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Detection of *OmpA* and bla_{PER-1} Genes and Biofilm Formation in *Acinetobacter baumannii* Clinical Isolates

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ABSTRACT

Background: Multi-drug-resistant *Acinetobacter baumannii* (MDR A. *baumannii*) infections are an ongoing problem in many hospitals, particularly in intensive care units. One of the most difficult infections to treat is *A. baumannii* because it forms a biofilm.
Objectives: To identify MDR *A. baumannii biofilm* producers from clinical isolates and detect some biofilm-related genes.
Materials and methods: During the period from October 2023 to February 2024, a total of 26 *A. baumannii* clinical isolates were collected from the laboratory of Ghazi Al-Hariri Hospital, Baghdad, Iraq. The automated VITEK®^{2} system was used to identify *A. baumannii* isolates phenotypically. The bla_{OXA51} gene confirmed *A. baumannii* isolates genotypically. The microtiter plate test and scanning electron microscopy were performed to assess biofilm formation. The presence of the biofilm-forming genes *OmpA* and bla_{PER-1} was evaluated.
Results: Overall, most of the specimens were from sputum (38.5%), and all the isolates were MDR with a high resistance percentage to imipenem at 100%, meropenem, ceftriaxone, and ciprofloxacin. The isolates showed 80.77% resistance to trimethoprim/sulfamethoxazole and 100% sensitivity to colistin. The results showed that among the 26 isolates, 31% were moderate biofilm formers, 54% were weak biofilm formers, and only 4 isolates were non-biofilm formers (15%). The occurrence rates of the biofilm-related genes *OmpA* and bla_{PER-1} were 16 (61.5%) and 1 (3.8%), respectively.
Conclusion: The current study suggests the presence of MDR *A. baumannii* isolates harbouring the biofilm *OmpA* and bla_{PER-1} genes.
Keywords: *A. baumannii*; Biofilm; *OmpA* gene; bla_{PER-1} gene.

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INTRODUCTION

Today, a rapidly growing and silent global crisis is antibiotic resistance. More bacteria are developing resistance to available treatments, leaving us with no treatment options [1]. *A. baumannii* is considered an opportunistic pathogen with a high rate of multidrug resistance (MDR) strains. Furthermore, treating *A. baumannii* can be costly and challenging. *A. baumannii* can cause various infections, including bacteraemia, pneumonia, urinary tract infections (UTI), burns, wound infections, and meningitis [2]. Recently, studies have reported high mortality rates in patients with coronavirus disease 2019 (COVID-19) due to the emergence of *A. baumannii* infections [3, 4].

A. baumannii's ability to form biofilms enables successful long-term persistence in healthcare facilities and improves its ability to escape the effects of antibiotics by evolving different mechanisms [5]. Many factors contribute to biofilm formation, which is a complex process involving substance aggregation, collagen adhesion, pili expression, and iron acquisition[6].

One of the genes involved in biofilm formation is the *OmpA* gene (outer membrane protein), which partially participates in the development of strong biofilms on plastic surfaces, stimulates the innate immune response and induces biofilm formation, which helps *A. baumannii* survive harsh conditions [7]. Studies done in different parts of Iraq have shown that biofilm-forming MDR *A. baumannii* isolates are common among patients [8].

On the other hand, among the several factors involved in biofilm formation is the Bap (biofilm-associated protein), which is encoded by the *bap* gene and responsible for intercel-

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lular adhesion, bacteria cell accumulation, and finally biofilm establishment [9]. One gene that contributes to biofilm formation is the beta-lactamase PER-1 (bla_{PER-1}) gene, which is a biofilm-associated protein that plays an essential role in *A. baumannii* adhesion and biofilm formation [10]. The bla_{PER-1} gene confers resistance to various antibiotics, including penicillin, cefotaxime, ceftibuten, ceftazidime, and monobactam. However, the bla_{PER-1} gene decreases resistance to carbapenems and cephamycin [11]. However, the quantitative correlation between biofilm and antibiotic resistance is unclear currently. Therefore, this study aimed to understand the association between biofilm, drug-resistant, and biofilm-related genes that can provide a valuable indication for the management and dissemination of biofilm MDR isolates, as they are difficult to treat.

