EGYPTIAN ACADEMIC JOURNAL OF

BIOLOGICAL SCIENCES

PHYSIOLOGY & MOLECULARBIOLOGY

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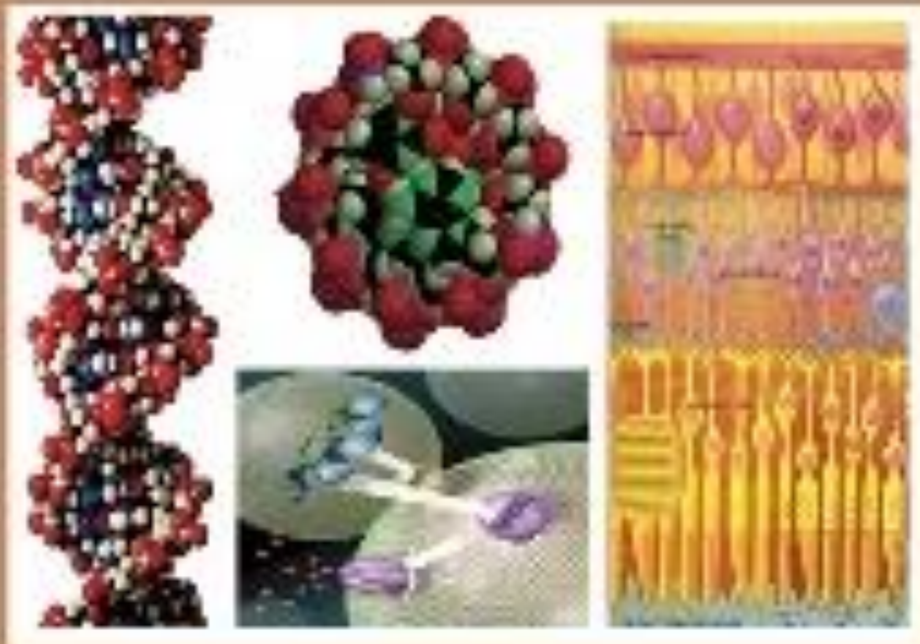

ISSN
2090-0767

WWW.EAJBS.EG.NET

Vol. 15 No. 2 (2023)

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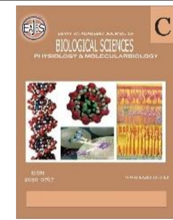
Citation: *Egypt.Acad.J.Biolog.Sci. (C.Physiology and Molecular biology) Vol. 15(2) pp379-389 (2023)*
DOI: 10.21608/EAJBSC.2023.319286

Egypt. Acad. J. Biolog. Sci., 15(2):379-389 (2023)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology
ISSN 2090-0767
www.eajbsc.journals.ekb.eg



Prevalence of Virulence Factor Genes Pvl, nuc, Sea, icaA in Methicillin-Resistant in *Staphylococcus aureus* In Al Najaf City

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ARTICLE INFO
Article History
Received:22/8/2023
Accepted:1/10/2023
Available:6/8/2023

Keywords:
Staphylococcus aureus , PVL, Nuc, Sea, icaA.

ABSTRACT

Staphylococcus aureus is a bacterium that is both common and capable of causing harmful infections in humans. *Staphylococcus aureus* colonies around 30% of the human population. Staphylococcus aureus has a variety of virulence traits that contribute to its capacity to colonies and infiltrate host tissues, avoid the host's immune system, and collect nutrition. Methicillin-resistant Staphylococcus aureus (MRSA) outbreaks have arisen as a major concern in healthcare facilities around the world. Sample collection began from September 2022 to January 2023, during which 300 samples were isolated from patients, including wound swabs, medial urine samples, semen, upper vaginal swabs, and burn swabs. The samples were selected from the patients of Al-Furat Al-Awsat and Al-Najaf Teaching Hospitals in Al-Najaf Governorate in the Guidance Department, and the included age groups ranged from (1-60) years. After diagnosing the samples by Gram stain, catalase test, and Coagulase test and cultures on a mannitol agar, the diagnosis was confirmed by the Vitek device. DNA was extracted by extraction kit. In the next stage, virulence genes were detected by PCR technique, and the result was PVL (20%), while Nuc was (90%), Sea was (63.3%), and finally, icaA was (36.6%). Correlations of virulence genes were also made, namely between age, sex and gene coexistence in the isolates, with Sea and Nuc being the most prevalent, followed by Sea with icaA.

