TURKISH REPUBLIC OF NORTHERN CYPRUS

NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

BIOFILM ASSESSMENT OF *STAPHYLOCOCCUS AUREUS* FROM DIFFERENT CLINICAL SPECIMENS

TAHA TALAL ABDULRAHMAN

MASTER OF SCIENCE THESIS

ADVISOR

ASSOC.PROF. DR. UMUT GAZI

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PROF. DR. PAYMAN A. HAMASAEED

MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY

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DECLARATION

Hereby, I declare that this thesis study is my own study, I had no unethical behaviors in all stages from planning of the thesis until writing there for, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by this thesis study and took these references into the reference list, and had no behavior of breeching patent rights and copyright infringement during the study and writing of this thesis

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TAHA TALAL ABDULRAHMAN

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DEDICATION

THIS THESIS IS DEDICATED TO:

MY BELOVED PARENTS

MY SUPERVISORS

MY DEAR BROTHERS

MY FRIENDS

ALL WHO HELPED ME

ÖZET

Staphylococcus aureus, kateterlerle ilişkili idrar yolu enfeksiyonları gibi klinik enfeksiyonların en önemli nedenidir ve zararlı patojenlerin birçok virülans faktörünü ifade ettiği ve ciddi enfeksiyonlara neden olduğu kabul edilmektedir. Bu çalışmada, Şubat 2021 ve Mayıs 2021 tarihleri arasında Erbil şehrinde farklı özel ve kamu hastanelerine başvuran hastalardan yaklaşık 127 klinik örnek alınmıştır. Morfolojik, kültürel ve biyokimyasal testlere dayalı olarak 75 *S. aureus* izolatı tanımlanmıştır (%59.05) 127 farklı klinik örnek arasında. Benzer şekilde, 16S rRNA genlerine dayalı olarak Polimeraz Zincir Reaksiyonları kullanıldı ve tüm izolatlar doğrulandı.

10 antimikrobiyallere karşı antimikrobiyal duyarlılık testi sonuçları, değişken duyarlılık paternleri gösterdi. En yüksek direnç penicillin karşı %94.67, en düşük direnç ise azitromisin her biri için %2.67 idi. Tüm izolatlar, farklı antimikrobiyallere önemli ölçüde farklı direnç modelleri gösterdi. Disk difüzyon yöntemlerini gerçekleştiren on antibakteriyel ajan için onaylanmış antibiyotik dirençli profiller. En yüksek direnç yüzdesi P'ye karşı %94,67 iken, bunu LEV (%70,67), CTX (%68), OFX (%54,67), AK (%50,67), CIP ve NOR (%48), CN (%24), ve TOB (%13.33), en düşük direnç yüzdesi (%2.67) ise AZM her birine karşı kaydedildi.

Tüm *Staphylococcus aureus* izolatları biyofilm gelişimi açısından tarandı ve sonuçlar yüksek düzeyde biyofilm üretimi gösterdi. Bulgularımıza göre, tüm *S. aureus* izolatları biyofilm açısından pozitif olarak test edildi, bunların %5.34'ü güçlü biyofilm oluşturan (*n*=4), %44.0'ı orta düzeyde biyofilm oluşturan (*n*=33) ve %50.66'sı olarak sınıflandırıldı. Biyofilmlerin zayıf oluşturucusu olarak sınıflandırılan (*n*=38).

Biyofilm gelişimi ile biyofilm gelişimi ile ilgili genler arasındaki ilişki, önemini belirlemek için incelenmiştir. *S. aureus* izolatlarında bu genlerin dağılımının şematik bir temsili. Tüm izolatlar arasında, dört genin tümü, hepsinin mevcut olduğunu gösteren değişen frekans seviyelerinde bulundu. Gösterildiği gibi, *ica*A ve icaC genlerinin, genler için pozitif test edilen *S. aureus* izolatlarının büyük bir yüzdesinde (yüzde 94.66 [*n*=71]) mevcut olduğu bulundu. Bulgulara göre, *ica*B ve *ica*D genlerinin popülasyonun sırasıyla yüzde 57.34 ve yüzde 86.67'sinde mevcut olduğu keşfedildi.

SUMMARY

Staphylococcus aureus is a most important cause of severe clinical infections, such as urinary tract infections associated with catheters, and is a harmful pathogen that expresses many virulence factors. In this study, 127 clinical specimens were taken from patients who admitted to the different private and public hospitals in Erbil city from February 2021 and May 2021. Based on morphological, cultural, and biochemical tests, 75 isolates of *Staphylococcus aureus* were identified (59.05%) among 127 different clinical specimens. Likewise, all isolates were confirmed by using 16S rRNA-based Polymerase Chain Reaction (PCR).

The antimicrobial susceptibility test results against 10 antimicrobials showed variable susceptibility patterns, which include Amikacin (AK), Azithromycin (AZM), Cefotaxime (CTX), Ciprofloxacin (CIP), Gentamicin (CN), Levofloxacin (LEV), Norfloxacin (NOR), Ofloxacin (OFX), Penicillin (P), and Tobramycin (TOB). The highest (94.67%) and lowest (2.67%) resistance rates were detected against and AZM, respectively. Resistance rates detected against other antimicrobials include LEV (70.67%), CTX (68%), OFX (54.67%), AK (50.67%), CIP (48%), NOR (48%), CN (24%), and TOB (13.33%).

All isolates of *Staphylococcus aureus* were screened for the developing of biofilms, and the results showed a high level of biofilm production. According to our findings, all *S. aureus* isolates tested positive for biofilms, with 5.34% (*n*=4), 44.0% (*n*=33) and 50.66% (*n*=38) classified strong, moderate and weak biofilm forming, respectively.

The relationship among the biofilm development and the genes related with biofilm synthesis was examined in order to determine its significance. Our findings revealed of the dispersion of these genes in *S. aureus* isolates. Among all isolates, all four genes (*ica*A, *ica*B, *ica*C, and *ica*D) were found at varying levels of frequency, indicating that they were all present. As has been shown, the *ica*A and *ica*C genes were found to be existing in the large percentage of *S. aureus* isolates (94.66%) tested positive for the genes. The *ica*B and *ica*D genes were present in 57.34% and 86.67% of the population, respectively, according to the findings.

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LIST OF ABBREVIATIONS

Abbreviations	Word details
Agr	Accessory gene regulator
BAB	Blood agar base
Bap	biofilm-associated protein
BHI	Brain Heart Infusion agar
bp	Base pair
BSIs	Bloodstream infections
CA	Community-acquired
CAPs	Surface-associated toxin
ccr	Cassette chromosome recombinase
CFU	Colony forming units
ClfA	Clumping factor A
ClfB	Clumping factor B
CLSI	Clinical and Laboratory Standards Institute
cna	Collagen-binding proteins
CoNS	Coagulase-negative *Staphylococcus*
DCFH-DA	Dye dichlorofluorescein diacetate
ddH_2O	Deionized distilled water
DTC	Direct tube coagulase
ECM	Extracellular matrix
eDNA	Extracellular deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
egc	Enterotoxin gene cluster
Embp	Extracellular matrix binding protein
EPS	Extracellular polymeric substances
ETs	Staphylococcal exfoliative toxin
fnbA	Fibronectin-binding protein A
fnbB	Fibronectin-binding protein B
FRAP	Ferric reducing antioxidant power
GP	Gram-positive ID card
HA	Hospital-acquired
HGT	Horizontal gene transfer
HP	Human plasma
ica	Intercellular adhesion
***ica*R**	Intercellular adhesion locus regulator
ICU	Intensive care unit
LB	Luria Bertani
MCRA	Modified Congo red agar
MDR	Multidrug-resistant
MFS	Major facilitator superfamily
MGEs	Mobile genetic elements
MHA	Mueller Hinton Agar
MIC	Minimum inhibitory concentration
MISA	Methicillin-intermediate *S. aureus*
MRSA	Methicillin-resistant *S. aureus*

MSA	Mannitol salt agar
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin-sensitive *S. aureus*
MTP	Microtiter plate
NC	Negative control
NIH	National Institutes of Health
OD	Optical density
ODc	Cutting optical density
PBP2a	Penicillin-binding protein
PBS	Phosphate buffered saline
PC	Positive control
PCR	Polymerase chain reaction
pH	Power of hydrogen
PIA	Polysaccharide intercellular adhesion
PNAG	β-1, 6- linked N-acetyl-D-glucosamine
PTSAgs	pyrogenic toxin superantigens
PVL	Panton Valentine-Leukocidin
QRDR	Quinolone-resistance-determining-region
QS	Quorum sensing
Rbf	Biofilm formation regulator
P1	Promotor 1
P2	Promotor 2
ROS	Reactive oxygen species
RP	Rabbit plasma
SasG	*S. aureus* surface protein G
S. aureus	*Staphylococcus aureus*
SAgs	Superantigens toxins
SCC*mec*	Staphylococcal chromosome cassette *mec*
SCV	Small colony variants
SEs	Staphylococcal enterotoxin
SOB	Super optimized broth
SPSS	Statistical Package for the Social Sciences
SRC	Scientific Research Center
SSS	Scalded skin syndrome
TAE Buffer	Tris-Acetate EDTA buffer
TE buffer	Tris–EDTA buffer
TG	Transglycosylase
Tm	Melting temperature
TP	Transpeptidase
TPTZ	2,4,6-tripyridyltriazine
TSST-1	Toxic shock syndrome toxin-1
UTI	Urinary tract infections
UVB	Ultraviolet B
VISA	Vancomycin-intermediate *S. aureus*
VRSA	Vancomycin-resistance *S. aureus*

1. INTRODUCTION

1.1 Scope of the study

Staphylococcus aureus is a pathogen that has been linked to a variety of human problems. The ability of *S. aureus* to colonization and infect various places throughout the tissue is dependent on the existence of a range of virulence factors (Tam and Torres, 2019). Among many of the virulence factors that contribute to *S. aureus*'s pathogenicity are adhesion proteins and the capacity to establish biofilms on biological and non - living things substrates. Numerous infections in humans are connected with *S. aureus* biofilms. Osteomyelitis, endocarditis, and other infections associated with surgical devices including prosthetic joints, endotracheal tubes, skeletal prostheses, vascular catheters, cardiac pacemakers, and heart valves are all possible medical indications. *S. aureus* biofilms on food-processing substrates are a serious problem for the food business, as they are a potential cause of contamination for food materials and human workers (Vergara et al., 2017). The seriousness and medical appearance of the disease are determined by the development of various toxins and adhesion proteins (Karahan et al., 2009).

Two components of the biofilm contribute significantly to the pathogenesis of animal and human infections: firstly, the bacterium' adherence to epithelial cells enables the insertion and release of several toxins (Archer et al., 2011). Second, reduced antimicrobial molecule penetration into the biofilm matrix inhibits the efficacy of antimicrobial treatment (Mah, 2012).

A biofilm has been described as an organized population of microbial cells contained in an extracellular polymeric matrix that adheres to a substrate (Achek et al., 2020b). The biofilm-formation mechanism is separated into numerous stages which start with staphylococcal cell adhesion to a biocompatible material and progress through accumulating and biofilm development. The dispersion step refers to the spread of germs from one surface to another (Beenken et al., 2004). Adherence to biotic and abiotic substrates is enhanced in staphylococcal biofilms by bacterial surface elements that recognized adhesive matrix molecules (MSCRAMMs) and the biofilm-associated protein (Bap) (Otto, 2008). The aggregation process seems to be

dependent on the *ica*ADBC operon-encoded polysaccharide intercellular adhesion (PIA) (Achek et al., 2020b). Biofilm production is a complicated mechanism that is influenced by numerous parameters such as nutritional supply, pH value, oxygen concentration, and surface qualities (Otto, 2008). Additionally, the production of biofilms in *S. aureus* is regulated by two genomic regions: *sar*A (staphylococcal accessory regulator) and *agr* (accessory gene regulator) mechanism for determining the presence of a quorum-sensing system (Zhang et al., 2018). According to microfluidic circulation cellular systems and time-lapse imaging, the creation of *S. aureus* biofilms involves a five evolutionary pathway as following: Attachment (a), proliferation (b), emigration (c), maturation (d), and dispersal (e) (Chen et al., 2020). Accumulating evidence indicates that three variables are involved in these complex processes: eDNA; poly (1, 6)-*N*-acetyl glucosamine (PIA/PNAG) that is stimulated by expression of the intercellular adhesion locus *ica*ADBC; and microbial surface elements identifying adhesive matrix molecules (MSCRAMMs), that have been demonstrated to perform tasks as extracellular matrix elements throughout early biofilm formation. Nevertheless, *ica*A/B/C mutants of the UAMS1 and USA300JE2 strains accumulated normally throughout the proliferation process, showing that these proteins play a significant involvement in the formation of ica-independent biofilms. *S. aureus* could indeed express a number of MSCRAMMs, including fibronectin binding proteins A and B (*fnbp*A and *fnbp*B), fibrinogen binding protein clumping factors A and B (*clf*A and *clf*B), biofilm associated protein (*bap*), serine aspartate repeat (*Sdr*) family, elastin binding protein (*Ebps*), collagen binding protein (cna), and laminin binding protein (eno) (Azara et al., 2017), and fibrinogen-binding protein (*fib*), with the proportional importance of each element seeming to differ depending on the stress or circumstance (Chen et al., 2020).

Aims of this study

This research was designed to include:

1. Isolation of *S. aureus* from clinical specimen and identification of isolates by

(phenotypically, microscopically and biochemical test) and molecular approaches.

2. Antimicrobial Susceptibility Test for isolates.

3. Detection and evaluation of biofilm development phenotypically through the microliter plate assay (MTP).

4. Molecular analysis for detecting expression of genes associated with biofilm formation (*ica*A, *ica*B, *ica*C, and *ica*D) by PCR.

2. LITERATURE REVIEW

2.1 *Staphylococcus* genus

Staphylococcus genus members are spherical, and have diameter about 0.5–1.0 μm. They may appear alone, in couples, in tetrads, or in simple terms-chain cells. They distinctively divide into more than one line and form an irregular grape-like cluster, which give positive test result to Gram reaction. They are non-motile, do not produce spore, and are usually not encapsulated or may gave restricted formation of capsules. Staphylococci emit catalase, which distinguishes them from the streptococci (Carroll et al., 2016). Staphylococci gradually ferment many carbohydrates, forming lactic acid without gas. Proteolytic action differs from all strains of staphylococci significantly. They belong to the phylum Firmicutes (with a low DNA G+C content of about 50 mol percent) (Whitman, 2009).

At least 45 species belong to the genus *Staphylococcus*, the four most frequently species of clinical significance are *S. aureus*, *S. epidermidis*, *S. lugdunensis*, and *S. saprophyticus*. Staphylococci grow on most microbiological culture media under aerobic or microaerophilic situations. They grow at 37°C, and at room temperature (20–25°C), they form the pigments in the best quantities. Colonies are round, flat, raised, and glittering on solid media. Staphylococci are relatively resistant to drying, temperature (50°C for 30 min.) and 10% sodium chloride (Carroll et al., 2016). All species are facultative anaerobes (proficient of growth both aerobically and anaerobically) (Harris et al., 2002). While many species do not cause any disease and live usually on the skin and mucous membranes of humans and other animals *S*ome can promote a great range of diseases in humans and animals, whether by toxin synthesis or by infecting other organisms (Stevens et al., 2015).