MATERIALS AND METHODS

Bacterial samples collection

A total of 52 *A. baumannii* clinical isolates were collected from the Ghazi Al-Hariri Hospital for Surgical Specialties Microbiology Laboratory, Baghdad, Iraq, from October 2023 to February 2024 from different sources, including Foley catheters, endotracheal tubes, sputum, blood, bed sores, urine, and wound swabs. However, this study only included 26 samples from inpatients with MDR, excluding samples from outpatients and those not classified as MDR. Informed consent was obtained from each participant enrolled in this study. The ethical approval was obtained from Mustansiriyah University, College of Science; ethical approval board under the number (BCSMU/1221/00040M).

A. bumannii identification

The suspected clinical isolates were cultured on MacConkey agar (Liofilchem, Italy) and HiChrom Acinetobacter Agar Base medium (HiMedi, India). After incubation at 37°C for 24 hours, the isolates were selected based on morphological characteristics and identified to the species level and antibiotic susceptibility patterns using the VITEK®^{2} compact system.

Identification of *A. baumannii* using the bla_{OXA51} gene

The bla_{OXA51} gene was used to identify *A. baumannii* at the genetic level using polymerase chain reaction (PCR). The genomic deoxyribonucleic acid (gDNA) as extracted from all bacterial samples as follows: a few colonies were taken from overnight culture on MacConkey agar, suspended in ddH2O (400μl) in Eppendorf tubes, and boiled for 10 minutes in a water bath (100°C) for cell lysis. After boiling, the samples were cooled on ice and then stored at −20°C for 20 minutes before being left at room temperature to thaw. Later, samples were homogenized with a vortex (10 seconds) and centrifuged (15000 rpm) at 4°C for 15 minutes. The supernatant from each sample was collected in a sterile Eppendorf tube and stored at −20°C [12]. The amplification reaction contained the following components recommended by the manufacturer: 5μl of gDNA, 12.5μl of master mix (Promega, USA), 1μl (10 μM) of the blaOXA51 forward primer 5'-TAATGCTTTGATCGGCCTTG-3' and 5'-TGGATTGCACTTCATCTTGG-3' [13] for the reverse primer, and ddH2O (3.5μl) to achieve a volume of 25μl with cycling conditions: initial denaturation at 94°C (5 minute); 30 cycles of denaturation at 95°C (30 second), annealing at 55°C (30 second), and extension at 72°C (30 second); a final extension at 72°C (10 minute) and a final hold at 4°C.

Biofilm assay in-vitro

All *A. baumannii* isolates were evaluated for biofilm formation ability using the microtiter plate method as described by a previous study [14]. Each isolate was cultured in 5 ml of TSB broth and incubated at 37°C for 24 hours. A 200 μl volume of broth turbidity equivalent to 0.5 McFarland was inoculated to each well in triplicate and incubated at 37°C for 48 hours. Three wells were inoculated with TSB only and served as negative controls. After incubation, the contents of each well were removed gently and washed with phosphate-buffered saline (PBS) (200 μl) three times to remove unadhered cells. The adhered cells were heat-fixed at 60°C for one hour, and then the plate was stained with 0.1% of crystal violet (-Aldrich, Gillingham, UK) by adding 150 μl to each and the stain was left for 15 minutes at room temperature. The unbounded stain was removed by washing with tap water, and ethanol (100 μl) was added. Biofilm biomass was determined by measurement of optical density (OD) using a spectrophotometer at a wavelength of 630 nm.

Scanning electron microscopy

A scanning electron microscope was used to detect *A. baumannii* strains' ability to form biofilm. Briefly, *A. baumannii* was grown in brain heart infusion (BHI) broth at 37°C for 24 hours. After incubation, *A. baumannii* was added in triplicate to a six-well plate containing a coverslip, volume was completed to 2 ml with BHI broth and incubated overnight. Three wells were used as a control by adding only BHI broth without bacteria. After the biofilm developed on a coverslip, media was removed and each well was washed with phosphate-buffered saline (PBS) (pH 7.5) twice to remove unattached cells. For biofilm mass assessment, coverslips were allowed to dry after washing and stained with crystal violet (0.1%) for 10 minutes, washed twice with PBS and measured via a microtiter plate reader at an OD of 630 nm. For SEM reading, coverslips were fixed with 2.5% glutaraldehyde for four hours and later washed twice with distilled water for 15 minutes. A series of ethanol alcohol dehydration was applied as follows: 50% (10 minutes), 75% (15 minutes), 80%, 90%, and 100%, 20 minutes for each. The coverslips were allowed to dry for 24 hours and the biofilm was coated with gold and examined using SEM (Axia, Holland) [15].

