BAŞKENT UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF MEDICAL MICROBIOLOGY

MASTER'S PROGRAM WITH THESIS

PHENOTYPIC AND GENOTYPIC ANALYSIS OF MACROLIDE-LINCOSAMIDE-STREPTOGRAMIN B RESISTANCE AMONG METHICILIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AND METHICILIN-SUSCEPTIBLE *STAPHYLOCOCCUS AUREUS* ISOLATES

NOURA SAED MAHMOUD AETEER

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THESIS ADVISOR:

DR. AYLIN ALTAY KOCAK

ANKARA - 2023

BAŞKENT UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

This study, which was prepared by Noura SAED MAHMOUD AEETER within the framework of the Department of Medical Microbiology Master's Program, was accepted as the Master's Thesis by the following jury.

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Thesis Jury Members (Title, Name - Surname, Institution) Signature

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MASTER'S THESIS STUDY ORIGINALITY REPORT

Date: 24/07/2023

Student's Name, Surname: NOURA AETEER

Student's Number: 22020134

Department: Department of Medical Microbiology

Program: Thesis Master's Program

Advisor's Title/ Name, Surname:

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ÖZET

Noura Saed Mahmoud Aeteer, Metisilin Dirençli ve Metisilin Duyarlı *Staphylococcus aureus* İzolatlarında Makrolid-Linkozamid-Streptogramin B Direncinin Fenotipik ve Genotipik Analizi, Başkent Üniversitesi, Sağlık Bilimleri Enstitüsü, Yüksek Lisans programı, 2023.

Metisiline dirençli Staphylococcus aureus suşları çoklu ilaç direnci nedeniyle ciddi bir tedavi sorunu oluşturmaktadır. Makrolid linkozamid streptogramin B (MLSB) direncine yol açan *erm* gen bölgelerinin hızlı iletimi, eritromisin gibi geleneksel makrolidlerin klinik uygulamasını ciddi şekilde sınırlamaktadır. Bu çalışmada Başkent Üniversitesi Hastanesi'nde izole edilen MRSA ve MSSA izolatlarının MLSB fenotipi ve genotipik direnç genlerinin karakterize edilmesi amaçlanmış ve MSSA ve MRSA izolatlarının direnç oranları karşılaştırılmıştır. Çalışmaya Başkent Üniversitesi Ankara ve Adana Hastanelerinden 2016-2022 yılları arasında toplanan 50 MSSA ve 50 MRSA izolatı dahil edilmiştir. İlk olarak *S. aureus* izolatları katalaz ve koagülaz testleri ile, MRSA izolatları ise sefoksitin disk difüzyon testi ile doğrulanmıştır. MLSB direncini belirlemek için izolatlar, disk difüzyon testi (D-test) kullanılarak klindamisin ve eritromisin direnci açısından çalışılmıştır. D-test sonuçlarına göre direnç fenotipleri saptanmış ve dirençli fenotipler polimeraz zincir reaksiyonu (PZR) kullanılarak direnç genleri açısından taranmıştır. *ermA, ermB, ermC* ve *msrA* genlerine spesifik primerler kullanılarak PZR amplifikasyonu yapılmıştır. 50 MRSA izolatından 25'i (%50) eritromisine dirençli, klindamisine duyarlı ve D-zonu pozitif olacak şekilde indüklenebilir direnç fenotipini (iMLSB) göstermiştir. İki (%4) izolat, yapısal direnç fenotipini (cMLSB) gösteren D-zonu negatif, klindamisin ve eritromisin dirençli saptanmıştır. Yirmi iki (%44) izolat ise S fenotipini gösteren D-zonu negatif, klindamisin ve eritromisin duyarlı saptanmıştır. Sadece bir izolat (%2) klindamisine duyarlı, eritromisin dirençli ve MSB fenotipine uygun olarak D-zonu negatif bulunmuştur. 50 MSSA izolatı 8'i (%16) eritromisine dirençli D-zonu pozitif iMLSB fenotipine sahiptir. İki (%4) izolat ise D-zonu olmaksızın her iki antibiyotiğe de dirençli cMLSB fenotipine sahiptir. S fenotipini gösteren D-zonu olmayan klindamisin ve eritromisin duyarlı 40 (%80) izolat saptanmıştır. Direnç genleri toplam 38 örnekten 28 MRSA ve 10 MSSA örneğinde incelenmiştir. *ermC*, 25 MRSA (%89,3) ve 4 MSSA örneğinde (%40) pozitif bulunmuştur. İkinci sırada saptanan gen *ermA* olmuştur; 4 MSSA izolatında (%40) saptanmış,

MRSA izolatlarında saptanmamıştır. MSB fenotipli bir MRSA örneğinde *msrA* geni pozitif olarak doğrulanmıştır. Tüm örnekler *ermB* geni için negatif bulunmuştur. Sonuç olarak, Ankara ve Adana'dan izole edilen izolatlarda gerçekleştirilen bu çalışmada, iMLSB fenotipi en yaygın fenotip olarak bulunmuş ve MRSA izolatlarında daha yaygın olduğu görülmüştür. MLSB direnci için direnç genleri arasında en sık görülen gen *ermC* olmuştur. ermA pozitifliği ise az sayıda saptanmış ve *ermB* geni hiç saptanmamıştır. Dolayısıyla *ermC* geninin bu bölgede daha yaygın olduğunu söyleyebiliriz.

Anahtar Kelimeler: *Staphylococcus aureus*, makrolid, linkozamid, streptogramin B, erm geni, msr geni.

Bu çalışma Başkent Üniversitesi Tıp ve Sağlık Bilimleri Araştırma Kurulu tarafından onaylanmış ve Başkent Üniversitesi Araştırma Fonunca desteklenmiştir (Proje No: KA22/361).

ABSTRACT

Noura Saed Mahmoud Aeteer, Phenotypic and Genotypic Analysis of Macrolide-Lincosamide-Streptogramin B Resistance Among Methicilin-Resistant *Staphylococcus aureus* and Methicilin-Susceptible *Staphylococcus aureus* Isolates, Baskent University, Institute of Health Sciences, Master's program, 2023.

Methicillin-resistant *Staphylococcus aureus* strains pose a serious treatment problem because of their multidrug resistance. The rapid transmission of the *erm* gene leading to macrolide lincosamide streptomycin B (MLSB) resistance severely limits the clinical application of traditional macrolides such as erythromycin**.** In this study, we aimed to characterize the MLSB phenotype and genotypic resistance genes of MRSA and MSSA isolates from Baskent University Hospital, and resistance rates of MSSA and MRSA isolates were compared. The study included 50 MSSA and 50 MRSA isolates collected between 2016 and 2022 from Baskent University in Ankara and Adana Hospitals. First, the *S. aureus* isolates were confirmed by catalase and coagulase tests, and the MRSA and MSSA isolates were confirmed by cefoxitin disc diffusion test. To determine the MLSB resistance, isolates were tested for clindamycin and erythromycin resistance using the disk diffusion (D test) test. According to D test, resistance phenotypes were detected and resistant phenotypes were screened for resistance genes using polymerase chain reaction (PCR). PCR amplification was made using primers specific for *ermA*, *ermB*, *ermC*, and *msrA* genes. Among 50 MRSA isolates, 25 (50%) isolates were resistant to erythromycin with D zone positivity and susceptible to clindamycin indicating inducible iMLSB phenotype. And 2 (4%) isolates were resistant to clindamycin and erythromycin without the D zone indicating constitutive cMLSB phenotype. Twenty-two (44%) isolates were susceptible to clindamycin and erythromycin with D-zone negative indicating S phenotype. Only one isolate (2%) was susceptible to clindamycin and has erythromycin resistance with absence of D-zone indicating MSB phenotype. Among all 50 MSSA isolates, 8 (16%) isolates showed iMLSB phenotype which were resistant to erythromycin with D zone positivity. Two (4%) isolates showed cMLSB phenotype which were resistant to both antibiotics without D zone. There were 40 (80%) isolates that were susceptible to clindamycin and erythromycin without D zone indicating S phenotype. We examined resistance genes in 28 MRSA and 10 MSSA isolates out of a total 38 isolates for resistance

genes. For MRSA, *ermC* was positive in 25 isolates (89.3%) and positive for 4 MSSA isolates (40%). The second common gene was *ermA*; it was detected in 4 MSSA isolates (40%) and was not detected in MRSA isolates. *msrA* gene was positive in one MRSA isolate with MSB phenotype. All samples were negative for the *ermB* gene. In conclusion, iMLSB phenotype was the most common phenotype and it was more common in MRSA isolates in the present study that were carried out in the isolates from Ankara and Adana, Turkey. Among resistance genes for MLSB resistance, the most frequent gene was *ermC*. *ermA* positivity was less and *ermB* was not detected. Therefore, we can say that the *ermC* gene is more common in this region.

Keywords: *Staphylococcus aureus,* macrolide, lincosamide, streptogramin B, *erm* gene, *msr* gene.

This study was approved by Baskent University Institutional Review Board (Project no: KA22/361) and supported by Baskent University Research Fund.

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LIST OF ICONS AND ABBREVIATIONS

ABE	Staphylococcal acute bacterial endocarditis
agr	AgrAC system
Avb TcR	T cell receptor
Cap	community-acquired pneumonia
CA-MRSA	community-associated MRSA
CLSI	Clinical and Laboratory Standards Institute
ClI	Clindamycin
cMLSB	constitutive macrolide lincosamide streptogramin B
CNS	central nervous system
Coa	coagulase
Cpc	capsular polysaccharide
Cps	coagulase-positive staphylococci
C3b	complement component c3d
D-Ala4	D-alanine 4
DNase	Deoxyribonuclease
Ecm	Extracellular matrix
Ery	Erythromycin
ETS	Exfoliative toxins
HA-MRSA	Healthcare-Acquired MRSA
HLA	Human leukocyte antigen
iMLSB	inducible MLSB

IL1-IL2	interlukin1-interlukin 2
IgG	immunoglobulin g
LTA	Lipoteichoic acid
MRSA	Methicillin resistant *S. aureus*
MSSA	Methicillin-sensitive *S. aureus*
MLSB	macrolide-lincosamide streptogramin B
MHC	major histocompatibility complex
MIC	Minimum inhibitory concentration
MHA	Muller-Hinton agar
NaCl	sodium chloride
PBP2	penicillin-binding protein 2
PTSAgs	pyrogenic toxin superantigen
PT	streptococcal pyrogenic exotoxin
Polyrbop	poly ribitol phosphate
S. aureus	*Staphylococcus aureus*
SES	staphylococcal enterotoxins
SAK	Staphylokinase
SSIS	Surgical site infection
SSTIS	skin and soft tissue infections
SFP	Staphylococcal food poisoning
SCCmec	staphylococci chromosome cassette mec
TCS	Two-component system
TSST-1	Toxic shock syndrome toxin -

TNF-a	Tumor necrosis factor-alpha
TMP-SMX	Trimethoprim-sulfamethoxazole
UTA	urinary tract infection
US	United States
VWbp	Von Willebrand factors binding protein
WTA	Wall teichoic acids
WGA	Whole genome sequencing
WHO	World Health Organization

1. INTRODUCTION

Staphylococcus aureus is one of the gram positive cocci bacteria which is 1 μm in diameter. They have usually grape-like appearance with clusters under microscope. They are non-motile, non-spore- forming and generally non-capsulated bacteria. They can grow on various culture media in 24 hours at 37ºC. The colony structure of *S. aureus* is round, 2-3 mm in diameter with smooth surface and opaque appearance and some strains have golden-yellow pigment [1]. It is an opportunistic pathogen which causes various infections, such as skin infections, abscess, wound infections, and also fatal diseases, such as endocarditis, osteomyelitis, or septicemia [2].

In 1928, Alexander Fleming discovered penicillin and won the Nobel Prize for his discovery. Twenty years later from the discovery of the penicillin, penicillin resistant staphylococci have spread all over the world. In 1959, methicillin which is a semi-synthetic penicillin and insensitive to penicillinases was presented for treatment. However, after a year, the isolation of methicillin-resistant *S. aureus* (MRSA) is done for the first time. After ten years, it became one of the global reasons of healthcare-associated MRSA outbreaks [3]. MRSA is one of the important health problems as an opportunistic pathogen [4]. The MRSA rates are increasing in the hospital and community settings in the USA, Asia, and some parts of Europe [4]. MRSA has also been found in Turkish hospitals as an important problem [4].

