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Detection of PgaABCD Proteins in Biofilm Producing *Acinetobacter Baumannii* Isolates

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ABSTRACT

Acinetobacter baumannii is a common cause of nosocomial infections. This bacterium is able to survive in hostile environments (desiccation, antimicrobial therapies, nutrient unavailability) beside colonization biotic and abiotic surfaces and form biofilm in hospitals and long-term care institutions. Biofilm is a three-dimensional structure of a multicellular complex in which the cells are embedded in an Extracellular Polymeric Substance (EPS) that was produced by the organism itself. Biofilm matrix contains proteins, ions, nucleic acids, and polysaccharide polymers. The main factor that leads to biofilm formation in *A. baumannii* is the creation of the exopolysaccharide poly-1,6-N-acetylglucosamine (PNAG), which is a virulence factor required for adhesion and aggregation by many Gram-negative bacteria. Many studies indicated that PNAG is an important factor to keep the integrity of *A. baumannii* biofilms in a more dynamic and stressful environment. A cluster of four genes (*pgaA, pgaB, pgaC*, and *pgaD*) are responsible for PNAG, which is encoded by pgaABCD locus. Therefore, the aim of this work was to detect proteins encoded by this locus using SDS-PAGE gel in *A. baumannii* isolates. Using PCR analysis to detect *16S rRNA* concluded that all 24 isolates showed a positive amplification with 242bp. *Acinetobacter baumannii* clinical isolates showed high resistance percentage to ampicillin-sulbactam (AMS) and ceftazidime (CAZ) with 100% and 91% respectively. Furthermore, the isolates showed 83.3% for cefepime (FEP) and 70.8% for amikacin (AK), while the isolates showed a variable resistance percentage toward other antibiotics. By using Congo red method indicated that 66.7% were positive to produce biofilm and 33.3 were non-forming biofilm. Protein sequences alignment showed 99%, 99%, 100% and 99% identity for PgaA, PgaB, PgaC and PgaD respectively with protein GenBank database.

KEYWORDS: *pgaABCD* locus; PNAG; *Acinetobacter baumannii*; Biofilm; poly-1,6-N-acetylglucosamine.

الخلاصة

تعد بكتيريا *Acinetobacter baumannii* من الأسباب الشائعة لعدوى المستشفيات. هذه البكتيريا قادرة على البقاء على قيد الحياة في بيئات قاسيه (الجفاف، العلاجات المضادة للميكروبات، عدم توفر المغذيات) ونموها على الأسطح الحيوية وغير الحيوية وتشكيل الأغشية الحيوية في المستشفيات وردهات الرعاية المركزه. الغشاء الحيوي عبارة عن هيكل ثلاثي الأبعاد لمركب متعدد الخلايا يتم فيه دمج الخلايا في (EPS)Extracellular Polymeric Substance تم إنتاجها بواسطة الكائن الحي نفسه. يحتوي خليط الغشاء الحيوي على البروتينات والأيونات والأحماض النووية وبوليمر متعدد السكريات. العامل الرئيسي الذي يؤدي إلى تكوين الأغشية الحيوية في *A. baumannii* هو تكوين متعدد السكريات الخارجية poly-1,6-N-acetylglucosamine (PNAG)، وهو عامل ضراوة ضروري للالتصاق والتجمع بواسطة العديد من البكتيريا سالبة لصبغة كرام. أشارت العديد من الدراسات إلى أن PNAG هو عامل مهم للحفاظ على سلامة الأغشية الحيوية لل *A. baumannii* في بيئة أكثر ديناميكية وإجهادًا. مجموعة من أربعة جينات (*pgaA* و *pgaB* و *pgaC* و *pgaD*) مسؤولة عن PNAG، والتي يتم ترميزها بواسطة locus *pgaABCD* لذلك كان الهدف من هذا العمل هو الكشف عن البروتينات المشفرة بواسطة هذا الlocus باستخدام هلام SDS-PAGE في عزلات *Acinetobacter baumannii*. باستخدام PCR للكشف عن *16S rRNA* خلص إلى أن الـ 24 عزلة أظهرت ناتج إيجابيًا مع 242bp. أظهرت عزلات *A. baumannii* السريرية نسبة مقاومة عالية لـ ceftazidime (CAZ) and ampicillin-sulbactam (AMS) بنسبة 100٪ و91٪ على التوالي. كما أظهرت العزلات 83.3٪ cefepime (FEP) و70.8٪ amikacin (AK)، بينما أظهرت العزلات نسبة مقاومة متغيرة تجاه المضادات الحيوية الأخرى. باستخدام طريقة Cong red، أشار إلى أن 66.7٪ كانت موجبة لإنتاج الأغشية الحيوية و33.3٪ كانت غير مكونة للأغشية الحيوية. أظهرت اصطفاف تسلسل البروتين 99٪ و99٪ و100٪ و 99٪ لـ PgaA و PgaB و PgaC و PgaD على التوالي مع قاعدة بيانات البروتين.

