Appendix (1)

Preparations of stock solutions of heavy metals:

1- stock solution of zinc:

$Zn(CH_3COO)_2.2H_2O$

Molarity = Weight / Molecular Weight × 1000 / Volume (ml)

0.5 = Wt. / 219.468 × 1000 / 200

Wt. = 109. 734 mg/ml

2- stock solution of cobalt:

$Co(CH_3COO)_2.4H_2O$

Molarity = Weight / Molecular Weight × 1000 / volume (ml)

0.5 = Wt. / 249.067 × 1000 / 200

Wt. = 124.534 mg/ml

3- stock solution of cadmium:

$Cd\ Cl_2$

Molarity = Weight / Molecular Weight ×1000 / Volume (ml)

0.5 = Wt. / 183.306 × 1000 / 200

Wt. = 91.653 mg/ml

4- stock solution of Mercury:

Molarity = Weight / Molecular Weight × 1000 / Volume (ml)

0.5 = Wt. / 271.5 × 1000 / 200

Wt. = 135. 75 mg/ml

Appendix (2)

Heavy metals concentrations in mg/ml and corresponding molar concentrations:

1- Zinc:

0.02 mg/ml	0.09 mM
0.04 mg/ml	0.18 mM
0.16 mg/ml	0.72 mM
0.32 mg/ml	1.45 mM
0.64 mg/ml	2.91 mM
1.28 mg/ml	5.83 mM
2.0 mg/ml	9.11 mM
2.2 mg/ml	10.02 mM

2- Cobalt:

0.02 mg/ml	0.08 mM
0.04 mg/ml	0.16 mM
0.16 mg/ml	0.64 mM
0.32 mg/ml	1.28 mM
0.64 mg/ml	2.56 mM
1.28 mg/ml	5.13 mM
2.0 mg/ml	8.02 mM
2.2 mg/ml	8.83 Mm

3- Cadmium:

0.005 mg/ml	0.02mM
0.01 mg/ml	0.05 mM
0.02 mg/ml	0.10 mM
0.04 mg/ml	0.21 mM
0.08 mg/ml	0.43 mM
0.16 mg/ml	0.87 mM
0.32 mg/ml	1.74 mM
0.64 mg/ml	3.49 mM

4- Mercury:

0.005 mg/ml	0.01 mM
0.01 mg/ml	0.03 mM
0.02 mg/ml	0.07 mM
0.04 mg/ml	0.14 mM
0.08 mg/ml	0.29 mM
0.16 mg/ml	0.58 mM
0.32 mg/ml	1.17 mM
0.64 mg/ml	2.35 mM

اسم الباحثة :- رفاه علي سمير

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عنوان الرسالة:-

A study on heavy metal and antibiotic resistance of staphylococus aureus isolated from clinical spicemeus.

دراسة المقاومة للمضادات الحياتية والمعادن الثقيلة في بكتريا *Staphylococcus aureus* المعزولة من إصابات مرضية مختلفة

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Place and Date of birth: Baghdad/ Al-Mansour/1981

Conclusions and Recommendations

4.1 Conclusions

- *S. aureus* represent a considerable pathogenic microorganism in the tested samples.
- High percent of isolated *Staph aureus* found to be resistance to Cefotaxime (a member of thired generation of cephalosporin) and that is may be due to irrational use of this important antibiotic.
- The tested isolates show a hazardous high percent of multiple resistance for antibiotics.
- The relationship between multiple antibiotic resistance and multiple heavy metal resistance indicats an environmental biohazared of heavy metal pollution in Iraq.
- May be there were two to three type of plasmids depanding on results obtained from curing experement. The genes that responsible for Resistance for some heavy metals and antibiotics may be located on the same plasmid DNA.

4.2 Recommendations

- New antibiotics should be used rationally in treatment of human infections.
- Further studies was needed to point the hazardous heavy metals pollution and its implication on the treatment of resistant pathogenic microorganism other than *S. aureus.*
- Further study for the ability for resisting antibiotics in bacterial cells treated with different concentrations of heavy metals

Introduction and Literature review

Introduction

Staphylococcus aureus has no long been recognized as a major human pathogen, its one of the most important species of family micrococaceae. Although this organism is frequently a part of the normal human microflora it can cause significant opportunistic infections under the appropriate conditions (Koneman *et al*., 1992).

Staphylococcus aureus was responsible for a wide range of infections, from mild skin infections to wound infections and bacteraemia. Although the introduction of antibiotics over the last 50 yr has lowered the mortality rate from *S. aureus* infections, the bacteria have developed resistance mechanisms to all antimicrobial agents that have been produced. (Hardy *et al.*, 2004).

S. aureus have Complex cell wall contain polymers of chains of polysuccharid cross linked with short peptides called (peptidoglycan), and units of phosphohydroxy alcohol which is hydroxyl group exchange with succarid and the amino acid (alanine) this unit calls (Teichoic acid) (Dziarski,1981; Bhattacherjee, 1988).

Lysostaphin protease is glycyl-glycin endopeptidase, which cleaves the pentaglycin cross-bridge of the staphylococcal peptidoglycan. Naturally, *S. aureus* cell walls do not hydrolyze by lysozyme. This criteria make the studies of the *S. aureus* some how difficult because lysostaphin is very expensive in comparison to lysozyme (Etienne *et al.*, 1998).

Some strains of *S. aureus* express many potential virulence factors that are lack in other strains. *S. aureus* infections can be treated with commonly used antibiotics (Todar, 2005). In recent years some strains of *S. aureus* have become

resistant to some antibiotics which means that it is not killed by antibiotics that's take great attention (CHIQ ,2005).

Heavy metals consist of a group of about 40 elements (Gadd and Griffitt, 1978). Many are essential for growth of both prokaryotic and eukaryotic organisms, and therefore are required low concentration. However, some metals like arsenic, mercury and cadmium, are not essential for growth and extremely toxic even at low concentration (Silver and Misra, 1984).

The trace heavy metals such as Cobalt, Zinc, Copper and Nickel play important roles in bacteria; they regulate a wide array of metabolic function as coenzyme or cofactors, as catalysts or acid in the enzymes and as structural stabilizer of enzymes and DNA binding protein (Hugher and Pool, 1991; Nies and Brown, 1997).

Understanding of metal resistance in Staphylococci has progressed rapidly in the last years with well-established cadmium, mercury, antimony and arsenic resistance system encoded by plasmids (Silver and Phung, 1996).

Little is known about transport of the resistance to zinc and cobalt (chromosomal encoded) ions in *S. aureus* (Xiong and Jayaswal, 1998).

Aims of study

1- Isolation and characterization of *S. aureus* taken from clinical specimens.

2- Study the profile of antibiotic resistance and tolerance to some heavy metals linked with antibiotic resistance.

3- Making a curing experement to demonstrate the relationship of antibiotic resistance and heavy metal tolerance with any cured plasmid could harboring such traits.

1.2 Staphylococcus

Clinically, the most important genus of the Micrococcaceae family is *Staphylococcus*. The *Staphylococcus* genus classified into two major groups: *aureus* and non-*aureus*. It can be distinguished from other species of *Staphylococcus* by a positive result in a coagulase test (all other species are negative).

The pathogenic effects of *Staphylococcus* are mainly asssociated with the toxins it produces. Most of these toxins are produced in the stationary phase of the bacterial growth curve.

Particularly, *S. aureus* has been found to be the causative agent in such ailments as pneumonia, meningitis, boils, arthritis, and osteomyelitis (chronic bone infection). Most *S. aureus* are penicillin resistant, but vancomycin and nafcillin are known to be effective against most strains (Ryan and Ray, 2004).

Of the non-aureus species, *S. epidermis* is the most clinically significant. This bacterium is an opportunistic pathogen which is a normal resident of human skin. Those susceptible to infection by the bacterium are Intra Vinous drug users, newborns, elderly, and those using catheters or other artificial appliances. Infection is easily treatable with vancomycin or rifampin (Houston Medical school, 1995).

1.2.1 *Staphylococcus aureus*

S. aureus is ubiquitous and can be a part of human flora found in the axillae, the inguinal and perineal areas, and the anterior nacres (Tolan, 2006). *Staphylococcus* (in Greek *staphyle* means *bunch of grapes* and *coccos* means granule) is a genus of Gram-positive bacteria. Under the microscope, they appear round (cocci), and form in grape-like clusters. (Ryan and Ray, 2004).

1.2.2 Characterization of *Staphylococcus aureus*

Microscopically, *S. aureus* is a gram-positive organism, cocci with a diameter of 0.5 to 1.7 µm. macroscopically; rapid growth on blood agar and other nonselective solid media characterize *S. aureus*. Individual colonies are sharply defined, smooth, opaque, and convex, translucent, with a diameter of 1 to 3 mm within 24 hours; they are β-hemolytic. The classic cream-yellow to golden pigmentation caused by carotenoids. *S. aureus* are nonmotile, non–spore-forming, and catalase positive. The cell wall contains peptidoglycan and teichoic acid. The organisms are resistant to temperatures as high as 50°C, to high salt concentrations, and to drying (Yu PKW, 1985; Holt *et al.*, 1994). The ability to clot plasma continues to be the most widely used and generally accepted criterion for the identification of *Staphylococcus aureus*. One such factor, bound coagulase, also known as clumping factor, reacts with fibrinogen to cause organisms to aggregate. Another factor, extracellular staphylocoagulase, reacts with prothrombin to form staphylothrombin, which can convert fibrinogen to fibrin. About 97% of human *S aureus* isolates possess both of these forms of coagulase (Tolan, 2006).

1.2.3 Pathophysiology

The organism can cause disease by 2 mechanisms, tissue invasion and toxin production. The toxins liberated by the organism may have effects at sites distant from the focus of infection or colonization (Tolan, 2006).

1.2.4 Pathogenesis of *S. aureus*

Staphylococcus aureus causes a variety of suppurative (pus-forming) infections and toxinoses in humans. One pathogenic species is *Staphylococcus aureus*, which can infect wounds. *S. aureus* is a major cause of hospital-acquired infections (nosocomial) and is being recognized with increasing frequency in

community-acquired infections. *S. aureus* is also implicated in toxic shock syndrome (Todar, 2005).Any *S. aureus* infection can cause the staphylococcal scalded skin syndrome, a cutaneous reaction to exotoxin absorbed into the bloodstream. It can also cause a type of septicaemia called pyaemia (Madigan and Martinko, 2005). *S. aureus* sometimes invade the skin to cause infection. This is more likely if you have a cut or graze which can allow bacteria to get under the surface of the skin. *S. aureus* is the cause of skin infections such as boils, pimples, impetigo, skin abscesses, styes, furunculosis and is a common cause of wound infections.

In some people, *S. aureus* can sometimes get into the bloodstream and travel to internal parts of the body to cause infections that are more serious. For example, blood poisoning (septicaemia), lung infection (pneumonia), bones infection (osteomyelitis), heart valve infection (endocarditis), urinary tract infections, deep-seated infections and meningitis etc as show in figure (1-1) (Todar, 2005). These serious infections are more likely to occur in people who are already unwell or debilitated, or who have a poor immune system. These infections needto be treated with antibiotics (EMIS and Patient Information Publications, 2005).

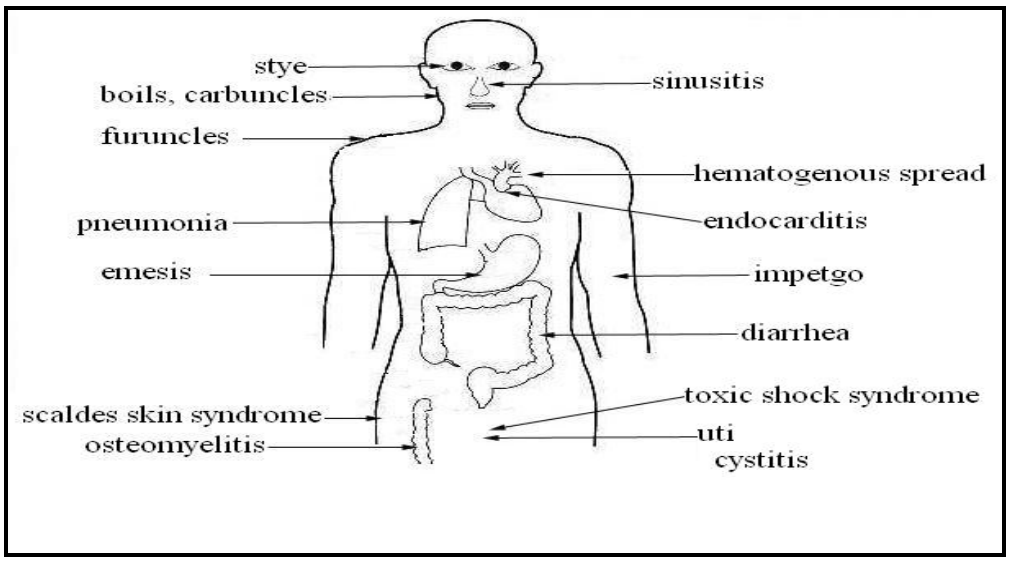

Figure: (1-1): Sites of infection and diseases caused by *Staphylococcus aureus* (EMIS, 2005)

1.2.5 Epidemiology

With the exception of natural valve endocarditis and some infections of peritoneal dialysis catheters, virtually all *S. epidermidis* infections are hospital acquired. In contrast, *S. saprophyticus* infections (urinary tract infections) are all acquired outside the hospital (Schaberg *et al*., 1982; Cohen *et al*., 1982).

S. *epidermidis* probably gain access to foreign bodies by direct inoculation during the insertion of the device. The molecular analysis of the abundant plasmid DNA in coagulase-negative staphylococci has been used successfully in outbreak investigations (Hiramatsu *et al*., 1997; Edmond *et al.,* 1996) and in differentiating infecting from contaminating culture isolates (Kluytmans *et al.,* 1997). *S. aureus* colonizes mainly the nasal passages, but it may be found regularly in most other anatomical locales (Todar, 2005).

Commonly *S. aureus* found on the skin and in the nose of healthy people staphylococci can get into the body and cause an infection. *Staph. aureus* is a common organism and can be found in the nostrils of up to 30% of persons. Person-to-person transmission is the usual form of spread and occurs through contact with secretions from infected skin lesions, nasal discharge or spread via the hands (Shinefield *et al.,* 2002)

1.3 *Staphylococcal* virulence Factors

Plasmid DNA is abundant in all species of coagulase-negative staphylococci (Peacock *et al.,* 1981), but only a few of the plasma-encoded genes have been identified. Resistances to such antibiotics as penicillin, macrolides, lincosamides, tetracyclines, chloramphenicol, trimethoprim, and aminoglycosides have all been associated with specific plasmids; plasmid-mediated resistance has been confirmed by the transfer of these plasmids to suitable plasmid-free recipients.

