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MOLECULAR CHARACTERIZATION OF *PGA* GENE TYPES A–D AMONG MULTI-DRUG RESISTANT STRAINS OF *ACINETOBACTER BAUMANNII*

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Abstract. This study aimed to explore the prevalence of *Acinetobacter baumannii* in clinical settings, its antimicrobial resistance, and biofilm formation ability in ventilator-associated pneumonia (VAP) patients, with a particular focus on the *pgaABCD* gene locus responsible for biofilm formation. A total of 53 isolates were collected over a 5-month period from patients suffering from pneumonia and lower respiratory tract infections. The isolates were identified, and their drug resistance profiles were evaluated using the VITEK automated system. Biofilm formation ability was assessed using the crystal violet assay. The presence of the *pgaABCD* gene was confirmed through PCR, and the sequences were analyzed to investigate gene prevalence and mutations. Among the 53 clinical samples, 29 isolates (54.7%) were confirmed as *A. baumannii*. Biofilm formation was detected in 62.1% of the isolates, with varying levels of biofilm production. All 29 isolates (100%) encoded both the *pgaA* and *pgaD* genes, while the *pgaB* and *pgaC* genes were present in 93.10% and 89.66% of the isolates, respectively. Multidrug-resistant (MDR) strains were prevalent among the clinical isolates, with high biofilm production ability. Sequencing of the *pgaABCD* genes revealed mutations contributing to the diversity of biofilm formation. This study emphasizes the strong relationship between the *pgaABCD* locus and biofilm formation in MDR *A. baumannii* strains. The high prevalence of biofilm-forming isolates underscores the challenges in treating infections caused by *A. baumannii*, especially in VAP patients. These findings highlight the need for biofilm-targeted treatment strategies to improve patient outcomes in healthcare settings.

Key words: *pneumonia, ventilator-associated pneumonia, A. baumannii, pgaABCD, virulence.*

МОЛЕКУЛЯРНАЯ ХАРАКТЕРИСТИКА ГЕНОВ *PGA* ТИПА A–D СРЕДИ ПОЛИРЕЗИСТЕНТНЫХ ШТАММОВ *ACINETOBACTER BAUMANNII*

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Резюме. Целью настоящего исследования было изучение в клинических условиях распространенности *Acinetobacter baumannii*, его устойчивости к противомикробным препаратам и способности к образованию биопленки у пациентов с ИВЛ-ассоциированной пневмонией (ИАП), основанное на исследовании локуса гена *pgaABCD*, ответственного за образование биопленки. Всего за 5 месяцев было собрано 53 изолята от пациентов, страдающих пневмонией и инфекциями нижних дыхательных путей. Изоляты были идентифици-

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рованы, а их профили лекарственной устойчивости были оценены с помощью автоматизированной системы VITEK. Способность к образованию биопленки была оценена с помощью теста с кристаллическим фиолетовым. Наличие гена *pgaABCD* было подтверждено с помощью ПЦР, а последовательности гена были проанализированы для оценки его распространенности и мутаций. Среди 53 клинических образцов в 29 изолятах (54,7%) было подтверждено наличие *A. baumannii*. Образование биопленки разной выраженности было обнаружено в 62,1% изолятах. Все 29 изолятов (100%) *A. baumannii* кодировали как гены *pgaA*, так и *pgaD*, тогда как гены *pgaB* и *pgaC* обнаружены в 93,10 и 89,66% изолятов соответственно. Среди клинических изолятов преобладали штаммы с множественной лекарственной устойчивостью (МЛУ) с высокой способностью к образованию биопленки. Секвенирование генов *pgaABCD* выявило мутации, способствующие различным типам образования биопленки. Настоящее исследование подчеркивает тесную связь между локусом *pgaABCD* и образованием биопленки в штаммах *A. baumannii* с МЛУ. Высокая распространенность изолятов, образующих биопленку, подчеркивает трудности в лечении инфекций, вызванных *A. baumannii*, особенно у пациентов с ИАП. Полученные результаты подчеркивают необходимость разработки стратегий терапии, воздействующей на биопленку, для улучшения результатов лечения пациентов в медицинских учреждениях.