2.2 *Staphylococcus aureus*

Even though *S. aureus* is normally considered to be a commensal bacterium found in approximately 30% of the human population, it could occasionally lead to bacteremia, infective endocarditis, and arthritis. Furthermore, it has the potential to

induce a variety of skin and soft-tissue infections, especially when the skin has been penetrated (Tong et al., 2015). *S. aureus* infection might transmit quickly and easily when an individual comes into touch with pus from an infected wound, or connection with goods utilized by an infected person (Kavanaugh and Horswill, 2016).

2.2.1 *Staphylococcus aureus* diseases

2.2.1.1 Infections of skin

Diseases caused by *S. aureus*, include small benign boils, impetigo, cellulitis, and folliculitis, as well as more severe, invasive soft-tissue infections, which are the most widespread source of skin infections in the America. Individuals suffering from atopic dermatitis, often recognized as eczema, are at high risk of developing this condition. It is most commonly seen in fertile, vigorous areas such as the armpits, hair, and scalp, among other locations. Large pimples that form in such places, especially if they are lacerated, could worsen the infection. As a result, staphylococcal scalded skin syndrome may develop, with an extreme condition appearing in neonates (Curran and Al-Salihi, 1980).

2.2.1.2. Food poisoning

Staphylococcal food poisoning (SFP) is an infection caused by the intake of foods containing enough amounts of preformed enterotoxin (or more). SFP signs manifest rapidly (2–8 hours) and include nausea, severe vomiting, and gastrointestinal pain, which may be associated with or without diarrhea. Typically, the sickness is self-limiting and resolves within 24–48 hours of beginning. Sometimes, it can be severe enough to require hospitalization, especially in infants, the elderly, or the disabled (Murray, 2005).

Food handlers with enterotoxin-producing *S. aureus* in their nostrils or on their hands are considered the primary cause of food contamination, through either direct contact or respiratory secretions. Indeed, *S. aureus* is a widespread commensal of human skin and mucosal membranes, with estimates ranging from 20%–30% for sustained colonization to 60% for intermittent colonization. Due to the fact that S.

aureus does not compete successfully with microbiota in raw foods, contamination is primarily caused by inappropriate handling of cooked or processed foods, followed by storage under circumstances conducive to *S. aureus* growth and synthesis of the enterotoxin (s). *S. aureus* is, nevertheless, found in food animals, and dairy cattle, sheep, and goats, particularly those with subclinical mastitis, are possible milk contaminants. Additionally, air, dust, and food contact surfaces can act as vectors for the spread of *S. aureus* to foods (Chiang et al., 2008).

Meat and meat items, poultry and egg products, milk and dairy products, salads, bakery products, notably cream-filled pastries and cakes, and sandwich fillings have all been implicated in staphylococcal poisoning. Salted foods, such as ham, have also been implicated, due to *S. aureus*'s ability to grow at low water activity (aw = 0.86) (Stewart, 2005). SFP is a widespread disease with a likely underestimated true prevalence due to a variety of factors, including misdiagnosis, unreported minor outbreaks, incorrect sample collection, and improper laboratory evaluation. Controlling this disease is critical for social and economic well-being. Indeed, it imposes a significant financial cost in terms of lost workdays and productivity, medical expenditures, and economic losses in the food industry, catering businesses, and restaurants (Argudín et al., 2010).

2.2.1.3 Bacteremia

A prominent source of bloodstream infections, *S. aureus* is often linked with skin or mucosal membrane wounds caused by surgery, injury, or the application of intravascular equipment particularly catheters, hemodialysis devices, or medications that are injected. Several organs may get infected after germs penetrate the bloodstream, including the heart and joints, producing infective endocarditis, septic arthritis, and bone infection (Rasmussen et al., 2011).

2.2.1.4 Infections of bone and joint

Major bone and joint infections caused by *S. aureus* include osteomyelitis, septic arthritis, and infection from a replacement joint surgery (Latha et al., 2019).

Orthopedic implant-related infections can be triggered by *S. aureus* biofilms that develop on medical equipment and are transplanted in the body. However, *S. aureus* biofilms could even form on other types of medical machines such as cardiac implants and vascular grafts as well as numerous catheters and cosmetic surgical implants.

When a medical machine has become contaminated, it should be eliminated altogether entirely because biofilms cannot be decimated by antimicrobial therapy (Nandakumar et al., 2013). The use of antibiotic-loaded, dissolvable calcium sulfate beads that are implanted with the surgical equipment could provide an alternative to postsurgical antibiotic therapy. These beads have the capability of releasing high doses of antimicrobials at the appropriate spot, thereby preventing the onset of the infection (Archer et al., 2011). Silver nanoparticles are being investigated as potential therapies for *S. aureus* biofilms since these compounds have demonstrated antagonistic impacts on *S. aureus* entrenched in biofilms (Chung and Toh, 2014).

2.3 Medications of *Staphylococcus aureus* infections

Penicillin is the alternative medication for treating *S. aureus* infection because it prevents the development of peptidoglycan cross-linkages that is responsible for the hardness and strength of a bacterial cell wall, leading in cell death in the bacteria. Penicillin resistance, on the other hand, is quite frequent in most locations, and penicillin-resistant β-lactam antibiotics, which have the similar mode of action as penicillin, were employed in most cases. Methicillin-resistant *S. aureus* (MRSA) strains are most recurrently linked with organizations such as hospitals where they have established resistance to the majority of β-lactam antibiotics used today. In order to combat this, vancomycin is frequently prescribed (Thwaites et al., 2018).

2.4 Antimicrobial resistant of *S. aureus* towards antimicrobials

Generally, pathogens have an extraordinary ability to establish and live in specifically configured regions of host and result in biological routine dysfunction. On the other hand, an overwhelming healthcare challenge is bacterial infections

related to evolving multidrug-resistant (MDR) and the absence of growth of innovative and active drugs. MDR microorganisms are associated with increased morbidity and fatality rates in a variety of frequent infectious illnesses (Ahmed et al., 2017). The reality that *S. aureus* has been immune to several types of antimicrobials in the clinical environment is indeed a difficult task that physicians face today while trying to treat *S. aureus* infections (Akanbi et al., 2017). Such resistance problems from a more than 50-year history of consistent resistance of *S. aureus* to several antibacterial agents presented over the years of medical practice. Abuse of antimicrobials, as well as indiscriminate using, leads to the transmission of resistance (Tavares, 2014). Antibiotic-resistance genes are located on plasmids and transposons (Werckenthin et al., 2001).

Staphylococcus aureus is an significant human pathogen with ability to obtain resistance to proliferation in both acute and public healthcare settings (Zetola et al., 2005). While most of the *S. aureus* infections identified in community settings were correlated with strains of MSSA (Adukwu et al., 2012) rather than the strains of MRSA, infections triggered by MRSA strains are associated with higher after-care prices than those instigated by MSSA strains (Gordon and Lowy, 2008). More than 50% of health-related MRSA strains are considered to be resistant to both β-lactam and non-β-lactam antibiotics, whereas strains of community-acquired *S. aureus* are susceptible to non-β-lactam antibiotics. (King et al., 2006). The development of community acquired infections, particularly strains of CA-MRSA, is of specific significance as they have molecular features losses from MRSA strains throughout the hospital outlined in a study of Chambers and Deleo (2009). Such features include; type IV allele of staphylococcal chromosome cassette *mec* (SCC*mec*) and genes coding the Panton Valentine-Leucocidin (PVL) virulence factor (King et al., 2006).

The PVL is a cytotoxin that causes tissue necrosis and leucocyte death (Loughrey et al., 2007), and both community-based MSSA and MRSA strains could possess the *pvl* gene (Rasigade et al., 2010). Antimicrobials work by blocking the essential bacterial functions including synthesis of cell wall (β-lactams and glycopeptides), metabolism pathways such as folate metabolic processes (sulphonamides and trimethoprim), protein biosynthesis (aminoglycosides,

tetracyclines, macrolides, lincosamides, chloramphenicol, mupirocin and fusidic acid), nucleic acid biosynthesis (hydroquinone), and RNA biosynthesis (rifampicin) (Wright, 2005, Tenover et al., 2006, Alekshun and Levy, 2007). The overuse of antimicrobial agents results in resistance through either the appearance of point mutations or via the acquiring of external resistance genes, resulting in alteration of the antimicrobial target and loss of the antimicrobial or decrease of the inner antimicrobial concentration of the cell (Tavares, 2014).

2.5 Virulence factors

2.5.1 Enzymes

Staphylococcus aureus releases a variety of enzymes, including coagulase (both bound and free coagulases), which clots plasma and covers the bacterial cell, most important to avoid phagocytosis from taking place. Hyaluronidase (also defined as spreading factor) is a digestive enzyme that splits down hyaluronic acid and aids its spread. *S. aureus* also generates deoxyribonuclease, which breakdowns DNA, lipase, which digests lipids, staphylokinase, which dissolves fibrin and aids in the transmission of the infection, and beta-lactamase, which aids in the progress of antimicrobial resistance (Dinges et al., 2000).

2.5.2 *Staphylococcus aureus* secreted toxins

Dependent on the strain, *S. aureus* have been able to secreting three groups of toxins are related with particular diseases:

1. Superantigens caused toxic shock syndrome (TSS) that is categorized by fever, erythematous rash, low blood pressure, shock, multiple organ failure, and skin peeling.
2. Exfoliative toxins which is caused staphylococcal scalded skin syndrome (SSSS), most frequently occur in infants and young children or as epidemics in hospital nurseries. The protease activity of the exfoliative toxins causes peeling of the skin observed with SSSS.

3. Some other Staphylococcal toxins affected the cell membranes like alpha toxin, beta toxin, delta toxin, and numerous component toxins (Becker et al., 2003).

More than 40 exotoxins have been identified as being generated by these bacteria, many of which have analogous activities and share significant molecular homology. A deeper examination at these exotoxins, which at first glance appeared to be redundant, demonstrated that each has its own set of characteristics. Exotoxins could be categorized into three major classes based on their established mechanisms: cytotoxins, superantigens, and cytotoxic enzymes.

Cytotoxins are the most common type of exotoxin. Cytotoxins have an effect on the membranes of host cells, leading in the destruction of target cells and the induction of inflammation. Super antigens are responsible for large cytokine production as well as the stimulation of T and B cell proliferation. Cytotoxic enzymes produced by mammalian cells cause cell death. Together, these exotoxins possess the facility to stimulus the host immune response, which is crucial for *S. aureus* infections (Tam and Torres, 2019).

2.5.3 Protein A

The Fc region of antibody is bound by a protein known as protein A, which is an immunoglobulin-binding protein. Some research utilizing mutations in protein-coding genes found that *S. aureus* had a reduced ability to survive in blood when the genes were changed (Patel et al., 1987).

2.5.4 Biofilm

Small single cells (planktonic) or large sessile aggregates are the two types of bacterial proliferation that might be observed. The second is known to as the biofilm mechanism of growth in the scientific community. Although there are many different descriptions of bacterial biofilms, all accept that biofilms are made of numerous bacteria that work together as a team to establish a community (Bjarnsholt et al., 2011). Biofilms were identified as immobile populations of bacterial cells integrated

in a matrix of extracellular polymeric constituents and displaying an transformed phenotype (Adukwu et al., 2012).

Biofilm production is a vital element in establishing chronic infection by opportunistic pathogen *S. aureus*, which is able to form biofilms on host surfaces including muscle, cartilage and heart valves and also on components in foreign bodies, like catheters and orthopedic instruments (Brady et al., 2008, Kiedrowski and Horswill, 2011). A variety of disorders, including cystic fibrosis, endocarditis, osteomyelitis, and different nosocomial diseases linked to central venous catheters, urinary catheters, heart valves or orthopedic instruments, are connected to the biofilm formation. Biofilms are well established for antimicrobial resistance and degradation of immune system defenses (Leid et al., 2002, Jones et al., 2001).

Bacterial biofilm possess excellent medicinal and industrial influences. It not only impacts human health and social life but similarly distresses the performance and efficiency of industrial organizations. Biofilm assists bacterial cells to implement a provisional lifestyle to survive, even under the adverse environmental state. Treatment options are limited due to biofilm recalcitrance, which usually involves destroying contaminated instruments and tissues. Such approaches are not suitable for patient care (Mootz, 2013).

The potential of bacteria to proliferate and develop biofilms is directly correlated with the capacity of bacteria to create an extracellular mucoid substance known as slime, which is polysaccharide in structure and consists primarily of glycosaminoglycan as its primary constituent (Nasr et al., 2012).

Biofilms are bacterial cell clusters that form when bacteria adhere to one another and proliferate on damp surfaces. Teichoic acids, host proteins, extracellular DNA (eDNA), and polysaccharide intercellular antigen may all be found in *S. aureus* biofilms, which are encased in a glycocalyx slime layer (PIA). Immunological response avoidance and antimicrobial resistance are critical in disease pathogenesis, and biofilms play a role in this for the reason that of their great resistance to antimicrobial therapy and the host immune response that they can induce.

Antibiotic-degrading enzymes, including β-lactamases, may be present in the biofilm matrix, which may inhibit antibiotics from penetrating the cell wall. Additional set of circumstances is that the biofilm matrix encourages the production

of viable cells, that are dormant bacterial cells that are very antibiotic-resistant (Archer et al., 2011). So biofilms can have four distinct effects on the body: they can aid in the development of antibacterial medication resistance, because persistent infections, modulate the host's immunological response, and contaminate medical equipment, among others.

It is also believed that the biofilm lifestyle helps microbes to survive in harsher environmental situations than they would normally (Lin et al., 2015). It is not the dispersed microbes inside the biofilm matrix that are of concern; rather, it is their structured representation that is controlled by a set of genes that consequences in the development growth, and dissemination of the biofilms. These biofilms are a self-formed moistened additional polymeric matrix that serves as the microorganisms' immediate habitat in their natural surroundings (Flemming et al., 2016).

Several scientific researchers have classified biofilms as an essential part of microbes rather than as an external constituent even though it has numerous regulatory and metabolic actions between the microbes and the microbe takes in the nutrients deemed necessary for their persistence, as well as enabling the bacterium to resolve detrimental environmental conditions as a result of nutrient shortage. Clinical research have concentrated on the involvement of biofilms in the transmission of infections, which can contribute for up to 60% of all illnesses in the human population (Chen and Wen, 2011).

Biofilms are related with a variety of chronic diseases that are effective of claiming the life of the patient, and their involvement in contaminating medical equipment in the context of hospitalized patients is widely recognized. It is also possible to find biofilms on a variety of biomaterials employed in medicine, including urinary catheters and orthopedic equipment (Campoccia et al., 2006). Additional aspects of the biofilm to consider are the amount to which microorganisms inside the additional polymeric matrix have developed antibacterial resistance that enables the pathogen resistant to routinely employed antibiotics (Carmen et al., 2005). Biofilms have a variety of characteristics that are influenced by their genetic makeup and otherwise their surrounding environment. As a necessary consequence, a varied group of genome and environmental factors influence the pathogen's ability to adapt to a diverse array of environments and

genetic variation is necessary for the advancement of new strains through horizontal gene transfer. Thus, biofilms are tightly controlled by a wide range of genetic and ecological factors and are in actuality the most important cause of bacterial infections worldwide (Watnick and Kolter, 2000).

