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Virulence Factors Enhancing Microbial Infection in Chronic Osteomyelitis

A Thesis

Submitted to the College of Science / AL-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

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Dedication

To the candle that burned to enlighten my way in life

My Mother

To the man who was the best supporter to me in my life

My Father

To the man who was always beside me

My Husband

To my little diamond

My son

Ali

To the people who were always encourage me in my life

My Brothers

Saif, Bashar, Mohammad,Marwaa and Noora

To my dearest friend who was always helping me

Maysaa

Rana

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Rana

Supervisors Certification

We , certify that this thesis entitled "**Virulence Factors Enhancing Microbial Infection in Chronic Osteomyelitis"** was prepared by "**Rana Adnan Mohsien"** under our supervision at the College of Science /Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in **Biotechnology** .

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Summary

In this study, twenty five swabs and twenty five biopsies were collected from twenty five patients suffering from chronic osteomyelitis (one biopsy and one swab from each patient) from Al-Yarmouk Teaching Hospital , Surgical Specialist Teaching Hospital and Baghdad Teaching Hospital in Baghdad during the period June 2010 to May 2011 .

Results showed that the most common affected gender with chronic osteomyelitis were males with 84% , and the highest incidence were recorded at the age group of 30-39 years distributed between (7) for males (28%) and (3) for females (12%) .

Depending on cultural , microscopic examination and biochemical characterization , results of both swab and biopsy specimens showed that 50% of the isolates were *Staphylococcus aureus* , 26% *Enterobacter cloacae* , 14% *Pseudomonas aeruginosa* , 6% *Escherichia coli* , and 4% *Klebseilla sp.*

Results of the antimicrobial susceptibility test showed wide variation in response to different (11) antibiotic used . All isolates of *S. aureus* were totally resistance to methicillin , vancomycin , cephalothin and tetracycline followed by clindamycin, gentamicin and penicillin with percentage of resistance of 83% . On the other hand, all *S. aureus* isolates were completely susceptible to ciprofloxacin , chloramphenicol , novobiocin and rifampicin .

A similar result was obtained, when three isolates of *Enterobacter cloacae* were resistant to ampicilllin, amoxicillin, cephalothin, tetracycline, vancomycin, rifampicin and penicillin , two of them resistant to

chloramphenicol and trimethoprim and only one isolate resisted gentamicin . All were susceptible to nalidixic acid and ciprofloxacin .

Regarding *Pseudomonas aeruginosa* and *E.coli*, results of antimicrobial susceptibility showed that they were susceptible to ciprofloxacin but resistant to all other groups of antibiotic . *Klebsiella sp*. isolate was susceptible to chloramphenicol and resistant to all other groups of antibiotic.

Depending on the antibiotic sensitivity results, four isolates of *S. aureus* were selected designated as (S_1 , S_3 , S_4 , S_7 due to their multiple resistances to antibiotics) to determining the minimum inhibitory concentrations (MIC) of four antibiotics (cephalothin, methicillin, tetracycline, vancomycin) . Results showed that the MICs of methicillin were 320µg/ml for S_1 , 80µg/ml for S_3, 40µg/ml for S_4 and 80µg/ml for S_7 . As well as, the MICs of vancomycin were 160µg/ml for S_1, 640µg/ml for S_3, 80µg/ml for S_4 and 40µg/ml for S_7 . In cephalothin , 320µg/ml for S_1, 80µg/ml for S_3, 640µg/ml for S_4 and 160µg/ml for S_7 . In tetracycline, 320µg/ml , 80µg/ml , 640µg/ml and 40µg/ml for S_1 ,S_3 , S_4 and S_7 , respectively .

In order to investigate the virulence factors of Staphylococcus osteomyelitis, four *S. aureus* isolates were found to be able for produce virulence factors like capsule , slime layer , biofilm , hyaluronidase and β-haemolytic of human blood . It was found that all *S. aureus* isolates were able to produce haemolysin enzyme , capsules, and the capsule stain method gave more clear and accurate scene than the negative stain method . Also they were able to produce slime layer by using Congo red agar method, it was useful and more specific method to differentiate between producing and non producing isolates .

As well as , able to produce biofilm; three of them were highly producers, while , the last revealed moderate production of biofilms . And production of hyaluronidase enzyme by using plate and turbidity reduction methods . In plate method, the diameters of hydrolyzed zones were 7 mm, 6 mm, 18 mm and 27 mm for S_1, S_3 , S_7 and S_4 isolates, respectively.

Plate method was faster and superior to distinguish between hyaluronidase producing from non producing isolates than turbidity reduction method which showed that four producing isolates caused reduction in the turbidity of medium .

List of Contents

Chapter One: Introduction and Literature Review

Chapter Two: Materials and Methods

Chapter Three: Results and Discussion

Chapter four: Conclusions and Recommendations

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Meaning
Api 20E system	Analytical profile index for *Enterobacteriaceae*
Api *Staph*.system	Analytical profile index for *S. aureus*
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
NCCLS	National Committee for Clinical Laboratory Standards
rpm	Revolution per minute
H.A.	Hyaluronic acid
min	Minutes
ELIZA	enzyme-linked immunosorbent assay
μm	Micrometer
μg/ml	Microgram per liter
N	Normality
OD	Optical density
MIC	Minimum inhibitory concentration
S_1 ,S_3 , S_4 and S_7	*Staphylococcus aureus* isolates
MSSA	Methicillin sensitive *Staphylococcus aureus*
MRSA	Methicillin resist *Staphylococcus aureus*

Chapter One

Introduction and Literature review

1.1 Introduction :

Infection of bone , which is also called osteomyelitis , can be described as acute , sub acute , chronic also haematogenous and exogenous according to the duration and source of infection . Infection of bone is caused by direct inoculation of bacteria as a result of trauma, by spread of bacteria from adjacent infected areas or by seeding of bacteria from infections elsewhere in the body via the bloodstream (Sax and Lew , 1999) .

Osteomyelitis can become chronic, and lead to the eventual death of the bone tissue, which is caused by loss of blood supply to the affected bone. This occurs when pus produced within the bone, causes bone abscess that deprives the bone from its blood supply (Twaij , *et al.* , 2001) . Chronic osteomyelitis can lead to permanent deformity, so it is important to treat the disease as soon as possible . There are many factors leading to infection, malnutrition, cancer, age extremes. Development of infection is dependent on number of organisms, virulence, and host resistance (Gregory, 2003) .

Twaij , *et al.*, (2001) found that the chronic osteomyelitis is polymicrobial , which means more than one infectious agent is involved. Many types of microorganisms, including viruses, and fungi, may cause osteomyelitis, but it is usually bacterial in origin .

Paediator , (2005) demonstrated that the bacterial osteomyelitis causes substantial morbidity worldwide, despite continued progress toward

understanding its pathophysiology and optimal management. *Staphylococcus aureus* and Gram negative bacteria are the most common organisms caused osteomyelitis. Another study done by Chamber, (2001) who found that the dominance of *S. aureus* that causing osteomyelitis and proposed that may be multifactorial , that have important role to cause infection , such as some enzymes, surface proteins, and toxins were produced as a virulence factors .

Some Staphylococcus enzymes are utilized as invasins because they act locally to promote bacterial invasion. Examples are extracellular enzymes that degrade tissue matrix , allowing the bacteria to spread , called spreading factor , this includes hyaluronidase . Akhtar & Bhakuni , (2004) found that the bacterial hyaluronidases are considered as virulence factors that facilitate the spreading of bacteria in host tissues by degradation of hyaluronic acid . As well as , other toxins that reported by Todar , (2004) degrade membrane components and the pore-forming toxins that insert a pore into eukaryotic cells membranes such as haemolysin .

Mack , *et al.,* (2000) found that slime layer production is considered to be a significant virulence factor for some strains of *Staphylococci* , and had a higher colonization capacity. There is no doubt that the slime layer is a fundamental and clinically-relevant feature of Staphylococcus osteomyelitis. Another virulence factor was studied by Thanh and Lee , (2002) who found that the bacterial capsule may inhibit binding of antibodies , thereby opsonization and phagocytosis do not occur . Also capsule protect cells from toxins , desiccation and play a role in bacterial adherence .

Another Staphylococcus biofilms research done by Wolcott and Ehrlich , (2008) who found that the microbial biofilms represent an important determinant of human chronic infections. In fact , it is now estimated that more than 65% of chronic infection are caused by bacteria growing in biofilms . Also, biofilm protects the bacteria from phagocytosis and the action of antibiotics .

Treatment of osteomyelitis is by appropriate antibiotics and surgery. A number of antimicrobial agents have been used to treat osteomyelitis and surgical procedures may involve removal of necrotic bone and tissue, restoration of blood flow or removal of foreign material (John and Zeller 2008) .

Aims of the study

The study was aimed to :

- Isolation and identification of bacteria causing chronic osteomyelitis.
- Detecting the most efficient antimicrobial agent against isolated osteomyelitis pathogens and determination of minimum inhibitory concentration (MIC) against the most common bacterial isolates .
- Detection of some virulence factors of bacterial isolates , included haemolysin enzyme , capsule , biofilm , slime layer and hyaluronidase enzyme production .

1.2 Literatures Review :

1.2.1 Osteomyelitis :

Osteomyelitis comes from osteo- derived from the Greek word , osteon meaning bone , myelitis - means inflammation of bone or marrow (Mader and Calhoun , 2003) , so, osteomyelitis is an inflammation of bone or bone marrow caused by a pathogenic organism . Although many types of microorganisms including viruses, and fungi, may cause osteomyelitis, it is usually bacterial in origin (Twaij , *et al.*, 2001) .

Kalantari , *et al.* (2007) sub classified osteomyelitis on the basis of the causative organism, the route, duration and anatomic location of the infection .

Sax and Lew (1999) described osteomyelitis as acute, sub acute, chronic also haematogenous and exogenous , according to the duration and source of infection. The infection may be limited to a single portion of the bone or may involve multiple areas .

Pressure sores may be associated with underlying osteomyelitis that is difficult to differentiate clinically from infection or colonization of adjacent soft tissue (Darouiche, *et al.*, 1994) . Durden , *et al.* (1987) demonstrated that bacterial infection of bones (osteitis) usually osteomyelitis, predominantly of bone marrow or joint may come via blood-stream usually from a septic focus elsewhere in the body, or it may be direct, via a wound .

Bacterial osteomyelitis causes substantial morbidity worldwide, despite continued progress toward understanding its pathophysiology and optimal management . The approach that cause osteomyelitis depends upon the route by which bacteria gained access to bone, bacterial virulence, local and systemic host immune factors and patient age (David and Mark , 1996) .

Although bones are usually well-protected against infection, bacteria can become the causative agents in several ways .

Bacteria can enter the bone through an open fracture, penetration by a sharp, contaminated object or orthopedic surgery . A wound and injury that permit bacteria to reach the bone directly, when the bone is infected , pus is produced within the bone , which may result in an abscess which then deprives the bone of its blood supply (Twaij , *et al.* , 2001) .

1.2.1.1 Classification of osteomyelitis :

Waldvogel , *et al*. (1970) described the first osteomyelitis staging system , classification of osteomyelitis was based on a number of criteria such as the duration and mechanism of infection and type of host response to the infection , the mechanism of infection can be haematogenous or exogenous osteomyelitis is caused by open fractures, or contiguous spread from infected local tissue while the haematogenous form results from bacteremia. Osteomyelitis can be classified as pyogenic or non pyogenic, also it was based on host response to disease .

Gierny, *et al.* (1999) proposed a classification system for chronic osteomyelitis based on host factors, anatomical criteria , the duration of symptoms and type of infection (Gregory, 2003) .

- **Acute haematogenous osteomyelitis :**

Is a serious disease characterized by an infection of bone marrow, cortex and periosteum (Dinesh , 2006), it is caused by a bacteremia which is a common occurrence in childhood therefore it is a specific clinical entity in children; males are affected twice as females, while this disease is decreasing in frequency in the developed world (Blyth , *et al*. , 2001) .

Bacteriological seeding of bone generally is associated with other factors such as localized trauma, chronic illness e.g. diabetes or malnutrition, etc . Bacterial seeding leads to an inflammatory reaction which can cause necrosis of bone and subsequent abscess formation (Gregory, 2003) .

Staphylococcus aureus causes the vast majority of cases of acute haematogenous osteomyelitis which is responsible for over (80-90)% of positive culture cases and the remaining (10-20)% of culture cases were caused mainly by *Pseudomonas aeruginosa* , Gram-negative enteric organisms and other more rare organisms e.g. fungi (Matusse , *et al.* , 2002).

- **Sub acute osteomyelitis :**

Sub acute osteomyelitis was first described by sir Benjamin Brodie in 1836 , it is a haematogenous infection of bone characterized by course longer than two weeks and paucity of systemic manifestation with local tenderness or swelling are the only clinical signs . The presumed cause for such a course is attributed to a combination of host resistance and low virulence of the infective organism (Tachdjian , *et al*., 1990) .

Subacute osteomyelitis can be difficult to diagnose because the characteristic symptoms and signs of indolent as well as non specific (Hayes, *et al*. ,1990) . A Brodie's abscess is a localized form of sub acute osteomyelitis that occurs most often in the long bones of young adults (Gergory, 2003) .

- **Chronic osteomyelitis :**

Chronic osteomyelitis is a superlative infection of bone that follows by long-term course of intermittent symptoms. Although acute osteomyelitis occurs in the first 6 weeks after the initial infection, chronic osteomyelitis

can flared years later . The primary cause of persistent infection was presence of dead bone to which systemic antibiotics cannot be effectively delivered therefore chronic osteomyelitis that is difficult to eradicate completely, the most common etiology of chronic osteomyelitis is trauma, in which bone is devitizalized from the outset because of its association with motor vehicle accidents (Carek , 2001) .

Chronic osteomyelitis is polymicrobial , which means more than one infectious agent is involved , the role of bacteria in chronic osteomyelitis is solely an opportunistic one, in this case , bacteria take advantage of bone that is already injured . Because of necrotic bone is present from the outset, therapy must be first and foremost focused upon the surgical elimination of all devitalized tissue (Bhandar, 1999) .

In chronic osteomyelitis, secondary infection are common, multiple organism may grow from culture, so increase frequency presence of Gram-negative enteric bacilli such as *E. coli, Klebsiella spp*. (James & Gruss, 1983) .

The dominance of *S. aureus* in causing osteomyelitis may be multifactorial , this includes the frequency of its asymptomatic carriage that range from 25% to 50% in the community (Chamber , 2001) . Chronic osteomyelitis may be due to the presence of intracellular bacteria (inside bone cells) once intracellular, the bacteria are able to escape and invade other bone cells . In addition, once intracellular, the bacteria becomes resistant to antibiotics, these combined facts may explain the chronicity and difficult eradication of this disease as in figure (1-1) . This results in significant costs and may even lead to amputation . Intracellular existence of bacteria in osteomyelitis is likely an unrecognized contributing factor to its chronic form .

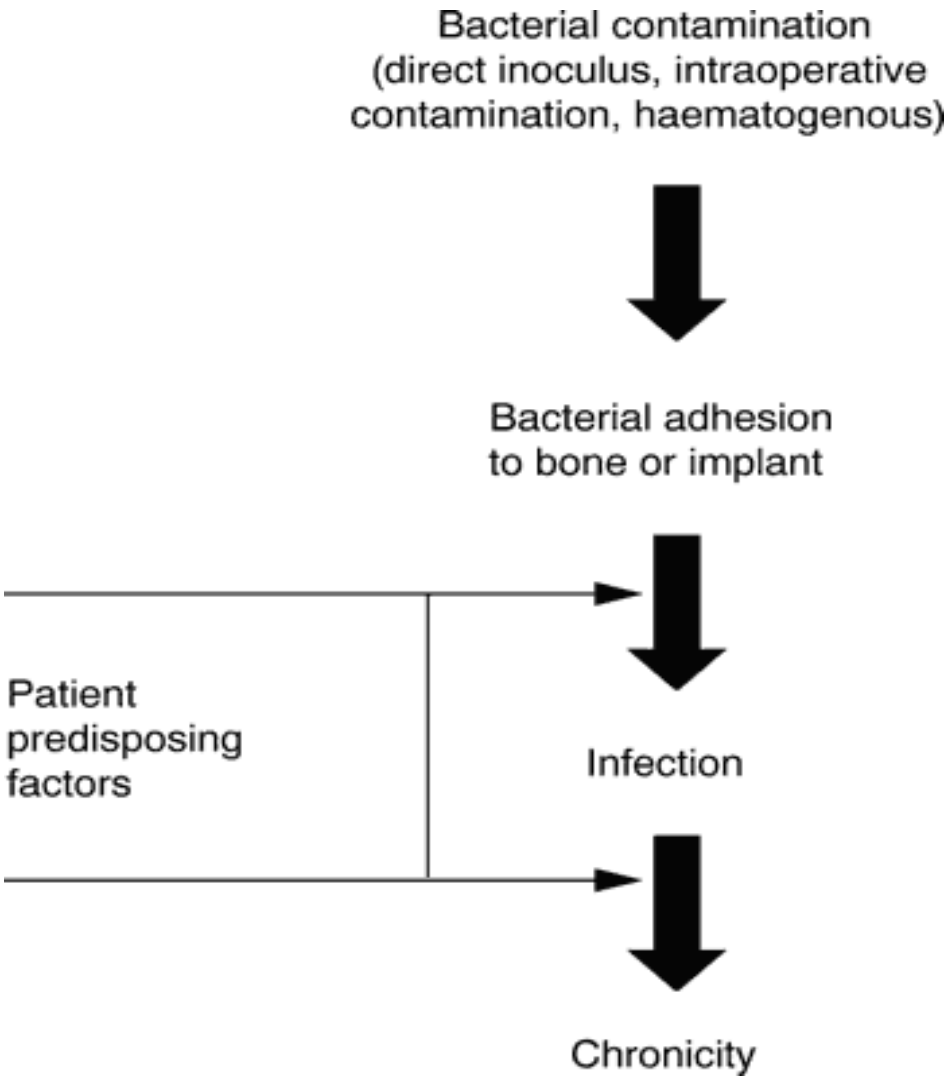

Figure (1-1) Pathogenesis of chronic bacterial osteomyelitis (Ciampolini and Harding, 2000) .

1.2.1.2 Nosocomial infections :

Infections that are hospital acquired (Garner, *et al*., 1996) , the most popular types of nosocomial infection causing bacteria are the Gram negative bacilli *Pseudomonas aeruginosa* and *Enterobacteriaceae* , they were responsible for 32% of nosocomial infection, the most common was

Escherichia coli, *Pseudomonas aeruginosa, Enterobacter spp.* and *Klebseilla spp.*(Weinstein , 1998) .