Ключевые слова: *пневмония, ИВЛ-ассоциированная пневмония, A. baumannii, pgaABCD, вирулентность.*

Introduction

Acinetobacter baumannii is a gram-negative coccobacillus that has been spotlighted as a formidable opportunistic pathogen in healthcare settings worldwide [9]. The Centers for Disease Control and Prevention (CDC) documented *A. baumannii* as a critical pathogen due to its resistance to various classes of antibiotics [29]. The organism is tremendously robust, can survive in harsh environments, and readily acquired antibiotic resistance, which makes it a persistent threat in hospital settings [11, 26].

One of the most critical challenges posed by *A. baumannii* is its association with ventilator-associated pneumonia (VAP), a common nosocomial infection in intensive care units (ICUs). VAP, often caused by drug-resistant *A. baumannii*, is linked to higher mortality rates and increased healthcare costs, particularly when there are delays in its recognition and treatment [21]. This pathogen is notorious for its rapid development of resistance to most antimicrobials, making it a frequent cause of lower respiratory tract infections in critically ill patients [10].

Recently, the effectiveness of first-line antibiotics against clinical isolates of *A. baumannii* has drastically decreased. The pathogen developed robust defense mechanisms against various antimicrobial agents, including cephems, aminoglycosides, fluoroquinolones, and carbapenems [15]. Presently, polymyxins, tigecycline, and ampicillin/sulbactam are often considered last-resort treatments for infections caused by *A. baumannii* [30]. The emergence of pandrug-resistant (PDR), extensively drug-resistant (XDR), and multidrug-resistant (MDR) strains underscores the growing concern in antimicrobial resistance stewardship [20].

A key factor contributing to the threat of *A. baumannii* in healthcare environments is its ability to form biofilms on various surfaces [28]. Biofilms significantly enhance antibiotic resistance through mechanisms such as impaired drug diffusion due to microbial aggregation and shields of exopolymeric substance (EPS) matrix [22]. Moreover, stress responses modify bacterial phenotypes and genotypes and physiological heterogeneity within the biofilm [32]. *A. baumannii* harbors the *pgaABCD* locus, which encodes proteins involved in synthesizing cell-associated poly-β-(1-6)-N-acetylglucosamine (PNAG), a critical virulence factor that protects the bacteria against innate host defenses [7, 19]. The *pgaB* gene, in particular, plays a crucial role in the exportation of PNAG, while *pgaC* and *pgaD* are essential for its biosynthesis [5, 16].

Recent studies have generated significant interest in understanding the relationship between virulence factors like PNAG and antibiotic resistance. Evidence suggests a strong correlation between the presence of such factors and increased drug resistance [18]. However, uncertainties remain regarding the risk factors and prognosis associated with *A. baumannii* infections. This study aimed to compare cases of VAP caused by *A. baumannii* and explore the relationship between drug resistance and biofilm formation, focusing on mutations in the *pgaABCD* locus among the isolates.

Materials and methods

Bacterial strains and phenotypic tests. This prospective observational study was conducted for a period of five months from January 2024 to May 2024, at the Department of Microbiology, Saveetha Dental College and Hospitals. A total of 53 (N) ICU patients with pneumonia and lower respiratory tract infections were included for this study for the characterisation of clinical isolates of *A. baumannii*. Clinical samples such as sputum, bronchoalveolar lavage (BAL) fluid, and endotracheal aspirates (ETA), collected under strict aseptic conditions were immediately sent to the microbiology laboratory. The samples were cultured on 5% blood agar and MacConkey agar, then incubated at 37°C for 16–18 hours. Identification of *A. baumannii* was performed using the VITEK automated system, which also determined the anti-

microbial resistance profiles. The identified isolates were preserved in glycerol stock at −80°C for further experimentation.

Identification of biofilm formers by crystal violet assay. Biofilm formation was evaluated using a 96-well microtiter plate (Himedia, Mumbai, India) as mentioned in an earlier report done by Kannan and Girija [17]. Each isolated colony was inoculated into 5 mL of BHI broth and incubated overnight at 37°C. The overnight cultures were then diluted 1:100 in fresh BHI broth, with 200 µL dispensed into each well of a microtiter plate. The plates were incubated for 24 or 48 hours at 37°C without shaking. After incubation, each well was washed three times with 200 µL of sterile phosphate-buffered saline (PBS; pH 7.4) to remove the planktonic cells. The biofilm was stained by adding 200 µL of 0.1% sterile crystal violet (CV, Merck) solution per well for 15 minutes. Plates were rinsed twice with distilled water and once with PBS, then dried for 30 minutes in an inverted position. After drying, 200 µL of 96% ethanol was added to dissolve the dye. Wells containing sterile medium served as blank controls. The contents of each well were transferred to a sterile polystyrene microtiter plate, and the optical density (OD) at 570 nm was measured using a microtiter plate reader (Robonic Elisa reader). All experiments were conducted in triplicate.

