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MOLECULAR DIAGNOSIS OF THE RESISTANCE OF GENES IN *Staphylococcus aureus* TO ANTIBIOTICS IN THE DIABETIC FOOT INFECTIONS

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MOLECULAR DIAGNOSES OF THE RESISTANCE OF GENES IN *Staphylococcus aureus* TO ANTIBIOTICS IN THE DIABETIC FOOT INFECTIONS

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October 2022

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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ABSTRACT

MOLECULAR DIAGNOSES OF THE RESISTANCE OF GENES IN *Staphylococcus aureus* TO ANTIBIOTICS IN THE DIABETIC FOOT INFECTIONS

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A total of 132 specimens were processed over a period of four months. *S. aureus* was identified using traditional biochemical tests and confirmed by the detected coagulase gene. MRSA isolates were characterized by seven conventional phenotypic methods using the methicillin resistance gene (mecA) detection test as a reference standard. Six phenotypic methods were used to identify β-lactamase production. The isolated MRSA were subjected to antibiotic susceptibility tests by seven methods. The MRSA isolates were subjected to staphylococcal chromosome cassette methicillin-resistance (SCCmec), by multiplex polymerase chain reaction (PCR). The presence of Panton-Valentine leucocidin (pvl) and toxic shock syndrome toxin (tst) genes was tested by monoplex PCR. The overall isolation rate of *S. aureus* was 17 (12.87%) isolates among various specimens. The *mec*A was recognized in 4 (23.52%) isolates (MRSA). Phenotypic methods including cefoxitin disc diffusion, chromogenic assay was highly effective for detecting MRSA. Biofilm forming by both MRSA strains indicates the high ability of these strains to persist in hospital environments which increases the risk of disease development in hospitalized patients. Additionally, resistance to antimicrobials in biofilm-forming isolates contribute to bacterial persistence which may lead to chronic infections and treatment problems.

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Keywords: Molecular diagnosis, *Staphylococcus aureus*, Resistance genes, Antibiotics, Diabetic foot ulcer

ÖZET

DİYABETİK AYAK ENFEKSİYONLARINDA *Staphylococcus aureus*'TAKİ GENLERİN ANTİBİYOTİKLERE DİRENCİNİN MOLEKÜLER TANILARI

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Dört aylık bir süre boyunca toplam 132 örnek işlendi. *S. aureus,* geleneksel biyokimyasal testler kullanılarak tanımlandı ve tespit edilen koagülaz geni ile doğrulandı. MRSA izolatları, referans standart olarak metisilin direnç geni (mecA) saptama testi kullanılarak yedi geleneksel fenotipik yöntemle karakterize edildi. B-laktamaz üretimini tanımlamak için altı fenotipik yöntem kullanıldı. İzole MRSA yedi yöntemle antibiyotik duyarlılık testlerine tabi tutuldu. MRSA izolatları, multipleks polimeraz zincir reaksiyonu (PCR) ile stafilokok kromozom kaset metisilin direncine (SKKmec) tabi tutuldu. Panton-Valentine lökosidin (pvl) ve toksik şok sendromu toksin (tşst) genlerinin varlığı monoplex PCR ile test edildi. *S. aureus*'un toplam izolasyon oranı çeşitli örnekler arasında 17 (%12,87) izolatlar. mecA, 4 (% 23,52) izolatlar (MRSA) tanındı. Sefoksitin disk difüzyon, kromojenik tahlilleri dahil olmak üzere, beslenme yöntemleri MRSA tespit için son derece etkili oldu. Her iki MRSA suşu tarafından oluşturulan biyofilm, bu suşların hastane ortamlarında devam etme kabiliyetinin yüksek olduğunu gösterir ve bu da hastanede yatan hastalarda hastalık gelişme riskini artırır. Ek olarak, biyofilm oluşturan izolatlardaki antimikrobiyallere karşı direnç, kronik enfeksiyonlara ve tedavi sorunlarına yol açabilecek bakteri kalıcılığına katkıda bulunur.

2022, 86 sayfa

Anahtar Kelimeler: Moleküler tanı, *Staphylococcus aureus,* Direnç genleri, Antibiyotikler, Diyabetik ayak ülseri

PREFACE AND ACKNOWLEDGEMENTS

This study was written with the title “Molecular diagnosis of the resistance of genes in *Staphylococcus aureus* to antibiotics in the Diabetic foot infections”. First of all, praise and thanks be to God alone (Glory be to him), for the shower of blessings throughout the period of my dissertation for successfully completing the thesis. I would like to extend my thanks, respect and appreciation to my advisor, Asst. Prof. Dr. İlker ŞİMŞEK and Co-Advisor Asst. Prof. Dr. Nawfal Hussain ALDUJAILI for their efforts, who did not spare me their knowledge and interest in this process. Also, I would like to extend my endless thanks to my father and mother before she is died and my wife who are supporting me throughout my education life until the moment. I would also like to thank the Turkish people and Çankiri Karatekin University, teachers and administrators alike, for their support. I would like to express my thanks and gratitude to my dear father and mother who are my eternal support. I will not be able to pay their rights, and my love for them cannot be described in words. I feel the need to thank my dear wife who put so much effort into supporting me by creating the right atmosphere for my studies. Thanks to my dear brothers, through whom I see my future bright.

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LIST OF SYMBOLS

-	Minus
%	Percent
+	Plus
°C	Degrees celsius
µL	Microliter
dL	Deciliter
g	Gram
L	Liter
m^2	Square-meters
Mg	Milligram
Min	Minutes
mL	Milliliters
Ng	Nanogram
Nm	Nanometre
Pmol	Picomole
U	Unit

LIST OF ABBREVIATIONS

Clf	Clumping factor
COA gene	Coagulase gene
CONs	Coagulase negative *staphylococci*
DFIs	Diabetic foot infections
DFUs	Diabetic foot ulcers
DNA	Deoxyribonucleic acid
FBD	Foodborne disease
HPV	Human papillomavirus
MBEC	Minimum biofilm eradication concentration
MBIC	Minimum biofilm inhibitory concentration
MHA	Muller Hinton Agar
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *S. Aureu*s
MSSA	Methicillin sensitive *S. Aureu*s
O.D	Optical density
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
PVL	Panton-valentine leukocidin
SAK	Staphylokinase
SSTIs	Soft skin tissue infections
TBE	Tris-borate-ethylenediaminetetraacetic acid
TCP	Detection of biofilm by tissue culture plate
TSST-1	Toxic shock syndrome toxin-1

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1. INTRODUCTION

Staphylococcus aureus (S. aureus) is a prominent human pathogen as well as a commensal organism. On the one hand, about 30% of healthy, asymptomatic people have *S. aureus* colonized (Zipperer *et al.* 2016). An even greater percentage of asymptomatic chronic and transitory *S. aureus* carriers is suggested by surveys of non-nasal cutaneous locations such the axillae and the perianal area. *S. aureus*, on the other hand, is a major contributor to bacteremia, infective endocarditis, osteomyelitis, pneumonia, infections linked to ingested medical devices, and infections of the skin and soft tissues (SSTIs) (Boldock *et al.* 2018).

Because germs may enter deeper tissue and spread throughout the body, invasive diseases including endocarditis, osteomyelitis, deep tissue abscesses, sepsis, and pneumonia can emerge as a result of SA-SSTIs. *S. aureus* is also the most common cause of surgery site infections (SSIs) (Olaniyi *et al.* 2016). Recurrent SSTIs are common, and the advent of multidrug-resistant *S. aureus* strains restricts the number of antibiotic medications that are currently accessible (Olaniyi *et al.* 2016).

Methicillin-resistant *Staphylococcus aureus* (MRSA), in particular, has become a significant clinical and epidemiological issue in hospitals. MRSA strains can develop resistance to the majority of lactam antibiotics as well as a variety of other antimicrobials, making infections challenging to treat and very expensive. There are now two fifth-generation cephalosporins with a comparable range that are typically effective against MRSA: ceftaroline and ceftobiprole (Mottola *et al.* 2016).

The bacteria included in the biofilm are also very resistant to antibiotic therapy (Soto 2014). *S. aureus* isolates were shown to have a high frequency of ESBLs determinants, which are frequently linked to the development of biofilms (Baldiris-Avila *et al.* 2020). No research on the frequency of ESBL-producing *S. aureus* isolates obtained from individuals with diabetic foot ulcers in Najaf has been reported.

2. LITERATURE REVIEW

2.1 Staphylococci

It is a gram-positive coccus that grows in a variety of planting media and forms colonies with a variety of hues, including white, dark yellow, and golden (Habib *et al.* 2015), and, to distinguish it from the species *Streptococcus*, is a catalase-positive *Staphylococcus* (Tong *et al.* 2015). The genus *Staphylococcus* is a member of the staphylococcal family Staphylococcaceae, which also contains the genera *Gemella*, *Salinicoccus*, *Jeotgalicoccus*, and *Macrococcus* (Becker *et al.* 2015).

Following the pattern established by the naming of *Streptococcus* five years earlier, the name was first used in 1880 by Scottish surgeon and bacteriologist Alexander Ogston (1844-1929). It is a combination of the word "Staphylo-," meaning "bunch of grapes" in Ancient Greek, and the suffix "coccus," meaning "spherical bacteria" in Ancient Greek (Kim *et al.* 2014).

Many of the at least 40 species of *Staphylococcus* that have been identified so far do not spread illness and are perfectly at home on the skin and mucous membranes of humans and other animals. Microorganisms belonging to the genus *Staphylococcus* were found in nectar, and they make up a small fraction of the soil microbiome (Francis *et al.* 2020).

The majority of staphylococci cohabit on human skin and mucous membranes, while certain other strains can result in surface abscesses and interior (systemic) damage, and sometimes may cause blood poisoning that may lead to death. The common types of food poisoning are caused by enterotoxins (fixed by heating) produced by some strains of *S. aureus*, the genus of staphylococci includes (44) species, but there are three clinically important types due to their association with injuries caused to humans (Planet *et al.* 2013, Almasaudi *et al.* 2017).

2.2 *Staphylococcus aureus*

One of the most diverse staphylococci harmful to humans, *S. aureus* bacteria are recognized from other staphylococci by their positive blood plasma coagulation enzyme (coagulase) (González-Martín *et al.* 2020).

S. aureus bacteria are defined as spherical-shaped cells with a diameter of approximately 1 micrometer, gram-positive, Immobile (Non-motile), non-spore forming and usually capsular with large yellow colonies on rich media (Jang *et al.* 2015). Where their colonies appear thin circular with shiny surfaces up to a diameter of one colony (3-2) mm and are dyed in a yellowish-golden color, and have the ability to decompose blood in the blood agar medium, and these bacteria live aerobic or anaerobic optionally or by fermentation producing lactic acid (Richardson 2019) but they grow in the form of it is preferred in aerobic conditions, and grows easily in many growing environments with optimal temperatures 30-37 oC (Grispoldi *et al.* 2021).

Now, there are more than 50 different species of *Staphylococcus*. These hardy microbes can survive in harsh conditions and can be found on the skin and in the mucous membranes of many animals, including humans. A major human and animal pathogen, *S. aureus* is a bacterium that can cause serious illness. Severe sepsis, foodborne disease (FBD), and skin infections are common human outcomes, and it is a common source of contamination in healthcare and childcare settings. Human papillomavirus (HPV) is also a major factor in mastitis in dairy animals, bone and joint diseases in poultry, and bumblefoot in cattle. Companion animals including dogs, cats, and horses can contract and spread *S. aureus* (Grace and Fetsch 2018).

S. aureus adapts in real time by mutating and acquiring mobile genetic components that boost its resistance and virulence. Since the 1960s, methicillin-resistant *S. aureus* (MRSA) strains have been an issue in hospitals due to their rapid spread and inability to be cured with methicillin (Malachowa and Deleo 2010).

Microscopy, culture, and biochemical analysis are the three mainstays of the microbiology lab's standard procedure for recognizing methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA); these steps are straightforward but often take at least 48 hours. Various community MRSA strains, responsible for life-threatening skin and respiratory infections, have arisen since the 1980s. In response to this unmet demand, quick molecular diagnostics have been developed for use as point-of-care testing in the clinic or as a laboratory adjunct to standard culture methods. Consequences to individual patient care and public health arise from inadequate or excessive use of empiric antibiotics and failure to execute proper infection control measures for MRSA-colonized patients within the first 48 hours after colonization (David and Daum 2010, Edgeworth 2011).

Depending on the sample and clinical environment, these new technologies can deliver data to physicians in as little as an hour or as long as 24 hours, and they should revolutionize the care of patients with, *S. aureus* and other bacterial illnesses, but adoption is sometimes gradual because of the disruptive nature of new technology, the costs of change, and the ambiguity around the best remedy in light of advancing science (Abdelgadeir 2015). Last but not least, stopping the spread of MRSA has been a focus for hospitals and other healthcare facilities for a long time. Recent efforts at the local and national levels have helped to reinforce fundamental and enhanced infection control treatments such universal hand hygiene, barrier nursing, decontamination, and others, leading to considerable decreases in transmission. However, the deployment of these new technologies will be helped by additional proof of the health economic, clinical, and antimicrobial resistance advantage. Increased understanding of reservoirs and transmission routes, made possible by developments in whole-genome sequencing, should allow for more precise treatment targeting, leading to better long-term control of endemic strains and the ability to detect and prevent the entry of novel strains (Edgeworth 2011).

S. aureus bacteria give positive results for the free plasma coagulase enzyme test, DNA analyzer enzyme (DNase), phosphatase, gelatinase, and give a negative result for the oxidase test (Corvec 2018).

S. aureus can live in wet and dry conditions, such as heating at a temperature of 60°C for 30 minutes, but it dies when exposed to phenolic, hypochlorite, hexachlorophene, and chlorhexidine, as it can grow in a wide PH range between (4.8-9.4) and PH (7.5) is the optimal for growth, these and other qualities have made it able to survive in dry pus and saliva samples for weeks (Xiang *et al.* 2020).

S. aureus can be recovered from almost any clinical specimen and is a significant cause of nosocomial infections. It also continues to grow in significance as a community-acquired pathogen, and rising drug resistance is a concern with this common isolate. Infections caused by *S. aureus* can range from being relatively mild to being life-threatening (Man *et al.* 2017). Taxonomy of an organism is according to their natural relationship, based on Berge's manual of systematic bacteriology (Shah *et al.* 2007). *S. aureus* are placed into:

Kingdom: Bacteria

Phylum: Bacillota

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Genus: *Staphylococcus*

Species: *Staphylococcus aureus*

2.3 *S. aureus* Pathogenesis

S. aureus despite being a typical component of the human skin, nose, throat, gastrointestinal system, and genital tract's natural flora, *S. aureus* is one of the most harmful kinds of staphylococci (Nair *et al.* 2014). Due to the presence of several surface antigens, enzymes, and toxins, these bacteria are able to infiltrate the tissues of the body

powerfully and have the potential to produce opportunistic infections that can vary from relatively small skin infections to fatal systemic disorders (Roy *et al.* 2018).

The likelihood of *S. aureus*'s virulence factors may result in minor skin lesions as well as more serious infections such pneumonia, mastitis, urinary tract infections, osteomyelitis, endocarditis, and in exceedingly rare cases, sepsis *S. aureus* is the culprit for meningitis (Al-Hasseny 2011, Al-Azawi 2013).

