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# Phenotypic and Genotypic Study of Biofilm Formation in *Acinetobacter baumannii* Isolated from Intensive Care Units

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ABSTRACT

**Background:** Biofilm formation supports the persistence of *Acinetobacter baumannii* in hospital environments and could reduce antimicrobial effectiveness. Therefore, phenotypic and genotypic studies of this bacterium in intensive care units (ICUs) could be helpful in controlling and containing infection. This study aimed to investigate the phenotypic and genotypic characteristics of biofilm formation in *A. baumannii* isolates from ICUs.

**Materials & Methods:** Between March and September 2021, 81 sputum-derived *A. baumannii* isolates were recovered from ICU patients. Species confirmation was performed by polymerase chain reaction (PCR) targeting the $bla_{\text{OXA-51-like}}$ gene. Antibiotic susceptibility testing was used to determine the phenotypic resistance pattern. Biofilm formation was assessed phenotypically for all isolates. The presence of *ompA*, *csuE,* and *bap* genes was determined by PCR.

**Findings:** Of the 81 patients, 42 (51.9%) were male, and 39 (48.1%) were female. Piperacillin-tazobactam (96.3%, n=78) and imipenem (93.8%, n=76) showed the highest resistance rates, while cefotaxime (56.8%, n=46) and gentamicin (46.9%, n=38) were the most effective antibiotics against the isolates. The biofilm formation ability could be described as follows: five (6.17%) isolates had no ability, while 76 (93.8%) isolates were able to form biofilm. Moderate biofilm formation was the most common biofilm pattern (n=42, 51.85%), followed by weak biofilm (n=32, 39.50%) and strong biofilm (n=2, 2.47%). Also, *bap*, *csuE*, and *ompA* were detected in 93.8, 86.4, and 82.7% of the isolates by PCR, respectively.

**Conclusion:** Phenotypic and genotypic information on biofilm formation ability of commonly-found isolates in ICUs could strengthen infection control measures and antimicrobial stewardship programs.

**Keywords:** *Acinetobacter baumannii*, Biofilms, Drug resistance, Intensive care units

CITATION LINKS

[1] Vázquez-López R, et al. Acinetobacter... [2] Yang CH, Su PW, Moi SH, Chuang LY. Biofilm formation in... [3] Al-Shamiri MM, et al. Phenotypic and... [4] Čiginskienė A, et al. Ventilator-associated... [5] Qi L, Li H, Zhang C, Liang B, Li J, Wang L, et al. Relationship... [6] Sung JY. Molecular characterization and... [7] Colquhoun JM, Rather PN. Insights into... [8] Shukla SK, Rao TS. Targeting hydrophobicity in... [9] Mahmood S. The prevalence of... [10] Pakharukova N, et al. Structural basis... [11] Nie D, et al. Outer membrane... [12] Oh MH, et al. AbOmpA in... [13] Sung JY, Koo SH, Kim S, Kwon GC. Persistence of... [14] Pal N, Sujatha R, Kumar A. Phenotypic and... [15] Arian Nejad M, et al. Characterization of... [16] Ahmed SH, Abdelwahab SF, Hasanen AM, Mohammed DS. Multidrug... [17] Asaad AM, Ansari S, Ajlan SE, Awad SM. Epidemiology of... [18] Khoshnood S, et al. Survey on... [19] Mahmoudi MA, Rezaei A, Poursina F, Faghri J. Detection of... [20] Clinical and Laboratory Standards... [21] Stepanović S, et al. Quantification of... [22] Kasperski T, et al. Extensive drug... [23] Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M. A modified... [24] Benamrouche N, et al. Phenotypic and... [25] Folliero V, et al. Evaluation of... [26] Sadr M, et al. Antimicrobial resistance... [27] Farzana R, Swedberg G, Giske CG, Hasan B. Molecular and... [28] Beigverdi R, et al. Status of... [29] Zeighami H, et al. Virulence characteristics of... [30] Shree P, et al. Biofilms... [31] Li Z, et al. Phenotypic and... [32] Saadati M, et al.The prevalence of... [33] Dutt Y, et al. The association... [34] Sharma S, et al. Comparative study of... [35] Wiradiputra MR, et al. Genomic insights into... [36] Liu H, et al. Biofilm-related... [37] Sherif MM, et al. Multidrug... [38] Viale AM, Evans BA. Microevolution in the... [39] Ghasemi E, et al. Phenotypic and... [40] Shenkutie AM, et al. Biofilm-induced...