Of considerable epidemiologic significance is the demonstration that certain aminoglycoside-resistance plasmids found in *S. epidermidis* can be transferred by conjugation to other *S. epidermidis* and to *S. aureus* organisms (Kinsman *et al.*, 1985; Peacock *et al,*. 1981). These conjugative plasmids also encode resistance to penicillin, trimethoprim, mupirocin, and disinfectants (quarternary ammonium compounds) and can mobilize the transfer of plasmids encoding resistance to macrolides, lincosamides, and chloramphenicol. Conjugative resistance transfer may help explain the rapid increase in resistance seen among hospital-associated *S. epidermidis* isolates (Berger, 1994; Firth *et al.*, 2000). *S. aureus* expresses many potential virulence factors:

(1) Surface proteins that promote colonization of host tissues.

(2) Invasins that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase).

(3) Surface factors that inhibit phagocytic engulfment (capsule, Protein A).

(4) Biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production).

(5) Immunological disguises (Protein A, coagulase, clotting factor).

(6) Membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin).

(7) Exotoxins that damage host tissues or otherwise provoke symptoms of disease (SEA-G, TSST, and ET).

(8) Inherent and acquired resistance to antimicrobial agents (Todar, 2005).

1.3.1 *Staphylococcal* Enzymes

1.3.1.1 Catalase: Hydrogen peroxide is produced by all staphylococcal strains and is converted into nontoxic H_2O and O_2 by the action of catalase (Mandell, 1975).

1.3.1.2 Coagulase: Coagulase is either extracellular or cell bound. It stimulates the conversion of fibrinogen to fibrin by binding to prothrombin. The reaction used to differentiate *S. aureus* from coagulase-negative staphylococci (Kawabata *et al.*, 1985).

1.3.1.3 Clumping Factor: *S. aureus* forms clumps when mixed with plasma through an interaction between fibrinogen and a bacterial cell surface compound called clumping factor (Hawiger *et al.*, 1982). Genetic evidence and molecular studies have demonstrated that coagulase and clumping factor are distinct entities of *S. aureus* (Mcdevitt *et al.*, 1992).

1.3.1.4 Hyaluronidase: Hyaluronidase hydrolyzes hyaluronic acids, a group of acid mucopolysaccharides present in the acellular matrix of connective tissue, its role in the pathogenicity of *S. aureus* (Hooper, 1997).

1.3.1.5 β-Lactamases: Most β-lactamases are plasmid coded. Their physiologic role in cellular metabolism in the absence of B-lactam antibiotics is unknown (Hooper, 1997).

1.3.1.6 Staphylokinase: Many strains of *S aureus* express a plasminogen activator called staphylokinase. This factor lyses fibrin. As with coagulase, there is no strong evidence that staphylokinase is a virulence factor, although it seems reasonable to imagine that localized fibrinolysis might aid in bacterial spreading (Todar, 2005).

1.3.1.7 Other Enzymes: *S. aureus* produces a nuclease that tested on a DNA substrate for taxonomic purposes, but in fact it is a phosphodiesterase with both exonuclease and endonuclease activity that cleaves nucleic acids into 3´-phosphomononucleotides. Abscess formation characterized by the disruption of protein and lipid constituants. Most *S. aureus* strains produce lipolytic enzymes (lipases) (Hooper, 1997).

1.3.2 *Staphylococcal* Toxins

S. aureus produces a variety of extracellular products that defined as toxins because they affect host cell function or morphology. Some of them express their detrimental effect by enzymatic action. Others such as enterotoxins and toxic shock toxin are potent cytokine inducers that act as superantigens and have opened a new field of pathophysiology of infectious diseases (Hawiger *et al.*, 1982; Bohach *et al.*, 1997). The *S. aureus* enterotoxin causes quick onset food poisoning. These microbes also secrete leukocidin, a toxin that destroys white blood cells and leads to the formation of pus and acne.

(1) α-Toxin: (Bohach *et al.*, 1997)

(2) β-Toxin: (Bohach *et al.*, 1997)

(3) γ-Toxin: (Bohach *et al.*, 1997)

(4) δ-Toxin: (Bohach *et al.*, 1997; Melish and Glasgow, 1970)

(5) Leukocidin: (Bohach *et al.*, 1997)

(6) Epidermolytic Toxins and the Staphylococcal Superantigen Family: (Bohach *et al.*, 1997; Melish and Glasgow, 1970)

(7) Toxic Shock Syndrome Toxin (Cone *et al.*, 1987; Parsonnet *et al.*, 1986)

(8) Enterotoxins: (Marrack and Kappler, 1990; Johnson *et al.*, 1991)

1.4 Bacteriolytic enzymes

1.4.1 Lysozyme

The term lysozyme to a bacteriolytic agent found in the tissue of a number of species of animals (Alderton *et al*., 2004). The enzyme is stable when heated in acid solution but very heat-labile in alkali. This enzyme attacks peptidoglycan by hydrolyzing the bond that connect N-acetylmuramic acid with carbon four of

N-acetylglucosamine. However, Schleifer and Kloos, 1975 claimed that lysozyme does not hydrolyze *S.aureus* cell wall (Prescott *et al.,* 1990).

1.4.2 Lysostaphin

It is commercially available protein preparation obtained from the culture filtrate of the organism *Staphylococcus staphylolyticus* (Schindler, 1996; Schindier and Schuhardt, 1975). Lysostaphin contain three enzymes capable of acting on bacterial cell wall peptidoglycan. The major component in lysostaphin is glycoglycin endopeptidase, which capable of specifically lysing *S.aureus* cells (Schindler, 1996).

This enzyme lyses staphylococcal cells by hydrolyzing glycylglycin bounds in the polyglycin bridges, which form cross-links between glycopeptide chains in the cell wall peptidoglycan of these organisms (Robinson *et al.,* 1979).

1.5 *Staphylococus aureus* plasmids

Multiple plasmids are frequently present in clinical isolates of *S. aureus*, and three broad categories of plasmids have been recognized (Novick, 1989).

Class I plasmids: These are small plasmids (5 Kb or smaller), phenotypically cryptic or encode a single resistance determinant. Rarely a plasmid carries two markers include Tc (tetracycline), Em (erythromycin), Cm (chloramphenicol), Sm (streptomycin), Km (kanamycin), B1 (bleomycin), Qa (quaternary amine), and Cd (cadmium). These plasmids utilize a rolling-circle replication via a single-stranded intermediate. Copy number is (15-60 copy/cell) (Novick, 1990). These plasmids divided into four groups, pT181, pC194, pSN2, and pE194 (Gruss and Erich, 1989; Brien *et al*., 2002).

Class II plasmids: Larger plasmids (15-30 Kb) which are characterized by the presence of multiple antimicrobial-resistance determinants such us resistance to β-lactam antibiotics, macrolides, and variety of heavy metal ions (Arsenic, Cadmium, Lead, and Mercury), which are frequently associated with transposable elements. They use the θ mode of replication (Paulsen *et al*., 1997; Udo *et al.*, 2001). Four to six copies are found per cell (Novick, 1990). These plasmids grouped into four families β, α, δ, γ which include pI524, PII147, PI258, pI1071 respectively which carry gene for betalactamase, heavy metals resistance and orphan which includes pSK1 (Firth and Skurray, 1998; Lacey, 1980).

Class III plasmid: The largest plasmids (30-60 Kb) are also multiresistance plasmids but are differentiated from those in class II by their ability to promote their own intercellular transmission via conjugation (Firth *et al.*, 2000). Class III plasmids can also mediate the independent mobilization of some suitably equipped class I plasmids, and facilitate the horizontal transfer of other non-self transmissible plasmids and or chromosomal segment by conjugation coduction (Firth and Skurray, 1998). These plasmids include pSK41, pG01, and pJE1. These plasmids carry resistance markers including CN (gentamicin), Pc (penicillin) and Qa (quaternary compound), some of which are transposable, and a number of insertion sequence (IS)-like elements (Thomas and Archer, 1989).

1.6 *Staphylococcal* resistance for antibiotics

The Gram-positive bacterium *Staphylococcus aureus* is an important human pathogen that has become increasingly resistant to a wide range of antibiotics over the last two decades. The emergence of multidrug-resistant isolates of methicillin-resistant *S. aureus* (MRSA) exhibiting also decreased

susceptibilities to glycopeptides (glycopeptide-intermediate *S. aureus*, GISA) represents a crucial challenge for antimicrobial therapy, antimicrobial susceptibility testing, and hospital infection control (Scherl *et al.,* 2007). Increased antibiotic resistance of common bacteria attributed in part to the widespread use of various antibiotic agents (Carrier *et al.,* 2002).

The introduction of penicillin offered an opportunity to successfully treat serious staphylococcal infections, but an enzyme produced by *S. aureus*, penicillinase (later known as ß-lactamase) was described. This enzyme was responsible for the clinical failures that appeared soon after the introduction of penicillin. During the early 1950s, a series of semi-synthetic penicillins (like Methicillin) were developed that were stable to destruction by bacterial ß-lactamases. One year after its introduction, the first methicillin resistant *S. aureus* (MRSA) was detected and the first clinical failure of methicillin for the treatment of *S. aureus* was described (Hardy *et al.,* 2004).

The resistance often is transferable (Noble *et al.,* 1992). The spread of resistance to antimicrobial agents in *S. aureus* is largely due to the acquisition of plasmids and/or transposons (Lyon and Skurray, 1987). Transfer of resistance between staphylococcal strains in the laboratory has been shown to occur via transformation, transduction, and conjugation (Townsend *et al.,* 1986). Resistance to methicillin in coagulase-negative staphylococci (*S. epidermidis, S. haemolyticus*) exhibits the same heterotypic expression, altered by changes in culture or environmental conditions, as do methicillin-resistant *S. aureus*. The heterotypy of resistance expression for coagulase-negative staphylococci, particularly *S. epidermidis*, is much greater than that seen for *S. aureus* (Tuazon *et al.,* 1975).

In addition to β-lactams antimicrobials, to which more than 50% of *S. epidermidis* and *S. haemolyticus* nosocomial isolates are resistant include

erythromycin, clindamycin, chloramphenicol, and tetracycline (Godfrey and Smith, 1958; Hill *et al.*, 1988).

Staphylococcus aureus has a proven ability to adapt to the selective pressure of antibiotics. At present, methicillin-resistant *S. aureus* (MRSA) strains with resistance to vancomycin are emerging and one of the most serious contemporary challenges to the treatment of *S. aureus* infections (Chang *et al.*, 2003; Crisostomo *et al.*, 2001). *S. aureus* isolates showed a special multiresistance pattern that included resistance to penicillin (P), streptomycin (S), tetracycline (TE), erythromycin (E), lincomycin and clindamycin (Lencastre *et al.*, 2000). To access the problem of antibiotic resistance we can use fusidic acid, which was an effective component of antibiotic combinations used to treat infections caused by *Staphylococcus aureus* (Brien *et al*., 2002).

S. aureus possesses a remarkable number of mechanisms for resisting antibacterial action. Aminoglycoside-resistant strains have been described with increasing frequency. Rifampin, which is remarkably active against *S. aureus*, cannot be used as a single agent because of a high one-step mutation rate of 10^{-7} to 10^{-8} to resistance (Moorman and Mandell, 1981). Resistance to fluoroquinolones has been found in methicillin-sensitive (Kaatz *et al.*, 1991). And methicillin-resistant strains (Murakami and Tomasz, 1989), and is becoming a major epidemiologic problem. Both altered gyrase and energy-dependent efflux mechanisms are implied (Kaatz *et al.*, 1991). The mechanisms of resistance for some antibiotics show in table (1-1) (Wu *et al.*, 1996).

Table (1-1): Mechanisms of resistance for some antibiotics (Wu *et al.*, 1996).

Mechanism of resistance for the antibiotics	Examples
Reduce permeability	Aminoglycosides
Active efflux	Tetracycline Fluoroquinolones
Alteration of drug target	Erythromycin Fluoroquinolones Rifampicin Tetracycline
Inactivation of drug	Aminoglycosides Chloramphenicol B-lactams
Sequestration of drug	B-lactams

1.7 Heavy metal resistance

Staphylococcus aureus is a common human pathogen associated with a number of diseases. Understanding of metal resistance in staphylococci has progressed rapidly in the last years, with well-established Cadmium, Mercury, Antimony, and Arsenic resistance systems encoded by plasmids (Nucifora *et al.*, 1989). While plasmid p1258 were encodes *S. aureus* resistance for Cadmium and Zinc (Naz *et al.*, 2005). The trace heavy metal ions such as Cobalt, Zinc, Copper, and Nickel play important roles in bacteria. They regulate a wide array of metabolic functions as coenzymes or cofactors, as catalysts or a type of acid in enzymes, and as structural stabilizers of enzymes and DNA-binding proteins (Nies and Brown, 1997). Although some heavy metals are essential trace elements, most can be, at high concentrations, toxic to all branches of life, including microbes, by forming complex compounds within the cell. Therefore, Increasing environmental concentrations of these heavy metals pose a challenge to bacteria (Beard *et al.*, 1997).

Because heavy metals are increasingly found in microbial habitats due to natural and industrial processes, microbes have evolved several mechanisms to tolerate the presence of heavy metals (by efflux, complexation, or reduction of metal ions) or to use them as terminal electron acceptors in anaerobic respiration (Silver and Phung, 1996). Many have speculated and have even shown that a correlation exists between metal tolerance and antibiotic resistance in bacteria. Because of the likelihood that resistance genes to both (antibiotics and heavy metals) may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment (Spain and Alm, 2003).

1.7.1 Mechanisms of bacterial resistance for the Heavy Metals

In high concentrations, heavy metal ions react to form toxic compounds in cells (Nies, 1999). To have a toxic effect, however, heavy metal ions must first enter the cell. Because some heavy metals are necessary for enzymatic functions and bacterial growth, uptake mechanisms exist that allow for the entrance of metal ions into the cell. There are two general uptake systems, one is quick and unspecific, driven by a chemiosmotic gradient across the cell membrane and thus requiring no ATP, and the other is slower and more substrate-specific, driven by energy from ATP hydrolysis. While the first mechanism is more energy efficient, it results in an influx of a wider variety of heavy metals, and when these metals are present in high concentrations, they are more likely to have toxic effects once inside the cell (Nies and Silver, 1995).

To survive under metal-stressed conditions, bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state (Nies, 1999; Spain and Alm, 2003). The molecular mechanisms involve a number of proteins, such as ion transporters, reductases,

glutathione-related cadystins and cysteine-rich metallothioneins, and low-molecular-weight cysteine-rich metal ligands (Silver and Phung, 1996).