INTRODUCTION

Acinetobacter baumannii (*A. baumannii*) is a Gram-negative coccobacillus, typical temperature to growth is mesophilic (37ºC), which has recently become a major cause of infections connected to healthcare globally. It is linked to high morbidity and mortality rates, prolonged hospital stays, and increased medical costs[1]. *A. baumannii* could

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cause various infections including bacteraemia, urinary tract infections (UTI), skin and soft-tissue infections, and bone infections. Ventilator-associated pneumonia in patients admitted to hospital intensive care units (ICUs) is one of the most prevalent illnesses associated with this bacterium [2]. Traumatic patients have been reported to develop *A. baumannii* infections [3-5]. Several infections caused by *A. baumannii* have been recorded in American service members who have just returned from the conflicts in Iraq and Afghanistan [6-8].

Antimicrobial resistance among *Acinetobacter* species has been reported more frequently, including multidrug resistance (MDR) to ampicillin-sulbactam (AMS), aminoglycosides, fluoroquinolones, and carbapenems [9][1]. *A. baumannii* isolates with reduced susceptibility to tigecycline and colistin (two drugs used as a last therapeutic option against these bacteria) have emerged recently [10]. The relatively low permeability of *Acinetobacter* to antibiotics and its acquisition of many different resistance genes acquired from frequent environmental exposures may be contributing factors to the capacity of *Acinetobacter* species for extensive antimicrobial resistance [11].

The major virulence factor in *A. baumannii* is the ability to form a biofilm that is defined as a complex of biological matrices made up of polysaccharide polymers, proteins, ions, and nucleic acids. The primary component of biofilms in *Staphylococcus epidermidis* and *Staphylococcus aureus* has been confirmed in publications to be Poly-(1-6)-N-acetyl glucosamine (PNAG) [12] [13]. An operon called *ica*ADBC regulates the ability of staphylococci to synthesize PNAG. Similar to the *ica*ADBC operon, the *pga*ABCD operon, which regulates PNAG biosynthesis, has been discovered in the genomes of *A. baumannii* and other Gram-negative bacteria [14] [15].

It was shown that deletion of the *pga* locus rendered the S1Δ*pga* strain of *A. baumannii* incapable of generating PNAG, whereas complementation with the *pgaABCD* genes completely recovered the wild-type PNAG phenotype. Furthermore, it was demonstrated that when the *A. baumannii pga* locus was expressed heterologous in *E. coli*, a large amount of PNAG was produced, in contrast to the absence of polysaccharides in cells carrying an empty vector [16]. Along with aiding in cell-to-cell adhesion, PNAG is a key component of virulence and protects bacteria from the body's natural defences, [17]. PgaC is an N-glycosyltransferase homolog of *IcaA*, according to the most in-depth investigation of conserved protein domains; meanwhile, PgaB is a lipoprotein with putative polysaccharide N-deacetylase domains that are comparable to those of IcaB, while PgaA and PgaD have no functional homologies [17]. Previous study in *E. coli* concluded that all genes of the locus *pgaABCD* have to be functional to enhance the attachment and formation of biofilm [14]. Therefore, the aim of this work was to detect proteins encoded by this locus using SDS-PAGE gel in *Acinetobacter baumannii* isolates.

MATERIALS AND METHODS

Acinetobacter baumannii isolation: Twenty-four isolates of *A. baumannii* were collected from the Medical City hospital, Ibn Al-balady Maternity & Children's Hospital, Hospital martyr Dhari Al-Fayad and Al-Imamain Al-Kadhimain Medical City over the period from October to December 2021. To avoid any risk of contamination, all samples were obtained using a sterile container in an aseptic environment.

***Acinetobacter baumannii* Isolates Identification:** All samples were cultured on culture medium as first step of identification, biochemical tests including oxidase, catalase and VITEK2 system was used. As well as molecular identification via *16S rRNA* was performed [18].

Genomic DNA extraction: Boiling and Freeze-thawing method was used according to Chen *et al.* (2022) for extract genomic DNA of *A. baumannii* and no chemical reagents were included. The frozen strain was transferred to a Tryptone soya broth (TSB) and incubated aerobically at 37°C for 24 h and then transferred to MacConkey agar plates to get the third generation of growth that was used for genomic DNA purification. Approximately Ten single colonies of *A. baumannii* were transferred into Eppendorf tube containing 400µl of ddH_2O. Then, the Eppendorf tubes were incubated for 10 min in boiling water, and immediately were cooled on ice. After those tubes were frozen for 20min at -20°C. Then, all samples were thawed at room temperature and mixed by vortex for 10s. Finally, the Eppendorf tubes were centrifuged for 15min at 4°C. The upper layer (Aqueous phase) that contains DNA was transferred to a new Eppendorf tube.