2.5.4.1 The biofilms composition

The biofilms are made up of microorganism cells and additional polymeric components that serve to provide nutrition to the microorganisms and to produce a suitable environment for their preservation. They also serve to facilitate genetic transfer and intracellular communications between the bacteria. Biofilms structurally consist of bacterial cells and extra polymeric constituents which contribute for 50 – 90 percent of their total organic carbon and are extremely hydrated as a result of the significant amounts of water present. In addition, bacterial biofilms contain biochemical compounds such as DNA, protein, lipids, and organic compounds that contribute to their overall hydration (Figure 2.1) (Sutherland, 2001).

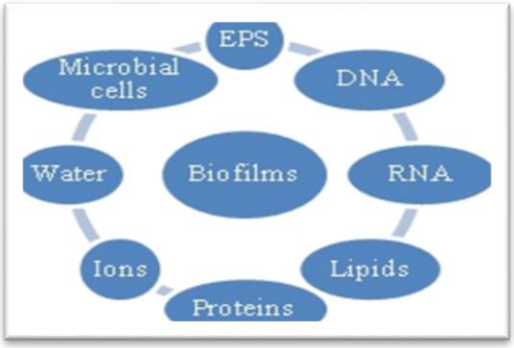


Figure 2.1 The components of bacterial biofilms.

2.5.4.2 Biofilm synthesis in gram positive bacteria

The biofilm development involves different stages:

1. **Primary adhesion**

Mechanisms such as electrostatic and hydrophobic interactions between bacteria and surfaces, as well as surgical instruments, are responsible for the adhesion of

germs to surfaces. In addition, several proteins, including the Ssp-1 and Ssp-2 proteins, assisted in the adherence of bacteria to a solid surface. An investigation on *S. aureus* has revealed the occurrence of adhesion proteins such as Aap and AltE, which are involved in the adhesion mechanism (Otto, 2008). Additional studies was found the function of DNase I in attachment of the bacterial cell to surfaces like glass and plastic (Qin et al., 2007).

2. Accumulation

In the presence of host proteases, intracellular accumulation is caused via the proteolytic process of cell wall-embedded accumulation-linked protein. In addition, teichoic acids and extracellular DNA have been identified as factors in adhesion that are necessary for structural preservation through binding molecules with one another (Rohde et al., 2005, Tormo et al., 2005).

3. Biofilm maturation

Throughout the pathway of biofilm development, adhesion mechanisms that bind bacteria together throughout proliferation take place, as well as disruptive events that cause channels to develop in the biofilm structure (O'Toole et al., 2000). In order for nutrition to enter cells in the deepest biofilm layers, the latter are required. Besides causing biofilm architecture, disruption events can also result in the separation of cell clusters from the biofilm that regulates biofilm development and seems to have crucial implications for *in vivo* biofilm infection because it may result in systemic dispersion. A great deal of investigation has been conducted into the genetics and physiology of sticky biofilm factors. In *S. aureus*, an exopolysaccharide known as polysaccharide intercellular adhesion (PIA), which is also named poly-N-acetyl glucosamine (PNAG) due to its biochemical makeup, is potentially the most significant adhesive biofilm component. PIA is produced, exported, and transformed by the outcomes of the *ica* gene locus, which includes the *ica*A, *ica*D, *ica*B, and *ica*C genes (Heilmann et al., 1996). The *ica*A and *ica*B proteins combine to generate an N-acetyl glucosamine transferase that is found in the membrane of the cell and is

responsible for adding N-acetyl glucosamine repeats from triggered N-acetyl glucosamine to the developing oligo-N-acetyl glucosamine chain.

It is thought that *ica*C represents the PIA exporter since the creation of longer chains necessitates the existence of this protein that, as according sequence investigation, forms a membrane-spanning transporter (PIA transporter). *ica*B in N-acetylates some of the N-acetyl glucosamine residues, which is found on the surface of the cell (Vuong et al., 2004). De-acetylation is critical because it inserts positive charges into the PIA polymer, which are necessary for the polymer to attach to the microbial surface. Several investigations have proven the role of PIA in the production of biofilms in both the laboratory and the animal (Rupp et al., 1999).

For a long time, it was supposed to be necessary for staphylococcal biofilm development; however, more contemporary investigations have discovered staphylococcal strains that are accomplished of *in vitro* and *in vivo* biofilm development however lack *ica* genes and, as a result, do not generate PIA. When it comes to cell–cell adhesion, these strains have particular proteins that take the place of PIA. Accumulation associated protein (Aap), extracellular matrix binding protein (Embp), protein A, fibrinogen-binding proteins (*fnbp*A and *fnbp*B), *S. aureus* surface protein G (SasG), and other biofilm adhesive proteins are among the developing range of biofilm adhesive proteins.

The processes by which these proteins participate to cell–cell adhesion are currently under extensive examination and they may involve the production of giant fibrils, including in the case of Aap, as well as the recruitment of other adhesion molecules (Otto, 2013). There were also reports of other polymers being involved in the production of staphylococcal biofilms. Teichoic acids are a type of acid that is detected on the surfaces of Gram-positive bacteria. They have been found to assist to the production of *S. aureus* and *S. epidermidis* biofilms. Almost certainly, they react with other surface polymers by electrostatic contact, so participating to the intricate network that comprises the surface of the staphylococcal cell itself. It is also made up of DNA produced from killed bacteria, which is referred to as extracellular DNA (eDNA) (Whitchurch et al., 2002). It is possible that the negative charge of DNA, similar to that of teichoic acids, will perform a critical impact in its interaction with other surface structures. Understanding of the extracellular biofilm matrix's structural

constituents is particularly important for understanding the probable involvement of biofilm organizing and detaching factors addressed in the following section, because enzymes that breakdown these structures may play a purpose in biofilm development (Otto, 2013).

4. Detachment and dispersal

The mature cells are expelled from the biofilm at the last stage of biofilm development. Numerous influences, such as physical power, nutrient scarcity and accumulation of waste byproducts, pH variations, and cessation of biofilm construction materials, all contribute to the detachment and distribution of mature biofilms into the surroundings, allowing them to detach from their respective surfaces and disperse into the surrounding environment (Boles and Horswill, 2011).

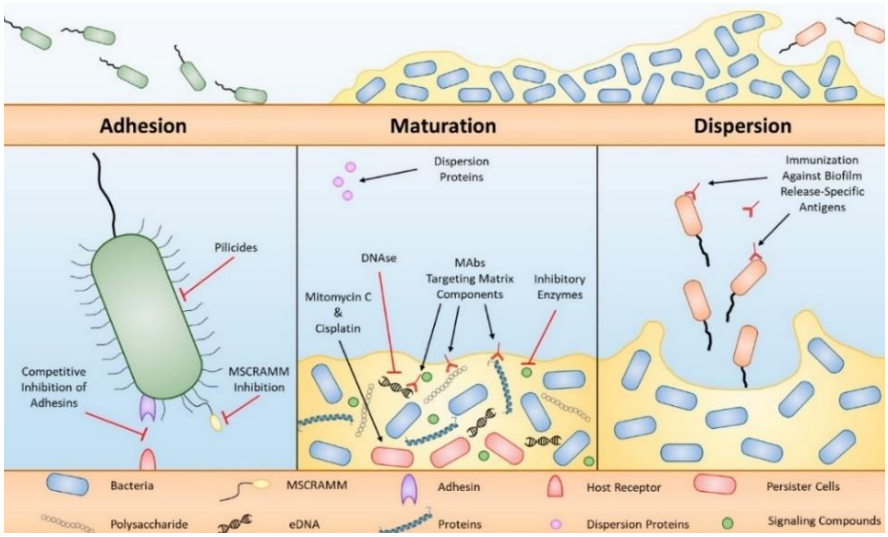


Figure 2.2 A graphic illustration of the improvement of a biofilm.

Beginning with the adhesion of planktonic cells to the surface (brown ovals), accompanied by the creation of an attachment to the surface (grey ovals), the development process is initiated (1). Afterwards, the microorganisms create a monolayer and establish an irreversible bond with one another by generating an extracellular matrix (2). Afterwards, a microcolony is established, within which multilayers develop (3). Throughout the later phases, the biofilm has matured and has begun to develop recognizable “mushroom” formations as a result of the

polysaccharides present (4). The biofilm (seen in yellow) will eventually spread as a result of the detachment of certain cells (5) (Shahmoradi et al., 2019).

2.5.4.3 Molecular regulation of biofilm formation

Biofilm forming regulation is enormously complex and requires several integrated regulatory processes that have yet to be entirely succinctly clarified. Biofilm-specific transcription regulators comprise the biofilm formation regulator (Rbf) is a member of the family of transcriptional regulators, which mediates, at the cell-cell interaction stage, the development of biofilm development in response to glucose and osmotic pressure, and intercellular adhesion locus regulator (IcaR) and teicoplanin-related locus regulator (TcaR), both of which adversely control the development of biofilms (Johnson et al., 2008).

Universal regulators involve *sar*A, which is essential for the production of biofilms, as the results of *sar*A mutation in a lower ability to build a biofilm, and the bi-constituent regulator *ArlRS*, a biofilm formation repressor. The function of the *agr* QS system seems to differ basing on the circumstances of strain production because *agr* disturbance can prevent, enhance or have no influence on the forming of biofilm (Figure 2.2) (Toledo-Arana et al., 2005, O'Neill et al., 2007).

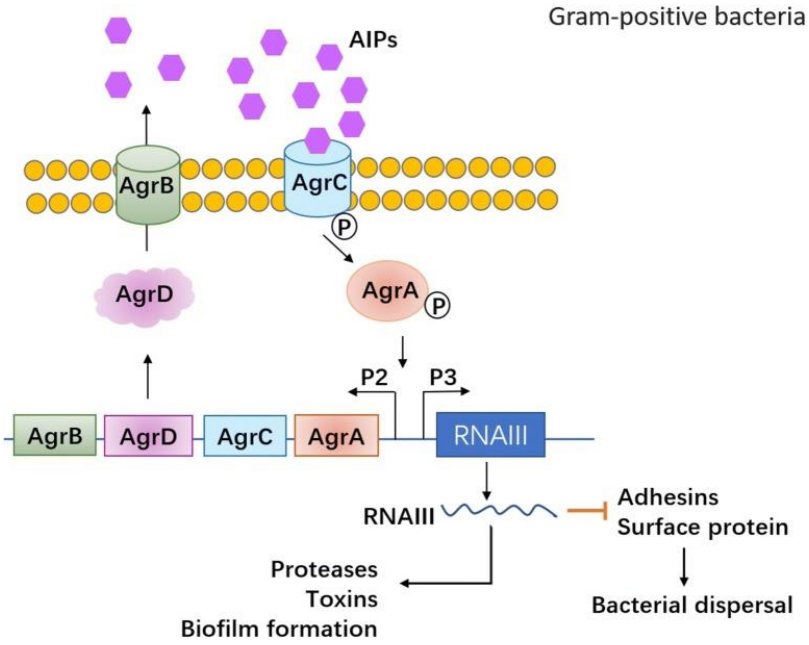


Figure 2.3 Quorum Sensing canonical signaling in Gram-positive bacteria and their function in the production of biofilms.

Several environmental factors, including anaerobic development, osmotic pressure, and glucose supply, affect *S. aureus* biofilm formation. The severe iron limitation is significant environmental stress faced by *in vivo* bacteria. The best-understood element involved in the active development of biofilms is the polysaccharide PNAG, which is produced *ica* operon byproducts. While the relationship among PNAG generation and biosynthetic processes might not be instantly apparent, environmental stressors that promote PNAG synthesis and stimulate biofilm development are also known to suppress central metabolic rate (O'Gara, 2007).

Agr mechanism has been established in Gram-positive bacteria as the most classic Quorum Sensing system. *Agr* mechanism has been well studied in *Staphylococcus*, the most prevalent Gram-positive bacteria, which is important and essential for generating virulence factors, comprising toxins and proteases. Agr operon controls the *Agr* system, which consist of four components *Agr*A, *Agr*B, *Agr*C, and *Agr*D. In Gram-positive bacteria, *Agr*D was the precursor to autoinducer peptides. *Agr*D is changed to the extracellular matrix by *AgrB*. When the abundance of the bacteria is small, the transmembrane protein *AgrC* is activated by autoinducer peptides. The phosphorylated *Agr*C stimulates *Agr*A faster, thereby facilitating the transcription of the target gene. *Agr*A might regulate two promoters.

One is P2, which controls the Agr proteins, and another is P3 that really could stimulate the transcription of RNAIII. RNAIII is the main regulator for modulating the expression of factors associated to Quorum Sensing and proteins involved in the biofilm development. RNAIII can be upregulated the expression of virulence factors particularly proteases, toxins, and degrading enzymes. On the other side, RNAIII may also suppress the cell attachment protein and surface protein expression, which may lead to bacterial dispersion. The bacterial swarming and disease can support by these dual functional functions of the Agr process. This will also include therapeutic targets for the production of antibiofilm drugs, such as autoinducer peptides, Agrs, and RNAIII (Toledo-Arana et al., 2005, O'Neill et al., 2007).

2.5.4.4 Biofilms and immune system

The communication among the immune response and bacterial pathogens developing biofilms was investigated, and the results revealed that the communities of pathogenic microbes within the extra polymeric matrix are extremely well guarded from a wide assortment of factors allowing the inserted pathogens to persist in harsh environments. The existence of exopolysaccharide alginate and the development of an additional polymeric matrix defends the bacterial cells from the mechanism of phagocytosis (Leid, 2009).

2.5.4.5 Biofilms and antimicrobial resistance

The bacterial population in biofilms increased the resistance to a diverse array of antibacterial drugs. The expression of various genes that encode a sequence of protein that imparts the bacterial population is one of the significant purposes for the rise in resistance to antibiotics. The extra polymeric matrix also protects the absorption of antibacterial drugs into biofilms (Percival, 2004). It has been discovered that certain harmful bacterial strains have established resistance to beta-lactam medications, which is owing to the existence of the beta-lactamase enzyme, which has accumulated in bacterial biofilms as a result of secretion or cell death (Fux et al., 2005). Biofilms were created to modify microbial communities to harsh environments, and as a result, the pathogen becomes more resistant to a widespread range of environmental influences, particularly antimicrobials (Rhoads et al., 2007).

Pathogens could survive in unfavorable environments because the bacterial population in biofilms may fall into hibernation when the conditions are adverse, allowing them to survive. These cells make up 0.1-10% of all biofilms, and they comprise a small population of biofilms that are inactive and well-preserved, and they are responsible of evading the action of antibacterial drugs. They also serve as the initiator cells, allowing the pathway to continue when the situations are becoming more desirable. Changes in gene expression within biofilms have the potential to lower antibacterial sensitivity while simultaneously increasing antibiotic resistance. Drugs target specific areas in the microbiome, and particular genes in biofilms are

effective of modifying these target sites in order to preserve the bacterial population within the biofilm (Roberts and Stewart, 2005, Percival et al., 2011).

