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Microbiological and Genetic Study on Non-Hemolytic *Streptococcus* spp. Isolated from X-Ray exposed Technicians and Subjection Isolates to Laser and Plasma Radiation.

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
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دراسة مايكروبايولوجية ووراثية على *Streptococcus* spp. غير المحللة للدم المعزولة من العاملين في مجال الاشعة السينية وتعريض العزلات لاشعتي الليزر و البلازما.

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مقدمة إلى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الإحيائية

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ريم نعيم ابراهيم العزاوي

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Summery

This study included isolation and identification of *Streptococcus* spp. from X-ray exposed patients and healthy persons. From a total of (144) throat samples, (122) swabs obtained from X-ray exposed patients and (20) swabs from healthy persons. The samples were collected from different hospitals and X-ray clinics in Baghdad during the period from October /2010 to June/2011.

Results of identification by the biochemical tests of X-ray exposed patients isolates showed that 44(35.4%) isolates were *Streptococus* spp., 43(27.4%) *Staphylococcus* spp., 15(12.2%) *Bacillus* spp.and 31(35%)isolates were belonged to the other microorganisms .While results of identification isolates from healthy persons showed that 9(45%) isolates were *Streptococcus* spp.,3(15%) *Staphylococcus* spp., 2 (10%) *Bacillus* spp. and 6(30%) isolates were belonged to the other microorganisms.

Identification of *Streptococcus* spp. isolated from patients exposed to x-ray by rapid ID32 system showed that (71.4%) isolates were *Streptococcus oralis, (*14.2 %) *S. viridians and* (14.2%) *S.salvairus.* While results of Identification of *Streptococcus* spp. isolated from healthy persons by rapid ID32 system showed that (57.1%) isolates were *Streptococcus oralis, (*28.5%) *S. feacalis, and* (14.2%) *S. mutans.*

Plasmid profile was determined for all *Streptococcus* spp. isolates. Results showed that all *Streptococcus* spp. exposed to X-ray lost their plasmid, while only two isolates of *Streptococcus* spp. isolated from healthy persons possessed a small size plasmid.

When the susceptibility of isolates toward 8 of commen antibiotics, was tested, results showed that several isolates of Streptococcus spp. isolated from healthy persons were resistant to most of the antibiotics used. The percentages of resistance were 92.8, 85.7, 50, 28.5 and 14.2% of isolates against ampicillin, cephalothin, bacitracin, erythromycin, tetracycline and gentamycin respectively. However *Streptococcus* spp. isolates exposed to X-rays patients were more resistant to same antibiotics.

The susceptibility to antibiotics of *Streptococcus* spp. isolates of X-ray exposed patients after exposed to diod laser 890nm was increased only toward bacitracin, While *Streptococcus* spp isolated from healthy persons were more resistance to cephalothin , cefixime and gentamycin ,despite the suscepibility to bacitracin was increased.

The ability of *Streptococcus* isolates for biofilm production was decreased after exposing to diod laser 890nm.

Almost same results in antibiotic susceptibility and biofilm production of *Streptococcus* spp. were obtained after exposure to Non-Thermal plasma, while biofilm production of X-ray exposured isolates was increased after exposure to Non-Thermal plasma.

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List of Abbreviations

Abbreviation	Words
A.A.H	Aquat Anim Health
AAP	American Academy of Pediatrics
ACOG	American College of Obstetricians and Gynecologists
API	Analytical Profile Index
BCD	barrier coronal discharge
CDC	Centers for Disease Control
CFU	Colony forming unit
CTE	Complete Thermal Equilibrium
DBD	Dielectric barrier discharge
EPS	Extracellular Polymeric Substance
EtO	Ethylene Oxide
FDA	Food and Drug Administration
GAS	Group A Streptococci
GBS	Group B Streptococci
GGS	Group G Streptococci
MRSA	Methicillin-Resistant *S. aureus* strains
NCCLs	National Committee for Clinical Laboratory standards
NTP	Non-Thermal Plasma
O.D	Optical Density
PC	Plasma Coagulator
P.R.R	Pulse Repetition Rate
PSR	Plasma Skin Regeneration technology
RF	Radio Frequency
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TP	Thermal Plasmas

U.S.N.R.C	United States Nuclear Regulatory Commission
UV	Ultraviolete

Chapter one

Introduction &Literature review

1-1: Introduction

Streptococcus bacteria are commonly found in human throat, mouth and in respiratory tract. It spreads easily in air and is responsible for sore throat, fever and sometimes pneumonia. This is a Gram positive bacterium. It contains pathogenic and commensal bacteria. Viridans ***Streptococcus*** spp are a commensal streptococcal bacteria, that are either α-hemolytic, producing a green coloration on blood agar, or nonhemolytic. They possess no Lancefield antigens. In general, pathogenicity is low. The organisms are most abundant in the mouth, and one member of the group, *S. mutans*, is the etiologic agent of dental caries. Others(*S.sanguis,S.mitis,S.oralis*) may be involved in other mouth or gingival infections. If they are introduced into the bloodstream, they have the potential of causing endocarditis, particularly in individuals with damaged heart valves. (Ryan and Ray, 2004)

X-Rays have high energy and short wavelength and are able to pass through tissue. Because staff in the X-Ray department work with X-Rays all the time they would, if they stayed beside every patient, over the course of time, be exposed to quite a high dose of radiation. This is why they go behind a shield when the X-Ray beam is switched on. The cumulative effect would be significant for them in a way that it is not significant for an individual patient. (Internet 1)

Since the invention of lasers fourty years ago,it had been developed rapidly and applications of lasers have been expanded from pure physics to biology, technology, chemistry, medicine, and allied field.(Chopra and Chawla,1992)