## Introduction

Hospital-acquired infections remain a major clinical problem, and *Acinetobacter baumannii* (*A. baumannii*) is considered an important nosocomial pathogen because of its increasing antibiotic resistance [1]. Biofilm formation helps this bacterium persist on hospital surfaces and medical equipment, which could facilitate its survival in clinical environments [2].

This bacterium is one of the most common bacteria isolated from intensive care units (ICUs), particularly from patients with ventilator-associated pneumonia (VAP) [3]. VAP-associated mortality rate in the ICU increases when patients are infected with extensively drug-resistant (XDR) *A. baumannii.* The bacterium has several strategies to survive in harsh conditions, one of which is the production of biofilms. Through this process, it could be protected from antibiotics [4].

A number of porins and pili, which are among the virulence factors in bacteria, are involved in the attachment of bacteria to different surfaces and the formation of biofilms.

The rate of biofilm formation in these bacteria is 5 to 24% higher than in other species, and studies have demonstrated that biofilm formation significantly increases antibiotic resistance of *A. baumannii* [5, 6]. *A. baumannii* could easily adhere to different surfaces through biofilm formation, and several genes are involved in this adhesion. One of the most important proteins expressed in this phenomenon is biofilm-associated protein (Bap), which is essential for the initial attachment and biofilm persistence [7, 8].

The *csuE* gene encodes the CsuE protein, which is located at the tip of the Csu pili and serves as the initial contact point with host cells.

Through this protein, pili could attach to biological (host cells) and non-biological (medical devices) surfaces. Specifically, the CsuE protein plays a key role in pili formation, adhesion, and biofilm development [9, 10].

Recent studies have highlighted OmpA as a major virulence determinant of *A. baumannii*, contributing to adhesion, induction of host cell apoptosis, and antimicrobial resistance. Novel insights into OmpA-mediated pathogenicity also highlight its potential as a therapeutic target [11, 12].

In recent years, drug-resistant *A. baumannii* infections have increased in ICUs, making treatment more challenging [13]. Therefore, it is important to investigate biofilm-formation factors and virulence determinants associated with resistance in these bacteria. The present study investigated the biofilm formation ability and the distribution of *bap*, *ompA*, and *csuE* among sputum-derived *A. baumannii* isolates recovered from ICU patients.

The *bap*, *ompA*, and *csuE* genes were selected because they are biofilm-associated genes in *A. baumannii* and collectively represent important stages of biofilm development, including adhesion, surface attachment, pili formation, and biofilm stability [2].

## Materials and Methods

The study included 81 *A. baumannii* isolates collected from sputum specimens of ICU inpatients at Hazrat Rasool Akram Hospital (Tehran, Iran) from March to September 2021. Isolates from outpatients were excluded.

Sputum samples were cultured on blood agar and MacConkey agar plates (Merck, Germany) and incubated aerobically at 37 °C for 24–18 hrs.

Presumptive *Acinetobacter* spp. isolates were identified based on colony morphology, Gram staining, and conventional microbiological and biochemical tests, including oxidase, catalase, motility, indole, citrate, and triple sugar iron (TSI) tests [14].

Sputum was selected as the sample source because during the study period, most of the available *A. baumannii* isolates recovered from ICU patients in the hospital laboratory were obtained from sputum samples. Therefore, to maintain consistency in the clinical

source of isolates and focus on respiratory isolates from ICU patients, only sputum-derived isolates were included in this study.

**DNA extraction and molecular identification:** For DNA extraction, overnight-grown *Acinetobacter* isolates were processed using the boiling method. Fresh colonies were suspended in 100 µL of sterile distilled water and boiled in a water bath for 10 min. The suspension was then frozen at −20 °C for 5 min and centrifuged at 10,000 rpm for 2 min. The supernatant was used as the DNA template in polymerase chain reaction (PCR). DNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Scientific, USA), and DNA quality was evaluated based on the A260/A280 absorbance ratio [15].

Molecular identification of *A. baumannii* was carried out by amplification of the intrinsic $bla_{\text{OXA-51-like}}$ gene. The primer sequences used in this study are presented in Table 1 [16-19]. Each PCR reaction was prepared in a final volume of 25 µL, containing 13 µL of 2X PCR Master Mix (Ampliqon, Denmark), 1 µL of each forward and reverse primer, 3 µL of DNA template, and 7 µL of sterile distilled water. Amplification was performed with initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 60 s, with a final extension step at 72 °C for 5 min [16].