Finally, the DNA samples were stored at -20°C until further experiments [19].

Identification of *A. baumannii* by convenient polymerase chain reaction (PCR): PCR was carried out to identify *A. baumannii* isolates by using a pair of primers to amplify 240 bp fragment of *16S rRNA* [18]. PCR was done in a total volume of 25µl/reaction including 12.5µl GoTaq® Green Master Mix (Promega, USA), 4.5µl ddH2O, 1µl of each primer (10 Picomoles) (Forward: 5' TTTAAGCGAGGAGGAGG3' and Reverse: 5'ATTCTACCATCCTCTCCC3'), and 5µl DNA template. The conditions of PCR were as follows: 94°C for 5min, followed by 30 cycles of 94°C for 30s, 57°C for 30s, and 72°C for 30s, with a final extension at 72°C for 7min. Amplicons were analysed by 1% agarose gel electrophoresis in 0.5× Tris/Boric acid/EDTA buffer (Promega, USA) run at 100V for 35min. 100 bp DNA Marker (Cleaver, UK) was used.

Antibiotic Susceptibility test (disc diffusion method): the Kirby-Bauer method for performing antibiotic susceptibility tests was used for twelve various antibiotics [20]. A few colonies from a pure and fresh *A. baumannii* cultures were transferred to a sterile test tube containing 5ml of normal saline (Pioneer, Iraq) and compared to 0.5 McFarland standards (1×10^8 CFU/ml). A portion of the bacterial suspension was transferred to Mueller-Hinton agar medium using a sterile cotton swab, and then the plates were allowed to dry. Using sterile forceps, antibiotic discs were placed on the inoculation plate. The plates were then inverted and incubated for 18-24 hours at 37C. The inhibition zones around the discs were measured by millimeter (mm) using a metric ruler, according to [21].

Detection of biofilm formation of *A. baumannii*: Congo Red method was used to detect biofilm. In this method, Congo Red Agar (CRA) was prepared by mixing brain heart infusion agar (37 g/L) (Himedia, India) and sucrose (5 g/L) (BDH, UK), All these materials were dissolved in up to 900ml of D.W. Congo red stain (0.8 g/L) (Himedia, India) was dissolved in 100ml of D.W and added to the medium above after autoclaving. When the medium was left to cool around 55 C, it poured into plates. Congo red medium plates were incubated aerobically at 37C for 24 hours. Colonies of bacteria appeared black and had a dry crystalline appearance indicating the creation of biofilm [22].

Detection of PgaABCD Protein in *A. baumannii*: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) method was used for detection of pgaABCD protein including [23].

Preparation of 10% ammonium per sulfate (APS): Dissolved 100mg of APS in 1ml of deionized water and prepared immediately.

Resolving gel solution (12%): According to of manufacturing leaflet, add 2.4ml of 40% Acrylamide, 2ml of Resolving buffer, 3.5ml of D.W, 80µl of 10% ammonium per sulfate (APS), 8µl of Tetramethylethylnediamine (TEMED). Mixed and poured then add 1ml of isopropanol on the top of the gel.

Stacking gel solution (4%): add 0.3ml of 40% Acrylamide, 0.75ml of Resolving buffer, 1.92ml of DW, 30µl of 10% ammonium per sulfate (APS), 3µl of Tetramethylethylnediamine (TEMED). Mixed and poured on the top of resolving gel, then inserted the comb.

SDS-PAGE running buffer (1X): The gel was transported to the tank with 1X SDS running buffer prepared by dissolving 3g of Tris base,14.4g of glycine, 1g of SDS, in 900 ml of DW then completed the volume to 1L. SDS-PAGE was run at 150V/60 minutes.

Staining solution: The gel was stained with Coomassie blue R250 for 30 minutes that was prepared by mixed 1.15 g/L of Coomassie blue R250, 400ml of methanol, 100ml of acetic acid, and completed the volume to 1L by D.W.

Destaining solution: After that, the gel was de-stained with a solution consisting of 400ml of methanol, 100ml of acetic acid, and 1L of DW.

Preparation of sample (Whole bacterial cell lysates): Samples were cultured in 5ml of Tryptone Soya Broth and incubated at 37ºC for 18-24 hours to get fresh primary cultures. Then, the growth of Acinetobacter cultures was estimated by measure the OD_{600} (600 nm) and for each sample 2 OD_{600} was harvested in Eppendorf tube by centrifugation at 14000xg for 10 minutes and the supernatant was discarded. The pellet for each sample was suspended in 100µL of 3x SDS loading buffer and boiled at 100ºC for 10 minutes. After that SDS-PAGE gel (SureCast™ HandCast System, Invitrogen) was used to separate the samples. 15µl

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of each sample were loaded in each well and 10µl of protein marker standard (SeeBlue® Plus2 Prestained protein standard, Invitrogen) was loaded as well. The gel was documented using scanner device.

