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Original Article

Molecular Detection of *Pap II*, *OmpA*, and *LuxR* Genes Responsible for Biofilm Formation in *Acinetobacter baumannii* Isolated from Hospitalized Patients

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Abstract

Background: The first pathogen to be designated a "red-alert" human pathogen is *Acinetobacter baumannii*, which is on the list of infections that must be treated urgently with new antibiotics. Infections due to this bacterium are on the rise, especially in patients admitted to hospital intensive care units. It can create biofilms on both biotic and abiotic surfaces. **Objectives:** This study aimed to detect biofilm formation by *A. baumannii* phenotypically and genotypically. **Materials and Methods:** A total of 250 samples were subjected to bacterial identification using the VITEK-2 compact system, which showed 42 *A. baumannii* isolates. Biofilm formation was phenotypically investigated using the microtiter plate method. **Results:** The results revealed three stages of biofilm formation: 5 (11.6%) nonbiofilm, 13 (30.2%) weak biofilm, 15 (34.9%) moderate, and 10 (23.3%) strong biofilm formation. The isolates from intensive care unit (ICU) patients had strong, moderate, weak, and nonforming biofilm ability in higher rates of biofilm producers compared with the isolates from samples of hospital wards. The polymerase chain reaction (PCR) products showed genotypically positive results as follows: *PapII* 12 (31.5%), *OmpA* 11 (28.9%), and *LuxR* 8 (21%) out of 38 positive samples of *A. baumannii* for all genes. **Conclusion:** Isolates of *A. baumannii* appeared in different stages of biofilm formation with a higher percentage rate in the ICU compared with hospitalized patients. The PCR products for isolates of *A. baumannii* showed that *PapII*, *OmpA*, and *LuxR* showed positive results.

Keywords: *Acinetobacter baumannii*, biofilm, ICU, *LuxR*, molecular detection, *OmpA*, *Pap II*

Introduction

Acinetobacter is a genus of Gram-negative bacteria, nonmotile coccobacilli.[1] This commensal bacteria is obligately aerobic, but in recent years it has developed into an opportunistic pathogen.[2] The nosocomial *Acinetobacter baumannii* causes numerous diseases, including septicemia, pneumonia, skin and soft tissue infections, blood infections, and even mortality.[3] One of the virulence factors that helps bacteria survive in hospitals and cause device-related diseases is the biofilm.[4] Biofilm is an arrangement of cells that differs from their planktonic counterparts in morphology, metabolism, and physiological characteristics.[5]

The bacteria that form biofilm are invariably associated with chronic humans.[2] Polysaccharides help the bacteria in the biofilm with intercellular adherence, and biofilm is most tightly regulated by quorum sensing and two additional component regulatory systems.[6] The majority of clinical isolates of *Acinetobacter* spp. exhibit an important trait called biofilm development, whereas surface microbial cell assemblages are encased in an extracellular polymeric matrix.[7] Epidemiological research studies show that *Acinetobacter* biofilms contribute to infectious disorders, such as cystic fibrosis, periodontitis in the bloodstream, and urinary tract infections due to their ability to stick to medical equipment.[8]

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It is well-recognized that *Acinetobacter* exhibits resistance to the bulk of antibiotics that are offered for sale (penicillins, amino-glycosides, cephalosporins, and quinolones), which poses a significant therapeutic challenge.[9] Pili and proteins of the outer membrane, as well as macromolecule secretions, such as extracellular deoxyribonucleic acid (DNA; eDNA), are all common elements that affect biofilm development.[10] It has been shown that a bloodstream isolation of *A. baumannii* produces a protein that is, related to a staphylococcal biofilm-associated protein (Bap), which is required for the development of biofilms on inorganic surfaces,[11] high-molecular-weight proteins from the Bap family can be found on the surface of bacterial cells.[7]

The study confirms earlier findings by demonstrating that the outer membrane protein A (*OmpA*) of *A. baumannii* contributes to the formation of strong biofilms on plastic surfaces.[12] The pili, which mediate adhesion and biofilm formation, are also a major factor in *A. baumannii's* capacity to produce biofilms.[13] This is in contrast to genome-wide analyses that show that *luxR* homologs typically outnumber *luxI* homologs[14] and that other species have a *luxR* homolog for recognizing AHL signals but lack *luxI* and as a result do not participate in signaling.[15]

Materials and Methods

Collection and samples

A total of 250 samples were taken from burns, injuries, and cerebrospinal fluid (CSF) infected adults and children in hospitals in Baghdad and Al-Mosul. Each sample was grown in Brain Heart Infusion Broth (BHIB), then, incubated for 24 h at 37°C. Each sample was inoculated on blood agar and MacConkey agar. VITEK-2 Compact System (BioMérieux, Marcy l'Etoile, France) was used to confirm the biochemical characterization of the isolates. Then, stored in glycerol stock at −20°C.

