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Phenotypic and Genotypic Detection of Biofilm Formation, and Capsular Polysaccharide and Relationship with Antibiotic Resistance of *Acinetobacter baumannii* Isolated from Different Clinical Sites in Salahdin City

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Abstract: *Acinetobacter baumannii* strains can have incredible antibacterial resistance. This species is resistant to several antibiotics, including aminoglycosides, tetracyclines, fluoroquinolones, β-lactams (including carbapenems), and trimethoprim-sulfamethoxazole, eventually becoming multidrug-resistant (MDR). In this study two hundred fifteen specimens were collected from different clinical sites from Tikrit city Hospitals. The total of 38 *Acinetobacter baumannii* isolates were isolated using Acinetobacter HICHROM agar, and confirmed by VITEK2 compact system, and *16s rRNA* gene. Antibiotic susceptibility test was performed. Phenotypic and genotypic biofilm and capsular polysaccharide formation were investigated. The result showed that this bacterium was resistant for more than two of drug classes. Also, (94.73%, n=36) of bacterial isolates were phenotypic biofilm formed. The bacterial isolates that produced capsule were (92.1, n=35). The molecular results were confirmed that (97.36%, n=37) of bacterial isolates were carried *bap* gen which one of the most crucial biofilm forming in *A. baumannii*. The *epsA* and *ptk* genes, which are complicated in capsule association and polymerization. Molecular detection of these two gens showed that (92.1%, n=35) of isolates carried these two gens. The result indicated that the isolates which resistant to more antimicrobial agents have ability to produced biofilm and capsular polysaccharide.

Keywords: *Acinetobacter baumannii*, Antibiotic resistance, Biofilm genes.

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Introduction

Acinetobacter baumannii is a kind of bacteria that is gram-negative and has a coccobacillus shape. It is catalase positive and oxidase negative (1, 2). This bacterium is ranked as the top pathogen on the critical relevance list of pathogens, indicating its status as a very concerning human pathogen (3). The ability of *A. baumannii* to acquire antibiotic resistance mechanisms enables it to flourish in hospital environments, hence promoting the worldwide dissemination of multidrug-resistant strains (4). Biofilm generation has emerged as a significant pathogenic characteristic of *A. baumannii* (5). A biofilm is a community of microorganisms that are adhered to a surface via an extracellular matrix (6). Bacterial biofilm formation amplifies various mechanisms of drug resistance, such as enzymatic degradation, drug exclusion, and permeability impairments. The penetration and efficacy of antibiotics are hindered by

the biofilm matrix, rendering the bacteria within it more resistant than those in a non-biofilm state. The *Bap* gene encodes a protein that has a crucial function in the adhesion of cells inside a tissue, the aggregation of bacterial cells, and the formation, upkeep, and growth of biofilm (7). Polysaccharide capsules are thought to be a key factor in *A. baumannii's* collection of traits that determine its ability to cause disease. They operate as a shield, protecting the bacteria from the immune system of the host, antimicrobial substances, other microorganisms, and harsh surroundings (8, 9). The *ptk* gene and *epsA* gene are both involved in the process of capsule biosynthesis in *A. baumannii* (10).

Materials and Methods

Isolation and identification of bacterial isolates

A. baumannii mainly causes infections in the respiratory tract, the urinary tract, and the bloodstream and can infect wounds and burns (11), for this reason the collection process has been adopted. Two hundred fifteen sample were collected from different clinical sites (wound swap, burn, sputum, and urine). The collection were from patient in Tikrit Teaching Hospital, Salaheddin Hospital, Al-Alam General Hospital, during the period July 2023 to theend of December, The samples were streaked on blood and MacConkey agar and incubated aerobically at 37°c for 24 hour. HICHROM agar for Acinetobacter was used as selective media, A. *baumanni* Identification by conventional biochemical tests: oxidase test, catalase test, urease test, IMViC test, and growth at 44c°on tryptic soy agar. Finally, the suspected isolates were identified by VITEK2 compact system (Biomerieux, France).

