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Prevalence, Antimicrobial Susceptibility, and Distribution of Biofilm Associated Virulence Genes in Multidrug-Resistant *Acinetobacter baumannii* Isolated from Various Environmental Sources in Baghdad Hospitals

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Abstract

It is estimated that about 20% of all hospital-acquired infections can be attributed to environmental contamination. *Acinetobacter baumannii* has been identified as the primary pathogen, posing a significant hazard to human health. This work aims to evaluate the frequency, and pattern of antibiotic resistance, as well as the correlation between biofilm production and the distribution of biofilm-related genotypes in multidrug-resistant *A. baumannii* samples isolated from a tertiary hospital environment in Baghdad, Iraq. Conventional methods, biochemical analysis, the VITEK-2 compact system, and *blaoxa-51* gene amplification were employed to identify suspected isolates. The biofilm-forming capability and antibiotic susceptibility were assessed using the microtiter plate technique and disk diffusion. Isolates were then subjected to PCR technique targeting genes associated with biofilms, including *omp*A, *csu*E, *bap* and *blaPER-1*. Of the 300 samples, 21.16% were identified and verified as *A. baumannii* samples by the presence of the *blaoxa-51* gene in all isolates. The highest distribution of environmental isolates was recorded in ventilator (21.8%) followed by pillow and bed linens (14.5%), trolleys, bedside tables (11.5%), floor (10.3%), food tables (9.2%), sink area (8%), Staff's hand (6.7%), incubators (5.7%), doors knobs (4.6%), surgical blades (3.4%), Curtains (2.2%) and the lowest distribution was observed in chairs (1.1%). The greatest percentage of resistance was observed against Cefepime and Ciprofloxacin (100%), Ceftazidime (97.7%), and Ceftriaxone (90%). The lowest degree was reported against Sulbactam, representing 33.33%. Out of all the isolates, a significant majority (91.95%) could form biofilms. These biofilm formers were further categorized based on their level of biofilm production, with 20% classified as weak producers, 27.5% classified as moderate producers, and 52.5% classified as strong producers. The *csu*E gene was the most common biofilm gene in 95.4% of the samples. The *omp*A gene was found in 83.9% of the samples, followed by the *bap* gene in 80.5%. The *blaPER-1* gene was the least prevalent, detected in only 10.6% of the samples. However, only biofilm-forming isolates contained *blaPER-1*, *bap* and *omp*A. Conversely, all biofilm-producing and non-biofilm-producing *A. baumannii* were found to harbor the *csu*E gene. In various samples obtained from hospital environments, our investigation revealed a substantial incidence of multi-drug-resistant *A. baumanii*. Additionally, we observed a significant presence of genes associated with biofilm formation. Therefore, it is crucial to enforce control measures to limit the dissemination and growth of *A. baumannii*.

Keywords: *Acinetobacter baumannii*, Antimicrobial Resistance, Molecular Epidemiology, Hospital Environments, Microbial Biofilm, Biofilm-Related Genes

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انتشار وحساسية المضادات الحيوية وتوزيع جينات الفوعة المرتبطة بالأغشية الحيوية في بكتريا الراكدة البومانية المقاومة للأدوية المتعددة المعزولة من مصادر بيئية مختلفة في مستشفيات بغداد

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الخلاصة

تقدر مساهمة التلوث البيئي في ما يصل إلى 20% من جميع الأمراض المكتسبة في المستشفيات. وقد تم تحديد بكتيريا الراكدة البومانية كعامل ممرض رئيسي، يشكل خطرًا كبيرًا على صحة الإنسان. يهدف هذا العمل إلى تقييم تكرار وأنماط مقاومة المضادات الحيوية، فضلا عن العلاقة بين إنتاج الأغشية الحيوية وتوزيع الجينات المرتبطة بالأغشية الحيوية في بكتريا الراكدة البومانية المقاومة للأدوية المتعددة المعزولة من مصادر بيئية مختلفة في ثلاث مستشفيات في بغداد، العراق. تم استخدام الطرق التقليدية والكيميائية الحيوية ,نظام VITEK-2 المضغوط وتضخيم جينات *blaoxa-51* للتحري عن العزلات المشتبه بها. تم تقييم قدرة البكتريا على تكوين الأغشية الحيوية وحساسيتها للمضادات الحيوية بأستخدام طريقة أطباق المعايرة الدقيقة وطريقة انتشار القرص، على التوالي. ثم خضعت العزلات بعد ذلك لتقنية تفاعل البلمرة المتسلسل التي تستهدف الجينات المرتبطة بالأغشية الحيوية والتي تتضمن*bap* ,*csu*E, *omp*A و *blaPER-1*. من بين 300 عينة تم الحصول على 21.16% من عزلات الراكدة البومانية وتم تأكيد وجود جين *blaoxa-51* في جميع العزلات. تم تسجيل أعلى توزيع للعزلات البيئية في أجهزة التهوية (21.8%)، تليها الوسائد وأغطية الاسرة (14.5%)، العربات، والطاولات الجانبية (11.5%)، الأرضيات (10.3%)، وطاولات الطعام (9.2%)، الاحواض (8%)، أيدي الموظفين (6.7%)، الحاضنات (5.7%)، مقابض الأبواب (4.6%)، الشفرات الجراحية (3.4%)، الستائر (2.2%)، وكانت ادنى نسبة في الكراسي (1.1%). تم الكشف عن اعلى معدلات المقاومة الحيوية ضد السيفيبيم و السيبروفلوكساسين (100%)، السيفتازيديم (97.7%)، السيفترياكسون (90%) بينما لوحظت أدنى معدلات المقاومة ضد مادة السولباكتام حيث بلغت 33.33%. من بين جميع العزلات، كانت نسبة كبيرة (91.95%) قادرة على تكوين للأغشية الحيوية، وتم تصنيف هذه العزلات بناءً على مستوى انتاج الاغشية الحيوية، حيث تم تصنيف 20% منها كمنتجات ضعيفة و 27.5% متوسطة و52.5% كمنتجات قوية. كان جين *csu*E هو الاكثر شيوعا، حيث بلغ 95.4% من العينات، تم العثور على جين *omp*A في 83.9% من العينات، يليه جين *bap* في 80.5% وكان جين *blaPER-1* هو الأقل انتشارًا، حيث تم الكشف عنه في 10.6% فقط من العينات. ومع ذلك، كانت العزلات المكونة للاغشية الحيوية تحتوي فقط على جينات*blaPER-1* و*bap* و *omp*A. في المقابل، تبين ان جميع أنواع الراكدة البومانية المنتجة وغير المنتجة للأغشية الحيوية تحتوي على جين *csu*E. في العينات المختلفة التي تم الحصول عليها من بيئات المستشفيات، كشفت تحقيقاتنا عن وجود نسبة كبيرة من بكتيريا الراكدة البومانية المقاومة للأدوية المتعددة. بالإضافة إلى ذلك، لاحظنا وجودًا كبيرًا للجينات المرتبطة بتكوين الأغشية الحيوية. وعليه، فمن الضروري تنفيذ تدابير الرقابة للحد من انتشار ونمو الراكدة البومانية.