These protein molecules either export the metal ions out of cells or detoxify or sequester them so that the cells can grow in an environment containing high levels of toxic metals. However, there is no common mechanism of resistance to all heavy metal ions (Nies and Brown, 1997). It has been test the minimal inhibitory concentrations (MICs) of several different metal ions for *Escherichia coli* on agar medium, and the most toxic metal (with the lowest MIC) was mercury, whereas the least toxic metal tested was manganese (Mergeay *et al*., 1985).

1.7.1.1 Resistance to Cadmium ions

Cadmium is a highly toxic divalent cation, Cadmium resistance is the most common resistance determinant found on resistance plasmids (R plasmids) of *Staphylococcus aureus*, occurring at frequencies of 80% or greater in some clinical collections (Nakahara *et al.*, 1977; Friberg *et al.*, 1971). It has been reported that certain strains of *Staphylococcus aureus* were resistant to inorganic ions including Mercury and Cadmium. Plasmid-determined resistance to Cadmium and Zinc has only been found with plasmids from *Staphylococcus aureus* (Cherian, 1974; Naz *et al.*, 2005).

Resistance to Cadmium was associated with a lower accumulation of Cd^{2+} ions by the plasmid-bearing resistant cells. Cadmium Accumulation by susceptible cells was energy dependent and had those characteristics usually associated with a transmembrane active transport system (Novick, 1969).

There was a specific interrelationship between Cadmium accumulation and manganese accumulation and retention. Cd^{2+} inhibited the uptake of Mn^{2+} and accelerated the loss of intracellular Mn^{2+} by the susceptible cells, but was without effect on Mn^{2+} transport in resistant *S. aureus* cells. Under similar conditions, there was no differential effect of Cd^{2+} on Mg^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, or Rb^{+}

accumulation or exchange between the susceptible and the resistant strains (Novick and Roth, 1968).

Cadmium resistance is associated with a lowered level of uptake of Cd^{2+} by the resistant-plasmid-containing cells (Silver *et al.*, 1976). Protoplasts of resistant cells retain the barrier to Cd^{2+} uptake (Chopra, 1971), suggesting that the cell membrane is involved in the barrier function. The process of uptake in susceptible cells is energy dependent, and it seems possible that the resistance barrier involves an active transport process such as known for physiologically required divalent cautions such as Mg2+, Mn2", and Zn2+ (Jasper and Silver, 1977).

1.7.1.2 Resistance to Zinc and Cobalt ions

Staphylococcal strains without plasmids show resistance to heavy metal ions, such as Zinc and Cobalt. This implies that a plasmid-independent chromosomal determinant might encode resistance to heavy metals such as Zinc and Cobalt. Although operons encoding Cobalt, Zinc, and Cadmium in *Alcaligenes eutrophus* (Nies, 1992) and Zinc in *Escherichia coli* (Beard *et al.*, 1997) have been investigated, relatively little is known about the transport and resistance mechanisms of Zinc and Cobalt ions in *S. aureus*. Zinc is one of the essential trace element. It is not biologically redox reactive and is thus not used in respiration. It is, however, important in forming complexes (such as zinc fingers in DNA) and as a component in cellular enzymes (Nies, 1999).

Bacterial cells accumulate Zinc by a fast, unspecific uptake mechanism and it is normally found in higher concentrations (but is less toxic) than other heavy metals (Nies, 1999). Uptake of Zinc ions is generally coupled to that of magnesium and the two ions may be transported by similar mechanisms in bacteria (Nies and Silver, 1995). Two general efflux mechanisms are responsible for bacterial resistance to Zinc. One is a P-type ATPase efflux1 system that

transports Zinc ions across the cytoplasmic membrane by energy from ATP hydrolysis (Beard *et al.*, 1997). The other mechanism involved in Zinc efflux is an RND-driven2 transporter system that transports Zinc across the cell wall (not just the membrane) of gram-negative bacteria and is powered by a proton gradient and not ATP (Nies, 1999).

1.7.1.3 Resistance to Mercury ions

Resistance of *Staphylococcus aureus* mediated by the penicillinase (Pc-ase) plasmid to divalent metal ions of Hg^{2+} and Cd^{2+} was found to be controlled by different mechanisms. The Hg^{2+} resistance of the Pc-ase plasmid-carrying organisms is based upon a process of changing the ion incorporated in the cell into a somewhat innocuous form. This process is independent of temperature and Seems to be controlled by an inducible enzyme. The killing effect of Hg salts was not influenced by the coexistence of other di-or monovalent ions such as MgC92, $CaCl_2$, $MnCl_2$, and NaCl. No vaporization of Hg, which explains the resistance mechanism such as that proposed by Komura et al. for R factor-mediated Hg resistance in enterobacilli, was found in the case of Hg resistance in staphylococci (Weiss *et al.*, 2001; Komura *et al.*, 1971).

It has been known since the report by (Novick, 1990) that the penicillinase (Pc-ase) plasmid in *Staphylococcus aureus* also carries genes determining resistance to several heavy metal ions as well as those to erythromycin and other antibiotics.The authors have been especially interested in the resistances to these metal ions, not only from the point of view of microbial genetics but also from the medical aspects. Plasmid-mediated resistances to these heavy metals have also been observed in enteric bacilli, especially in R factor-carrying organisms, and have recently been studied by several workers. Nevertheless, the mechanisms of resistance to heavy metal ions in staphylococci have not been studied as much as those in enteric bacilli or the mechanisms of staphylococcal penicillin resistance. (Komura and Izaki, 1971).

1.7.2 Correlation of Metal resistance and Antibiotic Resistance

Bacterial resistance to antibiotics and other antimicrobial agents is an increasing problem in today's society. Resistance to antibiotics is acquired by a change in the genetic makeup of a bacterium, which can occur by either a genetic mutation or by transfer of antibiotic resistance genes between bacteria in the environment (American Academy of Microbiology, 2000).

Products such as disinfectants, sterilants, and heavy metals used in industry and in household products are, along with antibiotics, creating a selective pressure in the environment that leads to the mutations in microorganisms that will allow them better to survive and multiply (Baquero *et al.*, 1998). Clustering of genes on a plasmid, if both or all genes clustered are useful to the organism is beneficial to the survival of that organism and its species because those genes are more likely to be transferred together in the event of conjugation (Lawrence, 2000).

Thus, in an environment with multiple stresses, for example antibiotics and heavy metals, it would be more ecologically favorable, in terms of survival, for a bacterium to acquire resistance to both stresses. If the resistance is plasmid mediated, those bacteria with clustered resistance genes are more likely to simultaneously pass on those genes to other bacteria, and those bacteria would then have a better chance at survival. There were studies on bacteria isolated from drinking water and found that a high percent of bacteria that were tolerant to metals were also antibiotic resistant (Calomiris *et al.*, 1984).

1.8 Curing of plasmid DNA

Plasmids have been observed in a wide variety of bacteria. In part, this is due to the development of new procedures that allow the detection, isolation, and molecular characterization of plasmid DNA. When working with some plasmid-containing bacteria, it is often desirable to obtain a plasmid-cured derivative. This allows a direct comparison to be made between the plasmid-containing and

plasmid-cured cell. Some plasmids undergo spontaneous segregation and deletion. However, the majority is extremely stable, and requires the use of curing agents or other procedures (elevated growth temperature, thymine starvation), to increase the frequency of spontaneous segregation (Caro *et al.*, 1984).

In view of the importance of plasmids in specifying antibiotic and metal resistance; metabolic properties; pathogenicity; host specificity and nodulation (Rhizobium spp.); conjugal properties, and replication-maintenance properties, reproducible procedures for obtaining plasmid-cured derivatives are necessary. (Trevors, 1985).

In addition it was stated that in certain organisms even the loss of a plasmid may not be adequate evidence to conclude that the trait is plasmid-encoded. This is because many plasmids are capable to integrate in to the bacterial host chromosome. If this occurs, the plasmid would not be present as a covalently closed circular (CCC) molecule (Caro *et al.*, 1984). Interchalating dyes such us acriflavine, acridine orange, ethidium bromide and quinacrine have been successfully used in curing plasmids of bacteria. The concentration of these dyes will depend on the organism and curing agent. Most effective concentration of a particular curing agent can vary considerably, in the range of 100 to 1000 fold. This depends up on the species being treated, curing agent efficiency, and the mode of action of the curing agent (Carlton and Brown, 1981).

Ethidium bromide has been extensively used to cure plasmid in wide variety of bacterial strains. In 1969, it was described that the use of ethidium bromide to eliminate plasmids in antibiotic resistant Enterobacteria and Staphylococci. Curing was usually observed at a high frequency, and the results obtained were more reproducible than with acridine dyes. Ethidium bromide was successfully cured penicillinase plasmids in *Staphylococcus aureus* strains (Robin and Rosenblum, 1971).

Materials and Methods

2.1 Materials

2.1.1 Equipments

The following equipments were used in this study:

Equipment	Company(Origin)
1- Autoclave	Gallen Kamp (England)
2- Balance	Ohans (France)
3- Compound Light microscope	Olympus (Japan)
4- Centrifuge	Harrier (U.K.)
5- Camera	Zenit (Russia)
6- Centrifuge (Biofuge B)	Hedraeces Christ (Germany)
7- Distillator	GallenKamp (England)
8- Oven	Memmert (Germany)
9- Gel electrophoresis unit	BioRad (Italy)
10- Hot plate with magnetic stirrer	GallenKamp (England)
11- Incubator	GallenKamp (England)
12- Laminar air flow	Memmert (Germany)
13- Millipore filter paper unit	Millipore and Whatman (England)
14- Micropipettes	Widget (Germany)
15- Portable Centrifuge	Hermle labortechnik (Germany)
16- pH-Meter	Metter-GmpH Tdedo (U.K.)
17- Power Supply	LKB (Sweden)
18- Sensitive balance	Delta Range (Switzerland)
19- Shaker Incubator	GFL (Germany)
20- U.V Transiluminator	Vilber Lourmat (France)
21- Vortex mixer	Buchi (Switzerland)
22- Water bath	GFL (England)

2.1.2 Chemicals

The following chemicals were used in this study:

Chemicals	Company (Origin)
1- Agarose	BDH (England)
2- Chloroform	
3- Ethanol	
4- Isoamyl alcohol	
5- Bromophenol blue	
6- Mercury Chloride ($HgCl_2$)	
7- Sodium hydroxide (NaOH)	
8- Lactose	
9- Galactos	
10- Mannitol	
11- Boric acid (H_3Bo_3)	
12- Cadmium Chloride ($CdCl_2$)	
13- Ethylene Diamine Tetra Acetic acid (EDTA)	
14- Hydrochloric acid (HCl)	
15- Zinc acetate $Zn(CH_3COO)_2.2H_2O$	
16- Cobalt acetate $Co(CH_3COO)_{2.}4H_2O$	
17- Phenol red	
18- Ethidum Bromide	
19- Crystal Violate	
20- Iodine	
21- Safranin	
22- Hydrogen Peroxide	FLUKA (Switzerland)
23-N,N,N,N-Tetramethylp-phenylene-Diamine Dihydrochlorid	
24- Sodium Chloride (NaCl)	
25- Tris-HCl	
26- Glycerol	Difco (USA)

2.1.3 Media

The following media were used in this study:

Type of media	Company(Origin)
1- Agar-agar	FLUKA (Switzerland)
2- Brain-Heart infusion agar	FLUKA (Switzerland)
3- Brain-Heart infusion broth	FLUKA (Switzerland)
4- DNase agar	Difco (USA)
5- Mannitol-Salt agar	FLUKA (Switzerland)
6- Mueller-Hinton agar	Difco (USA)
7- Nutrient agar	Difco (USA)
8- Nutrient broth	Difco (USA)
9- Staph 110 agar	FLUKA (Switzerland)
10- Urease base agar	FLUKA (Switzerland)

All media were prepared according to the manufacturer instruction, sterilized by autoclave under 15 psi at 121°C for 15 min and incubated at 37°C.

2.1.4 Enzyme

Lysozyme was used for plasmid isolation in this study.

2.1.5 Standard Strain

Strain	Source
Staphylococcus aureus ATCC 25923	Department of Biotechnology/ Al-Nahrain university

2.1.6 Reagents

The following indicators were used in API staph system:

Reagents	Company(Origin)
1- VP 1 & VP 2	BioMerieux (France)
2- NT 1 & NT2	BioMerieux (France)
3- ZYM A & ZYM B	BioMerieux (France)

2.1.7 Antibiotic Disks

The following antibiotic disks [Bioanalyse (Turkey)] were used for antibiotic sensitivity test for *S. aureus* strains (NCCLS, 1993).

Antimicrobial agent	Symbol	Disk concentration	Diameter of inhibition zone (mm)		
			R	I	S
1- Amikacin	AK	30 µg	≤14	15-16	≥17
2- Bacitracin	B	10 U	≤ 8	9-12	≥ 13
3- Carbenicillin	PY	100 µg	≤ 17	18-22	≥ 23
4- Cefotaxime	CTX	30 µg	≤ 14	15-22	≥ 23
5- Cephalexin	CL	30 µg	≤ 14	15-17	≥ 18
6-Chloramphenicol	C	30 µg	≤ 12	13-17	≥ 18
7- Fusidic acid	FA	10 µg	≤ 14	15-22	≥ 23
8- Gentamicin	CN	10 µg	≤ 12	13-14	≥ 15
9- Imipenem	IPM	10 µg	≤ 13	14-15	≥ 16
10- Streptomycin	S	10 µg	≤ 11	12-14	≥ 15
11- Tetracycline	TE	30 µg	≤ 14	15-18	≥ 19
12- Vancomycin	VA	30 µg	≤ 9	10-11	≥ 12

R: Resistance I: Intermediate S: Sensitive

2.1.8 Buffers and solutions

2.1.8.1 Bacterial diagnosis solutions

- **Gram stain**

  Prepared according to (Atlas *et al.*, 1995).

- **Catalase reagent**

  3% H_2O_2 was utilized according to method described by (Simbert and Krieg, 1981).

- **Oxidase reagent**

  Reagent prepared from 1% N,N,N,N-Tetramethyl p-phenylene – Diamine Dihydrochloride as in (Koneman *et al*., 1992).

- **Sugar fermentation media**

  1gm sugar added to 100 ml pepton water then mixed with phenol red

2.1.8.2 Antibiotic solutions

Solutions were prepared by dissolving one capsule (250 mg) of Tetracycline in 10 ml sterilized distilled water, while Gentamicin, Cefotaxime

and penicillin G were dissolved in sterile distilled water. Tetracycline stock solution prepared at concentration 25000 µg/ml, Cefotaxime stock solution prepared at concentration 10000 µg/ml, Gentamicin stock solution prepared at concentration 8000 µg/ml and Penicillin-G stock solution prepared at concentration 80000µg/ml, then stock solutions sterilized by filtration and kept at 4°C, until used (Maniatis *et al*., 1982).

