TURKISH REPUBLIC OF NORTH CYPRUS
NEAR EAST UNIVERSITY
HEALTH SCIENCE INSTITUTE

INVESTIGATION OF IN VITRO BIOFILM FORMATION AND CORRELATION WITH ANTIBIOTIC RESISTANCE PATTERN AMONG CLINICAL ISOLATES OF *STAPHYLOCOCCUS AUREUS*

AHMED HASSAN IBRAHIM

MASTER OF SCIENCE THESIS

MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY PROGRAM

ADVISOR

EŞREF ÇELIK MD ASSISTANT PROFESSOR

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APPROVAL

The Directorate of Health Sciences Institute, / INSTITUTE OF GRADUATE STUDIES

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Hereby, I declare that this thesis study is my study, I had no unethical behaviors in all stages from the planning of the thesis until writing there, 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 breaching patent rights and copyright infringement during the study and writing of this thesis

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Signature:

Date:

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ABBREVIATIONS AND SYMBOLS

AIP	Autoinducing peptide
AST	Antibiotic sensitivity Testing
AGR	Accessory Gene Regulator
CWA	Cell Wall-anchored
CRA	Congo Red Agar
CDC	Centre for Disease Control
CV	Crystal violet
eDNA	Extracellular Deoxyribonucleic Acid
ECM	Extracellular matrix
EPS	Extracellular Polysaccharides
ID	Identification
LTA	Lipoteichoic Acid
MSSA	Methicillin Sensitive *Staphylococcus aureus*
MRSA	Methicillin Resistant *Staphylococcus aureus*
OD	Optical Density
PCR	polymerase chain Reaction
PIA	polysaccharide intracellular adhesion
PSM	phenol soluble modulins
RNA	Ribonucleic acid
SAB	*S. aureus* bacteremia
SSTIs	Skin and Soft Tissue Infections
TCS	Two component systems
VISA	Vancomycin-Intermediate *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WTA	Wall teichoic Acid

ÖZET

Ahmed Hassan Ibrahim: *Staphylococcus aureus* Klinik İzolatları Arasında Invıtro Bıyofilm Oluşumunun Araştırılması ve Antibiotik Direnç Paterniyle Korelasyonunun Araştırılması

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Çalışmamızın amacı:*S. aureus* izolatlarının fenotipik Biyofilm oluşturma yeteneklerini ortaya çıkarmak ve Biyofilm oluşturan *S. aureus'un* antibiyotik direncinin, Biyofilm oluşumu ile ilişkisini araştırmaktır. Çalışmamız, Kuzey Kıbrıs Türk Cumhuriyeti'nde (KKTC) yakın doğu üniversite hastanesinin mikrobiyoloji laboratuvarında gerçekleştirildi. Çalışma için (Ocak 2020 ve Nisan 2021) arasında çeşitli hastane bölümlerinden yatan hasta ve ayaktan hasta örneklerinden toplam 67 örnek (Yara / İrin, Balgam, Aspirat, Kan ve İdrar) toplandı. Bakteriyel tanımlama ve Antibiyotik duyarlılık testi için Vitek 2 sistemi kullanıldı, Biyofilm oluşumu Kongo Kırmızı Agar ile değerlendirildi. Toplam 67 *S. aureus* izole edildi, bunların 38'i (% 56,7) MRSA ve 29'u (% 43,3) MSSA; Bunlardan 35'i erkek, 32'si kadındır, KKA yöntemiyle biyofilm oluşumu 56 (% 84,3), bunlardan 35'i (% 92,2) MRSA idi. Bunun aksine, 21'i (% 72.4) MSSA idi ve bunlardan en yüksek geri kazanılmış örnek Yara / irin 28'di (% 41.8). İzolatların daha yüksek bir oranı, *S. aureus'*un Tigesiklin'e (% 100) ve Gentamisine (% 100) duyarlı olduğunu göstermiştir. Ayrıca, biyofilm üretmeyen suşlara kıyasla biyofilm üreten suşlar arasında en yüksek çoklu ilaç direnci ve metisilin direnci oranları bulunmuştur. Bu çalışmanın bulguları, metisiline dirençli suşların daha fazla biyofilm ürettiğini ve neredeyse tüm antibiyotik sınıflarına yüksek derecede direnç gösterdiğini göstermektedir.

Anahtar Kelimeler: Metisiline dirençli *Staphylococcus aureus*, Biyofilm, Kongo Kırmızı Agar

ABSTRACT

Ahmed Hassan Ibrahim: Investigation of Invitro Biofilm Formation and Correlation with Antibiotic Resistance Pattern Among Clinical Isolates of *Staphylococcus aureus*

Advisor: Eşref Çelik, MD Assistant Professor

Near East University, Institute of Graduate Studies, Medical Microbiology and Clinical Microbiology Program, Master Thesis, Nicosia, 2021

The aim of our study is to reveal the phenotypic Biofilm forming abilities of *S. aureus* isolates and to investigate the relationship of antibiotic resistance of Biofilm forming *S. aureus* with Biofilm formation. The current study was carried out in the microbiology laboratory at the near east university hospital in the Turkish Republic of Northern Cyprus (TRNC). A total of 67 samples (Wound/pus, Sputum, Aspirate, Blood and Urine) for the study was collected Between (January 2020 and April 2021) from samples of inpatients and outpatients from various hospital departments. Vitek 2 system was used for bacterial identification and Antibiotic susceptibility testing, Biofilm formation was evaluated using CRA (Congo Red Agar). A total of 67 *S. aureus isolates* were isolated, of which 38(56.7%) were identified to be MRSA and 29(43.3%) MSSA; of these, 35 were male, and 32 were female, biofilm formation by CRA method was found to be 56(84.3%) of which 35(92.2%) were MRSA. In contrast, 21(72.4%) was MSSA, of which the highest sample recovered was Wound/pus 28(41.8%). A higher proportion of isolates showed susceptibility of *S. aureus* toward Tigecycline (100%) and Gentamycin (100%).in addition, the Highest rates of multidrug resistance and methicillin resistance were found among biofilm-producing strains in comparison to biofilm non producing strains. The findings of this study indicate that methicillin-resistant strains produced more biofilms and exhibited a high degree of resistance to almost all antibiotic classes

Keywords: Methicillin-resistant *Staphylococcus aureus,* Biofilm, Congo Red Agar

CHAPTER ONE

1.INTRODUCTION

This chapter covers the organism *Staphylococcus aureus* (*S. aureus*), its biology, pathogenesis, diseases it causes, biofilm production and constituents, antibiotic resistance, the study's goal and objectives, and its significance.

1.1 Background

S. aureus is a gram-positive commensal opportunistic pathogenic bacterium that represents a threat to public health. It was isolated from a surgical wound infection in 1880 by Alexander Ogston. In that year, Louis Pasteur demonstrated that animals infused with abscess from human *Staphylococcal* infections developed abscesses. The bacterium's natural habitats are the skin's surface and the mucosae of humans. The preferred location, particularly in adults, is the anterior nares squamous epithelium. *S. aureus* is responsible for bacteremia acquired in hospitals, bloodstream infections, surgical site infections, skin and soft tissue infections (SSTI), infectious endocarditis (IE), osteomyelitis (OM), device-related infections, pneumonia and breast implant infections are all caused by *S. aureus* bacteremia has a mortality rate of 20%-30% (Parastan et al., 2020).

The pathogenesis of *S. aureus* is due to two key factors: first, it is a natural pathogen of 30-50% of the population, so it is commonly available and can cause disease under certain conditions, and second, it can produce several virulence factors *S. aureus* colonizes 30-50 percent of healthy people in the USA and causes diseases ranging from mild to fatal. On the anti-*staphylococcal* antibody profiles, there are two types of *S. aureus* colonization: chronic carriers (20-25%) and 'other' (irregular carriers and non-carriers (75-80%). The risk of hospital-acquired infections is directly associated with *S. aureus* nasal carriage. Persistent colonization in the nose has a higher density of *S. aureus* than irregular

carriers with a single genotype, putting them at higher risk for hospital infections like, dialysis related infections, Bacteremia and surgical infections (Parastan et al., 2020).

S. aureus infections account for most pneumonia and bacteremia cases, which consumed 60% of total medical expenditures and resulted in a significant number of deaths. Endocarditis is a type of infection that frequently necessitates prolonged hospitalization, lasting an average of 26 days and resulting in higher medical costs per patient. On the other hand, diseases caused by surgical procedures can result in a 14-day hospital stay at a high financial price. According to estimates from the Netherlands and USA, *S. aureus,* which inhabits on mucous membranes, or the surface of skin is known to colonize many healthy people (25-35%) worldwide; more than 2 billion people are colonized with *S. aureus.* However, some organisms have developed resistance to the current antibiotics and are referred to as MRSA (Deurenberg et al., 2007).

According to the World Health Organization (WHO), MRSA is one of the gram-positive bacteria that has seen the most increased investment in new drugs, presenting a significant threat to the treatment of *S. aureus* diseases. MRSA infections are increasing in hospitals and the community worldwide, leading to an increase in medical costs. MRSA has multiple mechanisms of drug resistance and is resistant to the majority of currently available antibiotics (Qiu et al., 2020). Treatment options are limited due to MRSA's resistance to all beta-lactam antimicrobials. Since methicillin is a first-line and less expensive antimicrobial for MRSA infections, testing for *S. aureus* sensitivity to methicillin is critical for proper infection treatment without vancomycin, which may result in several therapeutic complications. Even though vancomycin has been in use since 1958, hospital samples have already revealed a decline in *S. aureus* susceptibility to the antibiotic (vancomycin-intermediate *Staphylococcus aureus*-VISA) (Batista et al., 2019).

1.2. *S. aureus* Biofilm

Biofilms are complex assemblages of bacteria embedded in an extracellular matrix of exopolysaccharides (EPSs), proteins, and macromolecules like DNA. They can grow on both living and non-living surfaces. Molecular methods and Scanning electron micrograph have confirmed that biofilms colonize wounds in studies. Biofilm shields the microbes from host immunity and prevents antibiotics from reaching the site of infection, causing wound healing to be hampered (Neopane et al., 2018). Biofilm production in *S. aureus* is maintained by the accessory gene regulator (agr) quorum sensing (QS) system, a chromosomal locus. Biofilm formation in *S. aureus* has led to diseases like infections related with implants, Endocarditis, osteomyelitis, cystic fibrosis lung infection, and chronic wound infection, Additionally, polymicrobial coexistence between *Candida albicans* and *S. aureus* is expected, resulting from increased virulence factors and microbial adhesion. *S. aureus* infections are costly, costing $450 million in the past. Antibiotic susceptibility is reduced in the presence of biofilm, and several approaches, including anti-biofilm drugs and vaccines, should be used to treat biofilm-implicated diseases (Parastan et al., 2020).

Biofilms have a considerable impact on healthcare, with biofilms thought to be responsible for 65 percent of nosocomial infections (Charankaur & Khare, 2013). When *S. aureus* biofilm infections occur, they can be challenging to treat with traditional methods and may require surgical elimination of the infection site or device removal. MSCRAMMs (microbial surface components that recognize adhesive matrix molecules) such as extracellular membrane proteins such as collagen-binding protein (CNA), elastin-binding protein (EbpS), fibronectin-binding proteins (FnbA and FnbB), fibrinogen-binding protein (Fnb), bone sialoprotein-binding protein (bbp), and clumping factor all participate in the adhesion stage of *S. aureus*. According to research, the intracellular adhesion (ica) operon is necessary for controlling biofilm production.