Susceptibility test

Thirty eight of *A. baumanni* isolates were tested for their resistance against 12 discs for all classes of antibiotics Bioanalyse (Turkey) according to the Clinical and Laboratory Standards Institute guideline (CLSI, 2023) on Muellar Hinton Agar and were incubated at 37C°for 24hr.

Biofilm formation

Biofilm formation was investigated using a microtiter plate assay according to (12). The isolates were grown overnight on a microtiter plate on Brain Heart infusion broth with 2% sucrose and incubated at 37C° for 24hr, to eliminate free bacterial cells, wash wells three times with normal saline PH=7.2, add 200 µl Methanol for 10 minutes, then 200µl crystal violet solution (0.1%) was added for 15 minutes. To remove unbounded dye, all wells were rinsed three times by distal water and left to dry at room temperature after removing crystal violet solution. 200µl ethanol 95% was added. The ELISA reader assessed each well's absorbance at 630 nm. Sterile BHI was used as a negative control (blank). The OD reading value of the blank was taken from the test values The Absorbance. The results were divided into four groups according to their optical thicknesses depending to (13). As shown in Table (1).

Table (1): Distribution The thickness of biofilm compared with OD reading value of the blank.

No.	Thickness of biofilm	Absorbance result
1	Strong biofilm	$4 \times ODc < ODT$
2	Moderate biofilm	$2 \times ODc < ODT \leq 4 \times ODc$,
3	Weak biofilm	$ODc < ODT \leq 2 \times ODc$
4	negative-biofilm	$ODT \leq ODc$

Capsular polysaccharide synthesis was investigated using negative staining with India ink and microscopic imaging (14). Resuspended in PBS, one bacterial colony was mixed 1:1 with India Ink stain and placed to a coverslip-covered microscope slide. Microscopic imaging utilizing an Olympus SC30 digital camera and 100× oil immersion lens.

Genomic DNA was isolated from overnight cultures using ABIO Pure, USA, the sensitivity of forensic DNA typing techniques can cause problems when evidence samples are inadvertently contaminated with DNA from another source, laboratory air and surfaces, apparatuses and equipment were assessed as potential sources of contaminating DNA. evaluated for purity and concentration using a Nanodrop spectrophotometer., The accepted ratio of DNA purity is between (1.8- 2), then the DNA extracts were confirmed and analyzed by gel electrophoresis, and genomic DNA was kept at -20°C.

The detection of *Acinetobacter baumannii* isolates by *16s rRNA*, and detection *bap, Ptk and EspA* gens using PCR according to (15). The primers sequences were listed in Table 2. The polymerase chain reaction master mix (25µl per reaction) included 1µM both reverse and forward primers, 12.5µl master mix, and 9.5µl nuclease-free water until the volume reached 22µl. Next, 3 µl of DNA was added to the mixture. Programs thermocycling conditions for the detection of genes (for all the primers) were shown in Table (3).

Table (2): Primers sequences and function.

Target gene	Seq. 5-3	Function	Annealing temp. C°	Product size (bp)	References
16srRNA	F-AGAGTTTGATCCTGGCTCAG R-TACCAGGGTATCTAATCCTGTT	Housekeeping gen	55	750	This study
Bap	F- TTATGATTTAAAAGAAAAGTTCAGAATCA R- TTTCTAATAAAGTGGCATATGTTGTTTTT	Biofilm formation	56	299	This study
Ptk	F -AGCCATAACCATAGCCAGCG R –ACTCGTGGTAAGAGCCCAAC	Capsular polysaccharide	60	304	This study
EpsA	F -AGCAAGTGGTTATCCAATCG R-ACCAGACTCACCCATTACAT	Capsular polysaccharide	55	451	This study

Table (3): PCR Programs thermocycling condition for detection of genes (for all the primers).