RESULTS AND DISCUSSION

Morphology and culture Identification of *A. baumannii* isolates: For initial identification of *A. baumannii* isolates, they were cultured on MacConkey agar, Blood agar and CHROM^{TM} agar: The colonies of *A. baumannii* appeared as small, smooth, and pale (non-lactose fermentative) on MacConkey agar as shown in Figure 1A. While, the colonies appeared as convex, opaque, white, and with no hemolysis area around colonies (non-blood hemolytic) on Blood agar since *A. baumannii* does not have enzyme for blood hemolysis as in Figure 1B. For Further identification and purification *A. baumannii* isolates were cultured on CHROM agar^{TM}, which is considered a selective media for *A. baumannii.* All the colonies appeared red. *Escherichia coli* was used as a negative control that appeared blue as in Figure 1C.

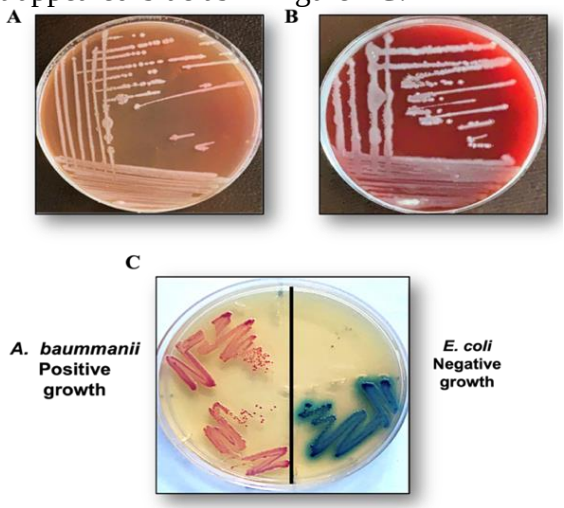

Figure 1. The *Acinetobacter baumannii* growth on different culture plates. (A) Colonies of *A. baumannii* on MacConkey agar. (B) Colonies of *A. baumannii* on blood agar. (C) Colonies of *A. baumannii* and *E. coli* on CHRMO agar.

All 24 isolates of *A. baumannii* showed a positive result for the catalase test. On other hand, they were negative for oxidase test. As well as IMVIC test was used and the isolates of *A. baumannii* showed the negative result for the Indole test, the same results appeared for Methyl red and Voges-Proskauer tests. However, a positive result was shown for the Citrate utilization test. In contrast, negative results were recorded for urease and motility tests respectively.

The VITEK 2 system was used to identify the clinical *A. baumannii* isolates. The Conventional PCR was used to detect housekeeping (*16S rRNA* gene) that is useful to determine the species of *A. baumannii.* The PCR amplified sizes were 242 bp as shown in Figure 2.

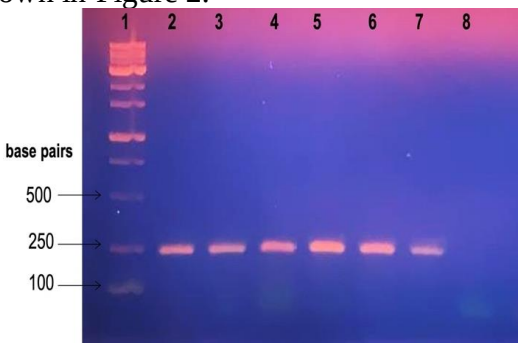

Figure 2. Genotypic detection of *A. baumannii* on agarose gel electrophoresis of *16s rRNA*. Agarose gel 1% was used with 100 V/cm /1hr and 0.05 µg/ml of ethidium bromide stain as a final concentration. Line 1:100 bp DNA ladder, line 2-7: positive result with 242 bp bands for *16s rRNA*, line 8: negative control. The UV light was used to visualize the DNA bands.

Antibiotic Susceptibility of A. baumannii isolates*:* Twelve antibiotics of eight classes were used to test antibiotic susceptibility of *A. baumannii* isolates as follows: the highest resistance percentage was 100% and 91% to ampicillin-sulbactam (AMS) and ceftazidime (CAZ) respectively. Furthermore, the isolates showed antibiotic resistance percentage as 83.3% for cefepime (FEP) and 70.8% for amikacin (AK), while the isolates showed a variable resistance percentage toward other antibiotics tested as in Figure 3.

