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**MOLECULAR STUDY OF THE BIOFILM FORMATION
ASSOCIATED WITH METHICILLIN-RESISTANCE OF
STAPHYLOCOCCUS AUREUS ISOLATED FROM CLINICAL
SAMPLES**

**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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IN
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**BY
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MOLECULAR STUDY OF THE BIOFILM FORMATION ASSOCIATED WITH
METHICILLIN-RESISTANCE OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM
CLINICAL SAMPLES

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May 2023

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ABSTRACT

MOLECULAR STUDY OF THE BIOFILM FORMATION ASSOCIATED WITH METHICILLIN-RESISTANCE OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CLINICAL SAMPLES

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May 2023

In this study, 132 samples were collected from different clinical sources. All samples were collected from Al-Imam Al-Sadiq Teaching Hospital and Mirjan Teaching Hospital in Babylon Governorate during the period from June to September 2022. Only 36 isolates were confirmed to be *Staphylococcus aureus* by biochemical tests, Vitek-2 detection, and molecular diagnosis using the *16SrRNA* gene. Methicillin-resistant *S. aureus* was phenotypically identified by methicillin disc assay and genetically confirmed by the *mecA* gene, all 36 were methicillin-resistant and possessed the *mecA* gene. The results of the antimicrobial resistance test by the disc diffusion method showed for 7 antibiotics from different groups, that 36 (100%) isolates are resistant to Methicillin and Penicillin, 33 (91.66%) isolates are resistant to Erythromycin, 21 (58.33%) isolates are resistant to Tetracycline, 18 (50%) isolates are resistant to Ciprofloxacin and Gentamicin, and 6 (16.66%) isolates are resistant to Clindamycin. The prevalence of biofilm producer bacterial isolates was 27 (75%); 6 (16.66%) produced strong biofilm, 21 (58.33%) produced moderate biofilm, and 9 (25%) isolates produced non/weak biofilm. Molecular detection results for *clfA*, *icaA*, and *bbp* genes responsible for biofilm formation showed that bacterial isolates possessed them by 41.66%, 50%, and 0%, respectively.

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Keywords: MRSA, Biofilm, *16SrRNA*, *icaA* gene

ÖZET

KLİNİK ÖRNEKLERDEN İZOLE EDİLEN *STAPHYLOCOCCUS AUREUS*'UN METİSİLİNE DİRENCİ İLE İLİŞKİLİ BİYOFİLM OLUŞUMUNUN MOLEKÜLER ÇALIŞMASI

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Bu çalışmada farklı klinik kaynaklardan 132 örnek toplanmıştır. Tüm örnekler Haziran - Eylül 2022 döneminde Babil Vilayeti'ndeki Al - İmam Al - Sadık Eğitim Hastanesi ve Mirjan Eğitim Hastanesi'nden toplanmıştır. Sadece 36 izolatın *16SrRNA* geni kullanılarak biyokimyasal testler, Vitek -2 tespiti ve moleküler tanı ile *S. aureus* olduğu doğrulanmıştır. Metisiline dirençli *Staphylococcus aureus* fenotipik olarak metisilin diski testi ile tanımlanmış ve genetik olarak *mecA* geni tarafından doğrulanmış, 36'sının da metisiline dirençli ve *mecA* genine sahip olduğu tespit edilmiştir. Disk difüzyon yöntemiyle yapılan antimikrobiyal direnç testinin sonuçları farklı gruplardan 7 antibiyotik için göstermiştir, 36 (%100) izolatın Metisilin ve Penisiline, 33 (%91,66) izolatın Eritromisine, 21 (%58,33) izolatın Tetrasikline, 18 (%50) izolatın Siprofloksasin ve Gentamisine, 6 (%16,66) izolatın ise Klindamisine dirençli olduğunu. Biyofilm üreticisi bakteri izolatlarının prevalansı 27 (%75) olup, bunların 6'sı (% 16.66) güçlü biyofilm üretmiş, 21'i (%58.33) orta derecede biyofilm üretmiş ve 9'u (% 25) non/zayıf biyofilm üretmiştir. Biyofilm oluşumundan sorumlu *clfA*, *icaA* ve *bbp* genleri için moleküler tespit sonuçları, bakteri izolatlarının sırasıyla % 41.66, % 50 ve % 0 oranında bunlara sahip olduğunu göstermiştir.

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Anahtar Kelimeler: MRSA, Biyofilm, *16SrRNA*, *icaA* geni

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LIST OF SYMBOLS

°C	Degrees celcius
μg	Microgram
μl	Microliter
μm	Micromolar
g	Gram
Mg	Milligram
Min	Minute
mL	Milliliter
mm	Millimolar



LIST OF ABBREVIATIONS

Agr	Accessory gene regulator
Bbp	Bone sialoprotein-binding protein
BHI	Brain heart infusion broth
<i>BlaZ</i>	Beta-lactam antibiotic resistance genes
CA-MRSA	Community-associated MRSA
CLSI	Clinical laboratory standards institute
DW	Distilled water
EbpS	Elastin-binding protein <i>Staphylococcus</i>
EDTA	Ethylene diamine tetra acetic acid
HA-MRSA	Hospital acquired MRSA
MAR	Multiple antibiotic resistance
<i>MecA</i>	Methicillin resistance gene
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSCRAMMs	Microbial surface components recognizing adhesive matrix
MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>
NaCl	Sodium chloride
OD	Optical density
PCR	Polymerase chain reaction
PVL	Panton-valentine leukocidin toxin
SCCmec	Staphylococcal cassette chromosome mec
TBE	Tris-borate edta (Ethylene diamine tetra acetic acid)
TSB	Tryptic soy broth
UTI	Urinary tract infection
WHO	World health organization

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1. INTRODUCTION

Staphylococcus aureus can cause a wide range of illnesses, from severe to mild. It can infect the skin, spreading to the hair follicles and even the deep tissues (Thomer *et al.* 2016). *S. aureus* has long been both a prominent commensal bacteria and an important opportunistic pathogen of humans. Bacteremia, sepsis, endocarditis, pneumonia, osteomyelitis, arthritis, meningitis, otitis media, toxic shock syndrome, urinary tract infections, and skin disorders are only some of the diseases it is responsible for in hospitals and the community at large (Tong *et al.* 2015, Dayan *et al.* 2016, Ibrahim *et al.* 2019, Rasheed *et al.* 2020).

S. aureus infection remains a global threat, especially when multidrug-resistant strains have emerged as a major healthcare problem worldwide due to limited options for antibiotics used in treatment (Bhatia and Zahoor 2007). These factors contribute to the pathogenicity of *S. aureus* by preventing the development of antibiotic resistance, toxins, bacterial invasion, and biofilm formation (Mwangi *et al.* 2019, Shaikh *et al.* 2019).

Although there are various studies on the nature of biofilm formation, the specifics of biofilm formation within *S. aureus* remain unknown, this is because *S. aureus* has made significant advances in the strategies of genetic regulation of the biofilm, which is one of the skills for evading the immune system and confronting antibiotics. *S. aureus*, which forms biofilms and is resistant to antibiotics, continues to pose challenges for the host (Schilcher and Horswill 2020).

Infections caused by bacteria resistant to most antibiotics are increasingly common, and some diseases are gaining antibiotic resistance to multiple types or classes, methicillin-resistant *S. aureus* (MRSA) in particular has become a serious health concern as a result of the rise in antibiotic resistance (Evangelista and Oliveira 2015, Neopane *et al.* 2018). *S. aureus* developed methicillin resistance due to a mutation in a chromosome-encoded protein called a penicillin-binding protein, phages are used to spread this kind of resistance among *S. aureus* bacteria, this is one of the few instances of chromosomally-mediated antibiotic resistance via phage transfer that is significant to medicine, however,

in some countries, MRSA has spread widely within the community, with increasing public health warnings about the overuse of antibiotics, which may result in multidrug-resistant strains (Ventola 2015, Sabbagh *et al.* 2019).

By irreversibly blocking peptidoglycan transpeptidase, beta-lactam antibiotics like penicillin, cephalosporins, monobactams, and carbapenems serve as cytostatics against bacteria (Bozcal and Dagdeviren 2017). Resistance to beta-lactam antibiotics can arise through the simultaneous operation of several different resistance mechanisms or from the more straightforward operation of a single mechanism, there are four possible ways through which bacteria develop resistance to β -lactam antibiotics (Munita and Arias 2016, Peterson and Kaur 2018). *S. aureus* develops resistance to beta-lactam antibiotics due to the *mecA* gene, which encodes a penicillin-binding protein variant (PBP2a or PBP2') with decreased affinity for β -lactam binding (penicillins, cephalosporins, and carbapenems), “thus, the bacteria can develop resistance to all beta-lactams that is used in the clinic for MRSA infections”. Due to this, vancomycin, a glycopeptide, is promoted extensively as an MRSA treatment option (Batabyal 2016, Straume *et al.* 2020). Both fibrinogen and bone sialoprotein (BSP) have been demonstrated to be binding partners for bbp, bbp is also implicated in the synthesis of binding proteins, which are essential for bacterial colonization and the development of biofilms that lead to infection (Yarwood *et al.* 2004, Vazquez *et al.* 2011). All *S. aureus* clinical strains include the *clfA* gene, and it is the primary virulence factor responsible for *S. aureus* clumping in blood plasma (O' Brien *et al.* 2002, Peacock *et al.* 2002). Multiple genes control *S. aureus* biofilm formation and early attachment, as its name suggests, *icaA* is controlled by the *ica* operon, Through the expression of polysaccharide intercellular adhesion (PIA), the genes *icaA*, *icaB*, *icaC*, and *icaD* in this operon play a vital role in the formation of *S. aureus* biofilms (O'Gara 2007, Rohde *et al.* 2007).

The Aims of this study:

This research set out to assess the prevalence of antibiotic resistance among *S. aureus* strains isolated from clinical samples and to examine the distribution of genes encoding for biofilm and adhesion.

1. Collection of samples from different clinical sites.
2. *S. aureus* isolation and identification utilizing biochemical and microscopic methods, confirmation of bacterial isolate diagnosis using the Vitek-2 Compact System, and molecular diagnosis using the *16SrRNA* gene.
3. Carry out antibiotic sensitivity tests using different types of antibiotics.
4. Phenotypic examination of biofilm composition of MRSA isolates using micro-titer plate assay.
5. Molecular detection of genes (*mecA*, *icaA*, *clfA* and *bbp*) using PCR.
6. Statistical analysis of the results.

2. LITERATURE REVIEW

2.1 General Characteristics of *Staphylococcus aureus*

Bacteria of the genus *S. aureus* are Gram-positive and range in size from 0.5 to 1.5 microns in diameter; they produce catalase and coagulase enzymes and may have a polysaccharide capsule, because they lack the ability to move and produce spores, Clusters of cells are sometimes likened to bundles of grapes (Ho *et al.* 2014). The coagulase produced by *S. aureus* is a virulence factor that plays a major role in biofilm development during *S. aureus* infections and is responsible for the potential toxicity of the bacteria to humans and animals, Staphylococci are gram-positive, aerobic bacteria of the genus *Staphylococcus*, which is a member of the class Bacilli, Order Bacillales, family Staphylococcaceae, polymerization of soluble monomeric fibrinogen into insoluble fibrin is catalyzed in vivo by coagulase bound to prothrombin, which activates staphylothrombin complexes (Crosby *et al.* 2016). A coagulase test is used to identify *Staphylococcus* species in clinical microbiology laboratories; this process has been hypothesized to be exploited by *S. aureus* to develop the biofilm structure (Zapotoczna *et al.* 2015).

Yellow, spherical, large (1-3 mm), convex, opaque, beta-hemolytic colonies are typical of methicillin-resistant *S. aureus* (MRSA), a multidrug-resistant bacterium that also displays penicillin and cephalosporin resistance; in contrast, colonies of methicillin-sensitive *S. aureus* (MSSA) are characterized by a lack of oxacillin resistance (Pillai *et al.* 2012).

2.2 *Staphylococcus aureus* Classification

Domain: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Genus: *Staphylococcus*

Species: *S. aureus* (Botelho *et al.* 2016).

2.3 Epidemiology of *Staphylococcus aureus*

The bacterium *S. aureus* is a leading cause of hospital- and community-acquired infections (Lakhundi and Zhang 2018, Kourtis *et al.* 2019). With the introduction and subsequent expansion of methicillin- and vancomycin-resistant *S. aureus* in the late 1970s, the prevalence of this microorganism has skyrocketed (Bendary *et al.* 2016, El-Aziz *et al.* 2018). According to prior researchs, methicillin-resistant *S. aureus* is quickly spreading in Iraq (Kareem *et al.* 2015, Kareem *et al.* 2020).

The opportunistic commensal bacteria *S. aureus* colonizes various sites in the human body, including the skin, nose, axilla, and inguinal regions (Boucher and Corey 2008, Taylor and Unakal 2020). Colonization with *S. aureus* occurs in 25% to 30% of otherwise healthy people (Feingold *et al.* 2012, Gnanamani *et al.* 2017). Moreover, 15% of the population is estimated to be chronic carriers of the bacteria (Taylor and Unakal 2020). People with protracted hospital stays, surgeries, indwelling catheters, dialysis, chronic metabolic illnesses, impaired immune systems, prior methicillin-resistant *S. aureus* (MRSA) infections, and skin infections are all at increased risk for bacterial colonization (Habeeb *et al.* 2014, Gnanamani *et al.* 2017). Patient-to-patient transmission of *S. aureus* occurs through close contact, the sharing of personal items, and other routes (Tong *et al.* 2015, Taylor and Unakal 2020). This increases the risk of infection at the surgical site, as well as infections of the lower respiratory tract and bloodstream (Gnanamani *et al.* 2017).

Since the bacteria in question are very adaptable, healthcare facilities tend to be plagued with this type of infection (Shore *et al.* 2011).

Because of this, hospitals use a wide range of methods for colonization control, colonization reduction, and infection removal, this method incorporates a wide range of strategies, including the application of various disinfectants, the use of local antibiotics (such as Mupirocin), and the use of systemic antibiotics, some hospitals infect patients' nostrils with the non-pathogenic strain 502A of *S. aureus* in an effort to boost the colonization density of *S. aureus* through competition (Gnanamani *et al.* 2017).

