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Phenotypic and Molecular Study of *Staphylococcus aureus* Isolated from Clinical Cases in Misan Province /Iraq

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By

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“Supervisor Certification”

This is certify that this thesis entitled **(Phenotypic and Molecular Study of *Staphylococcus aureus* Isolated from Clinical Cases in Misan Province /Iraq)** was prepared under my supervision at the Department of Biology, College of Science, University of Misan, as partial requirement of the degree of Master in Biology.

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Marwa

Dedication

To my Wonderful Fourteen Imams

(peace be upon them)

To the present in my conscience

(My Father)

To the incarnate tender

(My Mother)

To the source of sympathy and affection

(My brothers & sisters)

I dedicate my modest effort

Summary

A total of 274 samples, were collected from different clinical cases from two main hospitals in Misan Province during the period from December 2017 to April 2018.

Diagnostic results using microscopic examination, cultural characteristics, biochemical tests and Vitek2 system proved that 106 bacterial isolates belong to *Staphylococcus aureus*.

The susceptibility test was performed toward 18 antibiotics that utilize as conventional therapy for MRSA infections, which determined by agar disk diffusion method and the guidelines of CLSI (2017). The results showed that 100% of *S. aureus* were multi-drug resistance. Moreover from all these isolates 99(93.4%) were determined as MRSA by oxacillin antibiotic, while 93(87.7%) were determined as MRSA by cefoxitin antibiotic.

In molecular level, our primarily study focused on two genes that (*hlg* and *mecA*) for gamma hemolysin pattern and antibiotic resistance respectively. The detection of *hlg* gene indicated that 40(37.8%) from all *S. aureus* isolates in current study encoded γ-hemolysin protein. Whereas, the results of *mecA* gene detection for PBP2a(PBP′) illustrate that 89(89.9%) and 91(97.8%) from phenotypically positive MRSA isolates had this gene for oxacillin and cefoxitin antibiotics respectively.

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List of Abbreviations

Abbreviation	Key
Agr	**Accessory gene regulator**

CA-MRSA	**Community-acquired-MRSA**
ClfA	**Clumping factor A**
CFU	**Colony Forming Units**
CLSI	**Clinical and Laboratory Standards Institute**
DNA	**Deoxyribonucleic acid**
D.W	**Distilled Water**
ECM	**Extracellular Matrix**
e.g.	**exempil gratia**
ENT	**Ear, Nose and Throat**
Ets	**Exfoliative Toxins**
FDA	**Food and Drug Administration**
FnBPA	**Fibronectin binding protein A**
G	**gram**
HA-MRSA	**Healthcare-acquired-MRSA**
kDa	**Kilodalton**
MDR	**Multi-drug resistance**
MGEs	**Mobile Genetic Elements**
Ml	**Meleleter**
MRSA	**Methicillin Resistant *Staphylococcus aureus***
MSCRAMMs	**Microbial-Surface Components Recognizing Adhesive Matrix Molecules**
NFT	**Nitrofurantoin**
PBP2a (PBP′)	**Penicillin Binding Protein2a**
PCR	**Polymerase Chain Reaction**
PRSA	**Penicillin-Resistant *Staphylococcus aureus***
PVL	**Panton–Valentine Leukocidin**
S. aureus	***Staphylococcus aureus***
SAB	***S. aureus* bacteremia**

SCC	**Staphylococcal Cassette Chromosome**
SSTIs	**Skin and Soft Tissue Infections**
SSSS	**Staphylococcal Scalded Skin Syndrome**
TE	**Tris-EDTA-Buffer**
TSST-I	**Toxic Shock Syndrome Toxin 1**
UTIs	**Urinary tract infections**
UV	**Ultra violet**
VISA	**Vancomycin Intermediate *Staphylococcus aureus***
VRSA	**Vancomycin Resistant *Staphylococcus aureus***
WHO	**Would Health Organization**
µg	**Microgram**
µl	**Microleter**
µm	**Micrometer**
+ve	**Positive**
-ve	**Negative**

Chapter One

Introduction

&

Literature Review

1- Introduction and Literature review

1.1- Introduction

Staphylococcus aureus is one of the most common opportunistic human pathogens (Kim *et al*., 2018). It be found as normal flora in skin and mucous membranes such as anterior nasal, pharynx and gastrointestinal tract (Hanis *et al*., 2017). Nasal carriage is considered the most important site of *S. aureus* colonization (Kates *et al*., 2018). It persistently colonizes this site in about 10-35% of healthy individuals (Tong *et al*., 2015; Ezeamagu *et al*., 2018). The diseases causes by *S. aureus* ranging from superficial soft tissue and skin infections such as impetigo and abscess to severe and fatal systemic infections such as endocarditis, pneumonia, toxic shock syndrome and sepsis (Yang *et al*., 2017). Its pathogenicity is mainly related to a combination of toxin-mediated virulence, invasive capacity and antibiotic resistance (Carfora *et al*., 2015).

Mobile genetic elements (MGEs) play a central role in the evolution and the emergence of new strains of these bacteria (Jamrozy *et al*., 2017), which contribute to the acquisition of new genetic information through horizontal gene transfer (Naito and Pawlowska, 2016). *S. aureus* exhibits abundance of virulence factors some of which are encoded on the chromosome, while others are encoded on

extrachromosomal elements (Liu *et al*., 2015). The virulence factors including series of cell wall-associated proteins, enzymes and range of extracellular protein toxins including enterotoxins, toxic shock syndrome toxin 1 (TSST-1), exfoliative toxins (ETs) and hemolysins (Guo *et al*., 2017). The presence of hemolysin protein considered an important factor for the pathogenicity of this bacteria (Vandenesch *et al*., 2012). The alpha, beta, delta and gamma hemolysin types found in *S. aureus* that encoded by *hla*, *hlb*, *hld*, and *hlg* genes respectively (Hoseini *et al*., 2014).

S. aureus known for its ability to the resistance to most of antibiotics. The innate immunity considered the first line of defense against these bacteria, another line is the use of antibiotics, but the misuse or overuse of this antibiotics in part lead to the appearance of resistant strains of *S. aureus* (Alam *et al*., 2017). Its impact that enhanced by the development of antibiotic resistance most notably methicillin resistant *Staphylococcus aureus* (MRSA) (Paterson *et al*., 2014; Zhang *et al*., 2018). MRSA is a pathogenic *S. aureus* strain characteristic associated with increased patient morbidity and mortality in both community and hospital settings, so it has been generally regarded as a public health problem (Loftus *et al*., 2017; Price *et al*., 2017; Ezeamagu *et al*., 2018), thus it was classified into two different groups, community-acquired

(CA-MRSA) and Hospital-acquired (HA-MRSA) strains (Grumann *et al*., 2014; Aman and Adhikari, 2014).

The main reason responsible for this resistance returns to the expression of penicillin-binding protein2a PBP2a (PBP′) encoded by *mecA* gene (Rudkin *et al*., 2014). Additionally, the ability of *S. aureus* to produce various antibiotic deactivating enzymes has compounded the problems of antimicrobial therapy resulting in multiple resistance to these agents (Fair and Tor, 2014). These enzymes such as β-lactamase destroys the functional integrity of the β-lactam antibiotics by cleaving the β-lactam ring of the penicillin molecule (Waters *et al*., 2016). MRSA infections compared with the infections that caused by non-resistant strains of the same bacteria patients consume more resources of health-care (Mead, 2013; Waters *et al*., 2016), where their hospitalization is prolonged because most preferred drugs such as β-lactam antibiotics could no longer effectively produce bactericidal effects on this organism (Kong *et al*., 2016).

The aims of this study are:

Phenotypic and Molecular Study of *Staphylococcus aureus* Isolated from Clinical Cases in Misan Province /Iraq by:

1- Isolation and identification of *S. aureus* by using conventional and confirmatory techniques.

2- Detection of phenotypic traits related with their hemolysin and antibiotic resistance as virulence factors.

3- Estimation the occurrence of *hlg* and *mecA* genes in identified bacteria.

1.2- Literature Review

1.2.1- History of *S. aureus*

S. aureus is an important mammalian pathogen that recognized for its tendency to cause severe and invasive diseases (Kong *et al*., 2016). In 1878, Robert Koch first noted that various diseases were caused by Gram-positive cocci depending on whether they formed pairs, chains or clusters (Rodrigues *et al*., 2016). The staphylococci were initially identified as grape-like clusters of bacteria from a surgical abscess in a knee join by Alexander Ogston in 1880 (Khan, 2017). The name *Staphylococcus* out from (Greek term staphyl, abunch of grapes; kokkos, grain or berry) (Licitra, 2013). In 1884, Friedrich Rosenbach was able to grow this bacteria in pure culture and distinguished them based on pigmentation (Licitra, 2013). The pathogenic species *Staphylococcus aureus* produced a golden yellow pigment, in other hand the non-pathogenic type *S. albus*, (later renamed *S. epidermidis*), was generally white (Rosenstein and Götz, 2013).

Before invention the antibiotics pre 1940s, invasive *S. aureus*

disease was a significant cause of mortality. However the production of penicillin in 1942 reduced death rates caused by this organism (Bradley *et al*., 2015). Early in 1950s, strains were isolated that were resistant to penicillin (khan, 2017). Antibiotics was developed from penicillin called semi-synthetic penicillins such as methicillin to treat infections caused by *S. aureus* resistant to penicillin in 1959 (Harkins *et al*., 2017). But this bacteria in 1961 also resist this type of antibiotics (the resistance spread by phage 80a) (Alharbi *et al*., 2014). Jevons describes the first naturally occurring methicillin-resistant *S. aureus* (MRSA) in 1963 (Morgan and David, 2015).

In mid of 1980s the basis for methicillin-resistance described and PBP2a was characterized (Khan, 2017). Increased occurrence of community-acquired *S. aureus* are reported among athletic teams and compromised population from 2000 to present (Sowash and Uhlemann, 2017). During the period between 1960-2000 the resistance by MRSA to macrolides, tetracyclines, chloramphenicol, aminoglycosides and fluoroquinolones are reported (Aminov, 2010). Recently, methicillin-resistant *S. aureus* (MRSA) strains exhibiting intermediate and complete resistance to vancomycin have been isolated in hospitals and some MRSA strains are now endemic in various community niches (McGuinness *et al*., 2017).

1.2.2- General characteristics

S. aureus is a Gram-positive cocci ranging from 0.5 to 1.5 μm in diameter, normally arranged in grape-like clusters (Akanbi *et al*., 2017). They often golden-yellow pigmented cells (Gao *et al*., 2017). It non-motile, facultative anaerobic and expresses a polysaccharide capsule (Kuipers *et al*., 2016), also it non spore forming but is resistant to dry conditions (Nazari *et al*., 2014), which is essential to overcome to adverse environmental conditions and can be recovered from non-physiological environments even months after inoculation on any kind of surfaces (Kadariya *et al*., 2014; Zarpellon *et al*., 2015).

They grow most rapidly at 37°C but form pigment best at room temperature (20-25°C) also in temperature ranging from 18°C to 40°C , pH from 4.2 to 9.3 but the optimum 7 to 7.5 (Kadariya *et al*., 2014). This bacteria have the ability to grow at high saline concentrations, especially in media with 10% of NaCl (Cebrian *et al*., 2015).

Chemically, it distinguished from Streptococcal and other staphylococcal species based on positive catalase and coagulase, negative oxidase and non gas forming from carbohydrates (Askarian, 2014). The following is a scientific classification of *S. aureus* (Askarian, 2014).

Domain	Bacteria
Kingdom	Eubacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillale
Family	Staphylococcaceae
Genus	*Staphylococcus*
Species	*Staphylococcus aureus*

1.2.3- Virulence factors

Most bacterial pathogens that enter the host experience several different environmental conditions during the course of the infectious process and can also live as free organisms outside the host (Merikanto *et al*., 2012; Schatz and Vardi, 2018). All these are due to the possession of these organisms helpful factors called virulence factors (Bien *et al*., 2012). It can be defined as components of a pathogen that when deleted, specifically impairs virulence but not viability (Tuchscherr and Loffler, 2016), or as microbial products that permits a pathogen to cause disease (Segura *et al*., 2017). Bacteria express virulence factors as means of survival and not with the prime purpose to cause disease (Salini *et al*., 2015).

The remarkable ability of *S. aureus* to cause an enormous range of infections is due, in part, to its ability to produce multiple virulence

factors (Foster *et al*., 2014). It can express proteins to bind fibrinogen, fibronectin, laminin, vitronectin, collagen, elastin and thrombospondin to promote adherence and attachment to endothelial cells and basement membranes (Crosby *et al*., 2016). Collectively, these proteins are known as microbial-surface components recognizing adhesive matrix molecules (MSCRAMMs). MSCRAMMs are surface mediated adhesion proteins generally expressed during exponential growth because it critical step to establish the infection and their expression was controlled by the accessory gene regulator (Agr) system (Fernandes *et al*., 2017). Agr system is a system for coordinate the expression of most virulence factors in *S. aureus* (Pereira *et al*., 2018). These proteins such as fibronectin binding protein A (FnBPA), clumping factor A (ClfA) and Protein A (Bausier *et al*,. 2015). Protein A considered an important factor required for full virulence in this bacteria which binds to the F portion of antibody in the host (Lacey *et al*., 2016).