Isolation of bacterial genomic DNA. The MDR strains were identified based on the previous report done by Girija and Priyadharshini [12]. The genomic DNA of *A. baumannii* was obtained by cultivating the isolates in BHI broth at 37°C for 12 hours, followed by extraction using the Qiagen DNA extraction kit as per the manufacturer's instructions.

Prevalence of pgaABCD gene among MDR A. baumannii. The PCR reaction mixture included a 2X master mix (Takara), 3 µl of template DNA, 2 µl each of forward and reverse primers, and nuclease-free water to reach a total volume of 25 µl. The resulting PCR products were analyzed using agarose gel electrophoresis, compared against a 100 bp DNA ladder (Thermo Fisher Scientific, USA), and visualized under a UV trans-illuminator.

Confirmation of the pga gene amplicon by sequencing. The PCR products were sequenced using the Big-Dye terminator v3.1 Cycle sequencing kit (Applied Biosystem, USA), and the amplicons were analyzed with the 3730XL genetic analyzer. The obtained sequences were processed with Bio-Edit Sequence Alignment Editor v7.2.5. Nucleotide similarities and mutations were assessed using BLAST (Basic Local Alignment Search Tool). Multiple sequence alignments were performed using ClustalW software version 1.83.

Results

Isolation and identification of A. baumannii. Among the study population (N = 53), 29 isolates were identified as *A. baumannii*, with a prevalence rate of 54.7% (Fig. 1, cover II). All clinical isolates demonstrated MDR, exhibiting resistance to more than three classes of antibiotics as determined by VITEK analysis. High resistance rates were observed against cefepime (83.67%), meropenem (81.13%), imipenem (79.23%), and gentamicin (77.16%). Resistance to cefuroxime and cefoperazone was equally high at 75.21%, followed by piperacillin-tazobactam (73.11%), ampicillin (65.95%), and cefotaxime (59.13%). In contrast, all isolates were fully susceptible to colistin and tigecycline (0% resistance), underscoring their potential role in treating multidrug-resistant *A. baumannii* infections.

Determining the biofilm-forming ability of A. baumannii isolates. Among all *A. baumannii* isolates examined for biofilm formation, 18 (62.1%) were biofilm producers, while 11 (37.9%) were non-biofilm producers. The biofilm-producing strains were categorized into three groups: 3 (16.67%) were weak biofilm producers, 4 (22.22%) were moderate biofilm producers, and 11 (61.11%) were strong biofilm producers.

Frequency of pgaABCD gene among MDR A. baumannii. PCR analysis was performed to assess the presence of biofilm-associated genes *(pgaA*, *pgaB*, *pgaC*, and *pgaD)* across the 29 clinical isolates. All isolates (100%) were found to harbor both the *pgaA* and *pgaD* genes, while the *pgaB* and *pgaC* genes were present in 93.1% (n = 27) and 89.7% (n = 26) of the isolates, respectively. These results highlight a high prevalence of genes associated with biofilm formation in the clinical strains, underscoring their potential for biofilm production, which is a key factor for their virulence and persistence in clinical settings.

Table. Primer sequences and PCR conditions for *pgaABCD* gene types used in the study

Gene	Sequence, 5'→3'	Annealing Temperature	Amplicon size
***pgaA* F** ***pgaA* R**	ATTCAAAAGTCAGTTGATGGGC TTTTTTGTCCTTGCTCCAGC	56°C	460 bp
***pgaB* F** ***pgaB* R**	CCCCTGCTCATCATAATGTAAG GGTTTTGTTTAATGTGGCTGC	58°C	326 bp
***pgaC* F** ***pgaC* R**	CAGTGGTATGGCGTGATATT GGTACTGCAACAACACTGGT	57°C	178 bp
***pgaD* F** ***pgaD* R**	TTGATCAGCCTGAATATGTGA CACACATAGTCATAAATGAGG	54°C	145 bp