According to the Centers for Disease Control and Prevention (CDC), antimicrobial-resistant infections caused the deaths of 20,000 Americans in 2018 (Kourtis *et al.* 2019). While there have been reports of methicillin-resistant *S. aureus* (MRSA) in Singapore as far back as the 1980s (Htun *et al.* 2018), the first report of community-associated MRSA (CA-MRSA) occurred in 1981 (Chambers and DeLeo 2009). The incidence of MRSA increased from 0 to 70 percent in Korea (Chen and Huang 2014). A publication from the western Saudi Arabian city of Jeddah in 1994 contained the first report of methicillin-resistant *S. aureus* (MRSA) (Alrabiah *et al.* 2016). Methicillin-resistant *S. aureus* (MRSA) was found in 6 percent of *S. aureus* samples at Johns Hopkins Aramco Healthcare in the Eastern Province between 1999 and 2003 (Rabaan and Bazzi 2017). Data from north Jordan revealed that 19.8% of the population was nasally colonized with *S. aureus*, however only 5.8% of those cases were MRSA (Walton 2013). The incidence of sinusitis has been steadily increasing over the past few years (Vickery *et al.* 2019).

Researchers in Thi-Qar found that the isolation rate for methicillin-resistant *S. aureus* (MRSA) was 89.1% (Hashim *et al.* 2019), while researchers at Al-Hussain teaching hospital in Thi-Qar found that only 67.46% of MRSA isolates were collected from burn patients (Dakhil *et al.* 2015). MRSA is commonly found in patients with otitis media (71.4%), burn wounds (93%), and strep throat (94%) in Basrah hospitals (Aalaa'A and Abd Al-Abbas 2019). While it indicates that rates of MRSA were on the rise in research conducted in 2011 (28.24%) (Afati *et al.* 2014).

In Baghdad governorate, one study showed that the prevalence of MRSA was 78% (Al Ani and Al Meani 2018), while other study in Baghdad and Diyala, showed about 80.7% of *S. aureus* isolates diagnosed as MRSA (Saleem *et al.* 2016), also result study by Kareem *et al.* (2015) exhibited 82% of *S. aureus* isolate was diagnosed as MRSA. Another study in Hilla city, from 301 clinical samples in three main hospitals only 7.9% MRSA isolates were obtained (Al-Charrakh *et al.* 2015). MRSA prevalence in northern Iraq differed from that observed in the center and south, where MRSA prevalence among Kurdish healthcare workers (HCWs) was reported to be 22.5% *S. aureus* carriers and 61.0% MRSA carriers (Hussein *et al.* 2017). Only (2.04%) of pupils in a 2014 study exploring *S. aureus* nasal colonization in students were confirmed to be MRSA carriers (Habeeb *et al.* 2014).

The above studies show high rates in the spread of MRSA between the southern and central governorates, while relatively few percentages appear in northern Iraq, it may be because of their good health system and their healthy practice in avoiding infection (Alkhafaji and Alsaimary 2020).

2.4 Pathogenicity of *Staphylococcus aureus*

The exceptional virulence properties of *S. aureus* allow it to survive in the human body despite the tough environment inside, these bacteria control the expression of several virulence factors, leading to a devastating infection in the majority of healthy people (Beceiro *et al.* 2013). The pathogen *S. aureus* invades the host surface, colonizes, and causes severe infection if it comes into touch with a cut or scrape. In humans, *S. aureus* rapidly colonizes the skin and mucosal surfaces, causing potentially fatal infections, it is most commonly found in the nasal cavity, axillae, groin, neckline, vaginal wall, and gastrointestinal tract (Balasubramanian *et al.* 2017). Colonization is more successful in the nasal chamber, with only 20% of persons being permanently colonized by *S. aureus* versus the 30% who are temporarily colonized (Brown *et al.* 2014). Even though the human nasal chamber's natural defenses and resident microorganisms are effective against *S. aureus* invasion, once *S. aureus* comes into touch with nasal epithelial cells, it

can invade and bind to them via surface-bound proteins such as MSCRAMM molecules (Johannessen *et al.* 2012).

Because it is a living organism, *S. aureus* can continue to function both inside and outside of the host cell. According to Sabaté Brescó *et al.* (2017), infections typically start when *S. aureus* lands on an open cut or wound, host microorganisms and epithelial cells of the skin or mucosal tissue activate the immune system at the infection site in response to tissue injury or a bacterial toxin. When the complement system in the host serum recognizes foreign infections due to antibodies on them, neutrophils and macrophages migrate to the infection site, engulf them, and help eliminate them; this is the initial line of defense after *S. aureus* infection (Fournier and Philpott 2005).

Common ailments caused by *S. aureus* include toxic shock, food poisoning, keratitis, gastroenteritis, and urinary tract infections; bacterial meningitis in infants; infections of the skin and soft tissues; bloodstream infections; pneumonia; osteomyelitis; and endocarditis, where an infection takes place and the type of virus it is can play a role (Alfatemi *et al.* 2014, Chang *et al.* 2015, Tong *et al.* 2015, Ramessar and Olaniran 2019). Annually, MRSA is responsible for more cases and deaths in the United States than HIV/AIDS, viral hepatitis, and tuberculosis put together (Green *et al.* 2012).

Possible risk factors for CA-MRSA infections include, but are not limited to, race, occupation in the military, participation in contact sports, injection drug use (IDU), recent antibiotic use, and previous hospitalization; however, the relative importance of these and other potential risk factors may change depending on geographical and climatic considerations (Ruhe *et al.* 2007).

2.5 Virulence Factor of *Staphylococcus aureus*

Staphylococci have a wide variety of virulence factors that allow them to evade the immune system and cause more severe infections; almost all of these components were first identified in *S. aureus*, these include toxins, capsular polysaccharides, molecules

involved in biofilm formation (such as polysaccharide intercellular adhesin), surface proteins (such as Protein A, clumping factor, fibronectin-binding proteins), or iron-regulated surface determinants recently, antibiotic-resistant strains of *S. aureus*, such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), have arisen, making this bacteria even more dangerous (Oliveira *et al.* 2018, Turner *et al.* 2019).

Golden yellow is the signature color of *S. aureus* colonies (Gnanamani *et al.* 2017, Bitrus *et al.* 2018). Staphyloxanthin, the yellow pigment produced by the bacteria, acts as a defense mechanism against phagocytosis (Thomer *et al.* 2016, Gnanamani *et al.* 2017). Surrounding colonies on enhanced agar (blood agar with 5% sheep or horse blood) is typically a zone of bacterial growth (Plata *et al.* 2009, Gnanamani *et al.* 2017, Bitrus *et al.* 2018). Hemolysis is caused by the release of a large number of enzymes termed hemolysins (Gnanamani *et al.* 2017).

Surface proteins have a hand in adhesion, tissue invasion, and immune system evasion, Protein A from staphylococci is a key component of virulence factors that aid in evading the immune system, almost every single *S. aureus* isolate produces this protein, which acts as a superantigen by binding to immunoglobulins and preventing opsonization and phagocytosis (Balachandran *et al.* 2018). Adherence and evading immune reactions are facilitated by clumping factors A and B, which gain entry into host tissues by binding to fibrinogen, Staphylococci intake of iron is regulated by iron-regulated surface determinants, and fibronectin binding proteins infiltrate host tissues by binding specifically to fibronectin and elastin in the extracellular matrix (Scali *et al.* 2015).

The subject of numerous investigations, *S. aureus* The pore-forming toxins (PFTs), which are responsible for the death of cells by producing pores in eukaryotic membranes, have been divided into two classes, α -PFTs and β -PFTs, according to the structure of their transmembrane segments, *S. aureus* seven distinct PFTs are produced by *S. aureus*, one of which is a hemolysin and the remaining six being bicomponent leukocidins (Seilie and Bubeck Wardenburg 2017).

Panton-Valentine leukocidin (PVL) has been shown to be more than 100 times more effective than other Staphylococcal leukocidins, and studies have shown that different host cell types (monocytes, neutrophils, platelets, erythrocytes, and epithelial cells) are vulnerable to infection by different viruses (Kong *et al.* 2016). We discuss a wide variety of pyrogenic toxins, such as toxic shock syndrome toxin (TSST) and Staphylococcal enterotoxins (Kong *et al.* 2016, Ballhausen *et al.* 2017).

Some toxins have been established through study to be particularly significant in the development of biofilms, for instance, leukotoxin AB (LukAB) and hemolysin- (Hla or -toxin) were found to be important in the survival of biofilms by Scheer *et al.* (2015). Using a murine orthopaedic implant biofilm infection model, we evaluated the significance of these toxins and found that Hla and LukAB have a synergistic role in increasing macrophage dysfunction and inducing cell death. Since *S. aureus* biofilms are less likely to be phagocytosed by macrophages, they are better able to evade harmful host reactions (Scherr *et al.* 2015). Further research on the involvement of toxins in biofilm formation by Dastgheyb *et al.* (2015) revealed that phenol-soluble modulins (PSMs) limit biofilm formation by blocking the interactions of matrix molecules with bacterial cell surfaces (Dastgheyb *et al.* 2015). Leukotoxins consist of two different proteins that assemble into a barrel shape (Aman *et al.* 2010, Yamashita *et al.* 2011).

The *S. aureus* clumping factors produces bacterial adhesins termed clumping factor A (*clfA*) and clumping factor B (*clfB*), which aid in the establishment of an infection (Zecconi and Scali 2013). All *S. aureus* clinical strains contain the *clfA* gene, which O'Brien *et al.* (2002) say is the primary virulence factor that allows *S. aureus* to cluster in blood plasma (Peacock *et al.* 2002). A binding site is located on the carboxyl terminus of fibrinogen, Signal sequence (S), A domain (consisting of subdomains N1, N2, and N3), flexible repetition region (R), C-terminal cell wall (W), and membrane-spanning (M) region make up the 933-amino-acid ClfA protein (containing LPXTG motif), binding of ligands occurs in the A domain (McCormack *et al.* 2014). N2N3 subdomains contain the fibrinogen-binding region (residues 221-659) (Deivanayagam *et al.* 2002).

S. aureus strains that cause human infections are capable of producing four distinct leukotoxins: gamma (γ)-hemolysin (HlgACB), leukotoxin AB/GH (LukAB/GH), leukotoxin ED (LukED), and pantone-valentine leukocidin (PVL) (Yoong and Torres 2013). *S. aureus* produces a class of highly specialized serine proteases known as exfoliative toxins (ETs), sometimes known as epidermolytic toxins. Desmosome cadherins in the epidermis's outer layers are the targets of these proteases, which lyse them (Bukowsk *et al.* 2010, Mariutti *et al.* 2017). Staphylococcal enterotoxins are now recognized as superantigens (SAGs) because they cause symptoms similar to those caused by *S. aureus* in food poisoning, including nausea, vomiting, and diarrhea (SEs). The International Title Committee renamed the group SE in 2004 since several newly found toxins in this category did not exhibit these emetic qualities (Grumann *et al.* 2014).

Most people are familiar with *S. aureus* because of its most well-known intercellular polysaccharide, SaPII. However, *S. aureus* also carried variant surface antigens encoded by the (vSa-gene family), which encoded approximately 50% of virulence factors and toxin. Additional genes, such as prophages that code for antibiotic resistance, actively contribute to *S. aureus* (Kırmusaoğlu 2017). Class I plasmids have the highest copy number and are the smallest (5 kb), followed by Class II (40 kb) and Class III (80 kb) plasmids (60 kb), class III plasmids have (Tra) transport genes that allow for the transfer of plasmid conjugation between bacterial isolates, and these plasmids encode a large number of resistance genes (Shien 2014). In general, different permutations of virulence genes may produce varying degrees of infection severity (Luo *et al.* 2018).

The many secreted *S. aureus* enzymes either damage host molecules or interfere with host metabolism; this includes several proteases, which are mostly non-specific enzymes that degrade host proteins with a broad specific impact (Sause *et al.* 2016). Several cysteine proteases, including staphopain A and B, aureolysin, and glut amyl end peptidase, interact with complement components to protect bacteria from death (Jusko *et al.* 2012). Exfoliative toxins and the *S. aureus* protease family preferentially cleave desmosomal cadherins in the skin's stratum corneum for their biological effects (Bukowski *et al.* 2010). PantonValentine Leukocidin (PVL) and its -toxin, -toxin, play a critical role in pneumonia and lung injury, since both (PVL) and toxin (TSST) are pore-forming toxins that amplify

the host inflammatory response by inducing the production of pro-inflammatory cytokines and the lysis of inflammatory cells, which releases even more inflammatory mediators, therefore, these toxins can cause direct and indirect pulmonary harm (Becker *et al.* 2003).

2.5.1 Biofilm formation and the role in infections

Biofilms are characterized as a sessile microbial community in which cells are embedded in a protective extracellular polymeric matrix and stuck to a surface or to other cells, and their development style, compared to free-floating cells, results in distinct patterns of gene expression and protein synthesis (Parsek and Singh 2003, Archer *et al.* 2011, Kiedrowski and Horswill 2011).

Infection is aided by the formation of biofilms because they give protection from the body's defenses. However, "frustrated phagocytosis" and the inability to properly permeate the matrix may make it difficult for macrophages and other immune cells to eliminate biofilms (Scherr *et al.* 2014).

S. aureus uses wall teichoic acids (WTAs), sortase-attached surface adhesins, and other components and processes to cling to a biotic or abiotic surface during the initial attachment phase of biofilm formation, this is followed by the synthesis of extracellular matrix and cell proliferation, and finally the structuring and removal of the biofilm (Gross *et al.* 2001, Holland *et al.* 2011) (Figure 2.1).

Diseases like osteomyelitis and endocarditis may also be caused by biofilms, orthopedic devices, artificial heart valves, joint prostheses, and catheters are common examples of what they're used with (Kwiecinski *et al.* 2019, Balaure and Grumezescu 2020). Extracellular matrix provides multi-level protection (preventing medications from penetrating), altered metabolic states, and faster growth rate all contribute to biofilms' greater resistance to antibiotics than planktonic organisms (Kwon *et al.* 2008, Vermote *et al.* 2017). MSCRAMMs, or microbial surface components recognizing adhesive matrix

molecules, are said to be crucial in this first stage by Ghasemian *et al.* (2015). Polysaccharide intercellular adhesin (PIA) is generated throughout development and is a product of the *icaADBC* operon (Vergara *et al.* 2017, Yang *et al.* 2017). The next phase, known as "dispersion" facilitates recolonization of more accessible host areas (Balaure and Grumezescu 2020). Despite the prevalence of methicillin-resistant *S. aureus* (MRSA), only a handful of research has examined the relationship between biofilm production and the severity of human infection (Kwon *et al.* 2008, Nourbakhsh and Namvar 2016).