The basic structure of laser consists on amplifying medium with medium with an inverted population between two mirrors. And there are many kinds of amplifying media, which can be used in the laser to play the essential role of amplifying light through the population inversion and the stimulation an emission. (Saunders *et al*., 1980)

Plasma is a (partly) ionized gas, which contains free charge carriers (electrons and ions), active radicals and excited molecules. So-called non-thermal plasma. . In the past two decades non-thermal plasmas have made a revolutionary appearance in solid state processing technology. The recent trends focus on using plasmas in the health care for "processing" of medical equipment and even living tissues. The major goal of tissue treatment with plasmas is non-destructive surgery:controlled, high-precision removal of diseased sections with minimum damage to the organism. Furthermore, plasmas allow fast bacterial inactivation, which makes them suitable for sterilization of surgical tools and local disinfecting of tissues. (Boyd and Sanderson, 2003)

Although little attention has been given to the bactericidal effected of laser and plasma radiation particularly using low-power lasers and plasmas, they have demonstrated that He-Ne laser light and have no inhibitory action on cariogenic bacteria. (Okumotto *et al*., 1992)

Photodynamic therapy have been used to treat patient with post surgical infections and abscesses, the bacteria involved being *Pertostreptococcus anaerobes, Staphylococcus aureus and Streptococci spp*.(Karu *et al*.,1984)

In this study effect of low-level laser and Non Thermal plasmas radiation on *Streptococcus* spp. isolates with different exposure time were investigated through the following steps:

1-Isolation and identification of normal flora *Streptococcus* from healthy persons and X-Ray exposed patients.

2-Isolation of plasmid DNA from *Streptococcus* spp.

3-Determination of antibiotic sensitivity and biofilm formation of *Streptococcus* spp. after exposed to Diod laser 890nm and Non Thermal plasmas.

1.2- Literature review

1.2.1-Normal Flora of human body

Microbes that colonize the human body during birth or shortly thereafter, remaining throughout life, are referred to as normal flora. Normal flora can be found in many sites of the human body including the skin (especially the moist areas, such as the groin and between the toes), respiratory tract (particularly the nose), urinary tract, and the digestive tract (primarily the mouth and the colon), Table (1-1), On the other hand, areas of the body such as the brain, the circulatory system and the lungs are intended to remain sterile (microbe free) (Salyers and Whitt, 2000).

The mixture of organisms regularly found at any anatomical site is referred to as the normal flora, except by researchers in the field who prefer the term "indigenous micro biota". The normal flora of humans consists of a few eucaryotic fungi and protists, but bacteria are the most numerous and obvious microbial components of the normal flora (Todar, 2004).

The makeup of the normal flora depends upon various factors, including genetic, age, sex, stress, nutrition and diet of the individual. (Internet 2).

Anatomical Location	**Predominant bacteria**
Skin	staphylococci and corynebacteria
Conjunctiva	sparse, Gram-positive cocci and Gram-negative rods

Oral cavity	
Teeth	streptococci, lactobacilli
Mucous membranes	streptococci and lactic acid bacteria
Upper respiratory tract	
Nares (nasal membranes)	staphylococci and corynebacteria
Pharynx (throat)	streptococci, neisseria, Gram-negative rods and cocci
Lower respiratory tract	None
Gastrointestinal tract	
Stomach	*Helicobacter pylori* (up to 50%)
Small intestine	lactics, enterics, enterococci, bifidobacteria
Colon	bacteroides, lactics, enterics, enterococci, clostridia, methanogens
Urogenital tract	
Anterior urethra	sparse, staphylococci, corynebacteria, enteric
Vagina	lactic acid bacteria during child-bearing years; otherwise mixed

Table (1-1): Predominant bacteria at various anatomical locations in adults. (Todar, 2004)

1.2.2-*Streptococcus:*

Streptococcus is a genus of spherical Gram-positive bacteria belonging to the phylum Firmicutes and the lactic acid bacteria group. Cellular division occurs along a single axis in these bacteria, and thus they grow in chains or pairs, hence the name — from Greek στρεπτος streptos, meaning easily bent or twisted, like a chain (twisted chain).

Contrast this with *staphylococci*, which divide along multiple axes and generate grape-like clusters of cells. *Streptococci* are oxidase- and

catalase-negative, and many are facultative anaerobes. (Ryan and Ray, 2004)

In 1984, many organisms formerly considered Streptococcus were separated out into the **genera *Enterococcus*** and ***Lactococcus***. (Facklam, 2002)

They are widely distributed in nature some are numbers of the normal human flora, and *Streptococci* elaborate variety of extracellular substances and enzymes. *Streptococci* are hydrogenous group of bacteria. (Brooks *et al*., 1991)

They are Gram positive, non- sporulating, usually non-motile and most species are facultative in their oxygen requirements.Requiring nutritionally rich media for growth and sometimes 5% CO_2, homofermentative ,producing mainly lactate but no gas growth is usually restricted to temperature 25-45˚C(optimum 37˚C).(Holt *et al*.,1996)
The *Streptococci* are fastidious organisms that characteristically grow in small, translucent, convex colonies on the agar surface of media enriched with blood, serum, acetic fluid and the like. (Nolte, 1982).

1.2.2.1- Streptococcal classification

In addition to streptococcal pharyngitis (or *strep throat*), certain *Streptococcus* species are responsible for many diseases such as meningitis, bacterial pneumonia, endocarditis, erysipelas and necrotizing fasciitis. However, many streptococcal species are non-pathogenic.