Sequencing and MSA. The nucleotide sequences of the *pgaA*, *pgaB*, *pgaC*, and *pgaD* genes from clinical isolates were analyzed through Sanger sequencing. Multiple sequence alignments revealed notable variations among the genes involved in biofilm formation. The alignment displayed a high degree of sequence conservation across the four genes, with distinct single nucleotide polymorphisms (SNPs) and indels observed in *pgaB* and *pgaD* compared to *pgaA* and *pgaC*. Specifically, *pgaB* showed multiple substitutions and insertions were not observed in *pgaC*. Likewise, *pgaD* exhibited deletions and unique sequence regions, particularly in the N-terminal and C-terminal ends, suggesting divergence from the other genes. In several regions, *pgaB* had significantly longer stretches of sequence while *pgaD* was comparatively shorter (up to 103 bases), indicating gene size variability. Conserved motifs such as "TAAACAAAAC" were shared among multiple genes, hinting at potential regulatory and structural roles. These polymorphisms may play a role in biofilm-related functions and potentially influence antibiotic resistance or surface adhesion capabilities in the clinical isolates (Fig. 2).

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PgaC    ------------------------------------------------------------    0
PgaA    ----------------------------------TCCTTCACGATTTAAATATTTATA    24
PgaB    AACGGACCTCATTTATCACTATGTGTGGGTAGATCATGTTTCAGAAGTGAAAACTTTATT    60
PgaD    --ACAGCCACA-------------------------TTAAACAAAAC-----------    20

PgaC    ------------------------------------------------------------    0
PgaA    TCGAGCAATTGCATTGTTG-----------------ATATGTT----------GAGAAAA    57
PgaB    GCATTTACTTCTGTTAATAGGTCTCACCGCACTCATTTTAATTTTATGGGCAAGTTATAA    120
PgaD    ----------------------------CAACCTATTGTTTGTAAGGTATAGTTTGCA    50

PgaC    ------------------------------------------------------------    0
PgaA    CTCACTAACTTGTAAAGTC---------TGCAACATTTGCACTGTTTGTTCAGTTTTTTC    108
PgaB    CTCGCTTAGATTTCATGGAGATCATCGTCGCAGCAAGCCCCCGAATAGCTCTGTTGAGTT    180
PgaD    --GTTTTATTTTTCACATA-----------------------------------------    67

PgaC    ------------------------------------------------------------    0
PgaA    GCTCGCTTGC---------AGCAATTGCTGTGCCTTTTTATATGAGCCAATA--------    151
PgaB    GCTGGCCTCACAATTTATGGTCAGTACCGAATCATTATCAGAATTACAAAAGTCCCAGCG    240
PgaD    ------------------------------------------------------------    67

PgaC    ------------------------------------------------------------    0
PgaA    --------------GCAATTACATCCTAAACATATTCTTGTAAAACGGATGAAGATTTTG    197
PgaB    CATCATCTTACATTATGATGGAGCAGGGGACCGCTTCTTGTAATTCTGAAAATGATTCGG    300
PgaD    ------------------------------------------------------------    67

PgaC    ------------------------------------------------------ACCAA    5
PgaA    TTTGTTGTTGATAAGCATGCTCAACTGTAGCTAAAGCATCTACTGGTAAATTTATTAAAC    257
PgaB    TACTGACATAAATTGTCAGGCCGCAACTC--AACAGACCTTTTCGGGGCCTTGCTGCGAC    358
PgaD    --------------TTC---AGGCTGATC--AACAACACTCTCCGTTCCCTTA-------    101

PgaC    AAATAATTTCTAAAAACCCGACCACGATCATAACGATGGTCTATCCATAAACTTACTA--    63
PgaA    GATAAGCATATGCAACCCG--------TACCA------------------ACTGATCAGC    291
PgaB    CATTATCTCCTGGAAATCTAAGCCCGTTTTTAAT--TGCCCTTAAAATAAAATTTTTGGG    416
PgaD    ------------------------------------------------------------    101

PgaC    ----------------CA--AATTGGACAAGACATGTTCCACCTAATATCAC--GCCATA    103
PgaA    AGTTAAACCTTTAATATCGATCTTCGACAACTGATCTTTAGCATT---------CGCCAC    342
PgaB    CTTAAAACTTTTAAACGAAATTAATCGCAAAAAATTTTTCTTTTTAAAAAAGAACCCCCC    476
PgaD    ------------------------------------------------------------    101

