Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science

Bacteriological and Genetic Studies on Oxacillin Resistant *Staphylococcus aureus* Isolated from Some Hospital in Baghdad City

Dissertation

Submitted to the College of Science/ Al-Nahrain University as a partial fulfillment of the Requirements for the Degree of Doctorate of Philosophy in

Science/ Biotechnology

By

DhafarNajim Al-Ugaili

B.Sc. Biotechnology, 1998

M.Sc. Biotechnology, 2001

Supervised by

Dr. Ayad M. Al-Ubaidi
(Assit. Professor)

Dr. Salih A. Wohaieb
(Professor)

January 2013 **Raabea Awal 1434**

Supervisors Certification

We, certify that this dissertation entitled "**Bacteriological and Genetic Studies on Oxacillin Resistant *Staphylococcus aureus* Isolated from Some Hospital in Baghdad City"** prepared by "**DhafarNajim Al-Ugaili"** under our supervision at the College of Science/ Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Science/ Biotechnology.

Signature:
Supervisor: **Dr. Ayad M. Al-Obaidi**
Scientific Degree: Assistant professor
Date: / / 2013

Signature:
Supervisor: **Dr. Salih A. Wohaieb**
Scientific Degree: Professor
Date: / / 2013

In view of the available recommendations, I forward this dissertation for debate by the examining committee.

Signature:
Name: Dr. Hameed M. Jasim
Scientific Degree: Professor
Title: Head of Biotechnology Department
Date: / / 2013

Committee Certification

We, the examining committee certify that we have read this dissertation entitled "**Bacteriological and Genetic Studies on Oxacillin Resistant *Staphylococcus aureus* Isolated from Some Hospital in Baghdad City"** and examined the student "**DhafarNajim Al-Ugaili**" in its contents and that in our opinion; it is accepted for the Degree of Doctorate of Philosophy in Science / Biotechnology.

Signature:
Name:
Scientific Degree: Professor
(Chairman)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member)

I, Hereby Certify upon the decision of the examining committee.

Signature:
Name: **Khulood W. Al-Sammarrae**
Scientific Degree: Pro fessor
Title: Dean of College of Science
Date:

ACKNOWLEDGMENTS

At the beginning, thanks to Great Allah, Lord of the whole creation who gave me the faith and strength to perform this work.

I'd like to express my great respect and sincere gratitude to my supervisors Dr. Ayad Mohamed Ali Al- Ubaidi and Dr. Saleh Wohaieb for their guidance, useful advices and encouragement during the period of the research, I wish them all the best.

` I introduce my special thanks to Prof. Dr. Khulood Wohaieb Al-Sammarrae, Dean of College of Science / Al-Nahrain University.

My deep thanks and gratitude to the staff of Biotechnology Department / College of Science / Al-Nahrain University, especially to Mr. Zaid Naseif for his continuous help during my research work. My sincere and deeply thanks to Dr. Rehab Ramadan and Dr. Rabah NaJah for their continuous support and advices. Also I would like to express my appreciation to the staff members of Biotechnology Research Center at Al – Nahrain University for their assistance and advice during this study and especially to Mrs. Noor and Miss. Raghad.

A great appreciation to Mrs. Ameraa at Al-Kaddimia Teaching Hospital for her help during my work.

I would also like to express my sincere thanks and love to my friends at Biotechnology Department for their unending support, friendship, and encouragement.

A lot of appreciation to Mrs. Myada and miss. Lumma for sharing me the beautiful moments as well as the hard ones.

And most importantly, I would like to express my special thanks to my dearest friend Hana for her never ending love, support that help me during my toughest times, thank you.

Summary

Two hundred and six clinical specimens were collected from four different hospitals in Baghdad during the period (February to October 2010); these specimens included swabs from anterior nares, hands and ear swabs that were collected from visitors, hospitalized patients and the health care workers in these hospitals.

Diagnostic results show that 154 out of the 206 specimens gave positive bacterial cultures and 61 isolates are characterized as *Staphylococcus aureus* depending on the cultural and biochemical examinations. In addition, the antibiotic susceptibility profile for these isolates was examined against fifteen types of antibiotics of the following groups (β-lactam antibiotics, fluoroquinolones, aminoglycosides, macrolides, glycopeptide, phenicoles and tetracycline). The results showed that (77%) of the isolates were multi-resistant as determined by the resistance of such isolates to more than three types of antibiotics. Highest resistance was recorded against ticarcillin-clavulanic acid, penicillin, oxacillin, cefepime, cefoxitin, cephalothin and erythromycin with a percentage of (93.4%, 78.7%, 77.1%, 68.9%, 65.6%, 55.7% and 47.5%), respectively. Followed by moderate resistance against tetracyclin, gentamycin and amikacin (with the same percentage of resistance) then ciprofloxacin and chloramphenicol. On the other hand the tested isolates showed a high degree of susceptibility towards vancomycin, tobramycin and imipenem.

The isolates that exhibit oxacillin resistance in addition of being resistant to five or more of the antibiotics were selected to determine their MICs values against oxacillin using a concentrations ranging from (0.25μg/ml –1280 μg/ml) and as a result (23) isolates are chosen as their MICs values were (≥ 4.0) to test their hemolytic activity (hemolysis assay) to determine their ability to produce (*alpha-toxin)*. The results indicate that all the isolates except two of them were able to produce the toxin with variable degree of activity.

A confirmatory test was carried out for the selected isolates using PCR technique for further characterization up to the species level by the amplification of (***nuc***) gene (*Staphylococcus aureus* specific gene) and all the isolates are found to be positive for the presence of (***nuc***$^{+}$) gene as their agarose gel revealed the presence of DNA band of *nuc* gene with a molecular size of (300 bp.).

The gel electrophoresis of the plasmid DNA purified from these isolates was carried out; the results revealed that (11) of these isolates had a plasmid and (5) had two plasmids with a molecular size of (5200 bp.) and (10,000 bp.), while (5) of these isolates were found to have a plasmid with large molecular size (out of the maximum size of the leader) and one isolate shows three band of DNA plasmid with molecular size (4000 bp., 10,000 bp.) and the last band is out of the maximum size of the leader.

Detection of oxacillin-resistance gene represented by (***mecA***$^{+}$) revealed the presence of (19) positive isolates with the appearance of DNA band of about (200 pp.); A correlation between positivity of the isolates for cefoxitin resistant test and the molecular aspect for the presence of (***mecA***).

Results of the detecting the alpha-hemolysin specific gene (***hla***$^{+}$) showed that it was positive in (21) of the isolates as they appear to have a band with a molecular size of about (550 bp.), and these results were used to confirm the results of the (hemolysis assay). These results were almost considered as the first sign for the correlation between the presence of alpha-hemolysin gene and oxacillin- resistant trait.

The enhanced effect of zinc oxide nanoparticles on the antibacterial activity of oxacillin was studied and the effect was determined against specific selected isolate. A positive and induced effect for zinc oxide nanoparticle was detected upon the antibacterial activity of oxacillin through increasing the diameter of inhibition zone for the antibiotic as it recorded

(15mm) for the mixed activity to the antibiotic with nanoparticles compared with that of (10mm) for the zinc oxide nanoparticle alone and the disappearance of the inhibition zone for the antibiotic alone. These results might be considered as the first report in Iraq for the possibility to use the synergistic effect of nanoparticles with antibiotics to stop the progress resistance of bacteria against antibiotics used in the present time.

The antibacterial activity of the crude ethanolic olive leaf extract was evaluated alone or in combination with either oxacillin or zinc oxide nanoparticles against oxacillin resistant *Staphylococcus aureus* using disc diffusion method. The results revealed that olive leaf extracts (2.25-9 mg/disc) neither exhibited antibacterial activity when used alone, nor potentiated the antibacterial activity of zinc oxide nanoparticles (100µg/disc). However, in presence of the highest concentration of crude olive leaf extract (9 mg/disc), the antibacterial activity of oxacillin (1µg/disc) was improved when the diameter of inhibition zone increased from zero to a maximum of 14 mm, suggesting a potentiation of the antimicrobial activity of oxacillin against the already existing oxacillin-resistant strain of *Staphylococcus aureus*. If this unexpected finding can be reproduce by further studies using various concentrations of olive leaf extracts, then it may represent the first report in the available literature that signifies the potential utilization of olive leaf extracts in combination therapy against oxacillin-resistant *Staphylococcus aureus*.

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List of Abbreviations

Abbreviation	Meaning
ATS	American Thoracic Society
CAI	Community- Acquired Infections
CFSPH	Center for Food Security and Public Health
HAI	Hospital-Acquired Infections
Hla	Alpha-hemolysin gene
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MHB	Muller Hinton Broth
Mec	Methicillin Resistant gene
MGEs	Mobile Genetic Elements
NCCLS	National Committee for Clinical Laboratory Standa
Nuc	*Staphylococcus aureus* specific gene
NP	Nano-Particles
ORSA	Oxacillin Resistant *Staphylococcus aureus*
OLE	Olive leave Extract
PVL	Panton-Valentine leukocidin
PBP	Penicillin Binding Protein
SCC	Staphylococal Chromosomal Cassette
SCVs	Small-Colony Variants

1. Introduction and Literature Review

1.1 Introduction

One of the major ecological problems in a society is the problem of health-care facilities (hospitals, inpatient and outpatient departments). Hospital represents a special environment for serving health care to patients and as a work environment for medical and other staff (Farzana *et al*., 2008).

Nosocomial or hospital-acquired infections are those infections acquired in hospital by a patient who is admitted for a reason other than that infection (Ducel *et al*., 2002). The microbiological factor is the most risky one for immuno-compromised patients because it has the potential to start nosocomial infections (Frazana *et al.*, 2008). Any microbial group, bacteria, viruses, fungi, or parasites can cause a nosocomial infection, but bacteria are the most prevalent organisms. Among Gram positive bacteria, the common nosocomial causative agents belong to the genus of Staphylococci, Enterococci, Streptococci, and *Clostridium difficile*, while for Gram negatives, the most important agents were; *Escherichia coli*, *Klebsiella* spp, *Enterobacter*, *Proteus*, *Serratia*, *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Haemophilus* spp (Salmenlinna, 2002).

The natural bacterial world always coexists with humans and their extended environments (Urbana *et al.*, 2008). *Staphylococcus aureus is* an opportunistic bacterium frequently part of human micro flora causing disease when the immune system becomes compromised (De Sousa *et al*., 2004). Thus it has been recognized as one of the major human pathogens and is by far one of the most common nosocomial organisms (Balaky *et al.*, 2011). It permanently colonizes the epithelium of 20% of the population, transiently occurs in more than 60% and 20% never carry the organism (Foster, 2005).

With the fact that humans are a natural reservoir of *S. aureus* that colonizes the nares, axillae, vagina, and pharynx or damaged skin surfaces; Nosocomial *S. aureus* infections affect the bloodstream, skin, soft tissues and

lower respiratory tracts. Thus, it may be the cause of central venous catheter-associated bacteremia, ventilor-associated pneumonia it also causes serious deep-seated infections, such as endocarditis and osteomyelitis (Schito, 2006). Nasal carriage of *S. aureus* is considered a source of subsequent infection in health care settings (Paule *et al*., 2004). Infections caused by such microorganisms are the major causes of morbidity and mortality and they are currently among the ten main causes of death worldwide and the basic cause of death in 1% of cases (Ribeiro& Ustulin, 2011). Despite the availability of novel drugs as an approach to staphylococcal therapy, the bacteria seem to be able to rapidly develop resistance to these drugs (Diekeman *et al.*, 2004). In spite of advances in diagnosis and treatment, mortality and complication rates are still high with reports of in-hospital mortality of more than 20% (Wisplinghoff *et al*. 2008).

Methicillin-resistant *S. aureus* (**MRSA**) (which also may be called multi drug resistant *S. aureus* and oxacillin resistant *S. aureus* "**ORSA**") represents a serious problem in hospitals worldwide it increases infected patients mortality and morbidity and raising treatment costs and (Braoios *et al*., 2009).

Oxacillin-resistant strains represent an important health problem for infected individuals since the therapeutic options are restricted to glycopeptides, such as vancomycin. Even vancomycin appears to be losing its effectiveness and in spite of the high number of new agents with broad Gram-positive activity have been licensed, none has emerged as clearly superior (Kaul *et al*., 2008).

Hospital-aquired MRSA/ ORSA are usually associated with increased expression of multiple antibiotic resistance genes including those genes coding for aminoglyciside resistance (Deurenberg *et al.*, 2007). *S. aureus* developed resistance to β-lactam antibiotics through the acquisition of the *mecA* gene that encodes penicillin-binding protein 2a (PBP2a), which has a

significantly reduced affinity for β-lactam antibiotics, thereby conferring β-lactam resistance (Lee, 2010).

The entire genome of *S. aureus* was sequenced in 2001 (Kuroda *et al*., 2001) and ongoing molecular and genetic dissection of *S. aureus* revealed a large number of surface adhesions, secreted enzymes and toxins that make invasion possible.

Staphylococcus aureus harbor a number of mobilizable exogenous DNA stretches including insertion sequences, transposons, bacteriophages, and pathogenicity islands (also referred to as genomic islands) (Ruzin *et al.* 2001; Baba *et al*. 2002; Novick 2003), which contain specific determinants responsible for disease and antibiotic resistance. These exogenous elements explain the high capacity of *S. aureus* to undergo horizontal gene transfer and to exchange genetic elements with other organisms including both staphylococcal and non-staphylococcal genera.

Because gene exchange is a key player in evolution, this genetic plasticity is a probable explanation for the success of *S. aureus* as both a colonizer and a disease-producing microbe (Jacbsson, 2009)

The detection of *mecA*, by the Polymerase Chain Reaction (PCR) is considered a gold-standard technique for oxacillin resistance detection (Anand *et al*., 2009).

The usages of nanoparticles, which consist of metal oxides, represent a new class of important materials that are increasingly being developed for use in research and health-related applications (Jones *et al*., 2008).

Although the *in vitro* antibacterial activity and efficacy of metal zinc oxides which are used for pharmaceutical applications, have been known for a long time. Little is known about the antibacterial activity of ZnO nanoparticles against oxacillin- resistant *S. aureus* and no published data has documented at local academic level.

One of the important approaches for the containment of antibiotic resistance is the rational localization bioactive phytochemicals which have antimicrobial activity (Newman *et al*., 2000; Gottlieb *et al.,* 2002). In addition, drug synergism between known antimicrobial agents and bioactive plant extract has been reported (Esimon *et al*., 2006). The search for bioactive constituent of plant extracts with significant antimicrobial potentials and of interaction with the currently used antibiotics may represent a promising approach toward combating the rise in bacterial resistance to these antibiotics in medical practice.

Aims of the study

1- Isolating and identifying of multidrug oxacillin-resistant *S. aureus* from nosocomial infections.
2- Detection for the plasmid content of the bacterial isolates.
3- Molecular detection of oxacillin-resistant gene and alpha-hemolysin gene using PCR technique.
4- Studying the enhanced effect of ZnO nanoparticles on the antibacterial activity of oxacillin against *S.aureus*.
5- In *vitro* study for the antibacterial effect of olive leaves ethanolic extract alone or in combination with either oxacillin or ZnO nanoparticles.

1.2 Literature review:

1.2.1 Genus *Staphylococcus*:

Staphylococci, together with pneumococci and streptococci, are members of a group of invasive gram-positive pathogens, known as the pyogenic cocci, which cause various suppurative or pus-forming diseases in humans and other animals (Lyon and Skurray, 1987).

The name *Staphylococcus* (staphyle, bunch of grapes) was introduced by Ogston (1883) for the group micrococci causing inflammation and suppuration (Götz *et al*., 2006). In general, staphylococci are gram-positive spherical cells which are usually arranged in grape-like irregular clusters. They grow readily on many types of media and are active metabolically fermenting carbohydrates and producing pigments that vary from white to deep yellow (Brooks *et al*., 1998). Staphylococci are naturally observed as a part of the normal bacterial flora on normal skin and mucous membranes (Schjørring *et al*., 2002).

About 36 species and several subspecies are recognized in the genus *Staphylococcus* until 2006. The three main species of clinical importance are; Staphylococcus *aureus,* which causes infections in almost every organ and tissue of the human body with the fact that the most commonly affected part of the body due to *S. aureus* infection is the skin (Daum, 2007), *S. epidermidis* that causes infections associated with indwelling medical devices (Vadyvaloo and Otto, 2005) and *S. saprophyticus* causes urinary tract infections commonly associated with young girls (Horowitz and Cohen, 2007). In addition *S. lugunensis, S. haemolyticus*, *S. warneri, S. schleiferi* and *S. intermedius* are infrequently associated with pathogenesis in health-care settings (Brooks *et al*., 1998).

The laboratory diagnostics is based on culture and biochemical tests, typical morphology, and coagulase reaction, fermentation of mannitol and trehalose, and production of heat stabile nuclease (thermonuclease, for

S. aureus). The ability of coagulase to clot plasma is the most widely used method for identification of *S. aureus* and to differentiate it from *S. epidermidis* (Salmenlinna, 2002).

Among the staphylococcal species, coagulase-positive *S. aureus* and coagulase-negative *S. epidermidis* are of clinical importance (Ziebuhr, 2001).

Staphylococcus aureus are known to have an important influence to the following diseases: Boils, pimples, respiratory infections (pneumonia), osteomyelitis, meningitis, arthritis, scalded skin syndrome, toxic shock syndrome (TSS) and food poisoning (De Sousa and De Lencastre, 2004).

1.2.2 *Staphylococcus aureus* Virulence factors:

Staphylococcus aureus is a versatile pathogen that can express an array of virulence factors which result in their ability to multiply and spread across adjacent tissue (Dinges *et al*., 2000; Gould *et al.*, 2012). These virulence factors are extensive with both structural and secreted products playing a role in the pathogenesis of infection as described in figure (1-1).Also two noteworthy features of staphylococci are that a virulence factor may have several functions in pathogenesis and that multiple virulence factors may perform the same function (Gordon and Lowy, 2008).

The form and severity of the disease result from a complex interplay between the host defense and the activities of the virulence factor repertoire of the infecting strain (Salmenlinna, 2002). Furthermore, virulence factors of *S. aureus* strains can be structured into various classes defined by their cellular location and their function and one factor can serve in one or more of these activities and different factors are produced in different growth phase.

Most clinical *S. aureus* strains produce capsular polysaccharide of serotype 5 or 8. The capsule may inhibit binding of antibodies and thereby opsonization and phagocytosis helping *S. aureus* to evade the host immune

system during an infection (Thakker *et al*., 1998; Thanh and Lee, 2002; Foster, 2005). In addition, capsular polysaccharide may have a role in bacterial adhesion to polymer surfaces in medical devices (Muller *et al*., 1993).

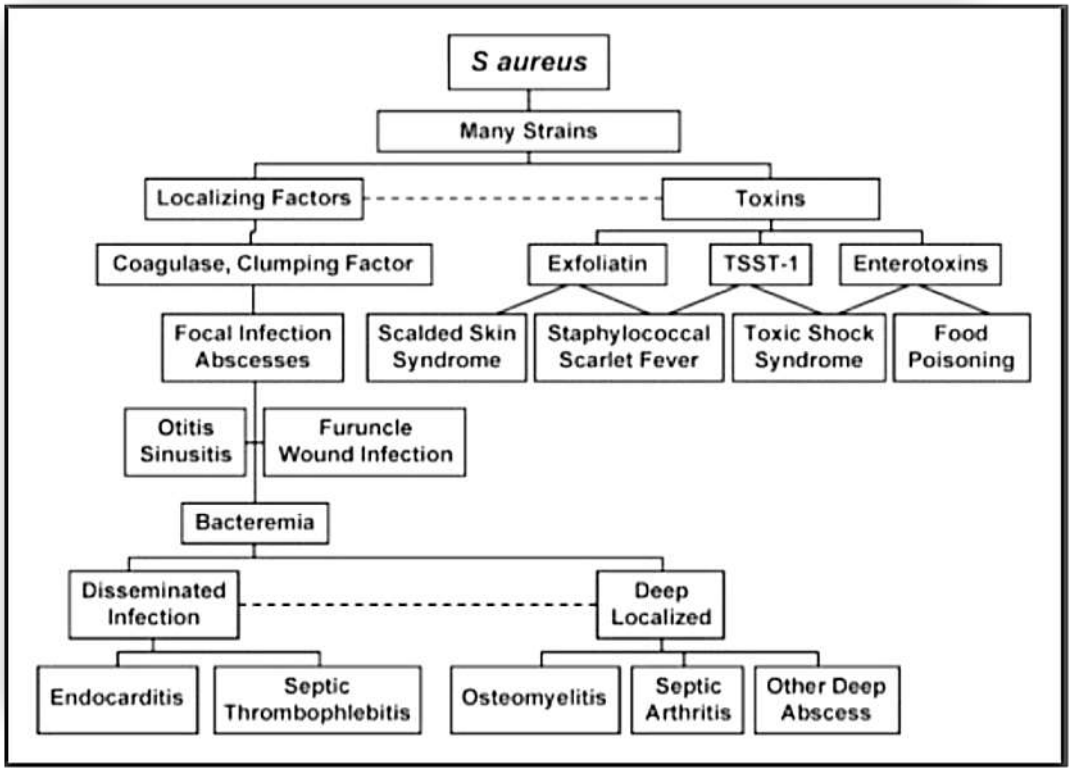


Figure (1-1): Correlation of staphylococcal virulence factors with disease (Todd, 2005).