1. Introduction

Hospital-associated infections (HAIs) represent a significant worldwide contributor to both mortality and morbidity rates [1, 2]. Bacteria play a major role in the cause of healthcare-associated infections (HAIs), and *Acinetobacter baumannii* is one of the most commonly encountered bacterial pathogens associated with HAIs [3]. It has been recognized as the primary cause of the preponderance of infections acquired in healthcare settings, particularly in intensive care units, and is the most medically significant [4]. Wound infections, meningitis, bloodstream infections, urinary tract infections, pneumonia, and bacteremia are all caused by *A. baumannii* [5, 6].

The extensive use of antibiotics has been the primary cause of the significant increase in the incidence of resistance to antibiotics in *A. baumannii* isolated in recent years. Consequently, various bacteria resistant to a broad spectrum of medications, including those frequently employed and those used to treat multiple types of infections, have emerged [7, 8]. According to the WHO, *A. baumannii* is the most significant disease in terms of importance and the greatest hazard to human health [9]. The capacity of *A. baumannii* to survive and disseminate extensively in hospital environments has been cited as the reason for its successful development as an endemic pathogenic organism in healthcare institutions [10, 11]. Research has consistently shown that *A. baumannii* is prevalent on various surfaces within hospital environments, with intensive care units being especially affected by this contamination [12-14]. Long-term persistence is considered an important factor in hospital transmission of this bacterium. In this context, patients become infected with *A. baumannii* after coming into contact with healthcare staff carrying the pathogen or through contaminated environments such as medical charts, pillows, laryngoscopes, bed curtains, bedside cabinets, telephones, door handles, mops, patient lifting equipment, floors, sinks, ventilation grates, keyboards, aspiration and ventilation equipment [15, 16].

A. baumannii's ability to survive in the hospital environment is affected by a variety of factors, such as the adhesion and colonization of epithelial cells, the formation of biofilms on both living and non-living objects (environmental surfaces and medical devices implanted in patients), and the ability to gain foreign genetic elements to improve its immunity to host selection pressures and therapeutic antibiotics [17, 18]. The capacity of *A. baumannii* to generate biofilm is primarily acknowledged as a successful approach to improving bacterial survival, dormancy in deeper layers of biofilm, adhesion to mucosal surfaces, and persistence in hospital environments under challenging conditions, including disinfectant, antibiotic, and desiccation exposure. This leads to infections that are difficult to treat and persist [19]. In device-related infections, biofilm formation is a pathogenic mechanism that enhances the survivability of inanimate objects, acting as a source for recurrent transmission [20,21].

B. The formation of the *A. baumannii* biofilm is dependent on a variety of virulence factors, such as outer membrane protein A (*Omp*A), CsuA/BABCDE chaperone-usher pili assembly system, and biofilm-associated protein (Bap) [22]. This discovery was recently identified. A virulence gene known as *bap* is essential for the production of biofilm and intercellular adhesion [23]. The development of biofilms is contingent upon enhancing cell-to-cell adhesion and cell-surface adhesion on both abiotic and biotic surfaces by *ompA* [24]. In addition, prior research has demonstrated that the *blaPER-1* gene's expression and occurrence promote the biofilm development and adhesion of bacteria to respiratory epithelial cells [25]. The most common pili in bacteria are Csu pili, which are crucial for forming biofilm on abiotic surfaces and for cell attachment [26]. The development of pilus and biofilm production is prevented by the inactivation of *csu*E [27, 28].

Offering an alternative viewpoint to enhance comprehension of opportunistic *A. baumannii* infections by determining the correlation between survivability factors in environmental isolates and the possibility of spreading from the environment to the body of the patient could be useful for minimizing *A. baumannii* infections in hospital environments. Although there is a significant amount of research on *A. baumannii* infections, especially in clinical settings, more thorough studies need to be conducted that specifically investigate the occurrence and distribution of biofilm-associated virulence genes in environmental isolates from hospitals in Baghdad. This research seeks to fill this gap by systematically examining these facets. Therefore, the primary objective of the current investigation is to ascertain the incidence, antimicrobial susceptibility, and correlation between biofilm productions and biofilm-related

genes in multidrug-resistant *A. baumannii* isolates from various environmental sources in tertiary hospitals in Baghdad, Iraq.

2. Materials and Methods

2.1 Sampling and isolation of A. baumannii

This research was conducted from July 2022 to February 2023. In Baghdad, Iraq, a total of 300 environmental samples were collected from four distinct medical facilities and two emergency departments within the three largest educational hospitals (Specialized Burn Hospital, Ghazi Al-Hariri Hospital for Surgical Specialties, and Baghdad Teaching Hospital) during the study period. The majority of these samples were randomly collected by swabbing bed linens, ventilator inlets, pillows, bedside tables, staff's hand, food tables, blankets, chairs, curtains, trolleys, baby incubators, bins, doorknobs, and surgical blades, as well as from the sink area and floor. Sterile swabs that have been previously moistened in five ml brain-heart infusion broth are followed by a (24-hour) incubation at (37°C). Subsequently, all enriched samples were sub cultured onto CHROMagar Acinetobacter, MacConkey agar, and Blood agar and incubated at 37°C for 24 hours. Afterward, the suspected colonies were confirmed as *A. baumannii* according to culture characteristics, Gram stain and biochemical identification such as indole production test, oxidase test, lactose fermentation test, Kligler iron agar (KIA), motility test, urease production test, Voges- Proskauer test, catalase test, hemolysin production, methyl red test, citrate utilization test and growth at 44°C [29]. The final confirmation was based on the existence of *blaOXA-51*-like genes, as previously described [30]. In addition, the Vitek-2 system was employed to precisely identify each isolate by the manufacturer's instructions, as outlined below:

2.2 Bacterial Identification by VITEK-2 Compact System

The purpose of this investigation was to verify the suspected *A. baumannii* colony among the Gram-negative bacteria. In brief, a group of colonies from an overnight culture of environmental isolates on MacConkey agar was selected and suspended in three milliliters of normal saline via DENSICHEK® Plus (BioMérieux Inc DensiCHEK™, France) to match the 0.5 McFarland Standard. The cassette barcodes of the instrument and the GN cards (BioMérieux, France) were scanned to ensure traceability. A standardized bacterial inoculum was used to inoculate the cards, and the cassette was inserted into the machine for verification. Finally, the code was manually transmitted from the filling to the loading door for automated processing from 5-8 hours of verification [31].