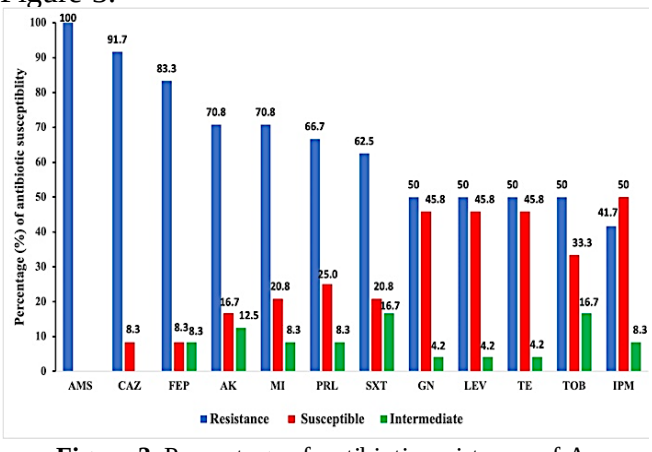

Figure 3. Percentage of antibiotic resistance of A. baumannii isolates. Ampicillin-sulbactam = AMS, Ceftazidime = CAZ, Cefepime = FEP, Amikacin = AK, Minocycline = MI, Piperacillin = PRL, Trimethoprim/ Sulfamethoxazole = SXT Gentamicin = GN, Levofloxacin = LVX, Tetracycline = TE, Tobramycin = TOB, Imipenem=IPM.

Detection of biofilm formation in *A. baumannii* isolates via Congo Red method

This method was used to detect biofilm by culturing all isolates on Congo red agar. The positive results were detected when colonies appeared in black, as shown in Figure 4A. Among of 24 isolates, it was found 16 isolates (66.7%) appeared as black colonies which identified as biofilm-forming isolates (positive results) and only 8 isolates (33.3%) as non-forming biofilm (negative results), as in Figure 4B.

Detection of A. baumannii pgaABCD protein using SDS-PAGE: PCR was used to amplify the four genes using primers listed in Table 1, then PCR products were subject to sequence. All gene sequences have been recorded in NCBI database and got the accession number, which listed in Table 2.

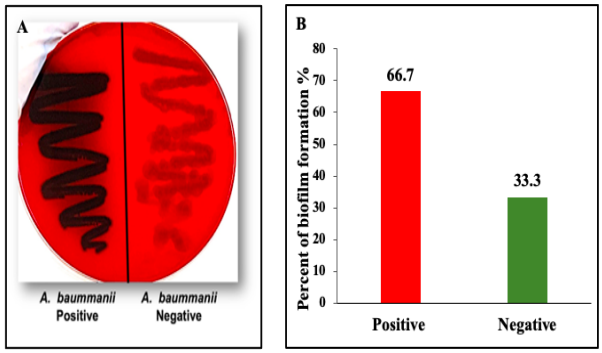

Figure 4. Biofilm formation on Congo red agar by *A. baumannii* isolates. Isolates were streaked on Congo red agar and incubated for 24 hr. at 37ºC. (A) Positive and negative results of *A. baumannii* on Congo red agar. (B) Percent of biofilm formation; positive and negative isolates of *A. baumannii* on Congo red agar.

Table 1. The sequence of the primers and PCR cycling conditions are listed

Genes	Sequence 5'→3'	Temp. C°	Products Pb	Reference
***PgaA*- F** ***PgaA*- R**	GCAAATGAATCCTTCCGATCCT ATAAAGGCCTACACCCGCTTC	57	1450	This study
***pgaB*- F** ***pgaB*-R**	TCAGATTCCGGCAGTATTTGC ACTTCTTGCCAGGCATTTTGC	57	1293	
***pgaC*-F** ***pgaC*-R**	TGATGGCTTGGACATGGATG TGATCTCCACGGAAGCCTCG	57	1188	
***pgaD*-F** ***pgaD*-R**	GACGTAGAAGTTTTAGATATACCT CCCTGCTCATCATAATGTAAGA	57	416	

Table 2. Accession numbers of genes recorded in NCBI database.

Gene name	Accession number
pgaA	ON790655
pgaB	ON790656
pgaC	ON790657
pgaD	ON790658

By using protein sequences via online Translate tool, the molecular weight was estimated via ExPASY online tool (https://www.expasy.org/). Predicted size of Pga proteins indicated that: PgaA was 94.4 kDa, PgaB was 68.9 kDa, PgaC was 48.5 kDa and PgaC was 19.2 kDa on the gel as in Figure 5.

