THE PREVALENCE OF (*OMPA, CSUE*) GENES AMONG BIOFILM PRODUCER *ACINETOBACTER BAUMANNII* ISOLATES

[1]Noor Alhuda A. K [2] S. S Mahmood
Researcher Professor
Department of Biotechnology, College of Sciences, University of Baghdad, Iraq
noornooralhda15@gmail suhadsaad22@gmail.com

ABSTRACT

***Acinetobacter baumannii* is a significant public health problem because it is capable of forming biofilms that may be responsible for the survival of this pathogen in the hospital environment. The aim of this study is to determine the role of (*OmpA, CsuE*) genes in biofilm production among *A. baumannii* isolates. A total of 100 clinical isolates of bacteria were collected from a different sources. All isolates were identified by biochemical test, specific selective media (CHROMagar) and polymerase chain reaction by detection presence of (*blaOXA51*) gene. only 60 isolates were diagnosed as *A. baumannii*, and to determine the biofilm production capacity two different methods were used Congo red agar method(CRA) and Microtiter plate method (MTP). MTP method result showed 85% of isolates were able to form biofilms, on the other hand, 33.33% of isolates had a biofilm production ability by showing bright black colonies on CRA. Conventional PCR was used to detect the presence of (*OmpA, CsuE*) genes and the result showed the prevalence of both genes in all isolates were 97%(58/60). It also shows a strong correlation between the presence of these genes and biofilm formation.**

Keywords: *Acinetobacter SPP*, PCR, Outer membrane protein A, Chaperone -usher pilus.

انتشار جينات (*OmpA،CsuE*) في عزلات *Acinetobacter baumannii* المنتجة للغشاء الحيوي

نور الهدى عبد الأمير كيطان سهاد سعد محمود
باحث أستاذ مساعد
قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، العراق

المستخلص

Acinetobacter baumannii تعد مشكلة صحية عامة كبيرة لأنها قادرة على تكوين أغشية حيوية قد تكون مسؤولة عن بقاء هذا العامل الممرض في بيئة المستشفى. الهدف من هذه الدراسة هو تحديد دور جينات (OmpA، CsuE) في إنتاج الأغشية الحيوية بين عزلات .A. baumannii تم جمع ما مجموعه 100 عزلة سريرية من البكتيريا من مصادر مختلفة. تم التعرف على جميع العزلات عن طريق الاختبار البيوكيميائي والوسط الانتقائي الخاص (CHROMagar) وتفاعل البلمرة المتسلسل عن طريق الكشف عن وجود جين (*blaOXA51*) تم تشخيص 60 عزلة فقط على أنها *A. baumannii*، ولتحديد قدرة إنتاج الأغشية الحيوية تم استخدام طريقتين مختلفتين (Congo red agar method(CRA و icrotiter plate method (MTP) أظهرت نتيجة طريقة MTP أن 85٪ من العزلات كانت قادرة على تكوين أغشية حيوية ، من ناحية أخرى فإن 33.33٪ من العزلات لديها قدرة إنتاج الغشاء الحيوي من خلال إظهار مستعمرات سوداء زاهية على .CRA تم استخدام تفاعل البلمرة المتسلسل التقليدي للكشف عن وجود جينات (*OmpA*، *CsuE*)وأظهرت النتيجة انتشار كلا الجينين في جميع العزلات بنسبة 97٪ (60/58). كما يظهر ارتباطًا قويًا بين وجود هذه الجينات وتكوين الأغشية الحيوية.

كلمات مفتاحية: *Acinetobacter SPP*, تفاعل البلمرة المتسلسل, Outer membrane protein A, Chaperone -. usher pilus

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INTRODUCTION.