Indeed, Streptococci are anecessary ingredient in Emmentaler ("Swiss") cheese. Streptococci are also part of the normal commensal flora of the mouth, skin, intestine, and upper respiratory tract of humans. (Schrag *et al*.2002)

As a rule, individual species of *Streptococcus* are classified based on their hemolytic properties. Alpha hemolysis is caused by an oxidation of iron in hemoglobin, giving it a greenish color on blood agar. Beta-only hemolysis is complete rupture of red blood cells, giving distinct, wide, clear areas around bacterial colonies on blood agar. Other streptococci are labeled as gamma hemolytic, actually a misnomer, as no hemolysis takes place, fig. (1-1). (Pattersom, 1996)

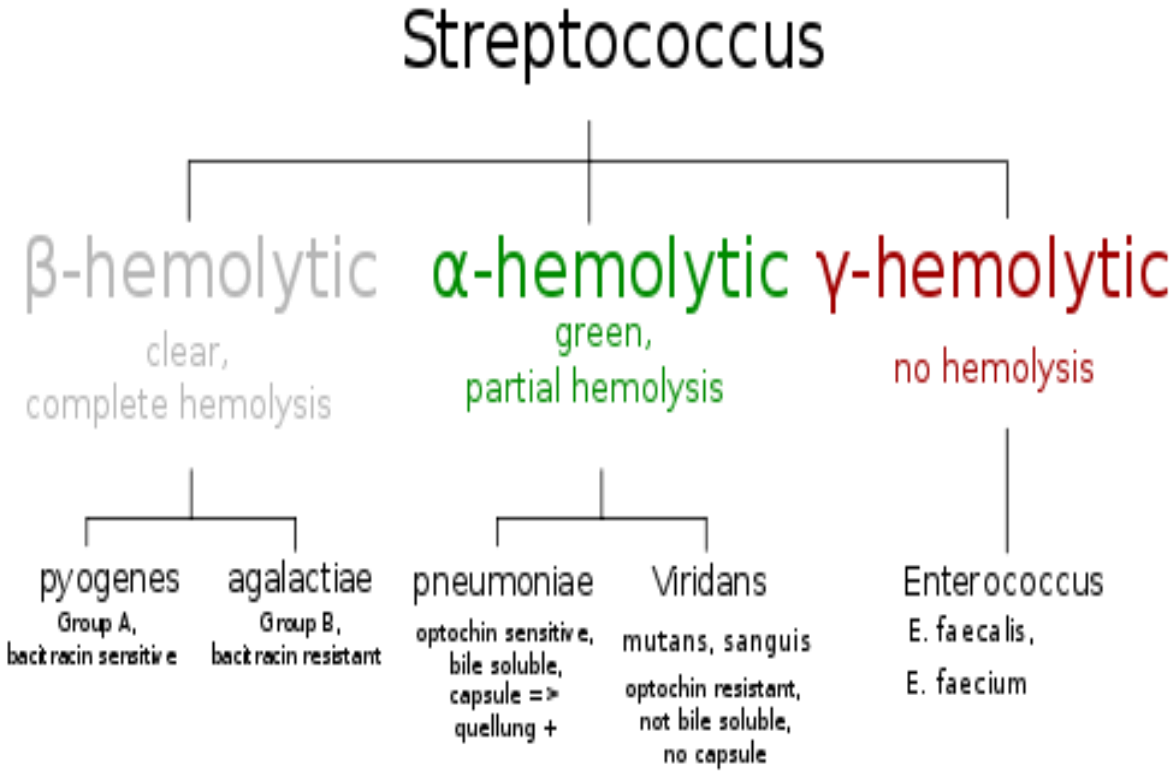


Figure (1-1): Hemolytic Activity of *Streptococci* (Pattersom, 1996)

Beta-hemolytic streptococci are further characterised via the Lancefield serotyping – based on specific carbohydrates in the bacterial cell wall. These are named Lancefield groups A to V (except I and J), although some species, such as *Streptococcus pneumoniae*, do not express Lancefield antigens, but these are alpha-hemolytic not the beta-hemolytic, fig. (1-2). In the medical setting, the most important groups are the alpha-

hemolytic streptococci, *S. pneumoniae* and *Streptococcus* Viridans-group, and the beta-hemolytic streptococci of Lancefield groups A and B (also known as “Group A strep” and “Group B strep”). (Facklam , 2002)

Figure (1-2): Alpha (left) and Beta (right) hemolytic *streptococci* grown on blood agar (Schrag *et al*.,2002)

1.2.2.1.1-The Viridans group: alpha hemolytic and no Lancefield Antigens

Viridans *Streptococcus* is a pseudotaxonomic non-Linnaenan term for a large group of commensal streptococcal bacteria that are either α-hemolytic, producing a green coloration on blood agar plates (hence the name "viridans", from Latin "vĭrĭdis", green), or nonhemolytic. They possess no Lancefield antigens. (Ryan and Ray, 2004)

In general, pathogenicity is low, Viridans streptococci can be differentiated from *Streptococcus pneumoniae* using an optochin test, as *Viridans* streptococci are optochin resistant; they also lack either the polysaccharide-based capsule typical of *S. pneumoniae* or the Lancefield antigens of the pyogenic members of the genus.(Pattersom, 1996)

Viridans streptococci include:

- *Streptococcus mutans*, a contributor to dental caries
- *Streptococcus mitis*, mostly found around cheek region
- *Streptococcus sanguinis*, no preference of locations

- *Streptococcus salivarius*, mostly found on the dorsal side of the tongue
- *Streptococcus salivarius ssp. thermophilus*, used in the manufacture of some cheeses and yogurts
- *Streptococcus constellatus*, occasional human pathogen, notable as colonies grown on blood agar smell strongly of caramel.