Detection of biofilm-related genes

Biofilm-related genes *OmpA* and bla_{PER1} were detected using PCR. The boiling method was performed to extract bacterial DNA. The specific primers are illustrated in Table 1. The PCR conditions for the *OmpA* and bla_{PER1} genes were initial denaturation at 95°C for 5 minutes (1 cycle); 30 cycles of denaturation at 95°C for 30 seconds, annealing temperature for both genes were 55°C for 30 seconds, and elongation at 72°C for 35 seconds; then a final extension at 72°C for 10 minute and a final hold at 4°C. The amplified DNA was subjected to 1% gel electrophoresis for DNA fragments visualization with Syber safe stain (Thermo Fisher, USA) and photographed.

Nucleotide sequence accession numbers

The sequence of the *OmpA* and bla_{PER1} genes was sent for sequencing to Macrogen DNA Sequencing Company (Seoul,

Table 1. Specific primer sequences used for amplification of *OmpA* and bla_{PER1} genes.

Gene	Primer sequence	Product size (bp)	Annealing temperature	Reference
Bla_{per-1}	F5'TGAATGTCATTATAAAAGCTGTAGTTACTGCC-3' R 5' TTAATTTGGGCTTAGGGCAGAAAGC-3'	927	55°C	This study (via SanpGene software)
OmpA	F5'ATGAAATTGAGTCGTATTGCACTTGCTAC-3'R5'- TTATTGAGCTGCTGCAGGAGC-3'	1071	55°C	This study (via SanpGene software)

Korea). The complete *OmpA* and bla_{PER1} sequences were deposited in the GenBank database under the accession numbers: PP921094 and PP894979, respectively.

The obtained data were presented as tables or figures and subjected to analysis using a one-way ANOVA test to compare the means of various groups with each other. The chi-square test was used to compare the categorical variables. A P-value of < 0.05 was considered a statistically significant difference. The statistical analysis was carried out using a statistical package for the social sciences (SPSS) version 20.

RESULTS

Isolation and identification

The distribution of the sample sources showed that most of the clinical isolates were from sputum (38.5%) followed by Foley catheter (23.1%). In contrast, only 3.8% were from urine and wound, as shown in Table 2.

Genotypic detection

All clinical isolates were tested via the automated VITEK®^{2} system and amplification of the bla_{OXA-51} gene for identification to species level. A bright band at 353 bp corresponding to the bla_{OXA-51} gene confirmed the identification of the *A. baumannii* clinical isolates (Figure 1).

Antimicrobial susceptibility pattern

All isolates showed resistance to most tested antibiotics. To detect the antimicrobial pattern of *A. baumannii* isolates, 18 antibiotics from different groups were tested. The results were as follows: 13 antibiotics, including imipenem, meropenem, cefpodoxime, ceftriaxone, tetracycline, cefepime, piperacillin, ofloxacin, ciprofloxacin, gentamicin, cefixime, ceftazidime, and piperacillin-tazobactam showed the highest resistance percentage (100%). While only 80.77% were resistant against trimethoprim/sulfamethoxazole. The only antibiotic that showed 100% susceptibility was colistin, as depicted in Figure 2.

Table 2. The distribution of A. baumannii samples according to source.*

Source of isolates	Number	%
Sputum	10	38.5
Foley catheter	6	23.1
Bed Sore	3	11.5
Endotracheal tube	3	11.5
Blood	2	7.7
Wound	1	3.8
Urine	1	3.8
Total	26	100

* Highly significant (P-value = 0.011), Chi-square 21.984

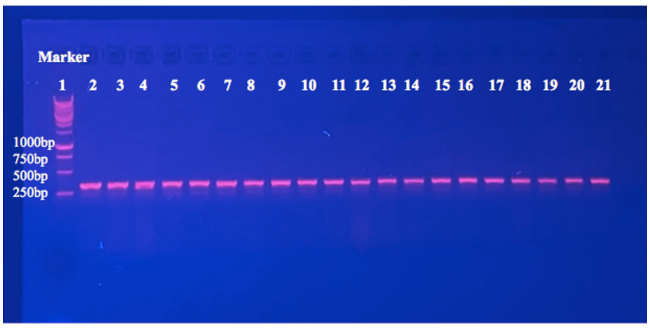

Figure 1. Electrophoresis of bla_{OXA-51} gene. The amplified bla_{OXA-51} gene from *A. baumannii* isolates on 1% agarose was run at 100 V/cm for 30 minutes with a final concentration of 0.125 μg/ml of SYBR Safe stain. Lane 1: 100 bp ladder marker, Lane 2-21: strains carrying the bla_{OXA-51} gene with the correct size of 353 bp.

