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Spa Typing for Detection of Genetic Variation of *Staph aureus* Isolated from Clinical Samples

A Thesis

Submitted to the Council of College of Medicine-University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master of Sciences in Medical Microbiology

By

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

﴿ فَبَدَأَ بِأَوْعِيَتِهِمْ قَبْلَ وِعَاءِ أَخِيهِ ثُمَّ اسْتَخْرَجَهَا مِن وِعَاءِ أَخِيهِ كَذَٰلِكَ كِدْنَا لِيُوسُفَ
مَا كَانَ لِيَأْخُذَ أَخَاهُ فِي دِينِ الْمَلِكِ إِلَّا أَن يَشَاءَ اللَّهُ نَرْفَعُ دَرَجَاتٍ
مَّن نَّشَاءُ وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ ﴾

صدق الله العلي العظيم

سورة يوسف ﴿٧٦﴾

Certification

We certify that this thesis entitled (***Spa* Typing for Detection of Genetic Variation of *Staph aureus* Isolated from Clinical Samples)**: was prepared under our supervision by "Ruqaya Fadhel Gazi Asewed " at the Department of Microbiology-College of Medicine/ Babylon University, as a partial fulfillments for the requirements for the degree of Master of Science in Medical Microbiology and this work has never been published anywhere.

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Dedication

For my mother's wishes when she asked God to complete my studies and obtain a master's degree, and God responded to them
For all the wishes my mother made for us
For my father's efforts and his hope that I will be something he can be proud of in the future, for it is something that has long been waited.
For my sister's love and support.
For my brothers encouragement and love.
For my husband's love, support and giving
To my friend Noor, for bearing with me, without your continued presence, I could not be able to complete this matter.

I Dedicate this Work

Ruqaya

Acknowledgement:

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Thank you for all

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Summary:

According to the big problem of bacterial resistance to antimicrobial drugs and the wide spread of *S.aureus* infections especially among nosocomial patients more than community patients and the overuse of antibiotics without a doctor descriptions all these problems lead to establish this study

This work aimed to study the genotyping method for *S. aureus* isolates by the application of *Spa* typing (variable number tandem repeat), and to find an alternative ways for antibiotic use by using lemon plant and (Gold, Titanium Dioxide) nanoparticles.

The study included 110 different samples collected from patients suffering from signs and symptoms of bacterial infection; they attended to the two main hospitals (Al- Hilla General Teaching Hospital and Al-Imam Al-Sadek Medical City) in AL_ Hilla City for the period from October 2021 to January 2022. Specimens were cultivated aerobically on different medium at 37℃ for 24hrs for bacterial diagnosis and identification.

Results of morphological and biochemical characterization tests revealed that out of 110 samples, 23 isolates of *S.aureus* were recovered, while 57 isolates belonged to other bacterial genera, and 30 isolates show negative culture result.

The isolates of *S. aureus* 23 (20.9%) were distributed as following: 5/33 (15.2%) isolates from burns and 8/25 (32.0%) from wounds, while 10/52 (19.2%) from urine.

Antibiotic susceptibility tests for all isolates were determined using disc diffusion test, as ten antibiotic types were applied; unfortunately, the overwhelmed *S.aureus* isolates that recovered in this study were resistant to most used antibiotics, where these isolates showed resistance to Trimethoprim (73.9%) while resistance to Clindamycin and Tetracyclin was (65.2%) and (60.9%) respectively. *S.aureus* showed less resistance to Azithromycin (43.5%) and to Amikacin (39%) while resistance to Ciprofloxacin was (34.8%) and for Trimethoprim Sulfomethoxazole was (30.4%) while the resistance percentage to Levofloxacin was (26%) and to Gentamicin (21.7%) while chloramphenicol showed the lowest resistance percentage by *S. aureus* constituting (17.4%).

Also, this study examined the effect of fresh lemon juices of both types sour and sweet in different concentrations (100%, 75%, 50% and 25%) on *S.aureus* isolates; there was statistically significant inhibitory effect ($P = 0.04$) on the growth; and these results showed that sour lemon had higher inhibition effect (91.3%) on *S. aureus* in (100% and 75%) concentrations; while in (50%) concentration inhibition was (82.6%) of bacterial growth. While sweet lemon showed lower effect in its different concentrations, in (100%) concentration made (74%) inhibition effect, while (75%) concentration inhibit (56.52%) of bacterial growth.

Moreover, AuNPs and TiO_2NPs inhibitory effects were examined against *S.aureus* isolates by making five double serial dilutions for each stock concentrations as (1/2, 1/4, 1/8, 1/16 and 1/32) and those concentrations were (2000 µg/ml and 400µg/ml) for AuNps and TiO_2NPs respectively; then after incubation for 24 and 48 hours the bacterial growth had been monitored and checked with spectrophotometer; and

it revealed that there was statistically significant inhibitory effect (P=0.005) on the growth especially with the first two dilutions (1/2, 1/4).

Additionally, AuNPs and TiO_2NPs effects were examined against biofilm formation by *S.aureus* isolates growth by making four double serial dilutions for each stock concentrations as (1/2, 1/4, 1/8, and 1/16); then after incubation the ability of *S.aureus* to form biofilm had been monitored and checked with spectrophotometer; results showed that TiO_2NPs had more inhibitory effect on the biofilm formation by its different dilutions compared with AuNPs concentrations which was having less effect and it revealed that there was statistically significant effect (P=0.008) on the biofilm formation especially with the first three dilutions.

Regarding, *Spa* genotyping was done and the result showed that out of 20 isolates identified, (8) different types among the 18/20 isolates were detected, and 2/20 isolates could not be typed; as the most commonly observed Spa were t304 (35%), t491 (15%) followed by t078 and t059 (10%).

Finally, based on phylogenetic relationships, *S.aureus* strains were classified into two clades. The first one contained 18 isolates and the second one contained 2 isolates, the most *Spa* types were included in clade A (18 isolates) where only 2 isolates were included in clade B, then the isolates in clade A were clustered into 3 different groups based on the variation in tandem repeats of the *Spa* gene, cluster 1 contain the t304, t078, t044 Spa types, cluster 2 contain t059, t4870 and t386 *Spa* types and cluster 3 contain t491 and t091 Spa types and clade B contain 2 *Spa* types (unknown).

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ABBREVIATIONS

agr	accessory gene regulator
AMR	Antimicrobial resistance
ANTs	aminoglycoside- nucleotidyltransferases
CLSI	Clinical and Laboratory Standards Institute
CPS	coagulase-positive staphylococci
DHFR	Di hydro folate reductase
ermA	Erythromycin methylases enzyme
ETA & ETB	Exfoliative toxins A and B
FQs	Fluoroquinolones
gyrA	Gyrase A
IgG	Immunoglobulin G
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
NCBI	National Center for Biotechnology Information
PAIs	pathogenicity islands
PFGE	pulsed-field gel electrophoresis
PIA	polysaccharide intercellular adhesin
PNAG	Poly -β(1 -6) - N-acetylglucosamine
PS/A	polysaccharide adhesion
ROS	reactive oxygen species
rRNA	Ribosomal riboxy nucleic acid
SAE	*S. aureus* exo polysaccharide
SERAMs	Secretable expanded repertoire adhesive molecules
SEs	Staphylococcal enterotoxins
sigB	sigma factor
SMX	Sulfamethoxazole
Spa	Staphylococcal protein A
tetM or tetO	Transposonal genes
WHO	World Health Organization

Chapter One

Introduction and Literatures Review

1.1.Introduction:

Staphylococcus aureus is one of the most important human colonizers that can cause infectious diseases. Colonization of human nose nares by *S. aureus* is a source and risk factor for staphylococcal disease, and invasive staphylococci infection can have its source in strains occurring naturally in the host (Newstead *et al*., 2020).

The capability of producing different virulence factors is the main reason for the high pathogenicity of *S. aureus*. In addition to its high pathogenicity, this bacterium also has high adaptive powers against environmental changes, as the horizontal gene transfer can convert non-pathogenic strains to pathogenic strains through the transfer of the virulence gene (Mrochen *et al*., 2020).

Staphylococcus aureus is a common opportunistic pathogen that causes a variety of infections due to the presence of many colonization factors and virulence factors. It is one of the most frequent causes of skin and soft tissue infections (SSTIs) such as skin abscesses, furuncles, impetigo, and wound infections. Some of them, especially in patients with risk factors (diabetics, patients during immunosuppressive or cancer therapy, patients with indwelling catheters, HIV/AIDS), may progress to severe infections and require hospitalization. *S. aureus* is also a leading cause of serious infections, such as bacteremia or infective endocarditis, which can have serious consequences for the patient. High morbidity and mortality are associated especially with the widespread occurrence of methicillin-resistant *S. aureus* (MRSA) strains. Resistance to all β-lactams (except for the latest generation of cephalosporins) and other antibiotics commonly used limits therapeutic options for treating staphylococcal infection (Shin *et al*., 2021)

Conventional medicinal practices utilize plants against various infections for over thousands of years now. 80% of the population in the developing nations are dependent

upon the easily accessible traditional medications to fulfill their primary medical needs. Indeed, plants are known to synthesize a wide array of compounds known as secondary metabolites or phytochemicals such as quinones, tannins, terpenoids, alkaloids, flavonoids, and polyphenols which have disease prevention properties and aid them in their self-defense and communication with other organisms in their environment. Plant extracts as medicines are inevitable substitutions for antibiotics prescribed by physicians. Plant derived compounds and extracts are commonly used in self-medication due to its easy availability, competence and nil side effects (Bhatia *et al*., 2021)

Interestingly, metallic nanoparticles (NPs) have been proven to be promising alternative to antibiotics. NPs interact with the important cellular organelles and biomolecules like DNA, enzymes, ribosomes, and lysosomes that can affect cell membrane permeability, oxidative stress, gene expression, protein activation, and enzyme activation. Since, NPs target multiple biomolecules concurrently; it becomes very difficult for bacteria to develop resistance against them (Colilla *et al*., 2020).

Genotypic typing methods are based on the analysis of chromosomal or extrachromosomal DNA. Plasmid analysis was the first DNA-based method to be applied to *S. aureus*. RFLP analysis with a variety of DNA and RNA probes has also been used to type bacterial strains (Shalon *et al*., 2021)

Protein A is an important virulence factor and a phenotypic determinant in *S. aureus*; thus, variations in the sequence of *spa* gene coding for this species-specific protein is beneficial in PCR typing for *S. aureus*. DNA-based typing methods are reportedly based on the principle that epidemiologically related bacterial isolates have genetic features that are different from those of other epidemiologically unrelated strains (Alkam *et al*., 2021).

Aim of the study:

Genotyping method for *S. aureus* isolates by *Spa* typing and sequencing of variable – number tandem repeat and the role in epidemiological study and finding an alternative treatment methods for resistance problem produced by *S. aureus* bacteria.

This Aim done by the following objectives:

1. Samples were taken from patients from different site (burn and wound swab, and urine).
2. Bacterial isolation and identification are done by culture media**,** microscopic morphology (Gram stain), and biochemical tests.
3. Study antibiotic susceptibility test of bacterial isolates.
4. Detection the antibacterial effect of Sour and Sweet lemon.
5. Application of inhibitory effect of Nanoparticles against growth and Biofilm formation.
6. *Spa* typing of *S. aureus* isolates by sequencing of VNTR region.

1.2. Literatures review:

1.2.1. General Characteristics of *Staphylococcus:*

The family Staphylococcaceae contains 98 validly published species housed within nine genera comprised of Abyssicoccus, Aliicoccus, Auricoccus, Corticicoccus, Jeotgalicoccus, Macrococcus, Nosocomiicoccus, Salinicoccus and Staphylococcus. Members of this family are Gram-positive, non-spore forming, spherical or coccoid cells with sizes ranging from 0.5 to 2.5 μm, non-motile, occurring singly, in pairs or tetrads, strictly aerobic to facultatively anaerobic, catalase-positive (typically), oxidase negative, and chemoorganotrophs capable of both aerobic respiration and fermentative metabolism (Schleifer *et al*., 2009).

Among Staphylococcaceae, the most populated genus is Staphylococcus with 55 validly published species and 23 subspecies. Their cells occur as grape-like clusters due to perpendicular division planes, their cell wall is made up of peptidoglycan with the predominant diamino acid l-lysine and cells grow in the presence of 10% NaCl (w/v) and at temperatures between 18–40 °C (Lory *et al*., 2014).

Staphylococci are tolerant to the high level of salt concentration, most strains are resistant to heat. Pathogenic staphylococci are commonly identified by their ability to produce coagulase, and thus clot blood (Medveďová *et al*., 2012).

1.2.2. Characteristics of *Staphylococcus aureus*:

In 1881, Ogston carried out experimental laboratory tests to investigate skin-associated infections caused by *S. aureus* by inoculating staphylococci into subcutaneous tissues of laboratory animals such as guinea pigs and mice, which induced

abscesses. The German physician Friedrich Rosenbach isolated and cultured staphylococci from humans in 1884, he studied their characteristics and categorized them according to the production of golden or yellowish colonies, naming the species aureus from the Latin word meaning golden, consequently, *S. aureus* was differentiated from *S. epidermidis* (previously known as *S. albus*) by their golden and white colonies, respectively, *S. aureus* divides by binary fission its cell division occurs at different planes, and its optimum growth occurs at temperatures ranging between 18-40°C (Rasheed *et al*., 2021).

S.aureus is a ubiquitous, versatile and highly adaptive pathogen that colonizes the skin and mucous membrane of the anterior nares, gastro- intestinal tracts, perineum, the genitourinary tracts and pharynx (den Heijer *et al*., 2013).

It is the causative agent of a wide range of infections in humans and animals with a significant impact on public health. Host specialization, ability to acquire and loss resistance and virulence genes as well as its zoonotic potential posed a significant public health implication (Luzzago *et al*., 2014).

S.aureus is a gram-positive non-motile, non-spore forming facultative anaerobe that is biochemically catalase and coagulase positive (Lindqvist, 2014).

It occurs as an irregularly grape-like cluster and sometimes singly or in pairs, typical colonies are smooth raised yellow to golden yellow color and hemolytic on blood agar containing 5% sheep or horse blood. While some members are important to human medicine, others are relevant to veterinary medicine as they are found in animals or food. Biochemically members of the genus are grouped into two; such as coagulase positive staphylococci and coagulase negative staphylococci, *S.aureus* being the most important member of coagulase positive staphylococci causing infection in both humans

and animals and are considered as the most pathogenic members of the genus staphylococci (Bitrus *et al*., 2018).

S. aureus is oxidase-negative therefore requiring certain important amino acid and B vitamins for growth and can also tolerate high salt concentration. The cell wall is made up of peptidoglycan which contains crosslinks of glycine residue that allows sensitivity towards lysostaphin (Lindqvist, 2014).

The stability and worldwide spread of this pathogen is due to its ability to rapidly acquire and loss resistance and virulence determinants from other members of the genus Staphylococci through horizontal transfer of mobile genetic elements MGEs (Bitrus *et al*., 2017).

Studies on whole genome sequence has revealed that the *S. aureus* genome is divided into a relatively stable core genome which is about 75-80% of the entire genome and a relatively less stable Mobile Genetic Elements (MGE) consisting of transposons, pathogenicity island, Staphylococcus cassette chromosomes, plasmids, bacteriophage and insertion sequence (Bitrus *et al*., 2018).

1.2.3. Pathogenesis of *Staphylococcus aureus*:

Staph. aureus is a leading cause of human infections worldwide ,the pathogen is also a commensal organism and approximately 30% of non-institutionalized individuals are colonized asymptomatically by *S.aureus* in the anterior nares, nasal carriage is associated with infection in susceptible individuals. The pathogen can cause peripheral to serious infections in almost all tissues, especially in immune compromised people, disease can manifest as skin and soft-tissue infections, pneumonia, surgical-site infections, bloodstream infections, endocarditis, septic shock, and many others, as well

as causing a myriad of infections. *S.aureus* is well-known for its ability to acquire resistance to antibiotics (Lu *et al*., 2015)

Staph. aureus is a major pathogen in human bloodstream infections. The bacterium is able to spread from an initial site of entry such as an indwelling central venous catheter to various organs including the lungs, bones, and heart valves. To establish itself on these sites, *S. aureus* expresses virulence factors involved in e.g. adhesion, immune evasion, and toxin production(Grønnemose *et al*., 2021)

Historically, *S. aureus* has been viewed solely as an extracellular pathogen, but research conducted during the past decades has demonstrated a pronounced ability for this bacterium to invade and colonize both professional and non-professional phagocytes such as endothelial cells (Rollin *et al*., 2017).

This behavior has since been linked to the pathogenesis of *S. aureus*, in particular its ability to spread via the blood to organs and its resilience against host response and antibiotic treatment e.g. in bloodstream infections such as endocarditis host response and antibiotic treatment e.g. in bloodstream infections such as endocarditis. Though the role of cellular invasion has not been completely clarified, it is believed to entail immune or antibiotic escape leading to subsequent relapse of infection from the intracellular reservoir. How the bacterium reemerges from the invaded endothelial cell or penetrates deeper into target tissues, however, remains to be revealed (Grønnemose *et al*., 2021)

Some groups of people are at higher risk of *S. aureus* colonization (up to 80%), including health care workers, diabetic persons, patients on intravenous drug individuals with weak immunity, patients with long hospital stays, recipients of surgical operations, indwelling catheter users, dialysis patients, individuals with chronic metabolic diseases, immune-compromised individuals, subjects with previous

methicillin-resistant *S. aureus* (MRSA) infection, and individuals with skin infections (Gnanamani A *et al*., 2017).

Carriers serve as sources of infection, transmission of *S. aureus* can take place from one person to another by close or direct contact, sharing personal items, food contamination, and fomite contamination such as doorknobs. *S. aureus* colonization in different parts of the body increases the risk of infection at the surgical site, as well as the infections of the lower respiratory and blood stream in hospitals. These infections are increased in healthcare facilities because the microorganism adapts quickly and effectively to the hospital environment. Therefore, various measures are taken in hospitals to manage and reduce colonization and subsequently eliminate infections. Examples of these strategies include the use of different disinfectants, local application of antibiotics (such as mupirocin), and use of systemic antibiotics (Rasheed *et al*., 2021).

S. aureus colonizes cell surfaces but is also an important human pathogen that can cause severe and invasive infections in almost every organ of the body, such as endocarditis, pneumonia, skin and wound infections, or osteomyelitis work has demonstrated that cell invasion of non- professional phagocytes essentially contributes to the infection development . Bacteria initiate the invasion process by adhering to host cell membranes via the expression of adhesins. As invasion of host cells is equally effective with live and killed bacteria, it has to be considered as not only a microbial pathogenicity mechanism, but also a strategy of the host defense system (Tuchscherr *et al*., 2015).

After host cell invasion, different post-invasion events are possible: the intracellular metabolically active bacteria can induce host cell activation and death. These effects cannot be attributed to a single virulence factor, but are most likely induced by the cumulative action of different bacterial components. The induction of

host cell death has been linked to the bacterial ability to escape from the phagolysosomes to the cytoplasm or reside in other membrane-bound compartments. By down regulation of cytotoxins the bacteria are also able to reside intracellularly for extended time periods. This bacterial pool may thus serve as a reservoir for persistent infections. All infection and post- invasion effects are dependent on the interplay between different factors on both the bacterial and the host cell side (Strobel *et al*., 2016).

1.2.4. Diseases caused by *Staphylococcus aureus*:

1.2.4.1. Staphylococcal Food Poisoning (SFP):

It is one of the most important foodborne diseases. It is caused by the ingestion of preformed staphylococcal enterotoxins (SEs), thermostable proteins produced by enterotoxigenic strains of coagulase-positive staphylococci (CPS) mainly *S. aureus*. The onset of symptoms occurs within a few hours causing nausea, vomiting and diarrhea, and the disease severity is SEs concentration dependent. Generally, the intoxications are self-limiting within 24 h; however, it might be fatal in children and in the elderly. To date, 28 SEs and SE-like toxins (SEls) have been reported in literature; however, only the five so-called classical enterotoxins SEA, SEB, SEC, SED, and SEE can be detected using commercial immune-assays (Chieffi *et al*., 2020).