1.2.2.1.2-Beta-hemolytic

Group A

Group A streptococci (GAS) cause a wide variety of human pathologic conditions, including pharyngitis, glomerulonephritis, myositis, carditis, necrotizing fasciitis,a toxic-shock-like syndrome involving multiple organ failure, and acute rheumatic fever. Invasive GAS infection can be characterized by severe tissue destruction and endothelial cell damage. (Congeni, 1992; Abolink and Sexton, 1994)

Other *Streptococcus* species may also possess the Group A antigen, but human infections by non-*S. pyogenes* GAS strains (some *S. dysgalactiae* subsp. *equisimilis* and *S. anginosus* Group strains) appear to be uncommon. (Ferrieri and Kaplan, 1992)

Group B

Streptococcus agalactiae, or *GBS*, causes pneumonia and meningitis in neonates and the elderly, with occasional systemic bacteremia. They can also colonize the intestines and the female reproductive tract, increasing the risk for premature rupture of membranes and transmission to the infant. The American College of Obstetricians and Gynecologists (ACOG), American Academy of Pediatrics (AAP) and the Centers for

Disease Control(CDC) recommend all pregnant women between 35 and 37 weeks gestation should be tested for GBS, Women who test positive should be given prophylactic antibiotics during labor, which will usually prevent transmission to the infant. (Schrag *et al*., 2002)

Group C

Includes *Streptococcus equi*, which causes strangles in horses and *S. zooepidemicus* - *S. equi* is a clonal descendent or biovar of the ancestral *S. zooepidemicus* - which causes infections in several species of mammals including cattle and horses. (Harrington *et al*., 2002)

Group D (enterococci) - variably hemolytic

Many former Group D streptococci have been reclassified and placed in the genus *Enterococcus* (including *Streptococcus faecalis*, *Streptococcus faecium*, *Streptococcus durans*, and *Sstreptococcus avium*). For example, *S. faecalis* is now *Enterococcus faecalis*.The remaining non-enterococcal Group D strains include *Streptococcus bovis* and *Streptococcus equinus*. (Ruoof, 1990)

Group G streptococci

These streptococci are usually but not exclusively beta hemolytic. *Streptococcus canis* is an example of a GGS which is typically found on animals but can cause infection in humans. (Auckenthaler *et al*, 1983)

Large colony-forming group G β-hemolytic streptococci (GGS) were first isolated in patients with puerperal sepsis in 1935. (Lancefield and Hare, 1935)

GGS are known to be commensals and pathogens in domestic animals. In humans, they may colonize the pharynx, skin, and gastrointestinal and female genital tracts.In recent years, GGS have been reported with increasing frequency as the cause of a variety of human infections, such as pharyngitis, cellulitis, meningitis, endocarditis, and sepsis. (Dickie *et al*., 1984).

Bacteremia attributable to GGS has been related to underlying conditions, such as alcoholism, diabetes mellitus, malignancy, intravenous substance abuse, or breakdown of the skin. (Lam and Bayer, 1983; Craven *et al*., 1986; Sylvetsky *et al*., 2002).

Non-hemolytic

Non-hemolytic streptococci rarely cause illness. However, weakly hemolytic group D beta-hemolytic streptococci and *Listeria monocytogenes* (which is actually a Gram positive bacillus) should not be confused with non-hemolytic streptococci. (Ruoof, 1990)

1.2.2.2 **Nomenclature of *Streptococcus.* (**Internet 3**)**

Kingdom:	Bacteria
Phylum:	Firmicutes
Class:	Bacilli
Order:	Lactobacillales
Family:	Streptococcaceae
Genus:	***Streptococcus***

Species
S. agalactiae, S. anginosus, S. bovis, S. canis, S. equi, S. iniae S. mitis, S. mutans, S. oralis,.S. parasanguinis, S. peroris S. pneumoniae, S. pyogenes, S. ratti, S. salivarius,S. uberis S. salivarius ssp. thermophilus, S. sanguinis, S. sobrinus, S. suis S. vestibularis, S. viridans

1.2.3:pathogenesis

The pathogenic streptococci in man cause various diseases like sore throat, scarlet fever, rheumatic fever etc. They are divided into groups. Group A streptococci is a pus producing bacteria in wound. Group B streptococci are responsible for neonatal diseases. This type of bacterium is also found in female reproductive organs and may cause premature rupture of membranes. The bacteria can be easily transmitted to the baby and may also cause some infections. Therefore pregnant women in the gestation period of 35 to 37 weeks need to diagnose for GroupB streptococci bacteria. Group C causes infection in animals particularly in horses. Strangles in horses is caused by Group C *streptococcus* bacteria. It is an upper respiratory tract infection most common in horses all over the world. This bacterium is highly contagious. Group c bacteria also cause death in chicken and mouse. (Mike, 2008).