Acinetobacter baumannii is the most widespread species 0f *Acinetobacter spp. it* is gram-negative bacteria, coccobacilli, non-fermenting, non-fastidious, aerobic, non-motile, oxidase negative, catalase-positive and occur in diploid-form or different-length chains (2*) A. baumanniiis* typically a pathogen associated with health care and has been identified as the cause of outbreaks and nosocomial infections in several studies (1). is bacterium being able to cause infection in a deferent site of the human body which is able to cause UTI, bloodstream infection, Pneumonia and wound infection (26). Antibiotics are used in the treatment of bacterial infectious illnesses. The overuse and misuse of antibiotics has resulted in a global health crisis characterized by treatment failure. The majority of the effective antibiotics become inactive as a result of the quick evolution of resistance. It has been noted by scientists that treatment failure can also result from persistence in addition to resistance. (9). The production of biofilm makes bacteria more resistance to antibiotics. 65-80% of human bacterial infections are estimated to be caused by biofilm production bacteria (17). Biofilms are microbial cells on biotic or abiotic surface areas, are surrounded by exopolysaccharide, biofilm formation is the most critical feature of pathogenesis in *A. baumannii*. (8). Determinants of virulence related to biofilm in *A. baumannii* include outer membrane protein OmpA, CsuA/BABCDE chaperone-usher pili assembly system (12). *A. baumannii* Outer membrane proteins (OMPs*)* include (CarO, OprD, Omp33, PstS and OmpA), all of these proteins have a role in formation of biofilm (7). OmpA is integral monomeric protein with β-barrel secondary structure and consist of eight to twenty- six antiparallel strands connected by 3short turns on the periplasmic side and 4 loops on the outer membrane surface (19). OmpA is main porin in *A. baumannii* because play an important role in invasion and attachment through interaction with fibronectin of epithelial cells also this protein involved in persistence, serum resistance, *A. baumannii* antimicrobial resistance, induction of programmed cell death (5) OmpA is a well-known virulence factor that plays a important role in A. baumannii survival and pathogenicity, including maintaining integrity of cell membrane, mediating drug resistance, and initiating biofilm formation (4). OmpA-deficient A. baumannii mutants formed weaker biofilms and were significantly less virulent than wild-type cells. They also demonstrated lower adhesion to human airway epithelial cells. (13). Csu pili Surface associated proteins are a key component in the irreversible adhesion of cells to a surface. Genes clustered together in a polycistronic *csu* operon assembly system called *csuA/BABCDE* encode chaperon usher pili (csu) (23). The main function of CsuA/BABCDE chaperone-usher pilus is essential for the beginning of biofilm formation on abiotic surfaces(28) in addition to biofilm production, csuE is involved in bacterial movement. (16) In view of the seriousness of this bacteria, the aim of this research was to find the relationship between these genes and one of the most important virulence factors production of biofilm Therefore, developing new and efficient methods is important for the avoidance and treatment of infections brought on by A. baumannii strains that produce biofilms.

MATERIALS AND METHODS

This study was approved by the ethical committee of the biotechnology department collage of science / university of Baghdad according to the Ref number (CSEC/1021/0068). The samples were collected after the approval of the patients. A total of 100 clinical samples were collected from different source such as wounds, burns, sputum, blood and urine and Distribution of clinical samples show in (figure 1) from the Medical City and the Burn Center at Yarmouk Hospital for the period from October 2021 to December 2022. All of these bacterial sample identified by using biochemical test, selective synthetic media (CHROMagar) and polymerase chain reaction (PCR) by using specific primer for *blaOxa51* gene as shown in table (2).