Biofilm formation by microtiter plate method

Inoculating 20 µL of bacteria in wells of 96-well polystyrene microtiter plate wells filled with 180 µL of BHIB with 0.1% glucose, plates were incubated at 37°C for 24 h to determine the amount of biofilm formation. The experiments were carried out in triplicates, washed three times with phosphate-buffered saline, then, stained by Crystal violet 0.1% remained at 15 min, followed by 200 µL of glacial acetic acid 33% added to each well left for 10 min then biofilm production measured the optical density (OD) by enzyme-linked immunosorbent assay reader, then, calculate the results as follows: OD_{630} (bacteria) divided on 3 then Biofilm = OD_{630} (bacteria) – OD_{630} (control). Biofilm-producing strains were scored as strong, moderate, and weak as mentioned.[12] The results were read as follows: weak: ODc < OD ≤ 2 × ODc, moderate: 2 × ODc < OD ≤ 4 × ODc, and strong: OD > 4 × ODc.[16]

DNA extraction:

Genomic DNA Purification Kit (Geneaid, Taiwan) was used. Primers from Promega (Madison, WI, USA) as shown in Table 1. Conventional polymerase chain reaction (PCR) is used to detect some biofilm genes (*PapII*, *OmpA*, and *LuxR*), PCR Programs are represented in Table 2.

Ethical consideration

Ethical approval for conducting this study was requested from the College of Health and Medical Sciences, Baghdad, Iraq.

Results

Isolation of *A. baumannii*

A total of 43 *A. baumannii* isolates with the standard strain ATCC 19606 (No. 24) were distributed from both sexes

Table 1: List of primers that were used in this study

Genes	Primer sequence from (5′ → 3′)	Product size (pb)	References
PapII	F: ATGCCTGAGATACAAA TTATTGCCAAGGATAATC R: AGGTGCTGAAGAATCA TCATTAC	561	[17]
LuxR	F: TGTGGATGATGTGCCG GAAA R: ATCCGGTAAGCATCGG TGTG	169	[18]
OmpA	F: GTTAAAGGCGAC GTAGACG R: CCAGTGTTATCTG TGTGACC	578	[19]

Table 2: PCR programs

Steps	*OmpA*		*PapII*		*LuxR*	
	Temperature (°C)	Time	Temperature (°C)	Time	Temperature (°C)	Time
Initial denaturation	94	5 min	95	5 min	95	10 min
Denaturation	94	1 min 34 cycles	95	30 s 33 cycles	95	15 s 39 cycles
Annealing	58	1 min	58	30 s	58	1 min
Extension	72	45 s	72	45 s	72	45 s
Final extension	72	5 min	72	10 min	72	5 min

