Iraqi Journal of Biotechnology, 2023, Vol. 22, No. 1, 163-172

Prevalence of some Virulence Genes in *Staphylococcus aureus* Isolated from Iraqi Infertile Male Patients and its Effects on Sperm Parameters

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Received: March 1, 2023/ Accepted: April 25, 2023/ Published: June 4, 2023

Abstract: *Staphylococcus aureus* is one of the most effective bacteria related with infertility and capable of immobilizing and agglutinating sperm cells. The aim of this study to identify of prevalence rate selected pathogenicity genes (*can*, *clfa*, *scn* and *hlg*) in *S. aureus.* This pathogen isolated from spermatozoa specimens of infertile males were attending Kamal Al-Sammrai Hospital / Baghdad during the period April 2020 until April 2021.Out of 120 cultures,25(20.83%)cultures utilized biochemical tests and microbiological assays to identify *S.aureus* isolates and confirmation by the Vitek 2 system. In addition, the molecular technique was based on The polymerase chain reaction assay was applied to identify according to the particular gene *gmk* encoding for (Guanylate Kinase).The results showed presence the *gmk* gene in all *S. aureus* isolates. The prevalence rate of examined virulence genes revealed that *clfa, cna, scn and hlg* genes present in 14 (56%), 14 (56%),13 (52%) and 7 (28%) among *S.aureus* isolates respectively. It is concluded that in general. *S. aureus* bacteria had negative effects on sperm parameters while in particular no effects of virulence genes in this bacterium on both sperm motility and sperm count.

Keywords: *Staphylococcus aureus*, virulence genes, sperm parameters

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Introduction

Infertility is a worldwide issue that impacts millions of couples. It is characterized as being unable to conceive after one year of unprotected sex (1) .In recent years, infertility has become a serious social, mental, and physical health problem worldwide (2).The several factors can contribute to a decrease in male fertility, including testicular failure, male genital abnormalities, immunologic issues, varicocele, genetic problems, endocrine disruptions, systemic diseases, cancer, genital tract illnesses, environmental agents, distorted lifestyle, and gonadotoxic factor exposure (3). Infections affect several parts of the male reproductive tract, including the epididymis, testis, and male genital tract glands. Urogenital infections can then have an impact on sperm cells at several stages of maturation, development, and transfer (4). *Staphylococcus aureus*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Chlamydia trachomatis*, and *Escherichia coli* have all been shown to impair sperm function in vitro, with S. aureus being the causal organism of seminal fluid illnesses (5).

Staphylococcus aureus is associated with multi drug resistance

infections and high levels of illness (6). The organism spreads illnesses by possessing virulent genes that, among other things, encode poisons and enzymes and other virulent components. Moreover, the pathogenicity of *S. aureus* has grown due to the presence of antibiotic resistance strains including Methicillin-Resistant *S. aureus* (MRSA) and Vancomycin-Resistant *S. aureus* (VRSA) (7).

Staphylococcus aureus is a common gram-positive bacterium that can be identified in both fertile and infertile males' spermatozoa specimens. According to other studies, *S. aureus* was predominated other bacteria in the spermatozoa specimens of infertile male with a high prevalence rate (8). Many reports indicate that co-incubating spermatozoa with *S. aureus* causes a reduction in spermatozoa movement and sperm agglutination (9). The additional chromosomal elements allow for the transmission of these genes among *staphylococcal* isolates, species, or other gram-positive bacterial species (10). The numerous of illnesses is a reflection of the virulence factors of the organism (11).