S. aureus also has an acute illness. Chronic infections caused by the *Staphylococcus aureus* can often be mediated by its capacity to stick to medical equipment and create biofilms (O'Neill *et al.* 2009). Coagulase-negative staphylococci have been viewed as benign while their coagulase-positive counterparts are considered opportunistic pathogens. Evidence is accumulating, however, that coagulase-negative staphylococci are a major source of nosocomial infections and can cause a wide range of pyogenic illnesses in immunocompromised patients and those with internal prosthetic devices (Planet *et al.* 2016).

S. aureus bacteria produce exfoliated toxin causing exfoliated skin syndrome, where this toxin causes peeling and shedding (Scalded) in the skin, affecting children aged four years and newborns, as well as the elderly with weak immunity, also called epidermolytic syndrome (Nishifuji *et al.* 2008). It also causes arthritis, inflammation of the membranes of the brain (meninges), liver abscess, prostate abscesses, inflammation of the urinary tract, tonsillitis, pharyngitis Tonsillitis and pharyngitis, sinusitis Nasal furuncles, the bacterium has a role in the development of endocarditis, which leads to high mortality among infected patients (Sousa *et al.* 2012).

S. aureus produces many indirect toxoid infections, causing food poisoning as a result of food contamination with enterotoxins produced by it, and then there are severe cases of severe vomiting and diarrhea, which may last from one to five hours, and the infection is usually without fever and quickly cured (Boynukara *et al.* 2007).

2.4 Virulence Factors

The clinical significance of *S. aureus* bacteria lies in the fact that it possesses many virulence factors that give it the ability to grow, multiply and invade host tissues, and thus it contributes significantly to its pathogenesis (Cheung *et al.* 2021).

The pathogenicity of *S. aureus* can be attributed to a number of virulence factors, including the capsule, enterotoxins, cytolytic toxins, and cellular components including protein A. Numerous cytolytic and exfoliative poisons have been discovered. Despite these virulence characteristics, *S. aureus* has a very high level of innate resistance and is thought to be an opportunistic pathogen (Shettigar and Murali 2020). The following are the most important of these factors.

2.4.1 *S. aureus* toxins

2.4.1.1 Alpha toxin (α-Toxin)

The majority of *S. aureus* strains produce a hemolysin, a 33 KDa polypeptide that is encoded on the bacterial chromosome. It poisons a variety of cell types, including erythrocytes, leukocytes, hepatocytes, platelets and cultured cells. Destroys the smooth muscle of blood vessels. It integrates into the hydrophobic regions of the host cell membrane, causing the formation of 1 and 2 nm ring-shaped holes (Berube *et al.* 2014). Osmotic swelling and cell lysis are caused by rapid K+ outflow and Na+, Ca++, and other small molecule influx. It is believed that a toxin is crucial to the tissue damage brought on by staphylococcal disease (Berube *et al.* 2014).

2.4.1.2 Beta toxin (β-Toxin)

Most *S. aureus* strains generate a 35 KDa heat-labile protein known as sphingomyelinase C as its beta toxin. This enzyme is toxic to a number of cells, including erythrocytes, leukocytes, macrophages, and fibroblasts, in addition to

preferring sphingomyelin and lysophosphatidylcholine (Tran *et al.* 2019). In sensitive cells, it catalyzes the breakdown of membrane phospholipids, with the degree of lysis determined by how much surface sphingomyelin is exposed. Despite the fact that its involvement in human illness is unknown, it is thought to work in conjunction with the toxin to cause the distinctive tissue damage and abscess development of staphylococcal infections (Astley *et al.* 2019).

2.4.1.3 Gamma toxin (γ-Toxin)

Gamma-toxin may cause the lysis of human lymphoblastic cells, sheep, rabbit, and human erythrocytes. A class of proteins that comprises leukocidin, γ-toxin, and other bicomponent toxins is encoded by the hlg and luk PV loci. The closely related Panton-Valentine leukocidin (PVL) and these ***γ***-toxins are the prototypical bicomponent poisons (Astley *et al.* 2019).

2.4.1.4 Delta toxin (δ-Toxin)

The majority of staphylococci and practically all strains of *S. aureus* generate a3 KDa polypeptide known as ***delta***-toxin. The toxin has a wide range of cytolytic activity and affects erythrocytes, other eukaryotic cells, various other mammalian cells, organelles, spheroplasts and protoplasts, as well as internal membrane structures (Astley *et al.* 2019). This rather non-specific membrane toxicity supports the theory that the toxin acts as a surfactant and damages cellular membranes by acting like a detergent. Additionally, when given in large doses to test animals, it has been demonstrated to be dermonecrotic and lethal. Phospholipids shield cells from ***δ***-toxin's damaging effects (Su *et al.* 2020).

2.4.1.5 Leukocidin

S. aureus is capable of producing a toxin that only affects polymorphonuclear leukocytes. Leukocidin should be a virulence factor since it may destroy leukocytes, which is a key defensive mechanism against staphylococcal infection (of which

neutrophils are one type) (Cotar *et al.* 2010). S and F, two different proteins, make up Panto Valentine Leukocidin (PVL). The B section of an A-B toxin is analogous to the S component. However, each of them is active in the metabolism of phosphatidylinositol and phospholipids, respectively (AL-Turaihi 2016). The two halves of the PVL bind to human neutrophils sequentially, despite the common belief that the S component binds first and then creates pores. Phosphatidylinositol is an essential signaling molecule in eukaryotic cells that regulates a number of cellular processes. Thus, it appears that this two-component toxin disrupts phospholipid metabolism and impairs normal cellular processes (Seilie and Wardenburg 2017).

2.4.1.6 Exfoliative toxins (A and B)

Two exfoliative toxins that are serologically different from one another have been discovered as exfoliative toxin A (ETA) and exfoliative toxin B (ETB). Staphylococcal scalded skin syndrome, which commonly affects infants and is defined by the intraepidermal separation of skin layers at the desmosomes, is brought on by them (Koosha *et al.* 2014). The sickness develops suddenly and often starts with a widespread erythema that quickly spreads throughout the whole body over the course of a few days. Later, huge flaccid sterile bullae emerge, which cause the stratum granulosum layer to separate. Between 7 and 10 days, pass from the beginning of the illness until recuperation. Areas of the skin do not develop permanent scars, and the causative toxins do not themselves pose a threat to the host's life (Los *et al.* 2013).

2.4.1.7 Toxic shock syndrome toxin-1

Toxic shock syndrome is mediated by toxic shock syndrome toxin-1 (TSST-1) and is characterized by fever, hypotension, rash, desquamation, and involvement of many organ systems. Not all staphylococcal isolates from patients with TSS have been found to produce TSST-1, but the majority of these isolates are known to produce enterotoxin B (Parsonnet *et al.* 2005).

2.4.2 Staphylococcal enzymes

2.4.2.1 Coagulase

Although certain strains of *S. aureus* do not generate measurable levels of coagulase, all strains seem to have the coagulase gene, which is the primary criteria utilized in the clinical microbiology laboratory for the identification of *S. aureus* (González-Martín *et al.* 2020).

The coagulase enzymes are found in two different forms in the strains of *S. aureus*; bound (also known as clumping factor) and free. While cell-free coagulase achieves the same result by reacting with a globulin plasma factor (coagulase-reacting factor) to form staphylothrombin, which catalyzes the conversion of fibrinogen to insoluble fibrin, coagulase-reacting factor (Cotar *et al.* 2010).

2.4.2.2 Catalase

The catalase enzyme, which is produced by all staphylococci, catalyzes the transformation of harmful hydrogen peroxide into water and oxygen (Becker *et al.* 2015). Different toxic forms of oxygen are produced as unintentional by products during the reduction of O_2 to H_2O in respiration. These reactive oxygen intermediates have many harmful effects on living organisms, ranging from DNA strand damage to peroxidation of membrane lipids. Hydrogen peroxide (H_2O_2) can accumulate during bacterial metabolism or after phagocytosis. Toxic oxygen products are destroyed by enzymes that bacteria have adapted to use. Catalase, an enzyme that targets H_2O_2, is the most prevalent member of this class (Ripolles-Avila *et al.* 2018).

2.4.2.3 Staphylokinase enzyme

One of the most important causes of death in the modern lifestyle is acute ischemic stroke, which is related to thrombosis in the blood vessels. Staphylokinase (SAK), a

fibrinolytic agent, which is produced mainly by *S. aureus*, is an indirect activator of plasminogen and belongs to the third generation of fibrinolytic enzymes (Sperber and Tatini 1975).

2.4.2.4 Hyaluronidase

Hyaluronic acids, an acidic mucopolysaccharide that holds together some body cells, especially those in connective tissue, are hydrolyzed by hyaluronidase. This digestion is thought to contribute to the tissue blackening of infected wounds and to aid the microorganism's spread from the site of infection (Vasvani *et al.* 2020).

2.4.2.5 Fibrinolysin

Almost all strains of *S. aureus* produce fibrinolysin, also known as staphylokinase, which is different from the fibrinolytic enzymes produced by streptococci and may break fibrin clots (Abdul Rahim and Rengaswamy 2022).

2.4.2.6 Lipase

It is thought that these enzymes are necessary for staphylococci to invade cutaneous and subcutaneous tissues and for superficial skin infections like furuncles (boils) and carbuncles to occur. All strains of *S. aureus* and more than 30% of the strains of coagulase-positive staphylococci produce a variety of lipases, which, as their names suggest, hydrolyze lipids (Hu *et al.* 2012).

2.4.2.7 Nuclease

Nearly all strains of *S. aureus* generate a thermostable nuclease (TNase), an enzyme that has been utilized as a diagnostic marker for this species. The TNase uses a calcium-dependent mechanism to break down single- and double-stranded DNA and RNA at the

5' position of phosphodiester linkages. This protein's primary role may be to transform nearby host tissues into the nutrients necessary for bacterial growth (Cotar *et al.* 2010).

2.4.2.8 Penicillinase

When penicillin was initially used therapeutically in 1941, more than 90% of staphylococcal isolates were sensitive to it; nevertheless, resistance to penicillin rapidly emerged, partly because the organisms were able to manufacture penicillinase (β-lactamase) (Qian *et al.* 2015). The enzyme breaks down the β-lactam ring of the penicillin molecule; its presence on transmissible plasmids guaranteed that it was widely distributed (Fernandes *et al.* 2013).

2.4.3 Other factors

2.4.3.1 Capsule

S. aureus clinical isolates that generate more than 90% of capsular polysaccharides (also known as the "slim layer"). Mucoid-type capsules strains generating these capsules are extensively encapsulated and are mucoid on solid medium, and these capsules may also be separated into two different categories based on colony shape (Mahon *et al.* 2018). These strains of microcapsules produce non-mucoid colonies on solid media and have a thin capsular coating. According to studies, the mucoid-type capsules were crucial antiphagocytic virulence factors that obscured C3b deposited on the bacterial cell wall and prevented phagocytic cells from recognizing it. In addition to shielding the bacteria from polymorphonuclear leukocytes, the capsule also prevents mononuclear cell growth in response to mitogen exposure (Aubais Aljelehawy *et al.* 2021).

2.4.3.2 Protein A

Although its role in staphylococcal infections is unclear, protein A is uniformly coated on the surface of most strains of *S. aureus* (but not coagulase-negative staphylococci). This protein is covalently linked to the peptidoglycan layer and has a unique affinity for binding to the Fc receptor of immunoglobulin (Ig)G1, IgG2, and IgG4. By doing so, it effectively prevents the antibody-mediated immune clearance (Foster 2019).

2.5 Laboratory Identification

Under aerobic or microaerophilic conditions, staphylococci grow easily on the majority of bacteriological medium. Although they develop most quickly at 37°C, room temperature (20–25°C) is the optimal for pigment formation. In a medium with 10% salt chloride, the bacteria may grow. On the enriched medium during culture, *S. aureus* may produce dark yellow, hemolytic colonies. On solid medium, colonies are rounded, smooth, elevated, and gleaming. Colonies of *S. aureus* often range from grey to deep golden yellow (Singh *et al.* 2014, Lagier *et al.* 2015).

S. aureus can be differentiated from other species of bacteria by using simple method, including gram stain morphology, cell morphology, production of catalase test and coagulase test, heat-stable nuclease, alkaline phosphatase and mannitol fermentation. *S. aureus* is a gram-positive stain. Catalase-positive bacteria where it produces bubbles when exposed to a solution containing hydrogen peroxide and it is also described as coagulase-positive staphylococci, where it secretes coagulase that caused citrated plasma to clot (Bennett and Monday 2003, Planet *et al.* 2016).

2.6 Epidemiology of Staphylococcal Infection

The epidemic is one of the most important aspects that must be taken note of, and adequately known for its importance to the individual and society, and for the purpose of controlling the spread factors, correcting the technical paths in confronting the

disease and preventing it (Rhee *et al.* 2015). As *S. aureus* naturally colonizes various body areas such as the nose, respiratory tract and skin, and this percentage increases in people working in hospitals and bacteria endemic to more than one site in the body is one of the most important sources of latent infections, the main problem is not knowing the link between the sites of bacterial settlement, the production of the disease however, it was possible to identify some breeds that have a latent ability to disease production, but there is no way to predict which strains may turn into pathological strains (Chen and Huang 2014, Schaumburg *et al.* 2014).

Understanding the behaviors of this bacterium is essential to comprehending how staphylococcal infections arise (Crossley 2016). Many newborns acquire *S. aureus* colonization in the anterior nares quite soon after delivery. Persons who often use needles, whether for insulin administration, allergy desensitization, or the use of illegal recreational drugs, have greater carrier rates than other people, despite the fact that this is not fully understood (Crossley 2016).

When an injury or skin break occurs, the carrier state is well-documented to be strongly correlated with the emergence of infections (David and Daum 2010). A patient's risk of acquiring a staphylococcal wound infection after surgery is much greater than it is for a patient who is not known to have nasal *S. aureus* colonization (Dayan *et al.* 2016).

In an intriguing study, volunteers who were either persistent carriers or non-carriers were artificially inoculated with a variety of *S. aureus* strains. While non-carriers quickly eliminated the inoculated strains, most persistent carriers selected their original resident *S. aureus* strain from the inoculation mixture. The researchers found that the *S. aureus* carrier state is substantially influenced by host features, and that an ideal (Sakwinska *et al.* 2010).

2.7 Immune Response Against *Staphylococcus aureus*

2.7.1 The innate immune response against *S. aureus*

Numerous diverse receptor systems and cells that are continually ready to detect and get rid of invasive microbial infections are housed in the innate immune system. Toll-like receptor (TLR)-2-mediated local synthesis of soluble mediators, including cytokines, chemokines, and antimicrobial peptides, controls the first contact with epithelial cells when *Staphylococcus aureus* enters the body through exposed epithelial surfaces, such as skin and mucosa (Bekeredjian-Ding *et al.* 2015).

The ultimate goal is a constant state of immune mediators and colonizing microorganisms. Intracellular microbial sensors and subsequent inflammasome activation are required for bacterial clearance after cell and tissue invasion. Tissue-resident mast cells and macrophages attract neutrophils, macrophages, and NK cells. The inflammatory response promotes the growth of IL-17-producing NKT, T cells, and T helper cells (Kaufmann and Dorhoi 2016). Local dendritic cells go to the lymph nodes where they classify the many stages of infection, from the epithelial barrier to intracellular and systemic infection, to improve the adaptive immune response (Bekeredjian-Ding *et al.* 2015).

2.7.2 Adaptive immune response against *S. aureus*

The ability of *S. aureus* to coexist as a major human pathogen and a commensal in humans is the result of a complex interplay between host and bacterial factors. Neutrophils have been shown to be an essential part of the innate immune response against *S. aureus*, particularly for control of systemic infection, in both animal models and in humans with acquired and congenital neutrophil dysfunction (Karauzum and Datta 2016).