In stationary phase, *S. aureus* produces large numbers of membrane-damaging exotoxins and various enzymes such as protease, nuclease, lipase, hyaluronidase, deoxyribonuclease and staphylokinase, to promote tissue invasion and spread of *S. aureus* within the host (Hu *et al*., 2012; kong *et al*., 2016). Also two common enzymes secreted by this bacteria is that, (i) catalase; it promotes the conversion of hydrogen

peroxide, a powerful and potentially harmful oxidizing agent, to water and molecular oxygen (Teo *et al*., 2015), so the main function of this enzyme is to prevent the accumulation of toxic levels of hydrogen peroxide formed as a byproduct of metabolic processes especially that of the electron transport pathway (Mustafa, 2014) and (ii) coagulase; a polypeptide that bind to and activate prothrombin, thereby converting fibrinogen to fibrin, thus promoting the clotting of plasma or blood in the host (Carfora *et al*., 2015).

Three toxin families are produced by this organism, namely (i) pore-forming toxins; such as Panton–Valentine leukocidin (PVL) toxin that mediates destruction of phagocytes (Liu *et al*., 2015), (ii) exfoliative toxins; which implicated in the disease staphylococcal scalded skin syndrome (SSSS) that occurs most commonly in infants (Mishra *et al*., 2016) and (iii) superantigens; such as toxic shock syndrome toxin I (TSST-I), to activate large numbers of T cells resulting in proliferation and cytokine release (Otto, 2012). The majority of toxin-encoding genes are located on mobile genetic elements (MGEs), resulting in a pronounced heterogeneity in the endowment with toxin genes of individual *S. aureus* strains (Jamrozy *et al*., 2017).

They also produce another types of toxins and enzymes such as alpha, beta, gamma and delta toxins that act on host cell membranes

(Swofford *et al*., 2014). That responsible for the damage of nearby organizations through affecting the red blood cell, platelet and neutrophil (Sit *et al*., 2017). The production of hemolysins by bacteria is dependent on their growth status in a particular medium (Stulik *et al*., 2014), and the extent of hemolysin production is positively correlated with the number of bacterial cells (Kong *et al*., 2016).

Another virulence factor in *S. aureus* is the biofilm formation that has high resistance to antibiotic treatments and host immune response (Archer *et al*., 2011). Collectively, studies on *S. aureus* gene regulation suggested that down regulation of virulence genes during colonization and up regulation during infection (Hwang *et al*., 2016).

As we know *S. aureus* have the ability to resist a wide range of antibiotics especially the β-lactam group. This resistance considers an important virulence factor (Ebrahimi *et al*., 2014). Resistance issue of this organism continues in threatening the world population despite the availability of antibiotics (Liu *et al*., 2015). Nosocomial *S. aureus* infections have been steadily increasing (Ebrahimi *et al*., 2014). This increase is due, in part, to the common use of implanted, intravascular medical devices (Inui and Bandyk, 2015). Additionally, there has been a significant increase in infections caused by MRSA and these strains have evolved resistance to other antimicrobials such as vancomycin (Capone *et*

al., 2016). At present, MRSA infections are not only seen in hospital, but also in community and livestock (Stefani *et al*., 2012). Prevalence of this infection underlies in its resistance mechanism (Sit *et al*., 2017).

1.2.4. Virulence genes

1.2.4.1- The *hlg* gene

The *hlg* gene, is a virulence gene consist of two parts (*hlgAB* and *hlgCB*) that encoding two functional γ-hemolysin subunits (HlgAB and HlgCB) respectively, is located in the core genome (Dunyach-Remy *et al*., 2016). HlgAB and HlgCB are major secreted *S. aureus* leukocidins (Spaan *et al*., 2014). This toxins present in 99.5% of human *S. aureus* strains (Dumont *et al*., 2011; Spaan *et al*., 2017). γ-hemolysins are bicomponent β-barrel pore forming toxins produced by *S. aureus* as water-soluble monomers (Alonzo and Torres, 2014), which assemble into oligomeric pores on the surface of lipid bilayers of target cell membrane by using chemokine receptors to induce pore formation (Alessandrini *et al.*, 2013). This exotoxin comprising at least six different combinations of proteins (Moraveji *et al*., 2014).

This protein toxins in a synergistic action with Panton–Valentine leukocidin (PVL) toxins causing cytotoxic changes in polymorphonuclear leukocytes and hemolysis of erythrocytes and leukocytes of human and

other mammalian species (Yamashita *et al*., 2011; Hoseini *et al*., 2014). The activities of both toxins result from their two protein components, HγI and Hγll for γ-hemolysin and LukF and LukS for leukocidin (Turner and Sriskandan, 2015). They play a role in the septic arthritis and could help community-acquired MRSA (CA-MRSA) to survive in human blood during infection, thus it considered as important factor for *S. aureus* immune evasion (Lacey *et al*., 2016).

1.2.4.2- The *mecA* gene

The *mecA* gene, is a gene found in bacterial cells which allows a bacterium to be resistant to antibiotics (Munita and Arias, 2016). This gene carrying on a mobile genetic element, which is a component of the larger Staphylococcal chromosomal cassette *mec* (SCC*mec*) region, conferring resistance to multiple antibiotics depending on the SCC*mec* type (Liu *et al*., 2016). At least six different types of SCC are recognized, this results in resistance to all beta-lactam antibiotics (Udo and Al-Sweih, 2017).

This gene encodes PBP2a(PBP'), the protein responsible for methicillin resistance in *S. aureus* (Rudkin *et al*., 2014), which has a low tropism to these antibiotics (Elhassan *et al*., 2015). This protein involved in the assembly of the cell wall peptidoglycan in the presence of high concentrations of beta-lactams that otherwise inhibit the endogenous

PBPs (Müller *et al*., 2015). So PBP2 continues to catalyze the synthesis of the bacterial cell wall even in the presence of many antibiotics (Fishovitz *et al*., 2014).

The majority of researches suggested that *mecA* gene presented in all MRSA strains (Elhassan *et al*., 2015). Thus it considered the corner stone responsible for producing MRSA phenomenon (Elhassan *et al*., 2015). The transcription of this gene is controlled by two regulatory systems that mecI-mecR1 and penicillinase blaR1-blaI-encoded regulatory elements (Chovanová *et al*., 2016).

1.2.5- Antibiotic resistance of *S. aureus*

The worldwide escalation of mortality rates due to antibiotic resistant bacterial strains poses a serious challenge to modern medicine and therapeutics (Kalita *et al*., 2016). Random mutations and plasmid-mediated horizontal gene transfer have introduced drug resistance capabilities to ever-increasing types of antibiotics, even in community associated strains which were previously described as being less drug-resistant (Bradley *et al*., 2015). As strains such as USA300 that acquired a variety of plasmids conferring additional drug resistance, in some cases comparable to hospital-associated strains (Kaïret *et al*., 2017), so the global emergence of multi-drug resistance (MDR) in bacterial

isolates has become a silent pandemic, affecting public health for common diseases e.g. urinary tract infections, superficial soft tissue infections, tonsillitis etc. (WHO, 2014).

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a MDR strain of *S. aureus,* resistant to penicillins, cephalosporins, carbapenems and macrolides (Mead, 2013). It is known for its ability to develop drug resistance quickly (Zhanel *et al*., 2015). Treatment of *S. aureus* infections is a major public health challenge due to variance in drug resistance between strains and wide range of clinical presentations, which span from simple to life-threatening (Singh *et al*., 2017).

Penicillin-resistant *Staphylococcus aureus* (PRSA) was reported shortly after the advent of mass produced penicillin and was pandemic in the United States through the 1950s and 1960s (Otto, 2017). Penicillin resistance has been developed due to the acquisition of gene for β-lactamase (penicillinase) by *S. aureus*, and was enhanced by excessive use of antibiotics and misuse, which inadvertently selected for bacteria expressing this gene (Bengtsson *et al*., 2017). To counter this, semi-synthetic penicillin derivatives were synthesized, such as methicillin, oxacillin, cloxacillin, dicloxacillin, flucloxacillin and nafcillin (Pawlowski *et al*., 2016). Methicillin was first introduced in 1959 to treat *S.aureus* infections resistant to penicillin (Harkins *et al*., 2017).

Erythromycin and other macrolide antibiotics, was proposed in the early 1950s as another penicillin alternative to treat resistant S*taphylococcus*. However, it was withdrawn after less than one year because of profuse acquisition of resistance (Mead, 2013). Vancomycin was the only antibiotic available for treating MRSA infections (Choo and Chambers, 2016). However, vancomycin resistant MRSA strains are emerged, including some CA-MRSA strains, have increasingly been reported, thereby causing public health concern (Zhang *et al*., 2018). Acquisition of high-level vancomycin resistance by *S. aureus* represents a major public health risk because this antimicrobial drug continues to be the first-line and most inexpensive therapy to treat MRSA despite concerns about its clinical efficacy (Panesso *et al*., 2015).

Although antibiotic susceptibilities may differ from strain to strain, empiric therapy is often used until cultures can be obtained and tested for resistance (Chopra *et al*., 2015). According to World Health Organization (WHO), it is estimated that patients infected with methicillin resistant *Staphylococcus aureus* (MRSA) are 64% more likely to die compared to people infected with non-resistant form of this bacteria (WHO, 2014). Therefore, there is an urgent need for new improvements in current methods and novel strategies are required to cope with the emergence of

antibiotic resistant bacterial strains in coming decades (Kalita *et al*., 2016; Singh *et al*., 2017).

There are several new antibiotics in developmental stages for MRSA encounter and some of which are undergoing phase II and III clinical trials for Food and Drug Administration (FDA) approval (Anselmo and Mitragotri, 2014; Molchanova *et al*., 2017). For example, Ceftobiprole and ceftaroline are new generation cephalosporins with strong affinity for PBP2a (Chan *et al*., 2016).

1.2.6- Mechanism of methicillin-resistance in MRSA

Although methicillin is no longer produced, the name MRSA has persisted and can be regarded as referring to resistance to virtually all β-lactam antibiotics (Waele *et al*., 2018). β-lactams damage bacteria by inactivation penicillin-binding proteins, enzymes that are essential in the assembly of the bacterial cell wall (Wanner *et al*., 2017). These antibiotics inactivate the four native penicillin-binding proteins found in *Staphylococcus* (Ezeamague *et al*., 2018). The weakened of bacterial cell wall as a result of antibiotic action lead to it became osmotically fragile and are easily lysed (Waele *et al*., 2018).

The staphylococcal β-lactamase protein, which cleaves the β-lactam ring structure, confers resistance to penicillin, but not to

semi-synthetic penicillins (Holmes *et al*., 2016). In MRSA, resistance to all β-lactam antibiotics, including the semi-synthetic penicillins, is conferred by PBP2' or PBP2a (76-kDa) (Ezeamague *et al*., 2018), that has a very low affinity for β-lactam antibiotics and is thought to aid cell wall assembly when normal penicillin-binding proteins are inactivated (Sit *et al*., 2017) . This protein encoded by the *mecA* gene that located on a mobile genetic element, the staphylococcal cassette chromosome mec (SCCmec) (Matys *et al*., 2015).

The confirmation of *mecA* gene presence, has until recently been the 'golden standard' for MRSA detection worldwide. This gets either by using the molecular detection of *mecA*, typically by polymerase chain reaction (PCR) or by PBP2a/PBP2′, usually using antibody detection with commercially available slide agglutination assays (Abrok *et al*., 2018).

1.2.7- Reservoirs site and Host range

The carriage of *S. aureus* in humans can occur at various body sites including; skin, anterior nares, perineum, axillae, vagina, different parts of the digestive system including pharynx, gastrointestinal tract, urinary tract and throat (Sollid *et al*., 2014; Habib *et al*., 2015). This carriage is usually asymptomatic (Tong *et al*., 2015). Although, the nares have been known as the main niche and reservoir of *S. aureus* in humans, several studies

have indicated higher prevalence of this bacteria in throat (Nazari *et al*., 2014). *S. aureus* can also colonize animals e.g. pigs, bats, dogs, cats, rabbits, parrots, cattle, horses and wild animal species (Monecke *et al*., 2016).

Various genetic analyses have shown that animal-associated *S. aureus* is not commonly found in human-associated lineages (Sharma *et al*., 2016). This reflects the presence of host specific barriers between *S. aureus* animal and human, but other studies have shown that both lineages are closely related to each other and only few particular genes or gene combinations may contribute to host specificity and adaptation (Pantosti, 2012). There is an exchange of genes encoding virulence factors between animal- and human- associated *S. aureus* lineages (Kraushaar *et al*., 2017). Clearly, acquisition of virulence genes by specific host-adapted isolates that enable them to colonize and infect new hosts, can be dangerous (Monecke *et al*., 2016). MRSA is increasingly recognized within the animal kingdom and a great concern has been raised due to its presence in the animal world, particularly in pigs, as these may serve as a reservoir for human infection and colonization (Gómez-Sanz *et al*., 2013).