INTRODUCTION

Methicillin-resistant Staphylococcus aureus (MRSA) isolates emerged after the introduction of methicillin by the acquisition of the mecA gene, which is located on a mobile SCCmec cassette chromosome. This genetic element confers resistance to most currently available beta-lactam antibiotics (Katayama *et al.,* 2000). The remarkable ability of S. aureus to cause a broad range of important infections in humans is due to its ability to produce multiple virulence factors that contribute to the pathogenicity and the ability to colonize the host, allowing this bacterium to adhere to surfaces/tissues, avoid or invade the immune system, and cause harmful toxic effects to the host (Costa *et al.,* 2013). One important feature of S. aureus is the ability to secrete toxins that play active roles in the weakness of the host immunity by disrupting host cells and tissues and interfering with the host immune system to release nutrients and facilitate bacterial spread. Leukocidins (Pvl) are virulence factors with two parts and pyrogenic super-antigenic poisons that can disrupt host cell membranes and influence immunological responses by activating immune cells (He *et al.,* 2018).

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The PVL is one of seven leukocidins produced by these bacteria. The PVL, a custom composed of two proteins named F, has 32KDa and S protein 38 KDa; these proteins are controlled by Lukf/PV genes (M'barki, *et al.,*2017). The expression of Pantone Valentine leucocidin (PVL) was originally considered one of the hallmark traits of CA-MRSA; however, reports of PVL-negative CA-MRSA have been increasing (Edslev *et al.,*2018). PVL is encoded by the lukF-PV and lukS-PV genes located in the genomes of a range of lysogenic bacteriophages. (StrauB *et al.,*2017). A thermostable nuclease (TNase) enzyme is produced by nearly all strains of S. aureus and has been used as a diagnostic criterion for this species. TNase hydrolyses single and double-stranded DNA and RNA at the 5' position of phosphodiester bonds by a calcium-dependent mechanism. The main function of this protein may be to convert local host tissues into nutrients required for bacterial growth (Cotar *et al.,* 2010). Staphylococcal enterotoxins (SEs) are produced when these bacteria contaminate food and are allowed to grow, and ingestion of which can cause food poisoning, it stimulates the vomiting center in the brain by binding to neural receptors in the upper gastrointestinal (GI) tract (Foulston *et al.,* 2014). Among staphylococcal enterotoxins, the sea (enterotoxin A) type is more resistant to heat and gastrointestinal proteolytic enzymes such as pepsin and trypsin (Pinchuk *et al*., 2010). (SEA) is the most common type in clinical and food-related S. aureus strains and it has been the incentive for most studies (Pourmand *et al.,* 2009). The genes of SEA are carried by temperate bacteriophages (De Oliveira *et al.,*2020). The process of biofilm formation is facilitated by the presence of the intercellular adhesion (ica) locus, which is comprised of the icaADBC operon. This operon consists of four genes that encode proteins necessary for the synthesis of polysaccharide intercellular adhesion (PIA) in Staphylococcus spp (Frank and Patel, 2007). The variability in the expression of ica genes for biofilm production is pronounced among different strains of S. aureus. While there are multiple genes that play a role in the process of biofilm formation, one of the key genes is the icaA gene. The gene under investigation is a specific gene that has been identified in S. aureus strains. This gene is associated with the capacity to produce biofilm, as indicated in the research articles by Omidi et al. (2020) and Arciola *et al.* (2015). The resultant of independent component analysis (ICA)The protein Agene is classified as a transmembrane protein.