Methicillin-resistant *S. aureus* strains are resistant to every antibiotic with β-lactam ring, such as carbapenems and cephalosporins. Only the novel MRSA-active antibiotics like ceftaroline can be effective against MRSA. Also, MRSA gains resistance against antibiotics without β-lactam ring such as erythromycin (ERY) and clindamycin (CLI). Resistance to CLI and ERY in *Staphylococci*, happens via ribosomal target site methylation and it is usually encoded by erm genes. When strains are resistant to ERY and CLI, this resistance is called constitutive, when strains are ERY resistant and susceptible to CLI in vitro, this resistance is inducible. Inducible resistance can cause difficulties in clinical treatments [5]. Beside nowel antimicrobials have been presented, clindamycin supports treatment choices with its great pharmacologic features for MSSA and MRSA. Also, for penicillin-allergic patients, clindamycin is a good alternative. However, drug

inactivation mechanisms, efflux or target site modification can cause resistance to MLSB antibiotics [6].

The aim of this study is the detection of phenotypic and genotypic MLSB resistance in MRSA and MSSA isolates in Baskent University Hospital and the comparison of the resistance rates of MSSA and MRSA isolates.

1.1. Classification

Staphylococcus aureus belongs to the family *Micrococcaceae*. Some studies about molecular taxonomy of *Staphylococci* showed that the *Staphylococcus* genus is in the *Bacillus-Lactobacillus-Streptococcus* cluster, and it is close to *Enterococcus, Bacillus,* and *Listeria* [7]. Taxonomically *the Staphylococcaceae* family is located in the middle of *Bacilliaceae* and *Listeriaceae* according to the 16S rRNA sequencing [8]. *S. aureus* is a coccoid bacterium of the Firmicutes phylum and until now it's the most clinically relevant species [9]. *Staphylococcus aureus* belongs to the genus *Staphylococcus* and it includes a lot of species such as *S. epidermidis*, *S. saprophyticus*, and *S. haemolyticus*. Staphylococci species can be classified biochemically by several tests such as, catalase, coagulase and oxidase [10,11]. *Staphylococcus* genus is complex and includes more than forty species with several subspecies [12].

1.2. Structure

1.2.1. Cell wall

Layers combined with peptidoglycan structure compose the cell wall. There are two different forms of teichoic acid that cover 40% of the cell wall; one is cell wall teichoic acid (WTA) which is combined in the cell wall and the other one is cytoplasmic membrane lipoteichoic acid (MAL) which is embedded into the lipid layer. In bacterial cells teichoic acids function in material transport [11]. WTA and MAL bind to the peptidoglycan layer covalently or implanted into the cell membrane. The 90% of the weight of the cell wall is carved out by peptidoglycan and teichoic acid structures, the remaining is comprised of surface proteins, exoproteins, and peptidoglycan hydrolases [13]. Cell walls of *S. aureus* contain murein teichoic acids and wall-associated surface proteins. Murein layer includes glycan fibers, which are cross-linked by peptide bridges. Glycan fibers are composed of N-acetylglucosamine and N-acetylmuramic acid structures [14]. Cell wall

teichoic acid is composed of polyribitol phosphate chains, in many *S. aureus* strains, attached with disaccharide connection part to C6-hydroxyl group of discontinious N-acetylmuramic acid sediments inside peptidoglycan. MAL combination differs less than WTA, and bacteria usually comprises polyglycerol phosphate bound to cellular membrane with a glycolipid anchor [15].

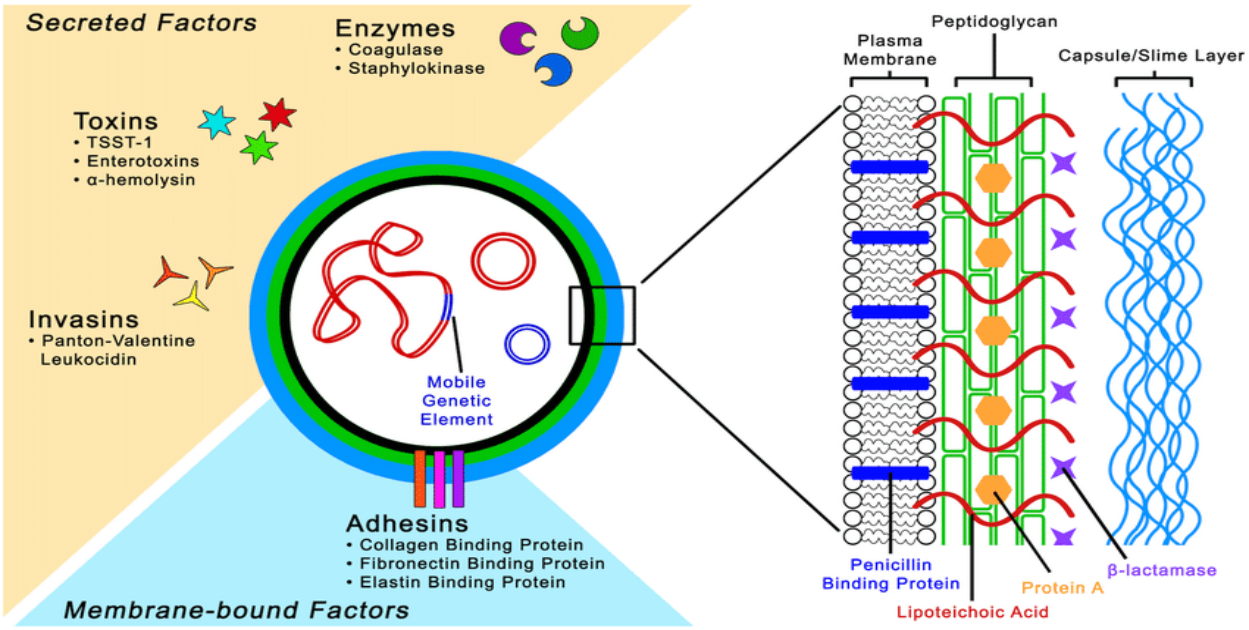


Fig 1. Cell structure and virulence factors of *Staphylococcus aureus* [16]

1.3. Pathogenesis of S*taphylococcus aureus*

Most strains have the ability to reduce the body defenses and invade and colonize the tissues by having a large number of cell-associated and extracellular factors [1]. *S. aureus* infections start with asymptomatic colonization or, more infrequently and particularly in the hospital settings, can occur from infected fomites or transmission from other patients. However, systemic *S. aureus* infection is dependent on the ability of bacteria to break the epithelial protective layer. For illustration, skin infections can begin as minor scratches of the skin and then convert to invasive also, the contamination that happen in indwelling medical devices gives another route of infection that happen frequently in the hospital settings [17]. *Staphylococcus* can produce disease by two causes; one of them is making toxins and the other is by growing in tissue and causing inflammation [18]. *S. aureus* can invade the tissues under the skin and cause local abscess lesions,

and if it enters to lymphatic or blood system it can cause septicemia due to the trauma or surgery; skin and mucous membranes are known as great barriers against local tissue invasion by *S. aureus.* It can cause a skin lesion called pyogenic abscess. *S. aureus* produces extracellular toxins: exfoliative toxin, toxic shock syndrome toxin-1 (TSST-1) and enterotoxin A-E [13]. Teichoic acid structures also help bacteria to adhere to host's epithelial cells and colonize. Additionally, bacterial surface elements (adhesive matrix molecules) have an important role in the later stage of nasal colonization [9].

1.3.1. Virulence of S*taphylococcus aureus*

S. aureus have the ability to produce many virulence factors, together with superantigen toxins, hemolytic cytotoxins, and surface proteins, hemolysins, leukocidins, proteases, enterotoxins, exfoliative toxins, and immune-modulatory factors. All factors are tightly controlled during growth. The agr system, recognized as the quorum-sensing system, has important role in the regulation of virulence factors [19]. *S. aureus* agr gene region is a quorum-sensing gene cluster that arranges several virulence and cell wall-related components [20]. It has two ingredients that is composed of a response regulator and a histidine kinase. Gene expression which encodes small RNA or RNA III, is controlled by the agr system, and the expression of several virulence factors like protein A, leukocidins and hemolysins are controlled by it. Also, RNA III encodes delta-hemolysin [19]. A lot of infections caused by *S. aureus* have relation to a number of virulence factors by letting them adhere to the surface, and attack or avoid the immune system, lastly, cause injury toxic effects [21].

1.3.1.1. Adherence factors (Adhesins)

The ability of *S. aureus* attachment to the host cell surface to start the colonization process can be made by numerous adhesins. One of the main classes of *S. aureus* adhesins contains proteins covalently linked to peptidoglycans, which particularly connect to the plasma or extracellular matrix (ECM) [21].

1.3.1.2. Toxin production

Staphylococcus aureus creates many exotoxins and exoenzymes. It is leading to the lysis of red cells, leukocidal action, and vasoconstriction with resulting tissue necrosis. Aggressins are possible components of the pathogenicity of *S. aureus* in humans [22]. *S. aureus* can produce more

than 40 recognized exotoxins, a lot of them have similar functions and same structure. Exotoxins can be divided into three groups: cytotoxins, superantigens, and cytotoxic enzymes. Cytotoxins effect the membranes of host cell and cause lysis and inflammation [23]. As pyrogenic toxin superantigens, TSST-1 and staphylococcal enterotoxins (SEs) have similar features in structure. They induce the T-lymphocyte release and T-cell-derived cytokine secretion such as TNF-α [24].

1.3.1.2.1. Enterotoxin

It is a protein that has a role in stimulating and release of a large amount of interleukin-1 and interleukin-2 and can cause vomiting and diarrhea. It is resistant to heat and so is not inactivated by brief cooking [18]. SEs are members of a big staphylococcal and streptococcal pyrogenic exotoxin family and they are phylogeneticly related with similar structures and functions. They result in syndromes like toxic shock and cause food poisoning, allergic situations and autoimmune diseases [25]. There are 6 immunologic types of enterotoxins: type A-I. They bind to class II MHC, alpha and/or beta chains [18, 26].

1.3.1.2.2. Toxic shock syndrome toxin (TSST)

Toxic shock syndrome is an acute systemic sickness presenting several symptoms like hypotension, fever, rash and desquamation [23]. In tampon-using menstruating women or in individuals with wound infection, toxic shock syndrome can be seen because of TSST, it is same with enterotoxin F. TSST causes toxic shock by resulting release of big amount of IL-1 and IL-2 [18]. According to CDC, TSS can effect several tissues/organs such as mucous membranes, muscular tissues, gastrointestinal, hepatic, hematologic, renal or central nervous system. Toxic shock syndrome is also divided into two types, as menstrual and non-menstrual TSS. About 50% of non-menstrual TSS are related with strains that produce TSST-1. Strains that producing SE-B or SE-C are associated with other type of TSS [23].

1.3.1.2.3. α-toxin

This toxin is a major cytotoxic agent that is released by *S. aureus* named as α-toxin or α-hemolysin (Hla) [20]. The α-toxin is a pore-forming hemolytic toxin that has the ability to cause cell membrane damage in several mammal cells. α-toxin monomers attach to cellular membrane and form pores [24]. The α-toxin causes cell membrane damage by two ways: making pores in the host cell cellular membranes and stimulating the secretion of chemokines and cytokines. Pore

formation causes host cell death with changes in ion gradients [11]. The α-toxin can cause the death of leukocytes and necrosis of tissues in vivo and marked necrosis of the skin and hemolysis [18].

1.3.1.2.4. Exfoliative Toxin

It is a protein made by staphylococci of phage group II, which can lead to scalded-skin syndrome in young children [18]. In *S. aureus,* exfoliative toxins (ET) are classified into four different antigenic types: ET-A, ET-B, ET-C, and ET-D. Different mobile genetic components encode every ETs. [23]. ET-A and ET-B are two important isoforms and they are linked to staphylococcal scalded skin syndrome and bullous impetigo [10].