Historically, SFP has been linked to improper food handling by operators acting as carriers. However, lately SFP was often linked to dairy products, in which the presence of enterotoxigenic *S. aureus* strains agent of mastitis in the herd can cause final product contamination, where SEs are produced when critical bacterial concentration and environmental temperatures are reached during the processing (Filipello *et al.*, 2020*).*

1.2.4.2. Skin Infections Caused by *Staphylococcus aureus:*

S.aureus is one of the leading causes of skin and soft tissue infections (SSTIs). Treatment of these infections is significantly hampered by the pathogen's propensity to acquire antibiotic resistance. In particular, community-acquired methicillin resistant *S.aureus* (CA-MRSA) skin infections are occurring with increasing frequency in healthy individuals with no identified healthcare-associated risk factors. It is estimated that 90% of CA-MRSA infections present as SSTIs. MRSA has become resistant to even last resort antibiotics (Lacey *et al*., 2016).

Those are caused by microbial invasion of the layers of skin and underlying soft tissues. SSTIs have variable clinical presentations, etiology and severity. Infections may occur at sites where the skin barrier has been breached, such as a wound or surgical site infection. However, infections may also appear without apparent breach of the skin barrier, such as folliculitis occurring at hair follicles, or furuncles and carbuncles forming at pores. The involvement of deeper layers such as the dermis and/or subcutaneous tissues leads to cellulitis. with the involvement of yet deeper tissues, such as underlying muscle leading to fasciitis (Kobayashi *et al*., 2015)

Skin and soft tissue infections are common and can affect all age groups, however, certain conditions such as trauma, immunosuppression, certain skin conditions and drug use may predispose an individual to SSTIs. *S.aureus* is capable of causing infections at all mentioned sites in the skin and in some instances outbreaks of *S.aureus* SSTIs can occur. These are mainly seen in cases where there is close body contact, in groups such as prisoners, athletes and soldiers. CA-MRSA strains from the lineage USA300 are the most common cause of skin infections and 97% of all MRSA SSTI cases were caused by this lineage. Many *S.aureus* SSTIs are self-limiting, however, complicated SSTIs can occur, and this often leads to the formation of a large

abscess. Abscesses can form in the dermis, epidermis and subcutaneous tissue and function primarily to contain the pathogen, preventing the spread of infection to adjacent healthy tissue (Chen *et al*., 2015)

Although abscess formation is part of the body's defence mechanism, they can cause significant pathology and lead to benign or malignant obstruction in tissues. They can rupture, releasing bacteria into the surrounding tissue and local inflammation at the site of the abscess can lead to painful swelling for the patient. An abscess begins as an acute localized inflammatory response to the invading bacteria. The abscess forms and becomes a collection of pus composed of live and necrotic neutrophils, tissue debris and live bacteria, encased in a fibrous capsule. Severe SSTIs may also lead to dermonecrosis of adjacent skin tissue (Lacey *et al*., 2016).

1.2.4.3. *Staphylococcus aureus* Bacteremia:

S. aureus can cause various infectious diseases, including pneumonia and skin and soft tissue infection, all of which further lead to bacteremia. In particular, loss of integrity of the skin barrier, via decubitus, surgical wounds, and diabetic foot, is the most common portal for *S. aureus* infection. Although several studies reported that intravenous drug abuse was one of the risk factors for endocarditis , local injection site for pain control, including acupuncture treatment , should also be considered a potential portal site (Horino and Hori., 2020).

S.aureus is a leading cause of community-acquired and hospital acquired bacteremia. *S aureus* bacteremia (SAB) can lead to seeding of virtually any body site and ensuing complications. These complications may result in severe disease, resulting in significant morbidity and death. Complications of SAB are common, occurring at

rates that range from 11% to 53%. Some complications more frequently require intensive care admission and carry poor prognosis because of the anatomic site or the difficulty in reaching a timely diagnosis. The risk factors that predispose to developing dissemination and seeding as a consequence of SAB bacteremia depend on the route of acquisition, site of infection, presence or absence of foreign material, pathogen characteristics, and host predisposition (Keynan and Rubinstein., 2013).

1.2.4.4*: Staphylococcus aureus* Pneumonia:

Historically, *S.aureus* was a rare cause of community-acquired pneumonia (CAP). *S.aureus* pneumonia accounts for <5 % of CAP. According to some studies, this percentage increases up to 30–50 % in case of hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), or healthcare-associated pneumonia (HCAP). CAP due to *S.aureus* has been more frequently described in children with cystic fibrosis, after influenza virus infection, and among intravenous drug users (IVDUs), while nosocomial acquisition was more common in severe patients with a longer length of hospital stay, admitted in burn units, and in patients undergoing intubation and mechanical ventilation, This entity has been associated with a high mortality rate (Woods and Colice., 2014).

In children, CAP is the leading cause of death, and also in the elderly (over 80 years of age) the incidence of pneumonia is high. The problem is further aggravated by the emergence and spread of methicillin-resistant *S. aureus* (MRSA). CAP caused by *S. aureus* is relatively rare compared to pneumococcal CAP, but often very severe. In contrast to CAP, in hospital-acquired pneumonia (HAP) *S. aureus* is the most frequent pathogen (Deinhardt-Emmer *et al*., 2019).

1.2.4.5*: Staphylococcus aureus* and Urinary Tract Infection:

Urinary tract infection (UTI) is one of the most common infectious diseases in humans both in the clinical and community settings. Its global incidence is estimated to be 250 million cases each year, *Escherichia coli* is the most prevalent causative organism of UTI, accounting for about 80% of bacterial isolates. However, involvement of Gram-positive bacteria cannot be ruled out in relation to UTI, *S.aureus* is one of such agents involved in the infection that is capable of invading the urinary tract, although *S. aureus* accounts to 0.5-6% of UTI, but if leave untreated infection can lead to severe life threatening condition (Yousefi *et al*., 2016).

Although mortality rates associated with UTI is usually low, antimicrobial therapy should not be overlooked. Increased antibiotic resistance has significantly limited the choice of therapeutic options available for treatment of staphylococcal infections and presents a particularly difficult challenge in these contexts. Due to rapid dissemination of MRSA and multi drug resistance (MDR), therapeutic options are limited when staphylococcal UTI is encountered; therefore, UTI caused by *S. aureus* requires special management (Goudarzi *et al*., 2018).

1.2.4.6. Toxic Shock Syndrome (TSS):

It is an acute, toxin-mediated illness characterized by fever, hypotension, multi-organ dysfunction, and a diffuse rash with desquamation. The disease can be rapidly lethal and is usually treatable, though physicians often fail to recognize this condition. The annual incidence has been suggested to range from 1.5–11 per 100,000 people (Cohn *et al*., 2018).

Cases occur most commonly at the extremes of age, with one study finding that the highest incidence occurred in adults aged > 45 years, followed by children < 5 years, and was lowest in persons aged 16–45 years. Another study found higher rates among children < 2 years of age and adults 65 years of age. TSS is more commonly seen in winter and spring, with a lower incidence in summer and autumn months (Gottlieb *et al.*, 2018).

It is attributed to staphylococcal super antigens that cause massive T-cell activation and cytokine release. TSS toxin 1 (TSST-1) is associated with 95% of menstrual TSS cases and 50% of TSS cases caused by non-menstrual infective foci. Although 24 different staphylococcal super antigens have been described, including staphylococcal enterotoxin (SE) and enterotoxin-like super antigens , SE types A, B, and C are implicated in remaining nmTSS cases, despite the lack of data from Europe (Sharma *et al.*, 2018).

1.2.5. Factors Contributing to *Staphylococcus aureus* pathogenicity:

The virulence of *S. aureus* is related to the potency of virulence factors that the bacteria possess, and the host immune response to the infection. *S. aureus* causes various infections by producing numerous virulence agents that enable it to cause diseases. Many conditions caused by *S. aureus* are mediated by integration of more than one type of virulence factor, including elements that enhance bacterial attachment to cells and tissues of the host, act as anti-phagocytic agents, and that are cell wall virulence factors (capsule, protein A, fibronectin-binding proteins, and clumping factors). In addition, other virulence factors include cytolytic exotoxins that attack immune cells and blood cells such as exotoxins (α, β, γ), and Panton- Valentine leucocidin. Furthermore, *S. aureus* expresses super antigen exotoxins such as enterotoxins (A-G), toxic shock

syndrome toxin (TSST-1), and exfoliative toxins (ETA and ETB). These toxins cause toxin-mediated clinical conditions. In general, producing these proteins enhances the microorganism‘s ability to cause various infections, ranging from simple to life-threatening infections (Rasheed *et al*., 2021).

Surface proteins are involved in adhesion, tissue invasion and evasion of immune defense. Among the virulence factors that contribute to evade immune response, staphylococcal protein A has a key role. Almost all *S. aureus* isolates synthetize this protein, which binds immunoglobulins, inhibits opsonization and phagocytosis, and acts as a superantigen (Balachandran *et al*. 2018).

Clumping factors A and B are also important for adhesion and immune defenses evasion. They bind to fibrinogen facilitating host tissues invasion. Fibronectin binding proteins are able to bind fibronectin and elastin present in the extracellular matrix, promoting invasion of host tissues. Iron regulated surface determinants are involved in staphylococcal iron uptake systems Biofilms are bacterial populations enclosed in an organic matrix. They promote adhesion to surfaces and provide protection against antibiotic and immune defenses (Seilie and Bubeck Wardenburg, 2017).

S.aureus have different host cell targets (monocytes, neutrophils, platelets, erythrocytes and epithelial cells). Panton-Valentine leukocidin (PVL) has been reported to be almost 100 times more potent than the other staphylococcal leukocidins. Among the toxins that act as super antigens, TSST-1 causes a serious illness with high mortality. Both, SEs and TSST-1, act as superantigens triggering T-cell activation and proliferation, and activating cytokine release and cell death. Exfoliative toxins (ETs) cause staphylococcal scalded skin syndrome characterized by destruction of desmosomal cell attachments (desmoglein-1) resulting in detachment of the epidermis (González-Martín *et al*., 2020).

1.2.5.1. Biofilm Formation by *Staphylococcus aureus*:

Many *S.aureus* infections involve formation of biofilms, that is, sessile communities of bacteria attached to surfaces and encased in extracellular matrix. Staphylococcal biofilms appear on implantable medical devices (catheters, prosthetic joints, implants, etc.), but also on host tissues in biofilm-like infections of chronic wounds, endocarditis, or osteomyelitis. As biofilms are resistant to host immune system and antibiotics, they contribute to the persistent and hard-to-treat character of staphylococcal diseases (Kwiecinski *et al.*, 2019).

First *S. aureus* attachment with surface takes place nonspecifically driven by electrostatic, hydrophobic and Lifshitz-Van der Waals forces by passive adsorption mechanism (Mohammed *et al*., 2018)

Second, bacteria adhere to host matrix via microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and secretable expanded repertoire adhesive molecules (SERAMs), third, formation of micro colonies of bacteria by attaching to each other produce extra polymeric substances. Fourth, maturation of the micro colonies in optimal growth conditions and biofilms are established with channels that enable nutrients to flow into the interior of the biofilms (Somerville, 2016).

The most known component of biofilm in *S.aureus* is the polysaccharide intercellular adhesin (PIA) or poly -β(1 -6) - N-acetylglucosamine (PNAG). PNAG is the agent of intercellular adhesion of bacterial cells and bacterial adhesion to external surfaces. Communities of bacteria in the biofilm escape immune responses and cause chronic diseases. The polysaccharide biofilms are called the other names based on the results of analysis of their components, including the capsular polysaccharide adhesion (PS/A) and *S. aureus* exo polysaccharide (SAE) (Parastan *et al*., 2020).

Biofilm forming bacteria produce matrix provide several benefits to the bacterial communities including; protection against immune cells, adhesion (facilitated by bacterial adhesins) and structure, and provide a survival strategy to the bacteria by positioning them to effectively use the available nutrients and prevent access to antimicrobial agents, antibodies and white blood cells; They have also been found to harbor a large number of antibiotic inactivating enzymes such as beta-lactamases hence creating an island of antimicrobial resistance (Katongole *et al*., 2020).

1.2.6. Antibiotics Susceptibility Profile of *Staphylococcus aureus* Isolates:

S. aureus develops resistance to antimicrobial agents by different means, such as horizontal gene transfer of different mobile genetic elements (MGEs), including bacteriophages, plasmids, Staphylococcus cassette chromosomes, transposons, and pathogenicity islands (PAIs) (Bitrus *et al.,* 2018).

All the aforementioned MGEs potentially carry antibiotic-resistant genes, which can be predicted based on the size of the plasmids possessed by bacteria. Small plasmids may carry genes resistant to tetracycline, erythromycin, and chloramphenicol, whereas large plasmids carry resistance genes against macrolides, beta- lactams, and aminoglycosides. On the other hand, larger plasmids carry genes that integrate with other MGEs and produce resistance to erythromycin, vancomycin, beta lactams, trimethoprim, and Spectinomycin (Rasheed *et al.,* 2021).

The development of antibiotic resistance is a key threat to humanity. The World Health Organization recently categorized global resistance threats, and among the organisms of high priority is *S. aureus* while treatment of staphylococcal infections has traditionally been with b-lactam antibiotics, but, as MRSA strains have spread globally,

alternative treatments involve vancomycin, daptomycin, and linezolid. While acquired resistance to vancomycin encoded by the vanA gene is rarely observed, vancomycin-intermediate *S. aureus* (VISA) is a growing concern as vancomycin treatment failure is prevalent in infections with such strains (Haaber *et al*., 2017).

Trimethoprim (TMP) (2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)-pyrimidine) is the well-known di hydro folate reductase (DHFR) enzyme inhibitor and widely prescribed antimicrobial agent . This synthetic antimicrobial drug was fully described by Roth *et al*. and has been used clinically since the 1960s. TMP belongs to a group of antibacterial agents called diaminopyrimidines. During the process of DHFR–TMP binding, the protonated aminopyrimidine group of TMP interacts with the carboxylate group of the enzyme, via a pair of N–H…O hydrogen bonds, generating a hydrogen bonded ring motif (Wróbel *et al*., 2020).

Trimethoprim is a competitive inhibitor of *S. aureus* (DHFR) enzyme, an enzyme in the essential folic acid pathway encoded by the chromosomal gene *dfrB*. It is usually administered with sulfamethoxazole, which inhibits another protein in the bacterial folic acid pathway, and the combination is used to treat urinary tract and soft tissue infections (Fowler *et al*., 2020).

Trimethoprim-sulfamethoxazole is an old antibiotic active against *S.aureus.* Trimethoprim is the main active component and bactericidal in itself, but the combination is highly synergistic. With increasing rates of MRSA infections in healthcare settings and in the community, trimethoprim-sulfamethoxazole has been suggested as a convenient treatment option. Trimethoprim-sulfamethoxazole is recommended for the treatment of uncomplicated skin and soft tissue infections but not for MRSA bacteraemia or pneumonia (Paul *et al*., 2015).

Sulfamethoxazole (SMX) and trimethoprim (TMP) are antibiotics that are most commonly used simultaneously. SMX is used for bacterial infections, such as urinary tract infections, bronchitis, and prostatitis. Besides, it is effective against both gram-negative and gram-positive bacteria, such as *E. coli* and *S. aureus*. TMP is an antibiotic primarily used for the treatment of bladder infections. Other uses of TMP are against middle ear infections and diarrhea in travelers. Common side effects of these two drugs include nausea, flushing, changes in taste, and skin rashes (Mehrabi *et al*,.2021*)*

Tetracycline antibiotics are well known for their broad spectrum of activity, spanning a wide range of Gram-positive and -negative bacteria, spirochetes, obligate intracellular bacteria, as well as protozoan parasites. The first Tetracyclines were natural products derived from the fermentations of actinomycetes. Tetracyclines remain in clinical use for the treatment of uncomplicated respiratory, urogenital, gastrointestinal, and other rare and serious infections (Grossman *et al*., 2016)

It can used in the treatment and prevention of bacterial infections and can be used for treatment of *S. aureus* caused infections such as skin and soft tissue infections (SSTIs). Two main mechanisms of resistance against Tetracyclines have been identified in *S. aureus*: active efflux, which is mediated by plasmid encoded *tetK* and *tetL* genes and ribosomal protection that is encoded by chromosomal or transposonal *tetM* or *tetO* genes (Khoramrooz *et al.*, 2017).

But the dissemination of tetracycline-resistant mechanisms has narrowed their utility, limiting use to only infections with confirmed susceptibility (Grossman *et al.*, 2016).

Aminoglycosides are used to treat different bacterial infections, including infections caused by *S. aureus*. This antibiotic class interrupts protein synthesis and binds to 30S ribosomal subunits. Resistance to aminoglycosides occurs via three

pathways, including mutations in the ribosomal binding site to antibiotics, modifications to aminoglycoside-modifying enzymes (AMEs) that result in drug inactivation, and the efflux pump system. Acquisition of these enzymes is important for aminoglycoside resistance acquired by staphylococci. Examples of the most clinically important AMEs are aminoglycoside acetyl transferases (AACs), aminoglycoside- nucleotidyltransferases (ANTs), and aminoglycoside phosphotransferases (APHs), which are encoded by genetic elements. Staphylococci bacteria develop resistance to different aminoglycoside antibiotics, such as gentamycin and kanamycin, by involving enzymes AAC (6') and APH (2‘) (Rasheed *et al*., 2021).

Aminoglycosides, such as gentamicin, interfere with protein synthesis by binding to the A-site of the 16S rRNA of the 30S subunit and promote inaccurate codon-anticodon recognition that ultimately decreases fidelity of translation, leading to the generation of mistranslated proteins. The bactericidal activity of aminoglycosides is attributed to the production of mistranslated proteins that may incorporate into the membrane, increasing its permeability and leading to uncontrolled small molecule diffusion and eventually increased uptake of aminoglycosides (Vestergaard *et al*., 2019).

In the following years, numerous semisynthetic aminoglycosides were synthesized with activity against resistance caused by different aminoglycoside modifying enzymes. Amikacin, one of the most successful semisynthetic aminoglycosides, was synthesized by acylation with the L-(−)-γ-amino-α-hydroxybutyryl side chain at the C-1 amino group of the deoxystreptamine moiety of kanamycin A .This antibiotic was introduced in 1977, and it is still used with great success to treat a variety of infections although the rise of aminoglycoside 6 -N-acetyltransferases type I are limiting its effectiveness (Ramirez *et al*., 2017)

Fluoroquinolones (FQs) are counted among broad-spectrum antimicrobials and are used to treat genitourinary, respiratory, gastrointestinal, skin and soft tissue infections.. Their mechanism of action is based on the drugs' ability to inhibit DNA gyrase and topoisomerase IV, and thus, DNA synthesis. FQs approved in Europe include ciprofloxacin, levofloxacin, moxifloxacin, ofloxacin, gemifloxacin, cinoxacin, enoxacin, flumequine, lomefloxacin, nalidixic acid, norfloxacin, pefloxacin, pipemidic acid, prulifloxacin and rufloxacin (Kuula *et al*., 2019).

Fluoroquinolone resistance mechanisms include mutational changes in the target site of topoisomerase IV and DNA gyrase and increased expression of endogenous efflux pump systems. Target-site resistance mutations have primarily been located in the DNA gyrase gene, *gyrA*, and the topoisomerase IV gene, *grlA*. Combinatorial mutations in topoisomerase IV and DNA *gyrase* produce higher levels of resistance than a single mutation in either of the targets. *S. aureus* harbors three chromosomally encoded efflux pump systems, namely, NorA, NorB, and NorC, whose overexpression leads to reduced susceptibility to fluoroquinolones. Fluoroquinolones generally have great activity against *S. aureus*, so accumulation of multiple mutations altering the target-site and/ or conferring overexpression of efflux pump systems is needed to exceed the clinical breakpoint (Vestergaard *et al*., 2019).

The mechanism of antibiotic resistance development in *S. aureus* to macrolide, lincosamides and Streptogramins-B occur via the methylation of their receptor binding site on the ribosomes. It is important to note that even though these classes of antibiotics have similar receptor binding site, they are structurally unrelated. Furthermore, the methylation that happens at their binding site is catalyzed by a methylases enzymes which is encoded by erythromycin methylases enzyme *ermA*, B and C whose expression is either inducible or constitutive. All the three classes of antibiotics are constitutive but only macrolide can induce expression of gene coding for erythromycin methylases *erm*

and is also mediated by an efflux pump system encoded by *mrsA*. This however, does not lead to resistance development to Streptogramins or lincosamides (Bitrus *et al.*, 2018).