MATERIALS AND METHODS

From September 2022 to January 2023, 30 isolates of S. aureus were isolated from pus swab, urine, seminal fluid, high vaginal swab and burn swab. The samples were selected from the patients who visited Al-Furet Al-Awsat Hospital and AL-Najaf Teaching Hospital in AL-Najaf City, Iraq for medical consultations. from both genders, ages ranging from (1-60) years. Vitek-2 microbiology machine (Biomatrix, USA) was used to confirm the S. aureus and bacterial isolates were incubated for 18-24 h at 37 °C after the cultivation on blood agar.

1. DNA Extraction:

Genomic DNA was extracted from bacterial isolates by using the Genomic DNA Extraction Mini Bacteria Kit (Favorgen). The extracted DNA was checked by using Nanodrop (THERMO. USA) which measured DNA concentration (ng/µL) and checked the DNA purity by reading the absorbance at (260 /280 nm).

2. PCR Assay:

Polymerase chain reaction by using master mix (Promega). A polymerase chain reaction assay was performed for the detection of a gene (*PVL, NUC, Sea, iCaA*) in *Staphylococcus aureus*. Table (1). The reaction conditions were as in the Table (2).

Table 1: Primers used in the study.

Primer		Sequence	Amplicon	*References*
Pvl	*F*	ATCATTAGGTAAAATGTCTGGACATGATCA	*433bp*	(Karmakar *et al* ,2018)
	R	GCATCAAGTGTATTGGATAGCAAAAGC		
Nuc	*F*	GCGATTGATGGTGATACGGTT	*270bp*	(Karmakar *et al* ,2018)
	R	AGCCAAGCCTTGACGAACTAAAGC		
Sea	*F*	TTG GAA ACG GTT AAA ACG AA	*120bp*	(Rasmi et al,.2022)
	R	GAA CCT TCC CAT CAA AAA CA		
iCaA	*F*	GAT TAT GTA A TG TGC TTG GA	*770bp*	(Rasmi et al,.2022)
	R	ACT ACT GCT GCG TTA ATA AT		

Table 2: PCR Programs.

Gene	Temperature(°C) /Time					Cycles number
	Initial Denaturation	Condition of one cycle			Final Extension	
		Denaturation	Annealing	Extension		
Pvl	94/2min	94/1min	51/45sec	72/45sec	72/4min	33cycle
Nuc	94/4 min	94/45 sec	60/30sec	72/45sec	72/4 min	35cycle
Sea	95/5min	95/1 min	55/45sec	72/1min	72/10min	40cycle
icaA	95/5min	95/1min	50/1min	72/1.5min	72/5min	40cycle

RESULTS AND DISCUSSION

The present study included 30 isolates from *Staphylococcus aureus* which were isolated from Urine, High Vaginal Swab, Pus, Wound and seminal fluid to Detection of Methicillin-Resistant S. aureus (MRSA) was used disk diffusion test by used oxacillin and cefoxitin according to (CLSI, 2021). Was MRSA (83.3%) from isolates. These results are roughly consistent with the study of Al-Maliki, (2009) which recorded a resistance rate of about 80.3%.

1. Detection of Virulence Genes:

To test the virulence genes of the isolates in this study, pvl, sea, icaA, and nuc were detected by PCR amplification (Table 2).