Pseudomonas aeruginosa is an opportunistic pathogen that accounts for 10.1% of all nosocomial infection and approximately 25% of all nosocomial infections through hospital acquired , it is contiguous osteomyelitis which can result from open wound or haematogenous spread from the primarily infection site .

Enterobacter bacteria have developed an acquired resistance to many commonly used antibiotics such as erythromycin, tetracycline ,clindamycin, penicillin and vancomycin (Richard, *et al*., 2000) .

E. coli is one of the four most common Gram negative bacteria and together with *Pseudomonas aeruginosa*, *Enterobacter spp.* and *Klebseilla spp.* cause 32% of nosocomial infections (weistein, 1998) .

1.2.1.3 Bacterial infection of bone :

Bacterial infection is the most common cause of chronic osteomyelitis.

1.2.1.3.1 Gram-positive bacteria :

- ***Staphylococcus aureus* :**

Is a facultative anaerobic , Gram-positive cocci which appears as grape like clusters when viewed though a microscope and has large, round, golden-yellow colonies, *Staphylococcus aureus* is the most common bacterial pathogen causing osteomyelitis, it is one of acute haematogenous infection cases and responsible for up to 90 % of cases healthy person .

Zimmerli, *et al*. (1998) explained the reason of this problem is due to production of Staphylococcus toxins which enhance the virulence and frequent colonization . In chronic osteomyelitis, *Staphylococcus aureus* is a

common cause of infection and treatment of chronic *Staphylococcus aureus* osteomyelitis in adults typically consists of surgical debridement and prolonged therapy with an intravenous antimicrobial agent active against the infecting *Staphylococcus aureus* strain .

Both of Cohen (1986) and Brock & Modigan (1988) have attributed the dominance of *Staphylococcus aureus* was due to production of virulence factors such as hyaluronidase, lipase, protease and possibly staphylokinase, facilitated dissemination by their enzymatic breakdown of connective tissues ground substance .

Another investigators mention that the production of protective glycocalyx coating protein, help or facilitate *S. aureus* to adherence to smooth surface (Baron , *et al.,* 1994 ; Jawetz , *et al*., 2010) so play a central role in its pathogenicity.

1.2.1.3.2 Gram-negative bacilli :

In chronic osteomyelitis, secondary infection are common, multiple organism may grow from culture, so increase frequency presence of Gram-negative enteric bacilli such as *E. coli* , *Klebsiella spp*. , *Proteus spp.* and others (James & Gruss, 1983) . Also Gram negative bacteria are frequently a cause of surgical wound infection usually contained different organisms (Culver , *et al.*, 1991) and reported that *E. coli* major once of Gram negative bacteria causing surgical wound infection then *Pseudomonas spp.* and *Enterobacter spp.* (Kasser , *et al.*, 1993) .

Many species of Gram-negative bacteria are pathogenic, meaning they can cause disease in a host organism .This pathogenic, capability is associated with certain components of Gram-negative cell walls , in particular the lipopolysaccharide (Salton , *et al*., 1996) . The incidence of

Gram negative bacilli causing osteomyelitis has increased especially in open fracture and the combination of Gram positive cocci such as *S .aureus* and Gram negative bacilli, such as *P. aeruginosa* are frequently occur in wound complicating open fracture (Edward , *et al*., 1978) .

1.2.1.3.3 *Enterobacteriaceae* :

The *Enterobacteriaceae* are Gram-negative bacilli include *Escherichia coli*, *Klebseilla spp.*, *Enterobacters spp.*, *Serratias spp.*, *Proteus spp.* and others account for 10-15% from organism that cause osteomyelitis (Kusisec , 1996) .

A- *Escherichia coli* :

Is Gram negative bacilli, facultative anaerobic and lactose fermenting, they are either non motile or motile by large intestine, *E. coli* is the most of frequent causes of some of the many common bacterial infections, include osteomyelitis (Mylonakis, *et al.* , 2007) .

In chronic osteomyelitis secondary infection due to Gram negative bacteria are frequently isolated specially in deeper infection are usually polymicrobial, aerobic Gram positive cocci and Gram negative bacilli e.g. *E. coli* (Wheat , *et al*., 1986) .

B- *Klebseilla spp.* :

Klebsiella spp. is Gram negative , non motile , encapsulated , lactose fermenting , facultative anaerobic , rod shaped , bacterium found in the normal flora of the mouth, skin, and intestines (Ryan , *et al* ., 2004) , it is clinically the most important member of *Enterobacteriaceae* .

Klebsiella have become important pathogens in nosocomial infections.The medically most important *Klebsiella* species was *Klebsiella pneumoniae*, accounts for a significant proportion of hospital-acquired urinary tract infections, pneumonia, septicemias, osteomyelitis and soft tissue infections (Podschun , *et al.* , 1998) . This organism colonize the gut, and is probably transmitted mainly by hand contamination of hospital acquired , *Klebsiella* infection are more virulent and refractory to both therapy and effective control of intra-hospital spread (Thomas , 1977) .

C- *Enterobacter spp.* :

It is Gram negative bacilli and important nonsocomial pathogens responsible for various infections, include bacteremia, lower respiratory tract infection, skin , soft tissue infection , septic arthritis , osteomyelitis and wound infection. *Enterobacter* bone and joint infection are usually implicated in septic arthritis and osteomyelitis in adults and children, they are usually more difficult to cure than those caused by *S. aureus* (Frase and Arnett , 2008) .

D- *Pseudomonas spp.* :

Is Gram negative bacilli, an opportunistic human pathogen , it can penetrate the wounds of feet and heels and relatively cause massive wound infections and osteomyelitis , especially in children (Ryan , *et al*., 2004) .

Pseudomonas aeruginosa was first associated with osteomyelitis following open wound of children feet also, osteomyelitis following trauma with contamination is usually associated with skin flora, especially *Staphylococcus aureus*, however infection following open wounds of the feet is recognized as a special category of problem. Infection occurs in

(3-15) % of children with open wounds , so in osteomyelitis, the second most common infection was *Pseudomonas aeruginosa* (Filagerald , *et al*., 1975) .

1.2.1.4 Virulence factors of *Staphylococcus aureus* :

The virulence of *S. aureus* infection is remarkable, given that the organism is a commensal that colonizes the nares , axillae , vagina , pharynx, or damaged skin surfaces. Infections are initiated when a breach of the skin or mucosal barrier allows *Staphylococci* access to adjoining tissues or the bloodstream .

Baselga , *et al*. (1994) explained that the virulence factors included surface proteins that promote colonization in host tissues, invasions that promote bacterial spread in tissues (leukocidin, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule) .

1.2.1.4.1 Haemolysin enzymes :

A substance that causes haemolysis is a haemolysin , it is a cytolytic enzyme secreted by many bacteria isolated from patients with osteomyelitis, the production of haemolysins is frequently associated with virulence in various bacterial species (Wojnicza, *et al*., 2007) , it is commonly produced by Gram positive and in most cases is considered to be virulence factor (Rowe , *et al.* , 1994) .

In Gram positive bacteria there is evidence of the involvement of haemolysins or cytolysins in the pathogenesis of infection e.g. streptolysin produced by *Streptococcus mutans* . The Staphylococcus ß-toxin acts as an haemolysin enzyme (Rogolsky and Breuil , 1979) .

1.2.1.4.2 Capsule production :

Certain bacteria possess a capsule around their cell wall, usually composed of polysaccharide and polypeptide in some organisms, they are manufactured by the cell membrane. They serve to protect cells from toxins , desiccation and antibodies, and play a role in bacterial adherence. Capsules can be used to identify certain organisms , e.g. *Staphylococcus aureus* .

About 90% of *S. aureus* isolates produce capsular polysaccharide (Hochkeppel , *et al* . , 1987) , which forms a layer on the outer surface of the peptidoglycan in the cell wall (Karakawa and Vann , 1982 ; Sompolinsky , *et al* . , 1985) . The *S. aureus* capsule was typical Gram positive cell wall (Giesbrecht , *et al*. , 1998) that inhibit binding of antibodies and thereby opsonization , and phagocytosis (Thakker, *et al* . , 1998; Thanh and Lee , 2002) .

Together with intercellular polysaccharide adhesins, *S. aureus* capsular polysaccharides enhance biofilm constitution by augmenting adhesiveness (Lowy , 1998) .

1.2.1.4.3 Slime layer :

It is an extracapsular, polysaccharide structure, which may influence virulence by increasing bacterial adhesion to host cells and inhibiting phagocytosis (Tarkowski , 1999) . Slime production is considered to be a significant virulence factor for some strains of *Staphylococci* (Ammendolia , *et al*. , 1999 ; Mack , *et al. ,* 2000) .

Some investigators reported that slime- producing *S. aureus* strains had a higher colonization capacity than its non-slime-producing variants did. Therefore, *S. aureus* slime may play a role in the establishment of infection (Baselga , *et al*. , 1993; Ammendolia , *et al*. , 1999) .

The importance of the role played by slime is further increased by its frequent association to reduced antibiotic susceptibility (Kloos & Bannerman, 1994) . Also the difficulty in eradicating a chronic infection associated with slime formation has been reported, and slime-producing bacteria has been shown to resist higher antibiotic concentrations than non-slime-producing bacteria (Gristina , *et al.* , 1987) .

Mayberry-Carson, *et al.* (1984) and Alderson, *et al*. (1986) suggested that a major contributing factor in bone and joint infection is the production of a bacterial glycocalyx or slime layer that promotes the adherence of bacteria to host tissues .

Several investigators explained that the glycocalyx forms the foundation of the multilayered biofilm containing bacterial microcolonies, there is no doubt that the glycocalyx is a fundamental and clinically-relevant feature of *Staphylococcal* osteomyelitis . However , it is not clear whether the glycocalyx promotes the initial adherence of bacteria or whether adherence is dependent on other factors , with glycocalyx production and biofilm formation being the consequence of attachment and the subsequent growth of bacteria. This scenario implies a two-stage process in which bacteria first attach to a substrate (e.g., bone) and then attach to each other as the biofilm grows and matures (Mayberry-Carson , *et al*., 1984 ; Evans, *et al.*, 1998) .

1.2.1.4.4 Biofilm production :

Microbial biofilms represent an important determinant of human chronic infections, bacterial biofilms involve a genetically-coordinated sequence of events, including initial surface attachment, microcolony formation and community expansion , this leads to a complex and structured architecture

protecting bacteria from host-defense mechanisms and killing by antimicrobials (Rebecca , *et al*., 2008) .

A biofilm is complex structure of microbial cells that are attached to a surfaces and substrate , interface , or to each other , are embedded in a matrix of extracellular polymeric substance . It can form on biotic or abiotic surfaces and can comprise of single or multiple microbial species , biofilm depth can vary , from a single cell layer to a thick community of cells surrounded by a thick polymeric milieu (Costerton ,*et al.*, 1995).

Among the most clinically significant bacterial pathogens is *S. aureus*, a leading cause of bone infections , most *S. aureus* clinical isolates showed the capacity to adhere to abiotic surfaces and to develop biofilms . Since *S. aureus* growing in biofilm is highly refractory to treatment, inhibition of biofilm formation represents a major therapeutic objective (Fitzpatrick ,*et al.*, 2005) .

The biofilm is associated with the production of an extracellular matrix , the main constituent of the sugar-based matrix of *S. aureus* biofilm is called the polysaccharide intercellular adhesion . *Staphylococcus spp*. that can produce a multilayered biofilm embedded within a glycocalyx, or slime layer. The glycocalyx develops on devitalized tissue and bone, or on medically implanted devices, to produce an infection (Akiyama , *et al.*, 1993; Ziran , 2007) .

Recent developments in research have seen the recognition of the association between biofilms and some chronic disease : osteomyelitis (Gristina , *et al*., 1985), otitis media (Hall-Stoodley, 2006) and cystic fibrosis(Costerton , 2001). In fact , it is now estimated that more than 65%

of chronic infection are caused by bacteria growing in biofilms (Wolcott and Ehrlich , 2008) .

1.2.1.4.5 Hyaluronidase :

Hyaluronidase (hyase) is a general term initially introduced by Karl Meyer in 1940 to describe enzymes that are able to breakdown primarily hyaluronic acid (H.A.) a major component of the extracellular matrix of human tissues (Rees, *et al* . , 2007) . For a long period of time the hyaluronidases were a group of poorly characterized , neglected enzymes (Kreil , 1995) .

Hyaluronidases , then have received attention and importance due to their regulatory function in hyaluronic acid metabolism , Meyer classified hyaluronidases into three different groups based on biochemical analysis and generated end products (Meyer , 1971) , the three groups include: (1) Mammalian hyaluronidases (testis type) , (2) Leech hyaluronidase , (3) Microbial hyaluronidases .

The hyaluronidases were first discovered at the beginning of the last century in 1928 by Duran-Reynals in extracts of mammalian testis and were identified as “spreading factors” due to their ability to facilitate the diffusion of antiviral drugs , dyes and toxins(Chain and Duthie , 1940) .

This enzyme plays an important role in processes of invasion by depolymerization of the mucoid ground substance H.A. of connective tissue. Additional evidence for the role of the enzyme in increasing the permeability of the host tissue has been contributed by the observation that hyaluronidase and “spreading factor” are identical (Hahn , 1943) , although all extracellular hyaluronidases are probably spreading factors, not all spreading factors are hyaluronidases (Kreil , 1995) .

The increasing in membrane permeability resulted in reduced viscosity of connective tissues and allows the injected drugs to diffuse farther and penetrate tissues more easily . According to Duran-Reynals (Duran- Reynals , 1942) the degree of invasiveness of bacteria is largely determined by the amount of spreading factor present, and the successive phases of infection induced by invasive bacteria include the hydrolysis of the mucoid ground substance of the connective tissue and spreading primarily through the interstitial system of the connective tissue .

The discovered enzymes differ in their molecular weight , substrate specificity or pH-optimum (Kreil , 1995) , the enzyme acts as an adjuvant and its ability to promote penetration and spread , was used to accelerate and increase absorption , dispersion of injected drugs, *e.g.* antibiotics, and improve the effectiveness of local anaesthesia and to diminish pain due to subcutaneous or intramuscular injection of fluids (Csoka , *et al.* , 1997) .

Also used as a spreading factor in several medical fields *e.g.* orthopaedia, surgery, ophthalmology (Meyer & Palmer, 1934) , dermatology, dentistry (Tam & Chan , 1985) , oncology (Muckenschnabel , *et al* . , 1998) , gynecology (Farr , *et al* . , 1997) also used in obstetrics, to accelerate the changes to the uterine cervix necessary for childbirth (Kavanagh , *et al.* , 2006) .

The application of hyaluronidases leads to diminished tissue destruction after subcutaneous and intramuscular injection of fluids(Frost , *et al.* , 1996; Pirrello , *et al.* , 2007) . Hyaluronidase was also investigated as an additive to chemotherapeutic drugs for augmentation of anticancer activity and there is evidence that hyaluronidase itself may have intrinsic anticancer effects and can suppress tumor progression (Muckenschnabel , *et al.* , 1998) .

A wide variety of microorganisms produce hyaluronidases , these enzymes produced by the Gram-negative organisms are periplasmic rather a role in pathogenesis (Spellerberg , 2000) while , in Gram-positive bacteria, the enzymes are often extracellular secreted and are thought to have a role in pathogenesis (Hynes and Walton, 2000; Makris , *et al*., 2004) .

Bacterial hyaluronidases are considered as virulence factors that facilitate the spreading of bacteria in host tissues by degradation of H.A. (Akhtar & Bhakuni , 2004) . Among the Gram positive bacteria , hyaluronidase production has been shown for pathogenic strains of *Staphylococcus aureus* (Skalka , 1985) .

In this study, the microbial hyaluronidases (bacterial hyaluronidases) had been studied .

- **Role in diseases :**

In microbes, hyaluronidases are virulence factors involved in pathogenesis and disease progression caused by the pathogen, often hyaluronidases directly interact with host tissues or conceal the bacteria from host-defense mechanisms. The enzymatic degradation of extracellular matrix components of host tissues facilitates the invasion of pathogens .

The enzyme degrading activities of microbes may facilitate adhesion, colonization and provide nutrients (Hynes and Walton , 2000). Disaccharides are the major end products of H.A. degradation and these can be transported and metabolized intracellularlly to supply needed nutrients (as a carbon source) for a pathogen as it replicates and spreads (Makris , *et al*. , 2004) .

Enhanced tissue permeability caused by the action of hyaluronidases appears to play a major role in some infections, pneumonia and other types

of sepsis such as bacteremia and meningitis (Matsushita and Okabe, 2001; Jedrzejas , 2004) .

1.2.1.5 Antimicrobial agents :

Chronic osteomyelitis has been a difficult problem for patients and doctors , appropriate antibiotic therapy is necessary to arrest osteomyelitis along with adequate surgical therapy , there are many factors involved in choosing the appropriate antibiotics include infection type, infecting organism, sensitivity results, host factors, and antibiotic characteristics .

Sensitivity of a microorganism to antibiotics requires that the organism should possess an appropriated target for the drug (e.g. receptor for the drug), the major mechanism has been mentioned early by which antimicrobial agents produce killing or inhibition of bacterial cell growth, organisms which lack an appropriate target for antibiotic action are said to possess intrinsic resistance (Brook , *et al.*, 2003) .

Resistance may develop in previously susceptible organisms in one of the two basic ways:

A- Mutation a spontaneous, random, permanent alteration in the DNA base sequence of the organism .

B- Transfer of genetic information from one organism to another, either through chromosomal recombination (transformation, transudation, conjugation or by extra-chromosomal–plasmid- transfer) .

Initially, antibiotics are chosen on the basis of the organisms that are suspected to be causing the infection , once the infecting organisms is isolated and sensitivities are established . In selecting specific antibiotics for the treatment of osteomyelitis, the type of infection, current hospital

sensitivity resistance patterns, and the risk of adverse reactions must be strongly appraised .

Antibiotic classes used in the treatment of osteomyelitis include penicillins, cephalosporins, other β-lactams (aztreonam and imipenem) , also vancomycin, clindamycin, rifampicin, aminoglycosides, fluoroquinolones and trimethoprim . Traditional treatments have used operative procedures followed by several weeks of parenteral antibiotics (Mader , *et al* . , 1999) .

Oral fluoroquinolones (lomefloxacin, levofloxacin, and ciprofloxacin) are safe, effective therapy for the treatment of chronic osteomyelitis , if they are given for a prolonged course as treatment for infections caused by susceptible Gram-positive as well as Gram-negative organisms, and in combination with adequate surgical debridement .

A great interest has been shown in the use of fluoroquinolones, because they can be taken by the oral route also, have a good bone distribution and a rapid efficacy on many Gram positive and negative bacteria (Rissing , 1997 ; Mader , *et al* . , 1999) , the efficacy of these treatments ranged from 65% to 95% (Dan , *et al* . , 1990) .