2.1.8.3 Heavy metal solutions

Heavy metals used were Zn $(CH_3COO)_2.2H_2O$, Co $(CH_3COO)_2.4H_2O$, $CdCl_2$ and $HgCl_2$ prepared as stock solution (see appendix (1)) and sterilized by filtration and kept at 4°C until used.

2.1.8.4 Curing solution

- **Ethidium Bromide solution 10 mg/ml (Bounchaud *et al*., 1969)**

  This solution was prepared by dissolving 0.2gm of ethidium bromide in 20 ml distilled water and stirred on magnetic stirrer for few hours to ensure that the ethidium bromide has dissolved then it was sterilized by filtration, and stored in a dark bottle at 4°C.

2.2 Methods

2.2.1 Collection of Isolates

One hundred thirty isolates of *s. aureus* were obtained from different clinical specimens such as urin, skin, wond, ear, blood and vagina which were collected from Al-Yarmouk and Al-Kadhmia hospitals from 74 femal and 56 male. Of these, 59 isolate were identified as *Staphylococcus* (34 isolate from female and 25 isolat from male), while, 30 isolat identefied as *S. aureus* (17 isolat from femal and 13 isolat from male).on the basis of their colony morphology, Gram's stain and positive results in coagulase, DNase, catalase, mannitol fermentation, and for the further confirmation the isolates were identified by API *staph* system.

2.2.2 Identification of *S. aureus* isolates

2.2.2.1 Morphological tests (Koneman *et al*., 1992)

On direct Gram's stained smears, *S. aureus* appeared, as Gram-positive cocci in grape like clusters. Colonies morphology was studies on brain-heart-infusion agar with 7.5% NaCl. Color, shape and size of colonies were recorded after 24 hours of incubation at 37°C.

2.2.2.2 Biochemical tests

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

  A single colony was placed on a clean glass microscope slide with a sterile toothpick, and then a drop of hydrogen peroxide (3%) was placed onto the colony. The production of gaseous bubbles indicates the presence of catalase.

- **Oxidase test (Atlas *et al.*, 1995)**

  This test was done by using moisten filter paper with few drops of a freshly prepared solution of N,N,N,N-Tetramethyl P-Phenylen-Diamine Dihydrochlorid. Aseptically a clump of cells was picked up from the slant growth with a sterile wooden stick and smeared on the moisten paper. The development of violet or a purple color within 10 seconds indicates a positive result.

- **Tube coagulase test (Atlas *et al.*, 1995)**

  It's performed by adding 0.1 ml of test culture with 0.5 ml of citrated plasma solution in the test tube. After incubation for 0.5, 1, 2, 4 and 24 hours the appearance of coagulation indicated the production of coagulase.

  Note: This test differentiate *S. aureus* from other species of staphylococcus.

- **DNase test (Harley and Prescott, 1993)**

  DNase agar plates were prepared according to the manufacturer. The bacterial strains were streaked on solidified medium and incubated at 37°C

overnight. 10 ml of (1N) HCl was added to each plate. The appearance of clear zone around the colonies indicated the positive results.

- **Sugar fermentation and acid production (Cruickshank *et al.*, 1975)**

  Few colonies of fresh culture of bacteria were inoculated to the sugar medium, for the sugars (Lactose, Galactose). After incubation at 37°C overnight, fermentation of sugar will lead to change the color of phenol red indicator from red to yellow.

- **Growth on Mannitol salt agar (Stukus, 1997)**

  The isolates of bacteria were cultured on Mannitol salt agar (MSA). This media permits the selection of *Staphylococci* due to the high salt concentration of the medium. Since *Staphylococcus aureus* ferments Mannitol it can be distinguished due to the change in color of the phenol red indicator in the medium from red to yellow.

- **Hemolysin production test (Cruickshank *et al.*, 1975)**

  The isolates of bacteria were streaked on freshly prepared blood agar medium (blood agar + 7% blood), the appearance of clear lyses zone around the colonies after 24 hours of incubation at 37°C, indicate the positive results.

2.2.2.3 Identification by API STAPH system (biomerieux)

Identification system for the genera *Staphylococcus* and *Micrococcus*, using standardized and miniaturized biochemical tests with a special adapted database.

- **Instruction for use**

  1- The organism was sub cultured onto blood agar for18-24 hour at 35-37°C.

  2- Purity of microorganism was checked.

3- The ampoules of API STAPH medium was opened under aseptic conditions.

4- A homogenized bacterial suspension was prepared.

5- Using a sterile pipette, the microtubes were filled with the inoculation API STAPH medium.

6- Anaerobic conditions for ADH and URE tests were performed by addition of mineral oil.

7- Strips were incubated at 35-37°C for 18-24 hour.

- **Reading the strips**

Adding 1 drop of each of the following reagents developed the following reactions:

1- VP test: VP1 and VP2 reagents:

After 10 minute, violet pink color indicates a positive reaction. Pale pink or light pink color indicates negative reaction.

2- NIT test: NIT1 and NIT2 reagents: After 10 minutes, red color indicates a positive reaction.

3- PAL test: ZYM A and ZYM B reagents: After 10 minutes, violet color indicates a positive reaction. Reading the reactions was performed by referring to (table 2). By using the analytical profile index the identification was made.

2.2.3 Maintenance of bacterial strains

Maintenance of bacterial strains performed according to (Maniatis *et al*., 1982) as follow: Colonies of bacteria were maintained for few weeks on nutrient agar media, plates were tightly wrapped in parafilm and stored in the refrigerator at 4°C. For longer storage, strains of bacteria were maintained in slants containing nutrient agar. These slants were prepared in 10 ml screw-capped bottles containing 3-4 ml of nutrient agar. Bottles were incubated with the bacterial strains at 37°C overnight then stored in the refrigerator for one month.

Types of tests included in API system for identification (Biomerieux).

Test	Substrate	Reaction/Enzyme	Results	
			Negative	Positive
0	No substrate	Negative control	Red	-
GLU **FRU** **MNE** **MAL** **LAC** **TRE** **MAN** **XLT** **MEL**	D-Glucose D-Fructose D-Mannose Maltose Lactose D-Trehalose D-Mannitol Xylitol D-Melibiose	(positive control) Acidification Carbohydrate utilization	Red	Yellow
NIT	Potassium nitrate	Reduction of nitrate to nitrite	NIT1 + NIT2 /10 min	
			Colorless-light pink	Red
PAL	B-naphthyl-acid phosphate	Alkaline phosphatase	ZYM A+ZYM B /10 min	
			Yellow	Violet
VP	Sodium pyruvate	Acetyl-methyl-carbinol production	VPI 1+VPI 2 /10min	
			Colorless	Violet-pink
RAF **ZYL** **SAC** **MDG** **NAG**	Raffinose Xylose Sucrose ∂-methyl-D-glucoside N-acetyl-glucosamine	Acidification due to carbohydrate utilization	Red	Yellow
ADH	Arginine	Arginine dihydrolase	Yellow	Orange-red
URE	Urea	Urease	Yellow	Red-violet

2.2.4 Antibiotic sensitivity test (Atlas *et al.*, 1995)

The disc diffusion method was used to test the antibiotic sensitivity of the selected isolate. A sterile cotton swab was dipped in to the inocula (freshly culture for 18 hour) and the entire surface of the brain heart infusion agar plates

was swabbed three times by rotating the plate approximately 60°after each streaking to ensure even distribution. Then the discs of antibiotics were applied on cultured media and incubated at 37°C. The zone of inhibition was observed after incubation for 18 hour.

2.2.5 Minimum Inhibitory Concentration (MIC) test (Atlas *et al.*, 1995)

Inocula of selected isolates were grown in 5ml nutrient broth, then 0.1ml of each culture were inoculated in series of 5ml fresh nutrient broth containing various concentrations of antibiotics or heavy metals solutions (8, 16, 32, 64, 128, 256, 512 and 1024 µg/ml for antibiotics) and (5, 10, 20, 40, 80, 160, 320, 640 and 1280 µg/ml for heavy metals) for each isolates of *S. aureus*, then all tubes incubated in 37°C for 24 hours. 100 µl from each tube were spread on brain heart infusion agar plates and all plates were incubated at 37°C for 24 hours. The lowest concentration of the antibiotics or heavy metals solutions that inhibited the growth of bacterial isolates considered as the minimum inhibitory concentration (MIC).

2.2.6 Plasmid DNA curing (Trevors, 1986)

Cells of the selected isolate were grown in 5ml of nutrient broth. 0.1ml samples of each culture were inoculated in series of 5ml fresh nutrient broth tubes containing various concentrations of ethidium bromide (50, 100, 200, 400, 600, 800 and 1000 µg/ml). All tubes were incubated at 37°C for 24 – 48 hours.

The growth density of the deferent tubes was measured visually and compared with the control to determine the effect of each concentration of curing agent on bacterial growth (Trevors, 1986). The lowest concentration of the curing agent that inhibits the growth of bacterial isolate considered as the minimum inhibitory concentration (MIC).

Sample was taken from the tube containing the highest concentration of ethidium bromide that still allows bacterial growth and diluted appropriately.

Then 0.1ml samples from proper dilutions were spread on nutrient agar plates and incubated overnight at 37°C to score the survived colonies.

2.2.7 Selection of Cured Cells (Trevors, 1986)

After treatment of bacterial isolate with standard curing agent and the isolation of survivors on nutrient agar, survivors were analyzed for the presence or absence of drug resistance as a result of elimination the plasmid by selecting 100 colonies of bacterial isolates from each treatment. These colonies were replica plated (using toothpick) on nutrient agar plate (master plates) and on nutrient agar plates containing an antibiotics and other nutrient agar plate containing a heavy metals to which the original isolate is resistant (Trevors, 1986).

If a colony was able to grow on the master plate but not on the selective agar containing the appropriate antibiotic or heavy metal, it means that, the cells of this colony are cured cells that lost plasmid responsible for resistance to the antibiotic or heavy metal. The percentage of the cured cells was determined.

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A study of antibiotic and heavy metal resistance in *Staphylococcus aureus* isolates from clinical specimens

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بسم الله الرحمن الرحيم

ُالله نورُ السمواتِ و الأرضِ مثلُ نورِه كمشكاةٍ فيها مصباحٌ المصباحُ في زجاجةٍ الزجاجةُ كأنها كوكبٌ دريٌّ يوقدُ من شجرةٍ مباركةٍ زيتونةٍ لا شرقيةٍ ولا غربيةٍ يكادُ زيتها يضئُ و لو لم تمسسهُ نارٌ نورٌ على نورٍ يهدي ُالله لنورهِ من يشاءُ ُو يضربُ ُالله الأمثالَ للناسِ و ُالله بكلِ شئٍ عليمٌ

صدق الله العظيم

سورة النور الآية (٣٥)

الإهداء

إلى روح والدي التي أحيت
منى نفسي و اوطاري
وإلى التي سقتني من شهد
الحنان
إلى الطير الذي انس بالتغريد
أسحاري
إلى أمي.. نزهة أبصاري
والى الفجر الذي رصع بالنداء
نواري
إلى أخواتي من هم جنة أحلامي
اهدي غض أزهاري

رفاه

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Supervisor Certification

I certify that this thesis was prepared under my supervision in the Collage of Science, Al-Nahrain

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Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents

and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

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Signature:
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Scientific Degree:
Date:
(Member)

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Name:
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Date:
(Member)

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Name:
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I hereby certify upon the decision of the examining committee

Signature:
Name: Dr. Laith Abdul Al-Aziz Al-Ani
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Results and Discussion

3.1 Isolation and Identification of *Staphylococcus aureus*

One hundred and thirty isolates collected from different sites of patients in Al-Kadhemia hospital and Al-Yarmook hospital from 74 female and 56 male during the period from 1/11/2005 to 15/4/2006. Of these, 59 isolates identified as *Staphylococcus* (34 isolates from female and 25 isolates from male), while 30 isolates identified as *Staphylococcus aureus* (17 isolates from female and 13 isolates from male), which represented 23% of total isolates, while the remaining isolates identified as different types of bacteria.

The high percentage of *S.aureus* might be due its role as the main cause of nosocomial infections (Wertheim *et al.,* 2004). It is also one of the most important infectious agents, which can cause an opportunistic infection because it is a part of body normal flora (Hiramatsu *et al.*, 2001). *Staphylococcus* has many surface antigens, toxins and enzymes especially protein lytic enzymes, which facilitate its invasion of body tissues and cause an infections (Zadik *et al.*, 2001).

3.2 Characterization of *S. aureus* isolates

3.2.1 Morphological characterization

Morphologically, the isolates show creamy, yellow or golden pigmented colonies on brain heart infusion agar (the diameter of single colony on Mannitol salt agar 0.5 – 1 mm). Moreover, the isolates show greenish colonies with β-heamolysis when cultured on blood agar. Microscopically examination demonstrated grape like clusters of cells with gram-positive reaction.

3.2.2 Biochemical tests

All isolates were given positive result for coagulase production test. Twenty-seven isolates produced acid from lactose and Galactose, three isolates gave negative results for fermenting lactose and galactose because a few isolates did not produce detectable acid from lactose and galactose, these results were in agreement with (Seifert *et al.*, 2003; Kloos *et al.*, 1974).

All isolates were catalase positive and have ability to ferment Mannitol aerobically and 23 isolates from 30 have ability to produce thermonuclease DNase. These isolates also gave negative results in oxidase test with production of β- blood haemolysis when cultured on blood agar, as show in table (3-1).

Table (3-1): The results of biochemical test for 30 *S. aureus* isolates.

NO. of isolates	catalase	oxidase	Free(tube) coagulase	Clumping factor	Heamolysin production	Sugar ferm.			DNase test
						L	G	M	
R1	+	-	+	+	+	+	+	+	+
R2	+	-	+	+	+	-	+	+	+
R3	+	-	+	+	+	+	+	+	-
R4	+	-	+	+	+	+	+	+	+
R5	+	-	+	+	+	+	+	+	+
R6	+	-	+	+	+	+	-	+	-
R7	+	-	+	+	+	+	+	+	-
R8	+	-	+	+	+	+	+	+	+
R9	+	-	+	+	+	+	+	+	+
R10	+	-	+	+	+	+	-	+	+
R11	+	-	+	+	+	+	+	+	-
R12	+	-	+	+	+	+	+	+	-
R13	+	-	+	+	+	+	+	+	+
R14	+	-	+	+	+	+	+	+	+
R15	+	-	+	+	+	+	+	+	+
R16	+	-	+	+	+	+	+	+	+
R17	+	-	+	+	+	-	+	+	-
R18	+	-	+	+	+	+	+	+	+
R19	+	-	+	+	+	+	+	+	+
R20	+	-	+	+	+	+	+	+	+
R21	+	-	+	+	+	+	+	+	+
R22	+	-	+	+	+	+	+	+	+
R23	+	-	+	+	+	+	+	+	+
R24	+	-	+	+	+	-	-	+	+
R25	+	-	+	+	+	+	+	+	+
R26	+	-	+	+	+	+	+	+	-
R27	+	-	+	+	+	+	+	+	+
R28	+	-	+	+	+	+	+	+	+
R29	+	-	+	+	+	+	+	+	+
R30	+	-	+	+	+	+	+	+	+

+: Bacterial growth, - : No bacterial growth, Ferm.: Fermentation,

L: Lactose, G: Galactose, M: Mannitol.