*A. baumannii* ATCC 19606 was used as a positive control, and a template-free reaction containing sterile distilled water instead of DNA was included as a negative control.

Amplified products were separated on 1% agarose gel (Pishgam, Iran) prepared in 1X Tris-borate-EDTA (TBE) buffer (Pishgam, Iran). DNA Safe Stain (Pishgam, Iran) was added before gel casting. PCR products, a DNA ladder (SMOBIO, Taiwan), positive control, and negative control were loaded into the wells. Electrophoresis was run at 100 V for 35 min, and the amplified DNA bands were visualized using a transilluminator (Pars Azma, Iran). The expected amplicon sizes are shown in Table 1.

**Antimicrobial susceptibility testing:** Disk diffusion testing was used to determine the antimicrobial susceptibility of all *A. baumannii* isolates, and the results were interpreted according to CLSI 2020 guidelines [20]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality-control strains, as recommended by CLSI. The following antibiotic disks (Padtan Teb, Iran) were used: ceftazidime (30 µg), gentamicin (10 µg), amikacin (30 µg), piperacillin-tazobactam (100/10 µg), ciprofloxacin (5 µg), cefepime (30 µg), imipenem (10 µg), ceftriaxone (30 µg), and cefotaxime (30 µg).

**Biofilm formation assay:** Biofilm production was evaluated by the crystal violet microtiter plate method with minor modifications to a previously described protocol [21]. For this assay, *A. baumannii* isolates were first cultured on Mueller–Hinton agar (Mer-

**Table 1)** Size and sequences of the primers used in this study

| Target Gene | Sequence (5′→3′) | Amplicon Size (bp) | Ref. |
|---|---|---|---|
| $bla_{\text{OXA-51-like}}$ | F: 5′-TAA TGC TTT GAT CGG CCT TG-3′<br>R: 5′-TGG ATT GCA CTT CAT CTT GG-3′ | 353 | [16] |
| *bap* | F: 5′-TGC TGA CAG TGA CGT AGA ACC ACA-3′<br>R: 5′-TGC AAC TAG TGG AAT AGC AGC CCA-3′ | 184 | [17] |
| *ompA* | F: 5′-GTT AAA GGC GAC GTA GAC G-3′<br>R: 5′-CCA GTG TTA TCT GTG TGA CC-3′ | 578 | [18] |
| *csuE* | F: 5′-CAT CTT CTA TTT CGG TCC C-3′<br>R: 5′-CGG TCT GAG CAT TGG TAA-3′ | 168 | [19] |

**Table 2)** Classification of biofilm formation ability using microtiter plate method

| Average OD* Value | Biofilm Production |
|---|---|
| OD ≤ ODc | Non-adherent |
| ODc <OD ≤ 2 × ODc | Weakly adherent |
| 2×ODc <OD ≤ 4 × ODc | Moderately adherent |
| 4 × ODc < OD | Strongly adherent |

*Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control [22, 23].

ck, Germany) and incubated overnight at 37 °C. The isolates were then transferred into tryptic soy broth (TSB; Merck, Germany) and incubated at 37 °C for 24 hrs in a shaker incubator.

To each well of a 96-well microtiter plate, 180 µL of TSB and 20 µL of bacterial suspension adjusted to 0.5 McFarland turbidity (1.5 × 10^8 CFU/mL) were added. *A. baumannii* ATCC 19606 was used as a positive control, and wells containing uninoculated TSB served as negative controls.

The plates were incubated statically at 37 °C for 48 h. After incubation, the wells were washed three times with phosphate-buffered saline (PBS) to remove non-adherent bacteria. Adherent cells were fixed with 150 µL of 96% methanol for 20 min and then left at room temperature.

The wells were stained with 150 µL of 1% crystal violet for 10 min and washed twice with double-distilled water. After drying, 150 µL of 96% ethanol was added to each well and left for 30 min.

The optical density (OD) of each well was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (BioTek ELISA Reader ELx800, USA).

Biofilm formation was classified according to Table 2, and each test was repeated three times for each isolate [22, 23].