In establishing an infection, *S. aureus* use a variety of molecules that are collectively termed **M**icrobial **S**urface **C**omponents **R**ecognizing **A**dhesive **M**atrix **M**olecules **(MSCRAMMs**) that mediate adherence to host tissues. MSCRAMMs bind molecules such as collagen, fibronectin, and fibrinogen, and different MSCRAMMs may adhere to the same host-tissue component.

Thus, it appears to play a key role in initiation of endovascular infections, bone and joint infections and prosthetic-device infections. Different *S. aureus* strains may have different constellations of MSCRAMMs and so may be predisposed to causing certain kinds of infections (Patti *et al*., 1994; Tung *et al*., 2000).

Staphylococcus aureus can form biofilms (slime) on host and prosthetic surfaces, which enabling it to persist by evading host defenses and antimicrobials (Donlan *et al*., 2002).

Staphylococcus aureus is also able to form Small-Colony Variants (SCVs), which may contribute to persistent and recurrent infection. *In vitro*, SCVs are able to "hide" in host cells without causing significant host-cell damage and are relatively protected from antibiotics and host defenses (Kahl *et al*., 1998).

Staphylococcus aureus produces a wide variety of exoproteins, most of them during the post exponential growth phase. These proteins degrade the host tissue to nutrients required for the growth of the bacteria and/ or allow the bacteria to penetrate deeper into the host tissue (Dinges *et al*., 2000).

The majority of the strains produce hemolysins, nuclease, proteases, lipases, hyaluronidase, and collagenase (Kollef and Micek, 2005). Alpha-hemolysin (or *alpha-toxin*) (α-toxin) is dermonecrotic, neurotoxic, and lysis mammalian cells especially red blood cells by forming a pore in the target membrane (Chanda *et al*., 2010).

Beta-hemolysin (β-hemolysin), acts as sphingomyelinase, gamma-hemolysin (γ-hemolysin), has leucocytolytic activity, and it has been suggested that delta-hemolysin (δ-hemolysin) has surfactant or channel forming properties (Dinges *et al*., 2000).

Important virulence factor in *S. aureus* is Panton-Valentine leukocidin (PVL), a member of the recently described family of synergohymenotropic toxins. PVL damages the membranes of host defense cells through the

synergistic activity of two separately secreted but non-associated proteins, LukS and LukF, causing severe abscesses, necrotizing pneumonia, and increased complications in osteomyelitis (Kuehnert *et al*., 2006).

1.2.3 *Staphylococcus aureus* Plasmids:

Plasmids are mobile extrachromosomal genetic elements bearing only non-essential genes which, however, may provide a benefit to the host under special environmental conditions (Plata *et al*., 2009). Consequently, epidemiologically related isolates can display different plasmid profiles. Moreover, many plasmids that have been reported in nearly all species of medically important bacteria carry resistance determinants contained in transposons that can be readily lost or acquired, quickly altering the composition of plasmid DNA (Udo *et al*., 1992; Trindade *et al*., 2003).

Apart from antibiotic resistance gene, plasmids often encode factors determining resistance to heavy metals, virulence factors and proteins facilitating survival in the presence of unusual nutrients (Wegrzyn, 2005).

Resistance based on transmissible plasmids affords the significant advantage of flexibility to the microorganism:

(i) Resistance to several antibiotics can be brought together in single plasmids.

(ii) Antibiotic resistance genes can be amplified when needed and de amplified when not needed.

(iii) Plasmids can be stored in a minimum portion of the microbial population and regained as needed.

(iv) Plasmids can serve as vectors to transfer genes.

(v) Plasmids serve an evolutionary role in the rearrangement of genetic parts both between and within organisms (Koch, 1981).

Plasmid-borne genes can undergo more radical evolutionary changes without affecting the viability of the cell, as they would be changed to

indispensable chromosomal genes, and established plasmid transfer mechanisms can provide recipient cells with new genetic material which has already been refined by selective pressures elsewhere. Moreover, plasmid-borne resistance has the disadvantage to the bacterium that it cannot easily function to alter the target of the antibiotic within the cell, since the structural genes for the cell's essential proteins in almost all cases are carried on the chromosome (Koch, 1981; Lyon and Skurray, 1987). Plasmids can, however, contribute to the development of chromosomal resistance in two ways. First, plasmids, either in part or in to, can integrate into the bacterial chromosome. Second, plasmids, together with bacteriophages, can act as vectors for transposable DNA elements or transposons (Kleckner, 1981).

The appearance of antibiotic-resistant staphylococci over the years was regarded as an inevitable genetic response to the selective pressure imposed by antimicrobial use (Lyon and Skurray, 1987; Chinedum, 2005).

Generally, bacteria are able to exchange genes in nature by three processes (conjugation, transduction and transformation). In Staphylococci, plasmids may be horizontally transferred through these mechanisms (Francia *et al*., 2004; Varella Coelho *et al*. 2009; Malachowa and Deleo, 2010). They carry resistances to antibiotics, metals, antiseptics, and disinfectants, as well as virulence genes, such as enterotoxins (Shalita *et al*., 1980; Bayles and Iandolo 1989; Omoe *et al*. 2003; Wegrzyn, 2005) and exfoliative toxins (Jackson and Iandolo 1986; Yamaguchi *et al*. 2001). Also, Staphylococci carry virulence plasmids originating from Bacillus (Gill *et al*. 2005) and Enterococcus (Clewell *et al*. 1985; Weigel *et al*., 2003; Sung and Lindsay 2007; Périchon and Courvalin 2009).

Clinical *S. aureus* strains often harbor multiple plasmids that are taxonomically grouped by replication mechanism and conjugation ability:

Class I plasmids are of the size of (1‑5 kb) and occur in high copy number (15‑50 per cell). They usually carry a single antibiotic resistance determinant.

Class II plasmids are of intermediate size and occur in intermediate copy number, and they usually code for β-lactamase and confer resistance to inorganic ions.

Class III plasmid consists of large conjugative plasmids (40‑60 kb) that carry multiple resistance determinants, exemplified by resistance to trimethoprim, gentamycin and ethidium bromide (Novick, 1989; Malachowa and Deleo, 2010).

Moreover, the conjugative plasmids encode their own conjugative horizontal transfer mechanism by *tra* genes that offer an advantage by which transfer of extrachromosal genetic information to other bacteria occurs (Kuroda *et al.*, 2001; Holden *et al.*, 2004; Gill *et al.*, 2005; Diep *et al.*, 2006), while Multiresistance plasmids between (20 and 30 kb) are common in staphylococci from several continents (Bayles and Iandolo 1989; Zuccarelli *et al*., 1990; Baba *et al*.2002; Toh *et al.* 2007).

1.2.4 *Staphylococcus aureus* alpha-toxin:

Pathogenesis of *S. aureus* depends partly on the production of an extensive repertoire of exotoxin and cell wall-anchored proteins that allow the organism to evade the innate immune system in order to establish and maintain infections. There are over 40 secreted proteins and enzymes that are known to cause or associate with *S. aureus* diseases (Foster, 2005; Nizet, 2007; Graves *et al*., 2010; Dong *et al*., 2012). Many of the known *S. aureus* exotoxins belong to the so- called group of membrane damaging toxins which attack target cells by disrupting their permeability barrier, either through channel formation or by detergent action or via lipase activity. As a result, the effect may be lethal if the cells are unable to compensate for leakage via active transport or by repairing their damaged membrane (Bhakdi and Jensen, 1991; Menestrina *et al*., 2001). Thus, *S. aureus* is able to induce apoptosis (programed cell death) in various cell types including epithelial cells,

endothelial cells, keratinocytes, osteoblasts as well as lymphocytes and macrophages (Jonas *et al*., 1994; Wesson *et al*., 2000).

Staphylococcal toxins can be divided into two groups according to their capacity for lysing cells, and those are: hemolysins or cytotoxins, which are capable of producing lesion directly to the outer membrane of target cells (Freer and Arbuthnott, 1983) and the so-called super antigenic toxins, which do not present direct lytic action, but can produce lesions through the overproduction of cytotoxins from activated T-cells and from monocytes/macrophages (Herman, 1991).

Staphylococcus aureus secrets five different membrane-damaging toxins, four hemolysins (alpha-, beta-, gamma- and delta-hemolysin) leukocidin and Panton-Valaninten Leukocidin (PVL) (Harshman *et al*., 1989; Kaneko & Kamio, 2004; Bartlett and Hulten, 2010).

The β-barrel family of pore-forming toxins is one group of staphylococcal cytotoxins that targets and kills mammalian cells by disrupting there membrane which result in the leakage of the cell's content and lysis (DuMont *et al.*, 2011).

Alpha-hemolysin (α-hemolysin, α-toxin and Hla) is a water-soluble monomer of 33.2 kDa that is produced by most *S. aureus* strains and representing one of its major cytolysin, it is a secreted pore-forming toxin encoded by *hla* gene that located on the chromosome (Brown and Pattee, 1980; Dong *et al*., 2012) with a cytolytic activity toward a variety of mammalian cell types including human erythrocytes, monocytes, keratinocytes, fibroblasts, platelet, epithelial cells and lymphocytes (Bernheimer *et al*., 1972; Walev *et al*., 1993; Menestrina *et al*., 2001).

Alpha-hemolysin is produced by nearly all virulent strains of *S. aureus* (Bhakdi and Tranum-Jensen, 1991; Pre´vost *et al*., 2001) and is implicated in several diseases caused by it. The role of α-toxin in their pathogenicity was

discovered when a mutant *S aureus* strain lacking *hla* display reduced virulence in invasive disease models (Wardenburg and Schneewind, 2008).

Staphylococcal alpha-toxin induces primarily necrotic cell death via a mechanism that involves binding to the cell plasma membrane, oligomerization of the toxin, and formation of pores (Essmann *et al*., 2003).

The fflux of intracellular potassium and the influx of sodium are followed by the loss of transmembrane potential, shutdown of ATP production, and predominantly necrotic cell death (Suttorp and Habben, 1988). These processes are accompanied by the release of proinflammatory and vasoactive mediators which contributing to the pathogenesis of staphylococcal infections (Lowy, 1998; Dinges *et al*., 2000; Cheung *et al*., 2002).

The cytolytic property of α-toxin has been studied extensively in rabbit erythrocytes and artificial membrane systems due to the fact that rabbit erythrocytes are extraordinarily susceptible to hemolysis by alpha-toxin at least 100 times more than other mammals and 1,000 times more than human erythrocytes (Cunha and Calsolari, 2008).

Like most staphylococcal extracellular proteins, α-toxin is not expressed constitutively but is tightly regulated by an array of extracellular and intracellular signals. At least three global regulatory loci; the accessory gene regulator ***(agr)***, the staphylococcal accessory gene regulator ***(sarA)***, and the staphylococcal accessory protein effector ***(sae)*** appear to coordinately control ***hla*** expression in *S. aureus in vitro* (Xiong *et al*., 2006). Furthermore, hemolysins, toxins, and enzymes facilitating tissue destruction and dissemination are expressed at the end of the exponential phase and during the stationary phase (Bayer *et al*., 1997). The *agr* system coordinates this sequential expression of virulence factors by repressing genes expressed during the early log phase and by activating genes expressed post-

exponentially. Both *agr* and *sar* are required for *S. aureus* pathogenicity (Vandenesch *et al*., 1991; Pragman and Schlievert, 2004).

Alpha-toxin possesses additional biological functions such as binding to a putative glycoprotein receptor on host cells, activation of intracellular signaling, and modulation of several processes (Bantel *et al*., 2001; Rose *et al*., 2002; Park *et al*., 2004). It was recently described that α- toxin facilitates the secretion of newly synthesized chemokines into the airway and exaggerates neutrophil-mediated inflammatory lung injury through syndecan-1 ectodomain shedding (Bartlett and Hulten, 2010).

1.2.5 *Staphylococcus aureus* Pathogenesis:

Pathogenesis is the ability of a microorganism to initiate disease. This includes entry, colonization and growth of the microorganism inside the host (Schjørring *et al*., 2002).

Staphylococci are ubiquitous microorganisms present on the skin of a high percentage of adults. However, several population groups are at serious risk of suffering pathogenic staphylococcal infections (Perez-Roth *et al*., 2001).

In 2007, Tang and his colleagues found out that, all species of staphylococci were identified as pathogen in blood stream infections. As well as, skin and soft tissue infections, post-operative wound infections and in ocular infections as mentioned by (Sharma, 2000). It is the primary cause of lower respiratory tract infections and surgical site infections (Richard *et al*., 1999) and the second leading cause of nosocomial bacteremia (Wisplinghoff *et al*., 2004), pneumonia, and cardiovascular infections (Klein *et al*., 2007).

Staphylococcus aureus, which represent the most pathogenic species of staphylococci, poses a significant public health threat it is human commensal that permanently colonizes the moist squamous epithelium of the anterior nares of 20% of the population (Peacock *et al*., 2001). Despite the fact that

nose is a favorite site for *S. aureus*, but it can also survive on the skin and the environment for long time (Zorgani *et al*., 2009). Other mucosal surfaces that harbor *S. aureus* include the throat, vaginal wall, and gastro intestinal tract. Yet, nasal carriage is probably most important because nose-picking could effectively disseminate the bacterium to other body surfaces and other hosts (Wertheim *et al*., 2006). Thus, a fundamental biological property of this bacterium is its ability to asymptomatically colonize healthy individuals who are considers being carriers with higher risk of infection (Chambers and DeLeo, 2009).

The primary mode of transmission of *S. aureus* is by direct contact, usually skin-to-skin contact with a colonized or infected individual, and by the contact with contaminated objects and surfaces which might also play a role (Muto *et al*., 2003; Miller and Diep, 2008).

Specifically, risk for colonization or infection with *S. aureus* is highest in patients with diabetes mellitus, intravenous drug users, and hospitalized patients undergoing hemodialysis, frequent catheter insertions and injections, postoperative surgical wounds, patients suffering chronic bronchopulmonary disorder, patients with acquired immunodeficiency syndrome, newborns, the elderly and generally immunocompromised persons (Levine *et al*., 1982; Lowy, 1998; Lodise *et al*., 2003 (b); Lindsay and Holden, 2004; Ohlsen, 2009).

It is established to be the leading cause of hospital-acquired infections (Trindade *et al*., 2003; Al-Ruaily and Khalil, 2011). This comes from the unique nature of *S. aureus* and its ability to adapt and survive in the environment that makes it one of the most successful pathogen known to man (De Lencastre *et al*., 2007).

The pathogen can cause a wide variety of infections which can be divided into three types:

(i) Superficial lesions such as wound infection.

(ii) Toxinoses such as food poisoning, scalded skin syndrome and toxic shock syndrome.

(iii) Systemic and life-threatening conditions such as endocarditis, osteomyelitis, pneumonia, brain abscesses, meningitis and bacteremia. (Jarvis, 2001; Guillemot *et al*., 2004).

Staphylococcal pathogenesis results from various bacterial activities mediated by virulence factors that facilitate attachment, colonization, cell-cell interactions, immune evasion, and tissue damage. It is commonly thought that bacterial adherence to host tissue is a prerequisite for colonization and infection (Holden *et al*., 2004).

Human staphylococcal infections are frequent, but usually remain localized at the portal of entry by the normal host defenses. The portal may be a hair follicle, but usually it is a break in the skin which may be a minute needle-stick or a surgical wound (Todar, 2011). *S. aureus* adheres and invades host epithelial cells using their (**MSCRAMMs**) (Liu, 2009; Makgotlho, 2009).

Subsequent survival, growth, and establishment of infection depend on the ability of the bacterium to circumvent host defense. The primary host response is mediated by polymorphonuclear leucocytes, which are attracted by expression of adhesion molecules on endothelial cells (Verdrengh and Tarkowski, 1997).

The cell wall components, peptidoglycan and teichoic acids, trigger signaling pathways leading to the release of cytokines (Ellingsen *et al*., 2002). Leucocytes and other host cellular factors can be destructed by locally acting bacterial toxins. *S. aureus* also produces toxins that directly attack human white and red blood cells. These toxins include the large family of leukocidins and α-toxin (also known as α-hemolysin) recently discovered phenol-soluble modulins (PSMs), and other hemolysins. The β-barrel structured leukocidins

form a pore almost exclusively in leukocytes, via a mechanism similar to that used by α-toxin (Joubert *et al*, 2007).

Virulent species can cause severe disease, which can be fatal. They can produce a complex arsenal of toxins and exhibit frequent and sometimes multiple resistances to antimicrobial agents. Most of these traits are located on mobile genetic elements (MGEs) on the genome (Monecke *et al*., 2011).

Different classes of MGEs have been identified in *S. aureus* and these include: bacteriophages, *S. aureus* pathogenicity islands, plasmids, transposons and staphylococcal cassette chromosome *mec* (SCC*mec*) and genomic islands (Malachowa and DeLeo, 2010).

1.2.5 Antibiotic resistance:

An antimicrobial refers to a substance that kills or inhibits the growth of microorganisms (Zhang *et al.*, 2010).Since the discovery of antimicrobial drugs in the 1923; many infectious diseases have been overcome. Typically, antimicrobials kill bacteria by binding to some vital compounds of bacterial metabolism, thereby inhibiting the synthesis of functional biomolecules or impeding normal cellular activities (Coates *et al.*, 2002). The evolutionary development of synthesized antibiotics has led to steadily increase in their use in both human treatment and animal disease management (McKeon *et al*., 1995). However, the prolonged therapy with antimicrobial agents and the extension of uses to areas other than prophylaxis and treatment of diseases have helped to create serious problems. Bacterial resistance to antibiotics has been recognized since the first drugs were introduced for clinical use (Chinedum, 2005). Resistance refers to the ability of a microorganism to survive the effect of drugs or other treatment, including antiseptics and debriding agents. In bacteria, resistance evolves through naturally occurring genetic mutation and the process of natural selection. The organism in question may develop the ability to destroy the antibiotic or to grow in its

presence (Holcomb *et al*., 2008). Bacterial resistance against conventional antibiotics is an escalating problem in modern medicinal treatment of infectious disease. This appears clearly in the growing number of immunocompramised patients who are suffering from hospital- acquired bacterial colonization (Berditsch *et al*., 2012).

Antibiotics have been called miracle drugs, but over 70 years of use, underuse, and overuse have resulted in frequent resistance in growing number of antibiotic-bacteria combinations (Barbosa *et al*., 2000; Lipsitch, 2001; Andersson, 2003).

The introduction of β-lactam antibiotics (penicillins and cephalosporins) in the 1940s and 1950s probably represents the most important event in the battle against infection in human medicine. Even before widespread global use of penicillin; the resistance was already recorded in *S.aureus* (Gaze, 2008).

Staphylococcus aureus is one of the most frequent bacterial pathogens in humans and one of the most important etiological agents of many hospital-acquired infections (HAIs) as well as community-acquired infections (CAIs) and poses a constant therapeutic problem clinicians (Bernardo *et al*., 2005 and Klein *et al*., 2007).

Methicillin (β-lactamase-resistant penicillin) and its derivatives becomes the drug of choice for the treatment of infections caused by *S. aureus*. However, the prolonged hospitalization and antibiotic therapy especially with β-lactam antibiotics predispose patients to the acquisition of methicillin / oxacillin- resistant *S. aureus* (**MRSA**/**ORSA**) (Hackbarth and Chambers, 1989; Tacconelli *et al*., 2008; Goto *et al*., 2009; Mattner *et al*., 2010).

Methicillin-resistant *S. aureus* (MRSA) is a strain of *S. aureus* which, by definition, is resistant to the semi- synthetic penicillins (i.e. methicillin, nafcillin, and oxacillin). As such, it is resistant to all other beta-lactam

antibiotics including other (penicillin, cephalosporins and cephamycins). Additionally, MRSA is often resistant to other classes of antibiotics including (aminoglycosides, macrolides and quinolones) or combination of these antibiotics. Thus, MRSA is not only methicillin resistant but also multiply-resistant as well and as described in figure (1-2) (Iyer and Kumosani, 2011; Onanuga & Temedie 2011).