The combination of a quinolone with other oral agents active against Gram-positive pathogens, such as clindamycin or rifampicin, offers a reasonable option for use in future studies in an effort to further improve cure rates for chronic osteomyelitis caused by Gram-positive pathogens (Rissing ,1997). Combination therapy including rifampicin are controversial although it is widely used in deep infections and when the response to standard antistaphylococcal antibiotic therapy alone is either poor or slow(Tan and File , 1996 ; Lowy , 1998) .

Rifampicin shows excellent antistaphylococcal activity against MSSA and MRSA strains including penetration into cells and biofilms , and ability to kill phagocytosed bacteria (Craig , 2004 ; Calfee , 2005) .

Krut , *et al.* (2004) found that rifampicin was effectively eradicated *S. aureus* also in non-phagocytic cells *in vitro* , which is suggested to be valuable in the treatment of invasive *S. aureus* infections , the combination therapy with rifampicin and vancomycin as compared to single-drug treatment in chronic Staphylococcus osteomyelitis although the drug combination was antagonistic *in vitro*(Dworkin , *et al*., 1990) .

Antagonism between rifampicin and other antimicrobials *in vitro* has substantially hampered the clinical acceptance of rifampicin in the treatment of Staphylococcus infections.

Several antibiotic regimens have been proposed for chronic osteomyelitis treatment. The first antibiotics, which showed a really good therapeutic efficacy, were β-lactams, (mainly the newer cephalosporins) used alone or in combination with other drugs .

Third generation cephalosporins are effective, they are mainly indicated for an ambulatory treatment of *Staphylococcus aureus* osteomyelitis (Guglielmo , *et al*. , 2000 ; Stengel , *et al*. , 2001) .

Chapter Two
Materials and Methods

2.1 Materials :

2.1.1 Apparatus and equipments :

Apparatus and equipment	Company	Origin
Electric oven	Gallen Kamp	England
ELISA	Bio Tek.	
Filter paper	Whatman	
Magnetic stirrer hotplate	Stuart	
Autoclave	Express	Germany
Incubator	Memmert	
Millipore filters	Sartorius Membarane	
Shaking incubator	GLF	
Water distillator		
Compound light microscope	Olympus	Japan
Digital camera	Mercury	China
Microtiter plate		
Morter		
pH- meter	Radiometer	Denmark
Vortex mixer	Stuart	UK
Electric sensitive balance	Delta Range	Switzerland
Micropipette	Gilson	France
Cooled centrifuge	Crison	Spain

2.1.2 Chemicals :

Material	Company	Origin
Congo red	Himedia	India
Sucrose		
Ethanol	Local market	Iraq
Acetic acid	BDH	England
Glycerol		
HCl		
∝ - naphthol		
Peptone water		
Sodium acetate		
Sodium chloride		

Hyaluronic acid	Sigma	USA
Tetra – methyl – p – Phenylene Diamine dihydrochloride		
KOH		

2.1.3 Biological materials :

Biological materials were obtained from Al-Yarmouk Teaching Hospital which included human plasma and human blood . While the samples (pus and biopsies) were obtained from Surgical Specialist Teaching Hospital , Al-Yarmouk Teaching Hospital and Baghdad Teaching Hospital in Baghdad .

2.1.4.1 Antibiotic discs : (Bioanalys / Turkey)

Antibiotic	**Symbol**	**Concentration (µg / disc)**
Ampicillin	AM	10
Amoxicillin	AMX	10
Chloramphenicol	C	30
Cephalothin	KF	10
Ciprofloxacin	CIP	5
Clindamycin	CM	2
Gentamicin	CN	10
Methicillin	ME	5
Novobiocin	NV	30
Nalidixic acid	NA	30
Penicillin	P	10
Rifampicin	RA	5
Tetracycline	TE	30
Trimethoprim	SXT	25
Vancomycin	VA	30

2.1.4.2 Antibiotic powders :

Antibiotics Powder	Source
Cephalothin	Ajanta – India
Methicillin	
Vancomycin	APM – Jordan
Tetracycline	

2.1.5 Media

2.1.5.1 Ready- to- use media:

All bellowed listed media used in this study were prepared according to the instruction on containers of their manufacturing companies:-

Media	Company	Origin
Bovine serum albumin	BDH	England
Blood base agar	Oxoid	
DNase agar		
Muller – Hinton agar		
MacConkey agar		
Mannitol salt agar		
Simmon's citrate agar		
Urea agar		
Agar-agar	Himedia	India
Brain heart infusion agar		
Brain heart infusion broth		
MR –VP broth		
Triple sugar iron agar (TSI)		
Trypticase soya agar		
Trypticase soya broth		
Nutrient agar	Difco	USA
Nutrient broth		

2.1.5.2 Laboratory prepared media:

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

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

2.1.5.2.2 Peptone water : (Collee, *et al.*,1996)

It was prepared by dissolving 20 gm of peptone and 5 gm of NaCl in a little of D.W. then completed the volume to 1000 ml of D.W. and distributed in test tubes (5 ml / tube) then sterilized by autoclaving at 121°C (15 Ib/inch^2) for 15 min .

2.1.5.2.3 Congo red agar : (Freeman , *et al.*,1989)

It was prepared by addition of autoclaved Congo red to autoclaved cooled 55°C media (which contained of 37g/l brain heart infusion broth , 10g/l agar and 50g/l sucrose) then mixed well , poured into plates and kept at 4°C until use . It was used for detection of slime layer production .

2.1.5.2.4 Brain heart serum albumin medium (BHSA) : (Smith and Willett , 1968; King , *et al*., 2004)

It was prepared by addition of hyaluronic acid (which sterilized by filtration through Millipore filters 0.2μm) to 100 ml of autoclaved cooled 50°C media (which contained of 37g/l brain heart infusion broth , 1.5% agar) and mixed well to give final concentration 400 μg/ml of H.A. then sterile bovine serum albumin was add to this medium with constant stirring to give a final concentration 1% . The final pH of each medium was 6.8 and it was poured to a depth of 3 to 4 mm . After

solidification , plates were kept at 4°C to provide a firm surface. This medium used for detection of hyaluronidase enzyme by plate method .

2.1.5.2.5 Medium of turbidity reduction assay: (Tam and Chan ,1983)

This medium was used for detection of hyaluronidase enzyme by turbidity reduction assay . Each one ml containing 0.25 ml of H.A. , 0.5 ml of D.W. and 0.25 ml of sterile bovine serum albumin in 0.5 M sodium acetate buffer with pH 3.1 then the media divided into tubes and freshly used .

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

2.1.6.1 Reagents:

2.1.6.1.1 Ready use reagents :

Reagents	Company / origin
Kovacs reagent	BDH / England
Methyl red reagent (MR)	
Catalase reagent (H_2O_2)	Iraq

2.1.6.1.2 Laboratory – prepared reagents:

- **Oxidase reagent:** (Collee, *et al*., 1996)

It was prepared by dissolving 1 g of tetramethyl-p-phenylene diamine dihydrochloride powder in a little of D.W. then completed the volume to 100 ml of D.W.

- **Voges–Proskaure reagent (VP):** (Collee, *et al*., 1996)

It was used with acetoin formation test which consisted of the following :

- First solution : 40% KOH , it was prepared by dissolving 40 gm of KOH in a little of D.W. then completed the volume to 100 ml of D.W.
- Second solution : It was prepared by dissolving 5 gm of α-naphthol in a little of absolute ethanol then completed the volume to 100 ml of absolute ethanol .

2.1.6.2 Stains:

2.1.6.2.1 Ready use stains:

Stains	Company / origin
India ink	BDH / England
Gram stain	Himedia / India

2.1.6.2.2 Laboratory – prepared stain :

- **Congo red stain:** (Freeman , *et al* ., 1989)

It was prepared by dissolving of 0.8gm of stain in a little of D.W. then completed the volume to 1000ml and autoclaved 121 °C (15 Ib/inch^2) for 15 min .

2.1.6.3 Solutions :

2.1.6.3.1 Ready - to - use solutions :

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

2.1.6.3.2 Laboratory – prepared solutions :

- **Antibiotics Stock Solutions :**

The four antibiotic solutions were prepared according to NCCLs , (2007) by dissolving 0.1 gm of methicillin , cephalothin , and vancomycin in 10 ml of D.W. and tetracycline in 10 ml of ethanol then sterilized by filtration through Millipore filters (0.2μm) .

- **Glycerol solution:** (Collee, *et al*., 1996)

It was prepared by addition of 20 ml of glycerol in a little of D.W. and the volume was completed with 100 ml of D.W. then autoclaved and stored at (4˚C) . It used for maintenance of isolates .

- **Bovine serum albumin solution(BSA) :** (Smith and Willett , 1968; King , *et al*., 2004)

It was prepared according to the manufacturing company by dissolving 0.6 gm of bovine serum albumin gradually in 10 ml of D.W. with gentle stirrer , and the pH was adjusted to 7.2 before sterilized by filtration through milipore filter 0.2 μm . It was used for detection of hyaluronidase enzyme .

- **Hyaluronic acid solution(H.A.) :** (Smith and Willett , 1968; King , *et al*., 2004)

It was prepared according to the manufacturing company by dissolving 0.2 gm of hyaluronic acid in D.W. then completed the volume with 100 ml of cold D.W. with gentle stirrer and the pH was adjusted to 7.2 then sterilized by filtration through milipore filter 0.2 μm . It was used as a substrate for hyaluronidase enzyme .

- **Acetic acid solution(2N) :** (Atlas , *et al.* , 1995)

It was prepared by addition of 11.3 ml of concentrated acetic acid (17.6 N) to a little of D.W. then completed the volume to 100 ml and stored in a dark bottle . It was used for detection of hyaluronidase production in plate method .

2.1.6.4 Buffers :

2.1.6.4.1 Ready- to - use buffer :

Phosphate buffer saline (PBS) pH = 7 , supplied by Biotechnology Research Center / Al- Nahrain University .

2.1.6.4.2 Laboratory – prepared buffers :

- **Sodium acetate buffer (0.5 M) :** (Atlas , *et al.* , 1995)

Prepared from two solutions :

- **Solution(A) acetic acid (0.5M) :**

Prepared by addition of 6.005 ml of acetic acid to a little of D.W. and completed the volume to 200 ml of D.W.

- **Solution (B) sodium acetate (0.5M) :**

It was prepared by dissolving 4.1 gm of sodium acetate in 100 ml of D.W. The buffer was prepared by mixing 184 ml from solution A with 66 ml of solution B , then the volume was completed to 500 ml with D.W. and the pH was adjusted to 3.1 .

2.1.7 Bacteriological kits: Bio Merieux / France

Api-20E and Api-*Staph.*

2.2 Methods:

2.2.1 Samples collection:

Twenty five specimens of osteomyelitis were collected from patients at Surgical Specialist Hospital , Al-Yarmook Teaching Hospital and Baghdad Teaching Hospital during June 2010 to May 2011 . For this purpose , a form was designed to be filled by each patient which included informations about age , sex , duration of infection , infection site , as shown in (Appendix) .

Specimens were obtained with sterile cotton swab and by biopsy . The pus was taken before the surgery by swabs from the osteomyelitis patients with an open infected wound and from deeper part of the infected bone , while , biopsies were taken from osteomyelitis patients during surgical management at the operation theater .

2.2.2 Sterilizing methods: (Baily *et al.,* 1990)

- **Wet-heat sterilization:**

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

- **Dry-heat sterilization:**

Electric oven was used to sterilize glassware at 160 °C for 3 hrs .

- **Membrane Filtration (Filtration) :**

Hyaluronic acid solutions, bovine serum albumin and antibiotic solutions were sterilized throughout (0.22) μm in diameter millipore filters .

2.2. 3 Isolation and characterization:

2.2. 4 Microscopic examination:

All specimens were subjected to the microscopic examination by staining with Gram stain .

- **Gram staining :** (Atlas , *et al.* , 1995)

Swab of each suspected isolate was fixed on microscopic slide , if swab was dry, it was hydrated in a drop of sterile saline , mixed , then smears were allowed to dry and all fixed smears were stained by Gram stain to examine (cell shape , grouping , size and Gram reaction) .

2.2.5 Cultural characteristics : (Collee, *et al*., 1996)

Biopsy was disrupted by morter , then inoculated on blood agar and MacConkey agar media , while pus was inoculated directly on blood agar and MacConkey agar media and were incubated aerobically at 37°C for 24 - 48 hrs .

After incubation , cultural characteristics were studied depending on colonies characteristics of suspected isolate (color, size, shape , edges and high) on the surface of MacConkey and blood agar media .

2.2.6 Biochemical tests :

- **Haemolysin production :**

Loopfull of suspected isolates were inoculated on human blood agar before incubation at 37ºC for 24-48 hrs to examine the ability of bacterial isolates to produce haemolysin . Presence of clear zones around the colonies represents β- haemolysis and considered as a positive result (Eaton and Gasson , 2001) .

- **Lactose fermentation :**

Suspected isolates were inoculated on MacConkey agar before incubation at 37ºC for 24-48 hrs to examine the ability of bacterial isolates to ferment lactose . Growth of pink or reddish colonies represents lactose fermenting and counted as a positive result (Collee, *et al*., 1996) .

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

A loopfull of suspected isolates were picked up from agar media and fixed on the surface of glass slide , a drop of 3% H_2O_2 was added . Appearance of bubbles indicated a positive result for catalase enzyme production .

- **Growth on mannitol salt agar (MSA) :** (Collee, *et al*., 1996)

A Suspected colonies were inoculated on sterile MSA and incubated for 24 – 48 hrs . After incubation , the presence of yellow color around the colony is an indicator for mannitol fermentation .

- **Coagulase test :** (Atlas, *et al.*, 1995)

Tube coagulase method which was carried out as follows: 1 ml of the human plasma was placed into two small test tubes , to the first one 0.1 ml of an overnight broth culture of suspected isolate was added , both tubes were incubated for 4 hours or overnight . Clot formation indicate that isolate produced coagulase enzyme (positive result) while the second tube used as a control .

- **Deoxyribonuclease test (DNase production) :**

A loop full of suspected isolate colonies from a primary culture was picked up by a sterile bacteriological needle and inoculated by spotting on

the surface of deoxyribonuclease (DNase) agar plate. The DNase plate was incubated for 18-24 hours at 37°C. After incubation , the plate was flooded with 1 N of HCl which led to precipitates the DNA and turns the plate cloudy. The appearance of a clear zone around the growth was an indication for DNase production and a positive result (Atlas, *et al.,* 1995) .

- **Indol test :** (Atlas, *et al.*, 1995)

It demonstrated the ability of suspected bacteria to decompose the amino acid tryptophan to indol which accumulated in the medium , it was performed by inoculating the suspected isolates into peptone water tubes prepared in (2.1.5.2.2) after incubation for 24-48 hours at 37°C , 0.5 ml of Kovacs reagent was added and mixed gently , the tubes were examined after one minute. The formation of a red colored ring on the surface of broth indicates a positive indol test .

- **Simmon citrate test :** (Atlas, *et al.,* 1995)

It was used for detection of the bacterial ability to utilize citrate as the sole of carbon and energy source for growth and an ammonium salts as the source of nitrogen . The surface of the slant of Simmons citrate agar medium was inoculated by suspected isolates and incubated at 37°C for 18-24 hours , changing the color of media from green to blue indicated a positive result .

- **Triple sugar iron agar (TSI):** (Atlas, *et al.,* 1995)

A tube of TSI agar medium(consist of 3 types of sugars : glucose , sucrose and lactose) was inoculated with suspected isolate by streaking the slope and stabbing the bottom of the TSI slant , and incubated at 37°C for 24 hours. The fermentation of any type of sugars was indicated by the change of the color of the indicator phenol-red from red to yellow color ,

this indicate the acid formation as a positive result while remaining the red color indicating as a negative result . TSI was also used for detection of CO_2 production by bubbles formation in the slant as a positive result. As well as, the appearance of black precipitate indicated of ferric sulfate formation .

- **Urease test:** (Atlas, *et al.*, 1995)

It was used to examine the presence of urease enzyme by inoculating the surface of sterile urea agar slants with tested bacteria and incubated at 37°C for 24 hrs . After incubation the appearance of pink color indicated a positive result while yellow color main a negative result .

- **Oxidase test :** (Collee, *et al*., 1996)

A freshly prepared oxidase reagent (tetramethyl paraphenyl diamine hydrochloride) was used (prepared in 2.1.6.1.2) .This test was done by using filter paper with a few drops oxidase reagent , a sterile wooden stick of suspected bacterial isolates was picked up from the slant growth and smeared on filter paper , the development of purple color within 5-10 seconds indicated a positive result .

- **Methyl red test (MR) :** (Collee, *et al*., 1996)

It was used to detect the production of sufficient acid during the fermentation of glucose . The MR – VP broth media was divided in 5 ml test tubes and sterilized by autoclaving , after inoculated with tested isolate , and incubated at 37°C for 48 hrs , 5 drops of methyl red reagent were added and mixed gently . The appearance of red color represented positive result , while appearance of yellow color represented negative result .

- **Voges-Proskuar test (VP) :** (Collee , *et al*., 1996)

The MR – VP broth media was divided in 5 ml test tubes and sterilized by autoclaving , after that , inoculated with tested isolate , then incubated at 37°C for 48 hrs . After that VP reagents which prepared in (2.1.6.1. 2) , (2drops from KOH and 6 drops of α-naphthol) were added to MR- VP broth . Shake well after addition of each reagent , the appearance of pink color during 2- 5 minutes indicated positive result for acetone production and negative result was yellow in color .

2.2.7 Maintenance of isolates :

Bacterial isolates were maintained according to Han , *et al.*, (1995) as follows:

- **Short – term storage (few weeks) :**

Bacterial isolates were maintained for period of few weeks when cultured on nutrient agar, and incubated at 37°C for overnight, the plates were tightly wrapped with parafilm and stored at 4 °C untile use .

- **Medium – term storage (1 – 3 months) :**

Bacterial isolates were maintained as stab culture for few months by inoculated the bacterial isolates in small screw capped bottles containing (5 – 8) ml of sterile nutrient agar as slants, then incubated at 37°C overnight , the bottles were tightly wrapped in parafilm and stored at 4°C.

2.2.8 Identification of bacteria by API system :

Identification of bacterial isolates was done at the Central Health Laboratory by using API 20E kits for identification of *Enterobacteriaceae* , while , API *Staph.* kits were used for identification of *Staphylococcus spp*. especially *Staphylococcus aureus* .

2.2.9 Antimicrobial sensitivity test: (Atlas, *et al.*, 1995)

The use of antimicrobial sensitivity test is essential for the selection of an appropriate drug for treatment of osteomyelitis . The disc diffusion method was used in this study depending on the Kirby-Bauer diffusion method (Bauer , *et al*., 1966) . In addition, some references refer cases of osteomyelitis due to *Staphylococci* must be treated as soon as possible and without waiting for a bacteriological reports .

