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Detection of some biofilm genes in *P. aeruginosa* and *S. aureus* isolated from clinical samples

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Abstract

Background. The biofilm an organized community of *P. aeruginosa* and *S. aureus* is usually associated with the transition from an acute to a chronic infection, and it is one of the bacteria's potent virulence factors**. Aims.** This study aimed to identify *P. aeruginosa* and *S. aureus* and determine whether the clinical isolates could form biofilms and their association with the biofilm genes. **Methods.** 122 samples were obtained from several clinical sources, and isolates were identified using morphological and biochemical tests furthermore, PCR was applied to identify the housekeeping genes (*rpsL*for *P. aeruginosa* and *16SrRNA* for *S. aureus*). **Results.** The results verified the existence of *P. aeruginosa* in 36 (29.5%) and *S. aureus* in 25 (20.4%) of the total sample size. Using a microtiter plate assay, the isolated bacteria's capacity to create biofilm was examined, the finding revealed that all isolates were biofilm producers with 55.5%, of *P. aeruginosa* and 64% *S. aureus* being strong biofilm producers, 30.5% and 32% were moderate producers of biofilms and 13.8% and 4% were weak producers of biofilms respectively. Additionally, a PCR assay was used to find out whether genes linked to biofilms were present (*algD* in *P. aeruginosa* and *ica*A in *S. aureus*). **Conclusion.** The results demonstrating that these genes were present in the isolates and were responsible for adhesion.

Keywords: *P. aeruginosa*, *S. aureus*, biofilm, *algD*, *ica*A, PCR

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Introduction

Pseudomonas aeruginosa is a common opportunistic infection that is Gram-negative and occurs in both water and soil (1). *P. aeruginosa* infections are mostly caused by bacterial biofilm, it involves urinary tract infections (UTI), respiratory mucosal infections, and keratitis (2). *Staphylococcus aureus* is a Gram-positive bacteria responsible for numerous infections in humans, encompassing invasive infections that can result in sepsis and toxic shock syndrome, as well as infections linked to *S. aureus* biofilms. These infections can affect the skin, respiratory system, bone articulations, and endocarditis (3).

The process for the formation of biofilms is complex and involves several steps. It starts with planktonic bacteria adhering to the surface to form a monolayer, then clonal growth /aggregation and forming microcolonies, maturation forming structures resembling mushrooms, ispersal (4).

The formation of biofilms is crucial for persistent infections in humans (5). The National Health Institute estimates that biofilms are responsible for around 80% of all microbial illnesses (2). Urinary tract and respiratory tract infections, keratitis, otitis media, skin infections, vaginal infections, and chronic wounds are among the diseases and infections associated with biofilms. Numerous forms of chronic biofilm-associated infections are caused by *P. aeruginosa*, *S. aureus,* and *Candida* spp. (6). As *P. aeruginosa*, *S. aureus*, and *Candida* species cause chronic biofilm-associated infections, treating these diseases with existing antimicrobials has grown more challenging (7) (8).In *P. aeruginosa* and *S. aureus* bacteria, genes responsible for biofilm formation are *algD* in *P. aeruginosa* and *ica*A in *S. aureus*. The first gene in the operon of *P. aeruginosa* is *algD*, which is completely controlled in its expression and is required for the synthesis of alginate (9). Alginate is a polymer that contains α-L-guluronic acid and β-D-mannuronic acid. It plays a crucial part in protecting and maintaining the structural stability of biofilms (10). The *ica*A gene is a particular gene linked to the ability to form biofilms in strains of *S. aureus*. (11) (12).

Transmembrane, a protein produced by the *ica*A gene, possesses the enzymatic activity of N-acetylglucosaminyl transferases, facilitating the formation of poly-N-acetyl glucosamine polymer (13).

Materials and Methods Sample collection

From October 2023 to February 2024, 122 clinical specimens were taken from different wards at AL-Yourmok Teaching Hospital and Central Teaching Hospital of Pediatric in Baghdad City. The specimens included individuals of various ages and sexes, including urine tract infections, blood, burn swabs, and wound swabs.