The results of API staph test, which considered as one of the most important test and the most precise one, further confirmation showed that all isolates give positive result for carbohydrate utilization, reduction of nitrate to nitrite, alkaline phosphatase production and acetyl-methyl-carbinol production, acidification due to sucrose utilization.

These isolates give negative results for acidification due to raffinose, xylose, α-methyl-D-glocoside and N-acetyl-glucosamine utilization, also give negative results for arginine dihydrolase production and urease production. These results was similar to standard characteristics results of *S. aureus* ATCC 25923 as show in figure (3-1).

Figure (3-1): Identification of *Staphylococcus aureus* isolates demonstrated by API STAPH system

3.3 Antibiotic sensitivity test of *S. aureus* isolates

Antibiotic sensitivity test performed with twelve types of antibiotics. The percentage of resistance were found 93.3%, 83.3%, 83.3%, 80%, 50%, 33.3%, 30%, 30%, 20%, 20% and 3.3% to the following antbiotics cfotaxime, carbenicillin, tetracycline, gentamicin, cephalixin, fusidic acid, chloramphinicol, bacitracin, vancomycin, streptomycin and imipenem was no resistance found to Amikacin , as show in table (3-2) and figure (3-2).

A study performed by Quentin *et al.* (2001) showed that results were found the percentage of resistance was benzyl penicillin: 90%, gentamicin: 13%, amikacin: 21%, erythromycin: 33%, tetracycline: 17%, fusidic acid: 14%, vancomycin: 0%. The differences may be attributed to the frequency and the specific exposure of each isolates to antibiotics. These results are approach to the results of Udo *et al.*(2003) which show *S. aureus* isolates were resistant to methicillin, gentamicin, streptomycin, erythromycin, and tetracycline. Vancomycin has a bactericidal effect on gram positive bacteria particularly Staphylococci, this antibiotic is a glycopeptid inhibits cell wall synthesis (Booth *et al.*, 2001; Henry and Chambers, 2001). This antibiotic has been the most reliable therapeutic agent against infections caused by *S. aureus* (Hiramatsu, 2001).

Recent reports indicate that *S. aureus* has continued to mutate and has developed intermediate resistance to vancomycin which is acquired by mutation and thickening of cell wall due to accumulation of excess amounts of peptidoglycan. This seems to be a common resistance mechanism for all Vancomycin Resistance *S. aureus* strains isolated in the world so far (Miller and Rudoy, 2000; Hiramatsu, 2001). Recently Sieradzki and Tomasz (2006), show that Vancomycin molecules can also paradoxically inhibit cell wall degradation.

Beta-lactam and vancomycin resistances in gram-positive cocci caused by altered cell wall binding sites with decreased affinity for the drug, the extensive

and often inappropriate, use of antibiotics in the world are the major factor for emergence and spread of antimicrobial resistance (Hand, 2000).

Table (3-2): Antibiotic sensitivity of the locally isolated *S.aureus*.

NO. of isolates	Types of antibiotics											
	VA	S	CN	CTX	C	TE	AK	PY	B	CL	IPM	FA
R1	R	S	R	R	R	R	S	R	R	R	S	R
R2	R	S	R	R	R	R	S	R	R	Int.	S	R
R3	S	R	S	R	S	R	S	R	R	R	R	Int.
R4	S	R	R	R	S	R	S	R	S	R	S	R
R5	S	R	R	R	S	R	S	R	S	R	S	R
R6	S	R	R	R	S	R	S	R	S	R	S	Int.
R7	S	S	R	R	S	S	S	S	S	S	S	S
R8	R	S	R	R	R	R	S	R	R	R	S	R
R9	S	S	R	R	S	R	S	R	S	S	S	S
R10	S	S	R	R	S	R	S	R	S	R	S	S
R11	S	S	R	R	S	R	S	R	R	R	S	R
R12	S	S	R	R	S	R	S	R	S	R	S	S
R13	S	S	R	R	Int.	R	S	R	R	R	S	R
R14	S	S	R	R	S	R	S	R	S	R	S	S
R15	S	S	R	R	S	S	S	R	S	S	S	S
R16	S	Int.	R	R	S	R	S	R	S	R	S	Int.
R17	S	S	S	R	R	R	S	S	S	S	S	S
R18	S	S	S	R	R	R	S	R	S	S	S	S
R19	S	S	S	R	R	R	S	S	S	S	S	S
R20	S	S	R	R	S	S	S	R	S	S	S	S
R21	S	S	R	R	S	R	S	Int.	S	S	S	S
R22	S	S	R	R	S	R	S	R	S	S	S	S
R23	S	S	S	R	R	S	S	R	S	R	S	R
R24	S	S	R	R	S	R	S	R	S	S	S	S
R25	R	R	S	S	S	R	S	R	R	S	S	R
R26	S	S	R	R	S	R	Int.	R	S	R	S	Int.
R27	S	Int.	R	R	R	R	S	R	S	Int.	S	Int.
R28	R	S	R	R	R	R	S	R	R	R	S	R
R29	R	R	R	S	S	R	S	R	R	S	S	Int.
R30	S	S	R	R	S	S	S	S	S	S	S	S

S: Sensitive, R: Resistant, Int.: Intermediate

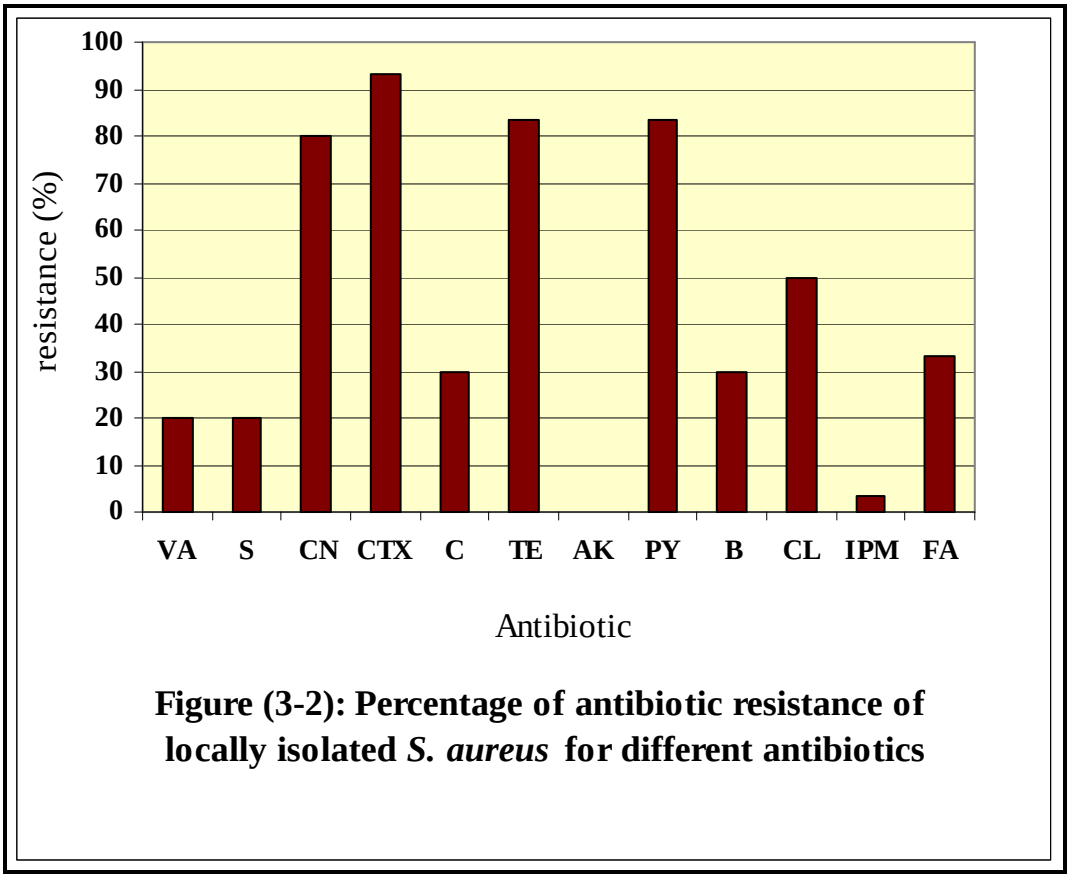

Figure (3-2): Percentage of antibiotic resistance of locally isolated *S. aureus* for different antibiotics

VA: Vancomycin, S: Streptomycin, CN: Gentamicin, CTX: Cefotaxime, AK: Amikacin, C: Chloramphenicol, TE: Tetracycline, PY: Carbenicillin, B: Bacitracin, CL: Cefalexin, IPM: Imipenem, FA: Fusidic acid

3.3.1 Multiple antibiotic resistances of *S.aureus* isolates

Multiple antibiotic resistances show in various isolates as presented in table (3-3).

There was no isolate resistant to only one type of antibiotic. Two isolates were resisting two types of antibiotics; the first was resistant to Gentamicin and Cefotaxime, the second one was resistance to Gentamicin and Tetracycline.

Five isolates were resistant three types of antibiotics; two isolates were resistant to Gentamicin, Cefotaxime and Carbenicillin; two isolates were resistant to Cefotaxime, Chloramphenicol and Tetracycline and the fifth was resistant to Gentamicin, Cefotaxime and Tetracycline.

Four isolates were resistant four types of antibiotics; three were resistant to Gentamicin, Cefotxime, Tetracycline and Carbenicillin; one was resistant to Cefotaxime, Chloramphenicol, Tetracycline and Carbenicillin.

Seven isolates were resistant five types of antibiotics; five isolates were resistant to Gentamicin, Cefotaxime, Tetracycline, Carbenicillin and Cephalexin; one isolate was resistant to Cefotaxime, Chloramphenicol, Carbenicillin, Cephalexin and Fusidic acid; the last one was resistance to Gentamicin, Cefotaxime, Chlioramphenicol, Tetracycline and Carbenicillin.

Three isolates were resistant six types of antibiotics; one isolate was resist to Streptomycin, Gentamicin, Cefotaxime, Tetracycline, Carbenicillin and Cephalexin; another one was resistant to Vancomicin, Streptomycin, Tetracycline, Carbenicillin, Bacitracin and Fusidic acid; last one was resistant to Vancomicin, Streptomycin, Gentamicin, Tetracycline, Carbenicillin and Bacitracin.

Five isolates were resisting seven types of antibiotics; two isolates were resistant to Streptomycin, Gentamicin, Cefotaxime, Tetracycline, Carbenicillin, Cephalexin and Fusidic acid; two isolates were resistant to Gentamicin, Cefotaxime, Tetracycline, Carbenicillin, Bacitracin, Cephalexin and Fusidic acid; the last was resistant to Streptomycin, Cefotaxime, Tetracycline, Carbenicilln, Bacitracin, Cephalexin and Imipenem.

Only one isolate was resistant to eight types of antibiotics which were Vancomycin, Gentamicin, Cefotaxime, Chloramphenicol, Tetracycline, Carbenicillin, Bacitracin and fusidic acid.

Three isolates were resistant to nine types of antibiotics; all of them were resistant to Vancomycin, Gentamicin, Cefotaxime, Chloramphenicol, Tetracycline, Carbenicillin, Bacitracin, Cephalexin and Fusidic acid.

Khan *et al*. (2000) showed that there were multiple antibiotic resistance of *S. aureus* isolates when these isolates resisting ampicillin, penicillin, erythromycin, lincomycin, azithromycin and ciprofloxacin in his study because

these isolates have multiple mechanisms for antibiotic resistanc like inactivation of antibiotics by enzymes, modification of target site, immpaired of penitration of drug target and present an efflux system.

Isolate R2 was chosen for plasmid curing because it had good growth and had resistant to eight types of antibiotics and the four types of heavy metals.

Table (3-3): Multiplicity of antibiotic resistance found in locally isolated *S.aureus*.

Multiplicity	Number of isolates	Pattern of antibiotic resistance
2	1	CN, CTX
2	1	CN, TE
3	2	CN, CTX, PY
3	2	CTX, C, TE
3	1	CN, CTX, TE
4	3	CN, CTX, TE, PY
4	1	CTX, C, TE, PY
5	5	CN, CTX, TE, PY, CL
5	1	CN, CTX, C, TE, PY
5	1	CTX, C, PY, CL, FA
6	1	S, CN, CTX, TE, PY, CL
6	1	VA, S, TE, PY, B, FA
6	1	VA, S, CN, TE, PY, B
7	2	S, CN, CTX, TE, PY, CL, FA
7	2	CN, CTX, TE, PY, B, CL, FA
7	1	S, CTX, TE, PY, B, CL, IPM
8	1	VA, CN, CTX, C, TE, PY, B, FA
9	3	CTX, PY, TE, CL, CN, B, FA, VA, C

CTX: Cefotaxime, PY: Carbenicillin, C: Chloramphenicol, CN: Gentamicin, CL: Cephalexin, TE: Tetracycline, VA: Vancomycin, S: Streptomycin, B: Bacitracin, FA: Fusidic acid, IPM: Imipenem

3.3.2 The minimum inhibitory concentration of antibiotics (MIC) of *S.aureus* isolates

The minimum inhibitory concentration (MIC) of the following antibiotics: Gentamicin, Penicillin-G, Cefotaxime and Tetracycline tested by the agar dilution method for the 30 isolates show the following results:

S. aureus isolates showed high percentage of resistance for the four types of antibiotics that tested against, 93.3% of *S. aureus* isolates showed resistance for Cefotaxime, of these 46.6% show high resistance level at 64 µg/ml while 13.3% of isolates showed a lower resistance level at 128 µg/ml, and there were 33.3% of isolates resisting 256 µg/ml of cefotaxim and the isolates R25, and R29 that represented 6.6% of the isolates showed no resistance.

Eighty percent of *S. aureus* isolates showed resistance for Penicillin-G. Of these, 50% showed high resistance level at 128 µg/ml. While 6.6% of isolates showed a lower resistance level at 512 µg/ml and there were 6.6% of isolates resisting 64 µg/ml, 16.6% of isolates resisting 256 µg/ml. While the isolates R1, R5, R10, R11, R13 and R23, which were, represented 20% of isolates showed no resistance as shown in table (3-4), table (3-5) and figure (3-3).