Streptococci are pathogenic bacteria and it causes:

1.2.3.1 Strep throat:

Strep throat is caused by the *Streptococcus pyogenes* bacteria, also called group A beta-hemolytic streptococci (GABHS). This infection is most common in children over three years of age and begins with a fever,

a red, swollen throat, and tonsils that can have a white coating of pus. Other symptoms often include swollen glands, decreased appetite and a decreased energy level. (Kumar *et al*., 2007)

1.2.3.2 Impetigo:

Impetigo is a highly contagious bacterial skin infection most common among pre-school children. People who play close contact sports such as rugby, American football and wrestling are also susceptible, regardless of age. Impetigo is not as common in adults. The name derives from the Latin *impetere* ("assail"). It is also known as school sores**.** (MacDonald, 2004)

It is primarily caused by *Staphylococcus aureus*, and sometimes by *Streptococcus pyogenes*. According to the American Academy of Family Physicians, both bullous and nonbullous are primarily caused by *Staphylococcus aureus*, with *Streptococcus* also commonly being involved in the nonbullous form. (Stulberg *et al*., 2002**;** Kumar *et al*., 2007)

1.2.3.3 Middle ear infection

Acute Otitis Media: The most common cause of fever is an acute ear infection or acute otitis media. This is an infection of the middle ear and mastoid cavity. Mastoiditis as used by the lay public actually refers to "Coalescent Mastoiditis" is a severe infection where the infection in the mastoid cavity starts to directly involve bone. Complications such as meningitis, brain abscess or spread into the soft tissues of the skull may occur. Acute otitis media can be caused by a number of different bacteria. The most common are*: Streptococcus Pneumonia, Heamophilus Influenzae* (this is not the flu virus*), Moraxella*

catarrhalis. Less commonly, *Mycoplasma pneumoniae, Streptococcus pyogenes, Staphylococcus aureus* along with other bacteria and viruses. (Beers and Abramo, 2004).

1.2.3.4 Scarlet fever (a result of a toxin produced by the organism)

Scarlet fever is a disease caused by an infection of the throat with group A beta-hemolytic streptococcal bacteria.**(**Kumar *et al*, 2007)

1.2.3.5 Rheumatic fever

Rheumatic fever is a serious inflammatory condition that can affect many parts of your body — heart, joints, nervous system and skin. The disease is twice as common in females as it is in males. Rheumatic fever is an inflammatory disease that occurs following a *Streptococcus pyogenes* infection, such as strep throat or scarlet fever. Believed to be caused by antibody cross-reactivity that can involve the heart, joints, skin, and brain, the illness typically develops two to three weeks after a streptococcal infection. Acute rheumatic fever commonly appears in children between the ages of 6 and 15, with only 20% of first-time attacks occurring in adults. The illness is so named because of its similarity in presentation to rheumatism. **(**Kumar *et al*., 2007)

1.2.3.6 Toxic shock syndrome**:**

Recently, severe invasive GAS infections associated with shock and organ failure have been reported with increasing frequency, predominantly from North America and Europe. (Jackson *et al*., 1990; Steven, 1992).

These infections have been termed streptococcal toxic-shock syndrome.Persons of all ages are affected; most do not have predisposing

underlying diseases (Francies and Warren, 1988; Holm, 1989; Sregmay *et al*., 1992).

This is in sharp contrast to previous reports of GAS bacteremia, in which patients were either under 10 or over 60 years of age, and most had underlying conditions such as cancer, renal failure, leukemia, or severe burns or were receiving corticosteroids or other immunosuppressing drugs (Brannestein, 1991)

The complications of current GAS infections are severe; bacteremia associated with aggressive soft tissue infection, shock, adult respiratory distress syndrome and renal failure are common; 30% to 70% of patients die in spite of aggressive modern treatments (Steven *et al*, 1989; Steven, 1992;Demers *et al*.,1993).

1.2.3.7 Pneumonia:

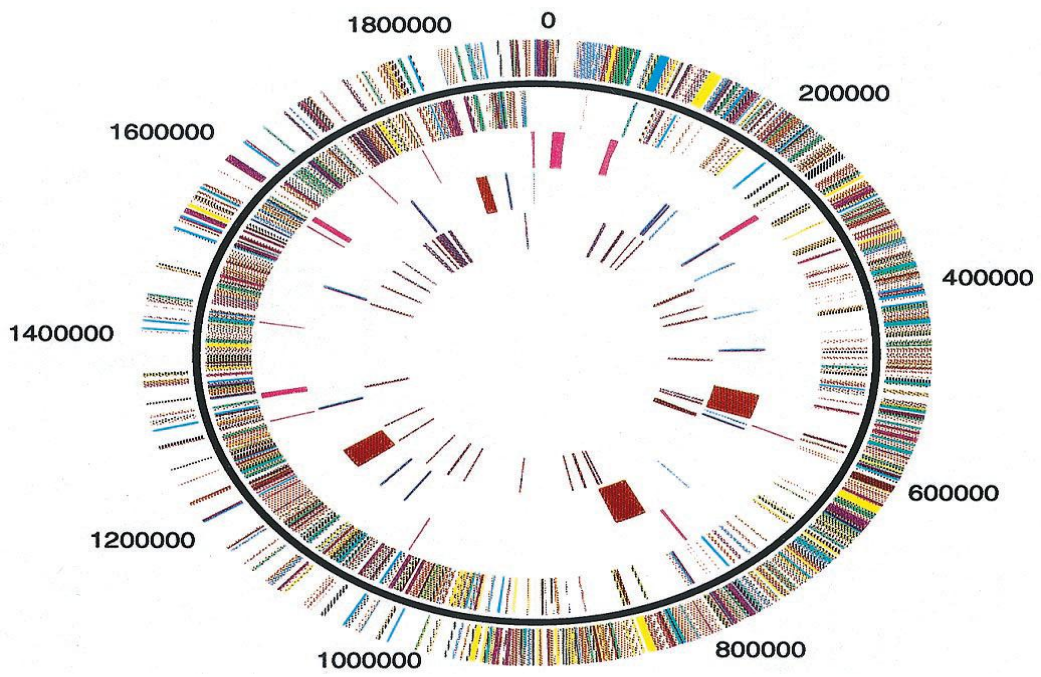
Pneumococci. The cells of these streptococci grow in pairs. *Streptococcus pneumoniae* causes bacterial pneumonia. This was once a major killer especially of the aged and infirm but today there is an effective vaccine and any infections that do occur usually respond quickly to antibiotics (Kumar *et al*., 2007).