This adhesion of clumping factors A and B encoded by *clfAB* genes enhances biofilm formation, aids in the colonizing of the host by *S. aureus*, and increases virulence by immune evasion by binding to soluble fibrinogen (12). The *S. aureus* MSCRAMM protein known as collagen adhesion (*Cna*), which binds collagen, likewise includes an A region with three subdomains at its N terminus. The ligand-binding domain of *Cna*, in contrast to the other MSCRAMMs, is made up of the IgG-folded subdomains N1 and N2, instead of N2 and N3. The IgG-like subdomains N2 and N3 of *ClfA* are closely connected to the N1 and N2 subdomains of *Cna*, which are variations of the IgG fold (13). A changeable number of BCNA repeating domains that differ from BSDR domains in order connect this N-terminal region of *Cna* to the cell wall-spanning W domain (14). Another distinction is that the repetitive B domains and the wall-spanning area are not connected by a flexible stalk in *Cna*. Other bacteria that express functional and structural homologues of *Cna* include *Enterococci*, *streptococci*, and bacilli (15).*S. aureus* has a variety of virulence factors, and released toxins are just one of them. These factors contribute to the pathogen's effectiveness (16).

The HlgAB has cytolytic action toward human and rabbit leukocytes and is especially effective at lysing human red blood cells (17). HlgCB, on the other hand, has minimal action targeting red blood cells (18). *S. aureus* secretes a variety of immune-modulating proteins through colonization. The *scn* gene's product, *staphylococcal* complement inhibitor (SCIN), effectively defends *S. aureus* by stifling innate immune system response carried out by neutrophils. The immune evasion cluster (IEC) contains the genetic coding for SCIN and other immune regulating proteins (19).

Materials and methods

Sampling

In this research, samples were involved 120 Iraqi infertile males with different ages. In additions, samples were collected from 50 healthy fertile males from April 2020 until April 2021, from patients Kamal Al- Samara'ay IVF Hospital of Baghdad city.

Semen collection

Male were masturbated to obtain fresh ejaculated specimens of human spermatozoa, which were then collected straight onto a clean, dry, and sterile plastic disposable Petri dish in a room designated specifically for this purpose. The sample was promptly transferred to the semen examination after 30 to 60 minutes for each individual with acquaintance during the abstinence period of three to five days. According to the WHO (2010) recommended procedures, each semen sample was given time to liquefy. The semen was thoroughly liquefied before being subjected to a macroscopic and microscopic examination for analysis using WHO2010 standards.

Bacterial isolation and identification

Using established techniques, the bacteria isolated from the cultivated plates were identified and described. Confirmation Depending on the inoculation onto mannitol salt agar and 24-48-hours incubation period at 37°C, It was determined that the isolates were *S. aureus*. Depending on the colony's size, shape, color, boundaries, and texture, colonies were identified. Gram staining was used to identify colonies with the typical morphology of *S. aureus*, after which specimens were subjected to biochemical testing like (Coagulase, Catalase, Oxidase). Following validated by the Vitek 2-GN system's pattern of biochemically profiles and finally molecular detection of *S. aureus* using one of the main housekeeping genes.

Molecular identification

Deoxyribonucleic acid extraction

The EasyPure Bacteria Genomic DNA Kit's procedure was followed to separate genomic DNA from bacterial growth. To assess the degree of specimen quality for usage in upcoming applications, a Quantus Fluorometer was utilized to measure the concentration and purification of Deoxyribonucleic acid that had been extracted. 199 µl of diluted Quanty Flour Dye were combined with 1 µl of DNA. DNA concentration readings were found following a 5-minute incubation period at room temperature.

Polymerase chain reaction cycle of *gmk and* virulence genes

The detection of housekeeping gene (*gmk*) (20) and Four virulence genes (*hlg, scn, cna,* and *clfA*) of *S. aureus* strains was carried out utilizing conventional PCR .Table (1) summarizes the primer sequences for the beginning. 4 µl of DNA, 1µl of each primer, 12.5 µl of Master Mix, and 6.5 µl of nuclease-free water were included in a reaction mixture (25 µl). According to table 2, the PCR conditions were initial denaturation at 95°C for 5 minutes (1 cycle), following by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The 1.5% agarose gel electrophoresis method was used to evaluate the PCR results, and the Gel Imaging System was used to view the ethidium bromide stained bands in the gel.