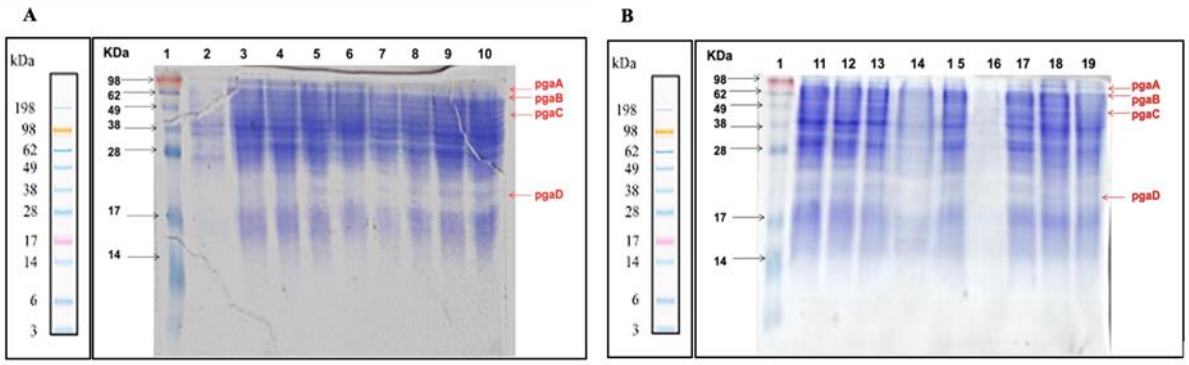

Figure 5. SDS-PAGE to detect *A. baumannii pgaABCD* protein. (A) Lane1, See Blue® Plus2 Pre-Stained protein standards (sizes in kDa are indicated); Lanes 2-10, soluble PgaA, PgaB, PgaC and PgaD proteins for isolates 1-9 of *A. baumannii*. (B) Lane 1, See Blue® Plus2 Pre-Stained protein standards (sizes in kDa are indicated); Lanes 11-19, soluble PgaA, PgaB, PgaC and PgaD proteins for isolates 10-18 of *A. baumannii*.

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Protein Alignment (PgaA, PgaB, PgaC and PgaD of A. baumannii): The protein sequences of PgaA, PgaB, PgaC and PgaD were submitted to protein blast in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to showed the similarity and identity of these proteins in GenBank database as in Figure 6 (PgaA), Figure 7 (PgaB), Figure 8 (PgaC) and Figure 9 (PgaD).

poly-beta-1,6 N-acetyl-D-glucosamine exporter porin PgaA, partial [Acinetobacter baumannii]

Sequence ID: WP_188147258.1 Length: 773 Number of Matches: 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 478 to 743 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
549 bits(1414)	0.0	Compositional matrix adjust.	261/266(98%)	264/266(99%)	0/266(0%)

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            M+NAQALGDI TWRKTTQNLVQYYPDDSGVIKSRKELEDR+RATISHS+T GQSKAEGRD
Sbjct  478  MQNAQALGDIPTWRKTTQNLVQYYPDDSGVIKSRKELEDRNRATISHSTTWGQSKAEGRD  537

Query  61   TVSEQNGLKDREMETRLNSPWINDNYRLFAWHQDRYGEYRFGDVHDQRYGVGAEWQANRK  120
            TVSEQNGLKDREMETRLNSPWINDNYRLFAWHQDRYGEYRFGDVHDQRYGVGAEWQANRK
Sbjct  538  TVSEQNGLKDREMETRLNSPWINDNYRLFAWHQDRYGEYRFGDVHDQRYGVGAEWQANRK  597

Query  121  ALSAIVSQSTDGGQAGVRLDWSQWLNDHWQYQLQYNSQADIPLQALDAGEDGQSYRAAVT  180
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Sbjct  598  ALSAIVSQSTDGGQAGVRLDWSQWLNDHWQYQLQYNSQADIPLQALDAGEDGQSYRAAVT  657

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Sbjct  658  WQKDESRQIGASYSLTDISDGNKQQEFSTFWRERLFDAPHHITYGTVRGFYGTNSQDQTA  717

Query  241  YFSPSSHYSAELNLSHDWVTWREYER  266
            YFSPSSHYSAELNLSHDWVTWREYER
Sbjct  718  YFSPSSHYSAELNLSHDWVTWREYER  743
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Figure 6. Sequences alignment of *A. baumannii* PgaA protein.

poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase PgaB, partial [Acinetobacter baumannii]

Sequence ID: WP_130115545.1 Length: 501 Number of Matches: 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 159 to 391 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
436 bits(1121)	2e-149	Compositional matrix adjust.	231/233(99%)	231/233(99%)	0/233(0%)

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Sbjct  159  MQASGLAEFASHSDDLHHGVLANPQGNEQPAATSYTYLKSQKRYETDVEYQQRILQDLKK  218

Query  61   SYAVLKKEVGVEPKAIIWPYGAVNEQLEKLSQEAGFIFSFSLGRDGMNRVSDSTFKRSLV  120
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Query  121  TNNPTAEQLTEGMINILNFEELDLFKQPRHFVSMDLKQLAASTNTQSDEklglllsklys  180
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Sbjct  279  TNNPTAEQLTEGMINILNFEELDLFKQPRHFVSMDLKQLAASTNTQSDEKLGLLLSKLYS  338

Query  181  lknntlILKPLDDQDGDGQYDIAYFPTTQLSVQQDILNRTLWQAQTRAEQSVI  233
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Sbjct  339  LKNNTLILKPLDDQDGDGHYDIAYFPTTQLSVQQDILNRTLWQAQTRAGQSVI  391
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Figure 7. Sequences alignment of *A. baumannii* PgaB protein.

