Genotypic estimation of the biofilm formation of *Staphylococcus aureus* (MRSA) isolates from dental staff

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Abstract

In this research, the six isolates of *Staphylococcus aureus* isolated from dental workers (dentists and assistants) were taken and diagnosed in another research which is under publication, to study its genetic estimation and its ability to form biofilms by plate Microtiter method. The results showed that all *Staphylococcus aureus* isolates have the ability to form biofilms because they own the genes *ica*A and *ica*D except the isolate number one which have the gene *ica*D so it gives a slight or thin biofilm . The isolates were also molecularly diagnosed using the Polymerase Chain Reaction (PCR) technique, and the results showed that 3\6 (50%) isolates of *S.aureus* bacteria were resistant to methicillin due to their possession of the *mec*A gene that encodes the resistance to the antibiotic methicillin. In addition, they possessed the *ica*A and *ica*D genes responsible for the formation of biofilms with percentages of (83.3 and 100)%, respectively, and it was found that (83.3%) of these isolates contained the *ica*A and *ica*D genes, but they did not contain the *qac*A\B gene., while they possessed the *Smr* gene with a percentage of (16.6%) of the total number of the six isolates under study.

Key words: MRSA , Biofilm , *mec*A , Adhesion gene , Dental staff.

Introduction

Particular attention should be paid to *Staphylococcus aureus* because it is most common cause of hospital – acquired in the world due to the greatest capacity of pathogenicity among *Staphylococcus* species (Yaslianifard *et al*., 2017). This species is G + ve coccus which colonizes the skin and nasal mucosa of healthy individuals , also can cause a wide range of infection from skin to soft tissue and systemic infections some are fetal disease(Sato *et al*.; 2019).One of the biggest problems in public health it is resistance to antibiotic (Hashemizadeh *et al*.; 2019).Methicillin–resistance *S.aureus* (MRSA) which resist multiple antibiotics and has emerged among persons in nursing homes , hospitals, etc (Zimmerli *et al*.;2009). MRSA is classified into hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (Guo *et al*.; 2020). The *mec* A gene is responsible for methicillin

resistance is gene carried by a DNA fragment known as staphylococcus cassette chromosome *mec* (SCCmec), which encodes penicillin binding protein (PBP-2a) which inhibits the action of β- lactam antibiotics such as methicillin (Rasheed and Hussein, 2020). Cell wall synthesis was inhibited by the action of PBPs,they involved in collecting of the peptidoglycan of bacterial cell wall because of their high affinity to β- lactams antibiotic (Aziz and Hassan, 2019). The slow affinity of penicillin binding protein which catalyzes transpeptidation of the peptidoglycan formation helps *S.aureus* stay alive in the presence of high concentration of antibiotics (AL-Saadi *et al.;* 2020).The resistant *Staphylococcus aureus* strains also have the ability to form biofilms in a dynamic and complex multi-layered matrix which is an important complication factor help the bacteria forming disease .The biofilm is a barrier against antibiotics and different adverse conditions ; also defend bacterial cells from the invasion of host – immune cells (Azmi *et al*.; 2019; Naorem *et al*.; 2020).Attachment , multiplication , maturation and dispesion are the dynamic biofilm formation steps ; and the density and number of bacterial cell were mediated by the outputs of adhesion genes *ica*A , *ica*B , *ica*D and *ica*C the proteins responsible for production of the intercellular polysaccharides (PIA) and capsular polysaccharide \ adhesion(PS\A) were encodes by these genes (Chen *et al*.; 2019; Khadija *et al* .; 2017).

Materials and methods

Samples were collected and *S.aureus* was diagnosed in a previous search (Prevalence of staphylococci among dental staff and their antibiotic resistance pattern) Under publication .

Biofilms formation of bacterial isolates

The Microtiter plate method was performed according to the method of Christensen *et al* ., (1985). we choose 6 isolates of *S.aureus* (Nad1, Nad2, Nad3, Nad4, Nad5, and Nad6), to detection their ability of biofilm formation, the absorbance (OD) was read at 630nm using Microplate reader 800 TS BioTek, USA. (Triveni *et al*., 2018).

Molecular assay:

DNA extraction:

The genome of *S.aureus* (Nad1, Nad2, Nad3, Nad4, Nad5, and Nad6), was extracted by using Miniprep ™ DNA isolation kit (Zymo \ USA) .