1.2.4- Genomic DNA of *Streptococcus*

In modern molecular biology and genetics, the **genome** is the entirely of an organism's hereditary information. It is encoded either in DNA or, for many types of virus, in RNA. The genome includes both the genes and the non-coding sequences.,fig.(1-3) (Ridley, 2006).

The genome sequence of an organism provides information about size of the genome, base composition, complete gene content, physiology and metabolism, content of virulence factors, and lateral gene transfer events.

Particularly interesting are the mechanisms of horizontal gene transfer and how these organisms gain new virulence genes to increase their pathogenic potential as well as how horizontal gene transfer mechanisms affect genome plasticity and evolution. Additionally, the sequence data provide new information about the evolution of these organisms and how they change in order to evade human host immune recognition. (Ferretti ,2001)



Figure(1-3): Circular representation of the *Streptococcus pyogenes* strain SF370 genome. (Ferretti, 2001)

Outer circle, predicted coding regions transcribed on the forward (clockwise) DNA strand. Second circle, predicted coding regions transcribed on the reverse (counterclockwise) DNA strand. Third circle, stable RNA molecules. Fourth circle, mobile genetic elements: burgundy, bacteriophage; blue, transposonsyIS elements; light cyan, transposonsyIS elements (pseudogenes). Fifth circle, known and putative virulence factors: purple, previously identified ORFs; brown, ORFs identified as a

result of genome sequence. The lines in each qconcentric circle indicate the position of the represented feature. Colors: dark gray, amino acid transport and metabolism; light gray, carbohydrate transport and metabolism; green, celC l division and chromosome portioning; olive green, cell envelope biogenesis, outer membrane; salmon, cell motility and secretion; tan, coenzyme metabolism; violet, DNA replication, recombination and repair; yellow, energy production and conversion; light pink, function unknown; rose, general function prediction only; light brown, inorganic ion transport and metabolism; light purple, lipid metabolism; light blue, nucleotide transport and metabolism; orange, posttranslational modification, protein turnover, chaperones; red, signal transduction mechanisms; transcription; green, translation, ribosomal structure and biogenesis; purple, virulence factors; magenta, stable RNA; burgundy, bacteriophage; medium blue, pseudogenes; brown, newly identified virulence factors; blue, transposonsyIS elements. (Ferretti, 2001)

1.2.5 Plasmids of *Streptococcus*

Over the past decades it became evident that virtuall in all bacterial species plasmids exist. These accessory genetic **21**elements are defined as autonomously replicating extrachromosomal DNA Plasmids typically account for only a small fraction of a bacterial genome corresponding roughly to a range between 1 and 200 kilobase pairs. However, extremely large megaplasmids with sizes far beyond 200kb have also been detected such as in *Rhizobium* and others. Plasmids of more than 50 kb might be characterized as large plasmids whereas plasmids used as tools in molecular genetics are often smaller than 10 kb. (Novick, 1980).

The existence of plasmids in the genus *Streptococcus* was first reported in 1972, and interest in the study of plasmids in this important group of bacteria has intensified ever since. (Courvalin *et al.*, 1972).

However, very little work appears to have been done with species of streptococci common to the human oral cavity. To our knowledge, with the exception of one strain of *S. sanguis* and a few isolates of *S. mutans* which apparently all harbor the same plasmid , no reports have appeared on the occurrence of extra chromosomal elements among the oral streptococci (Dunny *et al*., 1973;Yagi *et al.*, 1978)
Starting from 1978 many studies were carried out to elucidate the role and function of Plasmid DNA pool in Streptococci. . (Macrina *et al*., 1978)

1.2.6 Biofilm of *Streptococcus*:

A biofilm is an aggregate of microorganisms in which cells adhere to each other on a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). Biofilm EPS, which is also referred to as slime (although not everything described as slime is a biofilm, figure (1-4), is a conglomeration generally composed of extracellular DNA, proteins, and polysaccharides. Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial and hospital settings. (Hall *et al*., 2004; Lear and Lewis, 2012)

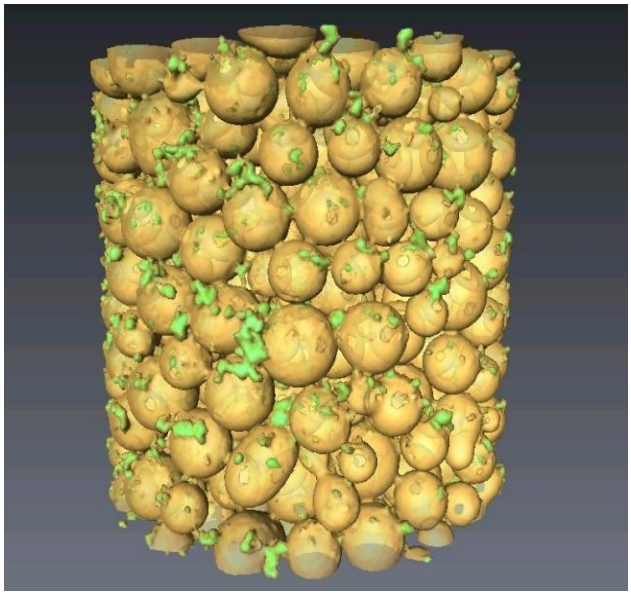


Figure (1-4): The Biofilm of *Streptococcus*.**(**Iltis *et al.*, 2011)

The microbial cells growing in a biofilm are physiologically distinct from planktonic cells of the same organism, which, by contrast, are single-cells that may float or swim in a liquid medium. Microbes form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentrations of antibiotics. (Hoffman *et al*., 2005; Karatan and Watnick, 2009).