2.3 Antibiotic Susceptibility Test

Antimicrobial susceptibility testing was conducted according to the Clinical and Laboratory Standard Institute guidelines using the Kirby-Bauer disk diffusion method [32]. Briefly, the McFarland 0.5 adjustment was implemented overnight to halt bacterial proliferation. A sterile cotton swab was employed to apply the bacterial suspension to the surface of the Muller-Hinton agar plate. The plates were permitted to remain at room temperature for 30 minutes after injection to absorb any excess moisture. The antibiotic disks were subsequently affixed to the agar surface using sterile forceps. A panel of thirteen distinct antibiotics (Himedia/India) was employed to evaluate *A. baumannii* isolates on Mueller Hinton agar plates. The anti-biotics, which consisted of Piperacillin (100mg), Ceftazidime (30µg), Ceftriaxone (30µg), Amikacin (30µg), Colistin (10µg), Ampicillin/Sulbactam (10µg), Imipenem (10µg), Erythromycin (15µg), Gentamicin (10µg), Ciprofloxacin (5µg), Trimethoprim/Sulfamethoxazole (25µg), Doxycycline (30 µg), and Cefepime (30 µg) were added to an inoculated media and incubated at 35ºC for 18-24 hours. Subsequently, the results were classed as resistant, intermediate susceptible or sensitive by the Clinical and Laboratory Standards Institute (CLSI) guidelines [32]. Multidrug resistance (MDR) is defined as the ability of a microorganism to

withstand the effects of at least one antibiotic from three or more distinct classes of antimicrobial agents. Extensively drug-resistant (XDR) isolates were defined as resistant to at least one agent in all antimicrobial classes except two or fewer [33].

2.4 Biofilm Formation Assay

With a modest modification, the biofilm production in environmental isolates was quantified using the microtiter plate technique, as described by Ghasemi *et al*. [34]. In conclusion, the bacterial isolates were cultivated in Tryptic-Soy Broth (TSB) at 37°C overnight while supplemented with 0.5% glucose (Li-ofilchem, Italy). At a ratio of 1:40, all cultures have been diluted in TSB with 0.5% glucose. Subsequently, 200uL was dispersed in triplicate into each well of microplates (SPL, Korea) and incubated at 37°C for 48 hours. Negative controls were considered in the well with 200 L of TSB with 0.5% glucose. The wells were flushed slowly with Phosphate Buffered Saline on multiple occasions, ensuring that adhering biofilms were not affected, followed by fixation for 20 minutes with methyl alcohol and subsequently air-dried at 20 to 25°C. After that, 0.1% safranin (Merck, Darmstadt, Germany) was used to stain the wells.

The optical density (ODw) value of each well was determined using an ELISA reader, with measurements obtained at a wavelength of 490 nm. The isolates were categorized into four categories using the following formulas: The biofilm was regarded as detrimental. The biofilm was considered feeble if the optical density (OD) was less than 0.062. Biofilm formation was moderate if OD < 0.124, strong if OD < 0.248, and moderate if OD < 0.062.

2.5 The Extraction of DNA

Genomic DNA was extracted from an overnight culture of environmental isolates using a QIAGEN Mini kit (QIAGEN, USA) in accordance with the manufacturer's prescribed protocols for Gram-negative bacteria.

2.6 Detection of blaoxa-51 gene

All isolates were PCR-screened for *blaOXA-51*, with primer information in Table 1. To create a 25µL reaction mixture, 2µL of extracted DNA template, 12.5µL of PCR master mixes, 1µL of forward and reverse primers, and nuclease-free water was added. Amplification processes were carried out employing a thermal cycler under these conditions: 5 minutes of denaturation at 94°C, 30 cycles of 45 seconds at 94°C, 1 minute of annealing at 52°C, 1 minute of extension at 72°C, and 6 minutes of extension at 72°C [30].

2.7 Gene Pattern Identification

As previously disclosed, PCR detected the *csu*E, *blaPER-1*, *omp*A, and *bap* genes in *A. baumannii* environmental isolates [35]. Table 1 lists the primer sequences used in this study. The PCR was conducted in a Thermocycler. The reactions were performed in a 25µL mixture with 5µL (5 ng) of extracted DNA, 1µL of each primer, and 12.5µL of PCR master mix (BioNeers, USA). PCR master mixes contain 0.4 mM dNTPs, three mM magnesium chloride, and 0.08 U/L Taq DNA polymerase. Biofilm-related gene PCR amplification involves 30 amplification cycles after a 4-minute denaturation phase at 94°C. Table 1 shows that each cycle included a 45-second annealing phase at 94°C, a 1-minute extension phase at 72°C, and a 1-minute annealing phase at a different temperature for each gene. Finally, the amplification was extended at 72°C for 5 minutes [36]. After PCR, the amplified DNA was separated on a 1.5% agarose gel (Merck, Darmstadt, Germany) and stained with ethidium bromide.

Table 1: PCR Primers used in this study.

Target gene		Sequence primer (5'➔3')	Amplicon size(bp)	Annealing temperature	References
***bla*OXA-51**	F	TAA TGC TTT GAT CGG CCT TG	353	52	**[30]**
	R	TGG ATT GCA CTT CAT CTT GG			
***omp*A**	F	CGCTTCTGCTGGTGCTGAAT	531	58	**[35]**
	R	CGTGCAGTAGCGTTAGGGTA			
bap	F	TACTTCCAATCCAATGCTAGGGAG GGTACCAATGCAG	1225	55	**[35]**
	R	TTATCCACTTCCAATGATCAG-CAACCAAACCGCTAC			
***csu*E**	F	ATGCATGTTCTCTG-GACTGATGTTGAC	976	60	**[35]**
	R	CGACTTGTACCGTGACCG-TATCTTGATAAG			
blaPER-1	F	ATGAATGTCATTATAAAAGC	927	55	**[35]**
	R	AATTTGGGCTTAGGGCAAGAAA			

2.8 Statistical Analysis

All data was verified and analyzed using SPSS software (version 27.0; Inc., New York, USA). Frequencies and percentages were employed to characterize the descriptive data of the bacterial isolates profile. Pearson's chi-square test was implemented to evaluate the relationship between categorical variables in the biofilm intensity results. Furthermore, to anticipate gene responses, multinomial logistic regression was implemented. A P-value of less than 0.05 was used to indicate statistical significance.

