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MOLECULAR DETECTION OF ADHERENCE AND BIOFILM FORMATION OF *Staphylococcus aureus* ISOLATED FROM BURN WOUND PATIENTS

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MOLECULAR DETECTION OF ADHERENCE AND BIOFILM FORMATION OF *Staphylococcus aureus* ISOLATED FROM BURN WOUND PATIENTS

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September 2021

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ABSTRACT

MOLECULAR DETECTION OF ADHERENCE AND BIOFILM FORMATION OF *Staphylococcus aureus* ISOLATED FROM BURN WOUND PATIENTS

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Master of Science in Biology

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In this study, fifty samples of burn wound infections were included. Specimens were collected from patients who were staying in hospitals with severe burn wounds during the period from October 2020 to February 2021, and specimens were taken from Al-Shaheed Ghazi Al-Hariri Hospital and the Specialized Burns Hospital in Iraq/Baghdad. Specific culture media, microscopic examination, and biochemical tests were used to identify the bacterial agents. The data was statistically analyzed by using the SPSS, version 23. The results showed 64 bacterial isolates, which were *S. aureus* 18 (28.0%), *S. epidermidis* 5 (8.0%), *Pseudomonas aeruginosa* 14 (22.0%), *Klebsiella pneumoniae* 15 (23.4%), *Escherichia coli* 6 (9.4%), *Acinetobacter baumannii* 3 (4.7%), *S. pseudintermedius* 1 (1.5%), *S. chromogenes* 1 (1.5%), and *S. haemolyticus* 1 (1.5%). From *S. aureus* isolates, while 2 (11.1%) strains showed moderate to producing biofilm and 7 (38.9%) strains showed weak biofilm formation, 9 (50.0%) showed nostrains, non-biofilm formation. PCR technology was used to detect ***ica***A, ***ica***B, ***ica***C, and ***ica***D genes, and the results of molecular examination of the prevalence of these genes were ***ica***A 11 (61.1%), ***ica***B 13 (72.2%), ***ica***C 9 (50.0%) and ***ica***D 9 (50.0%).

2021, 85pages

Keywords: Burn wound infections, *Staphylococcus aureus*, Polymerase chain reaction, ***ica*** genes

ÖZET

YANIK YARALI HASTALARDAN İZOLE EDİLEN *Staphylococcus aureus*'LARDA TUTUNMA VE BİYOFİLM OLUŞUMUNUN MOLEKÜLER TESPİTİ

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Bu çalışmaya elli yanık yara enfeksiyonu örneği dahil edildi. Ekim 2020-Şubat 2021 döneminde ciddi yanık yarası olan hastanelerde yatan hastalardan örnekler toplandı ve örnekler Al-Shaheed Ghazi Al-Hariri Hastanesi ve Irak/Bağdat'taki İhtisas Yanık Hastanesi'nden alındı. Bakteriyel ajanları tanımlamak için spesifik kültür ortamı, mikroskobik inceleme ve biyokimyasal testler kullanıldı. Veriler SPSS versiyon 23 kullanılarak istatistiksel olarak analiz edildi. Sonuçlar, *S. aureus* 18 (%28,0), *S. epidermidis* 5 (%8,0), *Pseudomonas aeruginosa* 14 (%22,0), *Klebsiella pneumoniae* 15 (%23,4), *Escherichia coli* 6 (%9,4), *Acinetobacter baumannii* 3 (%4,7), *S. pseudintermedius* 1 (%1,5), *S. chromogenes* 1 (%1,5) ve *S. haemolyticus* 1 (%1,5) olmak üzere 64 bakteri izolatı olduğunu gösterdi. *S. aureus* izolatlarında, 2 (%11,1) suş orta biyofilm oluşturma, 7 suş (%38,9) zayıf biyofilm oluşumu gösterirken 9 suş (%50,0) biyofilm oluşumu göstermemiştir. PCR teknolojisi ile ***ica***A, ***ica***B, ***ica***C ve ***ica***D genleri tespit edildi ve bu genlerin yaygınlığına ilişkin moleküler inceleme sonuçları ***ica***A 11 (%61,1), ***ica***B 13 (%72,2), ***ica***C 9 (%50,0) ve ***ica***D 9 (%50,0) olarak bulundu.

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Anahtar Kelimeler: Yanık yara enfeksiyonları, *Staphylococcus aureus*, Polimeraz zincir reaksiyonu, ***ica*** genleri

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LIST OF SYMBOLS

µg	Microgram
µL	Microliter
bp	Base pair
g	Gram
kb	Kilo Bite
mg	Milligram
mL	Milliliter
mm	Millimeter
N	Number
ng	Nanogram
rpm	Round per Minute
v/Amp	Volt/Ampere
β	Beta

LIST OF ABBREVIATIONS

ADH	Arginine DiHydrolase
API	Analytical Profile Index
AST	Antimicrobial Susceptibility Test
BHI	Brain Heart Infusion
CFU	Colony-Forming Unit
CLSI	Clinical and Laboratory Standard Institute
ddH_2O	Double Distilled Water
ELISA	Enzyme-Linked ImmunoSorbent Assay
GD	Genomic DNA
GIT	Gastrointestinal Tract
ica	IntercellularAdhesion
ID	Identity Document
IV	Intravenous
LPS	Lipopolysaccharide
MDR	Multiple Drug Resistance
MGEs	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *S. aureus*
MSSA	Methicillin-Sensitive *S. aureus*
NIT	Nitrates
OD	Optical Density
p value	Probability Value
PBP	Penicillin-Binding Protein
PBP-2a	Penicillin-Binding Protein 2a
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PVL	Panton-Valentine Leukocidin
SCC***mec***	Staphylococcal Cassette Chromosome ***mec***
SPSS	Statistical Package for the Social Sciences
TAE	Tris Acetate-EDTA
TCP	Tissue Culture Plate
Trim/Sulf	Trimethoprim/Sulfamethoxazole
URE	Urease
UTI	Urinary Tract Infection
VP	Voges Proskaue
VRSA	Vancomycin-Resistant *Staphylococcus aureus*
ZYM	Enzyme

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1. INTRODUCTION

2.1 Characteristics of *Staphylococcus aureus*

Itis a Gram-positive microorganism, and an imperative cause of community acquired infections and nosocomial infections. It caused several diseases like pneumonia, infections of soft tissue, blood stream infections, food poisoning, and urinary tract infection (UTI) (Havaei *et al*. 2012).

Inside hospitals, *S. aureus* could start infecting through surgical instruments, medical equipment... etc (Hogan *et al*. 2015, Tong *et al*. 2015).

S. aureus, colonizes asymptomatic people by approximately 30%, and could often be the source of illness. Specifically, *S. aureus* is the prominent source of many conditions, like bacteremia and a variety of skin and soft tissue diseases... etc (Tong *et al*. 2015).

Infections caused by *S. aureus* could spread from a burn wound that is infected through dealings with discharge and touching hands together, through direct and indirect transmission (Kuehnert *et al*. 2005). Also, *S. aureus* is an important source of medical implants for chronic biofilm infections, so the toxin inhibitor is part of the infection pathway (Kavanaugh and Horswill 2016).

Meta-analyses indicate, that bacteremia caused by methicillin-resistant *S. aureus* (MRSA) is more common from that caused by methicillin-sensitive *S. aureus* (MSSA) that is likely to be deadly, which leads to longer periods of stay in the hospital and increased utilization of hospital services (including medicines and personnel), this resulted in a threefold increase in care costs, while prevention of infection measures has helped reduce the occurrence of MRSA infections (Antonanzas *et al*. 2015).

S. aureus bacterium's capacity to form biofilms was recognized by its attachment to the surfaces and their incorporation into different extracellular polymeric substances, so that biofilm is one of the reasons Staphylococci are troublesome and commonly characterized as a group of cells embedded in an exo-polymer matrix which is attached to the surface. They are also known to be more resistant to treatment with antimicrobials and the defenses of the hosts (Flemming and Wingender 2010, Kiedrowski and Horswill 2011).

In addition to that, the intracellular adhesion locus is considered a group of final biofilm products produced by the *S. aureus* complex mechanism involving (Ellis *et al*. 2014).

1.2 Aim of the Study

The current study was designed to characterize the molecular characterization of adhesion and formation of biofilm of *S. aureus* isolates from burn wound patients by several criteria:

1. Isolation and identification of *S. aureus* bacterium, from burn wound patients.

2. Confirmation of these identifications by API and VITEK 2 compact systems.

3. Detection of antibiotic susceptibility profile and MIC of *S. aureus* isolates.

4. Conventional laboratory detection of adherence and biofilm formation.

5. Detection of molecular characterization of adherence and biofilm genes by conventional PCR.

The present study has medical importance through detection of virulence factors of *S. aureus* which valuable for bacterial treatments. Also, it has been classified as an applicable study for public health benefits.

2. LITERATURE REVIEW

2.1 Background of Burn and Wound Infections

Burn and wound infections are a general and important health problem all over the world (WHO 2010). Furthermore, burn wound infections are estimated to account for 1% of all cases arriving at the hospital for therapy and it is expected that 180,000 people die annually in the world from serious burn injuries (Jeschke *et al.* 2020, WHO 2020).

After the first phase of trauma, burn wound infections are a common complication, and are some important death cause individuals with burn. It is known which is greater than 70% of deaths occur in patients with burn wound infection because of the infection and that these patients remain at risk of death till the burn wound has fully healed (Rafla and Tredget 2011).

The first line of defense in case of microbial occupation is the skin, which becomes more susceptible to infection it has been burned. Burn wound infections are caused by a variety of microorganisms, like *Staphylococcus spp.*, *Enterococcus spp.*, *Pseudomonas spp.*, *Acinetobacter spp.*, fungi… etc (Norbury *et al.* 2016).

In burn centers, *S. aureus* is an important source of burn infections (Chen *et al.* 2012). MRSA's onset and spread in burn centers has a number of negative consequences, including prolonged hospitalization, bacteremia, or death (Issler-Fisher *et al.* 2015).

Study of the features of these infecting microorganisms and their antibiotic sensitivity is crucial for effective treatment of bacterial infections. So that, determining the antibiotic susceptibility of various strains allows for more targeted treatments and lower costs.

Treatment failure in burn patients is caused by a variety of causes, including antibiotic resistance and the creation of various virulence factors. As in the case of the *S. aureus*

strain, which produces Panton-Valentine Leukocidin (PVL) has an increased mortality rate compared to PVL-negative strains (Tong *et al.* 2015, Leistner *et al.* 2017).

2.2 Burn and Wound Epidemiology

Annually, about 500,000 Americans suffer from burn wounds that are needed for medical surgery. Some of them don't require hospitalization. However, about forty thousand of them are admitted, and 75% of them need specialized therapy in a specialized burn center (Gibran *et al.* 2013).

The first 24 hours of the burn, acute burn wound infections and their complications are still a cause of death. In the previous ten years, the majority of the main complications noted in patients' hospitalizations were pneumonia (3.5%), cellulitis (3.0%), and UTI (2.6%). Individuals who suffered a burn and had been on mechanical ventilation for 4 days or longer were more likely to develop pneumonia (Belenkiy *et al.* 2014).

2.3 Classification of Burn Wound Infections

It is based on depth. When a burn wound was diagnosed, the appearance, pressure on the skin, sensation, and pain, were required for evaluating depth (Toussaint and Singer 2014). Burns are usually dynamic actions, through that, some might develop within 2-4 days, and reach their peak within 72 hours (Evers *et al.* 2010).

2.4 Burn Classification

Burn wounds are classified according to the degree of tissue effects into three types (Kagan *et al.* 2013), which are:

1. 1st degree: it involves the outer skinonly. It looks pink to red, with no blisters on the skin, and completely dried. There is mild pain. Normally, it heals with no scarring in 5-10 days (Evers *et al.* 2010, Tolles 2018).

2. 2nd degree: it involves a dermis. When wet, it looks like red with blisters. The pains that are related to the superficial partial depth are acute. In most cases, healing takes 3 weeks with very few scars (Saranya *et al.* 2016).

3. 3rd degree: it involves the complete depth of the epidermis as well as subcutaneous tissues are included in the third degree. It ranges in color from white to black. There is little or no pain due to reducing sensation. Full-depth burn infections heal by shrinking and can take up to 8 weeks to recover. Skin grafting is required for full-depth burn infections (Saranya *et al.* 2016).

2.5 Microbiological Investigation of Burn Wound Infections

It is difficult to diagnose burn wound infections based just on clinical signs and symptoms. Samples of burn wound infections are taken either by a skin swab or a tissue biopsy for culture to detect if there are pathogens or not. To confirm microbial invasion, a quantitative culture of a tissue biopsy was performed on non-viable and non-burned tissues, which is the "gold standard" (Rennie *et al.* 2017).

2.6 Pathogenesis of Burn Wound Infections

2.6.1 Pathogenesis

Infectious complications in acute burned individuals, have been linked to thermal damage of the skin and concurrent suppression of immune responses (Chamania *et al.* 2012). With proteins, the surface of the burn wound is rich, which is an ideal environment for bacteria colonization and proliferation (Rahman *et al.* 2019).

The burn nature, area of the burn, and class and number of microorganisms that colonize the burn wound, have an effect on the risk of wound infections in coming times (Roberts *et al.* 2014).

Several Gram-positive bacteria (mainly *Staphylococcus*), aggressively colonize the wound within two days, if antibiotics are not applied (Aljghami *et al.* 2019).

During the following 5-7 days, burn wounds are colonized by different microorganisms, like Gram-positive and Gram-negative bacteria, and yeasts, which are obtained from GIT and the normal flora of the upper respiratory system of the host, or transferred by a medical crew (Wanis *et al.* 2016).

2.6.2 Virulence factors of bacteria

Virulence factors are molecules produced by bacterial cells that, through a variety of different mechanisms, promote infection and reproduction within the host. Certain virulence factors can be produced globally by a type of bacteria, while others can be acquired through mutation or acquired of mobile genetic elements (MGEs) (Gao *et al.* 2018), as described below:

A. Invasion of Host Cells and Immune System Evasion. Bacteria can move through cellular spaces to get through the epithelial surface of the host. Once inside the host cell, they could avoid phagocytosis, actively destroy phagosomes with toxins, prevent phagocytosis, or exit phagosomes and survive in the intracellular environment.

B. Biofilm formation. When sufficient numbers of bacteria are present in an environment that leads to changes in the production of virulence factors and the formation of biofilms, which allow bacteria to share nutrients and resist antibacterial agents (Deep *et al.* 2011).

2.6.3 Formation of biofilm

Biofilm is a surface-attached bacterial population that is the major bacterial complication of burn wound infection. It is surrounded by proteins, carbohydrates and/or DNA on the outside of the cells (Limoli *et al.* 2015).

Many pathogenic microorganisms make biofilms in response to many factors, like nutritional needs, or exposure to antibiotics or antiseptics (Lebeaux *et al.* 2014).

When the bacteria make a biofilm pattern, they go through a phenotypic change in their mode of action in which a huge number of genes are differentially regulated (Mataraciand Dosler 2012).

Practically, and in normal conditions, eighty percent of bacteria can produce biofilm. So, biological studies had shown that biofilm can form in many hard conditions (Rampelotto *et al.* 2013). However, studying the features of biofilm requires electron microscopy. This allows for high-resolution examination at a higher magnification than an optical microscope (Guggenbichler *et al.* 2011).

Biofilms are most commonly developed in hospitals, mostly on medical apparatuses such as catheters, ventilators, and other hospital tools. Furthermore, biofilms can also form on the teeth, middle ear, GIT, and respiratory tract (Gbejuade *et al.* 2015).

