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Molecular Detection of *rblB, and csgA* Genes of *Acintobacter baumannii* Isolated from Different Clinical and Environmental Samples

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Abstract: Multidrug-resistant *Acinetobacter baumannii* has emerged as a major problem around the world, posing a serious hazard to patients with burn and wound infections, In this study. Two hundred and fifty samples were collected from various sites in Baghdad/Iraq hospitals (Al-Yarmouk Teaching, burn center of Medical City, and Kamal Al Samray Hospitals) and divided into two groups based on their source: clinical samples (150) and environmental samples (100), collected and tested from October 2021 to March 2022. These samples were cultured on culture medium, and seven -foure *A. baumannii* isolates were identified by microscopic examination and biochemical tests. The identification of (74) isolates was verified using the VITEK-2 system and a molecular approach based on the *rplB* gene. In addition, antibiotic susceptibility testing was performed on allbacterial isolates, and the findings revealed that 36 isolates had Multi-Drug Resistance (MDR). and 40 isolates had the ability to form biofilm in the microtiter plate method 21 isolates gave strong biofilm, 15 isolates gave moderate biofilm, three isolate gave weak biofilm while the other one isolates are non-biofilm producer. This study found that 16 isolates carrying the *rblB* gene , and in all 16 *A. baumannii* isolates are carried (9) *csgA* gens, Sequencing analysis was applied to the PCR product of (8) positive samples from 16 isolates to *csuE* gene the isolates detect Sequencing and similarity of genetic relationship between locally isolates and high identity global isolates ingene bank.

Keywords: Multi-Drug Resistance, *csgA,rblB,* , *Acinetobacter baumannii*.

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Introduction

Acinetobacter baumannii is a non-fermenting *coccobacillus;* oxidase negative, aerobic *bacilli* and human opportunistic extracellular pathogen originating from hospital acquired infections to be known also as a nosocomial infection. The Gram-negative bacterium *Acinetobacter baumannii*, also known as “Iraqibacter” due to its emergence in US military treatment facilities in Iraq, has quickly become one of the most troublesome pathogens for healthcare institutions globally and currently tops the priority pathogens list for development of new antibiotics (1). The outstanding survival properties and antibiotic resistance of *A. baumannii* are strongly associated with its ability to form biofilms. The pathogen was shown to colonize various objects, including medical equipment and tools, hospital furniture, and even gowns and gloves of healthcare providers (2, 3, 4). *Acinetobacter baumannii* contain *rplB* housekeeping gene that encode to the 50S ribosomal protein L2. It is essential for ribosome activity and is a major component of the peptidyl transferase This protein

included in process activity (peptidyl transferase activity and binds to functionally important domains of 23S rRNA), one of the rRNA binding proteins, and contacts with the 16S rRNA in the 70S ribosome (5, 6). Therefore, *rplB* gene is very important housekeeping gene (7). As a class of important virulence factors in bacteria the Curli-specific gene *(csg).* The csgA gene, the primary subunit of the curli amyloid fiber, is produced in the cytoplasm and transferred to the cell surface as an unfolded protein, where it assembles into extracellular amyloid polymers after interacting with the *csgB* nucleator protein (8). proving the importance of *csgA* in biofilm development Thus, targeting *csgA* would be a novel therapeutic strategy for combating the threat of drug-resistant *A. baumannii* strains. (9). Various substances were identified for their antibacterial, anti-inflammatory, and anti-oxidant effects in multiple overviews on the bio-active ingredients from A. indica In light of this, the goal of this study is to molecularly identify the presence of the *csgA* gene in multi-drug resistant *A. baumannii* strains, as well as to conduct an in-vitro and in-silico docking investigation of A. indica bioactive compounds against *csgA* (10) and study comparison of locally isolated with global isolates documented in gene bank of *Acinetobacter baumannii* isolates.