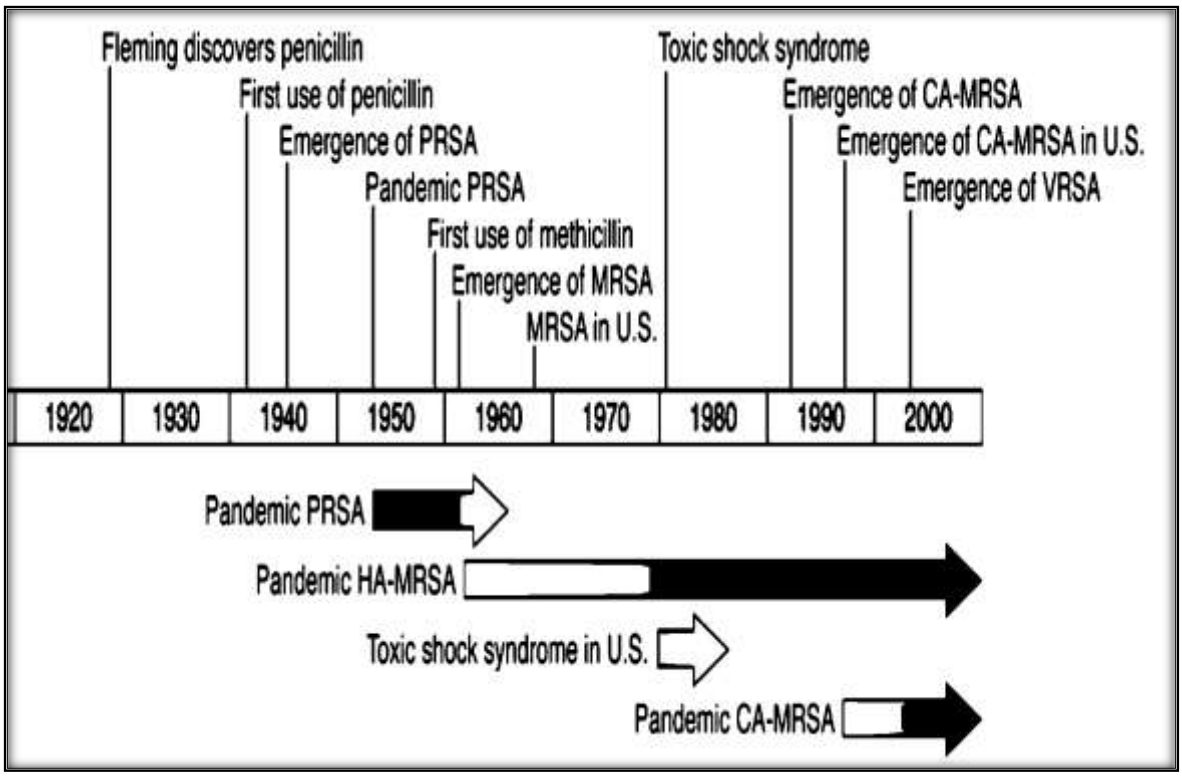


Figure (1-2): Emergence of antibiotic-resistant *Staphylococcus aureus*.

(Frank *et al*., 2009)

"Timeline indicates the year in which an event occurred or was reported"; "Arrows indicate approximate length of time for each pandemic /epidemic"

Multi-drug resistant strains of *S. aureus* have been reported with increasing frequency worldwide. Vancomycin, a glycopeptide, which was initially very effective in the treatment of Methicillin resistant *S.aureus* (MRSA) infections had been witnessed with intermediate resistance from MRSA strains (von-Eiff *et al*. 2001).

Moreover, the striking ability of this bacterium to acquire resistance against a wide spectrum of antibiotics is exemplified by the emergence and worldwide spread of methicillin resistance which renders *S. aureus* a subject of great concern in hospitals and other health care settings (Crisóstomo *et al.*, 2001; Hiramatsu *et al.*, 2002).

1.2.6 Methicillin resistant *Staphylococcus aureus*:

Emergence of bacterial strains which have been designated as methicillin-resistant *S. aureus* (MRSA) from 1960s to the present time has created clinical difficulties for nosocomial infections worldwide. And it was found to be associated with **C**omplicated **S**kin and **S**kin-**S**tructure **I**nfections (cSSSI) and serious hospital-acquired infections, especially bloodstream infections(BSIs) and ventilator-associated pneumonia (VAP) (Lodise *et al*.,2003 (a); Kuehnert *et al*., 2005; de Kraker *et al*., 2011). For this reason, *S. aureus* strains are considered as notable example of antibiotic resistance among Gram-positive bacteria (Liveromer, 2000).

Beta lactam antibiotics (e.g. penicillins, cephalosporins and pencillinase-insesetive β-lactams) damage bacteria by inactivation Penicillin Binding Proteins (PBPs), enzymes (transpeptidases) that are essential in the assembly of the bacterial cell wall (Pinho *et al*., 2001; Paez and Skiest, 2008; Plata *et al*., 2009). This process of inactivation of the cross-linking enzymes disrupts the normal physiological constitution of cell wall peptidoglycan and induces cell lysis and death (Zeba, 2005). There are four native PBPs found in staphylococci that are anchored on the cytoplasmic membrane whose

functions are the assembly and regulation of the latter stages of the cell wall biosynthesis. All of them are found to be inactivated by the action of these antibiotics (Bush and Mobashery, 1998; Goffin and Ghuysen, 2002; Palavecino, 2007).

Because of the weakened cell wall, treated bacteria become osmotically fragile and are easily lysed. The staphylococcal β-lactamase protein, which cleaves the β-lactam ring structure, confers resistance to penicillin but not to semi-synthetic penicillins such as methicillin, oxacillin, or cloxacillin (CFSPH, 2006).

There are three known mechanisms for which *S. aureus* becomes resistant to methicillin and those are:

(i) Hyperproduction of β-lactamases (Rello *et al.*, 2005).

(ii) Modification of normal penicillin-binding proteins (PBPs) (ATS, 2005).

(iii) The presence of an acquired penicillin-binding protein, PBP2a (Rayner and Munckhof, 2005).

Most of the known clinical bacterial isolates present the third mechanism. Resistance to methicillin is mediated by the bacterial acquisition of a novel mobile genetic element designed Staphylococal Chromosomal Cassette *mec* (SCC*mec*) which is one of the largest bacterial mobile elements known to date. Its size ranging from 21 to 67 kbp., and it is exclusive to staphylococci (Robinson and Enright, 2004). The SCC*mec* is considered to disseminate through horizontal transmission between staphylococcal species (Ito *et al*., 1999). The SCCmec carry *mecA* gene which encodes the alternative supplementary target protein (PBP2a) with low affinity for β-lactams including cephalosporins, carbapenems and β-lactamase inhibitor combinations. Therefore, it is capable of substituting the biosynthetic functions of the normal PBPs even in the presence of the β-lactams, thereby preventing cell lysis. PBP2a appears to be a rather poorly active enzyme

comparing to other native PBPs that synthesize highly cross-linked peptidoglycan. Also (SCC) can contain genes of resistance for non-beta-lactam agents causing multidrug resistant strains that found to be hospital associated (Hartman and Tomasz, 1984; Katayama *et al*., 2000; Berger-Bachi and Rohrer, 2002; Bou, 2007; Iyer and Kumosani, 2011).

PBP2a belongs to the group of high molecular mass (78 KDa|) family of PBPs and consists of a transpeptidase domain and a non-penicillin binding domain of unknown function (Goffin and Ghuysen, 1998).

1.2.7 The *mec* complex of MRSA:

Staphylococal Chromosomal Cassette *mec* is a variable genetic element with certain conserved features; it is integrated at a specific site in the staphylococcal chromosome (***attBcc***), which is located at the 3' end of the open reading frame X (***orfX***). The *orfX* encodes for a protein with an unknown function and is located near the origin of replication between a gene encoding a protein involved in purine synthesis (*purA*) and the protein A encoding gene (*spa*) (plata *et al*., 2009).

Staphylococal Chromosomal Cassette *mec* contains the *mec* operon composed of *mecA* and its regulatory genes, as well as the cassette chromosome recombinase complex *ccr* (Gill *et al*., 2005). The ***ccr*** locus is composed of the cassette chromosome recombines genes, ***ccrA***, ***ccrB*** or ***ccrC***, that are involved in the integration into the chromosome and in precise excision form the chromosome of the *SCCmec* element (Katayama *et al*., 2000; Burlage and Mahdi, 2008).

The variable regions of SCC*mec*, called **J**-region, contain integrated genetic elements, such as plasmids (pT181, pUB110 and p1258), transposons (Tn554) and insertion sequences (IS431, IS1272 and IS256) (Hanssen and Ericson Sollid, 2006).

The ***mecA*** gene is regulated by a repressor ***MecI*** and the transmembrane β-Lactam-sensing signal-transducer ***MecRI*** and as described in figure (1-3) both of which are transcribed divergently. In the absence of a β-lactam antibiotic, the transcription of ***mecA*** and ***mecRI*** is repressed by ***mecI***, otherwise ***mecRI*** which is a membrane protein that has an extracellular Penicillin-binding domain that, when bound by β-lactams, undergoes a conformational change inducing autocleavage of the intracellular protease domain. The active ***MecRI*** cleaves ***MecI*** which leads to derepression of ***mecA*** as well as ***mecI-mecRI*** operon which allows its transcription and so the resistance to methicillin (Lowy, 1998; Mallorqui-Fernandez *et al*., 2004).

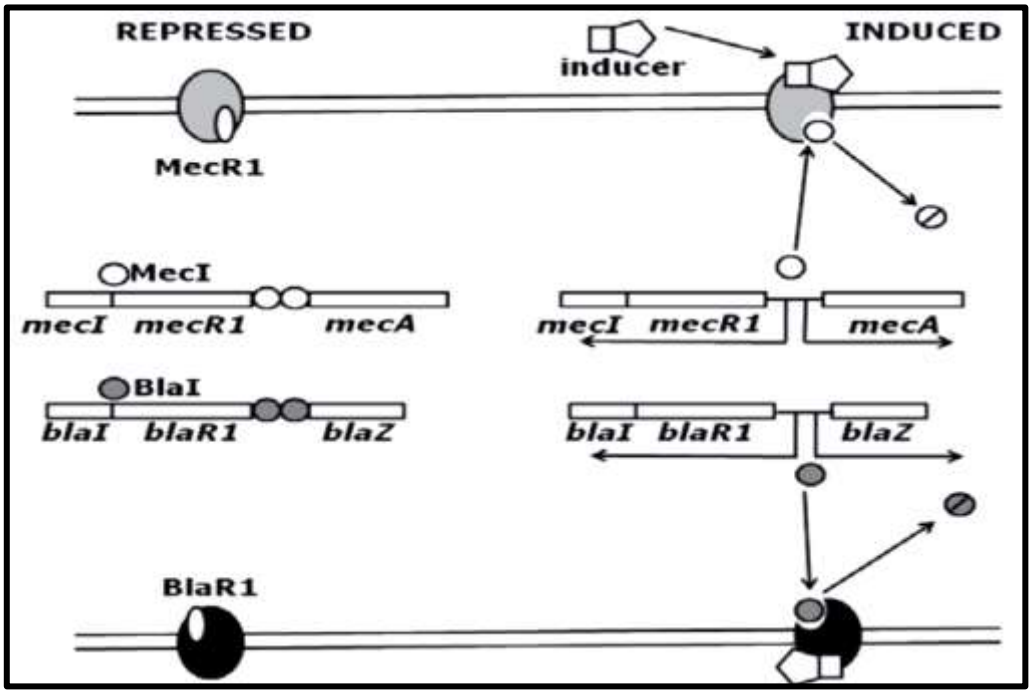


Figure (1-3): Two pathways regulating PBP2a and β-lactamase production (Zhang *et al*., 2001).

In addition to the regulation of ***mecA*** expression by its cognate ***MecI*** and ***MecRI*** regulators, it can also be regulated by structurally and functionally siminlar β-lactamase regulators; ***BlaI*** (a repressor) and ***BlaRI*** (a signal Transducer) are involved in the regulation of the expression of ***blaZ***, the gene coding for β-lactamase (Zhang *et al*., 2001).

The majority of clinical MRSA strains contain mutations in ***mecI***, however, in the vast majority of these cases, ***mecA*** expression is commonly regulated by ***BlaI*** (Rosato *et al*., 2003).

It has also been shown that certain genetic backgrounds of methicillin sensitive *S. aureus* (MSSA) strains carrying plasmid-borne, unregulated ***mecA***, are restrictive and selective against ***mecA*** expression because ***mecA*** is often mutated or deleted to avoid production of PBP2a in such strains. However, when the ***mec*** or ***bla*** regulatory genes are introduced together with ***mecA***, the system is tolerated (Katayama *et al*., 2000).

Until recently, eight *SCCmec* types were defined according to the *SCCmec* type and the chromosomal background determined by multilocus sequence typing (IWG-SCC, 2009). There are three types of SCC *mec* in HA-MRSA: types I, II and III, whereas CA-MRSA carry SCC*mec* types IV, V or VII (Mongkolrattanothai *et al*., 2003; Gould *et al*., 2012). All *SCCmec* elements carry genes for resistance to β-lactam antibiotics, as well as genes for the regulation of the expression of *mecA* (Matouskova and Janout, 2008).

Type I contains no additional resistance determinants, but types II and III contain resistance determinants in addition to *mecA*. These additional genetic elements account for the antimicrobial resistance to many antibiotics in addition to the *β*-lactam agents. The three *SCCmec* types contained in HA-MRSA have an identical chromosomal integration site and cassette chromosome recombinase genes which are responsible for horizontal transfer of SCC*mec* (Daum *et al.*, 2002).

1.2.8 Nanobiotechnology:

Nanotechnology can be defined as a research for the design, synthesis, and manipulation of structure of particles on the level of individual atoms and molecules, with dimension smaller than 100nm. It is a dynamically growing discipline of science, which has been particularly intensively researched from the beginning of the 1990s. (Zhang *et al*., 2008; Anita *et al*., 2010; Pulit *et al*., 2011).

A new branch of nanotechnology is nanobiotechnology that has emerged as integration between biotechnology and nanotechnology for developing biological synthesis and environmental-benign technology for synthesis of nanomaterials with specific functions (Sahayaraj and Rajesh, 2011).

'Nano' is a Greek word synonymous to "dwarf" meaning extremely small. The use of nanoparticles is gaining impetus in the present century as they possess defined physic-chemical properties including ultra-small size, large surface to mass ratio, high reactivity and unique interactions with biological systems (Zhang *et al.*, 2008). Thus, nanoparticles are being viewed as fundamental building blocks of nanotechnology (Thirumurugan *et al*., 2010; Taylor and Webster, 2011). Among them, the metallic nanoparticles are most promising as they contain remarkable antibacterial properties due to their large surface area (Gong *et al.,* 2007).

Metal oxides, such as (TiO2, ZnO, MgO and CaO) are generally regarded as safe materials to human beings and animals. They do not only exhibit strong antibacterial activity in small amounts even in absence of light but also they are stable under harsh process conditions (Stoimenov *et al*., 2002). In addition to the considerable antibacterial activities of inorganic metal oxide nanoparticles; they possesses selective toxicity to biological systems suggesting their potential application as therapeutics, diagnostics, surgical devices and nanomedicine based antimicrobial agents. This fact

received a great interest from researchers due to the growing microbial resistance against metal ions, antibiotics, and the development of resistant strains (Rai *et al.,* 2009).

Furthermore, the invention of the *scanning tunnel microscope* (STM), enabling the manipulation of atomic structures, is considered an exceptionally valuable achievement contributing to the development of nanotechnology (Bystrzewska-Piotrowska *et al*., 2009).

Biological methods of nanoparticles synthesis using microorganisms (Klaus *et al.*, 1999; Nair and Pradeep, 2002), enzymes (Willner *et al*., 2006) and plant or plant extract have been suggested as possible ecofriendly alternatives to chemical and physical methods. The use of plant for nanoparticles synthesis can be advantageous over biological processes by eliminating the elaborate process of maintaining cell structure (Shankara *et al*., 2004).

Today, nanotechnology is a science broadly applied in various disciplines, such as biotechnology, biomedicine, molecular medicine, pharmacology ecotoxicology, electronics and many others. Agriculture, veterinary science and the food industry also benefit from nanotechnology research to a comparable degree (Pulit *et al.*, 2011).

A number of nanoparticle-based therapeutic and diagnostic agents have been developed for the treatment of cancer, diabetes, pain, asthma, allergy, infections, and so on(Brannon-Peppas and Blanchette, 2004; Kawasaki and Player, 2005). These nanoscale agents may provide more effective and/or more convenient routes of administration, lower therapeutic toxicity, extend the product life cycle, and ultimately reduce health-care costs (Zhang *et al*., 2008).

1.2.8.1 Nanostructured zinc oxide:

Antibacterial properties of nano metal oxides have been discovered as a new generation of antimicrobial agent. Among metal oxide noparticles, ZnO nanoparticles, as one of the multifunctional inorganic nanoparticles, has many significant features, such as chemical and physical stability, high catalysis activity, effective antibacterial activity as well as intensive ultraviolet and infrared adsorption with broad range of applications as semiconductors, sensors, transparent electrodes, solar cells, etc. (Ghule *et al*., 2006; Matei *et al*., 2008). Also, ZnO has received considerable attention because of its unique optical, piezoelectric, and magnetic properties (Marcus and Paul, 2007).

Nano-ZnO particles are effective in inhibiting Gram-positive, Gram-negative bacteria and even spores that are high-temperature resistant and high-pressure resistant (Bahnemann *et al*., 1987; Hustvedt and Crews, 2005). Researchers have offered the use of zinc ion as superior disinfectant from hospitals infectious microorganisms (Reddy *et al*., 2007). Furthermore, ZnO NPs have selective toxicity to bacteria and only exhibit minimal effect on human cells, which recommend their prospective uses in agricultural and food industries especially with the growing need to find alternative methods for formulating new type of safe and cost-effective antibiotics in controlling the spread of resisted pathogens in food processing environment (Brayner *et al*., 2006; Jiang *et al*., 2009; Taylor and Webster, 2011). As well as, ZnO is listed as "generally recognized as safe" (GRAS) by the U.S. Food and Drug Administration.

Factors that may influence the antibacterial activity of ZnO are the concentrations of the metal oxides particles (Sawai *et al*., 1995), the particle size of the metal oxide powder (Zhang *et al*., 2007) and the specific surface area of the powder (Yamamoto *et al*., 1998). Thus, the antimicrobial activity of the nanoparticles is known to be a function of the surface area in contact

with the microorganisms. The small size and the high surface to volume ratio *i.e.*, large surface area of the nanoparticles, enhances their interaction with the microbes to carry out a broad range of probable antimicrobial activities (Rai and Bai, 2011). Therefore, nano-sized particles of ZnO have more pronounced antimicrobial activities than large particles, since the small size (less than 100 nm) and high surface-to-volume ratio of nanoparticles.

Nanoparticles (metals and metals oxides) bind to bacterial cell walls causing membrane disruption through direct interactions or through free radical production (Zhang *et al.*, 2007). Another possible mechanism for ZnO antibacterial activity is the release of Zn^{2+} ions which can damage cell membrane and interact with intracellular contents (Brayner *et al*., 2006).Mammalian cells are able to phagocytose nanoparticles, and can subsequently degrade these particles by lysozomal fusion, reducing toxicity and free radical damage. This may allow the selectivity of the same nanoparticle to promote tissue-forming cell functions, while also inhibiting bacterial functions that lead to infection (Arbab *et al*., 2005).

Because of its property as a highly safe compound, ZnO NP may be considered for combination therapy against *S. aureus*, due to its potential synergistic effect with important antibiotics (Thati *et al*., 2010). In addition to its greater effectiveness on resistant strains of microbial pathogens, less toxicity and heat resistance, ZnO NP provide mineral element essential to human cells and even small amount of it would exhibit strong activity (Padmavathy and Vijayaraghavan, 2008). Thus, levels of Zn above the essential threshold level inhibit bacterial enzymes including dehydrogenase and certain protective enzymes, such as thiol peroxidase and glutathione reductase (Anita *et al.*, 2010). Preliminary studies suggested that, among six nanoparticles tested, ZnO nanoparticles with relatively small particle size show a wide range of antibacterial effects on various microorganisms, including major pathogens, under normal visible light conditions (Jones *et al*.,

2008). Also, some data suggest the selective toxicity of the ZnO NPs toward cancer cells (Shantikumar *et al*., 2009).

1.2.9 Medicinal plant:

Finding healing powers in plants is an ancient thought that used as traditional medicine all over the world and predate the introduction of antibiotics and other modern drugs (Gangoué-Piéboji *et al*., 2006; Kaur and Arora, 2009; Das *et al*., 2010). The antimicrobial efficacy attributed to some plants in treating diseases has been beyond belief. It is estimated that local communities have used about 10% of all flowering plants on Earth to treat various infections, although only 1% have gained recognition by modern scientists (Lewis and Ausubel, 2006). Screening active compounds from plants has led to the discovery of new medicinal drugs which have efficient protection and treatment roles against various diseases including cancer (Sheeja and Kuttan, 2007) and Alzheimer's disease (Mukherjee *et al*., 2007). By and large, 60% of the world population and 80% of the population in developing countries rely on traditional medicine for curing many diseases (Shrestha and Dhillion, 2003).

