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Prevalence of Some Virulence factors Associated-genes in *Staphylococcus aureus* Isolated from Conjunctivitis Patients in Al-Najaf City, Iraq

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Abstract— The appearance isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), as well as vancomycin-intermediate *S. aureus* (VISA), in the community, becomes a big concern in worldwide public health in both developed as well as developing countries. Hence, the purpose of the current research aimed to examine phenotypic and genotypic detection of virulence factors in MRSA and VRSA and the frequency of VRSA and VISA isolates among conjunctivitis infections. The results showed that 6 (9.37%) of isolates were capsulated *S. aureus*by India ink staining, hence 67%of isolates showed the protease enzyme production. The results revealed that 57.8% of *S. aureus* isolates changed the color of DNase agar from blue to pink or rose, indicating that they were positive result.The results showed that 76.5% of *S. aureus* isolates produced gelatinase enzyme. The molecular results showed that all of *S. aureus* isolates do not contain the *cap5* gene, while *cap8* genedetected in 35% of *S. aureus* isolates.Also, result pointed out that 50%*S. aureus* of investigate isolates contained the *hla* gene. The findings of the molecular revelation of the *nuc* gene showed that 70% of *S. aureus* isolate gave positive for *nuc* gene.

Keywords: Virulence Factor, *hla*, *nuc*, *cap5*, *cap8*, protease, gelatinase, *Staphylococcus aureus*

1.INTRODUCTION

Staphylococcus aureus is a Gram-positive bacteria, non-motile, grape-like cluster, aerobic or facultative anaerobic, and catalase positive that can grow in a medium containing 10% NaCl at temperature ranging from 18°C to 40°C(Sadaka*et al*., 2017).*S. aureus* has positive results for free coagulase and phosphates, as well as DNase and gelatinase, but negative results for oxidase(MacFaddin, 2000).*S. aureus* are the most frequent cause of hospital-acquired illnesses (Nsofor*et al*., 2013). Because of the exposed nature of the eyes, ocular tissues are susceptible to *S. aureus* infection. *S. aureus* is the most prevalent Gram-positive bacteria that can cause eye infections, second only to *Pseudomonas aeruginosa*. It causes ocular disorders such as conjunctivitis, dacryoadenitisblepharitis, blepharoconjunctivitis, corneal ulcer, dacryocystitis,blepharitis, orbital cellulitis, and blepharoconjunctivitis (Khurana and Khurana, 2012).

The most common type of infective conjunctivitis is bacterial conjunctivitis (Agha, 2020). *S. aureus* is the most prevalent bacterial infection in conjunctivitis around the world (Cavuoto*et al*., 2008). MRSA is a major concern worldwide in the health sector and, increasingly lately, in the community (Rasheed and Hussein, 2020). MRSA infections acquired in hospitals and

communities are on the rise in many regions of the world, according to epidemiological studies (Abulreesh*et al*., 2017).

Antimicrobial resistance in *S. aureus* is a major veterinary and public health concern around the world, as multi-resistant strains pose a significant challenge to effective treatment(Garcés, 2012;Neopane*etal*., 2018).Antimicrobial multi-resistance is common in human medicine, and MRSA are common. The kind, intensity, and responsiveness to antibiotic therapy of a *S. aureus* infection are determined by the unique suite of virulence and antibiotic resistance related genes carried by the *S. aureus* strain generating the illness (Syed *et al*., 2011). Thisstudy aimed to highlight the importance of the prevalence of some virulence factors of*S. aureus* isolated from conjunctivitis infections.

2. Methods

Clinical Specimens

The total 80 specimens were collected from patients suffering from conjunctivitis and visiting Al-Furat Al-Awsat, Al-Hakeem Hospital and Al-Sadr Medical City as well as some chief clinical laboratories in Al-Najaf City-Iraq, private Laboratory in Al–Najaf province. Those swabs were collected frompatients of both sexes (female and male) and age range from 1 to 70 years suffering from different infections, during the period From August 2021 to October 2021.