Materials and methods

Sample collection

Two hundred and fifty samples were collected from various sites in Baghdad/Iraq hospitals (Al-Yarmouk Teaching, burn center of Medical City, and Kamal Al Samray Hospitals) and divided into two groups based on their source: clinical samples (150) and it were environmental samples (100), collected and tested from October 2021 to March 2022. These samples were cultured on culture medium, and seventy -foure *A. baumannii* isolates were identified by microscopic examination and biochemical tests. The identification of (74) isolates were verified by VITEK-2 system and a molecular approach based on the *rplB* gene.

Detection of biofilm production by microtiter plateassay

According to Kerkeni *et al*., (12), A.baumannii biofilm production was quantified using the microtiter plate method;,All isolates were grown over night in Brain Heart Infusion Broth at 37°C,Each isolates was transferred to tryptic soy broth (TSB) containing 1% glucose and mixed well by pipetting, A suspension of the bacterial isolate was adjusted to McFarland 0.5 turbidity standard. A volume (200 µl) of each isolates culture was added, in triplicate, to a sterile 96 wells microtiter plate with a flat bottom, The plate was covered with their lids and incubated under aerobic conditions at 37°C for 24h, After the incubation period, the planktonic cells were rinsed twice with distilled water to remove the unattached bacteria,The adhering bacterial cells in each well were fixed with 200 µl of absolute methanol for 20 min at room temperature,The adhering cells were stained by adding 200 µl of 0.1% crystal violet to each well for 15 min.,Once the staining reaction has completed, the excess stain was removed by repeated washing (2-3 washes) with distilled water,The plate was dried by leaving them at room temperature for approximately 30 min to ensure they were completely dry,Finally 33% acetic acid was added to fix the stain. Optical

density (OD) readings were determined using an ELISA auto reader at a wavelength of 630 nm, An average of OD values of sterile medium was calculated and subtracted from all test values. Cut off value (ODc) was calculated, which can provide categorization of isolates as biofilm producer or not (13). ODc: Average OD of negative control + (3 × standard deviation (SD) Negative control), OD isolate: Average OD of isolate – ODc. Classification of bacterial adherence based on OD values obtained for individuals bacterial:,Strong biofilm producer (4 × ODc< OD), Medium biofilm producer (2 × ODc< OD ≤ 4 × ODc), Weak biofilm producer(ODc< OD ≤ 2 ×ODc) ,Non-biofilm producer (OD ≤ ODc), (13).

Molecular study and DNA extraction

The estimation of the extracted DNA concentration was done by using the Quantus™ Fluorometer Single-Tube Format Protocol (11). Polymerase chain reaction master mix was prepared (with final volume 20 µl per one reaction) containing 10µM forward and reverse primers, 2 X of master mix, and 5µl of nuclease-free water was added until the volume reached to 17 µl. Then, 3 µl of DNA was added to mixture.

Table (1): Primer sets used in the present study

Primer Name	Sequence 5`-3`	Annealing Temp. (°C)	Product size (bp)
csgA-F	ACTCTGACTTGACTATTACC	58	200
csgA-R	AGATGCAGTCTGGTCAAC		
rplB-F	GTAGAGCGTATTGAATACGATCCTAACC	50	475
rplB-R	CACCACCACCRTGYGGGTGATC		

Results and discussion

Isolation and identification of *Acinetobacter baumannii*

In this study, the results revealed that a total number of *A. baumannii* was included in 74 (29.2 %) of the 250 clinical and environmental samples tested. From 250 samples inoculated on the Chromagar Acinetobacter, 74 isolate grew on the medium. CHROMagar Acinetobacter is a recently developed medium for selective and rapid identification of Acinetobacter spp (14). *Acinetobacter baumannii* is rod-shaped which grows well on MacConkey agar (without salt). Although officially classified as not lactose-fermenting, they are often partially lactose-fermenting when grown on MacConkey agar. Growth and purity of cultures of *Acinetobacter baumannii* were determined by culture on MacConkey agar and Blood agar. On MacConkey agar it's formed pale coloured, Non lactose fermenting colonies and on Blood Agar it's formed non-hemolytic colonies. There was only one type of colonies attesting to its purity(15). isolates of *A. baumannii* grow at 44°C were positive on blood agar medium after 24 hrs of incubation. A appropriate temperature for the growth of most Acinetobacter spp were 37oC whereas *A. baumannii* can grow well at high a temperature of 44 °C. The suspected all isolates of *A. baumannii* and were then subjected to the related biochemical tests. All isolates of *A. baumannii* were found to be catalase positive and oxidase / indole negative. Tests on Lactose fermentation and motility test gave negative results. The positive results for the test appeared in methyl red and Citrate utilization, finally we used the urease test which give negative results for *Acintobacter baumannii*(16).