Clindamycin belongs to the lincosamide class of antibiotics. It disrupts protein synthesis in the bacterial cell by binding to the 50S ribosomal subunit. Resistance to lincosamides occurs through methylation of its receptor binding site on the ribosome, consequently altering the target cell (Rasheed *et al*., 2021).

Chloramphenicol is a broad-spectrum, bacteriostatic antibiotic that interferes with protein synthesis by binding to the ribosomal 50S subunit. Chloramphenicol binds at the peptidyl-transferase center and thus inhibits peptide bond formation between the tRNAs at the A- and P-sites. The wide use of chloramphenicol has been hindered by its toxicity, because systemic administration of chloramphenicol is associated with irreversible aplastic anemia. Due to the severity of the adverse effects of chloramphenicol, it is now used primarily for topical applications, such as a treatment for staphylococcal conjunctivitis (Vestergaard *et al*., 2019).

Chloramphenicol acts by binding to 50S ribosomal subunits and blocking the action of peptidyl transferase. Resistance development to chloramphenicol by *S. aureus* occur through mutation in the *rpoB* gene that codes for the Beta subunit of RNA polymerase and action of an inactivating enzyme called chloramphenicol transferases which inactivates the drug (Bitrus *et al., 2018).*

Macrolides are a class of natural or semisynthetic products, express their antibacterial activity primarily by reversible binding to the bacterial 50S ribosomal subunits and by blocking nascent proteins' progression through their exit tunnel in bacterial protein biosynthesis. Generally considered to be bacteriostatic, they may also be bactericidal at higher doses (Jelić *et al*., 2016).

There are three recognized types of acquired macrolide resistance mechanisms in *S. aureus*: (i) methylation of the ribosomal target (*erm* gene); (ii) active efflux *msr* gene and (iii) inactivation of the macrolide (*mph/ere* gene). In the absence of these genes, mutations in ribosomal proteins have been implicated in macrolide resistance. As azithromycin is a synthetic analogue of erythromycin, it is presumed that these resistance mechanisms are active against both antimicrobials. However, molecular data confirming this association are limited for azithromycin, in particular for the efflux mechanism encoded by *msr*. Further, there are limited data available on the distribution of these macrolide resistance encoding genes following public health interventions using azithromycin (Bojang *et al*., 2019).

1.2.7. The Effect of Plant Extracts on Bacterial Growth:

A vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds as an alternative that can potentially be effective in the treatment of problematic bacterial infections. According to the World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs. Many plants have been used because of their antimicrobial traits, which are due to phytochemicals synthesized in the secondary metabolism of the plant. Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids, phenolic compounds, and flavonoids, which have been found in vitro to have antimicrobial properties (Manandhar *et al.*, 2019).

Sweet and sour lemon: Lemon (Citrus) is a flowering medicinal plant belongs to the family Rutaceae. It is a small evergreen tree native to Asia. Traditionally, the lemon is used to cure soothe sore throats and as an additive for flavoring to our foods. In Oman, it is used traditionally to reduce high blood pressure, mental health, respiratory

problems, arthritis and rheumatism. Recently, it has been a very good herbal medicine for the prevention of kidney stones. In addition, lemon fruit and leaves are used traditionally as wash for oral health to freshen your breath and to treat flaky dandruff, headaches and reduce asthma symptoms. Traditionally, in India, the lemon fruits are used for the treatment of dysentery and asthma. The essential oil of lemon showed fungi toxicity against some fungi. Citrus medical is relevant to treatment of diabetes and Alzheimer's disease. The crude extracts from different parts of the lemon showed anticancer and antibacterial potency (Al-Jabri *et al*., 2018).

Lemon is an important medicinal plant. It is a rich source of vitamin C and it is cultivated mainly for its alkaloids, which are having anticancer activities and the antibacterial potential in crude extracts of different parts (leaves, stem, root and flower) of Lemon against clinically significant bacterial strains has been reported. Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities (Mshelia *et al* ., 2017).

Citrus extract form lemon, lime and grape fruit had antibacterial activity against *Pseudomonas aeruginosa, Staphylococcus aureus, proteus, klebsiella, Escherichia coli* and that lime and lemon juice had better antibacterial activity and compared favorably to the commercial antibiotic disc (Tawfik *et al.*, 2010).

The use of natural ingredients in herbal medicines is considered safer than the use of modern chemical drugs, because the side effects of herbal medicines are relatively small if used appropriately, lemon fruit is a herbal plant that has the main content of alkaloid compounds which have the function as anticancer, antibacterial, antifungal, antiviral and antidiabetic. Alkaloid compounds that responsible for antibacterial is saponin. Lemon (Citrus limon) juice contains many bioactive compounds such as

flavonoids, carotenoids, limonoid, tannin, and terpenoids. The bioactive compounds contained in lemon (Citrus limon) each have an antibacterial (Nurfita , 2017).

Flavonoid compounds have properties that are effective in inhibiting the growth of bacteria, fungi, and viruses because flavonoid compounds include groups of phenol compounds that are able to denaturize bacterial cell proteins and damage bacterial cell membranes. Saponin compounds are antibacterial compounds that damage bacterial cell membranes. Besides that, lemon (Citrus limon) juice contains vitamin C which is useful as an antioxidant. The main ingredient in lemon (Citrus limon) juice is organic acid in the form of citric acid which is contained most in lemon (Citrus limon) juice. The citric acid content of citric acid provides a degree of acidity (pH) of the fruit becomes acidic. Acid pH is one of the factors that can inhibit bacterial growth which can cause the internal pH of bacterial cells to decrease and inhibit bacterial cell growth (Ekawati *et al*., 2019).

1.2.8. Inhibitory Effect of Nanoparticles against *Staphylococcus aureus:*

Nanotechnology is fundamental for drug delivery, with many latent applications in clinical medicine and research. The wide applications of nanotechnology in the field of biomedicine are enormous such as delivery of pharmaceuticals, diagnostic approaches as well as for therapeutic purposes. The choice of nanoparticles for drug delivery is highly favored by their unique chemical and physical properties that hold support for future development of treatment of diseases in drug delivery system with minimal side effect. Nanoparticles are particles used in nanotechnology, with size range of 1–100nm and have completely novel or advanced properties due to their high ratio of surface area to particle size as opposed to their larger counterparts. Metallic nanoparticles are the

subject of research efforts as new platforms for the target-specific delivery of therapeutic agents (Shittu *et al*., 2017).

Nanotechnology has enabled the use of nanoparticles (NPs) for the treatment of antimicrobial-resistant bacteria. The strength antimicrobial NPs' properties results from their large surface area to volume ratio, which also reduces the likelihood of antibiotic tolerance. NPs ranging from 10 to 100 nanometers (nm) in size are thought to possess unique physical and chemical features .Recently, NPs have been used as an alternative method for treatment of various antibiotic-resistant bacterial infections and may solve the problem of multidrug-resistant bacteria (Elbehiry *et al*., 2019).

Gold nanoparticles (AuNPs) have been reported for their most desirable properties as compared to any other noble metal-based nanoparticles, which wider their applications in various fields including catalysis, bio-imaging, biosensors, medicine, biology, and material chemistry (Hameed *et al*., 2020).

Gold nanoparticles (AuNPs) have been identified as an attractive candidate for delivery into their targets. The acceptance of AuNPs as an excellent candidate for drug delivery was due to its unique properties especially in the transport and release of the therapeutic agents to the target site. The therapeutic agents to be delivered could be small drug molecules or large biomolecules, like proteins, DNA, or RNA and effectiveness of their release is a prerequisite for efficient therapy (Shittu *et al*., 2017).

Gold nanoparticles are one of the few inorganic metal nanoparticles that can destroy both Gram-negative and Gram-positive bacteria, and have been shown to be effective against multi drug resistant bacteria (MDR). Pathogenic, biofilm-forming, and invasion bacteria were all blocked by the AuNPs, which also assisted the host immune response. Furthermore, these inhibitory effects are linked to the electrostatic interactions

between AuNPs and the cells they target. Metal nanoparticles' toxicity against pathogenic bacterial cells and biofilms has been linked to the production of reactive oxygen species (ROS) and bacterial membrane destruction (Abdulazeem *et al.*, 2021)

Titanium dioxide (TiO_2) is a multifunctional material, highly employed in the food (chewing gum with mint flavor, dairy products) and pharmaceutical (sunscreens and toothpaste) industries. However, most of the TiO_2 uses, including antibacterial activity, are usually UV-irradiation dependent , in particular for TiO_2 in the anatase phase (active under UV rays at a wavelength of 385 nm or shorter), which is a limiting factor for their potential applications. Furthermore, it has been reported that TiO_2 can be combined with selective elements, forming mixed oxide materials offering an effective method to enhance the physicochemical and antimicrobial properties of TiO_2. It has been reported that TiO_2 in the presence of UV-irradiation exhibits antimicrobial activity against *Escherichia coli*, *Salmonella typhi, Klebsiella pneumonia, Shigella flexneri and S. aureus* (Anaya-Esparza *et al*., 2019).

Titanium dioxide (TiO_2NPs) have also been explored as metallic NPs and have been successfully applied in MRSA therapy. Their application with different combinations of antibiotics, such as cephalosporins, glycopeptides, and azalides, shown anti-MRSA activity in a disk diffusion assay. Under UV photoactivation, TiO_2 NPs form free radicals that lead to their enhanced killing of MRSA (Vanamala *et al*., 2021).

TiO_2NPs can destroy microorganisms upon illumination of light due to its photocatalytic properties .Reactive oxygen species generated by TiO2NPs can oxidize the components of the cell membrane leading to destruction .Antimicrobial activity of TiO2 observed in the absence of light indicate that apart from the photocatalytic activity, direct contact and adsorption cells on to TiO_2nanoparticles may cause a loss of membrane integrity (Fernando *et al*., 2018).

1.2.9. *Staphylococcus aureus* Genotyping:

For bacterial typing to be more useful, the development, validation and appropriate application of typing methods must follow unified properties. The ability to quickly and reliably differentiate among related bacterial isolates is necessary for epidemiological surveillance systems. There are several typing methods used in laboratories today. These methods for bacterial phenotyping have a clear purpose in the confirmation and illustration of local and national healthcare-associated outbreaks due to bacterial strains these range from methods based on simple phenotypic features to DNA sequencing. Previously, the comparison of phenotypic characters, such as colony morphology, color, odor, antibiogram-based typing, biotyping, serotyping and the ability to grow in the presence of specific substances, were used for differentiation. These methods are becoming neglected because they require strict standardization of experimental conditions since phenotypes are extremely susceptible to the environmental conditions. Instead, more reliable genotyping methods have been developed (Chabuck *et al*., 2020; Mubdir *et al*., 2021)

The molecular characterization of bacterial strains is important for the detection of transmission routes and infection sources and for the monitoring of bacterial strain circulation among animal populations (Vitale *et al*., 2018).

Molecular typing of *S. aureus* can be done based on different typing techniques. In the past decade, numerous molecular techniques have been developed and used for the identification and comparison of *S. aureus* isolates in epidemiological studies. The conventional typing procedures have several drawbacks; for example, phage typing as well as genotyping methods using pulsed-field gel electrophoresis are labor-intensive and time-consuming and can be performed only in specialized laboratories (Javid *et al*., 2018).

Molecular typing of MRSA is an essential tool in the surveillance of healthcare-associated infections. Rapid identification of the strains will help control and prevention of MRSA, causing community-acquired or nosocomial infections (Rezai *et al*., 2020).

In recent years, numerous tools have become available for typing of *S. aureus*, ranging from fingerprint-based methods such as pulsed-field gel electrophoresis (PFGE), to PCR-based methods such as multilocus variable-number tandem repeat analysis, to sequence-based methods such as multilocus sequencing typing (MLST), and most recently, to whole genome sequencing (WGS) . The most widely used molecular typing method for defining MRSA epidemiology has traditionally been PFGE. However, PFGE results can be challenging to compare between laboratories and to interpret; furthermore, PFGE is low throughput, is not suitable for long-term epidemiological investigations and assesses a limited amount of the microbial genome. A low mutation rate of the sequence fragments of seven housekeeping genes makes MLST most suitable for long-term and global epidemiological studies. DNA sequencing of short sequence repeats of the polymorphic X region of the protein A gene, spa, consisting of a variable number of 21- to 27-bp sequences, is an alternative method for typing *S. aureus* (Park *et al*.,2017).

Despite the difficulties in reproducibility and interlaboratory reliability, many countries have established a nomenclature for their local pulsotypes through the standardization of PFGE protocols. Sequence-based methods, such as MLST and spa typing are highly reproducible among laboratories, can easily be standardized, and have common names worldwide, rendering these methods more advantageous compared to the PFGE method. Although MLST has a lower distinction power than spa typing, it is a superior method for monitoring clonal evolution. The spa typing method, on the other hand, not only provides an sufficient discriminative power but also has the advantage of being cost-effective as it targets a single locus (Gökmen *et al*., 2018).

S. aureus has many virulence factors that include the surface IgG binding protein A (*spa*) that its characteristic and function is to capture the Fc region of immunoglobulin of most mammalian species; therefore, prevent phagocytosis of the bacterial cells with the host immune system .The gene harboring and encoding protein A (*spa*) consists of some clear and distinct functions: Fc binding, X-region and a C terminus region, a sequence for cell wall attachment. The X-region of the spa gene contains 24-bp repeats with a different number, this allows the study of the genetic diversity in *S. aureus* strains as a molecular marker for epidemiological research of source of infects and the comparison of differences in virulent phenotypes between the strains. During recent decades, *S.aureus* has employed or developed a wide range of phenotyping and genotyping processes (AL-muhanna e*t al*., 2021).

The encoding region consists of a variable number of repeats with highly variable nucleotide sequences. Since it is sequence-based, the results produced in different labs are easily comparable (Van Mierlo *et al*., 2021).

The gene that encodes for protein A (*spa*) is the most widely used marker for molecular typing because it contains polymorphic units. *Spa* genes are also a good choice to be able to identify and distinguish *S. aureus* strain variability (Yunita *et al*., 2020)

Chapter Two

Materials and Methods

Materials and Methods

2.1. Materials

2.1.1. Laboratory Equipment and Instruments:

The main laboratory apparatus and equipment that used in this study are listed in Tables (2-1) and (2-2):

Table (2-1): Laboratory apparatus used in this study

Apparatus	Company/ Origin
Aerobic incubator	Memmert/Germany
Autoclave	Hirayama/Japan
Centrifuge	GFL/Germany
Digital camera	Sony/Japan
Elisa Reader	Biotech / USA
Gel electrophoresis apparatus	Cleaver/England
Hood	Labogene/Denmark
Incubator shaker	Julabo
Light microscope	Olympus/ Japan
Micro Centrifuge	GFL/Germany
Nano drop DNA	UK
PCR Thermal Cycler	Thermofisher / UK
Refrigerator	Arcelik/turkey
Sensitive electronic balance	A and D / Japan
UV- transilluminator	Cleaver/England
Vortex	Gemmy/Tawain
Water bath	GFL/ Germany

Table (2-2): Laboratory equipments used in this study

Equipment	Company/ Origin
Benson burner	Membrane/Germany
Disposable plastic cups	Bio-Hit/ Finland
Glass-wares	Himedia/ India
Micropipette	Afco/Jorden
Millipore filter 0.45µm,0.22 µm	Proway /China
PCR tubes 50µl and 100µl	Eppendorf / Oxford
Petri dishes	Himedia/ India
Plastic test tubes 10 ml	Afco/Jordan
Platinum wire loop	Himedia/ India
Sterile swab	Afco/Jordan

2.1.2. Chemical Materials:

The main chemical materials that were used in this study are listed in Table (2-3) as follow:

Table (2-3): Chemical materials

Materials	Company/ Origin
Ethanol (70%), Glucose, absolute alcohol	GCC /England
Glycerol, hydrogen peroxide	GCC /England
Gram stain kit	Sigma/ Germany
McFarland 0.5 standard	Biomérieux/France
Methyl Red	BDH
Oxidase, Alpha-naphthol, KOH	BDH
Phosphate buffer saline (PBS-pH =7.2) Tablets	B.D.H / England
TiO2 Nanotube (L>1um)	China
Zylole	GCC

2.1.3. Ready-made culture media:

The main ready-made culture media used throughout this study are listed in table **(2-4):**

Table (2-4): Ready-made culture media

Materials	Company/ Origin
Agar agar Blood agar base Brain heart infusion Agar Brain heart infusion broth, MacConky Agar Mannitol salt agar Müller-Hinton agar Nutrient agar Nutrient broth	Himedia /India.

2.1.4. Antibiotic Disks:

The antibiotic discs that are used in this study are shown in Table (2-5).

Table (2-5) Antibiotics disk used in the study (Bioanalyse / Turkey).

NO.	Antibiotic	Symbol	Potency (µg per disk)
1	Amikacin	AK	10
2	Azithromycin	AZM	10
3	Chloramphenicol	C	30
4	Ciprofloxacin	CIP	5
5	Clindamycin	DA	5
6	Gentamicin	CN	10
7	Levofloxacin	LEV	10
8	Tetracycline	TE	30
9	Trimethoprim	TMP	1
10	Trimethoprim/Sulfomethoxazole	SXT	30
11	Vancomycin	VA	10

2.1.5. Molecular-related Materials:

The Molecular related materials used in the present study are shown in Table (2-6)**:**

Table (2-6): Molecular related Materials

Item	Company	Country
100 bp Ladder, consists of : 11 double-stranded DNA fragments ranging in sizes from 100 to 1,500 bp with 100 bp increments. The 500, 1,000 and 1,500 bp bands are double to triple of the intensity of other fragments and brighter, for easier identification and comparison of molecular weight). While all other fragments seem with equal intensity on gel.	Bioneer Promega	Korea USA
Agarose	Promega	USA
Ethidium Bromide Solution, (10mg/ml).	Biobasic	Canada
Geneaid Genomic DNA Isolation Kit	Geneaid	UK
Green master mix 2X Kit, consist of: 1-Taq DNA polymerase. 2- dNTPs, 400µM for each. 3-Tris-HCl (pH 8.5-9.0), 10 mM. 4-KCl, 30 mM 5-$MgCl_2$, 3mM. 6-2eppendroffs of Nuclease free water 7-Stabilizer and tracking dye	Promega	USA
Nuclease free water.	Bioneer	Korea
Primer pairs	Bioneer	Korea
TBE Buffer (Tris-Borate-EDTA), 10X (pH 8.3)	Promega	USA
TE Buffer, 1X (pH 8.0)	Bioneer	Korea

2.1.6 Patients Specimens:

This study includes a total of one hundred and ten (110) different clinical specimens (wounds ,urine and burns) from patients who admitted to Al-Hilla General Teaching Hospital and Al Imam Al-Sadek Medical City for a period from October 2021 to Janurary 2022. These specimens were collected from out-patients and in-patients that admitted with unusual

symptoms that are suspected by physician to be a bacterial infection, were collected using clean, leak-proof container and sterile swabs with a proper way to avoid any possible contamination; taken and close it until transported to laboratory in college, specimens cultivated aerobically on different medium (Nutrient Agar, Blood Agar, MacConkey agar and Mannitol Salt Agar) and incubated at 37^{o}C for 24hrs for bacterial diagnosis; colonies of different morphology were isolated, subculture on other medium, gram stain were tested.

2.1.7. Ethical approval:

The necessary ethical approval was obtained by verbal consent from patients. This study was approved by the committee of publication ethics at University of Babylon\ College of Medicine\ Babylon Province, Iraq.

2.1.8. Study Design

Cross sectional study

2.1.9. Sample Collection

2.1.9.1. Urine Specimen Collection:

Fifty two fresh early mornings, mid-stream urine samples were collected in sterile urine containers, after advising the patients to clean and dry urethral region, to void the first portion of the urine stream into the toilet and later to pass urine into a container. While, urine samples collection from catheterized patients was done by clamping the catheter till the patient sensed the urge to urinate (conscious patient) or the bladder become palpable (unconscious patient), the catheter port was cleaned with 70% alcohol and

10 ml urine were collected using a needle and syringe, later on, the clamp was removed (Vandepitte *et al*, 1991).