Isolation and Identification of *S. aureus*

S. aureus was isolated and identified according to traditional biochemical diagnostic to, by using the routine methods e.g. according to MacFaddin (2000) andColle*et al.,* (1996).Vitek–2 compact system for confirm identification of bacterial isolates using a GP identification card (Guido and Pascale, 2005).

Phenotype Detection of Some Virulence Factors

Capsule Production Detection: It was performed with used India ink stain to detect the capsule production(Bottone*et al.,*1998).

Gelatinase test: Gelatinase test was performed by inoculating Staphylococcal Agar No.101 with bacterial growth culture and incubated at 37^{o}C for 24-48 hrs. Appearance clear zone around colonized, indicated the positive result when flooding plate with standard aqueous solution of ammonium sulphate.

DNase test: DNase test was performed by inoculating DNase agar with bacterial growth culture and incubated at 37^{o}C for 24-48 hrs, HCl (3.6%). Appearance clear zone around colonized, indicated the positive result (Collee *et al*.,1996).

Detection of Thermonuclease (TNase) Production: Toluidine blue-DNA agar was made and poured into Petri dishes, then punch out a maximum of 6 wells per plate. The samples made from tryptone soya broth cultures were placed in the wells. The plates were incubated in an upright

position at 35°C for 1 hour, 2 hours, and 24 hours under aerobic conditions. A favorable result was shown by the appearance of a pinkish zone around the well (Kaplan *et al*., 2004).

Protease Production: An overnight broth culture of bacterial isolate was spot-inoculated the skim milk agar, incubated at 37ºC for 24-48 hrs then the protease activity was seen as clear zone around the colonies (Collee *et al*., 1996).

Molecular Study

Extraction of DNA and PCR Conditions

The Promega company kit was used to extract whole DNA according to the manufacturer's instructions (Madison, WI, USA). Four virulence factors of *S. aureus* genes were chosen (Table 1). The Promega master mix was used to performed PCR component in a final volume of 25 µL. Each reaction had 12 µL of Master Mix, 2 µL of each primer, 5 µL of DNA as template and 4 µL of nuclease free water. The amplifications conditions for each primers. were 94℃ for 5 min followed by 50 cycles of amplification (denaturation at 94℃ for 30 sec, annealing at 55.5 ℃ for 30 sec, and an extension. at 72℃ for 30 sec), and a final .extension at 72℃ for 1 min. All PCR amplifications were carried out in a Veriti Thermal Cycler (Agilent, UK).

Table 1: Nucleotide sequences, and predicted sizes of some virulence genes.

Genes	Oligonucleotide sequence 5'→3'	Size of amplicon(bp)	References
nuc	**F**:GGCAATTGTTTCAATATTAC **R**:TTTTATTTGCATTTTCTACC	417	Appak, (2006)
hla	**F:** GGTTTAGCCTGGCCTTC **R**:CATCCACGAACTCGTTCG	550	Singh *et al.*, (2011)
cap5	**F**:GTCAAAGATTATGTGATGCTACTGAG **R**:ACTTCGAATATAAACTTGAATCAA	361	Verdier *et al.*, (2007)
cap8	**F:**GCCTTATGTTAGGTGATAAACC **R**:GGAAAAACACTATCATAGCAGG	173	Verdier*et al.*, (2007)

3. RESULTS AND DISCUSSION

Using traditional approaches such as growth characteristics, colony morphology, and biochemical testing, a total of 20 eye swab isolates were identified as *S. aureus*. *S. aureus* was found in 20/80 (23.5%) of the patients, with females accounting for 12.4 percent and males accounting for 11.1 percent. *S. aureus* was discovered to be extremely resistant to methicillin (MRSA) (89.0%), but less resistant to vancomycin (VRSA) (6.0%).