2. Detection of Panton-Valentine Leucocidin (Pvl gene):

PVL is one of the most important virulence factors of SA and it is encoded by LukS-PV and LukF-PV genes (karman, 2018). In the present study findings of the molecular identification of the *pvl* gene showed that 6/30 (20%) of S. aureus isolates gave positive band size (433bp) as shown in Figure (1). The findings of this study are inconsistent with those of a study conducted by Shariati and Validi, (2016) which indicated that 10.7% of isolates contained the *pvl* gene. In another study, also from 400 samples, only 139 were MRSA isolates, and 79 (56.8 %) were *pvl* gene positive (Bhatta *et al.,*2016). while in a China study, 15 (7.11%) of 211 strains were found to carry this *pvl* gene (Gao *et al.,*2019). PVL-SA-associated infections may be more aggressive and life-threatening. In a retrospective study of Hardy *et al*., (2019) among the six patients with PVL-SA bone and joint infections, there were 2 cases of necrotizing pneumonia, 2 cases of pericarditis and 1 death caused by cardiac tamponade. In Iraq partially in AL Anbar a study by (Hasan and Jasim, 2021) the result was quite different from ours where the *pvl* frequency was 63.5%. which was nearly the same as another study in Kurdistan where the rite of this gene was (64.3%). (Hami and Ibrahim,.2023). The findings of this study are inconsistent with those of a study conducted by (Shariati *et al.,*2016) which indicated that 10.7% of isolates contained the PVL gene. and agree with this study by (Hasan and Jasim, 2021) Based on the results, the PVL genes were found in 63.5% of the isolates.

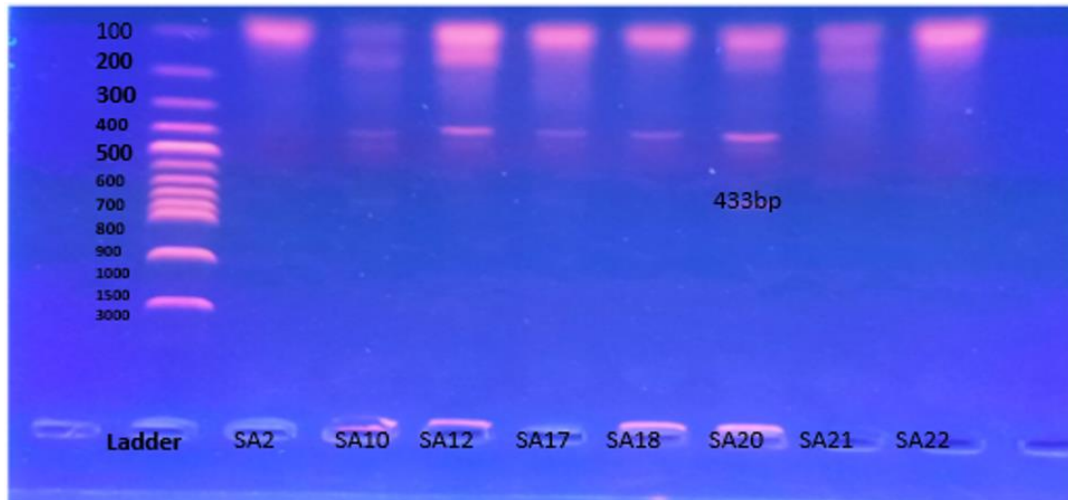

Fig. 1: The PCR product of pvl gene in 1% agarose gel electrophoresis, voltage (85 V), time (60 minutes) and 5 µL of PCR product loaded in each well. Lane M: DNA Ladder (100bp) Lanes 1,2,3,4,5,6,7,9,10,12,13,14,15,17: PCR product (positive case band (433 bp).

3. Detection of Thermostable Nuclease (nuc) Gene:

The findings of the molecular revelation of the nuc gene showed that (90%) of S. aureus isolate gave positive for nuc gene as shown in Figure (2). A comparison to other local studies conducted in Mosel showed nuc gene 70% from *S. aureus* isolates (Sheet *et al.,*2021). Also, studying in the same city (sheet *et al.,*.2021) was nuc gene found in all isolates S. aureus (100%). while was (77%) in a study in Basra (Sayhood *et al.,*2022). As well study In Kurdistan showed nuc gene (12.6%), (Hami and Ibrahim,.2023). Abd Jalil, (2010) suggested that the rapid nuc PCR assay is a suitable and practical tool for the routine detection and differentiation of S. aureus, MRSA and CoNS; which can be easily applied in microbiology laboratory procedures, Staphylococcus has the ability to produce a wide variety of exoenzymes, among these, nuclease is known to be an important virulence factor and are able to retain activity after incubation at 97˚C for 60 min. Nuclease is able to degrade the nucleic acid of the host by hydrolyzing phosphodiester bonds of DNA and RNA yielding 3′-mononucleotides.