1.3.1.2.5. Coagulase (Coa)

The pathogenicity of *S. aureus* is due to the ability of bacteria evading immune responses of host [20]. Coa binds to prothrombin then thereby becomes enzymatically active catalyzing the transformation of fibrinogen to fibrin coating the bacteria with fibrin and resulting resistance to opsonization and phagocytosis [10].

1.3.1.2.6. von Willebrand factor binding protein (vWbp)

A big multi-task glycoprotein called vWbp is created by *S. aureus* and causes blood clumping. It has an N-terminal domain with positive charge, a unique hydrophobic vWF motif, and a polar fibrinogen binding site. In addition, vWbp can activate prothrombin and it turns fibrinogen into fibrin. vWbp and Coa work together with clumping factor A [20].

1.3.1.2.7. Staphylokinase

It is a protein that is encoded by a bacteriophage with 15.5 kDa in size. The conformation of plasminogen is changed by dimerization of staphylokinase-plasminogen and it turns into a broad-spectrum proteolytic enzyme named plasmin. Plasmin induces bacteria to invade neighbor tissues [20].

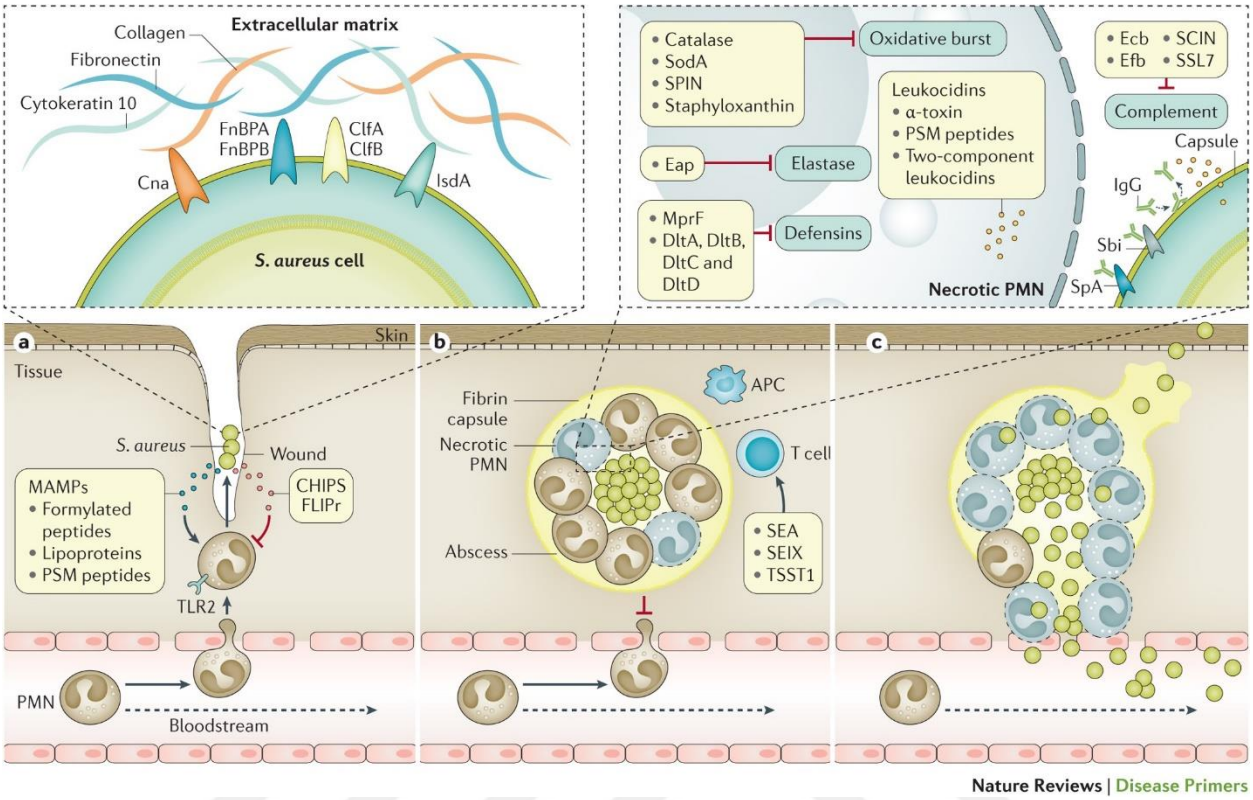


Figure 2: Stages of *Staphylococcus aureus* infection [9]

1.4. Clinical Manifestations

S. aureus can cause community or nosocomial infections such as mild skin, soft tissue infections and even fulminant sepsis. The treatment of invasive *S. aureus* infections is difficult, therefore serious spread control precautions are needed and a long period of antimicrobial treatment [27]. The abscess is a typical lesion of *S. aureus* infection that lead to central necrosis and then seen in the skin (furuncles and boils), may also circulate through the bloodstream [18]. Some syndromes and infections are mainly caused by *S. aureus*, for example, infections of the skin and soft tissue. In children *S. aureus* nasal carriage is varying from 45 percent to 70 percent and colonization is not a serious problem to the host, *S. aureus* may invade the host's natural defenses and get into underlying tissue that causes a different agent of local and aggressive infectious [28]. Skin and soft tissue infections are main clinical results and are known as furuncles (abscesses), boils and cellulitis that might progress to osteomyelitis or necrotizing fasciitis if continued for a long time [29]. Also, staphylococci can cause staphylococcal food poisoning. A cut or a wound on the skin, or any other reason might result in *S. aureus* infection. Transition to the blood circulation

is one of the most common reasons for septicemia, endocarditis, toxic shock syndrome, osteomyelitis and pneumonia [8].

1.4.1. Pneumonia

S. aureus can cause pneumonia in adult and pediatric communities. *S. aureus* pneumonia is common in MRSA-related diseases, and MRSA strains are increasing that results in severe pneumonia. In the past, MRSA infections were mostly nosocomial infections [21]. *S. aureus* enter the skin through an open wound, then into the upper airway, viral infection destructs the mucosal inner layer and make the patient prepare to get *S. aureus* pneumonia, and this process could occur routinely after getting an influenza infection [30]. It was beginning in the pandemic 1918 and involved a respiratory complication of influenza. It was subsequently a rare reason of community-acquired pneumonia, so *S. aureus* played a major role in patients in the hospital with respiratory infections [31].

1.4.2. Bacteremia

Studies report growing rates of bacteremia globally, especially the appearance of MRSA bacteremia in hospitals and in the community. The rate of *S. aureus* bacteremia has been expected to be 20-35 cases annually per 100,000 populations. There are some complications like metastatic illness with endocarditis, deep-seated foci of infection, severe sepsis and recurrences caused by *S. aureus* bacteremia [32].

1.4.3. Endocarditis

Endocarditis is caused by *S. aureus* as an opportunistic pathogen causing severe complications by entrance into the cardiovascular system, for example, infective endocarditis or thrombophlebitis, eventually cause organ failure and death. Most invasive diseases are caused by *S. aureus* strains, and some strains belong to specific clonal complexes (CC5, CC15, and CC30) may lead to bacteremia and have been related to endovascular complications and also hematogenous complication risk related to methicillin resistance [33]. Endocarditis has been detected between 11-35% in *S. aureus* bacteremia. Also, high mortality of *S. aureus* endocarditis is related to an increased incidence of extracardiac deep infections because of thromboembolic complications and metastatic spread [34].

1.4.4. Skin and soft tissue infections

Skin and soft tissue infections can be seen as benign (like uncomplicated cellulitis and impetigo) and also as life-threatening caused by *S. aureus*. The microorganism is isolated from purulent cellulitis, cutaneous abscesses and surgical site infections [31].

1.4.4.1. Impetigo

It is described as honey color-crusted sores and erosions (and infrequently vesicles) caused by *S. aureus* on the surface of the skin infection of the epidermis [35].

1.4.4.2. Cellulitis

Cellulitis caused by *S. aureus* like a warm and erythematous swelling plaque and it invades the dermis and subcutaneous layers of the skin [35].

1.4.4.3. Folliculitis

When *S. aureus* infects hair follicles, it causes folliculitis that has follicular-based erythematous papules and pustules [35].

1.4.4.4. Subcutaneous abscesses

Subcutaneous abscesses are edematous and erythematous lesions which are caused by deep *S. aureus* hair follicle and surrounding skin tissue infection [35].

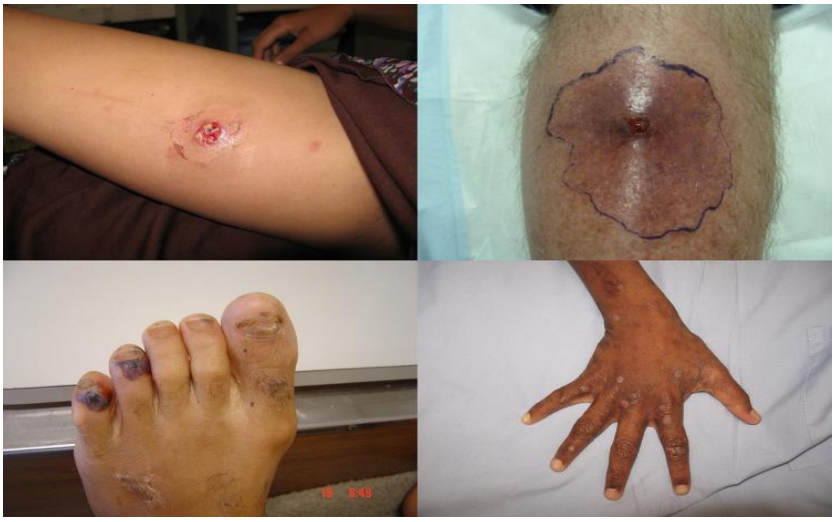


Figure 3: Skin and soft tissue infections of *S. aureus*: Abscess, cellulitis surrounding a pustule, embolic infarcts complicating infective endocarditis, impetigo complicating scabies infection [31].

1.4.4.5. Ulcers and wounds

Ulcers and wounds are caused by *S. aureus* look like open sores or craters on the skin. If it progresses to infection, it can leak purulent fluid and cause warmth and erythema in the deep dermis and subcutaneous tissues [35].

1.4.5. Urinary Tract Infection

In the community *S. aureus* is an uncommon reason for this disease but *S. aureus* UTI happens frequently in patients who use an indwelling urinary catheter. Fever is the main symptom of UTI. Additional UTI symptoms are hematuria, altered mental status, dysuria, suprapubic pain, and, rarely, flank pain. Although *S. aureus* UTI can be problematic in hospital setting where patients are often at increased risk of infection due to decreased immunity, prolonged hospital stay and most commonly the use of catheter which are common procedure in hospital for treatment of MRSA [31].

1.4.6. *Staphylococcal* food poisoning

Foodborne diseases especially staphylococcal food poisoning came after eating food contaminated with staphylococcal enterotoxins that are secreted by enterotoxigenic strains of *S. aureus*. These toxins have resistance to environmental conditions such as heat, freezing, drying, and low pH [36].

1.4.7. Bone and joint infections

S. aureus is a frequent pathogen after coagulase negative staphylococci in bone and joint infections like acute osteomyelitis and septic arthritis. Bone and joint infections can be caused by both MSSA or MRSA. *S. aureus* is a major cause of prosthetic joint infections; especially knee or hip prostheses infections are caused by MRSA [37].

1.4.8. Staphylococcal meningitis

Staphylococcal acute bacterial endocarditis complication leads to staphylococcal meningitis and also lead to meningeal seeding as the consequence of spread of *S. aureus* bacteria to central nervous system. Staphylococcal brain abscesses can result from open or closed neurosurgical trauma. Shunts in central nervous system can be infected and should be removed, which may result from MSSA or MRSA [37].