1.2.8- Transmission of *S. aureus*

S. aureus can be transmitted from person to person, as well as from animals to humans and vice-versa (Monecke *et al*., 2016). Typical transmission of *S. aureus* occurs mainly due to direct skin contact with colonized or infected people or contact with recently contaminated surfaces (Ferreira *et al*., 2011; Brown *et al*., 2014). If an individual is a carrier of *S. aureus*, this can enhance the risk of additional individual or surrounding contamination (Coll *et al*., 2017). *S. aureus* nasal carriers with rhinitis are able to disperse a high load of this bacterium to the environment (Goldstein *et al*., 2017). Hands play an important role in the transmission of *S. aureus* from surfaces to the nasal and other body sites and vice-versa (Ho *et al*., 2015; Haghverdian *et al*., 2018). Food may be contaminated with *S. aureus* ; handling or eating contaminated food is also a potential means of transmission (Trivedi *et al*., 2015). Also in the hospitals contaminated food can extend this organism to patients as well as to healthcare workers (EFSA, 2012).

Some studies have reported bidirectional transmission of MRSA (AVMA, 2014). Animal transmission to humans occurs through direct contact and environmental pollution and by dealing with the products of infected animals (Schmithausen *et al*., 2015), whereas human to animal transmission is still unclear (Weese, 2010; Schaumburg *et al*., 2015).

Moreover, host determinants, e.g. colonization status and immune impairment (Mulcahy and McLoughlin, 2016), as well as capability of *S. aureus* in colonization the corneal layer of the skin with different properties, e.g. low temperature, low pH, high osmotic concentration, nutrient restrictions, antibiotics and interference of commensal microorganisms, contribute to successful transmission of *S. aureus* (Leger *et al*., 2017; Relhan *et al*., 2017).

1.2.9- *S. aureus* colonization

Colonization by *S. aureus* occurs when the bacteria exist as a commensal on the surface of the skin or mucus without any signs or symptoms of infection (Liddle and Merchan, 2017). Because it considered a part of the normal microflora of the skin and mucosal surfaces of humans and animals (Nair *et al*., 2014; Habib *et al*., 2015). Thus the breakage of skin barriers promotes the transformation of *S. aureus* from a commensal colonizer to an invading pathogen (Leibler *et al*., 2017). So it's called an opportunistic pathogen (Walther *et al*., 2017). This means if given the opportunity *S. aureus* can cause infection, most commonly at sites of lowered host resistance such as damaged skin or mucosal membranes (Walther *et al*., 2017).

These bacteria possesses a large number of cell-associated and

extracellular virulence factors, some of which contribute to the ability of the organism to overcome the host immune defenses and to invade and colonize the tissue (Goldmann and Medina, 2017). *S. aureus* avoids host recognition or reduce the subsequent immune activation for survival in a human host (Geoghegan and Foster, 2015). It expresses variety of surface-associated as well as secreted proteins, which mediates attachment to mucus, plasma proteins, epithelial cells, endothelial cells and extracellular matrix (ECM) (Foster *et al*., 2014). Staphylococcal adhesins are covalently attached to peptidoglycan as well as non-protein materials such as wall teichoic acid (Kim *et al*., 2016), thus the success adherence of *S. aureus* to the host interaction partners may depend on the correct combination (Foster *et al*., 2014).

The difference in colonization means that host factors are important elements for successful bacterial colonization (Ballhausen *et al*., 2017). Additionally, the microflora of the host organ, e.g. anterior nares, is a harsh challenge for *S. aureus*, since the presence of certain bacterial competitors can hinder colonization (Lopez and Skaar, 2017).

Chapter Two

Materials

&

Methods

2- Materials and Methods

2.1- Materials

2.1.1- Equipments and Instruments

The equipment s and instruments used in this study are mentioned in the table (2-1).

Table (2-1): Equipments and Instruments used in this study

Equipments & Instruments	Company	Origin
Autoclave	Hirayama	Japan
Bottle	Simax	USA
Biosafety cabinet	Human lab	South Korea
Burner	Amel	Turkey
Compound light microscope	Nikon	Japan
Cooling Centrifuge	Eppendorf	Germany
Distillator	Lab Tech	South Korea
Electric oven	Precision	South Korea
Electrophoresis Unit	Cleaver	UK
Eppendorf Cooling Centrifuge	Hettich	Germany
Eppendorf tubes	BDH	UK
Flask	Lab glass	Japan
Gel documentation	Vilber	France
Incubator	Binder	Germany
Magnetic stirrer with hot plate	Glassco	India
Micropipette	Watson	Japan
Ph meter	Jenway	Germany
Petri dish	Plastilab	Lebanon
Refrigerator	Vestel	Turkey
Screw cup	Acon biotech	China
Sensitive balance	Sartorius	Germany
Sterilized loop	Unipak	Iran
Test tube	Samco	India
Thermocycler	Eppendorf	Germany
Transport swab	AFCO	Jordan
UV- Transilluminator	ELETTROFOR	Italy
Vitek 2 compact system	BioMérieux	France
Vortex mixer	Heidolph	Germany
Water bath	Memmert	Germany

2.1.2- Biological and Chemical materials

The materials and reagents used in this study are mentioned in the table (2-2).

Table (2-2): Materials and Reagents used in this study

Materials	Company	Origin
6X DNA Loading Dye	Biobasic	Canada
Agar-Agar	LabM	UK
Agarose	Promega	USA
Barium chloride BaCl2	Fluka	Switzerland
De-ionized water	Bioneer	South Korea
Ethanol absolute	Scharlau	Spin
Ethidium bromide	Promega	USA
Glycerol (C3H8O3)	Fluka	Switzerland
Hydrogen peroxide (H2O2) 30%	BDH	UK
AccuLadder 100 bp DNA size Marker	Bioneer	South Korea
Sodium chloride (NaCl)	BDH	UK
Sulphuric acid H2SO4	BDH	UK
Tris-Borate-EDTA Buffer (TBE buffer)	Biobasic	Canada
Tryptone Type-1	Himedia	India
Yeast extract	Himedia	India

2.1.3- Culture Media:

The culture media are listed in the table (2-3).

Table (2-3): Culture media used in this study

Media	Company	Origin
Blood agar base	Biomark	India
Mannitol salt agar	Biomark	India
Mueller-Hinton agar	Biomark	India
Nutrient agar	Biomark	India
Nutrient broth	Biomark	India

2.1.4- Antibiotic disks

The antibiotic disks used in present study were manufactured by the company of (Bioanalyse, Turkey) that mentioned in the table (2-4).

Table (2-4): Antibiotic disks used in this study

Antibiotic class	Antibiotic sub class	Antibiotic name	Symbol	Disk concentration
Penicillins	Aminopenicillin	Ampicillin	AM	10 µg
	Penicillinase-stable penicillins	Oxacillin Methicillin	OX ME	1 µg 5 µg
Cephems	Cephalosporin II	Cefoxitin	Fox	30 µg
	Cephalosporin III	Cefotaxime	CTX	30 µg
Carbapenems		Imipenem	IPM	10 µg
Glycopeptides		Vancomycin	VA	30 µg
Aminoglycosides		Gentamycin Amikacin	GM AK	10 µg 30 µg
Tetracyclines		Doxycycline Tetracycline	DXT TE	30 µg 30 µg
Macrolides		Erythromycin	E	15 µg
Lincosamides		Clindamycin	CD	2 µg
Fluoroquinolones		Ciprofloxacin Levofloxacin	CIP LEV	5 µg 5 µg
Ansamycins		Rifampin	RA	5 µg
Nitrofurantoins		Nitrofurantoin	NIT	300 µg
Folate pathway inhibitors		Trimethoprim sulfamethoxazole	TS	25 µg

2.1.5- Kits

The kits used in this study are listed in the table (2-5).

Table (2-5): Kits used in this study

Kit type	Purpose	Company	Origin
Gram stain	Differentiation of microorganisms	Syrbio	Syria
Master Mix(AccuPower® PCR PreMix)	For amplified primer by PCR	Bioneer	South Korea
Presto™ Mini gDNA Bacteria Kit	Extraction bacteria genomic DNA	Geneaid	Taiwan
Vitek-GB Kit	Identification of Gram-positive bacteria of the family Staphylococcaceae	BioMérieux	France

2.2- Methods

The general steps for this research are shown below in figure (2-1).

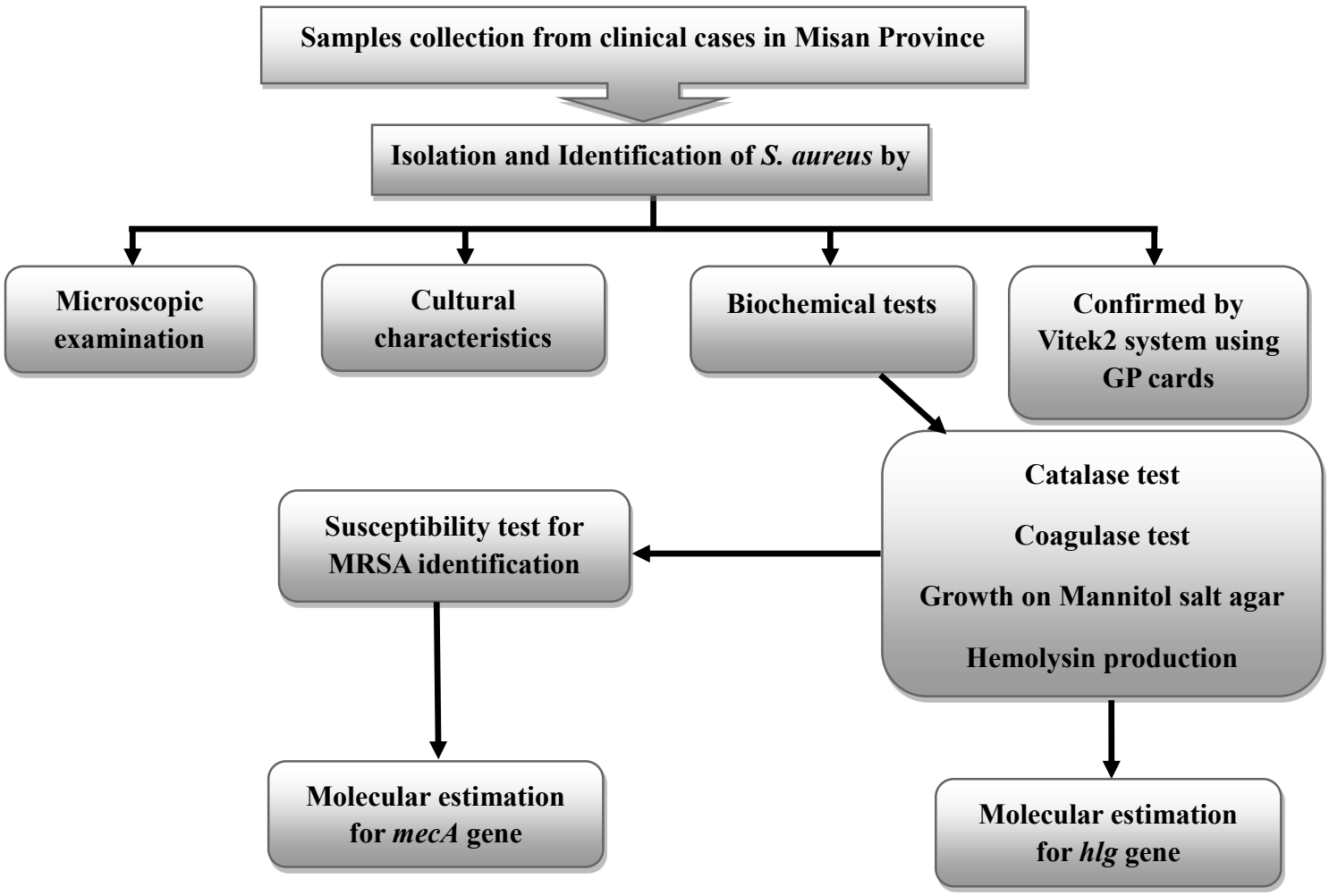

Figure (2-1): Scheme of the most important steps in the current study.

2.2.1- Samples collection

A total of 274 samples, were collected from different clinical cases as shown in table (2-6) from two main hospitals in Misan Province (Al Sadr Teaching Hospital) and (Al Zahrawi Surgical Hospital) during the period from December 2017 to April 2018. Collected swabs were streaked on mannitol salt agar then incubated at 37C°for 24-48 hr, the suspected colonies depending on the morphological bases were selected for the identification by conventional methods and Vitek2 system.

Table (2-6): Number of samples taken from clinical cases in Misan Province

Samples type	Samples number
Bedsore	22
Burn swab	80
Ear swab	15
Nasal swab	30
Oral swab	8
Throat swab	16
Urine	33
Vaginal swab	18
Wound swab	52
Total	274

2.2.2- Preparation of solutions and reagents:

2.2.2.1- Catalase reagent

Hydrogen peroxide (3%) prepared and it was used to identify bacterial ability to produce catalase enzyme (MacFaddin, 2000).

2.2.2.2- Ethidium Bromide (0.5%)

A stock solution was prepared by dissolving 0.05g of ethidium bromide powder in 10ml distilled water (D.W) in sterile dark bottle, the solution is mixed by vortex to be dissolve completely (Sambrook and Russell, 2006). It is used for electrophoresis as specific DNA stain.

2.2.2.3- McFarland standard (0.5)

McFarland standard solution consist of the following solutions according to (CLSI, 2017):

1- Solution A was prepared by dissolving 1.75g of BaCl2-H2O (Barium Chloride) in 100ml of distilled water.

2- Solution B was prepared by adding 1ml of H2SO4 to 100ml of distilled water.