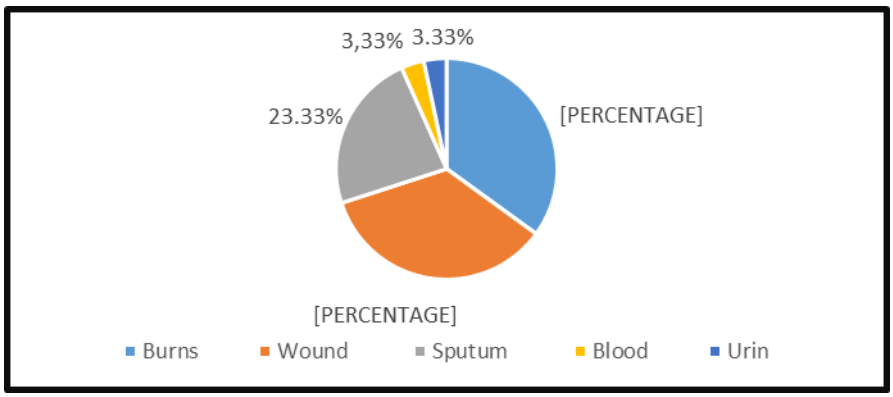

Figure 1. Percentages of clinical samples among different clinical sources

Biofilm detection methods

Congo red agar method(CRA).: Congo red agar which considered a qualitative. the medium is prepared by mixing 0.8 g of Congo red and 50 g of sucrose to 37 g of Brain heart agar. This technique is based on colonies on the Congo red agar (CRA) medium changing color. Red-pink colonies still contain non-biofilm producers while colonies with a black color suggest a biofilm producer. (14) the result of (CRA) method shown in figure (4).

Microtiter plate method (MTP).

Quantitatively detection of Biofilm Formation was done using the MTP test, a 0.5 McFarland standard was made from an overnight culture established in tryptic soya broth (Himedia / India) containing 1% glucose (Himedia / India), 200 µl were put to a 96-well tissue culture plate and incubated at 37°C for 24 hours. The supernatant from wells were removed and the plate was washed three times with phosphate buffer saline. The plate was then set aside to dry, after that, 200 µl of 96 % methanol was added to each well for 15 minutes at room temperature. air-dried each well's then adherent cells were stained with 0.1 percent crystal violet. To remove the extra color, the plate was rinsed twice with deionized water to remove excess dye, and then 200 µl of 96 % ethanol was added to re-dissolving adherent cells (10). An ELISA plate reader was used to measure the optical density of the adhering biofilm at 600 nm. Each test was carried out three times (3). Table 1 shows the criteria used to interpret the results (1).

Table 1.Analysis of the result of biofilm formation using microtiter plate method.

OD value	Intensity of biofilm
OD≤ ODc*	Non - producer
ODc < OD ≤ 2ODc	Weak
2ODc < OD ≤ 4ODc	Moderate
OD> 4ODc	Strong

***Mean OD of –ve control +3OD –ve control of**
OD =Optical density 600 nm, ODc =Optical density of cutt off DNA extracted from *A. baumannii*

Wizard® Genomic DNA Purification Kit manufactured by (Promega /USA) was used to extracted DNA from *Acinetobacter baumannii* isolates quantification of purity and concentration for extracted DNA by using Nano-drop spectrophotometer 260/288 (NAS-99/Taiwan) then stored at (-20).

Primers used in this study to detect (*blaOxa51, OmpA, CsuE*) genes.

Primers listed in the Table (2) were supplied by Alpha Company for detection *A. baumannii by blaOxa51* gene and biofilm related genes (*OmpA, CsuE*).

Table 2. Primer sets for detection the biofilm genes that used in this study

Genes		Primer sequence 5′→3′	Product size(bp)	References
blaOxa51	F	GCCATAACCAACACGCTTCA	178 bp	
	R	GGCAACCACCACAGAAGTAT		This study
OmpA	F	TGCTCCACAACCACAAGAGT	162 bp	
	R	GGCAACCACCACAGAAGTAT		
CsuE	F	CATCTTCTATTTCGGTCCC	168 bp	
	R	CGGTCTGAGCATTGGTAA		

Amplification of *(blaOxa51, OmpA/CsuE*) genes by PCR technique: A specific sets of primers Table (2) were used for detecting *A. baumannii* by blaOxa*51* and presence of *(OmpA/CsuE*) genes by using DNA extracted from *A. baumannii* and using reaction mixture with a total volume of 25 µl for every single gene: 1 µl forward primer and 1 µl reverse primer were added to 12.5 µl of Green Master Mix and. 4 µl of DNA was added to the mixture, finally 6.5 µl from Nuclease-free water was added to bring the total volume to 25 µl .The PCR reaction took place in Thermal Block, one round of denaturation at(95°C) for 5 minutes preceded the amplification of the genes. Following that, the reaction mixture was subjected to 35 cycles of denaturation at(95°C) for 30 seconds for each step ,annealing at (58 ,48 ,55°C) for 30 seconds for the (blaOxa*51*, *OmpA/CsuE*) genes respectively, and this step was followed by elongation at(72°C) for one minute, and finally, the reaction was completed with final extension at(72°C) for 5 minutes. The amplified products of PCR were electrophoresed in agarose gel.