Proper labeling to the collection container followed this, and then the specimen was processed within not more than 2 hrs of collection. A loop full urine samples was plated onto the culture media and incubated aerobically at 37 ºC overnight, further diagnostic processing was carried out for plates with positive bacterial culture (Collee *et al.*, 1996).

2.1.9.2: Wounds and Burn Swabs Collection:

The wound and burn specimens are generally collected from patients by using disposable swab, as it is introduced into the site of the lesion; swab must be collected without contamination.

Swab for culture should be placed in its cover immediately; this cover contains normal saline or phosphate buffer saline to maintain the swab wet and transformed to the laboratory during half hour of collection. The swab has been inoculated on culture media and incubated aerobically for 24hrs. at 37°C (Vandepitte *et al*, 1991).

2.2. Methods

2.2.1. Preparation of Solutions and Reagents:

2.2.1.1. Reagents:

2.2.1.1.1. Oxidase Reagent:

This reagent was prepared directly by dissolving 0.1gm of (tetramethyl-P-paraphenylene-diamine-dihydrochloride) in 10 ml of distilled water, to be store in a dark container. Every time used, the reagent has been freshly prepared to detect the ability of bacteria to produce an oxidase enzyme (Forbes *et al*., 2007).

2.2.1.1.2. Catalase Reagent:

This reagent is prepared by adding 3% of H_2O_2 to 1000 ml of distilled water, to be stored in a dark container and detect the ability of bacteria to produce catalase enzyme (Forbes *et al*., 2007).

2.2.1.1.3. Methyl Red Reagent:

A weight of 0.1 gm of methyl red was dissolved in 300 ml of 99% ethanol and then, the volume was completed to 500 ml by distilled water (MacFaddin, 2000).

2.2.1.1.4. Barritt's Reagent of Voges-Proskauer test:

A- Five gm of α - naphthol was dissolved in 100 ml of 99% ethanol alcohol, stored in a dark container in cool place.

B- A weight of 40 gm of KOH was dissolved in 100 ml of distilled water (Collee *et al*., 1996).

2.2.1.2. Solutions:

2.2.1.2.1. Phosphate Buffer Solution (PBS) (pH=7.3):

It has been prepared by dissolving one buffered tablet in 100 ml distilled water and sterilized by autoclave. In accordance with the instruction of manufacturer, after that was kept at 4^oC until used for only one week.

2.2.1.2.2. Agarose Gel Preparation:

The agarose gel was prepared according to the method of Sambrook and Rusell (2001) by adding 1-1.5 gm agarose to 100ml of 1x TBE buffer. The solution was heated to boiling (using water bath or burner) until all the gel particles dissolved. The solution was allowed to cool down reaching to 50-60^{o}C, and mixed with 0.5µg/ml ethidium bromide.

2.2.2. Preparation of Culture Media:

Some of general culture media was prepared according to the instructions of the company and sterilized by autoclaving at 121^{o}C for 15 minutes and 15 PSI.

2.2.2.1 Blood Agar Medium:

Blood agar medium was prepared according to manufacturer by dissolving 40 gm blood agar base in 1000 ml D.W. The medium was autoclaved at 121ºC for 15 min, cold to 50 ºC and 5% of fresh human blood was added. This medium was used as enrichment medium for cultivation of the *S. aureus* to determine their ability of blood hemolysis.

2.2.2.2. Mannitol Salt Agar Medium:

Mannitol salt agar was prepared according to manufacturer by dissolving 111g mannitol salt agar base in 1000 ml D.W. Then autoclaved at 121ºC for 15 min. This medium used for selective and differential isolation of *S. aureus.*

2.2.2.3. Nutrient Agar Medium:

Nutrient agar medium was prepared according to the method suggested by the manufacturing company by dissolved 28gm in 1000ml D.W. It was used for the cultivation of the bacterial isolates when necessary.

2.2.2.4. Brain Heart Infusion Agar Medium

This medium was prepared by dissolving 40 gm of medium to 1000 ml distilled water. The pH was adjusted to 7.2 and then sterilized by autoclave at 121 ^{o}C for 15minutes (MacFaddin, 2000).

2.2.2.5. Nutrient Broth:

This medium was prepared according to the method suggested by the manufacturing company by dissolving 37gm in D.W, then sterilized by autoclaved at 121C˚ for 15 minutes (MacFaddin, 2000).

2.2.2.6. MacConkey Agar (PH: 7.3)

This media was prepared according to the instruction of the industrialized company. McConkey agar is used to isolate most Gram negative bacteria and used to differentiate between lactose fermenter and non-fermenter bacteria.

2.2.2.7. Muller-Hinton Agar medium:

Muller-Hinton agar medium was prepared according to instruction of the manufacturing company by dissolving 38gm in 1000ml D.W. It was used to test the sensitivity of all bacterial isolates to antibiotics susceptibility test.

2.2.2.8. Brain Heart Infusion Broth:

This medium was prepared by dissolving 37gm of medium in 1000 ml distilled water, then sterilized by autoclave at 121^{o}C for 15 minutes. This medium used for the cultivation of *S. aureus.* (MacFaddin, 2000).

2.2.2.9. MR-VP Medium

MR-VP medium was prepared according to manufacture procedure and used to detect the partial and complete hydrolysis of glucose (McFadden, 2000).

2.2.2.10. Maintenance Medium:

The medium consisted of brain heart infusion broth as a basal medium, supplemented with 15% glycerol, after autoclaving at 121^{o}C for 15 minutes and cooling to 45^{o}C. It was distributed in 5 ml sterile test tube. This medium was used to preserve the bacterial isolates at (-20) ^{o}C for long term storage (Collee *et al*., 1996).

2.2.3 Laboratory Diagnosis:

2.2.3.1. Microscopic Examination and Colonial Morphology:

A single colony was taken from each primary positive culture and its identification depended on the morphological properties (colony size, shape, color and nature of pigments, translucency, edge, elevation and texture). Bacterial smear stained with Gram stain was used to check the cellular morphological properties of bacterial cells, including Gram reaction, shape, arrangement, etc.

2.2.3.2. Biochemical Tests:

2.2.3.2.1. Catalase Test:

Catalase is an enzyme that catalyses the release of oxygen from hydrogen peroxide. The nutrient agar medium was streaked with the selected bacterial colonies and incubated at 37 ºC for 24 hrs, then the growth was transferred by the wooden stick and it was put on the surface of a clean slide, a drop of (3% H_2O_2) was added. The formation of gas bubbles indicates a positive result (Forbes *et al*., 2007).

2.2.3.2.2. Oxidase Test:

This test depends on the presence of certain bacterial oxidases enzyme that would catalyze the transport of electrons between electron donors in the bacteria and a redox dye (tetramethyl-ρ-phenylene-diamine dihydrochloride), the dye was reduced to a deep purple color.

A strip of filter paper was soaked with a little freshly made reagent, and the colony to be tested was picked up with a sterile wooden stick and smeared over the filter paper. A positive result was indicated by an intense deep purple color which appeared within 5-10 seconds (Forbes *et al*., 2007).

2.2.3.2.3. Coagulase Test:

The method of Benson, (2001) was followed with some modifications. Several colonies of bacterial growth were transferred with a loop to a tube containing 5 ml of brain heart infusion broth. The tube was covered to prevent evaporation and incubated at 37°C in the incubator overnight. Then the tube mixed and centrifuged, 0.5 ml of the supernatant withdrawn and mixed with 0.5 ml of human plasma, then incubated in the

water bath at 37°C for several hours. If the plasma coagulates, the organism is coagulase-positive. Some coagulations occurred in 30 minutes or several hours later. Any degree of coagulation, from a loose clot suspended in plasma to a solid immovable clot, was considered to be a positive result, even if it takes 24 hours to occur.

2.2.3.2.4. Mannitol Fermentation Test:

Mannitol salt agar was inoculated with bacterial colonies then incubated at 37C° for 24 hours. The color changed from pink to bright yellow when the bacteria was mannitol fermenter and mean positive result, while unchanging color of the medium was a negative result.

2.2.3.2.5. Hemolytic Reaction:

Blood agar medium was streaked with a pure culture of bacterial isolate to be tested and incubated at 37Cº for 24-48 hrs. The appearance of a clear zone surrounding the colony is an indicator of β- hemolysis while the greenish zone is an indicator of α- hemolysis (Forbes *et al.*, 2007).

2.2.3.2.6. Methyl –Red Test:

This test employed to detect the production of sufficient acid during the fermentation of glucose that was shown by a change in the color of methyl red indicator. The test was performed by preparing (MR – VP broth) with 5 ml in each tube. The tubes were inoculated with bacterial colonies, then incubated for 24 hours at 37°C. After that 6-8 drops of methyl –red reagent was added. The change of color to orange- red indicated a positive result (MacFaddin, 2000).

2.2.3.2.7. Voges – Proskauer Test:

This test was used to detect the production of acetyle methyl carbinol from carbohydrates fermentation, or its reduction products. It was performed by preparing MR –VP broth 5 ml in each tube, inoculated with bacteria colony, then followed by incubation for 24 hours at 37^{o}C, after that 15 drops of 5% α- naphthol (reagent A) were added followed by 10 drops of 40% KOH (reagent B) shaken well and allowed standing up for 15 minutes before considering the reaction as negative. When positive, the culture turned red at the surface of the broth and the color spread gradually throughout the tube. The positive result indicated a partial analysis of glucose which produced acetyl methyl carbinol or its reduction product (Collee *et al.*, 1996).

2.2.3.3. Biofilm Formation:

2.2.3.3.1. Tissue Culture Plate Method (TCP):

This quantitative test described by Christensen *et al*. (1985) is considered the gold-standard method for biofilm detection. Organisms isolated from fresh agar plates were inoculated in 10 mL of BHI broth with 1% glucose. Broths were incubated at 37C^{0} for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well- flat bottom polystyrene tissue culture treated plates were filled with 200 µL of the diluted cultures.

The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37^{o}C for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were

fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) at 570nm of stained adherent biofilm was obtained by using Spectrophotometer and compared with results at table (2-7) (Mathur *et al.*, 2006).

Table (2-7): Tissue Culture Plate Method (TCP)

Mean OD Values	Biofilm Formation
< 0.120	None
0.120-0.240	Moderate
> 0.240	High

2.2.3.4. Antibiotic Susceptibility Testing (Disk Diffusion Test):

It was performed by using a pure culture of previously identified bacterial isolate. The most effective antibiotic for each bacterial isolate was determined as recommended by CLSI, (2020).

A- The inoculums to be used in this test was prepared by adding 5 isolated colonies grown on blood agar plate to 5 ml of nutrient broth and incubated at 35^{o}C for 18 hours and compared with (0.5) MacFarland standard tube (1.5 x 10^8 CFU/ml) .

B- A sterile swab was used to obtain an inoculums from the bacterial suspension. These inoculums were streaked on a Mueller-Hinton agar plate and left to dry.

C - The antibiotic discs were placed on the surface of the medium at evenly spaced intervals with flamed forceps or a disc applicator and incubated for 18 hours.

D -Inhibition zones were measured using a ruler and compared with the zones of inhibition determined by CLSI, (2020).

2.2.3.5. Plant Extract Collection:

Fresh sweet and sour Lemon fruits used in this study were obtained from the local market at Hilla City, Iraq, 2022. The fresh fruits were washed in running tap water in laboratory, surface sterilized with 70% alcohol, rinsed with sterile distilled water and cut open with a sterile knife and the juice pressed out into a sterile universal container separately and then filtered (using Millipore 0.45 filter paper) into another sterile container to remove the seeds and other tissues and used freshly without refrigeration (Hindi *et al*., 2013).

2.2.3.6. Effect of Plant Extract (Antimicrobial Activity of fresh Lemon Juice):

The screening of antimicrobial activity of fresh lemon juice on the tested bacteria was examined on Muller Hinton agar media using agar well diffusion method. Wells of 6 mm diameter and 5 mm depth were made on the solid agar using a sterile glass borer (CLSI, 2002; Prescott *et al*., 2002).

Approximately 20µl of each concentration (100%, 75%, 50% and 25%) of each extract were inoculated onto wells, after plate cultivation with spread technique of each microbial isolates. (The plates were performed in triplicates); uncultivated broth (used as a negative control) and results of antibiotic disk diffusion (used as a positive control). All plates were allowed to incubate at 37°C for overnight. After 24 hours of incubation, zone of inhibition for all isolates and concentrations were checked. The diameters of the zone of inhibitions were measured by measuring scale in millimeter.

2.2.3.7. Preparation of Nano Particles

2.2.3.7.1 Preparation of Gold Nano Particles

It is a chemical procedure were gold nanoparticles (AuNps) prepared as a solution, the size of gold nanoparticles is 35 nm, it is simple and result in a spherical gold nanoparticles which is testing transmission electron microscopy (TEM), Scanning Electron Microscopy (SEM). Material for synthesis Gold Nano particles include:

A-Chloroauric acid

B-Trisodium citrate dehydrate (TCD) as reducing agent.

For preparing the Gold Nanoparticles a stock solution was made from Chloroauric acid and trisodium citrate dehydrate**,** then to prepare this stock solution (2%Chloroauric acid) we dissolved 2g of Chloroa in 100ml deionized water , and for preparing 1%stock solution of Trisodium citrate dihydrate dissolve 1g in 100ml deionized water. After preparing the stock solutions, 150µl of Chloroauric acid solution plus 50ml of deionized water must heat up to 100°C then 500µl of Trisodium citrate solution was added (stirrer with heating at 100°C) until the clear color change into red color indicating the Gold Nano particles(Wang *et al*., 2003). Scanning electron microscopy was used to verify the uniformity of particle size and shape (30-35nm) and spherical shape.

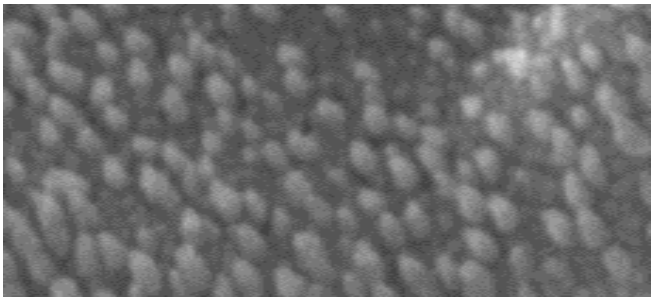

Figure (2-1)The Scanning electron microscope (SEM) image of Gold Nanoparticles

2.2.3.7.2. Preparation of TiO_2 Nanotube

Ten mg of TiO_2 nanotube, the size of TiO_2 was 3-50nm, dissolves in 10 ml D.W to prepare a suspension of 1000µg/ml, continuous ultra-sonication (over-night) for the suspension was achieved at the time of preparation and each time prior to use so as to re-disperse the particles in the solution (Kharisov et al., 2013). Transmission electron microscopy was used for material imaging and elemental composition analysis of TiO2 (Iran)

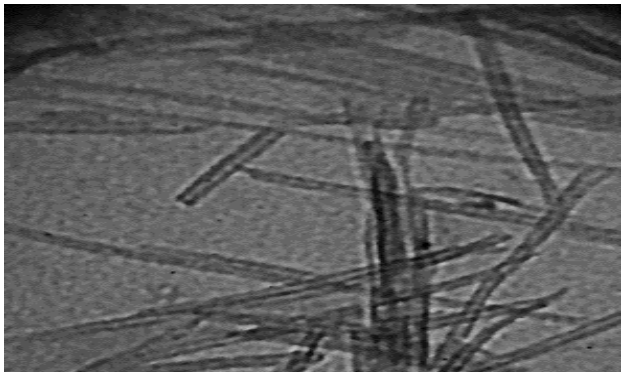

Figure (2-2) The transmission electron microscope (TEM) image of TiO_2 Nonoparticles

2.2.3.8. The Effect of AuNPs and TiO_2NPs on Bacterial Growth at 24 and 48 hours Incubation Time:

One replicate of nutrient broth used as a negative control in column number 1 and two replicates of positive control representing bacterial growth suspension (*Staph aureus*) in columns number 2,3 wells respectively of 96 well plate of 300 µl volume, while Columns 4 and 5 wells were filled with bacterial suspension that exposed to gold nanoparticles AuNPs with volumes of 150 µl for each one. columns 6 and 7 of wells replicate were filled with volumes of 200 µl for bacterial suspention and 100 µl of AuNPs, columns 8 and 9 wells replicate were filled with volumes of 150 µl for bacterial suspension and Titanium dioxide Nanoparticles for each one. while

10 and 11 columns of wells replicates were filled with 200 µl of bacterial growth suspension and 100 µl of TiO_2NPs.

Then, the plate was covered with a self-plastic lid and incubated once for 24 hours in the Incubator and the effect of AuNPS (Gold Nanoparticle) and Titanium dioxide Nanoparticles was read by ELISA reader at 405 nm. For 48 h the experiment was applied with the same conditions used in 24h experiment but the incubation period was 48 h (Al-Jbory *et al.*, 2019).

2.2.3.9. The Effect of AuNPs and TiO_2NPs on Bacterial Biofilm Production:

Procedure of biofilm production (2.2.3.3.1) mentioned above was repeated with the application of AuNPs and Tio_2NPs separately in order to detect their inhibitory effects on biofilm production. These nanoparticles were applied separately to the 96 well plate in a double serial dilution. Then after, optical density (OD) at 570nm of stained adherent biofilm was obtained by using Spectrophotometer and compared with OD that obtained in detection of biofilm production test (Chlumsky *et al*., 2020).

2.2.3.10 Genotyping Assays:

2.2.3.10.1. DNA Extraction:

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (UK). Chromosomal DNAs obtained were used as templates for all PCR experiments. The PCR reactions were carried out in a Thermal Cycler. Before PCR assay, DNA profile were performed by using bacterial DNA

and loading buffer without thermal cycling conditions, and according to the following steps:

Step1: Cell Harvesting/pre-lysis

One ml of bacterial suspension containing approximately (equal to 4.0 McFarland standard) was transferred to a 1.5ml micro centrifuge tube, centrifugation for 1 minute at 14-16,000×g and discard the supernatant.

- A volume of 200 µl of Gram Buffer added to 1.5 ml microcentrifuge tube then 200µl of lysozyme buffer was added to the Gram Buffer then vortex to completely dissolve the Lysozyme.
- Added 200µl of Gram Buffer to the sample in the 1.5 ml microcentrifuge tube, incubated at 37^{o}C for 30 minutes. During incubation invert the tube every 10 minutes.
- Added 20 µl of proteinase K then mix by vortex, incubate at 60^{o}C for at least 10 minutes. During incubation invert the tube every 3 minutes.

Step 2: Lysis

- A volume of 200 µl of GB buffer was added to the sample and mix by vortex for 10 seconds, then incubated at 70ºC for 10minutes or until the sample lysate is clear. During incubation, the tube was inverted every 3 minutes. At this time, the required Elution Buffer (200 µl per sample) incubated at 70ºC (for step 5 DNA elution).
- Following 70ºC incubation, 5 µl of RNase A (10mg ml) was added to the clear lysate and mixed by shaking vigorously.

Step 3: DNA Binding

- A volume of 200 µl of absolute ethanol was added to the sample lysate and immediately mixed by shaking vigorously. If precipitate appears, broke it up by pipetting.

❖ GD column was placed in a 2ml Collection Tube.

❖ All of the mixture (including any precipitate) was transferred to the GD Column and centrifuged at 14-16,000×g for 2 minutes.

❖ The 2 ml Collection Tube flow-through was discarded and placed the GD Column in new 2 ml Collection Tube.

Step 4: Washing

❖A volume of 400 µl of W1 Buffer was added to the GD Column.

❖Then, centrifugation at 14-16000×g for 30 second

❖The flow-through was discarded and placed the GD Column back in the 2 ml Collection Tube.

❖A volume of 600 µl of Wash Buffer (ethanol added) was added to the GD Column.

❖Centrifugation at 14-16,000×g for 30 seconds.

❖The flow-through was discarded and placed the GD Column back in the 2 ml Collection Tube.

❖Centrifugation again for 3 minutes at 14-16,000×g to dry the column matrix.