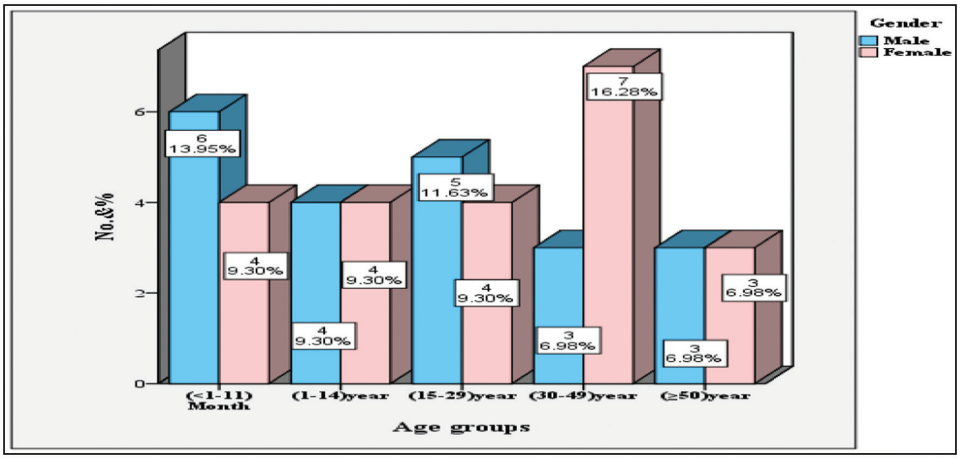

Figure 1: Distribution of sample according to age groups and gender

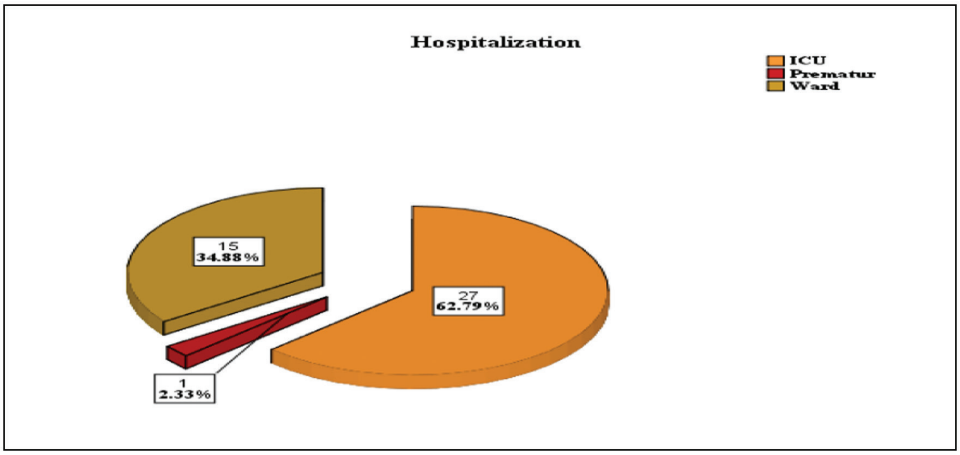

Figure 2: Distribution of samples according to hospitalized patients

22 isolates from female in rate (51.2%) and 21 isolates from male in rate (48.8%) arranged between 1 month and 50 year [Figure 1].

Distribution of the specimens according to hospitalized patients

The samples were collected from hospitalized patients: 27 samples (62.8%) from ICU, 15 samples (34.9%) from the ward, and 1 sample (2.3%) from the premature, and based on this, their percentages were as shown in Figure 2.

Distribution of the specimens according to sources of infections

The results are shown in Figure 3 a high percentage of *A. baumannii* in burn 13 (30.2%), blood 12 (27.9%), wound 8 (18.6%), 5 (11.6%), 2 (4.7%), CSF 2 (4.7%), and abscess only 1 (2.3%) isolates.

Quantitative biofilm formation by microtiter plate method

Biofilm formation of 43 isolates studied represented 5 (11.6%) isolates that did not form biofilm, 13 (30.2%) weak biofilm formation, 15 (34.9%) with moderate biofilm formation, and 10 (23.3%) with strong biofilm formation. Their percentages are shown in Figure 4.

Distribution of isolates according to classes of biofilm formation and hospitalization

A. baumannii was isolated from patients in higher percentages at a rate of 62.8% in the ICU compared with hospitalized patients (ward) at a rate of 34.9%. Moderate formation of biofilm higher at a rate of 23.3% (10 isolates) in addition to the standard strain ATCC19606, followed by strong and weak biofilm formation at a rate of 16.3% (7 isolates for each), and the last nonforming biofilm formation at a rate of 7.0% (3 isolates) in the ICU as shown in Table 3.

Distribution of isolates according to classes of biofilm formation and specimens

Biofilm formation by *A. baumannii* showed strong formation from blood culture samples at a rate of 14.0% (six isolates), followed by moderate formation from wound samples at a rate 11.6% (five isolates), followed by weak biofilm formation from burn samples at a rate