Morphological bacterial isolation and identification

Agar plates (Nutrient, MacConkey, Mannitol Salt, and Cetrimide) were used to culture all the specimens, incubated for 24 hr. at 37°C., then the isolates were identified by Standard microbiological techniques (14) as colony morphology, Gram staining, and biochemical reactions like as oxidase, catalase, and coagulase

Molecular Identification of *P. aeruginosa* and *S. aureus* using Polymerase chain reaction (PCR)

To conform the standard microbiological identification, the suspected isolates were molecularly identified using conventional PCR to detect the presence of the housekeeping genes: *16srRNA* gene in *S. aureus* and the *rpsL* gene in *P. aeruginosa*. The Wizard® Genomic DNA Purification Kit was utilized to extract genomic DNA from bacterial isolates. A nanodrop instrument was utilized to measure the concentration and purity of DNA, and agarose gel electrophoresis was used for examining genomic DNA run on 0.8 % agarose (30 min at 100 volts) stained with ethidium bromide (15). The PCR mixture was set up in a total volume of 25µl by using the Go Taq® Master Mix kit, including 12.5µl of the Master mix, 1µl for each primer as listed in (Table 1), 6.5µl from Nuclease-free water, 4µl from DNA, The program demonstrated in (Table 2) and (Table 3) was adopted. The procedure of gel electrophoresis was run in 1.5% agarose gel for 60 min. At 70 voltages.

Table (1): A list of primers used during the study

Primer name	Primers sequence 5′ ⟶ 3′	size (bp)	Reference
algD	F- ACGAAGTGGTGGCGAGTTC	126	(16)
	R-TGGTGTGCGGCATGAAGC		
rpsL	F- GCAAGCGCATGGTCGACAAGA	201	(17)
	R-CGCTGTGCTCTTGCAGGTTGTGA		
icaA	F- GAGGTAAAGCCAACGCACTC	151	(18)
	R- CCTGTAACCGCACCAAGTTT		
16S rRNA	F- GGGACCCGCACAAGCGGTGG	191	(18)
	R- GGGTTGCGCTCGTTGCGGGA		

Table (2): The PCR reaction condition of housekeeping gene (*rpsL*) in *P. aeruginosa*

Stage	°C	Time	No. cycle
Initial denaturation	95	5 min.	1
1-Denaturation	94	30 sec.	30
2- Annealing	57	30 sec.	
3- Elongation	72	30 sec.	
Final Extension	72	7 min.	1

Table (3): The PCR reaction condition of housekeeping gene (*16srRNA*) in *S. aureus*

Stage	°C	Time	No. cycle
Initial denaturation	95	5 min	1
1-Denaturation	95	20 sec.	30
2- Annealing	60	20 sec.	
3- Elongation	72	20 sec.	
Final Extension	72	5 min.	1

Biofilm formation assay

The formation of biofilms in 96-well microtiter plates (MTP) was tested according to the following steps (19): 180 µl of brain heart infusion broth with 1% glucose was poured into 96-well polystyrene microtiter plates, the overnight broth cultures of the isolates were inoculated into a microtiter plate (20 µl per well). Then the plate was placed in the incubator at 37°C for 24 hours in an aerobic environment. All plates underwent three rounds of distilled water washing and drying following incubation. 200 µl of methanol was applied to every well to fix the biofilms for 15 minutes at room temperature, followed by washing and air drying. 200 µl of 0.1% crystal violet solution was applied to the plates and left for 15 minutes. The plates were washed and dried for around 30 minutes at 37°C to make sure the wells were dry. 200 µl of absolute ethanol

was combined with glacial acetic acid (1:1) to resolubilize the dye for 10 minutes. Using a microtiter plate (MTP) reader, the optical density (OD) of every well was measured at 580 nm. Isolates were classified into four groups None, weak, moderate, and strong biofilm producers according to the (20).