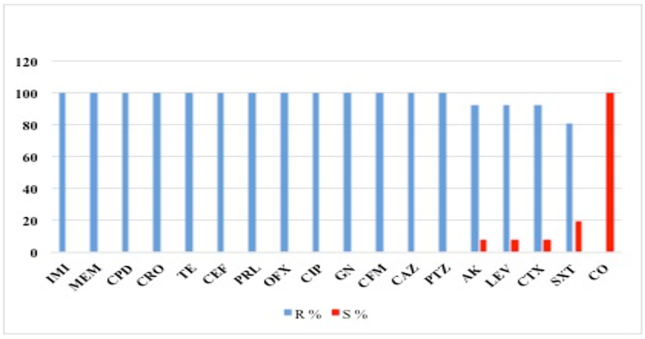

Figure 2. Percentage of antimicrobial susceptibility of *A. baumannii* isolates. IMI: imipenem, MEM: meropenem, CPD: cefpodoxime, CRO: ceftriaxone, TE: tetracycline, CEF: cefepime, PRL: piperacillin, OFX: ofloxacin, CIP: ciprofloxacin, GN: gentamicin, CFM: cefixime, CAZ: ceftazidime, PTZ: piperacillin-tazobactam, AK: amikacin, LEV: levofloxacin, CTX: cefotaxime, SXT: trimethoprim/sulfamethoxazole, CO: colistin, R: Resistance, S: susceptible.

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Table 3. Distribution of biofilm ability among A. baumannii tested isolates.*

Biofilm formation	Number	%
Moderate	8	31
Week	14	54
None	4	15
Total	26	100

* P-value = 0.049, Chi-square 10.5

Biofilm assay

Results indicated that among the 26 isolates, 54% (14) were weak, while 15% (4) were non-biofilm producers with a statistically significant (P-value = 0.049) difference (Table 3).

As shown in Figure 3A, tested *A. buamannii* isolates produced a biofilm on a six-well plate (upper raw), while the lower raw is the control with only media with no bacteria cultured. On the coverslip, the left side indicates biofilm mass-produced by tested *A. baumannii*, and on the right side is the control (Figure 3B). The SEM results uncovered the ability of *A. baumannii* to form a biofilm, as depicted in Figure 3C-D.

Distribution of biofilm-related genes

The PCR results showed the occurrence of the *OmpA* gene in 18 tested isolates (61.5%), and the bla_{PER-1} gene was identified in one isolate (3.8%) (Figure 4).

Out of the 22 positive biofilm isolates, the *OmpA* gene was present in 17 isolates and one negative biofilm isolate. The frequency of the bla_{PER-1} gene in biofilm and non-biofilm producing isolates was one in the positive biofilm producing isolate, as shown in Table 4.

The PCR products were sent for sequencing, and the sequences of the *OmpA* and bla_{PER1} genes were submitted to NCBI to obtain Accession Numbers as follows: - *A. baumannii* RKJ08 for *OmpA* under the accession number PP921094 and *A. baumannii* RKJ23 for bla_{PER1} with the accession number PP894979. The alignments of *A. baumannii* RKJ08 *OmpA* gene sequence showed 99% by 947 over 948 identities with *A. baumannii* strain W (GenBank: HG917405.1) with no Gaps 0%. After translation, alignment of *OmpA* protein presented 100% by 316/316 amino acids identity with outer membrane protein A (GenBank: ABO30516.1). In addition, the A. baumannii RKJ23 blaPER-1 gene sequence showed 99% identity to the A. baumannii IOMTU 442 bla_{PER1} gene (sequence ID: NG_049967.1) with 843 out of 844 matches, and no gaps. The *A. baumannii* RKJ23 bla_{PER1} gene encodes the PER protein, which belongs to the PER family of extended-spectrum class A beta-lactamases. It shares 99% similarity, with 280 out of 281 matches and no gaps, to the PER family of *A. baumannii* MDRA IOMTU449 (sequence ID: HAV6239208.1).

