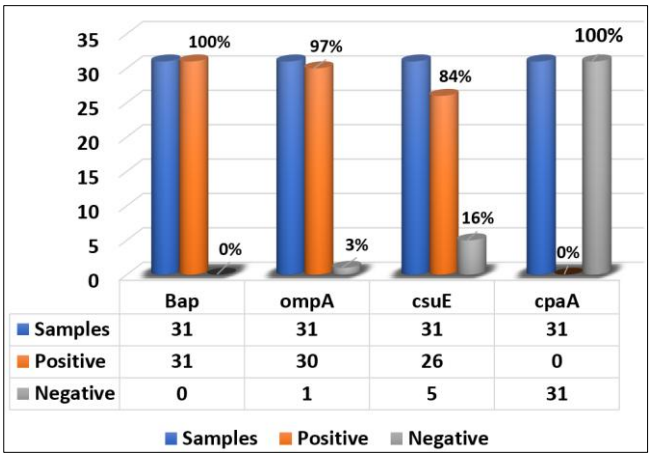
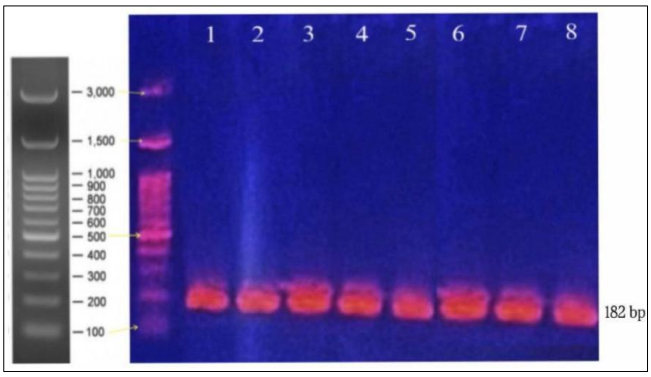
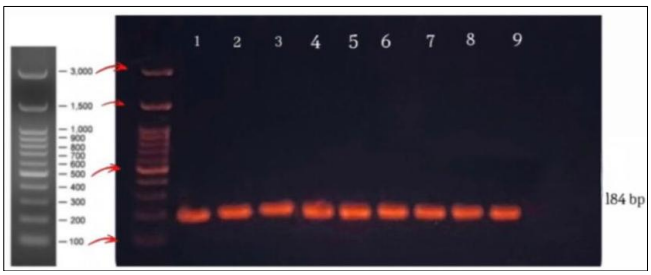


Fig 5: Percentage and distribution of biofilm-related genes in *A. baumannii* isolates



A



B

Fig 6: The gel electrophoresis picture of the biofilm-related genes in PCR products, using 1.5% agarose gel, 74V, 75min. Ladder (100-3000bp) **A:** PCR amplified products of *Bap* gene, (184bp). band size of bacterial isolates lane (no-1-9), **B:** PCR amplified products of *ompA* gene (182bp). band size of bacterial isolates lane (no-1-8)

A

B

Fig 7: The gel electrophoresis image of PCR product (biofilm-related genes, using 1.5% agarose gel, 74V, 75min. Ladder (100-3000bp) **A:** PCR amplified products of *csuE* gene, (976bp). band size of bacterial isolates lane (no-6-15) **B:** PCR amplified products of *capA* gene (669 bp amplicon) in *A. baumannii* isolates

The present result was consistent with the Iraqi study, which recorded the same percentage (100%) of *A. baumannii* isolates possesses a bap gene [10]. Also, another study conducted in Shanghai, China, reported that the *bap* gene was detected in 88.13% of the isolates [33]. A study conducted by Saadulla and Muhammed found that 92.45% of isolates tested positive for the ompA gene [34].

The study by Khoshnood in Iran showed results similar to current study that 86% the presence of the csuE gene [35]. The local study also showed close results in having *A. baumannii* isolates csuE gene 73.58 % [34].

The number of studies investigating the cpaA gene remains relatively limited. The present study is consistent with results of study done by Kinsella, the *A. baumannii* strain ATCC 19606 lacked the cpaA gene but it disagree with the local study which recorded 7.54% for the same gene [34].