3. Results

3.1 Epidemiology/isolate Collection

A total of 87 (21.16%) *A. baumannii* isolates were identified in hospital environmental samples during the investigation. These isolates were predominantly identified in hospitals and originated from several types of environmental sources within hospital settings. The morphological recognition of the organism that caused the illness was based on colonial morphology, selective media, Gram's staining, and microscopy, as illustrated in Table 2.

Table 2: Identification of *A. baumannii* by biochemical test.

NO.	Biochemical test	Results
1.	Oxidase test	-
2.	Hemolysin production	- γ hemolysis
3.	Microscopic shape	Coccobacilli
4.	Catalase production test	+
5.	Growth at 44°C	+
6.	Citrate utilization	+
7.	Lactose fermentation	-
8.	Gram stain	-
9.	Methyl red	+
10.	Urease production	Variable
11.	Voges- Proskauer	-
12.	Motility	Non motile
13.	Indol production	-
14.	Kliglar iron agar	No gas, Alkaline slant, No H2S, No change bottom,

+ = Positive result, - = Negative result

Finally, the Vitek-2 compact system, the standard biochemical identification system for bacteria, was employed to further confirm the identification of *A. baumannii* isolates. The results

of the Vitek2 compact system were consistent with those obtained from biochemical identifications, confirming that 87 (21.16%) of environmental isolates belonged to *A. baumannii* after 18 hrs of incubation with an accuracy of about 99 %, as illustrated in Figure 1.

bioMérieux Customer: Laboratory Report Printed 12:00 AST
System #: Printed by: System

Patient Name: 17
Isolate: 22 n-1 (Tobe reviewed)
Patient ID: aa137
Physician:
Isolate Number: 1

Card Type: GN Bar Code: 2410381403313815 Testing Instrument: 00001899F2A3 (16340)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0043051103500250
Organism Quantity: Selected Organism: Acinetobacter baumannii

Comments:	

Identification Information	Card: GN	Lot Number: 2410381403	Expires: Oct . 2023 12:00 AST
	Completed: October 15, 22 23:15 AST	Status: Final	Analysis Time: 4: 25 hours
Organism Origin	VITEK 2		
Selected Organism	99% Probability Acinetobacter baumannii		
	Bionumber: 0043051103500250	Confidence:	Excellent identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages:			
Contraindicating Typical Biopattern(s)			

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	+	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	+	14	GGT	-	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	+	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	+			

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline: Therapeutic Interpretation Guideline:

Figure 1: Biochemical results by using VITEK-2 Compact system.

Additionally, the *blaoxa-51* gene was identified in all of isolates, thereby verifying that the bacteria were *A. baumannii*, as shown in Figure 2.

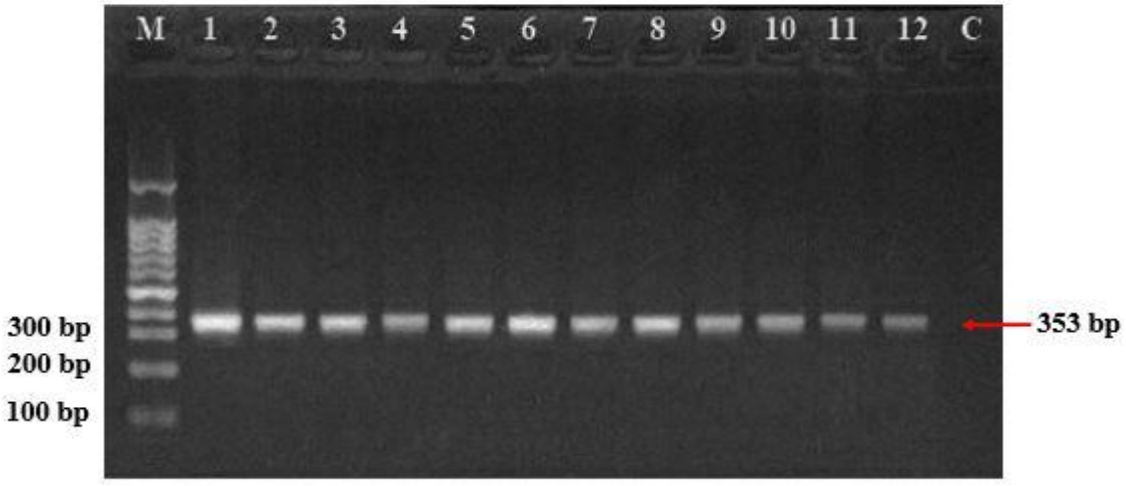


Figure 2: Agarose gel electrophoresis (1.5% agarose electrophoresis and staining for 1 hour at 75 V with ethidium bromide). Lane M = marker with 100 bp. Lanes. 1-12 represent positive samples for *blaoxa-51* gene with an amplicon size of 353bp. Lane C: Negative control.