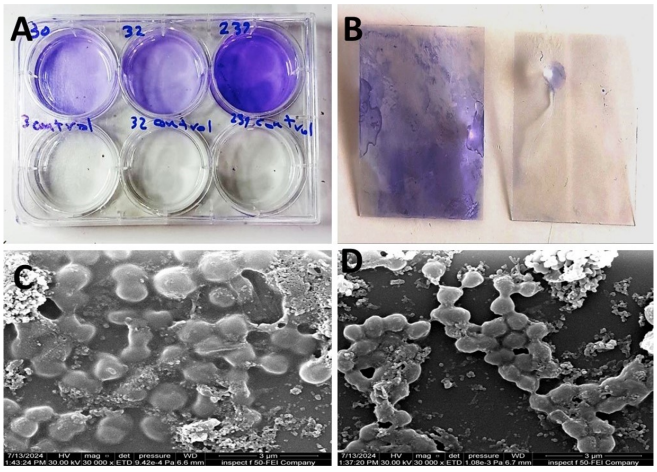

Figure 3. Biofilm formation. (A) Upper row: biofilm production by clinical *A. buamannii* isolates; lower row: control (only media with no bacteria); (B): Biofilm production on coverslip surface (left); control on (right), scanning electron micrograph of A. baumannii biofilm formation. (C): The SEM image shows moderate biofilm formation; (D): The SEM image shows weak biofilm formation. Magnification 30000x; Bars = 3μ.

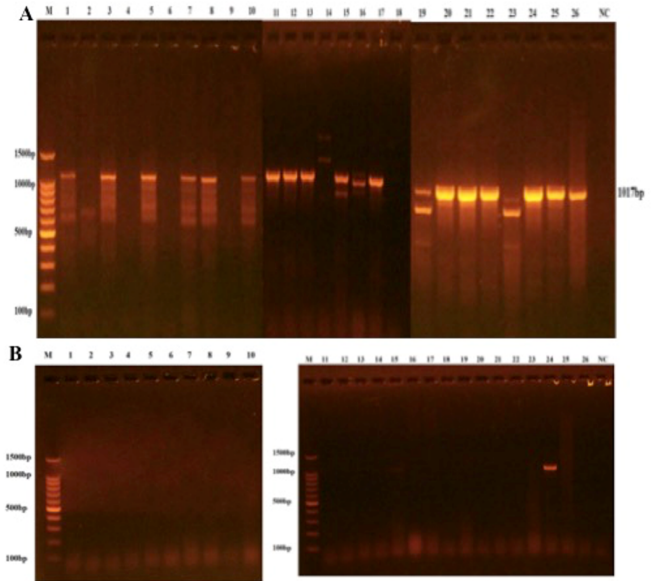

Figure 4. The polymerase chain reaction (PCR) assay for detection of *OmpA* and bla_{PER1} genes among *A. baumannii* isolates. 1% agarose was run at 100 V/cm for 30 minutes with ethidium bromide stain to detect amplified *OmpA* and bla_{PER1} genes. (A): Gel electrophoresis of amplified OmpA gene. Lane 1: Marker: 100 bp ladder marker. Lanes 2-27 resemble 1071 bp PCR products, Lane 28: negative control; (B): Lane 1: Marker: 100 bp ladder marker. Lanes 2-10 show 927 bp PCR products. On the right: Lane 1: Marker: 100 bp ladder marker; Lanes 1-16 show 927 bp PCR products and Lane 17: negative control.

Table 4. *OmpA* and bla_{PER1} genes distribution in positive and negative *A. baumannii* biofilm producing clinical isolates.

Group	Positive Biofilm	Negative Biofilm
Positive OmpA	17	1
Negative OmpA	5	3
Positive bla_{PER-1}	1	0
Negative bla_{PER-1}	21	4

DISCUSSION

The high prevalence of MDR *A. baumannii* isolates is a major threat to public health worldwide, which poses a growing challenge [16]. The result from this study agrees with another study that indicated a high prevalence of MDR *A. baumannii* in sputum samples (30.6%) [17]. However, our results disagree with other local studies reporting that most of the obtained *A. baumannii* were from wounds and urine samples [18]. The possible explanation is that the study sample size and source (i.e. in or outpatients) may affect the distribution of the sample source.

Antibiotic susceptibility profiles were conducted for 26 tested isolates of *A. baumannii*. All isolates showed resistance to most of the tested antibiotics. A study found that 11% of nosocomial isolates of *A. baumannii* were carbapenem-resistant, with a 52% rate of illness and death compared to 19% for patients infected with carbapenem-sensitive isolates [19].