Table (1): Oligonucleotide primer sequence and size amplicon.

Genes	Primer sequence (5'-3')	Product size(bp)	Reference
scn	F-ACAAGCTTGCCAACATCGAA R-TAGTGCTTCGTCAATTTCGTT	220	(19)
clfa	F-GTGGCTTCAGTGCTTGTAGG R-TGTCGTTTCCTGTTGTGCTG	207	(12)
hlg	F-GCCAATCCGTTATTAGAAAATGC R-CCATAGACGTAGCAACGGAT	938	(17)
cna	F-ACAGGTGGGTCAAGCAGTTA R-CACTACTTGTTCCCGCTTCA	198	(13)
gmk	F-GGCATCTTTGGCTTCAGCGTGT R-GCTATGTCACGCTGCATCACCT	488	(20)

Table (2): PCR program used in this study.

Steps	°C	M : S	Cycle
Initial denaturation	95	05:00	1
Denaturation	95	00:30	35
Annealing	60	00:30	
Extension	72	00:30	
Final extension	72	07:00	1

Effects of virulence genes on some semen parameters

The effect of virulence genes was studied on sperm count and sperm motility.

Statistical analysis

The mean and the standard error were taken into consideration using (IBM SPSS) version 28.0 of the mean. The probability was also investigated using an ANOVA table and a student T-test. The probability was calculated using chi-square analysis of Pearson for non-parametric information, to ascertain the connection between the investigated parameters; a Pearson's correlation was performed (34).

Results and discussion

Basic semen parameters distribution of the infertile and healthy fertile men groups

The microscopic semen parameters among the infertile and healthy groups demonstrated a highly significant variation, as shown in table (3).

Table (3): Semen microscopically analysis for the studied groups.

Parameters	Control group (n=50)	infertile group (n=25)	*P* value
Sperm concentration (million/ml)	49.58 ±2.93	25.32 ±2.33	0.001
Progressive motility (%)	57.20 ±2.27	20.83 ±2.27	0.001
Non progressive motility (%)	19.10 ±1.24	24.80 ±2.15	0.001
Immotile (%)	23.70 ± 1.97	54.40 ± 2.30	0.001
Normal morphology (%)	52.20 ± 1.91	35.40± 2.20	0.001

P < 0.001

The fertile control group's mean sperm concentration (49.580000m/ml) was significantly ($p < 0.05$) higher than the infertile men (25.320000m/ml).The percentage of progressive motility of fertile male (57.20 ± 2.27) was significantly ($p < 0.05$) higher as compared to infertile male group (20.83 ± 2.27) as showed in table (3). On the other hand, when comparing the percentages of non-progressive motility, it was found that the fertile male had (19.10 ± 1.24), whereas the infertile men group had (24.80 ± 2.15). Which

revealed a significant ($p<0.05$) difference in this percentage.

The mean of immotile sperm (23.70 ± 1.97) in fertile men was considerably different when compared to the infertile male group, the proportion of immotile sperm (54.40 ± 2.30). Results as reported in table (3) also revealed a highly significant ($p<0.05$) rise in morphologically normal sperm of control males (52.20 ± 1.91) as compared to infertile male group (35.4± 2.20). These results reported deterioration of semen parameters in infertile male group when compared with control group, which may be due to infection of the semen with *S. aureus.*

These findings were reported by other investigators they found that highly significant difference in microscopic semen parameters in healthy fertile men when compared with patients group (21). The role of virulence factor in the pathogenicity of pathogen in particular *S. aureus* increased the negative impact on seminal parameters (22). In the semen of an asymptomatic infertile male, bacterial infection was thought to be a primary contributor to infertility (23). There are several different explanations put forth for why infection in the reproductive system can lower male fertility (24). One of the primary pathways is the overproduction of reactive oxygen species (ROS), which causes testicular oxidative stress (OS) (25).