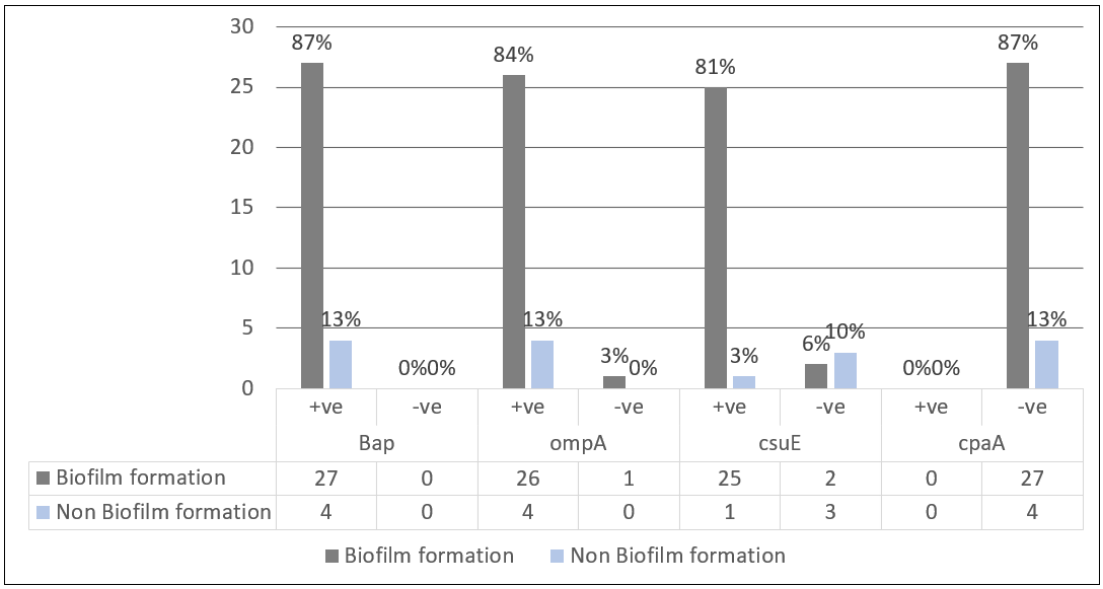
The results of the current study showed a high prevalence of the *bap* gene among *A. baumannii* isolates, which may explain the enhanced ability of these isolates to form robust biofilms and persist in clinical settings. This observation is consistent with the role of bap as a key virulence determinant that promotes bacterial adhesion and biofilm maturation [36].

4.3 Relationship biofilm formation and biofilm-related genes with MDR in *A. baumannii* isolates

The origins and dissemination of A. The infectious nature of *A. baumannii* is believed to be greatly influenced by the trait of biofilm formation [37]. In clinical strains of A. In the current study, we evaluated biofilm and antibiotic resistance genes and their relation to biofilm formation in *A. baumannii*.

Of the 31 isolates, 27 were biofilm formers; of these, 13 (41.94%) were strong biofilm formers, 9 (29%) were moderate, 5 (16.12%) were weak biofilm formers, and 4 (13%) were non-biofilm producer. Bap, ompA, and csuE genes were detected in 100% (27/27), 96% (26/27), and 92.5% (25/27) of the biofilm producers, respectively, according to the analysis of the relationship between biofilm production and biofilm-related genes. As shown in Figure 8. All biofilm-producing isolates possessed at least one of the tested genes associated with biofilm [38].

A significant correlation was shown between the presence of the bap gene and the ability of baumannii isolates to produced biofilm; all biofilm formers had the bap gene. Bap stimulates biofilm formation in Staphylococcus spp. and the expression of a particular biofilm phenotype on surfaces requires this surface protein. This is supported by the biofilm data presented in this work and elsewhere. These findings matter because A. The growth of *A. baumannii* on non-biotic surfaces is linked to nosocomial infection outbreaks [39].