The ica locus encodes the proteins necessary for the formation of the polysaccharide intercellular adhesion (PIA) molecule., which includes the gene icaADBC (Abdulrahim et al., 2019). because of its capacity to resist treatment by forming biofilms on medical devices implanted such as, catheters, joint prosthetics, and artificial heart valves the biofilm-producing pathogen *S. aureus* has gained notoriety for causing chronic infections (Moormeier & Bayles, 2017).

1.3. Biofilm formation and constituents

Biofilm formation is a multifaceted and complicated process. Attachment, maturation/accumulation, and detachment/dispersal are the three phases of the biofilm formation process. Bacteria that have developed into biofilms are then dispersed out of the biofilm endothelium in the final step. The elements secreted by the host, as well as the proteins obtained from lysis, eDNA, and polysaccharides are thought to make up the *S. aureus* biofilm matrix. This intricate structure envelops the matrix's cells. Each of these factors has different influences depending on the isolate and the surrounding conditions. Additionally, the matrix composition influences the effectiveness of many dispersal mechanisms. The major components of the biofilm matrix, as well as the factors that contribute to their formation, will be described briefly. Polymeric N-acetyl-glucosamine (PNAG) is a significant element of the biofilm matrix PIA, which is synthesized by enzymes carried in the icaADBC locus, is a crucial component of both *S. aureus* and *Staphylococcus epidermidis* biofilms. The ica locus encodes proteins responsible for the production, transfer, and alteration of PIA, which is made up of 1,6-linked N-acetylglucosamine polymers. However, various empirical studies have proven that strains of *S. aureus* can form biofilms in the absence of ica, and PIA polymer plays a vital role in biofilms' structural stability both in vivo and in vitro. (Kirmusaoglu, 2016).

In the absence of PIA, proteins and eDNA, which work as intercellular adhesins, were later named as elements of these biofilms (Lister & Horswill, 2014). *S. aureus* can adhere to inanimate surfaces via electrostatic and hydrophobic interactions in the absence

of human matrix molecules. The major autolysin, negatively charged teichoic acids and AtlA mediate Cells stick to polystyrene and glass surfaces (Xiang et al., 2017).

A generally recognized framework for biofilm development includes three stages, all of which differ according to the bacterial species, molecular products: attachment, maturation, and dispersal (Moormeier & Bayles, 2017). Figure 1 depicts these three phases. Planktonic cells attach to living or Non-living surfaces during the adhesion stage and multiply into adhesive clusters known as microcolonies. Bacterial cells produce an Extracellular matrix (ECM) as these microcolonies grow, which acts as a framework for the formation of this three-dimensional structure. When a certain cell density is reached, a mechanism is activated that causes ECM degradation, allowing cells encased in the biofilm to carve out and return biofilm formation at distant sites. *S. aureus* is thought to go through the same stages of biofilm formation as other bacteria (Le et al., 2014). Many biofilm studies have proven that when *S. aureus* biofilms develop into dense cell layers, detachment mechanisms become active. Subpopulations of the biofilm disperse, removing microcolonies in the remaining biomass (Periasamy et al.,2012).

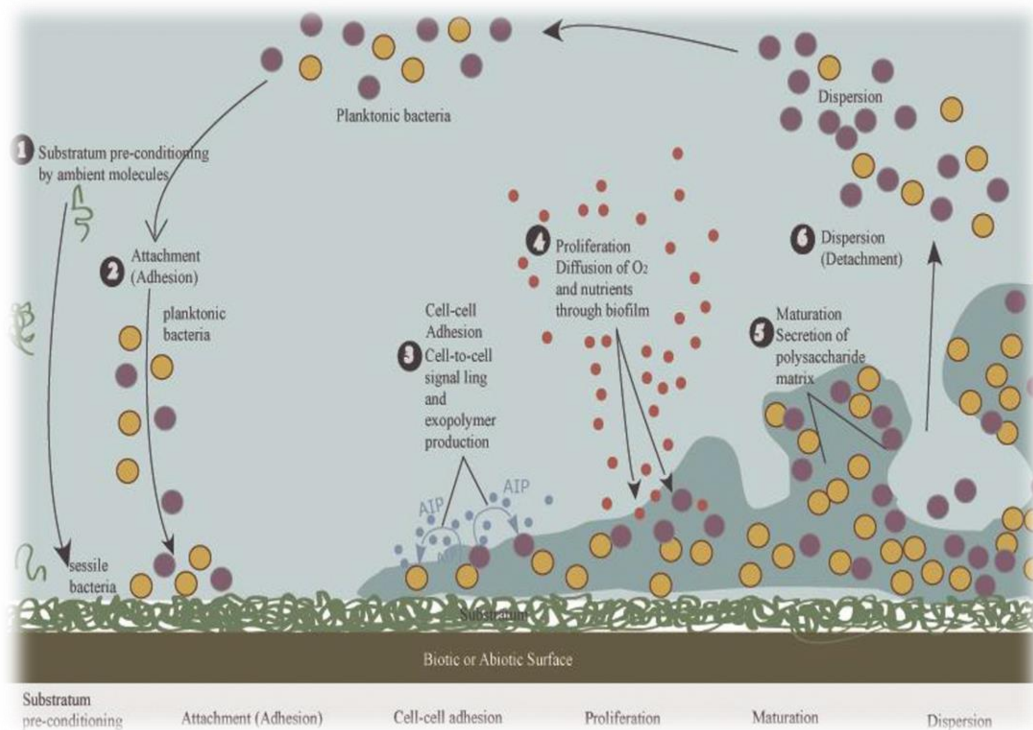


Figure 1. The Biofilm development stages adopted from Kirmusaoglu, (2016)

1.4. Aim and objectives of the study

The aim of our study is to reveal the phenotypic Biofilm forming abilities of *S. aureus* isolates and to investigate the relationship of antibiotic resistance of Biofilm forming *S. aureus* with Biofilm formation.

1.5. Significance of the study

This thesis will provide information about one method that can be used to detect biofilm production amongst *S. aureus* strains and learn their' antibiotic susceptibility, thereby addressing the proper use of antibiotics.

CHAPTER TWO

2. GENERAL INFORMATION

S. aureus is a gram-positive bacterium and part of the human nose and skin flora. It is associated with illnesses ranging from mild skin and wound infections to severe and even fatal infections in immunocompromised people. MRSA infection is a severe problem that could cause endocarditis, pneumonia and toxic shock syndrome (Sze & Kao, 2020). As the result of discharging various virulence factors and the development of multiple drug resistance to various antibiotics, it is a severe infectious agent in both public and hospitals. Antibiotic-resistant *S. aureus* poses primary threat worldwide (Haghi Ghahremanloi Olia et al., 2020*)*.

S. aureus capability to colonize and spread relies on the bacterium's ability to cling to the host's extracellular matrix components and serum proteins. Biofilm production was found to play a pivotal role in *Staphylococcal* infection pathogenesis in protecting the colony against environmental factors, antibacterial treatment, and the immune reaction to the hosts. Biofilms are individual or multiple layers of bacteria integrated into proteins, polysaccharides and DNA, which shield bacteria from environmental factors. Worse yet, bacteria in biofilms are of considerable concern because they represent up to 65% of human infections. High resistance (10 – 1000 times) to normal antibiotics. Except for the natural protection offered by the matrix material, bacteria within a biofilm undergo transcriptional changes to start quorum interaction, react to apparent strict stress factors, and stimulate mechanisms that protect cells against antibiotics and other antimicrobial risks. Biofilms are pervasive in our surroundings, as they are bacteria's preferred growth environment.

The persistence and threat of biofilmed diseases to the young, elderly, and immunocomp romised pose a significant problem for global health care systems. Unfortunately, there a

re no specific antimicrobial therapies for biofilm bacteria, which limits treatment success and contributes significantly to increased healthcare costs and poor patient outcomes (Haney et al., 2018).

Owing to its commensal presence with otherwise immunocompetent individuals, *S. aureus* is easily transmitted between individuals in the healthcare and in the community settings. Furthermore, it is a growing matter of concern due to its relation to hospital-acquired infections (HAI) and antibiotic resistance (Ward et al., 2018). Because of their capacity to colonize and cause illness in humans and animals, MRSA is a severe widespread outrage. New cases of MRSA infections have been reported in different environments following their initial presentation as hospital-associated pathogens, including the wider public (Community-associated-MRSA; CA-MRSA), among others (Papadopoulos et al., 2019).

2.1 *S. aureus* pathogenesis

Bone, joint infections, Skin infections, bacteremia, medical device infections and animal infections are all known to be caused by *S. aureus.* MRSA, is a toxin-producing bacterium that are found in hospitals. The onset of MRSA exacerbates the treatment outcome of *S. aureus* infection. Simultaneously, they are gaining attention in *S. aureus*. whose ability to infect is aided by a broad range of intrinsic virulence factors. The availability and spread of mecA have accelerated the global increase in antibiotic resistance, posing a significant public health impact. Consequently, discovering the mecA gene is crucial (2020, Li et al.) Similarly, Chung et al. (2021) examined virulence factors of *S. aureus* are linked to host cell surface attachment and invasion, immune avoidance, enterotoxin production, Hemolysis, type VII secretion system (T7SS), antimicrobial resistance, and a toxin-antitoxin (TA) system. Host cells' attachment to host glycoproteins and their invasion through the membrane due to host tissue damage contribute to bacterial infection penetration to the cell surface, which is an essential first step. Furthermore, *S. aureus* has a unique immune avoidance system that allows it to circumvent neutrophils in

various ways while also producing capsular polysaccharides that disrupt the host's primary defense system. Enterotoxins, exfoliative toxins A-B, hemolysins, and Panton-valentine leucocidin (PVL) are some of the toxins produced by *S. aureus,* according to Mahmoudi et al (2019). Certain *S. aureus* strains produce exfoliative toxins A and/or B, regulated by the eta and etb genes, The *Staphylococca*l scalded-skin syndrome (SSSS) has been linked to these toxins.

According to Poddighe & Vangelista (2020), human-specific *S. aureus* exotoxins can be divided into pore-forming toxins, enzymatic toxins and superantigens. Toxins that form pores include α-toxin and double-component leucocidins (LukSF-PV, HlgAB, HlgCB, LukED, and LukAB), which work by first recognizing a receptor determinant on the target cell's surface, then oligomerization (α-toxin forms heptamers, while leucocidins form octamers), and finally pore formation. ε-toxin, which causes keratinocyte lysis, and phenol-soluble modulins, which cause cell destruction, slime formation, and immune modulation, are examples of other toxins.