Molecular detection of biofilm genes using PCR

Using Uniplex PCR, the biofilm-producer isolates were chosen for the present study to identify the biofilm genes (*ica*A genes in *S. aureus*) and (*algD* gene in *P. aerugiosa*). Specific primer sequence and product size are mentioned in (Table 1). PCR mixture was performed using 12.5 µl of Go Taq® Master Mix kit, 1 µl (10pmol/µl) of each primer, and 4 µl of bacterial DNA extraction, the volume was completed by adding 6.5 µl of deionized distilled sterile water, bringing the final volume to 25 µl. All components of each PCR mixture were performed in a PCR thermal cycler apparatus. The program is demonstrated in (Table 4) and (Table 5) was adopted. The PCR results were electrophoresed on 1.5% agarose containing 0.5 mg/mL of ethidium bromide for 90 minutes at 70 volts using a 1 kb DNA ladder (Roche, Germany).

Table (4): The PCR reaction condition of biofilm gene (*algD*) in *P. aeruginosa*

Stage	°C	Time	No. cycle
Initial denaturation	95	3 min.	1
1-Denaturation	94	1 min.	35
2- Annealing	58	30 sec.	
3- Elongation	72	1 min.	
Final Extension	72	4 min.	1

Table (5): The PCR reaction condition of biofilm gene (*ica*A) in *S. aureus*

Stage	°C	Time	No. cycle
Initial denaturation	95	5 min.	1
1-Denaturation	95	20 sec.	30
2- Annealing	57	20 sec.	
3- Elongation	72	20 sec.	
Final Extension	72	5 min.	1

Results

Isolation and identification of *P aeruginosa* and *S aureus*

In this study, 122 clinical specimens were collected from wound swabs (n=32), blood (n=13), burn swabs (n=23), urine tract infections (n=26), and ear pus swabs (n= 28). *P. aeruginosa* and *S. aureus* isolates were identified using standard microbiological procedures, the study identified 63 isolates, which make up 51.6% of the 122 samples.

Among these, 38 isolates (31.1%) were classified as *P. aeruginosa* and 25 isolates (20.5%) as *S. aureus*. The results are shown in (Table 6). Based on the appearance of the bacterial isolates grown on the media, the diagnosis was made. *P. aeruginosa* isolates grew on MacConkey agar (a non-lactose fermenter) and produced colonies of the β-hemolysis type, while the bacterial colonies on Cetrimide agar medium appeared greenish-yellow. The isolates are gram-negative, positive for oxidase, positive for catalase, and negative for coagulase. The colonies of *S. aureus* isolates appeared on mannitol salt agar in yellow color (Mannitol fermenters), β-hemolysis type on blood agar, gram-positive, negative for oxidase, positive for catalase, and positive for coagulas.

Molecular detection and identification of *P. aeruginosa* and *S. aureus*

Genomic DNA was extracted from all bacterial isolates, as shown in (Figure 1), the presence of clear and well-defined bands indicated successful DNA extraction. The concentration of nanodrop ranged from 112.73 to 618.54 ng/µl and the purity ranged from 1.5 to 1.9.

Table (6): Prevalence of Bacterial Isolates by Injury Type

Injury type	No. Samples	*P.aeruginosa* (%)	*S.aureus* (%)	Total isolates (%)
Wound	32	14 (43.7%)	7 (21.8%)	21 (65.6%)
Blood	13	3 (23%)	0 (0%)	3 (23%)
Burn	23	11 (47.8%)	5 (21.7%)	16 (69.5%)
Urine	26	5 (19.2%)	3 (11.5%)	8 (30.7%)
Ear pus	28	4 (14.2%)	12 (42.8%)	16 (57.1%)
Total	122	38 (31.1%)	25(20.5%)	63(51.6%)