1.5. Epidemiology

The anterior nares which are the opening of nostrils are common location of colonization by *S. aureus* and are places where adults carry *S. aureus* at any time. We can divide the carriers to three different kinds which are about 30% of the people are prolonged carriers, the others about 50% are intermittent carriers and 20% are never colonized. Nasal carriers move the organism to its skin. If the skin is broken locally, it may lead to general infection [7]. Health care staff, diabetic patients, patients using intravenous drugs and individuals with weak immune system, also the patients who stay hospital for long time, patients with chronic metabolic diseases, dialysis patients, recipients of surgical operations, immune-suppressed patients and indwelling catheter users have an increased *S. aureus* colonization risk reaching 80% [11]. In addition, studies reported that the colonization rate in children is significantly higher than in adults [10]. In the United Kingdom, MRSA was detected for the first time in 1960s. However, full-genome sequencing of early MRSA isolates reports that it emerged in 1940s. In other words, it is earlier than the use of methicillin. In reality, it has been overused of penicillin instead of the introduction of methicillin that has a significant role in MRSA emergence [9]. Also at the same time, it has been isolated in Europe and the United States and the first semisynthetic anti-staphylococcal penicillinase-resistant penicillin even though methicillin is no used in therapy like the past, the MRSA may exchange by oxacillin-resistant *S. aureus*. The whole things began in the 1980s and a stable increase in MRSA prevalence was seen in the whole world with several epidemic strains that may responsible for hospital outbreaks described before [38].

A lot of countries in the Middle East suffer because of MRSA which has become known as a common pathogen of community infections. The epidemiology of *S. aureus* maybe because of introducing new strains, along with the international exchange of many clones [39]. In Asia *S. aureus* is a major problem because of its resistance to many antibiotics that are related to a lot of serious diseases. Between 1990 to 2000 the rate of MRSA in healthcare facilities becomes the highest. Also, CA-MRSA after 2000 appeared the highest of Asian countries, in some regions reaching rates of more than 50%. The highest rates of MRSA in either hospitals or communities are due to overuse or misuse of antibiotics that lead to the development of resistant strains of bacteria [40].

1.5.1. Epidemiology of MRSA in Turkey

In the 1980s, MRSA became a major clinical problem in Turkish hospitals. MRSA resistance varied between 7% and 55% across centers. Recently, data from various centers in Turkey for the past 5 years showed that the percentage of MRSA strains was 65.5%. All strains were reported to be susceptible to vancomycin and teicoplanin, but resistance to these drugs may develop. We believe that controlling for these factors and implementing good infection control procedures in Turkey will lead to a decrease in MRSA rates [41].

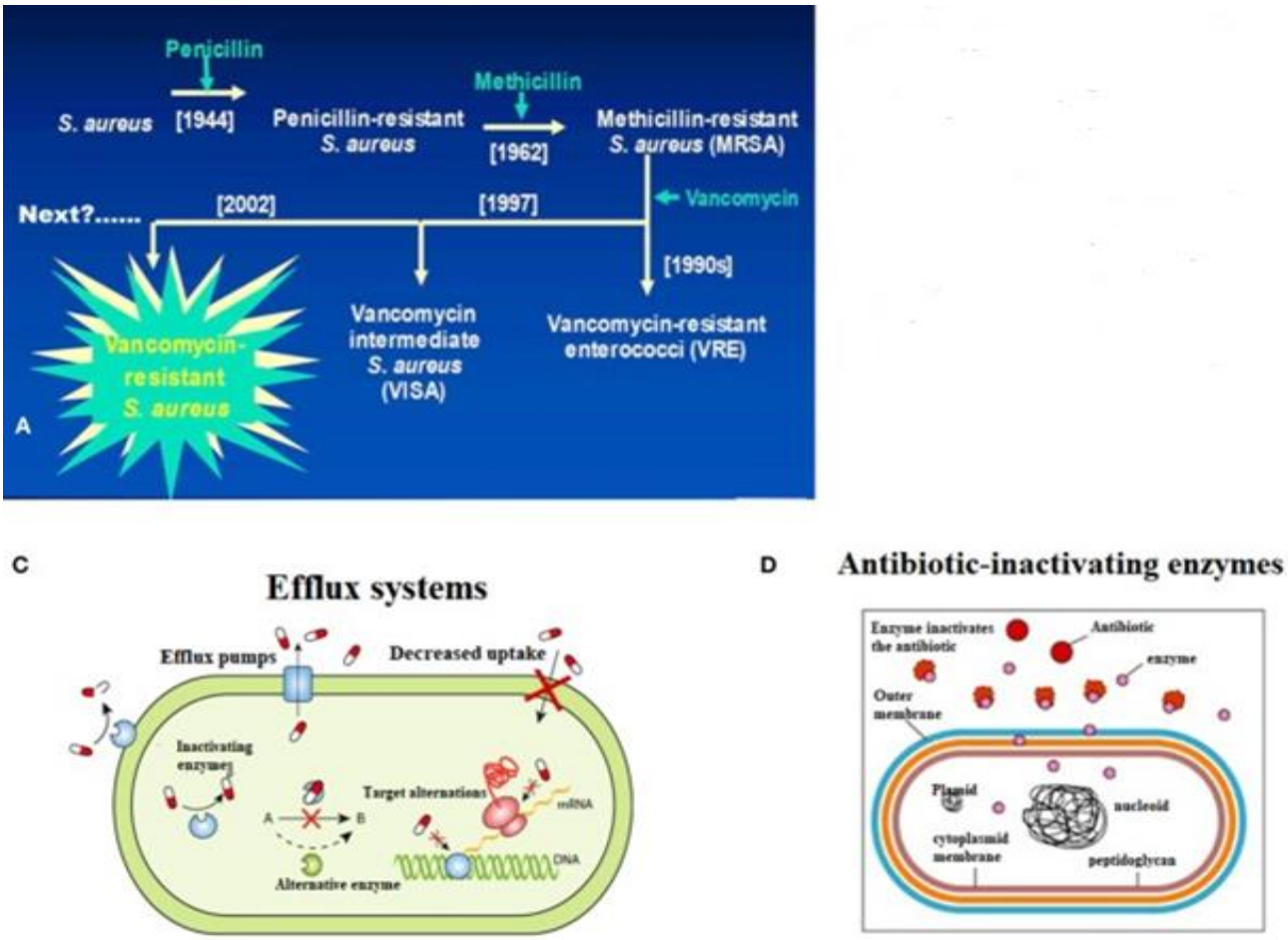


Figure 4: Evolution and mechanisms of drug resistance in *S. aureus* [42].

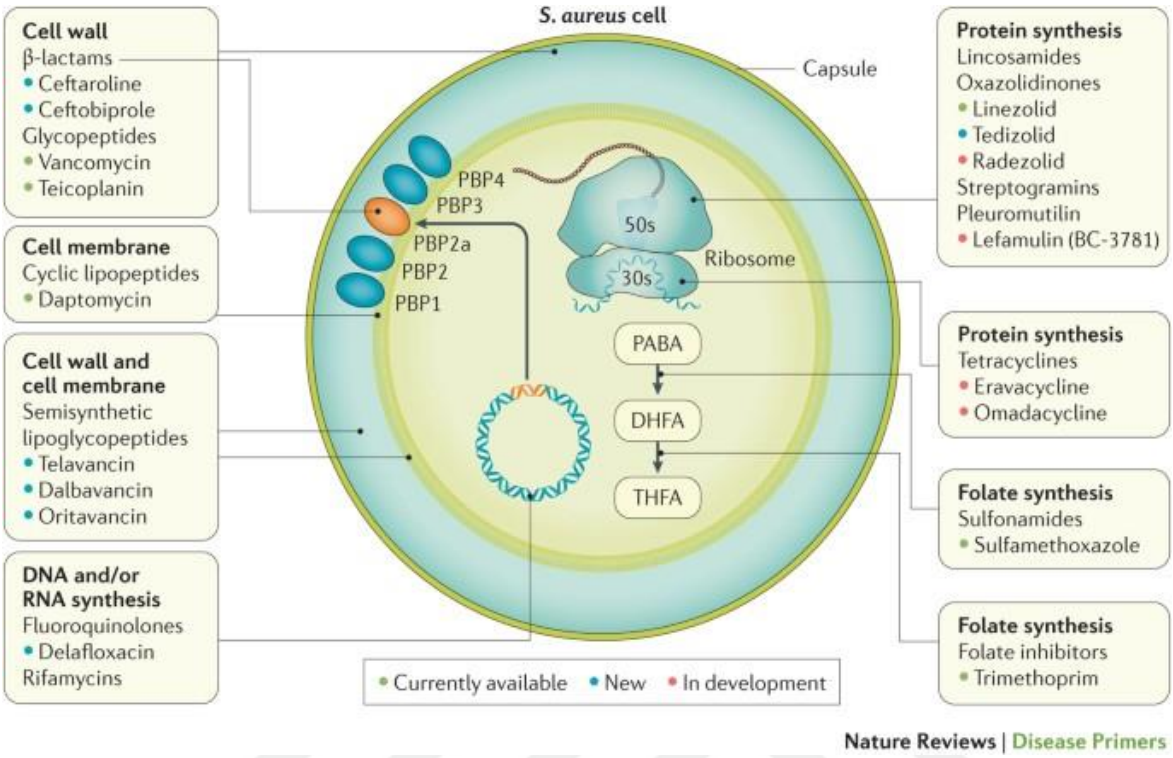


Figure 5: Targets of antimicrobials for MRSA treatment [9]

1.6. Laboratory Diagnosis of *Staphylococcus aureus*

1.6.1. Microscopy

1.6.1.1. Gram staining

Staphylococci are seen as purple grape-like clusters microscopically when they are examined from culture; however, they are seen as single cells or small groups of cells in clinical samples [43].

1.6.2. Culture

Clinical specimens are inoculated to enriched agar media supplemented with sheep blood. Staphylococci can grow on enriched general media in aerobic or anaerobic environments, making big, smooth colonies in 24 hours at 37ºC. *S. aureus* colonies gradually turn from white to yellow, especially when the cultures are incubated at room temperature. Most strains of *S. aureus* have an ability to make hemolysis on sheep blood agar [43].

S. aureus can also be selected on the mannitol-salt agar, which includes mannitol and 7.5% sodium chloride. *S. aureus* can ferment mannitol but most of the other staphylococci cannot. And 7.5% sodium chloride inhibits the growth of other organisms [43]. The strains of *S. aureus* have the capability to ferment mannose to grow and survive in high concentrations of NaCl. Microbial growth changes the pH value of the culture and pH change can be seen via an indicator; so the color changes from red to yellow [44].

DNase agar contains DNA, which acts as a substrate for deoxyribonuclease enzyme by depolymerizing the DNA led to a transparent halo around the colonies in the culture medium. *S. aureus* strains are DNase positive [44].

1.6.3. Biochemical tests

There are two methods for the coagulase test; slide and tube tests. Free coagulase is detected in tube coagulase test. If the result is positive, the plasma clots and if negative should be repeated in 24 hours because some strains need more than 4 hours to form a clot [45].

The slide test is used to detect bound coagulase by putting a drop of human or rabbit plasma on a slide with a colony of bacteria and mixed. Rapid clumping of the cocci shows positive test result. Latex agglutination tests for *S. aureus* is used to detect protein A and/or clumping factor. Slide agglutination tests are rapid methods, however about 15% of isolates are detected as negative. Therefore, negative isolates should be confirmed by tube agglutination method [45].

1.6.4. Diagnosis of antimicrobial resistance for *S. aureus*

1.6.4.1. Disk diffusion (Kirby-Bauer) test

A filter paper disc impregnated with an antibiotic suspension is used on a solid culture medium. Antibiotic discs form a transparent inhibition halo, if the bacteria are susceptible to antibiotic, and it is measured by the size inversely proportional to MIC [44]. According to CLSI these antibiotics are used for *S. aureus* antibiogram: penicillinase-stable penicillins, cephems, glycopeptides, lipoglycopeptides, lipopeptides, macrolides, aminoglycosides, fluoroquinolones, tetracyclines, lincosamides, nitrofurans, phenicols, folate pathway antagonists, ansamycins, oxazolidinones, streptogramins, pleuromutilins [46].

1.6.4.2. Gradient diffusion test

By this method, MIC value can be detected easily since it is easy to perform and has compatible results with gold standard methods, but gradient diffusion test is not a common method because it is expensive [44]. The method is used to inoculate MH agar with 2% NaCl at a seeding density equivalent to 0.5–1.0 McFarland standard. Antibiotic strips are placed on the agar plate and plates are incubated at 35°C for 24 hours. The results are evaluated with gradient to determine the MIC value [47].