Single colonies grown on nutrient agar for (18- 24) hrs. were transferred to tube containing 5 ml of normal saline and mixed well by vortex , then , bacterial growth was compared with McFarland tube No. 0.5 turbidity standard solution , which was equivalent to a bacterial inoculum concentration of (1.5×10^8) cell /ml .

By using sterile cotton swab, a touch of bacterial culture was transferred to Muller - Hinton agar and streaked three times by rotating the plate approximately 60° between streaking to ensure even distribution of the inoculum, the inoculated plates were placed at room temperature for 10 min to allow absorption of excess moisture, then the discs of antibiotic were fixed by sterile forceps on the surface of plates and gently pressed down on the surface of agar. Discs were arranged so as to avoid the development of overlapping of inhibition zones and the plates were incubated at 37 ºC for 18-24 hours.

After incubation , measured the diameters of inhibition zones (clear area around discs) were measured by ruler and the results were compared with the standards as in (NCCLs, 2007) .

2.2.10 Minimum inhibitory concentration (MIC) :

It was defined as the lowest concentration of antibiotic solution that completely inhibites the growth of the inoculums . MIC was determined by agar dilution method (NCCLs, 2007) . Agar dilutions were prepared using Muller-Hinton agar medium supplemented with two fold serial dilutions of vancomycin , cephalothin , methicillin and tetracycline stock solutions as prepared (2.1.6.3.2) ranging from (20-1280) µg/ml and poured into plates . Fresh isolates were inoculated in sterile nutrient broth , then incubated at 37 ºC for 24 hours under aerobic conditions . Then , by sterile cotton swabs each inoculum was delivered to the agar dilution plate in three different direction to ensure an even distribution of the inoculums.

All plates were incubated at 37 ºC under aerobic conditions for 24 hrs . Recorded the results of MIC after incubated for each antibiotic and compared the positive reaction with standard in (NCCLs, 2007) .

2.2.11 Detection of virulence factors of *Staphylococcus aureus* :

2.2.11.1 Haemolysin production test :

Staphylococcus isolates were inoculated on human blood agar prepared in (2.1.5.2.1) and plates were incubated at 37ºC for 24-48 hrs to examine the ability of bacterial isolates to produce haemolysin enzyme . The presence of clear zones around the colonies represented β-haemolysis as positive result (Eaton and Gasson , 2001) .

2.2.11.2 Detection of capsule production: (Alfred , 2005)

Two staining methods were applied in order to detect the production of capsule , negative and capsule staining . Negative stain by Indian ink which was done by mixing the bacteria with small amount of the ink and spreading a very thin film over the surface of the slide and examined

under the microscope . Appearance of dark background area around the cells , appearing capsules as transparent object surrounding the cells against the dark background is positive result . The capsule stain method was carried out as follow :

Two loopfull of the bacteria were mixed with a small drop of India ink , then the ink suspension of bacteria is spread on the slide and air-dried. The slide was gently heated – dried to fix the bacteria to the slide . The smear was stained with crystal violet for one minute before washing with water . Slide was blotted dry with bibulous paper , and examined with oil immersion objective .

The capsules appeared as a clear halo surrounding purple cells as a positive reaction .

2.2.11.3 Detection of slime layer production:

Congo red agar method developed by Freeman , *et al*. (1989) was used for this test . Congo red stain (prepared 2.1.6.2.2) was added to the cooled brain heart infusion agar and mixed well before poured into plates , then inoculated and incubated at 37 ºC for 24 hrs .The isolates which produced black colonies were regarded as slime positive , whereas those showing pink colonies were slime negative .

2.2.11.4 Detection of biofilm production:

It was carried out by Microplate method proposed by Pfaller , *et al*. (1988) using tissue culture plates of 96 flat bottomed wells. Each well was filled with 0.2 ml of 10^5 CFU/ml of a bacterial suspension in tryptic soya broth . After 48 hrs of incubation at 37 ºC , the contents were aspirated and plates were washed twice with phosphate buffer saline (pH = 7.2) . The wells were stained with 0.25% safranine for 30 sec , then the plates were read using an enzyme-linked immunosorbent assay

(ELISA) reader at 490 nm. Sterile tryptic soya broth was used as a negative control . All the experiments were repeated at least twice .

A three grade scale was used to evaluate the isolates biofilm producing ability: non biofilm producer when OD_{490} nm < 0.500 while , moderate biofilm producer when 0.500< OD_{490} nm < 1.500 and highly biofilm producer when OD_{490} nm > 1.500 .

2.2.11.5 Detection of hyaluronidase production:

Two different methods were applied in order to detect the production of hyaluronidase enzyme as follows :

- **Plate method :**

Brain heart serum albumin medium (BHSA) prepared in (2.1.5.2.4) was used in this method . The BHSA agar was poured to a depth of 3 to 4 mm , the final pH of each medium was 6.8 by using sterile pH paper . After solidification , plates were kept at 4 °C to provide a firm surface for wells making which were filled with 25µl of clear supernatant of overnight *Staphylococcal* broth culture , the plates were then incubated at 37 °C for 24 hrs.

After incubation , the plates were flooded with 2 N acetic acid prepared in (2.1.6.3.2) for 10 min . The hydrolyzed or clear zones of isolates were measured by determination of their diameters in millimeters , then the results were recorded , hydrolyzed zone produced by producing isolates while , non producing isolates remained without zones (Smith and Willett , 1968 ; King , *et al*., 2004) .

- **Turbidity reduction assay :**

The medium prepared in (2.1.5.2.5) was used . It contained H.A. , D.W. and dissolved bovine serum albumin in sodium acetate buffer. 0.5 ml of the supernatant of an 18-24 hrs bacterial broth culture which centrifuged at 8000 rpm for 20 min. at 4 °C (then diluted 1: 2 in saline) was added to the medium , mixed and incubated at 37 °C for 30 min . At the end of incubation time , the tubes were cooled in ice bath. To the above mixture , 0.1 ml of acetic acid (2 N) was added to precipitate the remaining H.A. Tube containing inoculating medium (without substrate) as control and another tube containing broth from hyaluronidase inactive cultures became turbid as a negative test , while tube containing broth from producing bacteria remained clear when addition of acetic acid as a positive test (Tam and Chan 1983) .

Chapter Three
Results and Discussion

3.1 Distribution of osteomyelitis patients according to age and sex:

Twenty five swabs and twenty five biopsies samples were taken from 25 patients suffering from chronic osteomyelitis , in three hospitals .

Results showed that high prevalence of males among osteomyelitis patients when 84% (21) of them , 16% (4) of females were infected, as shown in figure (3-1) .

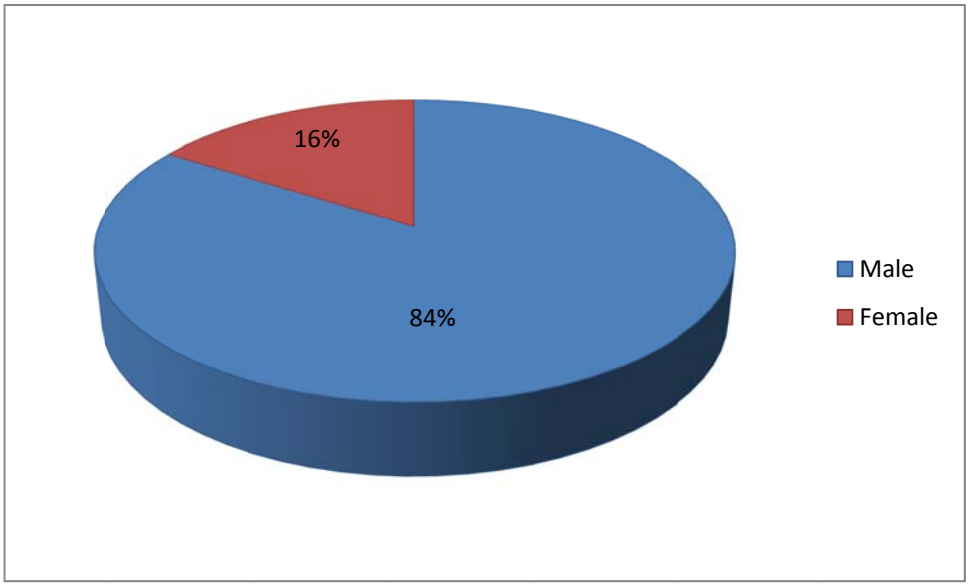

Figure (3-1): Sexes distribution of osteomyelitis among gender of patients .

Higher percentage osteomyelitis in male may be due to the fact that males are more exposed to trauma and accidents . This result was similar with that of AL-Kateeb, (2005) who found that the percentage of males was 80.36% and 19.64% females among osteomyelitis patients . Tice, *et al*. (2003) found that the percentage of males was 65% and females 35% from osteomyelitis patients .

Figure (3-2) shows the result of various osteomyelitis patients divided according to their age ranged from teenager to over sixty years old . The

sum of 12% was the prevalence of age group (10-19) distributed between 8% (2) were males and 4% (1) were females . Also, 8% (2) were the prevalence of males among age groups (20-29) and > 60 . As well as , 28% (7) of patients were males and 12% (3) were females among aged group (30-39) and 16% (4) males were found among aged group (40-49) and (50-59) among osteomyelitis patients .

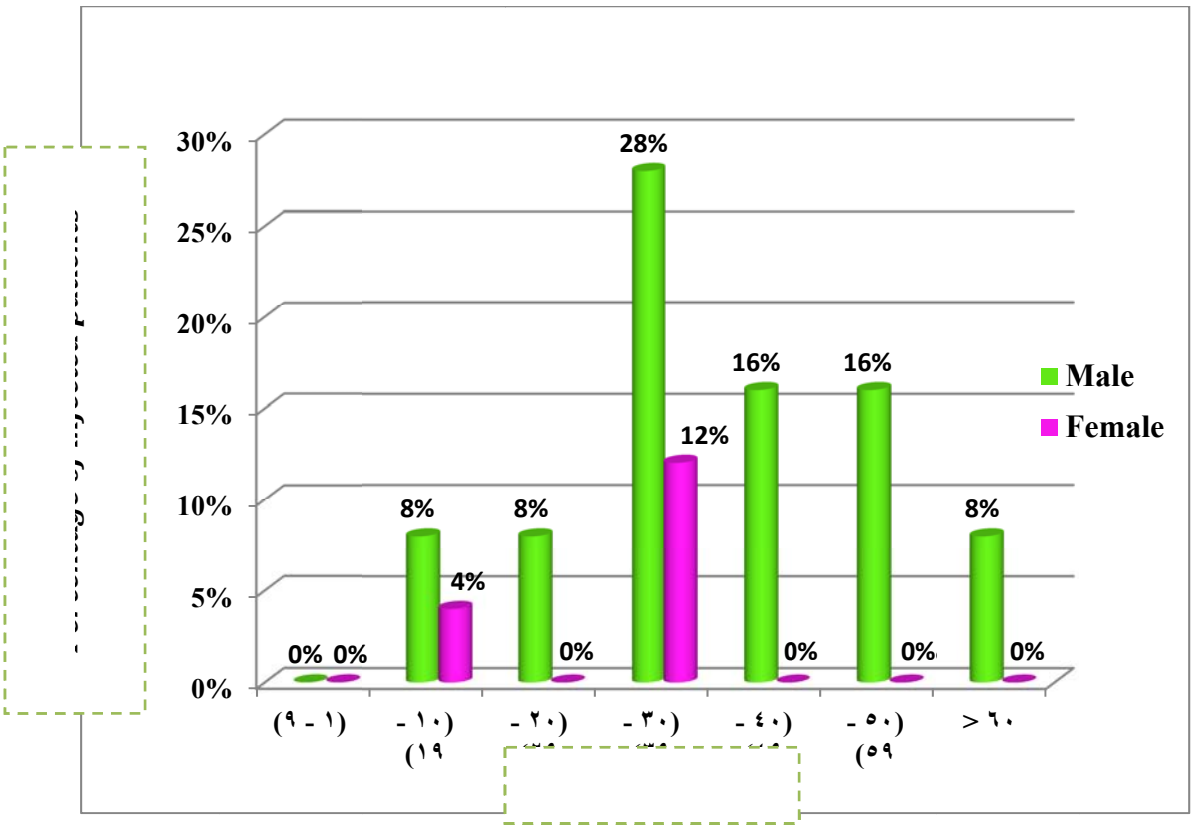

Figure (3-2) : Distribution of osteomyelitis according to the patients age groups .

This indicated that ages of osteomyelitis patients were varied , and high prevalence was found among the age group (30-39) years while , the low prevalence was found in the age group (10-19) years .

Such findings are close to those of AL-Shammary, (2009) who found a high prevalence of osteomyelitis in the age group (30-39) years (28.75%)

, while , this results disagreed with the study of Tice, *et al.* (2003) who reported highest percentage in the age of 51 years .

3.2 Identification of bacterial isolates :

Out of the 25 cases of osteomyelitis , fifty swab and biopsy samples were collected and cultured on blood agar medum , which allows growth of both Gram negative and positive bacteria , and MacConky agar medium was used for growth of Gram negative bacteria only .

3.2.1 Cultural characterization :

Results of cultural examination declared that the suspected isolates, which grown on blood agar , were varied in their ability to produce haemolysin . Half of the osteomyelitis isolates (all *S. aureus*) were found to be able to haemolyse red blood cells . Colonies of these isolates were circular in shape with entire margins and often raised with depressed centers . Among total isolates , (25) were able to grow on MacConky agar, some of them were lactose fermenting , producing pink colonies on this medium . While , the non–lactose fermenters displayed colorless translucent large colonies , shiny, convex and opaque , but others gave characteristic appearance, as a large mucoid colonies .

3.2.2 Microscopic characterization :

Microscopic characterization showed that 50% (25) of suspected isolates were rods as Gram negative while , the others 50% (25) were cocci arranged in clusters as Gram positive bacteria .

3.2.3 Biochemical findings :

Results indicated in table (3-1A) showed that half isolates gave positive results for catalase, citrate , TSI ,VP tests and negative results

for oxidase , urease , MR and indole tests . Their was (13) isolates found to belong to *Enterobacter cloacae* . Some isolates (3) gave positive results for TSI, catalase, MR and indole tests and negative results for VP, citrate , oxidase and urease, indicating they were belongs to *E. coli* , (2) isolates were positive results for TSI , catalase, MR and citrate but negative to oxidase , urease , VP and indol tests ; such characters are correlated with *Klebsiella sp.* while (7) of the isolates were belonged to *Pseudomonas aeruginosa* . They gave positive results for catalase, citrate, oxidase and urease , but negative results for MR , VP, indole and TSI tests .

The rest of the isolate gave positive results for coagulase, , DNase , β-haemolysis , mannitol salt agar and catalase tests and negative results for urease and oxidase tests , as shown in table (3-1B) so, these isolates (25) indicated that they were belongs to *Staphylococcaus aureus* .

Table (3-1A): Identification of Gram negative bacteria from osteomyelitis patients .

Bacterial isolate	Biochemical Test							
	Indole	**Methyl red (MR)**	**Vogas-proskuar (VP)**	**citrate**	**catalase**	**oxidase**	**urease**	**TSI**
Enterobacter cloacae	–	–	+	+	+	–	–	A/A
Pseudomonas aeruginosa	–	–	–	+	+	+	+	ALK/ ALK
Escherichia coli	+	+	–	–	+	–	–	A/A
Klebsiella sp.	–	+	–	+	+	–	–	A/A

Acid / Acid : A / A , ALK/ALK : Alkaline / Alkaline , negative test : – , positive test : +

Table (3-1B) : Identification of Gram positive bacteria from osteomyelitis patients .

Bacterial isolate	Catalase	Oxidase	Coagulase	Urease	DNase	Haemolysin	Mannitol salt agar
Staphylococcus aureus	+	_	+	_	+	β-haemolysis	+

Positive test : (+) , negative test : (-) .

On the other hand , results of the study insured that some bacterial isolates were obtained from both biopsy and swab samples .

Further diagnosis was done for Gram negative using API 20E system ; they were able to utilize arginine , citrate , gelatin , lysine and glucose . While diagnosis of Gram positive isolates were done using API *Staph.* system ; they were able to produce acetoin and produce acids from maltose , lactose, manitol and trehalose .

In chronic osteomyelitis, secondary infection is common, multiple organism may grow from culture, so increase frequency presence of Gram-negative enteric bacilli such as *E. coli, Klebsiella spp.* (James and Gruss, 1983) .

Depending on cultural and biochemical characterization, 50 isolates of bacteria causing osteomyelitis were obtained from different sites ; 25 of them (50%) were identified as *Staphylococcus aureus* , 13 (26%) *Enterobacter cloacae* , 7 (14%) *Pseudomonas aeruginosa* , 3 (6%) *Escherichia coli* and 2 isolates (4%) were *Klebsiella sp*. as shown in figure (3-3) .

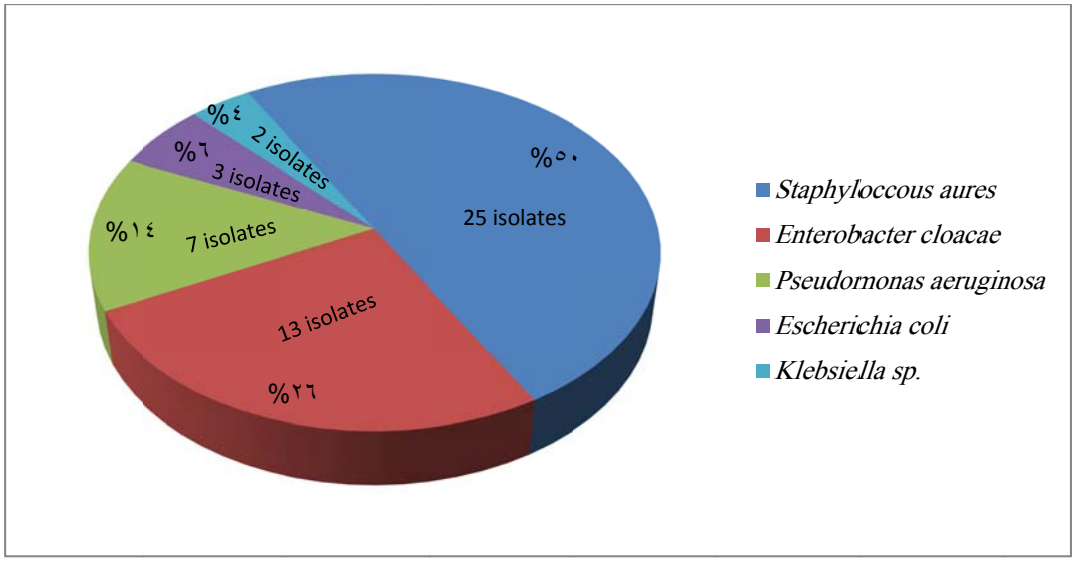

Figure (3-3): Percentages of bacterial isolates detected in the osteomyelitis patients .