Less is known about the adaptive immune system's function. However, deficiencies in adaptive immunity do not result in the pronounced susceptibility to *S. aureus* infection that neutrophil dysfunction imparts. Instead, emerging evidence suggests that both T cell- and B cell-mediated adaptive immunity can influence host susceptibility and control of *S. aureus*. Adaptive immunity can either benefit or harm the host, depending on the situation and location of the infection. *S. aureus* has also created methods to regulate adaptive immune responses in its favor. How adaptive immunity works during *S. aureus* infections, in order to fully understand how this role influences infection susceptibility and to appropriately create vaccines that trigger adaptive immune responses to protect against repeated infections, it will be imperative to better elucidate this role (Pidwill *et al.* 2021).

2.8 *Staphylococcus aureus* and Antibiotic Resistance

S. aureus is one of the most common causes of infections in hospitals. Especially infections associated with surgical wounds since it was first discovered by surgeon Alexander Ogston in Scotland in 1800 in an abscess resulting from contamination of surgical wounds and since the discovery of penicillin by Alexander Fleming in 1929 and the beginning of its use in 1936 (Foster 2019). Most staphylococci, including *S. aureus*, were considered sensitive penicillin was used until the beginning of 1944, but soon after the use of penicillin, especially in 1944, the percentage of resistance to penicillin (65%-85%) was recorded the production of beta-lactamase enzymes was linked to the resistance of staphylococci to penicillin, and for the purpose of avoiding penicillin resistance. Penicillin's fixed under the action of the beta-lactamase enzyme group (methicillin and Oxacillin) were used, as a result of the series of resistance shown by most strains of *Staphylococcus* to penicillin. The percentage of resistance reached approximately (80%-90%) of the total infections acquired from hospitals or from the community, and in 1961 it was confirmed that *S. aureus* show resistance to methicillin and resistant strains have been termed MRSA strains (Gajdács 2019).

Some studies have shown that antibiotic resistance is not limited to the beta-lactam group, but has extended to include many varieties of antibiotics such as

(aminoglycoside, Quinoline, sulfa compounds, clindamycin, macrolide, tetracycline, phenicol) and multiple drugs resistance (MDR) is an indicator of staphylococcal resistance to methicillin (NCCLS 2021), in addition, resistance to aminoglycosides has increased in recent years in parallel with the increase in resistance of *S. aureus* to methicillin. It was found in 1970 that *S. aureus* strains are resistant only to streptomycin, while in 1980 it was observed that resistance extended to include a large group of aminoglycoside antagonists, and in 1997 the first cases of resistance to the antibiotic (vancomycin) were recorded, which showed widespread success in the treatment of infections caused by MRSA. Strains of *S. aureus* resistant to vancomycin were called vancomycin resistant *S. aureus* strains (VRSA), prompting scientists to urge efforts to find new treatments with an effective effect (Linden 2016, Gajdács 2019).

2.9 Mechanism of Antibiotic Resistance

Antibiotic-resistant bacteria that are difficult or impossible to treat are becoming increasingly common and are causing a global health crisis. Antibiotic resistance is encoded by several genes, many of which can transfer between bacteria. New resistance mechanisms are constantly being described, and new genes and vectors of transmission are identified on a regular basis. This article reviews recent advances in our understanding of the mechanisms by which bacteria are either intrinsically resistant or acquire resistance to antibiotics, including the prevention of access to drug targets, changes in the structure and protection of antibiotic targets and the direct modification or inactivation of antibiotics.

Recent studies have indicated that the resistance of *S. aureus* bacteria to most antibiotics occurs due to the ability of these bacteria to produce many enzymes and genes that play an essential role in the resistance of bacteria to antibiotics. It was found that the resistance of bacteria to penicillin and cephalosporins results from their ability to produce the enzyme β-Lactamase, which breaks the β-Lactam ring of the penicillin molecule (Pantosti *et al.* 2007).

While it was found that bacterial resistance to methicillin occurs due to the possession of bacteria genes called *meca* gene that encodes a protein-binding penicillin substitute and thus becomes less familiar to bind with β-Lactam. It was also found that methicillin resistant *staphylococcus aureus* MRSA bacteria possess a special type of penicillin-binding protein (PBPs) called PBP2, which is not found in methicillin-sensitive *Staphylococcus* strains, which is characterized by its resistance to beta-Lactamase enzymes (β-Lactamase) produced by bacteria in order to possess this group of antibodies (acyl side chain). It is due to the possession by bacteria of the genes (VanA gene) which encode for the production of a peptidoglycan replacement enzyme that cannot bind with the antibiotic vancomycin (Gajdács 2019).

2.10 The Molecular Basis of Resistance of *S. aureus* to Antibiotics

2.10.1 Resistance to *S. aureus* for the methicillin antibiotic

The golden years for treating *S. aureus* infections were from 1946 to 1950, when penicillin was first used to treat infections caused by the bacteria. However, after a few years, penicillin activity began to decline due to the secretion of the penicillinase enzyme as a result of the emergence of *S. aureus* strains that were resistant to the antibiotic (Prescott 2018). Methicillin, a synthetic penicillinase-resistant antibiotic, was first introduced in 1959 and temporarily solved the problem in clinical practice. However, in 1960, it was discovered that some *S. aureus* strains were resistant to the new semisynthetic lactam antibiotics (methicillin, oxacillin, and flucloxacillin), and these strains came to be known as MRSA, especially in nosocom (Peacock and Paterson 2015).

The term "community-associated methicillin resistant S. aureus" refers to new MRSA strains that were discovered in the community between 1993 and 2003 that were phenotypically and genotypically different from the parent health care associated MRSA (HA MRSA) (CA MRSA) (David and Daum 2010).

MRSA has been consider one of the most important pathogens of injuries and diseases in the world for five decades, and MRSA is still increasing the importance of these strains comes as a result of their resistance to all types of beta-lactam antibiotics in addition to their resistance to many other types of antibiotics (Guo *et al.* 2020, NCCLS 2021).

MRSA's resistance to beta-lactam is due to the production of new proteins, namely pencillin binding proteins (PBPs) and known as (PBP2a) or (PBP2'), which usually does not resemble any of the group included in the types of pencillin binding proteins (PBPs), the mechanism of action of PBP2a is to reduce familiarity or homogeneity with beta-lactam antibiotics, and despite the presence of inhibitory concentrations of beta-lactam antibiotics, MRSA can complete the construction of the cell wall by relying on the inhibitor of PBP2A only (Jousselin *et al.* 2015).

The molecular basis for the resistance of *S. aureus* bacteria to methicillin is the presence of an additional piece of DNA on the chromosome of *S. aureus* bacteria called mec-A, which is in the range of 30-50 KBP, and this piece is not found in methicillin-sensitive *Staphylococcus aureus* (Jian *et al.* 2021).

PBP2a proteins are encoded by the mecA gene located on MRSA chromosomes, and in 1987 the mecA gene was introduced into some MSSA strains and the sequence of the nitrogenous bases of that gene was determined (Gasm Alseed 2019). MRSA is frequently discovered in diabetic foot ulcers, where the incidence can approach 40%. The presence of the mecA gene in *S. aureus* bacteria and in coagulation positive Staphylococci suggests that methicillin resistance is determined by gene determinants since these problems are one of the major causes of morbidity in diabetes people (mec determinants) and these determinants are free to transfer between staphylococcal species, and this explains the process of transmission and spread of resistance to methicillin between bacterial species (Silva *et al.* 2020).

(Hiramatsu *et al.* 2001) relied on mecDNA as a new class of genetic elements and identified the code (Staphylococcal chromosome cassettes mec (SCCmec)) to denote the

genetic elements of staphylococci, as two genes were distinguished that are highly effective in the process of forming new unions (Recombinase genes), namely ccrA and ccrB located in the chromosomal SCCmec. The first contains 499 amino acids and the second contains 542 amino acids, and there are also antibiotic resistance zones on the left and right of SCCmec, such as the site that carries complex antibiotic resistance genes (mecI-mecR1-mecA gene complex), which encodes resistance to various antibiotics, often carries the mecA gene responsible for resistance to methicillin from certain *Staphylococcus* bacteria and *S. aureus* bacterial strains (Chajęcka-Wierzchowska *et al.* 2015).

It can be concluded that the methicillin-resistant gene is mediated by new genetic elements SCCmec, which are fused and provoked by the genes of the new consortia ccrA and ccrB. Therefore, the study of the distribution of SCCmec helps to explain the exchange of important information between different species and *S. aureus*. As well as the knowledge of the mechanism of SCCmec regulation, may contribute to the production of a new drug therapy during which MRSA strains are converted to MSSA (Chajęcka-Wierzchowska *et al.* 2015).

It is believed that the group of fixed penicillin's (Penicillinase-Stable Penicillin's) which includes several antibiotics such as methicillin, oxacillin and naphcillin, possesses stimulating effects of *S. aureus* on antibiotic resistance, but was acquired by their transition from negative bacterial species for the coagulase enzyme (CONs) to *S. aureus* by genetic transmission mechanisms such as (bacterial conjugation, bacteriophage and genetic transformation) (McGough *et al.* 2020).

Also believe that the antibiotic cefoxitin or cephamycin has the potential to stimulate the regulation systems of the mecA gene more than penicillin's (McKinney *et al.* 2001), but Darini and Palazzo (2004) emphasize that cefoxitin does not stimulate the production of PBP2a in MSSA isolates, that is, it does not stimulate resistance in sensitive isolates, but it stimulates the inherent resistance in isolates with heterogeneous resistance.

2.10.2 Resistance of *S. aureus* to vancomycin antibiotic

The rise of *S. aureus* isolates that are resistant to the drug vancomycin recently represents a serious health concern (Hososaka *et al.* 2004, Pesavento *et al.* 2007). Where the first instance of this bacteria infection was discovered in Japan in 1997, followed by the registration of other cases on a larger scale in (2002) in the USA, France, Slovakia and other countries, which constituted a serious emergency and threatened with a major health disaster (Gardete and Tomasz 2014).

After the vancomycin antibiotic was classified as the best treatment for treating one of the most pathogenic types of bacteria on the human body, these bacteria were able to make this antibody unable to affect it like its predecessors of antibiotics of life, and this is what prompted the researchers to put this topic under constant discussion and research to find out the real reasons for this resistance and to stand on them (Gardete and Tomasz 2014).

And the exchange of chromosomal fragments, especially those carrying the trait of resistance to antibiotics that have the ability to be transmitted by different mechanisms that lead to the acquisition of resistance genes between different species, especially between bacteria *Enterococcus faecalis* and bacteria *S. aureus* (Melo *et al.* 2005). That the ability to transfer the resistance to antibiotics between different bacterial species is a real health problem, including the vancomycin antibiotic, which is used as an alternative to beta-lactam antibiotics to treat gram-positive bacteria especially *S. aureus* bacteria resistance to the methicillin antibiotic. Among the reasons behind this resistance, is what the two researchers mentioned (Tenover *et al.* 2004, Bhalakia 2005) that the cause of resistance to the vancomycin is the containment of bacteria *S. aureus* is based on plasmidine with a molecular size of 4 kbp and 120 kbp. While (Cong *et al.* 2020) that the reason for this resistance is the presence of jumping genes, specifically the jumping gene Tn1546, the carrier of the *vanA* gene responsible for coding for an enzyme that causes mutations on the peptidoglycan layer making the Vancomycin antibiotic unable to bind to its active site.

2.11 *Staphylococcus aureus* Biofilm

An organized community of bacteria known as a biofilm that adheres to various surfaces is found inside a self-produced matrix of polymeric materials (Gupta *et al.* 2016). According to the National Institutes of Health (NIH), bacterial biofilms are contagious by nature and can develop from nosocomial infections. Biofilms are linked to around 80% of all chronic illnesses and 65% of all microbiological infections (Jamal *et al.* 2018). Several steps are involved in the production of a biofilm, beginning with adhesion to a surface, followed by the development of a microcolony that results in the construction of a three-dimensional structure, and eventually maturation and separation (Yadav and Sanyal 2019). Due to its role in a number of infectious diseases, bacterial biofilm poses a serious danger to public health since it is more resistant to drugs and the human immune system (Jamal *et al.* 2015). It increased a microorganism's resistance to antibiotics and caused the human illness (Singh *et al.* 2017).

Bacterial cells of the same species within a bacterial culture may display variations in metabolism, gene expression, morphology, and physiology, enabling them to operate effectively within their community. These variations depend on their spatial connection in the biofilm matrix. For instance, a bacterial cell entrenched in the biofilm matrix could enter a latent condition to live on less nutrients and lower oxygen gradients (Tan *et al.* 2017).

A number of molecular mechanisms, such as slower growth rates, interactions between antimicrobials and biofilm matrix components, and the various activities of specific genetic determinants of antibiotic resistance and tolerance, contribute to the high level of recalcitrance that distinguishes biofilm communities. Even while alone, each of these mechanisms only partially explains the enhanced antimicrobial recalcitrance seen in biofilms, when combined, these defenses allow biofilm cells to survive even the most rigorous antimicrobial treatment regimens. The scientific evidence supporting the recognized biofilm resistance and tolerance mechanisms is compiled in this review of historical and current research (Hall and Mah 2017).

3. MATERIALS AND METHODS

3.1 Materials

In the Iraqi province of Babylon, from 1 January 2022 to 30 April 2022, 132 clinical samples were taken from AL-Hilla Teaching Hospital and Marjan Medical City. In the aforementioned hospitals' diabetic foot clinics, samples were taken using a sterile cotton swab from patients who had an infection with a diabetic foot ulcer. The BHI agar tubes were filled with the sterilized cotton swabs before being brought to the laboratory. This study did not contain any environmental isolates or additional samples from the same individual's appendix 1which show the instruments and equipments used in this study and the appendix 2 that show the biological and chemical materials were used in this study and the appendix 3 which show the Ready to made culture media which were used during this study and the appendix 4 which show the list of antibiotic discs which were used during this study

3.1.1 Polymerase Chain Reaction Materials

3.1.1.1 Primers

The primers that we used in this study show in Table 3.1.

Table 3.1 List of primers which were used during this our study

Gene		Sequence (5'-3')	Product size	Reference
mecA	F	GTAGAAATGACTGAACGTCCGATAA	310 bp	(de Castro and Von Zuben 1999)
	R	CCAATTCCACATTGTTTCGGTCTAA		
ermA	F	TCTAAAAAGCATGTAAAAGAA	645 bp	(Senon *et al.* 2021)
	R	CTTCGATAGTTTATTAATATTAGT		
blaZ	F	ACTTCAACACCTGCTGCTTTC	173 bp	(Nahar *et al.* 2017)
	R	TGACCACTTTTATCAGCAACC		
Spa	F	ATCTGGTGGCGTAACACCTG	1150- 1500 bp	(Shakeri *et al.* 2010)
	R	CGCTGCACCTAACGCTAATG		
** R: Reverse primer ** F: Forward primer				

3.2 Methods

3.2.1 *Staphylocoocus aureus* isolation and identification

Using sterile containers and transport swabs dampened with regular saline, all samples were taken. Each swab was immediately inoculated onto a mannitol salt agar plate and incubated aerobically for 24 hours at 37°C. This medium was created specifically to separate bacteria that are mannitol-fermenting (pink to yellow) from those that are not. All isolates that could ferment mannitol were subculture on mannitol salt agar and incubated for 24 hours at 37°C. According to regular biochemical tests, the suspicious *Staphylococcus* colonies, whose cells are clustered and Gram-positive, were further identified to species level (Mamza *et al.* 2016).