2.6.4 Multiple drug resistance of bacteria

It is the best way for a bacterium to fight antibacterial effects like preventing reproduction or bactericidal action. Through the extensive use of antibiotics, multiple drug resistance has developed over the years and problems have arisen with these resistant pathogenic microorganisms, such as the difficulty of treating infections (Shaikh *et al.* 2016). Various mechanisms of drug resistance include:

1. Adaptations in the cell wall, modification of the drug or enzyme, ribosome mutations, 16S rRNA methylation.

2. Higher expression of extended enzymes that could modify or degrade antibacterial and β-lactamase.

3. Drug specific effusion pump expressions.

2.6.5 Etiology of bacteria

Bacteria quickly invade unprotected wounds after a burn. Pathogens colonize the burn wound through the skin, gastrointestinal tract, and respiratory system flora. These germs can also be transmitted to a patient's wounds through contact with contaminated surfaces and health-care personnel's unclean hands (Mayhall *et al.* 2012).

Colonization of burn wound infection by fungi and yeasts, may subsequently occur due to the widespread antibiotics use. Therefore, many microorganisms had become more resistant to antibiotics (Guinea *et al.* 2014, Katz *et al.* 2014).

S. pyogenes, (classified as β-hemolytic Streptococci), and *Pseudomonas aeruginosa* were the most important pathogens of burn wound infections, and werethe main cause of death in many cases (Low 2013). While *S. aureus* became the main causative agent of these infections during the 1950s (Bahemia *et al.* 2015).

Infection rates had also increased in recent decades, due to more common microorganisms, such as fungi, yeasts, and some viruses. While infections of anaerobic bacteria are less common, they usually occur secondarily to electrical burn wounds or when open dressing are used instead of close dressings (Ortines *et al.* 2017).

2.7 Laboratory Diagnosis of Pathogenic Bacteria

Bacterial identifications by diagnostic microbiological labs are based on phenotypic features (Ramamurthy *et al.* 2014).

In medical microbiology, the investigator's experience is helpful in the early stages of identification. To make bacteria identification easier, it's important to keep in mind that their features can change. Furthermore, some features of species within the same genus may be different. *S. aureus* is a coagulase-positive bacterium, while *S. epidermidis* is a coagulase-negative bacterium (Becker *et al.* 2014).

2.7.1 Characteristics of the causative bacteria

All known properties of pathogenic microorganisms are considered while classifying them, but some differential and characteristic criteria are chosen and employed for identification (Vyzantiadis *et al.* 2012).

Essential investigations, like the study of the shapes of the cells, usually appear by bacterial Gram stain, growth in different conditions, different culture media (like blood agar, MacConkey agar... etc.), and biochemical tests (like catalase, coagulase, and oxidase). All of these make it viable to place pathogenic microorganisms, temporarily, in one of the major categories of medical interest (Smith and Hussey 2013).

2.7.2 Principles of bacterial identification

There are three major procedures for identifying bacteria. The first one depends on the investigator's experience in identifying the identity of an organism (Zhu *et al.* 2010).

Then a limited set of laboratory methods are used to confirm or refuse the theory. This is largely dependent on a consistent style of phenotypic characteristics. If the organism

cannot be identified using the first principle, the next step is to subject it to a battery of tests, such as those used in commercial identification systems (Tabssum *et al.* 2018).

Data was aggregated and compared with standard guides or used to create a description for a complete identification. The final procedure is a step-by-step procedure for determining final identification. The basic features of bacteria are determined by the main identification analysis, like Gram stain, and biochemical tests such as oxidase or catalase (Martiny *et al.* 2012).

This study's findings point to a secondary or even tertiary investigation to validate the problem's identity. This is a procedure that is systematic and does not rely on the investigator's knowledge or experience. Initial checks are among the system weaknesses. Wrong test setup and misinterpretation of results at this stage can lead the investigator down the wrong route, wasting time and money, and potentially leading to misidentification and inaccurate bacterial diagnosis (Ombelet *et al.* 2018, Barbe *et al.* 2017).

2.7.3 Microscopic appearance

Microscopic examinations and Gram staining show the shape, arrangement, and size of the bacteria as rods or cocos. After studying the forms of bacteria, Gram stain classifies bacteria into two types: Gram-positive bacteria and Gram-negative bacteria (Thakur and Guttikonda 2017).

2.7.4 Biochemical tests for identification of bacterial agents

Numerous biochemical tests might be used for the identification of microorganisms. Some of them, (like catalase, coagulase, glucose fermentation, and oxidase), are fast and not difficult to do, and they can also be used for primary classification (Capkin *et al.* 2015).

- **Catalasetest:** It is performed to determine whether or not catalase enzymes are present, by hydrolysis of H_2O_2 to produce O_2 and H_2O, as described in the chemical Equation (2.1).

$$H_2O_2 \rightarrow H_2O + O_2 \quad (2.1)$$

Many bacteria produce H_2O_2 as the oxidative final result of the aerobic decomposition of glucose (Mustafa 2014).

- **Oxidase test:** It is performed to know if the bacterial cell contains oxidase. This compound is usually only present in aerobic bacteria, which are able to use O_2 as the final hydrogen acceptor (El-Hadedy and Abu El-Nour 2012).

- **Coagulase test:** *S. aureus* is differentiated from other types of *Staphylococcus spp.* by this test. Furthermore, it is currently known that not all *S. aureus* are positive to coagulase test, and not all coagulase-positive Staphylococci are *S. aureus*. As a result, to better identification of *S. aureus*, other tests must be done in addition to a coagulase test (Kateete *et al.* 2010).

2.8 Gram-negative Bacteria

They are classified as the causative agents for burn wound infections such as, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and others.

2.8.1 *Pseudomonas aeruginosa*

2.8.1.1 General characteristics of *Pseudomonas aeruginosa*

It is an aerobic rod, a Gram-negative bacterium, of the family *Pseudomonadaceae* (Diggle *et al.* 2020). It is 0.5-1.5 μm wide, and 1.5-3.0 μm long (Ditmarsch *et al.* 2013).

It can grow at temperatures of 40-41 ºC and can't grow at a temperature of 4 ºC. Some groups belong to *Pseudomonas spp.* can grow at a temperature of 45 ºC (Striebich *et al.* 2014).

2.8.1.2 Pathogenecity of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa causes a lot of acute infections, in patients who have many clinical conditions. Itis one of the primary pathogenic bacteria which are responsible for the contamination of emergency clinics that occurs in the medical clinic, affecting the patient during his stay in the hospital.

Through a variety of virulence factors, such as LPS, exogenous sugars, flagella, cilia, toxins, and enzymes (proteases), It can often colonize sites of damaged tissues such as burns, surgical wounds, the respiratory tract (which causes pneumonia), and can cause Keratitis in the damaged eye (Alp *et al.* 2012, Alhazmi 2015).

2.8.2 *Klebsiella pneumoniae*

2.8.2.1 General characteristics of *Klebsiella pneumoniae*

It is a facultative anaerobic rod, a Gram-negative bacterium, of the family *Enterobacteriaceae*. It is non-motile and lactose fermentation (Hind *et al.* 2016).

2.8.2.2 Pathogenicity of *Klebsiella pneumoniae*

It is often associated with hospital-acquired pathogens that cause acute respiratory infections, such as pneumonia. These organisms also cause other infections, like diarrhea. The majority of infections in hospitals have high mortality if not properly treated. Lung infections, necrosis, soft mucous membranes, middle ear infections and pyogenic liver abscesses are also caused by *Klebsiella pneumoniae* (Ramos *et al.* 2014).

These pathogenic microorganisms are opportunistic pathogens that accompany people suffering from autoimmune disease and suffering from many infections of the urinary tract (Rajeshwari *et al.* 2010).

2.9 Gram-positive Bacteria (*Staphylococcus aureus)*

2.9.1 History of *Staphylococcus aureus*

The Scottish physician Sir Alexander Auguston, first demonstrated in 1880 that bacteria cause many purulent discharged diseases of the knee joint, he named it "*Staphylococcus*". The microbe's name comes from "staphyle", which is a Greek word for bunches of grapes, as well as "kokkos", which refers to a berry. In 1884, German physician Friedrich Julius Rosenbach was able to grow bacteria in pure culture. He succeeded in isolating colonies of the yellow microbe from the cysts and gave them the name *S. aureus*, "aureus" derived from the Latin word meaning gold (Licitra 2013).

Staphylococci are spherical microorganisms, generally arranged in a grape-like shape. They are commonly distributed in the environment and are often part of the normal human flora. There are about 39 species and 21 subspecies within the genus of *Staphylococcus*. The clinically important pathogens are *S. aureus*, *S. saprophyticus* and *S. epidermidis* (Murray *et al.* 2015).

2.9.2 General characteristics of *Staphylococcus aureus*

It is a Gram-positive bacterium that stains violet with Gram stain. It is cocci and the arrangement tends to be in groups described as a grape-like arrangement (Yusupov *et al.* 2013).

This organism could grow in up to 10% salt, and it is resistant to high osmolarity and detergents, as well as alcohol (Tsai *et al.* 2011). So, this pathogenic microorganism could grow in aerobic conditions or in anaerobic conditions (facultative bacteria), and

could grow at temperatures of 18-40 ºC. They are positive for the catalase test (all pathogenic Staphylococci), positive for the coagulase test (these bacteria can produce this enzyme as a defense mechanism), and therefore they are resistant to phagocytosis (Rasigade and Vandenesch 2014).

Staphylococcus can cause infections of the host like: bacteremia, lung inflammation, inflammation of both the small intestine and the colon, inflammation of bone or bone marrow, food poisoning, and infection of the skin. *S. aureus* can express several potential virulence factors, one of which is surface proteins that aid in the colonization of host tissues. There are several other types of virulence factors that inhibit phagocytosis, like capsules or protein A (Ramirez *et al.* 2020).

2.9.3 Epidemiology of *Staphylococcus aureus*

S. aureus (as well as MRSA), are present on human skin and mucous membranes, which are the major regions of this pathogenic microorganisms. It is approximate that about 15% of people carry *S. aureus* bacteria on their skin and mucous membranes (Liu *et al.* 2011).

S. aureus colonizes up to 80% of medical crew and people who use needles periodically, such as diabetics, patients receiving intravenous (IV) therapy, and immunocompromised patients. *S. aureus* can be transmitted from person to person by direct contact (Tong *et al.* 2015).

2.9.4 Pathogenecity of *Staphylococcus aureus*

The vast majority of frequent bacterial infections in people is *S. aureus*. It is considered a causative agent of multiple infections in humans, like bacteremia, inflammation of the endocardium (endocarditis), impetigo, inflammation of the hair follicles (folliculitis), boils, inflammation of subcutaneous connective tissue (cellulitis), inflammation of the mucous membrane of the mouth (stomatitis), inflammation of the bone or bone marrow

(osteomyelitis), inflammation of the joints (arthritis), lung infections (pneumonia)... etc (Tong *et al.* 2015).

Mechanisms for evading the host immune response include anti-phagocytic capsule production, sequestration of host antibodies or antigen masking by protein A, formation of biofilm, intracellular survival, and leukocyte chemotaxis prevention (Nanra *et al.* 2013).

Pneumonia, is associated with bacterial production of PVL, protein A, α-hemolysin, and infectionis more common after infection with influenza virus, as well as diagnosis of cystic fibrosis (Le and Otto 2015).

2.9.5 Molecular diagnosis of *Staphylococcus aureus*

The increase and development of antibiotic-resistant strains of these bacteria are serious complications for public health. Other *Staphylococcus* species may also be implicated in acute infections (Andersson *et al.* 2011).

Therefore, quick and accurate identification of the causative agent is a necessary condition for disease management as well as epidemiological surveillance. Current molecular methods depend on genomic and proteomic analysis, which is very useful compared to standard methods (Vogt *et al.* 2016).

To identify the isolated species of *S. aureus*, the most widely used molecular method is gene sequence amplification by polymerase chain reaction (PCR) (Egyir *et al.* 2014).

2.10 Antibiotic Resistance of *Staphylococcus aureus*

2.10.1 *Staphylococcus aureus* adaptation

S. aureus is a highly resistant bacterium that can remain alive on dried surfaces for a long time. It can withstand high levels of salt concentration, which is the main feature that distinguishes it from other types of bacteria in the selection of culture media (Chaibenjawong *et al.* 2010).

The dependence and increase of *S. aureus* pathogens is due to their ability to quickly acquire resistance and virulence from other organs of the genus *Staphylococcus* through horizontal transfer of MGEs (Abdelbary *et al.* 2017, Bitrus *et al.* 2017).

Furthermore, they encode a wide range of resistance genes, virulence genes, and immune evasion genes, facilitating the successful adaptation of MRSA and the emergence of new highly resistant pathogenic clones. These MGEs are lineage-specific in *S. aureus* and freely integrate, recombine, and move in and out of the genome via horizontal transfer (Lindsay 2014).

2.10.2 Development of antibacterial resistance in *Staphylococcus aureus*

It was first reported in the mid-1940s, when a strain of *S. aureus* developed resistance to penicillin by producing an enzyme called penicillinase, for which they got the name penicillin-resistant *S. aureus* associated with hospitals. Since then, penicillin-resistant *S. aureus* strains have been widely isolated in cases of bacteremia in the UK and USA, particularly in patients and healthcare workers (Aklilu *et al.* 2013, Abdelbary *et al.* 2017).

Subsequently, penicillin-resistant *S. aureus* has been identified among individuals in society. As a result, a scenario in which increased resistance to penicillin components was discovered from the late 1940s to the early 1960s, when a semi-synthetic analog of

penicillin called methicillin was introduced into medical centers as the drug of choice for treating infections of *S. aureus* (Jokinen *et al.* 2017).

Furthermore, the development of methicillin resistance in *S. aureus* was reported through a year of its introduction. This indicates that *S. aureus* provides a greater and more powerful copy for understanding the complexity of the adaptive progression of bacteria in the face of antibiotic (Hassoun *et al.* 2017).

These pathogens have demonstrated a new ability to respond rapidly to the challenges produced by new antibiotics by developing new antibacterial resistance mechanisms. Resistance developments in these pathogens occur through alteration of the drug target site, enzymatic inactivation of the antimicrobial agent, efflux pump, and sequestration of the antimicrobial agent. Other resistance mechanisms have evolved through the acquisition of resistance determinants, position selection, and spontaneous mutation (Bitrus *et al.* 2017).

MGEs such as SCCs, phages, integrins, integrative plasmids, transposons, and pathogenic islands make up more than 15% of *S. aureus*' genome. Bacterial resistance genes might be found in all of these genes (McCarthy and Lindsay 2012).

Plasmids ranging in size from 1 to 60 kb are found in most *S. aureus* strains, and they are known to carry a number of resistance genes which have the ability to be changed or adapted. Small plasmids carried resistance to Tetracycline, Chloramphenicol and Erythromycin, while large plasmids carried multiple drug resistance genes to aminoglycosides, β-lactams, and macrolides. In addition, large plasmids also combine with other MGEs (like transposons) to confer resistance to Spectinomycin, Trimethoprim, Erythromycin, β-lactams, and Vancomycin (Bitrus *et al.* 2017, Planet *et al.* 2017).

2.11 Methicillin-resistant *Staphylococcus aureus* (MRSA)

Antibiotic resistance in *S. aureus* was initially documented in the mid-1940s, when *S. aureus* isolates evolved resistance to penicillin by generating penicillinase (hydrolyzing enzyme) (Abdelbary *et al.* 2017).