3.2 Prevalence of A. baumannii

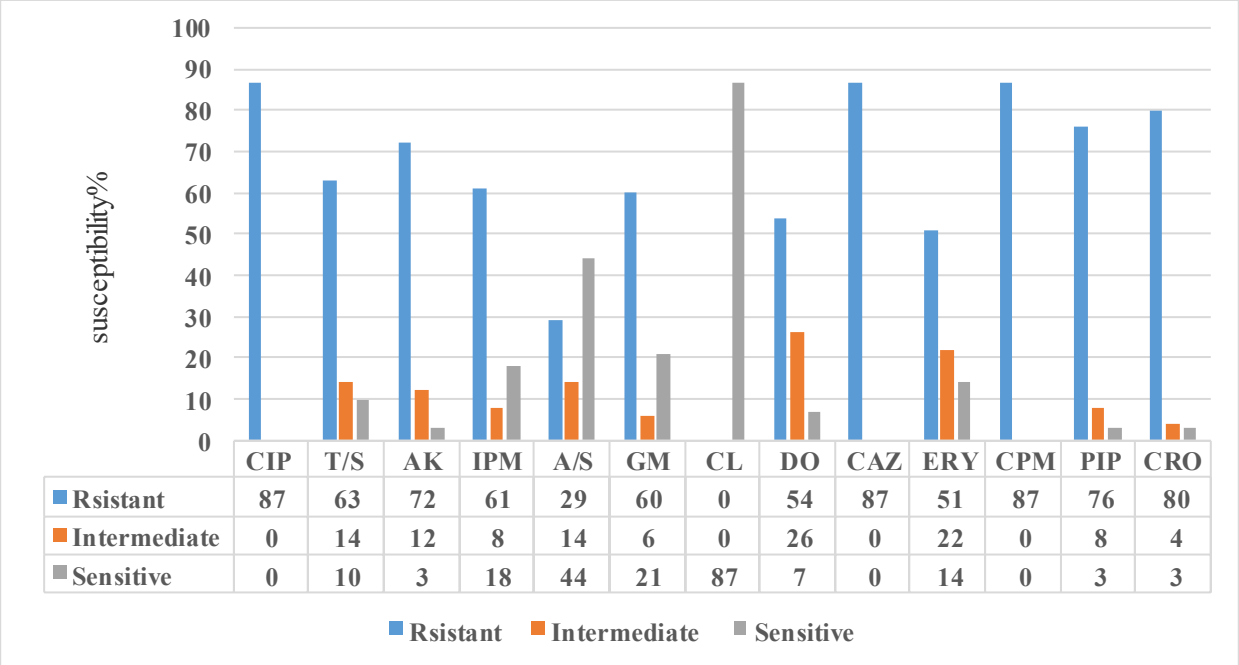
The distribution of *A. baumannii* isolates varied among the environmental samples collected, depending on the isolation sources in each hospital ward. The present results showed that the highest distribution was recovered from ventilator 19 (21.8%) followed by pillow and bed linens 13 (14.5%), trolleys and bedside tables 10 (11.5%), floor 9 (10.3%), food tables 8 (9.2%), sink area 7 (8%), staff's hand 6 (6.7%), incubators 5 (5.7%), doors knobs 4 (4.6%), surgical blades 3 (3.4%), Curtains 2 (2.2%) and finally chairs represented by 1 (1.1%), as illustrated in Table 3.

Table 3: The frequency distribution of environmental isolates according to the source of isolation.

Isolation site	Samples cultured No.	Positive culture No. (%)	Isolates No (%)	Hospital source		
				Hospital 1	Hospital 2	Hospital 3
Pillow& Bed Linens	40	33 (15)	13 (14.5)	6	4	3
Ventilator	56	42 (19.2)	19 (21.8)	8	5	6
Incubators	12	9 (4.1)	5 (5.7)	3	1	1
Hand Staff	21	13 (5.9)	6 (6.7)	2	1	3
Trolleys & Bedside Tables	35	29 (13.2)	10 (11.5)	4	3	3
Sink Area	25	17 (7.8)	7 (8)	3	2	2
Floor	30	23 (10.5)	9 (10.3)	5	3	1
Surgical Blades	13	8 (3.7)	3 (3.4)	1	1	1
Doors Knobs	16	10 (4.6)	4 (4.6)	2	1	1
Chairs	12	7 (3.2)	1 (1.1)	0	1	0
Curtains	13	8 (3.7)	2 (2.2)	2	0	0
Food Tables	27	20 (9.1)	8 (9.2)	4	1	3
Total	**300**	**219**	**87**	**40**	**23**	**24**

3.3 Antibiotic Susceptibility Testing

Notably, all isolates of *A. baumannii* were considered MDR as they demonstrated resistance to three or more classes of antimicrobial agents. Among the 87 test isolates, Cefepime and Ciprofloxacin demonstrated the highest resistance (100%), followed by Ceftazidime (97.7%), Ceftriaxone (90%), Piperacillin (87.35%), Amikacin (82.75%), Trimethoprim-Sulfamethoxazole (72.41%), Imipenem (70%), Gentamicin (69%), Doxycycline (62%), Erythromycin (58.62%) and ultimately Ampicillin-Sulbactam represented (33.33%), as illustrated in Figure 3.



	CIP	T/S	AK	IPM	A/S	GM	CL	DO	CAZ	ERY	CPM	PIP	CRO
Rsistant	87	63	72	61	29	60	0	54	87	51	87	76	80
Intermediate	0	14	12	8	14	6	0	26	0	22	0	8	4
Sensitive	0	10	3	18	44	21	87	7	0	14	0	3	3

Figure 3: Antibiotic susceptibility test: CIP (ciprofloxacin); T/S (trimethoprim/sulfamethoxazole); AK (Amikacin); IPM (Imipenem); A/S (ampicillin/sulbactam); GEN (Gentamicin); CL (Colistin); DO (Doxycycline); CAZ (Ceftazidime); ERY (Erythromycin); CPM (Cefepime); PIP (Piperacillin); CRO (ceftriaxone).

The most prevalent types of MDR included resistance Ceftriaxone, Imipenem, Cefepime, Trimethoprim-Sulfamethoxazole, Ciprofloxacin, Doxycycline, Ceftazidime, Piperacillin, Erythromycin, Gentamicin, and Amikacin with a frequency of 25% as shown in Table 4.

Table 4: Antibiotic Resistance Patterns.

No. of antimicrobial agents	Patterns of Antibiotic resistance	No. of isolates
5	Ceftazidime, Ciprofloxacin, Imipenem, Piperacillin, Ceftriaxone	8
7	Ciprofloxacin, Cefepime, Gentamicin, Amikacin, Imipenem, Ceftriaxone, Ampicillin-Sulbactam	14
8	Cefepime, Ceftriaxone, Erythromycin, Ciprofloxacin Trimethoprim/Sulfamethoxazole. Amikacin, Piperacillin, Gentamicin	20
9	Ceftazidim, Piperacillin, Ampicillin/Sulbactam, Cefepime, Imipenem, Gentamicin, Ciprofloxacin, Doxycycline, Amikacin	16
10	Ciprofloxacin, Cefepime, Imipenem, Ceftazidime, Trimethoprim-Sulfamethoxazole, Piperacillin, Erythromycin, Amikacin, Gentamicin, Ceftriaxone	22
11	Ceftriaxone, Imipenem, Cefepime, Trimethoprim-Sulfamethoxazole, Ciprofloxacin, Doxycycline, Ceftazidime, Piperacillin, Erythromycin, Gentamicin, Amikacin	25

3.4 Characteristics of Biofilm Producers

This study assessed the biofilm-forming ability of polystyrene using 96-well cell culture microtiter plates. The majority of environmental isolates could form varying degrees. The mean ODs for all strains were 0.407 ± 0.028 (Ranging from 0.016 to 0.866). Among the 87 test strains, 80 (91.95%) isolates were biofilm producers, of which 42 isolates (52.5%) were considered strong biofilm producers, 22 (27.5%) were classified moderate, and 16 (20%) were categorized as weak biofilm formers.