Chronic osteomyelitis is polymicrobial , which means more than one infectious agent is involved , the role of bacteria in chronic osteomyelitis is solely an opportunistic one . In this case, bacteria take advantage of bone that is already injured. Because of necrotic bone is present from the outset , therapy must be first and foremost focused upon the surgical elimination of all devitalized tissue (Bhandar, 1999) .

Results of this study came according with those of several researchers also found that *S.aureus* was the most isolated bacteria from the osteomyelitis patients . Highest percentage (60%) was reported by Mackowiack , *et al.* (1987) , (79.2%) by Jack and Irvin , (1960) , (40%) by Galanakis, *et al.*, (1997) and (26.02%) by AL-Dieny, (2006) .

On the other hand , Gristina, *et al.* (1985) found that highest percentage of infection (87%) was caused by *S. epidermidis* , while *S. aureus* was comented for only 2% in osteomyelitis patients .

In this study, *Enterobacter cloacae* was found to be second agent responsible for predisposing infection with a percentage of 26% . *Enterobacter* bone and joint infection are usually implicated in

osteomyelitis in adults and children (Frase and Arnett , 2008) . Jalaluddin, *et al*. (1998) found that *Enterobacter cloacae* was the most frequently isolated species , causing infections in hospitalized osteomyelitis patients .

AL-Kateeb, (2005) who found that high percentage was (31%) which was caused by *Pseudomonas aeruginosa* followed by (19%) for *Klebsiella spp.* and low percentage (1.7%) for *Enterobacter cloacae* in patients suffering from osteomyelitis .

A study done by Nathan, *et al*. (2003) found that hospitalized patients suffering from chronic osteomyelitis especially open fracture, being more predisposing to infections with Gram negative bacilli bacteria such as *Klebsiella spp*., *Pseudomonas spp*. and *E. coli* . While Bhandar, (1999) revealed that the high percentage (35%) of infection was with *Pseudomonas spp*. among osteomyelitis patients .

3.3 Antimicrobial susceptibility of bacterial isolates in osteomyelitis patients :

From a total of 50 Gram negative and positive bacterial isolates , 12 of them were selected to study their antimicrobial susceptibility toward antibiotics . They were 6 isolates of *S. aureus* , 3 of *Enterobacter cloacae*, and one of each of *E. coli*, *Klebsiella sp*. and *Pseudomonas aeruginosa* . Results showed that all *S. aureus* isolates were compeletly (100%) susceptible to ciprofloxacin, chloramphenicol, novobiocin and rifampicin. In contrast, all of these isolates were totally resistant to methicillin ,vancomycin, cephalothin and tetracycline followed by clindamycin, gentamicin and penicillin with the a percentage of 83% as in table (3-2A) .

These results were in agreement with study of Mohammad and Saeed, (2009) who found that *Staphylococcus* isolates were resistant to multiple antibiotics , including methicillin , tetracycline and vancomycin .

Brook , *et al.* (2003) stated that *S. aureus* easily develops antibiotic resistance either by mutation which causes permanent alteration in the DNA base sequence of the organism or by transfer of genetic information from one organism to another, either through chromosomal recombination (transformation, transudation, conjugation or by extra-chromosomal–plasmid- transfer) . In contrast , Korakaki, *et al*. (2007) found that 50% of *S. aureus* isolates were sensitive to ciprofloxacin, but all (100%) to vancomycin , while Mirnejad , *et al*. (2008) reported compeletly resistance to ciprofloxacin , and low percentage (40%) of resistance to vancomycin, methicillin , , cephalothin among osteomyelitis patients .

Table (3-2A): Antimicrobial susceptibility of Gram positive bacterial isolates .

Bacterial isolate and symbol	ME	P	VA	CIP	C	KF	TE	CN	NV	CM	RA
Staphylococcus aureus (1)	R	R	R	S	S	R	R	R	S	R	S
Staphylococcus aureus (3)	R	R	R	S	S	R	R	R	S	R	S
Staphylococcus aureus (4)	R	R	R	S	S	R	R	R	S	R	S
Staphylococcus aureus (5)	R	S	R	S	S	R	R	R	S	R	S
Staphylococcus aureus (7)	R	R	R	S	S	R	R	R	S	R	S
Staphylococcus aureus (10)	R	R	R	S	S	R	R	S	S	S	S

R: resistant , S: sensitive , ME:methicillin(5) , P:Penicillin(10) , VA:Vancomycin(30) , CIP:Ciprofloxacin(5) , C:Chloramphenicol (30) , KF: Cephalothin(10) , TE:Tetracycline(30) , CN: Gentamicin(10) , NV:Novobiocin(30) , CM:Clindamycin(2) , RA:Rifampicin(5) .

Rifampicin showed excellent antibacterial activity against methicillin sensitive *S. aureus* and methicillin resist *S. aureus* strains including penetration into cells and biofilms , and ability to kill phagocytosed bacteria (Craig, *et al.*, 2004; Calfee, *et al*., 2005) .

Rissing , (1997) stated that the combination of a quinolone with other oral agents was more effective in treatment infection with Gram positive pathogens, such as clindamycin and rifampicin, it will offers a reasonable option for improving the cure rates for chronic osteomyelitis treatment against Gram positive pathogens .

Results of the antimicrobial susceptibility of the three isolates of *Enterobacter cloacae* showed that they were resistant to ampicilllin, amoxicillin, cephalothin, tetracycline, vancomycin, rifampicin and penicillin , two of them resistant to chloramphenicol and trimethoprim, and only one isolate resisted gentamicin . But all were susceptible to nalidixic acid and ciprofloxacin as shown in table (3-2B) .

Table (3-2B) : Antimicrobial susceptibility of Gram negative bacterial isolates .

Bacterial isolate and symbol	**AM**	**P**	**C**	**CN**	**SXT**	**KF**	**NA**	**TE**	**VA**	**RA**	**AMX**	**CIP**
Enterobacter cloacae (6)	R	R	S	S	S	R	S	R	R	R	R	S
Enterobacter cloacae (12)	R	R	R	R	R	R	S	R	R	R	R	S
Enterobacter cloacae (13)	R	R	R	S	R	R	S	R	R	R	R	S
Pseudomonas aeruginosa	R	R	R	R	R	R	R	R	R	R	R	S
Escherichia coli	R	R	R	R	R	R	R	R	R	R	R	S
Klebsiella sp.	R	R	S	R	R	R	R	R	R	R	R	R

R : resist , S : sensitive , AM:Ampicillin(10) , P:Penicillin(10) , C:Chloramphenicol(30) , CN:Gentamicin(10) , SXT:Trimethoprim(25) , KF:Cephalothin(10) , NA:Nalidixic acid(30) , TE:Tetracycline(30) ,

VA:Vancomycin(30) , RA:Rifampicin(5) , AMX:Amoxicillin(10) , CIP:Ciprofloxacin(5) .

AL-Shammary, (2009) in this regard found compelete resistance of *Enterobacter cloacae* to rifampicin, ampicillin and tetracycline among osteomyelitis patients , and Mirnejad, *et al*. (2008) also revealed total resistance percentage of *Enterobacter cloacae* to amoxicillin, cephalothin and penicillin among osteomyelitis patients .

In contrast, the results were disagreed with study of AL-Attar, (2007) who found that the resistance to tetracycline was 25% for *Enterobacter* isolates which were sensitive to chloramphenicol .

Resistance of *Enterobacter spp.* and *S.aureus* to the β-lactam antibiotic is due to their production of β-lactamase enzyme that break down the β-lactam ring in the structure of β-lactam antibiotics, such as penicillins group and cephalosporins group.

Enteroboacter cloacae has also developed an acquired resistance to many commonly used antibiotics such as tetracycline , clindamycin, penicillin and vancomycin either by mutation or by transfer of genetic information from one organism to another, either through chromosomal recombination (transformation, transudation, conjugation or by extra-chromosomal–plasmid- transfer) (Brook , *et al.*, 2003) .

Furthermore, results of antimicrobial susceptibility of *Pseudomonas aeruginosa* and *E. coli* isolates showed that they were susceptible to ciprofloxacin but resistance to all other groups of antibiotic . *Klebsiella sp*. isolate was susceptible to chloramphenicol and resistance to all other groups of antibiotic .

Galanakis, *et al.* (1997) reported multi-resistance drug of Gram negative bacteria such as *Pseudomonas aeruginosa* and *E. coli* among

osteomyelitis patients , especially to nalidixic acid , tetracycline and penicillin , but susceptible to ciprofloxacin .

The results were in agreement with study of Mirnejad, *et al*. (2008) who found high resistance percentage of *E. coli* and *Klebsiella sp*. to amoxicillin , cephalothin and penicillin in osteomyelitis patients . As well as AL-Shammary, (2009) who revealed that high resistance percentage of *Klebsiella spp*. to rifampicin among osteomyelitis patients .

Traditional treatment have used operative procedures followed by several weeks of parenteral antibiotics (Mader, *et al.,* 1999) . Antibiotic classes used in the treatment of osteomyelitis include penicillins , β-lactamase , cephalosporins, other β-lactams, vancomycin, clindamycin, rifampicin, aminoglycosides, fluoroquinolones, trimethoprim .

3.4 Minimum inhibitory concentration (MIC) :

The MIC of methicillin, vancomycin, cephalothin and tetracycline were determined for four *S.aureus* isolates , results showed that MICs of methicillin were 320 for S_1 , 80 for each of S_3 and S_7 , and 40 µg/ml for S_4. For vancomycin , MICs were 160 for S_1 , 640 for S_3 , 80 for S_4 and 40 µg/ml for S_7 . In cephalothin, MICs were 320 for S_1, 80 for S_3 , 640 for S_4 and 160µg/ml for S_7. For tetracycline, MICs were 320, 80, 640 and 40µg/ml for S_1 ,S_3 , S_4 and S_7 , respectively, as shown in figure (3-4) .

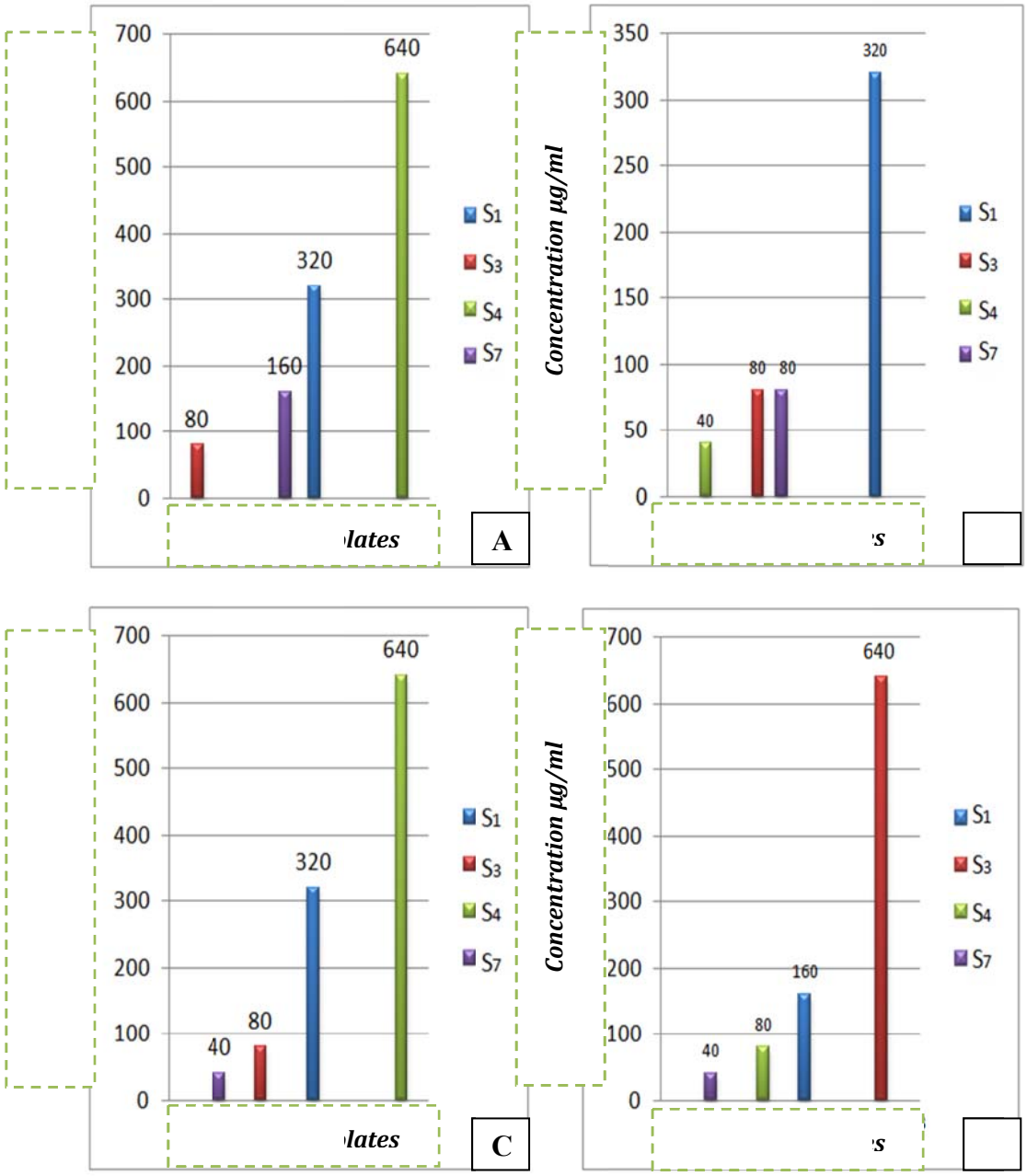

Figure (3-4) : MICs of antibiotics against *S. aureus* isolates from osteomyelitis patients : A- MIC of cephalothin to Staphylococcus isolates .

B- MIC of methicillin to Staphylococcus isolates . C- MIC of tetracycline to Staphylococcus isolates . D- MIC of vancomycin to Staphylococcus isolates .

The results were in close to the study of Zeidan , (2005) who found that MIC of all *S. aureus* isolates obtained from osteomyelitis patients , was 625µg/ml for each of tetracycline , cephalothin and vancomycin . While AL- Baldawi , (2005) found the MICs were 320µg/ml to each of

tetracycline , cephalothin and methicillin , but for another *S. aureus* isolates . They were 80µg/ml to each of methicillin, vancomycin, cephalothin and tetracycline .

Hans and Gunnar (2007) found that MICs of *S. aureus* isolates which obtained from bone infection were 40µg/ml to each of methicillin, vancomycin, and tetracycline . In contrast , the results were different from the study of Muhammad , *et al.* (2009) who found that MICs of *S. aureus* isolates were 20µg/ml to each of methicillin, vancomycin and cephalothin .

From antimicrobial susceptibility and MIC results of *S. aureus* isolates from osteomyelitis , four highly resistance isolates (S_1 , S_3 , S_4 and S_7) were further study their ability for production of some of virulence factors .

3.5 Production of virulence factors by *Staphylococcus aureus* :

3.5.1 Haemolysin production:

All four *Staphylococcus aureus* isolates from osteomyelitis patients showed complete haemolysis on blood agar due to β- haemolysin production as shown in figure (3-5) .

In this regard , Suheyla and Osman (2006) found that 58.9% of *Staphylococcus aureus* strains of osteomyelitis patients , possessed β-haemolysis characteristic . In contrast, Fanny, *et al.* (1993) in their studied on haemolysin as a virulence factors of *Streptococcus spp.* in osteomyelitis patients , found a percentage of 75% secreted haemolysins.

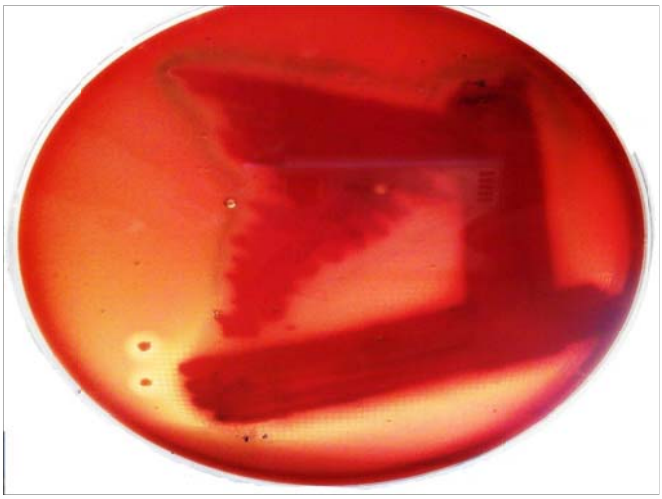

Figure (3-5): β-haemolysin producing *S. aureus* isolates on human blood agar medium incubated at 37°C for 24 hrs .

While Cavalieri, *et al.* (1984) found that *E.coli* contained potent exotoxin β-haemolysin as virulence factor in osteomyelitis patients and the percentage of haemolysin was 50% .

3.5.2 Capsule production:

Capsule formation is an important virulence factor of many bacteria , which can play a protective role for the pathogen such as *S. aureus* , the capsule may enable *S. aureus* to invade the bone tissue and cause osteomyelitis .

All four isolates of *S. aureus* obtained from osteomyelitis patients, were able to produce capsule in the two different used (capsule stain and negative stain methods) , as shown in figure (3-6) .

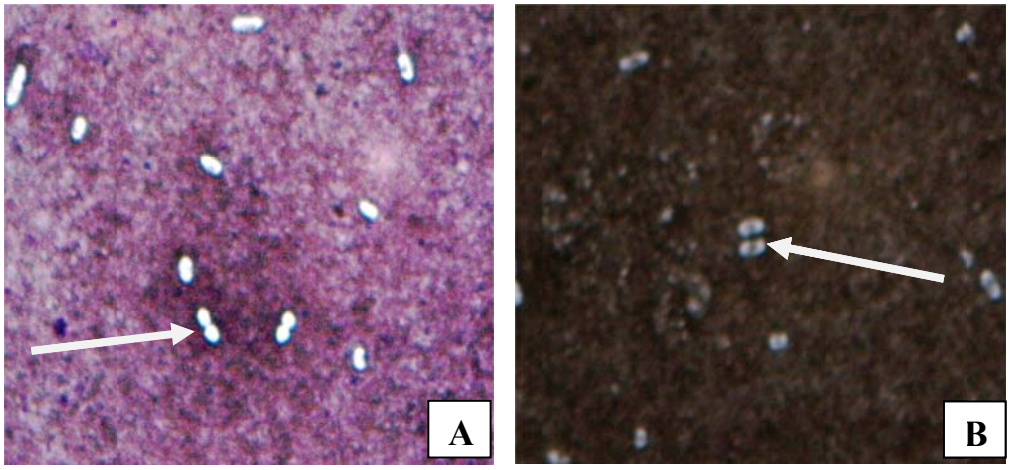

Figure (3-6): A-Capsule production by *S. aureus* isolates using the capsule stain method , the arrow shows transparent capsule around the bacteria . B- Capsule production by *S. aureus* isolates using the negative stain method (India ink) , the arrow shows transparent capsule around the bacteria .