Owing to their popular use as remedies for many infectious diseases, searches for plants containing antimicrobial substances are frequent (Betoni *et al*., 2006; Woods-Panzaru *et al*., 2009; Ali *et al*., 2011; Friedman *et al*., 2013).

Plants are rich in a wide variety of secondary metabolites, such as tannins, alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial properties (Lewis and Ausubel, 2006). According to the world health orginization, medicinal plants would be the best source for obtaining variety of drugs for treating infectious diseases due to their availability, fewer side effects and reduced toxicity (Lee *et al*., 2007). Furthermore, the emergence of multiple drug resistant strains of microorganisms due to

indiscriminate use of antibiotics to treat infectious diseases has generated a renewed interest in herbal medicine (Chopra *et al*., 1997; Hammer *et al*., 1999; Adwan *et al*., 2009).

1.2.9.1 Olive leaf (*Olea europaea* L.):

Family: Oleaceae.

Genus and specie: Olea europa (*O. europaea*).

Other Names: Oleae europaea, Oleae folium, Olivier

Latin Name: Olea europaea, Olea folium, Olea gallica, Olea lancifolia and Olea oleaster.

Common Names: Olive, Olive Leaf, Olive Tree and Olivier.

Properties: the medicinal parts are the dried leaves, the oil extracted from the Ripe fruits, and the fresh branches and leaves and clusters of flowers (Omar, 2010; Ghanbari *et al*., 2012

Throughout the history of civilization, the olive plant has been an important source of nutrition and medicine. Olive leaf from olive tree (*Olea europaea*) that is native to the Mediterranean countries, is a plant which can survive for hundreds of years and is known to naturally possess strong resistance to microbial attack (Pereira *et al*., 2007; Khalil *et al*., 2010; Keskin *et al*., 2012).

Olive leaf extract (OLE) is a dark brown, bitter-tasting liquid derived from the leaves of the olive tree (Sudjana *et al*., 2009). The first mention of olive leafs medicinal use in modern times was in 1843 when Daniel Hanbury from England reported a bitter substance from olive leaf tea was the agent responsible for healing malaria and associated fever (Benavente-Garcõa *et al*., 2000).

Olive leaf extract is very effective activity against various diseases, such as coronary artery disease, hypertension, high cholesterol level,

arrhythmia, cancer, diabetes, overweight, osteoporosis, herpes, flu and colds, and some bacterial, fungus and yeast infections (Erdohan and Turhan, 2011).

Olive leaf extract contains many different compounds collectively termed olive biophenols, which are thought to give the extract its varied therapeutic properties (Sudjana *et al*., 2009; Omar, 2010). The most abundant phenols in olive leaves are the non-polar oleuropein and ligstroside and the polar hydroxytyrosol and tyrosol. Phenolic compounds including oleuropein, tyrosol, hydroxytyrosol, caffeic acid, gallic acid, syringic acid, p-coumaric acid and luteolin, isolated from olive fruit and leaves, have shown antimicrobial activities against viruses, retroviruses, bacteria, yeasts, fungi and other parasites (Pereira *et al*., 2007; Keskin *et al*., 2012).

Increasing resistance to antibiotics, wide-spread use of immunosuppressing drugs and a rise in many infections all emphasize the necessity to find and develop new antimicrobial agents. Furthermore, *in vitro* research demonstrated the effectiveness of OLE against a wide range of pathogens, including *Escherichia coli*, *Streptococcus pyrogenes*, *Pseudomonas fluorescens*, *Helicobacter pylori*, *Compylobactor jujuni*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Salmonella typhimurium*, *Erwinia carotovora*, *Candida albicans* and *Plasmodium falciparum* (Sudjana *et al*., 2009; Ali *et al*., 2011; Keskin *et al*., 2012).

Like many natural products, variation due to differences, such as geographical location, plant nutrition, composition of soil and cultivar can influence the chemical composition and antibacterial activity of the olive leaf extract (Keskin *et al*., 2012).

Materials and Methods

2.1 Materials

2.1.1 Apparatus and equipment

Apparatus and equipment	Company	Origin
Water bath	Gallenkamp	England
Rotary evaporator		
Electric oven		
ELISA	BioTek	
Magnatic stirrer hotplate	Stuart	
Vortex mixer		
Soxhlet	Electrothermol	
VITEK 2 System	BioMerieux	France
Micropipette	Gilson	
Collection swab with media	Memmert	China
Microtiter plate	Mercury	
Digital camera		
Morter		
Incubator	Memmert	Germany
Milipore filters	Sartorius Membrane	
Shaking incubator	GLF	
Water distillator		
Mastercycler	Eppendrof	
Compound light microscope	Olympus	Japan
Deep freezer	Sanyo	

Power supply	LKB	Switzerland
Electric sensitive balance	Delta Range	
pH- meter	Radiometer	Denmark
Cooled centrifuge	Crison	Spain
Transilluminator UV	Ultraviolet product	USA

2.1.2 Chemicals:

Material	Company	Origin
Sodium chloride (NaCl)	BDH	England
Potassium chloride (KCl)		
Glycerol		
K_2HPO_4		
KH_2PO_4		
Ethidum bromide		
Tetra-methyl-p-phenylene dihydrochloride	Sigma	USA
Nano Zinc Oxide (35 nm)		
Ethanol	Local market	Iraq
Agarose	CennaGen	Iran

2.1.3 Biological materials

Biological materials, which include human plasma and human blood, were obtained from Al-Yarmook Teaching hospital and rabbit blood from Biotechnology Research Center / AL- Nahrain University. While samples (nose, ear and skin swabs) were obtained from Al-Kaddimia Teaching Hospital, Al-Yarmuk General Teaching Hospital, Al-Kindi General Teaching Hospital and Central Pediatric Teaching Hospital in Baghdad.

2.1.4.1 Antibiotic discs: (Bioanalys / Turkey)

Antibiotic	Disk potency	Resistant	intermediate	Sensetive
Oxacillin (OX)	1µg	≤10	11-12	≥13
Penicillin (p)	10µg	≤28	–	≥29
Vancomycin (VA)	30µg	–	–	≥15
Tobramycin (TOB)	10µg	≤12	13-14	≥15
Gentamycin (CN)	10µg	≤12	13-14	≥15
Ticarcillin-clavulanic acid (TIM)	75/10µg	≤22	–	≥23
Chloramphenicol (CL)	30µg	≤12	13−17	≥18
Cephalothin (KF)	30µg	≤14	15-17	≥32
Cefepime (FEP)	30µg	≤14	15-17	≥18
Erythromycin(E)	15µg	≤13	14-22	≥23
Cefoxitin (FOX)	30µg	≤21	–	≥22
Imipenem (IPM)	10µg	≤13	14-15	≥16
Tetracycline (TE)	30µg	≤14	15-18	≥19
Amikacin (AK)	30µg	≤14	15-16	≥17
Ciprofloxacin (Cip)	5µg	≤15	16-20	≥21

The value of sensitivity and resistance were registered from the Clinical and Laboratory Standards Institute (CLSI, 2011)[formerly known as (NCCLS)].

2.1.4.2 Antibiotic powder:

Oxacillin (Himedia / India).

2.1.5 Media

2.1.5.1 Ready-to-use media:

All the bellow listed media used in this study were prepared according to the instructions on their containers by the manufacturing companies:-

Media	Company	Origin
Blood base agar	Oxoid	England
DNase agar		
Muller – Hinton agar		
Mannitol salt agar		
Peptone		
Agar-agar	Himedia	India
Brain heart infusion agar		
Brain heart infusion broth		
Nutrient agar	Difco	USA
Nutrient broth		
Tryptic soya broth	Biolife	Italy

2.1.5.2 Laboratory Prepared Media:

All media were prepared according to the instruction of manufactures after they were brought to boiling to dissolve the constituents completely, the pH was adjusted as mentioned on the container and then sterilized by autoclaving at 121°C (15 Ib/inch^2) for 15 min, then poured in sterile petri dishes and incubated at 37°C for 24 hours to ensure sterilization.

2.1.5.2.1 Blood agar: (Collee *et al*., 1996)

It was prepared by the addition of 5% human blood to warm autoclaved blood agar base, mixed and poured into plates and kept at 4°C until use.

2.1.5.2.2 Luria−Bertani broth: (Atlas *et al*., 1995)

This broth medium contained the following components:

Component	Quantity(g)
Peptone	10
NaCl	10
Yeast extract	5

All components were dissolved in 950ml of D.W., pH was adjusted to 7.5 and the volume was completed to one liter before sterilization by autoclaving.

2.1.6 Preparation of reagents, stains, solutions, and buffers:

2.1.6.1 Reagents:

2.1.6.1.1 Ready use reagents:

Catalase reagent (H_2O_2) 3% Iraq.

2.1.6.2 Stains:

2.1.6.2.1 Ready use stains:

Gram stain kit (Himedia / India)

2.1.6.2.2 Laboratory – prepared stains:

Ethidium Bromid solution (10 mg/ ml): (Maniatis *et al*., 1982).

It was prepared by dissolving 1gm of ethidium bromide in 100 ml of distilled water and stirred on magnetic stirrer for few hours to ensure that the ethidium bromide has been dissolved. It was filtered and stored in dark container. As it represents a powerful mutagen, gloves and masks have been wearing during all preparation and handling steps.

2.1.6.3 Solutions:

2.1.6.3.1 Ready to use solutions:

Solutions	Company / origin
Mcfarland Turbidity Standard solution (NO. 0.5)	Central Health Laboratory/ Iraq
Normal saline	Iraq

2.1.6.3.2 Laboratory – prepared solutions:

1. Glycerol: (Collee, *et al*., 1996).

It was prepared by using **20** ml of glycerol and the volume was completed to 100 ml by D.W. in order to reach 20% of glycerol stock solution. Then, it was autoclaved and stored at 4˚C.

2. Oxacillin Stock Solution:

Oxacillin Stock solution was prepared according to (CLSI, 2011). This was carried out by dissolving 0.1 gm of the antibiotic in 10 ml of D.W. then sterilized by filtration through Millipore filters (0.2 μm). It was freshly prepared.

2.1.6.4 Buffers:

2.1.6.4.1 Ready to use buffer:

Buffer	Company / origin
TBE buffer	Promiga/ USA
Gel loading buffer	Promiga/ USA

2.1.6.4.2 Laboratory – prepared buffer:

- **Phosphate buffer saline (PBS) pH=7 (Cruckshank *et al*., 1975).**

It was prepared by dissolving NaCl (8 gm), KCl (0.2 gm), KH_2PO_4 (0.2 gm) and K_2HPO_4 (1.15 gm) in 950 ml of D.W. pH was adjusted to 7 and sterilized by autoclaving at (121˚C 15 Ib/in^2) for 15 min.

2.1.7 Kits:

Kits	Company / origin
Plasmid Miniprep Kit	Fermentas/ Germany
DNA Extraction Mini Kit	INtRON biotechnology/ Germany

2.1.8 Specific primers: (CinnaGen/ Iran)

2.1.8.1 *nuc* primer (Brakstad *et al*., 1992)

Primer	Primer sequence	Length (bp)	TA (˚C)
nuc F	F/ 5-GCGATTGATGGTGATACGGTT-3	21	65.7
nuc R	R/ 5-AGCCAAGCCTTGACGAACTAAAGC-3	24	68.4

2.1.8.2 *MecA* primer (Martineau *et al.*, 2000)

Primer	Primer sequence	Length (bp)	TA (˚C)
mecA F	F/ 5-AACAGGTGAATTATTAGCACTTGTAAG-3	27	61.4
mecA F	F/ 5-ATTGCTGTTAATATTTTTTGAGTTGAA-3	27	61.6

2.1.8.3 *hla* primer

Primer	Primer sequence	Length (bp)	TA (˚C)
hla F	F/5-GGTTTAGCCTGGCCTCT-3	17	59.7
hla F	F/5-CATCACGAACTCGTTCG-3	17	58.8

2.2 Methods:

2.2.1 Sterilizing methods:

2.2.1.1 Wet-heat sterilization:

Microbial culture media, solutions, buffers, and reagents were sterilized by autoclaving at 121°C (15 Ib/inch^2) for 15 min.

2.2.1.2. Dry-heat sterilization:

Electric oven was used to sterilize glassware at 160˚C for 3 hours or 180 ˚C. for 2 hours.

2.2.1.3. Membrane sterilization (Filtration):

Antibiotic solution was first dissolved in D.W., and then sterilized throughout (0.22) μm in diameter Millipore filters.

2.2.2 Samples collection:

Two hundred and six clinical samples were collected during the period from February to October 2010. These samples were obtained from

Al-Kaddimia Teaching Hospital, Al-Yarmuk General Teaching Hospital, Al-Kindi General Teaching Hospital and Central Pediatric Teaching Hospital.

The clinical samples were collected from different patients attending the four hospitals, health care workers such as staff-nurses, ward boys and indoor environment of these hospitals. Sterile gloves were worn while collecting samples to prevent skin bacterial contamination.

Samples from nose (both anterior nares), hand-swab (especially hands of the hospital personal) and ear-swab were collected using sterile swabs and placed into a transport medium; transported to the laboratory immediately.

2.2.3 Cultivation of the bacterial isolates:

All the swabs were first plated onto a complex differential blood agar medium. It provides a useful identification test depending on the hemolysis of blood cells which can be either complete breakdown of the cells with release of hemoglobin and a clearing of the red color from the surrounding medium around the colony (β-hemolsis) or the hemolysis can be partially breakdown, resulting in a greenish or green-yellow zone around the colony (α-hemolysis).

Bacterial cultures from blood agar were then grown on nutrient agar medium and identified by a single colony isolation technique according to the (shape, color, size, edges and heights of the colony).

2.2.4 Identification of the bacterial isolates

2.2.4.1 Cultural identification (G.F. brooks *et al*., 2010)

Identification of the *S. aureus* was carried out using conventional culture detection method by plating the tested isolates on mannitol salt agar medium that contains 7.5% NaCl, which is inhibitory to the growth of most bacteria other than the staphylococci. The medium also contains mannitol and

the pH indicators (phenol red) for detecting acid produced by mannitol-fermenting staphylococci (*S. aureus*) and differentiate it from nonmannitol-fermenting (*S. epidermidis*) because mannitol-fermenting staphylococci produce yellow zone surrounding their growth. Thus staphylococci that do not ferment mannitol do not produce a color change.

2.2.4.2 Microscopic identification: (Atlas *et al.*, 1995).

A loop full from a suspected *S. aureus* colony was fixed on a microscope slide, and then stained with Gram stain to examine (cell shape, grouping, size and Gram reaction).

2.2.4.3 Biochemical identification:

Identification of the clinical isolates was performed by traditional biochemical tests.

2.2.4.3.1 Catalase test (Atlas *et al*., 1995).

This test demonstrates the presence of catalase, which is an enzyme that catalyzed the decomposition of hydrogen peroxide (H_2O_2) to water and gaseous oxygen. The test was performed by aseptically pick up a mass of bacterial cells from the colony and deposit it on a clean microscope slide, then pipette a drop of H_2O_2 (3%) onto the mass of bacterial cells. The formation of bubbles (the release of oxygen) indicates a positive test.

2.2.4.3.2 Coagulase test:

Coagulase enzyme catalyzes the conversion of fibrinogen to fibrin blood plasma. The reaction is carried out by adding 0.1 ml of the tested bacterial isolate and 0.5 ml of citrated plasma solution to a test tube before incubated at 37˚C. The tubes are examined after 0.5, 1, 2 and 4 hours for coagulation. Formation of clot within four hours is an indicator for positive

result. The delayed or weak production of coagulase should be observed after 24 hours by placing the tubes at room temperature (Atlas *et al*, 1995).

2.2.4.3.3 Deoxyribonuclease test (DNase production):

The DNase catalyzes the hydrolysis of deoxyribonucleic acid (DNA). The test was performed by inoculating DNA agar plate with suspected bacterial isolate, and then incubated at 37˚C for 24-48 hours. After incubation, flood the plate with 1 N of HCl which led to, precipitates the DNA and turns the plate cloudy. The appearance of a clear zone around the growth was an indication for DNase production and a positive result. DNase-positive cultures show a rose pink halo around the area of growth.

2.2.4.4 Identification of bacteria with VITEK 2 System:

A specific number of the bacterial isolates were selected to confirm their identification and antibiotic susceptibility using the VITEK 2 System Provided by Al-Kaddimia Teaching Hospital.

2.2.5 Maintenance of bacterial isolates:

Maintenance of bacterial isolates was performed according to Maniatis *et al*. (1982) as follows:

2.2.5.1 Short term storage

Isolates of bacteria were maintained for few weeks on brain heart infusion agar, the plates were tightly wrapped in parafilm and stored at 4˚C.

2.2.5.2 Medium term storage

Isolates of bacteria were maintained for months as cultured prepared in small screw capped bottles containing 5-7 ml of nutrient agar as slants and stored at 4˚C.

2.2.5.3 Long term storage

Bacterial isolates can be stored for years in medium containing 20% glycerol at low temperature without loss of its significant viability; this was done by inoculating a single colony of bacterial culture into test tube contained 10 ml of sterile brain heart infusion broth and incubated at 37°C for 24 hours. Then 8.5 ml of the cell suspension was mixed with 1.5 ml of sterile glycerol 20% in item (2.1.6.3.2) .The tubes were tightly wrapped in parafilm and stored at (– 20) °C .

2.2.6 Antibiotic susceptibility testing:

2.2.6.1 Disc agar diffusion test:

The disc diffusion assay was performed using Kirby-Bauer method according to the (CLSI, 2011) guideline. All the *S. aureus* isolates that were previously identified by morphological and biochemical tests were plated by streaking on Muller-Hinton agar medium (with a turbidity equivalent to that of 0.5 McFarland tube containing approximately 1 to 2 x 10^8 cfu/ ml) by using a sterile cotton swab and rotating the plate between streaking to ensure even distribution of the inoculum. The inoculated plates were placed at room temperature for 10 minutes to allow absorption of excess moisture. Gently, each antibiotic disc was pressed down with sterile forceps to ensure complete contact with agar. Discs were arranged so as to avoid the development of overlapping of inhibition zones. Within 15 minutes of applying discs, the inverted plates were aerobically incubated at 37˚C for 24 hours (for cefoxitin, results may be reported after 18 hours) according to the (CLSI, 2005).

After incubation period, the diameter of zone of bacterial growth inhibition surrounding the disc (including the disc) was measured and compared with a standard of each drug according to CLSI published data. The zone margin should be considered the area showing no obvious visible growth

that can be detected with the unaided eye (CLSI, 2011). This gave a profile of drug susceptibility vis-à-vis antibiotic resistance (Chakraborty *et al*., 2011).

2.2.6.2 Determination of oxacillin MICs by Broth microdilution method

Minimum inhibitory concentrations were determined using the broth microdilution method. Oxacillin stock solution was prepared at a concentration of 1280 µg/ml in deionized water. The antibiotic was serially dilluted with 2.0% NaCl, as recommended by the NCCLS, to give working concentration of (0.125 – 64)µg/ml and bacterial suspension of 0.5 McFarland turbidity standard was added to all wells before incubation for 24 hours at 35 ± 2˚C. the MIC breakpoint of oxacillin (1µg/ml) for *S. aureus* is (≥ 4.0 - < 2) µg/ml.

2.2.7 Hemolysis assay:

Micro titer plate method described by Johenson *et al*. (1979) was used as follows:

Total hemolysis was assessed with rabbit erythrocytes, which are 100 times more sensitive to *S. aureus* α-toxin than human erythrocytes.

a) *Staphylococcus aureus* isolates were grown at 37°C in tryptic soya broth for 18 hours.
b) After centrifugation at 7000 rpm for 15 min. A supernatant of each tested isolate was transferred into new sterile test tube.
c) A portion of 100 µl of washed rabbit erythrocytes was added to the microtiter plates that was previously filled with 100 µl of the serially diluted (using PBS) bacterial culture supernatants.
d) The first well of each horizontal line was filled with (100 µl of PBS + 100µl of washed blood) which is considered as a negative control.
e) To the second well, (100) µl of washed blood + (100) µl of bacterial supernatant were added.

f) The microtiter plate was then incubated for 60 min at 37°C.

g) After incubation period, all the plates were kept at 4˚C for 2-18 hours, before reading the results as lysis of RBCs.

Data are expressed as mean units of hemolytic activity per milliliter of culture supernatant.

2.2.8 Extraction of the bacterial DNA:

The extraction of the chromosomal DNA was carried out for (23) *S. aureus* isolates that gave us the highest rate in antibiotic resistance and best efficiency in hemolysin production.

The extraction was carried out according to the instructions in the manual book of the company manufactured the extraction kit and including the following steps:

1- Fresh culture of the tested bacterial isolates was prepared by inoculating these isolates in LB medium and incubated it overnight.