MRSA is a major human pathogen, which is responsible for hospital and community-acquired infections (Aklilu *et al.* 2013). It is also, a source of skin and soft tissue infections (Lamy *et al.* 2012, Nowrouzian *et al.* 2013), and it is the second most common cause of bacteremia in humans (Purrello *et al.* 2012).

S. aureus acquired resistance to methicillin by horizontal transferring ***mec***A, which encodes a penicillin-binding protein (PBP) with little congruence to β-lactam antibiotics. The specific methicillin-resistant ***mec***A is located in the SCC***mec***, a 20-65 kb fragment, in *S. aureus* that mediates the horizontal transmission of methicillin resistance (Stojanov *et al.* 2013).

MRSA carries the ***mec***A gene on its cell chromosome, which is a component of the larger SCC***mec*** region, conferring resistance to multiple antibiotics depending on the type of SCC***mec***. The gene for microbe encodes penicillin-binding protein 2a (PBP-2a). PBP-2a is a PBP, or bacterial cell wall essential enzyme, that catalyzes the formation of peptidoglycan in the bacterial cell wall. PBP-2a has a lower affinity for binding to β-lactams (and other penicillin-derived antibiotics) when compared to other PBPs, so PBP-2a continues to stimulate bacterial cell wall synthesis even in the presence of several antibiotics. As a result, strains of *S. aureus* that make PBP-2a can grow in the presence of many antibiotics, and these strains are resistant to many antibiotics. MRSA strains tend to be resistant to Methicillin, Nafcillin, Oxacillin, and Cephalosporins (Rasigade and Vandenesch 2014).

3. MATERIALS AND METHODS

3.1 Subjects of the Study

The study involved fifty specimens of burn wound infections. Specimens were collected from patients who were staying in hospitals with severe burn wounds during the period from October 2020 to February 2021, and specimens were taken from Al-Shaheed Ghazi Al-Hariri Hospital and the Specialized Burns Hospital in Iraq/Baghdad. The standardized questionnaire involves a detailed history of patients, which includes sex, age, source of burn, duration of fever, and antibiotics given.

3.1.1 Ethical approval

Before including the patients in the report of this study, consent was obtained from every patient and from the hospital administration, according to Book No. 28997 issued by the Medical City Complex on 16/November/2020.

3.1.2 Sterilization

Reagents, solutions, and pigments were prepared dependent on the manufacturer's instructions which were used in this study. They were sterilized by using an autoclave at 121 ºC, under pressure of 15 pound/inch^2 for 15 minutes. As for the glass tools, they were sterilized in the electric oven at 180 ºC for 2 hours by using dry heat.

3.1.3 Laboratory preparation of culture media and maintenance media

3.1.3.1 Culture media preparation

The culture media used to isolate and identify bacteria were listed in (Table 3.1)

Table 3.1 Culture media

Media	Company	Origin
Agarose	Promega	USA
Blood agar	Himedia	India
Brain heart infusion	BHI	Italia
Brain heart infusion broth	Himedia	India
MacConkey agar	Himedia	India
Mannitol salt agar	Himedia	India
Mueller Hinton agar	Himedia	India
Chocolate agar	Himedia	India

Culture media were prepared under the guidance of the manufacturer, fixed in the container and the pH was set at 7.0. Then sterilized with an autoclave at 121 ºC 15 pound/inch^2 for 15 minutes, and then the petri dishes were incubated at 37 ºC for 24 hours, and stored in the refrigerator at 4 ºC until use.

3.1.3.1.1 Brain heart infusion broth (BHI broth)

It was used in microbiology for the artificial growth of *Streptococcus spp.*, *Staphylococcus spp.*, *Pneumococcus spp.*, *Meningococcus spp.*, etc. It is essentially a growth medium for fastidious microbes. It finds use in testing food and water safety, and antibiotic sensitivity testing (Stoll *et al.* 2008). The broth is prepared using an infusion of either bovine or porcine heart, in combination with several other essential nutrients. The nutrients include an amino-acid source such as gelatin or some other animal tissue. It contains salts, disodium phosphate, which is a buffer, and glucose, which acts as sugar source. Gelling agents like agar can also be used to build a strong medium. In clinical and industrial research, it is used for bacterial growth, yeasts, fungi, and more.

It is ready and dissolved 37 g of medium in 1000 mL of distilled water. Add 20% glycerol, about 1.5 mL of glycerol into a tube that contains 7.5 mL of medium, and then autoclave for sterilization. This is used for preserving isolates (Ali *et al.* 2019).

3.1.3.1.2 Blood agar

Blood agar was prepared by melting the weight of 21.25 g in 500 mL of distilled water, and sterilizedby autoclave. The mixture was cooled down to 45-50 ºC and 5% of sterilized human defibrinated blood was added, about 5 mL of blood per 100 mL of medium. After mixing well, it was poured into sterile petri dishes, cooled to 37 ºC and left to solidify at 25 ºC. To cultivate and isolate pathogenic microorganisms, and to discover their potential to produce different types of hemolysis (Niederstebruch *et al.* 2017).

3.1.3.1.3 MacConkey agar

It is useful for distinguishing between Gram-negative and Gram-positive bacteria. It was prepared by melting the weight of 51.5 g in 1000 mL of distilled water, then heat the mixture until the medium is completely dissolved. And then decontaminated by autoclave at 121 ºC, under pressure of 15 pound/inch^2 for 15 minutes and lowered to 55 ºC for cooling. After that, in petri dishes, they were poured, and put in an incubator at 37 ºC for 24 hours to ensure free contamination.

3.1.3.1.4 Mueller Hinton agar

Prepared by melting the weight of 38 g of the medium in 1000 mL of distilled water. For one minute, heat with frequent agitation until the medium is completely dissolved. Autoclave at 121 ºC for 15 minutes, then cool at room temperature. Pour cooled Mueller Hinton agar into sterile petri dishes on a flat, horizontal surface to give a uniform depth. Allow to cool at room temperature. Check for a final pH 7.3 ± 0.1 at 25 ºC. Store them at 2-8 ºC.

3.1.3.1.5 Mannitol salt agar

It was prepared by melting the weight of 111.02 g in 1000 mL of distilled water, boiling and sterilization at 121 ºC for 15 minutes in an autoclave. Cool it at the temperature of the room, reduced by 50 ºC. After that, in petri dishes were poured.

3.1.3.2 Maintenance media

Maintenance of the isolates was carried out on the basis of Manjana *et al.* (2016) and Al-Ugaili (2013), as follows:

3.1.3.2.1 Maintenance for a short period

Bacterial isolates were saved on the BHI agar for a few days. The plates were carefully covered in parafilm, and kept at 4 ºC (Tedeschi and De Paoli 2010).

3.1.3.2.2 Maintenance for a long period

At medium and low temperatures, bacterial isolates can be preserved for years without noticeably losing their viability. This was done by inoculating a single colony of bacterial culture into a sterile eppendorf tube which included 3 mL of sterile BHI with 20% glycerol (7.5 mL of the media was able to mix with 1.5 mL of glycerol and then sterilized by autoclave). These tubes are placed in an incubator prior to inoculation to verify that they are free of contamination. The tubes were carefully bound in parafilm and kept at -4 ºC (Kulkarni and Chitte 2015).

3.1.4 Preparation of reagents, solutions and stains

The reagents and chemicals which were used during this studywere listed below with their manufacturing companies (Table 3.2).

Table 3.2 Reagents and chemicals

Reagents and chemicals	Company	Origin
Absolute ethanol	ROMIL pure chemistry	UK
API Staph	Biomérieux	France
Crystal violet (0.1%)	RICCA	USA
Glacial acetic acid (GAA)	SCR	China
Glucose	Baniric	Spain
Glycerol	Sigma	USA
Gram stain	BDH	UK
Hydrogen peroxide (H_2O_2) 3%	BDH	UK
Methanol (99%)	Sharloo	Spain
Normal saline solution	S.D.I	Iraq
Oil immersion	Syrbio	Syria
Phosphate buffer saline (PBS)	HWA	UK

3.1.4.1 Catalase reagent

Hydrogen peroxide 3% was prepared from stock solution in a dark bottle and it was used for detecting the ability of bacteria to produce catalase enzyme.

3.1.4.2 Coagulase

Coagulase is an enzyme that clots the components of blood plasma in a mechanism that is similar to natural clotting. A coagulation test determines whether the bacteria produces this exogenous enzyme or not. Only important human disease-causing bacterium is *S. aureus*, which produces a blood clotting enzyme. Therefore, coagulase is an excellent index of *S. aureus* pathogenicity (Torres *et al.* 2010).

3.1.4.3 Oxidase reagents

Dissolving 1 g of N-N-N-N tetramethyl p-phenyl diaminedihydrochloride in 10 mL of distilled water. It was used directly after preparation or stored in a dark bottle. It was used for detecting the ability of isolates to produce oxidase enzyme.

3.1.4.4 Gram stain contents

The pigments consisting of the solution of crystal violet stain solution, 0.5% w/v, Gram's iodine solution, acetone-alcohol decolorizer, and safranin solution 0.5% w/v, were used in the pigmentation of bacteria to observe their microscopic properties and classify them as Gram-positive and Gram-negative bacteria (Chan *et al.* 2016).

3.1.4.5 API Staph system

The API Staph system was provided by (Biomérieux/France) and was used in this study to confirm the identification of *S. aureus* isolates.

3.1.4.5.1 Kit content

- API Staph strips.
- Incubation boxes.
- Ampoules of API Staph medium.
- Result sheets.

3.1.4.5.2 The composition of the API Staph (API Staph medium 6 mL)

- Yeast extract 0.5 g.
- Bactopeptone (bovine/porcine origin) 10 g.
- NaCl 5 g.

- Trace elements 10 mL.

- Demineralized water qsp 1000 mL (pH 7.0 - 7.4).

3.1.4.5.3 Reagents API Staph kit

- Voges-Proskauer VP1+VP2 for Acetyl-methyl-carbinol production.

- NIT1+NIT2 for the production of nitrates.

- Alkaline phosphatase ZYM A+ZYM B.

- Mineral oil.

- McFarland standard.

3.1.4.5.4 Material API Staph kit

- Pipettes.

- Ampoule rack.

- Ampoule protector.

3.1.4.5.5 Quantifluor dsDNA system

It contains sufficient reagents for 2,000 assays on a 200 µL scale. It includes:

- 25 mL of 20 X TE buffer (pH 7.5).

- 1 mL QuantiFluor® dsDNA dye.

- 100 µg Lambda DNA standard (100 µg/mL).

The protocol for quantitating dsDNA in a single tube using the Quantus™ fluorometer materials to be supplied by the user

- Nuclease-free water.

- Thin-walled 0.5 mL PCR tubes.

- Quantus™ Fluorometer.

3.1.4.6 Normal saline

NaCl sodium chloride (0.9 g) was disintegrated by 90 mL of distilled water, then the volume was filled by 100 mL of distilled water, and autoclaved at 121 ºC for 15 minutes (Kim *et al.* 2015).

3.1.4.7 Presto™ mini gDNA kit

- Gram **+** buffer

- GT buffer

- GB buffer

- W1 buffer

- Wash buffer (ethanol)

- Lysozyme
- Proteinase K (add ddH_2O)
- Elution buffer
- GD columns
- 2 mL collection tubes

3.2 Samples Collection

For diagnosis, according to the World Health Organization Standard Microbiological Diagnostic Criteria for *S. aureus* specimens were immediately transferred by transporting disposable cotton swabs to the microbiological laboratory.

The samples taken from the patients were based on the following information: gender, age, and antibiotics taken. Besides the above, some medical information from nurses observed about how to sterilize or administer treatment to these patients.

3.3 Isolation of *Staphylococcus aureus*

In vitro, under aseptic conditions, all swabs were placed on MacConkey agar with the purpose of ensuring that they were Gram-positive and also on blood agar to activate and detect the bacterial capacity to lysis red blood cells and on mannitol salt agar to identify genus of bacteria. These cultures were incubated for 24 hours at 37 ºC. Colonies were grown on blood agar, mannitol salt agar, and nutrient agar, and were examined by their shape, colour, size, and hemolytic activity.

3.4 Study Design

A cross-sectional study was designed that included 50 samples of burn wound infection, collected from patients of different ages of both sexes who were admitted (Figure 3.1).

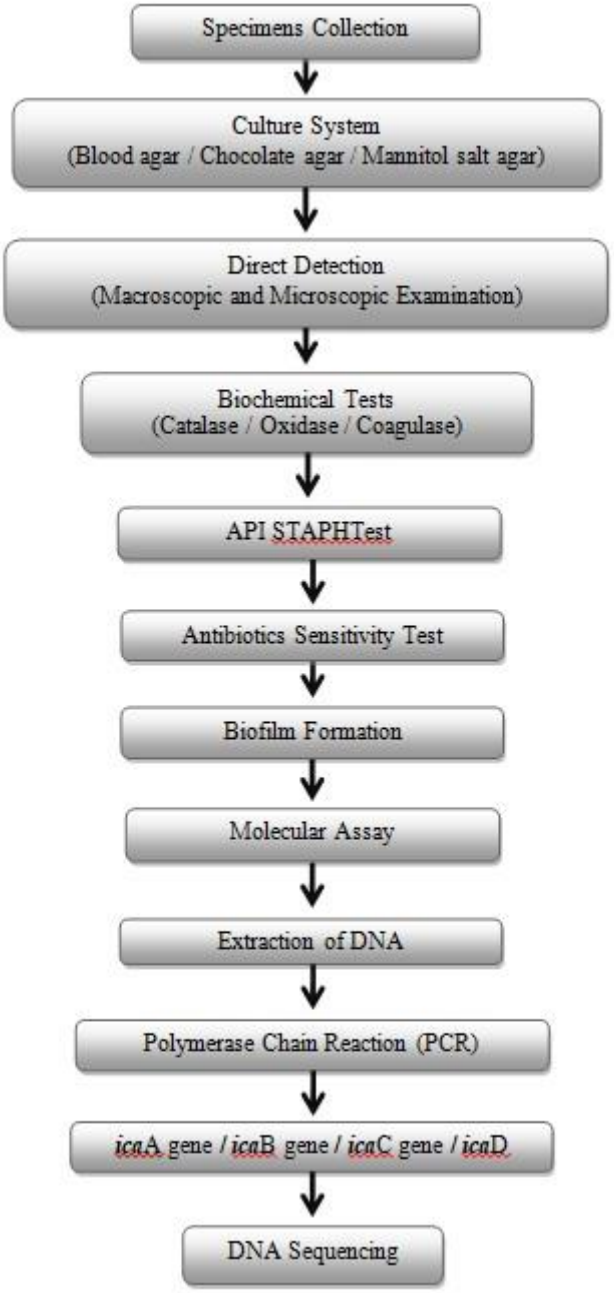

Figure 3.1 General design of the diagnosis of *S. aureus* bacteria

3.5 Identification of *Staphylococcus aureus*

S. aureus was identified according to morphological characteristics of bacterial culture and biochemical tests.

3.5.1 Morphological examination

3.5.1.1 Blood agar

The developing colonies of most isolates were identified by the type of hemolysis, agglutination, and iridescence appearance (Mahlen and Harrington 2011).

3.5.1.2 MacConkey agar

Growing colonies were identified according to their ability to ferment lactose. The positive result means fermentation of lactose by *S. aureus*.

3.5.1.3 Mannitol salt agar

The mannitol salt agar plates were streaked with pure bacterial colonies that were grown, and then incubated for 24 hours at 37 ºC. This medium is used to selectively isolate and incubate bacteria. The positive result is converting the cultivated medium to yellow (Brooks *et al.* 2010).