Additionally, *S. aureus* secretes a variety of enzymes, including betatoxin (sphingomyeli nase), exfoliative toxins (serine proteases), and awidearray of cofactor exoenzymes that aid in bacterial survival and spread

2.2. Biofilm formation in *S. aureus*

S. aureus first adheres to a solid surface, followed by cell-cell attachment, and then multiplies to form a multilayered biofilm encased in EPS. The formation of biofilms is entirely dependent on the IcaADBC operon, which codes for three membrane proteins (IcaA, IcaD, and IcaC) and one extracellular protein (IcaB) (Neopane et al., 2018). Many surface proteins have been inflicted in the slime formation process, including, *S. aureus* surface protein, fibronectin-binding proteins, biofilm-associated protein and *Staphylococcal* protein A. The formation of a *S. aureus* biofilm can delay the reepithelization of injured tissues, increasing the healing time. Biofilms of *S. aureus* have

been related to chronic wounds such as, pressure sores, diabetic foot ulcers and venous ulcers. A mature *S. aureus* biofilm must disperse in order for a wound infection to spread. *Staphylococci*, primarily *S. epidermidis* and *S. aureus*, cause the majority of biomaterial-associated infections. They are antimicrobial resistant, necessitating the removal of infected biomaterials and resulting in high morbidity and mortality (Mack et al., 2004).

Two-component systems and transcriptional and post-transcriptional regulators, including RNA, are all composed of the regulatory system that maintains the development of biofilms in *S. aureus* (Figueiredo et al., 2017). Similarly, Haghi Ghahremanloi Olia et al. (2020) reported that the icaADBC operon encodes PIA, which regulates biofilm production. This operon contains aa putative PIA exporter (icaC), PIA deacetylase (icaB), (icaA and icaD), and a regulatory gene (icaR). Additionally, *S. aureus* further expresses ClfA and ClfB, FnbA and FnbB, Cna, Bbp, Eno, and Ebp via MSCRAMMs that interact with host extracellular components (Haghi Ghahremanloi Olia et al., 2020).

Knobloch et al. (2002) revealed icaADBC genes in *S. aureus* and a closely resembling polysaccharide (PIA/PNSG), as well as slime formation in vitro in some ica-positive *S. aureus* strains. This phenotype is the subject of current research because elucidating the mechanisms by which *S. aureus* forms biofilms may result in the development of novel preventive measures (Knobloch et al., 2002). In routine microbiology laboratory conditions, where bacteria are normally cultivated planktonically in nutrient-rich situation, bacteria in the environment grow exclusively in nutrient-depleted environments, forming multicellular aggregations known as biofilms. Bacteria produce an ECM composed of carbohydrates, proteins and extracellular DNA (eDNA), which surrounds the cells in an adhesive matrix that allows them to survive in hostile or extreme environments. Due to their persistent recalcitrance to the host defense system and antimicrobials, human pathogen-produced bacterial biofilms have become increasingly important to study in recent years (Moormeier & Bayles, 2017).

According to Moormeier et al. (2017), During the course of biofilm development, there are five stages of growth: attachment, multiplication, exodus, maturation, and dispersion. (**Fig.2**)

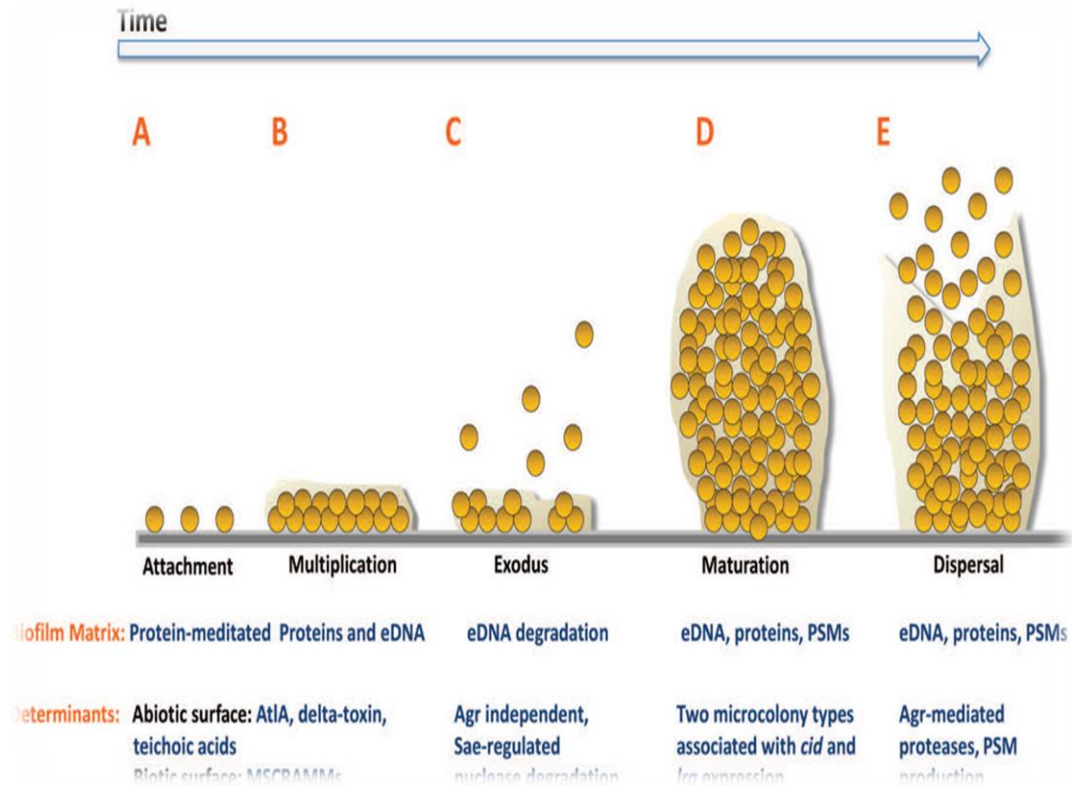


Figure 2. The stages of S. *aureus* biofilm development adopted from (Moormeier et al., 2017)

Attachment, multiplication, exodus, maturation, and dispersal are the five stages of *S. aureus* biofilm development. A. *S. aureus* cells adhere to Non-living and living surfaces via hydrophobic interactions or MSCRAMMs. B. After cell attachment, the biofilm develops into a viscous mixture of multitudes of cells made up of eDNA and a proteinaceous mesh. C. Once the biofilm attains confluence, a subset of cells is released via Sae-regulated nuclease-mediated eDNA lysis, allowing the formation of three-dimensional microcolonies. D. Microcolonies are produced when several specialized cell

groups persist during the exodus stage. This stage is characterized by rapid cell proliferation, which results in strong assemblages of proteins such as PSMs and eDNA. E. Slime matrix modulation and cell dispersal is mediated by protease activation and PSM production due to the presence of Agr proteins in the biofilm matrix. MSCRAMM is an abbreviation for microbial surface components that recognize adhesive matrix molecules, and eDNA is an abbreviation for extracellular DNA. PSM is an abbreviation for phenol soluble modulins, while Agr is an abbreviation for accessory gene regulator (Moormeier & Bayles, 2017).

2.2.1. Attachment phase

S. aureus planktonic cells adhere to a surface via a different of CWA proteins that are unique for various host matrix substrates, thereby initiating biofilm formation on biotic materials (Figure 2A). The MSCRAMMs are a well-characterized family of surface-imbedded proteins that share a cell wall targeting motif (LPXTG) However, each has a unique selectivity for the molecules of the host. such as, fibrinogen, collagen, fibronectin and cytokeratin. the serine-aspartate repeat family proteins (SdrC, SdrD, and SdrE), FnBPA and FnBPB, ClfA and ClfB, the collagen adhesin (Protein A), the plasmin-sensitive protein (Pls), and the SasG iron-regulated surface determinants (IsdA, IsdB, Is) (Moormeier & Bayles, 2017).

According to studies, early attachment can occur on inanimate or biotic surfaces. The attachment of *Staphylococci* to a non-living object, such as the naked plastic or metal surface of an indwelling medical device, is determined by the device's physicochemical characteristics. By contrast, bacterial surface components such as accumulation-associated protein (Aap), AtlE, lipoteichoic acids (LTA), and wall teichoic acids (WTA) are defined by the device's physicochemical properties (Speziale et al., 2014).

2.2.2. Multiplication phase

After adhering to a surface and being supplied with nutrients, the attached *S. aureus* cells will begin dividing and multiplying. However, newly formed daughter cells are prone to dissociation just before the formation of an ECM into which they can coat, particularly in the presence of shear forces associated with fluid flow. A number of *S. aureus* factors are known to contribute to maintaining cell-to-cell connections., thereby ensuring the stability of this immature biofilm. This stage of cell division and accumulation is referred to as the multiplication stage (Figure 2B). *Staphylococcus* species adhere to live and inanimate surfaces and produce EPS via the ica operon. EPS is an extracellular matrix (ECM) composed of PIA/PNAG, eDNA, and surface CWA proteins in an ica-free situation, and bacterial colonies develop (Kirmusaoglu, 2016).

CWA protein contributes to the growth and maturation of biofilms while also facilitating intracellular adhesion (Speziale et al., 2014). Intracellular adhesion polysaccharide/poly N-acetylglucosamine at this stage, biosynthesis occurs in clusters of cells, resulting in a multi-layered biofilm structure. *Staphylococca*l spp utilize surface proteins such as the pathogen's FnbA and FnbB, or another species' fibrinogen-binding protein SdrG/Fbe, as well as clumping factors A and B *S. epidermidis* that are CWA, which may aid intracellular Attachment and promote bacterial aggregation during ica-independent biofilm formation (Foster et al., 2014).

While these proteins appear to play a role in biofilm proliferation, their function in experiments utilizing flow cells in the absence of matrix components was not significant (Moormeier & Bayles, 2017). despite recent findings suggest that CWA proteins don't participate the multiplication stage, adding a protease during this stage has been shown to prevent biofilm production. This study indicates that a proteinaceous component is involved in cell accumulation. It is remarkable because recent research has been demonstrated that cytoplasmic proteins are used as matrix components in *S. aureus* biofilms. GAPDH and Enolase which are not generally thought of as biofilm-associated

proteins, were found to glitter 'as slime matrix molecules in these studies by sticking to the cells' surface in response to the biofilm environment's declining pH (Foulston et al., 2014).

2.2.3. Exodus phase

One of the experiments of biofilm development made using time-lapse microscopy was a broad and coordinated discharge of cells approximately six hours after the proliferation phase began. This 'exodus' stage of biofilm development is an early detachment process that occurs concurrently with microcolony production and results in biofilm reorganization (Figure 2C). Exodus occurs as a result of nuclease-dependent eDNA degradation and is unrelated to the mechanism by which Agr disperses following microcolony development (Moormeier & Bayles, 2017). Additionally, it has been demonstrated that a self-produced, secreted nuclease reduces the total biomass of *S. aureus* biofilms by degrading eDNA contained within the biofilm matrix. According to recent studies using time-lapse microscopy (Figure 2C), nuc-mediated eDNA lysis occurs very early in biofilm process and is involved in the exodus event (Lister & Horswill, 2014).

2.2.4. Maturation phase

Microcolony formation, which promotes area available for nutrient swap and waste disposal and facilitates the spread of biofilm cells to distal sites, is a critical component of biofilm maturation for any bacterial species. Additionally, Periasamy et al. (2012) provided an illustration of one model. It is believed that the formation of biofilm microcolony structures is a deduction process, with PSM-mediated dispersal attempting to disperse out channels from a dense tangle of slime cells. However, time-lapse microscopy observations of biofilm development demonstrate the formation of microcolonies from divergent foci of cells that persist in the basal layer shortly after exodus starts (Figure 2D).