PCR program	Temperature		Time	No. of cycles
steps	°C		m:s	Cycle
Initial Denaturation	95		05:00	1
Denaturation	95		00:30	30
Annealing	*16s rRNA*	55	00:30	30
	ptk	60		
	EpsA	55		
	Bap	56		
Extension	72		01:30	30
Final extension	72		07:00	1
Hold	10		10:00	1

Results and Discussion

Bacterial isolates

The results showed that only 38 isolates of *A. baumannii* shared the same morphological and biochemical characteristic as shown in Table (4). The result of VITEK2 compact system confirmed 38 isolates belong to

Acinetobacter baumannii. In this study, the 38 isolates were obtained from various clinical sites as shown in Figure (1), burn 18 isolates (47.36%), wound16 isolates (42.1%), Sputum 3 isolates (7.89%), urine 1 isolate (2.63%). This finding corroborated the research conducted by Narjis and Mahdi (16), which was documented in a study conducted in Baghdad, Iraq. A total of 28 isolates, accounting for 56% of the total, were obtained from burn patients. This finding is consistent with the study conducted by Raghda *et al*. (17), which reported a similar result of 54%. The number of isolates from wound and sputum cultures of *A. baumannii* was 10, accounting for 20% of the total. This proportion closely aligns with the findings of Ali and Suhad (18), who reported a 4% occurrence of *A. baumannii* in urin cultures.

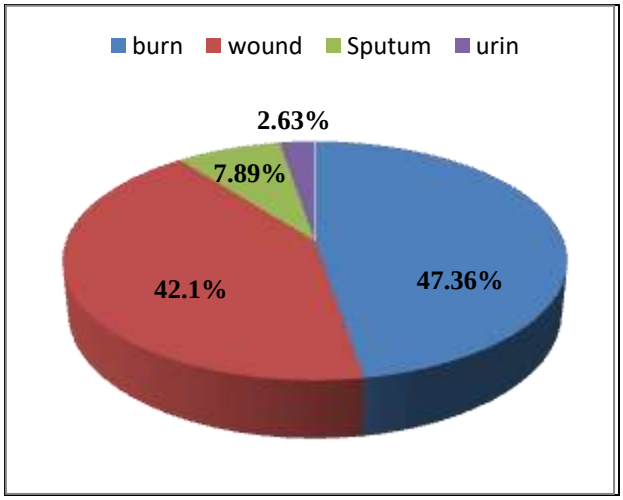


Figure (1): Distribution of *A. baumannii* according to the clinical sites.

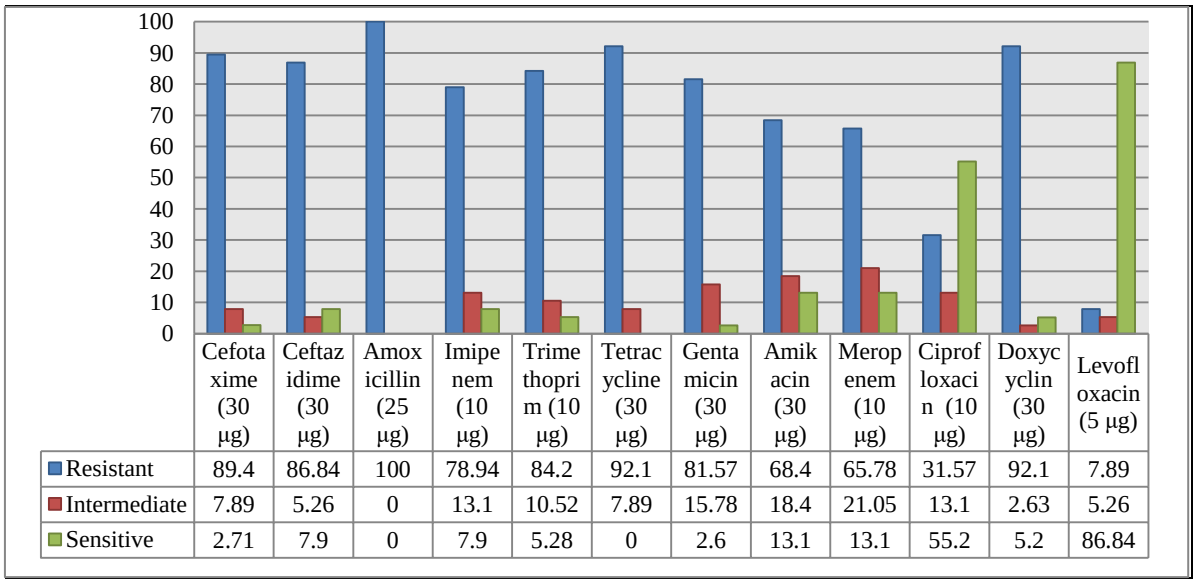
Table (4): biochemical tests or *A. baumannii.*