3.5.2 Microscopic examination

The bacterial isolation colonies were brushed with a burnt loop and located on a sterile glass slide. A droplet of saline was taken via the loop, and then the bacterial colony was coated on the glass slide. The glass slide was heated and the frost was allowed to dry. A droplet of crystal violet, the primary stain, was located on the smear. After one minute, the crystal violet was carefully washed off the glass slide. A drop of iodine was added,

and then, after one minute, the iodine was carefully washed off the slide. A droplet of 70% ethanol was located as the decolorizing agent and washed off after 15 seconds. Safranin, the counter stain, was added and after 60 seconds it washed off the glass slide. It was fully dried and it was then monitored under a microscope (Murray *et al*. 2015).

3.5.3 Biochemical tests

Several biochemical tests were done to facilitate the identification of *S. aureus* as follows:

3.4.3.1 Catalase test

A droplet of H_2O_2 (3%) was put on a slide. The bacterial species colony was put together with this reagent on the slide. The development of air bubbles showed positive results (Mustafa 2014).

3.5.3.2 Oxidase test

A filter paper had been treated with a slightly fresh 1% solution of "tetramethyl p-phenylene-diamine dihydrochloride", then picked up a single colony to smear on paper through a wooden stick. A positive result was displayed when a violet color appeared within five to ten seconds, but *S. aureus* was negative on this test (Cobos-Trigueros *et al.* 2017).

3.4.3.3 Coagulase test (glass slide method)

A normal saline solution (one drop) was located on a glass slide, and a loop full of suspected pathogen was emulsified on the saline drop. Then a drop of human undiluted plasma was located immediately adjacent to the drop of the bacterial suspension, and the two drops were thoroughly mixed. It has been tilted back and forth. The outcome was read within 10 seconds, the development of visible coarse clumping showed a

positive result but the negative result, or any slow reacting strain would be rediagnosed by the tube test (Malachowa *et al.* 2016).

3.4.3.4 Coagulase test (tube method)

One mL (normal 1/6 diluted salinesolution) of human plasma was inoculated in the test tube with a bacterial colony, and incubated for 24 hours at 37 ºC. The tubes were tested after one to four hours. A positive finding is coagulation. However, if negative, the tube will be incubated for 24 hours (Subhash 2012).

3.5.4 API Staph system

The API Staph system is used to confirm *S. aureus* isolates. This system consists of 20 diagnostic tests arranged in a strip containing several wells, each well bearing the name of a specific test (Shell *et al.* 2017).

3.5.5.1 Prepare the bacterial suspension

One pure colony, cultured on blood agar dishes for 18-24 hours, was transmitted to a test tube containing 5 mL of normal saline and infused well. The turbidity of the test organism was compared to the McFarland standard (0.5%).

3.5.5.2 Preparation of the strip

The inoculums container (tray as well as lid) was ready by supplying 5 mL of sterile distilled water to the tray wells that provide moisture for growth of bacteria. The strip was separated from its package and put in the tray. Then a sterile Pasteur pipette injected the strip with bacteria. Anaerobiosis was done by packing wells with mineral oil during the ADH and URE tests. Finally, tray was closed then incubated at 37 ºC for 18-24 hours.

3.5.5.3 Reading the results

The findings are read (10 minutes) after the addition of the following reagents:

1. A drop of VP1 and VP2 was added for the purpose of detecting the Voges-Proskaure test. The result is positive when the color is purple or pink.

2. A drop of NIT1 and NIT2 was added for the purpose of detecting a nitrate reduction test. The result is considered positive when a red color appears.

3. A drop of ZYM A and ZYM B was added for the purpose of detecting alkaline phosphatase test. The result is considered positive when the purple color appears. The result sheets are divided into 7 categories. Each one, consists of three numbered tests (1, 2, and 4). The results of each positive test were noted on the paper, whereas the negative tests were noted as zero. The sum of (1, 2, and 4) led to obtaining a code that consisted of 7 numbers which were interpreted by the index of the company to identify the genus as well as species of the bacteria tested.

3.6 Antimicrobial Susceptibility Test (AST)

3.6.1 Disc diffusion method (Kirby-Bauer method)

AST pattern for antibiotic resistance of *S. aureus*, was performed by using a disc spreading technique and Mueller Hinton agar as recommended in Clinical and Laboratory Standard Institute guidelines (CLSI 2019). 13 types of antibiotics were used by using Mueller Hinton agar medium, inoculated with 1.5×10^{8} cell/mL bacterial suspension (according to McFarland standard scale), On inoculated plates, antibiotic discs were put, then incubated for 24 hours at 37 ºC. The inhibition zone diameter was then calculated for each disc. The inhibition zones were measured and interpreted as sensitive, intermediate or resistant according to CLSI (2019) criteria (Balouiri *et al.*

2016, Chew *et al.* 2017). The *S. aureus* strain susceptibility test had been checked for antibiotic types (Table 3.3).

The antibiotic susceptibility test of *S. aureus* bacterium was tested against the following antibiotics as shown in (Table 3.3), by using Mueller Hinton agar according to CLSI (2019).

Table 3.3 Theantibioticdiscs and their potency

Antibiotic	**Symbol**	**Potency**	**Inhibition zone/diameter (mm)**			**Company/Origin**
			R	**I**	**S**	
Azithromycin	AZM	15 µg	≤ 13	14-17	≥ 18	Canda/Spain
Chloramphenicol	C	30 µg	≤ 12	13-17	≥ 18	Canda/Spain
Ciprofloxacin	CIP	5 µg	≤ 15	16-20	≥ 21	Canda/Spain
Clindamycin	CD	2 µg	≤ 14	15-20	≥ 21	Canda/Spain
Erythromycin	E	15 µg	≤ 13	14-22	≥ 23	Canda/Spain
Gentamycin	GM	10 µg	≤ 12	13-14	≥ 15	Canda/Spain
Levofloxacin	LVX	5 µg	≤ 15	16-18	≥ 19	Canda/Spain
Nitrofurantoin	F	300 µg	≤ 14	15-16	≥ 17	Canda/Spain
Penicillin	P	10 µg	≤ 28	-	≥ 29	Canda/Spain
Rifampicin	RD	5 µg	≤ 16	17-19	≥ 20	Canda/Spain
Tetracycline	TE	30 µg	≤ 14	15-18	≥ 19	Canda/Spain
Trim/Sulf	SXT	25 µg	≤ 10	11-15	≥ 16	Canda/Spain
Vancomycin	VA	30 µg	MIC	MIC	MIC	Canda/Spain

3.6.2 Vitek 2 compact system

The 18 representative *S. aureus* bacteria were confirmed by Vitek 2 compact system, which represents an advanced colorimetric technology to identify the bacteria and antibiotic susceptibility. The AST-GP card was used for AST of *S. aureus* isolates as this card reveals the minimum inhibitory concentration (MIC) of *S. aureus* isolates. (Figure 3.2) shows the Vitek 2 compact system.

Figure 3.2 Vitek 2 compact system

3.6.2.1 Procedure

The samples were achieved according to manufacture instructions as follows: A sterile disposable plastic loop was used to take pure colonies from culture media (blood agar media), and transfer a sufficient number of them to plastic (polystyrene) test tubes.

The age of colonies was 24 hours. Solutions containing 3 mL of 45% normal saline were placed in plastic (polystyrene) test tubes of the Vitek 2 compact system and inoculated with a loopfull of isolated colony. The test tube was inserted into a dens check machine for standardization of the colony to McFarland standard solution (1.5×10 cell/mL) by using a turbidity meter called the DensiChek$_{\mathbf{plus}}$. The McFarland turbidity range for bacteria is between 0.5-0.7.

The standardization inoculums were placed into the cassette of Gram-positive bacteria and a sample identification number entered into the software of the computer via barcode. Then, the cassette ID number was given. The cassette was installed in the stuffing unit. When the cards were loaded, the cassette was moved to the reader (incubator) unit. The temperature of incubation and optical reading of the cards are controlled by the device, which monitors and continuously transmits the test data to the computer system for analysis. The machine throws cards to the waste bin automatically.

3.6.2.2 Antimicrobial susceptibility test (AST)

Listed antimicrobials for normal reporting of *S. aureus* were included with the Vitek 2 compact system test card (AST-GP) for sensitivity testing, which are Cefoxitin Screen, Linezolid, Teicoplanin, Levofloxacin, Gentamycin, Erythromycin, Clindamycin, Nitrofurantoin, Vancomycin, Tetracycline, Tri/Sulf, Rifampicin, Benzylpenicillin, Oxacillin, Tobramycin, Moxifloxacin, Tigecycline, and Fusidic Acid. MIC Interpretive Standards or Breakpoint Values were set according to CLSI (2019).

3.7 Quantification of Biofilm Formation

Biofilm formation is a critical mechanism for bacteria's survival and persistence in the face of antibiotics and immune responses from the host. The biofilm of bacteria is in charge of the nonsuccess of a lot of medical apparatuses, therefore it is related to many contagious and non-contagious diseases (Donelliand Vuotto 2014).

Different approaches were applied to study bacterial biofilms. Semi-quantitative techniques like rolling on agar dishes or quantification of workable or whole cells after biofilm disturbance by sonication, swirling, wiping, washing, rinsing, scraping, or by using nitric oxide have been extensively described earlier. The differentiations between the different techniques have been well reviewed. Therefore, it is the gold standard procedure for biofilm detection (Portillo *et al.* 2013).

3.7.1 Micro-titer plate method

Briefly, a colony of *S. aureus* was isolated from a fresh blood agar and inoculated into 5 mL of brain heart infusion broth-glycerol medium. The broth was incubated overnight at 37 ºC. Log phase cultures of 0.5% McFarland were diluted 1:100 in freshly prepared BHI broth. A sterile multi-wall plate with 96 flat-bottom polystyrene wells was filled with 0.2 mL of diluted culture. Control organisms were also processed in the same method. The plate was incubated at 37 ºC for 24 hours. The substances were kindly

extracted from every well after incubation by tapping. To take out the planktonic bacteria, wells were washed by 0.2 mL of PBS (pH 7.2) three times. Biofilms composed of bacteria adhered to the wells were fixed with 99% methanol, and stained with 0.1% crystal violet, using 0.2 mL for each well. To take out the excess stains, wells were washed with 0.2 mL of PBS (pH 7.2) three times, then the plate was kept to dry. The spot adhered to the wells was dissolved by 0.2 mL for 10 minutes of 33% GAA.

The optical density (OD) of the stained adhesive biofilm was measured by using a micro-ELISA auto-reader (Human) at 630 nm wavelength (Figure 3.3). The test was conducted in triplicate. Biofilm production interpretation was done according to the described criteria, and bacteria were classified as non-biofilm producers, weak producers, moderate producers or strong producers of biofilm, and the average reading was $0.1 \geq 0.2 \leq 0.4$ (Guiton *et al.* 2012, Cole *et al.* 2014).

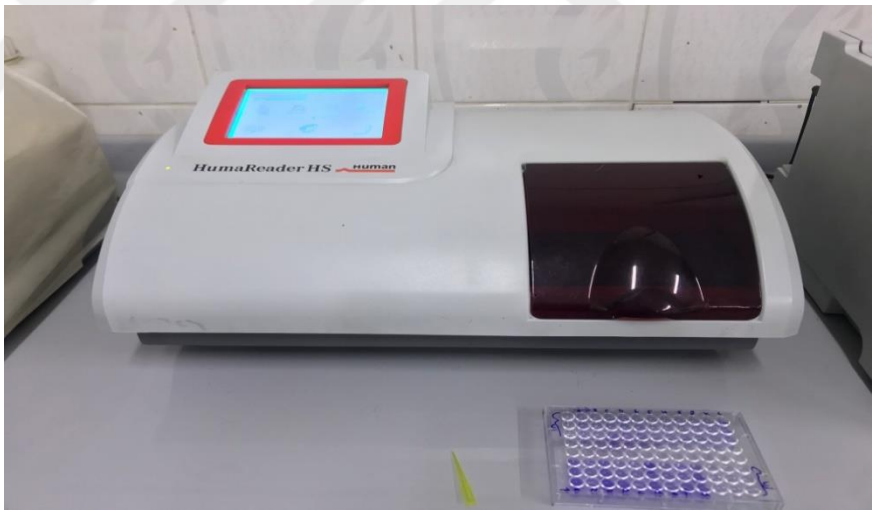

Figure 3.3 Micro-ELISA auto-reader (Human)

3.8 Molecular Assay

3.8.1 Bacterial DNA extraction and purification

Genomic DNA (GD) was isolated from bacterial growth according to the (Geneaid/Taiwan) protocol. Extraction was done in the following steps:

3.8.1.1 Suspension and protein digestion

For pellet cells, 1 mL was centrifuged from the overnight culture at 12,000 rpm for 3 minutes. The supernatant was thrown away. For suspension, 180 μL of GT buffer was added to the pellet and mixed by the vortex. For protein digestion, 20 μL of proteinase K was added to the suspension. Incubate the mixture at 60 ºC for 10 minutes at least.

3.8.1.2 Cell lysis

For cell lysis, 200 μL of GB buffer was added to the sample and mixed by vortex for 10 seconds. At 70 °C for at least 10 minutes, the mixture was incubated to ensure that the lysate sample was clear. After incubation, samples were spanned in a centrifuge for 10 seconds to take out the bubbles.

3.8.1.3 Binding

For DNA binding, absolute ethanol 200 μL was added to the sample lysate and mixed immediately by shaking vigorously. Next, the mixture (including any insoluble precipitate) was transferred to the GD column and centrifuged for one minute at 12,000 rpm. The GD collector tube that contains the flow-through was removed, and the GD column in the new GD collection tube was inserted.

3.8.1.4 Washing

For washing, 400 µL of W1 Buffer was added to the GD column and centrifuged at 12,000 rpm for 30 seconds, then the flow-through was discarded. The column of GD was placed back into the 2 mL collection tube, and 600 µL of wash buffer (be sure to add ethanol) was added to the column of GD. Centrifugation at 12,000 rpm for 3 minutes was performed, then the flow-through was discarded, and the GD column was placed back in the 2 mL collection tube. The empty column matrix was centrifuged at 12,000 rpm for 3 minutes to dry the column matrix.

3.8.1.5 Elution

The dried GD column was transferred into a clean 1.5 mL microcentrifuge tube. An aliquot of 100 µL pre-heated elution buffer 1 was added to the center of the column matrix. Waiting at least 3 minutes to allow elution buffer 1 to be completely absorbed. Centrifugation at 9,000 rpm for 3 minutes was performed to elute the purified DNA.

3.8.2 Quantitation of DNA

To detect the concentration of extracted DNA, a Quantus Fluorometer was used, as a result, to find out the quality of samples for downstream applications. For 1 µL of DNA, 199 µL of dilute Quantifluor dye was mixed. After 5 minutes of incubation, at the temperature of the room, DNA concentration values were detected.

3.8.3 Primers

The used primers in this study, had been provided by Macrogen/Korea, for PCR amplification of ***ica***A, ***ica***B, ***ica***C and***ica***D genes (Table 3.4).