Microcolonies were also detected rising from a basal membrane in another study that monitored biofilm formation at a lower magnification. As a result, and concerning the existing method, we now understand how Microcolonies with rapid growth emerge from a basal cell of sluggish growing cells. Conversely, these studies identified a variety of microcolony types, each with its own growth rate, gene expression pattern, and physical characteristics. The first had constitutive large-growth microcolonies, but their low oxygen level, or hypoxia, slowed down the microcolony's growth. (Figure 3A). (Moormeier et al., 2013).

2.2.5. Dispersal phase

Within the biofilm, a vibrant group actively shares and exchanges products critical for biofilm structure maintenance and allowing resident bacteria to thrive on the other hand, dispersal becomes possible as biofilms mature. Apart from passive dispersal caused by tensile stresses, many types of bacteria have learned to use external cues to determine whether it is safe to stay within the biofilm or if it is best to transition to a planktonic existence. Changes in nutrient availability, oxygen variations, an increase in toxic products, and other stressors all contribute to biofilm dispersal (Kostakioti et al., 2013).

The majority of research on *S. aureus* biofilms has focused on determining what causes biofilms to adhere and grow. However, in a recent study, Periasamy et al. (2012) examined the factors affecting biofilm dispersal control. Although Agr quorum sensing relies on cell concentration and the buildup of transcription factors known as autoinducers, which have been shown to control the diffusion of *S. aureus* biofilms, when an octapeptide pheromone is known as an auto-inducing peptide (AIP), reaches a critical concentration in *S. aureus*, it accumulates in the culture medium, where it binds to and activates the histidine kinase AgrC. AgrC begins the response regulator AgrA, which then initiates signaling from the P3 promoter of the agr operon, resulting in the production of a regulatory RNA molecule (RNAIII) that controls the expression of several virulence factors and genes associated with biofilm (Moormeier & Bayles, 2017).

When agr-depleted strains were compared to wild-type in the first experiment examining the Agr system's role in *S. aureus* biofilm development, it was discovered that agr-depleted strains formed more robust biofilms (Filmer et al., 2000). However, it was not until flow-cell studies evaluating the function of Agr quorum sensing during *S. aureus* biofilm development that the P3 promoter was identified in a subpopulation of cells primarily within microcolonies that appeared to fluctuate in waves over time, concurrent with biofilm detached cells (Yarwood et al., 2004). Since then, a study has been published demonstrating that AIP concentration results in increased P3 expression in microcolonies with low pressures. (Kim et al., 2016). While the initial report implied that Agr activity may contribute to biofilm dispersal, no Agr-induced factors involved in distribution were identified. Two consecutive studies, on the other hand, discovered that Agr-mediated detachment processes act in different ways. In one study, activation of P3 was associated with the dispersal of intact biofilms, one explanation is that it is due to a general increase in proteolytic activity, resulting in more extensive protein-based extracellular matrix degeneration. (Boles et al., 2010).

In another study, it was proposed that the production of phenol soluble modulin (PSM) peptides was involved in Agr-induced detachment and have been implicated in *Staphylococcal* biofilm dispersal and have been shown to be controlled by the Agr system through direct AgrA binding to the promoters of the PSM operons (Periasamy et al., 2012). Illustration in Figure 2E.

2.3. Types of biofilm development

Staphylococcus spp. biofilms are either ica-dependent or ica-independent, depending on the matrix structure of the biofilm. The first ica-dependent mechanism described is PIA or PNAG, PIA/PNAG is produced by the icaADBC operon (Figueiredo et al., 2017). While both types of biofilms can be found in MRSA and MSSA isolates, O'Neill et al. (2008) hypothesized that PIA biofilms were more common in MSSA isolates and ica-independent biofilms were more prevalent in MRSA isolates.

The actions of the charges, the positive charge on PIA and the negative charge on the bacterial surfaces, have succinctly revealed the intracellular attachment that occurs during this type of biofilm formation. It's worth noting that the icaADBC genes are present in all *S. aureus* strains. Except for *Staphylococcus haemolyticus* and *Staphylococcus saprophyticus,* a large number of *Staphylococcus* species have been identified as Ica loci, including *S. aureus* and *S. epidermidis* (Kirmusaoglu, 2016). On the other hand, biofilm formation that is not dependent on PIA can occur in the absence of ica. deleting the arlRS two-component is said to repress biofilm formation and result in solid attachment and PIA production. Fitzpatrick et al. (2005) defined biofilm as a group of microorganisms that adhere to one another on a solid surface, allowing microbes to float in liquid.

O'Neill et al. (2008) discovered a strong association between MSSA isolates and ica-dependent biofilms. However, the same study discovered that MRSA's glucose-dependent slime formation was largely independent of ica and was most likely made by proteins. As a result, the composition of *S. aureus* ica-independent biofilms has remained a mystery, as has the regulatory network that regulates biofilm production. The concentration of biofilms has been linked to the *S. aureus* surface protein G (SasG) and its homologue plasmin sensitive surface protein (Pls), FnBPA and FnBPB, and *Staphylococcal* protein A (Spa).

Additionally, Kirmusaoglu (2016) demonstrated that biofilm production is not a one-way mechanism involving PIA as the major biofilm development constituent; extra proteins exist. Elimination of icaADBC reduces PIA production and biofilm formation, but has no effect on bacterial virulence. Biofilm formation is possible in this case, as demonstrated in a catheter infection study that resulted in the formation of biofilms on isolates of *S. aureus* containing a mutant ica swarm. Fitzpatrick et al. (2005) discovered that while slime production was unaffected in icaADBC operon-depleted MRSA mutants, it was affected in icaADBC operon-deleted MSSA mutants.

The action of α-toxin, a hemolytic toxin, in aggregate promotes the formation of biofilms and cell-cell interactions in *S. aureus* binds to itself in the presence of DNA and biofilm matrix of biofilms to form a covalent bond, β-toxin, sphingomyelinase, causes hemolysis and lyses lymphocytes, Promoting the creation of a *S. aureus* biofilm. (Huseby et al., 2010).

2.4. Methods of biofilm detection

Numerous studies have shown that biofilms can form on different surfaces. To determine the formation of biofilms in *Staphylococcal* infections, phenotypic methods such as CRA tissue culture plate, tube method, electron microscopy, confocal scanning microscopy, and bioluminescent assay are applicable (Manandhar et al., 2018).

Mootz (2013) explained that regardless of the coating, biofilms form on surfaces. The researchers applied plasma to microtiter plates in a previously described method. After incubating the coated plates overnight at 4°C, the plasma was aspirated and other additives were added and the conditions adjusted. This method is referred to as microtiter plate biofilms. Flow cell biofilms are another technique for identifying biofilms. They involve supplementing bacteria in flow chambers for a predetermined period of time (48 hours) and detecting biofilm biomass. Sarkisian (2011) discovered that biofilms associated with clinical isolates of MRSA most likely originated from catheters in a study that classified and quantified biofilm production in unique clinical strains of MRSA. Catheters, particularly those used for urine, have a surface that is ideal for biofilm growth. Surprisingly, MRSA isolates from the nares produced a negligible amount of biofilm, indicating that the proper surface conditions must exist (Sarkisian, 2011). There appears to be a way to expand on Tang and Stratton's (2010) traditional method of detection and on the growth conditions for biofilm production. Finally, by measuring optical density (OD), bacterial films can be detected and classified as producing biofilms or not producing biofilms; these findings were confirmed by (Stepanovic et al., 2000).

Metzler (2016) developed a method for quantifying *S. aureus* biofilm production using crystal violet (CV). This approach has several advantages over other methods, including simplicity, reliability, and speed. Additionally, the assay enables classification of the strain as a biofilm producer at a high, moderate, or low level. The CV acts by staining all living and dead biomass, as well as the Extracellular polymeric substance matrix, in a non-specific manner. The assay can be used to determine an isolate's ultimate biofilm response due to the stain.

Numerous methods have been proposed for determining the relative formation of biofilms. Because biofilms grown inflow situations are considered to be more biologically critical to innate biofilms but also contribute significantly to slime morphology and structure, some researchers aim to develop biofilms in conditions that allow for a continuous flow of new media over the growing biofilm. For instance, the CDC Biofilm Reactor from Bio Surface Technologies grows biofilms on surface "coupons" with the recharged medium under streamlined conditions. Likewise, The Modified Robbins Device is a fluid flow cylinder used to retain suspension substances in order to examine biofilm growth under laboratory work. Flow cell chambers, which are examined using confocal microscopy to visualize adhered biofilms, are the most commonly used method for growing biofilms under circulating conditions (Haney et al., 2018).

CRA was used as a novel alternative method for detecting coagulase-negative staphylococci's slime production. It was found to be more precise than the Christensen method by Freeman et al. Nonetheless, it was limited in terms of black pigment formation variations. However, it is hypothesized that altering the agar component will improve the result of biofilm identification (Atshan, 2009). Moreover, different sugar preparations influence the formation of biofilms in *S. aureus* strains.

Croes et al. (2009) performed an analysis of several CRA screening methods described in a study. The morphology remained unaltered regardless of the agar medium used, whether it was brain heart infusion or trypticase soy. The majority (91 %) of *S.*

aureus strains had colonies with normal morphology (smooth round colonies), indicating that the majority of strains produced little or no slime. Without sucrose, colonies were colored (bright) red regardless of the agar medium used. Sucrose addiction to both agar media resulted in more dark colonies and made it more difficult to distinguish the dry crystalline morphology. Sucrose coloration of all colonies on brain heart infusion agar with Congo red ranged from red to Bordeaux red, whereas strains on trypticase soy agar with Congo red produced predominantly purple to black colonies. Color distinctions did not correlate with morphological distinctions. MSSA strains exhibited a more atypical, dry crystalline (rough) morphology (slime producing positive) than MRSA isolates, with 14% (22 of 156) and 0%, respectively, exhibiting an aberrant, dry crystalline (rough) morphology (slime producing positive). At a 0.1 percent glucose concentration, more than 60% of *S. aureus* strains associated with the multilocus sequence typing (MLST) clonal complex (CC) 8 produced significant amounts of biomass, compared to 0-7 percent for various other clonal lineages in the same study (Croes et al., 2009).

2.5. Antibiotic Resistance in *S. aureus*

S. aureus is a common cause of nosocomial infections such as surgical wound infections, bacteremia, and pneumonia, and it has developed resistance to a wide variety of antibiotics. Additionally, *S. aureus* is well-known for developing resistance to antibiotics. Penicillin-resistant *S. aureus* emerged in the late 1940s, and by the mid-1950s, resistance had spread to the point where the antibiotic was no longer effective in treating infections. MRSA was discovered in the early 1960s and has since spread globally. MRSA is now endemic in nearly all medical centers in developed countries, though recent data indicate that the rate of invasive MRSA infections in US health-care facilities is steadily declining. CA-MRSA, first surfaced mysteriously in the 1990s and has since spread to a number of countries worldwide, including the USA. In contrast to health-care-associated MRSA infections, which affect individuals with certain risk factors, CA-MRSA infections affect otherwise healthy individuals. Although resistance to beta-lactam antibiotics is

arguably the most serious problem in treating *S. aureus* infections, the pathogen can develop resistance to a variety of other antibiotics, including vancomycin, a key treatment for extremely drug-resistant severe *Staph* infections (Kobayashi et al., 2015).