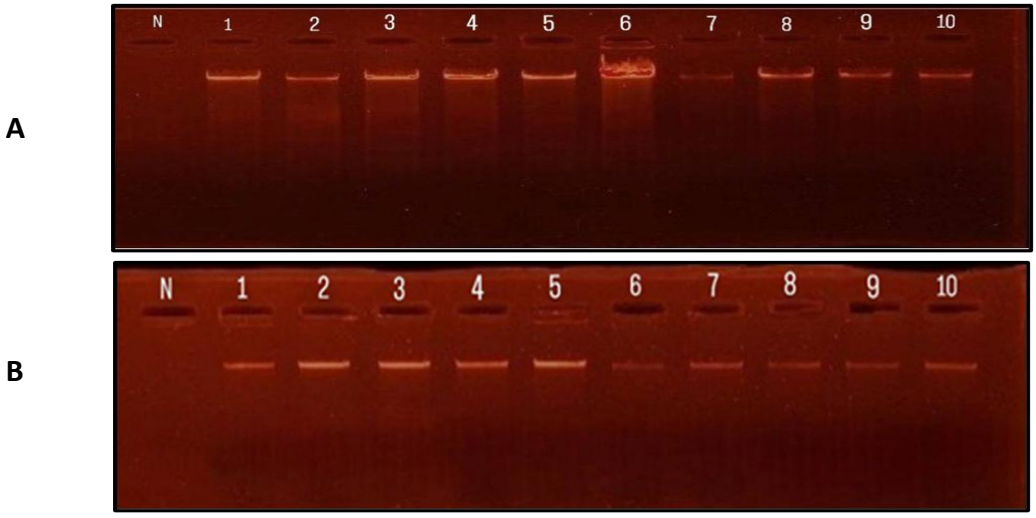


Figure (1): Agarose gel electrophoresis of some genomic DNA extracted from a) *P. aeruginosa* isolates and b) *S. aureus* isolates run on 0.8 % agarose (30 min at 100 volts) stained with ethidium bromide.

For detecting *rpsL* and *16SrRNA* genes, the results of the PCR technique through analyzing the DNA bands on 1.5% agarose gel electrophoresis showed that 36/38 (94.7%) *P. aeruginosa* isolates carried the *rpsL* gene with the size 201 bp and 25/25(100%) *S. aureus* isolates carried the *ica*A gene with product size 191bp (Figure 2).