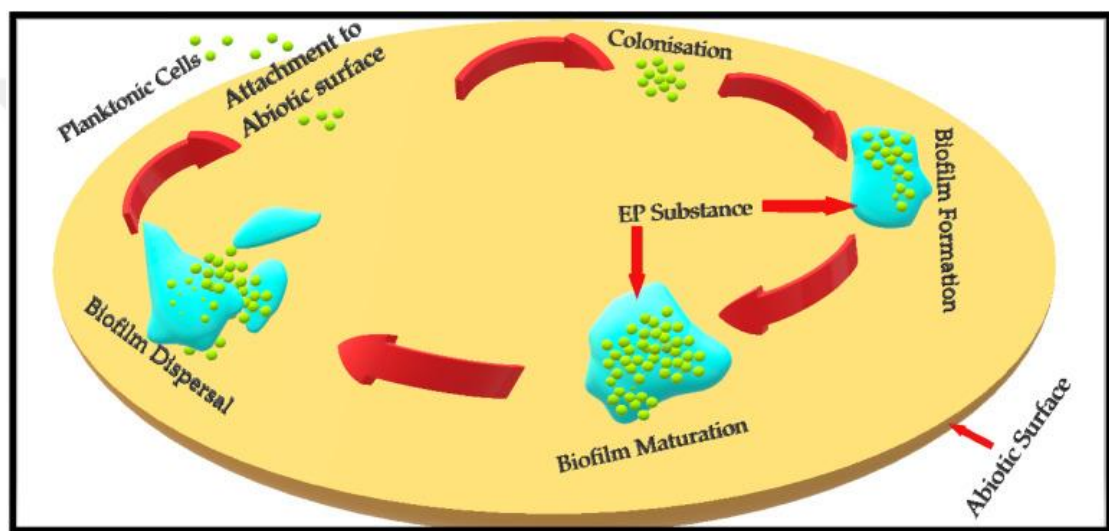


Figure 2.1 Depiction of *S. aureus* biofilm formation on an abiotic surface (Paharik and Horswill 2016, Idrees *et al.* 2020)

Biofilm development can be affected by a wide range of environmental and dietary factors, as such as pH, temperature, and dietary choices, and antibacterial medications, in order for biofilm to form, the pH must be just right. The ideal pH conditions for *S. aureus* to construct a persistent biofilm are those with low acidity, whereas high acidity or excessive alkalinity does not stimulate the formation of biofilm (Khalil and Sonbol 2014). Addition of glucose, sucrose, or NaCl to the culture medium has been found to stimulate biofilm formation in MRSA and MSSA isolates (Singh *et al.* 2017). NaCl, for instance, has a dose-dependent effect on biofilm aggregation, stability, and strength; as little as 7% NaCl can lead to the formation of a robust biofilm harboring *icaA* (Mirani *et al.* 2012).

The initial step in the formation of a biofilm is bacterial adherence to a surface; this is also the earliest form of bacterial survival adaptation (Cascioferro *et al.* 2014). *S. aureus* increases its ability to attach by producing a number of adhesion factors that enable it to adhere to biotic (such as soft tissue or bone) and abiotic (such as artificial or ecological) surfaces, or even to self-aggregate, biofilms can be formed by bacteria with this trait in any kind of setting, adhesion of bacteria is significantly affected by both physical and chemical factors, including cell surface charge, extracellular polymer, and hydrophobicity of the cell surface, most bacteria cells are negatively charged, so surfaces with a positive charge are better at keeping bacteria at bay (Hu *et al.* 2017). Bacterial adherence to hydrophobic and hydrophilic surfaces is mediated by macromolecules in *S. aureus* cell walls that respond to changes in temperature (Harris *et al.* 2002).

Recent research suggests that *S. aureus* and MRSA biofilms colonizing food-related objects and equipment may function as a hidden pathway for contaminating food handlers and others (Rodríguez-Lázaro *et al.* 2018). The biofilm-associated protein (bap) and the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are both important for *S. aureus* biofilm production (Otto 2012). Collagen-binding protein (cna), fibronectin-binding proteins (fnbA and fnbB), clumping factors (*clfA* and *clfB*), and bone sialoprotein-binding protein (bbp) are all examples of host extracellular matrix proteins that MSCRAMM proteins interact with (Atshan *et al.* 2012). Beenken *et al.* (2004) found that the *icaADBC* operon-expressed polysaccharide intercellular adhesin is essential for the accumulation stage of biofilm growth.

The synthesis of virulence factors in Gram-positive bacteria is regulated by an intricate regulatory network, it is widely known that *S. aureus*' accessory gene regulatory (*agr*) system and the staphylococcal accessory regulatory system (*sarA*) are prototypical examples of regulatory systems that regulate virulence factor synthesis and biofilm formation in different ways (Jenul and Horswill 2018). As a result, the *agr* and *sarA* systems in *S. aureus* function as molecular switches to govern biofilm formation, while an active *sarA* system promotes biofilm development, an active *agr* system inhibits the adhesion genes required for biofilm formation and promotes its dispersion (Vasudevan 2019).

2.5.2 *Bbp* gene

Most cases of osteomyelitis and septic arthritis in humans are caused by the bacterium *S. aureus*. However, *S. aureus* is also responsible for endocarditis, septicemia, and pneumonia, as well as osteomyelitis and soft tissue infections in diabetic foot infections (Persson *et al.* 2009). Osteomyelitis and septic arthritis both involve a glycoprotein called bone sialoprotein (BSP), which *S. aureus* binds to (Subhadharsini and Gopinath 2016). Several microbial surface elements that recognize sticky matrix molecules are members of the Sdr family, the 97-kDa protein known as Bbp from *S. aureus* strain O24 shares 76% and 96% of its amino acid sequences with the relevant domains in SdrE, respectively (Colque-Navarro 2010, Zheng *et al.* 2018). SdrE and platelet activation are related, but was inversely related to bone infection, The Pantone-Valentine leukocidin and methicillin (methicillin) resistance genes are associated with *S. aureus* gene *bbp* expression, however the location of this expression varies (Otsuka *et al.* 2006, Campbell *et al.* 2008).

It has been shown that Bbp binds to both fibrinogen and bone sialoprotein (BSP), the *bbp* is involved in the synthesis of binding proteins, which are crucial for bacterial colonization and the development of biofilms that lead to infection (Yarwood *et al.* 2004, Vazquez *et al.* 2011).

Bbp was found while looking for *S. aureus* surface proteins that are involved in the adhesion of *S. aureus* to bone tissue during osteomyelitis, and further investigation revealed that this MSCRAMM specifically linked to the bone sialoprotein component of the extracellular matrix (Vazquez *et al.* 2011, Francis 2015).

2.5.3 Clumping factors A (*clfA* gene)

Secreted virulence factors include the enterotoxins and surface-exposed virulence factors such as fibrinogen, protein A, and fibronectin-binding proteins that *S. aureus* can produce (Zhang *et al.* 2016). Clumping factors, A and B (*clfA*, *clfB*), which bind fibrinogen, are secreted by *S. aureus* on bacterial cells, allowing the germs to cling to cell surfaces, the

most endothelial lesions have fibrinogen as their host protein, *clfA* is expressed at all stages of bacterial growth, but *clfB* is only present during the initial logarithmic phase (Peacock *et al.* 2000).

Since fibrinogen is essential in the formation of platelet thrombi, the clumping factor is thought to be vital for colonization and the establishment of infections caused by *S. aureus*, the *clfA* gene is present in nearly all *S. aureus* strains and has a role in infection by encouraging bacterial binding to fibrinogen in soluble or immobilized forms (Karahan *et al.* 2011, Delfani *et al.* 2016). Staphylococcal adhesion to host extracellular matrix components is mediated by microbiological surface components that recognize sticky matrix molecules on the surface of *S. aureus* (Vazquez *et al.* 2011). Sortase enzymes catalyze the covalent attachment of these components to the peptidoglycan (Heilmann 2011). Along with the *ica* operon, which generates polysaccharide intercellular adhesion (PIA), these factors contribute to the formation of biofilms (Mirzaee *et al.* 2014). To avoid being destroyed by the immune system, MSCRAMMs can hide by binding to molecules including collagen (mainly via Cna), fibronectin (with FnbAB), and fibrinogen (with *ClfAB* and Fib) (Foster *et al.* 2014).

Moreover, one of the proteins necessary for *S. aureus* persistence inside a host is clumping factor A (*clfA*), a cell-wall anchored surface protein that likely takes part in *S. aureus*-mediated agglutination in the presence of fibrin(ogen) to escape host phagocytes (Palmqvist *et al.* 2004, Cheng *et al.* 2009, Cheng *et al.* 2011). Once *S. aureus* has adapted to host factors, abscesses occur as a result of *clfA*-mediated binding to fibrin (ogen) during infection (Cheng *et al.* 2011). *S. aureus* *clfA* ablation or mutation, on the other hand, has been shown to reduce human mortality and bacterial abscess formation in sepsis models in a number of laboratories (Josefsson *et al.* 2008, McAdow *et al.* 2011).

2.5.4 Ica operon (*icaA* gene)

S. aureus's initial biofilm and adhesion are regulated by a network of genes, the *ica* operon regulates the expression of the *icaA* gene, the genes *icaA*, *icaB*, *icaC*, and *icaD* in this operon play a crucial role in the formation of *S. aureus* biofilms by generating

polysaccharide intercellular adhesion (PIA), which also serves to hold the bacteria together and is important for the first attachment (O'Gara 2007, Rohde *et al.* 2007). As a result, it is hypothesized that lessening the expression of this operon, especially the *icaA* gene, may lessen biofilm formation and, thus, reduce infection rates (Archer *et al.* 2011, Otto 2019).

UDP-N acetylglucosamine is converted into phosphoinositide adenosine monophosphate (PIA) by N-acetylglucosaminyl-transferase, which is expressed from the *ica* locus and specifically from *icaA*, this gene's low enzymatic activity can be attributed to the small amount of polysaccharide produced by its expression alone, with an increase in N-acetylglucosaminyl-transferase activity, oligomers containing 10-20 b-1,6-N-acetylglucosamine residues are produced when *icaA* and *icaD* are expressed together (Oliveira and Cunha 2010, Gad *et al.* 2012, Namvar *et al.* 2013).

The expression of genes like the intracellular adhesion (*icaADBC*) operon and the microbial surface components recognizing adhesive matrix molecule (MSCRAMMs) operon likely affects this organism's behavior in vivo, including its resistance to treatment (Fey and Olson 2010, Rohde *et al.* 2010, Römling and Balsalobre 2012).

IcaA and *IcaD* are the two most important proteins in the PNAG biosynthesis pathway (Archer *et al.* 2011). In most clinical *S. aureus* strains, the *ica* operon is present and is responsible for the production of extracellular polysaccharides, the best-understood mechanism for biofilm development, the *ica* operon is further subdivided into the *icaA*, *icaD*, and *icaB* loci, each of which is responsible for the relevant pathogenic and virulent factors involved in polysaccharide intercellular adhesin synthesis (Idbeis and Khudor 2019).

2.6 Antibiotic Resistance in *S. aureus*

S. aureus is one of the common bacteria that can develop resistance to drugs very quickly, antibiotic resistance can arise from a number of different mechanisms, such as the

inactivation of antibiotic-activating enzymes, target modifications that reduce antibiotic affinity, antibiotic trapping, efflux pumps, etc (Piso *et al.* 2017). *S. aureus* can be classified into two subsets distinguished by their resistance evolution, MRSA, or methicillin-resistant *S. aureus*. Strains of *S. aureus* that have developed resistance to beta-lactam antibiotics, a class of broad-spectrum drugs that includes some penam-penicillin derivatives like methicillin and oxacillin, and cephalosporins like cepham, are particularly susceptible to MSSA-Methicillin. For example, *S. aureus* can easily infect them (Gurusami *et al.* 2013).

The rise of antibiotic-resistant bacteria is a major threat to global health; an estimated 23,000 people have lost their lives due to antibiotic-resistant germs because of the overuse of these drugs (Fymat 2017). Antibiotic-resistant diseases aren't limited to hospitals, either; they can pop up anywhere in the community, when bacteria develop resistance to antibiotics, they become resistant to treatment in general and can thrive in a wide range of environments. Bacterial resistance is a major public health concern (Wang *et al.* 2016, Margonis *et al.* 2018).

Aminoglycosides function as bactericidal antimicrobial agents by suppressing protein synthesis when they bind to the 30S ribosomal subunit, resistance to aminoglycosides can be generated through in vitro ribosomal subunit mutation, in a similar vein, it has been noted that acquiring enzymes that alter aminoglycosides can promote the formation of resistance to aminoglycosides (Wilson 2014, Walsh and Wencewicz 2016).

According to the studies, mutations in DNA gyrase (gyrA and B) and topoisomerase are necessary for the development of resistance to fluoroquinolones (ParC and ParE), the same is true for *S. aureus*, where a NorA-coordinated multidrug efflux pump system is necessary for resistance development via the efflux pump (Zeng *et al.* 2016, Foster *et al.* 2017). Tetracycline has a bacteriostatic effect and functions by attaching to 30S ribosomal subunits and preventing the tRNA from entering the acceptor site, thereby suppressing protein synthesis; *S. aureus* can develop resistance through either the efflux pump system or the ribosome's defense; TetM encodes for the ribosome's defense, while TetK codes for an efflux pump system (Jenner *et al.* 2013, Nguyen *et al.* 2014).

Antimicrobial resistance (AMR) in commonly occurring pathogenic bacteria, such as MRSA, is viewed as a severe health risk that increases healthcare costs, leads to treatment failure, and causes deaths (Prestinaci *et al.* 2015). The threat posed by AMR is more pressing in low- and middle-income nations where there is a lack of knowledge regarding the incidence and epidemiology of AMR bacteria in people, animals, and the environment, MRSA infections are particularly problematic since *S. aureus* has a history of displaying diverse resistance to various medications, notably to beta-lactam drugs (Lakhundi and Zhang 2018).

Bacteriostatic tetracyclines are among the most widely used classes of antibiotics (Emaneini *et al.* 2013). They specifically attack the 30s subunits of ribosomes and damage transfer RNA to halt protein synthesis (Bitrus *et al.* 2018). Tetracycline resistance can be generated in two ways: ribosome protection (encoded by the *tetM* and *tetO* genes) and the efflux pump system (encoded by the *tetK* and *tetL* genes and transported by plasmids) (Emaneini *et al.* 2013, Bitrus *et al.* 2018).

The lincosamide class of antibiotics includes clindamycin. By attaching to the 50S ribosomal subunit, it prevents the bacterial cell from synthesizing proteins (Adhikari *et al.* 2017). Lincosamide resistance emerges through methylating the ribosome's receptor binding site, altering the target cell. The methylase enzyme, which is encoded by the *erm* genes, regulates methylation (Bitrus *et al.* 2018).