Using the coagulase test and other biochemical tests, the isolates were identified as follows:

3.2.1.1 Coagulase test

S. aureus is identified and distinguished from coagulase-negative staphylococci using the coagulase test (Sperber and Tatini 1975, MacFaddin 1976, Kateete *et al*. 2010).

a. Slide coagulase

A dense suspension of staphylococci from culture were created on a spotless glass slide. The suspension were given a drop of citrated plasma and vigorously swirled. The tube coagulase test must be used to identify *S. aureus* isolates that do not produce bound coagulase. If staphylococci agglutination happens within 5 to 10 seconds, the results are positive.

b. Coagulase tube test

A tube test utilizing (0.5 Ml) of citrate-stabilized rabbit plasma diluted 1 in 10 was also used to measure the coagulase production by isolates. A positive test was identified by the development of a clot after 1, 4, or 24 hours when added to (0.5 mL) of trypticase soy broth inoculated with *S. aureus* isolate and incubated at 37°C.

3.2.1.2 Staphylokinase enzyme

Considering the very low level of production and immunogenicity concerns of natural SAK produced by *S. aureus*, attempts have been made to produce recombinant SAKs with high production levels, more fibrinolytic activities and low immunogenicity.

3.2.1.3 Catalase production

A sterile needle was used to place a 24-hour-old bacterial colony on a clean glass slide, after which a drop of 3% H_2O_2 solution was added. An instantaneous release of oxygen bubbles was the result, which was a positive indication.

3.2.1.4 Preservation and maintenance of *S. aureus* bacterial isolates

On nutritional agar slant, the bacterial isolates were kept at 4°C. The isolates were kept alive by re-culturing on fresh nutrient agar medium each month (short-term maintenance). For lengthy preservation, nutrient broth supplemented with 15% glycerol was utilized, and the isolates were kept frozen at -70°C (deep freeze) for a number of months (Mahmmoud 2020).

3.2.2 Subculture of cultures preserved and frozen

Stock cultures that have been preserved and frozen were subcultured on new blood agar plates and then incubated for 24 hours under aerobic conditions at 37°C (Mahmmoud 2020).

3.2.3 Antibiotics susceptibility testing

Disk diffusion on Muller-Hinton agar medium was used to test the antimicrobial susceptibility of bacterial isolates. The quality control strain was *Escherichia coli* ATCC 25922.

The cultures were incubated for 18 hours at 37°C under aerobic conditions in accordance with the Barry (1976) technique. The National Committee for Clinical Laboratories Standard criteria were used to quantify and interpret the bacterial growth inhibition zones surrounding the disks (NCCLS 2021).

3.2.4 Oxacillin salt agar medium (OXA)

Muller Hinton Agar (MHA) was mixed with sodium chloride (4%), and sterilized by autoclave, and then chilled to 50 degree. After that, oxacillin (0.6 mg) was added, the mixture was placed into Petri dishes, and it was kept there at 4°C until it was needed. This technique was used to find MRSA (NCCLS 2021).

3.2.5 Extraction of genomic DNA

Methicillin resistant *S. aureus* isolates have their DNA extracted in accordance with the instructions provided by Presto Mini gDNA bacterium Kits (Geneaid, Thailand).

Protocol

1. A 1.5 mL micro centrifuge tube containing 1 mL of overnight cultured bacterial cells was filled with the sample, which was then spun at a speed of 14–16,000 x g for one minute. The supernatant was then discarded.
2. After adding 180 µL of GT Buffer, a vortex was used to resuspended the cell pellet.
3. Proteinase K (20 µL) was added to the mixture (which suspended priory ddH2O).
4. For 10 minutes, the mixture-containing tubes were heated in a water bath at 60°C.
5. The sample was mixed with 200 µL of GB Buffer and vortexed for 10 seconds.
6. To make sure the sample lysate was clear, the mixture was incubated in a water bath at 70°C for 10 minutes.
7. A 200 µL of pure ethanol was immediately added to the mixture and violently shaken to combine.
8. After being transferred to the GD column, the mixture was centrifuged at 14–16,000 x g for two minutes.
9. After placing the GD column in a fresh 2 mL collection tube, the 2 mL collection tube containing the flow-through was discarded.
10. 400 µL of W1 Buffer were added to the GD column, which was then centrifuged at 14–16,000 x g for 30 seconds, discarding the flow-through.
11. Wash Buffer (600 µL) was added to the GD column, it was centrifuged at 14-16,000 xg for 30 seconds then was discarded the flow-through.
12. To dry the column matrix, the mixture was centrifuged one more for three minutes at 14–16,000 xg.
13. A 100 µL of pre-heated Elution Buffer was added to the center of the column matrix.
14. The column matrix was kept standing for 3 minutes to allow the full absorption of the elution buffer.
15. To elute the pure DNA, it was centrifuged at 14–16,000 x g for 30 seconds.
16. Stored at -20°C was the extracted DNA.

3.2.6 Calculating DNA purity and concentration

Following DNA extraction, the concentration and purity of DNA samples (Wu *et al.* 2017) were assessed using.

3.2.6.1 Nanodrop technique

Using the nanodrop technique, 1 µL of the extracted DNA was applied to the device to verify the concentration of DNA to protein and to determine its concentration and purity by observing the ratio of O.D. 260/280.

3.2.6.2 Preparation of primers

Following the instructions provided by the primer synthesiser firm, the primers (which had previously been lyophilized) were dissolved in free ddH_2O to a final concentration of 100 µm/µL, which served as a stock solution that could be stored at -20°C. The stock primers were generated at a concentration of 10 µm/µL to be used as a work primer.

3.2.7 GoTaq® G2 green master mix

GoTaq® G2 Green Master Mix is a high-quality Taq DNA Polymerase, deoxynucleotides, and reaction buffer solution that is prepared for usage in a 2X concentration. It includes all of the tools required for DNA amplification. An inert green dye and a stabilizer are included in the GoTaq® G2 Green Master Mix to enable direct loading of the finished goods onto a gel for analysis.

3.2.8 Agarose gel preparing

The agarose gel was prepared in accordance with the procedure outlined by Dean *et al.* (2001). In order to completely dissolve the 1 grams of agarose powder, 100 mL of (1X) trisborate-ethylenediaminetetraacetic acid (TBE) buffer was combined with agarose

powder. The mixture was then heated to boiling by microwaving for about 5 to 10 seconds. Then, 5 µL of RedSafe nucleic acid staining solution was added to allow for visibility after allowing the solution to cool to 45°C.

3.2.9 Electrophoresis

The electrophoresis equipment was configured to run for 42 minutes at 90 Volt, Constant Current, after the samples and Safe-Green 100bp Opti-DNA Marker had been loaded. The PCR products were finally fisualized using a 320 nm UV light source after the gel was put into the UVP system.

3.2.9.1 Loading the PCR products

Around 5 µL of each PCR product were placed into the corresponding hole in the 1.5% gel. About 5 µL of Safe-Green 100 bp Opti-DNA Marker, which served as a marker for figuring out the size of the PCR products, was added to the first hole in the gel's line. Table 3.2 show the PCR conditions which were used during this our study.

Table 3.2 List of PCR conditions which were used during this our study

Phase	Tm (^{o}C)	Time	Cycles
Initial denaturation	94°C	5 min	1X
Denaturation	94°C	30 sec.	35X
Annealing	52, 55, 57,58^{o}C	30 sec.	
Extension	72°C	1 min	
Final extension	72°C	5 min	1X

3.2.10 Agarose gel documentations

The 1.8% agarose gel was used to segregate the amplified PCR products, which were then seen with UV light utilizing the gel documentation system. Using the Cleaver gel documentation technique and a digital camera, the gel was finally captured on camera.

3.2.11 Biofilm formation

Biofilm detection using a Tissue Culture Plate (TCP). The semi-quantitative microtiter plate test (also known as the "biofilm assay") of *S. aureus*, which was first reported by Sharma *et al.* (2021). Table 3.3 Classification of bacterial biofilm formation by tissue culture plate method (TCP), was the most popular and was regarded as the gold standard test for spotting the production of biofilms:

1- Isolates from freshly prepared agar plates were added to TSB containing 1% glucose, cultured for 72 hours at 37C, and then diluted 1:100 with new TSB.
2- To test for non-specific media binding, 150 µL aliquots of the diluted cultures were placed in each well of a sterile, polystyrene, 96 well-flat bottom tissue culture plate. As a reference, only broth was used. Each isolate received a triple inoculation.
3- The tissue culture plates were kept at 37°C for a 24-hour incubation period. Following incubation, the plates were gently tapped to extract the contents of each well. To eliminate floating "planktonic" microorganisms, the wells were rinsed four times with phosphate buffer saline (PBS pH 7.2).
4- Biofilms created by'sessile' organisms that adhered to the plate were fixed by placing them at 37C for 30 min.
5- Crystal violet, 0.1% w/v, was applied to every well. Plates were retained for drying after being thoroughly washed in deionized water to remove any remaining stains.
6- To dissolve bound crystal violet, 150 l of a 20:80, v/v, sodium acetate mixture was added. After measuring the optical density (O.D.) at 630 nm, the data were analyzed using the Table 3.3.

Table 3.3 Classification of bacterial biofilm formation by tissue culture plate method (TCP)

4 Mean of OD value at 630nm	5 Biofilm formation
6 <0.120	7 Non
8 0.120-0.240	9 Moderate
10 >0.240	11 High

3.3 Statistical Analysis

To evaluate the data's statistical significance, a one-way ANOVA test was used. P values under 0.05 were deemed significant.

4. RESULTS AND DISCUSSION

4.1 Results

4.1.1 Isolation and diagnosis of *S. aureus*

The current study has proved the return of 17 isolates to the genus *S. aureus* by studying some of the characteristics of cultivation, microscopy and biochemical tests as the following:

4.1.1.1 Cultural characteristics

The developing colonies appeared on the mannitol salt agar medium in yellow color and on the Staphylococcus medium No.110 in orange color and as shown in Appendix 8 (A-B).

4.1.1.2 Microscopic characteristics of *S. aureus*

The results of microscopic examination showed that the isolated bacterial cells are spherical in shape, clustered in order, gram-positive in Gram staining.

4.1.1.3 Biochemical tests

Isolates developing on the selective medium were diagnosed based on biochemical tests where Table 4.1 and Figure 4.1 showed a response of 17 isolates by (100%) to each of the catalase test, coagulase, staphylokinase, DNase, gelatinase and mannitol fermentation, and the response of isolates to the clumping factor test varied with 11 isolates showing a positive result by (64.70%), and 13 isolates showed a positive urase test result (76.47%), while all isolates showed a negative oxidase test result.

Table 4.1 Showed a response of 17 isolates by (100%) to each of biochemical testes

Type of test	No. of +ve isolates	Percentage %
Catalase test	17	100%
Coagulase test	17	100%
Oxidase test	0	0%
Clumping Factor test	11	64.70%
DNase test	17	100%
Staphylokinase test	17	100%
Gelatinase test	17	100%
Urease test	13	76.47%
Mannitol Fermentation test	17	100%

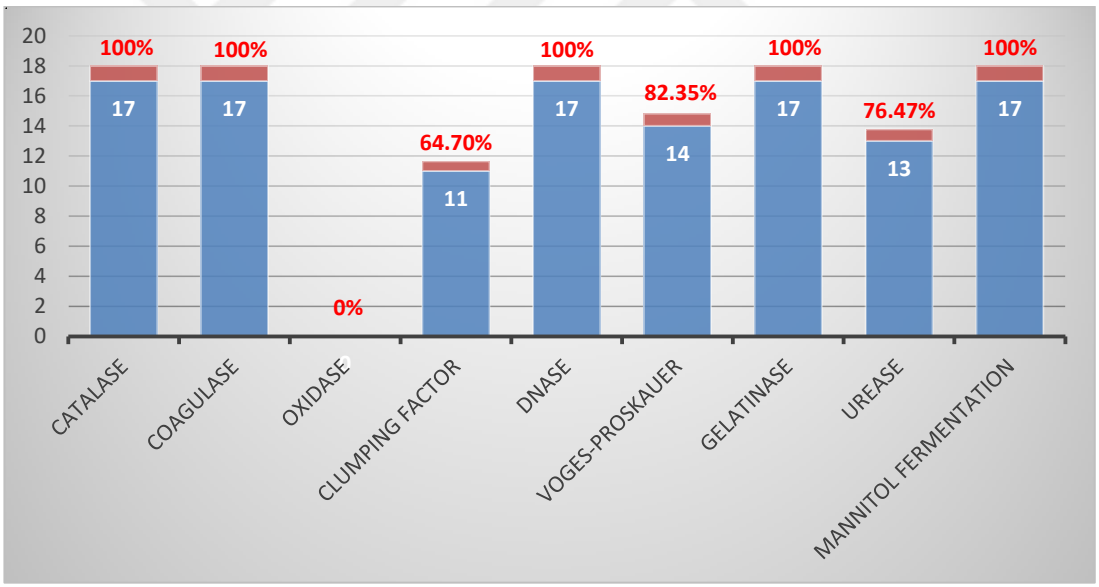

Figure 4.1 Showed a response of 17 isolates by (100%) to each of biochemical testes

4.1.2 Numbers and percentages of isolates of *S. aureus*

The 17 isolates were diagnosed (with an isolation rate of 12.87% of the total 132 samples) as shown in Table 4.2 that shows the places where the samples were collected and the highest percentage of isolation of *Staphylococcus aureus* bacteria from diabetic foot ulcer was (100%).

Table 4.2 Shows the places where the samples were collected

Source of samples	No. of samples	No. of isolates	percentage
Marjan medical city	78	11	64.70%
Al-hilla teaching hospital	54	6	35.30%
Total summation	132	17	100%

The results of the current study showed the highest percentage of isolation of *S. aureus* bacteria was from patients with diabetic foot ulcers whose age was limited to (60-69) years with an isolation rate of (58.82%) by 10 isolates, as shown in Table 4.3 that show the distribution of S. aureus bacterial samples and isolates according to ages.

Table 4.3 Shows the distribution of bacterial samples and isolates according to ages

Age group	No. of samples	No. of S. aureus isolates
50-59 years	20 (15.15%)	2 (11.76%)
60-69 years	65 (49.24%)	10 (58.82%)
70-80 years	26 (19.70%)	3 (17.64%)
75-80 years	21 (15.90%)	2 (11.77%)
Total summation	132 (100%)	17 (100%)

4.1.3 Investigation of the susceptibility of *S. aureus* to the production of beta-lactamase enzyme

The standard rapid iodine method was used to investigate the susceptibility of 17 isolates of *S. aureus* is under study on the production of beta-lactamase enzymes, Table 4.4 showed the presence of 15 productive isolates of beta-lactamase enzymes by (88.23%) out of a total of 17 isolates as the results in the current study showed the presence of 2 unproductive isolates of beta-lactamase enzymes by (11.77%), and

through the results it can be inferred the high percentage of production of beta-lactamase enzymes, Appendix 5 shows the standard rapid iodine method.

Table 4.4 Shows the isolates that produce beta-lactamase and non-produce

Hospital	Isolate produce lactamase	Isolate non-produce lactamase
Marjan M.C.	10 (66.66%)	1 (50%)
Al-hilla T.H.	5 (33.34%)	1 (50%)
Total summation	15 (100%)	2 (100%)

4.1.4 Antibiotic susceptibility test

The drug sensitivity of all *S. aureus* isolates under study was tested using 12 types of standard antibiotics, the results showed, as shown in Table 4.5 and Figure 4.2 that there is a discrepancy in the resistance of the isolates under study to the antibiotics used, and the results showed high sensitivity rate to aminoglycosides, including amikacin and cefdinir, was 17 isolates (100%) and 16 isolates (94.11%), respectively.