Determination of DNA concentration and purity:

The concentration and purity of the extracted DNA were determined by adding 200 µl from Tris-EDTA (TE) to 3,800 from D. water the mix 4000 µl, pull 10 µl ignore it and adding 10 µl from the dye (DNA Dye) pulling 200 µl of the mix for each sample. The series of the following tubes are prepared as follows (Table 1).

Table 1- The series of the following tubes.

	Blank	Standard	Sample
Mix	200 µl	200 µl	200 µl
DNA Extraction		2 µl	2 µl

Make vortex for second to mix, leaves on rake at room temperature for (5) min, extracted the value from the device immediately. The nanodrop spectrophotometer device (Nabi , korea) was used to measure the DNA concentration and purity ratio of absorbance at (260) nm \ absorption at (280) nm.

Agarose gel electrophoresis of DNA:

Electrophoresis was performed to determine the size of the DNA bundles extracted while the standard DNA was in the polymerase chain reaction on an agarose gel. According to the manufacturer's instructions, the extracted DNA was used as a template for PCR analysis, which was performed to detect the gene associated with the identification of S. aureus (Nad1, Nad2 and Nad3), 16S rRNA, *mec*A, *ica*A, *ica* D, *qac*A/B and *Smr* genes. The sequence of primers and amplification volume are described in Table (2).

Table(2): Primer sequences used in this study.

Gene	Primer	Nucleotide sequence (5' to 3')	PCR product (bp)	Reference
16S rRNA	F:	5'- AGAGTTTGATCCTGGCTCAG- 3'	1250	Srinivasan *et al.*, 2015
	R:	5'- GGTTACCTTGTTACGACTT- 3’		
*mec*A	F:	5'-GTAGAAACTGAACGTCCGATAA - 3'	125	Rasheed and Hussein ,2020
	R:	5'- CCAATTCCACATGTTTCGGTCTA- 3’		
*ica*A	F:	5'- ACACTTGCTGGCGCAGTCCA- 3'	188	Chen *et al.*,2019
	R:	5'- TCTGGAACCAACATCCAACA- 3’		
*ica*D	F:	5'- ATGGTCAACCCAGACAGAG- 3'	198	Keikhaie *et al.*, 2017
	R:	5'- AGTATTTTCAATGTTTAAAGC- 3’		
*qac*A\ B	F:	5'-CTTGGTATTGCAGGTGCTTT - 3'	1125	Lin *et al.*, 2020
	R:	5'- AATCCCACCTACTAAAGCAG- 3’		
Smr	F:	5'- ATAAGTACTGAAGTTATTGGAAGT- 3'	286	Lin *et al.*, 2020
	R:	5'- TTCCGAAAATGTTTAACGAACTA- 3’		

Results and Discussion:

Characterization of *S.aureus* isolates based on the genes encoding biofilm formation

Biofilm composition was detected in six pre–diagnosed *S.aureus* samples (Nad1, Nad2, Nad3, Nad4, Nad5, and Nad6), and biofilm formation was observed in Table (3).

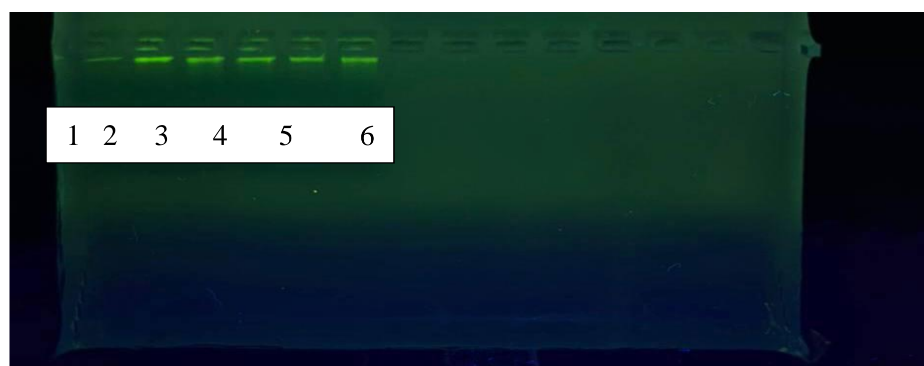
Table 3-Biofilm formation of isolate according to Duncan test.