In the clinical setting, ciprofloxacin, a fluoroquinolone-class broad-spectrum synthetic antibiotic, is administered orally or intravenously to treat bacterial infections that could potentially be fatal, DNA gyrase is an enzyme required for bacterial DNA replication; ciprofloxacin blocks this process in both Gram-positive and Gram-negative bacteria (Drlica and Zhao 1997). For this reason, it is recommended that ciprofloxacin be taken in combination with another antibiotic (Gündeş *et al.* 2000). For instance, when combined, quinolones and rifampicin are capable of totally wiping out MRSA strains (Günel and Erdem 2014).

The most striking characteristic of MRSA isolates is their MDR, or resistance to at least one agent in three or more classes of antimicrobials, only glycopeptide antibiotics, such as vancomycin, are effective against these isolates (Diaz *et al.* 2018). Many commonly used antibiotics fail to kill multidrug-resistant (MDR) MRSA isolates; this includes aminoglycosides, fluoroquinolones, macrolides, tetracycline, and chloramphenicol (Kot *et al.* 2020, Nataraj and Mallappa 2021).

Previous studies revealed that some developing countries might need alternatives to antibiotics. For instance, 88% of *S. aureus* infections in Nigeria are resistant to beta-lactam medicines. According to Akinkunmi and Lamikanra (2012), there are between 42% and 51% of MRSA cases in Pakistan. A different study found that in India and Pakistan, 95% of adults have bacteria that are resistant to β -lactam antibiotics (Reardon 2014). According to studies, MRSA results in 11,000–18,000 fatalities, 80,000 invasive infections, and limited treatment options per year in the US (Morgenstern *et al.* 2016).

2.7 Methicillin Resistant *S. aureus* (MRSA)

Hospital-acquired MRSA causes 64 percent more deaths than the non-resistant form of the diseases it spreads, making it a major public health concern, colonization with methicillin-resistant *S. aureus* (MRSA) has a pooled global prevalence of 1.3%, and it is believed that 30% of the general population is colonized with *S. aureus* (Hassoun *et al.* 2017, World Health Organisation 2021). Prevalence of methicillin-resistant *S. aureus* infections was found to be anywhere between 2.3% and 69.1% in countries of South and East Asia and the Western Pacific (World Health Organization 2014). Methicillin-resistant *S. aureus* (MRSA) caused poorer clinical outcomes than MSSA did (Van Hal *et al.* 2012). More than 80,000 people contract a life-threatening infection from MRSA each year, leading to 11,000 deaths (Pokharel *et al.* 2019).

Transmission of methicillin-resistant *S. aureus* (MRSA) between Staphylococcal species is hypothesized due to the fact that MRSA is generated when methicillin-susceptible *S. aureus* (MSSA) acquires the *mecA* methicillin resistance gene exogenously via the staphylococcal cassette chromosome *mec* (*SCCmec*), a mobile genetic element

(Tsubakishita *et al.* 2010). It is safe to assume that *S. aureus* is resistant to oxacillin and other beta-lactam antibiotics due to its demonstrated resistance to the penicillinase-stable penicillin family, which includes methicillin, oxacillin, nafcillin, cloxacillin, and dicloxacillin, this resistance is caused by *mecA* expression or other mechanisms, such as changes in the affinity of penicillin-binding proteins for o (Franklin *et al.* 2012).

S. aureus infections are notoriously difficult to treat due to the high rate of antibiotic resistance among *S. aureus* isolates, with methicillin-resistant *S. aureus* (MRSA) being the most clinically significant (Turner *et al.* 2019). Methicillin-resistant *S. aureus* (MRSA) infections are associated with a higher rate of hospitalization, mortality, and morbidity than those caused by methicillin-sensitive *S. aureus* (MSSA) (Ippolito *et al.* 2010). The prevalence of methicillin resistance among clinical isolates varies greatly among nations, ranging from a few percent in Scandinavia to well over half in the United States and China, to name just a few (Stefani *et al.* 2012). Hospital-acquired methicillin-resistant *S. aureus* (MRSA) infections are on the decline in the United States, Europe, China, and many other industrialized nations (Hu *et al.* 2016, Kourtis *et al.* 2019), but are on the rise in less developed places like Africa (Falagas *et al.* 2013).

2.8 Molecular Detection for MRSA

Molecular methods are used to diagnose MRSA, including:

2.8.1 *mecA* gene

The *mecA* gene complex has insertion sites for other mobile genetic elements (such plasmids and transposons) that make it easier for bacteria to pick up genes for antibiotic resistance, according to epidemiological research, hospital- and community-acquired MRSA infections are on the rise in many regions of the world (Abulreesh *et al.* 2017).

The *mec* gene complex, which consists of the methicillin resistance determinant and intact or truncated copies of the *mec* regulatory genes (*mecI*, *mecR1*, and *mecR2*), and the *ccr*

gene are the two critical loci that set staphylococcal cassette chromosome mec (*SCCmec*) (Katayama *et al.* 2000). Researchers have observed almost no variation in the sequencing of the *mecA* determinant among MRSA strains, despite the fact that there are a considerable number of variations in the genetics of *SCCmec* components (Hiramatsu *et al.* 2013).

Penicillin-binding protein 2a (PBP2a), an enzyme that crosslinks the peptidoglycans in the bacterial cell wall, is encoded by the *mecA* gene in the MRSA genome, the low-affinity of PBP2a to beta-lactam antibiotics like penicillins, cephalosporins (other than ceftaroline and cefobiprole), and carbapenems is the root cause of resistance to these drugs (Turner *et al.* 2019). Penicillin binding protein 2a (PBP2a), a hallmark of the MRSA phenomenon, has a poor affinity for all beta-lactam medicines but is encoded by the *mecA* gene present in all MRSA strains (Aguayo-Reyes *et al.* 2018).

3. MATERIALS AND METHODS

3.1 Materials

It involves the use of several materials mentioned below:

3.1.1 Laboratory equipment and apparatus

The apparatus and equipment's used throughout this study were shown in Table 3.1.

Table 3.1 Equipment's and Apparatus

No.	Equipment and Apparatus	Manufacture Company	Origin
1.	Autoclave	Daikyo	Japan
2.	Bunsen-burner	Amal	Turkey
3.	Centrifuge	Acculab	USA
4.	Compound Light microscope	Leitz	Germany
5.	Deep freezer	Angelantoni	Italy
6.	Disposable Plastic Petri dishes	Afco-Dispo	Jordan
7.	Disposable tips	Sterellin Ltd	UK
8.	ELISA-Reader	Biotek	USA
9.	Eppendorf Centrifuge	Hettich	Germany
10.	Gel Documentation	Bioneer	Korea
11.	Gel Electrophoresis devise	Cleaver	UK
12.	Hood	ESCO	Singapore
13.	Incubator	Memmert	Germany
14.	Micropipette(different size)	Eppendorf	Germany
15.	Millipore filters	Gallenkamp	UK
16.	Nanodrop	Implen	Germany
17.	Oven	Gallenkamp	UK
18.	PH Meter	LKB	Sweden
19.	PCR tube (0.2)	Biobasic	Canada
20.	Refrigerator	Hitachi	Japan
21.	Sensitive balance	Sartorius	Germany
22.	Spectrophotometers	Shimadzu	Japan
23.	Thermocycler	Agilent	USA
24.	Vitek 2	BioMerieux	France
25.	Vortex mixer	Grant	UK
26.	Water bath	Daiham	Korea

3.1.2 Biological and chemical materials

Biological and chemical materials that were used in this study are mentioned in Table 3.2.

Table 3.2 Biological and Chemical materials were used in the study

No.	Materials	Manufacture Company	Origin
1.	Agar-Agar	Himedia	India
2.	Agarose	Himedia	India
3.	Ethanol	B.D.H	UK
4.	Ethidium Bromide dye	Bioneer	Korea
5.	Glucose	B.D.H	UK
6.	Glycerol (C ₃ H ₈ O ₃)	Himedia	India
7.	Human blood and plasma	Blood bank	Iraq
8.	Hydrogen peroxide(H ₂ O ₂)	Himedia	India
9.	Methyl red	BDH	UK
10.	Nuclease Free Water	Bioneer	Korea
11.	Phosphate buffer saline (PBS)	Himedia	India
12.	Sodium chloride (NaCl)	B.D.H	UK
13.	Tris-Borate-EDTA Buffer	Promega	USA
14.	Urea	B.D.H	UK

3.1.3 Kit and other materials

Kit and other materials used for *S. aureus* DNA extraction and PCR technique are shown in Table 3.3.

Table 3.3 Kit and other materials for DNA extraction and PCR technique

No.	Type of Kit	Company	Origin
1.	DNA extraction Kit	Promega	USA
2.	DNA ladder	Promega	USA
3.	GoTaq® Green Master Mix	Promega	USA
4.	Gram stain	Syrbio	Switzerland
5.	Loading dye (6X)	Promega	USA
6.	Primers	Macrogen	Korea

3.1.4 Culture media

Culture media used in this study were listed in Table 3.4.

Table 3.4 Culture Media

No.	Media	Manufacturer	Origin
1.	Blood-agar-base	Himedia	India
2.	Brain-Heart Infusion Agar (BHIA)		
3.	Brain Heart Infusion Broth (BHIB)		
4.	Mannitol-Salt Agar (MSA)		
5.	Mueller Hinton Agar (MHA)		
6.	Nutrient Agar (N.A)		
7.	Nutrient Broth (N.B)		
8.	Tryptic Soya Broth (TSB)		
9.	Urea-agar-base		

3.1.5 Antibiotic discs

All Antibiotic discs had used in this study are listed below in the Table 3.5.

Table 3.5 Antibiotic discs were used in this study

No.	Antibiotic agent	Symbol	Concentration µg/ Disc	Company (origin)
1.	Ciprofloxacin	CIP	10	Bioanalyse (Turkey)
2.	Clindamycin	DA	10	
3.	Erythromycin	E	10	
4.	Gentamicin	CN	10	
5.	Methicillin	ME	10	
6.	Penicillin	P	10	
7.	Tetracycline	TE	10	

3.1.6 Primers (MacroGen, Korea)

All Primers Sequence had used in this study are listed below in the Table 3.6.

Table 3.6 Name and sequence of primers

Primer name	Sequence (5' to 3')	Amplicon size (bp)	Reference
<i>clfA</i> -F	GTAGGTACGTTAATCGGTT	1584 bp	(Peacock <i>et al.</i> 2002)
<i>clfA</i> -R	CTCATCAGGTTGTTTCAGG		
<i>icaA</i> -F	ACACTTGCTGGCGCAGTCAA	188 bp	(Rohde <i>et al.</i> 2001)
<i>icaA</i> -R	TCTGGAACCAACATCCAACA		
<i>bbp</i> -F	CAGTAAATGTGTCAAAAGA	1055 bp	(Peacock <i>et al.</i> 2002)
<i>bbp</i> -R	TACACGGTGTTGAAGTG		
<i>mecA</i> -F	ACTGCTATCCACCCTCAAAC	163 bp	(Sultan and Meani 2019)
<i>mecA</i> -R	CTGGTGAAGTTGTAATCTGG		
16S <i>rRNA</i> -F	GTAGGTGGCAAG CGTTATCC	228 bp	(Moore and Lindsay 2001)
16S <i>rRNA</i> -R	CGC ACA TCA GCG TCA G		

3.2 Methods

It includes a set of steps:

3.2.1 Sterilization methods

They include the following:

1. Wet-heat sterilization (Autocleaving)

The culture media and some solutions were autoclaved at 121 °C for 15 minutes under 15 pounds/inch².

2. Flaming sterilization

The loop and forceps are sterilized using a Bunsen burner, which is a type of laboratory stove.

3. Filter sterilization

A 0.22 and 0.45µm Millipore filter was used to sterilize solutions that are destroyed by high temperatures such as sugars, urea, and some dyes.

3.2.2 Collection of samples

From June 2022 to September 2022, 132 specimens were collected from human patients attended to hospitals in Babil Governorate (Imam Al-Sadiq Teaching Hospital and Mirjan Teaching Hospital). The specimens were included (9) nasal swab, (32) wound swab, (27) burn swab, (12) skin swab, (18) ear swab, (24) throat swab, and (10) urine culture.

All collected samples were inoculated in enriched medium of brain-heart infusion broth and incubated at 37 °C for 24 hours. then streaked in blood agar and mannitol salt agar (MSA) and incubated for an additional 24 hours to recognize the *S. aureus* from other Staphylococcus species by mannitol salt agar which is taken into consideration as a differential and selective medium for the identification, isolation, and purification of Staphylococcus, and for the detection of the ability of each *S. aureus* to ferment mannitol. The isolates had identified for their size, shape, and color by the microscope, and haemolytic activity on blood agar. The further biochemical tests were carried out to confirm the identity of the isolates, diagnosis, and identification of *S. aureus* (William *et al.* 2009).

3.2.3 Reagents and solutions

In this study some reagents and solutions were used, they are described below:

3.2.3.1 Reagents

They include the following:

Catalase reagent

This reagent is composed of a commercially available 3% hydrogen peroxide (H_2O_2) solution, which was used for detecting catalase enzyme production by bacterial isolates (Harley and Prescott 2007).

Oxidase reagent

Preparing the oxidase reagent required dissolving 1 gram of dimethyl-p-phenylenediamine hydrochloride in 100 milliliters of DW. Then, it was placed in a dark bottle and use to ensure the ability of bacteria to produce oxidase enzyme (Koneman *et al.* 2006).

Solutions

They include the following:

Ethanol 70%

It was prepared by adding 30ml of DW to 70ml of absolute ethyl alcohol.

Normal saline solution

It was prepared by adding 100 ml DW to g 0.85g of sodium chloride NaCl until it completely dissolved. The solution was sterilized by autoclaving (Freeman and Natanson 2005).

Tris-borate EDTA buffer (1X- TBE buffer)

TBE buffer (10X) was supplied by the provider (Promega, USA), consisting of Tris-base, boric acid, and EDTA. The PH was adjusted to 8.2; a (1X) working solution was made by adding 900 ml of distilled water to 100 ml of buffer.

McFarland standard solution

McFarland-standard solution No. (0.5) is the standard turbidity solution, which is the most common approach for standardizing or preparing inoculum, bacterial-suspension containing 1.5×10^8 CFU/ml was compared to this solution which has a specific optical density (O.D) to provide turbidity.

The following procedure indicates McFarland's preparation:

Solution (A): 100ml of distilled water with 1g of barium chloride (BaCl_2) dissolved in it .

Solution (B): 99 ml of distilled water was added to 1ml of concentrated sulphuric acid (H_2SO_4) to complete the volume to 100 ml.

0.5ml of solution (A) was added to 99.5 ml of solution (B) and stored in a dark bottle until used (Collee *et al.* 1996, Benson 2002).