Biochemical test	Result
Catalase test	+
Oxidase test	-
TSI	K/ no chang color
Indoletest	-
Methyle red	-
Vogas proskauer	-
Simmons citrate	+
Motility test	-
Urease test	-

Antimicrobial susceptibility test

The result of susceptibility test showed that *A.baumannii* isolates were highly resistant to antibiotics, all isolates showed highest resistance to amoxicillin (100%), and the lowest resistance against levofloxacin only 3 isolates, (7.89%) were levofloxacin resistant, 33 isolates (86.84%) were resistant to Ceftazidime, 30 isolates (78.94%) were resistant to Imipenem 34isolates (89.4%) resistant to Cefotaxime, 81.57% 3 1isolates to gentamicin, amikacin 26 isolates (68.4%),ciprofloxacin 12 (31.57%), 35 (92.1%) isolates against doxycycline,

Trimethoprim 32 isolate (84.2%)s, (65.78%) to meropenem 35 isolates, (92.1%) to tetracyclin. The isolates were distributed according to its resistant, sensitivity and intermediate as demonstrated in figure (2). These results agreed with Hussain *et al.*, (19) who reported in his local study that 78.6% resistant to Ciprofloxacin, 64.3%to Ceftazidime and Cefepime, and .with reached by Erfani *et al.*, (20) who reported that (95.33%) of *Acinetobacter baumannii* were resistant to Ceftazidime, Gentamicin (51.4%), Amikacin (87.5%) Ciprofloxacin (95.33). On the other hand this work disagreed with Kadhom *et al*. (21) who reported that (80%) of *A. baumannii* isolates resistant to AMX, and (50%) (LEV), and with (22) who described that all isolates were resistant to Amikacin, Gentamicin Meropenem.



	Cefotaxime (30 µg)	Ceftazidime (30 µg)	Amoxicillin (25 µg)	Imipenem (10 µg)	Trimethoprim (10 µg)	Tetracycline (30 µg)	Gentamicin (30 µg)	Amikacin (30 µg)	Meropenem (10 µg)	Ciprofloxacin (10 µg)	Doxycyclin (30 µg)	Levofloxacin (5 µg)
Resistant	89.4	86.84	100	78.94	84.2	92.1	81.57	68.4	65.78	31.57	92.1	7.89
Intermediate	7.89	5.26	0	13.1	10.52	7.89	15.78	18.4	21.05	13.1	2.63	5.26
Sensitive	2.71	7.9	0	7.9	5.28	0	2.6	13.1	13.1	55.2	5.2	86.84

Figure (2): Distribution of isolates according to its (resistant, sensitivity and intermediate) % to antibiotic.

Biofilm formation

The result of biofilm formation reveals that 36 (94.73%) of *A. baumannii* isolates were biofilm producer. Six (15.7) % isolates were strong biofilm producer, 19 (50%) isolates were moderate, and bacterial isolates distributed according to the ability to forming biofilm on three levels: strong, moderate, and 11(28.8%) were weak biofilm producer, 2(5.3%) isolates were negative biofilm. The distribution of biofilm showed as in figure (3). This study were similar to Iraqi study of Saadulla and Muhammed, (23) who found that 16.67% isolates formed no biofilm, 41.66%) were weak, 25% were moderate, and 16.67% (n = 2) were strong, and agreed with AL-falahat, and Al-Draghi, (24) a local study that reported 40 isolates (97.5%) have the ability to adhere and produce slim layer Whereas, one isolates with no change in OD over the control were detected as non-biofilm producer. On the other hand ,this study was disagree with Muhammad *et al*. (25) who showed that *A. baumannii* isolates produced biofilm as Strongl55%y, Moderately 25%, Weak 5%, and non- biofilm producers were, 15%.