3.5 Biofilm-Associated Genes

Biofilm-related genes were investigated in all of the *A. baumannii*. Out of 87 biofilm/non-biofilm producer isolates, 83 (95.4%) tested positive for the *csu*E, 73 (83.9%) for the *omp*A, 70 (80.5%) for the *bap*, and 31 (35.6%) for the *blaPER*-1, as detailed in Table 5.

Table 5: Correlation between biofilm production and biofilm-associated genes

Biofilm Strength	Biofilm-Related Genes								Statistical
	*omp*A		*bap*		*csu*E		*blaPER1*		
	N	%	N	%	N	%	N	%	
Strong (42)	42	100.0%	42	100.0%	42	100.0%	17	40.5%	Chi-square=17.38 Sig=0.043 *
Moderate (22)	19	86.4%	18	81.8%	20	90.9%	11	50.0%	
Weak (16)	12	75.0%	10	62.5%	14	87.5%	3	18.8%	
Non-biofilm (7)	0	0.0%	0	0.00%	7	100.0%	0	0.00%	
Total (87)	73	83.9%	70	80.5%	83	95.4%	31	35.6%	
Chi-Square	20.247		23.771		33.096		9.548		
Sig.	0.0001**		0.0001**		0.0001**		0.008*		

Moreover, all genes are identified in Figure 4. Among the positive isolates, the mean biomass of biofilms for *blaPER-1, csu*E, *omp*A, and *bap* were 0.429 ± 0.266, 0.381 ± 0.174, 0.319 ± 0.079, and 0.331 ± 0.411, relative to each. The strains harboring *csuE, blaPER, bap,* and *omp*A genes exhibit a higher propensity to develop strong biofilms than isolates without these genes

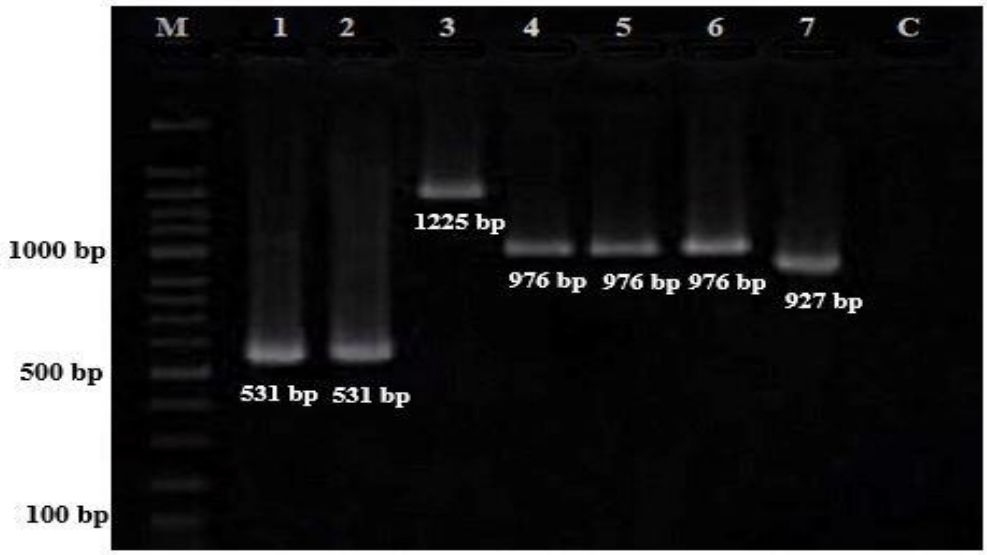


Figure 4: Result of amplified biofilm-related genes. Visualization was accomplished using 1.5% agarose electrophoresis and staining for 1 hour at 75 V with ethidium bromide. Lane M = marker with 100 bp. Positive samples for the *omp*A gene (531 bp) are shown in lanes 1 and 2. Lan 3: Positive *bap* gene (1225 bp). Lan 4, 5 and 6: Positive for *csu*E gene (976 bp). Lan7: Positive for *blaPER-1*gene (927 bp). Lane 8: Negative control.

Statistical analysis ($P < 0.05$) revealed a significant correlation between biofilm production and the presence of *blaPER-1, ompA, csuE,* and *bap* genes in the majority of the isolates. Additionally, the findings of this investigation demonstrated that 95.4% of isolates contained

the *csu*E gene, and there was no discernible correlation between the presence of this gene and the development of biofilm. (P < 0.01). Conversely, *blaPER-1, bap,* and *omp*A genes were significantly associated with biofilm formation in 35.6%, 80.5%, and 83.9% of the isolates, respectively. Notably, both biofilm/non-biofilm producer isolates were found to carry the *csu*E gene. Figure 5 summarizes that *blaPER-1*, *bap*, and *omp*A genes have only been identified in *A. baumannii* isolates capable of forming biofilms.

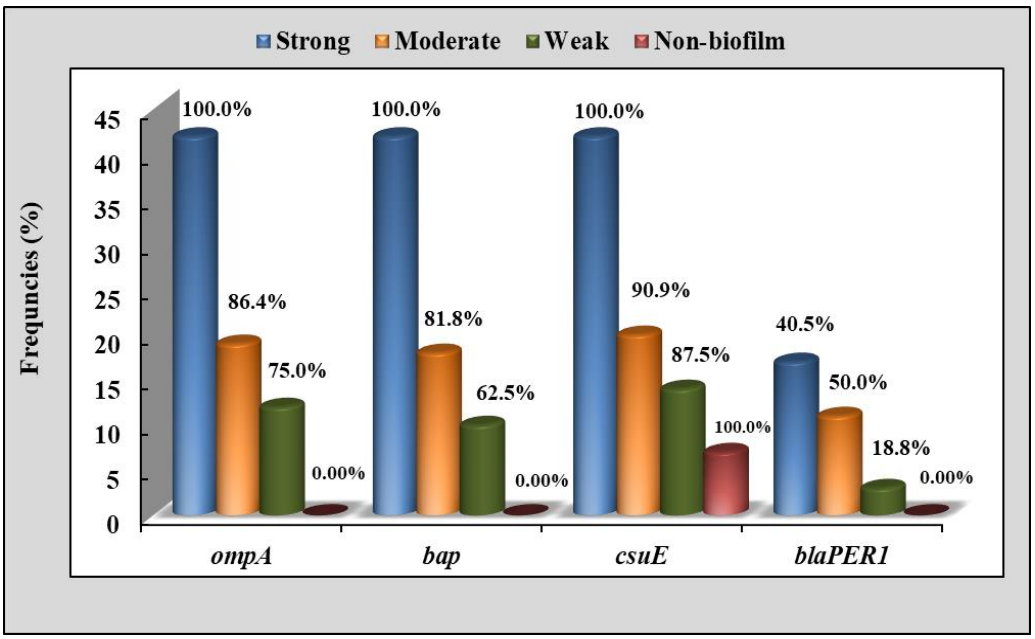


Figure 5: Frequency of biofilm-associated genes.