Isolate No.	Subset for alpha = 0.05 (Mean ± std)		
	A	B	C
Control	0.3633		
Nad1	0.5100		
Nad2		0.5467	
Nad5		0.6267	
Nad3			0.6467
Nad6			0.7967
Nad4			0.8000
Sig	0.55	0.93	0.56

The results showed that all isolates had the ability to form biofilm except isolate number one didn't form biofilm, Orjih *et al* .,(2021) showed that 13(26%) of *S.aureus* isolates was strong biofilm production while 22(44%) were moderate biofilm production and 15(30%) were non biofilm production when use tissue culture plate (TCP) method .

Genomic DNA isolation:

After isolation of genomic DNA of local isolates of *S.aureus* (Nad1, Nad2, Nad3, Nad4, Nad5, and Nad6), DNA transfer by electrophoresis using agarose gel 1% , the gel was exposed to UV rays at length 336nm in U.V translhumintor in order to detected the genomic DNA bands understudy (Fig.1). Agarose gel electrophoresis has proven an effective method for separating nucleic acids and it was found that the conventional concentration used for agarose is the most effective in separating DNA fragments between 100bp and 25 kb [Devor, 2010] . It is known that the DNA molecule is separated according to its size inside an agarose gel, with the distance traveled in inverse proportion to its molecular weight [Lee et al., 2012], so that, six bands of equal size appeared as they traveled equal distances and had large size this may be attributed to the small distances traveled in the agarose gel for all genomic DNA samples purified from local *S.aureus* isolates (Hisyan,2020).



Figure(1): Gel electrophoresis of genomic DNA extraction from Bacteria, 1% agarose gel at 5 vol /cm^2 for 1 houre.

Determination of DNA concentration and purity:

Extraction results of *S.aureus* (Nad1, Nad2, Nad3, Nad4, Nad5, and Nad6), were for concentration and purity determined by using nanodrop spectrophotometer at 260/ 280 nm. Concentration values of DNA rang between 3.4 to 176 ng/µl while purity ranged from 1.3 -2 at absorbant 260/ 280 nm, and for each of the second and sixth samples, the third and second samples, respectively (Tabble 4) .

Table 4- Concentration and Purity of DNA.

Sample ID	Nucleic Acid Conc. (ng/µl)	Purity at 260/280 (nm)
Nad1	6.0	1.4
Nad2	3.4	2.0
Nad3	8.7	1.3
Nad4	155	1.71
Nad5	76	1.9
Nad6	176	1.85

A good purity ranges from 1.80-2.00). Repeat for each sample.

Lara *et al.;* (2018), observed that DNA concentration was between 2.1 and 3.39 ng/µL, thus giving a lower standard deviation at concentration 107CFU/ml, while the purity was less than the ideal limit from 0.85 to 1.67 (at absorbance 260/280 nm) and from 0.18 to 0.44 (at absorbance 260/230 nm).

Specific PCR of genomic DNA:

The specific reaction was carried out for the purified DNA samples based on the 16S rRNA gene for three isolates of *S.aureus* (Nad1, Nad2, and Nad3), which were selected from the six previously mentioned isolates because the DNA was of equal sizes , the purified DNA product were obtained from *S.aureus* isolates of one size 1250bp in terms of the sizes of the accompanying DNA ladder as in Figure (2), and this is a definitive indication of the association of the nitrogenous bases of the primer of the 16S r RNA gene with the nitrogenous bases that complement it in one of the two purified DNA strands.

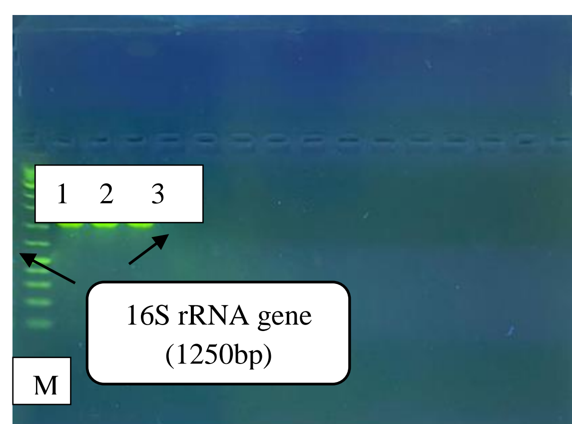


Figure 2-PCR product the band size 1250 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. M: DNA ladder (1000 PLUS).

The difference in the size of this gene shown in this study from it's size in other studies is often attributed to its design in other sizes, as in the primer used in size 257 bp by the researchers Omar and Mohammed (2021).