These results are in agreement with Booth *et al*. (2001) which found that 90% of isolates were resistant to β-lactame antibiotics. Ekiel *et al.* (1995) found that 91.5% of isolates were resistant to penicillin. Moreover, Cuny and Wittee, (1998) did not found any isolates of *S. aureus* sensitive to penicillin. Production of β-lactamase is the main cause of high resistance of *S.aureus* to β-lactam antibiotics since the β- lactame ring is the main constituent of β-lactam antibiotics molecules (Lowy, 2003).

S. aureus isolates which represented 83.3 % showed resistant for Tetracycline. Of these 56.6% showed high resistance level at 128 µg/ml. While 6.6 of isolates showed a lower resistance level at 256 µg/ml and there were 10% of isolates were resist 32 µg/ml, also 10% of isolates were resist 64 µg/ml. In

addition, the isolates R7, R15, R20, R23 and R30, which represented 16.6% of isolates, showed no resistance. The frequent use of Tetracycline to treat wound infections locally may lead to elevate the resistance percentage of *S.aureus* for this antibiotic. The mechanism of resistance for tetracyclins performed by ribosomal protection, active efflux and decreas aptake (Hardy *et al.*, 2004).

Eighty percent of *S. aureus* isolates showed resistance for Gentamicin. Of these, 56.6% of were resisting 32 µg/ml in high resistance level. While 10% of isolates showed a lower resistance level at 16 µg/ml and there were 13.3% of isolates showed resistance at 64 µg/ml, while isolates R3, R17, R18, R19, R23 and R25, which represent 20% of isolates, showed no resistance as shown in table (3-4), table (3-5) and figure (3-3).

Gentamicin was one of the aminoglycsid antibiotics. Aminoglycosids are irreversible inhibitor of protein synthesis (Henry and Chambers, 2001). That was in agreement with results reported by Kuroda *et al.* (2001) that about 45% of the total isolates of *S. aureus* carried a 35.5 kb plasmid and these isolates always showed resistance to gentamicin, tobramycin, kanamycin, amikacin, astromicin, and arbekacin the plasmid carried resistancehere may be transferred easily and that is explain the elevated percentage of resistance to Gentamicin. The introduction of antibiotics in treatment of infections over the last fifty years has lowered the mortality rate of *S.aureus* infections. In the other hand, the bacteria have developed resistance mechanisms to all antimicrobial agents that have been produced (Hardy *et al.*, 2004).

The *Staphylococcus* genome is composed of a complex mixture of genes, many of which seem to be acquired by lateral gene transfer. Most of the antibiotic resistance genes carried either by plasmids or by mobile genetic elements including a unique resistance island (Kuroda *et al*., 2001). It is possible that each resistance results fro more than one mechanism. However, the mechanism of plasmid-mediated resistance was known, there is striking similarity to that found in the *Enterobacteriaceae*. Resistance to

Chloramphenicol, Neomycin/Kanamycin and Streptomycin are due to inactivating enzymes and resistance to Tetracycline is due to decreased uptake (Lacey, 1975).

Table (3-4): MIC of the locally isolated *S. aureus* for some antibiotics.

No. of isolates	Antibiotics MIC of 30 *S. aureus* isolates (µg/ml)			
	Tetracycline	**Gentamicin**	**Cefotaxime**	**Penicillin-G**
R1	128	32	128	No resistance (8)
R2	128	32	256	256
R3	64	No resistance (8)	64	64
R4	128	32	64	128
R5	64	32	256	No resistance (16)
R6	128	32	64	512
R7	No resistance (16)	16	128	128
R8	128	64	64	128
R9	128	16	256	128
R10	128	32	256	No resistance (8)
R11	32	32	64	No resistance (8)
R12	64	64	256	128
R13	256	32	64	No resistance (16)
R14	128	64	64	64
R15	No resistance (8)	32	64	128
R16	32	32	128	128
R17	128	No resistance (8)	64	128
R18	128	No resistance (8)	64	256
R19	128	No resistance (8)	64	256
R20	No resistance (8)	32	256	128
R21	128	32	128	256
R22	128	32	256	128
R23	No resistance (16)	No resistance (8)	64	No resistance (8)
R24	32	32	64	256
R25	128	No resistance (8)	No resistance (16)	128
R26	128	32	256	128
R27	128	32	64	512
R28	256	64	256	128
R29	128	16	No resistance (8)	128
R30	No resistance (8)	32	256	128

Table (3-5): Resistance percentage of *S.aureus* isolates for different concentration of of antibiotics.

Antibiotic	**% Resistance of *S.aureus* for the following concentrations (µg/ml)**								**% Sensitive isolates**
	8	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	
TE	-	-	10	10	56.6	6.6	-	-	16.6
CN	-	10	56.6	13.3	-	-	-	-	20
CTX	-	-	-	46.6	13.3	33.3	-	-	6.6
P-G	-	-	-	6.6	50	16.6	6.6	-	20

CN: Gentamicin, P-G: Penicillin-G, CTX: Cefotaxime,

TE: Tetracycline

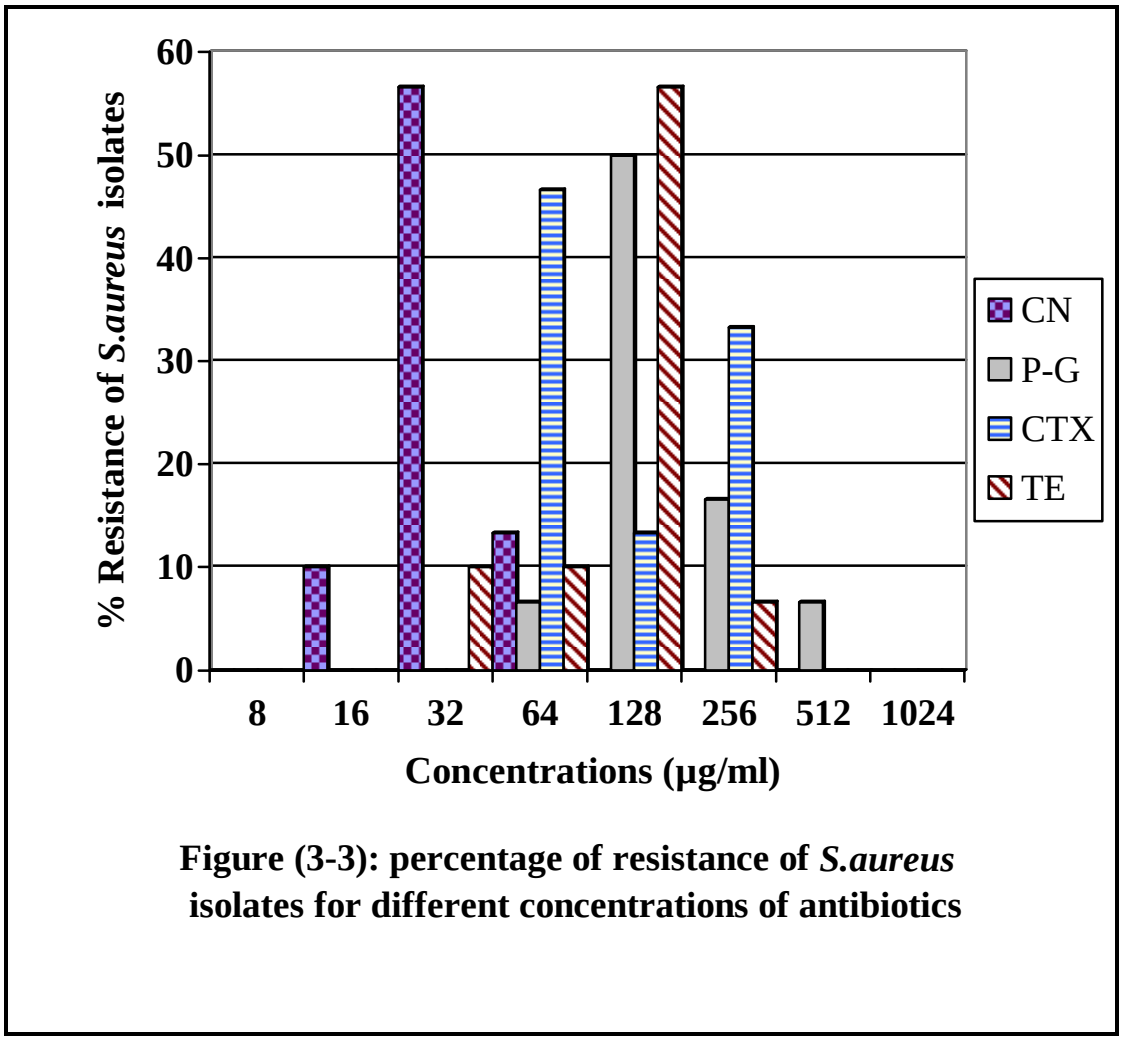

Figure (3-3): percentage of resistance of *S.aureus* isolates for different concentrations of antibiotics

3.4 Heavy metal resistance of *S.aureus* isolates

Thirty isolates tested for the resistance of some heavy metals, using agar dilution method. Bacterial isolates were cultured onto nutrient agar supplemented with different concentrations of Zinc, Cobalt, Cadmium and Mercury, these results were compared with control cultures. In the this study, thirty isolates of *S.aureus* showed a considerable resistance to the tested heavy metals. Some bacteria have evolved mechanisms to detoxify heavy metals, and some even use them for respiration in high concentrations, heavy metal ions react to form toxic compounds in bacterial cells that managed to survive under metal-stressed conditions, bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions.

These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state (Nies, 1999).

Table (3-6): MIC of locally isolated *S. aureus* for some heavy metals.

No. of isolate	Heavy metals MIC for 30 *S. aureus* isolates (mg/ml)			
	Zinc	**Cobalt**	**Cadmium**	**Mercury**
R1	2.0	1.28	0.02	0.02
R2	0.64	0.04	0.02	0.02
R3	1.28	0.64	0.04	0.005
R4	2.0	0.32	0.04	0.02
R5	No resistance	0.16	0.01	0.02
R6	0.32	0.16	0.02	0.005
R7	0.32	0.16	No resistance	0.02
R8	0.64	0.04	0.02	0.005
R9	1.28	0.32	No resistance	0.02
R10	0.32	No resistance	0.02	0.02
R11	0.64	0.16	0.08	No resistance
R12	0.64	0.32	0.16	0.02
R13	0.16	0.04	0.16	0.04
R14	0.32	0.04	0.02	0.005
R15	0.64	0.16	No resistance	0.02
R16	0.16	0.02	0.02	0.02
R17	0.32	0.02	0.04	No resistance
R18	No resistance	No resistance	0.04	0.02
R19	0.64	0.64	0.04	0.005
R20	No resistance	0.16	No resistance	0.005
R21	0.16	0.32	0.08	0.02
R22	0.64	0.32	0.08	0.04
R23	0.16	0.64	0.16	No resistance
R24	0.64	1.28	0.02	0.02
R25	No resistance	1.28	No resistance	0.02
R26	0.64	0.16	0.16	0.02
R27	0.64	0.16	0.02	No resistance
R28	1.28	0.16	0.16	0.005
R29	0.64	0.04	0.02	0.02
R30	0.64	0.04	0.16	0.02

3.4.1 Resistance of *S.aureus* isolates for Zinc ions (Zinc acetate)

There were 86.6% of isolates resist Zinc ions. About 40 % of the tested *S.aureus* isolates showed high resistance level (most of bacterial isolates resist it) at concentration 0.64 mg/ml, while 6.6 % of isolates showed lower resistance level at 2 mg/ml concentration. The remaining isolates showed the following results: 13.3 % of isolates were resist 0.16 mg/ml, 16.6 % of isolates were resist 0.32 mg/ml and 10 % of isolates were resist 1.28 mg/ml while 13.3 % of isolates showed no resistance when cultured at different concentrations. These results were shown in table (3-6) and table (3-7) also figure (3-4).

The highest zinc resistance among bacterial isolates, present in isolates R1 and R4 was found while isolates R13, R16, R21 and R23 showed lowest resistance of zinc ions. However, no resistance development detected in isolates R5, R18, R20 and R25 when tested at low concentrations. These results found to be near to results of Xiong and Jayaswal (1998) study on MIC of Zinc ions at *S.aureus* isolates when they found the MIC of *S. aureus* isolates between 0.5-10 mM, which determined by growing cells in 5 ml tryptic soy broth medium with appropriate concentrations of Zinc and Cobalt ions for 24 hours.

The molecular mechanism of resistance involves a number of proteins, such as ion transporters, reductase, glutathione related cadystins and systeine-rich metallothioneins, and low molecular weight cysteine-rich metal ligands (Silver and phung, 1996). These protein molecules either export the metal ions out of the cell or detoxify or sequester them so that the cells can grow in an environment containing high level of toxic metals. However, there is no common mechanism of resistance to all heavy metal ions (Nies and Brown, 1997).

3.4.2 Resistance of *S.aureus* isolates for Cobalt ions (Cobalt acetate)

There were 93.3% of the isolates resist Cobalt ions, 30 % of the tested *S.aureus* isolates showed high resistance level (most of bacterial isolates resist it)

at concentration 0.16 mg/ml. While 6.6 % of isolates showed lower resisting level at concentration 0.02 mg/ml. And the remaining isolates showed the following results: 20 % of isolates were resist 0.04 mg/ml, 16.6 % of isolates were resist 0.32 mg/ml, 10 % of isolates were resist 0.64 mg/ml and 10 % of isolates were resist 1.28 mg/ml of Cobalt ions, while, 6.6 % of isolates showed no resistance for Cobalt ions when cultured at different concentrations.

The highest Cobalt resistance among bacterial isolates presented in isolates R1, R24 and R25, while isolates R16 and R17 showed lowest resistance for Cobalt ions. However, no resistance detected in isolates R10 and R18, as shown in table (3-6) and table (3-7) also figure (3-4). These results found to be near to results of Xiong and Jayaswal (1998) study on MIC of Cobalt ions at *S.aureus* isolates when they found the MIC of *S. aureus* isolates between 0.5-5 mM, which determined by growing cells in 5 ml TSB medium with appropriate concentrations of Zinc and Cobalt ions for 24 hours.

The trace heavy metal ions such as Cobalt, Zinc, Copper, and Nickel play important roles in bacterial growth. They regulate a wide array of metabolic functions as coenzymes or cofactors, as catalysts and as structural stabilizers of enzymes and DNA-binding proteins (Nies and Brown, 1997). However, these trace heavy metal ions are toxic if exceed the normal physiological levels (Silver *et al.,* 1989). Increasing environmental concentrations of these heavy metals pose a challenge to bacteria. Therefore, bacteria have evolved mechanisms to regulate the influx and efflux processes to maintain the relatively steady intracellular level of the heavy metal ions. Different molecular mechanisms have been reported that are responsible for resistance to various trace heavy metal ions in bacteria (Lelie *et al.,* 1997).