With the advent of drug-resistant *S. aureus* in hospitalized patients worldwide, *S. aureus* has become a significant threat and burden, with rates of MRSA steadily increasing has developed into a significant global health threat (Chinnambedu et al., 2020). Until 2013, the WHO estimated that antibacterial drug resistance caused 25,000 deaths in the European Union, over 38,000 deaths in Thailand, and over 23,000 deaths in the USA. According to National Nosocomial Infections Surveillance System research, more than 60% of patients treated in intensive care units in the USA have been identified as having hospital-acquired MRSA infections. Another study conducted in a tertiary care facility in India discovered that 42 percent and 40% of *S. aureus* strains resistant to methicillin were discovered in 2008 and 2009. Numerous antibiotics, including second- and third-line agents, have developed resistance in *S. aureus*. MRSA infections have been associated with indiscriminate antibiotic use, operation theatre contamination in nosocomial settings, and repeated exposure in immunocompromised patients, among other factors. Vancomycin, daptomycin, and linezolid have been approved for the treatment of MRSA diseases. Tigecycline has also been shown to be effective against MRSA strains (Chinnambedu et al., 2020).

2.5.1. Methicillin Resistance and Mechanism in *S. aureus*

The mecA or mecC gene is found on the *Staphylococcal* chromosomal cassette and encodes penicillin-binding protein 2A (PBP2A) PBP2ALGA, an enzyme that crosslinks the peptidoglycans in the bacterial cell wall, conferring methicillin resistance. While the two enzymes are relatively inactive against beta-lactam antibiotics, which results in resistance to them. Vancomycin has been used as a first-choice antibiotic to treat MRSA infections for years. Outbreaks of multidrug-resistant, medium and high-level

vancomycin-resistant *Staphylococcus aureus* have occurred over the last two decades, putting a significant public health risk. (Cong et al., 2020).

2.5.2. Vancomycin Intermediate *S. aureus*

Vancomycin intermediate *S. aureus* (VISA) isolates have been identified throughout the world, and they have been associated with tenacious infection, hospitalization, vancomycin therapy prolongation, and failure. VISA strains are thought to have evolved from heterogeneous vancomycin-intermediate *S. aureus* (hVISA), which is defined as a *S. aureus* strain with a vancomycin MIC in the susceptible range (≤2 µg/ml) determined using routine methods but a subset of cells in the vancomycin-intermediate field (≥4 µg/ml) (Cong et al., 2020). The molecular mechanisms underlying the development of VISA are unknown at the moment. Computational studies aimed at discovering genetic variations that lead to vancomycin-intermediate resistance have combined proteomics, genomics, and genetic analysis to identify multiple mutations in genes involved in VISA development.

2.5.3. Vancomycin Resistant *S. aureus*

Vancomycin, the first glycopeptide antibiotic to be discovered, is still used as empiric therapy for MRSA infections. Japan first described a VISA with an MIC of 8 µg/ml in 1997. In 2002, in the United States, vancomycin-resistant *Staphylococcus aureus* (VRSA) was identified in a diabetic patient. Previously published in vitro studies suggested that vancomycin resistance in MRSA could be caused by a variety of mechanisms, the most common of which were decreased permeability and increased cell wall thickness, resulting in decreased vancomycin availability for intracellular target molecules. Resistance genes to vancomycin (vanA vanB, vanD, vanE, vanF, and vanG). Furthermore, according to a recent study, VISA cells have a thicker cell wall and a slower growth rate than fully susceptible cells. Although hVISA has MICs in the susceptible

range (2 µg/mL), a resistant subpopulation exists. VISA and infections are linked to an increased risk of vancomycin therapeutic failure, lengthy hospital stays, an increased likelihood of chronic infection and increased treatment costs. Despite the publication of a systematic review and meta-analysis on the prevalence of VISA and hVISA five years ago, a detailed study on the global prevalence of VRSA, VISA, and hVISA has not yet been published (Shariati et al., 2020).

CHAPTER THREE

3.MATERIALS AND METHOD

3.1. Material

3.1.1. Devices and Tool

Equipment	Company	Country
Incubator	WTB-Binder	Germany
VITEK 2 system	Biomerieux	France
Oven	Memmert	Germany
Medical Refrigerator	Sanyo	Japan
Autoclave	Sakura	Japan
Sensitive balance	Shimaduz	Japan
Centrifuge	Hettich	Germany
Congo Red Agar	Thermofisher	Germany
Disposable Petri dishes plate	The Science	USA
Blood Base media	Merck, KgaA	Germany
Safety cabinet II	DALTON	Japan
Inoculation loops	The Science	USA
Bacteria storage tubes	OR-BAK	Turkey

3.2. Method

3.2.1. Design of Study

The current study was carried out in the microbiology laboratory at the NEU hospital in the Turkish Republic of Northern Cyprus (TRNC). A total of 67 samples for the study was collected between January 2020 and April 2021 from samples of hospitalized patients from various hospital departments.

3.2.2. Samples Collection

This study included *S. aureus* strains isolated from a variety of clinical samples sent to the NEU Hospital Microbiology Laboratory. After collecting each sample, it was cultured on Blood agar (Merck, KgaA, Germany) and Eosin Methylene Blue (EMB) agar (Becton Dickinson, Sparks, MD 211 52, USA) and incubated at 35°C for 24-48 hours to obtain pure colonies. Only colonies that grew on Blood agar media were loaded into the Vitek 2 system for bacterial identification and antibiotic susceptibility patterns; then, when the Vitek 2 device identified *S. aureus*, the bacterial colonies were transferred and stored in bacteria storage tubes (OR-BAK, Ankara, Turkey) at -30°C until used.

3.2.3. Samples Isolation and Culturing

To revive the stored samples of *S. aureus* strains isolates were inoculated on Blood agar for growth and incubated for 24-48 hours at 35°C to get pure colonies, then CRA was prepared and pure colonies from blood agar were inoculated on CRA for biofilm detection and incubated for 24-48 hours at 35°C, colonies that were black were considered biofilm positive whereas colonies that showed red were considered biofilm negative. Both Blood agar and CRA were prepared as per the manufacturer's directions as follows:

3.2.4. Preparation of Blood Agar

1. 1000 ml of purified/distilled water is applied to suspend around 40 grams of the prepared medium.

2. Heat to a boil to totally remove the medium.
3. Sterilize at 15 lbs by autoclaving. (121°C) pressure for 15 minutes.
4. The medium is then withdrawn from the autoclave and cooled to around 40-45 °C.
5. The sterile defibrinated blood with 5 percent v/v is applied aseptically and well mixed.
6. Then the media is mixed well and poured into sterile petri dishes.
7. Replace each petri dish's lid and stack the plates in a fridge.

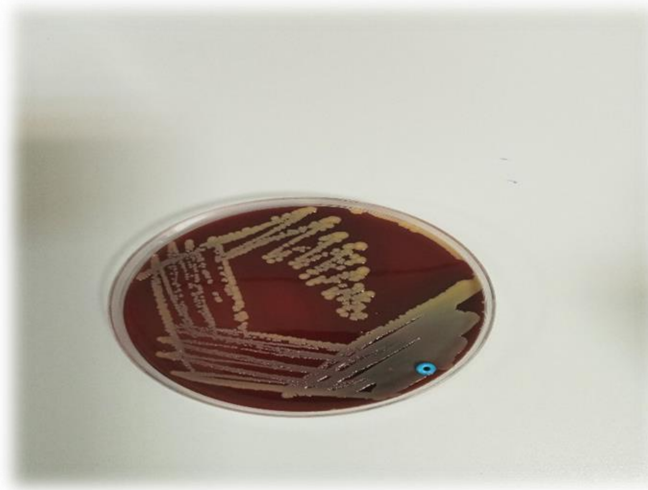


Fig. 3.1. *S. aureus* grown on Blood Agar incubated at 37°C for 24 hours

3.2.5. Preparation of Congo Red Agar

1. 1000 ml of purified/distilled water is applied to suspend around 40 gr of the Blood Agar Base-2 medium mixed well the contents of the medium.
2. 10 gr of glucose was weighed using analytical balance and added to the same flask and mixed well the contents.
3. 0.4 gr of congo red dye was measured and added to the same flask and mixed the contents very well.
4. Sterilize at 15 lbs by autoclaving (121°C) atm for 15 minutes.
5. The medium is then withdrawn from the autoclave and cooled to around 40-45 °C.

6. Then the media is mixed well and poured into sterile petri dishes.
7. Replace each petri dish's lid and stack the plates in a fridge

Three bacterial strains were used as controls for the experiment: *S. aureus* ATCC29213 was used as the positive control for biofilms, while *S. aureus* ATCC6538 and *S. epidermidis* ATCC11047 were used as negative Biofilm controls respectively. They were then incubated on CRA plates to determine whether they produce black colonies. For growth and biofilm formation, all control species were cultured on both Blood agar and CRA. The isolates were then incubated at 37°C for 24–48 hours.

3.2.6. Vitek 2 system for bacterial identification and antibiotic susceptibility testing.

Vitek® 2 compact is an automated microbial identification system developed in response to bioMerieux's extensive knowledge with microbial identification. Vitek 2® compact is intended to give ID/AST results in as little as 5 to 8 hours, using economical, ready-to-use Vitek® 2 ID/AST cards. Vitek®2 antibiotic susceptibility testing is used to identify bacteria and yeast. Additionally, the Vitek®2 is used to determine antibiotic susceptibility (AST) and resistance mechanisms. To transfer an adequate number of colonies of a pure culture, a sterile swab or applicator stick is used. Once transferred, the microorganism is suspended in 3.0 mL of sterile saline and the turbidity of the suspension is determined using a turbidity meter. Then, using an integrated vacuum apparatus, identification cards are inoculated with microorganism suspensions.

Fig.3.2.VITEK ® 2 antibiotic susceptibility testing system (Biomerieux, 2001)

3.2.7. Statistical Data analysis

All data acquired were statistically analyzed with a computer-based SPSS 20 software package. Frequency and cross-tabs analysis were used to test the totals. To discover an association between two variables, a Chi-square test was utilized with a significance level of $p<0.05$.

CHAPTER FOUR

4.FINDINGS

This thesis was conducted in the microbiology laboratory at the NEU hospital in the TRNC. A total of 67 samples for the study was collected between January 2020 and April 2021 from samples of hospitalized patients from various hospital departments and subjected to microbiological analysis to isolates *S. aureus* strains.

4.1. Congo Red Agar (CRA) preparation results

The results of CRA supplemented with glucose (10 gr) and Blood Base 2 media (40 gr/L) incubated for 24-48hours at 37°C is presented below (Fig.4.1).