2- About 2 ml from the bacterial culture were pelleted by centrifugation at 13,000 rpm for 1 min. and the supernatant was discarded.

3- The bacterial pellets was re-suspended completely with the remnant supernatant by vigorous vortexing to yield homogeneous solution.

4- To this suspension, 100µl of the pre-lysis (**detergent buffer**) andd 3µl of the **lysozyme** solution were added and mix it well by vortexing.

5- The pre-lysis suspension was incubated for about 30 min or more to allow the perfect enzymatically breakdown of the rigid cell wall (thick peptidoglycan layar), while the detergent buffer would ensure the complete lysis of the bacteria. The lysate was mixed by inverting the tube 5-6 times to ensure complete lysis of the cell wall.

6- Centrifugation of the pre-lysis suspension was carried out at 13,000 rpm for one min.. The supernatant was discarding and the cell pellet was resuspended by vortexing to ensure perfect pre-lysis of the cells.

7- The lysis of the bacterial cell was obtained by adding 200µl of the **lysis buffer**, 10µl of **proteinase K** solution and 5µl of **RNase A** solution followed by vigorous vortexing or pipetting. Then the mixture was incubated for 30 min. with inverting the tube about 5-6 times during the incubation period to get pure lysate.

8- At the end of the incubation period (complete lysis) an equilibration step that bind the genomic DNA to the column membrane was carried out by adding 250µl of the **binding buffer** to our lysate and mix by pipetting or inverting the tube gently 5-6 times, then spin down to remove any drops from inside the lid.

9- A homogenous solution was obtained by adding 250µl of 80% **ethanol** to the lysate and mixed by pipetting until the two phase disappeared, then spin it down to remove any drops from the inside lid.

10- Using a pipette, 750µl from the resulted mixture was transfered into the spin column that was inserted in 2 ml collection tube and centrifuge at 13,000 rpm for 1 min., then discard the flow-through and collection tube altogether.

11- After placing the spin column into a new 2 ml collection tube, 700µl of the **washing buffer** (mixed with ethanol) were added to the spin column and centrifuge at 13,000 rpm for 1 min. We discard the flow-through and again the centrifugation to ensure drying of the membrane because any residual ethanol would inhibit the subsequent reactions.

12- The spin column was transferred carefully and placed in a new 1.5 ml tube. To this, a 50µl of the **elution buffer** were added directly onto the membrane after incubation for about 1 min. at room temperature. Centrifugation was carried out at 13,000 rpm for 1 min.to elute the yielded DNA.

2.2.9 Plasmid DNA extraction:

Plasmid DNA was isolated according to the manual book of the used extraction kit and including the following steps:

1- From fresh culture of bacteria that was grown in LB broth mediumabout 5ml were pelleted by centrifugation at 10,000 rpm for 2 min.

2- After centrifugation, we discard the supernatant carefully using a micropipette to avoid any mixing of the precipitate.

3- The cells were re-suspend completely by adding 250 µl of **resuspention solution** (contain RNase) to the pellet and mix it well by vortexing or pipetting up and down until no cell clumps remain. Then, the suspension was transferred to a clean 1.5ml microcentrifuge tube (lysis will not occur if clumps of bacteria remain following an in efficient resuspension).

4- Alkaline lysis step was carried out by adding 250 µl of **alkaline lysis** solution and mix thoroughly by inverting the tube for several times (4-6) until the solution becomes viscous and slightly clear. During this step we did not use vortexing because it might cause shearing to the chromosomal DNA. In addition, long time incubation (over 5 min) was avoided as it may cause denaturation of the supercoiled plasmid DNA.

5- Neutralization of the lysate was obtained by adding 350 µl of **neutralizing** solution and mix it immediately and thoroughly by inverting the tube for several times (4-6) until a white precipitate forms (avoiding vortexing that shear the genomic DNA and lead to the contamination with the chromosomal DNA). This was followed by centrifugation at 14,000 rpm for 10 min. in order to pellet cell debris and chromosomal DNA.

6- After centrifuge, loading step was carried out through the transfer of the supernatant into a spin column assembled in a clean collection tube

using a micropipette to ensure not to disturb or transfer any white precipitate.

7- A centrifugation step at 14,000 rpm for 1 min. was carried out for the loading column. At the end of this step the supernatant was discarded and the column placed back into the same collection tube.

8- Column washing was accomplished by the addition of 500 µl of **washing buffer** (it was diluted with ethanol), followed by centrifugation at 14,000 rpm for 30-60 seconds and discarded the flow-through. This step was repeated for one more time to ensure drying of the column and removal of any residual ethanol.

9- Transfer the column into a new microcentrifuge tube. Then, the elution of the DNA was carried out by adding 75 µl of the **elution buffer** or TE buffer directly onto column membrane without any contact with the membrane and incubated for 2 min at room temperature, at the end of the incubation period. The column was centrifuged at 14,000 rpm for 1 min.

10- The yielded DNA was stored at the refrigerator for later examination by gel electrophoreses.

2.2.10 Spectrophotometer determination of DNA:

Ten micro liters of each DNA sample were added to 490µl of D.W. and mixed well. A spectrophotometer was used to measure the optical density at wave length of 260 nm and 280 nm. An O.D. of 1 corresponds to approximately 50 mg/ml for double stranded DNA. The concentration of DNA was calculated according to formula described in (Maniatis *et al*., 1982).

DNA concentration (µg/ml) = O.D. 260 nm **X** 50 X dilution factor.

2.2.11 Agarose Gel Electrophoresis: (Maniatis *et al.*, 1982)

To separate DNA fragments, agarose gel in different concentrations were used (about 0.8% for the extracted DNA and 1.2-2% for visual checking of specific PCR products). Agarose powder is mixed with electrophoresis buffer to the desired concentration, and then heated in a microwave oven until completely melted. The prepared gel was poured into a casting tray containing a sample comb and allowed to solidify at room temperature and still in its plastic tray; the gel was inserted horizontally into the electrophoresis chamber and just covered with (1X) TBE buffer. Samples containing DNA mixed with 1/10 of loading buffer were then pipeted into the sample wells. The lid and power leads were placed on the apparatus, and a current was applied. Generally, the gel was run at 5v/cm for about 1.5 hour to PCR product and 2 hours to plasmid DNA.

We could confirm that current is flowing by observing bubbles coming off the electrodes. DNA bands were visualized by U.V. trans illuminator at 365 nm wave length documentation system, and then photographed with digital camera.

2.2.12 detection of specific genes by polymerase chain reaction:

The detection of *S. aureus* specific species gene, as well as the genes encoding the resistance for oxacillin antibiotic and production of alpha-hemolysin that represents the most important virulence parameters associated with the pathogenicity and spread of *S. aureus,* was carried out by the amplification of specific sequences within the target gen using polymerase chain reaction technique.

The experiment was carried out using a mixture of specific sets of primers designated for each target gene that were mixed with the DNA sample (tamplate) and a mastermix reagent which contain (*Taq* plymerase,

PCR buffer, $MgCl_2$ and dNTPs), the final constituent was the deionized water. The reaction mixture was mixed and centrifuge for 3 seconds to collect the drops from walls in order to ensure the final volume of 25µl, and then transfer to thermal cycler to start the reaction according to the steps of specific program.

2.2.13 Preparation of crud ethanolic olive leaf extracts (OLE) (Keskın *et al*., 2012):

Olive leaves were collected from Al-Jadrhia garden and identified as Olea europaea by prof. Dr. Ali Al-Mossawy, Biology department, College of Science, Baghdad University. The dried olive leaves were powdered using a coffee grinder, and then extracted with 70% ethanol at a 20% w/v concentration. 50 grams of the processed plant were extracted in 250 ml of the ethanol (70%) using the soxhlet apparatus. The obtained extract was then evaporated to remove the solvent under reduced pressure by rotary evaporator at 40˚C by the rotary evaporator and the yield of extraction was approximately 3gm, i.e 6% (w/w) and was frozen at -20°C. to be used in subsequent analyses, the extract was dissolved in dimethyl sulfoxide and then sterilized by filtration to give an approximate concentration of 300 mg/ml. the whole leaf ethanolic extract was used in the present study.

2.2.14 Determination of plant antimicrobial activity: (Keskin *et al*., 2012)

Antimicrobial activity of the olive leaves extract was measured by the paper disc diffusion method with a slight modification. Sterile 6 mm filter papers discs are impregnated with 30 µl filter sterilized olive leaf extract of various concentrations (75,150, 225 and 300 mg/ml) to give a concentrations of (2.25, 4.5, 6.75 and 9.0 mg/disc, respectively). The agar plates inoculated with ORSA were incubated for 1 h before placing the extract impregnated paper discs on the plate. Following this, the sterile discs impregnated with the

extract was placed onto the surface of the agar plate. Oxacillin discs (1µg/disc) alone and in combination with the paper disc impregnated with the 300mg/ml OLE were also used as a positive standard references as to test the potential synergistic effect of the oxacillin-OLE combination, respectively. Plates were incubated at 35 ±0.1°C for 48 hours. After incubation, all plates were observed for zones of growth inhibition, and the diameters of these zones were measured in mm. All the analyses were performed under sterile conditions in duplicate.

2.2.15 Antimicrobial activity of ZnO Nanoparticles:

The potential synergistic antimicrobial effect of ZnO nanoparticles (35 nm) was measured using disc diffusion method as described by (Thati *et al*., 2010). Briefly, 6 mm diameter filter paper discs were impregnated with zinc oxide nanoparticles (100µg/disc). To determine combined effects, zinc oxide impregnated discs (100µg/disc) were further impregnated with 30 µl of various concentrations of crude OLE (75, 150 and 225 mg/ml). Also, saturated oxacillin discs (1µg/disc) were further impregnated with zinc oxide nanoparticles (100µg/disc).

The muller-Hinton agar plates inoculated for 1 hr before placing the zinc oxide impregnated discs on the plates. After incubation at 37ºC for 24 hr, the zones of inhibition (mm) were measured. Assays were performed in duplicate.

3. Results and Discussion

3.1 Isolation and identification of *Staphylococcus aureus*

A total number of 206 clinical samples were collected from different patients and hospital staff in various units from nearby hospitals. Depending on the conventional cultural procedures,154 samples showed positive culture and many of them were found to show beta haemolytic activity on blood agar due to the production of haemolysin by isolates, makes a clear zone surrounding the isolates.

The 154 growth positive cultures that give were further primary identified by Gram staining and the related biochemical tests. Upon that 61 appeared under microscope as G (+) with coccus shape mainly grouped in clusters in accordance with

On mannitol salt agar the suspected isolates were able to ferment mannitol and produced yellow color due to the acid production. They were catalase positive, due to the production of catalase enzyme which distinguishes them from *Streptococcus* spp., and coagulase positive, due to their production of coagulase enzyme that reacts with prothrombin and form staphylothrombin which causes blood to clot by converting fibrinogen to fibrin.

Coagulase positivity of isolates distinguishes *S. aureus* from other *Staphylococcus* spp. Thus, tubes with coagulase production is considered as "gold standard" for identification of *S. aureus* which describes the ability of tested isolates to produce nuclease enzyme, suggesting that these isolates have the ability to break down the DNA.

In addition, a confirmatory examination was carried out by using VITEK 2, a system enables rapid identification for the suspected isolates to the species level, in addition to its ability to perform antibiotic susceptibility pattern of the isolates against specific antibiotics given as data (kits) through highly computerized system that provided readable data at the end of the operation.

3.2 Antibiotic susceptibility profile

In recent years, *S aureus* become resistant to both new and traditional antibiotics and thus, treatment of antibiotic resistant bacteria represents a therapeutic problem. For this reason, investigating the susceptibility pattern is useful to determine the future challenges of effective therapy.

Susceptibility of *S. aureus* isolates was detected against 15 types of antibiotics, the tested pathogenic isolates were found to exhibit obvious level of resistance against the used antibiotics and the susceptibility pattern for these clinical *S. aureus* isolates are shown in figure (3-1) and appendix (1).

Oxacillin has been the agent recommended by the CLSI for phenotypic tests to predict resistance to penicillinase-stable penicillins (PSPs) due to its stability and superior sensitivity over other PSPs susceptibility tests.

The results of this study demonstrated that out of 61 tested *S. aureus* isolates that were isolated from hospital sources, 47 isolates showed a high level of resistance (77 %) to oxacillin, the target antibiotic, which considered as Oxacillin Resistant *S. aureus* (ORSA).

When plates of isolates exhibiting resistance to oxacillin were incubated for an additional 24 hours (to distinguish the intermediate resistant and hetero-resistant). The hetero-resistant *S. aureus* isolates were turned sensitive whereas intermediate resistance remained resistant.

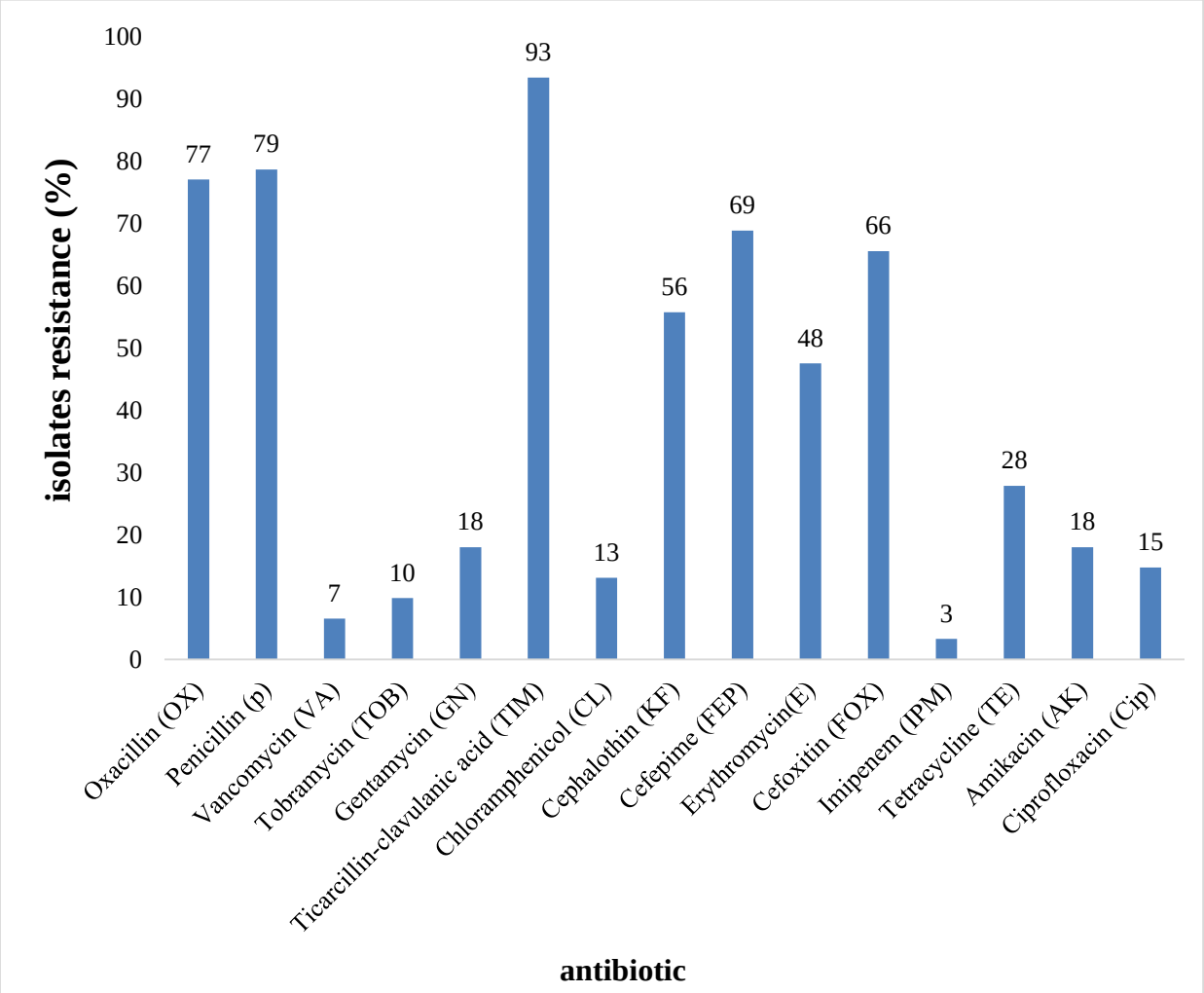


Figure (3-1) Antibiotic susceptibility profile of the tested *S. aureus* isolates to different antibiotics.

Drew *et al*. (1972) found that oxacillin in discs was less labile than methicillin in storage but oxacillin was less resistant to hydrolysis by staphylococcal *β*- lactamases.

Majority of ORSA isolates were found to be resistant to all *β*-lactam antibiotics used (Naji, 2008).

Furthermore, (78.7%) of the isolates showed resistant to penicillin which is closely to the percentage obtained by the oxacillin as the two antibiotics belonged to the same class of antibiotic that kill the bacteria by the inhibition of the cell wall synthesis. This may be due to the irrational use of this antibiotic. Such results are in agreement with the earlier study of Rushdy *et al*.(2007) who reported 100% resistance to penicillin and oxacillin by *S. aureus* isolates from clinical hospitals in Cairo and to those results obtained in Saudi Arabia by (Hamid, 2011). Also, agreed with the data published by Khan *et al*.(2007) who recorded (40%) resistance to the penicillin among their tested isolates.

The disc diffusion method currently recommended by the CLSI in (2011) for phenotypic detection of methicillin resistance in all staphylococci is the cefoxitin disc method. Cefoxitin which is a type of the cephamycin a subclass of *β*- lactams antibiotic, represents a surrogate for the accurate detection of *mecA*-mediated resistance to oxacillin and other (PSPs), i.e., methicillin and cloxacillin. Results of the study show that (65.6%) of the *S. aureus* isolates were found to be resistant to cefoxitin which also represents methicillin resistant among these isolates. Other investigators confirm similar findings whene cefoxitin DD was considered as a superior method to detect *mecA* mediated oxacillin- resistance (Baddour and Abu Elkheir, 2007; Tiwari *et al*., 2009; Anand *et al*., 2009).

On the other hand, vancomycin was found in this study as the most active antibiotic when (93.4%) of the tested isolates were sensitive to it. Moussa and Shibl(2009) and Al-Ruaily and Khalil (2011) obtained similar results when they reported that 100% of the tested clinical *S. aureus* isolates were sensitive to vancomycin as well as El-Azizi *et al*. (2005) showed that vancomycin and quinupristin/ dalfopristin were capable of killing 99.9% of MRSA. While the published data by Tiwari in (2007) show that, the emergence of vancomycin resistant strains in hospital environment may occur faster than expected which was not in agreement with the obtained results during this research. An

explanation for the results obtained in this regard may be due to that the vancomycin is represening the terminal drug of choice against MRSA/ORSA infections worldwide is rare in use in the Iraqi clinical centers and explain why locally isolated *S. aureus* strains during this research developed no resistance to vancomycin Al-Hamdani and Hamad (2012).

Maximum percentage (93.4%) of resistance was observed by the *S. aureus* isolates against Ticarcillin-clavulanic acid. This may be due to the effect of the clavulanic acid (β- lactamase inhibitors) that does not have antibacterial activity. Instead, they bind and inactivate β- lactamases, thereby protecting the antibiotics that are normally substrates for these enzymes.

High level of *S. aureus* sensitivity to aminoglycosides representing by gentamycin, tobramycin and amikacin antibiotics was found Their resistance percentage is (18%) for gentamycin and amikacin, while it is (9.8%) for tobramycin and that appear to agree with the results obtained by (Jacqueline *et al*., 2004) who indicated that despite aminoglycosides resistance among clinical MRSA isolates being widespread but gentamycin remains active against most MRSA strains. Moreover, khan *et al*. in (2007) reported no effect for amikacin within *S. aureus* isolates from hospital personal.

The isolates expressed high resistance against the first and fourth generation of cephalosporins represented by (55.7%) for cephalothin and (68.9%) for cefepime. This indicates that they are not being misused or commonly prescribed.

Nearly, all except (3.3%) the isolates were susceptible to imipenem. Ciprofloxacillin, the only floroquinolone used, shows relative efficacy that only (14.8%) of the tested isolates develop resistance against this type of antibiotic. This result closely agree with that observed by Naji (2008) khan*et al*.(2007) and with the findings of Al-Ruaily and Khalil in (2011), as they record ciprofloxacillin as the least active antibiotic during their work.

Chloramphenicol as a type of non β- lactams antibiotic appeared to be of high activity against the clinical isolates whene only (13.1%) of them were resistance to it. This came in accordance with the results of Daini and Akano, (2009) who reported that out of 28 tested *S. aureus* isolates from nose samples, only 5 isolates were found to exhibit resistance to chloramphenicol.