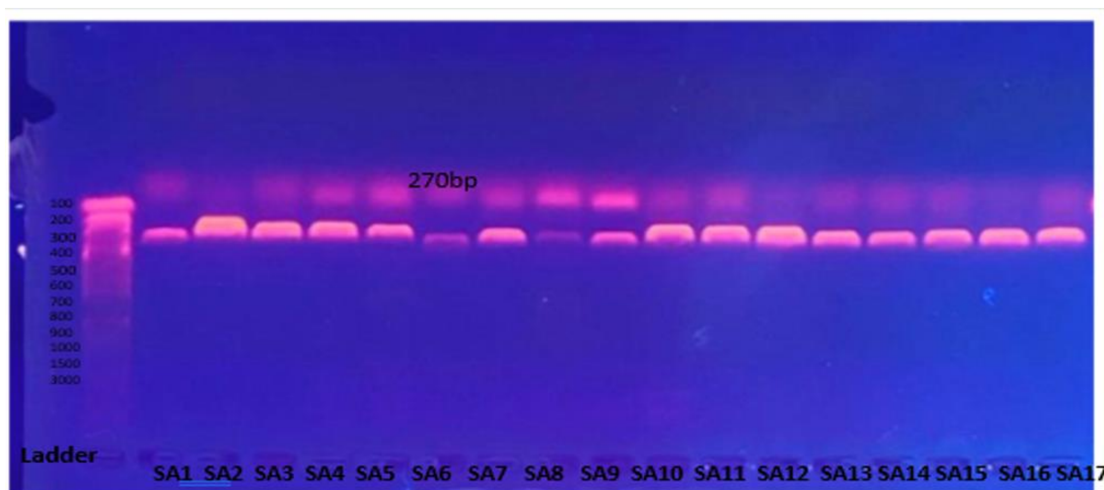

Fig.2: The PCR product of nuc gene in 1% agarose gel electrophoresis, voltage (100 V), time (90 minutes) and 5 µL of PCR product loaded in each well. Lane M: DNA Ladder (100bp) Lanes 1,2,3,4,5,6,7,9,10,12,13,14,15,17: PCR product (positive case band (270 bp).

4. Detection of *Staphylococcal enterotoxins* (*sea*) Gene Among *Staphylococcus aureus* Isolates:

Various studies show that 15–80% of S. aureus strains isolated from different sources can produce enterotoxin (Lowy *et al*., 1998). Among *Staphylococcal enterotoxins,* SEA (enterotoxinA) type is more resistant to heat and gastrointestinal proteolytic enzymes such as pepsin and trypsin (Pinchuk *et al*., 2010). The amplification results of the PCR study for SEA gene revealed that 19/30 (63.3%) of S. aureus isolate gave a positive band size (120 bp) as shown in Figure (3). In another study by Saadati *et al, (*2009) on healthy carrier samples using PCR technique, the frequency of sea gene was reported 25.3%. The high similarity of that result can be seen when comparing it with a local study in Baghdad and Diyala where the frequency of sea gene was (24.78%) of S. aureus isolates (Saleem et al,.2016). Another study's findings showed that sea gene was present in (30%) of MRSA (Valizadeh *et al*., 2016**).** Approaching our result, Cavalcante *et al*., (2021) reported that the prevalence of sea in S. aureus isolates collected from infected skin lesions of atopic dermatitis children was 76.4% in total *S. aureus* isolates, 73.9% in MRSA isolates, and 78.1% in MSSA isolate. Likely, in a study in Karbala Nasal swabs were collected from 332 food handlers were 38 S. aureus of which 16 (16%) sea genes (Alhashimi *et al.,*2017). Li *et al*., (2013) reported that the frequency of sea in S. aureus isolates from SSTIs in children was 0%, which was totally opposite to this study. Also, disagreed with (Karimzadeh and Ghassab, 2020) who did not detect sea genes in any of staphylococcus aureus isolates.