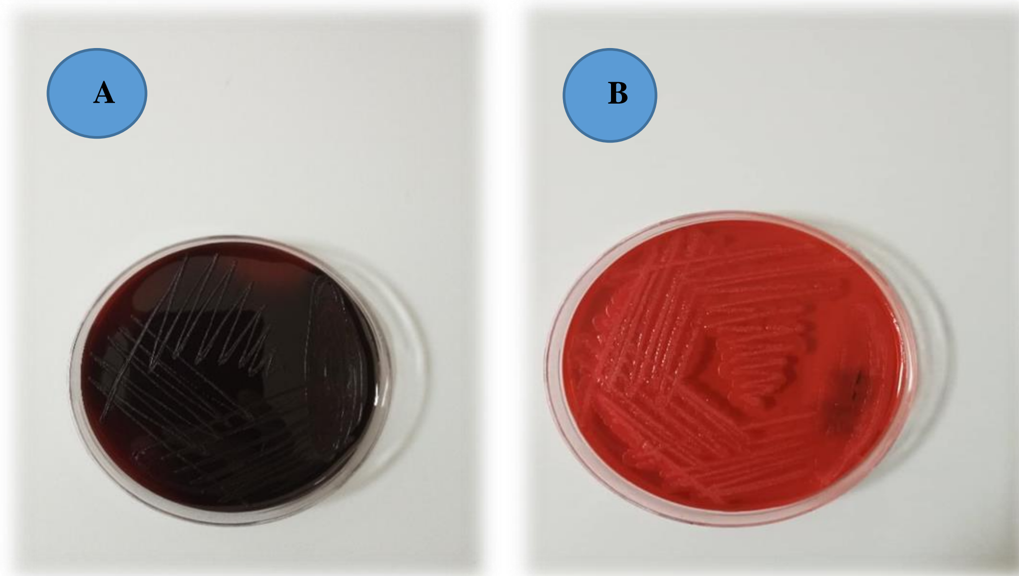


Fig. 4.1. CRA inoculated with the isolate after 48 hours incubation at 37°C(A-B)

A. *S. aureus* with crystalline black colonies indicating biofilm positive.

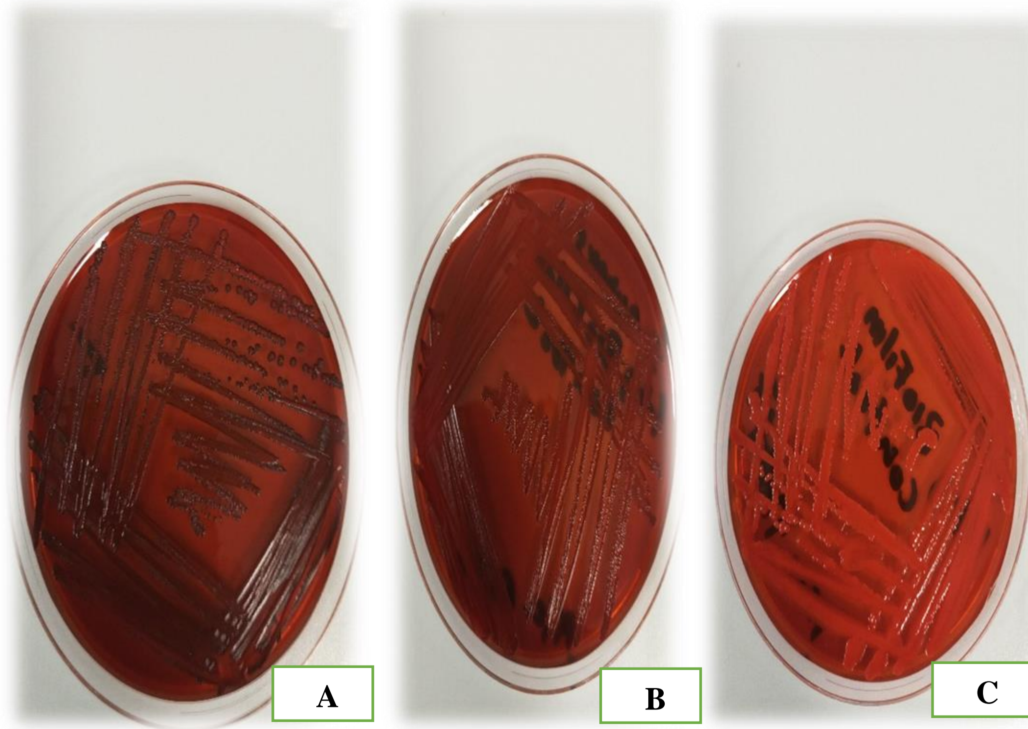
B. *S. aureus* with red colonies showing biofilm Negative.

Fig. 4.2. CRA inoculated with the isolate after 48 hours incubation at 37°C(C-D)

C. *S. aureus* with crystalline black colonies indicating biofilm positive.

D. *S. aureus* with red colonies showing biofilm negative.

Fig. 4.3. Three control strains of biofilms *S.aureus* and *S.epidermidis*(A-C)



A.*S. aureus* ATCC29213 was used as the positive control for Biofilms yielded positive.

B. *S. aureus* ATCC6538 was used as the negative control for Biofilm resulted negative.

C. *S. epidermidis* ATCC11047 was used as the negative control for biofilm resulted negative.

Three bacterial strains were used as controls for the experiment: *S. aureus* ATCC29213 was used as the positive control for Biofilms, while *S. aureus* ATCC6538 and *S. epidermidis* ATCC11047 were used as negative controls Respectively. The control strains used in this study are considered to be biofilm producers and non-biofilm producers and the results are presented below (Fig. 4.3).

Table 4.1. Gender distribution of patients

Gender	No of patients	Percentage
Male	35	52,2
Female	32	47,8
Total	67	100,0

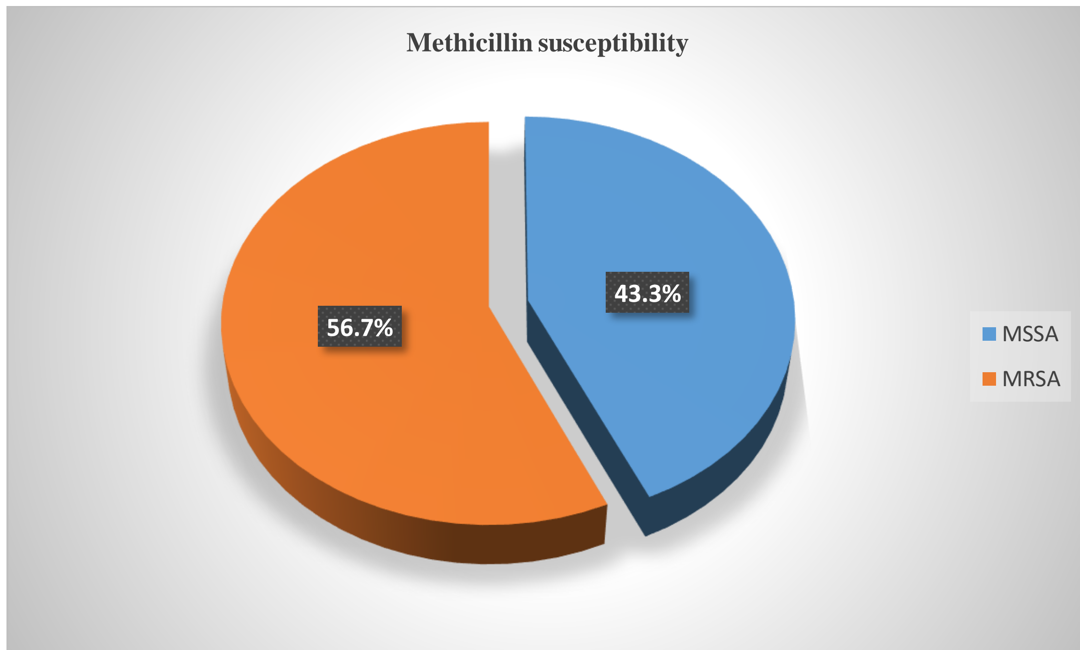
Distribution of *S. aureus* in 67 patients included in the study was dominant in male patients 35(52.2%) while 32(47.8%) were female (Table 4.1).

Table 4.2. Distribution of MRSA with in age

Methicillin susceptibility	Mean Age of Patients	No of patients	Std. Deviation
MSSA	44,24	29	28,50
MRSA	63,32	38	26,10
Total	55,06	67	28,59

The mean age of the patients with MRSA isolated was 63.32±26.10 (between 3-97 years), while the average age of patients isolated with MSSA was 44.24±28.50 (between 1-92 years). According to the data obtained, the frequency of MRSA infection increases significantly as the age gets older ($p=0.006$) as shown in Table 4.2.

Fig.4.4. Prevalence of MRSA and MSSA



Among a total of 67 *S. aureus* isolates 38 (56.7%) were identified to be MRSA by cefoxitin performed by Vitek 2 antibiotic susceptibility testing system and the remaining 29 (43.3%) to be MSSA as shown in Fig.4.4.

Table 4.3. Distribution of MRSA and MSSA in outpatients and inpatients

Patient's type	Number of MRSA (%)	Number of MSSA (%)	Total number (%)	p-value
Inpatients	29 (76.3)	14(48.3)	43 (64.2)	**0.018**
Outpatients	9 (23.7)	15(51.7)	24(35.8)	
Total	**38 (100)**	**29 (100)**	**67(100)**	

Out of 38 MRSA, 29 (76.3%) of them isolate recovered from inpatients and 9 (23.7%) from outpatients. The association between MRSA occurrences in inpatients was statistically significant (p=0.018), which demonstrated the fact that the possibility of finding MRSA in admitted patients was high as compared to the outpatients as shown in Table 4.3.

Table 4.4. Distribution frequency of *S. aureus* in different clinical samples

Clinical specimens	Number of *S. aureus*	Percentage (%)
Aspirate	9	13.4
Blood	8	11.9
Nasal swab	4	6.0
Sputum	10	14.9
Urine	8	11.9
Wound/Pus	28	41.8
Total	67	100

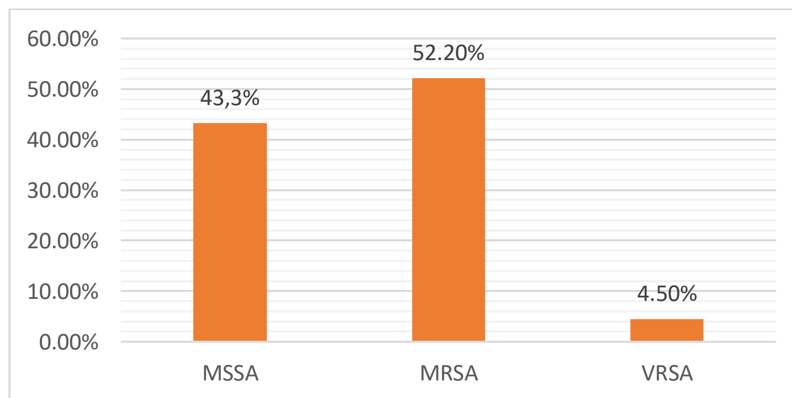
The study included a variety of clinical specimens such as aspirate, blood, nasal swab, sputum, urine, and wound/pus. The *S. aureus* obtained from these different specimens were 67 in number, of which the majority were from wound/pus swab 28 (41.8%) followed by sputum 10 (14.9%), aspirate 9 (13.4%), blood 8 (11.9%), urine 8 (11.9%), nasal swab 4 (6%), respectively.as shown in Table 4.4.