The result was similar to the finding of Hochkeppel, *et al.* (1987) who revealed that about 90% of *S. aureus* isolates from osteomyelitis patients, showed their ability to produce capsular polysaccharide .

Negative stains method using one stain India ink stain that do not penetrate the bacterial cell , it stains the background area around a cell but does not stain the bacterial capsule or the bacterial cell because the bacterial capsules are non-ionic, so neither acidic nor basic stains will adhere to their surfaces . The bacterial cells are negative charge as well as India ink stain , so the interaction does not occur between them and the cells does not stain with India ink .Therefore, the best way to visualize them is to stain the background by using India ink, this leaves the capsule as a transparent objects or clear halo surrounding the cells against black dark background (Atlas , *et al.* , 1995) .

This method reveals the shape of the cell and extracellular features such as capsules and in this method cells not heat fixed prior to the

application of the negative stain which is useful for determining cell morphology and size of the cells.

Avoiding heating was important for the capsule surrounding bacterial cells to be observed because heating severely shrinked and destroyed isolates make appearance of capsules difficults in stains, if the cells are not heat fixed to the slide, they can wash off during washing procedures. So, gentle heating was performed to fix the cells to the slide and this can be done when combining the methods for the simple stain such as Gram crystal violet and the negative stain (India ink) in method called capsule stain method (Alfred ,2005) that used 2 stains acidic and basic stain : India ink (acidic stain) that stains the background area around a cells and basic dye (positive charge) such as Gram crystal violet that interacts with the negative ions of the bacterial cells to stain them only . This leaves the capsule as a clear halo surrounding a purple cells in a field of black dark background .

The capsule stain method was more clear and accurate than the second one (negative stain) due to staining both of cell and its background that made the capsule more clear and accurate . Also, probability of loss some cells that were not heat fixed to the slide during washing procedures in negative stain method , thus the capsule stain method was better more than the negative stain method (Beveridge, *et al.,* 2007) .

The results were close to the study of Tollersrud , *et al*. (2000) who found the ability of capsule formation in most *Staphylococcus* isolates from osteomyelitis patients by using capsule stain method, also he used

the negative stain in addition to capsule stain method and showed the capsule stain was better than the negative stain method for detection of bacterial capsules .

3.5.3 Slime layer production:

Slime production may reflect the microorganism's capacity to adhere to specific host tissue and thereby to produce invasive microcolonies. Many researchers considered it to be a significant virulence factor for some strains of *Staphylococci* (Ammendolia , *et al*., 1999 ; Mack , *et al.,* 2000) . All four isolates of *S.aureus* from osteomyelitis patients , were able to produce slime layer by using Congo red agar method as shown in figure (3-7) . Colonies appeared as tightly bound to each other and had a black color and a rough surface, whereas non producing isolates were showed a smooth colony morphology and were loosely bound to each other and had a pink color .

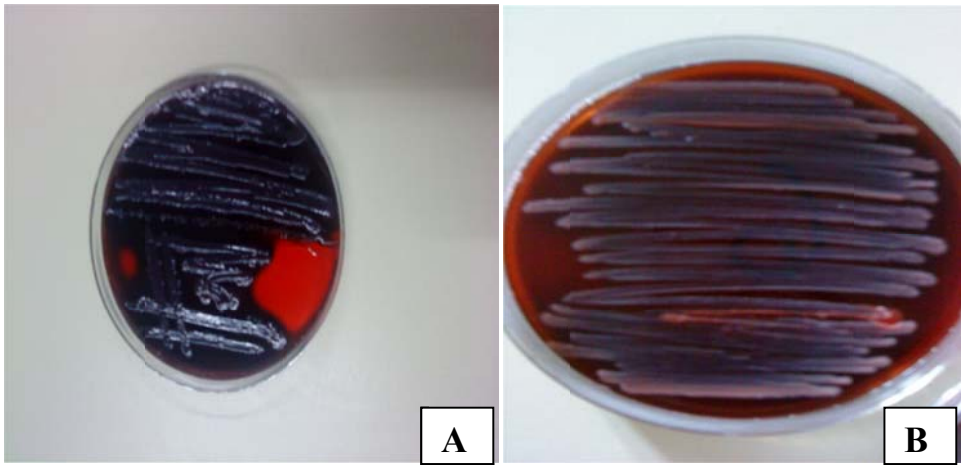

Figure (3-7): A- Slime layer production of *S. aureus* isolates shown the black colonies on Congo red agar . B- Non- producing isolates of slime layer shown the pink colonies on Congo red agar .

The advantage of using Congo red agar method which described by Freeman, *et al*. (1989) , that it was rapid , sensitive, and reproducible and has the advantage that colonies remain viable on the medium . Furthermore, it is not subject to the inter batch variation of media which affects the reproducibility of the other methods such as Christensen method .

This result was close to the study of Suhelya and Osman (2006) who showed that the rate of slime layer production positiveness was 77.8% for *S.aureus* isolates among osteomyelitis patients. As well as , Al-Baldawi, (2005) showed the ratio of slime layer producer isolates of *S.aureus* were 48.6% from osteomyelitis patients .

In contrast , this result disagreed with the results of AL-Tamemey, (2000) who showed 81% of *S. epidermidis* isolates from medical device, were able to produce slime layer . Seza and Fatma , (2007) found that clinical coagulase-negative *Staphylococci* isolates had a high frequency of slime production, particularly *S.epidermidis* isolates with 31% , was the most prevalent species isolated among clinical strains of coagulase-negative *Staphylococci* using Congo red agar method .

3.5.4 Biofilm production:

Biofilm-forming ability has been increasingly recognized as an important virulence factor in *Staphylococci* (Oliveira, *et al*., 2006) . Freeman, *et al.* (1989) found that testing for biofilm formation could be a useful marker for the pathogenicity of *Staphylococci* .

It was found that all four isolates of *S.aureus* from osteomyelitis patients, were able to produce biofilm . The results of three highly

producer *S.aureus* isolates showed that the OD_{490} nm value of ELIZA reader was more than 1.500 (1.500< OD_{490} nm) while, only one moderate producer *S.aureus* isolate was in value ranged between 0.5-1.5 (0.500< OD_{490} nm < 1.500) . Non producer isolate (control) showed the OD_{490} nm value less than 0.5 (0.500 > OD_{490} nm) as shown in table (3-3) .

Table (3-3) : ELIZA reader values revealed the biofilm producer *S. aureus* isolates by TCP method .

Staphylococcus isolates	Mean OD values	Biofilm formation
Control isolate	< 0.500	Non
S. aureus (7)	0.5-1.5	Moderate
S. aureus (1),(3),(4)	>1.500	High

Mathur, *et al*. (2006) found that using of TCP method was more sensitive (96.2%) and specific (94.5%) with high accuracy (97.3%) in terms of discriminating between biofilm producers and non-producer.

The tissue culture plate method (TCP) described by Christensen , *et al*. (1985) and Pfaller, *et al*. (1988) was most widely used and was considered as standard test for detection of biofilm formation . It was found to be most sensitive, accurate and screening method for detection of biofilm formation by *Staphylococci* and has the advantage of being a quantitative model to study the adherence of *Staphylococci* on biomedical devices .

The TCP method was more efficient procedure due to their ability to examine and distinguished between producing and non isolates in comparison with other methods as described by Christensen , *et al.*

(1982) who used tube method for strongly biofilm producing isolates but it was difficult to discriminated between moderate and biofilm negative isolates due to the variability in observed results by different observers. Consequently, high variability was observed and classification in biofilm positive and negative was difficult by tube method , in agreement with the previous reports, tube test cannot be recommended as general screening test to identify biofilm producing isolate .

The results close to the study of Suhelya and Osman (2006) who showed that the rate of biofilm production positiveness was 74.4% using TCP method for *S.aureus* isolates from osteomyelitis patients . As well as, Kien , (2008) found that 66.6% of *S.aureus* isolates using TCP method were able to form biofilm among patients with chronic disease .

The development in researches had revealed the association between biofilms and some chronic disease such as osteomyelitis (Cristina , *et al.,* 1985), otitis media (Hall-Stoodley, *et al.* , 2006) and cystic fibrosis (Costerton , 2001) . In fact, it is now estimated that more than 65% of chronic infection caused by bacteria were able to produce biofilms (Wolcott and Ehrlich, 2008) .

3.5.5 Hyaluronidase production:

Extracellular enzymes capable of breaking down hyaluronic acid in extracellular matrix of human tissue, also called spreading factor. Both of Cohen (1986) and Brock & Modigan (1988) have attributed the dominance of *Staphylococcus aureus* was due to production of virulence factors such as hyaluronidase, lipase, protease and possibly

staphylokinase, facilitated dissemination by their enzymatic breakdown of connective tissues ground substance .

By using two different methods , turbidity reduction assay (Tam and Chan , 1983) and plate method (Smith and Willett , 1968 ; King , *et al*., 2004) , it was found that all four isolates of *S.aureus* from osteomyelitis patients , were able to produce hyaluronidase enzyme .

The principle of using turbidity reduction assay for detection of hyaluronidase was depend on reduction in turbidity of media when inoculated with hyaluronidase producing *S.aureus* isolates , after 30 min of incubation, the medium was appeared as a clear broth (positive result) while , non-hyaluronidase producing isolate , medium was remained turbid broth (negative result) .

The second method (plate method) was performed by using BHSA media which contained 2 mg / ml of H.A. and 1% bovine serum albumin . Wells were made and filled with supernatant of overnight *S.aureus* cultures, then addition of acetic acid on the plated surface, caused appearance of hydrolyzed zones around of the wells (Smith and Willett , 1968; King , *et al*., 2004) which indicator of positive result whereas non producing isolates appeared without zones .

The diameter zone of producing *S. aureus* isolates were 27mm , 18mm, 7mm and 6mm for *S. aureus* isolates (4) ,(7) ,(1) and (3), respectively, as shown in figure (3-8) . This mean that *S. aureus* isolates(4) and (7), respectively, were highly producer for hyaluronidase than the *S. aureus* isolates(1) and (3) that produced the enzyme in

moderate level according to the diameters sizes of hydrolyzed zones in plates , the ratio was 50% : 50% .

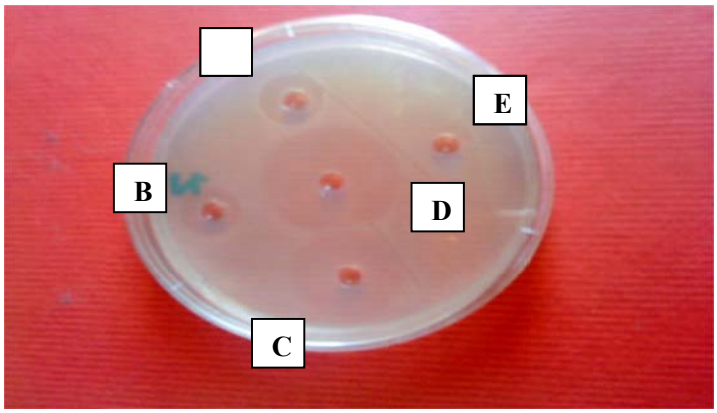

Figure (3-8) : Production of hyaluronidase enzyme by *S. aureus* isolates using plate method showed the symbol [A] represented *S. aureus* (1) (7mm) , the symbol [B] represented *S. aureus* (3) (6mm) , the symbol [C] represented *S. aureus* (7) (18mm) , while the symbol [D] represented *S. aureus* (4) (27mm) and the symbol [E] represented the control isolate (non former zone) .

The non-degraded substrate by the enzyme precipitates as a conjugate with the albumin, leaving a hydrolyzed zone around those colonies which produce soluble enzymes that attack the hyaluronic acid .

Plate method was rapid screening for distinguish between hyaluronidase producing and non producing isolates so , it was qualitative test , whereas, turbidity reduction assay was quantitative test , it also used to distinguish between enzyme producing and non producing isolates but it could not differentiate among producing isolates which could produced it in high or moderate level .

Hence, the plate method was more accurate and easy than turbidity reduction assay due to appearance of hydrolyzed zones that revealed

not only the enzyme producing isolates but also revealed which one were able to produce the enzyme in high level than those produced it in moderate level according to the zone size (Sabuj , *et al*., 2009) .

These results were disagreed with study of AL-Damalogy, (2008) who revealed the ability of 72.7% of *Pseudomonas maltophilia* isolates to produce hyaluronidase enzyme .

In other hand , the results were in closed with study of Mark, *et al*. (2009) who found that only *S.aureus* isolates which cause bone infections , were possess hyaluronidase enzyme . This would suggest that hyaluronidase represented yet another potential virulence factor employed by *S.aureus* to cause disease and may represent a diagnostically important characteristic for distinguishing *S. aureus* .

Chapter Four
Conclusions and Recommendations

4.1 Conclusions:

- In chronic osteomyelitis , males with the percentage of 84% were found to be affected than females 16% .
- The most common age group affected by osteomyelitis was 30-39 years .
- The high prevalence rate of osteomyelitis infection was found to be *Staphylococcus aureus* 50% followed by 26% of *Enterobacter cloacae* , 14% of *Pseudomonas aeruginosa* , 6% of *Escherichia coli* and 4% of *Klebseilla sp.*
- Four isolates of *S. aureus* were compeletly resistance to each of methicillin , vancomycin, cephalothin and tetracycline, but compeletly susceptible to ciprofloxacin, chloramphenicol, novobiocin and rifampicin .
- *Enterobacter cloacae* isolates were compeletly resistance to each of ampicilllin, amoxicillin, cephalothin, tetracycline, vancomycin, rifampicin and penicillin but , compeletly susceptible to nalidixic acid and ciprofloxacin .
- Both of *Pseudomonas aeruginosa* and *E.coli* isolates were compeletly resistance to all antibiotics except ciprofloxacin .
- *Klebsiella sp.* isolate was compeletly resistance to all antibiotics except chloramphenicol .
- The results of MICs were ranged (40 - 640 μg/ml) for each of methicillin , vancomycin , cephalothin and tetracycline .

- Complete haemolysis of human blood due to β- haemolysin enzyme production indicated high virulence of the bacterial isolates which were able to :
- Produce capsules, and the capsule stain method gave more clear and accurate scene than the negative stain method .
- Produce slime layer by using Congo red agar method, it was useful and more specific method to differentiate between producing and non producing isolates.
- Produce biofilm; three of them were highly producers, while , the last revealed moderate production of biofilms .
- Produce hyaluronidase enzyme by using plate and turbidity reduction methods . In plate method, the diameters of hydrolyzed zones were 7 mm, 6 mm, 18 mm and 27 mm for S_1, S_3 , S_7 and S_4 isolates, respectively .
- Plate method was faster , turbidity reduction assay was superior to distinguish between hyaluronidase producing from non producing isolates. Results showed that the four *S. aureus* isolates caused reduction in the turbidity of medium .

4.2 Recommendations:

- An *in vitro* and *in vivo* study for application of hyaluronidase enzymes as an antitumor agents .
- Studying some of bacterial toxins such as Staphylococcus toxins to be applied as vaccines against osteomyelitis disease.
- Studying of other organisms like viruses and fungi that cause osteomyelitis .
- Future studies were recommended to study other virulence factors in *Staphylococcal* osteomyelitis like bone sialoprotein , surface associated protein (SAP) and exofoliative toxin.
- Detection of hyaluronidase enzyme by molecular methods using specific PCR .

References

- **Acha, P.N. ; Tom, H. and Szyfres, B.** (2003). Zoonoses and Communicable Disease Common to Man and Animals. Vol. 1. (3Rd ed.). Bacteriocoin and Mycosis . Washington DC. PAHO. Scientifics and Technical Publication. **580**: p. 257-265.
- **Akhtar, M.S. and Bhakuni, V.** (2004). *Streptococcus pneumoniae* hyaluronate lyase. An overview. Current Sci. **86**: 285-9 .
- **Akiyama, H. ; Torigoe, R. and Arata, J.** (1993) . Interaction of *Staphylococcus aureus* cells and silk threads *in vitro* and in mouse skin. J. Dermat. Sci. **6:** 247–257.
- **AL- Baldawi , M. S.** (2005) . The Study of Bacterial Adherence and Resistance in Orthopaedic Prosthetic Infections . Master in Biotechnology . College of Science , Baghdad University .
- **AL-Attar, N. S. M.** (2007) . Study on Pathogenecity of *Enterobacter cloacae* Isolated From Wound Infections in Some of Baghdad Hospitals . Master in Microbiology , College of Science , Baghdad University .
- **AL-Damalogy, A. S.** (2008) . Biochemical and Molecular Study of Hyaluronidase Produced by Local Isolates of *Pseudomonas spp.* Doctor of Philosophy (Ph.D) in Microbiology . College of Science , Baghdad University .
- **Alderson, M. ; Speers, D. ; Emslie, K. and Nade, S. (**1986) . Acute haematogenous osteomyelitis and septic arthritis - A single disease.

An hypothesis based upon the presence of transphyseal blood vessels. J. Bone Surg. Am. Vol. 68 . p: 268–274 .

♣ **AL-Dieny, I. M.** (2006) . Bacteriological and Serological Study of Osteomyelitis Patients . Master in Biotechnology , College of Science , Baghdad University .

♣ **Alfred, E. B**. (2005). Benson's Microbiological Applications . Laboratory Mannual in General Microbiology (9^{Th} ed.). pp: 97-100 . Mc Graw Hill . USA .

♣ **AL-Kateeb, A. N.** (2005) . Isolation of Aerobic and Anaerobic Bacteria From Patients With Osteomyelitis and Study Some of The Virulence Factors . Master in Microbiology . College of Science , AL-Mustansiriyah University .

♣ **AL-Shammary , Z. A.** (2009) . Spectrum of Bacteria Causing Acute and Chronic Osteomyelitis . Master in Medical Microbiology . College of Medicine , Baghdad University .

♣ **AL-Tamemey, A. A.** (2000) . Isolation and Purification of Slime Layer of *Staphylococcus epidermidis* From Medical Device . Master in Microbiology . College of Science , Baghdad University .

♣ **Ammendolia, M.G. ; Dirosa, R. ; Montanaro, R. ; Arciola, C.R. and Baldassarri, L .**(1999) . Slime production and expression of the slime-associated antigen by *Staphylococal* clinical isolates. J. Clin. Microbiol. **37**: 3235 - 3238.

♣ **Atlas, R. N. ; Brown, A. E. and Paks, L. G**. (1995). Laboratory Manual of Experimental Microbiology. (1^{St} Ed.). Mosby. USA.