Step 5: DNA Elution

❖The dried GD Column was transferred to a clean 1.5 micro centrifuge tube And 100 µl of preheated Elution Buffer was added to the center of the column matrix and let stand for 3-5 minutes or until the Elution Buffer is absorbed by the matrix.

❖Centrifugation at 14-16,000×g for 30 seconds to elute the purified DNA.

2.2.3.10.2. Detection of DNA concentration or purity by Nano drop:

The extracted DNA was checked by using nanodrop spectrophotometer, which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260/280nm) as following steps:

1. After opening up Nanodrop software, chosen the appropriate application (Nucleic Acid, DNA).
2. A dry wipe was taken to clean instrument pedestals several times. then carefully pipette 2 µl of ddH2O on to the surface of the lower measurement pedestals for blank system.
3. The sampling arm was lowered and clicked OK to initialized the nanodrop, then cleaning off the pedestals and 1 µl of extracted DNA carefully pipette onto the surface of the lowered measurement pedestals,then concentration and purity of extracted DNA was checked (wilfinger *et al*., 1997).

2.2.3.10.3. DNA Amplification by Polymerase Chain Reaction (PCR):

For amplification of the polymorphic X region of the *Staphylococcus* protein A gene (spa), a PCR was performed in a total volume of 25 µl (Primers 1.5 x2, DNA 3 µl, Master mix 12.5 µl, nuclease free water 6.5 µl), then DNA amplification was carried out with the thermal cycler by the application of primers with their sequences and amplification condition listed at tables (2-8 and 2-9).

Table (2-8): PCR condition of primers used in this study

PCR condition		
Stage	Temp.(ºC)	Time (sec.)
Initial denaturation	94	5 min
Denaturation	94	45 sec
Annealing	60	45 sec for 35 cycles
Initial extension	72	90 sec
Final extension	72	10 min

Table (2-9): Primers used in this study with their sequences

Primer		**Sequence**	**Product size(bp)**
***spa*-1113**	**F**	(5′-TAAAGACGATCCTTCGGTGAGC-3′)	200-500
***spa*-1514**	**R**	(5′-CAGCAG TAGTGCCGTTTGCTT-3′)	

2.2.3.10.4. Agarose Gel Electrophoresis:

Agarose gel prepared at (Sambrook and Russell, 2001) then the tapes were placed across the ends of the gel tray. The comb was fixed at one end of the tray for making wells used for loading DNA samples. The agarose gel was poured gently into the tray, and allowed to solidify at room temperature for 30 minutes.

Then the comb was removed gently from the tray and the tapes were also removed from the ends of the tray. The latter was fixed in

electrophoresis chamber which was filled with TBE buffer as (5 ml TBE for each 45 ml distilled water) about 300 ml covered the surface of the gel.

In the 1st. well 5 µl ladder were added (100 bp ladder was run along-side the samples as a molecular weight marker), then 5 µl of each PCR products were added respectively in each well.

The electric current was used at 60 volt for half to 1hrs. UV transilluminator was used at 320 or 336 nm for the observation of DNA bands, and the gel was photographed using digital camera.

2.2.3.9.5. DNA Sequencing and *Spa* Analysis.

All sequencing reactions were carried out with an ABI Prism BigDye Terminator cycle sequencing ready reaction kit at Macrogen Company, Korea, by using the ABI 3100 Avant Genetic Analyzer in accordance with the instructions of the manufacturer (Applied Biosystems).

Spa types were assigned by using StaphType software (version 1.4; Ridom GmbH, Würzburg, Germany), as described by Harmsen et al. (2003). Moreover, sequence annotations (repeat score) of studied isolates was done according to Kreiswirth Method (Shopsin *et al*., 1999; Koreen *et al*., 2004).

2.2.3.11. Statistical Analysis:

All study data were analyzed by the use of Chi-square through SPSS version 18.0. The 95% confidence interval of a proportion was used to calculate the significances. P-value that was obtained as less than the 0.05 the level of significance was considered statistically significant.

Chapter Three

Results and Discussion

3.1. Isolation and Identification of *Staphylococcus aureus* Isolates:

3.1.1. Isolation of *Staphylococcus aureus*:

A total of 110 clinical specimens were collected during this study, among them, 23 isolates of *S. aureus* were recovered, while 57 isolates belonged to other bacterial genera, and 30 isolates show negative culture result.

The isolates of *S. aureus* 23 (20.9%) were distributed as following: 5/33 (15.2%) isolates from burns and 8/25 (32.0%) from wounds, while 10/52 was (19.2%) isolates from urine; as shown in (Table 3-1).

Table (3-1): Numbers and percentages of *S.aureus* isolates recovered from different clinical specimens

Sample Type		Culture		Total
		Positive for *Staphylococcus aureus*	**Negative for *Staphylococcus aureus***	
Burns	**Count**	**5** **15.2%**	**28** **84.8%**	**33** **100.0%**
Wounds	**Count**	**8** **32.0%**	**17** **68.0%**	**25** **100.0%**
Urine	**Count**	**10** **19.2%**	**42** **80.8%**	**52** **100.0%**
Total	**Count**	**23** **20.9%**	**87** **79.1%**	**110** **100.0%**

In this study the existence of *S.aureus* isolates was found in 23 samples isolated from 110 different clinical samples and represented 20.9% from all total samples percentage, these were classified according to the source of collection as follow.

S. aureus isolates were present in (8) wound samples from (25) wound samples and this result represent (32.0%). while *S. aureus* isolates found in (10) samples only from (52) urine samples and this result represent (19.2%), on other hand (5) *S. aureus* isolates were obtained from (33) burns samples and this result represent (15.2%)

S. aureus isolates from wounds were (32.0%), and this result is similar to AL-Enawey *et al*., (2020) who found that the result of *S. aureus* from wound were (32.0%), and this percentage is also closely similar to the result obtained by Bessa *et al*. (2015) who found that isolation rate of *S. aureus* is (37%), but it is more than Adhikari *et al*. (2020) whose found that percentage of *S.aureus* were (16.5%), but less than Pal *et al*. (2019) who found that *S. aureus* is the most common pathogen, with a prevalence percentage (69.9%).

The high prevalence of wound infections caused by *S. aureus* may be due to this infectious agent is most likely associated with endogenous source as it is a member of the skin and nasal flora, and also with contamination from the environment, surgical instruments, or from hands of healthcare workers (Pal *et al*., 2019).

As shown in Table (3-1), *S. aureus* isolates recovered from urine (19.2%) which was closely to Al-Naqshbandi (2019) who obtained 15 isolates of *S. aureus* from urine sample in a percentage (18.99%), also was close relative to Marami *et al.,* (2019) who found the percentage of *S. aureus*

was (11.1%), but was more than the result obtained by Sharma *et al*,. (2019) which was (5.0%).

In present study, most patients were catheterized, this is the justification for high result in present study and this can be explained by its ability to attach to and aggregate onto uroepithelial cells, *S. aureus* is therefore, an important causative pathogen of UTI (Kitano *et al*., 2021).

The Prevalence of *S.aureus* in UTI was lower when compared with other causes like *E.coli* and *Klebsiella* , but they are many factors increase the incidence rate of UTI infection caused by *S.aureus* like age, female, gender, married individuals, diabetes and hospitalization patients and this may explained the high detection rate. UTI cause by *S. aureus* are produce ascending urinary tract colonization and infection (Selim *et al*., 2022).

S.aureus isolates from burns (15.2%) which were closely relative to the result obtained by Chen *et al.*, (2021) in percentage (21.68%) and less than the result obtained by Mahmoudi *et al*., (2019) that was (62.5%), and Al-Byti (2019) which was (33.33%), and this is may be due to *S. aureus* is the predominant species responsible for burn infections. Because this organism is easily transmitted via close contact whether from patients' relatives, medical staffs or even from sites of its normal resident, and it requires long-term treatment. This organism is easy to be detected but difficult to be eliminated, especially during progression to systemic infection (Chen *et al*., 2021).

3.1.2. Identification of *Staphylococcus aureus* Isolates:

The identification of *Staphylococcus* is done according to (Forbes *et al.*, 2007), by depending on colonial morphology (shape, odor and mannitol fermentation on Mannitol salt agar) and microscopic examination that

include the morphology of bacterial cell is investigated by gram-stain. After staining, specific biochemical tests were done to each isolates for final identification as shown in Table (3-2).

Table (3-2): Diagnostic features of *Staphylococcus aureus* isolates:

Tests	***S. aureus***
Gram stain	Gram-positive
Shape of bacteria	Cocci grape-like clusters
Catalase	+ve
Oxidase	-ve
Mannitol fermentation	+ve
Coagulase	+ve
Hemolysis (Blood agar) (Beta hemolysis)	+ve
Voges-proskauer	+ve
Methyle red	+ve

3.2. Detection of Antimicrobial Agents Effect on *Staphylococcus aureus*:

3.2.1. Susceptibility of *Staphylococcus aureus* to Antibiotics:

In this study, Ten (10) antibiotic types were applied for all *S. aureus* isolates for testing their susceptibility and to identify the most effective one

against *S.aureus*; unfortunately, the overwhelmed *S. aureus* isolates that recovered from clinical specimens were resistant to most antibiotics that used in present study, and these results in details were as follow; as shown in figure (3-1): *S. aureus* isolates showed high resistance to Trimethoprim (73.9%) while resistance to Clindamycin and Tetracyclin was (65.2%) and (60.9%) respectively. *S.aureus* show less resistance to Azithromycin (43.5%) and to Amikacin (39%) while resistance to Ciprofloxacin was (34.8%) and for Trimethoprim Sulfomethoxazole was (30.4%) while the resistance percentage to Levofloxacin was (26%) and to Gentamicin (21.7%) while chloramphenicol showed the lowest resistance percentage by *S. aureus* constituting (17.4%).

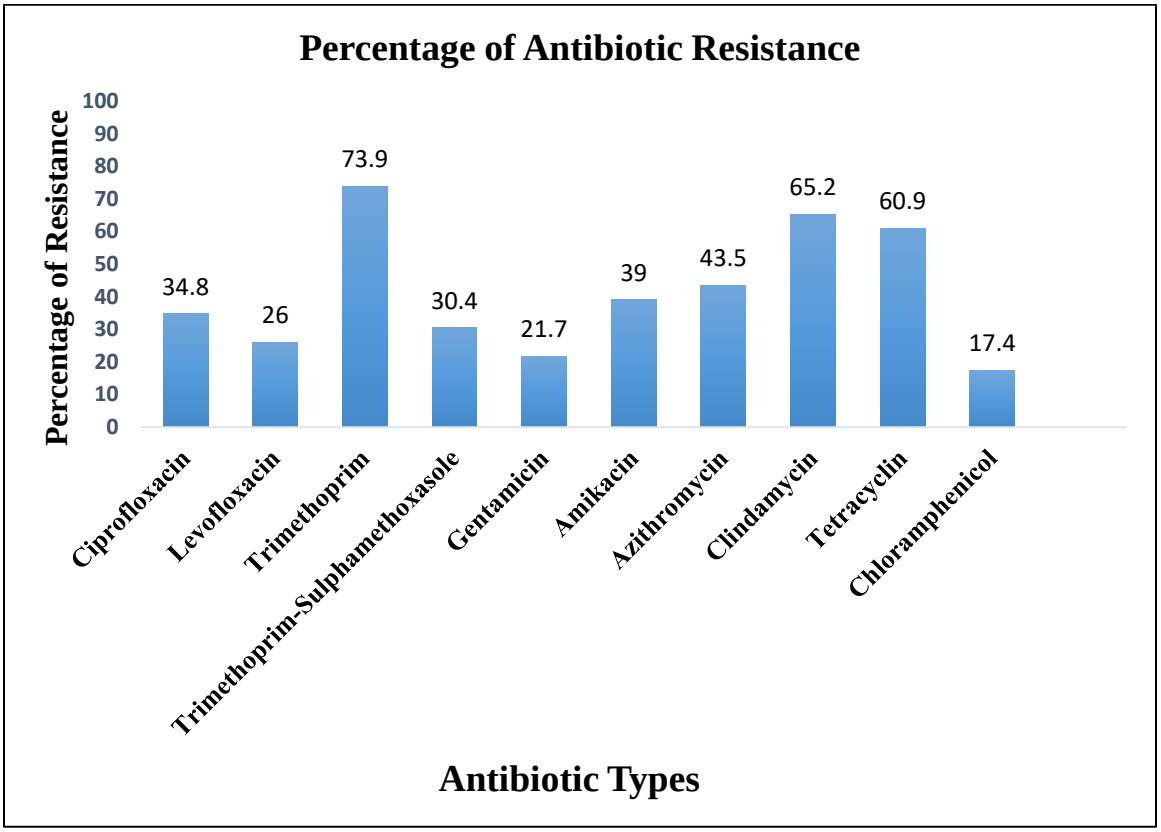

Figure (3-1): Percentages of antibiotic resistance among *S.aureus* isolates to different antibiotic types by disk diffusion method

Results of this study revealed that *S.aureus* showed a highly resistance values to most of the commonly used antibiotics in hospitals where development of antibiotic resistance is often related to the overuse, and misuse of the antibiotics prescribed. As Iraq is one of the developing countries where all types of antibiotics are sold over the counter, an attitude that encourages self-medication resulting in this antibiotic resistance problem. Also this finding was alarming as infection due to multi-drug resistant *S.aureus* isolates are difficult to treat (Ruppe *et al.,* 2019).

Results revealed that (73.9 %) of *S. aureus* isolates were resistant to Trimethoprim while resistance of *S. aureus* isolates to Trimethoprim Sulfomethoxazole combination was (30.4%) which is the same result obtained by Mohammed *et al*. (2018) who found *S.aureus* isolates resistance to Trimthoprim Sulfomethoxazole (30.4%), and this result was greater the resistance result obtained by Matallah *et al*. (2019) who showed intermediate susceptibility (5.26%).

Trimethoprim and sulfamethoxazole have a greater effect when given together than when given separately, because they inhibit successive steps in the folate synthesis pathway (Wormser *et al*., 2012). As, Sulfamethoxazole, a sulfonamide drug, is a structural analogue of para-aminobenzoic acid and inhibits synthesis of the intermediary dihydrofolic acid from its precursors. Trimethoprim is a structural analogue of the pteridine portion of dihydrofolic acid that competitively inhibits dihydrofolate reductase and, consequently, the production of tetrahydrofolic acid from dihydrofolic acid. This sequential blockade of 2 enzymes in one pathway results in an effective bactericidal action (Eyler and Shvets, 2019).

Resistance to Trimethoprim (TMP) in *S. aureus* can either from mutations in the chromosomal gene *dfrB*, or from the introduction of other

naturally resistant genes (*dfrA*, *dfrG*, and *dfrK*) via plasmids (Fowler *et al*., 2018).

S.aureus showed resistance rate to Clindamycin in a percentage (65.2%) and this result was closely relative to the result obtained by Kishk *et al*. (2020) who found resistance rate to Clindamycin in a percentage (50%), and greater than Mohanty *et al*. (2019) who found resistance rate to clindamycin (44.3%), this may be due to Clindamycin resistance can be constitutive or inducible depending on the presence or absence of macrolide inducer. Erythromycin is an inducer of clindamycin resistance by stimulating the production of erythromycin ribosome methylase (*erm*) that induce the expression of clindamycin resistance. Constitutive clindamycin resistance isolates, where methylase is always produced, are resistant to both erythromycin and clindamycin due (*erm*) gene expression. Inducible clindamycin resistance isolates display resistance to erythromycin but falsely susceptible to clindamycin on disc diffusion method. Modification of the target site, efflux pump and drug inactivation are the main mechanisms of macrolide and lincosamide resistance in clinical isolates (Kishk *et al*.,2020)

Moreover, *S. aureus* isolates showed resistance rate (60.9%) to tetracycline and this result was closely related to the result obtained by Omwenga *et al*. (2021) who found that (74%) of *S. aureus* isolates were resistant to Tetracyclin, and more than Bastidas *et al*. (2019) who found resistance percentage (19.4%); Resistance to tetracyclines is usually attributed to one or more of the following: the acquisition of mobile genetic elements carrying tetracycline-specific resistance genes, mutations within the ribosomal binding site, and/or chromosomal mutations leading to increased expression of intrinsic resistance mechanisms. Three general class-

specific mechanisms have been well described: efflux, ribosomal protection, and enzymatic inactivation of tetracycline drugs (Grossman, 2016).

In the present study bacterial isolates of *S. aureus* exhibited resistance to Azithromycin in (43.5%) percentage and this result was more than Gui *et al*. (2014) who found that the antibacterial effect of Azithromycin was 63.5%, and less than Cogen *et al*., (2018) who obtained that *S. aureus* strains increased from 7% before azithromycin use to 52.5% after azithromycin initiation. The problem in staphylococcal infections is an increasing resistance to macrolide because of their extensive use against Gram-positive bacteria and due to its ability to rapidly acquire resistance, where the macrolide resistance genes are found on plasmids, transposons, and genomic islands and can be easily transferred horizontally between strains and species; thus it is better to limit their irregular usage to reduce resistance and maintain the beneficial activity of the drug in treating different infectious agents (Febler *et al*., 2018).

The percentage of resistance of *S. aureus* for amikacin and Gentamicin were (39%) and (21.7%) respectively. These results were less than the result obtained by Darvishi *et al*. (2016), who found that the isolates had resistance rate (71.42%) for Amikacin, and less than Gebremedhn *et al*.,(2016) who found that all bacterial isolates showed resistance (100%) to Amikacin, and this result was less than the result obtained by Rahi *et al*. (2020) who found the resistance percentage to gentamicin was (78.57%) while this result was less than Preeja *et al*. (2021) who found that bacterial isolates have showed resistance to gentamicin (40.7%).

Most common cause of staphylococcal resistance to aminoglycosides is due their thick peptidoglycan, and as with all antibiotics,

resistance to aminoglycosides is becoming increasingly prevalent. Repeated use of aminoglycosides, especially when only one type is employed, leads to an increased incidence of resistance (Watkins *et al*., 2019). Also, Gentamicin is more active than amikacin against most type of gram-positive bacteria as *Staph aureus*; some strains of *S.aureus* can inactivate amikacin by phosphorylation and adenylation (Aronson, 2016).

Another opinion show that Amikacin is unaffected by many of the modifying enzymes that inactivate gentamicin and tobramycin and is consequently more active against staphylococci (Roger *et al*., 2011).

In the present study resistance to Levofloxacin and Ciprofloxacin have been studied and the percentage of resistance of *S. aureus* to those antibiotics were found to be (26%) and (34.8 %) respectively. and this result was closely relative to the result obtained by Rahi *et al*., (2020) who had found that the resistance to Levofloxacin (32.14%). While this result was more than Preeja *et al*., (2021) who found Levofloxacin resistance (12.3%) , Whereas this result was less than the result obtained by Darvishi *et al*. (2016), who found that the isolates had resistance rate (85.71%) for Ciprofloxacin. Whilst Gebremedhn *et al*. (2016) who obtained that reistance to Ciprofloxacin was (16.7%) and this result was less than the result obtained in this study.

In this study *S. aureus* showed resistance percentage (17.4%) to chloramphenicol and this result was closely relative to the percentage obtained by Younis *et al* (2018) who found that resistance of *S. aureus* isolates was (18.75%), but less than Wu *et al* (2019) who found the resistance to chloramphenicol was (38.0%). But Adhikari *et al* (2020)

discovered that resistant of *S. aureus* to chloramphenicol was (6.4%). This makes it a good option for treatment of different staphylococcal infections.

3.2.2 Effect of Sour lemon and Sweet Lemon on *Staphylococcus aureus* Growth:

This study examined the effect of lemon juice of both types sour and sweet in different concentrations (100%, 75%, 50% and 25%) for each one on *S. aureus* isolates and their effect was expressed in percentage value; as revealed in figure (3-2) and there were statically significant differences (Pvalue = 0.04) between the inhibitory effects of the sour lemon. The 1st.two concentrations of sweet lemon also when compare them with effect of antibiotics. While there was no significant differences (Pvalue = 0.085) between inhibitory effect of sour and sweet lemon.