Table 3.4 Primers for molecular study

Primer name	Sequences (5'-3')	Amplicon size (bp)	Reference
icaA-F	5'-GAGGTAAAGCCAACGCACTC-3'	151	Atshan *et al.* 2013
icaA-R	5'-CCTGTAACCGCACCAAGTTT-3'		
icaB-F	5'-ATACCGGCGACTGGGTTTAT-3'	140	Atshan *et al.* 2013
icaB-R	5'-TTGCAAATCGTGGGTATGTGT-3'		
icaC-F	5'-CTTGGGTATTTGCACGCATT-3'	209	Atshan *et al.* 2013
icaC-R	5'-GCAATATCATGCCGACACCT-3'		
icaD-F	5'-ACCCAACGCTAAAATCATCG-3'	211	Atshan *et al.* 2013
icaD-R	5'-GCGAAAATGCCCATAGTTTC-3'		

3.8.3.1 Optimization of primers

To check the optimum annealing temperature of the primer, the DNA template was amplified with the same primer pair, forward and reverse. When annealing at 58 ºC. PCR amplifications were done at volumes of 20 µl containing 10 µl Go Taq. Green Master Mix (2X); 1 µl per primer (10 pmol); 6 µl of nuclease-free water and 2 µl of DNA template (Table 3.5). PCR cycling was performed with PCR Express (Thermal Cycler by Thermo Fisher Scientific, USA), with the following temperature program: denatured at 94 ºC for 4 minutes followed by 30 cycles of denaturation at 94 ºC for 30 seconds; annealing at 58 ºC for 30 seconds; and extension at 72 ºC for 30 seconds. A final extension incubation of 7 minutes at 72 ºC was included, followed by 10 minutes. Incubation at 4 ºC was used to stop the reactions.

3.8.3.2 Reaction setup and thermal cycling protocol

PCR component and calculations were given in (Table 3.5).

Table 3.5 PCR component calculation

No. of reaction	18	rxn	**Annealing temperature of primers**		58	
Reaction volume/run	20	µl	**Number of primers**		4	
Safety margin	5	%	**No. of PCR cycles**		30	
Master mix components	**Stock**	**Unit**	**Final**	**Unit**	**Volume**	
					1 sample	**18.05 samples**
Master mix	2	X	1	X	10	180.5
Forward primer	10	µM	1	µM	1	18.1
Reverse primer	10	µM	1	µM	1	18.1
Nuclease free water					5	90.3
DNA	ng/µl		ng/µl		3	
Total volume					20	
Aliquot per single rxn	17 µl of Master mix per tube and add 3 µl of Template					

3.8.3.3 Agarose gel electrophoresis

To confirm the presence of the amplification, agarose gel electrophoresis was used. That is done after PCR amplification. PCR was completely dependent on the parameters of the extracted DNA.

3.8.3.3.1 Solutions

1X TAE buffer, loading dye, DNA ladder marker, Ethidium bromide 10 mg/mL.

3.8.3.3.2 Preparation of agarose

1. 1X TAE (100 mL) was taken in a flask.

2. 1.5 g (for 1.5%) agarose was added to the buffer.

3. It was heated to boiling by microwave, until all the gel particles were dissolved.

4. 1 µl of Ethidium bromide (10 mg/mL) was added to the agarose.

5. The agarose was stirred in order to mix and prevent bubbles.

6. At 50-60 ºC, the solution is left to cool.

3.8.3.3.3 The casting of the horizontal agarose gel

The agarose solution was poured into the gel tray after both edges were sealed with cellophane strips. It was left to harden for 30 minutes at room temperature. The comb had been removed gently, and the gel was put in the jelly tray. The tray had been filled with 1X TAE electrophoresis buffer till the buffer arrived to a thickness of 3-5 mm on the surface of the gel.

3.8.3.3.4 DNA loading

The products of PCR were directly loaded. To the product of PCR, 5 µL was loaded directly into the well. Electrical power was turned on at 100 v/mAmp for 60 minutes. DNA moves from the cathode to the plus anode poles. Ethidium bromide-stained bands in the gel were visualized using a gel imaging system. DNA concentration ranges from 12-20 ng/µL.

3.9 Statistical Analysis

Statistical analysis was done by using the Statistical Package for the Social Sciences (SPSS) version 23. Categorical variables were presented as frequencies and percentages.

4. Results

4.1 Isolation and Identification of *Staphylococcus aureus*

4.1.1 Bacterial isolation

The results of bacterial culture showed that out of all 50 burn (14 cases) and wound (36 cases) swabs, there were 64 isolates of different types of pathogenic bacteria (Table 4.1). Only 18 (36%) of specimens detected the growth of *S. aureus* bacteria. These results may be due to many reasons, like the patient's use of medical hygiene practices or the administration of antibiotics (Table 4.2).

Table 4.1 Distribution of specimens according to their source (N=50)

Source		**Burn**	**Wound**	**Total**
Gender	Male	10 (20%)	22 (44%)	32 (64%)
	Female	4 (8%)	14 (28%)	18 (36%)
Age group (years)	1-15	6 (12%)	8 (16%)	14 (28%)
	16-35	7 (14%)	14 (28%)	21 (42%)
	36-50	2 (4%)	13 (26%)	15 (30%)

Table 4.2 Distribution of bacterial isolates

Source	**No. of specimens**		
	No. of *S. aureus*	**Other bacterial species**	**Total**
Burn	8 (16%)	6 (12%)	14 (28%)
Wound	10 (20%)	26 (52%)	36 (72%)
Total	18 (36%)	32 (64%)	50 (100%)

4.1.2 Bacterial identification

The isolates were recognized by a combination of macroscopic and microscopic appearance, biochemical assays, and confirmatory assays. 26 (41%) of isolates were Gram-positive bacteria and 38 (59%) were Gram-negative bacteria (Table 4.3).

Table 4.3 Genus distribution of bacterial isolates

Gram stain	Type of bacteria	Burn isolates	Wound isolates	Total
Gram-posative bacteria (N=26)	*S. aureus*	8 (44.4%)	10 (55.6%)	18 (28.0%)
	S. epidermidis	1 (20.0%)	4 (80.0%)	5 (8.0%)
	S. pseudintermedius	0 (0%)	1 (100%)	1 (1.5%)
	S. chromogenes	0 (0%)	1 (100%)	1 (1.5%)
	S. haemolyticus	0 (0%)	1 (100%)	1 (1.5%)
Gram-negative bacteria (N=38)	*Pseudomonas aeruginosa*	8 (57.0%)	6 (42.9%)	14 (22.0%)
	Klebsiella pneumoniae	7 (46.7%)	8 (53.3%)	15 (23.4%)
	E. coli	4 (66.7%)	2 (33.3%)	6 (9.4%)
	Acinetobacter baumannii	2 (66.7%)	1 (33.3%)	3 (4.7%)
Total		33 (51.5%)	31 (48.5%)	64 (100%)

4.2 Phenotypic Diagnosis

4.2.1 Growth on mannitol salt agar

The results of all 18 (36%) positive for mannitol salt agar of burn wound patients were diagnosed as *S. aureus*, because they changed the media from red to yellow, and the high percent of salt enhances Staphylococci to evolve carotenoid pigments and can change the medium of the colony to yellow due to mannitol fermentation, while other white colonies were non-pathogenic. Staphylococci do not have fermentation of mannitol (Figure 4.1).

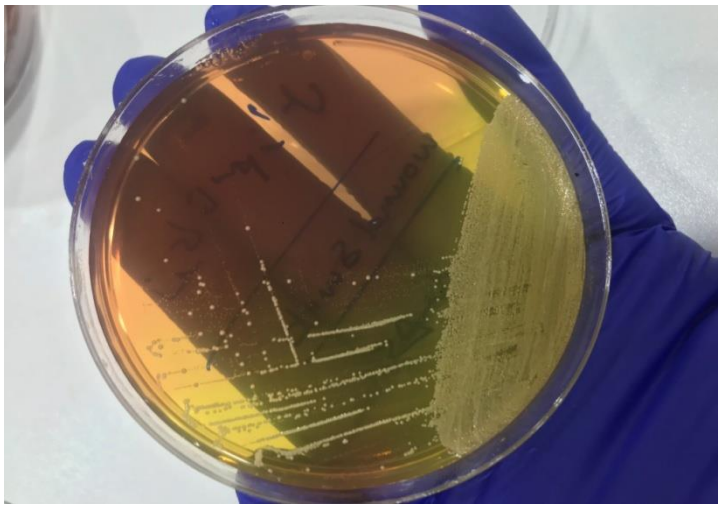

Figure 4.1 *S. aureus* colonies on mannitol salt agar under 37 ºC for 24 hours

4.2.2 Growth on blood agar

In this research, the colonies of *S. aureus* appeared on blood agar in various shapes; white to cream or golden colonies, convex surface, smooth, developing, and large colonies after 24 hours of incubation at 37 ºC. Often the colonies were small and fine. Most of the isolates were surrounded by areas of clear β-hemolysis while the other isolates were not (Figure 4.2).

Figure 4.2 Typical *S. aureus* colonies on blood agar at 37 ºC for 24 hours

4.2.3 Growth on chocolate agar

In this research, *S. aureus* colonies appeared on chocolate agar in various shapes. The colonies were large, round and slightly or pale yellow in colour after 24 hours of incubation at 37 ºC (Figure 4.3).

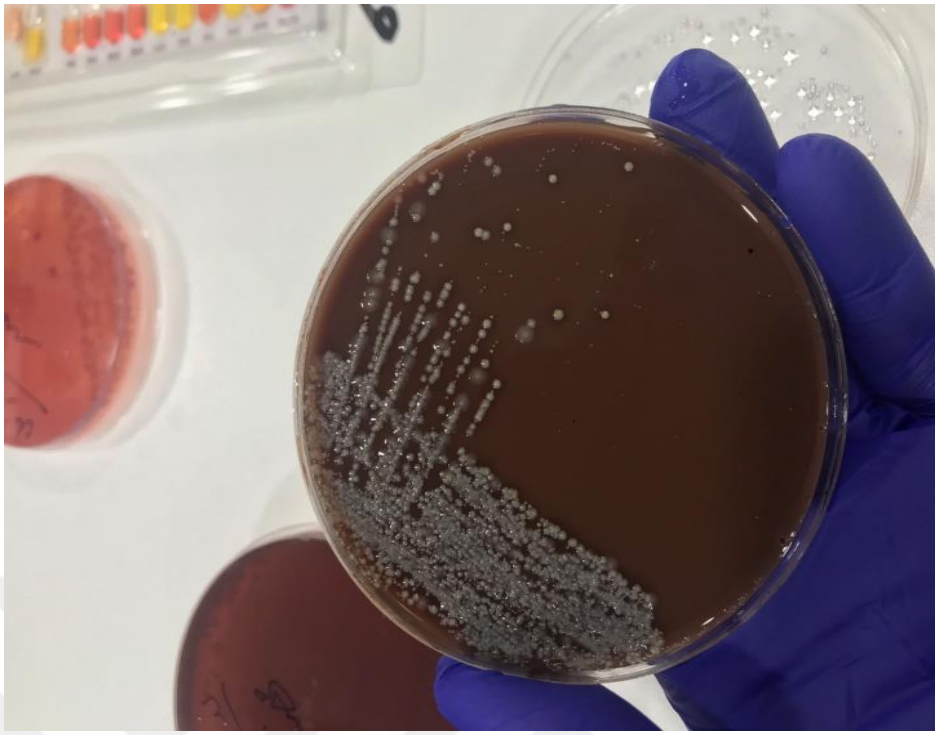

Figure 4.3 Typical *S. aureus* colonies on chocolate agar at 37 ºC for 24 hours

4.3 Microscopic Examination

Total of patients' isolates (burn wound) 18 (36%) isolates by Gram stain (grape-like clusters) gave positive results. Pure cultures of the suspected colony of *S. aureus* with Gram stain were shown as Gram-positive bacteria (purple) arranged in single cells, in pairs, or forming irregular cocci groups. These clusters occur because bacterial cells divide into three levels by binary fission, and the two daughter cells do not separate completely and remain attached to each other.

4.4 Biochemical Identification

4.4.1 Catalase test

The results showed that out of 50 clinical isolates from a patient with burns, only 26 (52%) isolates were catalase-positive, while 24 (48%) isolates were catalase-negative.

A positive result appears when the oxygen (O_2) gas forms in the bubbles, showing that the bacteria contain catalase enzyme (Figure 4.4). Negative results mean no formation of the gas.

Figure 4.4 *S. aureus* detection by catalase test (slide test method)

4.4.2 Coagulase test tube

The results recommended that out of 50 clinical isolates from a burn wound patient, only 18 (36%) isolates were coagulase-positive, while 32 (64%) isolates were coagulase-negative (Figure 4.5).

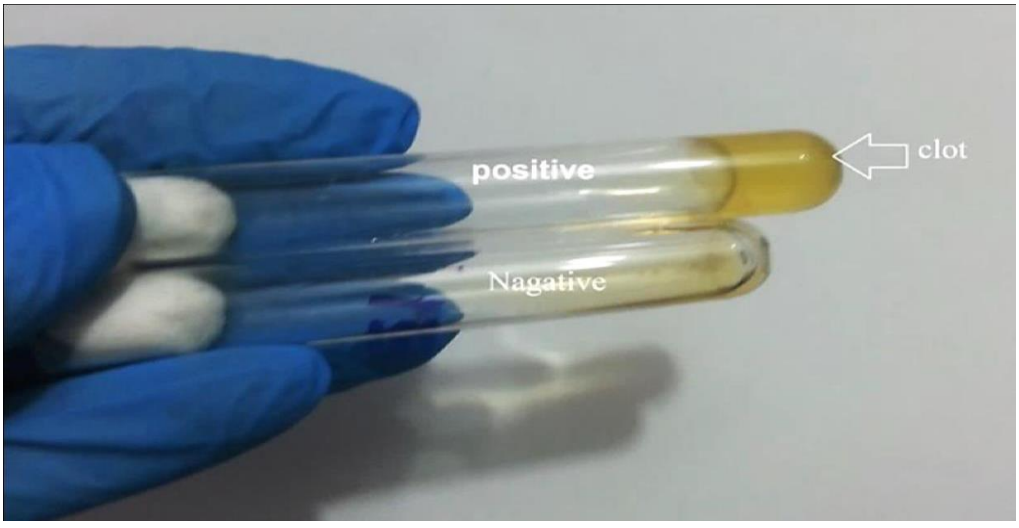

Figure 4.5 The tube coagulase test

4.5 Diagnosis by API Staph System

The analytical profile index (API) was used to support the detection of previously identified *S. aureus* isolates by classical biochemical assays. All 18 (36%) results of the API Staph system were in agreement with those obtained from conventional bacterial biochemical identification (Figure 4.6).

Figure 4.6 Detection of *S. aureus* by the API Staph system

4.6 Antibiotic Susceptibility

4.6.1 Antibiotic resistant

S. aureus was 90% resistant to Penicillin G in burn isolates (Figure 4.7). Resistance to Penicillin G and other β-lactam antibiotics may be due to β-lactamase enzymes that break the lactam ring and inhibit the antibiotic's activity.