3.2.4 Biochemical tests

They include:

1. Oxidase test

This test indicates whether or not the bacteria under investigation have the ability to manufacture the enzyme oxidase. Using a wooden stick, a bacterial colony was

transferred from an overnight incubated nutrient agar culture and placed on the oxidase reagent, which had been made in three drops and deposited on a filter paper that had been placed on a clean plate (petri dish). Finally, in a favorable reaction, the colony's color should change to purple after 10-30 seconds, while in a negative reaction; the colony's color should not change (Prescott 2002).

2. Catalase test

This test was used for determining the capability of *S. aureus* to catalase enzyme productions which converts H_2O_2 to oxygen and water. A single isolated bacterial colony was looped out of the culture plate and placed on a clean glass slide. after that 2 drops of 3% concentration of H_2O_2 were added to the cells in the slide and mixed. The formation of gas bubbles indicates a positive result (Brooks *et al.* 2007).

3. Urease test

This test was used to determine the capability of bacteria to produce urease enzyme. By using a loop, a colony of a bacterium to be tested was inoculated in tubes containing urea medium prepared and then incubated for 24-48 hours. The Positive result is given by a change of medium from yellowish orang to pink color due to bacteria produce urease that hydrolyzes the urea to ammonia that raises the pH and causes the phenol red that is a pH indicator to turn to pink. The non-production of pink color is an indication that the bacteria are unable to hydrolyse urea, and it is urease negative (Collee *et al.* 1996).

4. Coagulase test

This test is used for the detection of the ability of *S. aureus* to produce coagulase enzyme, as follows:

In this test, two drops of normal saline were put onto the slide and emulsified with one or two colonies of the test bacteria using a sterile loop, after that 1 drop of human plasma

was dropped on the mixture on the slide and mixed carefully. Observation of clumping within 10-15 seconds is indicating a positive result. Negative control was prepared by mixing plasma with normal saline without bacteria (Brooks *et al.* 2007, Anyanwu and John 2013).

3.2.5 Preparation culture media

They include the following:

3.2.5.1 Ready-made media

The media used in the study was prepared according to the manufacturer's instructions, while the other media was prepared according to laboratory references. All of these media were sterilized in an autoclave for 15 minutes at 121°C with a pressure of 1 bar/inch.

1. Mannitol salt agar

Mannitol salt agar used in this study were prepared (110 gm\ L) as the following: pH adjusted as demanded by pH-meter; Sterilized by autoclaving at 121 °C for 15 min at 15 pound/square, then distributed into Petri dishes or tubes as needed, incubated for 24 hrs.' at 37 °C to ensure sterility and stored as 4 °C until use. This medium used for selective isolation of *S. aureus* (Tille, 2016).

2. MacConkey agar

MacConkey agar used in this study were prepared (51.5 gm\ L) as the following: pH adjusted as demanded by pH-meter; Sterilized by autoclaving at 121 °C for 15 min at 15 pound/square, then distributed into Petri dishes or tubes as needed, incubated for 24 hrs.' at 37 °C to ensure sterility and stored as 4 °C until use.

3.2.5.2 Lab-prepared media

These media include:

1. Blood Agar

The media was prepared by following instructions producer in the leaflet of the company, sterilized with autoclave, then 5ml of sterile defibrinated human blood was applied to every 100ml of the medium after cooling to 50 °C, mixing well, and then pouring into a sterile Petri-dish. This media is sufficient for the cultivation, detection of haemolytic activity, isolation of bacteria, and the kind of haemolysis (Benson 2002).

2. Urease medium

Urea base agar is prepared according to the instruction of the manufacturer. The pH was adjusted to 6.8-6.9 and autoclaved. The medium was cooled to 45-50 °C, then 5ml of sterile-filtered (using 0.45 µm Millipore filter) of 40% urea solution was aseptically added to each 95ml of Urea base agar, mixed well and distributed into sterile tubes and allowed to set in the slope position. This medium was used for detecting the ability of bacteria for urease enzyme production (Atlas 2006).

3. Skim – Milk agar

This medium was used to know the ability of *S. aureus* to make pigments, the medium was prepared by adding 5g/12.5 ml D.W of sterilized (by heating in the autoclaving for 5 min at 121°C) skim milk to previously sterilized and cooled to 45 °C of (2.45 gm of nutrient agar dissolved in 87.5 ml of distilled water), then mixed well then poured in a sterile Petri-dish (Collee *et al.* 1996, Benson 2002).

4. Tryptic soya broth (TSB)

Tryptic soy broth (TSB) was prepared according to the instruction of the manufacturer company (Himedia/India), sterilized by autoclaving (121°C/15 psi) for 15 min, cooled to room temperature and filter-sterilized glucose solution was added at a final concentration 1%. It used in the detection of biofilm formation.

3.2.6 Identification of isolates

They include the following:

3.2.6.1 Microscopic examination

The colony that suspected *S. aureus* was examined under the microscope by using a Gram stain reaction which was achieved by a loopful of sterile distilled water placed in the middle area of the slide. A bacterium loop was mixed with the sterile DW and diffused over the slide, let the smear to dry in the air and heat-fixed by moving the slide quickly over the flames of the burner 3 times. Then the slide was stained with gram-stain and examined under oil immersion to detect their response to the shapes, stain, and arrangement of the cells (Benson 2002).

3.2.7 VITEK 2 diagnostic system

The automatic VITEK 2 system (BioMerieux SA, France) including VITEK 2 DensiCHEK standard, VITEK 2 cassette, and VITEK 2 BCL card was used for identification of *Staphylococcus* spp. according to the manufacturer's instructions. There were 64 plastic wells on the card. Based on the results of 43 colorimetric substrates, bacterial species are phenotypically identified in this technique. Different metabolic activities are measured by substrates, including acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitors (Pincus 2006).

According to the manufacturer's instructions:

1. The isolates under investigation were grown on a Nutrient agar medium and incubated at 37 °C for 24 hours.
2. Three ml of normal saline (aqueous 0.45% to 0.50% NaCl and pH 4.5 to 7.0) were put in clear plastic test tube.
3. Isolated colony was put in test tube used for identification to make suspension using an applicator stick or a sterile swab
4. The density was measured by a Densi Chek device whose density was in the range (0.5-0.63).
5. After completing the sample preparation, the identification card inserted into the hole next to the tube carrying the bacterial suspension, which placed in a special rack (cassette), and the transfer tube is fitted into the appropriate suspension tube.
6. The filled cassette was transferred to the VitEk-2 device to identify the bacteria through 64 biochemical tests.
7. Results are given after incubation, approximately (4-6) hours of placing the sample in the device, depending on the type of bacterial isolates.

3.2.8 Preservation of bacterial isolates

- Short-term storage: Storage for short periods (2-3 weeks) on a nutrient agar plate, stored at 4 °C, sealed to prevent contamination.
- Mid-term storage: Only several months; stored in the medium of nutrient agar (2-3ml) placed in a screw cup then stored at 4 °C.

- **Long-term storage:** It can be stored for up to one year by adding 15% of glycerol to a screw-cup containing bacteria in a liquid medium and stored at -20 °C (Sharma *et al.* 2011).

3.2.9 Antibiotic susceptibility test

The modified Kirby-Bauer method is used for the determination of the resistance and sensitivity of *S. aureus* to a variety of antibiotics (Morello *et al.* 2006), as follows:

1. Preparation of Mueller-Hinton agar plates.

The Mueller-Hinton agar used in this experiment was set up per the manufacturer's guidelines. Once the flask reached 45 °C, it was poured into the Petri dishes, allowed to cool until the plates hardened, and then stored at 4 °C.

2. Inoculum's preparation (Turbidity standard)

To prepare the Inoculum (3-5 colonies have been moved to 5 ml of standard saline tubes) to obtain culture with 1.5×10^8 CFU/ml by adjusting to turbidity standard of McFarland 0.5.

3. Inoculation of the test plates

- For plate inoculation, a sterile swab was dipped into the inoculum. The excessively high Inoculum was reduced by spinning the swab several times and pressing firmly over the fluid amount of the liquid, against the internal wall of the tube.
- Muller-Hinton agar plates the inoculum was inoculated over the whole sterile agar surface by streaking the swab. This step was repeated twice, and each time the plate was rotated by 60°C. Eventually, to ensure the complete distribution of the inoculum the swab was moved over the edge of the agar surface.

- The plates were abandoned to dry at room temperature for a few minutes with the cover of the plates closed.
- Selected antibiotic discs were placed by using sterile forceps on the inoculated agar plate. Before dispensing discs on the agar surface, they should be warmed to room temperature; Discs must press down gently with forceps.
- Agar plates were put in incubator at 37 °C for 18-24 hours within 30 minutes after the application of disks.

4. Reading the results

The Petri dishes were tested for the presence of a bacterial growth inhibition zone (clear rings) around the antibiotic discs after 18-24 hours of incubation. If there is no inhibition zone was found, the organism was considered resistant to the antimicrobial agent in that disc. If there was an inhibition zone around the disc, the zone diameter was measured (by millimeters) then by comparing their sizes with the numbers or values given in (CLSI 2021), (Table 3.7).

Table 3.7 Standards inhibition zones (CLSI 2021)

Antimicrobial Agent		Sensitive S	Intermediate I	Resistance R
Ciprofloxacin	CIP	≥ 21	16-20	≤ 15
Clindamycin	DA	≥ 21	15-20	≤ 14
Erythromycin	E	≥ 23	14-22	≤ 13
Gentamicin	CN	≥ 15	13-14	≤ 12
Methicillin	ME	≥ 22		≤ 21
Penicillin	P	≥ 29		≤ 28
Tetracycline	TE	≥ 19	15-18	≤ 14

3.2.10 MRSA detection

- ❖ Methicillin and Penicillin disc diffusion is a significant approach to identifying MRSA. Cultures were performed on an MHA plate, with incubation durations of 18 to 24 hours and temperatures of 35 to 36 °C.
- ❖ PCR Methods, strains of *S. aureus* harboring *mecA* gene defined as MRSA (Brown *et al.* 2005).

3.2.11 Biofilm formation assay (quantitative)

The microtiter plate test was used to detect biofilm development for all *S. aureus* isolates (Christensen *et al.* 1985, Amaral *et al.* 2005, Atshan *et al.* 2012). Procedure entails the following steps:

1. Culture and preparation of inoculum for *S. aureus*

Individual *S. aureus* isolates were cultured overnight in Tryptic soy broth (TSB) supplemented with glucose (1%). Bacterial cultures were diluted into 5ml of sterile normal saline (NaCl 0.85%), mixed thoroughly, and adjusted to the McFarland turbidity standard (0.5). Bacterial isolates (1.5×10^8 CFU/ml) from each culture were placed in three wells (triplicate testing) of a polystyrene microtiter plate (180µl from TSB supplemented with 1% glucose and 20l from Bacterial cultures) and incubated at 37°C for 18-24 hours.

2. Staining procedure for biofilm and reading the result

A. The culture plate content were removed by aspiration and wells were washed three times with PBS (pH7) to remove loosely adherent cells.

B. The remaining bacterial attachment were fixed at 40°C for 15min., wells were stained with 125µl of 0.1% Crystal violat solution for 5min. at room temperature.

C. Wells were washed with PBS (pH7), and the biofilm production (quantitative analysis) was performed by adding ethanol (95%) for 15min.

D. An ELISA microtiter plate reader was used to quantify the absorbance of the Crystal Crystals present at 570 nm.

E. The control was performed with Crystal violat binding to the wells exposed only to the culture medium without bacteria. The following formula was used to determine the biofilm's adherence as in the Table 3.8.

Table 3.8 The calculated OD values (Sultan and Nabel 2019)

Biofilm Formation	Interpretation
< 0.120	Non/Weak
0.120-0.240	Moderate
> 0.240	Strong

3.2.12 Molecular techniques:

These techniques include:

3.2.12.1 Protocol of DNA extraction

To get the DNA, we followed Promega's protocol for extracting it from MRSA isolates. Before beginning the extraction process, *S. aureus* isolates were cultured on the medium of the brain and heart infusion broth and incubated for (18-24 hours) at a temperature of (37°C), then the following sequential steps were taken:

- One ml of *S. aureus* bacteria grown on heart-brain infusion broth medium was transferred by Micropipette into sterile Eppendorf tubes size (1.5 ml).
- Centrifuged (13000 rpm/2 min) and remove supernatant.
- Added 600µl nuclei lysis solution and incubate at (80 °C/ 5 min) then cool to room temperature.
- Added 3µl of RNase solution and incubate (37 °C/ 15-60 min).
- Added (200µl) of protein precipitation solution by Vortex.
- Incubate on ice for 5 min, and then centrifuge the mixture at 13000 rpm for 5 minutes.
- Transferred supernatant to a new tube containing (500 µl) of isopropanol. Then centrifuging for 2 minutes.
- Added (500µl) of ethanol 70%.
- Centrifuged for 2 minutes then carefully aspirate the ethanol.
- Placed the tube on clean absorbent paper and allow the pellets to air-dry for 15 min.
- In the tube, added 100µl of DNA Rehydration Solution and rehydrate the DNA by incubating the solution overnight at room temperature or 4°C.

3.2.12.2 Prepare agarose gel electrophoresis

- The electrophoresis tray is cleaned and a suitable comb was prepared.

- Fill a container with 1× TBE.
- Dissolved 1.5 g of agarose in a 100 mL of (1× TBE) buffer.
- Put it in the microwave until clear and dissolves agarose completely; then let it cool down a little.
- Agarose was chilled to around 50°C before adding 3µL of Ethidium bromide dye.
- Apply the comb and pour the agarose and leave to cool for (30-60 min).
- The gel placed in the electrophoresis container, adding sufficient buffer to cover the gel almost 1 mm (Voytas 2001).

3.2.13 Estimation of DNA concentration and purity

Using a Nanodrop spectrophotometer, the concentration and purity of the DNA samples were determined, 1µl of elution buffer from Genomic DNA extraction kit as a blank, and then added 1µl of each sample was used to measure the purity (260/280 ratio), and concentration, a ratio of 1.7-2 was considered pure and a concentration of 10-100ng/µl was accepted (Clark and Christopher 2000, Desjardins and Conklin 2010).

3.2.14 Polymerase chain reaction (PCR) technique

Conventional polymerase chain reaction (PCR) is performed to amplify different fragments of genes (Bartlett 2003). In this study, it was used for the detection of the *mecA*, *icaA*, *clfA*, and *bbp* genes in all MRSA isolates.