No of *A. baumannii* isolates =31

Fig 8: Correlation of biofilm formation and biofilm-related gene

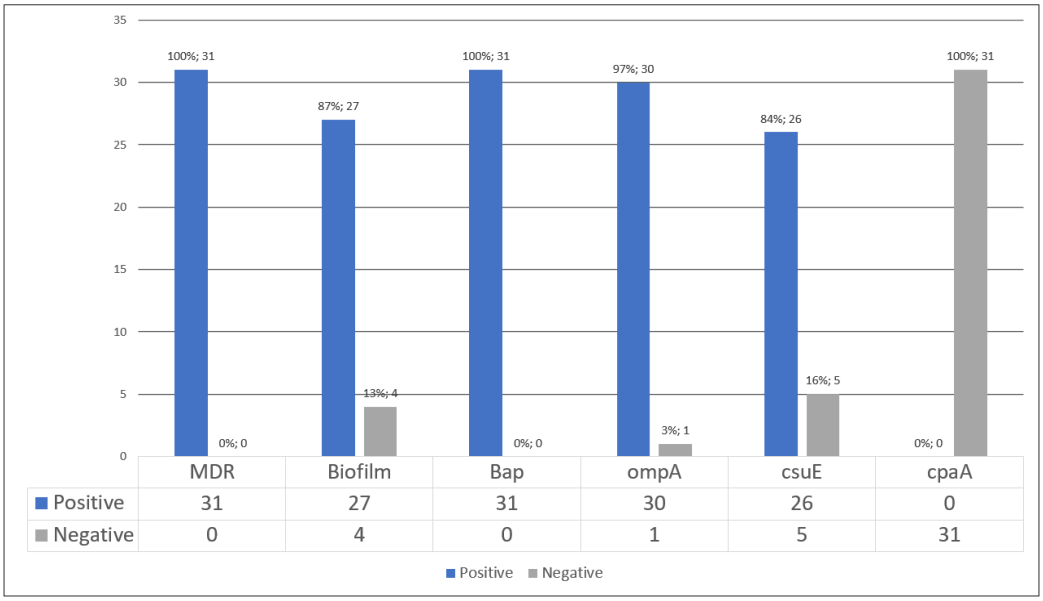
Also, the widespread distribution of ompA can be attributed to its essential biological functions, ompA is one of the factors of virulence affecting and decisive for *A. baumannii*, playing an important role in influencing the immune response, epithelial cell adhesion, and biofilm maturation. Biofilm formation mediated by OmpA enhances bacterial survival in hospital environments and contributes significantly to antimicrobial resistance by limiting antibiotic and also contributes to the apoptosis of cells [40]. The most common pili in bacteria, Csu pili, play a crucial role in cell adhesion and biofilm development. The csuE gene is a part of the chaperone-usher pili assembly system. [41].

Although the present study did not detect the cpaA gene in any of the *A. baumannii* isolates, previous research has identified it as an important virulence factor in *A. baumannii*. It encodes a zinc-dependent metalloprotease that disrupts host coagulation proteins, promoting bacterial [34].

However, biofilm formation is not directly linked to the cpaA gene. This result is in line with an Iranian investigation that found a link between the cpaA gene and the coagulation system as a mechanism of *A. baumannii* pathogenicity, but no significant correlation (p>0.05) between the presence of the gene and *in vitro* biofilm development [47].

Biofilm-related genes, are closely linked to enhanced virulence and play a critical role in promoting biofilm formation. This biofilm structure acts as a protective barrier that limits antibiotic penetration and interferes with host immune responses [42]. In addition, recent studies have demonstrated a strong association between the expression of these genes and the emergence of Multidrug-Resistant (MDR). Consequently, the ability of *A. baumannii* to form biofilms represents a key factor underlying its persistence in hospital environments, posing a significant challenge for infection control and therapeutic management [43].

This study's higher frequency of the bap, ompA, and csuE genes suggests that all of these genes are essential for the development of biofilms and antibiotic resistance, as shown in Figure 9.



No of *A. baumannii* isolates =31

Fig 9: Biofilm production and biofilm-related gene correlations with MDR in isolates of *A. baumannii*

MDR and XDR strains can develop due to biofilm formation happening on biotic and abiotic surfaces. This is because the bacteria within a biofilm niche can develop antimicrobial resistance through various molecular mechanisms. They can do this through horizontal gene transfer, plasmid transformation and DNA uptake. The current study also found a link between strong biofilm formation and antibiotic resistance patterns; findings indicated that all isolates that exhibited strong biofilm formation were MDR, this result agreed with Khoshnood reported that all MDR and XDR *A. baumannii* isolates showed strong capacity to produce biofilm [35].

Conclusions

Blood samples yielded the largest percentage of *A. baumannii* isolates from a variety of clinical sources, including burn swabs, wound swabs, sputum, blood, and urine samples from patients with UTIs. Furthermore, the current study shows a significant decline in the effectiveness of widely used clinical antibiotics against *A. baumannii* isolates, underscoring the concerning emergence of multidrug- and extensively drug-resistant phenotypes. It also revealed a strong correlation between biofilm formation and multidrug-resistant *A. baumannii* isolates.

Also, the presence of biofilm-related genes (bap, ompA, and csuE) was detected in *A. baumannii* isolates, where the bap gene was present in all isolates at a rate of (100%), followed by the ompA gene at a rate of (97%), and the csuE gene was (84%), while the presence of the cpaA gene was not found in any of the isolates.

Acknowledgments

We, the authors, extend our sincere thanks to the College of Science/Department of Biology at the University of Basra, for their assistance in completing this research.

Funding

This study was done without any external funding.

Conflicts of interest

No conflicts of interest are disclosed by the writers.

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