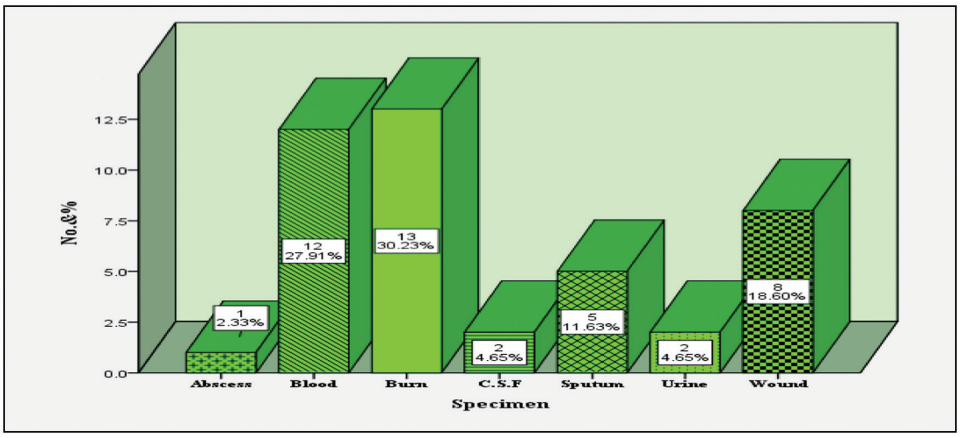

Figure 3: Distribution of samples according to the type of specimen

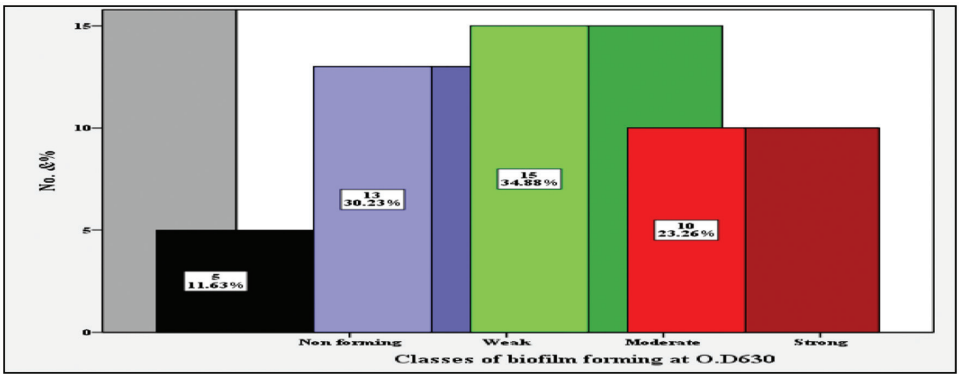

Figure 4: Distribution of sample according to classes of biofilm formation at OD_{630}

Table 3: Distributions of isolates according to classes of biofilm formation and hospitalization

Hospitalization		Classes of biofilm formation at OD_{630}				Total
		Nonforming	Weak	Moderate	Strong	
ICU	*N*	3	7	10	7	27
	Percentage (%)	7.0	16.3	23.3	16.3	62.8
Premature	*N*	1	0	0	0	1
	Percentage (%)	2.3	0.0	0.0	0.0	2.3
Ward	*N*	1	6	5	3	15
	Percentage (%)	2.3	14.0	11.6	7.0	34.9
Total	*N*	5	13	15	10	43
	Percentage (%)	11.6	30.2	34.9	23.3	100.0

16.3% (seven isolates), and last nonforming biofilm from sputum samples at a rate 4.7% (two isolates) as shown in Table 4.

Distribution of isolates according to classes of biofilm formation and age groups

A. baumannii is distributed in different ages ranging from 1 day to 60 years the first category, < 1–11 months gives higher strong biofilm formation at a rate of 11.6% (five isolates), followed by 30–49 and >50 years categories, which moderate biofilm formation at a rate of 9.3% (four isolates) respectively, followed by 15–29 years categories give weak biofilm formation at a rate of 9.3% (four isolates), and the 15–29 and 30–49 years that give nonforming biofilm at a rate of 4.7% (two isolates) as shown in Table 5.

Distribution of isolates according to classes of biofilm formation and gender

The results revealed biofilm formation by *A. baumannii* in male strong biofilm with rate of 14.0% (six isolates) and nonforming biofilm at a rate of 7.0% (three isolates). While females have shown moderate formation at a rate of 20.9% (nine isolates), and weak formation at a rate of 16.3% (seven isolates) as shown in Table 6.