While the bacteria showed high resistance to the glycopeptides. Glycopeptide group such as vancomycin was 4 isolates (23.52%), and the rates of resistance to folate pathway inhibitors such as trimethoprim and sulfamethoxazole are 12 isolates (70.58%), and the results showed that the antibiotics quinolones, fluoroquinolone group such as ciprofloxacin is one of the most important therapeutic options in the treatment of infections caused by *S. aureus* bacteria, as the resistance rates reached 8 isolates (47%). The results showed that the resistance rate to aminoglycosides such as gentamicin and levofloxacin 6 isolates (35.3%) together, and to the β-lactams group such as amoxicillin/clavulanic acid and moxifloxacin 4 isolates (23.5%), and the rates of resistance to doxycycline and nitrofurantoin are 3 isolates (17.6%). To the one generation of penicillin's penicillinase-stable penicillin's, represented by methicillin where the rates of resistance to it came 6 isolates (35.29%) were resistant to oxacillin

and cefoxitin by the discs diffusion method and also showed 5 (45.4%) isolates with a positive result using the HI-Comb MIC test amid Chromagar MRSA and PCR technique.

Table 4.5 Resistance ratios of 17 isolates of *S. aureus* bacteria for antibiotics

Names of antibiotic	Symbol	R	I	S
Amikacin	AK	0	0	17
Cefdinir	FED	0	1	16
Amoxicillin/ Clavulanic acid	AMC	4	2	11
Ciprofloxacin	CIP	8	2	7
Gentamicin	CN	6	2	9
Vancomycin	VA	4	4	9
Methicillin	MET	6	4	7
Moxifloxacin	MFX	4	2	11
Levofloxacin	LEV	6	1	10
Doxycycline	DO	3	2	12
Nitrofurantoin	F	3	7	7
Trimethoprim and sulfamethoxazole	TMP	12	0	5
R= Resistance	I= Intermediate		S= Sensitive	

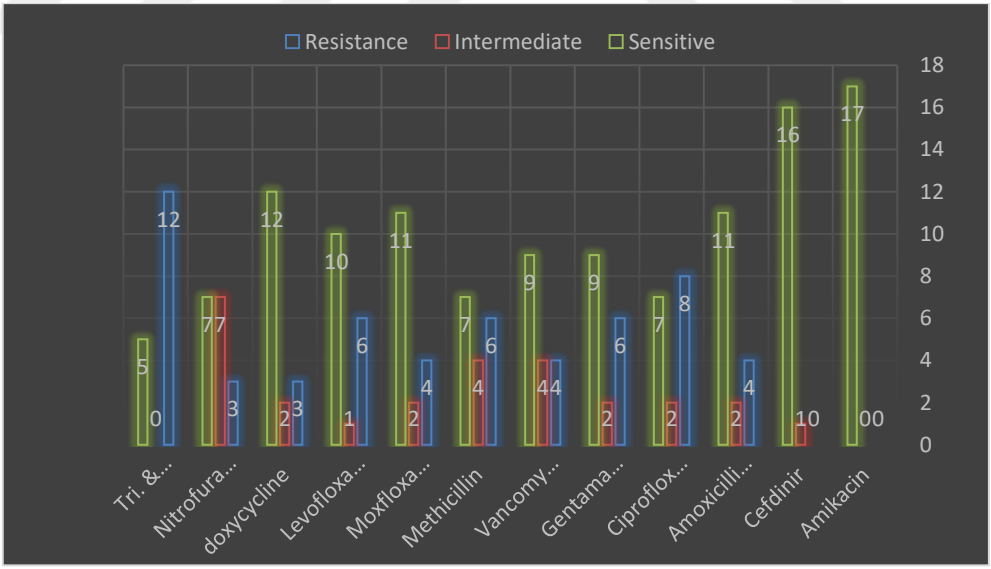

Figure 4.2 Resistance ratios of 17 isolates of *S. aureus* bacteria for antibiotics

4.1.5 Investigation of methicillin resistant staphylococcus aureus (MRSA)

4.1.5.1 The discs method of sensitivity test

The methicillin-resistant trait of all *S. aureus* isolates was investigated for the (17) isolates by using the oxacillin and cefoxitin drug sensitivity test discs (anti-methicillin discs were not used in this test), the results of the current study showed that the percentage of clinical isolates resistant to oxacillin and cefoxitin is (29.41%) by 15 isolates, and therefore these isolates were considered resistant to MRSA as a preliminary investigation, as shown in Table 4.6.

Table 4.6 Numbers and percentages of isolates of S. aureus methicillin-resistant based on the method of sensitivity test discs

Antibiotic types	**Symbol**	**Isolates number (51) isolate**		
		R	**I**	**S**
Oxacillin	OX	5	9	3
Cefoxitin	FOX	5	6	6
R= Resistance	I= Intermediate		S= Sensitive	

4.1.5.2 The method of the minimum inhibitory concentration (MIC) of cefoxitin and oxacillin

The minimum inhibitory concentration of oxacillin and cefoxitin resistant isolates was investigated using the HI Comb MIC test for methicillin and oxacillin, as shown in Appendix 7 (A and B) and Table 4.7.

Table 4.7 The minimum inhibitory concentration MIC of Cefoxitin and Oxacillin

Isolates Numbers	MIC for Methicillin			MIC for Oxacillin		
	R	**I**	**S**	**R**	**I**	**S**
	> 60 µg/mL	–	≤ 8 µg/mL	> 4 µg/mL	–	≤ 2 µg/mL
RS1	≥ 240			≥ 256		
RS2	50			2		
RS3	7			3		
RS4	40			3		
RS5	7			2		
RS6	120			60		
RS7	8			≤ 3		
RS8	7			1		
RS9	8			3		
RS10	180			3		
RS11	8			20		
RS12	6			128		
RS13	40			≥ 256		
RS14	8			3		
RS15	20			3		
RS16	120			2		
RS17	7			3		

4.1.5.3 CHROM Agar MRSA

All (17) isolates *S. aureus* in the study of (17) isolates have been grown on the medium of CHROM Agar MRSA, as (6) isolates showed (35.29%) its ability to resist methicillin and grow on the medium of chromium agar (MRSA) and is distinguished by giving the pink color of the isolates of the (MRSA), as shown in Appendix 8 (A and B).

4.1.5.4 Polymerase chain reaction (PCR)

4.1.5.4.1 Molecular detection of MRSA isolates by using PCR technique

All isolates of *S. aureus* have been tested confirmed that it contains the *mec*A (methicillin resistant) gene using (PCR) technique, as the (9) by (52.94%) isolates (positive for the previous methods) showed that they contain these genes (mecA, blaZ, ermA and spa) and therefore these isolates were considered resistant to methicillin MRSA as will be shown in the Figures (Figure 4.3, Figure 4.4, Figure 4.5, Figure 4.6).

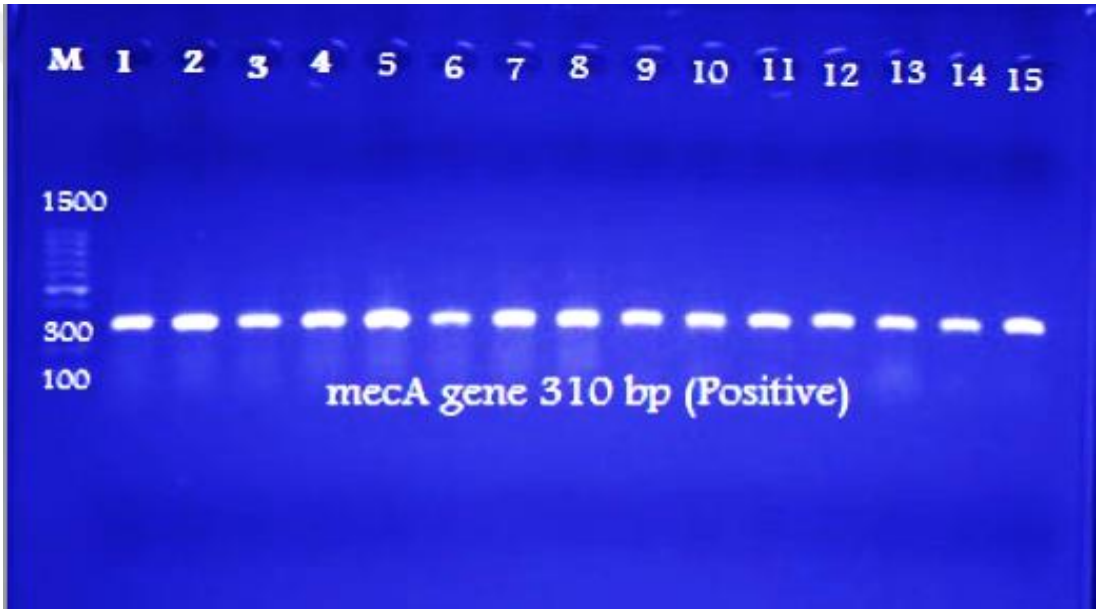

Figure 4.3 Safe red-stained agarose gel of monoplex PCR amplified products from isolated DNA of MRSA isolates and amplified with one gene primer. At 75 volts for one hour, the electrophoresis was carried out. Lanes 1,2,3,5,6,7,8, 9,10,11,12,13,14 and 15 containing the *mecA* gene (310 bp) provide good findings with Lane (M), a DNA molecular size marker (100-1500 lader).

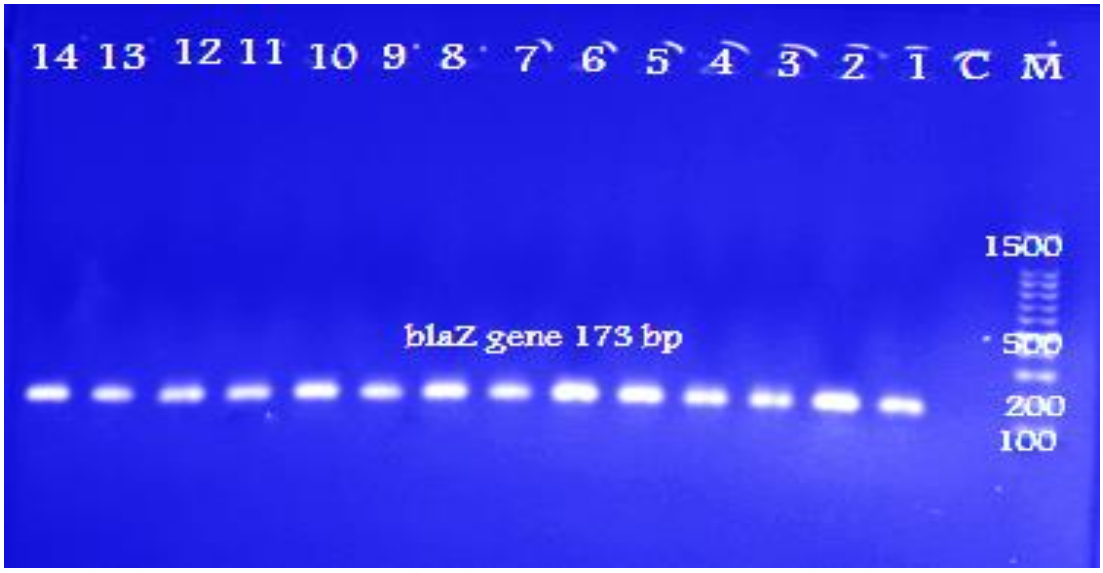

Figure 4.4 PCR products from MRSA isolates' isolated DNA that were amplified with a single gene primer were detected on a safe red-stained agarose gel. 75 volts were used for the electrophoresis for a whole hour. Lanes 1 to 14 denote positive results with the *blaZ* gene (173 bp), Lane (M), a DNA molecular size marker (100 bp ladder), and Lane (C), a control result that is negative (Control negative)

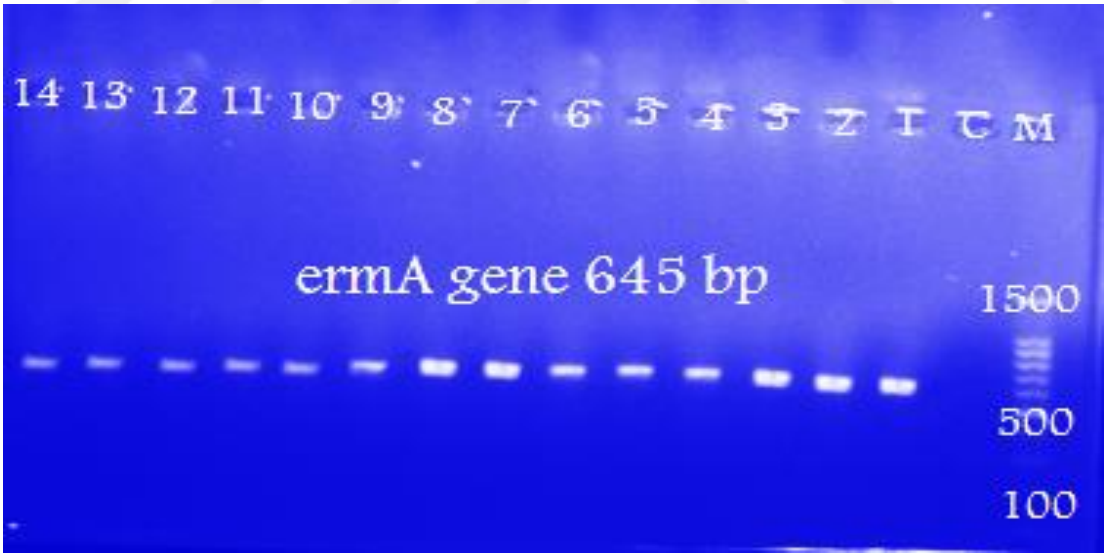

Figure 4.5 PCR results from extracted DNA of MRSA isolates amplified using a single gene primer are shown on a safe red-stained agarose gel. For one hour, the electrophoresis was run at 75 volts. Lane (M), *ermA* gene (645 bp), Lanes 1,2,3,4,5,6,7,8,9,10,11,12,13 and 14 indicate positive findings, Lane (M), DNA molecular size marker (100 bp ladder), and lane C (Control negative).