Then mixing 0.5ml of A solution with 9.5ml of B solution in the test tube. This solution used for comparison with the tubes containing bacteria about $1.5x10^8$ (CFU/ml), such as susceptibility test using antibiotic disks.

2.2.3- Preparation of culture and diagnostic media

All cultures media which mentioned in table (2-3) were prepared according to the manufacturer's instructions, boiling to dissolve the media completely and sterilized by autoclave at 121°C (15 Ibs pressure) for 15 min, incubated for 24 hr after pouring in sterile petri dishes was done to detect the contamination then stored at 4°C until use.

While the following media prepared as:

2.2.3.1- Blood agar

As instructed by the company equipped it has been prepared by suspend 40g of the blood agar base powder in 1000ml of D.W, heated to boiling and sterilized in autoclave at 15 Ibs pressure (121°C) for 15 min, then 10% of fresh human blood was added after cooling to 45-50°C. It used to show the colonial morphology and hemolysin production (Collee *et al*., 1996).

2.2.3.2- Lauria broth

It was prepared by dissolve 10g Tryptone, 5g Yeast extract and 5g NaCl in 800ml D.W. The pH is adjusted to 7.2, then the complete into 1000ml by distilled water and sterilized by autoclaving for 15 min. Lauria agar prepared by adding 20g of agar-agar for one liter of Lauria broth. It is used for DNA extraction (Sambrook and Russell, 2006).

2.2.4- Isolation and Identification of *S. aureus*

The swabs samples were directly inoculated on to mannitol salt agar and incubated at 37°C for 24-48 hr. The isolates were diagnosed depending on Holt *et al*. (1994), Collee *et al*. (1996) and MacFaddin (2000) to diagnose some of their microscopic, cultural and biochemical characteristics as following:

2.2.4.1- Microscopic examination

Smears from the fresh colonies were cultured on the selective medium (Mannitol salt agar) stained with Gram's stain and examined under the microscope to observe the shape, arrangement and the interaction with the dye.

2.2.4.2- Cultural characteristics

Colonies of the isolates grown on solid medium (Mannitol salt agar) was described according to their shape, pigmentation, edge and the change in the color media.

2.2.4.3- Biochemical tests

2.2.4.3.1- Catalase test

Catalase production was examined by putting a loop full of bacteria on sterilized slide. One drop of 3% hydrogen peroxide was added, bubble formation indicates to the positive results (MacFaddin, 2000).

2.2.4.3.2- Coagulase test

The procedure involves a loop full of bacteria from a blood agar plate (or 0.5 ml of broth culture) was mixed with 0.5 ml of a one to five dilution of human plasma and incubated at 37°C, examined after half an hour/4 hr then after 24 hr (MacFaddin, 2000).

2.2.4.3.3- Growth on Mannitol salt agar

The samples were inoculated onto mannitol salt agar (which contains a high salt concentration (NaCl 7-10 %), the plates incubated aerobically at 37ºC for 24-48 hours. Then colonial morphology examined (Collee *et al*., 1996).

2.2.4.3.4- Hemolysin production

A loop full of an overnight growth from mannitol salt agar was cultured on blood agar by streaking method, incubated at 37°C for 18-24 hr. Then examined to identify the pattern of hemolysis appeared around the colonies resulting from blood cells lysis (Collee *et al*., 1996).

2.2.5- Identification of bacteria by Vitek2 system

The identified isolates were confirmed with the automatic Vitek2 system according to Manufacturers's instructions using ID-Gram Positive Cocci cards (ID-GPC cards; bioM΄erieux, France) as following:

1- The VITEK-2 ID-GPC cards were taken out the refrigerator and allowed to achieve room temperature before opening the package liner.

2- A smart carrier containing room for 10 tubes and 10 cards were placed.

3- Colonies from a fresh Lauria agar plate were suspended in 3ml 0.45% NaCl by vortex and the turbidity was measured by a Densichek Master and adjusted to 0.50 – 0.63 CFU/ml.

4- The prepared suspension was placed at the positions in cassette.

5- The ID-GPC card bar code labels were scanned and the cards were placed in the card positions next to the inoculum tubes.

6- The Smart Carrier containing the inoculums tubes and ID-GPC cards, were placed in Vitek2 system where each test card was automatically filled with a bacterial suspension and automatic identification testing was performed. The software analyzed the data and reported the results after 4 hr.

2.2.6- Preservation and Maintaining of *S. aureus*

2.2.6.1- Short-term preservation

Nutrient agar was dispensed into tubes in 10ml amounts autoclaved and left slanted to solidify. The slant surface was inoculated with bacteria by streaking and incubated at 37°C, for 18-24 hr and stored in 4°C. The bacteria were transferred to new media every month for the activation of isolates and avoid the contamination (Collee *et al*., 1996).

2.2.6.2. Long-term preservation

Nutrient broth (2.4ml) supplemented with glycerol 15% (0.6ml) was previously inoculated with bacteria one day before and stored at -20°C. This will preserve bacteria samples for one year (Collee *et al*., 1996).

2.2.7- Susceptibility test for MRSA identification

1- Preparing the inoculum:

The inoculum was prepared by using broth or saline suspension of isolated colonies that were selected from an 18-24 hr culture. The suspension was adjusted to match the 0.5 McFarland tube standard to give approximately number of bacteria equal to $1.5x10^8$ (CFU/ml).

2- Inoculation of plates:

A sterile cotton swab dipped into bacterial suspension, was pressed against the side of the tube to remove any excess fluid, streaked on the surface of Muller-Hinton agar for several times in a different directions to ensure complete cover of the plate. After allowing the plates to dry for approximately 5 min to ensure the moisture absorption. Antibiotic disks were applied onto the surface and pressed gently to ensure complete contact with the agar surface. Finally, the plates were incubated at 35-37°C for 16-18 hr.

3- Susceptibility testing:

Susceptibility to the antibiotics was determined by the agar disk diffusion method for Bauer *et al*. (1966), and the guidelines of the national committee for Clinical and Laboratory Standards Institute (CLSI, 2017). Also the diameters of inhibition zone were measured and results interpreted according to CLSI (2017).

2.2.8- Identification of the genes by PCR

The genes in this study are identified as following:

2.2.8.1- Extraction of bacterial chromosomal DNA

All the confirmatory isolates were screened for chromosomal DNA content according to Presto™Mini gDNA Bacteria Kit protocol.

2.2.8.2- Detection of DNA content by Agarose Gel Electrophoresis

Agarose gel electrophoresis at 65 volt for 1hr was used for detection of DNA where the visible bands were illustrated by UV Transilluminator (Sambrook and Russell, 2001). 5µl of extracted DNA mixed with 2µl loading dye, then loaded in agarose gel electrophoresis.

2.2.8.3- Master Mix

The components of the master mix were mentioned in table (2-7), were prepared by the company of Bioneer, South Korea.

Table (2-7): Master Mix (AccuPower®PCR PreMix) used in this study:

Component	Reaction volume 20µl reaction
Taq DNA polymerase	1 U
dNTPs (dATP, dCTP, dGTP, dTTP)	Each µM
Tris-HCL(PH 9.0)	10 mM
KCl	30 mM
MgCl2	1.5 mM
Sterilizer and tracking dye1	Trace

2.2.8.4- Primers used in this study

The specific primers for *hlg* and *mecA* genes have been choosen as in table (2-8), were prepared by the company of Bioneer, South Korea.

Table (2-8): Primers used in this study

Gene	Primer sequences (5' → 3')	Product length (bp)	Refrences
hlg	F: GCCAATCCGTTATTAGAAAATGC R: CCATAGACGTAGCAACGGAT	937	(Cotar *et al*., 2010)
mecA	F: CGGTAACATTGATCGCAACG R: TTTGCCAACCTTTACCATCG	985	(Stotts *et al*., 2005)

2.2.8.5- Preparation of primers solution

The oligonucleotide primers were suspended by dissolving the lyophilized product after spinning down briefly with TE buffer molecular grade 1X (pH=8) depending on manufacturer's instruction as stock suspension. Working primer tube was diluted with TE buffer molecular grade, the final picomoles depended on the procedure of each primer.

2.2.8.6- Polymerase Chain Reaction protocol

The protocol used depending on Bioneer manufacturer's instructions. All PCR components were assembled in PCR tube and mixed by cooling microcentrifuge at 50 rcf (850 rpm) for 10 sec.

The steps were conducted as following:

1- Template DNA and primers were thawed before use.

2- Template DNA and primers were added into the AccuPower®. Taq premix tubes as in table (2-9), for getting 20ml reaction volume.

Table (2-9): The mixture of PCR for 20 µl/reaction

Mixture solution	
De-ionized water	6 µl
Master mix	5 µl
Forward primer	2 µl
Reverse primer	2 µl
DNA template	5 µl
Final volume 20 µl	

3- The lyophilized blue pellet was completely dissolved and spin down either by using centrifuge 15 sec, vortex or by pipette up and down several times.

4- The Eppendorf PCR tubes were placed in the thermocycler and the right PCR cycling program parameter conditions were conducted in table (2-10).

Table (2-10): PCR cycling program parameter conditions

Genes	**Temperature °C/ time**					**Cycle number**
	Initial denaturation	**Cycling conditions**			**Final extension**	
		Denaturation	Annealing	Extension		
Hlg	95/5 min	95/1 min	55/1 min	72/1 min	72/7 min	35 cycles
mecA	95/5 min	95/45 sec	55/1 min	72/2 min	72/7 min	35 cycles

5- Seven micro-liters of amplified PCR product were loaded to the agarose gel wells with DNA ladder (marker) to one of the wells and running by Gel Documentation.

2.2.8.7- Agarose preparation

Agarose gel electrophoresis (1%) was preparing by dissolving 1g in 100ml 1X TBE buffer, left to cool at 50°C and 5µl of ethidium bromide was added to agarose and poured on preparing tray. Comb was removed after hardening of agarose leaving wells (Sambrook and Russell, 2006).

2.2.8.8- Agarose Gel Electrophoresis

TBE 1X buffer was added to the electrophoresis tank, tray with agarose was immersed in electrophoresis tank. Each well is loaded with 7µl of DNA sample. Then standard molecular weight of DNA ladder is loaded in a first well. Electrophoreses run at 65 volt for 2 hr, the gel was visualized by Gel Documentation (Mishra *et al*., 2010).

Chapter Three

Results

&

Discussion

3- Results and Discussion

3.1- Isolation and Identification of *Staphylococcus aureus* by conventional methods and Vitek2 system

A total of 274 samples from different clinical cases table (3-1) were collected for isolating and diagnosis of *Staphylococcus aureus* to evaluate the prevalence of this bacteria, compare antimicrobial susceptibility and molecular identification of some virulence factors such as hemolysin and antibiotic resistance through study of *hlg* and *mecA* genes respectively.

Table (3-1): Number of *S. aureus* isolates taken from clinical cases in Misan Province

Samples type	Samples number	*S. aureus* isolates	Percent %
Bedsore	22	9	40.9
Burn swab	80	35	43.7
Ear swab	15	6	40
Nasal swab	30	14	46.6
Oral swab	8	3	37.5
Throat swab	16	4	25
Urine	33	8	24.2
Vaginal swab	18	5	27.7
Wound swab	52	22	42.3
Total	274	106	38.6

The bacterial isolates firstly identified by microscopic study of colonial properties and the biochemical tests as initial identification as in

table (3-2), then the diagnosis of the isolates were confirmed by Vitek 2 system table (3-3).

Table (3-2): Microscopic and Biochemical tests for *S. aureus* isolates

Primary test	Result
Gram stain	+ve
Catalase test	+ve
Coagulase test	+ve
Growth on Mannitol Salt agar and mannitol fermentation	+ve
Hemolysin Production	+ve

The colonies of *S. aureus* are grown on culture media once revealed the typical characteristics being described by referential studies. Microscopically the cells were arranged in irregular (grape-like) clusters and stained purple by Gram stain as in figure (3-1).

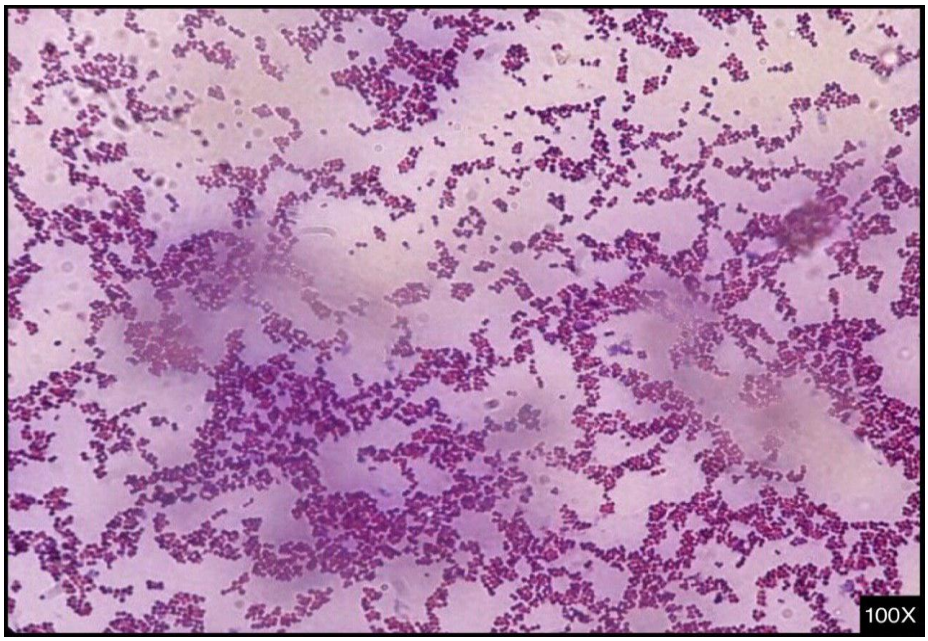

Figure (3-1): Gram stain of *Staphylococcus aureus.*

Also in current study, the results of biochemical tests for *S. aureus* diagnosis indicated that 100% of the isolates response to Catalase test as positive result. Which used to differentiate between Staphylococcal species especially *S. aureus* (catalase positive) and Streptococcal species (catalase negative) (Reiner, 2010). For Coagulase test all these isolates revealed a positive result as shown in figure (3-2). Which used to distinguish *S. aureus* from other *Staphylococcus* species (Taylor and Unakal, 2017).