Study and analysis of nitrogen bases sequences of the specific PCR products:

The objective of conducting a technique sequences for the purpose of determining the sequences of the nitrogenous bases (nucleotides) of the DNA molecule obtained from the previous reaction in which agarose was used at a concentration 2% and the bands were exposed to ultraviolet radiation at wavelength (302) after staining them with a dye red stain , the products of the specific replication reaction (specific PCR) were sent in the form of purified DNA from three local isolates belong to the *S.aureus* and entered it in to a program Basic Local Aligment Search (Blast) and available in the National Center Biotechnology information at (http:// www.ncbi.nlm.nih.gov) and Bio Edit program to analyze these sequences and show their affinity with the existing sequences registered in the genebank. The result of the analysis of the three samples showed a significant similarity of 99% between these sequences and the sequences of isolates of *S.aureus* bacteria recorded in the GenBank with the numbers MT154222.1, MZ047202.1, and MT154222.1 as shown in Figures 3-5 and Table 5.

Conventional diagnosis of bacteria based on phenotypic characteristics is generally not as accurate as molecular diagnosis, particularly using bacterial 16S rRNA gene sequencing [Clarridge, 2004], so that, the classification of bacteria was based on the 16S rRNA gene become a major tool in the determination of relationships between bacteria, and therefore the similarity limit is currently accepted at 98.65% for species identification , and his method is accurate and convenient for scientific classification and identification of prokaryotes [Goodfellow *et al*., 2014].

On the other hand, only three regions were observed in the first and second isolates, 394, 305, 391 and 300, 334, 438 respectively, in which the nitrogenous bases were transvertion, while two regions were observed in the third isolate, one of them was the nitrogenous bases transition at location 492, and the other was the transvertion at position 565, and these regions did not have any effect on the genetic code of this gene.

These results may be interpreted on the basis that 16S rRNA genomic sequencing is the rapid and accurate method adopted in the diagnosis of bacterial isolates as it provides accuracy in identifying nucleotide differences in the RNA operon in a single genome [Raina et al., 2019].

Table 5- Trinsvertion and Transition mutation of 16S ribosomal RNA gene in *Staphylococcus aureus* isolates understudy.

	Gene: 16S ribosomal RNA gene					
No.	**Type of substitution**	**Location**	**Nucleotide**	**Sequence ID with compare**	**Source**	**Identities**
Nad	**Transvert**	**394**	**G\C**	**ID: MT15422**	***Staphylococcus***	**99%**

1	ion			2.1	*aureus*	
	Transvert ion	305	G\C			
	Transvert ion	391	G\C			
Nad 2	Transvert ion	300	G\C	ID: MZ04720 2.1	*Staphylococcus aureus*	99%
	Transvert ion	334	G\T			
	Transvert ion	438	G\C			
Nad 3	Transitio n	492	G\A	ID: MT15422 2.1	*Staphylococcus aureus*	99%
	Transvert ion	565	G\C			

The result showed that 3/6 (50%) isolates of *S.aureus* were harboring *mec*A gene (MRSA) in size 125bp as shown in Fig.3, this gene explain the trait of multidrug resistance (MDR), which is one of the major health problems worldwide, and indicates to that *mec*A gene has been horizontally transferred in *S.aureus* strains more frequently that may be acquired from other *Staphylococcus species*, Sultan and Almeani(2019) showed that all 89(100) isolates of *S.aureus* harboring *mecA* gene (MRSA), while Saeed *et al*.;(2020) showed that *mec*A gene was detected in 33.3% of tested isolated (10/30) of *S.aureus*.