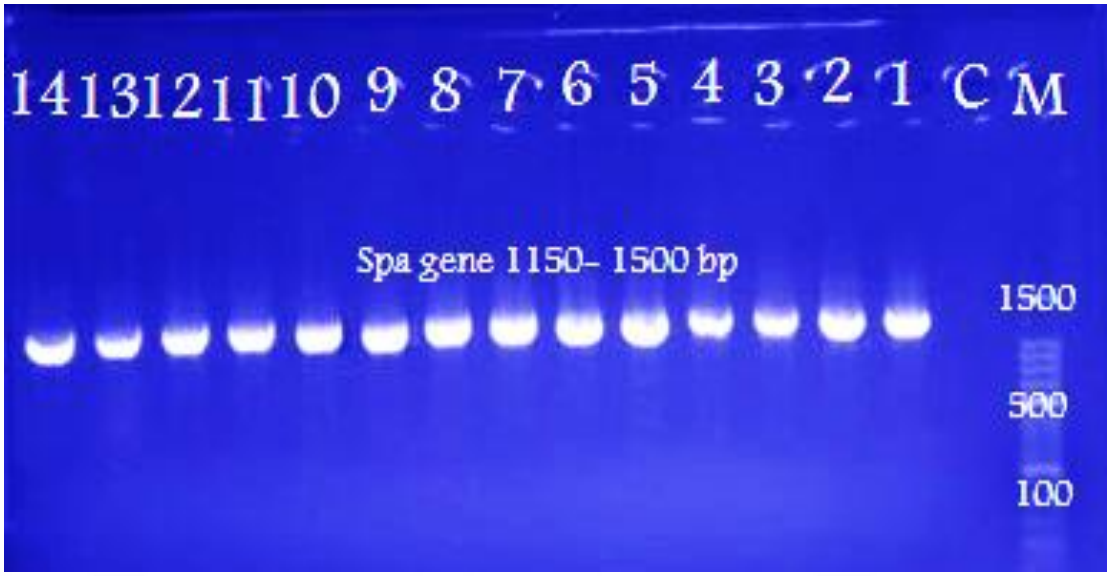

Figure 4.6 PCR results from extracted DNA of MRSA isolates amplified using a single gene primer are shown on a safe red-stained agarose gel. For one hour, the electrophoresis was run at 75 volts. Lane (M), a DNA molecular size marker (100 bp ladder), Lanes 1,2,3,4,5,6,7,8,9,10,11,12,13 and 14 demonstrate positive findings with *Spa* gene (1150-1500 bp), lane C, control negative, and Lane (M), a DNA molecular size marker (Control negative)

4.1.6 Detection of virulence factors of *Staphylococcus aureus*

This research found evidence of the formation of the enzymes hemolysin, DNase, lipase, protease, and gelatinase.

Hemolysin; by cultivating the isolates on blood agar, hemolysin production was discovered; positive findings showed up as a haemolytic zone surrounding the colonies. All (17) *S. aureus* isolates exhibited hemolytic activity, with the lysis being of β type, the appendix (9).

Lipase; All of the isolates were lipase producers when they were cultured on egg yolk agar, and the hydrolyzed clear zone around the colonies was deemed a positive result appendix. These enzymes work by assisting bacteria in invading host tissues by hydrolyzing cell membranes.

All of the isolates grown on egg yolk agar, a selective and differentiating medium used for the isolation and identification of *Staphylococcus* species, appear dark grey-black shiny colonies surrounded by a zone of clearing 2–5 mm colonies identified as *S.aureus*, while black colonies that are not shiny and infrequently produce clearing are identified as *S.epidermidis*.

Protease; on skim milk agar plates, protease production was found. Casein is the main protein in milk and its presence gives milk its distinctive white color. Protease hydrolyzes casein to form more soluble, transparent derivatives. The findings of this investigation demonstrated that the majority of the isolates were protease producers, with the hydrolyzed clean zone around the colonies being deemed a favorable outcome.
On skimmed milk agar plates a number of pigmented colonies appeared from white to deep orange, *S. aureus* appeared as golden-yellow colonies, *S. epidermidis* as white colonies appendice. The human pathogen *S. aureus* is known for its gold pigment, which serves as a barrier to oxidation-based clearance. Golden staphyloxanthin is the main carotenoid pigment produced by this organism via a biochemical route that has been extensively studied.

Additionally, staphyloxanthin functions as a virulence factor. It protects the microorganism from being killed by reactive oxygen species created by the host defense system by acting as an antioxidant.

Gelatinase; All *S. aureus* isolates were shown to be gelatinase makers, according to the findings of a test that included stabbing the isolates into a gelatinase medium and watching the medium liquefy.
These findings show that staphylococcal isolates may create a range of enzymes that aid in tissue invasion of their hosts.

DNase; when the isolates were cultured on DNase agar to determine DNase production, the presence of a yellow zone around the colonies was regarded as a successful outcome. The findings demonstrated that 17 *S. aureus* isolates (100%) produced DNase.

The majority of *S. aureus* pathogenic isolates generate DNase. DNase damages the host DNA, which makes the staphylococci that contain it more invasive and harmful.

4.2 Discussion

4.2.1 Isolation and diagnosis of *Staphylococcus aureus*

In this study, samples were collected from diabetic foot infections for the purpose of identifying the foci of contamination with *S. aureus* and the consequent diagnostic, preventive and curative measures. The bacterial isolates under study were diagnosed have been preliminarily diagnosed through the study of certain culture, microscopic and biochemical characteristics and examinations.

The anthers were transplanted into a medium blood agar to obtain typical colonies, the colonies were smooth, slightly raised, shiny, with diameters ranging from 1-3 mm, surrounded by a decomposition Halo (β-hemolysis) type, and the developing colonies also appeared on the medium of Staphylococcus No. 110 medium in orange and on a mannitol salt agar medium in yellow, pale yellow, golden, and reached diameters of approximately 1-2 mm, in addition, the color of the medium changed from red to yellow. As a result of mannitol fermentation and acid production, these results were consistent with what was stated (Stukus *et al.* 1997, MacFaddin and MacFaddin 2000).

The results of microscopic examination showed that the isolated bacterial cells are spherical in shape, clustered in a binary or quadruple arrangement or in the form of chains, and positive for gram stain, and this corresponds to what was mentioned before (Lowy 1998, Seetha *et al.* 2004, Brooks *et al.* 2014).

Table 4.1 of biochemical tests showed the response of 17 isolates (100%) to each of the catalase test, coagulase test, staphylokinase test, DNase test, gelatinase test and mannitol fermentation test, while all isolates showed a negative result of the oxidase test

and this was close to what was reported in each of the (MacFaddin and MacFaddin 2000, Prescott and Harley 2002).

The response of isolates to the agglutination factor test varied, as 11 isolates (64.70%) showed a positive result. The difference in the results of the bound coagulase and the free coagulase is due to several reasons, including that the glass slide method works to identify the coagulase bound to the outer surface of the bacterial cell wall, which is responsible for converting fibrinogen to fibrin (MacFaddin and MacFaddin 2000). While the tube method works to determine the free coagulase, and when a negative result is obtained by the glass slide method this isolation lacks the associated coagulation factor, but it is not necessarily unproductive for the plasma coagulant enzyme. Therefore it was necessary to conduct a tube test to obtain confirmed results, and the results sometimes depend on the effectiveness and activity of the isolation (Matthews and Dickenson 2001).

Table 4.2 showed that the highest percentage of isolation of *S. aureus* bacteria reached 11 isolates (64.70%) from Marjan Medical City, and the reason for the high percentage of these bacteria in diabetic foot ulcer samples can be attributed to the fact that the defensive means of the infected are penetrating and less resistant and the long duration of stay for diabetic foot ulcers in the hospital, as a result of the presence of *S. aureus* bacteria as a normal flora in the body, this was confirmed by (Novak 2000), as well as being one of the main causes of hospital infections in hospitals (Hososaka *et al.* 2004). As studies have shown that the resulting infections are mostly caused by *S. aureus* is a symbiosis that takes advantage of immunodeficiency and causes disease (Kowalski *et al.* 2005), while others get infected while staying in the hospital, they get *S. aureus* is endemic and highly resistant to antibiotics (Gormley 2007).

The severity of the isolation rate lies in the degree of infection and often the cases of foot ulcers consist of a high percentage of isolated bacteria at the level of the countries of the world, especially the third world countries. As it is inversely proportional to the amount of progress. In the statistics of the WHO (2000) during which (80%) of the cases of (second degree foot ulcers) were included, causing a deaths at very high rates

due to the fact that the defensive means of the infected are penetrating and less able to resist, the magnitude of the association between the increase in the settlement of *S. aureus* and the production of toxoids in various cases of diabetic foot ulcers was also confirmed. It can be concluded that the isolate rate in the current study is not without danger, as it is high compared to other isolation rates and other studies, if the isolation rate is of *S. Aureus*, the foot ulcers in the current study is higher than the percentage of isolation in the Al-Hassnawi study and his team (Al-Hassnawi *et al.* 2013), which amounted to (4.3%) and the Al-Fuadi study (Al-Fuadi 2010), which amounted to (0%). The percentage was very close to the percentage in the Hammoushi and Mahmoud (2004) and the study Al-Khudairi (2008), which amounted to (40%) and (41.7%), respectively, and since foot ulcers are cases of penetration of immune barriers, any presence of *Staphylococcus aureus* bacteria. The toxin-producing S. aureus is a dangerous indicator, and with the high rate of colonization, the risk levels rise with it.

The results of the current study showed that the second highest isolation ratio is the isolation ratio of *S. aureus* of diabetic foot abscess, which were (38.63%) of total summation of samples that were collected, and the percentage of isolation was *S.aureus* of diabetic foot infections in the current study is higher than the isolation rate in the study Al-Khudairi (2008), which amounted to (37.5%), and lower than the isolation rate in the Zidan (2007), which amounted to (50%), and higher than the isolation rate in the Al-Hassnawi *et al.*(2013), which amounted to (10.8%), counting *S. aureus* is one of the most important main causes in the production and formation of wound abscesses and because of its inherent ability to invade and attack the process of causing disease and then causing abscesses in large areas of the body occurs (Murphy *et al.* 2001). Cluster infections occur when they penetrate immune barriers such as skin and mucous barriers or when foreign bodies enter and the person who is infected may already suffer from a weakness in the body's immune systems (CDC 2001).

The isolation ratio was *S. aureus* of the diabetic foot absces in the current study is lower than the percentage of isolation in the study of Murphy *et al.* (2001), which amounted to (40%) for the years (1999-2001) and also lower than the percentage of (Barry 2001), which amounted to (60%). The skin is a natural plant for commensal bacteria, but the

high rates of settlement and colonization increases the likelihood of the presence of opportunistic strains whose obvious effect appears when the ability to this was confirmed by Murphy *et al.* (2001), and thus the incidence rates of skin infections and abscesses in various areas of the body are more or less affected with the rates of settlement and colonization and are directly affected by the amount of factors possessed by bacteria. The percentage of bacterial isolation from the skin can be counted conditionally depending on the degree of virulence of the isolates. The state of health of people and the amount of influence of surrounding conditions. It is not possible to isolate between the proportions taken from the skin and other related ratios, such as the percentage of isolation from wounds or burns, because of the strong correlation.

The process of settlement in different skin areas is controlled by several factors, such as adhesion factors associated with the surface such as capsular structure, extracellular enzymes and exotoxins, and these products allow *S. aureus* bacteria adheres to eukaryotic cell membranes and thus resists the process of phagocytosis. Thus obtaining the process of lysis of eukaryotic cells (Lyse eukaryotic cells) and then the release of products of host immune molecules occurs (Chambers 1997) due to the presence of the multifactorial nature of cluster mixtures and the repetition of the adhesion process of *Staphylococcus Aureus* bacteria and the frequency of production of exogenous proteins make it difficult to know which individual factor plays a role in determining the colonization process or which factor is the main contributor to the pathogenesis of the disease (Murphy *et al.* 2001).

The ages of the infected showed that the highest percentage of isolation of *S. aureus* bacteria in the current study was one of the most common diabetic foot infections for patients aged between (50-60 years) and (54.92%), and this percentage in the current study was higher than the percentage of isolation in the Hammoushi and Mahmoud (2004), which amounted to (33.0%).

4.2.2 Investigation of the susceptibility of *S. aureus* to the production of beta-lactamase enzyme

The standard rapid iodine method was used to investigate the susceptibility of 17 isolates of *S. aureus* is under study on the production of beta-lactamase enzymes, it is an indirect method, as it depends on the amount of penicilloic acid produced as a result of the decomposition of penicillin G and the breakdown of the amide bond in the beta-lactam ring. The degradation process occurs by beta-lactam enzymes, as penicilloic acid reduces iodine to iodide which does not have the ability to react with starch to form the color complex, and then the process of shortening the color of the starch complex with iodine occurs, and the color changes from blue to white (Noman *et al.* 2021).

Table 4.4 showed the presence of 15 isolates productive isolates of beta-lactamase enzymes by (88.23%) out of a total of 17 isolates as the results in the current study showed the presence of 2 isolates unproductive isolates of beta-lactamase enzymes by (11.76%). Through the results it can be inferred the high percentage of production of beta-lactamase enzymes from diabetic foot infections. This percentage is higher than the percentage recorded in the study Al-Hassnawi *et al.* (2013), which amounted to (4.5%), and through the results it can be inferred that the production of beta-lactamase enzymes is high, and the reason can be attributed to the high percentage of *S. aureus* produced beta-lactamase enzymes to the abuse of beta-lactam antagonists, which may be the cause of stimulating the production of beta-lactamase enzymes (Zhang *et al.* 2020).

The proportion of isolates produced by beta-lactamase enzymes in the current study is relatively high, and referring to several studies, it is noted that *S. aureus* has shown a successive series of resistances since the introduction of penicillin in 1943 and until now, the resistance rate in most countries of the world has reached more than (80%) (Al-Rawahi *et al.* 2008), in the UK the percentage of resistance to penicillin was recorded for hospitalized patients (97%), in the USA (98%), in Sweden (91%) and in some Arab countries such as Saudi Arabia (94%) and the kingdom of Jordan (95%), and the percentage of resistance in Norway (92%) and Singapore (96%) (Noskin *et al.* 2005), and Yokota (1999) also indicated a high proportion of isolate *S. aureus*

resistance to penicillin by (90.9%), while Medeiros (2001) found that the percentage of resistance to penicillin in his study was (88.8%) and by comparing the results in this study with the rates recorded in many countries of the world, It turns out that the percentage does not differ much from its counterparts (Khan *et al.* 2019).

The standard rapid iodine method has been widely successful in covering a high percentage of *S. aureus* strains produced the beta-lactamase enzyme, but this method cannot be counted from the exact methods because it must be taken into account that some strains produce (latent) amounts of beta-lactamase enzymes, which are characterized by slow effectiveness with a stimulating antibiotic such as penicillin, and it is possible to stimulate this enzyme to production by adding a certain percentage of sodium chloride salt (Brown *et al.* 2005). In this method, no emphasis was placed on this aspect, which may lead to the loss of a partial assessment of the amount of the latent enzyme, due to the fact that the positive result of the test was determined by the change in the color of the experimental medium from blue to white by five minutes, but the amount of latent beta-lactamase produced needs a sufficient period of time to determine it. This period is controlled by several factors such as the characteristics of the catalyst, the Ionic content of the medium, the acidity of the medium, the amount of Ionic binding of the beta-lactamase enzyme with the bacterial cell, and the effectiveness of the bacterial strain, and these factors together interact with each other affecting the duration of the response.

The production of beta-lactamase enzymes accounted for the isolates of *S. aureus* in the current study (88.23%), based on these results, according to the NCCLS (2021), any strain from *S. aureus* strains producing beta-lactamase enzymes are strains resistant to penicillin, ampicillin, amoxicillin, ticarcillin, carbenicillin and mezlocillin, and with reference to the above. It can be inferred how dangerous the high rate of production of the beta-lactamase enzyme is because it is possible to count the strain producing the beta-lactamase enzyme as a strain resistant to at least six types of beta-lactam antibiotics, and this resistance forms a pressure pharmacologically. It reduces the schedule of antibiotics provided to the patient, and in general, the high percentages of *Staphylococcus* isolate. *S. aureus* produced the enzyme beta-lactamase in this study may

be due to many reasons, some of which are at the local level, such as the use of antibiotics at random and without consulting a doctor. The lack of controls preventing the dispensing of the drug without a prescription, as well as the lack of health awareness in the use of antibiotics, and there are reasons at the cellular and molecular level related to the bacteria themselves, as some researchers conducted a statistical study in Britain on the resistance of *S. aureus* to penicillin and for the period between (1942-1953) and (1965-1986), when finding that the percentage is more than (70%), and that the percentage of resistance for the years (1990-2000) ranges between (80%-90%) the causes of resistance were attributed to the production of beta-lactamase enzymes and four types of them were distinguished and named (A, B, C and D), and their genetic code is carried on some plasmids or jumping factors that can merge with the chromosome, and it was found that the ratio has passed a certain level of permanence over six decades, indicating the persistence of resistance (Lyon and Skurray 1987, Goerge *et al.* 2017).