Table 4.5. Distribution of MRSA in gender

Gender	MSSA (%)	MRSA (%)	Total (%)	p-value
Male	16 (55.2)	19 (50)	35 (52.2)	**0.675**
Female	13 (44.8)	19 (50)	32 (47.8)	
Total	**29 (100)**	**38 (100)**	**67 (100)**	

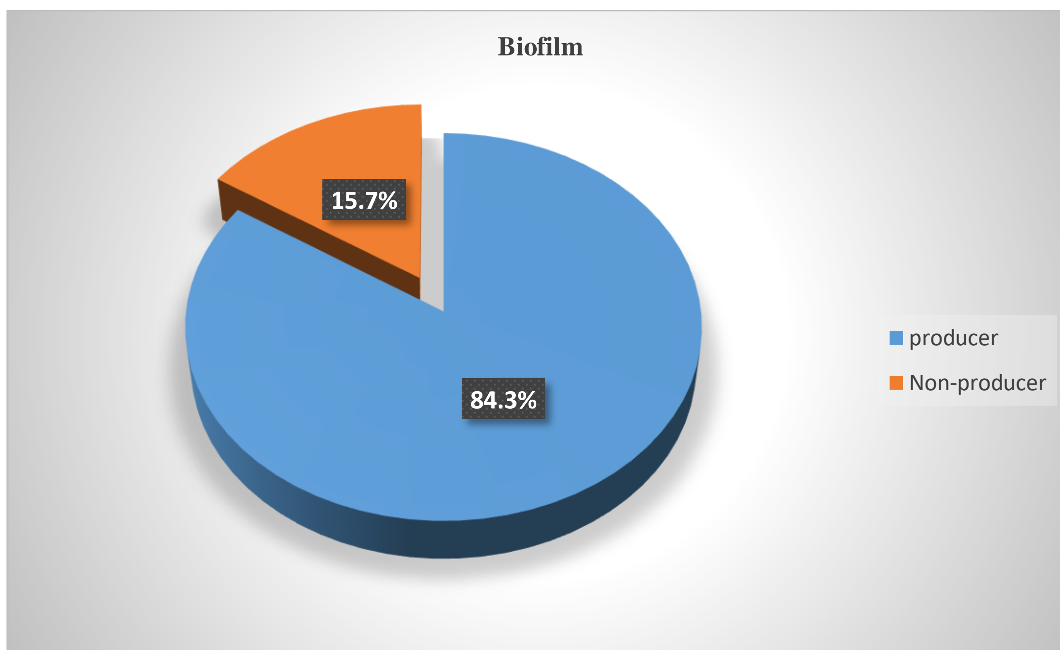
Among 67 *S. aureus* isolates 29 were MSSA and 38 were MRSA of these 35 were male and 32 were female patients no significant relationship between gender and growth of MRSA was identified (p=0.675) as presented in Table 4.5.

Fig.4.5. Distribution of strains (%)



Among 67 *S. aureus* strains recovered 52.2% was MRSA,43.3% comprised of MSSA while 4.5% were VRSA.

Fig.4.6. Rate of biofilm in CRA method



A total of 67 *S. aureus* isolates undergoing CRA method demonstrated 56 (84.3%) as biofilm producer and the rest 11 (15.7%) as a non- biofilm producer.

Table 4.6. Correlation between biofilm production and methicillin-resistance

Biofilm (CRA)	Methicillin susceptibility		**Total** (%)	**p-value**
	MRSA (%)	**MSSA (%)**		
Producer	35 (92.1)	21 (72.4)	56 (83.6)	**0.034**
Non-producer	3 (7.9)	8 (27.6)	11 (16.4)	
Total	**38(100)**	**29 (100)**	67(100)	

A statistically significant relationship was found between methicillin resistance and biofilm formation in *S. aureus* isolates. Accordingly, significantly higher biofilm formation was observed in MRSA (92.1%) compared to MSSA (72.4%) isolates (p=0.034) as shown in Table 4.6.

Table 4.7. Distribution frequency of *S. aureus* in hospital departments

Departments	No of patients	Percentage (%)
Brain Surgery	1	1.5
Cardiology	16	23.9
Chest disease and Allergy	3	4.5
Dermatology	6	9.0
Infectious diseases	14	20.9
ENT	2	3.0
General surgery	2	3.0
Gyno and Obstetrics	2	3.0
Intensive care	11	16.4
Neurology	2	3.0
Orthopedics and Traumatology	3	4.5
Pediatrics	2	3.0
Plastic surgery	1	1.4
Urology	3	4.5
Total	67	100.0

Among 67 *S. aureus* isolates the highest number were received from patients applied to the cardiology unit 16 (23.9%) followed by infectious disease and intensive care 14 (20.9%), 11 (16.4%), respectively. furthermore, the least number of samples were recovered from plastic surgery and brain surgery 1 (1.4%), 1 (1.5%), respectively.as shown in Table 4.7.

Table 4.8. Distribution of biofilm with in age

Biofilm	Mean Age	No of patients	Std. Deviation
Negative	42,73	11	31,92
Positive	57,48	56	27,55
Total	55,06	67	28,59

The mean age of the patients with biofilm positive isolated was 57.48±27.55 (between 3-97 years), while the average age of patients isolated with negative biofilm was 42.73±31.922 (between 1-92 years). According to the data obtained, the chance of biofilm formation increases with age but there is no statistically significant difference in age of patients and biofilm formation ($p=0.118$) (Table 4.8).

Table 4.9. AST pattern of *S. aureus* from different clinical specimens

Antibiotics	MSSA	MRSA	P value
Benzylpenicillin	22/27 (81.5)	33/34 (97.1)	0.055
Gentamicin	0/29 (0)	0/35 (0)	-
Ciprofloxacin	1/29 (3.4)	7/36 (19.4)	0.054
Levofloxacin	1/29 (3.4)	5/38 (13.2)	0.174
Clindamycin	10/29 (34.5)	27/36 (75.0)	**0.001**
Linezolid	1/27 (3.7)	0/35 (0)	0.435

Daptomycin	0/28 (0)	2/35 (5.7)	0.305
Teicoplanin	1/28 (3.6)	2/35 (5.7)	0.584
Vancomycin	0/28 (0)	3/36 (8.3)	0.171
Tetracycline	5/29 (17.2)	17/36 (47.2)	**0.011**
Tigecycline	0/29 (0)	0/35 (0)	-
Fosfomycin	0/29 (0)	3/34 (8.8)	0.151
Fusidic acid	0/27 (0)	3/34 (8.8)	0.166
Mupirocin	1/27 (3.7)	0/32 (0)	0.458
SXT	3/29 (10.3)	2/38 (5.3)	0.372

***SXT:** Trimethoprim/Sulfamethoxazole

All *S. aureus* isolates were tested for their sensitivity against 16 commonly used antibiotics. Resistance rates of the MRSA isolates were significantly higher towards benzylpenicillin 33 (97.1%), clindamycin 27 (75%) and tetracycline 17 (47.2%) compared to MSSA isolates. The resistance rates of MSSA isolates were also the highest to benzylpenicillin 22 (81.5%), clindamycin 10 (34.5%), and tetracycline 5 (17.2%). But lower rates of resistance were observed in linezolid 1 (3.7%) towards MSSA, however, linezolid showed no resistance towards MRSA 100% susceptibility, making the most effective antibiotic for severe MRSA infections and can be used as empiric therapy. On the other hand, ciprofloxacin, levofloxacin, daptomycin, mupirocin and trimethoprim/sulfamethoxazole showed less resistance towards both MRSA and MSSA. Furthermore, the MRSA isolates showed a statistically significant resistance pattern against the following antibiotics: clindamycin and tetracycline compared to MSSA ($p<0.05$; Table 4.9). Almost all isolates were sensitive to tigecycline and gentamycin 100%. Interestingly, MRSA isolates were even resistant to vancomycin and teicoplanin 3 (8.3%), 2 (5.7%), respectively. On the other hand, no MSSA was found resistant to vancomycin but it was resistant to teicoplanin 1 (3.6%) as shown in Table 4.9.

CHAPTER FIVE

5.DISCUSSION AND RESULTS

Nosocomial infections are a severe and persistent issue in the hospital setting. MRSA is a significant human pathogen. It causes various diseases in humans, ranging from skin infections to severe infections like pneumonia, soft tissues, bones, heart valves, and even fatal septicemia. In recent years, the number of infections caused by MRSA isolates has dramatically risen, and they are associated more frequently with mortality than with other bacterial infections. *S. aureus* is one of the most causes of bacteremia and, despite appropriate treatment, currently has a death rate of 20-40 percent at 30 days. Due to the resurgence of antimicrobial resistance in *S. aureus*, these infections have become more dangerous and costly in recent 20 years because of the extensive use of antibiotics (Piechota et al., 2018).

A biofilm consists of any population of microorganisms that are syntrophically linked to form a cellular matrix composed of extracellular polysaccharide (slime) that both sticks the members of the consortium to the internal surface of the organism and prevents it from being affected by the host resistance and antimicrobial treatments. Antoni van Leeuwenhoek was the first to find animalcules on the tooth's surface using a basic microscope, and this was widely accepted as a discovery in biofilms. Adhesion to the inner surface of the biofilm is produced by the biofilm-associated protein, and fibronectin-binding proteins, *S. aureus* surface protein G, which combine to create a polysaccharide-containing extracellular matrix (Sharma et al., 2021).

In this study, the CRA method was used to detect biofilm production by *S. aureus.* A standard microbiological method was used to isolate *S. aureus* from the strains for the isolation of the test organism. A total of 67 *S.* aureus isolates were incorporated into this

study, together with three control strains *S. aureus, and S. epidermidis,* as positive and negative biofilm controls, respectively.

The present study encountered difficulties in producing the required colonies on the first set of the experiment. The cultural method failed to yield positive black colonies, an indication that a biofilm had formed. This was attributed mainly to a lack of adequate sugar (polysaccharide) in the media and the test organism's inability to produce enough, the primary component of biofilm; extracellular. Periasamy et al. (2011) demonstrated that ECM contributes to intracellular aggregation, which aids in proliferation; additionally, the authors showed that the matrix of Staphylococci contains numerous secreted polymers such as exopolysaccharide, teichoic acid, and specific proteins, as well as DNA from lysed cells.

Among 67 isolates of *S. aureus*, 35 (52.2%) and 32 (47.8%) were obtained from male and female patients, respectively. In another study conducted by (Sharma et al., 2021), male patients were dominant 60.65%, 39.35% in males and females, respectively. In our current study, the prevalence of MRSA was found to be high 38 (56.7%) compared to MSSA 29 (43.3%), with a similar number reported by (Belbase et al., 2017). MRSA 36 (47.4%), and MSSA 17 (22.4%) but a higher number was written by (Piechota et al., 2018) MRSA 73 and MSSA 57. And a lower number of MRSA was reported, that is, 26.12% (Pandey et al., 2013). Of the 38 (56.7%) MRSA strains, 3 (4.5%) were resistant to vancomycin which is comparable to the results of Jahanshahi et al., (2018).