2.5.4.6 Basis of biofilm resistance against antibiotics

Antimicrobial are often used to cure *S. aureus* biofilms, including protein biosynthesis inhibitors that attack the cell wall and plasma membrane, and also DNA and RNA biosynthesis inhibitors and antibacterial agents such as Cu^{2+}, which cause cell membrane breakdown and subsequently cell death. It has been hypothesized that the widespread use of pharmaceuticals for the therapy of microbial illnesses is significant for the emergence of multidrug-resistant germs including such MRSA (Melo et al., 2016).

It seems the mechanism to be multifactorial by which biofilms withstand antimicrobials. Both restricted treatment options recalcitrance and contribute to biofilm, which typically involves destroying contaminated instruments and tissues. These approaches are not sufficient for patient care, and additional investigations is necessary to clarify the molecular pathways of *S. aureus* biofilm formation to enhance treatment approaches for chronic infections (Darouiche, 2004). Antibiotic resistance in the bacterial biofilms can rise to a level around 10–1,000 times higher than during planktonic development. The explanations may be:

1. Formation of the typical EPS structure within the biofilm, which reduces the penetration of antimicrobials to their target.
2. Slow development and low metabolic action of the specific gene expression have revealed that basic cellular pathways are down-regulated, and aerobic energy formation is removed toward anaerobic fermentation.
3. Heterogeneity; it has been presumed that a small subpopulation of cells in a biofilm could survive elevated concentrations of an antibacterial substance owing to a specific physiological state (Mengi et al., 2013).

Various pathogenic strategies have been suggested for biofilms, and these comprise:

1. Attachment to a solid surface;

2. Labor division improve the metabolic efficiency of the population;
3. Evade host defenses mechanism particularly phagocytosis;
4. Obtain a high bacterial density;
5. Exchange genes that can contribute to bacteria being more virulent;
6. Produce a high toxins concentration;
7. Protect the bacterial cells from antimicrobials;
8. The detachment of microbial aggregates transmits organisms to other sites (Kirmusaoğlu, 2016).

2.5.4.7 The Biofilms on medical devices

The presence of biofilms on surgical equipment has been demonstrated in investigations in which the equipment were either evaluated after being removed from patients or examined in animal or laboratory settings (Raad et al., 1993) as presented in table (2.1).

Table 2.1 Biofilms on medical devices

Central venous catheters
Central venous catheter needleless connectors
Contact lenses
Endotracheal tubes
Intrauterine devices
Mechanical heart valves
Pacemakers
Peritoneal dialysis catheters
Prosthetic joints
Tympanostomy tubes
Urinary catheters
Voice prostheses

According to Table (2.2), a wide range of microorganisms can generate both pure-culture and polymicrobial biofilms, including a diversity of dangerous bacteria and fungi that are well-known to science.

Table 2.2 The types of microorganisms that develop biofilms from indwelling medical devices

Indwelling medical device	Organisms
Central venous catheter	Coagulase-negative staphylococci, *Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Candida albicans*
Prosthetic heart valve	Viridans *Streptococcus,* coagulase-negative staphylococci, enterococci, *Staphylococcus aureus*
Urinary catheter	*Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, Proteus mirabilis*
Artificial hip prosthesis	Coagulase-negative staphylococci, *β*-hemolytic streptococci, enterococci, *Proteus mirabilis, Bacterioides* species, *Staphylococcus aureus,* viridans *Streptococcus, Escherichia coli, Pseudomonas aeruginosa*
Artificial voice prosthesis	*Candida albicans, Streptococcus mitis, Streptococcus salivarius, Rothia dentrocariosa, Candida tropicalis, Streptococcus sobrinus, Staphylococcus epidermidis, Stomatococcus mucilaginous*
Intrauterine device	*Staphylococcus epidermidis, Corynebacterium* species, *Staphylococcus aureus, Micrococcus* species, *Lactobacillus plantarum*, group B streptococci, *Enterococcus* species, *Candida albicans*

3. METHODOLOGY AND RESEARCH DESIGN

3.1 Experimental location

All the experiments was conducted between February 2021 and May 2021 in the Microbiology Lab in the Salahaddin University-Erbil, College of Education, and Department of Biology.

3.2 Equipment's and apparatus

The equipment's, instruments and their manufacturers lists performed in this research are shortened in Table (3.1).

Table 3.1 Equipment and apparatuses, company, and origin.

No.	Apparatuses	Company	Source
1.	Autoclave	Memmert	Germany
2.	Benchtop UV-Transilluminator	BioTech	USA
3.	Bunsen Burner	WLD-TEC	England
4.	Centrifuge	Eppendorf	Germania
5.	Cooled Centrifuge	Sigma-Aldrich	USA
6.	ELISA EL-800 Reader	BioTech	USA
7.	Electrophoresis system	BioTech	USA
8.	Hood	BioTech	USA
9.	Hot plate	Memmert	Germania
10.	Incubator	Gallenkamp	UK
11.	Magnetic Stirrer	Memmert	Germania
12.	Microfuge	Eppendorf	Germania
13.	Micropipettes	Eppendorf	Germania
14.	Microwave oven	LG	Korea
15.	NanoDrop Spectrophotometer	Thermo	China
16.	Refrigerator	LG	Korea
17.	Sensitive Balance	Sartorius	Germania
18.	Thermal cycler PCR	ALPHA	UK
19.	Vortex	Fisher Scientific	USA
20.	Water bath	GFL	Germania

3.3 Chemical materials and bacterial cultural media

3.3.1 Chemical materials

The chemical materials, bacterial cultural media and their reported supplier used in the studies are provided in Table (3.2).

Table 3.2 Chemical materials, bacterial cultural media, company, and origin.

No.	Chemicals and cultural media	Company	Origin
A.	**Chemicals**		
1.	Absolute alcohol	Sharlau	Spanish
2.	Agarose	GeNet Bio	Korea
3.	Crystal Violet	BDH	England
4.	EDTA	BDH	England
5.	Egg Yolk Emulsion	Merk KGaA	Germany
6.	Glacial acetic acid	Sharlau	Spanish
7.	Hydrogen peroxide	Sharlau	Spanish
8.	Methyl green	BDH	England
9.	Peptone	Oxoid	UK
10.	Sodium chloride (NaCl)	Sharlau	Spanish
11.	Sodium hydroxide (NaOH)	Sharlau	Spanish
12.	Tryptone	Oxoid	UK
13.	Urea solution	LAB M	UK
14.	Yeast extract	Oxoid	UK
B.	**Bacterial cultural media**		
15.	Nutrient agar	Oxoid	England
16.	Nutrient broth	Oxoid	England
17.	Blood agar base	Oxoid	England
18.	Urea agar base	Oxoid	England
19.	DNase agar	Oxoid	England
20.	Mannitol salt agar	Oxoid	England

3.3.2 Bacterial culture media preparation

3.3.2.1 Blood agar

Blood agar base (BAB) was prepared as manufacturer directions, after autoclaving, and cooling blood agar base medium to 50–55˚C, and then aseptically 5-

7% of blood O^+ was added (Atlas and Synder, 2014). This medium has been used for the detection of hemolysis activities.

3.3.2.3 Luria Bertani broth

The Luria Bertani (LB) broth was produced through soaking 10.0 g tryptone, 5.0 g yeast extract, and 10.0 g sodium chloride in 950 mL sterilized distilled water; the pH was adapted to close 7 with 1.0 N sodium hydroxide and the volume brought to one liter, then autoclaved typically for 20 min. at 121°C (15 Ib/inch^2) and was used for activation of old culture and colonies that used for extraction of genomic DNA (Mary et al., 2009).

3.3.2.4 Urea agar base

In 95.0 mL of ddH_2O, 2.4 g of urea agar base was suspended and sterilized at 115°C for 20 minutes through autoclaving. Leave to decline the temperature to 50°C and add 5.0 mL of sterile 40% urea solution. Ten milliliter amounts were distributed into clean containers and let to establish in the slope situation. This medium was used to determine the capacity of *S. aureus* of hydrolyzing urea from ammonia as a by-product, thus turning the medium alkaline (Atlas and Synder, 2014).

3.3.2.5 DNase agar with methylene green

Forty-two grams of DNase agar was suspended in one liter of ddH_2O, then add 0.05 g of methylene green, and it was heated to melt the contents. It was sterilized for 15 min through autoclaving at a temperature of 121°C around 15 Ib/inch^2. Mix well and pour onto clean plates (Bamigboye et al., 2018).

3.3.2.6 Mannitol salt agar

One liter of deionized water was be used to dissolve 111.0 g of agar powder. Make a thorough mix. For 1 min, constantly stirring, bring the mixture to a boil till totally melted. Put it in the autoclave for 15 min at 121°C for sterilization.

3.3.2.7 Gelatin medium

This agar was used to detect the capacity of a microorganism to form the extracellular proteolytic enzymes-gelatinases which hydrolyze gelatin; it was prepared by suspending 65 g in 1000 mL of preheated water. Brought to warm to completely dissolve the medium. At the pressure of 15 lbs (121 °C), sterilize for 15 minutes by autoclaving. Then mixed well and poured into petri dishes (Alfred and Heidi, 2015).

3.4 Antimicrobial disks

The antibacterial susceptibility test has been carried out toward the ten antibiotics employing the disk diffusion technique, in dependence of the guidelines of Clinical and Laboratory Standards Institute (CLSI) references (CLSI, 2020), as demonstrated in Table (3.3).

Table 3.3 Antibiotic disks used for antimicrobial susceptibilities in this research.

No.	Antimicrobial Name	Symbol	Disk potency (µg)	Company
1.	Amikacin	AK	30	Bioanalysis
2.	Azithromycin	AZM	15	Bioanalysis
3.	Cefotaxime	CTX	30	Bioanalysis
4.	Ciprofloxacin	CIP	5	Bioanalysis
5.	Gentamicin	CN	10	Bioanalysis
6.	Levofloxacin	LEV	5	Bioanalysis
7.	Norfloxacin	NOR	10	Bioanalysis
8.	Ofloxacin	OFX	5	Bioanalysis
9.	Penicillin	P	30	Bioanalysis
10.	Tobramycin	TOB	10	Bioanalysis

3.5 Valuation of biofilm formation by microtiter plate assay

The microtiter plate (MTP) test is most extensively performed and was measured as a typical evaluation for the purpose of measuring the production of a biofilm (Achek et al., 2020b). In the proposed investigation, we used the MTP technique to

check all strains for their potential to establish biofilms. Using brain heart infusion broth supported with 2% glucose and 2% sucrose, all isolates were cultivated nightly at 37°C in the presence of oxygen It was necessary to dilute the culture by one hundred percent in new medium before inoculating sterilized dry dipped polystyrene microtiter plates with 200 µL of the diluted suspension. The tissue culture plates were incubated without shaking for 48 hours at 37°C. Following incubation, the contents of every well was mildly detached through pressing the plates and wells was rinsed three times with 300 µL of PBS (pH 7.2) to eliminate 'planktonic' bacteria, dehydrated in an upstanding situation and stained for 45 min with 300 µL of 1% crystal violet solution. Following the staining, plates were washed three times with PBS buffer. The ELISA auto reader was used to assess the optical density (OD) of colored adherent bacteria at 570 nm (OD_{570nm}). In this study, the optical density (OD) results were interpreted as an indicator of bacteria adhering to surfaces and producing biofilms.

3.5.1 Classification of bacterial adherence

To fix the background staining of the microtiter plate, the mean OD values obtained for blank tests were deducted from the mean OD values obtained for each sample. The clinical isolates have been classified into four groups. Excel software has made the average OD values and standard deviations. In order to measure the data, we utilized categorization (Table 3.4) depending on the OD values that were accessed (Achek et al., 2020b).

Table 3.4 Classification of bacterial adherence by MTP.

No.	OD results	Biofilm status
1.	OD ≤ODc*	Non-biofilm producer
2.	ODc <OD ≤2×ODc	Weak biofilm producer
3.	2×ODc <OD≤4×ODc	Moderate biofilm producer
4.	4×ODc <OD	Strong biofilm producer

*: ODc: cutting optical density, OD: optical density.

3.6 Genomic DNA extraction by kit

3.6.1 Sample preparation

Genomic DNA was obtained from high purity cultures using the GeneAll® ExgeneTM for Clinic Cell SV mini kit, which was purchased from GeneAll® (Songpa-gu, Seoul, KOREA). Bacterial cells could be provided by developing the culture for 12 – 24 hrs. at 37°C with continuous agitation till the cells attain the log phase of their growth cycle. Bacterial cells collected for extraction could be used immediately or preserved at -20°C or -80°C for future usages in a variety of applications. Bacteria that are gram positive must be cured with lysozyme or lysostaphin to destroy their rigorous and layered cell wall. When dealing with pathogenic bacteria, excessive caution must be implemented.

Start preparing the enzyme contents: merely prior to actually using the enzyme, solubilize it with buffer GP. When the enzyme solution has been kept at -20°C, it must be diluted once more per aliquot and kept frozen.

1. Pellet cells (~ $2*10^9$ CFU/mL) in a 1.5 mL centrifuge tube through centrifugation for 1 minute at 14,500 rpm. Remove the supernatant.
2. Using 180 μL of the formulated enzyme solution (30 mg lysozyme/mL buffer GP), vigorously dissolve the pellet. Incubate at 37˚C for 30 minutes.
3. Add 20 μL of Proteinase K buffer (20 mg/mL) and 200 mL of Buffer BL to a mixing tube and thoroughly mix by vigorous vortexing or pipetting the mixture.
4. Initially, was incubated for 30 min at 56°C, followed by a second 30 min at 70°C.
5. Spin the tube down gently to eliminate any droplets that have collected from the inside of the lid.
6. Liquids that have accumulated from the inside of the lid's inner surface should be removed by carefully spinning the tube.
7. Completely transport all of the mixture to the SV column with attention, centrifuge for 1 minute at >8,000 rpm, and change the collection tube in favor of a fresh one.
8. Adding 600 μL of Buffer BW and centrifuge for 1 minute at >8,000 rpm before removing the collection tube and replacing it with a sterilized one.

9. Add 700 µL of Buffer TW on the surface. Centrifuge for 1 minute at a speed of >8,000 revolutions per minute. Remove the pass-through and re-insert the SV column into the collecting tube to complete the process.
10. Centrifuge for 1 minute at 14,500 rpm to remove any remaining wash buffer. Transfer the SV column into a new 1.5 ml tube and shake well.
11. Adding 200 µL of Buffer AE (TE buffer or sterile water) to the spin column. Incubate at room temperature for 1 minute. Centrifuge at 14,500 rpm for 1 minute.

3.6.2 Determination of DNA concentration

To determine the concentration and pureness of DNA, after calibrating the device and establishing the subsequent blank to zero absorption, the NanoDrop spectrophotometer (Scientific Research Center (SRC), SUE) was prepared to start taking the OD of a DNA sample and perform a DNA concentration and purity assessment using 1 µL of genome DNA. A ratio of 1.8 to 2.0 indicates the existence of pure DNA, whereas a proportion greater than 2.0 indicates the existence of RNA in the DNA sample. A protein-to-DNA proportion of less than 1.8 implies that there is protein in the DNA sample.