RESULTS AND DISCUSSION

Collected of bacterial sample: All 100 bacterial isolates were identified by using a biochemical test and results showed that only 60 bacterial isolates were identified as *A. baumannii*, based on the results of indol, oxidase Simmon citrate and catalase tests. The results were confirmed by using selective media (CHROMagar) and the result shows in Figure (2)**.**

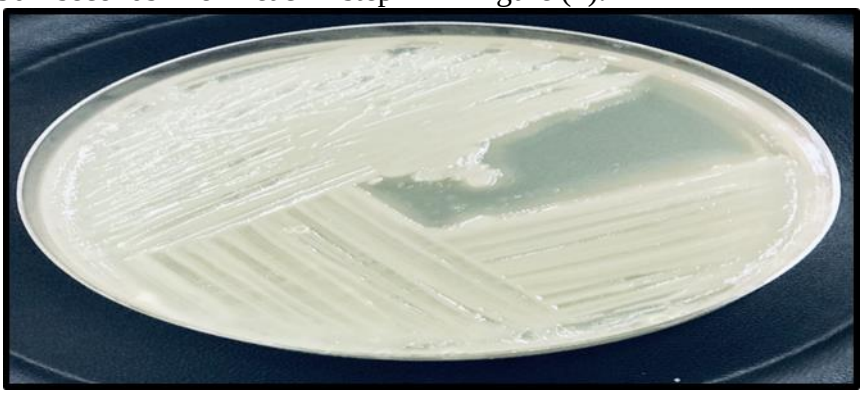

Figure 2. Phenotype appearance of *A. baumannii* on CHROMagar

Molecular identification of *A. baumannii* by *blaOxa51:* All 60 bacterial isolates that showed a positive result for biochemical tests and CHROMagar were subjected to confirmatory diagnosis by using a special primer for *blaOxa51* gene by using a specific primer, the polymerase chain reaction was used to determine the presence of a gene. The amplified products of PCR were electrophoresed in 2.5% agarose gel and 85 volts for 90 minutes and the results showed that all isolates contained the gene with size (178 bp). Previous research shows that all clinical isolates of *A. baumannii* have *blaOxa51*, which has not been found in any other *Acinetobacter spp* (20). as shown in Figure (3).

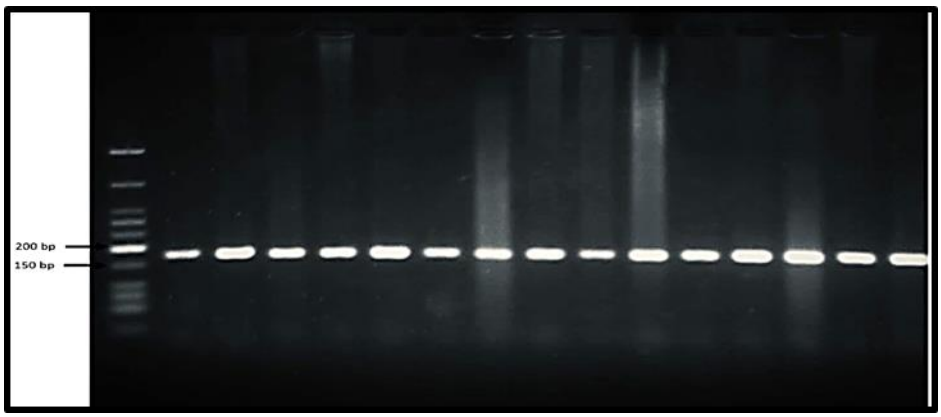

Figure 3. Gel electrophoresis pattern for detection of *blaOxa51* gene (178 bp) for isolates (1-15) on 2.5% agarose gel and 85 volts for 90 minutes in presence of low molecular weight DNA Ladder (25 -766 bp).