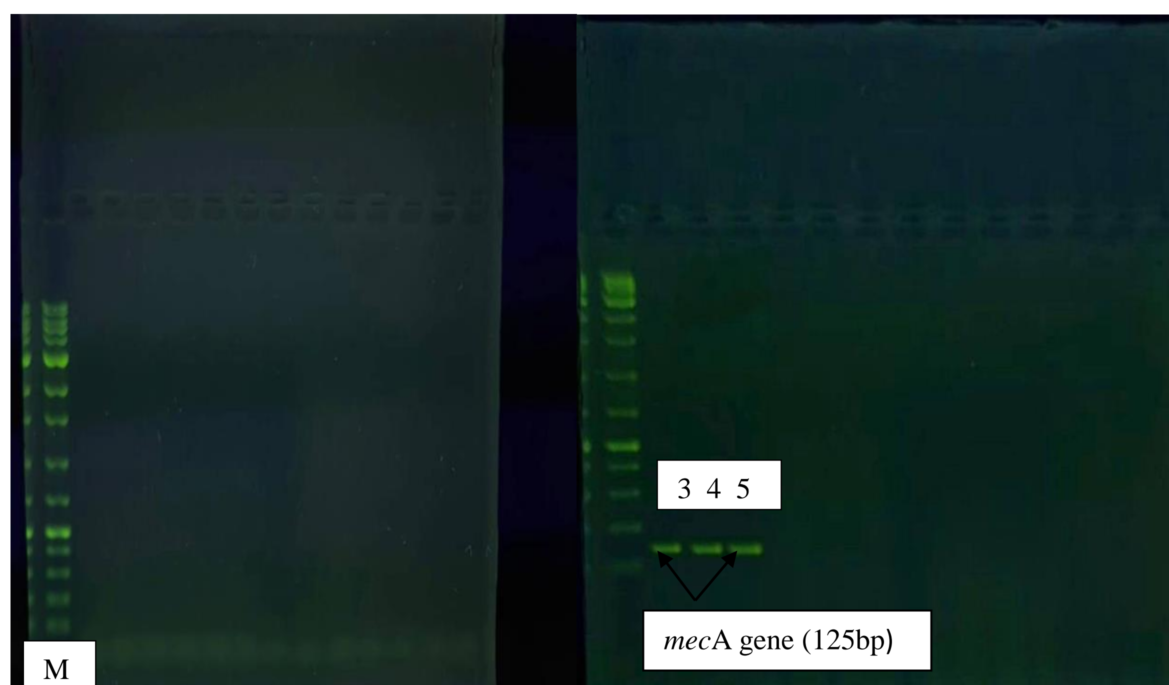
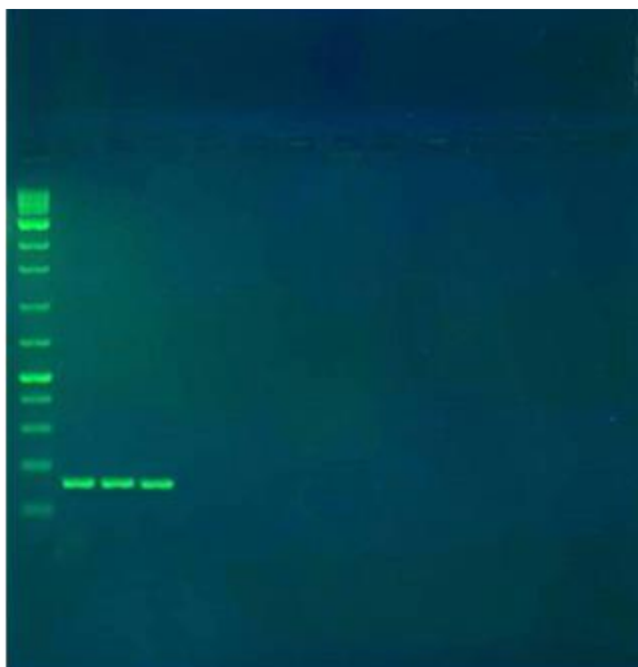


Figure (3) PCR product the band size 125 bp, the product was electrophoresis on 1.5% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. M: DNA ladder (1000 PLUS).



Nad2,3,4,5,6 isolates were found to *S. aureus* possesses the gene *ica*A(Fig.4), which is known to encode biofilm formation. After using the sequence of this gene, it was found that two(Nad2,3) of the five (Nad2,3,4,5,6) isolates showed a significant similarity of 99 and 96% with the sequences of this gene for the two standard isolates *S.aureus* CP071943.1 and *S.aureus* CP017679.1 registered in GenBank, respectively , it was also shown in the first isolate that the transition of the nitrogenous bases constituting this gene was obtained at the 637022 location, and on the contrary, the transition of the nitrogen bases occurred in only two location 2712049 and 2712045 in seconed isolate , as is evident in Table (6).