When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behavior in which large suites of genes are differentially regulated. (Parsek, 2007).

In nature, and in many medical situations, colonies of bacteria construct and live in a **biofilm**, made up principally of capsule material. A biofilm usually consists of a consortium (mixture) of bacteria living in a matrix of slime which is secreted by one of the bacterial members. Dental plaque is an example of a natural biofilm, as is a slimy mass of bacteria attached to

a rock in a mountain stream. In medical situations, bacteria in a biofilm may have certain advantages over planktonic counterparts. For example, biofilm bacteria may be less susceptible to phagocytosis, drugs, or neutralizing antibodies (Todar, 2004).

1.2.7 Antibiotic sensitivity of *Streptococcus*

The oral cavity, a home to a wide variety of microorganisms, is a large reservoir of antibiotic-resistant bacteria, Fig. (1-5). Antibiotic resistance among the oral microflora has been well documented, and it is gaining increasing attention, especially among the viridans group streptococci (VS). VS are a major cause of morbidity and mortality associated with bloodstream infections, shock, and respiratory distress syndrome in febrile neutropenic patients and plays a leading role in the etiology of infective endocarditic. Their portal of entry is oral and gastrointestinal mucosal lesions and intravascular catheters (Doern *et al*., 1996; Rosser *et al*., 1999; Wisplinnghoff *et al*.,1999; Thomas,2003; Smith *et al*., 2004 ;Rotimi *et al*., 2005)

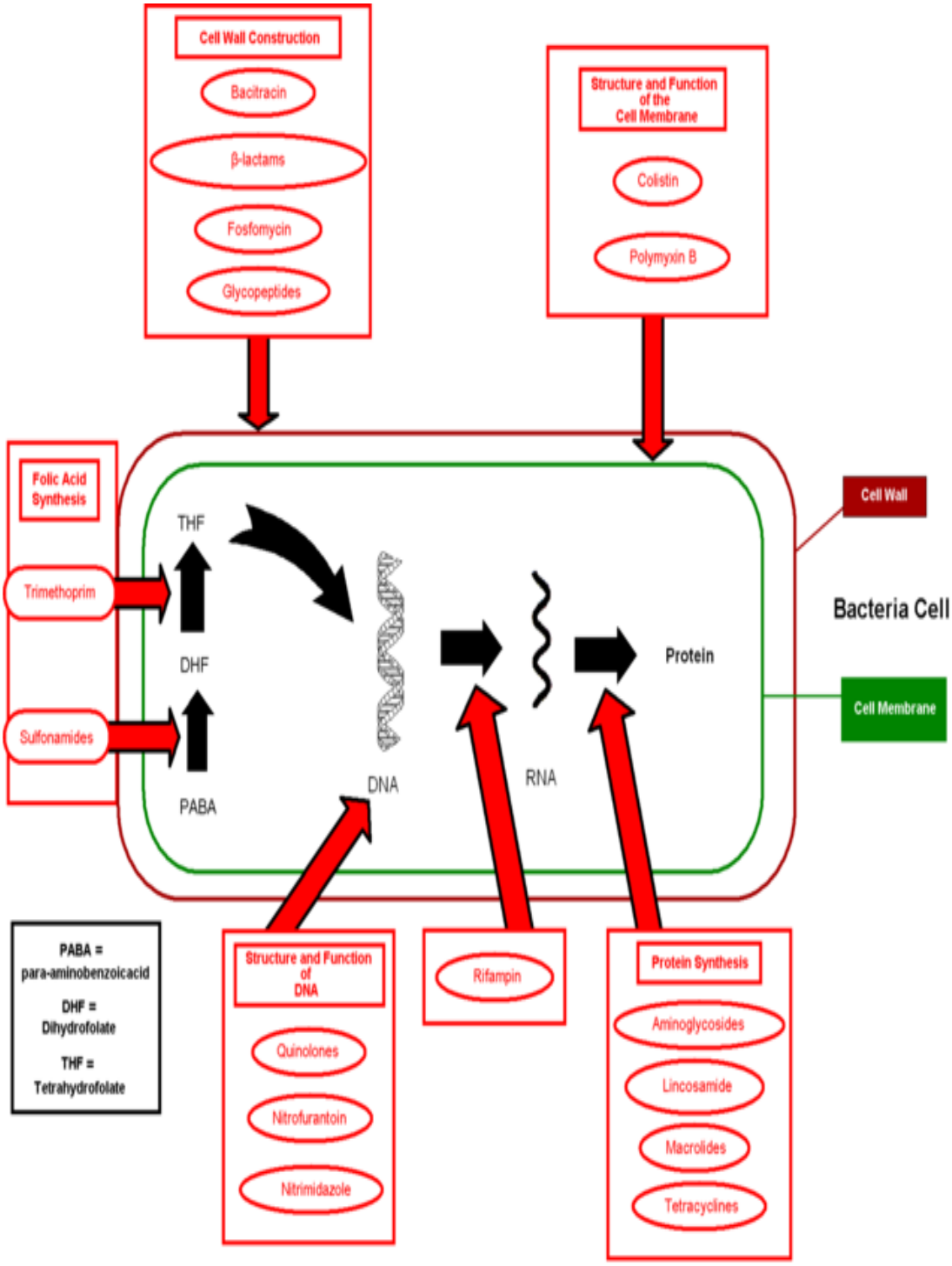


Figure (1-5): The molecular goal of antibiotic on bacterial cell**. (Rghu *et al.*, 2007)**