3.4.3 Resistance of *S.* aureus isolates for Cadmium ions (Cadmium Chloride)

There were 83.3% of isolates resisting Cadmium ions. About 33.3% of the tested *S. aureus* isolates showed high resistance level (most of bacterial isolates resist it) at 0.02 mg/ml. while, 3.3 % of isolates showed lower resistance level at 0.01 mg/ml. The remaining isolates showed the following results: 16.6 % of isolates were resist 0.04 mg/ml, 10 % of isolates were resist 0.08 mg/ml, and 20 % of isolates were resist 0.16 mg/ml, while 16.6 % of isolates showed no resistance for cadmium ions when cultured at different concentrations.

The highest Cadmium resistance among bacterial isolates present in isolates R12, R13, R23, R26, R28 and R30, while isolate R5 showed low resistances for cadmium ions. Moreover, isolates R7, R9, R15, R20 and R25 showed no resistance for all concentrations. These results were shown in table (3-6) and table (3-7) also figure (3-4).

Doyle *et al*., (1974) reported that Cadmium had a significant repressive effect on growth in bacterial media containing 40 and 80 µg/ml of Cadmium for *S.aureus* isolates. These results were in agreement with our results obtained in this study.

Olsan and Thornton (1982) suggest that bacterial population could withstand a small input of cadmium ions (several µg/ml) in environment without showing significant change in number of bacterial cell. The Cadmium content of the cells increased with increases in Cadmium content of media (Tornabene and Edwards, 1972). Cadmium enters *S. aureus* through a Mn^{2+}-specific active transport system and accumulates to toxic levels (Crupper *et al*., 1999).

Novice and Roth (1968) and Chopra (1971) reported that certain isolates of *S.aureus* carried resistance factors to some inorganic ions including Mercuric, Cadmium, Arsenate and Lead, also they reported that penicillinase plasmids In *S.aureus* carried resistance factors to some inorganic ions including Arsenate,

Lead, Mercuric and Cadmium. Brien (2002) rported that there was a Plasmid encodes a ß-lactamase, resistance to Cadmium and resistance to fusidic acid.

A plasmid-mediated metal resistance mechanism in *Staphylococcus aureus* is governed by the *cadB* operon, with two genes designated *cadB* and *cadX*. It has been suggested that *cadB* provides protection by enabling cells to bind Cadmium in their cell membranes. Chromosomal DNA mediated Cadmium resistance gene *cadD* in *Staphylococcus aureus* has shown sequence similarity with the *cadB*-like gene from *Staphylococcus lugdunensis* (Naz *et al*., 2005).

3.4.4 Resistance of *S*.aureus isolates for Mercury ions (Mercury Chloride)

There were 86.6 % of isolates resist Mercury ions, 56.6 % of the tested *S.aureus* isolates showed high resistance level at 0.02 mg/ml, while 6.6 % of isolates showed low resistance level at 0.04 mg/ml concentration, and the remaining isolates, which represented 23.3 % showed resistance at 0.005 mg/ml, and 13.3 % of isolates showed no resistance when cultured at different concentrations.

The highest Mercury resistance among bacterial isolates shown in isolates R13 and R22, while isolates R3, R6, R8, R14, R19, R20 and R28 showed low resistance for Mercury ions. However, no resistance detected in isolates R11, R17, R23 and R27. These results were shown in table (3-6) and table (3-7) also figure (3-4).

These results are in agreement with Kondo *et al*.(1974) who reported that the maximal concentration of $HgCl_2$ under which *S. aureus* isolates were able to grow was 20 µg/ml (0.02 mg/ml). As far as, the results obtained in the present study are taken in to consideration, the killing effect of $HgCl_2$ on Staphylococci seem to be much different from those of $CdCl_2$. and the resistance mechanism of Staphylococci to the Mercury ions differ from that of Cadmium ions.

Curing and transfer experiments revealed that the 26-kb plasmid encoded resistance to Cadmium, Mercuric chloride, Propamidine isothionate and Ethidium bromide (Udo *et al.,* 2001).

However, since bacteria are very likely to be confronted with toxic Mercury concentrations, Mercury resistance determinants are very widespread (Silver and Phung 1996).

The mechanism of resistance of *S.aureus* to Mercury considered belonging to the category that the detoxication of noxious substances introduced into bacterial cells by some interacellular mechanisms, which somehow change them into non-noxious form by reduce Mercury ions to a less toxic oxidation state by the bacterial cell (Nies, 1999; Komura *et al.,* 1971). This type of resistance considered as a main mechanism for resisting Mercury ions. Hg ions are rapidly transferred into bacterial cells, and more than 90% of the ions are removed from the culture media after 24 hour when the media containing $HgCl_2$ (Kondo *et al*., 1974).

Table (3-7): Resistance percentage of locally isolated *S.aureus* for different concentrations of heavy metals.

Heavy metal	**% Resistance of *S.aureus* isolates for the following Concentrations (mg/ml)**										**% Sensitive isolates**
	0.005	**0.01**	**0.02**	**0.04**	**0.08**	**0.16**	**0.32**	**0.64**	**1.28**	**2.0**	
Zn	-	-	-	-	-	**13.3**	**16.6**	**40**	**10**	**6.6**	**13.3**
Co	-	-	**6.6**	**20**	-	**30**	**16.6**	**10**	**10**	-	**6.6**
Cd	-	**3.3**	**33.3**	**16.6**	**10**	**20**	-	-	-	-	**16.6**
Hg	**23.3**	-	**56.6**	**6.6**	-	-	-	-	-	-	**13.3**

Zn: Zinc Co: Cobalt Cd: Cadmuim Hg: Mercury

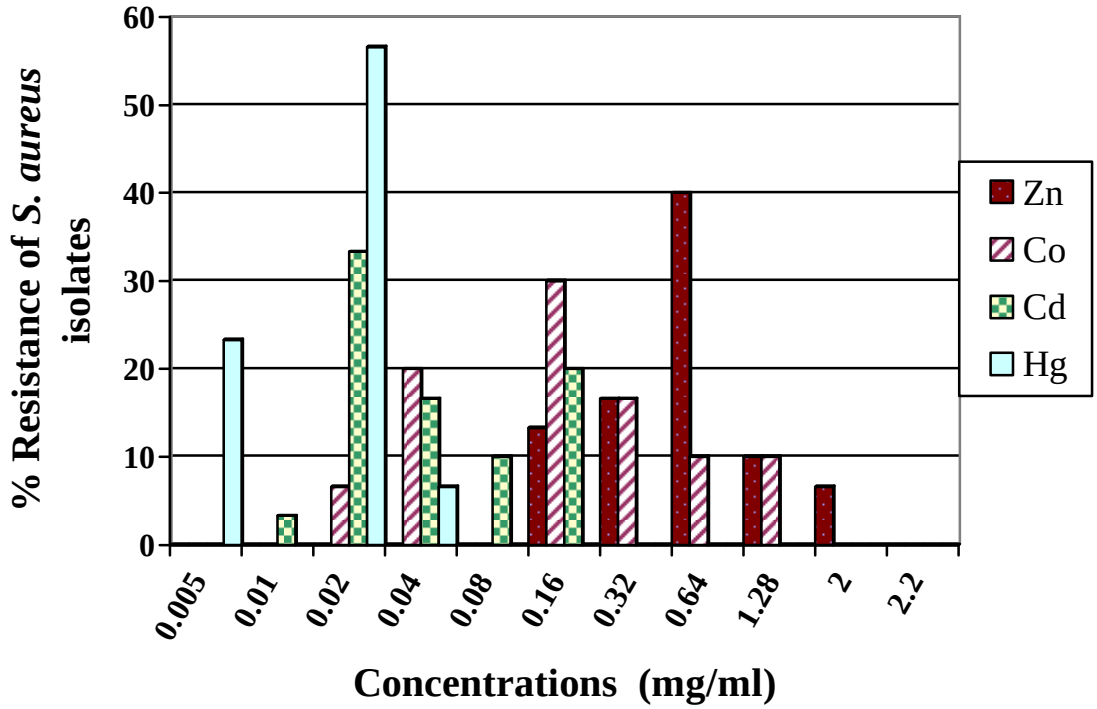

Figure (3-4): percentage of resistance of *S.aureus* isolates at different concentrations of four types of heavy metals

3.4.5 Multiple resistances of heavy metals

The thirty chosen resistant *S.aureus* isolates screened for the development of more than resistance features in a way to demonstrate double, triple and quadruple metal resistance. The tables (3-8) showed the percentage for each type of resistance; quadruple metal resistance represented the highest frequency among the 30 isolates followed by triple resistance and then double resistance to Zinc, Cobalt, Cadmium and Mercury. While single resistance were completely absent for all types of heavy metals. About 60% of isolates were resist to all types of metal ions, which used in this study. While, 30% of isolates were resist to three types of metal ions; of these 13.3% resist Zn, Co and Cd, 10% of the isolates resist Zn, Co and Hg, and 3.3% of the isolates resist Co, Cd and Hg. Moreover, 10% of isolates were resist to two types of metal ions; of these 6.6%

resist Co and Hg, and 3.3% of the isolates resist Cd and Hg, but 0% of isolates showed no resistance to any one type of metal ions as show in table (3-10).

quadruple, Triple and double resistance indicate a very strong genetic link between different heavy metals resistance. The resistance to some heavy metals ions like (Hg and Cd) is mediated by same plasmid that determines resistance to drug (Nakahara *et al.,* 1977). The *cadA* operon has been reported to provide Cadmium resistance in *Bacillus subtilis*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Pseudomonas putida*, *Listeria monocytogenes*, and *Helicobacter pylori*. The *cadA* homolog *zntA* has been reported in *Escherichia coli*, which responsible for Zn, Cd, and Pb (Naz *et al*., 2005).

Mercury resistance was frequently linked with other heavy metal resistance (Timoney *et al.*, 1978). Plasmid-independent chromosomal determinant might encode resistance to heavy metals such as zinc and cobalt (Nies, 1992). While Cadmium and Mercury resistance might encode by plasmid (Silver and Phung, 1996). Chromosomal resistance factors can move to plasmids by means of transposition and become mobilizable to other bacteria (Liebert *et al.*,1999; Summers, 2002).

Table (3-8): Percentage of multiple heavy metal resistance on locally isolated *S.aureus*

Type of resistance	heavy metals	% Resistance isolates
1- Quadruple	Zn, Co, Cd, Hg	60%
2- Triple	Zn, Co, Cd	13.3%
	Zn, Co, Hg	10%
	Zn, Cd, Hg	3.3%
	Co, Cd, Hg	3.3%
3- Double	Zn, Co	0%
	Co, Hg	6.6%
	Zn, Cd	0%
	Co, Cd	0%
	Zn, Hg	0%
	Cd, Hg	3.3%

Zn: zinc, Co: cobalt, Cd: cadmium, Hg: mercury

3.5 Relationship between heavy metals resistance and antibiotics resistance

S. aureus isolates which represented 94.4 % (17 from 18-quadruple heavy metal resistance *S. aureus* isolates) was resistant to Tetracycline at concentrations ranged between (32 -256 µg/ml) this percentage representing 56.6 % of the total isolates. While only the isolate R30 showed no resistance, which represented 5.5% from these isolates, and 3.3% from total isolates. In addition, 94.4 % (17 from 18-quadruple heavy metal resistance *S. aureus* isolates) was resistant to Cefotaxime at concentrations ranged between (64-256µg/ml) this percentage representing 56.6 % of the total isolates. Only isolate R29 showed no resistance, which represented 5.5% from these isolates, and 3.3% from total isolates.

S. aureus isolates which represented 88.8 % (16 from 18-quadruple heavy metal resistance *S. aureus* isolates) was resistant to Gentamicin at concentrations ranged between (16-64µg/ml) this percentage representing 53.3 % of the total isolates. While isolates R3 and R19, showed no resistance, which represented 11.11% from these isolates, and 6.6% from total isolates. Also, 88.8% (16 from 18-quadruple heavy metal resistance *S. aureus* isolates) resisted Penicillin-G at concentrations ranged between (64-256µg/ml) this percentage representing 53.3 % of the total isolates. While isolates R1 and R13, showed no resistance, which represented 11.11% from these isolates, and 6.6% from total isolates.

Plasmids might be capable of encoding resistance to antibiotics specifically related to heavy metals (Silver, Mercury, and Copper resistance (Gilbert and Mcbain, 2003). Genes encoding for metal and antibiotic resistance may be located on the same plasmids and/or transposons, conferring co-resistance (Liebert *et al.*,1999; Summers, 2002). Lacey showed that both of genes for determining the synthesis of penicillinase and for the control of its production very probably carried by one plasmid. Some years later, plasmid DNA corresponding to the phenotypic properties of penicillinase production and metal-

ion resistance was isolated, and this established beyond any doubt that the genes formed part of plasmid (Lacey, 1975).

3.6 plasmid profile

Gel electrophoresis has been done to show the plasmid profile of *S.aureus* isolate R2 before and after curing but there were no results obtained because staphylococci, in contrast to many other bacterial species, are relatively resistance to lytic action of lysozyme, a readily available, inexpensive lytic enzyme (Tomasz and Vollmer, 2003). Consequently, great deal of attention has recently been given to a powerful lytic enzyme of bacteria; lysostaphin, which has a narrow antibacterial spectrum but a high activity against *S.aureus* (Strominger and Ghuysen, 1967). Lysostaphin has been used successfully to isolate plasmid DNA from *S.aureus* and is now indispensable in the preparation of plasmid DNA from this organism. However, enzyme lytic for *S.aureus* as lysostaphin is relatively expensive and in limited supply (Horinouchi *et al.*, 1977).

3.7 Curing of plasmid DNA of *S.aureus* isolate R2 with Ethedium bromide

One isolate had been chosen designated as isolate R2 resistance because it have multi drug and metal resistance and it showing effective growth among the 30 *S.aureus* isolates. Table (3-9) showed that 100 µg/ml of Ethedium bromide was the less concentration which have noticeable inhibitory effect on bacterial growth for the isolate R2 compared with control growth. From this concentration, appropriate dilutions were prepared and spread on brain heart infusion agar plates, which represented as master plate. Then 100 single colonies were taken from master plate and tested on to selective media containing specific antibiotic (Gentamicin, Penicillin-G, Tetracycline and Cefotaxime) or heavy metal (Zinc, Cobalt, Cadmium and Mercury) in order to determine the cured colonies, which cannot grow on this antibiotic or heavy metal containing media.