Isolation and identification of bacterial isolates

From 120 cultures only 25(20.83%) *S. aureus* isolates. There may be a causal link among staphylococcal infection and infertility in male (26). A similar study found that the *S. aureus* infection rate in the spermatozoa specimens from males with infertility issues was 20.6%. Most importantly, it was discovered that *S. aureus* infection was strongly associated with poor sperm parameters and decreased spermatozoa movement (27). Other study conducted by Basima and Ama, (28) demonstrates that *staphylococcus spp*. was present in 18% of infertile male patients. Orji et al, (29) revealed the *S. aureus* was most frequent bacteria (37.1%).All isolates were recognized as *S.aureus*, as shown in table (4).

Table (4): The biochemical tests of 25 *S.aureus* isolates.

Biochemical test	Results
Blood agar	100%gray-golden colonies(alpha and beta hemolysis)
Mannitol salt agar	100% yellow colonies
Catalase	100% Bubbles (+)
Oxidase	100% No Purple color (-)
Coagulase	100% (+)

Positive: (+); Negative: (-)

As a final step in organism identification, the colorimetric ID-GP VITEK 2 system utilizes a fluorogenic methodology barcoded with data on the type of card, expiry date, lot number, and unique card identification number, probability about 93% - 98% belong to genus *S.aureus.*

Identification of *S.aureus* using the *gmk* gene

The accuracy of this research test and method utilized for identifying this genus was verified by the PCR findings figure (1), which revealed that the *gmk* gene (488bp) exists in all 25 S. aureus figure (1) identified by the previous identification methods.

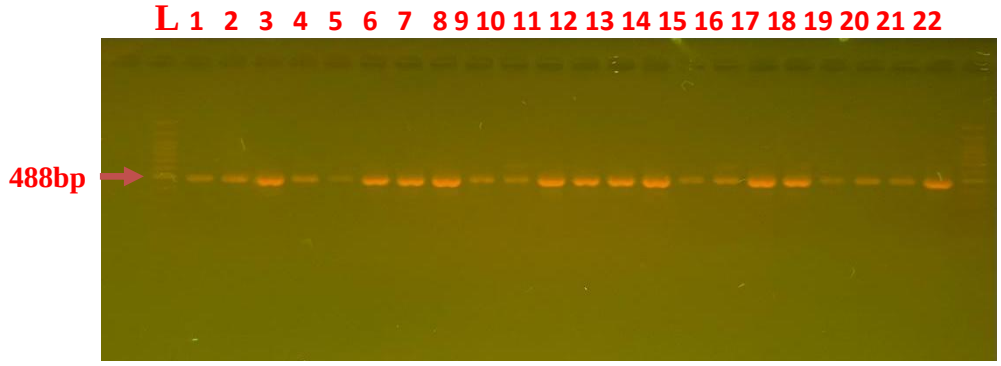

Figure (1) Agarose gel electrophoresis of amplified PCR product of *gmk* gene in *S.aureus* isolates. LaneL: 100 bp DNA ladder;Lanes 1-22: amplicons of *S.aureus* isolates (1-22). (Expected amplicon size: 488bp, agarose (1%), TBE buffer (1x), 70-volt for1.5 hrs. stained with gel stain.).

The molecular identification of virulence genes in *S.aureus*

The results found that the proportions of the genes (*cna*,*clfA*, *scn* and *hlg*) were [14 (56%) , 14 (56%) , 13 (52%) and 7(28%) respectively as shown in figures (2,3,4 and 5) .Because it produces a variety of virulence factors that play in colonization and the invention of the host, which can result in subsequent infections, *Staphylococcus aureus* is an important type of bacteria that played a role to the severity of the infection in humans. These virulence factors are associated with a variety of virulence genes.