PgaC    CC--------------------------------------------------------    105
PgaA    AT-----------CTT-----GAGCTTCAGCATACAAAACCGATAATAAAATACGCCCAT    386
PgaB    CCCCTGTCCTTTAATTAAGGGGCCCTTCAAAAATCTATAAAATTAAGGAAACGGGGGGAA    536
PgaD    -------------TT--------------------------------------------    103

PgaC    --ACTGAACC--------------                                       113
PgaA    CAACTGACCTTTTGAATAAAAA--                                       408
PgaB    AAACCCCCCTTAAAAAAACAAAAA                                       560
PgaD    ----------------------                                         103
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Figure 2. Multiple sequence alignment of partial gene sequences *(pgaA, pgaB, pgaC,* and *pgaD)* from *A. baumannii* clinical isolates, highlighting nucleotide variations including substitutions and deletions

Discussion

A. baumannii species has become increasingly common in ICUs over the past two decades, causing serious infections [13]. Ventilator-associated pneumonia (VAP) is a prevalent nosocomial infection that poses a significant challenge in hospitalized patients, particularly those in intensive care units (ICUs) [25]. *A. baumannii* is one of the most common pathogens responsible for VAP and contributes significantly to both morbidity and mortality, especially in immunocompromised patients [8]. The increasing prevalence of *A. baumannii* in VAP cases can be attributed to its resistance to multiple classes of antibiotics [24]. These associations underscore the need for alternative therapies and the rapid identification of *A. baumannii* in healthcare settings to improve patient outcomes.

In this study, the sample collection period was 3 months, which is longer than the 47-day collection period reported by Chang et al. [6] for endotracheal tube aspiration samples. This extended duration may reflect differences in study design or patient populations. Identification and antimicrobial susceptibility testing of *A. baumannii* were conducted using the VITEK 2 automated system, which efficiently identified a significant number of non-fermenting gram-negative rods within 3 hours. Rapid identification is clinically critical, as it is associated with reduced mortality, earlier initiation of appropriate antimicrobial therapy, shorter hospital stays, and lower healthcare costs [3]. The quick turnaround time provided by the VITEK 2 system highlights its value in managing the infections, particularly in critically ill patients where timely treatment is crucial.

In our study, the prevalence of *A. baumannii*-associated VAP was 54.7% (29 out of 53 samples), demonstrating the high incidence of this infection in the ICU setting. *A. baumannii* has emerged as a leading pathogen responsible for VAP, contributing to the high morbidity and mortality rates among critically ill patients [23]. The bacterium's multidrug-resistant (MDR) nature complicates treatment and limits therapeutic options for VAP patients [4]. The high mortality rates associated with *A. baumannii*-related VAP highlight the urgent need for effective treatment strategies and robust infection control measures in ICUs.

In this prospective study, all 29 *A. baumannii* isolates were identified as MDR, highlighting its critical role as a major pathogen in VAP within ICUs. The high levels of antimicrobial resistance observed in *A. baumannii* complicate clinical management. Our findings revealed significant resistance rates to 11 commonly used antibiotics, with resistance to imipenem and meropenem at 79.23% and 81.13%, respectively. Gentamicin, ampicillin, and cefoperazone/sulbactam showed resistance rates of 77.16%, 65.95%, and 75.21%, respectively. Cefepime and piperacillin/tazobactam exhibited resistance rates of 83.67% and 73.11%, respectively. Although cefuroxime had the lowest resistance rate (59.13%), it was still significant. Notably, *A. baumannii* exhibited complete sensitivity to colistin and tigecycline, underscoring the importance of these antibiotics in treating MDR infections. These resistance patterns are consistent with previous reports, emphasizing the need for novel therapeutic strategies to address MDR *A. baumannii* infections [14].

A. baumannii infections are of particular concern due to the high rates of MDR observed in clinical settings, exacerbated by the bacterium's ability to form biofilms, which further complicates treatment and eradication efforts [31]. In our study, all isolates (100%) demonstrated biofilm production, a rate considerably higher than the 48.8% reported in other clinical isolates [27]. This finding aligns with previous research, which suggests a strong correlation between biofilm formation and MDR strains [2]. The biofilm-forming ability of *A. baumannii* likely contributes to its persistence in hospital environments and resistance to antimicrobial therapies, underscoring the need for targeted strategies to combat biofilm-associated infections.