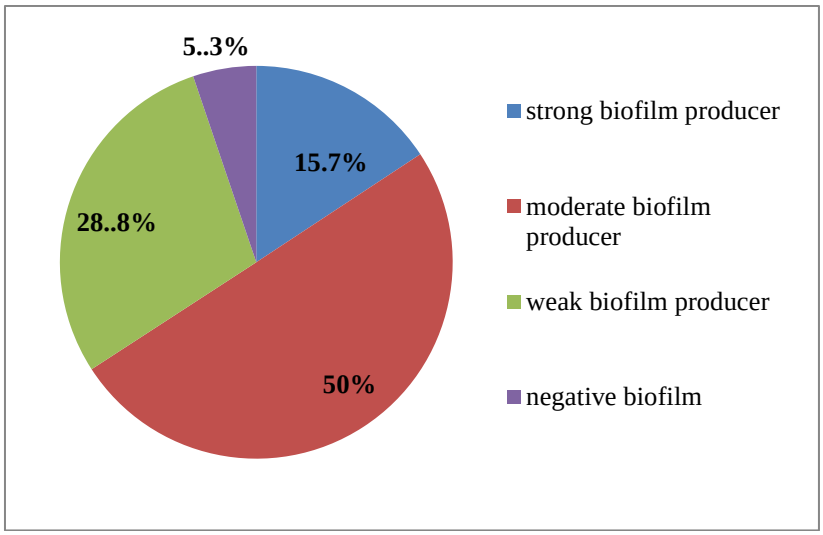


Figure (3): Biofilm formation by *Acinetobacter baumannii* isolates.

Figure (4): Bacterial capsule under microscope shown dark background with translucent zones that represented bacterial cells.

Capsular polysaccharide production

The total of 35(92.1) of *A. baumanniii* isolates were picked using India ink staining. Cells containing capsule prevent large particles of dye to penetrate the cell and thus provide a negative background for analysis. When viewed under microscope, the cells looked in the form of translucent zones on a black background (Figure 4). This work was established with a local study of Hussain *et al.* (19). Who showed that all isolates under study (100%) were capsule producers. This contradicts AL-kareem, (26), which found capsules in just 50% of isolates. The results generated from this study have confirmed that capsule is used by this bacterium to resist antibiotics, as non-capsulated strains showed high level of sensitivity to the antibiotics.

DNA extraction

All extracted DNA values ranged from 58 to 141 ng/l. Gel electrophoresis in 1% agarose for 60 minutes at 75 volts proved DNA purity. Figure (5) showed thick DNA outlines.

Molecular detection of *16s rRNA, bap ptk* and *EspA gens*

General housekeeping the first-step traditional PCR technique employed 16srRNA. The usage of *16S rRNA* genes in bacterial species arrangement is widely known since they are well-kept across species and all organisms have single species-specific regions for bacterial documentation. Comparing the molecular weight of the PCR products

to 1500 bp DNA ladder showed that all 38 isolates were *A. baumannii*. This research was comparable to Iraqi study (16), which found that a *16S rRNA* gene primer identified all isolates, and upset with Abdumohsin, and Al-Daraghi (27) who described that (21.3%) gave positive results specific *16s rRNA*. (97.36%, n=37) bacterial isolates were carried bap gen which one of the most central biofilm producing in *A. baumannii*, this result was established with Li, *et al.* (28) that described in which 96% of isolates carried the biofilm *bap* gene and upset with Rouhi, *et al.* (29) that reported the frequency of the *bap* gene was (53.42%).