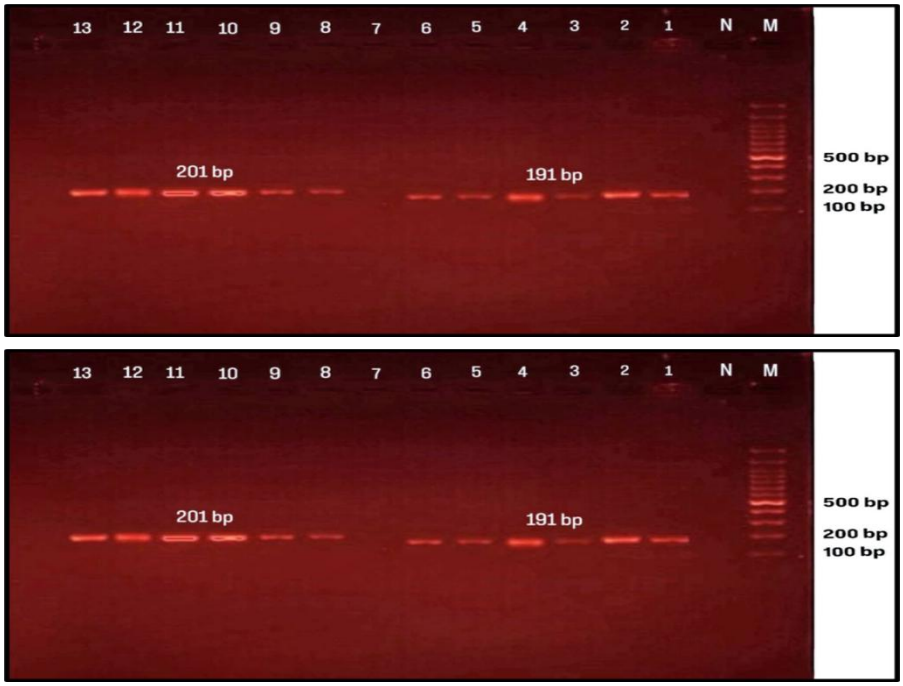


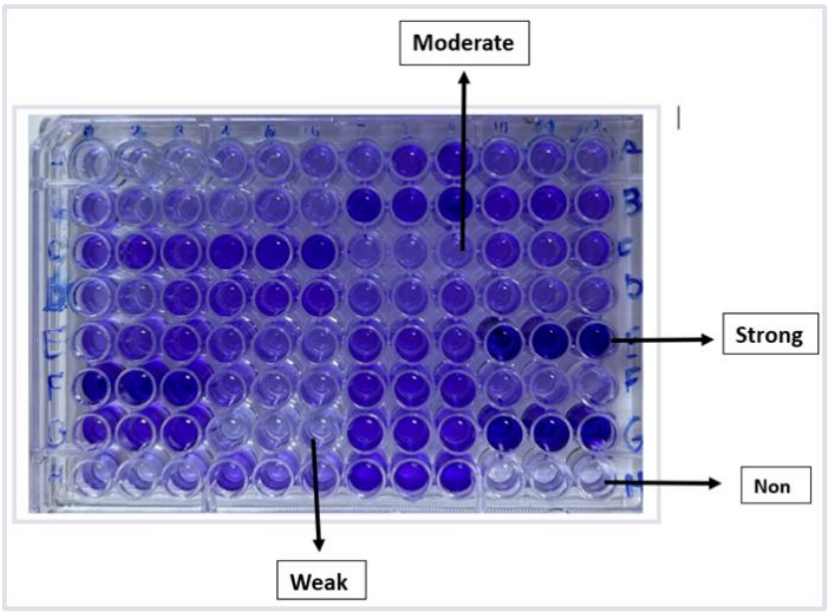
Figure (2): Agarose gel electrophoresis of amplified PCR product to identify the *16srRNA* gene and the *rpsL* gene, 1.5% agarose was run on for 60 minutes at 70 volts (stained with ethidium bromide), lane M: DNA . ladder; N: Control negative; lanes 1-6: *16srRNA* gene (191 bp) in *S. aureus*; lanes 8-13: *rpsL* gene (201 bp) in *P. aeruginosa*

Biofilm-formation assay

The present study used a microtiter plate (MTP) and a microplate spectrophotometer to measure the capacity of 36 *P. aeruginosa* isolates and 25 *S. aureus* isolates to adhere to the different surface and form a biofilm, which is one of the most significant virulence factors of the bacteria. Distribution of *P. aeruginosa* and *S. aureus* isolates according to biofilm formation based on determined cutoff value. The results are shown in (Table 7), (figure 3) and (figure 4).

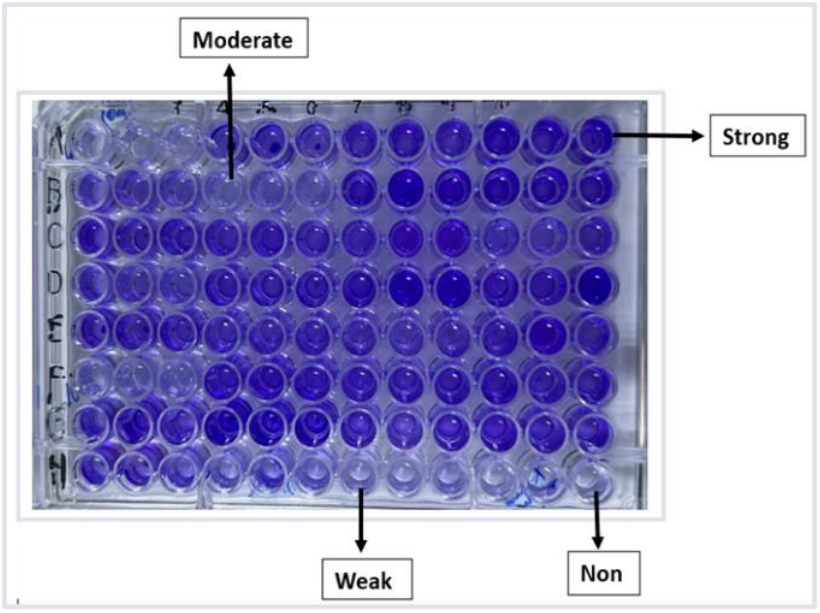
Table (7): Distribution of *P. aeruginosa* and *S. aureus* isolates according to biofilm formation based on determined cutoff value.