**Genotypic analysis of biofilm formation:**

To detect biofilm-associated genes, PCR amplification was performed for *bap*, *ompA*, and *csuE*. DNA templates were prepared from all isolates by the boiling method, as described earlier. PCR reactions for *bap*, *ompA*, and *csuE* were performed using the same reaction mixture described above. Amplification was conducted in a SensQuest Labcycler thermal cycler (Germany) using the following gene-specific programs:

1. The *ompA* reaction included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 50 s, 56 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min [16].
2. The *bap* reaction started with denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, with a final extension at 72 °C for 5 min [17].
3. The *csuE* reaction included initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 48 °C for 45 s, and 72 °C for 50 s, with a final extension at 72 °C for 6 min [18].

*A. baumannii* ATCC 19606 was used as a positive control for amplification of the *bap*, *ompA*, and *csuE* genes, and a template-free reaction containing sterile distilled water was included as a negative control.

The resulting amplicons were examined using the agarose gel electrophoresis protocol described earlier.

**Statistical analysis:** Data normality was checked with the Shapiro–Wilk test. According to the distribution pattern of each variable, group comparisons were conducted using suitable parametric or non-parametric tests. The threshold for statistical significance was set at $\alpha = 0.05$. Statistical processing and figure generation were carried out in GraphPad Prism Version 9.

## Findings

PCR-based confirmation verified 81 isolates as *A. baumannii*. Of the 81 patients, 42 (51.9%) were male, and 39 (48.1%) were female. The

**Table 3)** Antibiotic resistance rates of *A. baumannii* isolates (N = 81)

| Antibiotic | Resistant Isolates, N (%) |
|---|---|
| Amikacin | 72 (88.9) |
| Ceftriaxone | 48 (59.3) |
| Ceftazidime | 73 (90.1) |
| Cefotaxime | 35 (43.2) |
| Imipenem | 76 (93.8) |
| Piperacillin-tazobactam | 78 (96.3) |
| Cefepime | 74 (91.4) |
| Ciprofloxacin | 64 (79.0) |
| Gentamicin | 43 (53.1) |

mean ± SD of the participants' age was 69.80 ± 14.58 years, with an age range of 19–95 years.

**Antimicrobial susceptibility:** According to the results obtained, piperacillin-tazobactam (96.3%, n=78), imipenem (93.8%, n=76), cefepime (91.4%, n=74), and ceftazidime (90.1%, n=73) showed resistance rates of over 90%. In addition, the highest antibiotic susceptibility rate was found for cefotaxime (56.8%, n=46), followed by gentamicin (46.9%, n=38). The resistance rates of the isolates to the tested antibiotics are presented in Table 3.

**Phenotypic assay of biofilm production:** Among the 81 *A. baumannii* isolates, 76 (93.8%) isolates showed the ability to form biofilm, whereas five (6.17%) isolates did not form biofilms. Based on biofilm formation intensity, moderate biofilm formation was the most frequent biofilm pattern observed in 42 isolates (51.85%), followed by weak biofilm formation in 32 isolates (39.50%) and strong biofilm formation in two isolates (2.47%). For each isolate, the assay was repeated three times, and the classification of biofilm production was done based on the mean OD value.

**Association between biofilm biomass and antimicrobial resistance by class:** Biofilm biomass (OD at 490 nm, crystal violet assay) was compared between resistant and susceptible isolates to each antibiotic

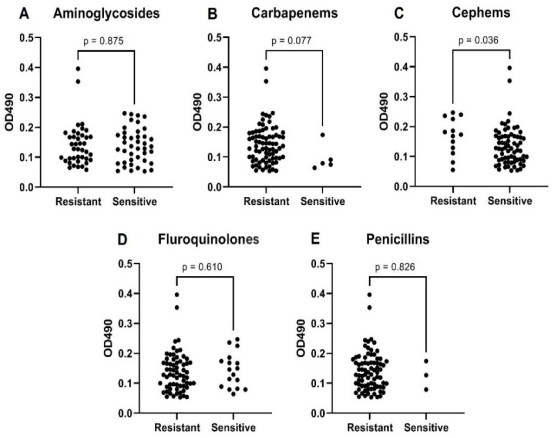


**Figure 1)** Crystal violet microtiter assay comparing biofilm biomass (OD at 490nm) between resistant vs susceptible isolates across antibiotic classes; points represent mean of triplicates. *P*-values are related to two-tailed Mann–Whitney U tests.

class (Fig. 1). Regarding cephems, resistant isolates showed higher OD values at 490 nm than susceptible ones [Median (IQR): 0.17 (0.12 – 0.23) vs 0.13 (0.09 – 0.17) for resistant and sensitive respectively, $p$= .036].