1.6.4.3. Broth dilution tests

Broth dilution test is used to detect the MIC value of antibiotics. This test analyzes the growth of bacteria in the medium with serial concentrations of the antibiotics. After the incubation, the turbidity of the liquid cultures is examined. Broth dilution method can be applied as macrodilution or microdilution. In the macrodilution method, glass test tubes are used [44].

The microdilution test involves inoculating broth with serial two-fold antimicrobial dilutions with bacteria from an isolated culture and incubating the mixture for 16–24 hours. Turbidity in the wells shows growth bacteria with antibiotic concentration; as a result, the antibiotic MIC can be detected via the standards of CLSI or EUCAST [48].

1.6.4.4. Molecular methods

The *mecA* gene encoding PBP2a mediates the resistance to methicillin in *S. aureus*. *mecA*-PCR is the molecular gold standard for the diagnostic detection of MRSA. The *mecA* gene is contained together with *ccr* genes encoding for mobility, regulatory genes (*MecI/MecR1/MecR2*), and other antimicrobial resistance genes in a mobile genetic element called the staphylococcal cassette chromosome *mec* (*SCCmec*). Also, there are some limitation for molecular methods: they cannot determine actual phenotypic resistance behavior, are unsure of MICs, and only detect known resistance genes, so they cannot detect new resistance or genes. In addition, bacteria sometimes have resistance genes but remain susceptible to antibiotics [48].

1.7. Antimicrobial Resistance of *Staphylococcus aureus* and Treatment

Penicillin had been used for the treatment of infectious diseases since 1940s; and resistant strains of *S. aureus* to penicillin had been noticed in hospitals for the first time in 1942. About

80% of *S. aureus* isolates from both hospital- and community-acquired were penicillin-resistant. MRSA strains are found internationally at present time, and most of them are multidrug-resistant [49]. The other choice of the drug for the treatment of *S. aureus* was the penicillinase-resistant penicillin called oxacillin or methicillin. The first intermediate vancomycin resistant isolate has been detected in Japan in 1996 and the glycopeptide agent vancomycin is the best choice of treatment against infections with MRSA [10].

S. aureus becomes resistant to several antibiotics that happen by gaining of mobile gene fragments via horizontal gene transfer. Mutations can cause resistance by changing antibiotic binding sites molecularly and also by expressing more endogenous efflux pumps [50].

1.7.1. Penicillinase resistance

Penicillin includes a β-lactam ring and belongs to β-lactam antibiotics, which can bind to penicillin-binding-protein in the bacteria cell wall, inactivate it and inhibit the synthesis of bacteria cell wall [11]. There are three mechanisms of penicillinase resistance; first is disruption of β-lactam ring by β-lactamases/penicillinases, second is change in penicillin binding protein that attenuate their affinity for penicillins, third is a permeability barrier prevents penetrating antibiotic from entering cells. Penicillin activity is blocked if β-lactamases hydrolyze the β-lactam ring of the antibiotic [8]. The methicillin which is a penicillin derivative and it was presented in 1961 to prevent enzymatic inhibition by penicillinases; however, in a short time methicillin resistance was detected in *S. aureus* [11].

1.7.2. Methicillin resistance

S. aureus gains resistance to methicillin by acquiring *mecA* gene, which encodes a modified penicillin-binding protein-2a (PBP2a) that has decreased affinity to β-lactam antibiotics. Thus, even in the existence of β-lactam antibiotics, modified PBP2a of MRSA isolates have an ability to replace normal penicillin-binding proteins biosynthesis function, thereby prevents cell lysis. *S. aureus* strains with PBP2a production have resistance against all β-lactam antibiotics [51]. MRSA is one of the most common drug-resistant bacteria found in many parts of the world, containing the United States, Europe, the Middle East, North Africa, and East Asia [42]. About 35% of hospital strains in the United States today are staphylococci [52]. Despite adequate treatment, MRSA causes 20% to 40% mortality within 30 days [2].

1.7.3. Vancomycin and other glycopeptide resistances

In hospitalized patients with severe MRSA infections, a glycopeptide antibiotic called vancomycin is used to treat them [50]. In recent years, vancomycin resistance to *S. aureus* has emerged.

1.7.4. Macrolide, lincosamide and streptogramin B resistance

In staphylococci, resistance to macrolide, lincosamide, and streptogramin B (MLSB) correlated with methicillin resistance. The transmission of erm genes leading to MLSB resistance severely limits the usage of macrolides like erythromycin in hospital settings [3]. More than 80% of MRSA strain with the msr gene and msr gene simultaneously exhibit resistance to MLSB antibiotics [3]. Macrolides, lincosamides, and streptogramins were presented in 1952 for the first time called as MLS. They inhibit the protein synthesis by targeting the 50S ribosomal subunit of bacteria [53]. Although the chemical properties of macrolide, lincosamide, streptogramin B are far apart, they have a similar mode of action [4]. Erm genes result in conformational modification of 23S rRNA by encoding methylases and finally MLSB antibiotics cannot bind to their target [4]. *msrA* gene encodes efflux pumps and cause MSB resistance and *lnu* gene encodes drug modifications [54]. Various mechanisms are used for MLSB resistance; usually the resistance mechanism is expressed by the *erm* (erythromycin ribosome methylase) genes [2]. Macrolides are rarely used against MRSA infections in developed countries however they can still be used in MSSA infections [50]. There are two different phenotypes for MLSB resistance in *S. aureus*. One is inhibition of the binding of antibiotics to ribosomal target site which is made by two ribosomal modifications by 23S rRNA methylases and is expressed mostly by *ermA*, *ermB*, or *ermC* genes that are present on plasmids or chromosomes. The second resistance type decreases the level major for binding to ribosomes that mediated by *msrA* [53]. MLSB resistance expression can be constitutive (c) or inducible (i). MLSB should not be used to treat constitutive or inducible resistant phenotypes [3].

In iMLSB resistance, bacteria produce methylase when there is an inducing agent that can develop resistance to macrolides with 14-membered and 15-membered-ring. Nevertheless, iMLSB resistant staphylococci are not resistant to 16-membered macrolides, streptogramin B and lincosamides. The continual secretion of the methylase is stimulated by the mutations in the 5’

upstream sequences of the *erm* gene and bacteria gain resistance to macrolides, streptogramin B and lincosamides [6].

iMLSB resistance is detected by a special disk diffusion test that includes erythromycin induction of clindamycin resistance (D-zone test) [54]. The strains with erythromycin resistance and clindamycin susceptibility have the efflux phenotype (MSB) [6]. There are 40 *erm* genes and they are consisted of 14 classes, however the significant classes are *ermA*, *ermB*, and *ermC* for the occurrance of MLSB resistance [3].

S. aureus resistance to methicillin and macrolides is caused by several mechanisms; modifying the antimicrobial target via erm genes or throwing out the macrolides from the bacteria via *msr* genes. The existence of these mechanisms limits the treatment options for MRSA infections [3]. The resistance mechanisms to macrolide antibiotics can be constitutive MLSB (cMLSB), inducible MLSB (iMLSB), or macrolide and streptogramin B (MSB) in MRSA and MSSA isolates. MRSA strains generally have constitutive resistance to MLSB antibiotics that shows multi-antimicrobial resistance. The difference of cMLSB and iMLSB resistance phenotype rates in MSSA strains, is not high, but the iMLSB phenotype is more common. Whereas the MSB phenotype in MRSA and MSSA strains, is relatively rare. MRSA strains are resistant to MLSB antibiotics because of the genes *ermC* or *ermA*. Among MSSA strains, the *ermB* gene follows the *ermC* gene. Investigations about the msr gene rates in MRSA and MSSA strains is not much. However, the studies reported that the rates of *msrA* gene is higher comparing the *msrB* gene [3].

Distribution of these genes changes according to the location. The MLSB resistance in the Middle East, is usually caused by the the *ermC* gene, and *ermA* gene follows it. However, in Egypt and China, *ermB* gene is more common. Besides, the *ermA* gene has the highest rate in South America. In the constitutive and also inducible phenotypes *ermA* gene is detected. In Europe, the *ermC* gene is predominant, however the second frequent gene is *ermA* gene [3].

1.7.5. Treatment

In the nosocomial staphylococci infections, vancomycin and teicoplanin as glycopeptide antibiotics are chosen for the treatment. However, it was reported that *S. aureus* has gained resistance to vancomycin and teicoplanin [45]. Vancomycin is the most reliable antibiotic against MRSA infections [13]. Some β-lactam antibiotics highly stimulates the manufacturing of

virulence-associated extracellular proteins, propounding that *S. aureus* infection findings might be exacerbated if a β-lactam antibiotic is used for the treatment of patients. On the other hand, some antibiotics that inhibit protein-synthesis, like quinupristin/dalfopristin, linezolid and clindamycin were indicated that virulence factors were inhibited by them, which is why these antibiotics are recommended for toxic syndromes of *S. aureus* [24]. Quinupristin/dalfopristin, daptomycin, minocycline, linezolid and vancomycin have a stable and high activity against MRSA. The effects of trimethoprim-sulfamethoxazole (TMP-SMX) against MRSA is variable. Rifampin is also a strong anti-staphylococcal antibiotic, however its activity for MRSA infections has not been illuminated [37]. New types of antibiotics are necessary to overcome this threat. The oxazolidinone linezolid has become the first antibacterial drug that targeting gram-positive bacteria since glycopeptides in the 1950s. In 2003, a lipopeptide daptomycin was presented for clinical usage in the United States and is available in Europe since early 2006 [53].

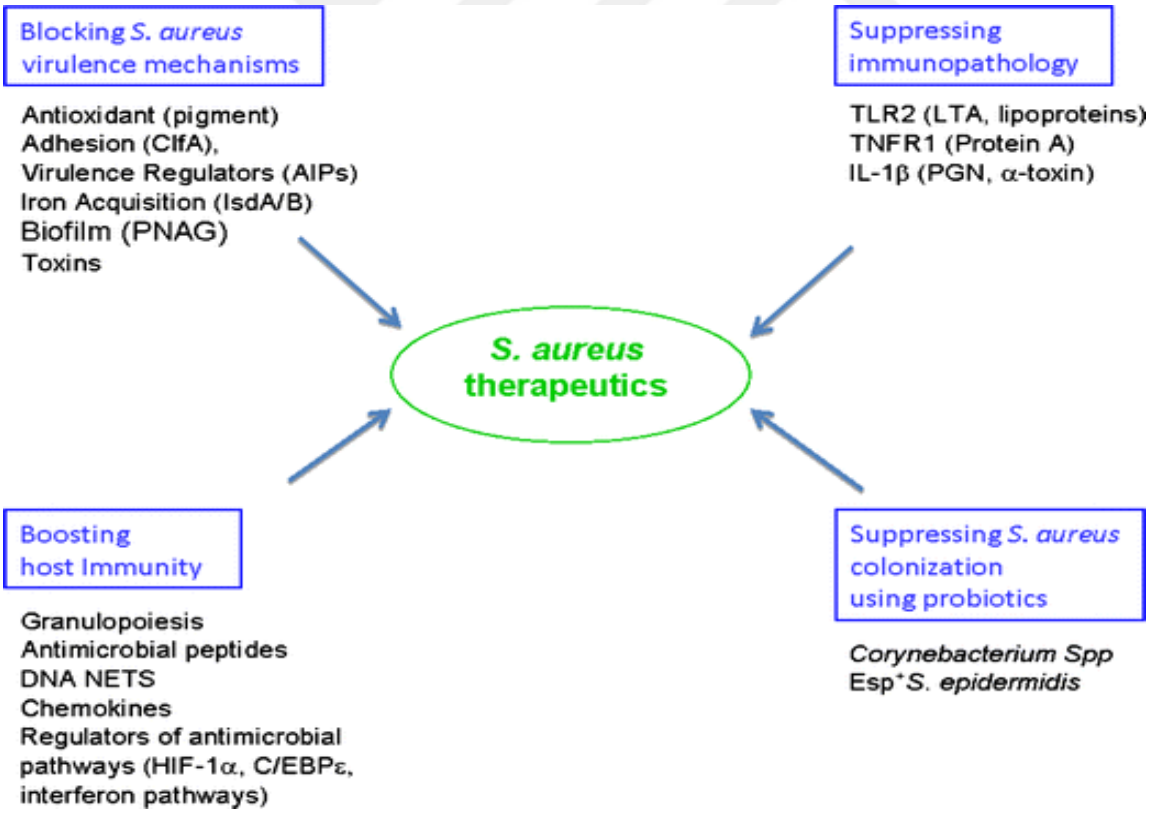


Figure 6: New anti-staphylococcal mechanisms that do not target essential *S. aureus* genes [55]

1.8. Prevention

Various prevention methods are necessary to avoid transmission and infection of MRSA [9].