3.7 PCR protocol for confirming genetically *S. aureus* identity

For more confirmation of the identity of the isolates, these were chosen for molecular typing and for detecting each of 16S rRNA gene in *S. aureus* isolates.

3.7.1 Primers

Primers which described in Table (3.5), was used in the present study has been ordered from Macrogen (Korea), were prepared by adding the recommended volume in the datasheet of free nuclease water to produce 100 µM from lyophilized primers (stock solution). Then 10 µM concentration were prepared and used as a workable solution in Polymerase Chain Reaction (PCR) reaction. All primer aliquots were stored at –20°C.

Table 3.5 The designed oligonucleotide primers for genes amplification.

Gene name	The nucleotide sequence	Amplicon size (bps)	References
16S rRNA	F5'-CAC CTT CCG ATA CGG CTA CC-3' R5'-GTT GAC TGC CGG TGA CAA AC-3'	372	(Hamasalih and Abdulrahman, 2019)
*ica*A	F5'-ACA CTT GCT GGC GCA GTC AA-3' R5'- TCT GGA ACC AAC ATC CAA CA-3'	188	(Chen et al., 2020)
*ica*B	F5'-CCC AAC GCT AAA ATC ATC GC-3' R5'-ATT GGA GTT CGG AGT GAC TGC-3'	173	(Hamasalih and Abdulrahman, 2019)
*ica*C	F5'-CTT GGG TAT TTG CAC GCA TT-3' R5'-GCA ATA TCA TGC CGA CAC CT-3'	209	(Hamasalih and Abdulrahman, 2019)
*ica*D	F5'- ATG GTC AAG CCC AGA CAG AG -3' R5'-CGT GTT TTC AAC ATT TAA TGC AA -3'	198	(Chen et al., 2020)

3.7.2 Optimization of primer melting temperature

The estimated melting temperature (*Tm*) can be determined using the Equation (3.1A) for primers containing less than 25 nucleotides.

$$"\boldsymbol{Tm} = 4(\boldsymbol{G}+\boldsymbol{C}) + 2(\boldsymbol{A}+\boldsymbol{T}) \ldots\ldots\ldots \mathbf{3.1A}"$$

The estimated melting temperature (*Tm*) can be determined using the Equation (3.1B) for primers containing less than 25 nucleotides;

$$"\boldsymbol{Tm} = 64.9 + 41 \times (\boldsymbol{G}+\boldsymbol{C}-16.4)/2(\boldsymbol{A}+\mathbf{T}+\boldsymbol{G}+\boldsymbol{C}) \ldots\ldots\ldots \mathbf{3.1B}"$$

Wherever G, C, A, and T are the digit of nucleotides in the primer sequences.

3.7.3 Detection of 16S ribosomal RNA gene

Polymerase chain reaction was conducted to increase 16S rRNA gene. The purified genomic DNA, primers, and PCR master mix was melted at 4°C. PCR contents was established in the entire volume of 25 µL (0.2 mL sterile PCR tubes) include 12.5 µL of 2× Master mix, 1.5 µL (10 ng/µL) of each forward and reverse primers, 2 µL (50 ng/µL) of template DNA and the remain volume was

accomplished with free nuclease water, then spin down. Positive control (PC) (*S. aureus* ATCC 25923). PCR reaction tubes had been placed in the thermocycler machine, and DNA of 16S rRNA gene were amplified depending on the PCR program, which was mentioned in Table (3.10) and was set in the thermocycler. PCR run results were alienated on a 1.2% agarose gel, and the bands of amplicon products were visualized with Red safe dye.

3.7.4 Detection of biofilm related gene in *S. aureus*

All strains of bacterium *S. aureus* that were found to be dependent on the development of biofilm classification were evaluated with PCR for the presence of some biofilm-related genes, which involved four biofilm-related genes known as intracellular adhesion and abbreviated in (*ica*A, *ica*B, *ica*C, *ica*D) (Table 3.6).

Table 3.6 PCR Program used for amplifying the biofilm-associated genes in *Staphylococcus aureus*.

Genes	Stages						
	No. of cycles	Denaturation		Annealing		Extension	
		Temperature	Time	Temperature	Time	Temperature	Time
16S rRNA	35	95°C	30 sec	59°C	45 sec	72°C	60 sec
***ica*A**	30	94°C	30 sec	56°C	60 sec	72°C	45 sec
***ica*B**	40	95°C	30 sec	58°C	30 sec	72°C	45 sec
***ica*C**	40	95°C	30 sec	55°C	40 sec	72°C	45 sec
***ica*D**	30	94°C	30 sec	55°C	40 sec	72°C	45 sec

3.7.5 Agarose gel electrophoresis

It was essential to construct the agarose by dissolving 1.2 g of it in a 250 mL conical flask, which contained 100 mL of 1× TAE buffer swirled to mix well. The mixture melted in a microwave oven. Leaving it to mildly decrease the temperature and down to about 50–55°C and Red safe dye at 5 µL/100 mL of agarose gel was added. The proper comb inserted into the tray and the agarose poured slowly into the tank to a depth of about 1 cm, the gel allowed to get solidified at lab temperature. Carefully the comb taken out, and the gel places in the electrophoresis tank with the wells closest to the cathode end. The gel covered with 1× TAE running buffer

ensuring that the gel was just submerged. Desired DNA samples to be loaded and pipetting up 0.2 volumes of loading dye, then the sample and loading dye were mixed by filling and emptying the pipette a few times. The ready mixture loaded into a well. Gel tank forced to close, power supply turned on, and gel running at 5 Volts per centimeter of its length. Then, the voltage was increased to 75-100 volts, and the electrophoresis could proceed for a sufficient time. The improvement of the gel is regulated by comparing it to the marker loading dye. The gel running ceased when the bromophenol blue dye in the loading dye track 3/4 the length of the gel. The current of power supply turned off, On a UV transilluminator, DNA bands were captured on camera under UV illumination at (240-366 nm) wavelengths. A photo documentation camera captured the gel image.

3.8 Methods

3.8.1 Sterilization methods

All solutions, media, and chemical materials were sterilized through autoclaving for 15 min at 121°C (15 pounds/inch^2), and the oven was used to sterilize glassware at 180°C for two hrs., Bunsen burner was used to sterilize inoculating loops. On the other hand, the solutions, sugars, and other chemical materials affected by heating were sterilized by filtration (0.45 µm).

3.8.2 Specimen's collection

A total of 127 non-repetitive human samples individuals submitted to Erbil Hospitals were interviewed for this study, they were investigated in Erbil city during the period February 2021 till May 2021 and all specimens directly at same time transferred to the lab for cultivation.

3.8.3 Collection and clinical isolation of *S. aureus*

A total of 127 clinical samples were obtained from individuals for this cross-sectional investigation. Following immediate culture on nutrient agar for 24 hr. at 37°C, all samples were isolated using conventional microbiological methodologies

such as Gram stain, catalase and coagulase tests, as well as biological experiments and molecular approaches. A stock culture of the bacteria was maintained on the surface of BHI agar slants at 4°C as a starting point for even more investigative process.

3.8.4 Bacteriological methods

Suspected colonies of *S. aureus* were recognized employing conventional techniques; a small amount of bacterial suspension was distributed over a microscopic slide, and a uniform thickness was produced; the prepared smear was air-dried and then fixed using the benzene burner gas fire. The fixed smear was colored with Gram stain and investigated with an oil immersion lens using the light microscopy (Ali et al., 2018).

3.8.5 The biochemical tests

3.8.5.1 Catalase

The presence of catalase is demonstrated when substrate (H_2O_2) is added to a colony or loopful of bacteria. The presence of catalase is demonstrated by the formation of air bubble of free oxygen gas O_2, and the absence of bubble development indicates a negative catalase assessment (Arikan et al., 2021).

3.8.5.2 Urease

A loopful of a high purity culture of the exam species was inoculated onto the prepared medium; the surface of the agar slant has been cultured with the checked species as a result of the inoculation. Cap was did leave loosely on and the experiment tube was incubated for 18-24 hrs. at 35°C in room temperature with no added oxygen (Atlas and Synder, 2014).

3.8.5.3 Coagulase

Direct tube coagulase (DTC) testing was conducted in test tubes comprising 1.0 mL of 10 percent human plasma with anti - coagulant EDTA and blood samples

culture broth that had been pre-treated for 18 hrs. A 1:10 dilution was formulated by mixing ten drops (0.25 mL) of blood culture broth in 2.5 mL of 0.9 percent saline for an 18 hrs. incubation period. Four drops of a 1:10 dilution of the blood culture broth were introduced to 1 mL of plasma, and the tube was tilted to 90°C for one, two, and four hrs. to check for clot development. Negative plates have been left at room temperature nightly and then re-examined the next day. This process is required for some *S. aureus* isolates in order to produce a postponed clot that is quickly lysed at 37°C through the species' staphylokinase. It was then incubated for another 24 hrs. before being reexamined (Arikan et al., 2021).

3.8.5.4 DNase with methyl green

It was done by streaking only one colony of *S. aureus* on DNase agar, then incubated at 37ºC for 24 hrs. Two types of inoculation that can be done. DNase causes the secretion of methyl green, which transforms the medium translucent across the checked organism, if it is positive for DNase, whereas if there is no breakdown of DNA, the medium persists green, indicating that the experiment is negative for DNase (Arikan et al., 2021).

3.8.5.5 Mannitol fermentation

In order to isolate and count of *S. aureus* from clinical and nonclinical samples, Mannitol salt agar (MSA) is had been using as a selective agar. Its high salt concentration (7.5% sodium chloride concentration) allows *S. aureus* to grow selectively in MSA, allowing for the growth of other bacteria in the medium. S. aureus organisms have the ability to withstand high saline concentrations, which allows them to thrive on MSA media. MSA also contains the pH indicator phenol red, as well as the sugar mannitol, which is a carbohydrate. If an organism is capable of fermenting mannitol, an acidified natural consequence will be produced, which will cause the phenol red that has been incorporated into the agar to transform a yellow pigment. The bacteria *S. aureus* are capable of fermenting mannitol (Hansen, 2019).

3.8.5.6 Gelatin hydrolysis

The gelatin hydrolysis characteristics of microorganisms inoculated into tubes containing nutritional gelatin medium were investigated. The tubes were incubated at 37°C for 48 hrs. As negative controls, tubes that had not been inoculated were run alongside the inoculated tubes. After leaving the culture tube at 4°C overnight, the liquefaction of the culture media was noticed at the end of the incubation time (Alfred and Heidi, 2015).

3.8.6 Storage and maintenance of *S. aureus* isolates

A single colony of *S. aureus* isolates have been cultured on nutrient agar and incubated for 24 hrs. at 37°C, then 0.8 mL of LB broth was applied to each plate and the growth was collected, then moved to the Eppendorf tube containing fifteen percent of sterilized glycerol stored at –20°C (Vitko and Richardson, 2013).

3.8.7 Antimicrobial susceptibility patterns of *S. aureus*

3.8.7.1 Preparing and standardizing inoculum suspension

Antimicrobial sensitivity assessment process was performed out utilizing disk diffusion method depending on the CLSI (CLSI, 2017), against the 16 antibiotic agents. *S. aureus* inoculums were uniformly distributed across the surface of the test lawn to create a lawn of the bacteria under investigation (~10^8 CFU/mL) according to the 0.5 McFarland standard solution (Cavalieri et al., 2005).

3.8.7.2 Preparing for plate inoculation

Mueller Hinton agar plate heated up to room temperature to absorb any excess moisture into the medium, and so this step could be accelerated by placing the plates in the incubator with their lids ajar for 10-15 min. The appropriate depth of the MHA plate should be 4 mm. The suspension of the species has been vortexed to ensure that it is well mixed. Then the suspension was dipped into the new, clean cotton-tipped swab. The remaining liquid was withdrawn from the tube by pushing it on the side of it (Hudzicki, 2016).

3.8.7.3 Culturing of the *S. aureus* inoculum on the plate

Starting with the swab at the top of the MHA plate inoculate the surface. The whole plate was filled by streaking back and forth from edge to edge. The plate was take tums around 60° and the swabbing process was replicated, rotating another time and swabbing the whole surface a third time. This makes sure that the inoculum is equally distributed.

3.8.7.4 Applying the antimicrobial disks

The disks were applied that contain the antimicrobial agents within 15 minutes of inoculating the MHA plate, and the findings were evaluated in accordance with CLSI recommendations (CLSI, 2017). Typically, up to 5 disks on a 100 mm plate were applied. It was necessary to apply stressful pressure to every disc to achieve full and uniform connection with the agar.

4. RESULTS

4.1 Identification of *Staphylococcus aureus*

Based on morphological features, cultural characteristics, biochemical tests, and molecular approach, from 127 clinical specimens including urine (*n*=43), sputum (*n*=29), pus (*n*=14), burn (*n*=18), and wound (*n*=23), 75 isolates (59.05%) were recognized as *S. aureus*. These isolates were from samples were taken for this study, comprise 39 male patients (52%), and 36 female patients (48%), as mentioned in Figure (4.1). To support and confirm the identification of *S. aureus* isolates through a microbiological standard method, and all 75 isolated were recognized via cultural and biochemical tets, reidentified as *S. aureus*.

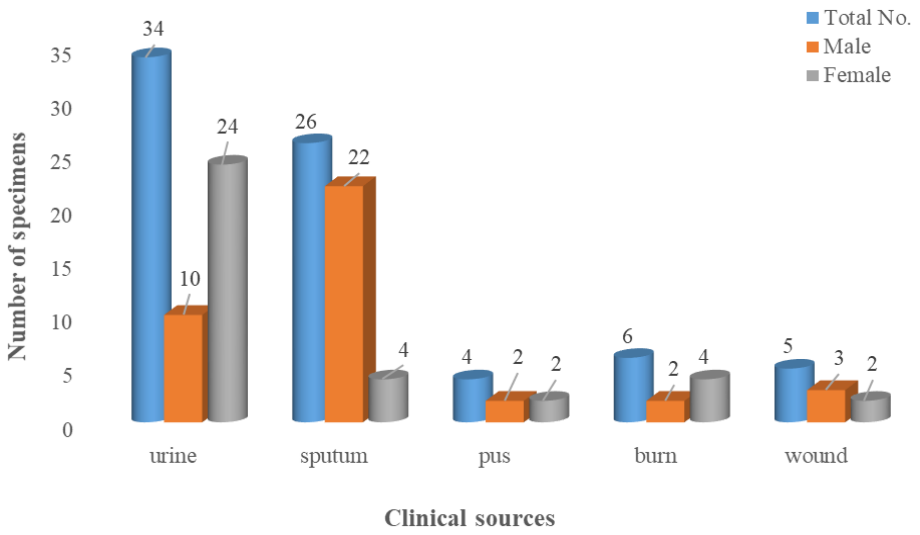


Figure 4.1 The sources distribution of *Staphylococcus aureus* isolates among patients

The Gram-positive identity of all 75 isolates of *S. aureus* was determined; further culture features and biochemical experiment findings are listed in Table (4.1). All *S. aureus* samples had been assessed for the occurrence of the specific 16S rRNA gene in order to describe and confirm the *S. aureus*. This was done to additionally validate the identification of the isolates. The presence of the 16S rRNA gene in all of the isolates confirmed that they were all *S. aureus*. All of investigated samples (*n*=75) tested positive for coagulase when tested with human plasma (HP).