It is noteworthy that genes involved in adhesion are crucial for *S. aureus* strains' ability to create biofilms (30).In this research, *clfA* (56%) and *cna* (56%) were the most common virulence genes. Nourbakhsh *et al* (31) revealed 41.4%, and 18.3% for *clfA*, and *cna* genes respectively. On the other hand, Ghasemian *et al* (32) revealed the prevalence rate of 100 % and 78%, for *clfA* and *cna* genes respectively. Elboshra *et al* (33) revealed that from 65 S. aureus isolates. The cna gene was found in 19 isolates (29.2%), while five isolates (7.6%) were *hlg* positive.

Figure (2): Agarose gel electrophoresis of amplified PCR product of *cna*gene in *S.aureus* isolates. LaneL: 100 bp DNA ladder;Lanes 1-22: amplicons of *S.aureus* isolates (1- 22).(Expected amplicon size: 198bp,agarose (1%), TBE buffer (1x), 70 volt for1.5 hrs. stained with gel stain.).

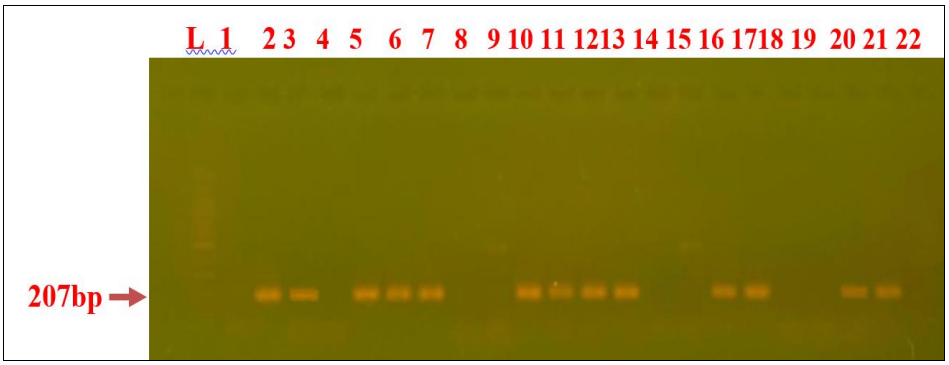

Figure (3): Agarose gel electrophoresis of amplified PCR product of *clfa*gene in *S.aureus* isolates. LaneL: 100 bp DNA ladder;Lanes 1-22: ampliconsof *S.aureus* isolates (1-22). (Expected amplicon size: 207bp, agarose (1%), TBE buffer (1x), 70-volt for1.5 hrs. stained with gel stain.)

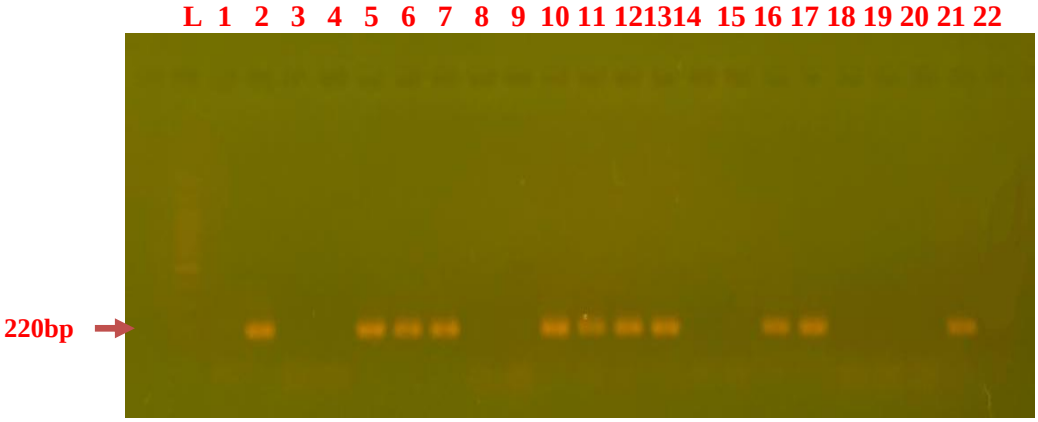

Figure (4): Agarose gel electrophoresis of amplified PCR product of *scn*genein *S.aureus* isolates. LaneL: 100 bp DNA ladder; Lanes 1-22: amplicons of *S.aureus* isolates (1- 22).(Expected amplicon size: 220bp, agarose (1%), TBE buffer (1x), 70 volt for1.5 hrs. stained with gel stain.).