Positive result **Negative result**

Figure (3-2): Coagulase test of *S. aureus.*

S. aureus grow on Mannitol salt agar as in figure (3-3), because it has the ability to grow at high NaCl concentration and fermentation of mannitol and change its color from red to yellow in the presence of phenol red as a pH indicator. The mannitol fermentation positive to distinguish it from *Staphylococcus epidermidis* (Taylor and Unakal, 2017).

Figure (3-3): *S. aureus* on Mannitol salt agar.

The isolates were grew on blood agar media and show their hemolytic activity. Hemolysis zone appeared around colonies refers to the ability of bacteria to hemolyse the red blood cells, and the results are interpreted as alpha, Beta, gamma and delta, figure (3-4) shows ß-hemolysis pattern.

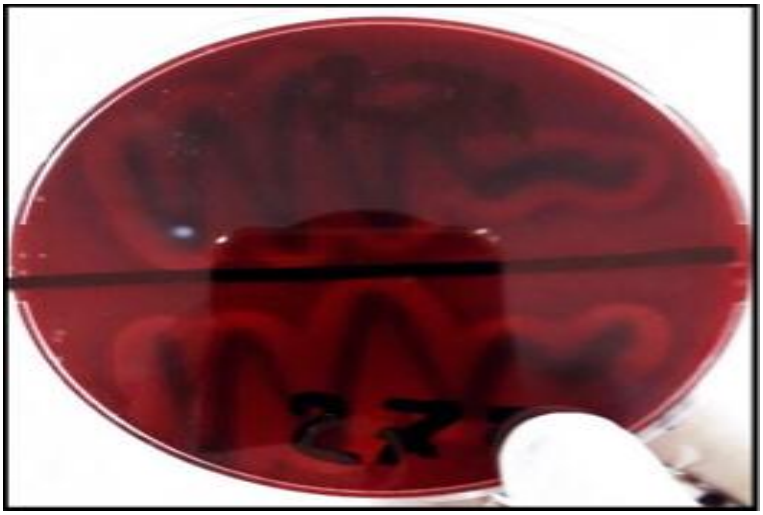

Figure (3-4): Hemolytic activity of *S. aureus.*

S. aureus colonies on solid media often grey to deep golden-yellow pigmented colonies, as a result of carotenoid pigments that form during their growth (AL-Kazaz *et al*., 2014), no pigment is produced anaerobically and in broth (Gao *et al*., 2017; Mohammed *et al*., 2017) and they round, smooth, raised and glistening (Kahl *et al*., 2016).

The confirmation of *S. aureus* identification was performed using Vitek2 system as in table (3-3).

Table (3-3): Distribution of Vitek 2 results in different classes of probability

Classes of probability	No (%) of identification
Excellent	21(19.8)
Very good	19(17.9)
Good	40(37.7)
Acceptable	17(16)
Low discrimination	9(8.5)

Probability of excellent identification = 96-99%

Probability of very good identification = 93-95%

Probability of good identification = 89-92%

Probability of acceptable identification = 85-88%

Probability of low discrimination with extra tests = 50%

The automated Vitek2 system (Version: 07.01) can provide rapid and accurate identification results for gram-positive cocci in few hours based on fluorescence technology using ID- GPC cards.

Previous studies such as (Francis *et al*., 2005; Bubeck Wardenburg

et al., 2008; Zhang *et al*., 2016) demonstrated that *S. aureus* species are the main reason for skin and soft tissue infections such as impetigo, furunculosis, bedsore, surface and surgical wounds, abscess and further systemic infections such as urinary tract infections (UTIs) and pneumonia. So table (3-1) illustrates some of these cases and the number of samples taken from each case in addition to the isolates of *S. aureus*.

As shown in table (3-1) the highest percentage of *S. aureus* isolates was in the nasal area that (46.6%), because it is the main site responsible for the colonization of *S. aureus*. This rate is less than the rate of Ouedraogo *et al*. (2016) study where it reached to 69.4%, whereas it more than the rate of Eibach *et al*. (2017) was 35.2%.

The colonization of *S. aureus* in this area was classified by Alsterholm (2012) to three types, that may be temporary, called intermittent carrier and often make up 60% of the population, the persistent carrier forming percentage reached to 29%, and the third form was the non-carrier group which 20%. Whenever the rate of colonization is higher in the patients, the risk of *S. aureus* infections become more, especially diabetics, patients with immunodeficiency syndrome, dialysis patients, and eczema patients (Brown *et al*., 2014).

The percentage of patients in this study can be considered within the normal range because patients exhibit a higher carrying rate for these

bacteria than the normal rate of colonization in the nose area, this fact was noted by Isidre *et al*. (2017) study by the analysis of isolated strains of patients they infected with bacteremia and found that they were identical to those isolated from the nose area and that the source of 80% of *S. aureus* bacteria causing the bacteremia is endemic bacteria in the nasal carriage and this also observed by Bergin *et al*. (2015).

The colonization process was obtained by extracellular compounds called MSCRAMMs especially ClfA that acts as a link to fibrinogen and thus the adhesion process is occur (Foster *et al*., 2014). *S. aureus* has been found to have a variety of ability to produce surface compounds that allow it to colonize such as protein adhesions, polysaccharides intercellular adhesions and biofilm formation (Porayath *et al*., 2018). In addition to the role of ClfA in nasal colonization, studies have shown that the resulting infections are mostly the result of *S. aureus* that take advantage of the chances of immune weakness and lead the disease to occur (Hwang *et al*., 2016), while others get infected while staying in the hospital so they get *S. aureus* endemic and highly resistant to antibiotics and this resistance be silent (Kaur and Chate, 2015). The method of transmission is supposed to be carried out by hospital care supervisors and Puah *et al*. (2016) confirmed that the risk is not high or low proportion, but the amount of genetic linked to the latent virulence factors

of some *S. aureus* isolates, where has been found that some of them are more dangerous than others and suggested that the incidence of infection for some people usually occur with strains of internal origin, this fact confirmed by Rong *et al*. (2017) where referring to the fact that the process of carrying *S. aureus* does not constitute a danger to the wounds of operations and does not cause the infection to the wounds, this mean the rate of carrying bacteria in the nose area is not the criterion for the prediction of infection, while other sources assured that the origin of the source of infection remains unclear (Thomas *et al.*, 2017), and on this basis the increase or decrease in the percentage is still under study.

The results also accounted that the second highest rate of *S. aureus* isolation that from burns was (43.7%) which was higher than the rate of Saaiq *et al*. (2015) that 18.6%, while percentage in study conducted by Amissah *et al*. (2017) was more than current study that 45%.

Burning cases are often associated with a high percentage of isolated bacteria in the worldwide, especially the third world countries, which are inversely proportional to the amount of progress (Chen and Huang, 2014), during statistical study for WHO (2012) revealed that 80% of the cases of burns (second degree burns), which cause a very high mortality rate, because the defensive means of the injured were penetrated and less ability to resistance, also confirmed that the amount of

the connection between the increase of colonization and the production of toxins in cases of burns of various types. So can be concluded that the isolation rate in the current study is not without risk because the presence of *S. aureus* that produced toxins is a dangerous indicator and the degree of danger increases with the rise of colonization.

Data in the current study showed that the ratio of isolation from wounds reached to (42.3%), it higher than the rate of Almeida *et al*. (2014) study which revealed that 20% of wound was *S. aureus* isolates and it less than the percentage in study conducted by Ahmed (2016) that 65%.

S. aureus is one of the main reasons for the production and formation of wound infections. Because of its potential ability for invasion and attack the disease, *S. aureus* caused infections in large areas of the body (Corey *et al*., 2016). Its infections occur when immune barriers, such as skin and mucous barriers were broken or when foreign things enter the body and the person may already have a complications in the immune system (Bekeredjian-Ding *et al*., 2017).

The colonization process in high percentage of skin areas are controlled by number of factors, such as adhesion factors associated with the surface, capsule, external cell enzymes and external toxins (Anderson *et al*., 2016), these products allow *S. aureus* bacteria to stick with the eukaryotic cell membranes and thereby resist the phagocytosis and lyse

this cells then triggered the release of the host's immune molecules (Malachowa *et al*., 2016). Because of the multivariate nature of *S. aureus* infections, repetition of adhesion to these bacteria and the replication of external proteins, it is difficult to identify the individual factor that plays a role in determining the colonization process or the factor that contributes mainly to the occurrence of the disease (Spaan *et al*., 2017).

The percentage of *S. aureus* isolates for bedsore also called (pressure ulcer) in this study was (40.9%), it more than the rate of Nourbakhsh *et al*. (2018) study was 23.2%, while it less than that reported by Buzzi *et al*. (2016) that 66%.

The bedsores can affect any part of the body, but more susceptible areas of the body to the pressure ulcers are the bony areas around coccyx, Knees, heels, ankles, and elbows (Nordqvist, 2017). It exacerbated in stages, identifying them in the early stage enables treatment and reduces the risk of complications (Bhattacharya and Mishra, 2015). The tendency to develop pressure ulcers associated not only with prolonged hospital staying for patients, but also to the hospital infections, due to the spread of this bacteria in the community and among individuals, also the ulcers are likely increased over time when the pressure was continued (Dana and Buman, 2015). In addition to wound site and associated microflora,

may have the ability to affect the bacterial composition of pressure ulcers (Liang *et al.*, 2017).

In the current study, we found *S. aureus* as the probable pathogen of ear infections in (40%) of cases, which is higher than previously reported study for Duarte *et al*. (2017) that 30.6% and lower than Hwang *et al*. (2015) study was 54.1%.

This difference can reflect demographic population changes and/or changes in the nature of *S. aureus* strains occurring in the community (Becker *et al*., 2014). Local modifications in the microbial assemblies of the external ear may also result from the selective pressure of experimental antibiotic therapy (Ouidri, 2018). As the potential causative factor for 1 of 3 cases in ear infections, *S. aureus* bacteria should be given in antimicrobial coverage decisions at the start of empiric treatment (Duarte *et al*., 2017).

The presence of *S. aureus* in the oral specimens from patients with lymphoma, meningitis and hepatitis in our study in rate (37.5%), which a timely reminder that it is not restricted indeed to the usually cited body niches (nose, throat, perineum). This percentage rises in study for Petti *et al*. (2014) was reached to 43%, while it be less than study for McCormack *et al*. (2015) was 10%.

S. aureus have long been recognized as constituents of the oral flora, however their role in oral health and disease remains contentious (Hua *et al*., 2013). Reported isolation rates for *S. aureus* vary with the population studied, in study conducted by McCormack *et al*. (2015) the carriage rates of 24-84% in healthy adult dentate oral cavities and an incidence of 48% among the denture-wearing population. In addition, a number of distinct oral infections (eg, angular cheilitis, parotitis, staphylococcal mucositis) are caused by this microorganism (Blomqvist *et al*., 2015). Raphel *et al*. (2016) also suggested that *S. aureus* may have a role in dental implant failure.

It seems likely that in line with infections caused by *S. aureus* at other body sites, a number of oral staphylococcal infections are probably as a result of cross-infection from a variety of sources (Khan, 2017). This reinforces the importance of standard infection control provision for all patients when undergoing dental examination or treatment. However, the lack of appropriate use of diagnostic microbiology services by dental practitioners and the absence of a regular surveillance system, including oral infection, make it difficult to detect transient infections (Serra *et al*., 2015). Also the importance of appropriate and timely microbiological sampling, because the proportion of isolates contains a group of this

bacteria, indicating resistance to frequently used antibiotics (McCormack *et al*., 2015).

The percentage of *S. aureus* isolates for vaginal samples in this study was (27.7%). In study conducted by Kosnik *et al*. (2017) found that the levels of IL-8 were significantly higher in women colonized vaginally with *S. aureus* than in women not so colonized, this indicated that *S. aureus* in the vagina can contribute to vaginal inflammation and could have adverse effects on vaginal health beyond being a risk factor for toxic shock syndrome (Karina *et al*., 2012).

Whereas in the current study, the rate of *S. aureus* colonization in throat was (25%) which lower than nasal swab rate. This ratio less than Kosnik *et al*. (2017) study which was 31.3% and more than the study of Joore *et al*. (2013) which was 11%. While the percentage in study for Esposito *et al*. (2014) is almost agree with this study was 25.9%.