The effect of Ethedium bromide as interchalating dyes preferential inhibition of plasmid replication. The most effective concentration of the particular curing agent can vary considerably, in the range of 100- to 1000 fold. This depends upon the species being treated, curing agent efficiency, and the mode of action of the curing agent (Carlton and Brown, 1981).

Table (3-9): Different concentrations of Ethedium bromide and its inhibitory effects on bacterial growth for isolate R2 compared with control growth.

Bacterial isolate	Concentrations of Ethedium bromide (µg/ml)									
	control	20	50	100	200	250	400	600	800	1000
R2	+++	++	+	+ -	-	-	-	-	-	-

+++ Heavy growth,++ Good growth,+ Moderate growth, +- light growth, - No growth

Depanding on curing experement which indicated that may be there were two types of cured colonies; colonies lost resistance for Zinc, Cobalt, Cadmium, Penicillin-G and Tetracycline, colonies lost resistance for Zinc, Cobalt, Cadmium, Penicillin-G, Tetracycline and Cefotaxime this indicated loosing for more than one type of plasmid in the last type of colonies of *S. aureus* isolates. While there were no loss of Genamicin and Mercury resistance, which indicated that these markers are not, located on plasmid DNA (located on chromosome or on mega plasmid). That means that may be there were two to three type of plasmids depanding on results obtained from curing experement as shown in table (3-10).

Isolates from the 1960s to 1970s were commonly found to carry multiresistance plasmids conferring resistance to penicillin and heavy metals or other inorganic ions. Such β-lactamase-heavy-metal resistance plasmids characteristically contain the β-lactamase-encoding transposon Tn*552* (transposon 552) or a derivative and operons mediating resistance to arsenical, cadmium, and/or mercuric ions (Firth *et al.,* 2000).

If the resistance is plasmid mediated, those bacteria with clustered resistance genes are more likely to simultaneously pass on those genes to other bacteria, and those bacteria would then have a better chance at survival. In such a situation, one may suggest an association with antibiotic resistance and metal tolerance (Spain and Alm, 2003).

If both or all clustered genes clustered are useful to the organism, it is beneficial to the survival of that organism and its species because those genes are more likely to be transferred together in the event of conjugation. Thus, in an environment with multiple stresses, for example antibiotics and heavy metals, it would be more ecologically favorable, in terms of survival, for a bacterium to acquire resistance to both stresses.

Clinical staphylococci commonly carry one or more plasmids, ranging from small replicons that are phenotypically cryptic or contain only a single resistance gene, to larger episomes that possess several such determinants and sometimes additionally encode systems that mediate their own conjugative transmission and the mobilization of other plasmids (Skurray and Firth, 1997). Udo *et al.* (2003) showed that his isolates carried two plasmids of approximately 26 and 2.8 kb.

Table (3-10): Number of cured bacterial colonies that lost resistance to antibiotics and heavy metals after treatment with Ethedium bromide.

Resistance phenotype	***Staphylococcus aureus***	
	Wild type	**Cured cells**
Zn, Co, Cd, p-G, TE, CTX	100 % resistance	3 % sensitive
Zn, Co, Cd, P-G, TE	100 % resistance	97 % sensitive
Hg	100 % resistance	100 % resistance
CN	100 % resistance	100 % resistance

Zn: zinc, Co: cobalt, Cd: cadmium, Hg: mercury, P-G: Penicillin-G, TE: Tetracycline, CTX: Cefotaxime, CN: Gentamicin

Summary

One hundred and thirty bacterial isolates were collected and identified (from 74 female and 56 male) and thirty *Staphylococcus aureus* isolates were obtained from the overall isolates. Seventy-four isolates (from 17 femal and 13 male).

The thirty *S. aureus* isolates tested for antibiotic sensitivity, 93.3% of them found to be resistant for Cefotaxime. While, 83.3% showed resistance for Carbenicillin, 83.3% for Tetracycline, 80% for Gentamicin, 50% for Cephalexin, 33.3% for Fusidic acid, 30% for Chloramphenicol, 30% for Bacitracin, 20% for Vancomycin, 20% for Streptomycin and 3.3% of isolates resist Imipenem while, there was no resistance found for Amicacin.

S. aureus isolates also showed multiple antibiotic resistance. Such that, two isolates were resist two types of antibiotics. Five isolates were resist three types of antibiotics. Four isolates were resist four types of antibiotics. Seven isolates were resist five types of antibiotics. Three isolates were resist six types of antibiotics. Five isolates were resist seven types of antibiotics. Only one isolate was resist eight types of antibiotics. Three isolates were resist nine types of antibiotics.

The minimum inhibitory concentration of thirty *S. aureus* isolates were determined for four types of antibiotics, which were Teracycline, Gentamicin, Cefotaxime and Penicillin-G, 83.3% of the isolates were resisting Tetracycline at concentrations ranged between (32µg/ml-256µg/ml), 80% of the isolates were resisting Gentamicin at concentrations ranged between (16µg/ml-64µg/ml), 93.3% of the isolates were resisting Cefotaxime at concentrations ranged between (64µg/ml-256µg/ml), 80% of the isolates were resisting Penicillin-G at concentrations ranged between (64µg/ml-512µg/ml).

Resistance of *S. aureus* isolates heavy metals ions were tested; 93.3% of isolates found to be resistant for Cobalt ions (Co^{2+}) at concentrations ranged between (0.02-1.28 mg/ml), 86.6% resisted Zinc ions (Zn^{2+}) at concentrations

ranged between (0.16-2 mg/ml), 86.6% resisted Mercury ions (Hg^{2+}) at concentrations ranged between (0.005-0.04 mg/ml). While, 83.3% of isolates resisted Cadmium ions (Cd^{2+}) at concentrations ranged between (0.01-0.16 mg/ml) and

When multiple resistance for heavy metals were tested, 60% of isolates found to be resistant for Zn, Co, Cd and Hg ions in duadruple resistance. Regarding triple resistance Zn,Co and Cd were resisted by 13.3% of *S. aureus* isolates. 10% of bacterial isolates resisted Zn,Co and Hg ions, while (Zn, Cd and Hg) and (Co, Cd and Hg) multiple resistance found in 3.3% of the tested *S. aureus* isolates. Regarding double resistance; 6.6% of isolates resisted Co and Hg, 3.3% resisted Cd and Hg, while (Zn and Co), (Zn and Cd), (Co and Cd) and (Zn and Hg) double resistance were not found for all *S. aureus* isolates. In addition, single resistance for only one heavy metal was not found.

present resultes revealed a relationship between antibiotic and heavy metal resistance; i.e. 94.4% of quadruple heavy metal resistance of *S. aureus* isolates resisted (64-256µg/ml) of Cefotaxime, 94.4% resisted (32-256µg/ml) of Tetracycline, 88.8% of the isolates resisted (16-64µg/ml) of Gentamicin, and 88.8% of them resisted (64-512µg/ml) of Penicillin-G.

Ethidium bromide was used as a curing agent with freshly growing *S. aureus* to study resistance features link with antibiotic and heavy metal resistance. Results showed two groups of cured colonies, group lost resistance to Zinc, Cobalt, Cadmium, Penicillin-G and Tetracycline. While, The second group lost their resistance to Zinc, Cobalt, Cadmium, Penicillin-G, Cefotaxime and Tetracycline, these results could indicates the presence of more than one type of plasmids. On the other hand, all the cured colonies still showing the resistance to Gentamicin and Mercury, it could be concluded that these markers are not located on plasmids and may be located on chromosomal DNA or on mega plasmid.

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الخلاصة

- شملت هذه الدراسة عزل و تشخيص مائة و ثلاثين عزلة جرثومية (٧٤ عزلة ماخوذة من اناث و٥٦ عزلة ماخوذة من ذكور) عزلت من عينات اخذت من مناطق مختلفة لاجسام مرضى راجعوا مستشفى اليرموك و مستشفى الكاظمية في مدينة بغداد. تبين ان ٥٩ عزلة هي مكورات عنقودية *Staphylococci* (٣٤ عزلة ماخوذة من اناث و٢٥ عزلة ماخوذة من ذكور) و كانت ٣٠ عزلة منها مكورات عنقودية ذهبية *Staphylococcus aureus* (١٧ عزلة ماخوذة من اناث و١٣ عزلة ماخوذة من ذكور). وعند فحص حساسية الاخيرة للمضادات الحياتية كانت 93.3% منها مقاومة لمضاد Cefotaxime. بينما 83.3% منها قاومت مضادي Carbenicillin وTetracycline كلا على حدة. وأظهرت نسبة 80% منها مقاومة لمضاد Gentamicin و 50% منها قاومت مضاد Cephalexin، بينما 33.3% منها قاومت fusidic acid و 30% منها قاومت مضادي Chloramphenicol و Bacitracin كلا على حدة. كما و اظهرت نسبة 20% منها مقاومة للمضادين Vancomycin و Streptomycin كلا على حدة. و كان اشد المضادات تأثيرا هو Imipenem و Amikacin اذ قاومت الاول نسبة ضئيلة بلغت 3.3% من عزلات *S. aureus* و لم تكن هناك عزلة مقاومة للمضاد الثاني.
- عند فحص المقاومة المتعددة للمضادات الحياتية في عزلات *S. aureus* تبين مقاومة ثلاث منها لتسع مضادات حياتية و عزلة واحدة قاومت ثمانية مضادات حياتية و خمسة مقاومة لسبعة مضادات و ثلاثة مقاومة لستة مضادات و سبعة مقاومة لخمسة مضادات و أربعة مقاومة لأربعة مضادات و خمسة مقاومة لثلاثة مضادات و عزلتين فقط مقاومة لمضادين.

- التركيز المثبط الأدنى لعزلات *S. aureus* تم تحديده لاربعة انواع من المضادات الحياتية وهي Tetracycline, Gentamicin, Cefotaxime وPenicillin-G. اذ ان 83.3% من العزلات قاومت مضاد Tetracycline ضمن التراكيز الواقعة بين (32-256µg/ml). ثمانون بالمئة من العزلات قاومت مضاد Gentamicin ضمن التراكيز الواقعة بين (16-64µg/ml). وحوالي 93.3% من العزلات قاومت مضاد Cefotaxime ضمن التراكيز الواقعة بين (64-256µg/ml). وثمانون بالمئة من العزلات قاومت مضاد Penicillin-G ضمن التراكيز الواقعة بين (64-512µg/ml).
- وعند اختبارمقاومة عزلات *S. aureus* لاربعة انواع من المعادن الثقيلة ظهرت 93.3% منها مقاومة لايونات الكوبلت (Co^{2+}) ضمن التراكيز الواقعة بين (0.02-1.28 mg/ml). بينما قاومت 86.6% منها ايونات الزنك ضمن التراكيز الواقعة بين (0.16-2 mg/ml). و 86.6% منها قاومت ايونات الزئبق ضمن التراكيز الواقعة بين(0.005-0.04 mg/ml) . و اظهرت 83.3% من العزلات مقاومة لايونات الكادميوم ضمن التراكيز الواقعة بين (0.16 mg/ml-0.01).
- و فيما يتعلق بتعدد المقاومة للمعادن الثقيلة فقد اظهرت 60% من عزلات *S. aureus* مقاومة رباعية للمعادن الاربعة المذكورة اعلاه. فيما اظهرت 30% العزلات مقاومة ثلاثية لايونات المعادن الثقيلة، منها 13.3% قاومت ايونات الزنك و الكوبلت و الكادميوم معا. بينما اظهرت 10% منها مقاومة لايونات الزنك و الكوبلت و الزئبق معا. لكن اظهرت نسبة ضئيلة و هي 3.3% من العزلات مقاومة لايونات (الزنك و الكارميوم و الزئبق) معا و ايونات (الكوبلت و الكادميوم و الزئبق) معا. و كذلك اظهرت 10% من العزلات مقاومة ثنائية

لنوعين من ايونات المعادن الثقيلة حيث قاومت 6.6% من عزلات *S. aureus* الكوبلت و الزئبق معا فيما قاومت 3.3% منها الكادميوم و الزئبق معا. ولكن لم يلاحظ وجود مقاومة لايونات (الزنك والكوبلت) معا وايونات (الزنك والكادميوم) معا وايونات (الكوبلت والكادميوم) معا وكذلك ايونات (الزنك والزئبق) معا. ولم يلاحظ وجود مقاومة أحادية لنوع واحد من المعادن الاربعة المذكورة اعلاه.

- اظهرت هذه الدراسة علاقة وثيقة بين المقاومة للمضادات الحياتية و المقاومة للمعادن الثقيلة، فعند فحص العزلات ذات المقاومة الرباعية للمعادن الثقيلة ظهر ان 94.4% منها قد قاومت مضاد Cefotaxime ضمن التراكيز الواقعة بين (64-256µg/ml) و94.4% منها قاومت مضاد Tetracycline ضمن التراكيز الواقعة بين (32-256µg/ml)، واظهرت 88.8% منها مقاومة لمضاد Gentamicin ضمن التراكيز الواقعة بين (16-64µg/ml)، و 88.8% من العزلات اظهرت مقاومة لمضاد Penicillin-G ضمن التراكيز الواقعة بين (64-512µg/ml) .
- ولغرض دراسة عوامل مقاومة المعادن الثقيلة و مقاومة المضادات الحياتية عوملت افضل العترات نموا بمادة Ethedium bromide كمادة محيدة للبلازميدات فأظهرت النتائج قسمان من الخلايا المحيدة، حيث فقد القسم الاول من الخلايا مقاومة لكل من ايونات الزنك و الكوبلت و الكادميوم و كذلك المضاد Penicillin-G و المضاد Tetracycline بينما فقد القسم الثاني من الخلايا مقاومة لكل من ايونات الزنك و الكوبلت و الكادميوم و المضادات Penicillin-G و Cefotaxime و Tetracycline مما يدل على وجود أكثر من نوع من البلازميدات في العزلات المفحوصة. و من جهة اخرى حافظت العزلات كافة

على مقاومتها لايونات الزئبق و مضاد Gentamicin مما يدعو الى الاستنتاج بان هذه العوامل المسؤولة عن مقاومة هذه العناصر قد لا تكون محمولة على البلازميدات و ربما تكون موجودة في الحامض النووي الكروموسومي منقوص الاوكسجين DNA او على mega plasmid.

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

دراسة المقاومة للمضادات الحياتية والمعادن الثقيلة في بكتريا *Staphylococcus aureus* المعزولة من إصابات مرضية مختلفة

رسالة
مقدمة إلى كلية العلوم جامعة النهرين
و هي جزء من متطلبات نيل درجة الماجستير في علوم التقانة الإحيائية

من قبل
رفاه علي سمير
بكلوريوس علوم الحياة – الجامعة المستنصرية (٢٠٠٣)

ربيع الثاني ١٤٢٨
نيسان ٢٠٠٧