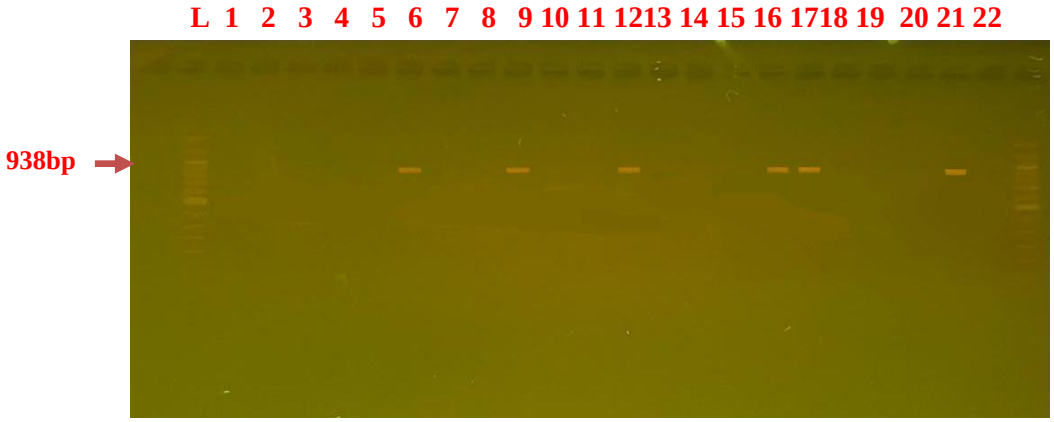

Figure (5): Agarose gel electrophoresis of amplified PCR product of *hlg* gene in *S.aureus* isolates. LaneL: 100 bp DNA ladder;Lanes 1-22: ampliconsof*S.aureus* isolates (1- 22).(Expected amplicon size: 938bp,agarose (1%), TBE buffer (1x), 70 volt for1.5 hrs. stained with gel stain.)

Effects of virulence genes on some semen parameters

As observed from results in Table 5 and 6. No statistically significant differences were found in the positive Isolates for virulence genes compared to negative isolates for virulence genes on both sperm motility and sperm count ($P > 0.05$).

Table (5): Means of sperm counts.

Genes		Mean	Std. Error of Mean	Probability
cna	**+ve**	29285714.29	3395906.67	0.835
	-ve	28272727.27	3244830.60	
scn	**+ve**	29153846.15	2414821.10	0.892
	-ve	28500000.0	4220261.37	
clfa	**+ve**	28714285.71	3428113.52	0.953
	-ve	29000000.0	3196589.09	
hlg	**+ve**	30714285.71	3577138.29	0.356
	-ve	28111111.11	2965916.28	

Table (6): Means of sperm motility.

Genes		Mean	Std. Error of Mean	Probability
cna	**+ve**	21.92	3.28	0.612
	-ve	19.55	3.20	
scn	**+ve**	18.46	2.15	0.264
	-ve	23.64	4.22	
clfa	**+ve**	21.54	2.57	0.743
	-ve	20.0	4.05	
hlg	**+ve**	22.86	3.25	0.906
	-ve	20.0	2.94	

Conclusions

In general, *S.aureus* bacteria had negative effects on sperm parameters while in particular no statistically significant differences were found in the Isolates positive for virulence genes compared to isolates negative for virulence genes on both sperm motility and sperm count.

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