1.8.1. Hand hygiene

WHO has recommended that hand hygiene is important in health-care facilities and there is a need for detailed instructions regarding appropriate hygiene using alcohol / water and soap, to decrease MRSA transmission [9].

1.8.2. Active surveillance

Most of the individuals with MRSA colonization are symptomless, to determine the carriers of MRSA several screening methods such as active surveillance programs are used [9].

1.8.3. Contact precautions and isolation

It is used to decrease the contamination in different facilities, especially health-care workers should take some precautions such as using disposable lab coats and gloves while helping MRSA colonized patients [9]. Patients with MRSA should be isolated from other patients by placed in isolated rooms, they can be settled alone or with other patients that have the same condition. Also, MRSA can survive on inanimate objects or surfaces for that we must clean the areas with disinfectants where MRSA patients stay [52].

1.8.4. Vaccines

A vaccine could stop the spread of infections and also weaken *S. aureus* colonization and protective immunity caused by long exposure to *S. aureus* antigens, which leads to decreasing the over-use of antibiotic treatment and infection control measures. There were two monovalent vaccine trials that have been studied before, however these vaccine candidates could not stimulate protecting immunity in the late clinical stages. The StaphVax was developed by Nabi Biopharmaceuticals in Maryland, USA. The StaphVax contains CP5 and CP8 antigens which are capsular polysaccharides. V710 vaccine was developed by Merck in New Jersey, USA. V710 contains iron regulated surface determinant protein B antigen(IsdB), observed preventive in animal models but failed to prevent in placebo controlled phase 3 trials. Although scientists study about a preventive vaccine for *S. aureus,* a vaccine will not be developed in the near future [9].

2. MATERIALS AND METHODS

2.1. Materials and Devices

Pasteur oven (Nüve, Türkiye)

Heat Block (Biosan, Türkiye)

Centrifuge (Hettich, Germany)

Autoclave (Hirayama, Japan)

Biosafety Cabinet (Heraeus, Germany)

PCR Cabinet (BLF PCR, Yenimahalle, Ankara/Turkey)

Vortex (Velp, Italy)

Deep freezer (Arçelik, Türkiye)

Thermal Cycler (Labnet, Finland)

Weight (SCALTEC, USA)

McFarland device (Fisher Scientific, USA)

Incubator (Nüve, Türkiye)

Microwave (ARCELIK, Istanbul Turkey)

Electrophoresis gel tank (Portsmouth, NH USA)

Electrophoresis power source (Cleaver, Ankara)

1.5 mL microcentrifuge tube

0.5- 10 µl sterile filtered pipette tip (Eppendorf, Germany),

Automatic pipettes 10-100-1000 µl (GILSON-France)

Balloon jug

Glass test tube

Petri dishes

Sterile saline

Sterile plastic loop

Collection swab [cotton swab wooden stick]

5% Sheep Blood Agar (RTA, Türkiye)

Mueller Hinton Agar (Condo Lab, Spanish)

5% Skim-milk medium (BD Difco, USA)

EDTA (SRL, Italy)

Agarose (Sigma, USA)

PCR Mastermix (Solis Biodyne, Germany)

Ethidium Bromide (AppliChem, Germany)

Tris Base (Sigma Alorich, USA)

Boric Acid (SRL, Italy)

Gel loading dye (SNP Biotechnology, Turkey)

0.2 ml PCR tube with flat cap

Antibiotic Discs:

Clindamycin (Bioanalyse, Yenimahalle -Ankara/ Turkey)

Erythromycin (Bioanalyse, Yenimahalle -Ankara/ Turkey)

Cefoxitin (Bioanalyse, Yenimahalle -Ankara/ Turkey)

2.2. Phenotypic Tests

Fifty MSSA and 50 MRSA isolates from Baskent University Ankara and Adana Hospitals that were collected between 2016-2022 were included in the study. Firstly, *S. aureus* isolates were confirmed by catalase and coagulase tests. MRSA and MSSA isolates were confirmed by disc diffusion test via cefoxitin disc according to CLSI 2021.

2.2.1. Coagulase test

2.2.1.1. Slide test

1. Physiological saline solution was dropped on two sides of a slide. One bacterial colony was mixed with each drop using the loop.

2. Human or rabbit plasma was dropped into one of the suspensions, and mixed gently.

3. Clumping in the preparate was observed within 10 seconds.

4. No plasma was added to the second suspension and used as negative control.

2.2.1.2. Tube test

1. Plasma was diluted in 1 to 10 in physiological saline.

2. It was taken into three test tubes and labeled as T (Test), P (Positive Control), and N (Negative Control). 0.5 ml of the diluted plasma was pipetted into each tube.

3. 100 µl from test tube was added into the tube "T", 100 µl of *S. aureus* culture into the tube "P" and 100 µl of sterile broth into the tube "N" and mixed.

4. The tubes were incubated at 35-37ºC.

5. The tubes were examined for clotting after 2-3 hours incubation. If no clotting was observed, they were examined again at room temperature in 24 hours.

2.2.2. Preparation of mueller hinton agar

1. 38g was measured from Mueller- Hinton agar powder.

2. 1 liter of distilled water was measured and mixed in a flask with the Mueller hinton agar powder.

3. The flask was put in the autoclave at 121ºC for 15 minutes.

4. In the final step sterilized mueller hinton agar was poured in to the plates at room temperature and waited until solidified then stored in the refrigerator at +4ºC.

2.2.3. Confirmation of methicillin resistance

1. Bacterial suspension was prepared as 0.5 McFarland and inoculated on to the Mueller Hinton agar.

2. Cefoxitin antibiotic discs (30 µg) were taken out from the refrigerator to the room temperature and settled on to the agar plates.

3. The plates were incubated at 35-37ºC for 18-24 hours.

2.2.4. D test for MLSB resistance

1. Bacterial isolates were inoculated on blood agar to obtain fresh cultures.

2. In next day a single colony was taken from the culture and 0.5 McFarland bacterial suspension was prepared and inoculated on to the Mueller Hinton agar.

3. Erythromycin (15 µgr) and clindamycin (2 µgr) antibiotic discs were settled on Mueller-Hinton agar with 15-20 mm distance between them according to CLSI 2021.

4. After that the plate was placed in the incubator at 35-37ºC for 18-24 hours.

5. The results were evaluated after the incubation. Erythromycin resistance with D-zone in clindamycin was named as iMLSB, both erythromycin and clindamycin resistance was named as cMLSB and erythromycin resistant-clindamycin susceptible isolates without D-zone was named as MSB phenotypes.

2.3. Genotypic Tests

Clindamycin and/or erythromycin-resistant MSSA (n=10) and MRSA (n=28) isolates were taken to the genotypic tests.

2.3.1 DNA isolation

DNA isolation was made by boiling method that described before [56].

1. 1-2 colonies were taken from fresh cultures of MLSB-resistant isolates with a disposable sterile loop and suspended in 1 ml of sterile deionised water in 1.5 ml centrifuge tubes.

2. Tubes were incubated in the heat block at 95ºC for 10 minutes.

3. The tubes were transferred into ice for 2-3 minutes, then they were centrifuged at 14,000 rpm for 10 minutes after cooling.

4. The upper phase was transferred to a clean tube and kept at -20ºC until PCR studies.

2.3.2. *erm* and *msr* genes amplification by PCR

PCR amplification [6] was performed by using *ermA*, *ermB*, *ermC* [57] and *msrA* primers [58]. *S. aureus* RN1551, *S. aureus* 6520, *S. aureus* FPR3757, *S. aureus* 15114 reference strains were used as positive controls for *ermA*, *ermB*, *ermC* and *msrA* genes, respectively.

PCR protocol:

DNAse/RNase free water: 10 µl

5X Mastermix: 4 µl

Forward Primer: 4 µl

Reverse Primer: 4 µl

Target DNA: 2 µl

Total volume: 20 µl

Table 1: Primer sequences for PCR

Gene Name	Primers Sequences
erm A1	3'TCTAAAAAGCATGTAAAAGAA'5
erm A2	3'CTTCGATAGTTTATTAATATTAGT'5
erm B1	3'GAAAAGGTACTCAACCAAATA'5
erm B2	3'AGTAACGGTACTTAAATTGTTTAC'5
erm C1	3'TCAAAACATAATATAGATAAA'5
erm C2	3'GCTAATATTGTTTAAATCGTCAAT'5
msr A1	3'GCAAATGGTGTAGGTAAGACAACT'5
msr A2	3'ATCATGTGATGTAAACAAAAT'5

Table 2: PCR conditions for *ermA* gene

	Temperature	Time	
Initial denaturation	94ºC	5 min	
Denaturation	93ºC	30 sec	x35
Annealing	50ºC	30 sec	
Elongation	72ºC	1 min	
Final extension	72ºC	7 min	

Table 3: PCR conditions for *ermB* gene

Initial denaturation	94ºC	5 min	
Denaturation	93ºC	30 sec	x35
Annealing	52ºC	1 min	
Elongation	72ºC	1 min	
Final extension	72ºC	7 min	

Table 4: PCR conditions for *ermC* gene

Initial denaturation	94 ºC	5min	
Denaturation	93 ºC	30sec	x35
Annealing	50 ºC	1min	
Elongation	72 ºC	1min	
Final extension	72 ºC	7min	

Table 5: PCR conditions for *msrA* gene

Initial denaturation	95 ºC	3 min	
Denaturation	93 ºC	30 sec	x35
Annealing	55 ºC	2 min	
Elongation	72 ºC	90 sec	
Final extension	72 ºC	7 min	

2.3.3. Preparation of agarose gel

Amplicons were loaded on to the 1.5% agarose gel with ethidium bromide and observed via UV light.

1. 1.2 g gel powder was added to 60 ml of 0.5x TBE solution.
2. After the heating process, 2 µl Ethidium bromide (10 mg/ml) was added in the gel.
3. The PCR products were loaded after half an hour when the gel solidified.

2.4. Statistical Analysis

Since the resistance rates of the MSSA and MRSA isolate groups in the study was compared, "Significance test of the difference between two percentages in independent groups" or "2x2 Chi-square tests in independent groups" or "Fisher's exact test" was used. Type I error probability was taken as $\alpha=0.05$ in all hypothesis tests and SPSS v25.0 package program was used for statistical evaluations. G*Power 3.1.9 program was used while calculating the required sample size to test the research hypothesis.

2.5. Review Board Approval

This study was approved by Baskent University Institutional Review Board (Project no: KA22/361) and supported by Baskent University Research Fund (Appendix 1).

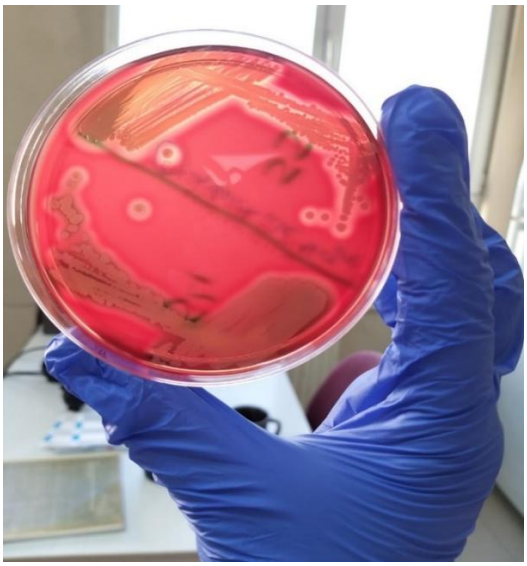
3. RESULTS

The clinical samples of inpatients were collected and sent to Microbiology Laboratories between March 2016 and October 2022. A hundred *S. aureus* isolates were included in the study. Thirty-three of them were blood samples, 35 of them were tissue biopsy samples, 13 of them were body fluid samples, 12 of them were wound/pus samples, 4 of them were sputum samples, 2 of them were deep tracheal aspirate samples and 1 was respiratory secretion sample.