poly-beta-1,6-N-acetyl-D-glucosamine synthase [Acinetobacter baumannii]

Sequence ID: WP_005128352.1 Length: 415 Number of Matches: 1

See 5 more title(s) See all Identical Proteins(IPG)

Range 1: 67 to 375 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
632 bits(1631)	0.0	Compositional matrix adjust.	309/309(100%)	309/309(100%)	0/309(0%)

```
Query  1    RYALQTKYPNFEVIAVNDGSSDSTAEILDELAAQDARLRVVHLAENQGKAVALRSGVLVS  60
            RYALQTKYPNFEVIAVNDGSSDSTAEILDELAAQDARLRVVHLAENQGKAVALRSGVLVS
Sbjct  67   RYALQTKYPNFEVIAVNDGSSDSTAEILDELAAQDARLRVVHLAENQGKAVALRSGVLVS  126

Query  61   KYEYLVCIDGDALLHPHAVLWLMQPFLNFPRIGAVTGNPRILNRSSILGKLQVGEFSSII  120
            KYEYLVCIDGDALLHPHAVLWLMQPFLNFPRIGAVTGNPRILNRSSILGKLQVGEFSSII
Sbjct  127  KYEYLVCIDGDALLHPHAVLWLMQPFLNFPRIGAVTGNPRILNRSSILGKLQVGEFSSII  186

Query  121  GLIKRAQRTYGRIFTVSGVIAAFRKTALVRVGFWSDDKITEDIDISWKLQMDHWDIQYIP  180
            GLIKRAQRTYGRIFTVSGVIAAFRKTALVRVGFWSDDKITEDIDISWKLQMDHWDIQYIP
Sbjct  187  GLIKRAQRTYGRIFTVSGVIAAFRKTALVRVGFWSDDKITEDIDISWKLQMDHWDIQYIP  246

Query  181  QALCYIYMPETFKGLWKQRLRWAQGGVEVLLEYIPKMFKLRLRRMWPVMLEALVSIIWSY  240
            QALCYIYMPETFKGLWKQRLRWAQGGVEVLLEYIPKMFKLRLRRMWPVMLEALVSIIWSY
Sbjct  247  QALCYIYMPETFKGLWKQRLRWAQGGVEVLLEYIPKMFKLRLRRMWPVMLEALVSIIWSY  306

Query  241  VMIMIFILFFVGLFVDLPQQFQINSLMPQWYGVILGGTCLVQFLVSLWIDHRYDRGRLFR  300
            VMIMIFILFFVGLFVDLPQQFQINSLMPQWYGVILGGTCLVQFLVSLWIDHRYDRGRLFR
Sbjct  307  VMIMIFILFFVGLFVDLPQQFQINSLMPQWYGVILGGTCLVQFLVSLWIDHRYDRGRLFR  366

Query  301  NYFWVIWYP  309
            NYFWVIWYP
Sbjct  367  NYFWVIWYP  375
```

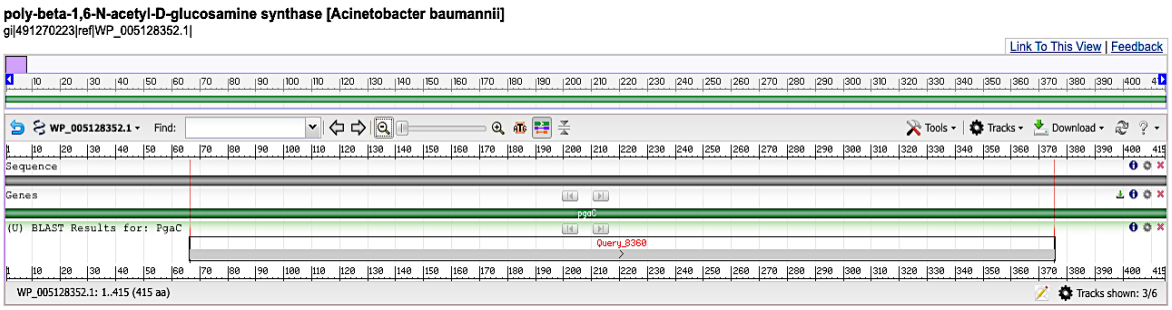

Figure 8. Sequences alignment of *A. baumannii* PgaC protein.

poly-beta-1,6-N-acetyl-D-glucosamine biosynthesis protein PgaD [Acinetobacter baumannii]