P	CD	E	AZM	CIP	TE	C	SXT	RD	LVX	F	GM	VA
90	73.3	56.7	50	23.3	23.3	14.3	14.3	13.7	13.3	13.3	10	3.3

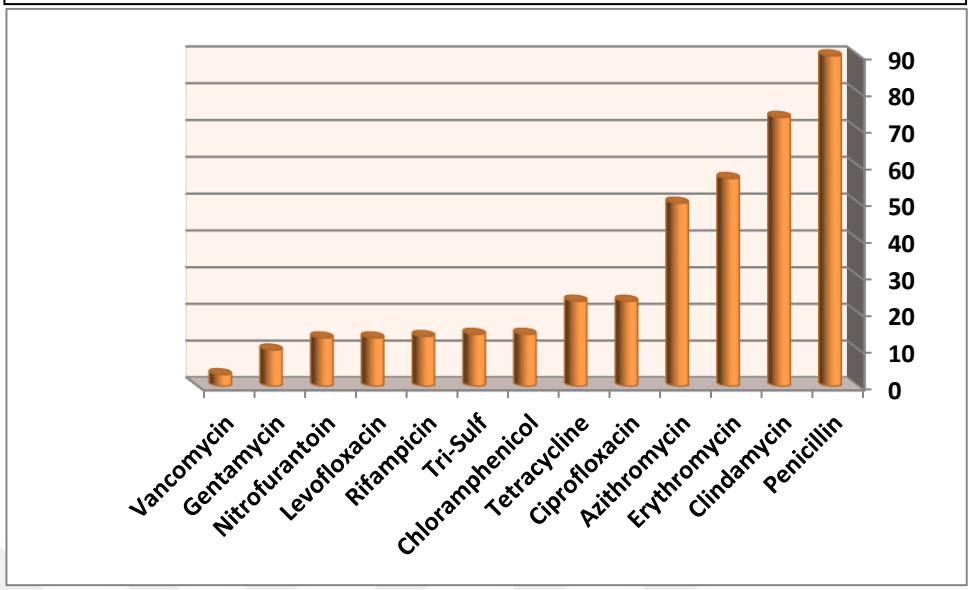

Figure 4.7 Percentages (%) of antibiotic resistance of *S. aureus* isolates

The results showed that the percentage of Vancomycin resistance of burn wound isolates was 3.3% (Figure 4.7).

Some results of studies found that clinical isolates of *S. aureus* seem to have high resistance to Vancomycin by 44%, and confirm that the frequency of VRSA occurrence in sputum samples was the highest among other samples. This supports the idea that the level of Vancomycin resistance in people with respiratory diseases has increased significantly compared to other patients.

The results indicated that resistance of *S. aureus* to Ciprofloxacin was 23.3% in burn wound isolates (Figure 4.7).

The present study indicated a 50% resistance to Azithromycin in burn wound patients (Figure 4.7).

Clindamycin is useful for treating skin infections as well as soft tissue infections. In the current study, Clindamycin resistance was 73.3% for isolates of burn wounds as shown in (Figure 4.7).

In the presented study, Nitrofurantoin showed 13.3% resistance to burn wound infections. But Levofloxacin showed the same resistance rate of 13.3% (Figure 4.7).

The results, which were listed in (Figure 4.7), showed a significant increase in Erythromycin resistance up to 56.7% for burn wound isolates and the observed resistance in isolates of patients to Erythromycin could be explained by the fact that Erythromycin was used as a potential therapeutic option for diseases caused by *S. aureus* in patients with hypersensitivity to Penicillin.

In the presented study, Gentamycin showed 10% resistance to burn wound infection (Figure 4.7).

The results presented in (Figure 4.7) showed resistance to Tetracycline by 23.3%. Consequently, all 18 *S. aureus* isolates had low resistance to Chloramphenicol 14.3%, Rifampicin 13.7% and Tri-Sulfa 14.3% (Figure 4.7).

4.6.2 Antibiotic sensitivity

The current study shown in (Figure 4.8), recorded the highest percentage of sensitivity to Vancomycin at 96% and Gentamycin at 90%, which were the most effective in burn wound isolates. Therefore, it can be used as a treatment for burn wound infections (Figure 4.9).

P	CD	E	AZM	CIP	TE	SXT	C	RD	F	LVX	GM	VA
10	26.7	43.3	60	76.7	76.7	85.7	85.7	86.3	86.7	86.7	90	96.7

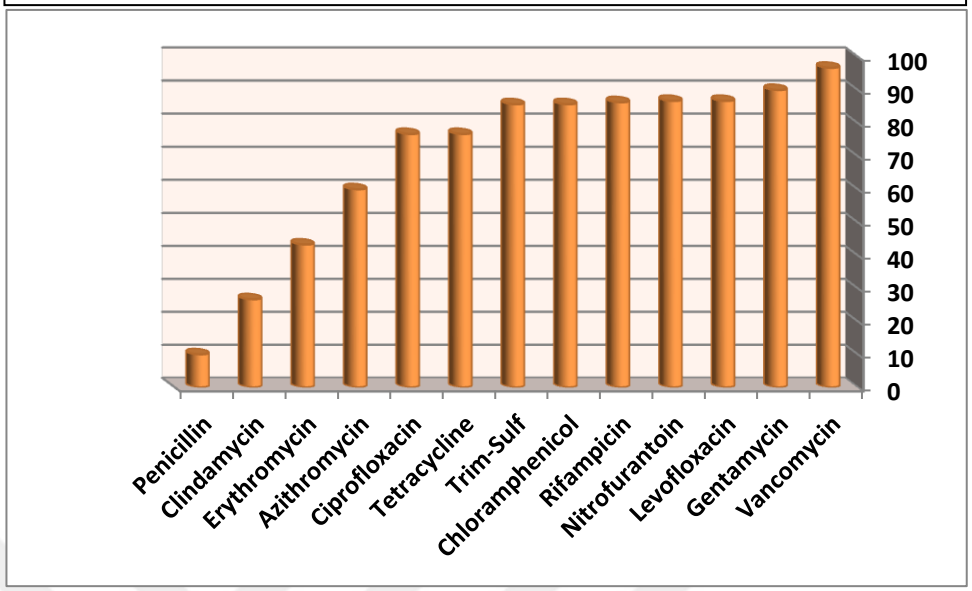

Figure 4.8 Percentages (%) of antibiotic sensitivity of *S. aureus* isolates

The emergence of some penicillin-sensitive isolates associated with burn wound patients was shown in (Figure 4.10). It might be due to the difference in geographical location, clinical sample locations, genetic origin, as well as the assembly of the isolated strains.

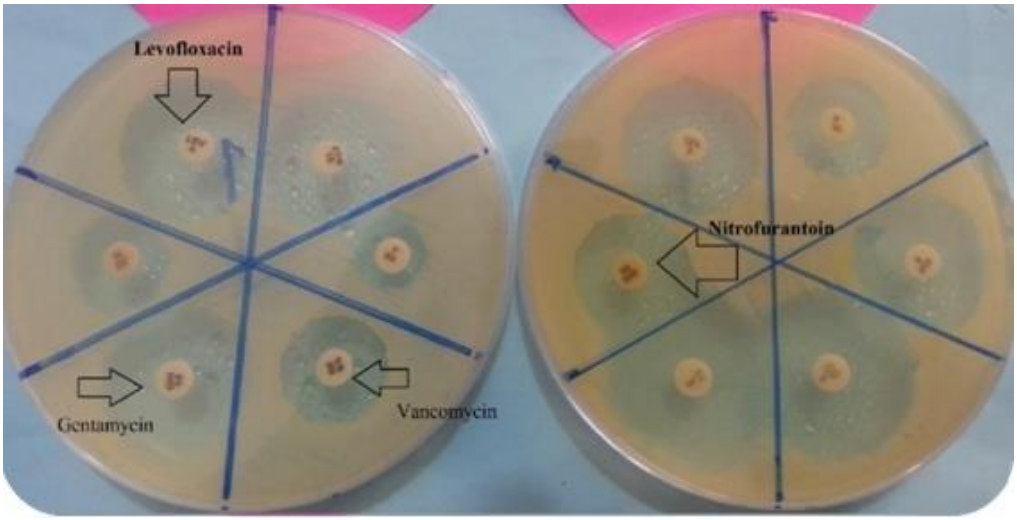

Figure 4.9 Most effective antibiotic for inhibition of clinical isolates

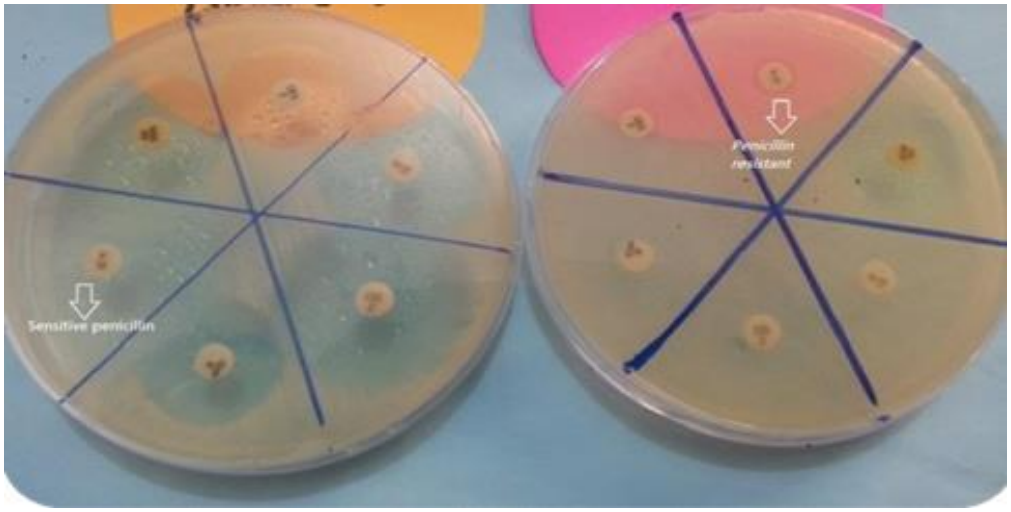

Figure 4.10 Penicillin-sensitive isolates related to burn wound patients

4.6.3 Intermediate antibiotics

The results of the current study recorded the following ratios, 10% for Ciprofloxacin and Azithromycin, 6.7% for Tetracycline, and 3.3% for Erythromycin from burn isolates as shown in (Figure 4.11).

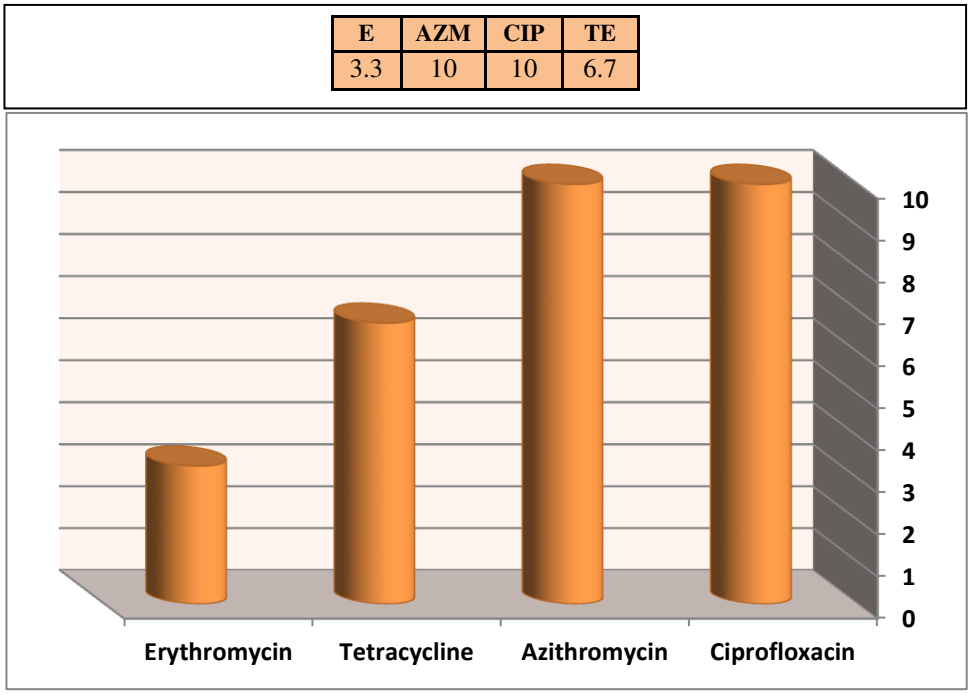

Figure 4.11 Percentages (%) of intermediate antibiotics for isolates of *S. aureus*

4.7 Biofilm Formation

In the 50 specimens of *S. aureus* collected from patients with burn wound infections, biofilms were found in 18 (36%) of the isolates by the Tissue Culture Plate (TCP) method (Table 4.4 and Figure 4.13). From *S. aureus* isolates, 2 (11.1%) strains showed moderate to produce biofilm, and 7 (38.9%) strains showed weak biofilm formation, and 9 (50.0%) showed strains non-biofilm formation by the TCP method. Using the TCP method showed 0 (0.0%) strains strong enough to produce biofilm (Figure 4.12).

Table 4.4 Biofilm production results for isolates of *S. aureus* by the TCP method

Samples codes	Biofilm average
A	0.100
B	0.086
C	0.089
D	0.110
E	0.098
F	0.069
G	0.110
H	0.080
I	0.070
K	0.079
L	0.130
M	0.220
N	0.089
O	0.240
P	0.130
Q	0.088
S	0.100
T	0.100

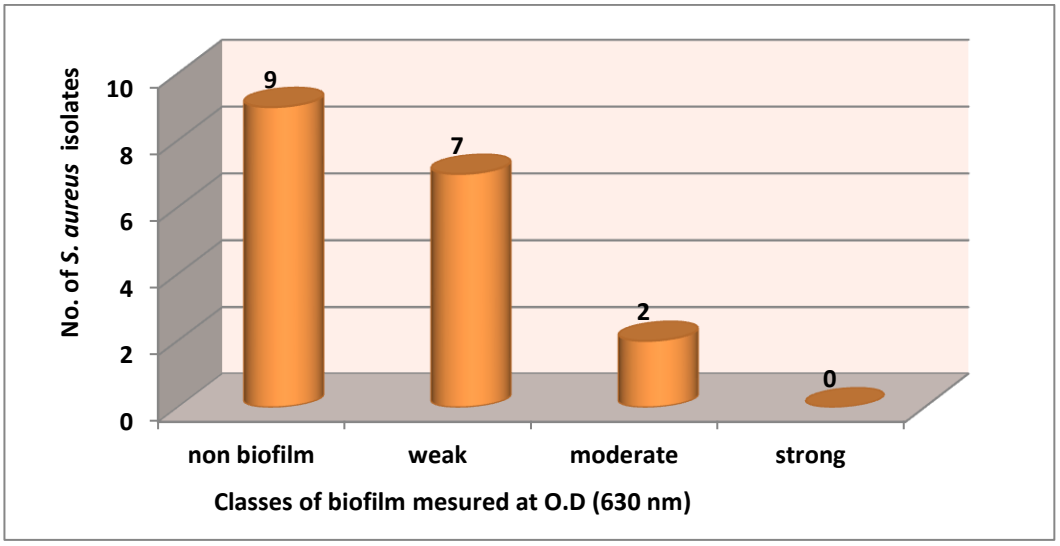

Figure 4.12 Distribution of biofilm production among *S. aureus* isolates

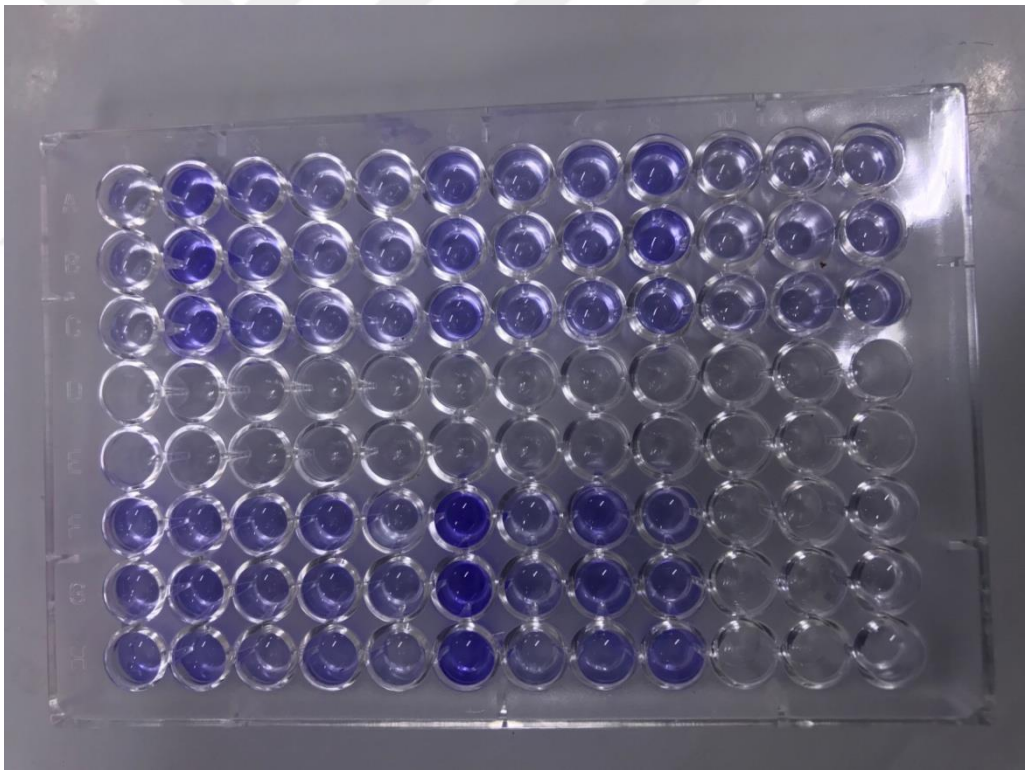

Figure 4.13 Biofilm production of *S. aureus* isolates (TCP method)

4.8 Identification of *Staphylococcus aureus* by the Vitek 2 Compact System

The final identification was performed with the automated Vitek 2 compact system by using AST-GP card, which contained 64 biochemical tests. The results demonstrate that all 18 (36%) isolates for *S. aureus* were confirmed with ID message confidence levels ranging from excellent (the best probability percentage was 96%) (Appendix 1). This technique is characterized by fast detection of bacteria without the need for many culture media as well as reduced culture contamination. A new tool for the rapid identification of bacteria from human clinical specimens. Automated bacterial identification in the clinical laboratory provides a rapid and reliable diagnosis for most pathogens involved in infectious diseases with a highly acceptable level of identification accuracy. Additionally, automated identification enables the interpretation of AST for the correct treatment of patients.