The isolates were identified as *A. baumannii* and have been confirmed by using VITEK 2 Compact, Antibiotic Susceptibility Testing. The development of *Acinetobacter baumannii* to become extensively drug-resistant and multidrug-resistant are rated as a significant curative issue which can be clarified by several theories; one of them is the incorrect use of antibiotics, besides, the extensive use of antibiotics. This made these bacteria to be the focus of the researchers' attention. Susceptibility to antibiotics was determined by VITEK 2 system and determined by using the disk diffusion method as illustrated in section ntimicrobial susceptibility patterns were determined by disk diffusion method on Mueller–Hinton agar (MHA) (Biolab, Hungary) according to CLSI guide-lines. The following antimicrobial disks. The Vitek 2 system automatically measures a turbidity signal for each test well containing an antibiotic, every 15 minutes for up to 18 hours. These data are used to generate growth curves and by comparing with a control, the minimum inhibitory concentration (MIC) of each antibiotic is estimated. MIC results (µg/ml) are translated into clinical categories (Susceptible, Intermediate, and Resistant) by comparing with Breakpoints for susceptibility category determination recommended by the clinical and laboratory standards institute (CLSI) guidelines. The results showed that only 36 isolates are MDR, according to reports all (74) isolates are resistant to ceftazidime (94.59%) and are more sensitive to colistin (82.42%) and Trimethoprim/ Sulfamethoxazole (74.32%), Also, the results showed that 30 isolates are resistant to meropenem and 20 isolates are resistant to Imipenem, and they were 38 isolates resistant to Piperacillin and 36 isolates resistant Ciprofloxacin.

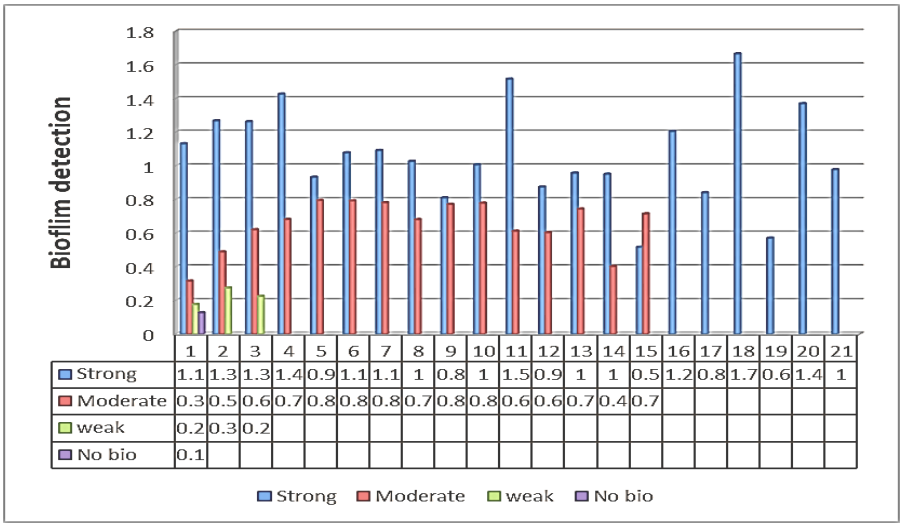
Table (1): Antimicrobial Susceptibility of 74 *A. baumannii* Isolates to different antimicrobial agents.