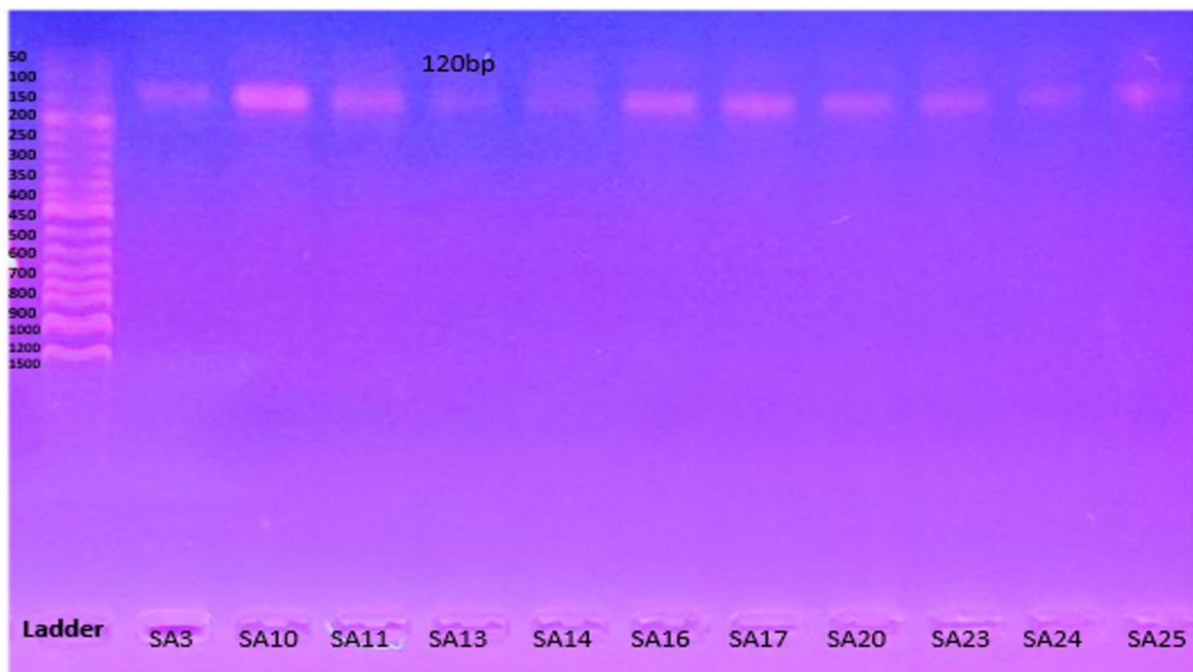

Fig. 3: The PCR product of sea gene in 1% agarose gel electrophoresis, voltage (85 V), time (45 minutes) and 5 μL of PCR product loaded in each well. Lane M: DNA Ladder (100bp) Lanes

5. Detection of Intracellular Adhesion-A (icaA) Gene Among *Staphylococcus aureus* Isolates:

Slime layer production, as the major part of biofilm formation, plays a remarkable role in bacterial colonization of exterior surfaces (O′Gara and Humphreys, 2001). Several methods have been recognized for examining slime layer production, including (the CRA method) used in this study (Ozkurt *et al*., 2009). Genetically the amplification results of PCR study for *ica*A revealed that 11/30 (36.6%) of S. aureus isolate gave positive results as shown in Figure (4). In

addition, our results showed that gene (icaA) was present in all biofilm-producing strains, indicating the important role of *ica* genes as virulence markers in staphylococcal infections (Arciola *et al*., 2001).