Phenotypic Detection of Capsule Production, Protease and Some Virulence Factors

It is well known that the pathogenicity of *S. aureus* is associated with many virulence factors (enzyme and toxin). In this study, some of them were detected by traditional phenotypic methods while others detected by phenotypic and then genotypically by using monoplex PCR techniques. These tests were applied on 20 isolates, 18 isolates identified as MRSA and 2 isolates identified as MSSA. The investigated enzymes were protease, DNase, and gelatinase.

Capsule Production Detection

The capsule polysaccharide of *S. aureus* is a key antiphagocytic virulence component (Lei and lee, 2020).*S. aureus* virulence is controlled by a complex regulatory network that regulates the production of numerous virulence factors. To research virulence gene regulation in *S. aureus*, we've been employing capsule as a model virulence factor(Lei and lee, 2020).

Indian ink stain was utilized to detect capsulated *S. aureus* isolates in our study. Table (2) shows that 9.37% of isolates were capsulated *S. aureus* and 90.62% were non-capsulated *S. aureus*. The findings revealed that a large majority of isolates were not capsulated.

The contact between external *S. aureus* adhesions and eukaryotic cell receptors is aided by the absence of CP expression, which promotes bacterium internalization into the eukaryotic cell milieu(Tuchscherr*et al*., 2010).

The ability of *S. aureus* to encapsulate itself has been shown to be a critical characteristic for the organism's survival in the host system, as the capsulated organism can prevent itself from being phagocytosed. On the basis of serological or agglutination reactions with monospecific antisera, 11 distinct capsular types have been identified Capsular serotypes 5 and 8 were shown to be the most prevalent in *S. aureus* isolates from human sources, out of 11 capsular serotypes(Tollersrud*et al*., 2008).

Detection of Enzyme Production

Various virulence factors generated in *S. aureus* isolates were investigated, including hemolysin, DNase, gelatinase, and protease enzymes.

Protease Production

The results showed high level of protease production isolates, hence 67% showed the isolation of protease enzyme production. This result agreed Fathi, (2007) who has reported that 50% of isolates produce protease. Additionally, this result agreement with Hamid and Al-meani, (2021) who pointed out that 60percent of *S. aureus* had protease enzyme.

On the other hand, this result different with the finding of with the local study performed by AL-Amery *et al*., (2011) who has found high level of protease production isolates, hence showed that 83.33% of isolates protease production.These findings demonstrated the significance of this enzyme in the induction of Staphylococci pathogenesis. *In vitro* investigations have revealed that

proteases of staphylococci may break and destroya variety of essential host proteins, such aselastin, plasma proteinase inhibitor, and the heavy chains of all human immunoglobulin classes, implying that they are involved in pathogenicity(Kantyka*et al*., 2009).The change of *S. aureus* from an adhesive pathogen to an invasive pathogen has been linked to extracellular proteases. The emergence of drug-resistant bacteria has required a quest for novel drug sources(Mashezha*et al*., 2020).Proteinase K can be utilized to promote biofilm dispersal by cleaving surface proteins, according to a previous study proteins (Shukla and Rao, 2017).

Thermo Stable DNase Production

According to the findings, 57.8% of *S. aureus* isolates generated positive results on DNase agar by altering the color from blue to pink or rose, whereas only 42% of *S. aureus* isolates were non-producers. This result agrees with Khwen*et al*., (2021) that showed only 66% of *S. aureus* producing this enzyme.

This result also indicates*S. aureus* has a high variety of virulence factors agreeing with Zecconi and Scali, (2013) that revealed that this bacteria has a wide range of virulence factorsthat contribute to *S. aureus'* ability to cause infection, such as enzyme, cell-surface protein, toxin, and factors that aid in evading innate immune defense.On the other hand, in recent study pointed out all isolates 100% of *S. aureus* produced DNase enzymes (Hamid and Al-meani, 2021).

Gelatinase Production

The results showed that 76.5% of *S. aureus* isolates produced gelatinase enzyme and 23% of isolates not produce gelatinase enzyme (table 2). This result showed high number of *S. aureus* isolates produce gelatinase enzyme but not all isolate different with the result of Ghafoor and Yassin, (2020) and Hamid and Al-meani, (2021) who pointed out that 100%of *S. aureus* isolates generatedgelatinase enzyme.