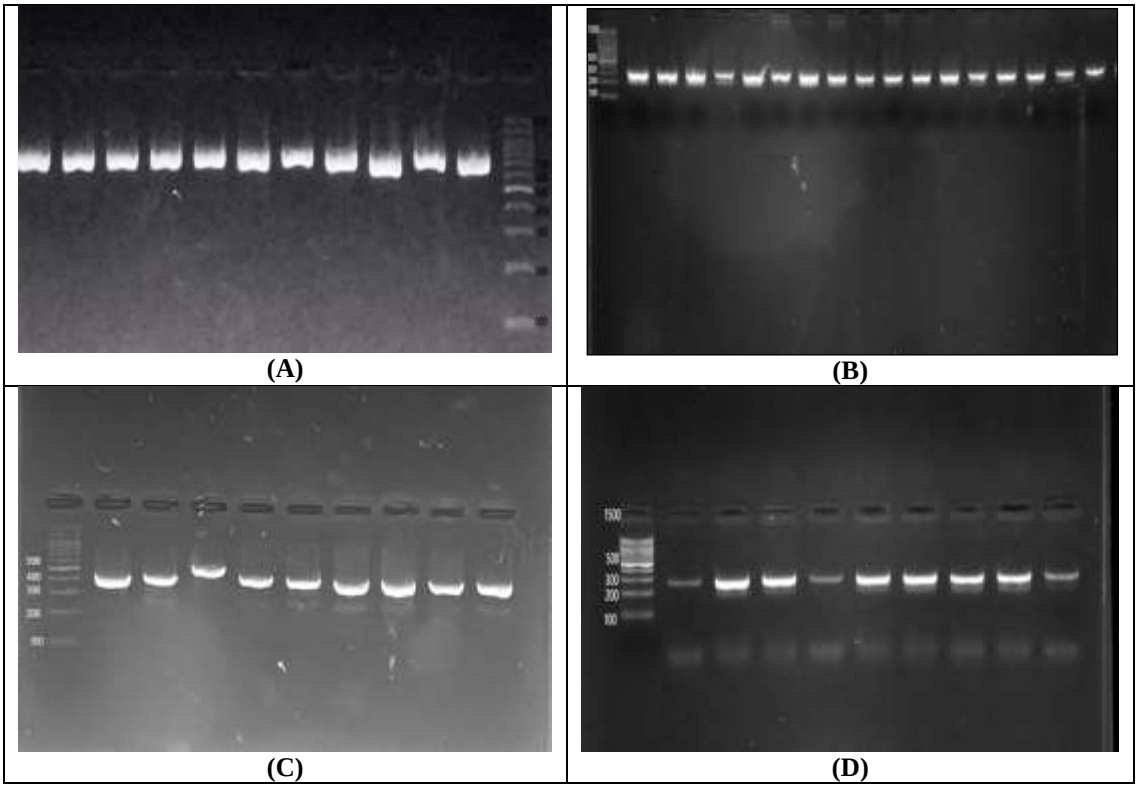


Figure (5): DNA bands of *A. baumannii* amplified on 2% agarose for 60min at 75 volts labeled with red safe (A)*16s rRNA* (B) *Ptk* gene (C) *Esp* gene (D) *Bap* gene.

The capsule is encoded by the *eps*A and *ptk* genes, which are involved in capsule assembly and polymerization. Haitham *et al.*, (30) investigations of these two genes showed that (92.1%, n=35) of isolates carried these two genes this result agreed with Zeighami *et al*., (31) who informed that (95%) of *A. baumannii* isolates carried *epsA* and *ptk* genes and Russo *et al.*, (32) which found these genes necessary for capsule positivity. These genes are essential for a capsule-positive phenotype and characterize capsule's principal function. This study showed that the rate of bacterial isolates which able to forming biofilm and have capsular polysaccharide, on the other hand have great ability to be antimicrobial resistant, and the strong biofilm forming isolates have the biggest ability to resistant.

Conclusion

This study explained that the vast majority of *Acinetobacter baumannii* isolates have ability to produced tow

virulence factor which represented by biofilm formation and capsular polysaccharide as reported microorganisms in a biofilm are resistant due to the following suggested factors polymeric matrix that can restrict distribution of antibiotics , interaction of antibiotics with a polymeric matrix that drops their activity, enzyme-mediated resistance such as β-lactamase, alterations in metabolic activity inside the biofilm, genetic changes on target cells or beating the target sites extrusion of antibiotics using efflux and the presence of outer membrane structure ,and the result indicated that the isolates which first class resistant to more antimicrobial agents have ability to produced strong biofilm, followed by the moderate, and the weak biofilm formation, on the other hand the bacterial isolates that negative biofilm formation are less resistant to different classes of antibiotics. Also the bacterial isolates that able to forming capsular polysaccharides involved in the resistance to antimicrobial agents, and it is important to point out that the bacterial isolates that lacks to CPS was sensitive to more antibiotics.

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