Our study explored the potential link between the phenotypic and genotypic resistance profiles of *A. baumannii* isolates and their capacity to form biofilms. We observed a strong association between MDR and biofilm formation, consistent with findings from other researchers who noted a similar connection [1]. Further investigation into the mechanisms underlying this association is essential for developing effective strategies to combat *A. baumannii* infections.

Additionally, our study found a strong correlation between the presence of the *pgaABCD* operon in *A. baumannii* and its role in biofilm formation, reflecting a significant homology within the pga locus. The *pgaA* protein is crucial for transporting poly-N-acetylglucosamine (PNAG) outside the cell, contributing to the biofilm matrix. *pgaB* promotes cell-to-cell adhesion, stabilizing the biofilm, while *pgaC* catalyzes PNAG synthesis, and *pgaD* supports efficient biofilm formation [7].

In this study, all 29 isolates (100%) encoded the *pgaA* and *pgaD* genes, while the *pgaB* and *pgaC* genes were present in 93.10% and 89.66% of the isolates, respectively. These findings align with previous research reporting a 100% prevalence of the *pgaB* gene in clinical isolates [18]. The presence of the *pgaABCD* operon, primarily linked to biofilm formation, is also associated with increased antimicrobial resistance. Biofilms act as physical barriers, reducing antibiotic efficacy and leading to persistent infections. The frequent detection of the *pgaABCD* operon in MDR strains suggests that biofilm formation contributes to the resistance profiles ob-

served in these isolates. This connection emphasizes the need for novel therapeutic approaches targeting biofilm formation alongside conventional antibiotic treatments.

Limitations of the study include small sample size and less time period of study might not have provided a significant result on the diversity of *pga* types A–D and MDR profiles. There may be a minor variation in pga gene clusters among the clinical strains, complicating the understanding of their role and association with the pathogenesis. Periodical monitoring and identification of more pga based genetic determinants beyond A–D, which may be further studied upon gene sequencing.

Conclusion

This study examined the prevalence, antimicrobial resistance, and biofilm formation of *A. baumannii* isolates from ventilator-associated pneumonia (VAP) patients, revealing the significant burden of *A. baumannii* infections in healthcare settings. The strong correlation between the presence of the *pgaABCD* gene types, playing a vital role in biofilm formation, underscores the need for biofilm-targeting strategies. Overall, this research provides crucial insights into the clinical impact of the virulent and resistant traits of *A. baumannii* in VAP and warranting the immediate need in its management in the health care settings.

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Illustrations for the article "Molecular characterization of *pga* gene types A–D among multi-drug resistant strains of *Acinetobacter baumannii*" (authors: Supreeta M.S., Kannika Parameshwari K., Smiline Girija A.S., Vijayashree Priyadharsini J.) (pp. 536–542)

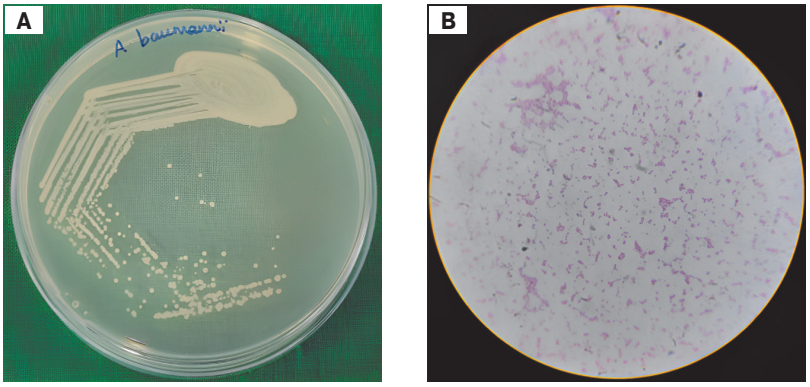

Figure 1. Isolation and identification of *A. baumannii* from the respiratory samples of the patients with VAP
Note. A. Typical *A. baumannii* colonies on the nutrient agar plate. B. Gram staining showing the typical gram negative coccobacillary forms