A trend toward higher OD values at 490 nm was observed for carbapenems; however, it was not statistically significant ($p$= .077). No significant differences were detected for aminoglycosides, fluoroquinolones, and penicillins ($p$= .875, .610, and .826, respectively).

**Genotypic assay of biofilm formation:** The genes involved in biofilm formation were studied by PCR. The *bap* gene was observed in 93.8% (n=76) of the isolates, and 93.8% (n=76) of the isolates were able to produce biofilm. Identification of the *ompA* gene using specific primers resulted in the observation of the expected band at 578 bp in 82.7% (n=67) of *A. baumannii* isolates, and the frequency of the *csuE* gene was 86.4% (n=70). These results are shown in Fig. 2.

## Discussion

*A. baumannii* remains a major nosocomial pathogen in ICU settings [24]. Because all isolates analyzed here were recovered from sputum specimens of ICU patients, the findings should be interpreted mainly in relation to

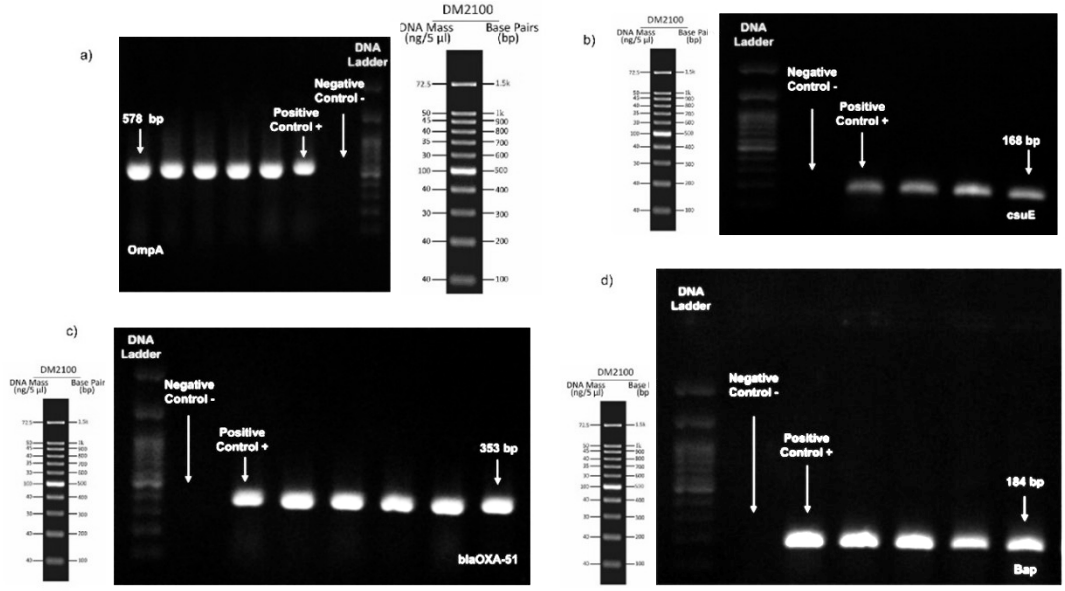


**Figure 2)** Agarose gel electrophoresis (%1) of PCR products. a, *ompA* (~578 bp); b, *csuE* (~168 bp); c, *blaOXA-51-like* (~353 bp); d, *bap* (~184 bp). The DNA ladder (M) and the positive (PC) and negative (NTC) controls are shown and labeled in the figure.

respiratory infections in critically ill patients. In this context, the simultaneous presence of marked antimicrobial resistance and frequent biofilm production is important, as it may favor persistence of *A. baumannii* in ICU environments and complicate infection-control efforts. This is clinically relevant because *A. baumannii* is implicated in ventilator-associated pneumonia [4], and biofilm formation contributes not only to its survival on medical devices and abiotic surfaces but also to reduced antimicrobial susceptibility [5, 7, 25].

Among the isolates examined here, the highest resistance was observed to piperacillin-tazobactam (96.3%), imipenem (93.8%), cefepime (91.4%), and ceftazidime (90.1%). This pattern is comparable to the findings of a study by Sadr et al. (2021) in general hospitals in Tehran, Iran, who reported 100% resistance to piperacillin-tazobactam and cefepime in *A. baumannii* isolates [26]. In another study in Bangladesh, Farzana et al. (2022) reported high resistance rates to ciprofloxacin, imipenem, gentamicin, and amikacin [27]. Overall, these findings show that resistance to commonly used antibiotics remains an important concern among *A. baumannii* clinical isolates.