Antibiotic	Resistant	Intermediate	Sensitive	P-value
Piperacillin/tazobactam(PTZ)	38 (51.30%)	2 (2.70%)	34 (45.94%)	0.0001 **
Colistin(cs)	5 (6.75%)	9 (12.16%)	61 (82.42%)	0.0001 **
Trimethoprim/Sulfamethoxazole (TMP/SMX)	17 (22.97%)	2 (2.70%)	55 (74.32%)	0.0001 **
Tobramycin(TM)	24 (32.43%)	13 (17.56%)	37 (50.0%)	0.00027**
Piperacillin (PRL)	38 (51.35%)	0 (0.0%)	36 (48.64%)	0.0001 **
Meropenem(MEM)	30 (40.54%)	13 (17.56%)	31 (41.89%)	0.0152 *
Minocycline (MNO)	5 (6.75%)	4 (5.40%)	65 (87.83%)	0.0001 **
Imipenem (IMI)	20 (27.02%)	21 (28.37%)	33 (44.59%)	0.116 NS
Cefepime(CPM)	49 (66.21%)	0 (0.0%)	25 (33.78%)	0.0001 **
Ceftazidime(CAZ)	70 (94.59%)	0 (0.0%)	4 (5.40%)	0.0001 **
Ciprofloxacin(CIP)	36 (48.64%)	3 (4.05%)	35 (47.29%)	0.0001 **
Gentamicin(GM)	30 (40.54%)	0 (0.0%)	44 (59.45%)	0.0001 **
Ticarcillin	25 (33.78%)	13 (17.56%)	36 (48.64%)	0.0044 **
Ticarcillin /clavulanate(TIM)	23 (31.08%)	10 (13.51%)	41 (55.40%)	0.0441 *
P-value	0.0001 **	0.0001 **	0.0001 **	---
*** (P≤0.05), ** (P≤0.01).**				

Detection of biofilm production by microtiter plate assay

In this study, determining the ability of 40 *A.baumannii* isolates to adhere and produce a slime layer (Biofilm formation, showed that 40 isolates (97.5%) have the ability to adhere and produce slim layer with

significant differences in thickness degrees (strong, moderate and weak) where the variation in biofilm thickness may be due to differences in isolates ability to produce biofilm. Whereas, one isolates with no change in OD over the control were detected as non-biofilm formers, which represented (2.5%). These isolates could not adhere and produce a slim layer. The results in figure (2) that 21 isolates gave strong biofilm, 15 isolates gave moderate biofilm, and three isolate gave weak biofilm.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Strong	1.1	1.3	1.3	1.4	0.9	1.1	1.1	1	0.8	1	1.5	0.9	1	1	0.5	1.2	0.8	1.7	0.6	1.4	1
Moderate	0.3	0.5	0.6	0.7	0.8	0.8	0.8	0.7	0.8	0.8	0.6	0.6	0.7	0.4	0.7						
weak	0.2	0.3	0.2																		
No bio	0.1																				

Figure (2): The percentage of antibiotic susceptibility.

Babapour *et al*. (17) and Fallah *et al*. (18) used a microtiter plate approach to test biofilm formation of A.baumannii isolates and found a higher biofilm formation method. Babapour *et al*. (17) reported *A. baumannii* in 156 clinical samples obtained from Tehran-based hospitals. The number of bacteria with positive biofilm was 66.66 % and 73.72 %, respectively. At least 92 % of the biofilm-forming isolates were immune to several drugs(17). AL-Mousawi *et al*., (19) used phenotypic approaches to evaluate the biofilm formation potential of *Acinetobacter baumannii* recovered from clinical samples in Baghdad hospitals. They found substantial differences amongst 83 *Acinetobacter baumannii* isolates. Seventy-four isolates (89.15%) exhibit the ability to attach and form a thin layer with considerable thickness variability (strong, moderate and weak). While nine isolates (10.84 percent) were unable to attach and generate a thin layer. Sajjad, (20) indicated that 30 isolates (83.33 percent) can attach and generate a thin layer with significant thickness differences (strong, moderate, and weak), suggesting that the variance in biofilm thickness could be related to differences in isolates' ability to produce biofilm.