Table 4: Distribution of sample according to classes of biofilm formation at OD_{630} and specimen

Specimen		Classes of biofilm formation at OD_{630}				Total
		Nonforming	Weak	Moderate	Strong	
Abscess	*N*	0	0	1	0	1
	Percentage (%)	0.0	0.0	2.3	0.0	2.3
Blood	*N*	1	3	2	6	12
	Percentage (%)	2.3	7.0	4.7	14.0	27.9
Burn	*N*	1	7	3	2	13
	Percentage (%)	2.3	16.3	7.0	4.7	30.2
C.S.F	*N*	0	0	1	1	2
	Percentage (%)	0.0	0.0	2.3	2.3	4.7
Sputum	*N*	2	1	2	0	5
	Percentage (%)	4.7	2.3	4.7	0.0	11.6
Urine	*N*	0	1	1	0	2
	Percentage (%)	0.0	2.3	2.3	0.0	4.7
Wound	*N*	1	1	5	1	8
	Percentage (%)	2.3	2.3	11.6	2.3	18.6
Total	*N*	5	13	15	10	43
	Percentage (%)	11.6	30.2	34.9	23.3	100.0

Table 5: Distribution of isolates according to classes of biofilm formation and age groups

Age groups		Classes of biofilm formation at OD_{630}				Total
		Nonforming	Weak	Moderate	Strong	
<1–11 months	*N*	1	1	3	5	10
	Percentage (%)	2.3	2.3	7.0	11.6	23.3
1–14 years	*N*	0	3	3	2	8
	Percentage (%)	0.0	7.0	7.0	4.7	18.6
15–29 years	*N*	2	4	1	2	9
	Percentage (%)	4.7	9.3	2.3	4.7	20.9
30–49 years	*N*	2	3	4	1	10
	Percentage (%)	4.7	7.0	9.3	2.3	23.3
≥50 years	*N*	0	2	4	0	6
	Percentage (%)	0.0	4.7	9.3	0.0	14.0
Total	*N*	5	13	15	10	43
	Percentage (%)	11.6	30.2	34.9	23.3	100.0

Distribution of isolates according to hospitalization specimens

A. baumannii samples scored higher rates in blood culture, burn, and wound infections at a rate of 20.9% (nine isolates), 18.6% (eight isolates), 14.0% (six isolates), respectively, from lying ICU patients as shown in Table 7.

Detection of *PapII*, *OmpA*, and *LuxR* by PCR

PCR products of 38 *A. baumannii* represented the percentage of positive results of *PapII* 12 (31.5%), *OmpA* 11,(28.9%), and *LuxR* 8 (21%) genes as seen in Table 8 and Figures 5–7.

Discussion

(1) *A. baumannii*, an opportunistic pathogen, has become a significant source of concern, as it frequently causes healthcare-associated infections, notably infections of the lower respiratory tract, the bloodstream, and the urinary system.[20] Results shown similarly with Azizi *et al.*[21] who determined high infections by *A. baumannii* in the ICU. The results are similar to other research in a world-determined *A. baumannii* in different infection sites, especially patients who are lying down and their surroundings are among the main sources of biofilm in ICUs for hospitalized patients,[22] the results are also similar to Iraqi research.[23]

(2) *A. baumannii* is a major contributor to nosocomial infections because of its aggressiveness and growing drug resistance. The capacity of *A. baumannii* to produce biofilms aids in its survival in unfavorable environmental conditions, such as hospital environments and medical devices. In this study, the results showed similar to other Iraqi studies giving

Table 6: Distribution of isolates according to biofilm formation ability and gender

Gender		Classes of biofilm formation at OD_{630}				Total
		Non forming	Weak	Moderate	Strong	
Male	*N*	3	6	6	6	21
	Percentage (%)	7.0	14.0	14.0	14.0	48.8
Female	*N*	2	7	9	4	22
	Percentage (%)	4.7	16.3	20.9	9.3	51.2
Total	*N*	5	13	15	10	43
	Percentage (%)	11.6	30.2	34.9	23.3	100.0

Table 7: Distribution of isolates according to hospitalization by specimen

Specimen		Hospitalization			Total	Sig.
		ICU	Premature	Ward		
Abscess	No.	0	0	1	1	
	%	0.0%	0.0%	2.3%	2.3%	
Blood	No.	9	1	2	12	0.136
	%	20.9%	2.3%	4.7%	27.9%	
Burn	No.	8	0	5	13	0.166
	%	18.6%	0.0%	11.6%	30.2%	
C.S.F	No.	1	0	1	2	
	%	2.3%	0.0%	2.3%	4.7%	
Sputum	No.	3	0	2	5	
	%	7.0%	0.0%	4.7%	11.6%	
Urine	No.	0	0	2	2	
	%	0.0%	0.0%	4.7%	4.7%	
Wound	No.	6	0	2	8	0.116
	%	14.0%	0.0%	4.7%	18.6%	
Total	No.	27	1	15	43	
	%	62.8%	2.3%	34.9%	100.0%	