3.2.15 Preparation of primers

Forward and reverse primers were obtained in a lyophilized form and prepared according to manufactures instructions by dissolving in deionized water to get a concentration of 100 pmol/μl and stored at -20°C until being used a working solution of 10 pmol/μl

The total volume of the polymerase chain reaction mixture consists of 25 μL and as shown in Table 3.9.

The optimum conditions and temperature for the polymerase chain reaction were adjusted and the DNA was amplified for each gene separately. As shown in Table 3.10.

Table 3.9 Components of the reaction mixture of monoplex PCR

Contents of reaction	Volume
Promega Green Master Mix	12.5μL
Forward-primer	1.5 μL
Reverse-primer	1.5 μL
DNA template	2.5μL
Nuclease free water	7 μL
Total	25 μL

Table 3.10 Thermal cycler programs used in this study

Gene	Temperature (°C) / Time					Cycle No.
	Initial denaturation	Cycling condition			Final extension	
		Denaturation	Annealing	Extension		
<i>clfA</i>	95°C/5 Min	95°C/45 Sec	56°C/60 Sec	72°C/60 Sec	72°C/3 Min	35
<i>icaA</i>	94°C/4 Min	94°C/30 Sec	56°C/30 Sec	72°C/1 Min	72°C/10 Min	35
<i>bbp</i>	95°C/5 Min	95°C/60 Sec	55°C/45 Sec	72°C/60 Sec	72°C/4 Min	30
<i>mecA</i>	94°C/5 Min	94°C/1 Min	54°C/1 Min	72°C/1 Min	72°C/7 Min	35
<i>16SrRNA</i>	95°C/5 Min	95°C/1 Min	64°C/45 Sec	72°C/1 Min	72°C/10 Min	16

3.2.16 Agarose gel electrophoresis

In (1.5%) agarose gel 5 μ L of PCR products and Promega DNA ladder (100-1500bp) were carefully loaded in the wells and the electric current was matched (70 volts for 90 minutes).

The gel was observed under a UV trans-illuminator to detect DNA band (PCR Products) and compare with ladder (100-1500 bp).

3.2.17 Statistical analysis

The effect of various factors on research parameters was determined using the statistical analysis system- SAS (2018) application. The Chi-square test was employed in this study to compare percentages (0.05 and 0.01 likelihood) in a significant manner.

4. RESULTS AND DISCUSSION

4.1 Isolation and Identification of *Staphylococcus aureus*

They include the following:

4.1.1 Isolation of bacteria

The current study included isolating *S. aureus* bacteria from different clinical sources (wounds, sore throat, burns, nasal, ear, skin, and urinary tract infections), where 132 samples were collected from patients attending the different hospitals in Babylon city (Al-Imam Al-Sadiq Teaching Hospital and Mirjan Teaching Hospital) during the period from 20/6/2022 to 20/9/2022 (Table 4.1). After performing phenotypic, microscopic, biochemical, and molecular tests, 36 isolates belonging to *S. aureus* were obtained.

Table 4.1 Numbers and percentage of *S. aureus* isolates recovered from different clinical specimens

Source of specimens	Total number of specimens	Number of <i>S. aureus</i> isolates	Percentages of <i>S. aureus</i> isolates
Nasal	9	3	(33.33%)
Wound	32	11	(34.37%)
Burn	27	6	(22.22%)
Skin	12	3	(25%)
Ear	18	2	(11.11%)
Throat	24	4	(16.66%)
Urine	10	7	(70%)
Total	132	36	27.27%

4.1.2 Morphological identification

After initial diagnosis based on phenotypic traits by growing them on mannitol salt agar (MSR) and blood agar, all isolates grown on mannitol salt agar (MSR), which is a selective and differential medium for *S. aureus*, appeared round, glistening, smooth, raised and mucoid. It was subsequently confirmed as *S. aureus* by its ability to test for

coagulopathy. The tubular coagulation test is the traditional gold standard for distinguishing *S. aureus* from other *Staphylococcus* (Harley 2016, Arber 2017).

4.1.3 Microscopic identification

Microscopic examination was conducted of bacterial isolates of *S. aureus* by stained with Gram stain to revealed their response to the stain. The Gram-positive bacteria appeared under the microscope arranged in pairs or in groups and non-spores as mentioned in (Carroll *et al.* 2016).

4.1.4 Biochemical identification

The results of biochemical tests showed that all isolates of *S. aureus* gave a negative result for the oxidase test, and positive for the coagulase tests, urease, hemolysis, gram stain, and catalase. It can produce the enzyme "catalase" and is able to modify hydrogen peroxide (H_2O_2) to water and oxygen (Ford 2014, Goldman and Green 2015) (Table 4.2).

Table 4.2 Results of the biochemical test used for *S. aureus* identification

Biochemical test	Mannitol fermentation	Oxidase	Coagulase	Urease	Hemolysis	Gram stain	Catalase
<i>S. aureus</i>	+	-	+	+	+	+	+

4.1.5 VITEK-2 compact system identification

The System Compact Vitek-2 was used to confirm the diagnosis of bacterial isolates isolated from clinical samples (wounds, sore throat, burns, nose infections, ear infections, skin infections, and urinary tract infections). This system provides 64 biochemical tests needed to diagnose bacterial isolates.

After determining the cultural and microscopic characteristics of the bacterial isolates expected to return to the bacteria *S. aureus* was first diagnosed based on biochemical tests, and the diagnosis was confirmed with a system Vitek2 showed that the number of isolates of *S. aureus* bacteria from nose swabs 3 isolates, wound swabs 11, burn swabs 6, skin swabs 3, ear swabs 2, throat swabs 4 and urine 7 isolates.

4.1.6 Molecular identification

All isolates of *S. aureus* (36) were diagnosed using the gene-specific primer *16SrRNA* to detect the *16SrRNA* gene in its genome to confirm its diagnosis by polymerase chain reaction (PCR) using a Cyclor Thermal device, the PCR products' results were confirmed by comparing their molecular weight with a 100 bp DNA ladder by analyzing the bands on gel electrophoresis in 1.5% agarose, with a PCR product size single amplicon 288 bp band in the agarose gel, the results showed that all isolates (36) belong to *S. aureus* because they possess the *16SrRNA* gene (Figure 4.1).

A study done by Shaymaa and Darem (2016) reported that all isolates included study appear positive result for *16SrRNA* and another study by Omar and Mohammed (2021), showed that all *S. aureus* isolates (150) had a positive result for *16SrRNA*. This gene was also adopted by Shamkhi *et al.* (2019), Kazar and Abbas (2021), and Rasheed *et al.* (2022) to diagnose MRSA isolates from different clinical sources. This method is one of the modern methods of diagnosing pathogens; it has high efficiency and short time (Srinivasan *et al.* 2015).

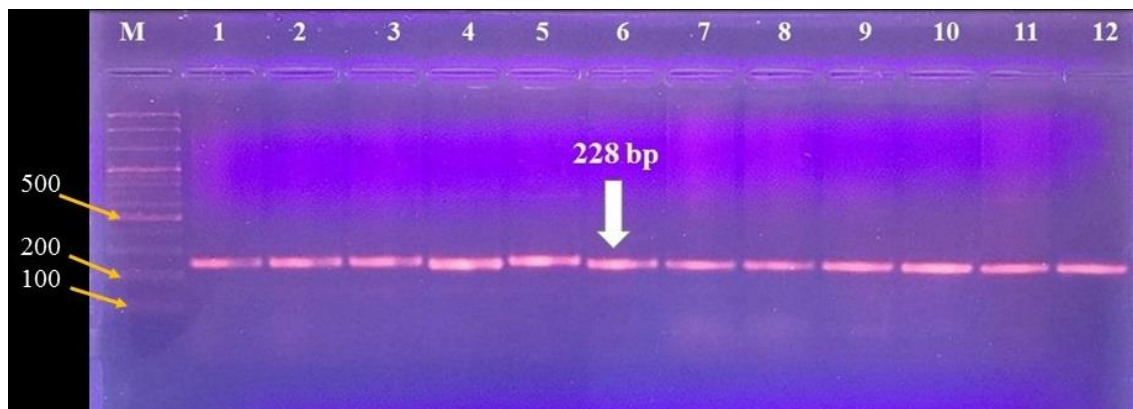


Figure 4.1 Gel electrophoresis of PCR product for *16SrRNA* gene in *S. aureus* with amplicon size of 288 bp. Agarose 1.5%, 70 V for 90 min. Line M: DNA marker (100bp)

Many PCR-based molecular approaches have recently been developed as an alternative option for reliable identification (Emel *et al.* 2010). The most frequent approach to identifying and classifying bacteria, including staphylococci, involves the amplification of *16S rRNA* gene sequences (228) bp (Shah *et al.* 2007).

4.2 Antibiotic Susceptibility

To perform the antibiotic susceptibility test of bacterial isolates, the method described below was used:

4.2.1 Kirby-Bauer disk diffusion susceptibility test

Most strains of MRSA have proven resistant to multiple classes of antibiotics, including beta-lactams, aminoglycosides, macrolides, lincosamides, and, most recently, fluoroquinolones.

To determine the potential resistance of *S. aureus* isolates to seven antibiotics distributed among the antimicrobial groups (Fluoroquinolones, Lincosamides, Macrolides, Aminoglycosides, Pencillines and Tetracyclines), all 36 isolates were subjected to an antimicrobial susceptibility test based on the CLSI (2021), (Appendix 2) which was

carried out using the widely used Kirby-Bauer disk diffusion technique (Hoelzer *et al.* 2011).

The study results shown in Figure 4.2 and Table 4.3 showed that 36 (100%) bacterial isolates are resistant to the antibiotics of the penicillin group represented by (Methicillin and Penicillin), 33 (91.66%) isolates resistant to the antibiotic Erythromycin, 21 (58.33%) resistant to the antibiotic Tetracycline, 18 (50%) resistant to Ciprofloxacin and Gentamicin, 6 (16.66%) resistant to the antibiotic Clindamycin.

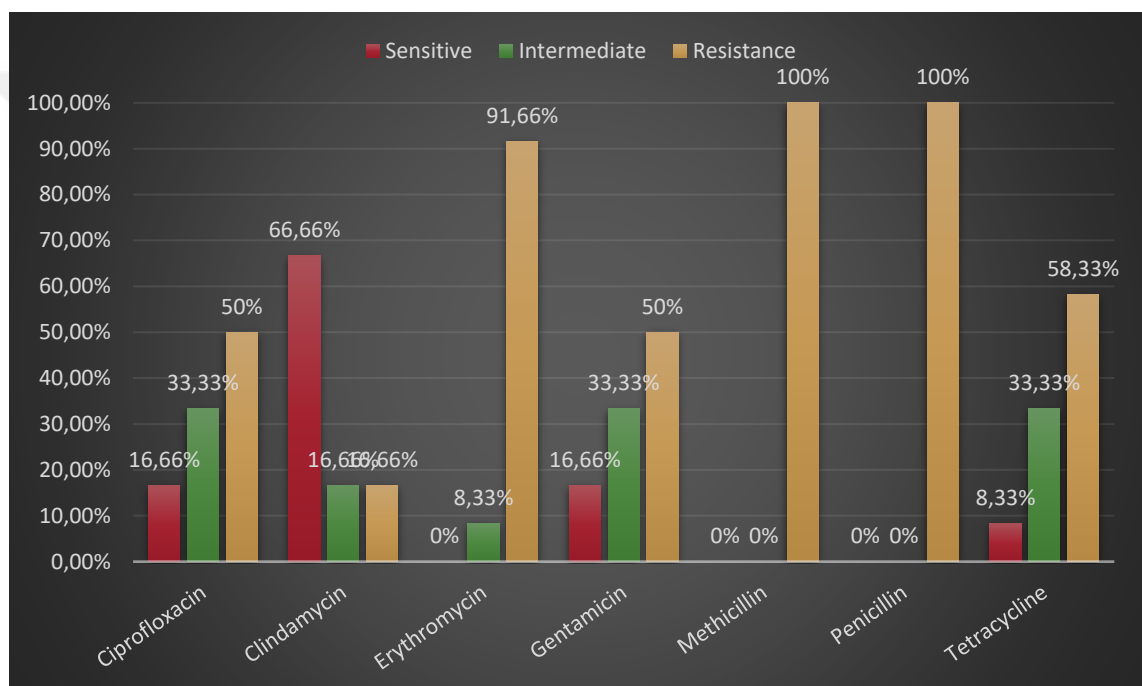


Figure 4.2 Percentage result of antibiotics susceptibility test against *S. aureus* (MRSA) isolates

The results of this study for the antibiotic Methicillin are in agreement with previous studies, including the local results reached by researchers Aziz and Hassan (2019) in which the percentage of isolates resistant to the antibiotic methicillin reached (100%), and the results of our study are also consistent with the results of other studies that indicated the presence of high resistance to this antibiotic, including the study presented by Rafiq *et al.* (2017) and the results by Hatem (2017) the resistance to this antibiotic was (93.42%) and (95%), and also the results of the current study converge with the results another study by Saeed *et al.* (2019) in Pakistan and the results reached by Ranjkesh *et*

al. (2020) in Iran and as the rate of resistance to this antibiotic was (76%) and (100%), respectively. In this study, the antibiotic Methicillin did not agree with the results of the study reached by AL-Sheikh and Yosif (2014) if the resistance of bacterial isolates to this antibiotic was (55.26%).

As for the antibiotic Penicillin, the results of this study agree with the results obtained by Habeeb (2013), and Alkaabi *et al.* (2016), where was the proportion of isolates resistant to this antibiotic (100%), and the results of present study also similar the results of the local another study of Al Ani and Al Meani (2018), where the percentage of isolates resistant to the antibiotic reached (97%), and the results of this study of the penicillin antagonist are consistent with the results the study by Jafari-Sales *et al.* (2020); also, another study in Iran by AlMayahi and Srhan (2021) stated that 100 % of isolates were resistant.

MRSA strains that display extensive resistance to beta-lactam antibiotics were recognized in the early sixties and persist to this day as a cause of serious infections, the mechanism of resistance against these antibiotics is due to two mechanisms of resistance: production of the enzyme beta-lactamase, encoded by *blaZ*, which confers resistance to penicillin, and production of an additional penicillin-binding protein (PBP2a), encoded by *mecA*, with reduced affinity for nearly all other β -lactams (Khan *et al.* 2019). The persistent increase in β -lactam-resistant antibiotics may be attributed to the misuse or overuse of these antibiotics due to their high efficacy and low toxicity (Sultan *et al.* 2018).