rplB gene was used to detect A. baumannii and *csgA* gene using a polymerase chain reaction (PCR) technique

Detection of *rblB,* and *csgA* virulence genes by Polymerase Chain Reaction (PCR) technique PCR assay was used to determine the virulence genes in all 16 *A. baumannii* isolates. The presence of the rplB gene was

confirmed by PCR analysis in all 16 (100 percent) as shown in Figure (3), According to Hamzah (21), the rplB gene is unique to this species, and its detection provides a rapid and easy technique to identify *A. baumannii*, and is more accurate than biochemical identification. Because *Acinetobacter baumannii* is the most clinically relevant member of the genus, the ability to distinguish it quickly from other members of the genus will be tremendously beneficial. This gene was found to be highly selective for *A. baumannii*, allowing for species-level identification of these bacteria, which was validated by PCR amplification with specific primers (21). *A. baumannii* isolates in this investigation, confirming the usual diagnostic of culture, biochemical tests, and Vitek-2 assays. Furthermore, as shown in Figure, the generated amplicons of this gene emerged clearly on agarose at 475 bp during the electrophoresis technique. In addition, a PCR assay was used to detect a single gene using particular primers. The findings of this analysis showed that 9 isolates (56.2%) carried the (*csgA* gene), asshown in Figure (4), studies show that CsgA genes among 60% and 18% isolates respectively (22). Darvishi M., (23) detected csga, virulence genes in 70% isolates, Momtaz *et al*. (24) reported that the prevalence of csga, virulence genes the *A. baumannii* of clinical infections in Iran were 12.39%.

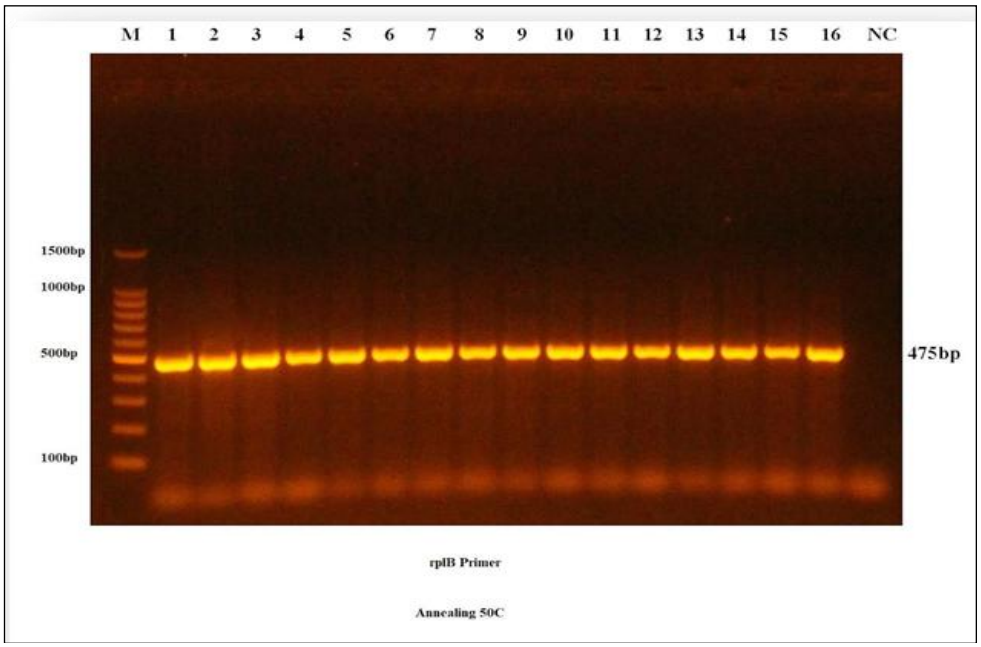


Figure (3): Results of the amplification of *rplB gene* of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-1S resemble 475bp PCR products.