The recognition about throat carriage of *S. aureus* is important, because it has been associated with treatment failure in the extermination of this bacteria among those with uncomplicated and complicated carriage (Ammerlaan *et al*., 2011). In the case of failing eradication treatment due to persisting throat carriage, one could consider a tonsillectomy in order to achieve successful *S. aureus* treatment (Labordus-van Helvoirt, *et al*., 2018).

In study conducted by Wardyn (2012) have shown that individuals may have an exclusive throat colonization, and may be absent from the limited screening of anterior nares, also yields of throat swabs were higher than the yield of nasal swabs, also in study done by Nair *et al*. (2014) explained that, the throat was the most common site of *S. aureus* colonization, and the perianal region was the second most common.

The nares were colonized in 32% of patients, a prevalence resembling that found in other sites of the body (Kumar *et al*., 2015). Thus, culture surveys of the nares alone greatly underestimate the prevalence of *S. aureus* colonization (Curry *et al*., 2016). This data thus do not support the concept that the nose is the primary site of *S. aureus* colonization, rather, it was found to colonize frequently in each sites cultured among the patients with skin and soft tissue infections (SSTIs) (Brown *et al*., 2014).

This observation was independent of background and not a function colonization at extranasal sites (Harkins *et al*., 2017). Extranasal colonization of *S. aureus* also takes place in several locations, including the skin, rectum, axillae, vagina, pharynx, and gastrointestinal tract (Aryee and Edgeworth, 2017).

The rate of *S. aureus* in urine samples in this study was reached to (24.2%). In study accounted by Megged (2014) the ratio was high that

reached to 32%, while in other study done by Yousefi *et al*. (2016) the percentage was lower than current study that 16.6%.

S. aureus is a rare cause of urinary tract infection (UTI) in the community, accounting for only 0.5 to 1% of positive urine cultures (Walker *et al*., 2017). *S. aureus* associated with UTI is more frequent in patients with an indwelling urinary catheter (Nicolle, 2014). Phillips *et al*. (2012) conducted a longitudinal study of patients at a long-term veteran care facility with documented *S. aureus* bacteriuria and found that 33% of the patients with this bacteria isolated from their urine had UTI symptoms, and 13% were bacteremia.

Importantly, when *S. aureus* is isolated from urine in patients without an obvious urinary focus, it can reflect *S. aureus* bacteremia (SAB), and thus, it is imperative to perform blood cultures for any patient with *S. aureus* bacteriuria (Tong *et al*., 2015). *S. aureus* UTI was associated with longer stays in health care facilities, recent antibiotic use and urinary catheterization (Rowe and Juthani-Mehta, 2013). Because catheters are a frequent cause of UTI, it is important to reduce the use of urinary catheters for individuals only who have a clear indication of it and remove the device as soon as clinically possible (Nicolle, 2014), also patients with UTI associated with catheter (once SAB is excluded) receive 10 to 14 days of appropriate antibiotics, as determined by culture

sensitivity results (Abbo and Hooton, 2014).

3.2- The susceptibility test of *S. aureus*

Results of susceptibility test showed that all isolates 100% of *S. aureus* were multi-drug resistance (MDR) when done toward 18 antibiotics from the following groups: Penicillins, Cephems, Carbapenems, Glycopeptides, Aminoglycosides, Tetracyclines, Macrolides, Lincosamides, Fluoroquinolones, Ansamycins, Nitrofurantoins and Folate pathway inhibitors which used as a traditional treatment for the infections of these bacteria as in the table (3-3) that represent the ratios of resistance in *S. aureus* isolates in this study.

Table (3-4): Drug susceptibility pattern of *S. aureus* isolates

Antimicrobial agents	Resistance (%)	Sensitive (%)
Methicillin	106(100)	0(0.0)
Ampicillin	104(98.1)	2(1.9)
Oxacillin	99(93.4)	7(6.6)
Cefoxitin	93(87.7)	13(12.3)
Cefotaxime	89(84)	17(16)
Erythromycin	85(80.2)	21(19.8)
Tetracycline	84(79.2)	22(20.8)
Doxycycline	60(56.6)	46(43.4)
Trimethoprim/sulfamethoxazole	76(71.7)	30(28.3)

Gentamicin	67(63.2)	39(36.8)
Amikacin	52(49.1)	54(50.9)
Rifampin	65(61.3)	41(38.7)
Levofloxacin	63(59.4)	43(40.6)
Ciprofloxacin	52(49.1)	54(50.9)
Clindamycin	54(51)	52(49.1)
Vancomycin	53(50)	53(50)
Imipenem	50(47.2)	56(52.8)
Nitrofurantoin	41(38.7)	65(61.3)

The following figure (3-5) shows the sensitivity test by agar disk diffusion method, where the resistance isolates are shown by observing the growth despite the presence of the antibiotics.

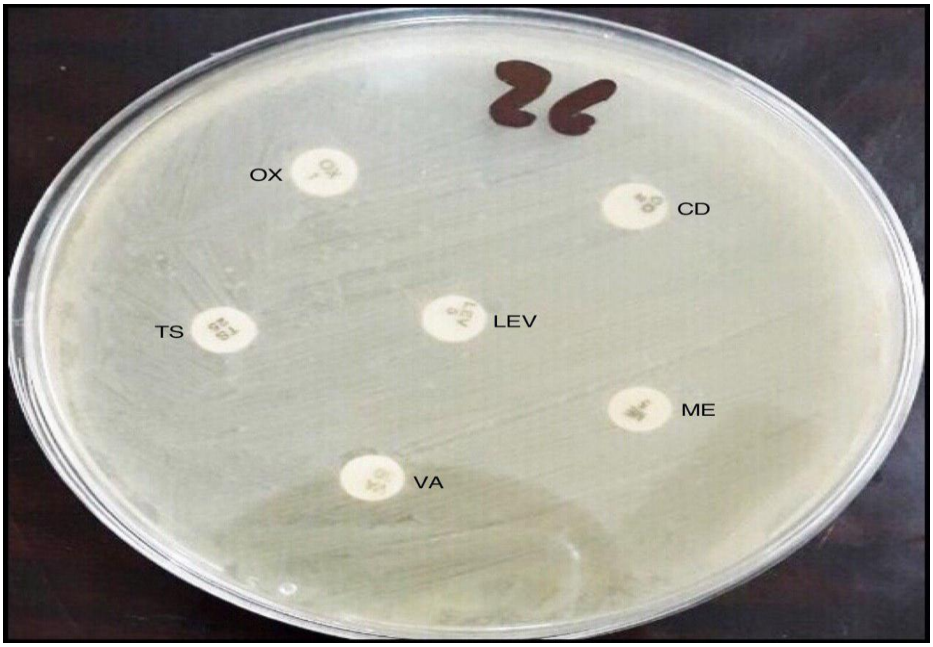

Figure (3-5): Disk diffusion method of Susceptibility test for MRSA detecion.

The results in table (3-3) exhibit a high levels of resistance to ß-lactam antibiotics: Methicillin, Ampicillin, Oxacillin, Cefoxitin, Cefotaxime, which showed resistance in percentage (100, 98.1, 93.4, 87.7, 84%) respectively, which is higher than the rates of (Akanbi *et al*., 2017) that (96.7, 80, 73.3, 67.7, 66.7 %) respectively, but they are almost compatible with the ratios of (Mohammed *et al*., 2017) that (100, 100, 92, 81.7, 84%) respectively.

The resistance in this study is not much different from that of previous studies shown a rise in resistance to most of ß-lactam group. This point is noticeable, because it may lead to failure in the treatment, prolonged illnesses, increased expenses for health care, and in serious cases, risk of death if humans are infected with such strains (Tanwar *et al*., 2014).

The results of the current study accounted that the rate of resistance to Erythromycin was (80.2%). This antibiotic has been listed within the list of antibiotics which has a high resistance by *S. aureus*, therefore the incidence of resistance was reached in some studies to 95.7% (Xie *et al*., 2016) which was higher than that reached in this study. On the other hand Bouchiat *et al*. (2015) enrolled 62.5% which considered low as compared with the present study. The resistance of *S. aureus* to erythromycin is common by the production of RNA methylase enzyme that encoded by

erm gene (Akanbi *et al*., 2017).

This study revealed that, resistance rates 79.2% and 56.6% to Tetracycline and Doxycycline respectively which were higher than the resistance rate obtained by Akanbi *et al*. (2017) that 56.7 and 50% for tetracycline and doxycycline respectively, and lower than Onanuga and Awhowho (2012) study were 66 and 59.3% for tetracycline and doxycycline respectively. These two antibiotics must be grouped together as the group of tetracycline, due to cross-resistance between them (Walter *et al*., 2017). The resistance to these antibiotics by the bacterial isolates encoded by *tetK* and *tetM* respectively that carried on plasmids, this means it acquired horizontally (Alipiah *et al*., 2014).

In the present study, the rate of Trimethoprim-sulfamethoxazole resistance was (71.7%), it less than the ratio of Nurjadi *et al*. (2015) study that reached to 92% and higher than the rate of Paul *et al*. (2015) study that 38%. The resistance encoded by *drfB* and *sulA* genes (Alipiah *et al*., 2014). Recent trials have demonstrated that non-inferiority of Oral Trimethoprim/sulfamethoxazole compared to intramuscular benzathine benzylpenicillin or oral clindamycin for treatment of uncomplicated skin and soft tissue infections such as impetigo, cellulitis and simple abscesses (Harris *et al*., 2018).

S. aureus recorded in this study, the percentage of resistance to Gentamicin and Amikacin is that (63.2 and 49.1%) respectively, which more than the rates in Bhatt *et al*. (2014) were 57.8 and 15.7% for gentamicin and amikacin respectively and less than that in Khosravi *et al*. (2017) were 84.5 and 77.6% for gentamicin and amikacin respectively.

The resistance caused by aminoglycoside-modifying enzymes that encoded by *eg , aac, aph* genes carried on plasmid. These antibiotics can be considered as antibiotics that have strong therapeutic impacts and can be utilized in the treatment of infections resulting from *S. aureus* that resistance to many antibiotics.

Rifampin was revealed a resistance that (61.3%), which was lower than that obtained by Liu *et al*. (2015) that 70.5%, whereas higher than the the rate revealed by Kumar *et al*. (2015) that 52%. The causes of rifampin resistance that mutations within bacteria lead to alterations in the *rpoB* gene, which encodes the ß-subunit of the RNA polymerase enzyme (Akanbi *et al*., 2017).

The resistance toward Levofloxacin and Ciprofloxacin in this study were (59.4, and 49.1%) respectively, the results were higher than that reported by Mohammed *et al*. (2017) that 46% for both, corresponding they less than that achieved by Akanbi *et al*. (2017) they accounted rate of resistance 86.7 and 66.7% respectively. The *gyr* gene mutations have a set

of mutations that encode high-level Fluoroquinolones resistance and a second set that encodes intermediate resistance levels (Farhat *et al*., 2017).

The results of this study also revealed that, the rate of resistance to Clindamycin reached to (51%). The incidence of resistance in this study was lower than in the study of Xie *et al*. (2016) which reached to 92.3%, while in the study conducted by Mendes *et al*. (2015) the percentage of resistance was less than this study which 33.4%.

The *ermA* and *ermC* genes that carried on plasmid is responsible mainly for clindamycin resistance (Abbas *et al*., 2015). This antibiotic has a large therapeutic importance in the treatment of infections caused by *S. aureus,* but the resistance stimulating by erythromycin lead to a failure in the treatment by Lincosamide antibiotics (Miklasińska-Majdanik *et al*., 2018).

S. aureus isolates showed a resistance percentage to Vancomycin (50%), this result agree with the study of Akanbi *et al*. (2017). In the study of Mandal *et al*. (2015) the rate reached to 15%, whereas all isolates were susceptible to vancomycin in the study of Liu *et al*. (2015).

Several heterogeneous vancomycin-resistant strains (eg, VRSA, VISA) have been well described for their resistance mechanism by acquisition of *vanA* operon (Stryjewski and Corey, 2014). The *vanA*

operon works to change the effectiveness of combinations of DD-peptidase enzyme who play a role in the building of bacterial cell wall in which the C-terminal d-Ala residue is replaced by d-lactate or d-serine (Walter *et al*., 2017). The resistance to this antibiotic is very few and in limited levels (Mandal *et al*., 2015).

Whereas toward Imipenem antibiotic the ratio of resistance was (47.2%) which lower than the percentage in Akanbi *et al*. (2017) study which accounted to 96.7%, while the rate in the study of Yu *et al*. (2015) reached to 17.5%, whereas in Mohammed *et al*. (2017) the isolates was 100% susceptible for imipenem.

The resistance for this antibiotic occur by the production of β-lactamase enzyme encoded by *blaZ* gene, that hydrolyze this antibiotic or through changes in the bacterial cell wall porins that lead to reduce the permeability of this drug into these bacteria, both mechanisms can occur by mutations or plasmid transfer (Munita and Arias, 2016; Foster, 2017). Imipenem used to treat serious infections caused by *S. aureus*, so the emergence of resistance toward this antibiotic limits therapeutic options for the infections caused by these bacteria and others (Foster, 2017).

The lowest rate of resistance has been detected toward Nitrofurantoin antibiotic which was (38.7%), but remainder higher than that reported by Onanuga and Awhowho (2012) that reached to 34.8%

and the result was greatly more than that which reported in the study of Looney *et al*. (2017) which was only 2.7%.