Among used aminoglycoside antibiotics, erythromycins that act by inhibition of protein synthesis showed relative activity against the tested *S. aureus* isolates in such a way that (45.9%) of them were sensitive. Al-Hamdani and Hamad in (2012) reported the development of moderate susceptibility to erythromycin among the tested *S. aureus* isolates. For tetracycllin, the percentage of the resistant was (27.9%), which are in accordance with that of Lobo-Sanchez *et al.*(2011) who recorded resistance of (24%) among *S. aureus* strains isolated from hospitalized patients, while Abdul-Barei and Jabar (2011) recorded (50%) sensitivity among *S. aureus* isolates towards tetracycllin.

Seventy seven percent of the total number of isolates was found to exhibit multiple resistances as determined by the resistance of such isolates to more than three types of antibiotics (table 3-1). This was in accordance with the findings of Chakraborty *et al*. (2011) who detected high level of resistance among clinically isolated *S. aureus* strains to β-lactam antibiotics, quinolones aminoglycosides, macrolides, tetracycline and chloramphenicol. Which may be due to different mechanisms of inhibition exhibited by these classes of antibiotics against pathogenic *S. aureus* isolates as a result of the antibiotic's selective exposure.

Table (3-1): Multiple antibiotic resistances of oxacillin resistant *S. aureus* isolated from nosocomial infections.

No.of antibiotic	Resistant isolates	
3	Number	Percentage (%)
4	2	3.3
5	3	4.9
6	7	11.5
7	7	11.5
8	8	13.1
9	6	9.8
10	6	9.8
11	4	6.6
13	2	3.3
14	1	1.6
	1	1.6

The minimal inhibitory concentrations (MICs) of oxacillin for selected *S. aureus* isolates that show multi-resistance (n=47) were determined by using the method of microdilution broth (MHB). MIC values were defined as the lowest drug concentration where no bacterial growth was observed.

The antibiotic concentrations are ranging from (1280 µg/ml –0.25*µ*g/ml). Of the 47 screened *S. aureus* isolates, 17 of them recorded MIC values ranged between (0.25- 1.0) µg/ml each, 7 isolates with an MIC value of (2) µg/ml, 3 with (4) µg/ml each, 7 with (8) µg/ml each, while only one of the tested bacterial isolates had MIC value (16) µg/ml. Moreover, 5 isolates found to have an MIC value of (32) µg/ml and 6 of (64) µg/ml. Only one isolate shows complete

resistance to oxacillin at all concentration used. These results are found to be in accordance with the findings of Japoni *et al*. (2004).

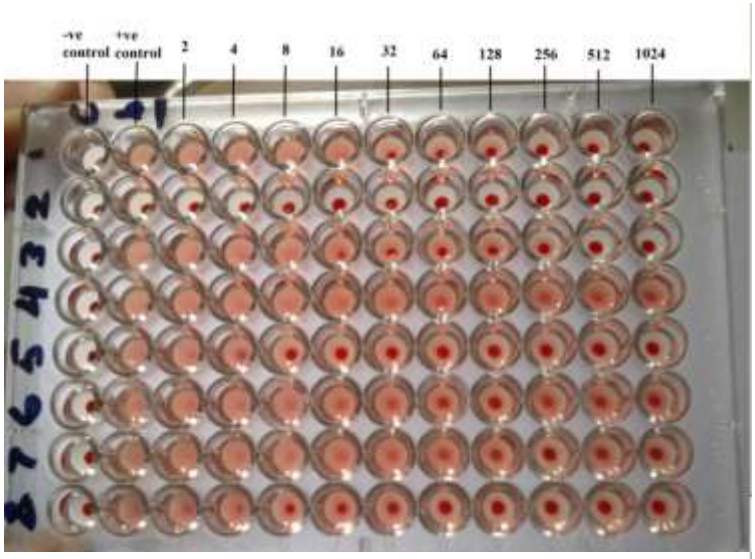
3.3 Hemolysin production by *S. aureus* isolates

Alpha- toxin is one of the key virulence factors in *S. aureus* infections, and the level of alpha-toxin expressed by a particular strain of *S. aureus* directly correlates with the virulence of the strain (Wardenburg*et al*., 2008).

During the study work, determination of hemolysin production by the *S. aureus* isolates was done by using microtiter plate method. This method was more easy and un expensive. Results showed that most of the isolates exhibited complete rabbit blood lysis, while only two of them represented by (S51 and S82) were found to produce partial blood hemolysis as shown in figure 3-2(A, B and C).

These results confirm that there is a correlation between alpha-hemolysin production and *S. aureus* pathogenesis; which appeared clearly in the high resistant isolates. Beta-lacta m antibiotics are ineffective during the stationary phase of bacterial growth—or, conversely, they are only effective in rapidly growing organisms

A:



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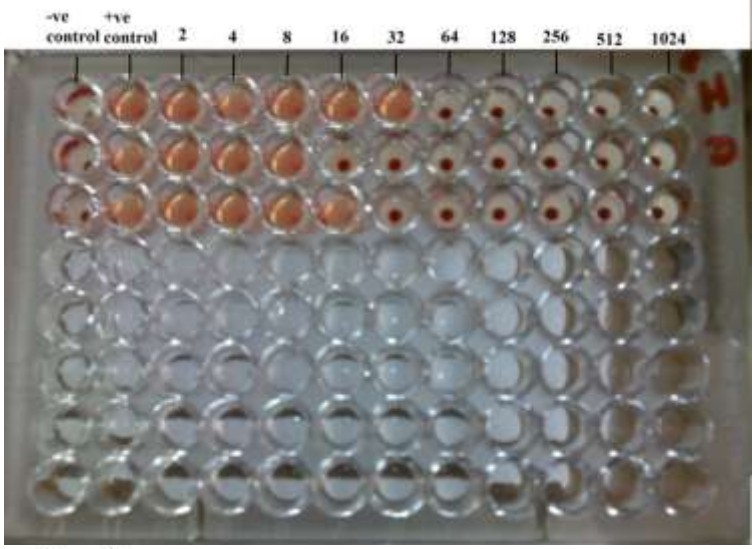


C:

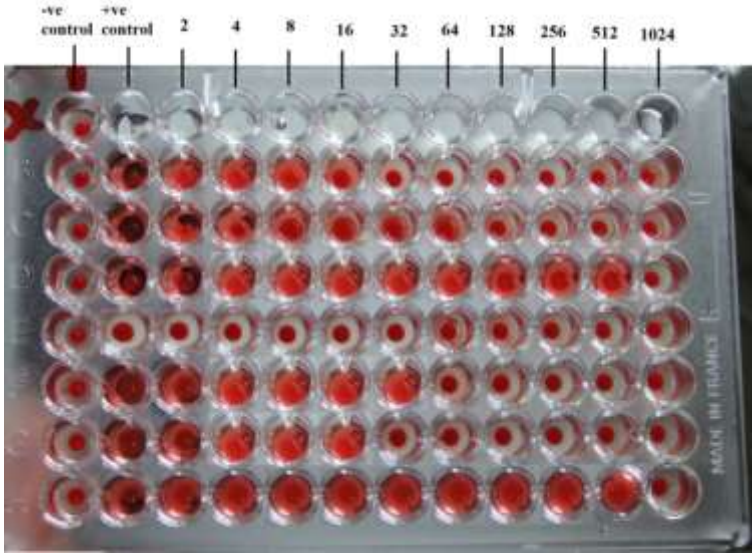


Figure (3-2) A, B and C: Hemolysis of rabbit RBCs by microtiter plate method.

Wells of first and second columns represent negative control (PBS+RBCs) and positive control (RBCs+ bacterial sample).

3.4 Molecular identification of *S. aureus* specific gene:

The selected isolates that were previously identified by morphological and biochemical characteristics in addition to the VITEK 2 System as *S. aureus* and tested for antibiotic susceptibility, were further characterized by amplifying a conserved region of the thermostable nuclease (TNase) - encoding *nuc* gene using gene-specific primers defined in the methodology chapter for the confirmation up to the species level.

The *nuc* primer set recognized all staphylococci belonged to S. *aureus*, but not other bacteria tested.

All the selected isolates (n=23) expressed *S. aureus* specific sequence genes in their PCR products which confirmed the assumption that all the isolates are *S. aureus* and as shown in figure 3-3(A, B and C).

The amplified PCR product appears as a single DNA band with a molecular size of about (300bp) when compared with the size marker 100 bp. Ladder. This result is slightly close to the previous published result by Rushdy *et al*. (2007) who detected a (276 bp) DNA fragment in all tested *S. aureus* isolates. Also it came to be in accordance with the finding of Brakstad (1992) who observed that the obtained quantity of PCR product varied with the number of *S. aureus* (CFU) isolated from the specimen in such a way that the *nuc* PCR detected < 20 viable *S. aureus* cells which ensure the high specificity and sensitivity of this test Brakstad *et al*. (1992).

Results of the study also confirm the efficacy of the classical biochemical and morphological methods for identification of the studied isolates to the species level that was earlier mentioned by Chakraborty *et al*. (2011) as well as Al-Ruaili and Khalil (2011).

Published data indicate that treatment with antibiotics does not interfere with the detection of the *nuc* gene as long as minimum quantities of the target DNAsequences are still present in the clinical specimens.

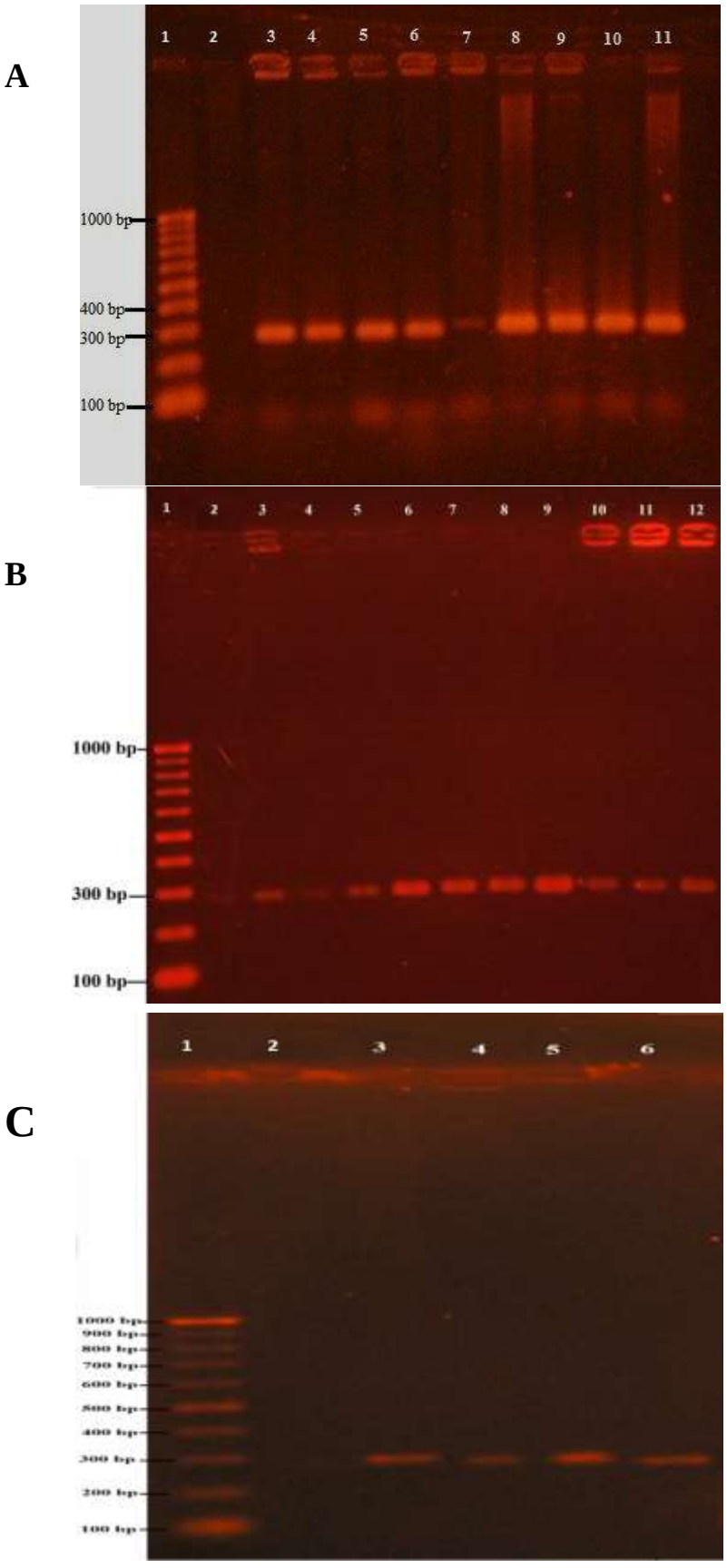


Figure (3-3) A, B and C: Agarose gel electrophoresis of PCR reaction for *nuc* gene. Band were fractionated by electrophoresis on 1% agarose gell for 1.5 hr, 5 v/cm, Tris borate buffer and visualized under U.V. light after staining with ethidium bromide.

A, B and C; Lane1:100 bp. DNA ladder.

A, B and C; Lane 2: negative control.

A; Lane 3-11 PCR product for *S. aureus* specific *nuc* gene of nine isolates arranged as (S5, S10, S12, S14, S21, S34, S35, S41 and S45).

B; Lane 3-12: PCR products for *S. aureus* specific *nuc* gene of ten isolates arranged (S76, S79, S81, S82, S86, S87, S88, S90, S100, and S102).

C; Lane 3-6 PCR product for *S. aureus* specific *nuc* gene of four isolates (S50, S51, S56 and S67).

3.5 Plasmid profile of the *S. aureus* isolates:

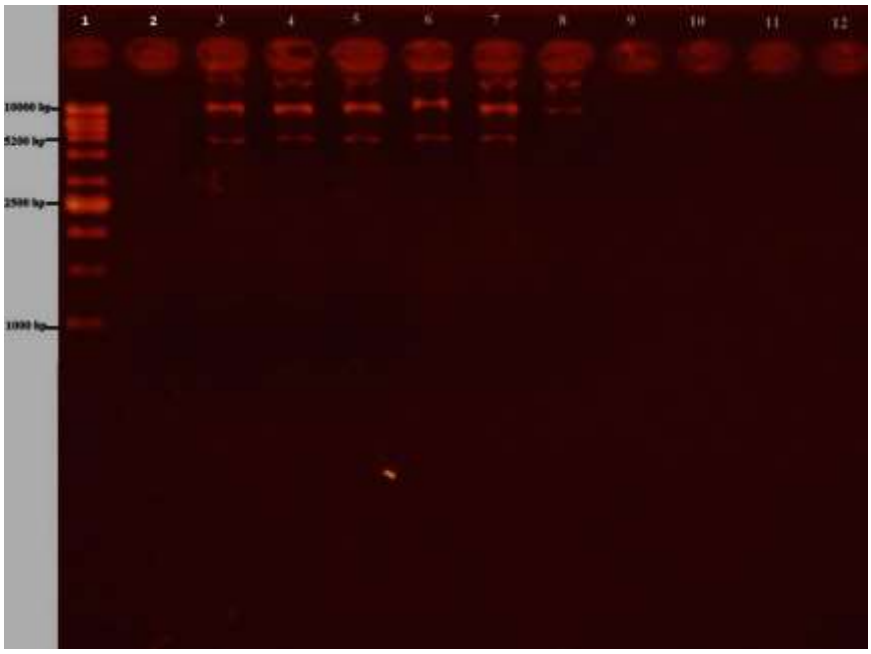
Plasmids profiles are often used as epidemiological markers for nosocomial outbreaks of *S. aureus* (Schaberg *et al*., 1985). It is the first molecular technique used for investigation of MRSA as it differentiates isolates according to the number and size of plasmids, measured by electrophoresis.

Results of this study detected that the DNA bands were matched with those for 1000 bp. ladder marker in the range (5000- 10000) bp. The approximate molecular size of each plasmid is consequently obtained by comparing with the molecular size of the marker.

A total number of 23 isolates of multiple resistant *S. aureus* were screened for plasmids and 11 isolates of them produced one or two bands representing their plasmid content. These isolates show plasmid patterns as illustrated in figure (3-4 A); in such a way that they appear to produce two bands, one at (10000) bp. and the second band at (5200) bp except for the isolate S45that produces one band at (10000) bp.

Each one of these ORSA isolates resists a number of antibiotic that reached 14 type out of 15 for the (S35) isolate and include resistant to tetracycline, chloramphenicol, gentamycin, erythromycin, amikacin and vancomycin for isolates (S10 and S45). This might determine the location of genetic determinants for antibiotic resistance in these isolates.

A:



B:

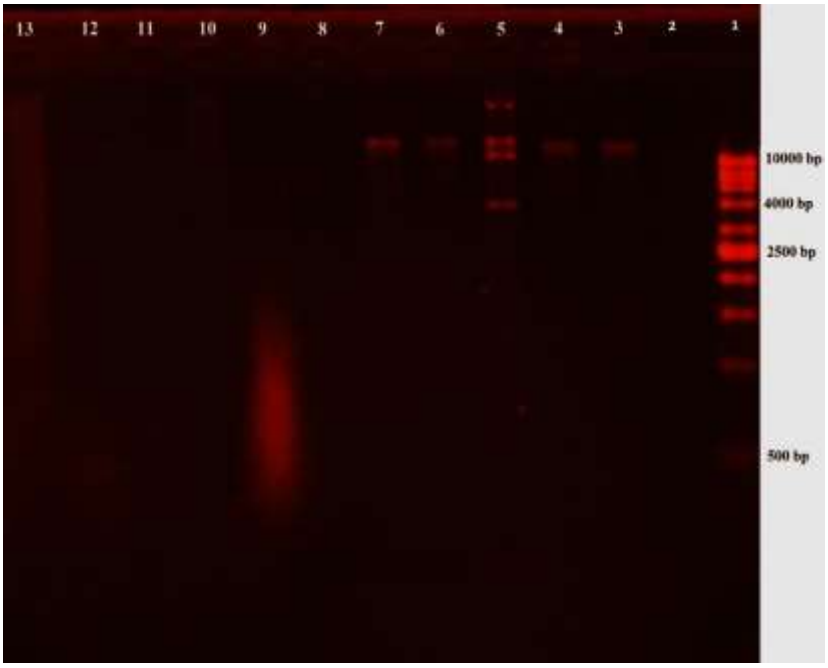


Figure (3-4) A and B: Agarose electrophoresis for plasmid profile of *S. aureus* from clinical samples. Band were fractionated by isolated electrophoresis on 1% agarose gell for 2 hr, 5 v/cm, Tris borate buffer and visualized under U.V. light after staining with ethidium bromide.

A and B; Lane 1: DNA marker, Lane 2: Negative control.
A; Lane (3-8): the *S. aureus* isolates (S5, S10, S14, S21, S35 and S45).
B; Lane (3, 4, 5, 6 and 7): *S. aureus* isolates (S51, S56, S67, S76 and S79).

So closed findings were obtained by Daini and Akano (2009) whene they recorded that 16 out of 34 *S. aureus* isolates harbored plasmids were found to be related to their antibiotic resistance with a molecular size ranging from (0.28 to 25.12) kb.

In figure (3-4 B), only five isolates are found to have plasmids, the results appeared as a band with a molecular size greater than (10000)bp. except the isolate (S67) that produced three bands; the first band appeared more than the marker size, the second is at (10000) bp. while the third band at (4000) bp.

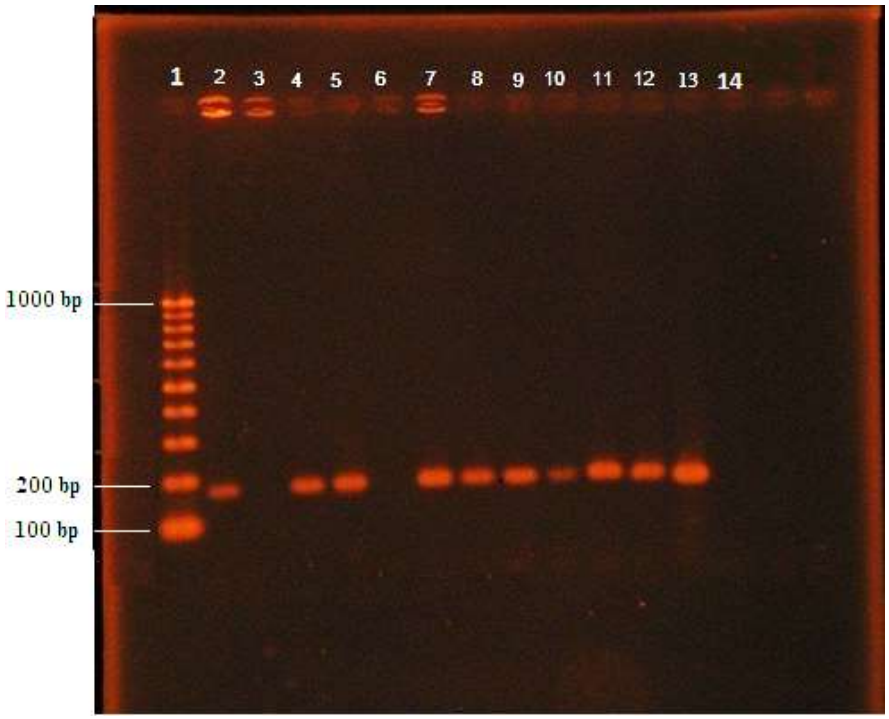
Plasmids are not detected in 12 of the resistant isolates which indicate that their antibiotic resistance is located on the chromosome. This may suggest a clear potential for inter species spread of antibiotic resistance.