Table (2): **The Percentage of Some Enzymes Produced by *S. aureus***

Virulence Factors	Positive %	Negative %
Protease	43(67%)	21 (32.8%)
DNase	37(57.8)%	27(42%)
Gelatinase	49(76.5)%	15(23%)

Gelatinase is a key enzyme that improves bacterial pathogenicity by breaking down gelatin into basic units from amino acids. In addition, Gelatinase plays a significant role in the dissemination of malignant tumors by degrading extracellular matrix membrane components including collagen, gelatin, and proteoglycan substances.Given that *S. aureus* produces gelatinase, gelatinase-responsive antibiotic reagent release can target pathogenic *S. aureus* while leaving beneficial bacteria in the microbiome alone(Qiu*et al*., 2021).Urease and gelatinize enzymes, for example, are virulence factors that help transmit infection to surrounding tissues and hence have a role in bacterial disease(Nelson-Sathi*et al*., 2015).

Molecular Detection of MRSA Virulence Factors

Detection of Microcapsule Gene (*cap5* and *cap8*)

Specific PCR primers were used for the detection of capsule types gene *cap5* and *cap8* gen as shown in figure (1).The current results showed that all of *S. aureus* isolates do not contain the *cap5* gene, while *cap8* detected in (35%) of *S. aureus* isolates.

The current result agrees with Ambroggio*et al*., (2018) who determined that *S. aureus* isolates that do not generate *cap5* or *cap8* are non-typeable (NT).NT isolates of *S. aureus* are generate non-mucoid colonies on solid media (Cocchiaro*et al.,* 2006). These results reveal that *S. aureus* may turn on or off capsule production expression depending on the bacteria's *in vivo* microenvironment (Suligoy*et al*., 2020). The ability to produce inflammation was diminished due to the intracellular position (Tan *et al*., 2019). However, other workers had demonstrated lower percentage of *cap5* possessing isolates (Marraffini*et al*., 2006).However, in a persistently infected host, lack of capsule expression can contributeto *S. aureus* persistence. The genes *cap5* and *cap8* are virulence factors that allow *S. aureus* to evade phagocytosis and spread through the body(Nanra*et al*., 2013).*Cap5* and *cap8* are generated by 75 to 80% of human *S. aureus*isolates and play a critical role in staphylococcal infection pathogenesis(O'Riordan and Lee, 2004).