4.2.3 Detection of the ability of *Staphylococcus aureus* to be sensitive to antibiotics

Drug sensitivity was tested for all isolates of *S. aureus* is being studied using 12 types of standard antibiotics, and as shown in Table 4.5, this group of antibiotics was selected for its common use in the treatment of some mixtures resulting from infection with *Staphylococcus Aureus* bacteria, in order to find out how resistant the *Staphylococcus Aureus* bacteria are antibiotics and the danger of the spread of such resistance, which may extend to a wide number of different antibiotics (CDC 2007).

The results of the drug sensitivity screening test in the current study showed high levels of resistance to most aminoglycoside and cephalosporin III antibiotics, as the percentage of sensitive to amikacin sulfate and cefdinir reached (100%) and (94%), respectively. The percentage of sensitive to amikacin sulfate and cefdinir in the current study was consistent with the percentage of Miller *et al.* (2005). The percentage of Noskin *et al.* (2005) and the percentage of Al-Hassnawi *et al.*(2013), as the percentage in their studies reached (100%), it was higher than the percentage of Khudairi (2008), which amounted

to (90.8%). The percentage in the current study does not differ much from its counterparts, as it showed an increase in the rates of resistance to some beta-lactam antibiotics, as many studies have shown the presence of (90%) of *S. aureus* bacteria is resistant to penicillin. As a result of the secretion of the enzyme penicillinase mainly or through the production of beta-lactamase enzymes (Enright *et al.* 2002), Fuda *et al.* (2004) also confirmed that resistance to penicillin compounds includes not only the production of penicillinase and beta-lactamase enzymes, but also the production of penicillin binding proteins (PBPs) located in the cytoplasmic membrane attached to the cell wall, These proteins have enzymatic efficacy such as transpeptidases and carboxypeptidases, and these proteins are the target of both antibiotics (Aminoglycosides and cephalosporins).

As they work to change the target for beta-lactam antibiotics and thus produce bacterial resistance to beta-lactam antibiotics. The study of Fuda *et al.* (2006) confirmed the role of plasmids in the production of beta-lactamase enzymes and their responsibility for encoding some penicillin binding proteins (PBPs). Many studies have also pointed to the knowledge of the mechanisms of resistance to beta-lactams and the knowledge of the genes responsible for that resistance. This was confirmed by an extensive study by Lyon and Skurray (1987), as resistance to antibiotics was linked to the sources responsible for that resistance. As it was found that most of the resistance shown by *Staphylococcus* strain *S. aureus* of plasmid origin.

The isolates of *S. aureus* bacteria in the current study also showed resistance to the trimethoprim/sulfamethoxazole antibiotic by (70.58%), and this percentage was higher than the percentages in the Khudairi study (2008) and lower than the study Al-Hassnawi (2012), which amounted to (66.2%) and (93.1%), respectively. This antibiotic is among the anti-beta-lactam with a synergistic effect, and is characterized by its high resistance to enzymes beta-lactamase, due to the fact that the use of amoxicillin alone has been met with high resistance by most strains of *S. aureus* has increased its efficiency by adding clavulanic acid. The mechanism of resistance to this antibiotic does not differ from its predecessors of beta-lactam antibiotics, as it is resisted by changing the target of the beta-lactam antibiotic, and resistance may occur as a result of reducing the

permeability of the antibiotic through the bacterial cell membrane (Ray and Ryan 2010, Panchal *et al.* 2020). The bacterial isolates in this study shown a marked variation in their resistance to beta-lactam antibiotics and this may be due to the diversity in the mechanisms of resistance to *S. aureus* isolates, resistance to beta-lactam antibiotics appears through several mechanisms, such as the production of beta-lactam inhibitory enzymes, or by changing the binding sites of penicillin binding proteins (PBPs) (Hiramatsu *et al.* 2001), and the other reason depends on the virulence factors of the bacteria themselves, as some isolates show a greater amount of virulence than others, as well as the difference in the sources of sample collection. The difference in test conditions and the type of techniques used in the study, all together, there may be a difference in resistance levels (Brown *et al.* 2005).

The results of the current study indicated an increase in the percentage of resistance to the ciprofloxacin antibiotic, reaching (47%). these results were consistent with Leclercq (2002) and the study of Schmitz *et al.* (2000), which indicated an increase in the percentage of resistance to isolates of *S. aureus* for the ciprofloxacin antibiotic, while the results were inconsistent with Al-Hassnawi (2012), who indicated that the resistance rate was only (25%) in isolates of *S. aureus*. The resistance shown by the isolates of *S. aureus* compared the ciprofloxacin antibiotic to its ability to produce the RNA methylase enzyme encoded by the *cip* gene (Jorgensen 2004, Jørgensen *et al.* 2005).

As for the resistance to aminoglycoside antibiotics represented by gentamicin, it reached (35.29%), and it may be due to the presence of the gene responsible for resistance to this antagonist, which encodes to mutating events at the target site 30S, to which the antagonist binds, causing resistance to this antibiotic (Hanaki 2004), and the resistance rate in the current study was lower than the rates recorded in the study of Khudairi (2008) and higher than Al-Hassnawi (2012), which amounted to (49.2%) and (13.6%), respectively.

The current study showed that the percentage of resistance to vancomycin was (29.4%), since 5 isolates under study showed a low resistance to the vancomycin antibiotic, this percentage in the current study was relative with many studies in Iraq, including the

study of (Alsamarai *et al.* 2015) and the study of Al-Khudairi (2008). In the city of Nasiriyah and Najaf, and the study of Al-Hassnawi (2012) in the city of Babylon, which showed that the percentage of resistance to the antibiotic vancomycin was (25%), and in another study in Egypt showed a high resistance of (80%) to the vancomycin antibiotic (Ghazal *et al.* 2011, Muhammad and Al-Mathkhury 2014). The results were identical to what was stated in the NCCLS (2021), as it was confirmed that there is widespread resistance within the medical data. Except for some isolated single resistances by patients who have been using vancomycin for long periods, while the percentage in the current study differs with the percentage in the Zidan (2007), which amounted to (10%) by four isolates out of 40 isolates resistant to this antibiotic and isolated from bone marrow inflammation, and also differs with the percentage of resistance in the study Al-Geobory (2011), which amounted to (2.27%).

That the *S. aureus* resistance of vancomycin is caused by the acquisition of the *van* gene, which alters the binding effectiveness of the peptidase enzyme (D, D-peptidase), which plays a role in building the bacterial cell wall, and resistance to vancomycin is very high and at wide levels (Clark *et al.* 2005).

The cause of resistance may be due to changes in the manufacturing pathway of the antigen-sensitive cell wall leading to an increase in its thickness, fewer cross-links, or an increase in the D-Ala end. D-Ala induced a change in the target site of the vancomycin antagonist (Hanaki 2004, Reipert *et al.* 2003), or resistance may be caused by the transfer of genes encoded by the resistance trait of this antigen and carried on conjugate plasmids or jumping genes from other bacterial strains and species *to Staphylococcus aureus* bacteria acquired resistance to this antibody during a short period of (Jacobs 2005, Chambers 2001).

4.2.4 Detection of methicillin sesistant of *S. aureus* (MRSA)

4.2.4.1 Method of sensitive test discs

The methicillin-resistant trait of all s isolates was investigated. *S. Aureus* using the drug sensitivity test tablets for oxacillin and cefoxitin (the methicillin antagonist was not used in this test). As a result of the effect of the methicillin antagonist on the test conditions and giving false results led to the failure of the investigation of resistance using this antagonist (Brown *et al.* 2005).

The oxacillin antibiotic is used more than the methicillin antagonist in the investigation of methicillin resistant *S. aureus* due to its high resistance to storage conditions and its high ability to investigate isolates of staphylococci with heterogeneous resistance (Kuok *et al.* 2017).

The results of the current study showed that the percentage of clinical isolates resistant to oxacillin and cefoxitin is (29.4%) (by 5 isolates) and therefore these isolates are considered resistant to methicillin MRSA.

The oxacillin antagonist is stable under storage conditions, and the cefoxitin antibiotic is in fact a good promoter of the *mecA* gene responsible for resistance to the methicillin antibiotic (Weigelt *et al.* 2010). Accordingly, oxacillin-and cefoxitin-resistant isolates are a preliminary indication of methicillin resistant (MRSA).

Methicillin-resistant strains in vitro can be considered resistant to all types of beta-lactam antibiotics because most clinical cases have been documented as resistant to methicillin. Despite showing sensitivity to beta-lactam antibiotics in vitro, and therefore it is wrong to count laboratory-sensitive strains clinically sensitive (NCCLS 2021, Brown *et al.* 2005) . As MRSA works to resist beta-lactam antibiotics by the production of the enzyme (PBPs) a axis that closes the binding site of beta-lactam antibiotics with

(PBPs) and thus extends resistance to all β-lactam antagonists (Hiramatsu *et al.* 2001, Kohner *et al.* 1999).

The danger of MRSA strains lies in their endemism within hospitals, the speed of their spread in communities and their transformation into a silent mode, and the high percentage of their resistance, and the spread process is not limited to a specific country or a certain region. They are widespread in industrial countries such as the United States and in many other countries such as Greece, Italy, France, Spain and Portugal, and this was confirmed by Barry (2001). It was noted that many factors interfere with MRSA and increase the rate of its danger, especially hospital infections, and these factors include the areas of settlement of these strains are people working and lying in the hospital the patient's health status and the antibiotic schedule provided to the patient and these factors may interact. Santos e al. (1999) pointed out the fact of these factors and emphasized the need to take them into account when studying the effect of MRSA isolates at any of the levels. The proportion of MRSA in the current study is higher than in the UK, which is (39.6%, 40.1%, 39.3% and 39.2%) for the years (1990, 2000, 2004 and 2005) respectively (Kuehnert *et al.* 2006). Lower than the percentage recorded by Klein *et al.* (2007) in a survey of hospitals in the United States of America for the period 1999-2005 amounted to 62%, and higher than the percentages recorded in the Khudairi study (2008). The study of Al-Fuadi (2010) and the study of Al-Hassnawi (2012), which amounted to (18.5%), (32.3%) and (%29.5) respectively.

4.2.4.2 The method of the minimum inhibitory concentration of methicillin and oxacillin (MIC)

The minimum inhibitory concentration of all MRSA isolates diagnosed by the method of oxacillin and cefoxitin sensitive test disks was (17) isolates investigated using the HI Comb MIC test for methicillin and Oxacillin, and the minimum inhibitory concentration levels were determined based on NCCLS (2021). Assuming that the breakpoint (b.p) represents the optimal concentration of the antibiotic at which it reaches the serum and gives the highest level of therapeutic effects, but if the MIC level is less than (b.p) isolation is sensitive to this antibiotics.

The results shown in showed the resistance of the isolates under study for both methicillin and oxacillin by (54.90%) and (29.41%), and the results recorded high levels of MIC ratios for both antibiotics, which were between (60 - ≥240) micrograms/milliliter for methicillin antibiotic and (20 - ≥256) micrograms/milliliter for oxacillin antibiotic compared to the values of (b.p) and the mic ratios in the current study correspond to the ratios in the study Al-Hassnawi (2012), which amounted to (20 - ≥256) micrograms/ milliliter for oxacillin. The ratios in this study differ from the ratios recorded in the study of Raymond and Albert (2000), as the MIC ratios reached a level of 32 micrograms/ milliliter for methicillin and 16 micrograms/milliliter for oxacillin, and the ratios in the current study were lower than the ratios in the study of Ockubo (2001), as the levels of MIC ratios of (512-4) micrograms/milliliter for oxacillin, while the ratios in the current study were higher than the ratios in Al-Fuadi (2010), as the levels of mic ratios were of (32-4) micrograms/milliliter for oxacillin. This may be due to the type of test used in the current study, as the HI Comb MIC test is characterized by high speed and accuracy in investigating MRSA methicillin resistant isolates.

The high levels of MIC of some MRSA isolates may be due to the endemicity of MRSA in hospitals more than in communities, as the MIC method is characterized by its high ability to be sensitive to the amount of low resistance, as Brown (2005) confirmed the accuracy of the results of this method and its high sensitivity to low resistance levels. In comparison with previous results, it is noted that the MIC method in this study agreed in its results with the method of allergy screening tablets, so these two methods can be relied on in diagnosing MRSA methicillin resistant isolates.

4.2.4.3 Method of planning on the planting CHROMagar medium MRSA

Isolates of methicillin resistant *S. aureus* (MRSA) were investigated using the optional CHROMagar medium, since all isolates of *S. aureus* is under study. The number of (17) isolates on the chromagar MRSA medium, where 8 isolates (47.05%) showed their ability to grow and give them pink colonies of MRSA isolates, while the CHROMagar medium inhibits the growth of methicillin sensitive *S. aureus* (MSSA), and therefore the

CHROMagar medium is considered MRSA is an optional medium for isolating and differentiating methicillin resistant *S. aureus* srtains (MRSA) and even within a few levels.

The sensitivity and specificity ratio is about 100% for the MRSA CHROM agar medium (Schreiber *et al.* 2021). It is characterized by ease of use and speed in giving results depending on the color after an incubation period of 18-24 hours. It is not affected by certain conditions compared to the method of planning on the implant medium supported by the antibiotic methicillin or oxacillin. They are accompanied by special conditions such as lowering the temperature and using a direct vaccine (not dense), and the duration of incubation does not exceed 24 hours (NCCLS 2021) due to the fact that the low temperature of 30C. It is not extreme allows resistant isolates to grow well, as well as this period is sufficient to enable MSSA to grow even by a very small amount, which affects the correctness of the diagnosis, which may lead to erroneous results, and the direct vaccine (not dense) encourages methicillin-resistant bacteria to grow, and reduces the chances of sensitive bacteria growing (Brown 2001).

The percentage of MRSA isolation in the current study diagnosed using the MRSA CHROM agar medium is higher than the percentages recorded in the study of Van JC *et al.* (2006) and the study of Cesur *et al.* (2010), which amounted to (9.3%) and (35.7%), respectively. The high isolation of MRSA compared to other studies is a high risk in cases of disease with therapeutic complications, as MRSA isolates endemic in hospitals are characterized by the amount of control over the treatment provided. As it is a pressure to select qualitative and specific treatments because the relationship between resistance to methicillin and beta-lactam antibiotics and multiple resistance to other antibiotics is an interrelated and the table of antibiotics presented to him (Griffiths *et al.* 2004).

4.2.4.4 Confirmatory diagnostics for the investigation of MRSA isolates by using the polymerase chain reaction technique (PCR)

All isolates of *S. aureus* have been tested under study to determine its ability to resist methicillin using PCR technology. This technique was used to diagnose the mecA gene responsible for active and latent resistance to MRSA strains (Huletsky *et al.* 2005). The results showed that the percentage of isolates containing the *mec*A gene (MRSA) was (54.90%) by (28) isolates. As the amplification output of the *mec*A gene for MRSA isolates was investigated by examining the gel under ultraviolet radiation and comparing it with the standard ladder DNA Ladder (100-1500bp). Where it was found that one single package with a molecular weight (310 bp) is base pair, and this result is identical to what was mentioned (Santos *et al.* 1999).