3.6 Detection of *mecA* gene by PCR:

ORSA as well as MRSA isolates cause high rates of morbidity and mortality in hospitals in many geographical areas (Griffiths *et al*., 2004). The correct identification of ORSA/ MRSA using conventional methods is complicated because different populations of staphylococci express different levels of resistance. The susceptibility testing are highly dependent on the experimental conditions and often more than one method would need to be performed for obtaining an accurate susceptibility profile. Therefore, PCR-based identification is used in this study for accurate detection of *mecA* gene, which mediates oxacillin resistance in staphylococci using the primers defined in methodology.

When all the *nuc* positive isolates (n=23) that were characterized as ORSA by the (oxacillin DD and MIC test) were subjected to PCR to detect the presence of *mecA* gene, 19 out of the 23 oxacillin- resistant *S. aureus* isolates were obtained as shown in figures 3-5 (A and B).

A:



B:

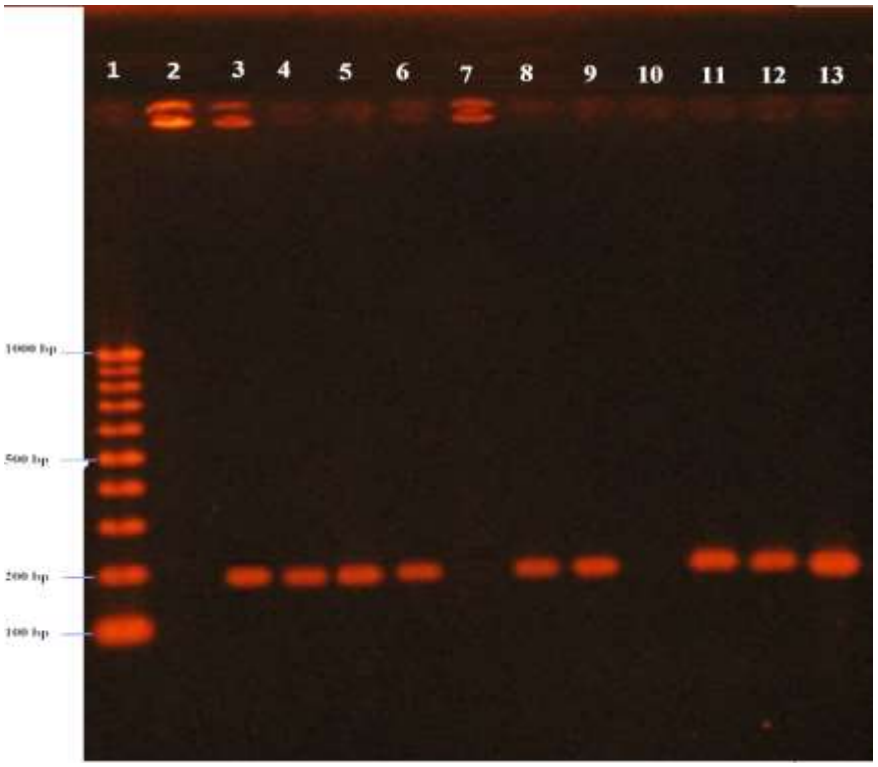


Figure (3-5) Agarose gel electrophoresis of PCR reaction for *mecA* gene. Band were fractionated by electrophoresis on 1% agarose gell for 1.5 hr, 5 v/cm, Tris borate buffer and visualized under U.V. light after staining with ethidium bromide.

Figure A: Lane 1 (100 bp.) DNA ladder
Lane (2-13): PCR product of twelve suspected MRSA isolates from clinical samples (S5, S10-ve, S12, S14, S21-ve, S34, S35, S41, S45, S50, S51 and S56).
Lane 14: negative control (methicillin susceptible *S. aureus*).
Figure B: Lane 1: (100 bp) DNA ladder.
Lane 2: Negative control (methicillin susceptible *S. aureus*).
Lane (3-13): PCR product of eleven suspected MRSA isolates from clinical samples (S67, S76, S79, S81, S82-ve, S86, S87, S88-ve, S90, S100 and S102).

The PCR product appeared as a single DNA band with a (200) bp. in size that was close to that obtained by Martineau *et al*. (2000); Motlagh and Anvari (2010).

By comparing the performance of these PCR assays for *mecA*$^{+}$ isolates with those of classical methods of susceptibility testing and discrepant confirmed the observation between oxacillin and cefoxitin when the disc diffusion method was used.

A correlation between phenotypic and genotypic method of 100% for cefoxitin resistance is detected. These results confirm the previous findings of Boubaker *et al.* (2004) and Urbaskova *et al*. (2004) who founed that cefoxitin (DD) tests was correlated better with the presence of *mecA* than with the results of (DD) tests using oxacillin. Anand *et al*.(2009) demonstrates that sensitivity and specificity of the cefoxitin (DD) test was (100%), and that the results of cefoxitin (DD) is in a accordance with the PCR for *mecA* gene considering cefoxitin which is a more potent inducer of the *mecA* regulatory system than are the penicillins.

The four *mecA*-negative tested isolates were not detected by the cefoxitin disc, but detected by the oxacilin disc. Thus, the sensitivity in cefoxitin DD test is found slightly better than that for oxacillin disc. In this regard, Ekrami *et al.* (2010) found almost closely related findings, while Louie *et al*. (2000) found

that differentiation between MRSA and BORSA strains was very difficult by using phenotypic tests.

The cefoxitin test is the preferred method for testing *S. aureus*, *S. lugdunensis* and coagulase negative staphylococci for resistance to penicillinase-stable pencillins. Cefoxitin is used as a surrogate for detecting oxacillin resistance and reporting oxacillin as susceptible or resistant is based on the cefoxitin result (CLSI, 2011).

Data of this work found cefoxitin as a potential alternative to *mecA* detection test which comes in agreement with the results obtained by Jain *et al*. (2008), Tiwari *et al*. (2009) and Mathews *et al*. (2010) during their work as they indicate that cefoxitin is a good surrogate marker for methicillin-resistance.

False positivity of oxacillin (DD) method during work may be due to hyper production of β-lactamase which may lead to phenotypic expression of oxacillin-resistance (borderlineoxacillin resistance). This is corroborated by the fact that all the isolates that are resistant to oxacillin but sensitive to cefoxitin are negative for *mecA* gene because they do not possess the usual genetic mechanism for such resistance.

Laboratory methods for detecting resistance of any isolates are influenced by different variables such as temperature, period of incubation, inoculum density and salt concentration used in culture medium. Therefore, accurate detection of oxacillin/methicillin resistance is difficult due to the presence of two subpopulations (one susceptible and the other resistant) that may coexist within a culture of staphylococci.

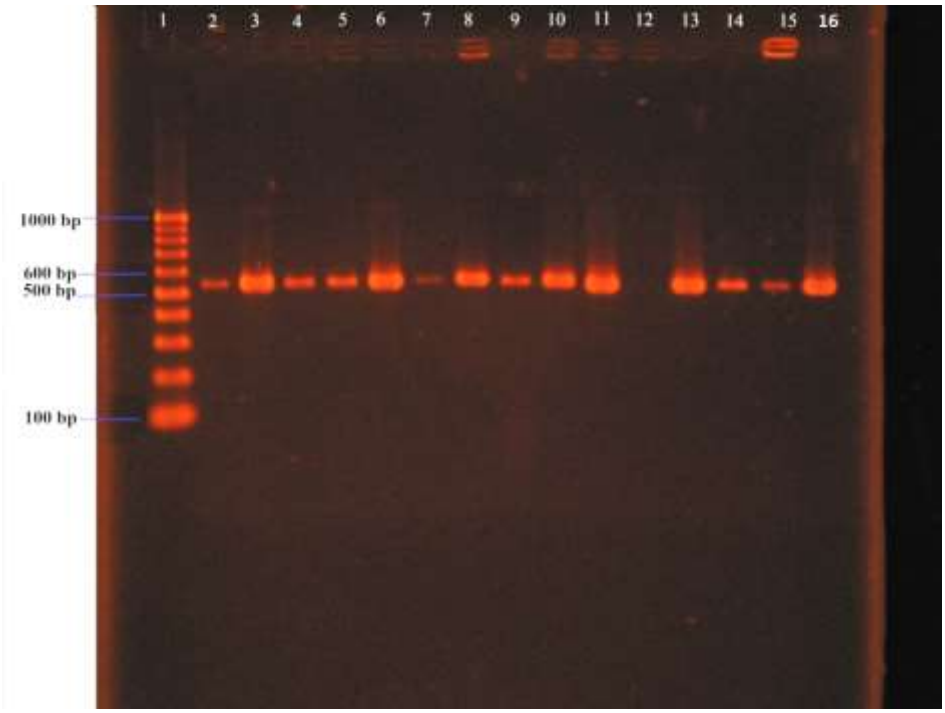
All the cells in a culture may carry the genetic information for resistance, but only a small number may express the resistance *in vitro*. This phenomenon is termed heteroresistance. Cells expressing hetero-resistance grow more slowly than the oxacillin-susceptible populations and may be missed at temperatures above 35˚C; methicillin, or nafcillin at 33-35˚C (maximum of 35˚C) for a full 24 hours before (CDC, 2005).

3.7 Detection of *hla* gene by PCR

Extracellular protein toxins contribute to the pathogenesis of a wide variety of *S. aureus* infections and α-toxin which represents the major hemolysin of *S. aureus* is a pore-forming protein which is shown to evoke the typical apoptotic alters such as oligonucleosomal DNA fragmentation. Detection for the presence of this virulence factor among the tested bacterial isolates was carried out using specific primers designated for the amplification of specific sequence within the gene. The extracted DNA from isolates that were previously grown in the presence of (4μg) of oxacillin are used as a template for the amplification of alpha hemolysin gene.

Results in figure 3-6 (A and B) revealed the presence of the α-hemolysin gene (hla^+) in 21 of the 23 isolates which represent a clear marker that local isolates are among the most pathogenic hospital isolates that in addition of being multidrug resistant; they contained one of the most important virulence factors responsible for their epidemic distribution among hospital environment. Such results came in accordance with those of Subrt *et al*. (2011) as they found that sub inhibitory concentrations of seven cell wall active antibiotics have been shown to stimulate virulence gene expression in *S. aureus*, and this may alter the progression of infection and thus rendered antimicrobial therapy unreliable. Furthermore; Ohlsen *et al.* (1998) observed that β-lactam antibiotics have proven to be unfavorable because even sub-inhibitory concentrations of methicillin (for example), led to an increase in α-toxin expression through a stimulatory effect on exoprotein synthesis, and for this reason they established that "methicillin-induced alpha-toxin expression is a common phenomenon of alpha-toxin-producing strains".

A:



B:

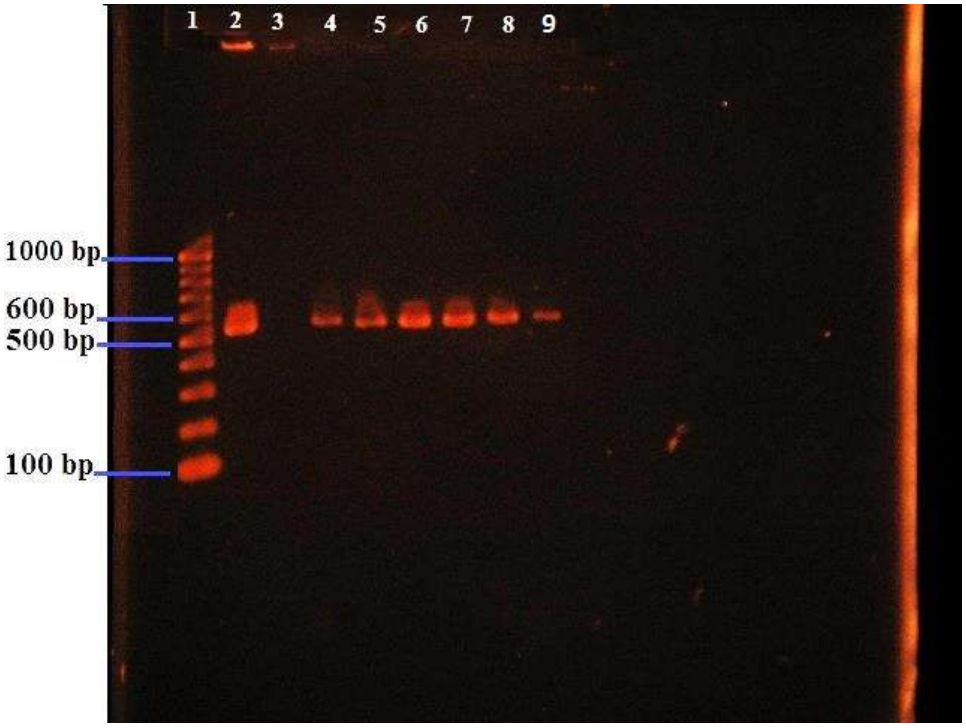


Figure (3-6): Agarose gel electrophoresis of PCR reaction for *hla gene*. Band were fractionated by electrophoresis on 1% agarose gell for 1.5 hr, 5 v/cm, Tris borate buffer and visualized under U.V. light after staining with ethidium bromide.

Figure A: Lane (1):100 bp DNA ladder.

Lane (2-16): PCR product of fifteen suspected MRSA isolates from clinical samples (S5, S10, S12, S14, S21, S34, S35, S41, S45, S50, S51-ve, S56, S67, S76 and S79).

Figure B: Lane 1: (100 bp) DNA ladder.

Lane (2-9): PCR product of eight suspected MRSA isolates from clinical samples (S81, S82-ve, S86, S87, S88, S90, S100 and S102)

Lane (10): negative control.

Parsonnet *et al*. (1994) demonstrated that, beta-lactam antibiotics may fail in infections associated with toxin-producing organisms as these cell-wall–active agents. In contrast to protein-synthesis inhibitors, they failed to suppress toxin production. They can increase alpha-hemolysin owing, in part, to lysis of the organism and release of intracellular toxin.

The amplified PCR product during the study gave clear band of about (550bp.) compared with the 100bp. DNA molecular marker.

As shown in figure 3-6 (A) only isolate (S51) was found to be negative as it didn't show any amplification for alpha- hemolysin gene. While isolate (S82) in the figure 3-6 (B), gave negative result as no band was producing with the DNA marker. These two *S. aureus* isolates were previously detected as no hemolytic activity ones during the hemolysis assay by microtiter plate.

3.8 Antibacterial activity of ZnO nanoparticles:

During the study, the antimicrobial activity of nano-ZnO suspension was tested against selected *S. aureus* isolate (S79)888 that gave positive result for the presence of ($mecA^+$) gene and (hla^+) gene in addition to their wide range of resistance to the majority of tested antibiotics. The zinc nanoparticles of (35) nm in size were used alone and in combination with oxacillin antibiotic by the disc diffusion method. The zinc nanoparticles content were used as (100) µg/disc as

described by Thati *et al*. (2010), and it had been chosen to confirm that the effect produced was due to its potential synergistic effect with the antibiotic but not to the effect of the zinc nanoparticles alone.

Presence of an inhibition zone was measured after 24 hr of incubation, and the synergetic effect of the Zinc nanoparticles and oxacillin was observed by increasing diameter of inhibition zone (mm) around the oxacillin antibiotic disc compared with that of the oxacillin alone.

Presence of a clear inhibition zone indicates that the mechanism of the biocidal action of ZnO involves disrupting the membrane.The antimicrobial ability of nano-ZnO might be referred to their small size which is 250 times smaller than that of a bacterium. This makes them easier to adhere with the cell wall of the microorganisms causing its destruction and leads to the death of the cell. Also, the high rate of generation of surface oxygen species from ZnO leads to the death of the bacteria.

As shown in figure (3-7) and table (3-2), the zinc oxide nanoparticles enhanced the antibacterial activity of oxacillin against the selected bacterial isolate of *S. aureus* in such a way that upon using the standard oxacillin antibiotic disc no inhibition zone was obtained around the disc. Furthermore, ZnO nanoparticles alone were recorded to produce an inhibition zone of (10) mm, while combination of nano- ZnO with oxacillin gave an inhibition zone of (15) mm around the (oxacillin-ZnO) disc. That result is in accordance with the findings of Thati*et al*. (2010) as they recorded an increase in the activities of two tested antibiotics that include (oxacillin and methicillin) used in the presence of nano sized zinc oxide against *S. aureus* isolate from a clinical source.

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Figure (3-7): Synergistic effect of ZnO nanoparticles (35 nm) and oxacillin against oxacillin resistant *S. aureus* isolate.

Table (3-2): Combined antibacterial effect of oxacillin and Zinc oxide nanoparticles against oxacillin-resistant *S. aureus*.

Drug concentration	**Inhibition zone diameter (mm)***
Oxacillin (1µg/disc)	NA
Zinc oxide (100 µg/disc)	10
Oxacillin (1µg/disc) + Zinc oxide (100 µg/disc)	15

* Zone of inhibition, including the diameter of the filter paper disc (6mm): each value represents the mean of two readings.
NA= no activity.

Hence, the enhanced effect of nano-ZnO upon the antibacterial activity of oxacillin is observed by the notable increase in the diameter of inhibition zone around the disc. This may be related to the synergistic effect of antibiotic-zinc nanoparticles combination.

At the concentration tested, zinc nanoparticle significantly improved antibiotic efficacy against *S. aureus.* Ansari *et al*., (2012) published a report on the antibacterial properties of ZnO nanoparticles as a promising new unconventional antibacterial agent that could be helpful to confront methicillin-resistant *S. aureus* and other drug-resistant bacteria. The results also agreed with those of Jones *et al*., (2008) who suggested that nanoparticles of ZnO gave significantly higher antibacterial effects on *S. aureus* than did other tested metal oxide nanoparticles.

3.9 Antibacterial activity of medicinal plant extract

3.9.1 Olive leaf extract and oxacillin antibiotic:

The *in vitro* antibacterial activity was expressed as the mean diameter of inhibition zones (mm) produced by the extract alone and in combination with the oxacillin. Results presented in figure (3-8) and table (3-3) show that neither oxacillin alone (1µg/disc) nor increasing concentrations of OLE to (2.25-9) mg/disc produced any inhibition zone when challenged alone with ORSA. However, when oxacillin was combined with the highest concentration of OLE (9) mg/disc, a pronounced inhibition zone (14 mm) was produced.

Several studies have demonstrated that OLE possesses potent antibacterial activities against oxacillin-resistant *S. aureus* (Pereira *et al*., 2007; Omar, 2010; Ghamberi *et al*., 2012; Keskin *et al*., 2012). This was not the picture revealed in the present study, which suggest that OLE alone lacked any antibacterial effect.

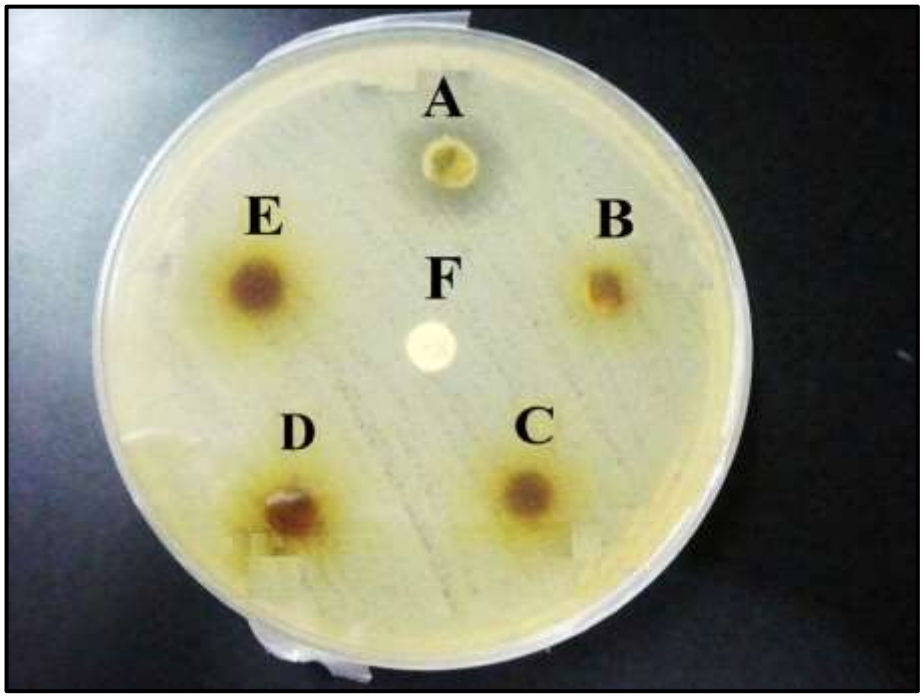

Figure (3-8): Combined antibacterial activity of olive leaves ethanolic extract with oxacillin against oxacillin resistant *S. aureus* isolate.