One of the most detected genes that are used to identify *A. baumannii* is the bla_{OXA-51} gene, which is encoded via chromosomal DNA and is considered a reliable method to detect *A. baumannii*. A recent study showed that *A. baumannii* strains carrying the bla_{OXA-51} gene and bla_{OXA-23} genes are more resistant to antibiotics than strains carrying NDM-1 and KPC genes, and there is a correlation between strains carrying the bla_{OXA-51} gene and bla_{OXA-23} genes and biofilm formation [20].

The biofilm plays a vital role in the pathogenesis contributes to the infection persistence and improves antibiotic resistance. Studies from our region have investigated the prevalence of biofilm-producing *A. baumannii* isolates in hospitals and indicated a high prevalence of MDR *A. baumannii* biofilm producer isolates [21]. Many factors control *A. baumannii* biofilm production such as cell density, environmental factors, and surface expressed factors [6]. With all these factors, formed biofilm decreases drug diffusion through bacterial cells and increases the bacterial ability to persist in harsh conditions in addition to MDR.

A useful tool that is used to visualize biofilm formation with high-resolution images and high magnification is conventional SEM. Our results indicate the formation of thin and less dense cell aggregation in weak biofilm producer isolates when compared with moderate biofilm producer isolates, where adhered cells form a cloud and adhesion between bacterial cells is stronger [20].

The *OmpA* gene was more prevalent in the tested isolates. A local study showed that 52% of the studied clinical isolates were biofilm producers, and the *OmpA* gene was prevalent in 86.7% of the studied isolates [22]. The *OmpA* is considered a multifunctional protein involved in adhesion, aggressiveness, and biofilm formation, and might induce apoptosis in human epithelial cells [23]. The relation between the *OmpA* gene and antibiotic resistance is not clearly understood yet. However, some studies have shown a correlation between the *OmpA* gene and antibiotic resistance. A study generated a clone with a non-functional *OmpA* gene that showed susceptibility more than 8-fold to chloramphenicol, 8-fold to aztreonam, and 3-fold to nalidixic acid when compared with the wild-type [24]. Another study reported that a mutation in the OmpA-like domain of *A. baumannii* increased susceptibility to trimethoprim by more than 3.5-fold, tetracycline by 2.3-fold, and other antimicrobial agents by 2-fold, except tigecycline, compared to the wild strain [25]. The *OmpA* gene may be involved in pumping compounds from the periplasmic space through the outer membrane and pairing with inner membrane efflux systems like the major facilitator superfamily (MFS) efflux pumps or resistance–nodulation–division (RND) systems, which lack the OMP component [26].

Meanwhile, one of the bap genes is the bla_{PER-1} gene, which encourages *A. baumannii* clinical isolates to form biofilm and adhere to respiratory epithelial cells as reported [27]. A study reported that bla_{PER-1} was not detected in more than 55% of the tested isolates, and the OmpA gene was the most frequent (100%) [28]. Another study showed that the distribution of bla_{PER-1} and *OmpA* genes were 39% and 91% respectively [29]. *A. baumannii* sticks to plastic surfaces more when the bla_{PER-1} gene is present than when biofilm forms [30]. This may explain why the bla_{PER-1} gene is not very common.

However, there are some limitations to consider in this study. The small number of clinical isolates tested is one limitation, and the lack of testing for other genes involved in biofilm production is another. Furthermore, we collected all samples from single hospital rather than from multiple hospitals. Moreover, there is a lack of comparison between inpatients and outpatients. Despite these limitations, this study provides valuable insights, and researchers can overcome them.

CONCLUSION

The most frequent source for *A. baumannii* was sputum. The MDR *A. baumannii* gene is also very common in clinical isolates, and the *OmpA* gene is more common than bla_{PER-1} in tested isolates. Further studies are required in the future to confirm the association between biofilm, antibiotic resistance, and other biofilm-related genes.

ETHICAL DECLARATIONS

Acknowledgments

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Ethics Approval and Consent to Participate

This research was carried out in line with the Declaration of Helsinki. The study was approved by the Mustansiriyah University, College of Science; ethical approval board under the number (BCSMU/1221/00040M). All patients who participated in this study were provided with informed consent.

Consent for Publication

Not applicable (no individual personal data included).

Availability of Data and Material

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing Interests

The author declares that there is no conflict of interest.

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Authors' Contributions

Jabbar RK contributed to samples collection and experimental performance. Abdulsattar BO and Ibrahim SA equally contributed to this work by designing the experiment, analyzing the data, and writing the original draft. All authors read and agreed to the final version of the manuscript for publication.

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