Biofilm formation	Bacterial isolates (n=61)			
	No.(%) of *P. aeruginosa*	OD 580 Limits	No.(%) of *S. aureus*	OD 580 Limits
Strong	20(55.5%)	0.521-3.827	16(64%)	0.505-2.165
Moderate	11(30.5%)	0.217-0.465	8(32%)	0.217-0.436
Weak	5(13.8%)	0.131-0.169	1(4%)	0.126
Non-adherence	0(0%)	0.047-0.085	0(0%)	0.056
%	36 (100%)	---	25 (100%)	---



*Non (control -)

Figure (3): The results of biofilm formation using a microtiter plate (MTP) for *P. aeruginosa*



*Non (control -)

Figure (4): The results of biofilm formation using a microtiter plate (MTP) for *S. aureus*

Detection of biofilm genes (*algD*) for *P. aeruginosa* and (*ica*A) for *S. aureus* by conventional PCR

PCR is utilized to determine which gene in the isolates is responsible for biofilm formation. The conventional PCR technique was also used to detect the presence of biofilm genes: *algD* (126 bp) in *P. aeruginosa*, and *ica*A (151bp) in *S. aureus* associated with housekeeping genes using specific primers for each gene. The presence of the target genes was confirmed by the PCR findings, as indicated in (Figures 5 and 6 respectively) by comparing the molecular weight of the PCR products on 1.5% agarose gel electrophoresis with the 100 bp DNA ladder.

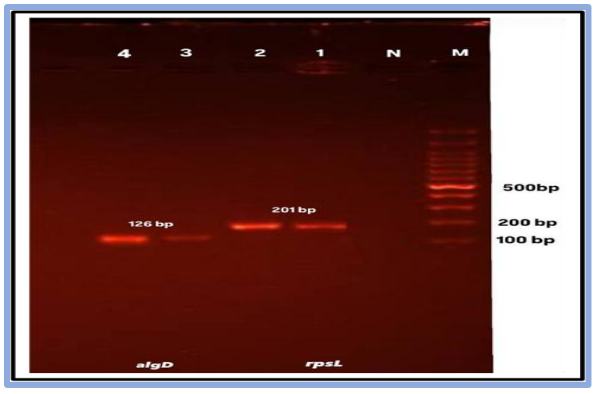


Figure (5): Amplified PCR products for *algD*, *rpsL* genes of *P. aeruginosa* in 2%agarose, 70 volts for 90 minutes. M: 100bp DNA ladder N: Control negative; lanes 1-2 *rpsL* amplicon (201 bp); lanes 3-4 algD amplicon (126 bp).

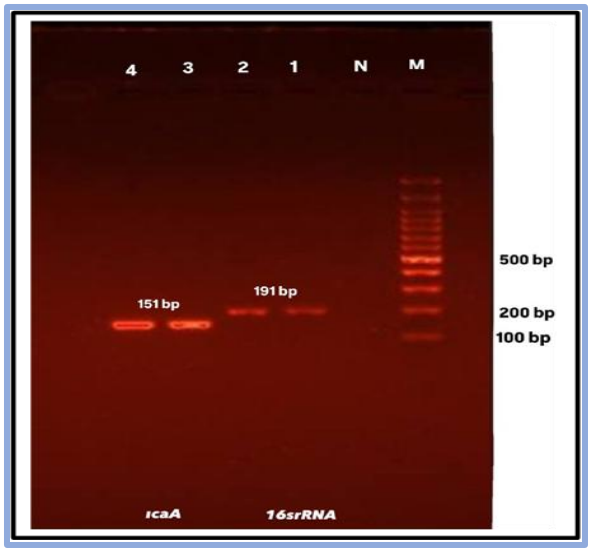


Figure (6): Amplified PCR products for *16srRNA*, *ica*A genes of *S. aureus* in 2%agarose, 70 volts for 90 minutes. M: 100bp DNA ladder N: Control negative; lanes 1-2 *16srRNA* amplicon (191 bp); lanes 3-4 *ica*A amplicon (151 bp).