- **Baily, S. ; Baron, E. and Finegold, S.** (1990). Diagnosis Microbiology (8^{Th} ed.). The C.V. Mosby. U.S.A.
- **Baron, E. J. ; Chang, S. R. ; Howard, H. D. ; Miller, N. J. and Truner, A. J.** (1994). Dissemination by haematogenous spread , Medical microbiology. A short course, Wiley-Liss. Chapter-7, pp : 77- 78 , 127-128.
- **Baron, E.J. ; Peterson, R. and Finegold, S.M.** (1994). Hospital Epidemiology, Conventional and Rapid Microbiology Methods for Identification of Bacteria and Fungia. (9^{Th} Ed.). In Bailey & Scott's Diagnostic Microbiology. Mosby. Chapter- 6 . pp: 41 , 97-108.
- **Baselga, R. ; Albizu, I. and Amorena, B.** (1994) . *Staphylococcus aureus* capsule and slime as virulence factors in ruminant mastitis. rev. Vet. Microbiol. **39** : 195-204.
- **Baselga, R. ; Albizu, L. ; Cruz , M. ; Delcacho, E. ; Barberan, M. and Amorena, B.** (1993) . Phase variation of slime production in *Staphylococcus aureus*: implications in colonization and virulence. Infect. Immun. **61**: 4857-4862.
- **Bauer, A.W. ; Kirby, W. M. M. ; Scherris, J.C. and Turck , N.** (1966). Antibiotic susceptibility testing by a standardized single disc method . Am. J. Clin. Path. **45**:493-496.
- **Beveridge, A. ; Tass, T. and Doduge, S.** (2007) . The demonstration of bacterial capsules and slime. J. Pathol. Bacteriol. **63(4) :** 673-85.

- **Bhandar, M**. (1999) . High and low pressure Pulsatile lavage of Contaminated tibial Fractures : an *in vitro* study of bacterial adherence and bone damage . J. Orthop. Trauma. **13**(**8**): 526-533.

- **Blyth, M. J. ; Kincaid, R. ; Craigen, M.A. and Bennet, G.C.** (2001). Changing Epidemiology of Acute and Sub Acute Haematogenous Osteomyelitis in Children. **83**: 99-102 .

- **Brock, T.D. and Modigan, M.T.** (1988) . Biology of Microorganism. (5^{Th} Ed.) . Prentice Hall. Inc. New Jersey.

- **Brook, I. ; Yocum, P. ; Shah, K. ; Feldman and Epstein, S.** (2003). Increased antimicrobial resistance in organisms recovered from otitis media with effusion. J. Laryngol. Otol. **117(6)** : 449-53.

- **Calfee , D.P.** (2005) . Rifamycins. Vol. 1. (6^{Th} Ed.). In : Mandell, G.L. ; Bennett, J. E. ; Dolin, R. (eds). Mandell, Douglas, and Bennett`s Principles and Practice of Infectious Diseases. Philadelphia . Churchill Livingstone . pp. : 374-387.

- **Carek, P. J.** (2001) . Diagnosis and Management of Osteomyelitis . Am. Farm. Phys. **63(12)** : 2413-2720.

- **Cavalieri, S. J. ; Bohach, G. A. and Snyder, I. S.** (1984) . *Escherichia Coli* α-Haemolysin , Characteristics and The Role in Pathogenesis. Microbial. Rev. **(48)** : 326-343.

- **Chain, E. and Duthie , E.S.** (1940) . Identity of hyaluronidase and spreading factor. British J. of Experimental Path. Pp. : 21, 324–338.

- **Chamber , H.F.** (2001) . The changing epidemiology of *Staphylococcus aureus* . Emerg . Infect. Dis. **7** : 178- 182 .

- **Christensen, G. D. ; Simpson, W. A. ; Bisno, A. L. and Beachey, E. H.** (1982) . Adherence of Slime–Producing Strains of *Staphylococcus epidermidis* to Smooth Surfaces. Infect. Immun. **(37) :** 318–26 .
- **Christensen, G. D. ; Simpson, W. A. ; Younger, J. A. ; Baddour, L.M. ; Barrett, F. F. and Melton, D. M.** (1985) . Adherence of Coagulase Negative *Staphylococi* to Plastic Tissue Cultures . A Quantitative Model for The Adherence of *Staphylococci* to Medical Devices. J. Clin. Microbiol. (**22) :** 996–1006.
- **Ciampolini , J. and Harding , K.G. (2000) .** Pathophysiology of chronic bacterial osteomyelitis. Why do antibiotics fail so often? UK. Med. J. Rev. **76 (898)** : 479-483 .
- **Cohen, M.L.** (1986) . *Staphylococcus aureus*. Biology. Mechanism of Virulence . Epid. J. Pediatr. **5**: 796 .
- **Collee, J. G. ; Miles, R. S. and Watt, B.** (1996). Tests for the Identification of Bacteria. In: Mackie and MacCartney . Practical Medical Microbiology by Collee , J. G. ; Fraser, A. G. ; Marmion , B. P. and Simmons , A. (14 Th Ed.). Churchill. Livingstone.
- **Costerton , J.W.** (2001) . Cystic fibrosis pathogenesis and the role of biofilm in persistent infection . Trends Microbiol. **9** : 50-52 .
- **Costerton, J.W. ; Lewandowski , Z. ; Caldwell , D.E. ; Korber, D.R. and Lappin-Scott, H. M.** (1995). Microbial biofilms . Annu. Rev. Microbiol. **49***:* 711–745.
- **Craig , W.A.** (2004) . Rifampicin and Related Drugs. In: Gorbach , S.L. ; Bartlett , J.G. and Blacklow, N.R. (eds) . Infectious Diseases. (3Rd Ed.) . Lippincott Williams and Wilkins. Philadelphia . p. : 277-280 .

- **Csoka, A. B. ; Frost, G. I. ; Wong , T. and Stern , R.** (1997) . Purification and Microsequencing of Hyaluronidase Isozymes From Human Urine. FEBS Lett. **417 (3)** : 307-310.
- **Culver, D.H. ; Haron, T.C. and Gaynes , R.P.** (1991). Surgical wound infection rates by wound class, operative procedure and patients risk index . Am. J. Med. **91**: 1525-1575.
- **Dan, M. Y. ; Siegman-Igra, S. ; Pitlik and Raz, R.** (1990) . Oral ciprofloxacin treatment of *Pseudomonas aeruginosa* osteomyelitis. Antimicrob. Agents Chemother. **34:** 849-852 .
- **Darouiche, R.O. ; Landon, G. C. ; Kimia , M. and Markowski, J.** (1994). Osteomyelitis Associated With Pressure Sores. Harrison s' (14^{Th} Ed.) . J. Arch. in term Med. **154** : 753-8 .
- **David , W.H. and Mark , P. M.** (1996). Bacterial osteomyelitis in adults: Evolving considerations in diagnosis and treatment . Am. J. Med. **101(5)** : p. 550-561 .
- **Dinesh, D.M.S**. (2006). Acute Hematogenous Osteomyelitis in Children. Nigerian. J. Orthopedics and Trauma. **5(2):**41-44.
- **Duran- Renald , F**. (1942). Mechanism of invasion . Bact. Rev. **6** : p. 197 .
- **Durden, B.I. ; Reid, T.M. and Turk , D.C.** (1987). Bone and Joint Infections. In: A New Short Textbook of Medical and Parasite Infection. British library Cataloguing in Publication (2^{Ed} Ed.) . Chapter 22 . pp: 299-303.

- **Dworkin, R. ; Modin, G. ; Kunz, S. ; Rich, R. ; Zak, O. and Sande, M.** (1990) . Comparative efficacies of ciprofloxacin, pefloxacin and vancomycin in combination with rifampicin in a rat model of methicillin-resistant *Staphylococcus aureus* chronic osteomyelitis. Antimicrob. Agents Chemother. **34**:1014-1016 .
- **Eaton, T. J. and Gasson, M. J.** (2001). Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl. Environ Microbial. **67(4)** : 1628-35.
- **Edward, M.S. ; Gddenburg, M.I. and Scoles, K.**(1978). An etiologic shift in infantile osteomyelitis . The emergence of the group B *Streptococcus*. J. Pediat. **93**: 578-581.
- **Evans, R. P. ; Nelson, C. L. ; Bowen, W. R. ; Kleve, M. G. and Hickmon, S. G.** (1998) . Visualization of bacterial glycocalyx with a scanning electron microscope. Clin. Orthrop. Relat. Res. **347:** 243–249 .
- **Fanny, T. ; Arnold, U. and Teal, E.** (1993) . Study of Virulence Factors of *Streptococcus spp.* in Osteomyelitis Patients . Iran. J. Med. Sci. **(36) :** 64- 66 .
- **Farr, C. ; Menzel, J. ; Seeberger, J. and Schweigle, B.** (1997). Clinical pharmacology and possible applications of hyaluronidase with reference to hyase 'Dessau'. Wien. Med. Wochenschr. **147** : 347-50.
- **Filagerald, R.H. ;Yan, D. and Gowan, J.D.E.**(1975) . Puncture wounds of the foot . Orthopedic clin. North. Am. **6**: 965-972 .
- **Fitzpatrick, F. ; Humphreys, H. and O'Gara, J.P.** (2005) .The genetics of *Staphylococcal* biofilm formation – will a greater understanding of

pathogenesis lead to better management of device-related infection? Clin. Microbiol. Infect. **11**: 967–973 .

- **Frase, S.L. and Arnett, M.** (2008) . *Enterobacter* Infections. J. Med. Infec. Dis.
- **Freeman, D. J. ; Falkiner, F. R. and Keane, C.T**. (1989). New method for detecting slime producing by coagulase negative *Staphylococci*. J. Clin. Pathol. **42:** 872-874.
- **Frost, G.I. ; Csoka, T. and Stern, R**.(1996) . The hyaluronidases: a chemical biological and clinical overview. Trends in Glycoscience and Glycotechnology . p : 8 , 419–434.
- **Galanakis, N. ;Giamarellou, H. ; Moussas, T. and Dounis, E.** (1997). Chronic osteomyelitis caused by multi-resistance Gram negative bacteria. J. of Antimicrob. Chemother. **39** : 241-246.
- **Garner, J.S. ; Jarvis, W.R. ; Emori, T.G. and Hughes, T.C.** (1996) . Nosocomial Infections. pp. :1-20 .
- **Gaston, M. A.** (1988) . *Enterobacter* : an emerging nosocomial pathogen. J. Hosp. Infect. **11:**197-208 .
- **Gierny, G. ; Mader, J.T. and Pennick, H.A.** (1999) . Clinical Staging System of Adults Osteomyelitis . Contemp. Orthop. **10**:17-37.
- **Giesbrecht, P. ; Kersten, T. ; Maidhof, H. and Wecke, J. (**1998) . *Staphylococcal* cell wall : morphogenesis and fatal variations in the presence of penicillin . Microbiol. Mol. Biol. Rev. **62:**1371-414 .
- **Gregory, D.** (2003) . Osteomyelitis Canale Campbell's Operative Orthopedics. (10^{Th} Ed.) . Chapter 16 .

- **Gristina , A.G. ; Hobgood, C.D. ; Webb, L.X. and Myrvik, Q.N.** (1987) . Adhesive colonization of biomaterials and antibiotic resistance. Biomaterials. **8**: 423-426.
- **Gristina , A.G. ; Oga , M. ; Webb, L.X. and Hobgood , C.D.** (1985) . Adherent bacterial colonization in the pathogenesis of osteomyelitis . Sci. **228** : 990-993 .
- **Guglielmo, B.J. ; Luber, A.D. ; Palotta, D. and Jacobs, R.A.** (2000). Ceftriaxone therapy for *Staphylococcal* osteomyelitis. Rev. Clin. Infect. Dis. **30**: 205-207.
- **Hahn , L.** (1943) . Biochem. Z. p: 83-315 .
- **Hall-Stoodely , L.** (2006). Direct detection of bacterial biofilms on the middle ear mucosa of children with chronic otitis media . Indian J. Med. **296**: 202-211 .
- **Han, P. J.** (1995). Principles of Haematology. Edited by L. H. Young and W. B. Publisher. London. pp: 310 – 325.
- **Hans, O. H. and Gunnar, L.** (2007) . Identification of Cephalosporin-Resistant *Staphylococcus aureus* With the Disc Diffusion Method . J. Antimicrob. Agents and Chemother. Vol. 1. **(5) :** p. 422- 426 .
- **Hayes, C. S. ; Heinrich, S. D. ; Craver, R. and Macewen, G. D.** (1990). Sub acute Osteomyelitis. J. Orthopedics . **13**: 363-366.
- **Hochkeppel , H.K. ; Braun , D.G. ; Vischer , W. ; Imm , A. ; Satter , S. ; Staeubli , U. and Kaplan , E.L.** (1987). Serotyping and electron microscopy studies of *Staphylococcus aureus* clinical isolates with monoclonal antibodies to capsular polysaccharide types 5 and 8. J. Clin. Microbiol. **25** : 526-30 .

- **Hynes, W.L. and Walton, S.L.** (2000) . Hyaluronidases of Gram-positive bacteria . FEMS Microbiology Letters. **183**: 201–207 .
- **Jack, L.W. and Irvin, C.** (1960) . Acute Hematogenous Osteomyelitis . J. Bone Joints surg. Am. **42** : 691-704.
- **Jalaluddin, S. ; Devaster, J.M. ; Devasster, J.M. ; Scheen, R. ; Gerared, M. and Butzler, J.P.** (1998). Epidemiological Study of Nosocomial *Enterobacter aerogenes* Isolates Belgian in a Hospital. J. of Clin. Microbiol. **36(7)** : 1846-1852 .
- **James, E.T.R. and Gruss, J.S.** (1983). Closure of osteomyelitis and traumatic defects of the leg by muscle and myocutaneous flaps. J. Trauma . **23**: 411-414 .
- **Jawetz, E. ; Melnick, J. L. and Adelberg, E. A.** (2010). Review of Medical Microbiology. (25Th Ed.). Appletion and Lange. U.S.A.
- **Jedrzejas, M. J.** (2004) . Extracellular virulence factors of *Streptococcus pneumoniae*. Frontier in Bioscience. **9**: 891–914 .
- **John, L. and Zeller, M.D.** (2008) . Osteomyelitis. Treatment of Orthopedics Implant Related *Staphylococcal* Infection. Vol. 299. JAMA. **279(7)** :1537-1541.
- **Kalantari, N. ; Tacherikalani, M. ; Parvanen, N. and Mamishi, S.** (2007). Etiology and Antimicrobial Susceptibility of Bacteria Septic Arthritis and Osteomyelitis. Iran. J. Pup. Health. Vol. 36. (**3)** : p 27-32.
- **Karakawa , W. and Vann , W.** (1982) . Capsular polysaccharide of *Staphylococcus aureus* . Semin. Infect. Dis. **4**: 285-93 .

- **Kasser, J.R. ; O'Roouke, E. and Kozakewich, H.** (1993). Vascularized fibular grafts in the treatment of osteomyelitis and infected nonunion. Clin. Orthop. **293**: 256.
- **Kavanagh, J. ; Kelly, A.J. and Thomas, J.** (2006) . Hyaluronidase for cervical ripening and induction of labour. Cochrane Database Syst. Rev.
- **Kien , R. H.** (2008) . Investigating Bacterial Biofilms in Chronic Rhinosinusitis : An *In Vitro* Study , *In Vivo* Animal Study and Examination of Biofilms in Human CRS . Master of Surgery , Faculty of Health Science , Adelaide University .
- **King, S. J. ; Allen, A. G. ; Maskell, D. J. ; Dowson, C. G. and what-more, A. M.** (2004). Distribution , Genetic diversity and variable expression of the gene encoding hyaluronate lyase with the *Streptococcus suis*. Pop. J. bacteriol. **186(14):** 4740- 4747.
- **Kloss ,W.E. and Bannerman, T.L.** (1994) . Update on clinical significance of coagulase-negative *Staphylococci*. Clin. Microbiol. Rev. **7:** 117-140.
- **Korakaki, E. A. ; Aliqizakis, A. ; Manoura, E. ; Hatzidaki, E. ; Saitakis, M. ; Anatoliotaki, E. ; Velivasakis, S. ; Maraki, R. and Giannakopoulou, C.** (2007) . Methicillin-resistant *Staphylococcus aureus* osteomyelitis and septic arthritis in neonates : Diagnosis and management. Jap. J. Infect. Dis. **(60)** : 29-31 .
- **Kreil, G.** (1995) . Hyaluronidases—a group of neglected enzymes. Protein Sci. **4**: 1666–1669.

- **Krut, O. ; Sommer, H. and Kronke, M.** (2004) . Antibiotic-induced persistence of cytotoxic *Staphylococcus aureus* in non-phagocytic cells. J. Antimicrob. Chemother. **53**:167-173 .
- **Kusisec, J.M.** (1996) . Bone and joint infections caused by Gram negative bacteria. Acta. Med. Grotic. **50(1):** 37-44 .
- **Lowy, F. D.** (1998) . *Staphylococcus aureus* infections. Engl. J. Med. **339:**520-32 .
- **Mack, K.D. ; Bartscht, S. ; Dobinsky, M.A. ; Horskotte, H. ; Kiel, K.M. ; Knobloch, A. and Schafer, P.**(2000) . *Staphylococcal* factors involved in adhesion and biofilm formation on biometarials. Handbook of Bacterial Adhesion. Principles Methods and Applications. Humana Press. Totowa, N. J. p. 307-330 .
- **Mackowiak, P. A. ; John, S. and Smith, J.W.** (1987) . Teichoic acid antibodies in chronic *Staphylococcal* osteomylitis. Ann. Intern Med. **89(4):** 494- 6 .
- **Mader, J.T. ; Shirtliff, M.E. ; Bergquist, S.C. and Calhoun, J.** (1999). Antimicrobial treatment of chronic osteomyelitis. Clin. Orthop. **360:** 47-65.
- **Mader, J.T. and Calhoun, J.** (2003). Generial Concepts. Necrotizing Soft Tissue Infection , Bone , Joint , and Necrotizing Soft Tissue Infections. Med. Microbiol. Chapter 100 .
- **Makris, G. ;Wright, J.D. ; Ingham, E.I. and Holland, K.T.**(2004) . The hyaluronate lyase of *Staphylococcus aureus*—a virulence factor? Microbiol. **150:** 2005–2013.
- **Mark, E. ; Hart, G. ; Morgan, J. ; and Anna, J.** (2009) . Genotypic and Phenotypic Assessment of Hyaluronidase Among Type Strains of a

Select Group of *Staphylococcal* Species . Int. J. Microbiol. Vol. 5 . **(5) :** 754 - 755 . USA .