In this study, a bacterial isolates showed a moderate resistance rate to Ciprofloxacin of (50%), this result converged with the results of the study presented by AlMohana *et al.* (2012) when the percentage of bacterial isolates resistant was (45.4%), and other studies such as a study by Yaseen *et al.* (2013), and Mujawar *et al.* (2019), also study presented by Ahmed and Ahmed (2022), they found that the percentage of isolates resistant to this antibiotic was (60%), (48%), and (50%), respectively. But Al Ani and Al Meani (2018), and AlHasnawy *et al.* (2019), documented different resistance levels of (6%) and (4.7%), respectively. Despite popular belief, ciprofloxacin resistance is not linked to the *SCCmec* gene (Ender *et al.* 2007). The widespread availability of ciprofloxacin in the form of an

oral suspension that floods the local market has contributed to the rise in ciprofloxacin resistance, which has been linked to its use to treat a wide variety of illnesses, including gastroenteritis, urinary tract infections, endocarditis, and lower respiratory infections, bone and joint infections, prostatitis, and especially enteric fever (Abdullah *et al.* 2012).

As for the antibiotic Clindamycin, the resistance (16.66%) agreed by Rafiq *et al.* (2017), Hatem (2017), Raheema (2020) and also agreed with the findings of Gatta and Ali (2021) where the percentage of isolates resistant to the antibody was (26.32%), (35%), (34%) and (14.28%), respectively. While other studies reported a different result of penicillin resistance rate presented by Alkaabi *et al.* (2016), and Dogramachy (2018), in which the rate of resistant isolates was (92.2%) and (75%) respectively. The majority of *S. aureus* isolates resistant to clindamycin possessed *SCCmec* types I or V, which were previously thought to cause solely β -lactam antibiotic resistance (Deurenberg and Stobberingh 2008). Numerous repeat infections with MRSA isolates among patients with medicine resistance resulted in an attempt to benefit from clindamycin treatment as an alternate antibiotic until cure like this infection (Prabhu *et al.* 2011).

Moreover, Erythromycin resistance was (91.66%), the results of some studies conducted by previous researchers indicated that there are high levels of resistance to erythromycin, which are consistent with the results of our study. Among these studies, Alkaabi *et al.* (2016), Nikbakht *et al.* (2017), and Jafari-Sales *et al.* (2020), also Study Sami and Qassim, (2022) in which the resistance of the isolates to the antibiotic was (92.6%), (86.2%), (83.63%), and (91.1%) respectively. While the results of the current study of this antagonist did not agree with the results obtained by the researcher Habeeb (2013), and Raheem *et al.* (2019), which was (37.5%) and (37.5%) isolates, respectively. Efflux pump, enzyme synthesis, and ribosome modification are three key mechanisms that *S. aureus* uses to evade the antimicrobial effects of macrolides, causing the bacterium to become increasingly resistant to treatment (Reveles *et al.* 2016). As an alternative treatment for sickness caused by these bacteria, erythromycin was administered in patients with hypersensitivity to penicillin, which may explain the high rate of resistance in patient isolates to erythromycin (Elsherife *et al.* 2011).

Gentamicin resistance reached an average of (50%) of MRSA isolates. The results of several studies gave a resistance rate close to resistance Gentamicin, such as the study of Rahimi (2016), and the study by Goudarzi *et al.* (2017), also study Jaddoa and Al-Mathkhury (2018), who recorded resistance rates (47.8%), (60.9%) and (51.51%), respectively. While Dogramachy (2018), and Rasheed and Hussein (2020) in their studies different results from the results of the current study for this antibiotic with resistance rate (12.5%) and (17.86%), respectively. When it comes to Staphylococcal infections, Aminoglycosides like Gentamycin play a crucial role in treatment, but some strains can remain resistant, the main method of resistance to these classes is the deactivation of antibiotics by modifying enzymes that are encoded by genetic elements, and mutations that alter the structure of the ribosome, thereby shortening the binding with drug or affecting the cellular membrane energization (Jensen and Lyon 2009, Rahimi 2016).

As for antibiotic resistance to Tetracycline was (58.33%), which was similar to the results of studies conducted by Al-Hassnawi *et al.* (2012), and Sulaiman and Turkey (2015), also results obtained by Tajik *et al.* (2019) which reached (59%), (56%) and (61.5%). While other studies recorded results that differed from the results of our study, like Mohammadi *et al.* (2014), and Al Ani and Al Meani (2018) studies with a resistance rate of (26%) and (6%) respectively. Since the introduction of mechanisms that promote resistance and can carry different genes of resistance on plasmids and chromosomes, methicillin-resistant *S. aureus* (MRSA) has been recognized as a distinct class of *S. aureus* that demonstrates the property of multi drug resistance (MDR) (Kaur and Chate 2015).

Table 4.3 Antibiotic susceptibility of *S. aureus* (MRSA)

Antimicrobial Agent		Sensitive S	Intermediate I	Resistance R	P-value
Ciprofloxacin	CIP	6(16.66%)	12(33.33%)	18(50%)	0.0007 **
Clindamycin	DA	24(66.66%)	6(16.66%)	6(16.66%)	0.0001 **
Erythromycin	E	0	3(8.33%)	33(91.66%)	0.0001 **
Gentamicin	CN	6(16.66%)	12(33.33%)	18(50%)	0.0007 **
Methicillin	ME	0 (0%)	0 (0%)	36(100%)	0.0001 **
Penicillin	P	0 (0%)	0 (0%)	36(100%)	0.0001 **
Tetracycline	TE	3(8.33%)	12(33.33%)	21(58.33%)	0.0001 **
P-value		0.0001 **	0.0001 **	0.0001 **	---
* ($P \leq 0.05$), ** ($P \leq 0.01$).					

4.2.2 Multidrug resistance (MDR) of MRSA

Most of the MRSA isolates showed multiple resistance at a rate of 94.44% for a group of different antibiotics, if 14 isolates of MRSA (S2, S4, S9, S11, S14, S16, S21, S23, S25, S26, S27, S28, S33, and S35) were resistant to 4 Antibiotics, 8 bacterial isolates (S5, S10, S13, S15, S17, S22, S29, and S34) are resistant to 5 antibiotics, 12 bacterial isolates are (S6, S7, S8, S12, S18, S19, S20, S24, S30, S31, S32, and S36) resistant to 6 antibiotics, as in the Table 4.4.

These results agree with the local findings by Ali and Abd Al Mayahi (2021), and the local study by Abd Zaid and Kandala (2021), which showed that the percentage of MRSA isolates with multiple antibiotic resistance (MDR) was (86.6%) and (94.9%), respectively, and also agree with another study in Egypt by Abdel-Maksoud *et al.* (2016), also in Iran Moosavian *et al.* (2020), the percentage of MDR isolates was (85.8%) and (78%), respectively. While the result of our study incompatible with other studies by Ahrabi *et al.* (2019), and Aljeldah *et al.* (2022), recorded different rates of resistance of bacterial isolates to multiple-antibiotics (31.66%) and (48.45%), respectively. A high rate of MRSA resistance to multiple-antibiotics in high rates is due to the increased ability of bacteria and their preparations to develop their methods and tools and their ability to confront antibiotics more than any other bacterial type as a result of their presence in environments where antibiotics are used randomly and outside health controls (Abushalla 2020).

Table 4.4 Patterns of multiple antibiotic resistance of MRSA isolates

No. of isolate	CIP	DA	E	CN	ME	P	TE
1.	S	S	R	I	R	R	I
2.	I	S	R	I	R	R	R
3.	I	I	R	I	R	R	I
4.	I	S	R	I	R	R	R
5.	R	S	R	S	R	R	R
6.	R	R	R	R	R	R	I
7.	R	S	R	R	R	R	R
8.	R	R	R	R	R	R	I
9.	S	S	R	S	R	R	R
10.	R	S	R	R	R	R	S
11.	I	S	I	R	R	R	R
12.	R	I	R	R	R	R	R
13.	S	S	R	R	R	R	R
14.	I	S	R	I	R	R	R
15.	I	R	R	R	R	R	I
16.	I	S	R	I	R	R	R
17.	R	S	R	S	R	R	R
18.	R	R	R	R	R	R	I
19.	R	S	R	R	R	R	R
20.	R	R	R	R	R	R	I
21.	S	S	R	S	R	R	R
22.	R	S	R	R	R	R	S
23.	I	S	I	R	R	R	R
24.	R	I	R	R	R	R	R
25.	S	S	R	I	R	R	R
26.	I	S	R	I	R	R	R
27.	I	I	R	R	R	R	I
28.	I	S	R	I	R	R	R
29.	R	S	R	S	R	R	R
30.	R	R	R	R	R	R	I
31.	R	S	R	R	R	R	R
32.	R	R	R	R	R	R	I
33.	S	S	R	S	R	R	R
34.	R	S	R	R	R	R	S
35.	I	S	I	R	R	R	R
36.	R	I	R	R	R	R	R

*Ciprofloxacin (CIP), Clindamycin (DA), Erythromycin(E), Gentamicin (CN), Methicillin (ME), Penicillin (P), Tetracycline (TE)

4.2.3 The multiple antibiotic resistance index (MAR)

The MAR index is a tool for analyzing health risks in the study environment and is effective in ascertaining the prevalence and development of bacterial resistance in a specific population as a result of the use of several antibiotics (Davis and Brown 2016). The MAR index for the isolates under study was calculated according to the formula a/b

formula for each isolate, where (a) is the number of antibiotics to which the organism registered resistance, and (b) is the total number of antibiotics used (Subramani and Vignesh 2012).

The results showed, as shown in Figure 4.3, that 94.44% of the MRSA isolates had a MAR index level ranging between (0.57-0.85). A results of this study agree with a results presented by Morshdy *et al.* (2019) in Egypt, and the results of the local study by Al-Mayahi and Srhan (2021), where the MAR index ranged between (0.9-0.36) and (0.48-0.83), respectively. MAR values > 0.2 suggest "high-risk" sources of contamination and ineffectiveness of antibiotics since antibiotics are frequently used carelessly; Bacterial plasmids harboring antibiotic-resistance genes are commonly associated with high MAR values, which can disrupt treatment plans. Because of this, patients may need more time in the hospital than expected (Sandhu *et al.* 2016, Bhuvaneshwari 2017).

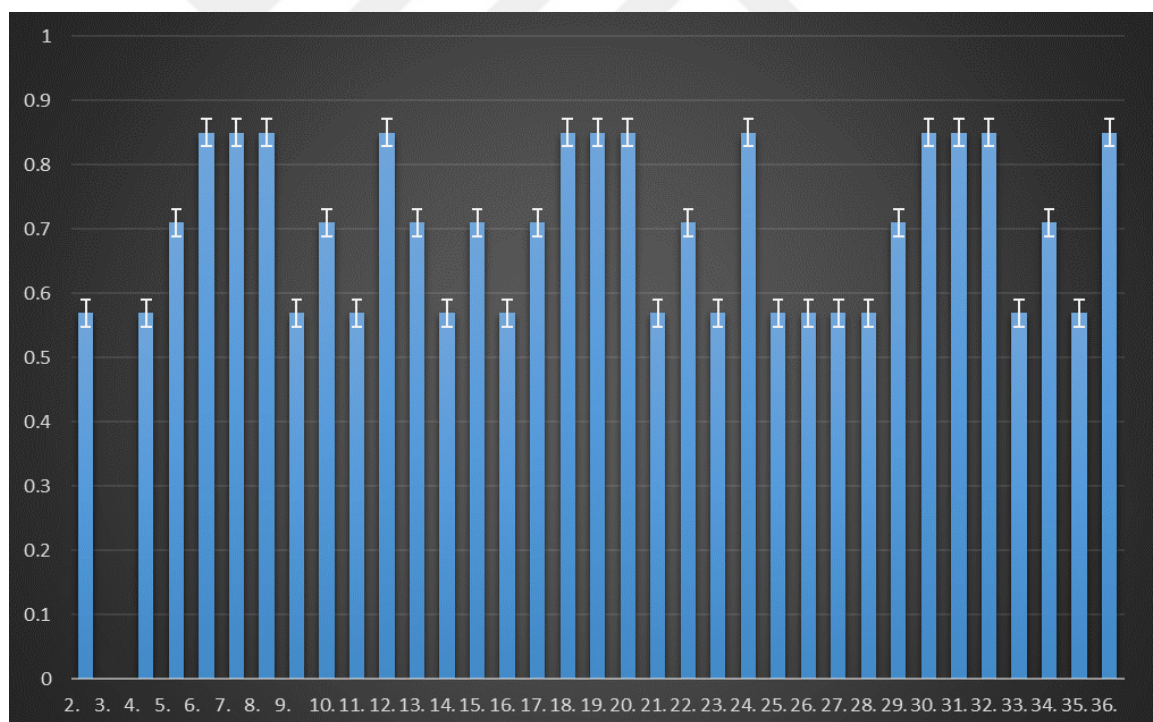


Figure 4.3 MAR index values for 36 MRSA isolates

4.3 The Biofilm Assay of MRSA

The method was used:

4.3.1 Phenotype detection of the biofilm production (micro-titer plate)

One of the most important processes in eradicating infection is determining the mechanism of biofilm formation as it provides defense against antibiotics and host immune response (Gupta *et al.* 2016).

Detection of the efficiency of MRSA isolates in biofilm formation was carried out using a 96-hole micro-titer plate method, and the optical density was measured using an ELISA device at a wavelength of 570 nm. The results showed that a high percentage of bacteria were biofilm producers in varying proportions, 6 (16.66%) isolates have the ability to produce strong biofilm, 21 (58.33%) bacteria have the ability to Moderate biofilm production, 9 (25%) Non/Weak biofilm producing isolates, as in Figure 4.4 and Table 4.5.



Figure 4.4 Detection of biofilm formation of *S. aureus* isolates by microtiter plate method using crystal violet stain

In previous studies conducted by previous researchers, the results indicated a high rate of biofilm-formation in *S. aureus* bacteria that converged and agreed with a results of this study, including the researcher's study Alledani (2016) and the results of the researcher's study Tawfeeq, Heba K (2016), where the proportion of bacterial isolates produced for biofilm (80.6%) and (90%), respectively. Previously, closest to our findings, Goudarzi *et*

al. (2019), and Hezam (2019) reported that more than 72% and 93% of their clinical *S. aureus* strains collected from clinical samples were biofilm producers, in Egypt study by Mashaly and Badr (2022) showed about 76.5% of isolates have ability to formation biofilm, while this finding was inconsistent with the result recorded by Belbase *et al.* (2017) in which the percentage of biofilm-producing isolates reached (46.1%), as well as the results by Pandey *et al.* (2020) in which the percentage of biofilm-forming isolates was (25.6%). So far, several phenotypic and molecular approaches have been used to detect biofilms by bacteria, and among these methods, MTP has been presented as a suitable standard method for biofilm detection with the highest sensitivity and specificity and is recommended to be used simultaneously with molecular methods (Dadgar *et al.* 2019). As for the difference in the percentages of bacterial isolates under study for biofilm production, it is due to the difference in the type of strain and the source of isolation, as the isolates were from multiple infections, from both sexes and at different ages.