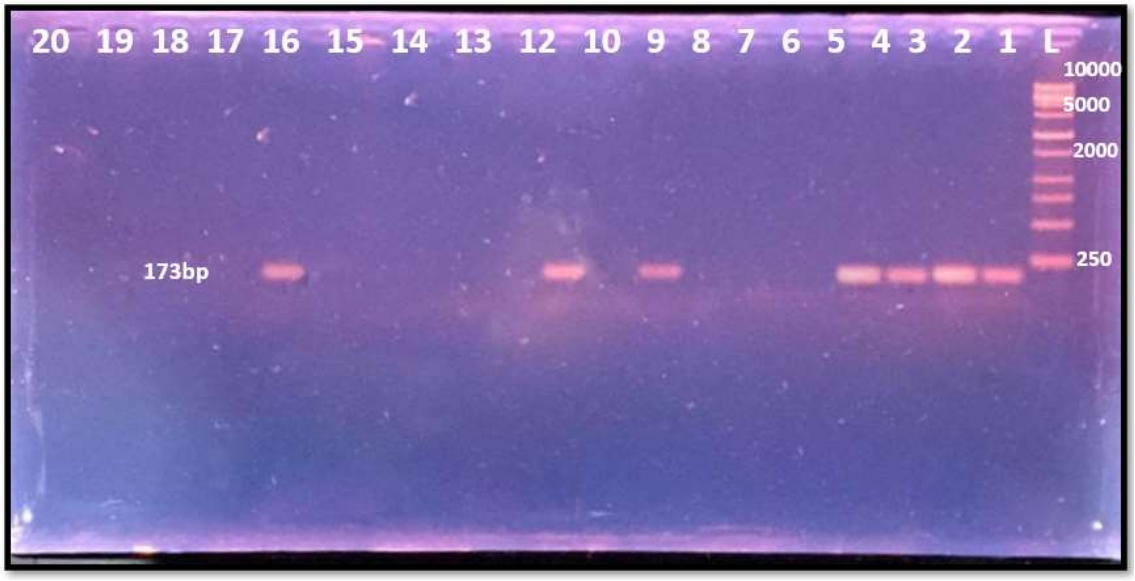

Figure (1): The PCR product of *cap8* gene in 1% agarose gel electrophoresis, voltage (85 V), time (90 minute) and 5 µL of PCR product loaded in each well. Lane L: DNA Ladder (10000bp) Lanes 1,2,3,4,9,12.16: PCR product (positive case band 173 bp).

Detection of alpha Hemolysin*(hla*gene)

Specific PCR primer was used for the detection of hemolysin type α- gene *hla* as shown in figure (2). The results showed that 50%*S. aureus* of investigate isolates contained the *hla* gene.

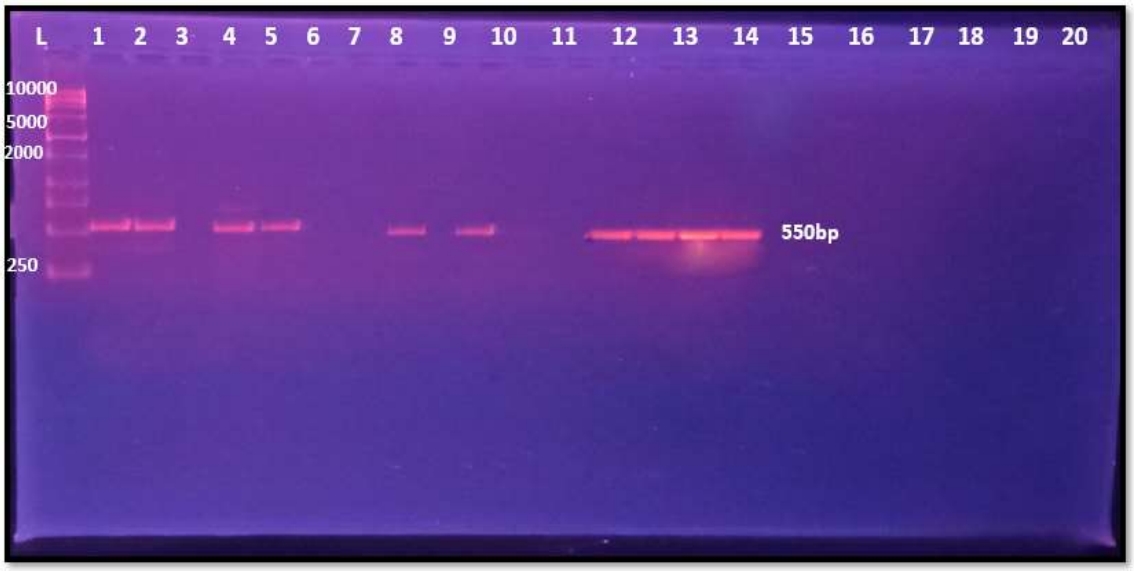

Figure (2): The PCR product of *hla* gene in 1% agarose gel electrophoresis, voltage (85 V), time (90 minute) and 5 µL of PCR product load1ed in each well. Lane L: DNA Ladder (10000bp) Lanes 1,2,4,5,8,9,12,13,14,15: PCR product (positive case band 550 bp).

The alpha (α) gene is found on the chromosome of bacteria and the*hla* geneencodes one of the most well-known pore-forming cytotoxin-hemolysin.α -hemolysin (Hla) toxin is the most emphasized and characterized virulence factor (Wang *et al.,* 2016).Hlais a virulence factor found in *S. aureus* that encourages disease by inducing the destruction of both innate and adaptive immune cells,excessive inflammation, and tissue injury, among many other biological targets(Joyner *et al*., 2020).