- **Mathur, T. ; Singhal, S. ; Khan, S. ; Upadhyay, D. J. ; Fatma, T. and Rattan , A.** (2006) . Detection of Biofilm Formation Among The Clinic Isolates of *Staphylococci* . An Evaluation of Three Different Screening Methods . Indian J. of Med. Microbiol. **24 (1) :** 25-9 .
- **Matsushita , O. and Okabe , A.** (2001) . *Clostridial* hydrolytic enzymes degrading extracellular components. Toxicon. **39:** 1769–1780.
- **Matusse, P.A. ; Jones, S.R. and Smith, J.W.** (2002) . Diagnostic value of sinus tract cultures in chronic osteomyelitis . JAMA. **239** : 2772- 2775 (Abstract).
- **Mayberry-Carson, K. J. ; Tober-Meyer, B. ; Smith, J. K. ; Lambe, D. W. and Costerton, J. W.** (1984) . Bacterial adherence and glycocalyx formation in osteomyelitis experimentally induced with *Staphylococcus aureus*. Infect. Immun. **43:**825–833.
- **Meyer, K.** (1971) . Hyaluronidases in The Enzymes. Vol. 5. (3Rd ed.) . Boyer, P. D. Ed. Academic Press. London. Pp: 307-320.
- **Meyer, K. and Palmer, J. W.** (1934) . The polysaccharide of the vitreous humor. J. Biol. Chem. **107(3)**: 629-634.
- **Mirnejad, R. ; Fallahi, S. ; Kiani, J. ; Jeddi, F. ; Khoobdel, M. ; Jonaidi, N. and Alaeddini, F.** (2008) . Epidemic assessment of bacterial agents in osteomyelitis and their antibiotic resistance pattern determination. Iran. J. Boil. Sci. (**8):** 478- 481 .
- **Mohammad, R. P. and Saeed, N.** (2009) . *Staphylococci* . J. of Chinese Clin. Medicine . Vol.4 . Chapter 6 . pp. 67 .

- **Muckenschnabel, I. ; Bernhardt, G. ; Spruss, T. and Buschauer, A. (**1998) **.** Pharmacokinetics and tissue distribution of bovine testicular hyaluronidase and vinblastine in mice : an attempt to optimize the mode of adjuvant hyaluronidase administration in cancer chemotherapy. Cancer Lett. **131 (1):** 71-84.
- **Muhammad, A. ; Yameen, H. ; Nasim, N. ; Akhtar, S. I. ; Imran, J. and Abdul, H.** (2009) . Antibiotic susceptibility profile of methicillin-resistant *Staphylococci* isolated from nasal samples of hospitalized patients . African J. of Microbiol. Research. Vol. 4 . **(3) :** pp. 204-209 .
- **Mylonakis, P.J. ; Rao, S. ; Wilkins, J. ; Moore, T.M. and Harvey, P.J.** (2007) . Analysis of 61 cases of vertebral osteomyelitis. J. Clini. Orthop. Relat. Res. Mars. **264**:178-183.
- **Nathan, L. ; Michael, P. and Marinis, P. M.** (2003). Primary Bacterial Pyomyositis Associated With Septic arthritis Caused by *Streptococcus pyogenes*. J. Amjor Orthopedics .
- **National Committee for Clinical Laboratory Standards (NCCLs).** (2007) . Performance Standards for antimicrobial susceptibility testing. Villanova . PA. USA.
- **Oliveira, M. ; Bexiga, R. ; Nunes, S. F. ; Carneiro, C. ; Cavaco, L. M. and Bernardo, F.** (2006) . Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates . Vet. Microbiol. **(118) :** 133- 40 .
- **Paediator, J.** (2005) . Acute osteomyelitis and septic arthritis in children. Int. J. Antimicrobiol. Agent . **41**: 59-62.

- **Pfaller, M. A. ; Davenport, D. ; Bale, M. ; Barrett, M. ; Koontz, F. and Massanari, R. M.** (1988) . Development of the quantitative micro-test for slime production by coagulase negative *Staphylococci*. Eur. J. Clin. Microbiol. Infect. Dis. **7:** 30-33.
- **Pirrello, R. D. ; Ting, C.C. and Thomas, S. H.** (2007) **.** Initial experiences with subcutaneous recombinant human hyaluronidase. J. Palliat. Med. **10 (4)** : 861-864.
- **Podschun, R. ; Tom, S. and Ullmann, U.** (1998) . Clin. Microbiol. Rev. **11(4)** : pp. 589-603.
- **Rebecca, A. ; Brady, R. ; Jeff, G. ; Leid, S. ; Jason, H. ; Calhoun , J. ; William, C. ; Mark, E. and Shirtliff** . (2008). Osteomyelitis and the role of biofilms in chronic infection . FEMS. Immunol. Med. Microbiol. **52(1)** : 13-22 .
- **Rees, M.D. ; McNiven, T.N. and Daviies, M. J.** (2007) . Degradation of extracellular matrix and its components by hypobromous acid. Biochem. J. **401:** 587-90 .
- **Richard, P. N. ; Tom, A. and Greenberg, M.T.**(2000). Ciprofloxacin, Lomfloxacin or Levofloxacin as Treatment for Chronic Osteomyelitis Antimicrobial Agents and Chemotherapy. Vol. 44 . (**1)** : P 164-166 .
- **Rissing, J. P.** (1997) . Antimicrobial therapy for chronic osteomyelitis in adults. Role of the quinolones. Clin. Infect. Dis. **25**: 1327-1333 .
- **Rogolsky, D. J. and Breuil, C.** (1979) . Penicillinase (β-lactamase) formation by blue –green algae. Arch. Microbiol. **112**: 219-223 .

- **Rowe, G. E. ; Has, W. and Welch, R.** (1994) . Assay of Hemolytic Toxins Methods. Enzymol. **235**: 657-675.
- **Ryan, K. J. ; Ray, C. G. and Graw, H.** (2004). Sherris Medical Microbiology. (4^{Th} ed.) . pp: 8385-8394 .
- **Sabuj, S. ; Satyaranjan, M. ; Anindita, N. ; Sashi, K. P. ; Prasana, K. P. ; Sashi , K. D. and Poluri , E.** (2009) . Isolation, Screening and Characterization of Hyaluronidase Producing Bacteria . Iran. J. Pharm. Sci. **5(2**): 95-102 .
- **Sague, G.M. and Henry, N.K.** (1994). Pediatrics bone and joint infection . Diagnosis and anti-Microbial Management Pediatrics. Clin. North. Am. **43(4)**: 933-47.
- **Salton, B.G. ; Adam, A. and Cumberland, N.** (1996). Antibiotic release from impregnated pellets and beads. J. trauma. **26**: 3-35.
- **Sax, H. and Lew, D.** (1999) . Osteomyelitis . Curr. Infec. Dis. Rep. **1**: 261-66.
- **Seza, A. and Fatma, Ö.** (2007) . Slime production and antibiotic susceptibility in *Staphylococci* isolated from clinical samples **.** Turk. J. Med. Sci. Vol. 102. **(1) :** pp. 29-33 .
- **Smith**, **R. F. and Willett, N. P.** (1968) . Rapid plate method for screening hyaluronidase and chondroitin sulfatase- producing microorganisms. Appl. Microbiol. Vol. 16 . **9** : pp 1434- 1436. U.S.A
- **Sompolinsky , D. ; Samra , Z. ; Karakawa , W.W. ; Vann , W.F. and Malik , Z.** (1985) . Encapsulation and capsular types in isolates of *S. aureus* from different sources and relationship to phage type . J. Clin. Microbiol. **22** : 828-34 .

- **Spellerberg, B.** (2000) . Pathogenesis of neonatal *Streptococcus agalactiae* infections. Microbes Infect. **2 (14):** 1733-1742.
- **Stengel, D. ; Bauwens, K. ; Sehouli, J. ; Ekkernkamp, A. and Porzsolt, F.** (2001). Systematic review and meta-analysis of antibiotic therapy for bone and joint infection. Lancet Infect. Dis. **1**: 175-188.
- **Suheyla, T. and Osman, K.** (2006) . Determination of Some Virulence Factors in *Staphylococcus spp.* Isolated From Various Clinical Samples. Turk. J. Med. Sci. **(30) :** 127-132 .
- **Tachdjian, S.S. ; Kirshner, S.A. ; Mahnke, L. A. and Frank, D.W**. (1990). Osteomyelitis. Invest**.** Orphthalmol.Vis. Sci. **34**: 2699-2712.
- **Tam, Y. C. and Chan, E.C.S.** (1983). Modifications enhancing reproducibility and sensitivity in the turbidimetric assay of hyaluronidase. J. Microbiol. Methods. **1**: 255-60.
- **Tam, Y.C. and Chan, E.C.S.** (1985). Purification and characterization of hyaluronidase from oral *Peptostreptococcus* species. Infect. Immun. **47**: 508-12.
- **Tan, J.S. and File, T.M.** (1996) . Management of *Staphylococcal* and *Streptococcal* Infections. Clin. Podiatr. Med. Surg. **13**: 793-816 .
- **Tarkowski, A.** (1999) : Pathogenetic events in experimental *Staphylococcus aureus* infections. pp : 102-119. U.S.A
- **Thakker, M. ; Park, J. S. ; Carey, V. and Lee, J. C.** (1998) *Staphylococcus aureus* serotype 5 capsular polysaccharide as antiphagocytic and enhances bacterial virulence in a murine bacteremia model. Infect. Immun. **66:** 5183-9.

- **Thanh, T. and Lee, C. Y.** (2002) . Overproduction of type 8 capsular polysaccharide augments *Staphylococcus aureus* virulence. Infec. and Immun. **70:** 3389-3395 .
- **Thomas,C.G.A**. (1977) . Gram- Positive Cocci, Medical Microbiology. (3Rd ed.). Baillere Tindall London.
- **Tice, P. A. ; Alan, D. ; Hoaglund, A. ; David, A. and Shoult, Z.**(2003). Risk factor and treatment out comes in osteomyelitis . J. of Antimicrob. Chemother. **51**: 1261-277.
- **Todar, K.** (2004) . Bacterial Protein Toxins (Bacterial Toxigenesis) **.** Text Book of Bacteriology. Sci. Mag. **(304)** : p. 1421 .
- **Tollersrud, T. ; Kenny, K. ; Reitz, A. J. and Lee, J. C.** (2000) . Genetic and serologic evaluation of capsule production by isolates of *Staphylococcus aureus* and other *Staphylococcus spp.* from Europe and the United States. J. Clin. Microbiol. **(38):** 2998-3003 . USA .
- **Twaij, J.H.; AL- Najar, A.R. and AL- Hashemi, E. H .** (2001). Clinical and bacteriological analysis of osteomyelitis. Iraqi J. Comm. Med. Vol.14 . **(2)** : pp. 201-205.
- **Waldvogel, F.A. ; Medoff, G. and Swartz, M.N.**(1970) . Osteomyelitis. A review of clinical features , therapeutic considerations and unusual aspects. J. Med. **282**: 198-206 , 260-6 , 316-22.
- **Weerd, J.L.L. ; Kinapen, C. and Miedema, J**.(1997). Spinal osteomyelitis cased by *Proteus mirabilis* in a child . **156**: 33-34.
- **Weinstein, R.** (1998). Special Issue . Nosocomial Infection. J. Emerging infec. Dis. pp : 4-3 .

- **Wheat, L. J. ; Allen, S.D.; Henrt, M.; Kluebler, T. and Norton, J.** (1986). Diabetic Foot Infections. Bacteriology Analysis Arch. Med. **146** : 1935 -40.
- **Willams, J.D.** (1999). β-Lactamase and β-Lactamase inhibitors. J. Antimicrobal. Agent . **12 (1)**: 53-57 .
- **Wojnicza, D.; Klaka, M.; Adamski, S. and Jankowski, F.** (2007). J. Microbiol. **52(4)**: 429-436 .
- **Wolcott , R. D. and Ehrlich , G.D.** (2008) . Biofilms and chronic infections . JAMA. **299**: 2682-2684 .
- **Zeidan , I. A.** (2005) . Bacteriological and Genetic Study on Different Clinical Samples of *Staphylococcus aureus* Resistant to Vancomycin . Master in Biotechnology . College of Science , Baghdad University .
- **Zimmerli ,W. ; Widmer, A.F. ; Blatter, R.F. and Ochsner, P.E.** (1998) . Role of rifampicin for treatment of *Staphylococcal* infection . UK .
- **Ziran, B.H.** (2007) . Osteomyelitis. J. Trauma. **62 (6)** : 559–560 . USA .

The Questionnaire , AL-Nahrain University / Collage of Science / Department of Biotechnology (MSc. Research) :

Name of patient :

Sex :

Age :

Historial disease :

Other diseases :

Site affected :

History of recurrent attacks :

History and preview surgery :

Was the patient on antibiotic now ?

الملخص

تضمنت هذه الدراسه جمع خمسون عينه من عينات المسحة القطنية والخزع النسيجيه من خمسه وعشرون مريض يعانون من مرض خمج العظام المزمن من مستشفيات مختلفه فى بغداد : مستشفى اليرموك التعليمى ، مستشفى الجراحات التخصصي ومستشفى بغداد التعليمى خلال الفتره من 2010/6/1 الى 2011/5/1 .

ولقد اظهرت النتائج ان اكثر الاجناس تاثرا بالمرض هو جنس الذكور بنسبه 84% وكانت اعلى نسبه حدوث للاصابه متمثله بالفئه العمريه 39 - 30 سنه متوزعه ما بين سبعه ذكور (28%) وثلاثه اناث (12%) .

اعتمادا على الفحوصات المجهريه ، الزرعيه والخصائص البايوكيميائيه ، كانت نتائج عينات كلا من المسحات القطنية والخزع النسيجيه قد اظهرت ان 50% من العزلات تعود الى *Staphylococcus aureus* 26% ، *Enterobacter cloacae* 14% , *Pseudomonas aeruginosa* 6% , *Escherichia coli* و 4% *Klebseilla sp.*

اما نتائج فحص الحساسيه للمضادات الحياتيه المختلفه فقد اوضحت ان هنالك تغاير كبير فى استجابه البكتريا لاحد عشر مضاد عندما كانت كل عزلات بكتريا *S. aureus* ذات مقاومه تامه ضد methicillin , vancomycin , cephalothin و tetracycline يتبعها clindamycin و gentamicin و penicillin بنسبه 83% وبالعكس كانت كل تلك العزلات ذات حساسيه تامه ضد chloramphenicol , ciprofloxacin , novobiocin و rifampicin .

وكانت النتائج مشابهه بالنسبه لعزلات *Enterobacter cloacae* عندما كانت ثلاثه من هذه العزلات ذات مقاومه عاليه ضد ampicilllin , amoxicillin , cephalothin و vancomycin , rifampicin , tetracycline و penicillin وعزلتين منها ذات مقاومه عاليه ضد chloramphenicol و trimethoprim وعزله واحده قاومت gentamicin ، فى حين كانت تلك العزلات الثلاثه حساسه ضد nalidixic acid و ciprofloxacin .

اما فيما يخص نتائج فحص الحساسيه لكلا من عزلتى *Pseudomonas aeruginosa* و *E.coli* فهى تمتلك حساسيه عاليه ضد ciprofloxacin لكنها ذات مقاومه عاليه ضد كل مجاميع

المضادات الاخرى بينما كانت عزله *Klebseilla sp.* حساسه ضد chloramphenicol ولها مقاومه عاليه ضد كل مجاميع المضادات الاخرى .

اعتمادا على نتائج فحص الحساسيه للمضادات فقد تم اختيار اربعه عزلات من بكتريا *S. aureus* وذلك لتحديد التركيز الادنى المثبط للنمو البكتيرى (MIC) لاربعه مضادات وهى vancomycin , methicillin , cephalothin و tetracycline بشكل منفصل لكل مضاد وكانت نتائج ال MIC لمضاد methicillin هى 320 للعزله S_1 بينما كان 80 للعزله S_3 كذلك كان 40 للعزله S_4 وللعزله S_7 كان 80µg/ml . اما نتائج ال MIC لمضاد vancomycin كانت 160 ، 640 ، 80 و 40µg/ml للعزلات S_1 ، S_3 ، S_4 و S_7 على التوالى .

وكانت نتائج ال MIC لمضاد cephalothin هى 320 ، 80 ، 640 ، 160µg/ml للعزلات S_1 ، S_3 ، S_4 و S_7 على التوالى . اما بالنسبه لنتائج ال MIC لمضاد tetracycline فهى 320 ، 80 ، 640 و 40µg/ml للعزلات S_1 ، S_3 ، S_4 و S_7 على التوالى .

لغرض البحث فى عوامل الضراوه لبكتريا *S. aureus* المسببه لمرض خمج العظام المزمن ، فقد كانت اربعه من عزلات *S. aureus* لها القابليه على انتاج بعض عوامل الضراوه مثل المحفظه ، الطبقه الحيويه ، الطبقه اللزجه وانزيم الهيالورونيديز ولها القابليه على تحليل دم الانسان بسبب انتاجها لانزيم الهيمولايسين من نوع بيتا .

وعند دراسه قابليه العزلات الاربعه البكتيريه على انتاج المحفظه ، كانت طريقه ال capsule stain اكثر دقه ووضوحا من طريقه ال negative stain وكذلك قدرتها على انتاج الطبقه اللزجه باستخدام طريقه احمر الكونغو اكار وهى طريقه مفيده واكثر خصوصيه للتمييز بين العزلات المنتجه وغير المنتجه للطبقه اللزجه وكان لها القدره على انتاج الطبقه الحيويه فلقد اظهرت النتائج ان ثلاثه من هذه العزلات كانت ذات انتاجيه عاليه بينما اظهرت العزله الاخيره انتاج معتدل للطبقه الحيويه بالاضافه الى قدرتها على انتاج انزيم الهيالورونيديز باستخدام طريقتين مختلفتين هى : طريقه الاطباق وطريقه خفض العكوره ، ففى طريقه الاطباق كانت اقطار مناطق التحلل قد تم تحديدها وهى (27mm , 18mm , 6mm , 7mm) للعزلات S_1 ، S_3 ، S_7 و S_4 على التوالى .

وكانت هذه الطريقه اسرع وافضل للكشف عن العزلات المنتجه وغير المنتجه للانزيم من طريقه خفض العكوره التى اوضحت ان العزلات الاربعه المنتجه قد تسببت فى خفض عكوره الوسط السائل .

بسم الله الرحمن الرحيم

وفوق كل ذى علم عليم

صدق الله العظيم

سوره يوسف : جزء من الايه ٧٦

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

عوامل الضراوه المحفزه للاصابه الميكروبيه في مرض خمج العظام المزمن

رسالة

مقدمة الى كلية العلوم - جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الأحيائية

من قِبل

رنا عدنان محسن

بكالوريوس علوم الحياة- كلية التربية (ابن الهيثم) – جامعة بغداد - 2001

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