In this study, the highest number of samples were received from patients applied to the cardiology unit 16 (23.9%) followed by infectious disease and intensive care 14 (20.9%), 11 (16.4%), respectively. A study carried out by (Horváth et al., 2020) reported that the highest number of samples were recovered from the following wards: internal medicine unit (42.2%), intensive care unit (18.0%), hematology (15.7%), with fewer isolates recovered from cardiology (10.3%), surgery ward (6.5%), transplant clinic (3.9%) and pulmonology ward (2.9%), this may be due to differences in geographic locations.

Furthermore, a maximum number of *S. aureus* and MRSA were isolated from wound/pus swab 28 (41.8%), followed by sputum 10 (14.9%), aspirate 9 (13.4%), blood 8 (11.9%), and urine 8 (11.9%) that allied with the result of (Ansari et al., 2014), elucidating the role of organisms in pyogenic infection (Pandey et al., 2013).

This study discovered that patient age was a risk factor for MRSA infection in admitted patients. The mean age of patients with MRSA infection was 63.32±26.10 (range: 3-97 years), whereas the average age of patients with MSSA infection was 44.24±28.50 (between 1-92 years). According to the data obtained, the prevalence of MRSA infection increases significantly with age (p=0.006), which is in agreement with the findings of (Kshetry et al., 2016), who found that 29 strains of MRSA were isolated from adults and 18 strains were isolated from pediatric patients, with the difference being statistically insignificant (p>0.05). Similarly, 30 MRSA strains were isolated from males and 17 females (Kshetry et al., 2016).

While MRSA isolates were more prevalent in male and female subjects 35 and 32, respectively, no significant relationship between gender and MRSA growth (p=0.675). In our study, a more substantial proportion of MRSA isolates were obtained from hospitalized patients 29 (76.3%). Colonized health care workers in hospitals are the primary source of MRSA infection in hospitalized patients, resulting in increased infection rates. However, the isolation rate of MRSA was low among outpatients 9 (23.7%). Additionally, admitted patients who became colonized during their hospital stay might act as secondary sources of community-acquired MRSA infections. The higher rate of MRSA infection in admitted patients was statistically significant (p=0.018), which is consistent with (Belbase et al., 2017) findings of 54.5% and 41.9%, respectively, in inpatients and outpatients. 66.9% and 33.1%, respectively, as outpatients and inpatients, in another study (Ansari et al., 2014). This difference could be explained by a prolonged hospital stay, instrumentation, and other invasive devices, as well as the fact that *S. aureus* is mostly associated with nosocomial infections.

Numerous studies have been conducted on producing biofilms by *Staphylococcus* species using various methods (Croes et al., 2009; Walker and Horswill, 2012; Metzler, A., 2016;). It was revealed in this study the technique used was capable of detecting the formation of biofilms between isolated strains. The current study evaluated the production of biofilms/ESPs by 67 *S. aureus* strains by producing black biofilm colonies on CRA. Out of 67 cultures inoculated on CRA, 56 (84.3%) were identified as *S. aureus* producing biofilm, which is comparable to the results of Sharma et al., (2021), which identified 53 (80%) as *S. aureus* having biofilm. However, (Haghi Ghahremanloi Olia et al., 2020) reported a higher rate of biofilm production, 57(95%), which could be explained by the imprecision with which this method identifies moderate biofilm-producing strains (Hassan et al., 2011).

Because biofilms are protective, bacteria growing in them are intrinsically resistant to a wide variety of antibiotics. Positive biofilm producers were detected in 92.1 % of MRSA samples and 72.4 %of MSSA samples. A statistically significant relationship between methicillin resistance and biofilm formation in *S. aureus* isolates ($p=0.034$), consistent with the results of (Khasawneh et al., 2020), indicated that 90.9% of MRSA and 71.4% of MSSA isolates were resistant to methicillin. According to a study conducted by (Grinholc et al., 2007), only 45-47% of MRSA strains and 66-69% of MSSA strains could form biofilms in vitro. Certain strains have been reported to produce no biofilm despite the presence of a locus. Biofilm formation is widely regarded as a significant factor in the virulence of antibiotic-resistant bacteria, particularly MRSA. Further phenotypic and genotypic characterization of the ica locus genes is required to better understand the mechanism of biofilm production in staphylococcal infections (Darwish & Asfour, 2013).

The mean age of patients isolated with positive biofilm was 57.48±27.55 (range: 3-97 years) in the current study, whereas the average age of patients isolated with negative biofilm was 42.73±31.922. (Between 1-92 years). According to the data collected, the

likelihood of developing a biofilm increases with age, but there is no statistically significant association between patient age and biofilm formation (p=0.118).

Most antibiotics used in *Staphylococci* infections are increasingly resistant to multi drugs. The development of MRSA among *S. aureus* strains led to problems in the treatment of these infections. Monitoring *S. aureus'* antimicrobial susceptibility patterns is of prime significance to understanding new emerging resistance trends and treating infections in hospitals and the community (Ansari et al.,2014).

This study found that commonly used antibiotics were more resistant to MRSA than to MSSA; the highest resistance rates were observed for benzylpenicillin 33 (97.1%), clindamycin 27 (75%), and tetracycline 17 (47.2%). In this study, a high proportion of isolates (97.1%) were penicillin-resistant. This was expected, as only a minority of *S. aureus* strains do not produce beta-lactamases. In a study carried out by (Ansari et al., 2014), a comparable rate of resistance to penicillin was observed (94.7%).

MRSA is commonly treated with Clindamycin, an antibiotic. Other types of antibiotics, like macrolides, can also lead to macrolide-resistant strains of *Staphylococcus aureus.* Resistance to macrolides, on the other hand, can occur due to mutation of the 23S rRNA encoded by the erm gene, known as MLSB resistance, and is also referred to as clindamycin resistance or MLSB resistance (due to efflux mechanism encoded by the msrA gene) Failure could occur if the treatment is not effective against a strain of bacteria that contains an erm gene, which can inducible resistance. In our study, we identified 27 (75%) MRSA resistant strains and 10 (34.5%) MSSA Resistant strains towards clindamycin which agrees with the findings of (Horváth et al., 2020), indicating that clindamycin resistance is present in 79.1% of cases.

In our study, the two most effective antibiotics are tigecycline and gentamycin with 100% susceptibility towards all isolates, followed by linezolid, mupirocin, and

daptomycin as shown in Table.4.9. According to the findings of this study, *S. aureus* forms a biofilm. This finding is clinically significant because biofilm formation is associated with the pathogenicity of organisms that cause device-related implant infections and high resistance to antibiotics; additionally, the CRA used in this study to detect biofilm was a reliable method; moreover, the prevalence rate of isolation of MRSA from hospitalized patients with *S. aureus* positive cases was high.

In hospital settings, the wound/pus was the primary source of *S. aureus* and MRSA. The study's gender-based evaluation revealed a higher prevalence of MRSA in male patients than in female patients, with the mean age of patients isolated with MRSA being 63.32±26.10 (between 3-97 years), compared to 44.24±28.50 for patients isolated with MSSA (between 1-92 years). Tigecycline and gentamycin (100%) were the drugs of choice for treating *S. aureus* infections, including MRSA, followed by linezolid, mupirocin, and daptomycin. MRSA strains exhibited multidrug resistance and were unusually resistant to vancomycin, the drug of choice, indicating that MRSA is a vibrant organism. As a result, we recommend that this threat be mitigated through the implementation of sound infection control policies, regular surveillance of the antibiotic profile of *Staphylococcus* isolates to establish antibiotic policies, and the reasonable use of antimicrobial agents. Additionally, because this study only qualitatively presents biofilm in isolates, additional research is recommended that further research be conducted on the molecular mechanisms involved. There is a need for detailed information on the molecular mechanisms underlying biofilm formation and its relationship to other microbial processes such as virulence and antibiotic resistance.

With the advent of molecular methods such as the polymerase chain reaction (PCR), which amplifies the genes responsible biofilm formation, will significantly aid in validating the methods used in this study and reinforcing other methods used elsewhere. These are phenotypic techniques that include CRA and TCP assay. The recommendation to expand the use of molecular techniques for biofilm detection will not only aid in

characterizing the genes involved in biofilm formation. but it will also enable in determining whether such genes are associated with other organisms' activity, such as resistance.

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Near East University / Faculty of Medicine	**Medical and Clinical microbiology**	**Master (M.Sc.)**	**Cyprus**	**2020-2021**

Masters Thesis	
Title:	**Investigation of invitro biofilm formation and correlation with antibiotic resistance pattern among clinical isolates of** ***Staphylococcus aureus***

Advisor:	Eşref Çelik MD Assistant Professor

Job Experience

Duty	Place	Duration
Pharmacist Assistant	Mogadishu-Somali-Turkish Research and Training Hospital	2017-2019
Working in Laboratory	Ilhan Clinic	2016-2017
Working in Microbiology Lab / Near East Hospital	Near East Hospital	2020-2021

Courses and Certificate

Name	Name of the Institution where take place	Year
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Online Webinar on Emerging Trends in Allied Health sciences (Attendance)	B.N. Patel Institute of Paramedical and science, India	2021
e-symposia on COVID-19: One Year Later (Attendance)	Keystone symposia,	2018
Virtual conference on Immunology aspects of coronavirus: Epitope Prediction and *insilico* vaccine design organized by LLB-School	LLB-School, University of Cambridge, United Kingdom	2020

Course of pharmacology	Mogadishu-Somali Turkish Research and Training Hospital	2018
International Webinar "From Bench to Bedside: The Story of How Medicines are made (online attendance)	Johnson and Johnson lnc., USA	2020
Virtual webinar on Development of Novel Vaccines for SARS-COV-2(Attendance)	Somali Medical Association (SMA), Somalia	2021

Computer Knowledge

Program	Use proficiency
SPSS	Good
Common Computer Programs and Skills	Excellent

Other languages

Languages	Speaking	Writing	Reading
Somali	Excellent	Excellent	Excellent
English	Excellent	Excellent	Excellent
Arabic	Good	Excellent	Excellent
Turkish	Good	Excellent	Excellent

MRSA and Biofilm 2

ORİJİNALLİK RAPORU

%14	%5	%14	%
BENZERLİK ENDEKSİ	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCİ ÖDEVLERİ

BİRİNCİL KAYNAKLAR

1	Derek E. Moormeier, Kenneth W. Bayles. " biofilm: a complex developmental organism ", Molecular Microbiology, 2017 Yayın	%3
2	"Posters", Clinical Microbiology and Infection, 2011 Yayın	%1
3	Raziey Parastan, Mohammad Kargar, Kavous Solhjoo, Farshid Kafilzadeh. "Staphylococcus aureus biofilms: Structures, antibiotic resistance, inhibition, and vaccines", Gene Reports, 2020 Yayın	%1
4	doi.org İnternet Kaynağı	%1
5	Ravichandran Swathirajan Chinnambedu, Ragavan Rameshkumar Marimuthu, Suhas	%1

Solomon Sunil, Pradeep Amrose et al. "Changing antibiotic resistance profile of Staphylococcus aureus isolated from HIV patients (2012–2017) in Southern India", Journal of Infection and Public Health, 2020