Furthermore, screening for hydrogen peroxidase was conducted through testing all isolates to breaking down the H_2O_2 and producing bubbles, and was found that all isolates have the catalase enzyme which responsible for splitting H_2O_2 and bubble producing.

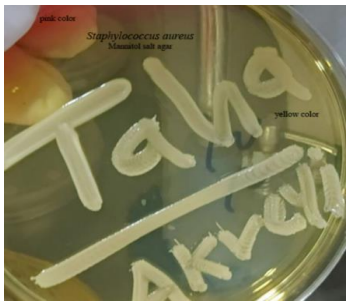
Table 4.1 The diagnosis characterization of *Staphylococcus aureus* strains is accomplished by the use of phenotypic, cultural, biochemical, and molecular investigations.

Biochemical tests	Results of tested isolates	
	Positive *n.* (%)	Negative *n.* (%)
Fermentation of mannitol sugar*	75 (100)	0 (0)
Tube coagulase*	75 (100)	0 (0)
Catalase	75 (100)	0 (0)
Gelatinase	75 (100)	0 (0)
Hemolysis	β Hemolysis 56 (74.66)	….
	α Hemolysis 2 (2.67)	….
	γ Hemolysis 17 (22.67)	….
DNase	75 (100)	0 (0)
Unique 16S ribosomal RNA gene	75 (100)	0 (0)

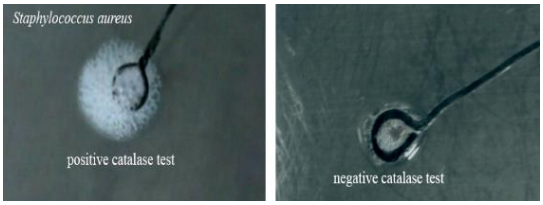
*: Mannitol fermentation: yellow colonies; HP: human plasma.

In the current study, the entire sample of *S. aureus* isolates demonstrated the potential to breakdown gelatin, confirming the existence of the gelatinase enzyme in the bacteria. Out of 75 strains of *S. aureus* were tested for hemolytic activity, 56 isolates of *S. au*reus (74.66%) displayed the capacity to produce β–hemolysin, two isolates (2.67%) expressed α–hemolysin, and 17 isolates (22.67%) exhibited γ–hemolytic function. Deoxyribonuclease (DNase) is a DNA-degrading enzyme. Numerous bacteria are capable of producing the DNase enzyme (e.g. *S. aureus*). This ability is illustrated by culturing bacteria on agar media containing DNA and a dye that changes color when the degraded DNA was presented. This research investigated the efficacy of DNase, one of the most often used phenotypic approaches for identifying *S. aureus* and all isolates became positive for the test.

According to the findings of both standard biochemical tests and the PCR approach, there is statistical confidence in each method at 100% (Figure 4.2).

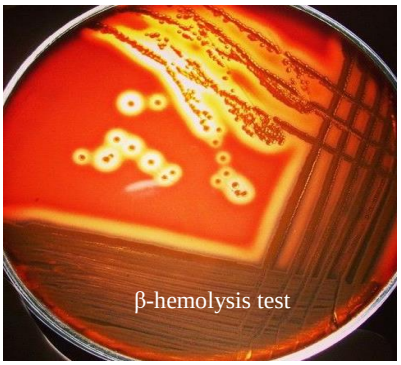


Mannitol fermentation on mannitol salt agar

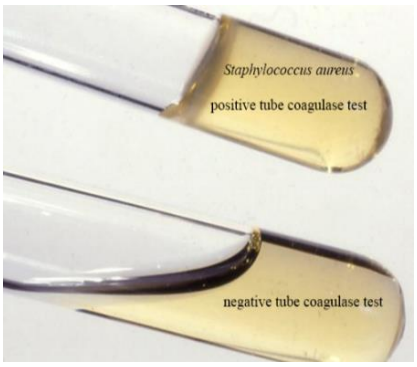


Catalase test

γ-hemolysis test

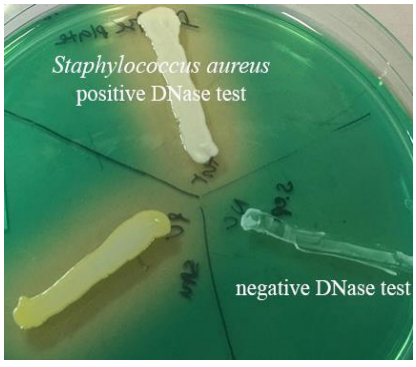


β-hemolysis test



Tube coagulase test

Gelatine liquification test



DNase test

Figure 4.2 Biochemical test results for conventional identification of *Staphylococcus aureus*.

4.2 Molecular identification of *Staphylococcus aureus*

Throughout this experiment, microbial genomic DNA was collected from whole cells employing the GeneAll® ExgeneTM for Clinic Cell SV small kit, which is available from GeneAll® (Songpa-gu, Seoul, KOREA). The quality of the extracted genomic DNA was checked using a 0.8 percent agarose gel to ensure that it was not contaminated with other DNA (Figure 4.3). A PCR assay was achieved on all *S. aureus* isolates to provide additional confirmation of their identities. Using the uniformly conserved 16S rRNA gene (Figure 4.4), particularly constructed primers were utilized to differentiate the isolates. All of the isolates tested positive for the 16S rRNA gene (Figure 4.4).

Figure 4.3 Image of gel electrophoresis of the extracted genomic DNA of *Staphylococcus aureus* isolates. Lane NG: negative control; lanes 1, 2, 3, 4, 5 and 6 represented the appearance of successfully extracted demonic DNA.

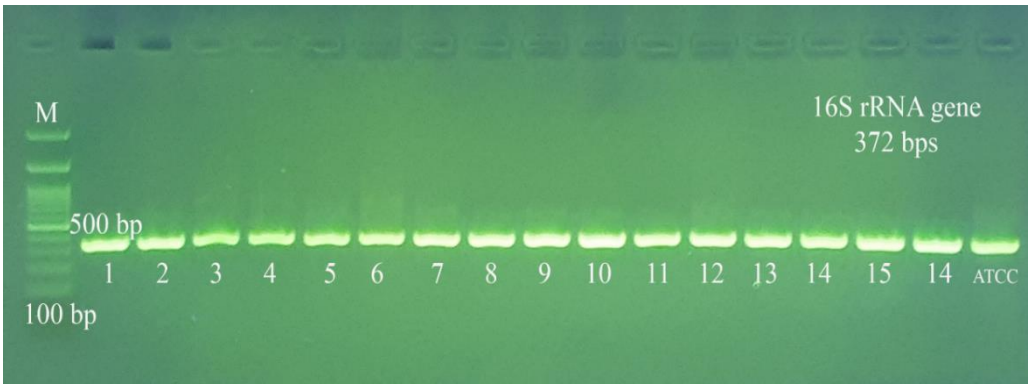


Figure 4.4 PCR amplifying byproducts for the 16S rRNA gene of *Staphylococcus aureus* were separated on an agarose gel. M: The DNA ladder (100 bp), lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and positive amplification of 372 bp for the 16S rRNA gene, using *Staphylococcus aureus* ATCC 25923 as positive amplification of 372 bp for the 16S rRNA gene.

4.3 Antibiotic resistance profiles of *Staphylococcus aureus* towards a variety of antibacterial compounds

All isolates showed significantly different resistance patterns to different antimicrobials. Antibiotic resistant profiles approved for ten antibacterial agents performing disk diffusion methods (Table 4.2). The highest resistant percent was 94.67% against P, followed by LEV (70.67%), CTX (68%), OFX (54.67%), AK (50.67%), CIP and NOR (48%), CN (24%), and TOB (13.33%), while the lowest resistant percent (2.67%) was recorded against each of AZM. On the other hand, some isolates of S. aureus revealed the intermediate resistant to 7 antibiotics, and among them, the AK, CTX, CIP, LEV, NOR, P, and TOB antibiotics.

Table 4.2 Antibacterial resistance profiles of *Staphylococcus aureus* isolates were investigated toward ten different antibacterial agents.

Antibacterial agents	Resistance *n.* (%)	Intermediate *n.* (%)	Sensitivity *n.* (%)
AK	38 (50.67)	4 (5.33)	33 (44.00)
AZM	2 (2.67)	0 (0.00)	73 (97.33)
CTX	51 (68.00)	14 (18.67)	10 (13.33)
CIP	36 (48.00)	2 (2.67)	37 (49.33)
CN	18 (24.00)	0 (0.00)	57 (76.00)
LEV	53 (70.67)	4 (5.33)	18 (24.00)
NOR	36 (48.00)	6 (8.00)	33 (44.00)
OFX	41 (54.67)	0 (0.00)	34 (45.33)
P	71 (94.67)	2 (2.67)	2 (2.67)
TOB	10 (13.33)	6 (8.00)	59 (78.67)

*: AK: amikacin, AZM: azithromycin, CTX: cefotaxime, CIP: ciprofloxacin, CN: gentamicin, LEV: levofloxacin, NOR: norfloxacin, OFX: ofloxacin, P: penicillin, and TOB: tobramycin.

The table (4.3) displays that the all 75 isolates of *S. aureus* in the present work were distributed in 9 groups as an antibiograms ranges and the first multi-resistant group was resisted to nine antimicrobials while the most sensitive group was did not

revealed any resistance to antimicrobial, and the other groups ranged from more resistant to lower resistant as group for 9, 8, 7, 6, 5, 4, 3, 2, and 1 antimicrobials, respectively; on the other hand, the resistant percentage against antimicrobials was ranged from 0.0 to 90 %.

Table 4.3 Antibiogram groups of *Staphylococcus aureus* isolates to different antimicrobial agents.

Antibiogram no.	No. of isolates	Resistant %	Antimicrobials									
			AK*	AZM	CTX	CIP	CN	LEV	NOR	OFX	P	TOB
1	28	90	R	S	R	R	R	R	R	R	R	R
2	8	80	R	S	I	R	R	R	R	R	R	R
3	2	60	R	S	I	R	R	R	I	R	R	I
4	2	50	I	S	I	R	R	R	I	R	R	I
5	2	40	I	R	I	I	R	R	I	R	I	I
6	11	30	S	S	R	S	S	R	S	S	R	S
7	12	20	S	S	R	S	S	S	S	S	R	S
8	8	10	S	S	S	S	S	S	S	S	R	S
9	2	0	S	S	S	S	S	S	S	S	S	S

*: AK: amikacin, AZM: azithromycin, CTX: cefotaxime, CIP: ciprofloxacin, CN: gentamicin, LEV: levofloxacin, NOR: norfloxacin, OFX: ofloxacin, P: penicillin, and TOB: tobramycin.

It has been demonstrated that antimicrobial resistance in *S. aureus* is regularly a consequence of the prevalent consumption of antimicrobials in agricultural production, hospitals, and the wider community. As a result, the research has definitionally revealed high levels of antimicrobial resistance between many pathogenic *S. aureus* isolates, making it essential for our health societies to instill excellent antimicrobial preservation practices. Because the single utilization of antimicrobials induces resistant microorganisms to establish and distributed through evolutionary pressure, healthcare organizations must also focus their medication on appropriate antibacterial sensitivity experiment findings and prevent unnecessary blind therapy as much as possible. Keeping the effectiveness of existing drugs intact and thus preventing the emergence of drug-resistant microbial organisms is critical to maintaining public health.

4.4 Detection of biofilm production by phenotypic methods

4.4.1 Biofilm formation through MTP assay

All of the biofilm-forming isolates were divided into four groups: non-producers (OD less than ODc), weak producers (ODc < OD < 2ODc), moderate producers (2ODc < OD < 4ODc), and strong producers (OD greater than 4ODc) (Achek et al., 2020a). In this study, after crystal violet staining, the OD average of microplate measurements after crystal violet staining varied widely from 0.216 to 0.827. The average of NC was 0.054. As of this writing, the ODc570 of biofilm development was 0.139. Researchers categorized them into four groups depend on their capacity to synthesis the biofilms: non–biofilm producers (-), $OD_{570} \leq 0.098$; weak biofilm producers (+), $OD_{570} \leq 0.196$; moderate biofilm producers (++), $0.196 < OD_{570} \leq 0.391$; and strong biofilm producers (+++), $OD_{570} \geq 0.391$. According to our findings, all *S. aureus* isolates tested positive for biofilms, with 5.34% of them being classified as a strong biofilms forming (*n*=4), 44.0% being classified as a moderate biofilms forming (*n*=33), and 50.66% being classified as a weak former of biofilms (*n*=38) (Table 4.4).

Table 4.4 MPM assessment test for evaluating *Staphylococcus aureus* isolates from biofilm formation.

The condition of biofilm development	Technique of MTP evaluation *n.* (%)
Strong	4 (5.34%)
Moderate	33 (44.00%)
Weak	38 (50.66%)
None	0 (0.00%)

4.4.2 Detect the presence of genes involved in the development of biofilms

The *ica*A, *ica*B, *ica*C, and *ica*D genes were detected using a PCR screening test. The main objective of this experiment is to genotypically characterize the biofilm-associated genes (Figure 4.5 to 4.8).

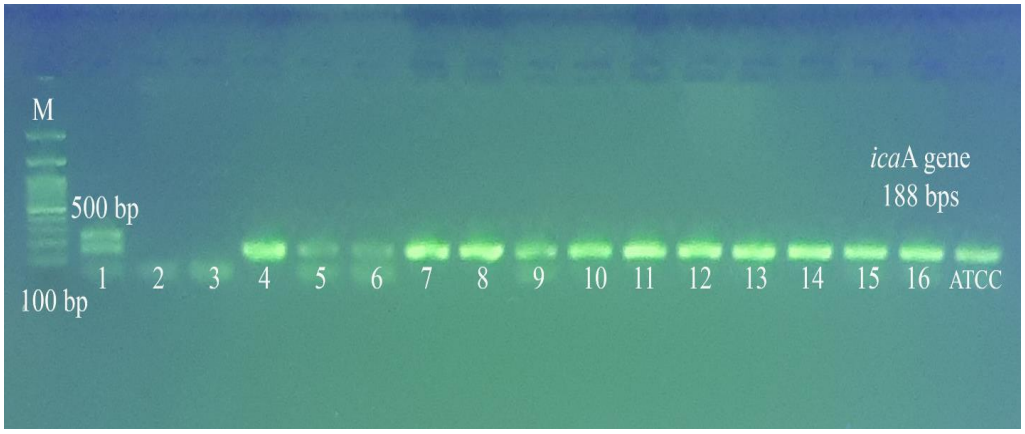


Figure 4.5 PCR detection of the *ica*A gene in *S. aureus* isolates. Lane M: DNA ladder (100 bp); lanes 1, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and ATCC 25923: *S. aureus ica*A (188 bp) positive isolates; lanes 2, and 3: negative for *ica*A gene.