These results showed that Sour Lemon had higher inhibition effect on *S. aureus* in (100% and 75%) concentrations; as the inhibition for *S. aureus* growth was in a percentage (91.3%) for both concentrations, while in (50%) concentration sour lemon made inhibition effect of (82.6 %) of bacterial growth while (25%) concentration made (52.2%) inhibition effect of *S. aureus* growth. While sweet lemon showed lower effect in its different concentrations in (100%) concentration made (74%) inhibition effect on *S. aureus* while (75%) concentration of sour lemon inhibit (56.52 %) of bacterial isolates growth while (50%) concentration of sweet lemon inhibit (30.43%) of *S. aureus* growth and (25%) concentration inhibit (13.04%) of bacterial growth.

The result of this study showed compatibility with Moosavy *et al*,. (2017) result who found the lemon had a significant inhibitory effect against *S. aureus*, and also show agreement with Hindi *et al*. (2013) who showed

that The extract from the juice of *C. limon* presented the highest antimicrobial activities, as it inhibited 13 isolates (out of 15 isolates used) of the bacteria under the study whether Gram positive or negative with inhibition zone ranging from (10-30mm) and with Aburowais *et al.*, (2017) who found that Lemon juice showed inhibition against all the test organisms (Gram positive and Gram negative) with a maximum zone of inhibition against *S. aureus*. Also this study agreed Aziz *et al* ., (2018) who suggested that 50% of citrus fruit juices have a potential antimicrobial effect against selected enteric pathogens especially *E. faecalis*, who use fresh lemon juice as a compensating chemical irrigation solution.

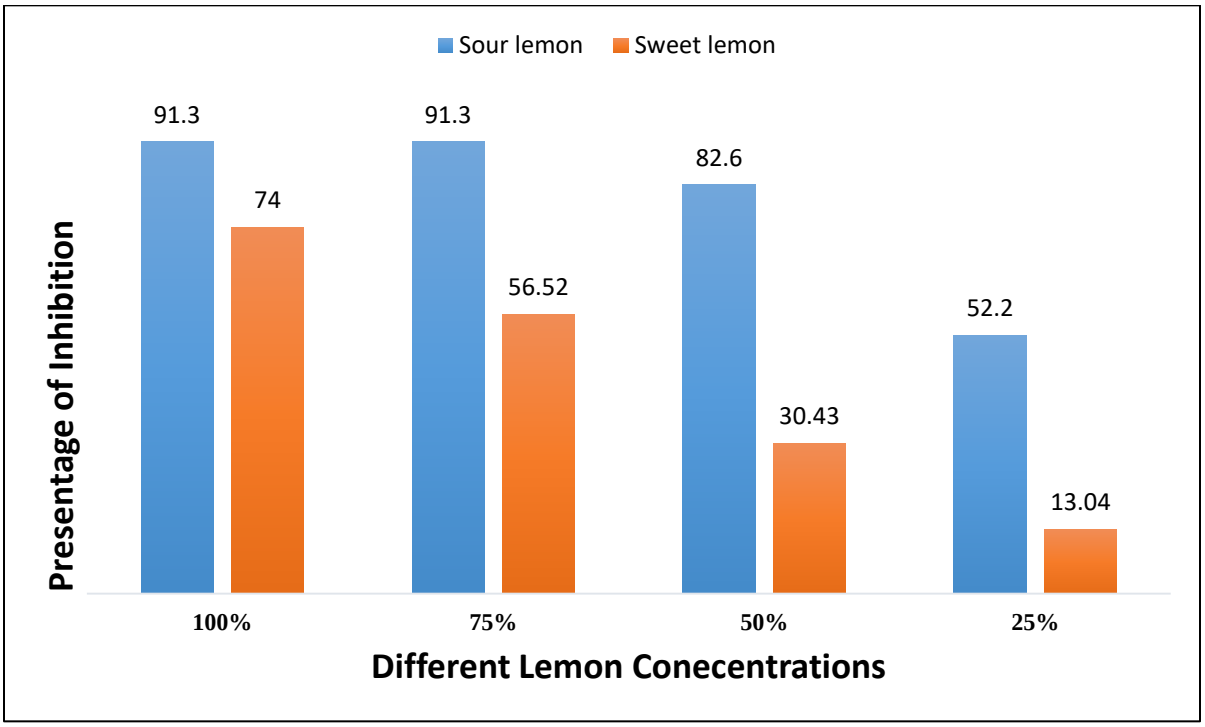

Figure (3-2): Inhibitory effect of Fresh Lemon (sour and sweet) Juices on the growth of *S. aureus*

The Gram-positive bacteria showed higher susceptibility values than gram-negative bacteria because the cell wall of gram-positive bacteria

contains a polysaccharide which is a water-soluble polymer, which acts as incoming positive ions. This soluble nature shows that the gram-positive cell wall is more polar. The flavonoid compounds contained in lemon juice are non-polar, causing disruption of the cell wall function as a giver of cell shape and protects the cells from osmotic lysis. Disruption of the cell wall will cause lysis of the cell (Batubara *et al*., 2019).

The Gram- negative outer membrane acts as a barrier, preventing the penetration of numerous environmental substances, including antimicrobial substances into the organism. The periplasmic space is also known to contain enzymes capable of breaking down foreign molecules attempting to gain entry into the microorganism (Oikeh *et al*., 2016).

3.2.3 Effect of (Gold nanoparticles and Titanium dioxide nanoparticles) on *Staphylococcus aureus* Growth.

In this study AuNPs and TiO_2NPs effects were examined against *S. aureus* isolates growth by making five double serial dilutions for each stock concentrations as (1/2, 1/4, 1/8, 1/16 and 1/32) and those stock concentrations were (2000µg/ml) and (400µg/ml) for AuNps and TiO_2NPs respectively; then after incubation for 24 and 48 hours the bacterial growth had been monitored and checked with spectrophotometer; results were cleared as follow and it revealed that there was statistically significant inhibitory effect ($P = 0.005$) on the growth especially with the first three dilutions, as shown in figure (3-3)

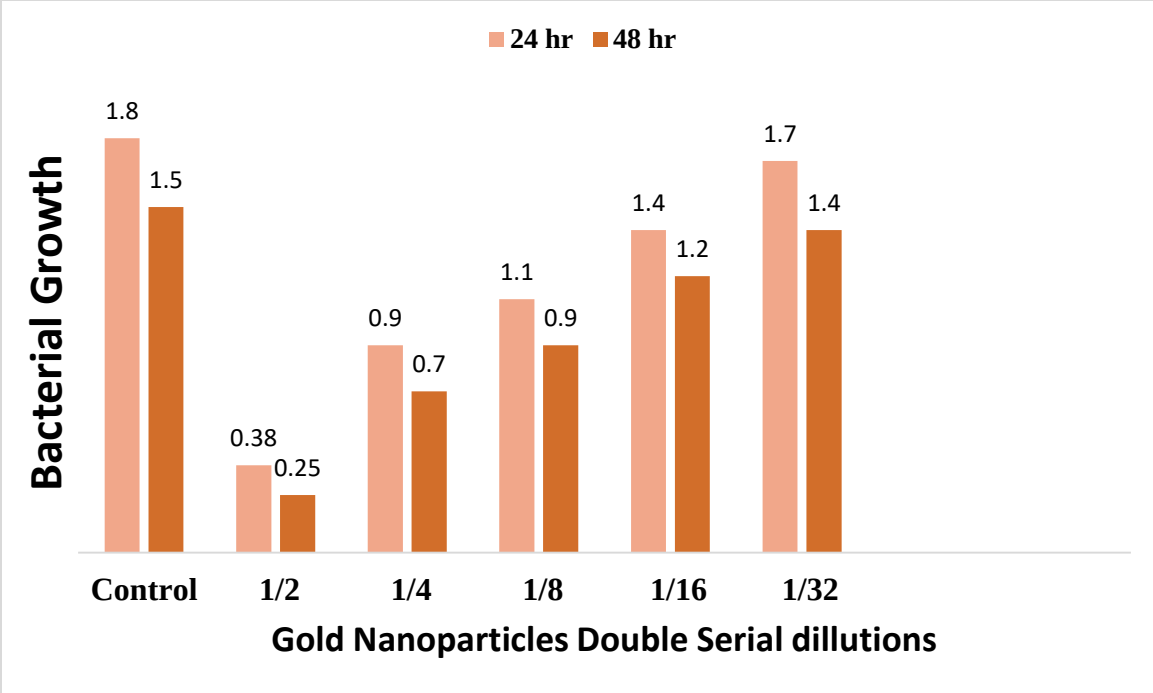

Figure (3-3): *S. aureus* growth inhibition by double serial dilution of Gold (AuNPs) Nanoparticles

In this figure AuNPs show significant inhibition with p value = 0.007 and also showed that the highest concentration of AuNPs (1/2) made the highest inhibition effect for bacterial growth and this inhibition minimized the bacterial growth from (1.8) to (0.38) after 24hours of incubation while after 48 hours of incubation bacterial growth minimized from 1.5 to 0.25. While the lowest concentration of AuNPs that made by diluted the nanoparticles for (1/32) showed the lowest inhibition effect on *S.aureus* growth after 24 and 48 hours of incubation, this study agreed with Zhang *et al*., (2015) who found that AuNPs only been showed inhibition at a very high concentration; also agreed with Mohamed *et al*., 2017 who found that the MIC (minimum inhibitory concentration) is relatively high. The reason might be attributed to the thicker wall of Gram- positive that reduces the penetration rate of AuNPs through cell wall and hence reduces the

antibacterial activity of AuNPs at lower concentrations. Also this study showed agreement with Qiu *et al*., (2021) who showed a marked inhibitory effect on the growth of *S. aureus* in a concentration dependent manner.

AuNPs' antibacterial efficacy may be attributed to a variety of pathways. The key mechanism proposed is related to oxidative stress caused by ROS (it was previously understood that replacing each $Au2^{+}$ ion results in the release of one free electron). Superoxide radicals, hydroxyl radicals, hydrogen peroxide, and single oxygen are all ROS that can destroy bacteria's proteins and DNA. Second, electrostatic interactions between nanoparticles and bacterial cell membranes or cell membrane proteins can cause physical damage, leading to bacterial cell death (Abdulazeem *et al*., 2021).

While regarding TiO_2NPs cause significant inhibition with p value = 0.005 especially with the first two dilutions, and this was relatively a similar inhibition effect to that produced by AuNPs on *S.aureus* growth by the same serial double dilutions and results showed that when TiO_2NPs concentration diluted to (1/2) appeared the highest inhibition effect on bacterial growth that minimized the growth of bacterial isolates from (2) to (0.7) after 24 hours of incubation and from (1.5) to (0.4) after 48 hours of incubation. Whereas the lowest concentration of TiO_2NPs that prepared by diluting to (1/32) showed the lowest inhibition effect on *S.aureus* growth represented by minimizing the bacterial growth from (2) to (1.9) and (1.5) only after 24 and 48 hours of incubation respectively, as in figure (3-4).

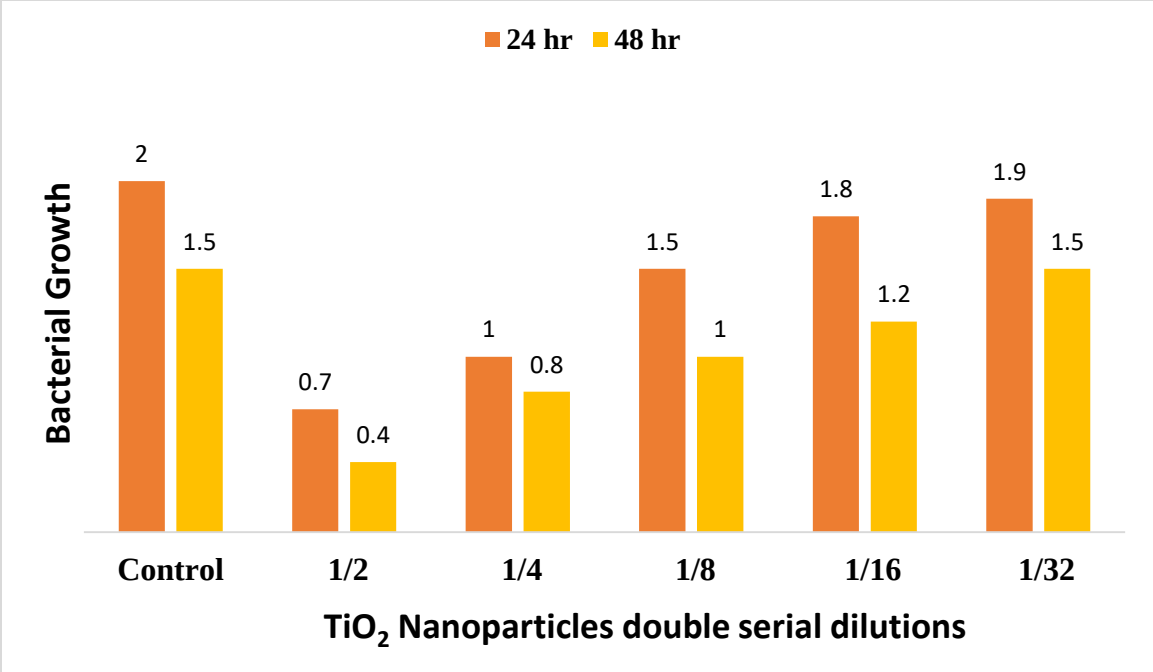

Figure (3-4): *S.aureus* growth inhibition by double serial dilution of (TiO_2NPs) Nanoparticles

This result agreed with Babaei *et al.* (2016) who showed the decrease in the growth of *S. aureus* in the presence of titanium dioxide nanoparticles. Also agreed with Anandgaonker *et al.* (2019) who found that TiO2 nanoparticles that synthesized at two different densities (100 µl, 50µl) showed good antibacterial activity, 100 µl concentration of TiO_2 nanoparticles is better as compared to 50 µl for inhibiting growth of bacterial test organism (*S.aureus* and *E. coli*), the TiO_2 nanoparticles proved to be very active on the tested Gram-positive strains, this differential sensitivity of Gram-negative and Gram-positive bacteria toward nanoparticles could be explained by the fact that the liquid medium is probably favoring the close interaction between the suspended nanoparticles and the Gram-positive microbial cells, which could better attach and anchor to the surface of the microbial cell, causing structural changes and damages leading to cell death.

Moreover this study show compatibility with Mahdy *et al*. (2017) who found that basically the growth of *S. aureus* inhibited compared to the control.

Several studies have believed the germicidal mechanisms of TiO_2 nanoparticles involving release of positively charge ions to reaction medium linked to (negative charges) thiol group (-SH) of the proteins on the cytoplasmic membrane, this reaction lead to capture the cell wall and increased permeability beside it cause deform the structure of cellular components such as DNA, ribosomes and cellular enzymes and finally death of microbial cell (Mahdy *et al*., 2017).

3.3. Biofilm Formation by *Staphylococcus aureus*:

Biofilm formation on polymeric surfaces was tested in the semi quantitative microtiter plate test using nutrient broth supplemented with 1% glucose. This assay was repeated as triplicate to increase the accuracy of assay. According to mean of OD value at 570nm the results were interpreted as none, moderate and high biofilm producer when the mean of OD value were (<0.120, 0.120-0.240, and >0.240) respectively.

The results revealed that all *S. aureus* isolates were biofilm producer (95.65%), high biofilm formation were account for (86.96%) while there were 2 isolates that express moderate and one isolate that was weak-biofilm formation. As shown in Table (3-3).

Table (3-3): Number and Percentages of Biofilm production by *S. aureus* isolates

Bacterial isolate No.	Biofilm (OD at 570nm)		
	Strong	Moderate	Weak
S. aureus (23)	20 (86.96%)	2 (8.70%)	1 (4.34%)

Crystal Violet (CV) assay was first described by Christensen, and then has been improved constantly to be suitable for all biofilm quantification. CV is a basic protein dye that stains negatively charged surface molecules and extracellular matrix of polysaccharides. Viable cells, dead cells, and extracellular matrix can be dyed by CV, which proved that CV assay has an advantage in measuring the amount of biofilm but not the functional biofilm (Xu *et al*., 2016).

This results somewhat agree with Torlak *et al* (2017), Who revealed that 90.6% isolates of *S. aureus* have the ability of biofilm formation, and also agreed with Abdelraheem *et al* (2021) who showed that the percentage of biofilm producing *S. aureus* isolates was (81.6%). But this result was more than the result obtained by El-Nagdy *et al.* (2020) who showed that (72.7%) of *S. aureus* were biofilm producer, and more than Elkhashab (2018), who revealed that 60% of the isolates were positive biofilm forming.

The difference in biofilm production may be attributed to differences in virulence capacity of bacteria to form biofilm and the number of bacterial cells that succeeded in adherence, type of specimen, geographical origin and the genetic makeup of the *S. aureus* isolate. Also environmental factors like

growth medium, type of surface (rough/smooth), porosity and the charge of the surface affect biofilm formation. Regarding studying possible risk factors for biofilm formation by *S. aureus*, the source of the isolates and history of antibiotic administration shows significant association with biofilm formation (Abdelraheem *et al*., 2021).

3.3.1 Effect of (Gold and Titanium dioxide Nanoparticles) on Biofilm Formation by S*taphylococcus aureus:*

In this study AuNPs and TiO_2NPs effects were examined against biofilm formation by *staph aureus* isolates growth by making four double serial dilutions for each stock concentrations as (1/2, 1/4, 1/8, and 1/16) and those stock concentrations were (2000µg/ml) and (400µg/ml) for AuNps and TiO_2NPs respectively; then after incubation for 24 hours the ability of *S. aureus* to form biofilm had been monitored and checked with spectrophotometer.

Results showed that TiO_2NPs had more effect on the biofilm formation by its different dilutions compared with AuNPs concentrations, which was having less effect on biofilm formation produced by *S.aureus* , results were cleared as follow and it revealed that there was statistically significant effect (P =0. 008) on the biofilm formation especially with the first three dilutions, as shown in figure (3-5).

In this figure AuNPs cause significant inhibition with p value = 0.004 and also showed that the highest concentration of AuNPs after diluted for (1/2) made the highest inhibition effect for bacterial biofilm production and this dilution minimized the bacterial biofilm formation from (0.3) to (0.1) after 24hours of incubation. While the lowest concentration of AuNPs that

made by diluted the nanoparticles for (1/16) showed the lowest inhibition effect on biofilm formation by *S. aureus* after 24 hours of incubation.

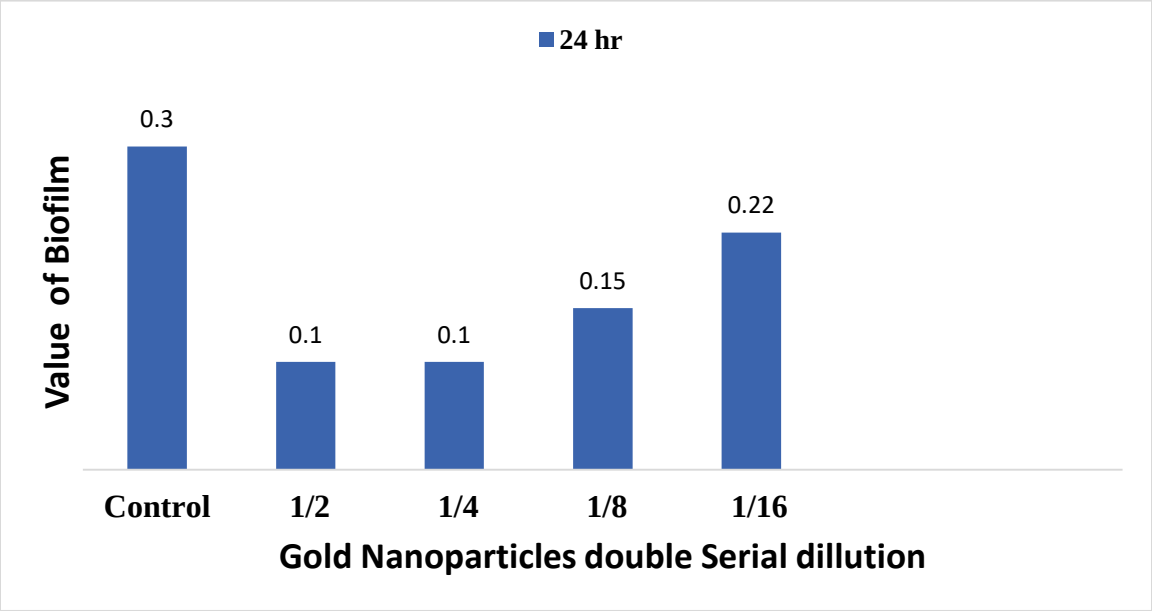

Figure (3-5): *S. aureus* biofilm inhibition by double serial dilution of Gold (AuNPs) Nanoparticles

This result show compatibility with Yu *et al*., (2016) who found that AuNPs showed significant inhibitory effect only when its concentration reached to 10 and also 5 of AuNPs strongly inhibited biofilm formation. Hence, AuNPs also strongly attenuated biofilm formation of the pathogenic bacterium, and also agreed with Villa-García *et al*., (2021) who showed that AuNPs behaved like an agent with antimicrobial activity *of S. aureus* biofilm; results are confirmed that prove AuNPs may serve as potential therapeutic agents against the biofilm-forming bacterial pathogens since the essentially inert nature of Au makes it an attractive material as an

antimicrobial agent, and agreed with Boda *et al*., (2015) who found that AuNPs revealed around 80%–90% decline in bacterial survival.