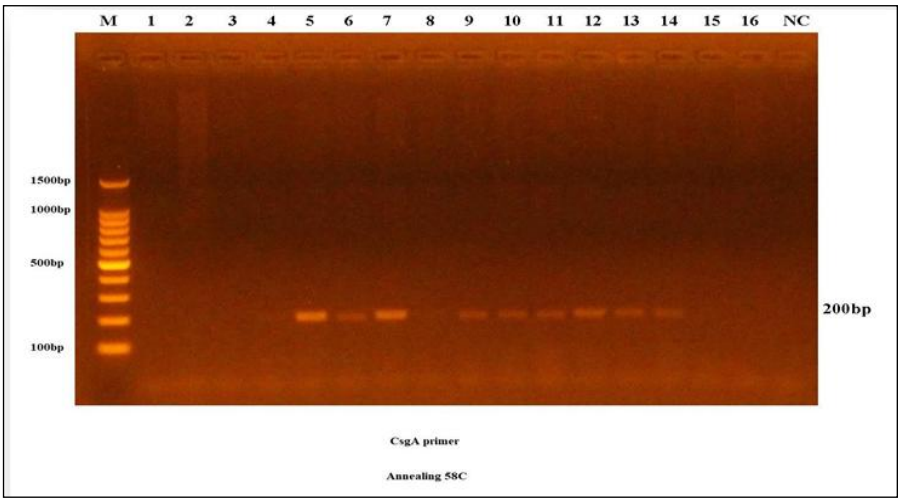


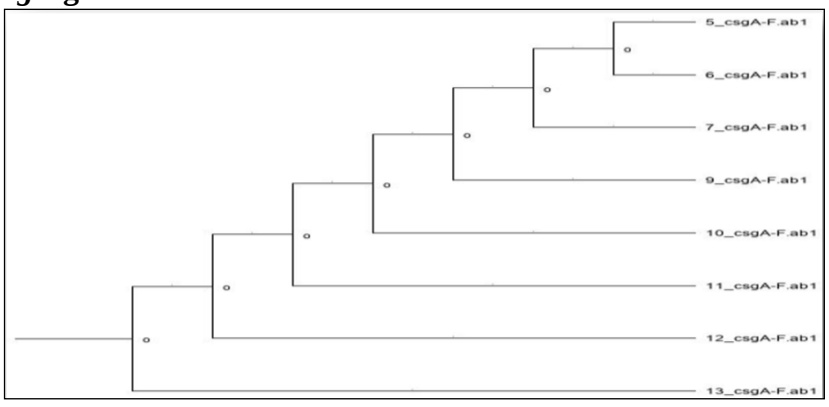
Figure (4): Gel Electrophoresis for PCR Amplification of *A. baumannii csgA* Gene on 1.5%agarose gel, 100 V for 75 min. lane (M): DNA ladder marker, lane 1- 18: amplified products for bacterial isolates.

Sequencing analysis

Sequencing of csgA gene analysis

Sequencing of *csgA* gene of all isolates was done by sending PCR products of amplification of *csgA* gene to Macrogen Corporation-Korea using ABI3730XL, automated DNA sequences. Then sequenced DNA in NCBI GenBanK database and the results were analyzed by using Geneious 9 software to draw phylogenetic tree. Sequencing analysis was applied to the PCR product of 8 positive samples from 16 isolates. Phylogenetic tree of the *csgA* gene carried out by using Geneious 8 software for all (16) locally isolates of multidrug resistance *A. baumannii* from sources of isolation included, wound, and Burns infections. The results in phylogenetic tree showed similarity by sequence analysis between them 100% of isolates as shown in Figure (3).

- Tree of *csugA* gene:



Conclusion

The present study showed that *rplB* gene is very important housekeeping gene for molecular detection of *A. baumannii* at the genus and species level, *csgA* that have been diagnosed in the collected isolates and have a role in the formation of the biofilm, which is a

major cause of antibiotic resistance, sequence analysis of *csgA* showed that the genetic sequence of isolates collected from hospitals in Baghdad has the same genetic sequence found in the gene bank for *csgA* genes.

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