A: olive leaves extract (9 mg/disc) + standard oxacillin antibiotic disc.

B: olive leaves extract (2.25 mg/disc)

C: olive leaves extract (4.5 mg/disc)

D: olive leaves extract (6.75 mg/disc)

E: olive leaves extract (9 mg/disc)

F: oxacillin antibiotic disc.

Al-assadi et al. (2008) reported a similar lack of an inhibition zone for ethanolic OLE that was collected from Al-Basrah governorate in Iraq against *S. aureus*. It is not clear at present, the reasons for this discrepancy between the finding of the present study and the above-mentioned studies.

Table (3-3): Antibacterial effect of olive leaf extract (OLE) alone or in combination with oxacillin against oxacillin-resistant *S. aureus*

Drug concentration	Inhibition zone diameter (mm)*
Oxacillin (1µg/disc)	NA
Olive leaf extract (OLE) (2.25 mg/disc)	NA
OLE (4.5 mg/disc)	NA
OLE (6.75 mg/disc)	NA
OLE (9 mg/disc)	NA
Oxacillin (1µg/disc) + OLE (9 mg/disc)	14

* Zone of inhibition, including the diameter of the filter paper disc (6mm): each value represents the mean of two readings.
NA= no activity.

However, the fact that the combination of OLE and oxacillin produced an obvious inhibition zone needed to be confirmed and further studies about various concentrations of OLE are is needed also. It would be a quiet interesting novel finding if this is proved to be a fact.

No comparable report in the available literature reported that OLE can potentiate the antibacterial activity of oxacillin against ORSA *in vitro*, which is already resistant to this antibiotic. Therefore, this sporadic positive finding should not be over interpreted in the midst of the present study negative findings concerning the antibacterial potential of OLE.

Accordingly, results of this study suggested that, under the present experimental conditions, OLE exhibited controversial effects against ORSA *in vitro*. While it failed to show antibacterial activity when applied alone to the agar plate, yet it potentiated the antibacterial activity of oxacillin when used in combination against ORSA. Further studies are needed to investigate the significance of this proposed OLE- induced enhancement of the antibacterial activity of oxacillin, and the potential application of OLE in combination therapy against ORSA.

3.9.2 Olive leaves extract and nano- ZnO:

The integration of green chemistry principles to nanotechnology is one of the key issues in Nano science research to avoid adverse effects in medical applications that might resulted from the use of toxic chemicals. For this reason, the combined antibacterial activity of olive leaves ethanolic extract and ZnO nanoparticles were tested against our selected *Staph aureus* isolates and as described in figure (3-9) and table (3-4).

Results showed the appearance of an inhibition zone at all concentration tested with a maximum inhibition zone of (11) mm for ZnO nanoparticles alone. Such results produced no any evidence for an enhanced activity of the olive leaves extract in presence of nano-ZnO.

It may be concluded that the present results might be the first report to the possible utilization of nano-ZnO and plant extract against highly resistant *S. aureus* isolates in Iraq.

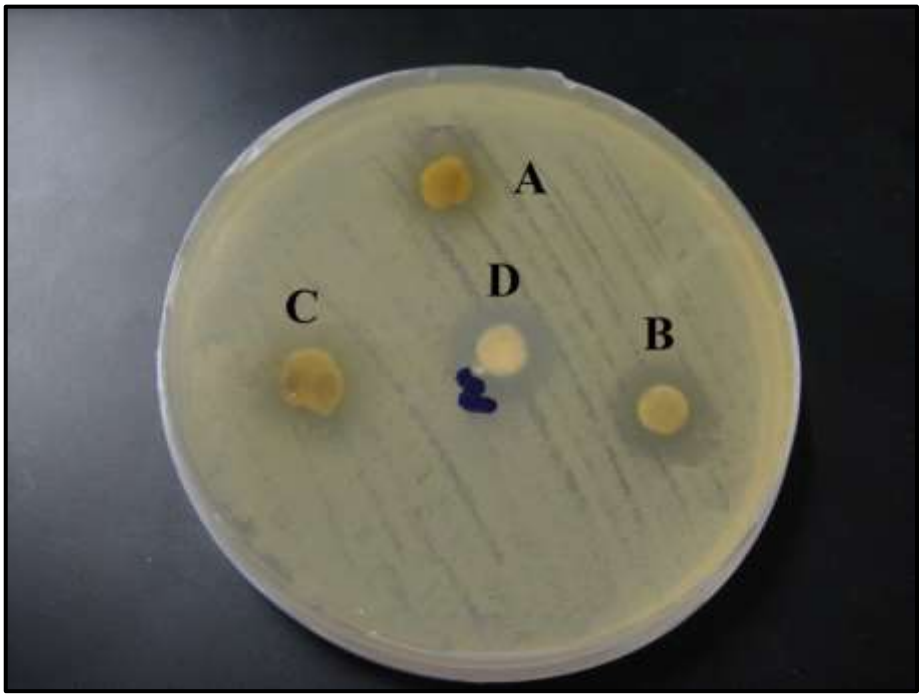

Figure (3-9): Combined antibacterial activity of olive leaves ethanolic extract with nano-ZnO (100) µg/disc against oxacillin resistant *S. aureus* isolate.

A: olive leave extract (6.75 mg/disc) + nano- ZnO (100 µg/disc)

B: olive leave extract (4.5 mg/disc) + nano- ZnO (100 µg/disc)

C: olive leave extract (2.25 mg/disc) + nano- ZnO (100 µg/disc)

D: nano- ZnO (100 µg/disc).

Table (3-4): Combined antibacterial activity of olive leaves ethanolic extract with Zinc oxide naoparticles against oxacillin-resistant *S. aureus*.

Drug concentration	Inhibition zone diameter (mm)*
OLE (2.25mg/disc) + Zinc oxide (100 µg/disc)	**8**
OLE (4.5 mg/disc)+ Zinc oxide (100 µg/disc)	**8**
OLE (6.75mg/disc) + Zinc oxide (100 µg/disc)	**10**
Zinc oxide (100 µg/disc)	**11**

* Zone of inhibition, including the diameter of the filter paper disc (6mm): each value represents the mean of two readings.

4.1 Conclusions:

- Molecular technique found to be the most sensitive method in detecting *S. aureus* at both genus and species level with 100% accuracy in detecting ORSA.
- The multidrug ORSA strains most often contained one or more plasmids.
- Cefoxitin as antibiotic disc provides more specific and sensitive test for accurate phenotypic detection of ORSA.
- The occurrence of alpha toxin gene is more frequent in those strains that resist high number of antibiotics.
- Zin oxide nanoparticles show enhanced synergistic effect when it is mixed with oxacillin against the tested isolate and provided a good antimicrobial activity when it was tested a lone.
- Crude thanolic olive leaf extract at concentrations of (2.25-9) mg/disc alone showed no antibacterial activity against ORSA *in vitro*. However, when OLE (9) mg/disc was combined with oxacillin (1)µg/disc, a marked potentiation of the antibacterial activity of oxacillin was observed against ORSA *in vitro*
- The obtained vancomycin-resistant *S. aureus* were isolated at very low frequency from the hospitals environments in Baghdad due to the rarely use of vancomycin as a therapy for staphylococcus infections by the physicians.

4.2 Recommendations:

- The replacement of the routinely used oxacillin discs (1μg) by cefoxitin (30μg) durig the susceptibility test method for staphylococci which provide easier-to-read test and result in an equivalent detection (sensitivity and specificity) of oxacillin resistance in *S. aureus*.
- As alpha-toxin plays significant role in the pathogenesis of *S. aureus* infections. It may potentially serve as an important target for the development of anti-virulence chemotherapy.
- Further investigation should be carried out to elucidate the role of ZnO nanoparticles and its mechanism in enhancing antibiotics activity.
- Additional studies on the safety/toxicity properties of ZnO nanoparticles to detect their effects on eukaryotic cells are needed.
- Studying the evaluation of exact drug-plant ratio to obtain maximum interaction between plant extract and the antimicrobial drug.
- Studying the antibacterial effect of various active constituents of olive leaf extracts (both water and ethanolic).
- Using more advanced accurate methods for the diagnosis of ORSA in Iraqi hospitals specially PCR technique to differentiate between antibiotic sensitive and resistant strains that are independent on physical and chemical conditions.

Table (1-1): Mobile genetic elements (MGEs) identified in Staphylococcus aureus (Malachowa and Deleo, 2010)

MGE	Description	Examples	Notes
Bacteriophages encoding toxins	Lytic: complete bacterial lysis Temperate: long-term relationship with cells Chronic: release progeny without killing the host	Staphylococcal enterotoxin A (SEA), PVL (luk-PV) Staphylococcal complement inhibitor (SCIN), chemotaxis inhibitory protein (CHIP) staphylokinase	Widespread in *Staph aureus* 1–4 per strain Appear to have the greatest impact on *Staph aureus* diversity and evolution
Pathogenicity and composite islands (SaPIs)	Related to phages but lack genes for constructing capsid heads and tails necessary for horizontal transfer	Encode TSST, superantigens (SEB, SEC), MDR transporters, fusidic acid resistance genes	14–17 kb in size 0–2 per strain ≥16 sequenced
Plasmids and transposons	SCCmec are large pieces of DNA that insert in the orfX gene in *Staph aureus*. Sau1 type 1 RM systems	SCCmec types I–XI	SCCs encode the meticillin resistance gene mecA. Can encode antibiotic resistance and/or virulence determinants
Staphylococcal cassette chromosome mec (SCCmec)	Not limited to encoding methicillin resistance	SCC mercury	Encode resistance to mercury chloride
SCC (non-mec)	Not limited to encoding methicillin resistance		Flanked by a broken transposase gene upstream, and a partial type 1 RM system downstream
Genomic islands		Three families: vSA_, vSA_, vSA_. Responsible for phenol-soluble modulins (PSMs), possible pro-inflammatory activity, enterotoxins and bacteriocin production	Flanked by a broken transposase gene upstream, and a partial type 1 RM system downstream

Table (3-1): Susceptibility of *Staph aureus* isolates to 15 differen antibiotics.

Antibiotic	Resistans	%	Intermedate	%	Sensitive	%
Oxacillin	47	77.1	1	1.6	11	18
Penicillin	48	78.7	1	1.6	12	19.7
Vancomycin	4	6.6	9	14.8	48	78.7
Tobramycin	6	9.8	0	0.00	55	90.2
Gentamycin	11	18	7	11.5	43	70.5
Ticarcillin-clavulanic acid	57	93.4	0	0.00	4	6.6
Chloramphenicl	8	13.1	8	14.8	45	73.8
Cephalothin	34	55.7	9	14.8	18	29.5
Cefepime	42	68.9	3	6.6	16	26.2
Erythromycin	29	47.5	4	6.6	28	45.9
Cefoxitin	40	65.6	3	4.9	18	29.5
Imipenem	2	3.3	0	0.00	59	96.7
Tetracycline	17	27.9	4	6.6	40	65.6
Amikacin	11	18.0	2	3.3	48	78.7
Ciprofloxacin	9	14.8	1	1.6	51	83.6

جمعت (٢٠٦) عينة طبية من اربع مستشفيات في بغداد للفترة من شباط وحتى تشرين الثاني عام ٢٠١٠، أخذت العينات من المرضى الوافدين لهذه المستشفيات بالأضافة الى عدد من العاملين الاصحاء في هذه المستشفيات ، تضمنت العينات مسحات من التجويف الداخلي للأنف ومن اليد (وخاصة من ايدي العاملين في المستشفى) ومن الأذن.

أظهرت نتيجة التشخيص ان (١٥٤) عينة أعطت نتيجة موجبة للزرع البكتيري كان من بينها (٦١)عزلة شخصت بأنها بكتريا المكورات العنقودية الذهبية بأستخدام طرق التشخيص الزرعية والكيموحيوية كما وتم أختبار الحساسية الدوائية لهذه العزلات اتجاه (١٥ نوع من المضادات الحياتية) شملت (مضادات مجموعة البيتا لاكتام ، الكوينولينات، الامينوكلايكوسيدات، الماكرولايدس، التتراسايكلينات والكلورامفينكولات) التي تعمل على قتل أو تثبيط النمو البكتري بآاليات مختلفة. أظهرت النتائج ان (٧٧%) من المجموع الكلي للعزلات يمتلك صفة المقاومة المتعددة من خلال مقاومتها لثلاثة أو أكثر من المضادات مع نسبة عالية من المقاومة لمضادات Ticarcillin-clavulanic acid, penicillin, oxacillin, cefepime, cefoxitin, cephalothin and erythromycin وبنسبة مئوية مختلفة بلغت (٩٣.٤%، ٧٨.٧%، ٧٧.١%، ٦٨.٩%، ٦٥.٦%، ٥٥.٧%، ٤٧.٥%) ،على التوالي، تلتها مقاومة متوسطة لمضادات gentamycin, tetracyclin, amikacin وبنسبة(١١، ١٧ و ١١) ثم ciprofloxacillin و chloramphenicol و من جهة أخرى فقد أظهرت العزلات قيد الدراسة درجة عالية من الحساسية الدوائية لمضادات vancomycin, tobramycin و imipenem.

تم انتخاب العزلات التي أظهرت مقاومة لمضاد oxacillin ولعدد من المضادات الأخرى لغرض اجراء تجربة التركيز المثبط الأدنى للنمو البكتيري **(MIC)** تجاه مضاد oxacillin وبأستخدام تراكيز تراوحت بين (١٢٨٠ - ٠.٢٥) مايكروغرام /مل وكمحصلة تم اختيار ٢٣ عزلة والتي كانت قيمة التركيز المثبط الأدنى لها (٤≥) لأختبار مقايسة الحالة الدموية بأستخدام كريات الدم الحمر من الأرانب للكشف عن قابلية هذه العزلات على انتاج سم ألفا- هيمولايسين، جميع العزلات بأستثناء عزلتين كانت منتجة للسم بنسب متفاوتة.

أجري تشخيص تأكيدي للعزلات بأستخدام تقنية تفاعل سلسلة البلمرة لمعرفة عائديتها الى نفس النوع من البكتريا عن طريق تضخيم جين ***(nuc)***، وأظهرت جميع العزلات نتيجة موجبة بوجود الجين عند (٣٠٠ زوج قاعدة) في وسط الهلام.

لدى تحليل المحتوى البلازميدي لهذه العزلات وجد انه (١١) من هذه العزلات يحتوي على بلازميد و (٥) منها أحتوت على بلازميدين أحدهما يبلغ (٢٥٠٠) زوج قاعدة والثاني (١٠,٠٠٠) زوج قاعدة فيما ظهر أن (٥) منها احتوت بلازميد حجمه اكثر من (١٠,٠٠٠) زوج قاعدة فيما أظهرت عزلة واحدة فقط ثلاثة أنواع من البلازميدات وبأحجام بلغت (١٠,٠٠٠ ، ٤٠٠٠ وأكثر من ١٠,٠٠٠) زوج قاعدة).

أعطى الكشف عن جين المقاومة للأوكساسيلين (***mecA***$^{+}$) نتائج موجبة في(١٩) عزلة حيث ظهرت حزمة من الدنا ذات حجم (٢٠٠) زوج قاعدة ، وكانت النتائج هذه متطابقة بين الطرق المظهرية والجينية كون جميع العزلات المقاومة لمضاد الـ cefoxitin كانت موجبة لجين ***mecA*.**

أظهر الكشف عن وجود جين الألفا- هيمولايسين (***hla***) أظهر وجود (٢١) عزلة موجبة للجين من خلال وجود حزمة ذات حجم جزيئي (٥٥٠) زوج قاعدة، وبهذا فقد كانت النتيجة هذه مؤكدة لما سجل في أختبار مقايسة الحالة الدموية.

تمت دراسة التأثير المحفز لجزيئات اوكسيد الزنك النانوية على الفعالية المضادة للبكتريا لمضاد الأوكساسيلين،و تم تحديد التأثير على عزلة محددة ومنتخبة . وحدد التاثير الأيجابي لجزيئات اوكسيد الزنك النانوية اعتماداً على الفعالية المضادة للبكتريا مقارنة بمضاد الأوكساسلين دون اية أضافة من خلال زيادة قطر هالة التثبيط والتي بلغت (١٥) ملم للفعالية المشتركة مابين خليط المضاد الحيوي والجزيئات النانوية بالمقارنة مع فعالية الجزيئات النانوية دون أضافة (١٠)ملم فيما لم يعطي استخدام المضاد الحيوي بدون اضافة اي نتيجة موجبة. يمكن أعتبار هذه الدراسة الأولى من نوعها في العراق في مجال دراسة امكانية استخدام الجزيئات النانوية مع المضاد الحيوي للحد من تطور مقاومة البكتريا للمضادات الحيوية في الوقت الحاضر.

قدرت الفعالية المضادة للبكتريا للمستخلص الخام لأوراق الزيتون بدون اية اضافة و التأثير التأزري للمستخلص مع مضاد الأوكساسيلين أوجزيئات أوكسيد الزنك النانوية بأستخدام نفس العينة قيد الدراسة والتي تمثل عزلة بكتريا المكورات العنقودية المضادة للأوكساسيلين وبأستخدام طريقة التثبيط بالأقراص. أظهرت النتائج ان مستخلص اوراق الزيتون وبالتراكيز المستخدمة (٢٥.٢-٩)ملغم/ قرص لم يعطي اي تأثير مثبط على البكتريا عندما استخدم بدون اي اضافة ولم يكن له أي تأثير محفز للتأثير المضاد البكتيري لجزيئات الزنك النانوية (١٠٠)مايكروغرام/قرص عندما استخدم كمزيج. بالرغم من ذلك فأن الفعالية ضد المايكروبية لمضاد الأوكساسيلين ازدادت، وبالأعتماد على قطر منطقة التثبيط من صفر الى (١٤) ملم عندما قيست بوجود التركيز العالي(٩)مايكروغرام/قرص لمستخلص أوراق الزيتون مما يعد تأثير محفز لفعالية المضاد ضد بكتريا المكورات العنقودية الذهبية المقاومة للأوكساسيلين. اذا ظهرت لنا مثل هذه النتائج الغير متوقعة لأستخدام تراكيز مختلفة لمستخلص أوراق الزيتون في دراسات اخرى فمن الممكن اعتبار ان هذه الدراسة بداية لدراسة التأثر التأزري لمستخلص أوراق الزيتون مع العلاج المستخدم ضد بكتريا المكورات العنقودية المقاومة لعقار الوكساسيلين.

بسم الله الرحمٰنْ الرحيْم

﴿فأما الزبد فيذهب جفاءً وأما ماينفع الناس فيمكث في الأرض﴾

صدق الله العظيم

سورة الرعد: ﴿الآية ١٤

الأهداء

إلى من غرس في روحي حب العلم وبذل الكثير لأحقق ماوصلت اليه اليوم..

ومضى دون أن اوفيه حقه... مثلي الأعلى

والدي الحبيب.. رحمه الله

إلى منبع الحنان والطيبة .. نهر العطاء الذي لاينضب.....

الى التي تحملت الكثير من أجلي.....نور عيني

والدتي الحبيبة.. حفظها الله

إلى من قاسمتني حب أبي وأمي...سندي في الحياة التي لم تبخل علي بما تستطيع....

أختي الغالية

إلى من شجعني لأكمل الطريق وكان عوناً لي في كل أوقاتي..الى الأمل الذي احيا به ومن أجله...

زوجي الحبيب

إلى مصدر سعادتي وغاية أملي...من أعيش من أجلهم..فلذات كبدي...أطفالي

فرح و محمد

ظفر

وزارة التعليم العالي والبحث العلمي
جمهورية العراق
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

دراسة بكتريولوجية و وراثية لبكتريا *Staphylococcus aureus* المقاومة لمضاد الأوكساسيلين والمعزولة من بعض مستشفيات مدينة بغداد

أطروحة

مقدمة إلى مجلس كلية العلوم\ جامعة النهرين وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في **العلوم \ التقانة الأحيائية**

من قبل

ظفر نجم العكيلي

بكالوريوس تقانة أحيائية\ جامعة النهرين، ١٩٩٨

ماجستير تقانة أحيائية\ جامعة النهرين، ٢٠٠١

بأشراف

د. أياد محمد علي العبيدي
(أستاذ مساعد)

د. صالح احمد وهيب
(أستاذ)

ربيع الأول ١٤٣٤

كانون الثاني ٢٠١٣