Sequence ID: EIY0852597.1 Length: 154 Number of Matches: 1

Range 1: 26 to 141 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
196 bits(498)	8e-62	Compositional matrix adjust.	112/116(97%)	115/116(99%)	0/116(0%)

```
Query  1    NKTANYTLQTIGWFCLMWLLFPLVSLFLWIFEGHLIYDYVWVDHVSKVKTllhllllligl  60
            NKTANYTLQTIGWFCLMWLLFPLVSLFLWIFEGHLIYDYVWVDHVS+VKTLLHL LLIGL
Sbjct  26   NKTANYTLQTIGWFCLMWLLFPLVSLFLWIFEGHLIYDYVWVDHVSEVKTLLHLFLLIGL  85

Query  61   salililWASYNWLKFHGDDRRSKAPNSSVELLASQFMVSTESLSKLQKSQRIILH  116
            SALILILWASYNWL+FHGDDRRSKAPNSSVELLASQFMVSTESLS+LQKSQRIILH
Sbjct  86   SALILILWASYNWLRFHGDDRRSKAPNSSVELLASQFMVSTESLSELQKSQRIILH  141
```

Figure 9. Sequences alignment of *A. baumannii* PgaD protein.

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Acinetobacter baumannii has been identified as an ESKAPE pathogen (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), a group of pathogenic organisms with a high percentage of resistance to antibiotics [24]. This bacterium has the capability to live under a broad variety of conditions, enhancing its spreading in hospitals, on side of such intriguing resistance development [25].

The high level of antibiotic resistance in this study such as the resistance of Ampicillin-sulbactam (AMS) 100% and Ceftazidime (CAZ) 91.7% supported the finding of Rizk and Abou El-Khier (2019), which concluded that the Colistin was the most efficient antibiotic to *A. baumannii* infection [26]. The results of aminoglycoside resistance agree with Li *et al.* (2021) who concluded that *A. baumannii* has resistance percentages of 54% to Gentamicin, and 52% to Tobramycin [27], but disagree about Amikacin found to have a 52% resistance percentage. The presence of aminoglycoside modifying enzymes (AMEs) is a common reason for *A. baumannii* resistances [28]. The result of this study agreed with Rizk and Abou El-Khier (2019) who found that 50% of the study isolates were Gentamicin resistant [26].

At the same time, the results of Rizk and Abou El-Khier (2019) disagree about the resistance of *A. baumannii* for Amikacin (AK) that notice appeared in 50% of isolates [26]. The results of resistance of Imipenem (IPM) disagree Babapour *et al.* (2016) with that reported *A. baumannii* has a resistance of 41.7% for Imipenem [29].

Pathogenic isolates of *A. baumannii* grow as either dispersed cells or as matrix enclosed communities forming biofilm complex so all scientific research concluded that all *A. baumannii* species possess the ability to form biofilm probably as a result of the pathogen's ecological success in the hospital being facilitated by the biofilm mode of growth [30]. The results of this study agreed with [31], which concluded that out of all 52 isolates, only 33 isolates appeared to have the ability to produce biofilm. While contradicts who recorded a high level of isolates that can produce biofilm as 60 (32%) from 156 isolates [29] [32]. The advantage of the Congo red approach is that colonies remain viable on the medium and it is quick, sensitive, and reproducible [33].

Wang *et al.* (2004) concluded that all genes of the locus *pgaABCD* have to be functional to enhance the attachment and formation of biofilm [14]. Therefore, the SDS-PAGE was used to separate protein bands according to differences in their molecular weight as in Figure 5. This is considered the first step to detect the proteins. Further purification is needed. Furthermore, specific protein detection is important by using a synthesized antibody specifically for each protein of the four tested proteins.

Acinetobacter baumannii PgaA is an outer membrane protein with 812 amino acids. The molecular weight of *A. baumannii* PgaA is 94.4 kDa. The second protein is *A. baumannii* PgaB, which is a 609 amino acids protein with polysaccharide deacetylase domain. The molecular weight of *A. baumannii* PgaB is 68.9kDa. The third protein is *A. baumannii* PgaC, which is N-acetylglucosaminyltransferase protein with 415 amino acids and molecular weight 48.5kDa. Finally, *A. baumannii* PgaD is a small inner membrane with a 154 amino acids protein. The predicted molecular weight of *A. baumannii* PgaD is 19.2kDa [16].

Alignment of protein sequences showed 99%, 99%, 100% and 99% identity for PgaA, PgaB, PgaC and PgaD respectively with protein GenBank database, which suggested a further investigation at amino acid substation is needed and this may effect on the protein structure and function.

PNAG is consider as one of the crucial and conserved factors that found in different bacteria. In addition, PNAG is important to maintain *A. baumannii* biofilm integrity. PNAG is consider as a target for active and passive immunization from various infections such as *staphylococcus* [16].

Conclusions

This work concluded that *A. baumannii* possessed the ability to form biofilm. *Acinetobacter baumannii* had multi antibiotic resistance. It has been showed that synthesize of PNAG commonly appear in *A. baumannii* isolates in Baghdad.

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Author Contributions: All authors contributed equally in writing original draft preparation, all authors have read and agreed to the published version of the manuscript.

Informed Consent: All patients gave their written informed consents before inclusion.

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