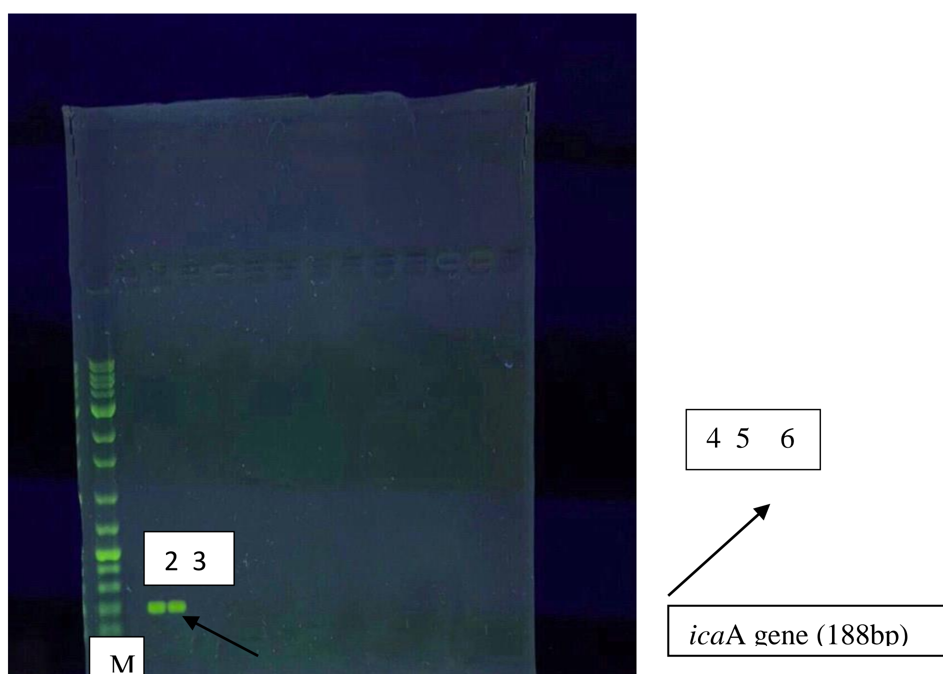


Figure (4) PCR product the band size ~188 bp. The product was electrophoresis on 1.5%

agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. M: DNA ladder (1000 PLUS)

Table 6- Trinsvertion and Transition mutation of IcaA gene in *Staphylococcus aureus* isolates understudy.

Source: *Staphylococcus aureus*									
No. Of sample	**Type of substitution**	**Location**	**Nucleotide**	**Nucleotide change**	**Amino acid change**	**Predicted effect**	**Sequence ID with compare**	**Gene**	**Identities**
Nad2	Transition	637022	A\G	GCC\ACC	Alanine\ Threonine	Missense	**ID: CP071943.1**	**IcaA**	**99%**
Nad3	Transvertion	2712049	G\C	ACT\AGT	Threonine\ Serine	Missense	**ID: CP017679.1**	**IcaA**	**96%**
	Transvertion	2712045	T\G	AAT\CAT	Asparagine\ Histidine	Missense			

The results of electrophoresis by polymerase chain reaction technology showed that all six (100%) isolates possessed the *ica*D gene (Fig.5), and when comparing the sequence of its nucleotides with its standard analogs in the GenBank, it was found that they matched by 99, 100 and 100% for only three (Nad 1,2,3) isolates of *S. aureus* registered with CP047924.1, CP047924.1 and CP047924.1, respectively, and one region in which the nitrogenous bases were replaced was of the transitional type at location 2088261 only (Table 7), this meanse these regions did not affect on the genetic code of this gene.

Biofilm formation genes at *ica*A (188 bp.) were found in (83.33%) of *S.aureus* but (100%) contained *ica*D (198 bp.) gene only and (83.33%) contain both *ica*A and *ica*D gene . Sulaiman and Abdulla, (2018) showed that biofilm formation genes at *ica*A (188 bp.) were found in 35.7% of *Staph. aureus*, *Staph. hominis*, and *Staph. xylosus* but 42.8 % of *Staph. aureus*, *Staph. epidermidis*, *Staph. hominis*, and *Staph. xylosus* contained *ica*D (198 bp.) gene only. Keikhaie et al .,(2017) showed that from 40 isolates of *S.aureus* ,12 (30%) of them were contain *ica*A gene and 8 (20%) isolates were positive for *ica*D gene and 5 (12.5%) isolates were contained both genes .