Detection of thermostable endonuclease (*nuc*gene)

The findings of the molecular revelation of the *nuc* gene showed that (70%) of *S. aureus* isolate gave positive for *nuc* gen as show figure (3).

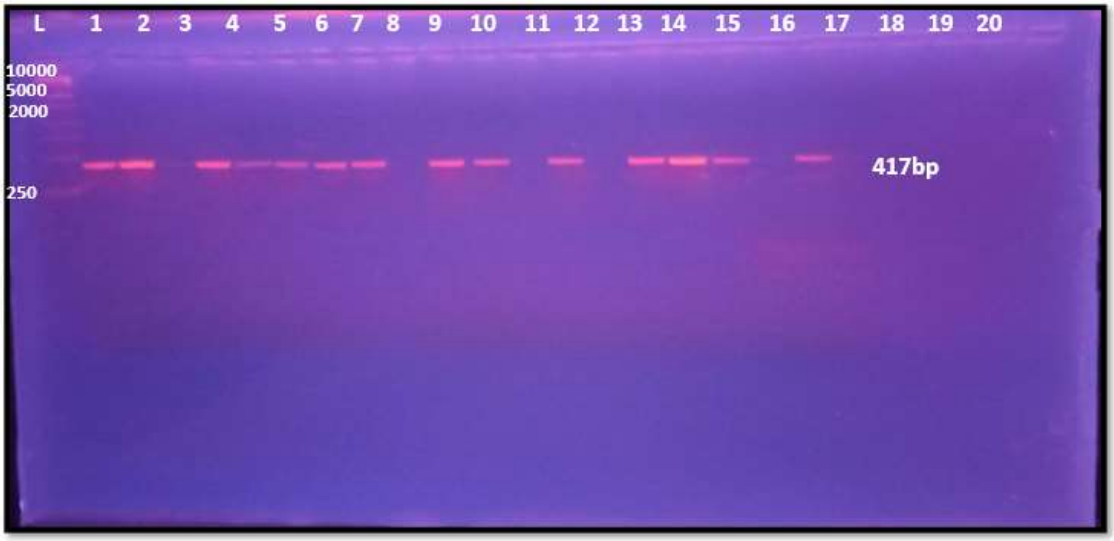

Figure (3): The PCR product of *nuc* gene in 1% agarose gel electrophoresis, voltage (85 V), time (90 minute) and 5 µL of PCR product loaded in each well. Lane L: DNA Ladder (10000bp) Lanes 1,2,3,4,5,6,7,9,10,12,13,14,15,17: PCR product (positive case band (417 bp).

The *nuc* gene serves as a marker, and the presence of the heat resistant nuclease gene (nuc) is highly linked to enterotoxin generation, and it may be used to detect infection with enterotoxin producers *S.aureus*(Brakstad*et al.,*1992).AbdJalil, (2010) suggested that the rapid *nuc*PCR assay is a suitable and practical tool for the routine identification and characterization of *S. aureus,* MRSA, and CoNS; which can be easily applied in a microbiology laboratory procedure,*Staphylococcus* is capacity to generate a large rangeof exoenzymes, among these, nuclease is known to be an important virulence factor and can maintain operationafter incubation at 97˚C for 60 min. Nuclease can destroy the host's nucleic acid by hydrolyzing phosphodiester bonds in DNA and RNA, resulting in 3′-mononucleotides.

Conclusion:

*S.aureus*is the commonest Gram-positive bacteria isolated from Conjunctivitis patients. MRSAisolates are commonest *S.aureus* isolated from Conjunctivitis infections. MRSAisolatesappeared *cap8, nuc*and*hla*genes in methicillin resistant isolates,while no results for*cap5*genes. Virulence factors phenotype detection was highly prevalent of gelatinase, Dnase, and protease among MRSAisolates.

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