Al-Humam (2016), found the benefit of using the Vitek 2 compact system for comparing biochemical characteristics of *S. aureus* isolates from humans, and the identification by the system at the species level was accurate.

4.8.1 Antibiotic susceptibility test (AST)

The results of the AST showed that a total of 18 isolates obtained in this study were resistant to at least 18 of the antibiotics used. The highest resistance percentage was observed against Benzylpenicillin at 100%, against Oxacillin at 88.8%, against Erythromycin and Clindamycin at 55.5%, against Tetracycline at 50%, against Trim/Sulf at 38.8%, against Fusidic Acid at 33.3%, against Gentamycin, Tobramycin, Levofloxacin and Moxifloxacin at 27.7%, and against Rifampicin at 22.2% (Table 4.5 and Figure 4.14).

Also, a high percentage of *S. aureus* isolates were sensitive to Linezolid, Teicoplanin, Tigecycline, and Nitrofurantoin at 100%, to Vancomycin at 94.4%, to Rifampicin at 77.7%, to Gentamycin, Tobramycin, Levofloxacin, and Cefoxitin at 72.2%, to

Moxifloxacin and Fusidic Acid at 66.6%, to Trim/Sulf at 61.1%, to Tetracycline at 50%, to Erythromycin at 44.4%, to Clindamycin, and to Oxacillin at 11.1% (Table 4.5 andFigure 4.14).Vancomycin 5% showed an intermediate effect against isolates.

Figure 4.14 and Table 4.5 demonstrates the sensitivity pattern of an 18 *S. aureus* isolate against regularly used antibiotics.

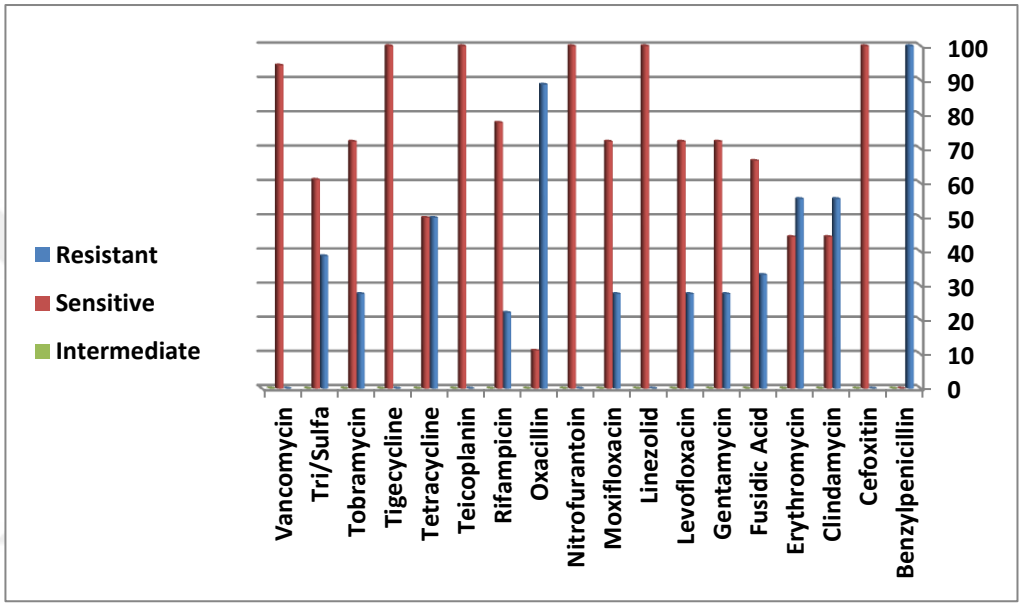

Figure 4.14 Percentages (%) of antibiotic susceptibility tests

Table 4.5 Antibiotic susceptibility test on different *S. aureus* isolates

Antibiotics	Antibiotics sensitivity		
	Resistant	**Sensitive**	**Intermediate**
Benzylpenicillin	18 (100%)	0 (0.0%)	0 (0.0%)
Cefoxitin	0 (0.0%)	18 (100%)	0 (0.0%)
Clindamycin	10 (55.6%)	8 (44.4%)	0 (0.0%)
Erythromycin	10 (55.6%)	8 (44.4%)	0 (0.0%)
Fusidic Acid	6 (33.3%)	12 (66.7%)	0 (0.0%)
Gentamycin	5 (27.8%)	13 (72.2%)	0 (0.0%)
Levofloxacin	5 (27.8%)	13 (72.2%)	0 (0.0%)
Linezolid	0 (0.0%)	18 (100%)	0 (0.0%)
Moxifloxacin	5 (27.8%)	13 (72.2%)	0 (0.0%)
Nitrofurantoin	0 (0.0%)	18 (100%)	0 (0.0%)
Oxacillin	16 (88.9%)	2 (11.1%)	0 (0.0%)
Rifampicin	2 (22.2%)	14 (77.8%)	0 (0.0%)
Teicoplanin	0 (0.0%)	18 (100%)	0 (0.0%)
Tetracycline	9 (50.0%)	9 (50%)	0 (0.0%)
Tigecycline	0 (0.0%)	18 (100%)	0 (0.0%)
Tobramycin	5 (27.8%)	13 (72.2%)	0 (0.0%)
Trim/Sulf	7 (38.9.0%)	11 (61.1%)	0 (0.0%)
Vancomycin	0 (0.0%)	17 (94.4%)	1 (5.6%)

4.9 Molecular Study

Recently, several molecular methods based on PCR have been presented as an alternative method for accurate identification. The diagnostic gene (for ***ica***A, ***ica***B, ***ica***C and ***ica***D genes) was used in the present study to detect *S. aureus* isolates. PCR analysis was applied to all cluster study *S. aureus* isolates. The ***ica***A, ***ica***B, ***ica***C and ***ica***D genes were screened with 151 bp, 141 bp, 209 bp, and 211 bp, respectively, by using PCR.

Figures (4.16, 17, 18 and 19) showed the PCR results for ***ica***A, ***ica***B, ***ica***C and ***ica***D genes, respectively. As shown in the figures, ***ica***A was found in 11 (61.1%) isolates, ***ica***B was found in 13 (72.2%) isolates, ***ica***C was found in 9 (50%) isolates, and ***ica***D was found in 9 (50%) isolates (Table 4.6 and Figure 4.15).

Table 4.6 Distribution of biofilm genes in the different isolates of *S. aureus*

Gene	No. of positive isolates	No. of negative isolates	Total
*ica*A	11 (61.1%)	7 (38.9%)	18 (100%)
*ica*B	13 (72.2%)	5 (27.8%)	18 (100%)
*ica*C	9 (50.0%)	9 (50.0%)	18 (100%)
*ica*D	9 (50.0%)	9 (50.0%)	18 (100%)

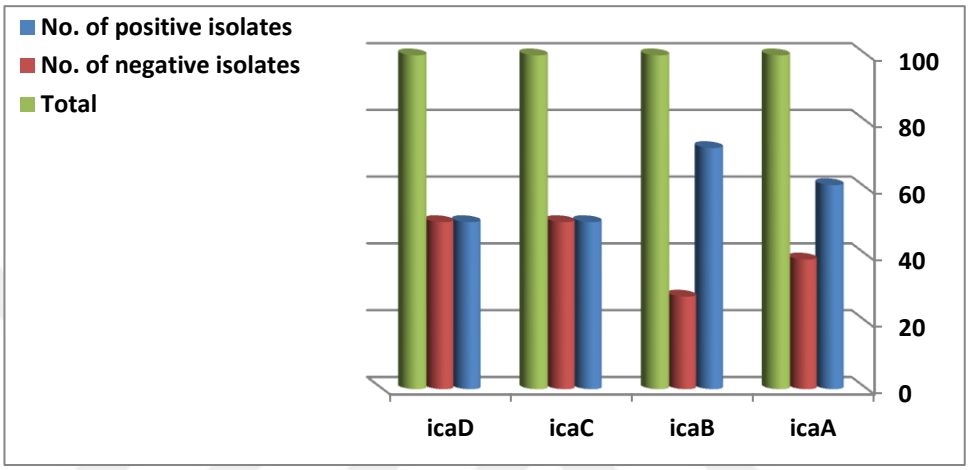

Figure 4.15 Percentages of biofilm genes in 18 isolates of *S. aureus*

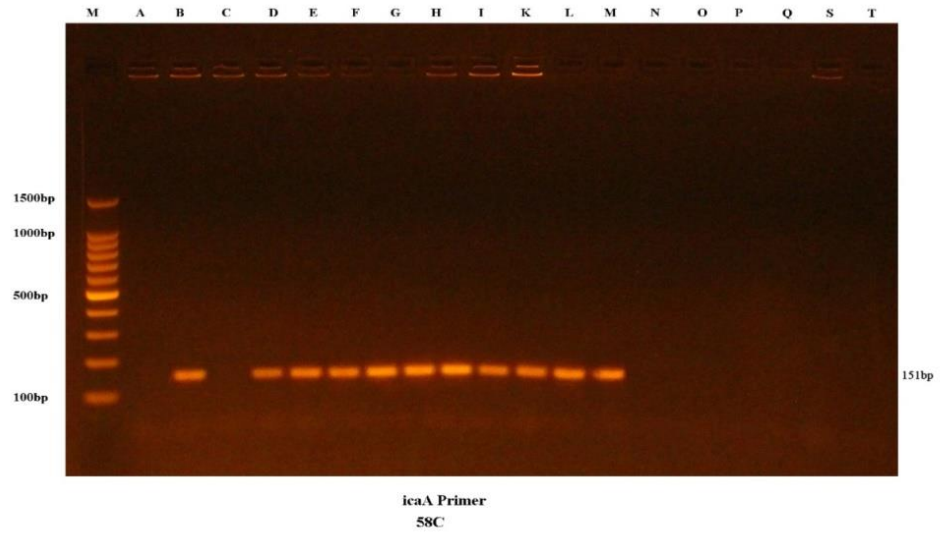

Figure 4.16 Image of agarose gel electrophoresis showing the amplified products of the ***ica***A gene in *S. aureus* isolates. The first line (M) is a positive control for a 100 bp line in the DNA ladder. (A, C, D, E, F, G, H, I, K, L, and M) are positive results (151 bp).

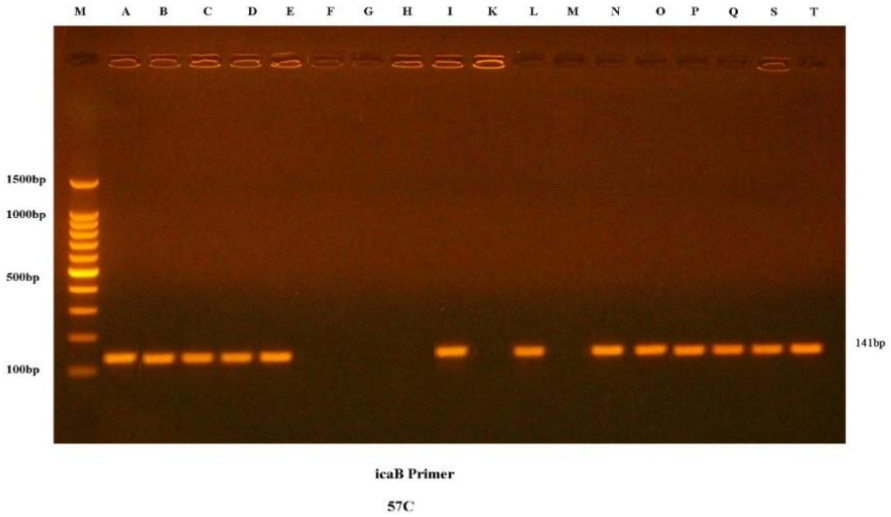

Figure 4.17 Image of agarose gel electrophoresis showing the amplified products of the ***ica***B gene in *S. aureus* isolates. The first line (M) is a positive control for a 100 bp line in the DNA ladder. (A, B, C, D, E, I, L, N, O, P, Q, S, and T) are positive results (141 bp).

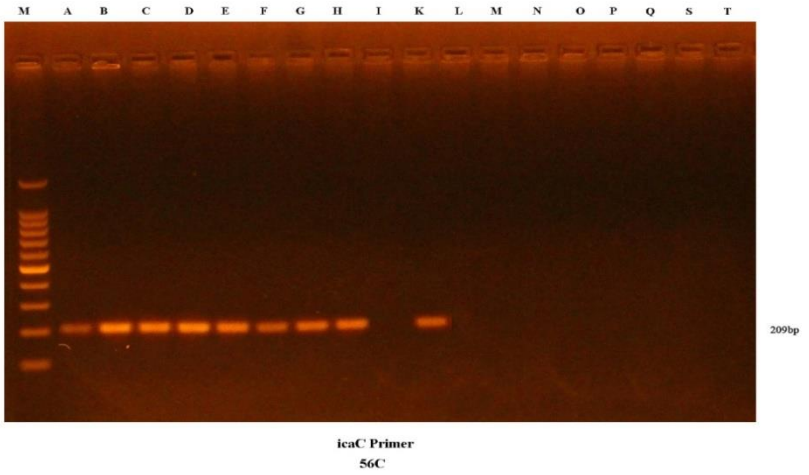

Figure 4.18 Image of agarose gel electrophoresis showing the amplified products of the ***ica***C gene in *S. aureus* isolates. The first line (M) is a positive control for a 100 bp line in the DNA ladder. (A, B, C, D, E, F, G, H, and K) are positive results (209 bp).