Multiple logistic regression modeling was employed to assess the association between the production of microbial biofilms and the total frequencies of genes involved in biofilm development in environmental isolates using (non-biofilm, strong, moderate, and weak groups) as a reference category variable, as indicated in Table 6. The present results revealed that *csu*E, *omp*A, and *bap* exhibit a higher capacity to develop strong biofilms than *blaPER1*, as follows: *csu*E (OR, 95% CI: 6.000 (2.696–13.355), with effect size B = 1.792, P = 0.017), followed by *bap* (OR, 95% CI: 4.200 (2.107–8.3718.371), with effect size B = 1.435, P = 0.0001), and *omp*A (OR, 95% CI: 3.500 (1.843–6.648), with effect size B = 1.253, P = 0.0001).

Table 6: Nominal Regression Models of biofilm-related genes.

						95%CI	
Biofilm related genes	**Biofilm Intensity**	**B**	**Std. Error**	**Sig.**	**OR**	**Lower Bound**	**Upper Bound**
***omp*A**	**Strong**	1.253	0.327	0.0001*	3.500	1.843	6.648
	Moderate	0.460	0.369	0.213	1.583	0.769	3.262
bap	**Strong**	1.435	0.352	0.0001*	4.200	2.107	8.371
	Moderate	0.588	0.394	0.136	1.800	0.831	3.899
***csu*E**	**Strong**	1.792	0.408	0.0001*	6.000	2.696	13.355
	Moderate	1.050	0.439	0.017*	2.857	1.208	6.757
	Weak	0.693	0.463	0.134	2.000	0.807	4.955
blaPER1	**Strong**	1.735	0.626	0.006*	5.667	1.661	19.336
	Moderate	1.299	0.651	0.046*	3.667	1.023	13.143

a. The reference category is: Weak for split file VAR00001 = *omp*A.
b. The reference category is: Weak for split file VAR00001 = *bap*.
c. The reference category is: Non-biofilm for split file VAR00001 = *csu*E.
d. The reference category is: Weak for split file VAR00001 = *blaPER1*.

B: size effect (size predict). OR: odds ratio. 95% CI: 95% Confidence Interval.

4. Discussion

A. baumannii is an opportunistic pathogen that has emerged as one of the most resilient organisms capable of surviving in diverse environments, leading to heightened challenges in healthcare settings. Consequently, hospital settings were the primary reservoirs of infectious disorders caused by pathogenic microbes. However, the current more severe issue is the dissemination of antimicrobial-resistant microbes among community members and the environment [37, 38].

In a hospital environment, the greatest risk of pathogen transmission is posed by the actions of healthcare personnel, such as the contamination of their hands and garments after contact with a patient. Most previous investigations have focused on *A. baumannii* isolates from hospitals, even though the hospital environment is becoming an increasingly significant reservoir of *A. baumannii* [39]. A limited number of investigations have concentrated on isolates from population or environmental sites [40, 41].

The microorganism's ability to rapidly adapt to selective environmental stresses is underscored by the increasing global prevalence of *A. baumannii* strains resistant to most antimicrobial treatments [42]. Antibiotic-resistant gram-negative bacilli, such as MDR *A. baumannii*, are difficult to cure. Consequently, the capacity of MDR *A. baumannii* to endure and thrive in various healthcare environments has established it as a substantial global contributor to nosocomial infections [43-45].

Acinetobacter baumannii has been subjected to rigorous experiments to evaluate its susceptibility to various antibiotics. As illustrated in Figure 3, all isolates exhibited resistance to at least one antibiotic in three or more categories, as indicated by the findings of the present study. Consequently, the prevalence of MDR in isolates was 100%, consistent with comparable investigations conducted in Iran [46]. Cefepime, Ciprofloxacin, and Ceftazidime exhibited the highest resistance, with a total of 100%. This pattern agrees with the results of Noori *et al*. [47], who reported a complete resistance to Ceftazidime, Ciprofloxacin, and Piperacillin, which is extremely similar to the current findings.

Nevertheless, Bastian *et al*. [48] found that resistance to ceftazidime and ciprofloxacin was lower in their study than ours, even though they investigated a similar rate of resistance to piperacillin. Furthermore, Ghadiri *et al*. [46] reported that resistance to Doxycycline, Erythromycin, and Colistin was 61.11%, 56.67%, and 0%, respectively, which agrees with the current findings. Ceftriaxone and imipenem exhibited a high resistance rate in a separate study, while ampicillin/sulbactam and colistin exhibited a lower resistance rate, consistent with the current findings [49].

The emergence of elevated resistance rates resulted from the overutilization of antibiotics to treat *A. baumannii* infections in patients [50]. As a result, it is imperative to establish guidelines for implementing infection control protocols to prevent the transmission of these

strains in hospital settings [51]. The emergence of MDR isolates tends to be a particular hotspot in Iraq, which raises serious concerns in the environment of hospitals.

Numerous studies have demonstrated that the production of biofilms is responsible for *A. baumannii'*s high resistance to many antibiotics and its resilient survival capacity in harsh conditions [52,53,54]. The ability to establish biofilm is mainly regarded as a successful strategy to enhance survival, stick to surfaces, dormancy, and long-term presence in hospital environments under stressful conditions [36]. Indeed, previous epidemiological investigations have demonstrated that biofilm development is a crucial virulence factor that allows *A. baumannii* strains to persist and spread chronically over time in nosocomial infections and hospital environments [55-57].

Intriguingly, the present investigation discovered that 92% of environmental isolates could establish biofilms with varying capacities (Table 6). The results are consistent with the findings of previous studies conducted by Khosravy *et al*. [58] and Smitran *et al*. [59], which demonstrated that (90%) and (91.9%) of *A. baumannii* isolates could form biofilms, respectively. Conversely, other authors have also reported a greater capacity for biofilm formation. All isolates were discovered to have the ability to generate biofilm in the research conducted by Khalil *et al*. [60]. In Jordan, Ababneh *et al*. [61] conducted an additional study in which all isolates could form biofilms.