Nitrofurantoin (NFT) resistance is mainly mediated by mutations in *nfsA* and/or *nfsB* genes, both of which encode oxygen-insensitive nitroreductases responsible for NFT resistance (Shakti and Veeraraghavan, 2015). This antibiotic is important for the treatment of uncomplicated urinary tract infections (Cassir *et al*., 2014). NFT resistance is slow and uncommon, making it an important drug for treating urinary tract infections resistant to common and recent antibiotics such as cephalosporins, fluoroquinolones, aminoglycosides and carbapenems (Sekyere and Asenate, 2018).

In this study, *S. aureus* isolates show a contrast in antibiotic resistance due to the diversity in the mechanisms of resistance of *S. aureus* isolates, as it appears antibiotic resistance through several mechanisms such as the production of ß-lactamase enzymes that inhibit those antibiotics, or by changing the PBP2a (Holmes *et al*., 2016).

The reason for the other may depends on the virulence factors of bacteria, this confirmed by the study of Kumar *et al*. (2015) where they show some isolates of *S. aureus* have amount of virulence more than others, as well as the difference in the sources of sampling, the difference in the tests circumstances and type of techniques that used in the study, all

these combined may lead to a difference in resistance and sensitivity levels (Fair and Tor, 2014).

Also revealed that most of *S. aureus* isolated from patients with burns, surgical site infection were resistant to most antibiotic that used in this study. The probable reason for that might be due to high contamination of the burns and surgical wards, while the isolates cultured from nasal swabs and ear swabs were resist to less number of antibiotics, that could possibly because they obtained from outpatients in ENT ward who had less exposure to antibiotics, therefore the level of previous exposure to the antibiotics might not be high as those from burns and surgical wards they were hospitalized.

MRSA and other endogenous MDR pathogens can be transmitted by skin contact between patients, health personnel, hospital environment and surfaces, unless infection control measures are applied (Mehta *et al*., 2014). The poor healthy conditions of the hospital and lack of routine surveillance of antibiotic susceptibility of circulating bacterial strains might have contributed to spread of resistant bacteria in the hospital (Tadesse *et al*., 2018). Studies have shown an upward pattern in the incidences of *S. aureus* isolates with multiple antibiotic resistance (Skórczewski *et al*., 2014). It has also been reported that *S. aureus* isolates with multiple antibiotic resistance attributes have a negative

impact on the treatment of staphylococcal infections, especially in elderly, children and immune-compromised individuals (Alipiah *et al*., 2015).

In the current study, out of 106 *S. aureus* isolates 99(93.4%) and 93(87.7%) were identified as MRSA determined by oxacillin and cefoxitin respectively, because for susceptibility testing now typically uses oxacillin and/or cefoxitin antibiotics (Wu *et al*., 2016). Oxacillin has been proposed as a representative antibiotic for testing susceptibility not only to methicillin but also to all β-lactams (Loftus *et al*., 2017). Cefoxitin also used with oxacillin for MRSA detection, because it is a powerful influence of *mecA* gene that shows to be less affected than oxacillin by isolates have the ability to produce penicillinase excessively (Wu *et al*., 2016), and tests using cefoxitin give more reproducible and accurate results than tests with oxacillin (Shah *et al*. 2017).

3.3- Molecular estimation of *hlg* & *mecA* Genes

Though several virulence-associated genes in *S. aureus* are known, our primarily study focused only on two genes that (*hlg, mecA*) genes were chosen because they determined within the most common genes among invasive isolates. Polymerase chain reaction technique (PCR) has been used to amplify these genes from genomic DNA of *S. aureus* isolates. DNA was extracted from all confirmed isolates in current study and the detection of the extracted DNA by Agarose Gel Electrophoresis.

The table (3-5) represents the results of molecular estimation for these genes.

Table (3-5): The results of estimation for *hlg* and *mecA* genes

Gene	No of positive result	Percent %
hlg	40	37.8
mecA by Oxacillin	89	89.9
mecA by Cefoxitin	91	97.8

3.3.1- Estimation of *hlg* gene:

The results of *hlg* gene for γ-hemolysin in this study revealed that 40(37.8%) isolates from 106 isolates that encoded this protein figure (3-6 A, B) and the appendix.

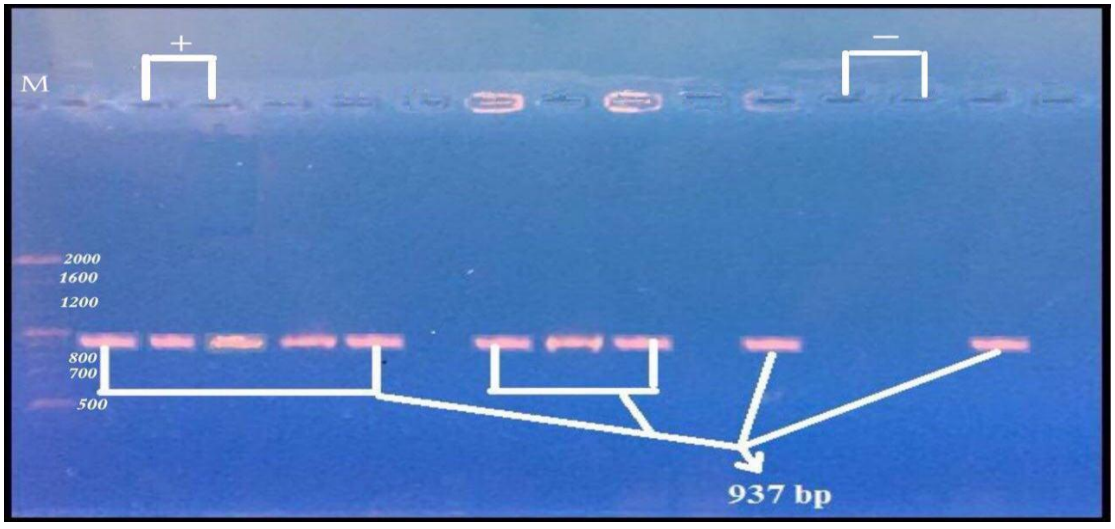

Figure (3-6 A): Ethidium bromide-stained Agarose Gel Electrophoresis of *hlg* gene of *S. aureus* isolates, Lane M DNA molecular size Ladder (100-2000)bp, Lane with positive marker refer to the isolates that have *hlg* gene, Lane with negative marker refer to the isolates that not have this gene.

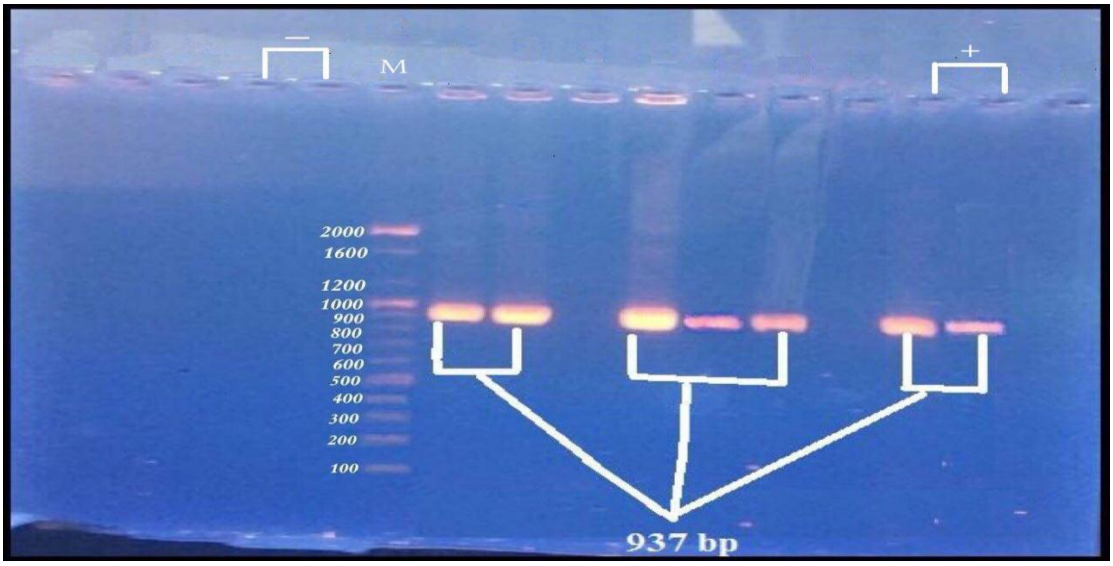

Figure (3-6 B): Ethidium bromide-stained Agarose Gel Electrophoresis of *hlg* gene of *S. aureus* isolates, Lane M DNA molecular size Ladder (100-2000)bp, Lane with positive marker refer to the isolates that have *hlg* gene, Lane with negative marker refer to the isolates that not have this gene.

The study of Xie *et al*. (2016) indicated that the low *hlg* gene rate in *S. aureus* strains could be a toxin characteristics of these bacteria in the region, thus studies conducted by many researchers revealed that high percentage of this gene in *S. aureus* genome such as Knox *et al*. 2012; Lim *et al*. (2012); Cebrian *et al* (2015); Xie *et al*. (2016) and Wang *et al*. (2016) they were (57.4, 61, 80.1, 87.5, 98.4%) respectively, whereas in studies for another researchers such as Tarabees *et al*. (2016); Elboshra (2017); Azizov *et al*. (2014); Mohammed *et al*. (2017) and Spaan *et al*. (2017) the percentages were lower than current study which were (5.9, 15, 21.9, 31.4, 35%) respectively.

Gamma-hemolysin is highly prevalent in human *S. aureus* isolates (Moraveji *et al*., 2014). As clinical epidemiology is inadequate in the context of high prevalence, a functional approach is needed to assess the role of γ-hemolysin in staphylococcal virulence (Spaan *et al*., 2014). This toxin considered the only leukotoxin can lyse red blood cells with high efficiency (Yoong and Torres, 2013). In turn, γ-hemolysin (Hlg) promotes the survival of *S. aureus* in blood (Malachowa *et al*., 2011). Therefore, the presence of adhesion of *S. aureus* species together with the ability to lyse erythrocytes will presumably boost the species virulence and hence increase the severity of infection (Tarabees *et al*., 2016). The expression of γ-hemolysin is highly up-regulated during culture in blood and during phagocytosis by neutrophils (Malachowa *et al*., 2011).

3.3.2- Estimation of *mecA* gene:

The results of *mecA* gene detection illustrates that 89(89.9%) and 91(97.8%) from phenotypically positive MRSA isolates had this gene for oxacillin and cefoxitin antibiotics respectively figure (3-7 A, B) and the appendix.

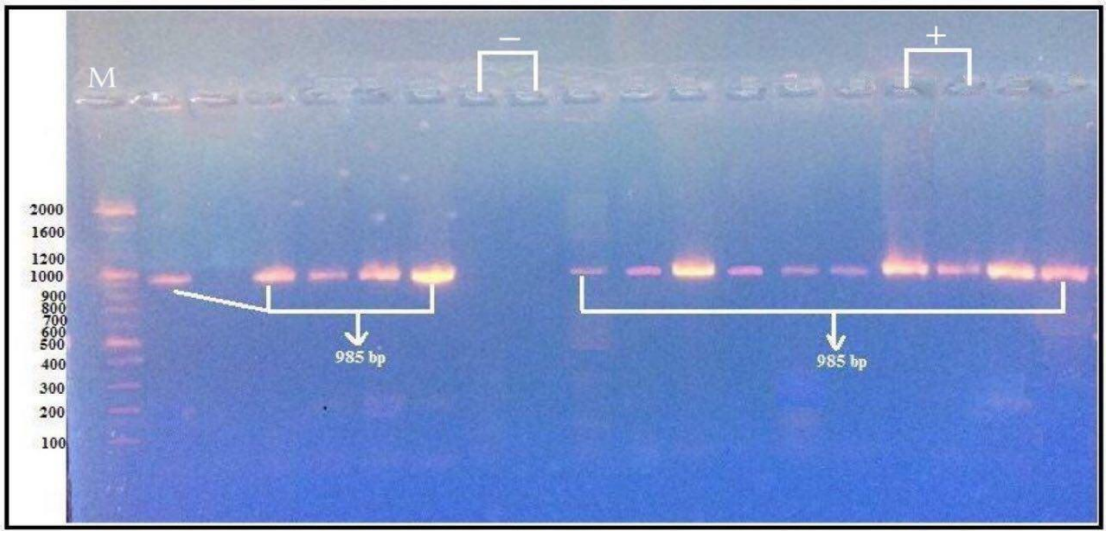

Figure (3-7 A): Ethidium bromide-stained Agarose Gel Electrophoresis of *mecA* gene of *S. aureus* isolates, Lane M DNA molecular size Ladder (100-2000)bp, Lane with positive marker refer to the isolates that have *mecA* gene, Lane with negative marker refer to the isolates that not have this gene.