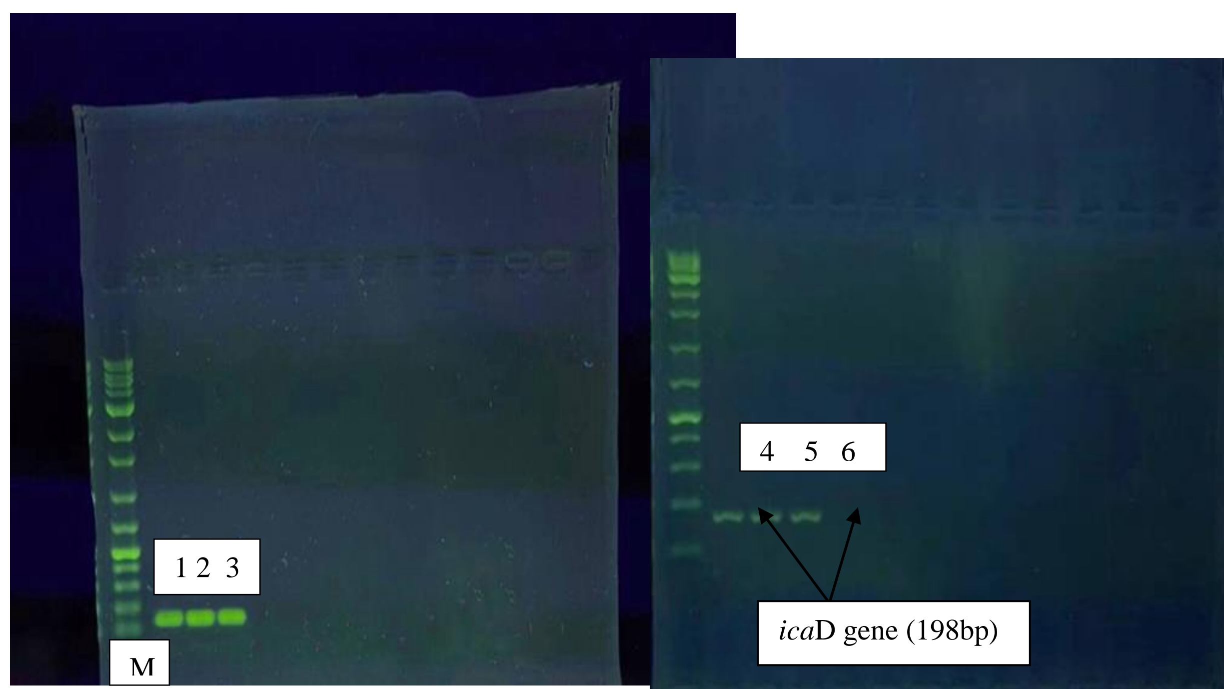


Figure (5) PCR product the band size ~198 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm². 1x TBE buffer for 1:30 hours. M: DNA ladder (1000 plus).

Table 7- Trinsvertion and Transition mutation of ICAD gene in *Staphylococcus aureus* isolates understudy.

Source: *Staphylococcus aureus*									
No. Of sample	**Type of substitution**	**Location**	**Nucleotide**	**Nucleotide change**	**Amino acid change**	**Predicted effect**	**Sequence ID with compare**	**Gene**	**Identities**
Nad1	Transition	2088261	**A\G**	**ATT\GTT**	**Isoleucine\ Valine**	Missense	**ID: CP047924.1**	**ICAD**	**99%**
Nad2	-----	**-------**	------	-------	------	------	**ID: CP047924.1**	**ICAD**	**100%**
Nad3	-----	**-------**	------	-------	------	------	**ID: CP047924.1**	**ICAD**	**100%**

AS for *qac*A/B gene the results showed that no one of isolates harboring this gene Fig.(6). Wong et al., (2013) reported zero prevalence of *qac*C/smr and *qac*A/B genes associated with disinfectant genes in methicillin-resistant *S.aureus* isolated in porcine although another gene associated with disinfectant resistant such as *qac*G was detected.

Figure (6) PCR product the band size 1125 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. M: DNA ladder (1000 PLUS).

As for *smr* gene the result showed 1/6 (16.6%) Nad2 isolates to *S. aureus* possesses this gene (Fig.7). Damavand et al.,(2017) showed that the *smr* gene was identified in 38 (31.7%) isolates of *S.aureus* from which, 19 (50%) *smr* alone.Bacterial efflux systems are a large classes of transported responsible for the uptake of necessary nutrient and ions , also removal of harmful substance , and communication between cells and environment (Amr *et al* ., 2020)

Figure (7) PCR product the band 286 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. M: DNA ladder (1000 PLUS).

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