The distribution of isolates among the collected environmental samples varied depending on the isolation sources. According to Table 3, the ventilator had the highest distribution levels of *A. baumannii* isolates (21.8%), followed by pillows and bed linens (14.5%), and chairs (1.1%) had the lowest levels (1.1%) in the current study. The present findings are consistent with the research conducted by Barbara *et al*. [62], which determined that the highest percentage of *A. baumannii* environmental isolates was from ventilators (37.5%), followed by pillows and bed linens (25%). The situation above adversely affects hospital individuals, particularly those in surgical wards or intensive care units. Therefore, the high prevalence of *A. baumannii* in various environmental sources within hospitals may suggest insufficient infection control protocols.

The isolates were classified into four categories based on their biofilm production capabilities: non-biofilm, weak, moderate, and strong biofilm producers. Table 6 illustrates that 42 (48.27%) isolates exhibited a strong capacity for biofilm formation. Previous research has demonstrated that 45.4% of *A. baumannii* isolates establish a robust biofilm [63].

Acinetobacter baumannii has developed extraordinary antibiotic resistance due to the accumulation of numerous resistance genes in its genome. Numerous molecular and epidemiological investigations have been conducted on the extensive repertoires of biofilm-related genes. These genes allow the organisms to form biofilms and adhere to a diverse array of surfaces and environments [34, 64,65]. The PCR results obtained indicated that the majority of isolates exhibited a strong association between the frequency of virulence genes, including *omp*A, *csu*E*, bap*, and *blaPER1,* and the development of biofilms.

According to current findings, the *omp*A*, csu*E*, bap*, and *blaPER1* genes were simultaneously present in all strong biofilm-forming isolates (Table 6). Also, these genes were present in some moderate biofilm producers and some weak producers. The findings are consistent with previous investigations conducted in various countries [35, 66]. Yang *et al*. [67] conducted an additional investigation that demonstrated that *A. baumannii* strains that contain

the *bap, omp*A, and *csu*E genes have a greater propensity to form strong biofilms than strains that lack these genes.

However, the gene with the highest prevalence was *csu*E 83 (95.40%), with *omp*A 73 (83.90%), *bap* 70 (80.50%), and *blaPER1* 31 (35.60%) following in that order. These results are consistent with previous investigations that reported that the most prevalent gene associated with biofilm in *A. baumannii* isolates was *csu*E. Consequently, *csu*E was identified in 93.2% and 91.3% of isolates in the Monfared *et al*. [68] and Li *et al*. [69] studies, respectively. However, the present findings suggest that environmental isolates that lack the *cau*E gene are also capable of establishing biofilms. Elbehiry *et al*. [70] also discovered that the development of *A. baumannii* biofilms was not reliant on cause, which is consistent with our findings. In the same vein, Khoshnood *et al*. [71] discovered that the *csu*E gene was more frequently present in strong biofilm producer strains than in weak or non-formers, but it was not necessary for biofilm formation. This is supported by the fact that it encodes proteins essential for initiating biofilm production and improves biofilm mass and matrix amount formation [72].

The *omp*A of *A. baumannii* is a critical virulence factor that facilitates biofilm production, adhesion, and invasion on abiotic surfaces [73,74]. In the current investigation, molecular analyses demonstrated that the *omp*A gene was present in 83.90% of environmental isolates (Table 6). Similar results were observed in Iran and China, with 81% and 89.6% positive isolates, respectively [35, 69]. Furthermore, analytical investigation verified a robust correlation between biofilm production by isolates and the occurrence of the *omp*A.
Biofilm-related proteins are expressed on the surfaces of bacterial cells; numerous *A. baumannii* strains that possess the *bap* gene can establish biofilms on both living and non-living surfaces [75]. *bap* serves several functions, including the preservation of the structure of fully formed biofilms and the facilitation of initial biofilm adhesion and maturation [76]. Our results indicated that *bap* was detected in 80.50% of the analyzed isolates, as illustrated in Table 6. Taiwan and China reported comparable outcomes of 81% and 85.81.7%, respectively [63,69]. Additionally, the statistical investigation demonstrated a substantial association between the occurrence of *bap* and the production of microbial biofilms. Previous research demonstrated a considerable difference in biofilm formation capabilities between isolates containing the *bap* gene and those without it [77]. Hence, the current observations have verified that *bap* is a critical factor in biofilm development.

blaPER-1 was the least prevalent gene in our research, as it was detected in 35.6% of the tested isolates, as detailed in Table 6. Qi *et al*. [78] discovered that *blaPER-1* was present in 30.2% of the tested isolates, which is consistent with the current research. It is noteworthy that prior investigations have verified a positive correlation between the *blaPER-1* and the development of microbial biofilms [79]. Additionally, previous research has indicated that *A. baumannii* with *blaPER-1* exhibited considerably higher biofilm production than those lacking the gene [80]. Nevertheless, Sherif *et al*. [81] conducted a study that uncovered no correlation between *blaPER-1* and biofilm production. Therefore, it is probable that *blaPER1* improves the adhesion capacity of cells that express this gene, although it may not consistently contribute to biofilm production [82].

To reduce the incidence of opportunistic *A. baumannii* infections in hospital environments, it may be advantageous to establish a correlation between the survivability factors of environmental isolates and the likelihood of their transmission from the environment to the patient's body.

Conclusion

Our results in the present investigation indicated that hospital environments could be a substantial source of multidrug-resistant *A. baumannii* isolates. Many *A. baumannii* strains exhibited robust antimicrobial resistance to various antibiotic classes. Our findings demonstrated that the majority of environmental *A. baumannii* isolates exhibited strong biofilm-forming capabilities. Our study also identified that *bap*, *blaPER-1*, and *ompA* are recognized as biofilm-related genes in different strains of *A. baumannii*, which may promote biofilm production and aid in environmental adaptation. In contrast, the *csu*E gene did not exhibit any correlation with the development of biofilm. The persistence and potential dissemination of multidrug-resistant and biofilm-producing bacteria, as well as their associated genes, in hospital environments, pose a substantial public health concern. Consequently, the propagation of *A. baumannii* infections in hospital environments is significantly reduced by the early identification and implementation of appropriate control strategies.

Declaration

- I confirm that I am the sole author of all the Tables and Figures included in the manuscript.
- There are no conflicts of interest.

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