In this study, *ica*A gene prevalence was similar to another local study done in Baghdad by Al-Hadban *et al.,* (2017) where it was 40% of the total number of S. aureus isolates. In the same manner a study also in Baghdad was *ica*A gene 46.15% (Mohammed and Radif, 2020). Bissong and Ateba, (2020) showed that the biofilm-forming gene *ica* gene was detected among 75.3% of the isolates, with icaA being the most prevalent (63.6%). As icaA was found in 49.2% of the isolates (Rasmi *et al.,*2022). Also, in a study in Kurdistan in 2023 the prevalence of *ica*A gene was (75%) from isolates (Hami and Ibrahim,.2023). The fundamental step in *S. aureus* biofilm formation is initial attachment, which is accomplished by the expression of different Microbial Surface Components Recognizing Adhesive Matrix Molecules (Otto *et al*., 2014). *S. aureus* initially adheres to each other and then widens to structurally dynamic biofilm structures during the later phases of adherence. The maturation of the biofilm matrix into multi-layered patterns is initiated by the polysaccharide intercellular adhesin (PIA), synthesized from β-1, 6- linked N-acetyld-glucosamines (PNAG) (Periasamy *et al*.,2012). Encoded by four core genes, namely icaA, icaD, icaB and icaC, besides a regulatory gene, called icaR (McKenney *et al*., 1998). These genes encode the corresponding proteins ICAA, ICAD, ICAB, and ICAC. The production of slime is facilitated by the coexpression of icaA and icaD gene, It has been shown that strains harbouring the icaADBC cluster are potential biofilm producers (Atshan *et al*., 2012).