Discussion

Isolation and identification of *Pseudomonas aeruginosa* and *S aureus*

The current study revealed that 38 (31.15%) clinical isolates were recognized as *P. aeruginosa*. This finding was higher than the rates reported by (21) and (22) which were 20%, and 24.5%, respectively. Many studies conducted in Baghdad with a similar nature by (23) and (24),(25), and (26), found *P. aeruginosa* in 30%, 35%, 37.7%, and 50.6% of their samples respectively.

The current results showed that 25 (20.5%) clinical isolates were recognized as *S. aureus*. This finding was less than (27) and (28) who found a rate of *S. aureus* 35.5% and 64% of the samples, respectively. And higher than (29) found a rate of *S. aureus* (17.3%).

Over all this finding may be explained by the highest prevalence of this bacteria in the contaminated environment and nature.

Molecular detection and identification of *P. aeruginosa* and *S. aureus*

Confirmatory identification of 38 *P. aeruginosa* isolates and 25 *S. aureus* isolates was done by detecting the housekeeping gene (*rpsL* and *16S rRNA*) using the PCR technique.

Numerous genes have been documented to function as internal controllers with stable expression in a variety of conditions, including *rpsL* Among the 13 housekeeping genes studied, these studies indicated that *rpsL* was the most stable and therefore the best housekeeping gene under various conditions (30, 31, 32).

The *16S rRNA* gene was identified in all isolates (100%) of *S. aureus*. These findings agree with other studies indicating that identifying clinical isolates of *S. aureus* through the detection and sequencing of this gene is an effective means with (29), (33), and (34). but not in agreement with (35).

Biofilm-formation assay

The results of *P. aeruginosa* in this study were consistent with the results of (36) indicating that 100% of isolates form a biofilm, but these results are not consistent with others like (37). In addition, the results of *S. aureus* in this study were consistent with (38) and (39) who demonstrated that all *S. aureus* isolates (100%) can form biofilms. But the results were not consistent with (40) who found 70.1% and (41) who found that 52.6% of *S. aureus* isolates from humans were adherent.

Detection of biofilm genes (*algD*) for *P. aeruginosa* and (*ica*A) for *S. aureus* by conventional PCR

According to studies on the relationship between the *algD* gene and *P. aeruginosa* biofilm formation, all biofilm formers have the *AlgD* gene. This finding is consistent with the findings (42) and (43) revealed that 100% of the *P. aeruginosa* isolates that form biofilms had the *AlgD* gene. And with a study conducted by (44) showed that 96% of the isolates that produced biofilm, contained the *algD* In addition, the study demonstrated that all biofilm-producer - strains of *S. aureus* carried the *ica*A gene Our results were similar to the findings of (45), and (46) who indicated that all biofilm producer - strains carried the *ica*A gene. The capacity of *S. aureus* to form biofilms is also dependent on the synthesis of polysaccharide intercellular adhesion molecules, which are encoded by the ica locus, which is made up of the *ica*A, *ica*B, *ica*C, and *ica*D genes. Amplified by PCR of (47). The *ica*A gene demonstrates the inherent biofilm producing nature of the isolates. And disagree with (27) who found that (81.3%) possess the *icaA* gene. A study by (11) demonstrated that, although the contribution of multiple genes in the formation of biofilms, the *ica*A gene was the particular gene found to be necessary for the generation of biofilms in *S. aureus* isolates.

Conclusion

The study concluded that the presence of biofilm genes is required for the ability of *P. aeruginosa* and *S. aureus* to form a biofilm

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