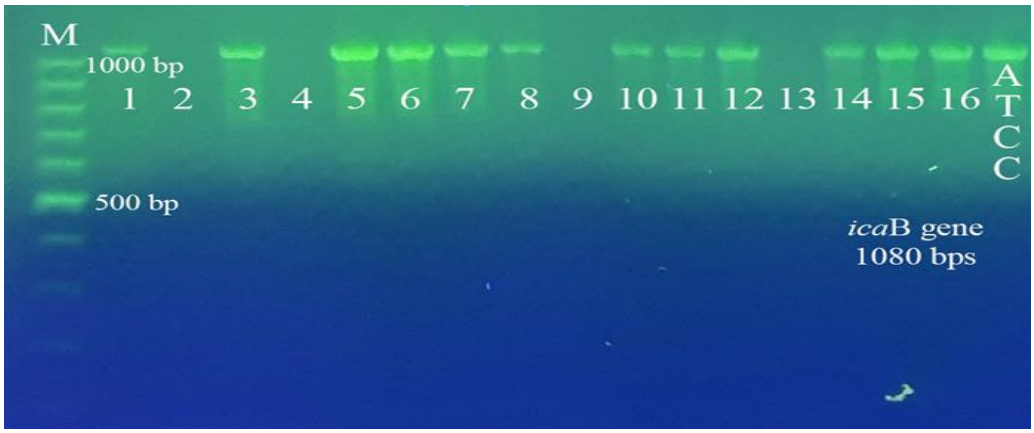


Figure 4.6 Agarose gel electrophoresis with PCR amplicons of the *ica*B gene. Lane M: DNA ladder (100 bp); lanes 1, 2, 5, 6, 7, 8, 10, 11, 12, 14, 15, 16 and ATCC 25923: *S. aureus ica*B (1080 bp) positive isolates; lanes 2, 4, 9, and 13: negative for *ica*B gene.

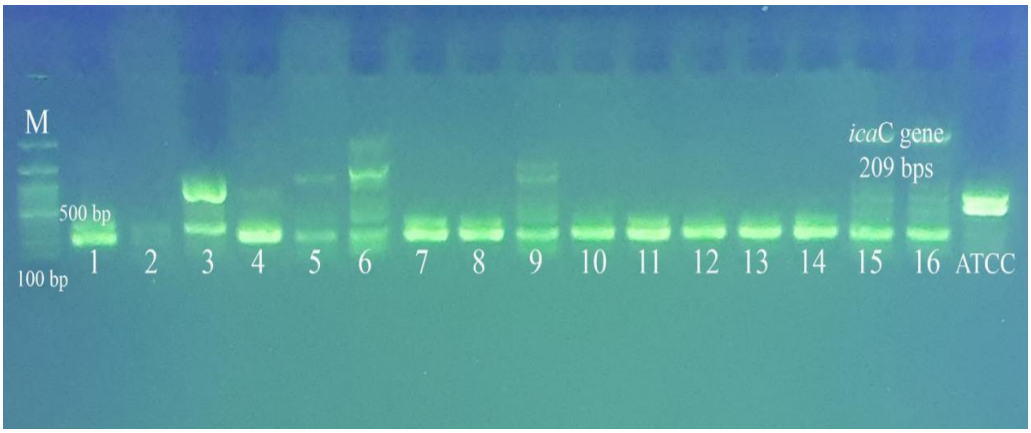


Figure 4.7 Representative gel image of molecular biofilm *ica*C gene by PCR among *S. aureus* isolates; lane M: 100 bp DNA ladder; lanes 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 positive for *ica*C (209 bp), lane 2 and ATCC 25923: negative for *ica*C.

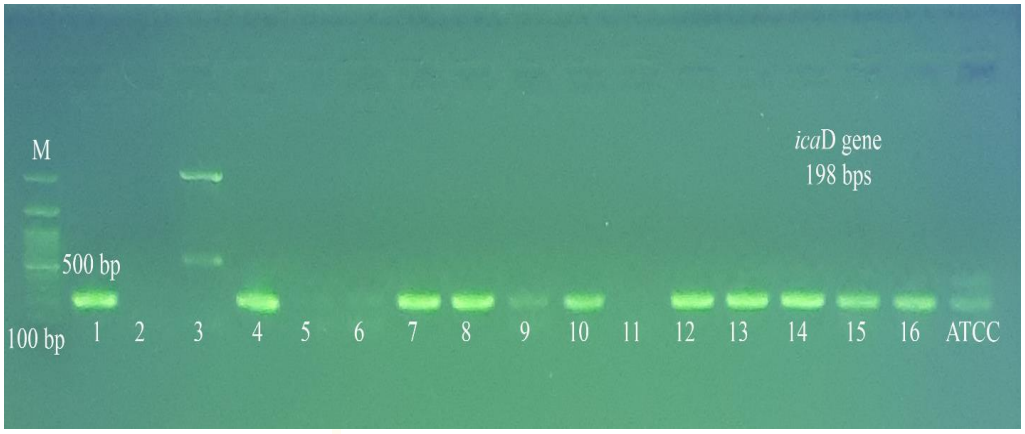


Figure 4.8 Electrophoresis in 1.2% agarose gel PCR amplicons achieved by using unique primers for the *ica*D gene in S. aureus clinical isolates. Lane M: DNA ladder (100 bp); lanes 1, 4, 7, 8, 9, 10, 12, 13, 14, 15, 16 and ATCC 25923: positive for *ica*D gene (198 bp); lanes 2, 3, 5, 6, and 11: negative for *ica*D gene.

The relationship among the biofilm development and the genes related with biofilm development was examined in order to determine its significance. A schematic representation of the dispersion of these genes in *S. aureus* isolates is shown in Table (4.5). Among all isolates, all four genes were found at varying levels of frequency, indicating that they were all present. As has been shown, the *ica*A and *ica*C genes were found to be present in the large percentage of *S. aureus* isolates (94.66 percent [*n*=71]) tested positive for the genes. The *ica*B and *ica*D genes were discovered to be present in 57.34 percent and 86.67 percent of the population, respectively, according to the findings. Fifty-five percent of the isolates that were biofilm formers were found to contain all of the biofilm-associated genes that were being evaluated in the present research.

Table 4.5 Correlations among biofilm-associated genes and the evaluation of biofilm synthesis by the MTP experiment in *Staphylococcus aureus* isolates were investigated.

Isolates	Phenotypic biofilm detection (MTP Assay)	Biofilm-associated genes			
		***ica*A**	***ica*B**	***ica*C**	***ica*D**
1	0.535 (+++)	+	+	+	+
2	0.301 (++)	-	-	-	-
3	0.247 (++)	-	+	+	-
4	0.212 (++)	+	-	+	+
5	0.169 (+)	+	+	+	-
6	0.309 (++)	+	+	+	-
7	0.163 (+)	+	+	+	+

8	0.172 (+)	+	+	+	+
9	0.165 (+)	+	-	+	+
10	0.158 (+)	+	+	+	+
11	0.183 (+)	+	+	+	-
12	0.19 (+)	+	+	+	+
13	0.223 (++)	+	+	+	+
14	0.161 (+)	+	+	+	+
15	0.159 (+)	+	+	+	+
16	0.23 (++)	+	-	+	+
17	0.227 (++)	+	-	-	+
18	0.239 (++)	+	+	+	+
19	0.237 (++)	+	+	+	+
20	0.207 (++)	+	-	+	+
21	0.383 (++)	+	-	+	+
22	0.204 (++)	+	+	+	+
23	0.321 (++)	+	+	+	+
24	0.161 (+)	+	+	+	+
25	0.155 (+)	+	-	+	+
26	0.166 (+)	+	+	+	+
27	0.231 (++)	+	-	+	+
28	0.196 (++)	+	-	+	+
29	0.378 (++)	+	+	+	+
30	0.204 (++)	+	-	+	+
31	0.185 (+)	+	-	+	+
32	0.441 (+++)	+	+	+	+
33	0.125 (+)	+	+	+	+
34	0.172 (+)	+	-	+	+
35	0.158 (+)	+	-	+	+
36	0.205 (++)	+	-	+	+
37	0.182 (+)	+	-	+	+
38	0.535 (+++)	+	+	+	+
39	0.301 (++)	-	-	-	-
40	0.247 (++)	-	+	+	-
41	0.212 (++)	+	-	+	+
42	0.169 (+)	+	+	+	-
43	0.309 (++)	+	+	+	-
44	0.163 (+)	+	+	+	+
45	0.172 (+)	+	+	+	+
46	0.165 (+)	+	-	+	+
47	0.158 (+)	+	+	+	+
48	0.183 (+)	+	+	+	-
49	0.19 (+)	+	+	+	+
50	0.223 (++)	+	+	+	+
51	0.161 (+)	+	+	+	+
52	0.159 (+)	+	+	+	+
53	0.23 (++)	+	-	+	+
54	0.227 (++)	+	-	-	+
55	0.239 (++)	+	+	+	+
56	0.237 (++)	+	+	+	+
57	0.207 (++)	+	-	+	+
58	0.383 (++)	+	-	+	+
59	0.204 (++)	+	+	+	+
60	0.321 (++)	+	+	+	+
61	0.161 (+)	+	+	+	+
62	0.155 (+)	+	-	+	+

63	0.166 (+)	+	+	+	+
64	0.231 (++)	+	-	+	+
65	0.196 (++)	+	-	+	+
66	0.378 (++)	+	+	+	+
67	0.204 (++)	+	-	+	+
68	0.185 (+)	+	-	+	+
69	0.441 (+++)	+	+	+	+
70	0.125 (+)	+	+	+	+
71	0.172 (+)	+	-	+	+
72	0.158 (+)	+	-	+	+
73	0.205 (++)	+	-	+	+
74	0.182 (+)	+	-	+	+
75	0.441 (+++)	+	+	+	+
ATCC	0.289 (++)	+	+	-	+
Positive	75	71	43	71	65
Percent	**100**	**94.66**	**57.34**	**94.66**	**86.67**

Our findings revealed that the *ica*A, *ica*B, *ica*C, and *ica*D genes perform a significant function in the synthesis of biofilms in the presence of bacteria. In the present research, we detected that all isolates form biofilm in the MTP experiment, with levels varying from weak (*n*=38) to moderate (*n*=33) to strong (*n*=4) biofilm formation.

5. DISCUSSION

5.1 Phenotypical identification of *Staphylococcus aureus* isolates

From 127 clinical specimens, including urine, sputum, pus, burn, and wound, 75 isolates (59.05%) were identified as *S. aureus*. These isolates were obtained from samples collected for this research which included 39 male patients (52%) and 36 female patients (48%), as illustrated in Figure (4.1). To disprove and confirm the identification of *S. aureus* isolates using a microbiological standard approach, and all 75 isolates were confirmed as *S. aureus* using cultural and biochemical tests. All 75 *S. aureus* isolates were identified as Gram-positive; other culture characteristics and biochemical assay results are given in Table (4.1). When all isolates of *S. aureus* were tested with human plasma, all of the studied samples (100%) proved positive for each of tube coagulase (HP), catalase and DNase tests. The current investigation indicated that all sample of *S. aureus* isolates was capable of degrading gelatin, demonstrating the presence of the gelatinase enzyme in the bacteria. To confirmation the *S. aureus* isolates (*n*=50) were tests to observing the presence of gram positive cocci, Hoseiny and Zaker (2019) isolate 50 clinical *S. aureus* strains with using *S. aureus* ATCC 25923 as the standard strain and was performed the catalase, coagulae, DNase examinations and growth on both mannitol salt agar for detection of mannitol fermentation and blood agar for hemolysis. They found that all 50 isoates (100%) positive for catalase, coagulae, and DNase tests, with the ability to ferment the mannitol with β-hemolysis on blood agar.

Out of 75 *S. aureus* isolates examined for hemolytic activity, 74.67% of isolates were found to be capable of producing β–hemolysin, 2.67% were found to express α–hemolysin, and 22.66% of isolates demonstrated γ–hemolytic action. Similar to findings of Karmakar et al. (2016), 40% of *S. aureus* isolates were able to generate hemolysin in the lysis area around their growing on blood agar media. Similarly, hemolytic ability was determined in 57 different clinical samples of *S. aureus* by Tang et al. (2013), with five bacterial isolates (8.77%) exhibiting no capacity to hydrolyze blood on blood agar and 52 distinct clinically strains (91.23%) of *S. aureus* exhibiting robust hemolytic activity. Our consequences approve with the findings of Jimenez et al. (2014), they collected 354 samples from different sources

and cultured directly on mannitol salt agar and blood agar to detect the hemolysis type and mannitol fermentation, in addition to detection catalase and tube coagulase tests. They stated that only 222 isolates (62.71%) showed β-hemolysis and amnnitol fermentation with positive results for both catalase and tube coagulase tests.

5.2 Molecular detection of *Staphylococcus aureus* isolates

A PCR assay was achieved on all *S. aureus* isolates to provide additional confirmation of their identities. Using the uniformly conserved 16S rRNA gene (Figure 4.4), particularly constructed primers were utilized to differentiate the isolates. All of the isolates tested positive for the 16S rRNA gene (Figure 4.4). In the study by Rocchetti et al. (2018), the PCR amplifying process was achieved on 371 strains, which comprised in the research of them, in the situation of the 85 isolates of *S. aureus* recognized through conventional microbiological tests, there was 100% conformity with the molecular identification outcomes, with amplification of the 16S rRNA gene. To confirm the identification of *S. aureus*, Javid et al. (2018) subjected 114 cultures to molecular classification by PCR using species-specific 16S rRNA gene amplification.

Thirty-nine (34.2%) isolates, which were identified as *S. aureus* performing microbiological standard experiments, shown the existence of the 16S rRNA gene by the amplification of 270 bp band of species-specific 16S rRNA gene. In the research of Al–Alak (2018) that conducted on 36 clinical samples for identification of *S. aureus* using 16S rRNA gene, and they found that all isolates (100%) carry the 16S rRNA gene at 228 bp bands, which all isolates identified previously based on the cultural and biochemical tests. Twenty-two isolates (6.21%) were identified among 354 samples depending on the convential tests by Jimenez et al. (2014), and these isolates that identified as *S. aureus*, undergo molecular test for confirm the identity using 16S rRNA gene and all strains revealed the 273 bp band specific for *S. aureus*. Likely, Yahya Ahmed et al. (2021) collected 400 samples from different sources and out of all isolates, 137 samples (34.25%) were confirmed as *S. aureus* using 16S rRNA housekeeping gene result which corresponds to 756 bp band size. Similarly, 46 isolates of methicillin-resistant *S. aureus* (MRSA) were collected by Tahir and

Hamim (2021) and identified through conventional PCR technique via the 16S rRNA gene amplification, which previously identified by standard microbiological techniques.