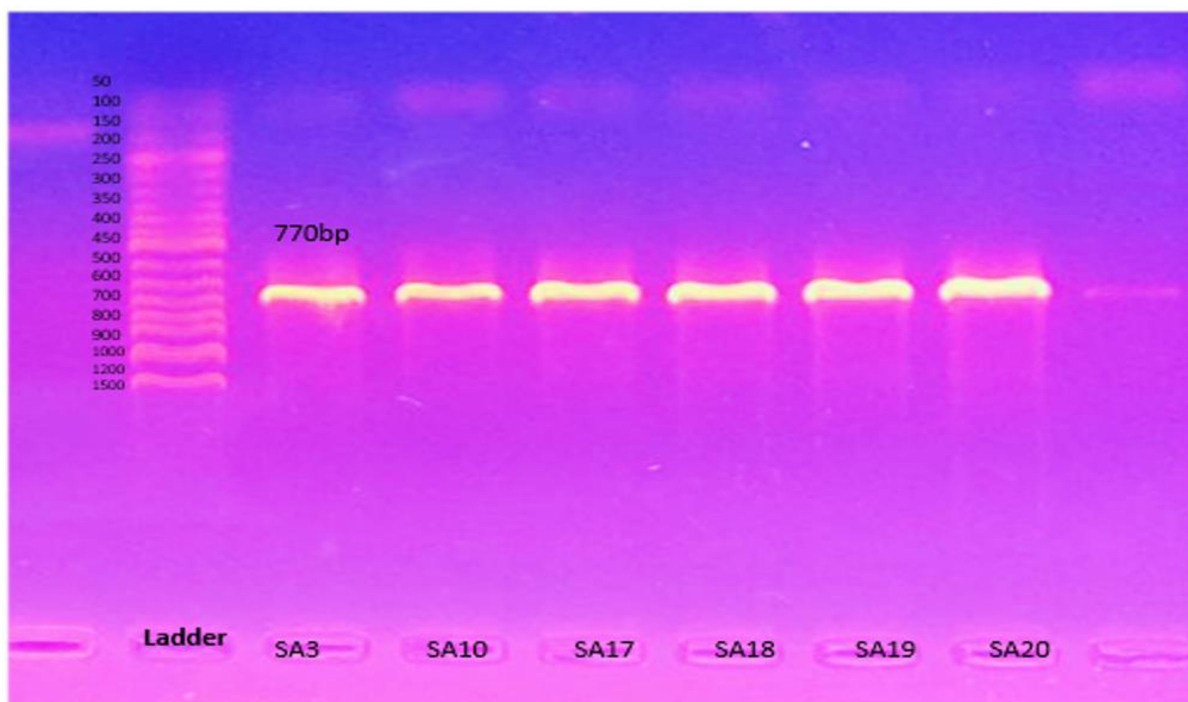

Fig. 4: The PCR product of icaA gene in 1% agarose gel electrophoresis, voltage (85 V), time (45 minutes) and 5 µL of PCR product loaded in each well. Lane M: DNA Ladder (100bp) PCR product (positive case band 770 bp).

6. Coexistence of Virulence Genes Among *S. aureus* isolates:

According to Table 3, which shows the Coexistence of virulence genes in *S. aureus* isolates, the *Pvl* gene was found simultaneously with *Nuc* gene in five isolates (16.6%), and the same genes were found in addition to *Sea* gene in two isolates (6.6%),

while *Pvl* gene was found with *Sea* gene in four isolates (13.3%). See table below *icaA* gene was found with *Sea* in 11 isolates (36. .6%), similarly, it was found in Egypt by Rasmi *et al.,* (2022), where the coexistence of *Sea* gene and *icaA* was (40.7%). Table (3) shows the data presented in the study indicates that the coexistence between Sea and Nuc exhibited the highest percentage at 50%, while the coexistence between Sea and icaA was observed at a slightly lower percentage of 36.6%. The coexistence of *Sea* gene with *Nuc* gene was recorded in 15 of the isolates (50%). This agreed with the study of Kayan and Victor (2019), where the *Nuc* gene acts as a marker and the presence of the heat-resistant *Nuc*lease gene (*Nuc*) is strongly associated with the production of enterotoxin and it can be considered as an indicator of infection with enterotoxin producer *S. aureus*.

Table 3: Coexistence of virulence genes among *S. aureus* isolates.

Virulence genes	Distribution No. (%)
Pvl+Nuc	5(16.6%)
Pvl+Nuc+Sea	2(6.6%)
Pvl+Nuc+Sea+icaA	2(6.6%)
Pvl+Sea	4(13.3%)
Pvl+icaA	5(16.6%)
Sea+icaA	11(36.6%)
Nuc+icaA	9(30%)
Sea+Nuc	15(50%)

The possession of *S. aureus* for numerous virulence determinants, for example, but not limited to; the biofilm formation ability, surface proteins, and exotoxins, is linked to the potential of *S. aureus* to initiate and maintenance of various infections. Unarguably the higher the virulence factors coexistence incidence, the more the virulent isolates are. There was no statistically significant correlation found between the presence of virulence genes (Sea, Pvl, Nuc and icaA) and the age groups of the patients (P>0.05). Furthermore, it was observed that there was no statistically significant disparity between the examined virulence genes and the gender of the participants, with the exception of the Pvl gene (refer to Table 4).

Table 4: Correlation between virulence genes and patient gender and age groups.

	PVL		P*	*Sea*		P*	Ica A		P*	*Nuc*		P*
	+	-		+	-		+	-		+	-	
Gander												
Male	5	4	0.0284	4	5	0.562771	4	5	0.796812	7	2	0.1440
Female	2	19		7	14		7	14		20	1	
Age group												
1-20	0	4		2	2		4	1		4	0	
21-40	4	16	0.434	6	14	0.7408	11	8	0.5716	17	3	0.434
41-60	2	4		2	4		3	3		6	0	

***Chi-square test; P-value was set to 0.05 Gene present (+); gene absent (−)**

Conclusion

The current study revealed various distributions of detected virulence genes of *S. aureus* which may be attributed to differences in the sites of isolation that exhibit variation of virulence factors according to their growth and survival requirements. Furthermore, the high prevalence of *nuc* gene encourages the utilization of this gene for genetic identification of *S. aureus* which also showed concurrence in 50% of isolates harboring *sea* gene

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