Figure 4. formation of biofilm on congo red agar (1) A biofilm producers are represented by colonies that are black in color. (2) Non-biofilm producers are retained in red-pink colonies

Detection of the biofilm-producing *A. baumannii* isolates by CRA and MTP methods.

Due to its multidrug resistance characteristic, *A. baumannii* has been one of the most important isolates of the medical community in latest years and The capacity to produce a biofilm is one of the most important virulence factors involved in the pathogenicity of A. baumanni. (18,24) Bacteria produce Biofilms to provide a safe cover against stressors of the environment such as antimicrobials, disinfectants, drying, heat, and attacks of the immune system (6). The result of biofilm detection utilizing two different methods: CRA, MTP showed that most isolates were able to form biofilms. Primary detection of bioflm obtained by CRA (conformational method) and the results were obtained showed (33.3 %,20/60) of isolates had a biofilm phenotype ability by creating a black colony on Congo Red agar, whereas (66.6 %, 40/60) isolates were recognized as a non-biofilm producer and retained in red – pink colonies as showed in figure (4) compared to (15 %, 9/60) non-biofilm producer by using the MTP assay. MTP assays identified that (85%,51/60) of *A. baumannii* isolates are able to form biofilms; wounds isolates had the highest proportion of biofilm producers (95%, 20/21) while isolates from burns and sputum had (85.7% ,18/21) and (78.5 %,11/14) respectively, and 50 % (1/2) identified as biofilm- weak producers obtained from both blood and urine respectively as showed in figure (5). However, The CRA approach had no connection to the MTP method. Furthermore, the ability of *A. baumannii* isolates to produce biofilms in two assays did not correlate with patient variables such as age, clinical specimen type and gender. Our findings support prior research that shown that more than 75% of *A. baumannii* isolates form biofilms. (22), (21).

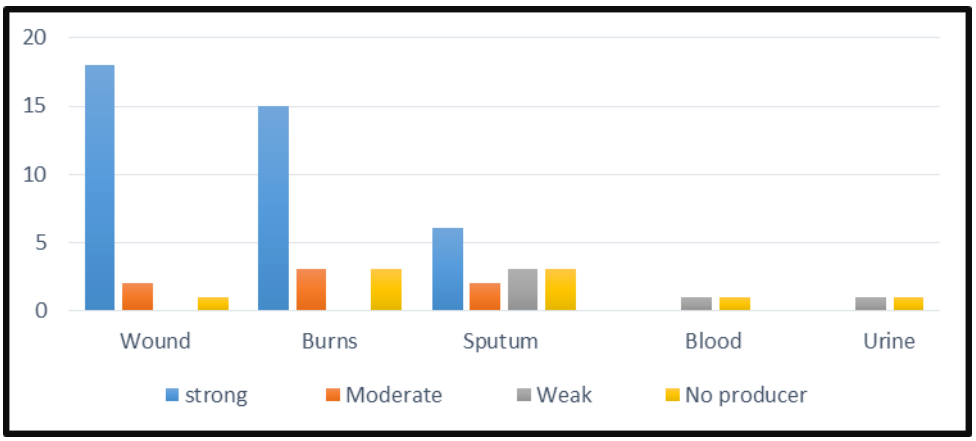

Figure 5. biofilm production capacity among different source of clinical samples by using MTP method