Previous studies in Iran, including a systematic review by Beigverdi et al. (2019) and an ICU-based study by Zeighami et al. (2019), have also reported high levels of imipenem resistance among *A. baumannii* isolates, which is consistent with the present study findings [28, 29]. This finding highlights the need for regular monitoring of local antimicrobial resistance patterns and rational antibiotic prescribing based on susceptibility results. Differences in antimicrobial resistance patterns between studies may be related to differences in sample type, hospital setting, patient population, antimicrobial prescribing practices, infection-control measures, sample size, and laboratory methods. Bacteria with the ability to form biofilms tolerate harsh environmental conditions better than planktonic bacteria and are more resistant to various antibiotics and disinfectants, making these infections much more difficult to treat and control [30].

Li et al. (2021) in a study in southwestern China reported biofilm formation in 99% of strains after crystal violet staining, whereas Zeighami et al. (2019) in Iran found that all

*A. baumannii* isolates were biofilm-positive [29, 31]. These findings are close to the result of the present study, in which 76 (93.8%) isolates were able to form biofilm. Saadati et al. (2021) in Iran also reported biofilm formation in 84% of *A. baumannii* isolates [32]. Previous studies have shown that biofilm-forming isolates could show resistance to a wider range of antibiotics [25, 33]. Therefore, the high frequency of biofilm formation in the isolates should be considered when interpreting the antimicrobial resistance findings of this study.

The frequent detection of *bap* in the present collection agrees with previous studies conducted in Korea and Iran, including Sung et al. )2016( and Saadati et al. )2021( [13, 32]. Among the examined isolates, *bap* was detected in 76 (93.8%) isolates, which is close to previous reports. The high frequency of this gene is important because *bap* has been reported to be associated with bacterial attachment to surfaces and biofilm stability [13, 34, 35]. Therefore, the high prevalence of *bap* should be considered in conjunction with the high rate of biofilm formation observed in this study.

Previous studies in Iran, mainland China, and Egypt, including Zeighami et al. (2019), Saadati et al. (2021), Liu et al. (2016), and Sherif et al. (2021), have reported high frequencies of the *ompA* gene among *A. baumannii* clinical isolates, suggesting that this gene is commonly detected in isolates associated with biofilm formation and antimicrobial resistance [29, 32, 36, 37]. In this study, *ompA* was detected in 67 (82.7%) isolates, indicating that this gene is also common among sputum-derived ICU isolates. OmpA is one of the major outer membrane proteins of *A. baumannii* and has been reported to be associated with biofilm formation, pathogenicity, host immune response, membrane permeability, and antibiotic resistance [38]. The high frequency of *ompA* is in line with the high rate of biofilm formation observed among the isolates.

In earlier clinical reports, Yang et al. (2019) in Taiwan, Ghasemi et al. (2018) in Iran, and Shenkutie et al. (2020) in Hong Kong have reported frequent detection of *csuE* in *A. baumannii* isolates [2, 39, 40]. In this study, *csuE* was detected in 70 (86.4%) isolates, indicating that this gene is also common among sputum-derived ICU isolates. The *csuE* gene is one of the most frequently-studied biofilm-associated genes in *A. baumannii*, whose presence has also been discussed in relation to antimicrobial resistance [2, 36]. Therefore, the high frequency of *csuE* is consistent with the high rate of biofilm formation observed among the isolates.

The exclusive use of sputum samples should be considered when interpreting the findings. Because all isolates were obtained from respiratory specimens of ICU patients, the observed antimicrobial resistance and biofilm-related gene patterns may not be generalizable to *A. baumannii* isolates from other clinical sources, such as blood, urine, wounds, or catheter-related samples. Additionally, most isolates were resistant to several antibiotics; therefore, the number of susceptible isolates was small in some comparison groups. This imbalance may limit the robustness of comparisons between antibiotic susceptibility patterns and biofilm production. Therefore, statistically significant findings in these analyses should be interpreted with caution.

## Conclusion

Sputum-derived *A. baumannii* isolates from ICU patients frequently showed biofilm production ability, along with high detection rates of *bap*, *ompA*, and *csuE*. Marked resistance to several commonly used antibiotics was also observed. These findings support continued surveillance for biofilm-forming and resistant *A. baumannii* isolates in ICU settings, implementation of effective

infection-control practices, and antimicrobial stewardship.

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