(*) HS: Highly Sig. at P = 0.05 S: Sig. at P = 0.05 NS: Non Sig. at $P > 0.05$

Table 8: PCR products showed that *PapII, OmpA*, and *LuxR* out of 38 *A. baumannii* for all genes

Gene	*N*	Percentage (%)
Pap II	12/38	31.5
Lux R	8/38	21
OmpA	11/38	28.9

three types of biofilm formation strong, moderate, weak, and the last one nonbiofilm formation also observed moderate biofilm formation at higher rates in most Iraqi research.[23] The results are near to the results of the local study,[24] but disagree with Jawad *et al.*,[25] which showed a rate of 94% high production of biofilm and a moderate rate of 10%.

(3) In a Taiwanese study conducted on clinical samples of *A. baumannii*, the results appeared among (154) clinical samples, 15.6% were weak biofilm producers, whereas moderate and strong biofilm formations appeared in 32.5% and 45.4%[10,26] was discovered that 66 (70.1%) of the *A. baumannii* isolates produced biofilms and 28 (29.9%) did not. Out of 66 biofilm-producing bacteria, isolates from 16.2%, 34.3%, and 20.2% were deemed to be weak, moderate, and strong biofilm producers.

(4) Hospital-acquired infections and *A. baumannii*, a disease that has recently become more prevalent, are closely related.[26] According to numerous studies, the production of biofilms is the cause of *A. baumannii's* high resistance to different antibiotics as well as its strong capacity to survive in extreme environments.[24]

In the current study, it was investigated how the clinical specimens of *A. baumannii* linked to each other in terms of the development of biofilms, antibiotic resistance, and related genes, and examined a phenotype profile with antibiotic resistance, biofilm formation, and biofilm production capacity, and found that antibiotic resistance was strongly correlated with these traits. Some of the drug-resistant bacteria were more capable of forming biofilms when exposed to specific antibiotics; penicillin-resistant strains, for instance, showed more capability to do so.[27]

Pap's pili system has been integrated into the development of *A. baumannii* biofilms and shares 80% of its genetic makeup with *Escherichia coli* pili.[28] Pap and other virulence factors of *A. baumannii* have been linked to

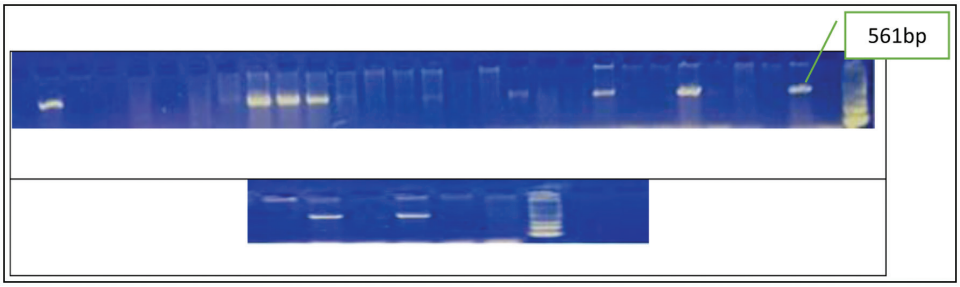

Figure 5: Electrophoresis of PCR amplification produces biofilm *PapII* gene *A. baumannii*, expected amplification products (561 pb), ladder (MW = 100–1000 bp), agarose 1.5%, 150 V, 30 min