5.3 Antimicrobial susceptibility of *Staphylococcus aureus* isolates

All isolates showed significantly different resistance patterns to different antimicrobials. Antimicrobial resistant profiles was performed for ten antibacterial agents performing disk diffusion methods (Table 4.2). The highest resistant percent was 94.67% against P, followed by LEV (70.67%), CTX (68%), OFX (54.67%), AK (50.67%), CIP and NOR (48%), CN (24%), and TOB (13.33%), while the lowest resistant percent (2.67%) was recorded against each of AZM. Khoramrooz et al. (2017) reported that 26.44% of 121 *S. aureus* isolates resistant against each of tobramycin, kanamycin, amikacin, and gentamycin, 47.93%, 20.65%, and 3.3% of *S. aureus* isolates were resistant to tetracycline, doxycycline, and minocycline, respectively. Antibacterial resistance assessments are dependent on this evidence by Boada et al. (2018) among the 765 evaluated isolates of *S. aureus*, the high resistant amounts were detected for penicillin, and then measured for azithromycin, erythromycin, and clindamycin, with resistant ratios of 87.1%, 11.6%, 11.2%, and 9.7%, respectively. The basis of the resistance mechanism to oxacillin and methicillin is gene encoding a PBP2 homolog known as PBP2a which is resisting to antibiotic action. The reason for this is due to the active transpeptidase (TP) of the PBP2a site of serine that is not accessible to β–lactams. In this way, the enzymes can take over peptidoglycan biosynthesis if the housekeeping PBP2 TP site is inactivated. Despite fact, the activity of PBP2 transglycosylase (TG) is vital for biosynthesis of peptidoglycan because the moiety of PBP2a is not working. Thus, the biosynthesis of peptidoglycan is, therefore, a collaborative effort between the two proteins in the inactivation of the PBP2 TP site (Foster, 2017). Disc diffusion studies were performed by Jimenez et al. (2014) to detect the susceptibility of the isolates of *S. aureus* toward five diverse antibiotics, and revealed that all isolates resist to ampicillin and penicillin, and the remain isolates showed various range of resistant against all antibiotics.

An investigation were performed by Cavalcante et al. (2015) about antibacterial sensitivity profiles and different antimicrobials was used against 100 strains of *S. aureus* (90 from nares and 10 from skin lesions), and 24 were categorized as a MRSA. Ciprofloxacin, chloramphenicol, linezolid, rifampin, teicoplanin, tigecycline, and trimethoprim-sulfamethoxazole were all found to be effective against all of the isolates. Antibiotic resistant percent was discovered for the antibiotics erythromycin (40%), clindamycin (15%), tetracycline (12%), mupirocin (8%), and gentamicin (7%). Antibiotic susceptibility testing which carried out by Bhowmik et al. (2019) against *S. aureus* isolates, it was established that the microorganisms were less resist to linezolid (83.46%), after that revealed less resist toward minocycline (42.25%), clindamycin (38.58%), doxycycline (36.66%), tigecycline (25.35%) and tetracycline (23.94%), however erythromycin (0.042%) exhibited very low level of efficiency toward *S. aureus*. The data on the antimicrobial susceptibility of Aslantas and Demir (2016) were performed on 112 isolates of *S. aureus* and the various rates of resistance to penicillin (45.5%), ampicillin (39.3%), tetracycline (33%), erythromycin (26.8%), trimethoprim-sulfamethoxazole (5.4%), oxacillin (4.5%), enrofloxacin (0.9%), and amoxicillin-clavulanic acid (0.9%) were detected.

According to the antibiograms of Cavalcanti et al. (2019), the isolates of *S. aureus* revealed higher resistant percent toward penicillin (83.6%), after that, the isolates of *S. aureus* has been shown the resistant percent to cefoxitin (26.2%), ciprofloxacin (24.6%) and amoxicillin-clavulanate (13.1%). All isolates were susceptible to gentamicin and sulfamethoxazole-trimethoprim. Kates et al. (2018) were conducted antibacterial sensitivity screening on all isolates positive for *S. aureus* (58/621, or 9.3%), and they discovered resistance to the majority of the antimicrobials evaluated. Resistance to erythromycin was found to be prevalent in 51.7 % (*n*=37) of the population, accompanied by oxacillin at 43.1% (*n*=25) and levofloxacin at 41.4% (*n*=24). The prevalence of clindamycin resistance was found to be 22.4% (*n*=16). Neither isolates were found to be resistant to vancomycin, daptomycin, or quinupristin/dalfopristin, among other antibiotics. Twenty-six (44.8%) of the isolates encountered the description of MDR, possessing acquired non-susceptibility to at least one agent in three or more antibacterial subgroups as a result of their acquisition of non-susceptibility. (Magiorakos et al., 2012). Our results

were supported by data that recorded by Bai et al. (2021), which they found that penicillin-resistant rate was 100%. No resistance to teicoplanin, linezolid, and vancomycin. The percentages of antibacterial resistance to ciprofloxacin, trimethoprim/sulfamethoxazole, gentamicin, levofloxacin, clindamycin, erythromycin, rifampin, and tetracycline were 14.8, 11.9, 12.5, 13.2, 76.6, 77.7, 5.9, and 32.7%, respectively. Likely, in the antimicrobial sensitivity screening findings, Wang et al. (2020) were discovered that the majority of *S. aureus* isolates were susceptible to linezolid, rifampin, and gentamicin, and therefore resistant to penicillin (92.45%), erythromycin (49.95%), clindamycin (45.28%), and tetracycline (32.08%). A total of 18 isolates of *S. aureus* were found to be multidrug resistant. Particularly concerning was the fact that 38.89% of MDR *S. aureus* strains were resistant to erythromycin, clindamycin, and chloramphenicol. Antibiotic susceptibility was performed by Wolters et al. (2020) on 28 isolates of *S. aureus* taken from numerous clinical specimens, and the highest rates of routine antibacterial resistant rates were detected for penicillin (100%, 28/28), tetracycline (57%, 16/28) and trimethoprim/sulfamethoxazole (39%, 11/28).

It has been demonstrated that antimicrobial resistance in *S. aureus* is regularly a consequence of the prevalent consumption of antimicrobials in agricultural production, hospitals, and the wider community. As a result, the research has definitionally revealed high levels of antimicrobial resistance between many pathogenic *S. aureus* isolates, making it essential for our health societies to instill excellent antimicrobial preservation practices. Because the single utilization of antimicrobials induces resistant microorganisms to establish and distributed through evolutionary pressure, healthcare organizations must also focus their medication on appropriate antibacterial sensitivity experiment findings and prevent unnecessary blind therapy as much as possible. Keeping the effectiveness of existing drugs intact and thus preventing the emergence of drug-resistant microbial organisms is critical to maintaining public health (Ugwu et al., 2015). Antibiotic resistance is frequently gained through horizontal gene transfer (HGT) from external sources, while Deurenberg and Stobberingh (2009) have also documented chromosomal mutations and antibiotic distribution. *S. aureus* established methicillin resistance by gaining the *mecA ge*ne which codes for PBP2a with lower affinity to bind β-lactams. Patients

who could not tolerate vancomycin became cured with minocycline, trimethoprim-sulfamethoxazole, clindamycin, and fluoroquinolones (the commonly utilized form of quinolone antimicrobials). Quinolone antibiotics block other bacterial topoisomerase enzymes, including DNA gyrase (bacterial topoisomerase II) and topoisomerase IV (Hashem et al., 2013). Quinolones work by connecting to DNA-gyrase and topoisomerase IV complexes. The quinolones cause a conformation switch in the enzyme immediately after binding.

5.4 Detection of biofilm production among *Staphylococcus aureus* isolates

5.4.1 Biofilm formation assessment by MTP assay

Achek et al. (2020a) categorized them into four groups depend on their capacity to synthesis the biofilms: non–biofilm producers (-), $OD_{570} \leq 0.098$; weak biofilm producers (+), $OD_{570} \leq 0.196$; moderate biofilm producers (++), $0.196 < OD_{570} \leq 0.391$; and strong biofilm producers (+++), $OD_{570} \geq 0.391$. According to our findings and classification of biofilm status by Achek et al. (2020a), all *S. aureus* isolates tested positive for biofilms, with 5.34% of them being classified as a strong biofilms forming (*n*=4), 44.0% being classified as a moderate biofilms forming (*n*=33), and 50.66% being classified as a weak former of biofilms (*n*=38) (Table 4.4).

All strains studied of *S. aureus* were evaluated for their morphologic development of biofilm by Serray et al. (2016) using the microtiter plate test, and all of the strains were biofilm positive; 21 isolates (39.62%) were strong, 20 isolates (37.74%) were moderate, and 12 isolates (22.64%) were low biofilm formation within 24 hrs. The biofilm-producing capability of 112 isolates of *S. aureus* categorized into four groups depend on their capacity to produce biofilms as mentioned by Kim et al. (2016): weak ($OD \leq 0.3$), moderate ($0.3 < OD \leq 0.6$), strong ($0.6 < OD \leq 0.9$), and very strong ($OD > 0.9$). Most strains of *S. aureus* (93/112, 83.03%) have been categorized as strong or moderate in their capacity to establish biofilms. On the other hand, Achek et al. (2020a) were found that 23 isolates (41.88%) among of 55 isolates of *S. aureus* were establish to be biofilm-producing. Achek et al. (2020b) were collected the 39 clinical samples and perform a tissue culture plate to detect the phenotypic formation of biofilm and found that 10.5%

were strong biofilm former, 5.3% moderate, 36.8% weak, and 52.6% non-biofilm former.

5.4.2 Biofilm associated genes detection in *Staphylococcus aureus*

The relationship among the biofilm synthesis and the genes related with biofilm development was examined in order to determine its significance among them. Our findings of the dispersion of these genes in *S. aureus* isolates is shown in Table (4.5). Among all isolates, all four genes were found at varying levels of frequency, indicating that they were all present. As has been shown, the *ica*A and *ica*C genes were found to be present in the large percentage of *S. aureus* isolates (94.66% [*n*=71]) tested positive for the genes. The *ica*B and *ica*D genes were discovered to be present in 57.34% and 86.67% of the population, respectively, according to the findings. Fifty-five percent of the isolates that were biofilm formers were found to contain all of the biofilm-associated genes that were being evaluated in the present research. The present outcomes were confirmed by Achek et al. (2020a), which detected the *ica*ACDB genes that responsible for biofilm and slime formation, and found that all isolates harbored icaACD genes. The PCR technique was applied by Serray et al. (2016) to the 53 MRSA strains. The *ica*D gene was revealed in 100% of both high-virulence strains and low-virulence strains. All the strains produced slime, so in all MRSA isolates, there was a relation among biofilm development and the existence of the *ica*D gene. Ghasemian et al. (2016) found that the occurrence of the biofilm-related genes *ica*A, *ica*D, *ica*B, and *ica*C genes in MSSA isolates was 71%, 54%, 69%, and 71%, respectively. While in the MRSA isolates, the rate of recurrence of these genes was 76%, 69%, 64%, and 74%, respectively.

Out of 112 *S. aureus* isolates for detection of biofilm synthesis and biofilm-associated genes, Aslantas and Demir (2016) were recorded the near consequences of our obtained results, who found that 112 isolates assessed for biofilm formation by CRA method, and they found that 43 (38.4%) isolates form biofilm in strong status, moderate 36 (32.1%), and non-biofilm former was 33 (29.5%), while using MTP assay, 75 (67%) isolates were strong 27 (24.1%), moderate 48 (42.9%), weak 0 (0%), and non-biofilm former was 37 (37%). Both *icaA* and *icaD* were detected in 97

(86.6%) isolates, and *bap* was detected in 15 isolates (13.4%). From 64 isolates of *S. aureus*, 26 (40.6%) isolates were harbor the three biofilm-associated genes were examined (*ica*A, *ica*D, and *bap*), and the *ica*A gene alone was discovered in 85.9% and *ica*D in 84.3% of isolates (Budri et al., 2015).

The development of biofilms is caused by a variety of genes and environmental factors. The *ica*A, D, B, and C operons, which generate PIA, were found to be the primary process for biofilm development in almost all of the research published. A second process for biofilm establishment in *S. aureus* has been discovered that is autonomous of the ica operon. *ica*A, *ica*D, *ica*B, and *ica*C are the four ica operon biogenesis genes, and *ica*R is a transcribed repressor gene (Bimanand et al., 2018). A research project discovered that a mutation in the ica genes of *S. aureus* resulted in decreased biofilm growth and PIA production (Nathan et al., 2011). A further process of ica-independent biofilm development is facilitated by the biofilm related protein (Bap), and this process is susceptible to the proteinase k inhibitor (Cucarella et al., 2004). Our findings revealed that the *ica*A, *ica*B, *ica*C, and *ica*D genes perform a significant function in the synthesis of biofilms in the presence of the bacterium *S. aureus*. In the present research, we detected that all isolates form biofilm in the MTP experiment, with levels varying from weak (*n*=38) to moderate (*n*=33) to strong (*n*=4) biofilm formation. Comparable to those reported elsewhere, with 50% of clinical *S. aureus* isolates forming a biofilm, which is comparable to the current findings (Kouidhi et al., 2010). A biofilm was formed in 60% of the *S. aureus* isolates tested using the CRA method, and PCR recognition of biofilm-associated genes, *ica*A and *ica*D, demonstrated that both genes were prevalent in 78% of the isolates tested using the CRA method. (Salehzadeh et al., 2016).

6. CONCLUSIONS, RECOMMENDATIONS AND FUTURE WORKS

1. CONCLUSIONS

Though our interpretation of Staphylococcus aureus contact with humans has vastly continued to improve until the bacteria's revelation, there are nevertheless many unanswered questions concerning the molecular determinants associated. The bacteria *S. aureus*, despite this, continues to be an effective colonizer and a significant human pathogen. The process of *S. aureus* colonization and/or infection of the host is respectively complex and multifactorial. These findings describe the responsibility of *S. aureus* cell wall supported proteins in securing the bacteria's adhesion and/or immune evasion, as demonstrated in this research paper.

A. High prevalence of infections with *S. aureus* is observed among clinical specimens taken from patients.
B. High prevalence of isolates of *S. aureus* were formed biofilm, which detected phenotypically and genotypically.

2. RECOMMENDATIONS AND FUTURE WORKS

A. Focus more on bacterial biofilm-related studies to avoid the spread of biofilm-related genes among all bacterial isolates responsible for human infections.
B. Distance from more using of antibiotics and discover a natural material to reducing the biofilm-forming among bacterial isolates.

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Ministry of Higher Education and Scientific Research
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وەزارەتی خوێندنی باڵا و توێژینەوەی زانستی
زانکۆی سەلاحەددین-هەولێر
کۆلێژی پەروەرد
بەشی بایۆلۆجی

No.: 141
Date: 23-06-2021

Approval of Research Ethical Committee

Herewith we declare that Ethical Committee-Scientific Research Office in Research Center of Salahaddin University-Erbil has processed the submitted research proposal of (MSc. Student **TAHA TALAL ABDULRAHMAN**) master student in (Near East University). His research entitled (**BIOFILM ASSESSMENT OF *Staphylococcus aureus* FROM DIFFERENT CLINICAL SPECIMENS**) approved to be conducted in the setting of our research center.

ASST. PROF. DR. KAZHAL M. SULAIMAN
Head of Biology Department
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PROF. DR. HIKMAT M. ALI
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