Reduction in the biofilm formation by bacterial cell with increasing AuNPs concentrations might be due to the toxicity of AuNPs towards the bacterial cells (Ahiwale *et al*., 2017)

While regarding TiO_2NPs cause significant inhibition with p value = 0.008 with its all four dilutions, and this was relatively had more inhibition effect to that produced by AuNPs on biofilm formation by *S.aureus* by the same serial double dilutions and results showed that when TiO_2NPs concentration diluted to (1/2) appeared the highest inhibition effect on biofilm production that minimized the biofilm formation of bacterial isolates from (0.28) to (0.07) after 24 hours of incubation. Whereas the lowest concentration of TiO_2NPs that prepared by diluting to (1/16) showed the lowest inhibition effect on *S.aureus* biofilm production represented by minimizing the bacterial biofilm formation from (0.28) to (0.15) after 24 hours of incubation, as in figure (3-6).

This result show compatibility with Altaf *et al*., (2021) who showed that the bioflms of tested Gram +ve bacteria (*S. aureus*) were maximally inhibited by TiO2NPs. The addition of 8, 16, 32, and 64 mg TiO_2-NPs in culture media decreased the formation of biofilms of *S. aureus*. At the highest tested concentration (64 TiO_2NPs), more than 80% inhibition was recorded.

Additionally agreed with results obtained by Fatima *et al*., (2021) who found biofilms of the tested gram positive strains of TiO_2 NPs by CV assay, only 1000 or 2000 µg/mL resulted in significant destruction of mature biofilms, suggesting that TiO_2NPs are more efficient against developing

biofilms at 250–500 µg/mL, but they can also destroy biofilms at higher concentrations of 1000 or 2000 µg/mL.

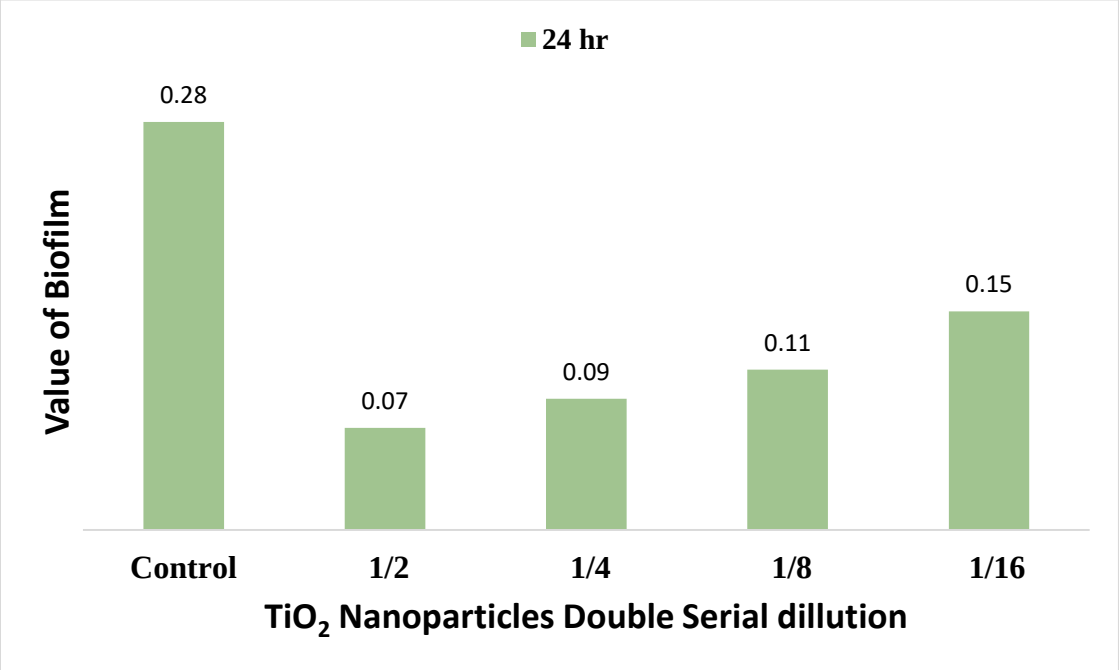

Figure (3-6): *S.aureus* biofilm inhibition by double serial dilution of (TiO_2NPs) Nanoparticles

Moreover this study show agreement with Shawkat *et al*., (2021) who obtained that these TiO2NPs are completely capable of suppressing biofilm formation, and preventing further growth of the microbial biofilm of *S. pneumoniae, S. aureus, S. epidermidis, E. coli, P. aeruginosa, K. pneumoniae, and C. albicans.* The test results indicated that in the absence of the TiO_2 NPs, strong biofilms will be form on the plates, but adding the particles would strongly inhibit biofilm formation and maturation. TiO_2 demonstrated that it could effectively inhibit the growth of all isolated bacterial strains.

The possible mechanism of the anti-biofilm activity of metal nanoparticles is that TiO_2 NPs adhere to the surface of the biofilm and interact with the bacterial cell membrane to cause oxidative stress and kill the bacteria (Jin *et al*., 2021).

3.4. Molecular SPA Typing:

Out of 20 isolates (100%) showed the *Spa* Gene PCR products with different size from (200-500) bp with single PCR band reflecting the number of 24bp repeat units within *Spa* Genes as shown in Figure (3-7).

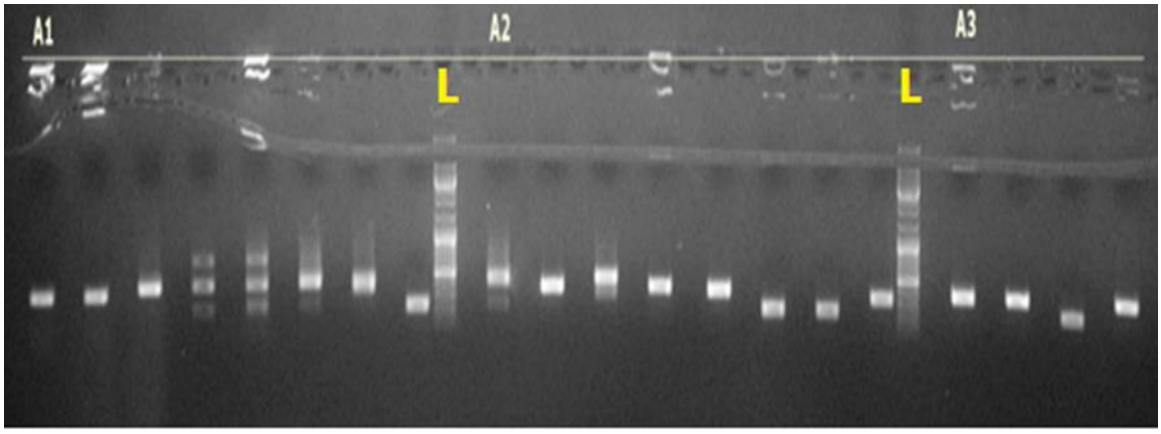

Fig. (3-7): Agarose gel electrophoresis of PCR products obtained by using Spa-specific primers. Lanes A1-D3 represent the identified spa gene products with variable sizes (200-<500bp). L: Ladder, GeneRuler DNA Ladder was used as 100bp DNA ladder.

Out of 20 isolates identified, (8) different types among the 18/20 isolates were detected, and 2/20 isolates could not be typed; as the most

commonly observed *Spa* were t304 (35%), t491 (15%) followed by t078 and t059 (10%) as shown in Table (3-4).

Table (3-4): Number and percentages of *Spa* types among the studied *S.aureus* isolates.

Spa types	Number	Percentage
t304	7	35%
t491	3	15%
t386	1	5%
t078	2	10%
t059	2	10%
t044	1	5%
t14870	1	5%
t091	1	5%
Unknown*	2	10%
Total	20	100%

* not typeable strains

S.aureus protein A is a surface protein encoded by the Spa gene, the gene consist of the variable number tandem repeat (VNTR) region (Xr), So the *Spa* typing is based on ampilification and sequencing of the Xr region, which consist of a variable number of 24bp repeats (cases of 21bp and 27bp repeats) where each repeats has a unique sequence (mernelius, 2015).

The different *Spa* types arise from point mutations in the repeats, as well as from deletion and duplications of the repeats, therefor the sequence

of each *Spa* type is unique and there are *Spa* types of variable length as shown in the results of single locus sequence figure (3-8).

1_spa t304:(frame 1)

5'flank.....Y1[11](24.84).....C2[10](26.26).....F1[21](29.26).....
21 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGC**A**AAGAAGAC
AATAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGC

M1[17](29.81).....B1[34](26.64).....Q1[24](27.43).....B1[34](26.64).....
117 **A**AAGAAGACGGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAT
GGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT

L1[22](30.07).....O1[25](26.84).....3'flank.....
213 **A**AAGAAGACGGCAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGTAAGAAGATG
GTAACGGAGTACATG

2_spa - t304:(frame 1)

5'flank.....Y1[11](24.84).....C2[10](26.26).....F1[21](29.26).....
21 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGC**A**AAGAAGAC
AATAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGC

M1[17](29.81).....B1[34](26.64).....Q1[24](27.43).....B1[34](26.64).....
117 **A**AAGAAGACGGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAT
GGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT

L1[22](30.07).....O1[25](26.84).....3'flank.....
213 **A**AAGAAGACGGCAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGTAAGAAGATG
GTAACGGAGTACATG

3-3_spa t491:(frame 1)

5'flank.....T1[26](24.96).....J1[23](29.49).....G1[12](29.00).....
27 CTAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAACAAAAAACCTGGT**A**AAGAAGAC
GGCAACAAACCTGGC**A**AAGAAGACAACAACAAGCCTGGT

B1[34](26.64).....B1[34](26.64).....G1[12](29.00).....G1[12](29.00).....
123 **A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAC
AACAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGT

J1[23](29.49).....A1[2](26.91).....G1[12](29.00).....J1[23](29.49).....
219 **A**AAGAAGACGGCAACAAACCTGGC**A**AAGAAGACAACAAAAAACCTGGC**A**AAGAAGAC
AACAACAAGCCTGGT**A**AAGAAGACGGCAACAAACCTGGC

3'flank.....

315 AAGAAGACGGCAACGGAGTACATG

4_spa t491:(frame 1)

5'flank.....T1[26](24.96).....J1[23](29.49).....G1[12](29.00).....
27 CTAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAACAAAAAACCTGGT**A**AAGAAGAC
GGCAACAAACCTGGC**A**AAGAAGACAACAACAAGCCTGGT

B1[34](26.64).....B1[34](26.64).....G1[12](29.00).....G1[12](29.00).....
123 **A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAC
AACAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGT

J1[23](29.49).....A1[2](26.91).....G1[12](29.00).....J1[23](29.49).....
219 **A**AAGAAGACGGCAACAAACCTGGC**A**AAGAAGACAACAAAAAACCTGGC**A**AAGAAGAC
AACAACAAGCCTGGT**A**AAGAAGACGGCAACAAACCTGGC

3'flank.....
315 AAGAAGACGGCAACGGAGTACATG

5_spat491:(frame1)

5'flank.....T1[26](24.96).....J1[23](29.49).....G1[12](29.00).....
27 CTAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAACAAAAAACCTGGT**A**AAGAAGAC
GGCAACAAACCTGGC**A**AAGAAGACAACAACAAGCCTGGT

B1[34](26.64).....B1[34](26.64).....G1[12](29.00).....G1[12](29.00).....
123 **A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAC
AACAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGT

J1[23](29.49).....A1[2](26.91).....G1[12](29.00).....J1[23](29.49).....
219 **A**AAGAAGACGGCAACAAACCTGGC**A**AAGAAGACAACAAAAAACCTGGC**A**AAGAAGAC
AACAACAAGCCTGGT**A**AAGAAGACGGCAACAAACCTGGC

3'flank.....
315 AAGAAGACGGCAACGGAGTACATG

6S_spa t304:(frame 1)

5'flank.....Y1[11](24.84).....C2[10](26.26).....F1[21](29.26).....
21 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGC**A**AAGAAGAC
AATAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGC

M1[17](29.81).....B1[34](26.64).....Q1[24](27.43).....B1[34](26.64).....
117 **A**AAGAAGACGGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAT
GGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT

L1[22](30.07).....O1[25](26.84).....3'flank.....

213 **A**AAGAAGACGGCAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGTAAGAAGATG
GTAACGGAGTACATG

7G - t304:(frame 1)

5'flank.....Y1[11](24.84).....C2[10](26.26).....F1[21](29.26).....
21 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGC**A**AAGAAGAC
AATAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGC

M1[17](29.81).....B1[34](26.64).....Q1[24](27.43).....B1[34](26.64).....
117 **A**AAGAAGACGGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAT
GGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT

L1[22](30.07).....O1[25](26.84).....3'flank.....
213 **A**AAGAAGACGGCAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGTAAGAAGATG
GTAACGGAGTACATG

8S_spa t386:(frame 2)

5'flank.....U1[7](26.73).....J1[23](29.49).....E1[13](28.41).....
22 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAACAACAAACCTGGT**A**AAGAAGAC
GGCAACAAACCTGGC**A**AAGAAGACAACAACAAACCTGGT

3'flank.....
118 AAGAAGACGGCAACGGAGTACATG

9S_spa (RC) - *:(frame 2)

5'flank.....E1[13](28.41).....L1[22](30.07).....O1[25](26.84).....
-
268 TAAAAGATGGTAAAAAACCAAAAA**A**AAGAAGACAACAACAAACCTGGT**A**AAGAAGACG
GCAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGT

3'flank.....
-364 AAGAAGATGGTAACGGAGTACATG

10S_spa- t078:(frame 2)

5'flank.....Z1[4](24.58).....F1[21](29.26).....G1[12](29.00).....
25 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGT**A**AAGAAGACA
ACAACAAGCCTGGC**A**AAGAAGACAACAACAAGCCTGGT

U2[41](27.60).....D1[20](28.68).....M1[17](29.81).....G1[12](29.00).....
121 **C**AAGAAGACGGCAACAAGCCTGGT**A**AAGAAGACAACAACAAACCTGGC**A**AAGAAGAC
GGCAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGT

G1[12](29.00).....M1[17](29.81).....3'flank.....
217 **A**AAGAAGACAACAACAAGCCTGGT**A**AAGAAGACGGCAACAAGCCTGGTAAGAAGACG
GCAACGGAGTACATG

11S_spa (RC) - *:(frame 1)

5'flank.....Q1[24](27.43).....*[204](22.10).....Q1[24](27.43).....
-
126 AAGAAGACAACAACAAGCCTTTAA**A**AAGAAGATGGCAACAAGCCTGGT**A**AAGAAGACAA
CAAAAAACATGGT**A**AAGAAGATGGCAACAAGCCTGGT

Q1[24](27.43).....B1[34](26.64).....L1[22](30.07).....O1[25](26.84).....
-
222 **A**AAGAAGATGGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGACG
GCAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGT

P1[33](27.69).....O1[25](26.84).....3'flank.....
-
318 **A**AAGAAGATGGCAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGTAAGAAGATGGT
AACGGAGTACATG

12S_spa t304:(frame 0)

5'flank.....Y1[11](24.84).....C2[10](26.26).....F1[21](29.26).....
23 CTAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGC**A**AAGAAGACA
ATAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGC

M1[17](29.81).....B1[34](26.64).....Q1[24](27.43).....B1[34](26.64).....
119 **A**AAGAAGACGGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAT
GGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT

L1[22](30.07).....O1[25](26.84).....3'flank.....
215 **A**AAGAAGACGGCAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGTAAGAAGATG
GTAACGGAGTACATG

13S_spa t304:(frame 0)

5'flank.....Y1[11](24.84).....C2[10](26.26).....F1[21](29.26).....
23 CTAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGC**A**AAGAAGACA
ATAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGC

M1[17](29.81).....B1[34](26.64).....Q1[24](27.43).....B1[34](26.64).....
119 **A**AAGAAGACGGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAT
GGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT

L1[22](30.07).....O1[25](26.84).....3'flank.....
215 **A**AAGAAGACGGCAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGTAAGAAGATG
GTAACGGAGTAATGT

14S_spa t059:(frame 0)

5'flank.....Y1[11](24.84).....H1[19](26.52).....O1[25](26.84).....
26 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGC**A**AAGAAGAC
AATAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGT

3'flank.....
122 AAGAAGATGGTAACGGAGTACATG

15S_spa t059:(frame 1)

5'flank.....Y1[11](24.84).....H1[19](26.52).....O1[25](26.84).....
21 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGC**A**AAGAAGAC
AATAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGT

3'flank.....
117 AAGAAGATGGTAACGGAGTACATG

16S_spa- t044:(frame 2)

5'flank.....U1[7](26.73).....J1[23](29.49).....G1[12](29.00).....
19 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAACAACAAACCTGGT**A**AAGAAGAC
GGCAACAAACCTGGC**A**AAGAAGACAACAACAAGCCTGGT

B1[34](26.64).....B1[34](26.64).....P1[33](27.69).....B1[34](26.64).....
115 **A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAT
GGCAACAAGCCTGGC**A**AAGAAGACAACAAAAAACCTGGT

3'flank.....
211 AAGAAGACGGCAACGGAGTACATG

17S_spa - t078:(frame 2)

5'flank.....Z1[4](24.58).....F1[21](29.26).....G1[12](29.00).....
22 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGT**A**AAGAAGACA
ACAACAAGCCTGGC**A**AAGAAGACAACAACAAGCCTGGT

U2[41](27.60).....D1[20](28.68).....M1[17](29.81).....G1[12](29.00).....
118 **C**AAGAAGACGGCAACAAGCCTGGT**A**AAGAAGACAACAACAAACCTGGC**A**AAGAAGAC
GGCAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGT

G1[12](29.00).....M1[17](29.81).....3'flank.....
214 **A**AAGAAGACAACAACAAGCCTGGT**A**AAGAAGACGGCAACAAGCCTGGTAAGAAGACG
GCAACGGAGTACATG

18S_spa- t304:(frame 0)

5'flank.....Y1[11](24.84).....C2[10](26.26).....F1[21](29.26).....
20 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGC**A**AAGAAGAC
AATAACAAGCCTGGT**A**AAGAAGACAACAACAAGCCTGGC

M1[17](29.81).....B1[34](26.64).....Q1[24](27.43).....B1[34](26.64).....
116 **A**AAGAAGACGGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAT
GGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT

L1[22](30.07).....O1[25](26.84).....3'flank.....
212 **A**AAGAAGACGGCAACAAGCCTGGC**A**AAGAAGATGGCAACAAACCTGGTAAGAAGATG
GTAACGGAGTACATG

19S Spa t14870:(frame1)

5'flank.....Y1[11](24.84).....C2[10](26.26).....E1[13](28.41).....
21 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAATAACAAGCCTGGC**A**AAGAAGAC
AATAACAAGCCTGGT**A**AAGAAGACAACAACAAACCTGGT

O1[25](26.84).....3'flank.....
117 **A**AAGAAGATGGCAACAAACCTGGTAAGAAGATGGTAACGGAGTACATG

8G_spa_t091:(frame2)

5'flank.....U1[7](26.73).....J1[23](29.49).....F1[21](29.26).....
25 TAAACGATGCTCAAGCACCAAAAG**G**AGGAAGACAACAACAAACCTGGT**A**AAGAAGAC
GGCAACAAACCTGGC**A**AAGAAGACAACAACAAGCCTGGC

M1[17](29.81).....B1[34](26.64).....G1[12](29.00).....J1[23](29.49).....
121 **A**AAGAAGACGGCAACAAGCCTGGT**A**AAGAAGACAACAAAAAACCTGGT**A**AAGAAGAC
AACAACAAGCCTGGT**A**AAGAAGACGGCAACAAACCTGGC

A1[2](26.91).....G1[12](29.00).....J1[23](29.49).....3'flank.....
217 **A**AAGAAGACAACAAAAAACCTGGC**A**AAGAAGACAACAACAAGCCTGGT**A**AAGAAGAC
GGCAACAAACCTGGCAAGAAGACGGCAACGGAGTACATG

Fig (3-8): Sequence Annotations (repeat score) of studied isolates according to Kreiswirth Method (Kreiswirth *et al*., 2004).