Table 4.5 Number and percentage of *S. aureus* (MRSA) and the degree of their biofilm formation by microtiter plate

Biofilm production	Percentage (%)	P-value
Strong	6 (16.66%)	0.0050 **
Moderate	21 (58.33%)	
Non/Weak	9 (25%)	
** (P<0.01).		

4.3.2 Biofilm production and antibiotics resistance

Antibiotic resistance ratios were compared with biofilm-forming isolates, and the results revealed a high statistical significance with p-value ≤ 0.01 for antibiotic resistance with biofilm production as shown in the Table 4.6.

Table 4.6 Biofilm formation and antibiotic resistance of *S. aureus* isolates

Biofilm Antibiotic	Total of Resistance isolates	Weakly	Moderately	Strongly	P-value
Ciprofloxacin	18	9(50%)	9(50%)	0 (0%)	0.0001**
Clindamycin	6	3(50%)	3(50%)	0 (0%)	0.0001**
Erythromycin	33	9(27.27%)	21(63.63%)	3(9.09%)	0.0001**
Gentamicin	18	9(50%)	6(33.33%)	3(16.66%)	0.0007**
Methicillin	36	9(25%)	21(58.33%)	6(16.66%)	0.0009**
Penicillin	36	9(25%)	21(58.33%)	6(16.66%)	0.0009**
Tetracycline	21	3(14.28%)	12(57.14%)	6(28.57%)	0.0006**
P-value		0.0001 **	0.0076 **	0.0061 **	---
** (P≤0.01).					

Biofilms are surface-bound bacterial communities that exhibit a wide variety of growth, gene expression, and protein production characteristics because of the extracellular polymer matrix in which they are embedded, bacteria in a biofilm use chemical signals for inter-bacterial communication, biofilms allow microbial pathogens to remain in hosts, where they can cause extensive tissue damage and chronic inflammation by spreading preexisting infections (Li and Lee 2017). Therefore, biofilm formation on medical equipment, human tissues, and organs has consequences for both economics and public health. Antibiotic resistance is a consequence of bacterial biofilm colonies, which lead to persistent infections (Cepas *et al.* 2019).

4.4 Molecular Detection

In this study, the molecular detection of genes (*mecA*, *bbp*, *clfA*, and *icaA*) responsible for biofilm formation in MRSA isolates was carried out using PCR technology.

4.4.1 Detection of *mecA* gene

S. aureus resistant to the antibiotic methicillin is one of the most important types of bacteria that cause many infections and inflammations in communities and hospitals, and the reason for its resistance to this antibiotic is the presence of the *mecA* gene, which

produces the penicillin-binding protein (PBP2a) (Carretto *et al.* 2018, Turner *et al.* 2019, Ismail *et al.* 2021).

To confirm its presence, polymerase chain reaction technology using a thermocycler was utilized to conduct a molecular detection of the *mecA* gene in 36 *S. aureus* bacterial isolates, and the results showed that all 36 (100%) of them be positive for the presence of this gene, as in the Figure 4.5.

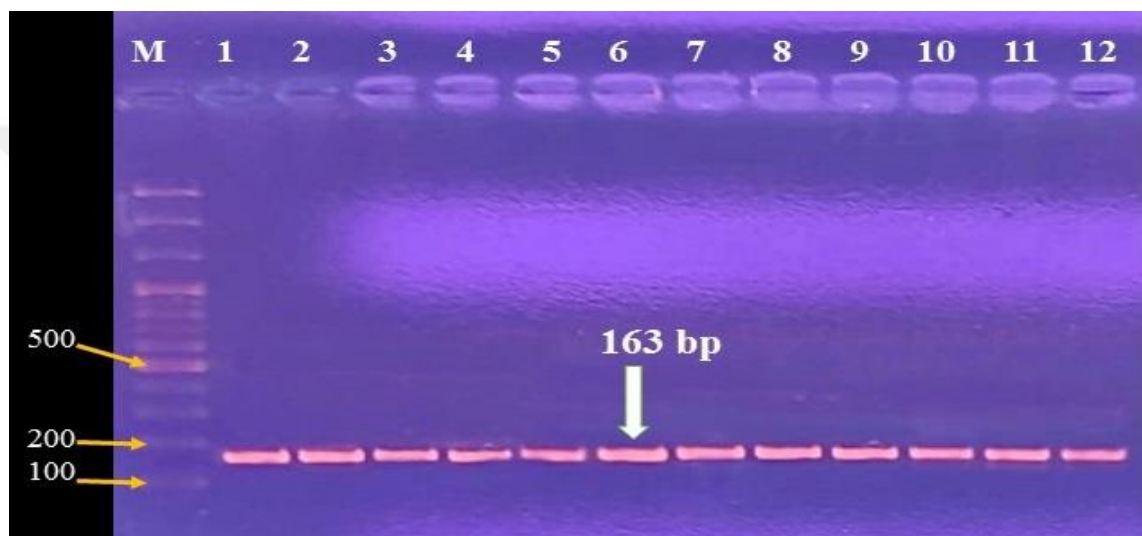


Figure 4.5 Gel electrophoresis of PCR product for *mecA* gene in *S. aureus* with amplicon size of 163 bp. Agarose 1.5%, 70 V for 90 min. Line M: DNA marker (100bp)

The comparison between the phenotypic method and the PCR examination shows a perfect match between the results of the phenotypic detection and the results of the molecular detection, in this study, the results are consistent with the findings of Mahdiyoun *et al.* (2016) in Iran, in which the percentage of gene presence in isolates reached (100%), as well as similar to the results of local studies by Al-Musawi *et al.* (2019), and Alsaimary (2020), which give positive results (93.22% and 100%) respectively, and also 100% gave the study by Ali and Abd Al Mayahi (2021), another study in Egypt by Ibrahim *et al.* (2022) showed the rate of gene presence in study isolates about (95.28%). But the results of our study did not agree with the results presented by Abulreesh *et al.* (2017) in Saudi Arabia and the results of the study reached by the researcher Havaei *et al.* (2017) where the percentage of isolates possessing the gene was

(44%) and (45%) respectively. The *mecA* gene is located on the staphylococcal cassette chromosome *mec*, which is a movable genetic element that is integrated into the chromosome (*SCCmec*). The methicillin-resistant phenotype is dependent on the expression of the *mecA* gene, which is widely dispersed throughout *S. aureus* and other Staphylococcal species (Ito *et al.* 2012). It's possible that the Staphylococci's exceptionally complex beta-lactam resistance mechanism, which includes both native and acquired genes, accounts for the observed diversity in phenotype and genotype pattern, different MRSA strains may also have unique auxiliary genes or use alternative strategies (Miragaia 2018). The resistance to beta-lactams is most often caused by a mutation in the *mecA* gene, but other genetic factors may also be considered (Elhassan *et al.* 2015).

4.4.2 Detection of *bbp* gene

Molecular detection of the *bbp* gene, which has a size of 1055 bp was carried out using PCR technology and using a thermal cycler. The results revealed the absence of a gene pathway in the isolates of MRSA (36) under study. The results of this study are in agreement with the results of other previous studies, including the study obtained by Ghasemian *et al.* (2016), Eftekhari *et al.* (2017) in Iran and the study of the researcher Firoozeh *et al.* (2020) and the local study by Shahid *et al.* (2020) as the percentage of bacterial isolates that possess the gene was (0%), respectively. While another study that differed with the current study recorded results at low rates and among these results are the findings by Atshan *et al.* (2012) in Malaysia and the results of the local study conducted by Al Ani and Al Meani (2018), and the results of study by Haghi *et al.* (2020) as the rate of isolates possessing the gene was (10%), (21%) and (5%), respectively. Bone sialoprotein-binding protein (Bbp), it is a cell wall glycoprotein and which facilitates the attachment of *S. aureus* cells to the osteomyelitis bone matrix, it has been established that *bbp* and osteomyelitis are related (Otsuka *et al.* 2006, Persson *et al.* 2009, Aung *et al.* 2011). The presence of the *bbp* gene is a clear evidence of the virulence of *S. aureus* isolates, specifically isolates of osteomyelitis infections, as it is one of the most factors that increase their virulence and enhance the infection resulting from these infections, and this explains the lack of the gene in the isolates under study, as there were no isolates of inflammation bone and marrow.

4.4.3 Detection of *clfA* gene

The results of molecular detection of the *clfA* gene, which is one of the clumping and virulence factors that have an effective role in biofilm formation in *S. aureus* bacteria and increases its pathogenicity (Kadhum *et al.* 2022) for the isolates under study, as the results showed that 15 (41.66%) isolates out of a total of 36 an isolate of MRSA possessed the *clfA* gene, it included 5 (33.33%) isolates from wounds, 3 (20%) isolates from urine, 3 (20%) isolates from burns, 2 (13.33%) isolates from the skin, and 2 (13.33%) isolates from the nasal as shown in Figure 4.6.

The results of our study agreed with other studies like Serray *et al.* (2016), and Gowrishankar *et al.* (2016) in which the percentage of isolates resistant to the gene was (43.39%) and (58.7%), respectively. Also, the results presented by Kandala *et al.* (2017), and Hosseini *et al.* (2020) was (51.16%) and (64.7%). They are convergent with the results of this study for this gene. While the results of the present study differed from the results of other studies that revealed the gene presence was (94.9%) and (94.7%), respectively, including the study by Cheraghi *et al.* (2017) and Goudarzi *et al.* (2019).



Figure 4.6 Gel electrophoresis of PCR product for *clfA* gene in *S. aureus* with amplicon size of 1584 bp. Agarose 1.5%, 70 V for 90 min. Line M: DNA marker (100bp)

4.4.4 Detection of *icaA* gene

Molecular detection of the *icaA* gene, which encodes for biofilm production of methicillin-resistant *S. aureus* isolates was carried out. The results of the detection of the gene of 188 bp in size by PCR technology and using the heat cycler showed that 18 (50%) isolates possess the *icaA* gene, it included 6 (33.33%) isolates from wounds, 4 (22.22%) isolates from urine, 3 (16.66%) isolates from burns, 3 (16.66%) isolates from the skin, and 2 (11.11%) isolates from the nasal as shown in the Figure 4.7.

The results of the present study are consistent with the results presented by Zalipour *et al.* 2016 and the results of the researcher's study Asadpour (2018), as the percentage of isolates that possess the gene reached (63.3%) and (53.9%), and also the results of our study converged with the results by Sedaghat *et al.* (2018) and the results reached by Ali *et al.* (2021), which reached the percentage of resistant isolates (72%) and (69%), respectively. While results of the current study did not agree with other studies that indicated different rates of isolates possessing the *icaA* gene, including the researcher's study Bimanand *et al.* (2018) and the results of study by Hiawy and Mukharmish (2019), which amounted to (15.6%) and (15.8%) on the straight. Molecular investigation of the *icaA* gene is related to the production of biofilms, and the absence of the gene in the isolates under study does not mean that they are unable to produce biofilms. The phenotypic examination of the isolates showed higher rates of biofilm formation, and it is necessary to rely on both methods to reach accurate results (Noha *et al.* 2015).

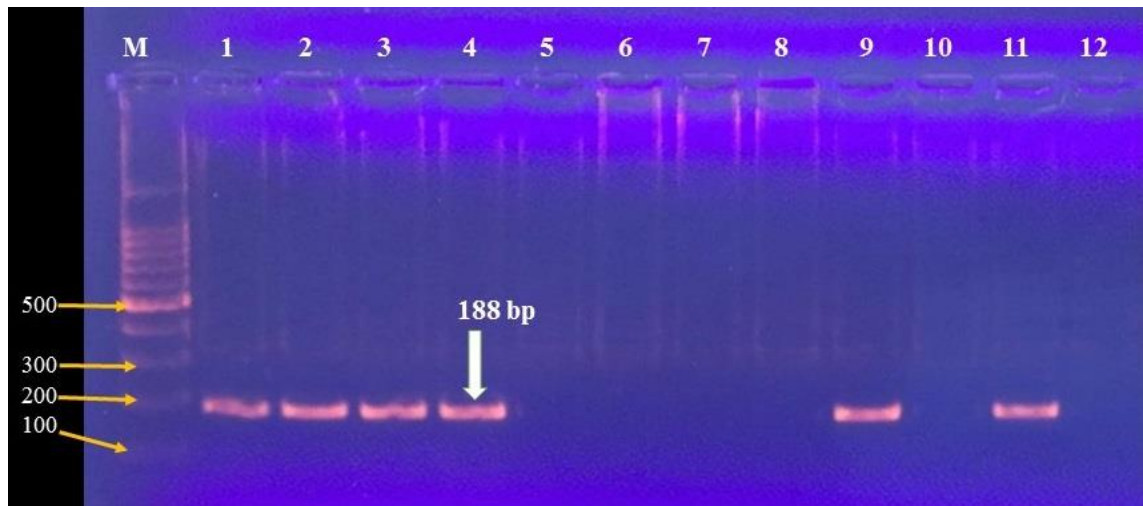


Figure 4.7 Gel electrophoresis of PCR product for *icaA* gene in *S. aureus* with amplicon size of 188 bp. Agarose 1.5%, 70 V for 90 min. Line M: DNA marker (100bp)

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

1. Most of the MRSA isolates isolated from different clinical sources showed multiple antibiotic resistance, the highest resistance to Penicillin and Erythromycin, and the least resistance to Clindamycin (16.66%).
2. Most of the bacterial isolates resistant to antibiotics were biofilm-forming.
3. Effectiveness of using the genetic determinants of methicillin-resistance *mecA* gene in the detection of MRSA.
4. The genes *clfA*, and *icaA* were the most prevalent in the study's MRSA isolates while gene *bbp* did not record any appearance among the isolates.

5.2 Recommendations

1. Irresponsible and widespread usage of antimicrobials is a major contributor to the rise of multidrug resistance; a nationwide awareness campaign is needed to curb this occurrence.
2. Study of the gene-expression of virulence factors responsible for biofilms in methicillin-resistant *S. aureus* isolated from various clinical sources.
3. Determining *mecA* is essential to identify *S. aureus* isolates as methicillin-resistant *S. aureus* (MRSA).
4. Comparing a genetic sequence of MRSA in Iraq with its sequence in neighboring countries, and how this affects virulence and its spread.

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APPENDICES

APPENDIX 1. Ethical statement

APPENDIX 2. Inhibition zone of *S. aureus* isolates grown on Muller-Hinton agar for sensitivity test.



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