All samples were sent from different departments such as cardiovascular surgery, nephrology, cardiology, chest diseases, emergency, anesthesia, general surgery, orthopedics and traumatology, pediatric nephrology, plastic surgery, neonatal, obstetrics and gynecology, dermatology, pediatric cardiology, burn treatment, neurology, infectious diseases, otolaryngology, geriatrics, general internal medicine, oncology, neurosurgery, urology, pediatric infectious diseases, endocrinology.

3.1. Phenotypic Results

Among all included isolates, 50 of them were methicillin-sensitive *S. aureus* (MSSA) and the other 50 were methicillin-resistant *S. aureus* (MRSA), all of them were confirmed by catalase and coagulase tests. We also confirmed methicillin resistance by using cefoxitin discs according to CLSI standards.



Picture 1: *S. aureus* growth on blood agar

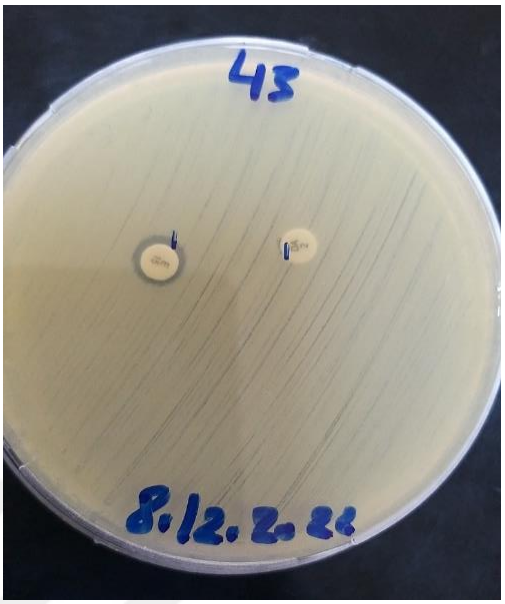
Picture 2: Cefoxitin susceptibility confirmation

3.1.1. D-Test results

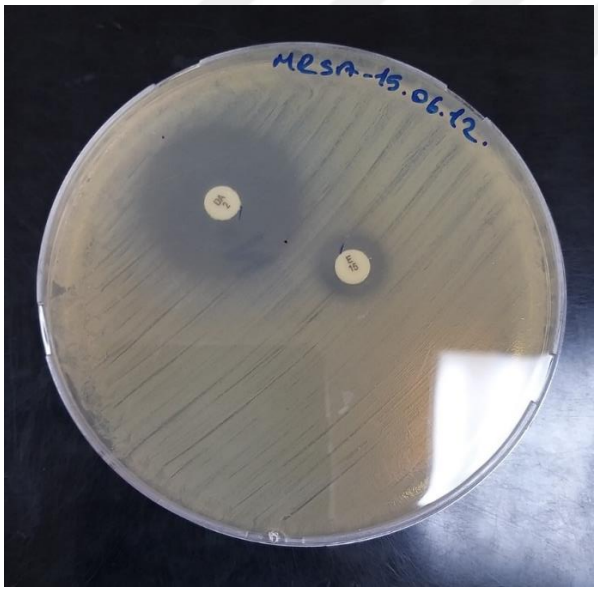
Both MSSA and MRSA isolates were tested for erythromycin and clindamycin susceptibility for the MLSB resistance according to CLSI 2021. Among 50 MRSA isolates 25 (50%) isolates were resistant to erythromycin with D-zone positive and susceptible to clindamycin indicating inducible MLSB (iMLSB) phenotype. Two (4%) isolates were resistant to clindamycin and erythromycin without the D-zone indicating constitutive MLSB (cMLSB) phenotype. Twenty-two (44%) isolates were susceptible to clindamycin and erythromycin with D-zone negativity. Only one isolate (2%) was susceptible to clindamycin and has erythromycin resistance with absence of D-zone indicating MSB phenotype.

Among all 50 MSSA isolates; 8 (16%) isolates showed iMLSB phenotype and 2 (4%) isolates showed cMLSB phenotype. There were 40 (80%) isolates that were susceptible to both erythromycin and clindamycin without D-zone.

Picture 3: D-test, inducible MLSB resistance



Picture 4: D-test, constitutive MLSB resistance



Picture 5: MSB phenotype

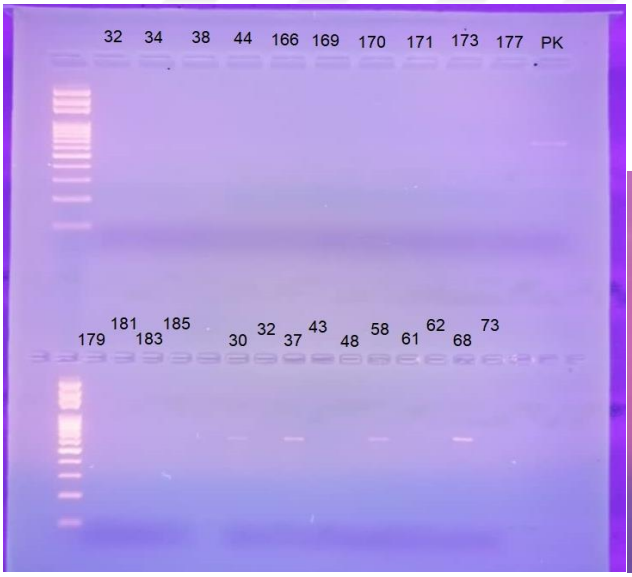
Picture 6: Erythromycin/clindamycin susceptible

The phenotypic difference between methicillin-susceptible and resistant groups is statistically significant with 95% confidence due to the iMLSB and erythromycin/clindamycin susceptible phenotypes ($p<0.05$).

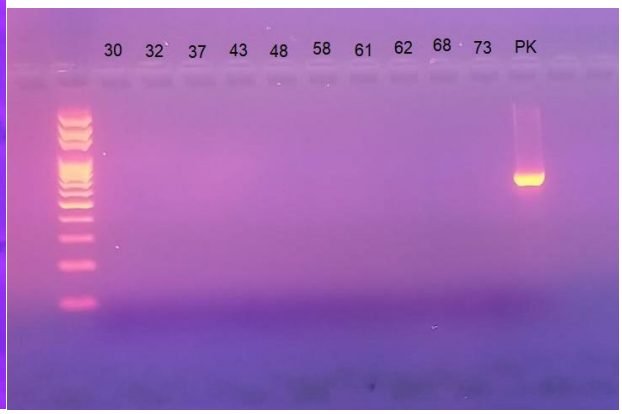
3.2. Genotypic Results

We studied the resistance genes *ermA*, *ermB*, *ermC* and *msrA* in 28 MLSB resistant isolates of MRSA and 10 MLSB resistant isolates of MSSA from a total of 38 isolates. The most common gene was *erm C* due to 25 positive isolates (89.3%) for MRSA isolates and positive for 4 MSSA isolates (40%). The difference in the number of *ermC* gene region positive isolates between the methicillin-susceptible and resistant groups is statistically significant with 95% confidence ($p<0.05$).

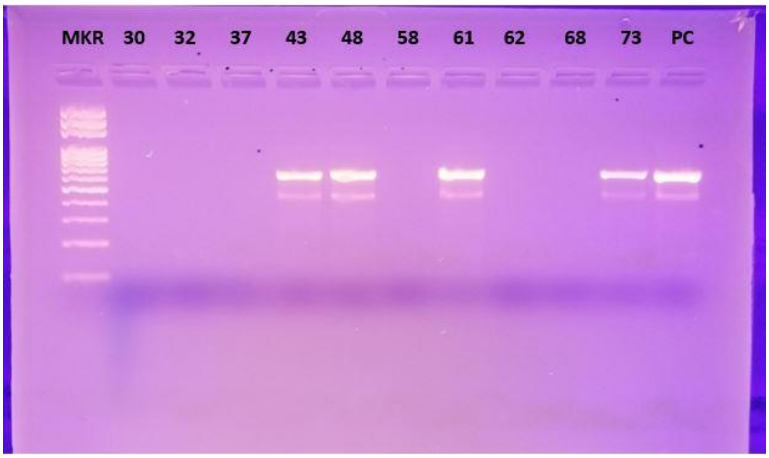
The second common gene was *ermA*; it was detected in 4 MSSA isolates (40%) and was not detected in MRSA isolates. The difference in the number of *ermA* gene region positive isolates between the methicillin susceptible and resistant groups is not statistically significant with 95% confidence ($p=0.117$). *ErmB* gene was not detected in any of the isolates. In addition, *msrA* gene was confirmed in one MSB phenotype isolates. The difference in the number of *msrA* gene region positive isolates between the methicillin-susceptible and resistant groups is not statistically significant with 95% confidence ($p=1.000$).



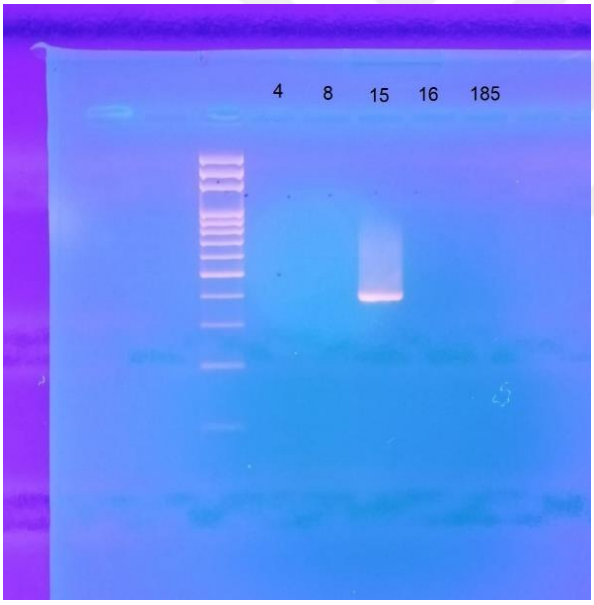
Picture 7: *ermA* positive PCR products and PC (645 bp)



Picture 8: *ermB* gene PC (639 bp)



Picture 9: *ermC* positive PCR products and PC (642 bp)



Picture 10: *msrA* positive PCR product (399 bp)

4. DISCUSSION

We studied a hundred samples of *S. aureus* isolates that were tested for susceptibility to erythromycin and clindamycin. Fifty of them were MRSA and the other 50 were MSSA and also confirmed in our study by using cefoxitin disc. In the 50 MRSA isolates, the inducible phenotype was detected in 25 isolates (50%), the constitutive phenotype in 2 isolates (4%), the MSB phenotype in 1 isolate (2%) and 22 isolates (44%) were susceptible to erythromycin and clindamycin without D zone. In 50 MSSA isolates tested 8 (16%) isolates showed iMLSB phenotype which is resistant to erythromycin with D zone positive. And 2 (4%) isolates are cMLSB that give resistance to both antibiotics with D zone negative. There are 40 (80%) isolates that are susceptible to clindamycin and erythromycin without D zone. In a recent study by Nahar et al. in 2023 iMLSB resistant MRSA isolates were detected more than iMLSB resistant MSSA (58.6%, 23.5%) respectively, these results are similar to our results [59]. Mahesh et al. found out in 2022 that of 140 *S. aureus* isolates, 33.6% were MSSA and 66.4% were MRSA. iMLSB phenotype rate was 29.3%, and cMLSB phenotype rate was 26.4%. And only 8 (17%) iMLSB isolates were found in MSSA strains similar to our results [60]. In the study of Assefa in 2022, 605 of 3064 *S. aureus* isolates were iMLSB resistant (19.8%) [61]. iMLSB phenotypes in MRSA strains were highest in Egypt, Nigeria and Libya as 77.8%, 75.0%, 66.2%, respectively. The lowest occurrence of the phenotypes iMLSB among MRSA isolates was reported in Coteˆd'Ivoire's study with 3.9%. The iMLSB phenotype was not detected in MSSA strains in 2007 in Libya and in 2017 in Cote d'Ivoire, cMLSB phenotypes was showed in MRSA and MSSA strains as 0–75% 0–60%, respectively [61].