This method based on sequencing of polymorphic Xr region. The highly conserved regions flanking the Xr region enables annealing of the

primers necessary for amplification and sequencing, then analysis of sequence data and the attribution of *Spa* types of isolates, that was performed using the ridom Staph types software as shown in Table (3-5).

Table (3-5): Spa typing of *S. aureus* isolates.

Strain Name	start pos[1]	repeat units[2]	len in bp[3]	repeat seq[4]	Spa type[5]
1_spa	45	9	216	Y1:C2:F1:M1:B1:Q1:B1:L1:O1 r11:r10:r21:r17:r34:r24:r34:r22:r25	t304
2_spa	45	9	216	Y1:C2:F1:M1:B1:Q1:B1:L1:O1 r11:r10:r21:r17:r34:r24:r34:r22:r25	t304
3_spa	51	11	264	T1:J1:G1:B1:B1:G1:G1:J1:A1:G1:J1 r26:r23:r12:r34:r34:r12:r12:r23:r02:r12:r23	t491
4_spa	51	11	264	T1:J1:G1:B1:B1:G1:G1:J1:A1:G1:J1 r26:r23:r12:r34:r34:r12:r12:r23:r02:r12:r23	t491
5_spa	51	11	264	T1:J1:G1:B1:B1:G1:G1:J1:A1:G1:J1 r26:r23:r12:r34:r34:r12:r12:r23:r02:r12:r23	t491
6s_spa	45	9	216	Y1:C2:F1:M1:B1:Q1:B1:L1:O1 r11:r10:r21:r17:r34:r24:r34:r22:r25	t304
7G_spa	45	9	216	Y1:C2:F1:M1:B1:Q1:B1:L1:O1 r11:r10:r21:r17:r34:r24:r34:r22:r25	t304
8S_spa	46	3	72	U1:J1:E1 r07:r23:r13	t386
9S_spa	292	3	72	E1:L1:O1 r13:r22:r25	*
10S_sp	49	9	216	Z1:F1:G1:U2:D1:M1:G1:G1:M1 r04:r21:r12:r41:r20:r17:r12:r12:r17	t078
11S_spa	150	9	216	Q1:*:Q1:Q1:B1:L1:O1:P1:O1 r24:r204:r24:r24:r34:r22:r25:r33:r25	*
12S_spa	47	9	216	Y1:C2:F1:M1:B1:Q1:B1:L1:O1 r11:r10:r21:r17:r34:r24:r34:r22:r25	t304
13S_spa	47	9	216	Y1:C2:F1:M1:B1:Q1:B1:L1:O1 r11:r10:r21:r17:r34:r24:r34:r22:r25	t304
14S_spa	50	3	72	Y1:H1:O1 r11:r19:r25	t059

15S_spa	45	3	72	Y1:H1:O1 r11:r19:r25	t059
16S_spa	43	7	168	U1:J1:G1:B1:B1:P1:B1 r07:r23:r12:r34:r34:r33:r34	t044
17S_spa-1113f	46	9	216	Z1:F1:G1:U2:D1:M1:G1:G1:M1 r04:r21:r12:r41:r20:r17:r12:r12:r17	t078
18S_spa	44	9	216	Y1:C2:F1:M1:B1:Q1:B1:L1:O1 r11:r10:r21:r17:r34:r24:r34:r22:r25	t304
19S_spa	45	4	96	Y1:C2:E1:O1 r11:r10:r13:r25	t14870
8G_spa	49	10	240	U1:J1:F1:M1:B1:G1:J1:A1:G1:J1 r07:r23:r21:r17:r34:r12:r23:r02:r12:r23	t091

[1]Starting coordinate of repeats in sequence, [2] Number of repeat units; [3] Length of entire VNTR; [4] Kreiswirth, Ridom nomenclature, ***** indicates a sequence that is a likely spa repeat, but does not exist in this database, [5] *spa* type name.

The results in this study agree with (Mohammed *et al*., 2021) which found that the tested isolates belong to t304 (30.3%), but disagree within this study it found the *Spa* type t037 detect in (19.4%), but in present study this type was not detectable.

The difference in the *Spa* type that reported in the local region or neighboring countries may be due to cross border patients' motility or migration from one to other country during years (Mohammed *et al*., 2021).

Pomorska *et al.* (2021) detected 52 different *Spa* types among 616 MRSA strain, the most common type included t003, t586, t014 and t002, which not typable in present study. It is important to note that the dominance of just one *Spa* type (or few) in some region does not mean that the other Spa types are not present in these part of area.

Spa Typing Method is based on the sequencing of only single locus ranging from 200-600 bp in size. The Polymorphic X region in the *Spa* gene is relatively stable and has discrimination power between the level of PFGE and MSLT (Khademi *et al.*, 2016).

Spa Tandem repeats were calculated by Bioinformatic analysis, results revealed partial identity; they were detected by the stating coordinate of the repeat in the sequence alignment, number of repeats units, length of entire variable number tandem repeat; so the *Spa* typing represents the excellent tool for national and international surveillance as well as for short term local epidemiology (Kareem *et al.*, 2020).

Additionally, Mohammed *et al.* (2021) found that *Spa* typing of 36 *S.aureus* isolates revealed (11) different *Spa* type t304 detect in (30%), t044 (8%), t386 (5%) and t14870 in (2.8%), which agree with the results detect in present study.

Based on phylogenetic relationships, *S. aureus* strains were classified into two clades. The first one contain 18 isolates and the second one contain 2 isolates as shown in (Fig 3-9), the most *Spa* types were included in clade A (18 isolates) where only 2 isolates were included in clade B, then the isolates in clade A were clustered into 3 different groups based on the variation in tandem repeats of the Spa gene, cluster 1 contain the t304, t078, t044 Spa types, cluster 2 contain t059, t4870 and t386 Spa types and cluster 3 contain t491 and t091 *Spa* types and clade B contain 2 *Spa* types (unknown).

A highly diversity in *Spa* genes obtained from different sources was compared to the consensus and control obtained from NCBI, results revealed that some chains take place in nucleotide sequence compared to control *Spa* gene and most isolates display different genetic variation; Khademi *et al.*

(2016) showed that any 2 *Spa* types that had a majority of identical repeats or differed in a single deletion or insertion of the nucleotide sequence fit into the same cluster.

The different genetic cluster group were shown dendogram and phylogenetic tree, found that the different genetic cluster may exhibited the same type of *Spa* tandem repeats, this may be sue to the similarity between more than one type of *Spa* repeats, The discrimination between *S.aureus* isolates is possible by determining the repeats sequence number within the x-region of *spa* gene (Kareem *et al*., 2020).

The huge alteration in the *Spa* pattern could be recognized with each *Spa* sequence containing a different pattern, so there was also no genetic relation regarding infection sources.

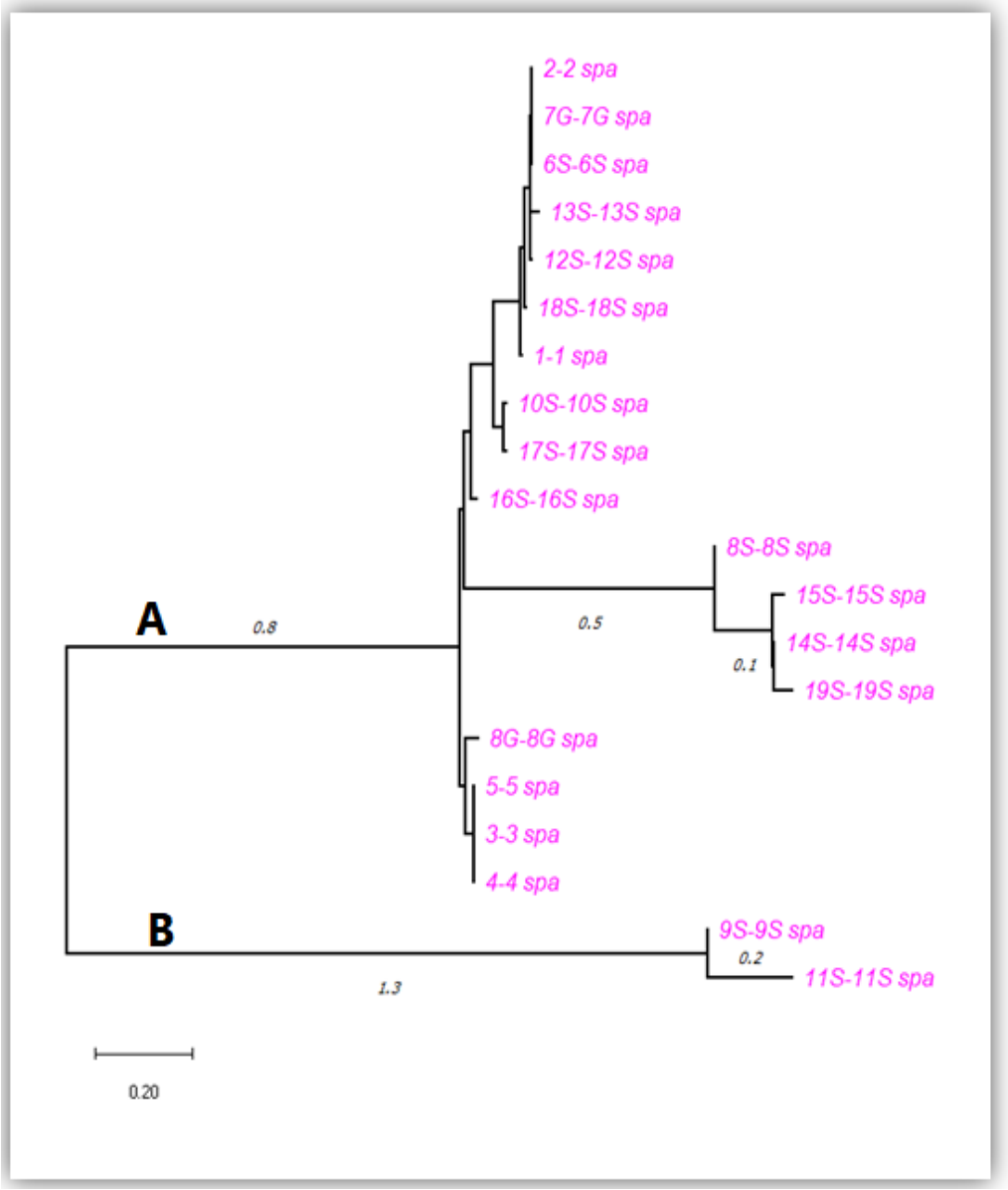

Figure (3-9): phylogram of of *S. aureus* isolates using *spa* sequences. In this analysis, the Maximum Likelihood method and Tamura-Nei model (1,2) were used to build tree. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches)

S= the number of *S.aureus* isolates.

Conclusions
And
Recommendations

Conclusions:

1- The highest percent of *S.aureus* was found in wound and urine followed by wound swabs.

2- Gentamicin and Chloramphenicol are effective antimicrobial treatment against most *S. aureus* isolates.

3- The use of alternative methods (Lemon plant Juices and nanoparticles) are advantageous for the treatment of *S. aureus* infections.

4- Spa genotyping is a beneficial method for typing of *S. aureus* isolates and showing the genetic relation regarding infection sources and showing phylogenetic relationships.

Recommendations:

1- Encourage the application of alternative treatment materials against MDR *S. aureus* isolates.

2- Use other genotypic methods such as pulsed-field gel electrophoresis and / or multi locus sequence typing that provides more discriminating power.

3- Correlations between genotyping and antibiotic resistance patterns of *Staph*. *aureus* should be studied.

4- Further studies should investigate the combination of Nano particles and antibiotics against hospital strains for the development of new materials and substances for medical applications.

5- The prevalence of *S.aurus* spa genes type and their relationship with clinical severity in human and the expression of this gene should be further investigated.

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الخلاصة :

وفقا للمشكلة الكبيرة المتمثلة في المقاومة البكتيرية للأدوية المضادة للميكروبات والانتشار الواسع لعدوى بكتريا المكورات العنقودية الذهبية خاصة بين مرضى المستشفيات أكثر من مرضى المجتمع والإفراط في استخدام المضادات الحيوية دون وصف الطبيب ، كل هذه المشاكل أدت إلى إقامة هذه الدراسة .

يهدف هذا العمل إلى دراسة طريقة التنميط الجيني لعزلات *S. aureus* عن طريق تطبيق تصنيف *Spa*(تكرارات المتغايرة العدد بالترادف) ، وإيجاد طرق بديلة لاستخدام المضادات الحيوية باستخدام نبات الليمون والجسيمات النانوية (الذهب ، ثاني أوكسيد التيتانيوم).

اشتملت الدراسة على 110 عينة مختلفة تم جمعها من مرضى يعانون من علامات وأعراض عدوى بكتيرية. حضروا إلى (مستشفى الحلة التعليمي العام ومدينة الإمام الصادق الطبية) في مدينة الحلة للفترة من أكتوبر 2021 إلى يناير 2022. تم زراعة العينات على اوساط مختلف عند 37 درجة مئوية لمدة 24 ساعة. لتشخيص وتحديد نوع البكتيريا.

أظهرت نتائج اختبارات التوصيف المظهري والكيميائي الحيوي أنه من أصل 110 عينة ، تم ايجاد 23 عينة سريرية من *S.aureus* ، بينما كانت 57 عزلة تنتمي إلى أجناس بكتيرية أخرى ، و 30 عينة سريرية أظهرت نتيجة سلبية.

توزعت العينات ((20.9%) 23 *S. aureus* على النحو التالي: 33/5 (15.2٪) من الحروق و 25/8 (32.0٪) من الجروح ، بينما 52/10 (19.2٪) من الادرار .

تم تحديد اختبارات الحساسية للمضادات الحيوية لجميع العزلات باستخدام اختبار انتشار القرص ، حيث تم تطبيق عشرة أنواع من المضادات الحيوية. لسوء الحظ ، كانت عزلات المكورات العنقودية الذهبية مقاومة لمعظم المضادات الحيوية المستخدمة ، حيث أظهرت هذه العزلات مقاومة لمضادات Trimethoprim (73.9%) بينما كانت مقاومة Clindamycin و Tetracyclin (65.2%)و (60.9%) على التوالي. أظهرت بكتريا S.aureus مقاومة أقل للأزيثروميسين (43.5%) ولأميكاسين (39%) بينما كانت المقاومة للسيبروفلوكساسين (34.8%) وللتريميثوبريم

سلفوميثوكسازول (30.4%) بينما كانت نسبة المقاومة لليفوفلوكساسين (26%) وللكاسين. الجنتاميسين (21.7%) بينما الكلورامفينيكول أظهر أقل نسبة مقاومة للمكورات العنقودية الذهبية شكلت (17.4%).

كما فحصت هذه الدراسة تأثير عصير الليمون الطازج بنوعيه الحامض والحلو بتركيزات مختلفة (100% ، 75% ، 50% و 25%) على عزلات المكورات العنقودية الذهبية. كان هناك تأثير مثبط ذو دلالة إحصائية (P = 0.04) على النمو ؛ وأظهرت هذه النتائج أن الليمون الحامض كان له تأثير تثبيط أعلى (91.3%) على بكتريا *S. aureus* بتركيزات (100% و 75%). بينما في (50%) كان تثبيط التركيز (82.6%) من نمو البكتيريا. بينما أظهر الليمون الحلو تأثيرا أقل في تراكيزه المختلفة ، في (100%)أظهر تركيز مثبط بنسبة (74%) بينما تركيز (75%) ثبط (56.52%) من نمو البكتيريا.

علاوة على ذلك ، تم فحص التأثيرات المثبطة لـ TiO2NPs و AuNPs ضد عزلات المكورات العنقودية الذهبية عن طريق اجراء سلسلة من خمس تخافيف مزدوجة (2/1 ، 4/1 ، 8/1 ، 16/1 و 32/1) وكانت تلك التراكيز (2000 ميكروغرام / مل و 400 ميكروغرام / مل) لـ TiO2NPs و AuNPs بالتتابع , بعد الحضن لمدة 24 و 48 ساعة ، تمت مراقبة النمو البكتيري وفحصه باستخدام مقياس الطيف الضوئي ؛ وأظهرت النتائج أن هنالك تأثير مثبط ذو دلالة إحصائية (P = 0.005) على النمو بشكل خاص مع التخفيفين الأوليين (2/1 ، 4/1).

بالإضافة إلى ذلك ، تم فحص تأثيرات AuNPs و TiO2NPs ضد تكوين الأغشية الحيوية بواسطة بكتريا المكورات العنقودية الذهبية عن طريق إجراء أربعة تخفيفات متسلسلة مزدوجة لكل تركيزات المخزونة (2/1 ، 4/1 ، 8/1 ، و 16/1) ؛ ثم بعد الحضانة تم رصد قدرة بكتريا المكورات العنقودية البرتقالية على تكوين غشاء حيوي وفحصها باستخدام مقياس الطيف الضوئي. أظهرت النتائج أن TiO2NPs كان لها تأثير مثبط أكبر على تكوين الأغشية الحيوية من خلال تخفيفاته المختلفة مقارنة بتركيزات AuNPs التي كان لها تأثير أقل وأظهرت وجود تأثير معنوي إحصائيًا (P = 0.008) على تكوين الغشاء الحيوي خاصة مع التخفيفات الثلاثة الأولى.

لاحقاً تم إجراء التنميط الجيني *Spa* واظهرت النتائج أنه من بين 20 عزلة تم تحديدها , تم اكتشاف (8) أنواع مختلفة من عزلات 20/18، ولم يمكن تصنيف 20/2 عزلة, وكانت أكثر أنواع الـ *Spa* التي تمت ملاحظتها هي (35٪) t304 ، (15٪) t491 وتليها (10٪) t059 و t078

بناءً على العلاقات التطورية ، تم تصنيف سلالات المكورات العنقودية الذهبية إلى جزأين. احتوت الأولى على 18 عزلة والثانية احتوت على 2 عزلة ، تم تضمين معظم أنواع الـ *Spa* في Clade A (18 عزلة) بينما تم تضمين 2 عزلة فقط في Clade B ، ثم تم تجميع العزلات في Clade A في 3 مجموعات مختلفة على أساس الاختلاف في التكرارات الترادفية لجين الـ *Spa* ، تحتوي المجموعة 1 على أنواع الـ *Spa* t304 و t078 و t044 ، وتحتوي المجموعة 2 على أنواع الـ *Spa* t059 و t14870 و t386 وتحتوي المجموعة 3 على أنواع الـ *Spa* t491 و t091 ، وتحتوي المجموعة B على نوعين من الـ *Spa* (مجهولة غير معروفة).

وزارة التعليم العالي
والبحث العلمي
جامعة بابل
كلية الطب

تنميط البروتين A للكشف عن التغايرات الجينية لبكتريا المكورات العنقودية المعزولة من عينات سريرية

رسالة
مقدمة الى مجلس كلية الطب / جامعة بابل
كجزء من متطلبات نيل درجة الماجستير في علوم الأحياء المجهرية الطبية
من قبل

رقية فاضل غازي أسيود
بكالوريوس تحليلات مرضية/كلية العلوم الطبية التطبيقية/جامعة كربلاء

2017

بأشراف

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