Molecular detection of (*OmpA, CsuE*) genes

Molecular detection of (*OmpA, CsuE*) genes**.** was performed on 60 isolates of *A. baumannii* by using a conventional PCR technique and specific primers listed in Table (2) and gel electrophoresis using agarose gel (2.5%) and 85 volts for 90 minutes. The result obtained showed the percentage of (*CsuE, OmpA*) in *A. baumannii* were 97%(58/60) With presence of genes size (168 ,162 bp) respectively as shown in Fig(6A,6B), these result investigated there was a significant correlation between the ability to form biofilms and the presence of (*CsuE, OmpA*) genes in *A. baumannii* isolates. The genes encoding (*OmpA, CsuE*) are known to be important in the production of biofilms in *A. baumannii*. Our findings revealed that the (*CsuE, OmpA*) were found in all biofilms producers, as well as in some weak producers and moderate producers. Also (88% ,8/9) of isolates are unable to produce biofilm and contain both genes because in addition to the existence of these genes, there may be other factors play a vital role in the capacity of biofilm formation such as nutrition, environmental factors, bioflms may only produce under certain specific conditions or bioflm-associated genes might involvement in other physiological processes rather than bioflm formation (25). This research provides information on the most important genes that play a key role in the formation of biofilms, which is one of the most essential virulence factors in *A. baumannii* bacteria thus providing strategies to control the infections correlated to biofilm formation. Existing evidence in this field has shown different results regarding the correlation of *(OmpA, CsuE*) with the biofilm formation, these results agreed with the work of Khoshnood, Monfared (11), (15) which showed a high prevalence of biofilm related genes (*OmpA and CsuE*) but other studies showed the percentage of (*CsuE, OmpA*) are 68%,81% respectively. (27, 28).

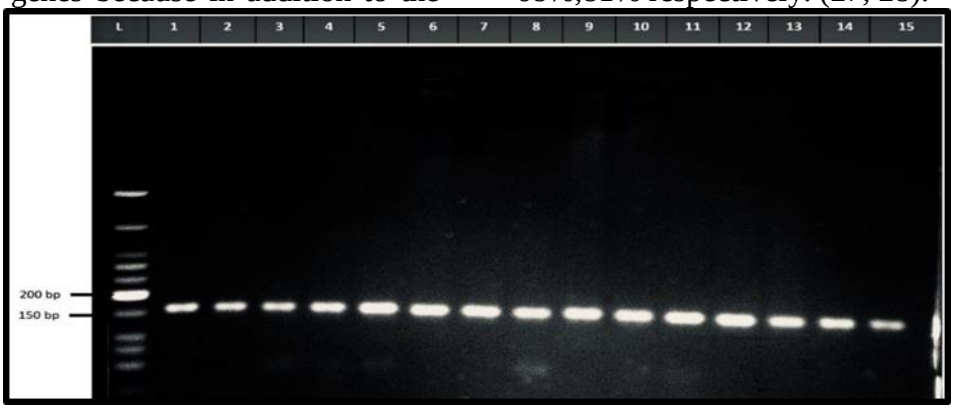

Figure 6A. gel electrophoresis pattern for Detection presence of *CsuE* gene (168 bp) for isolates (1-15) on 2.5% agarose gel and 85 volts for 90 minutes in presence of low molecular weight DNA Ladder (25 -766 bp).=

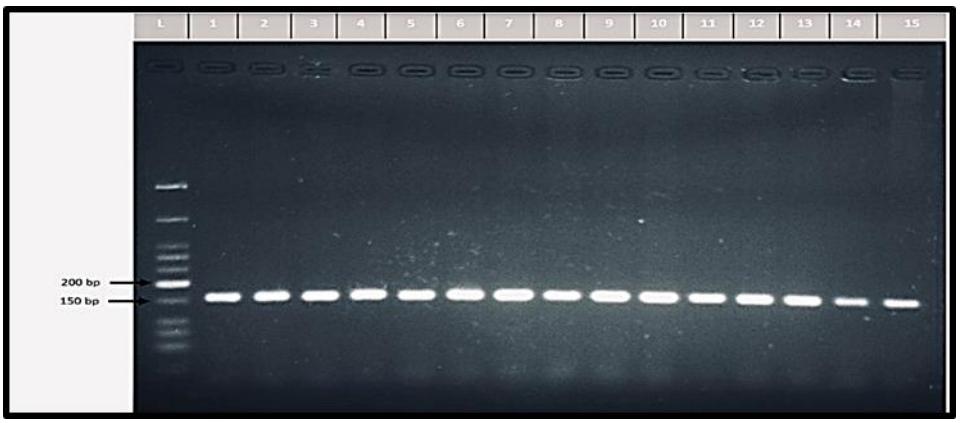

Figure 6B. gel electrophoresis pattern for Detection presence of *OmpA* gene (162 bp) for isolates (1-15) on 2.5% agarose gel and 85 volts for 90 minutes in presence of low molecular weight DNA Ladder (25 -766 bp).

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