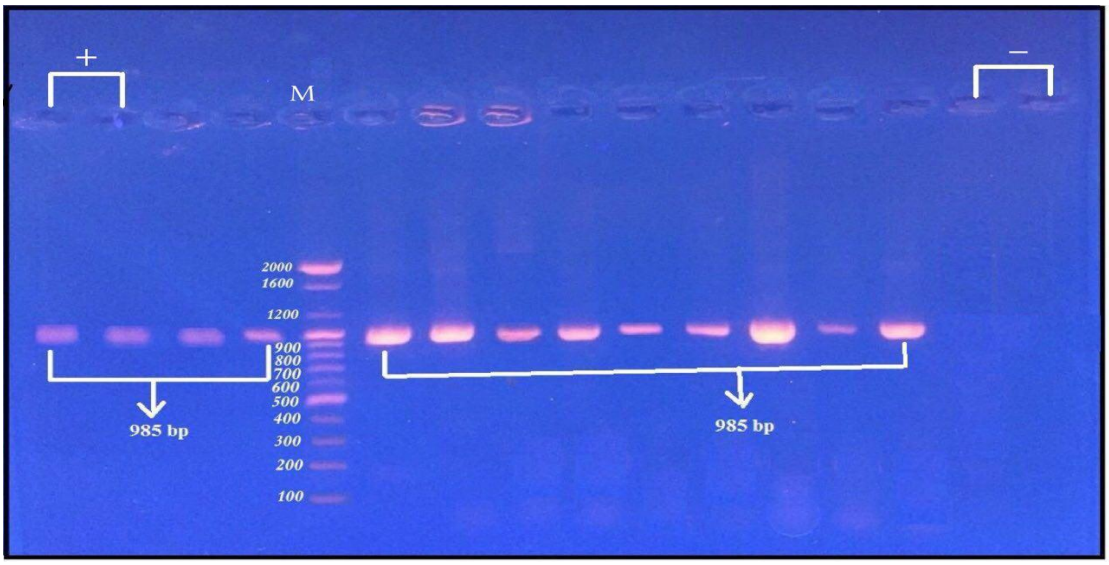

Figure (3-7 B): Ethidium bromide-stained Agarose Gel Electrophoresis of *mecA* gene of *S. aureus* isolates, Lane M DNA molecular size Ladder (100-2000)bp, Lane with positive marker refer to the isolates that have *mecA* gene, Lane with negative marker refer to the isolates that not have this gene.

As shown in this study, the occurrence of *mecA* gene was higher than that obtained by other researchers such as Martini *et al*. (2017); Abbas *et al*. (2015); Rajabiani *et al*. (2014); Pournajaf *et al*. (2014) and Elkenany (2018) which were (18.8, 29, 39.2, 45.1,54.5%) respectively. While other studies revealed that the percentage of *mecA* gene incidence has been more than this study such as Elhassan *et al*. (2015); Kuar and Chate (2015); Eshetie *et al*. (2016); Becker *et al*. (2016) and Ito *et al*. (2012) which were (90.2, 95, 96, 98.1, 98.3%) respectively.

According to the results in table (3-5) which showed that the percentage of the presence of this gene within *S. aureus* isolates that positive to the cefoxitin antibiotic was higher than that presence in the isolates of these bacteria that positive to the oxacillin antibiotic, thus we can concluded that cefoxitin antibiotic is more reliable than oxacillin in MRSA identification, and this confirmed by study conducted by Sahai and Chauhan (2014), Vyas *et al*. (2015) and Sharma *et al*. (2017). Therefore it currently recommended by CLSI (2017) for MRSA detection.

Also, the results revealed that cefoxitin disc diffusion test is in concordance with the PCR for *mecA* gene. Thus, the susceptibility test can be an alternative to PCR for detection of MRSA, this confirmed by

Chowdhury *et al*. (2013), Rezazadeh *et al*. (2014) and Patil and Ghorpade (2016).

S. aureus which phenotypically positive MRSA while gave a negative results to *mecA* gene this could be referred they have an alternative mechanism instead of this gene for antibiotic resistance including the alteration of the penicillin binding proteins (PBPs) leading to hyper-production of methcillinase or ß- lactamase (CLSI, 2017), subsequently this explain why not all isolates that resistant to β-lactams not have this gene.

The studies has been revealed that 90% of *S. aureus* resistant to penicillins due to β-lactamase (penicillinase) production (Xu *et al*., 2017), also Yu *et al*. (2015) confirmed that resistance to penicillins is not only by these enzymes, but also to the production of trans-membrane proteins in the plasma membrane associated with the cell wall, which represent enzymatic activity such as Transpeptidases and carboxypeptidases a target of penicillins and cephalosporins that encoded by *MecR1* and *BlaR1* genes respectively (Manjunath and Padma, 2012), where it works to change the target of ß-lactam antibiotics (Akindolire, 2013).

Munita and Arias (2016) affirmed the role of plasmids in the production of β-lactamase enzymes and their responsibility on the encoding of some PBP2a. Wanner *et al*. (2017) has been linked between

the resistance of antibiotics and the sources responsible for this resistance, where they found that most of the resistance shown by the strains of *S. aureus* has plasmid origin.

Conclusions

&

Recommendations

Conclusions

The findings of this study are as following:

1. The spread of MRSA strains with high resistance to most antibiotics were increasingly prevalent in the hospitals, including that causing hospital acquired infections.

2. The greatest antibiotic resistance in this study was returned to the isolates that taken from burns and wounds.

3. Cefoxitin antibiotic is more reliable than oxacillin antibiotic and can be an alternative to PCR for MRSA detection.

4. The study showed high prevalence of *mecA* gene compared with that of *hlg* gene for γ-hemolysin.

Recommendations

The present study recommends the following:

1. The necessity of adopting the antimicrobial sensitivity test to investigate MRSA isolates in the hospital laboratories in Misan Province.

2. The dependence on cefoxitin antibiotic instead of oxacillin antibiotic to detect the isolates that resistant to ß-lactam especially MRSA.

3. Emphasize the use of antibiotics when absolutely necessary.

4. The investigation of more virulence genes in *S. aureus* than was studied in current study to identify its pathogenicity more broadly.

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Appendices

Appendix: List of *S. aureus* isolates, MRSA detection by Oxacillin & Cefoxitin antibiotics and *hlg* & *mecA* genes results.

Type of isolate	No. of *S. aureus* isolate	*hlg* gene	Oxacillin	Cefoxitin	*mecA* gene
Urine	1	+ve	R	R	+ve
Urine	2	+ve	R	R	+ve
Wound	3	+ve	R	R	+ve
Urine	4	+ve	R	S	-ve
Burn	5	-ve	R	R	+ve
Urine	6	-ve	S	R	+ve
Oral swab	7	+ve	R	R	+ve
Wound	8	-ve	R	S	+ve
Bedsore	9	+ve	R	R	+ve
Nasal swab	10	+ve	S	S	-ve
Ear swab	11	+ve	R	R	+ve
Wound	12	-ve	R	R	+ve
Nasal swab	13	+ve	R	R	+ve
Nasal swab	14	+ve	R	R	+ve
Burn	15	-ve	R	R	+ve
Throat swab	16	-ve	S	R	-ve
Nasal swab	17	-ve	R	R	+ve
Burn	18	-ve	R	R	+ve
Burn	19	+ve	R	R	+ve
Burn	20	-ve	R	R	+ve
Ear swab	21	+ve	R	R	+ve
Vaginal swab	22	+ve	R	R	+ve
Nasal swab	23	-ve	R	R	+ve
Wound	24	-ve	R	R	+ve
Wound	25	+ve	R	R	+ve
Vaginal swab	26	-ve	S	R	+ve
Nasal swab	27	-ve	R	R	+ve
Burn	28	+ve	R	R	-ve
Burn	29	+ve	R	R	+ve

Nasal swab	30	-ve	R	R	+ve
Burn	31	-ve	R	R	+ve
Burn	32	+ve	R	R	+ve
Bedsore	33	-ve	R	R	+ve
Wound	34	-ve	R	R	+ve
Urine	35	+ve	R	R	+ve
Burn	36	+ve	R	R	+ve
Wound	37	+ve	R	R	+ve
Oral swab	38	-ve	R	R	+ve
Nasal swab	39	-ve	R	R	+ve
Burn	40	+ve	S	R	+ve
Burn	41	-ve	R	R	+ve
Ear swab	42	+ve	R	S	-ve
Bedsore	43	-ve	R	R	+ve
Throat swab	44	-ve	R	R	+ve
Nasal swab	45	+ve	R	R	+ve
Urine	46	-ve	R	R	+ve
Burn	47	-ve	R	R	+ve
Burn	48	-ve	R	R	+ve
Burn	49	-ve	R	R	+ve
Wound	50	-ve	R	S	-ve
Vaginal swab	51	-ve	R	R	+ve
Bedsore	52	-ve	R	R	+ve
Wound	53	-ve	R	R	+ve
Burn	54	-ve	R	R	+ve
Burn	55	-ve	R	R	+ve
Wound	56	-ve	R	R	+ve
Wound	57	+ve	R	R	+ve
Throat swab	58	-ve	R	S	-ve
Bedsore	59	-ve	R	R	+ve
Wound	60	-ve	R	R	+ve
Ear swab	61	+ve	S	S	-ve
Wound	62	-ve	R	R	+ve
Ear swab	63	+ve	R	R	+ve
Burn	64	-ve	R	R	+ve
Nasal swab	65	-ve	R	R	+ve

Burn	66	-ve	R	R	+ve
Wound	67	-ve	R	R	+ve
Vaginal swab	68	+ve	R	R	+ve
Bedsore	69	-ve	R	R	+ve
Burn	70	+ve	R	R	+ve
Burn	71	-ve	R	R	+ve
Burn	72	-ve	R	R	+ve
Wound	73	-ve	R	R	+ve
Burn	74	-ve	R	R	+ve
Urine	75	-ve	R	S	-ve
Nasal swab	76	+ve	S	R	+ve
Burn	77	-ve	R	R	+ve
Burn	78	-ve	R	R	+ve
Wound	79	-ve	R	R	+ve
Wound	80	-ve	R	R	+ve
Nasal swab	81	-ve	S	R	+ve
Burn	82	+ve	R	R	+ve
Burn	83	-ve	R	R	+ve
Bedsore	84	+ve	R	R	+ve
Wound	85	-ve	R	R	+ve
Burn	86	+ve	R	R	+ve
Nasal swab	87	+ve	R	S	-ve
Burn	88	-ve	R	R	+ve
Ear swab	89	+ve	R	R	+ve
Burn	90	-ve	R	R	+ve
Urine	91	-ve	R	R	+ve
Bedsore	92	-ve	R	R	+ve
Wound	93	+ve	R	R	+ve
Throat swab	94	+ve	R	R	+ve
Oral swab	95	+ve	R	R	+ve
Vaginal swab	96	+ve	R	R	+ve
Burn	97	-ve	R	R	+ve
Wound	98	+ve	R	S	-ve
Burn	99	-ve	R	R	+ve
Burn	100	-ve	R	R	+ve
Burn	101	-ve	R	R	+ve

Wound	102	+ve	R	S	-ve
Nasal swab	103	-ve	R	R	+ve
Wound	104	-ve	R	R	+ve
Bedsore	105	-ve	R	R	+ve
Burn	106	-ve	R	R	+ve

الخلاصة

جمعت 274 عينة من حالات سريرية مختلفة من إثنين من المستشفيات الرئيسية في محافظة ميسان خلال الفترة من كانون الأول 2017 إلى نيسان 2018.

أثبتت نتائج التشخيص المجهرية والزرعية والإختبارات الكيموحيوية والفايتك ان 106عزلة بكتيرية تنتمي الى المكورات العنقودية الذهبية (*Staphylococcus aureus*).

أختبرت حساسية عزلات هذه البكتريا تجاه 18 مضاد حيوي مستخدمة كعلاج تقليدي لإصاباتMRSA بالإستناد إلى طريقة إنتشار القرص وعن طريق الإجراءات المبينة في المبادئ التوجيهية للجنة الوطنية لمعهد المعايير السريرية والمختبرية (CLSI, 2017). أظهرت النتائج أن 100% من عزلاتها مقاومة متعددة للمضادات الحيوية. علاوة على ذلك تم تحديد 99(93.4%) من جميع عزلات هذه البكتريا على أنها MRSA عن طريق مقاومتها لمضاد الأوكساسلين, بينما 93(87.7%) منها حددت على أنها MRSA عن طريق مقاومتها لمضاد السيفوكستين.

أما على المستوى الجزيئي فقد إقتصرت دراستنا بالدرجة الأولى على إثنين من الجينات وهما ((*hlg* و *mecA*) للتحلل الدموي گاما ومقاومة المضادات الحيوية على التوالي. بينت نتائج الكشف عن جين *hlg* أن 40(37.8%) من بين كل عزلات المكورات العنقودية الذهبية في هذه الدراسة تشفر لبروتين التحلل الدموي. في حين أن نتائج الكشف عن جين *mecA* لتشفير PBP2a(PBP′) أشارت إلى أن 89(89.9%) للأوكساسلين و 91(97.8%) للسيفوكستين من هذه البكتريا محددة على أنها MRSA في التشخيص المظهري تمتلك ذلك الجين.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة ميسان

كلية العلوم

دراسة مظهرية وجزيئية للمكورات العنقودية الذهبية المعزولة من حالات سريرية في محافظة ميسان/ العراق

رسالة مقدمة الى

مجلس كلية العلوم / جامعة ميسان وهي جزء من

متطلبات نيل درجة الماجستير في علوم الحياة

من قِبل

مروة عبدالحسين حسان

بكالوريوس علوم حياة/ جامعة ميسان

(2016)

بإشراف

أ.م.د. زاهد سعدون عزيز

محرم 1440 هـ

أيلول 2018 م