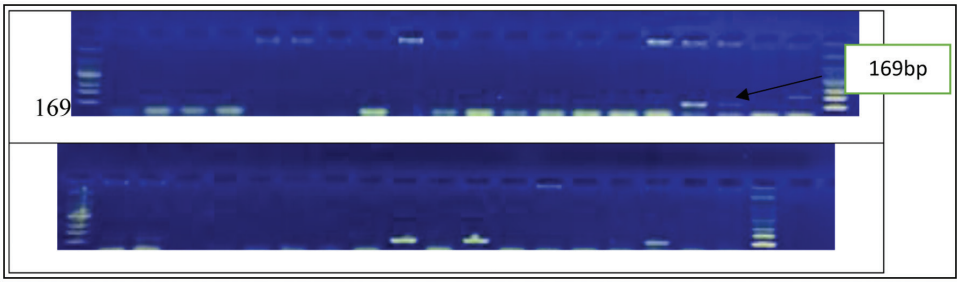

Figure 6: Electrophoresis of PCR amplification produces biofilm *LuxR* gene *A. baumannii*, expected amplification products (169 pb), ladder (MW = 100–1000 bp), agarose 1.5%, 150 V, 30 min

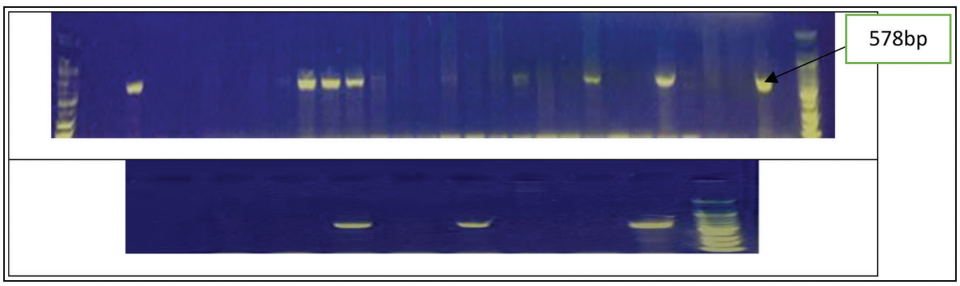

Figure 7: Electrophoresis of PCR amplification produces biofilm *OmpA* gene *A. baumannii*, expected amplification products (578 pb), ladder (MW100–1000bp), agarose 1.5%, 150 V, 30 min

adhesion and biofilm development. It has been discovered that the pap operon is essential for the movement of bacteria from the urinary bladder to the kidney because it encodes proteins that are similar to Pap pili in *E. coli.*[29]

Facilitating adhesion and biofilm formation is the usher-chaperone assembly mechanism, including gene *OmpA*, which is associated with biofilm formation. *OmpA*, the most prevalent surface protein on the pathogen, also contributes to biofilm development and complement resistance. *OmpA* also encodes a porin protein that contributes to biofilm formation.[30]

To promote bacterial survival in both the inside and the outside of the host, two essential stress survival techniques and possibly significant virulence-associated factors are required. *A. baumannii* may survive and thrive in difficult situations and settings because of its capacity to build biofilms. It has been demonstrated that *A. baumannii* may create biofilms on both biotic and abiotic surfaces, including epithelial cells and the glass used in intensive care units.[31]

In addition, *A. baumannii* had *LuxR* gene made up 21% of the test isolates. Number sensing one of the key characteristics of the bacterial cells that promotes connection and communication within the surroundings is QS. Autoinducers (AIs) are small, diffusible signaling molecules produced by Quorum Sensing (QS) that interact with receptors of nearby cells to activate and induce the expressions of specific genes, allowing cells to respond to stimulation in a coordinated manner.[32] The QS system in bacteria has been identified as the LuxI/LuxR system in Gram-negative bacterium, which uses an acyl-homoserine lactone as an autoinducer type-I (AI-1) and a QS molecule present in both the Gram +ve and Gram −ve bacterium.[33] It has been discovered that *Acinetobacter* spp. has an AI-I acyl-homo-serine lactone (AHL) QS system. The production of *N*-(3-hydro-xydo-decanoyl)-L-HSL (AHL) occurs in *A. baumannii*. The signaling molecules permeate the membrane in the second and third steps of the process. It responds to the AHL receptor and a baR found on the outer surfaces of nearby bacterial cells. The transcription

of genes related to pathogenicity and biofilm is then activated once the receptor signals complex is retrieved from the cell surface and attaches to the promoter regions.[28]

Conclusion

Isolates of *A. baumannii* appeared in different stages of biofilm formation with a higher percentage rate in the ICU compared with hospitalized patients (ward), whereas the age <1–11 months showed higher strong biofilm formation and the 15–29 and 30–49 years showed nonforming biofilm compared with the standard strain ATCC19606. The PCR products for isolates of *A. baumannii* showed that *PapII*, *OmpA*, and *LuxR* showed positive results.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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