In 2015, Ozansoy et al. from Turkey reported that 39.1% of *S. aureus* isolates were MRSA and 60.9% were MSSA. In MRSA samples cMLSB and iMLSB resistances were 7.6% and 56.3%, respectively. 1.3% of isolates were MS phenotype and 34% of isolates belonged to erythromycin/clindamycin susceptible phenotype. Among the MSSA samples iMLSB was 8.9% and cMLSB was 2.9%, MSB was 1.2% and erythromycin/clindamycin susceptibility was 87% [62]. Özbek et al found in 2021 that cMLSB resistance (49%) was higher than iMLSB (19%) and MSB phenotype was not detected [63]. In the study of Uyar Gulec et al. iMLSB resistance was determined in 25%, structural resistance in 42.9% and MSB phenotype resistance in 3.5% of MRSA strains in 2010. This rate was 15.3% structural resistance and 7.7% inducible resistance in MSSA. No resistance was observed in the MSB phenotype in MSSA strains [6].

There are differences between our study and Modukuru et al's study which reported that 165 *S. aureus* isolates were susceptible to erythromycin and clindamycin in a total of 339 isolates. Rest of them (174 isolates, 56.3% MRSA and 43.7% MSSA) had resistance to erythromycin or clindamycin, or both. Among MRSA strains, 76.6% were cMLSB, 64.5% were iMLSB, and 43.75% were MSB phenotype. Among 76 MSSA isolates; 23.40% was cMLSB, 35.48% iMLSB and 56.25% was MS phenotype. This study shows that iMLSB phenotype had higher rate in MRSA isolates comparing to MSSA isolates [64]. In the study of Nagarkoti et al., 60 isolates were found as erythromycin-resistant in 312 *Staphylococcal* isolates comprising 65% *S. aureus* and 71% of them were representing MRSA, among them cMLSB, iMLSB, MS phenotypes were 12%, 44%, 44%, respectively. Among MSSA isolates cMLSB resistance was 35.7%, iMLSB 7.2% and MS 57.1% [65]. There are differences between our results and Pereira et al's study in 2016 which reported that 22 (21.4%) MRSA and 37 (35.9%) MSSA were detected. It was detected that among MRSA isolates 22.7% of them belonged to erythromycin/clindamycin susceptible phenotype, 68.2% cMLSB, 4.5% iMLSB, 4.5% MSB phenotype. Among MSSA isolates erythromycin/clindamycin susceptible phenotype was 67.6%, cMLSB 10.8%, iMLSB 10.8%, MSB 4.5% [66]. Abouelnour et al.'s study from Egypt obtained that in MRSA isolates iMLSB rate was 25.2%, cMLSB 30.8% and MS 4.7%. About MSSA results iMLSB phenotype was 18.7%, cMLSB 12.4%, MS 8.4% [67]. In the study of Tandon et al. in 2018; among 604 isolates, 36.4% were MRSA and 11.4% of them were reported as iMLSB phenotype [68]. Zeki et al. in 2015 reported that in a total of 63 *S. aureus* isolates 32 (50.8%) were MRSA and 31 (49.2%) were MSSA. They reported that among MSSA isolates, 29 strains had resistance to erythromycin and 18 strains had resistance to clindamycin and 10 strains had resistance to both erythromycin and clindamycin [69]. Timsina et al. in 2020 showed that in 64 *S. aureus* isolates, 17 (26.6%) were MRSA and 15 (23.4%) of them have iMLSB resistance. iMLSB resistance were higher in MRSA isolates (76.4%) than MSSA isolates (4.2%) [70]. Khodabandeh et al. in 2019, among 106 MRSA isolates the rate of cMLSB resistance was 56.2%, iMLSB resistance was 22.9%, and MSB resistance was 16.6% [71]. Goudarzi et al. reported in 2020 that in MRSA and MSSA isolates, cMLSB phenotype was found (30.2%, 24.4%), however iMLSB and MS phenotypes were detected only in MRSA isolates [72]. In the study of Antonio et al. between 1990 and 2019, 3544 MSSA and 819 MRSA isolates were detected in blood stream infections [73]. Aetrugh et al. in 2022 reported that the distribution of isolates showing iMLSB phenotype was 19.4% for MRSA and

6.4% for MSSA isolates [74]. The studies from literature show that according to the region iMLSB and cMLSB resistance rates are variable. In general, iMLSB resistance is higher than cMLSB resistance.

We studied the resistance genes in 28 samples of MRSA and 10 samples of MSSA isolates with MLSB phenotypic resistance from a total of 100 samples. In our study, it was found that the *ermC* gene is the most common gene (89.3%) in MRSA isolates and positive for 2 iMLSB MSSA isolates and *ermC* gene was detected in 2 cMLSB MRSA isolates and 2 MSSA isolates. The second common gene was *ermA* because it was positive in 4 MSSA samples with iMLSB. And *ermB* gene was negative for all isolates. The other detected gene was the *msrA* gene which was positive for one isolate in MRSA samples. The results of Nagarkoti et al. in 2019 agreed with our results due to in 39 *S. aureus* isolates, *ermC* gene was found as 36% which was the most common gene in the study and *ermB* gene was detected as 5%. However, the *ermA* gene was not detected. Also *msrA* and *msrB* genes were detected in 2.6% and 5.1% of *S. aureus* isolates [65]. Pereira et al. reported in 2016 that among 44 *S. aureus* isolates with cMLSB and iMLSB phenotypes had 38.6% *ermC* gene and 9.1% had *ermA* gene [66]. In the study of Assefa in 2022, the rate of *ermC* gene was 70% and the *ermA* gene was detected as 67.9% in Egypt, which is very different from our result. They also reported that another common gene was *msrA* in Egypt with 70% detection rate [61]. Nahar et al. in 2023 found *ermC* gene 14.3% in MSSA and 11.5% in MRSA isolates. The *ermA* gene predominated in both MSSA (70.1%) and MRSA (86.9%) isolates, different from our study [59]. This result found higher *ermC* rate in MSSA isolates. Mazloumi et al. in 2021 detected the *ermC* gene as the frequent gene in *S. aureus* isolates (43.5%) and *ermA* gene had the lowest frequency among MRSA and MSSA isolates and 7% of these strains were positive for the *msrA* gene. They detected the *ermB* gene as the most frequent gene among *S. aureus* (44.6%) isolates. In addition, *ermB* (57.1%) and *ermC* (53.1%) genes were found to have a high frequency in MRSA isolates [75]. Abouelnour et al. obtained in 2020 that *ermA* (29%) and *ermC* (18.7%) were widespread genes carried by the isolates, however *ermB* (4.7%) was carried by a few isolates [67]. Tandon et al. in 2018 found among inducible resistant isolates, 25 *ermC* (84%) isolates and *ermA* and *ermB* genes were not detected [68]. Timsina et al. reported in 2020 that 15.6% were *ermA* positive, 3.1% were *ermB* and 18.7% were *ermC* positive [70]. Khodabandeh et al. reported in 2019 that 81.8%, 63.6% and 54.5% of 11 isolates with iMLSB phenotype, *ermC*, *ermB* and *ermA* genes were detected, respectively. The rates of *ermA*, *ermB*, *ermC*, *msrA* and *msrB* genes were 25.9%, 18.5%,

44.4%, 0.0% and 0.0%, respectively in cMLSB phenotype isolates [71]. In the study of Goudarzi et al. in 2020, the results showed that the *ermC* gene was detected as 40.7%, *ermB* gene rate was 14% and *ermA* gene rate was 8.1% among all gene regions studied [72]. In the study of Antonio et al. in 2019 MLSB resistance was detected in 35 isolates, related with genes *ermA* and *ermC* [73]. Uyar Gulec et al. detected *ermA* as the most common gene in *S. aureus* isolates [6]. Yildiz et al. in 2014 reported that in 225 erythromycin-resistant isolates, 48 had *ermA*, 20 had *ermC*, and among MRSA isolates 64 had erythromycin intermediate resistance. Of which these isolates, 36 were positive for *ermA*, so the most common resistance gene was *ermA* [76]. In the study of Gulaydin et al. in 2023, iMLSB, cMLSB, MS and erythromycin and clindamycin susceptible phenotypes were 10%, 0%, 6.66% and 83.33%, respectively. Also, the *ermC* gene with a positive D-zone was detected in one isolate [77]. In the literature, *ermC* and *ermA* genes are the most common genes. Most of the studies found ermC gene higher than ermA, in some studies *ermA* gene is higher than *ermC*. The least common genes are *ermB* and *msr* genes. So our results are compatible with the literature.

5. CONCLUSION

In this study, a hundred *S. aureus* isolates that were collected from Baskent University Ankara and Adana Hospitals between 2016-2022 were included into the study. Fifty isolates belonged to MSSA and the other 50 were MRSA isolates. In MRSA isolates iMLSB phenotype was more common than cMLSB and MSB phenotypes; in MSSA samples iMLSB phenotype was more common than cMLSb and erythromycin/clindamycin susceptible phenotype was more common than both of them. In genotypic analysis the highest rate belonged to *ermC* gene and it was followed by *ermA* and *msrA* genes and the *ermB* gene was not detected.

To conclude, methicillin-resistant *Staphylococcus aureus* strains produce an important healthcare problem since they might have multi-drug resistance, and MLSB resistance is related with methicillin resistance. The transition of the *erm* genes between bacteria causes MLSB resistance and restricts the usage of macrolides. Therefore, phenotypic and genetic analysis to detect the frequency of resistance genes should be done for the epidemiological information.

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APPENDIX 1: REVIEW BOARD AND APPROVAL

Evrak Tarih ve Sayısı: 28.09.2022-164485

1993
BAŞKENT ÜNİVERSİTESİ
Tıp ve Sağlık Bilimleri Araştırma Kurulu

Sayı : E-94603339-604.01.02-164485
Konu : Proje Onayı 28.09.2022

DAĞITIM YERLERİNE

Tıbbi Mikrobiyoloji Anabilim Dalında görev yapmakta olan Dr. Öğr. Üyesi Dr. Aylin Altay Koçak'ın danışmanlığında Sağlık Bilimleri Enstitüsü / Tıbbi Mikrobiyoloji Anabilim Dalı, Tıbbi Mikrobiyoloji Tezli Yüksek Lisans Programı öğrencisi Aeteer Noura Saed Mahmoud'in sorumluluğunda yürütülecek olan KA22/361 nolu "Phenotypic and Genotypic Analysis of Macrolide-Lincosamide-Streptogramin B Resistance Among Methicilin-Resistant *Staphylococcus* aureus and Methicilin-Susceptible *Staphylococcus* aureus Isolates" başlıklı araştırma projesi Kurulumuz tarafından uygun bulunmuştur. Projenin başlama tarihi ile çalışmanın sunulduğu kongre ve yayımlandığı dergi konusunda Kurulumuza bilgi verilmesini rica ederim.

Not: Çalışma bildiri ve/veya makale haline geldiğinde "Gereç ve Yöntem" bölümüne aşağıdaki ifadelerden uygun olanının eklenmesi gerekmektedir.

— Bu çalışma Başkent Üniversitesi Tıp ve Sağlık Bilimleri Araştırma Kurulu tarafından onaylanmış (Proje no:…) ve Başkent Üniversitesi Araştırma Fonunca desteklenmiştir.

— This study was approved by Baskent University Institutional Review Board (Project no:…) and supported by Baskent University Research Fund.

[redacted]

Dağıtım:
Sağlık Bilimleri Enstitüsü Müdürlüğüne
Tıbbi Mikrobiyoloji Anabilim Dalına

Bu belge, güvenli elektronik imza ile imzalanmıştır.

[redacted]