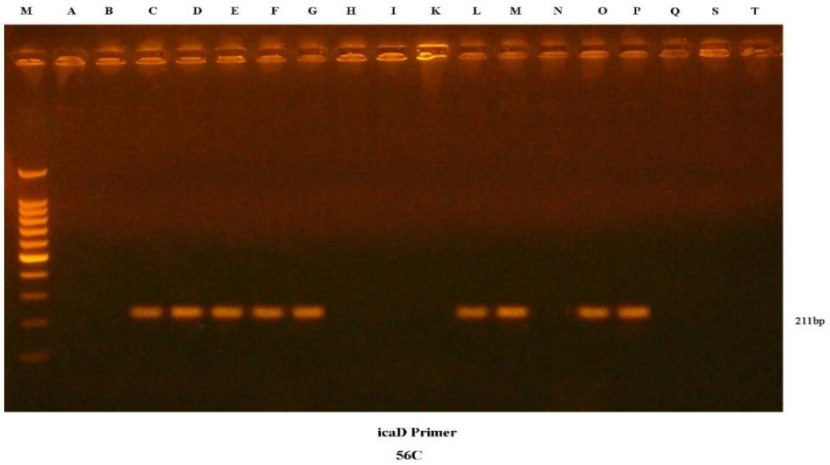

Figure 4.19 Image of agarose gel electrophoresis showing the amplified products of the ***ica***D gene in *S. aureus* isolates. The first line (M) is a positive control for a 100 bp line in the DNA ladder. (C, D, E, F, G, L, M, O, and P) are positive results (211 bp).

5. DISCUSSION AND CONCLUSIONS

1.1 Antibiotic Susceptibility Test

5.1.1 Antibiotic resistant

The results of patient isolates are consistent with results obtained from other local studies (in terms of their resistance to antibiotics) by Hatem (2017), Salman and Ali (2017) who showed that the isolates showed high levels of resistance to Penicillin, which was more than 90%, and the study data was consistent with other local studies by Dogramachy (2018) as well as Al-Dahbi and Al-Mathkhury (2013). In those studies, resistance to Penicillin was 100%. But compared to other countries, Penicillin resistance was 100%, 85%, 83.5% and 71.9%, such as Palestine, Southern China, Malaysia, and Western Nepal, respectively (Sengupta *et al.* 2013).

After Penicillin resistance emerged and a new strain called MRSA emerged in the 1960's, Vancomycin became the drug of choice in many health care settings for life-threatening MRSA infections. Although Vancomycin was the most effective therapeutic agent against MRSA infection, there was a worrying emergence of strains of *S. aureus* less sensitive to Vancomycin and other glycopeptides.

So, in patients with burn wound infection, *S. aureus* resistance to Vancomycin increases due to cell thickening and prior exposure to the antibiotic Vancomycin. Gade and Qazi (2013), reported that Ciprofloxacin has been proposed as a possible alternative to Vancomycin, but misuse of antibiotics had led to the emergence of resistant isolates.

In percentage terms, the difference in patient isolates appeared to be very small and was not a distinguishing factor. Zahmatkesh *et al.* (2016), indicated that ***nor***A and ***nor***B play an essential role in creating resistance to Ciprofloxacin in clinical isolates of *S. aureus* and identification of these genes might indicate a successful model for treating *Staphylococcal* infections.

The study findings were in complete disagreement with the results of the other local study by Salman and Ali (2017), who reported that resistance to Ciprofloxacin was 12%, but there was agreement with Al-Khafaji (2018) and Hatem (2017), who reported that resistance to Ciprofloxacin was 20% in *S. aureus* isolates.

The research data somewhat approximates the results described by Al-Alak *et al.* (2016) who reported that the rate of resistance to Azithromycin reached 70% and Al-Taai*et al.* (2017) who reported that the rate of resistance to Azithromycin was 60%.

The study indicated that *S. aureus* isolates which were founded were different from other isolates taken from other countries by obtaining resistance genes for different classes of antibiotics as a result of excessive use and different antibiotics at random without consulting a clinician. Going to speculate with the data collected, health workers might be colonized by a number of drug-resistant *S. aureus*.

Attempting to reduce the unreasonable use of drugs by people may be the main reason for stopping many of the new antibiotic-resistant variants. School students as well as occupational health services accompanying hospital habitats should be instructed in the constant use of decontamination tools and the use of personal preventive methods to stop the transmission of pathogenic agents, including several specific antibiotic-resistant strains, primarily MRSA isolates.

For patients' isolates, these results approached those of Al-Daherie and Abbas (2015), who reported Clindamycin resistance of 80%, but did not agree with Abbas *et al.* (2014), who stated that resistance to this antibiotic was 27%.

An antibiotic like Clindamycin is used as an alternative for patients with an allergy to Penicillin due to its good oral absorption, and is an important option in ambulatory therapy. Resistance develops through several different mechanisms, but the most well-known mechanism is modification of the target site affected by ***erm*** genes, which could be demonstrated as proximal (***c***MLSB phenotype) or inducer (***i***MLSB phenotype). ***erm***

codes for the MLSB antibiotic target variable methylase enzyme (modification of drug-binding site in rRNA).

Regarding patient isolates, the present results did not agree with other local studies conducted by Al-Kaabi (2013) that studied burn wound specimens at the Burns Specialist Hospital in Baghdad, which showed that the results of examining some specimens against Levofloxacin amounted to 33.33%. These results were also no agreement with the results of Hallabjaiy *et al.* (2014), who reported that the resistance to Nitrofurantoin was 31.58%. In addition, the results of Babakir-Mina *et al.* (2012) in the north of Iraq, were the closest to the study results presented, among other local studies that recorded Nitrofurantoin resistance of 2.5%. A local study by Mohammed and Flayyih (2016), also reported that 92.5% of the isolates were sensitive to Levofloxacin, which means the resistance is 7.5%, so it was the closest for this study.

Although the action of Nitrofurantoin is not very well known, its effects appear to require an enzymatic reduction in the bacterial cell, and the reduced derivatives seem to be able to bind to ribosomal proteins. Therefore, antibacterial activity has also been noticed in situations where the functions of Nitroreductase are suppressed, suggesting that Nitrofurantoin could sometimes act in part without decreasing the active metabolites. The oral Nitrofurantoin absorption rate is 40 to 50%, and it is increased when taken with food. Nitrofurantoin concentrations in serum are low to unnoticeable, but their concentrations in urine are 50-250 milligram/liter.

Shakti and Veeraraghavan (2015), report that Nitrofurantoin acts on multiple targets in the bacterial cell and that resistance has not developed as rapidly as other drugs with a single bacterial target. The resistance was caused by mutations in the genes encoding Nitrogenases (***nfs***A and ***nfs***B) that were responsible for the high-level resistance to Nitrofurantoin. This antibiotic was included in this study because in the patients' medical history there were other diseases, including UTIs, so it was tabulated to get a broad concept about resistance.

Erythromycin is commonly used for treatment of infections related to various bacteria, such as *S. aureus*. It has been shown to reduce the development of pro-inflammatory cytokines in various situations.

The results were in agreement with those of other local studies by Hatem (2017), which indicated 75% resistance to Erythromycin, but there was no agreement with Phaku *et al.* (2016), who recorded the Erythromycin resistance rate was 20%.

Gentamycin belongs to the aminoglycosides that play a critical role in the treatment of *Staphylococcal* burn wound infections, but some strains are still strong against it. The main method of resistance to these classes was inactivation of antibiotics by modifying the enzymes encoded by genetic elements.

The results of the current study were in agreement with other local studies by Al-Taai *et al.* (2017), Salman and Ali (2017), which reported that the resistance of Gentamycin against *S. aureus* was 22.3% and 18%, respectively. Also, there was no agreement with the results of Hatem (2017), who stated that the resistance to the Gentamycin antibiotic was 90%.

In patients' isolates, these results approached other local studies by Salman and Ali (2017), who reported that resistance to Tetracycline was 16.23%, but there was no agreement with Yasin (2013), who reported that resistance to Tetracycline was 0%.

The study believed that resistance to Tetracycline depends on two main mechanisms. These include active efflux that arises from the acquiring of plasmid-sited ***tet***K and ***tet***L genes as well as ribosomal defense through elongation factor-like proteins expressed by chromosomal or transposonal ***tet***M or ***tet***O determinants.

Doxycycline might be a potential alternative to Tetracycline resistance in many situations. It is used in oral and injectable formulations.

5.1.2 Intermediate antibiotics

The emergence of some intermediate isolates of antibiotics could be considered as a first step towards the generation of new strains of *S. aureus* that are more virulent and more adapted to different environments. Local studies conducted by Al-Hamdani and Hamad (2012), showed close results to this study, where the median Ciprofloxacin isolates were 8.33%, but the same study also showed a high level of intermediate Erythromycin isolates by 33.33%. The presented study results did not record any antibiotic value for Vancomycin, but Al-Meani and Al-Ani (2018), indicated in their study that the percentage of intermediate Vancomycin isolates was 3%. The difference in the results of studies could be explained by a different number of isolates.

Other studies were conducted by Al-Taai *et al*. (2017) that showed intermediate 40%, 30%, 10% and 0% to Tetracycline, Erythromycin, Azythromycin and Vancomycin, respectively. Naqvi *et al*. (2015), showed an identical proportion (10%) to the current study that linked Ciprofloxacin in burn wound patients.

5.2 Biofilm Formation

There were significant ($p < 0.05$) or non-significant differences in the ability of *S. aureus* to biofilm production.

Our findings were inconsistent with a study Mirzaee *et al.* (2014a), and they found that 29 (46%) MRSA strains were able to produce robust biofilm formation from 63 *S. aureus* isolates.

It almost agrees with Mirzaee *et al.* (2014b), who found 3 (25%) strains were averaged from 31 samples.

The present study reports a high prevalence of ***ica*** genes, but the presence of biofilm-related genes is not always associated with biofilm production. Thus, the difference

between phenotypic and genetic characterization may be due to heterogeneity in the genetic origins rather than the presence or absence of genes required for the formation of biofilms.

5.3 Molecular Study

The current results were in agreement with those of another global study by Ghasemian *et al.* (2015) that indicated the prevalence of ***ica***A in isolates was 16 (73%), from 22 *S. aureus* isolates.

The latest findings matched those of a previous global survey by Ghasemian *et al.* (2016) that indicated the prevalence of ***ica***B in isolates was 49 (76%), from 64 isolates of *S. aureus*.

The target results agreed with those of another global investigation by Goudarzi *et al.* (2019), that indicated the prevalence of ***ica***C and ***ica***D in isolates was 26 (53.3%), from 48 isolates of *S. aureus*.

According to the above, the following observations were discovered:

S. aureus was the most common isolated from burn wound infections, followed by *Pseudomonas aeruginosa*, *S. epidermidis*, *S. pseudintermedius*, *S. chromogenes*, *S. haemolyticus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Acinetobacter baumannii*. Most isolates of *S. aureus* can produce virulence factors such as hemolysin, urease, and proteases.

The high prevalence of virulence genes (***ica*** genes) was in *S. aureus* isolates of burn wound patients. Most bacterial isolates from burns and wounds showed multiple drug resistance (MDR). An antibiotic susceptibility test was performed for isolates of *S. aureus* that showed multiple antibiotic resistance.The production of biofilm from *S. aureus* isolates explains its resistance to antibacterial agents.

Isolated *S. aureus* bacteria were more sensitive to Vancomycin than to others antibiotics. *S. aureus* isolates were more resistant to Penicillin through burn wound patients.

The ELISA technique and molecular techniques, like PCR revealed the same sensitivity in relation to diagnosis of *S. aureus* bacteria and biofilm formation.

In recent years, one of the most widely used molecular methods has been PCR, which gives fast and reliable results, and molecular diagnosis of species. With the widespread use of this method, the rapid and timely diagnosis of lethal bacteria in burn wounds that adversely affect human health is very important. Supporting and developing such studies on basic examinations will be useful in preventing possible health problems in the future and in accelerating treatment by diagnostic methods in a short time. In this context, it is expected that the results obtained and the methods used in this thesis will be useful in future studies for the detection of *S. aureus* in burn wounds. Eliminating and eliminating *S. aureus* bacteria and reducing potential health problems requires various and multiple treatments, such as high quality personal health management, dismantling all sources of bacterial contamination, avoiding indiscriminate use of antibiotics (which lowers bacterial resistance), early diagnosis of bacterial infections, and scientific medical treatments. Finally, advanced molecular and genetic studies should be conducted for more involved epidemiology.

The current study recommended that necessary studies be performed on exogenous and endogenous *S. aureus* in patients with burn wounds and other clinical sources. Further studies are required to conduct continuous monitoring of the β-lactamase producing strains. It is necessary to deal well with burn wounds from medical interventions to reduce the production of biofilms. Study of other virulence genes associated with plasmids of *S. aureus* in burn wound isolates.

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APPENDICES

APPENDIX 1.Vitek 2 compact system report

APPENDIX 1.Vitek 2 compact system report

ASCo

bioMérieux Customer:

Microbiology Chart Report

Patient Name: Patient ID: 475
Location: Physician:
Lab ID: 475 Isolate Number: 1

Organism Quantity:
Selected Organism : Staphylococcus aureus

Source: **Collected:**

Comments:	

Susceptibility Information	**Analysis Time:** 9.00 hours			**Status:** Final	
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Cefoxitin Screen	POS	+	Teicoplanin	2	S
Benzylpenicillin	>= 0.5	R	Vancomycin	1	S
Oxacillin	>= 4	R	Tetracycline	>= 16	R
Gentamicin	<= 0.5	S	Tigecycline	0.5	S
Tobramycin	<= 1	S	Fosfomycin		
Levofloxacin	0.25	S	Nitrofurantoin	<= 16	S
Moxifloxacin	<= 0.25	S	Fusidic Acid	<= 0.5	S
Inducible Clindamycin Resistance	POS	+	Mupirocin		
Erythromycin	>= 8	R	Rifampicin	>= 32	R
Clindamycin	<= 0.25	*R	Trimethoprim/Sulfamethoxazole	<= 10	S
Linezolid	2	S			

+= Deduced drug *= AES modified **= User modified

AES Findings		
Confidence:	Consistent	
Phenotypes flagged for review:	BETA-LACTAMS	MODIFICATION OF PBP (mecA)
	MACROLIDES/LINCOSAMIDES/STREPTOGRAMINS	MLSB INDUCIBLE

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CURRICULUM VITAE

Personal Information

Name and Surname **:**Mohannad Ahmed Jawad AL-ZAIDI

Education

Diploma	Al-Dour Technical Institute Departmentof Medical Laboratory Technology	2003-2004
MSc	College of Health and Medical Technology Departmentof Medical Laboratory Technology	2006-2007

Work Experience

Year	**Institution**	**Position**
2008-Present	Medical City Complex Al-Shaheed Ghazi Al-Hariri Hospitalin	Manager of laboratory intensive care unit

Academic Activities

1. Attended a Training Course in "Patient Safety in ICU" held in Cooperation with Irshad Center 26/January/2014-9/February/2014, in University of Jordan.
2. Completed a one month Training Course of Laboratories in Rafik Al-Hariri University Hospital/Lebanon-Beirut on March/2013.
3. A Technical Training Course for "ESCHEILER Blood Gas and Electrolyte Analyzer"in Germany-Kiel on July/2012.
4. Course of Internet and Computing Core Certification (IC3) on August/2012.
5. Course of the TOEFL in Baghdad University on April/2011.