The percentage of MRSA in the current study is higher than the percentages recorded in the study of Warren *et al.* (2004). As the percentage of MRSA isolation reached (21%) in 1994 and (29%) in 2003, and the percentage of MRSA isolates recorded in this study is higher than the percentages recorded in a research study prepared by Panhotra *et al.* (2005). As the percentage of recorded MRSA isolates which isolated from some Greek hospitals less than (0.5%) for the years (1999-2004). The percentage of MRSA was higher than the percentage of Khudairi (2008), which amounted to (18.5%), and all the mentioned percentages were calculated using the PCR technique, which is called the gold standard method for its accuracy in diagnosis, the PCR technique is considered one of the best diagnostic methods. Currently for its high accuracy and specificity in the diagnosis of in a study by Brown (2005), the sensitivity of the PCR technique in the diagnosis of MRSA strains was calculated using laboratory methods (98.1%).

4.2.5 Antibiotic resistance of MRSA isolates

4.2.5.1 Antibiotic susceptibility test

The drug sensitivity of the 6 MRSA isolates was investigated using 12 different standard antibiotics. This test was chosen to find out the resistance of MRSA isolates to various antibiotics and the correlation between their resistance to the group of beta-lactam antibiotics and the rest of the other antibiotics, and the sensitivity to some antibiotics that may be offered as a treatment for infections resuling from infection. MRSA isolates are resistant to practically all beta-lactam antibiotics represented by penicillin's and cephalosporins (Katayama *et al.* 2000, NCCLS 2021).

The amount of correlation between the ability to produce the beta-lactamase enzyme and the resistance arising from the production of (PBP2a) has been inferred through mutations. In some of which the deletion of the organizations responsible for the reproduction of mecA may occur, and by the process of deletion. The ability to express the mecA gene is lost, as well as occurs in the absence or loss of the plasmid encoded by the beta-lactamase enzyme that maintains the stability of the mecA gene, and in practice this relationship was confirmed by the deletion of the pyk20 plasmid for the mecA strain N315. *S. aureus*, and found that the stability of the mecA gene depends on the presence of plasmid regulating the process of reproduction of the mecA gene (Katayama *et al.* 2003).

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

1. The predominance of Gram-positive bacteria in diabetic foot ulcer contamination over other bacteria.
2. The bacterial species isolated in this study were distinguished by their high antibiotic resistance reflecting the ill-considered use of antibiotics in the treatment of patients in the province.
3. Methecillin resistant strains of MRSA are increasingly spreading within hospitals among *S. aureus* bacteria causing nosocomial infections.
4. The HiComb MIC test for measuring the minimum inhibitory concentration is of relatively high efficiency for the examination of sensitivity to antibiotics and may be adopted in the future.
5. The results provide evidence that *S. aureus* (MRSA and MSSA) had high levels of resistance to the antibiotics: amikacin, cefdinir, Amoxicillin/ Clavulanic acid, doxycycline, Nitrofurantoin, methicillin, Moxifloxacin and vancomycin.

5.2 Recommendations

1. Use these antibiotics (amikacin, cefdinir, Doxycycline, Amoxicillin/ Clavulanic acid, Levofloxacin and Moxfloxacin) as a treatment option for infections caused by *S. aureus* bacteria and the MRSA strain after conducting additional experiments for this purpose.
2. The necessity of adopting the planning method on the chromagar MRSA implant medium in laboratory work for ease of use and speed in giving results.
3. The use of the Primer design prefix technology and its adoption in research as it has reliable results and provides ideal conditions for the designed prefixes used in the PCR technology.

4. Using Real-time PCR technology to investigate the genes responsible for virulence factors in MRSA bacteria, especially the toxins (mecA, Spa, blaZ and ErmA) to determine their gene expression.
5. Application of this approach (detection of resistant for antibiotics) on other microorganisms especially other gram-positive bacteria, since the DNA extraction from such cells being no more difficult.

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APPENDICES

APPENDIX 1. The Instruments and equipments used in this study

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APPENDIX 9. Hemolysin producing *S. aureus* colonies on blood agar medium

APPENDIX 1. The Instruments and equipments used in this study

Type of equipment	Manufacturing company	Origin
Autoclave	Hirayama	Japan
Bench centrifuge	Hettich	Germany
Cold centrifuge	Hettich	Germany
Distillator (Water distiller)	GFL	Germany
Digital camera	Sony	Japan
Deep freezer	GFL	Germany
Electric oven	Memmert	Germany
Electrophoresis unit	Labnet	Taiwan
Eppendorf centrifuge	Hettich	Germany
Gel documentation system	Biometra	Germany
High speed cold centrifuge	Hettich	Germany
Incubator	Memmert	Germany
Laminar flow	Cruma	Spain
Magnetic stirrer	Labtech	Germany
Micropipette set (1-1000µl)	Eppendorf	Germany
PCR system	GeneAmp	Singapore
pH-meter	LKB	Sweden
Shaker water bath	Memmert	Germany
Standard loop 0.01 mL	Himedia	India
Sensitive balance	Memmert	Germany
Vortex	Thermolyne	USA

APPENDIX 2. The biological and chemical materials were used in this study

Materials	M. Company	Origin
Agarose	Promega	USA
Boric acid	Fischer	USA
Chloroform	BDH	England
C-reactive protein kit	Spinreact	Spain
DNA loading buffer	Promega	USA
ELISA kits (INFγ, IL-4and IL17A)	PeproTech	USA
Ethylenediamine tetra-acetic acid (EDTA)	AppliChem	Germany
Ethidium bromide	Sigma	USA
Ethanol (96%)	BDH	England
Fetal calf serum	Gibco	USA
Ficoll	Flow Laboratories	Scotland
Glycerol ($C_3H_8O_3$)	Fluka	Switzerland
Hanks balanced salt solution	Sigma	USA
Hydrochloric acid (HCl)	BDH	England
Hydrogen peroxide (H_2O_2) 3%	Himedia	India
Isopropyl alcohol	Mast Diagnostic	USA
Lysostaphin	Sigma	USA
Lysozyme	Sigma	USA
Methyl red	BDH	England
Molecular grad water	Promega	USA
N-Lauroylsarcosine sodium salt	BDH	England
α-Naphthol (C10H8O)	BDH	England
Phenol	Scharlau	Turkey
Seakem Gold Agarose	LaboShop	Switzerland
Sma I 20,000 units/mL	Biolabs	England
Sodium deoxycholate	BDH	England
Sodium dodecyl sulfate (SDS)	AppliChem	UK
Sodium chloride (NaCl)	BDH	England
Staphylococcus aureus enterotoxin-A (SEA)	Sigma	USA
Tris-HCl solution	AppliChem	UK
Tris-Borate-EDTA Buffer (TBE buffer	Promega	USA
Tris- EDTA (TE) buffer molecular grad	Promega	USA
Trypan blue	Pharmacia Fine Chemical Uppsala	Sweden

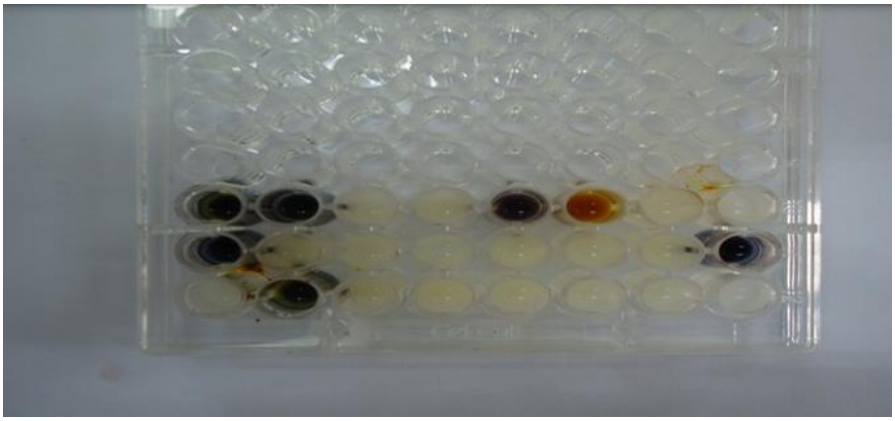
APPENDIX 3. Ready to made culture media which were used during this study

Medium	Manufacturer	Origin
Blood agar	Himedia	India
Brain heart infusion broth	Himedia	India
Mannitol Salt Agar	Himedia	India
Muller-Hinton agar	Himedia	India
MR-VP broth	Oxoid ltd	Ireland
Nutrient broth	Himedia	India
Nutrient agar	Himedia	India
RPMI 1640 medium	Gibco	USA
Trypticase soy broth	Biolife	Italy
Trypticase soy agar supplemented with 5% sheep blood	Biolife	Italy

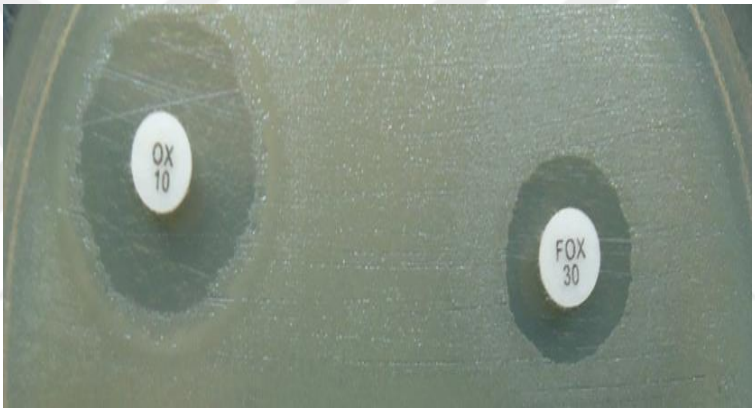
APPENDIX 4. List of antibiotic discs which were used during this study

Antibiotic class	Antibiotic subclass	Antibiotic Name	Symbol	Content	Company and Origin
Penicillin's	Penicillinase-stable penicillin's	Oxacillin	OX	1 µg	Bioanalyse (Turkey)
		Methicillin	ME	5 µg	Bioanalyse (Turkey)
β-lactams /β-lactamase inhibitor combinations		Amoxicillin-clavulanic acid	AMC	30 µg	Basingstoke (England)
Cephems (parenteral)	Cephalosporin III	Cefdinir	FED	30 µg	Bioanalyse (Turkey)
Quinolones	Fluoroquinolone	Ciprofloxacin	CIP	5 µg	Bioanalyse (Turkey)
		Levofloxacin	LEV	50 µg	Bioanalyse (Turkey)
		Moxifloxacin	MFX	50 µg	Bioanalyse (Turkey)
Folate pathway inhibitors		Trimethoprim	TMP	5 µg	Bioanalyse (Turkey)
Glycopeptides	Glycopeptide	Vancomycin	VA	30 µg	Bioanalyse (Turkey)
Aminoglycosides		Amikacin	AK	50 µg	Bioanalyse (Turkey)
		Gentamicin	CN	10 µg	Bioanalyse (Turkey)
Nitrofuran		Nitrofurantoin	F	5 µg	Bioanalyse (Turkey)
Tetracyclines		Doxycycline	DO	5 µg	Bioanalyse (Turkey)

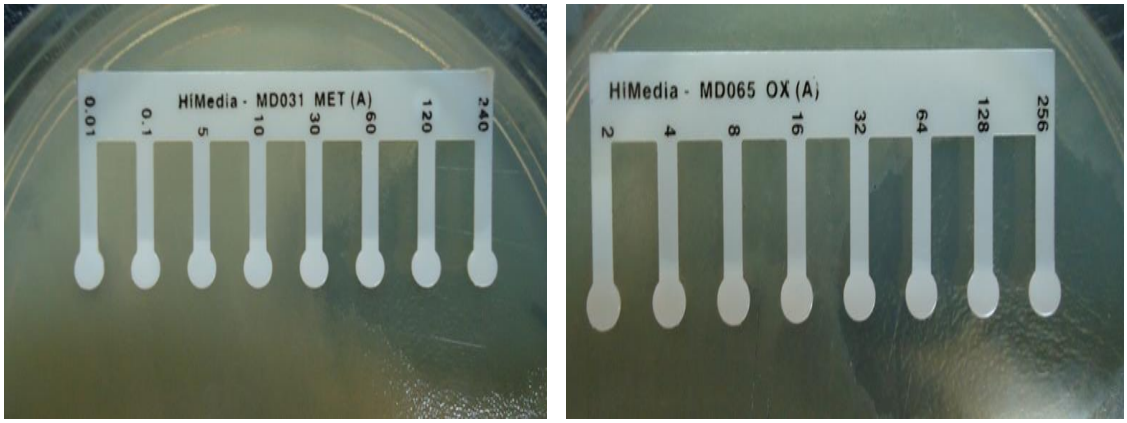
APPENDIX 5. Standard rapid iodine method



APPENDIX 6. The method of sensitivity test disks to investigate MRSA isolate

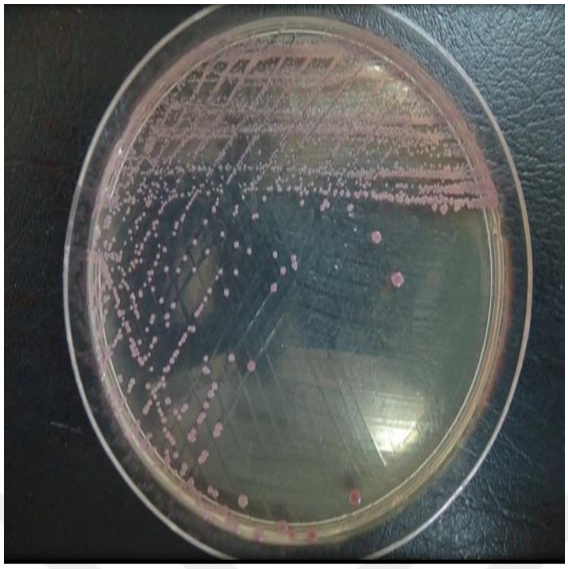


APPENDIX 7. The method of the minimum inhibitory concentration

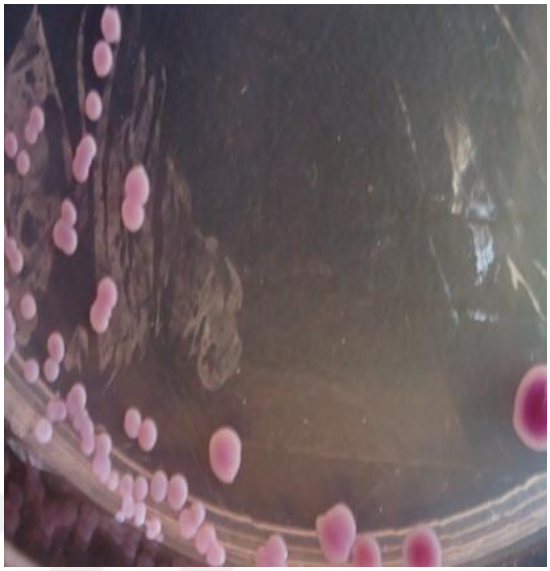


A/ Minimum inhibitory concentration HI-Comb MIC test for methicillin antibiotic
B/ Minimum inhibitory concentration HI-Comb MIC test for Oxacillin antibiotic

APPENDIX 8. MRSA isolates growing on CHROM agar MRSA



A



B

APPENDIX 9. Hemolysin producing *S.aureus* colonies on blood agar medium

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