A major factor associated with infective endocarditis is orodental procedures, which may lead to hematogenous dissemination of VS into many extraoral sites. (Amrouche *et al*., 2004)

Penicillin and, to a lesser extent, erythromycin are the mainstays of therapy for infections caused by VS, and VS have been uniformly susceptible to β-lactam antimicrobial agents. (Bonventura *et al*., 1998; Wisplinnghoff *et al*., 1999)

However, antimicrobial resistance among VS is becoming an obstacle to the antibiotic therapy of, for example, endocarditis. (Smith *et al*., 2004; Rotimi *et al*., 2005)

The emerging increasing rate of microbial resistance to β-lactam antimicrobial agents, macrolides, and tetracyclines among VS poses a serious clinical problem in the management of therapy .(Rosser *et al*., 1999; Wisplinnghoff *et al*.,1999; Amrouche *et al*.,2004; Bancescu *et al*.,2004)

Study by Kastner and colleagues(2001) found that macrolide resistance developed in VGS in children treated for URTI ,Initial pretreatment cultures taken Antibiotics: azithromycin or clarithromycin, 1 week post-treatment 60% of patients had at least one macrolide-resistant organism, 6 weeks later 87% of patients treated with azithromycin were colonized with macrolide-resistant VGS (60% in clarithromycin group) .VGS remain susceptible to a group of antibiotics :(Vancomycin, Linezolid, Daptomycin)but VGS have developed resistance to penicillin and macrolide (MLS) antibiotics. Streptococci will also be found in gastrointestinal tract. The risk of getting the disease is higher in women.

Pregnant women need to diagnose for this bacterium since it can be easily transmitted to the infants.

Medicine for streptococci: Antibiotics and vaccines are developed to control *streptococcus* bacteria. Enthromycin and penicillin have highest resistance capacity against this bacterium. However early treatment by giving antibiotics can help control the diseases. (Smith *et al*., 2004)
In the past, viridans group streptococci were considered to be uniformly susceptible to β-lactam antimicrobial agents, macrolides, and tetracyclines. However, the emergence of strains resistant to β-lactams and other antibiotics is a cause of concern and could compromise currently used prophylactic and therapeutic antibiotic regimens. In one study, after the introduction of penicillin prophylaxis, resistance to penicillin increased from 0% in 1989 to more than 80% in 1994. (Krcmery *et al.*, 1995; Carratalá *et al.*, 1995; Doern *et al.*, 1996; Pfaller *et al*., 1999)

1.2.8- X--ray

X-radiation (composed of **X-rays**) is a form of electromagnetic radiation. X-rays have a wavelength in the range of (0.01 to 10 nm).corresponding to frequencies in the range 30 petahertz to 30 exahertz (3×10^{16} to 3×10^{19})Hz and energies in the range (120 eV to 120 keV). They are shorter in wavelength than UV rays and longer than gamma rays. In many languages, X-radiation is called **Röntgen radiation**, after Wilhelm Conrad Röntgen,1895 who is usually credited as its discoverer, and who had named it X-radiation to signify an unknown type of radiation .Correct spelling of X-ray(s) in the English language includes the variants x-ray(s) and X ray(s).(Oxford English Dictionary,1989). X-

RAY is used as the phonetic pronunciation for the letter x(Novelline and Robert,1997)

X-rays from about(0.12 to 12) keV (10 to 0.10 nm wavelength) are classified as "soft" X-rays, and from about (12 to 120) keV (0.10 to 0.01 nm wavelength) as "hard" X-rays, due to their penetrating abilities. (Holman *et al*., 1996)

Hard X-rays can penetrate solid objects, and their most common use is to take images of the inside of objects in diagnostic radiography an crystallography. As a result, the term *X-ray* is metonymically used to refer to a radiographic image produced using this method, in addition to the method itself. By contrast, soft X-rays hardly penetrate matter at all; the attenuation length of 600 eV (~2 nm) X-rays in water is less than 1 micrometer. X-rays are a form of electromagnetic radiation, just like visible light. In a health care setting, a machine sends individual x-ray particles through the body. A computer or special film is used to record the images that are created,.Structures that is dense (such as bone) will block most of the x-ray particles, and will appear white. Metal and contrast media (special dye used to highlight areas of the body) will also appear white. Structures containing air will be black, and muscle, fat, and fluid will appear as shades of gray. (Mettler, 2005)

1.2.8.1- Properties of X-Ray

X-Rays are defined as weightless package of pure energy (Photon) that are without electrical charge and that travel in waves along a straight line with a specific frequency and speed. The Properties of X-Ray are divided into 4 heading:

1.2.8.1.1 Physical Properties

X-Ray are electromagnetic radiations having a wavelength between 10 a to 0.01A **,** in free space they travel in a straight line,speed-1,86,000 miles/sec(same as that of visible light),they are invisible to eye**,** cannot be heard and cannot be smelt**,** they cannot be reflected, refracted or deflected by magnetic or electric Field**,** they show properties of interference, diffraction and refraction similar to visible light, they produce an electric field at right angles to their path of propagationThey produce an magnetic field at right angles to the electric field and path of propagation, they do not require medium for propagation**,** the X-Ray can penetrate liquids, solids and gases. the degree of penetration depends on quality, intensity and wavelength of X-Ray beam. X-Rays are absorbed by matter, the absorption depends on the anatomic structure of the matter and the wavelength of the X-Ray beam. X-Rays interact with materials they penetrate and cause ionization, when X-Rays fall upon certain materials visible light will be emitted called fluorescence**.** X-Rays have the property of attenuation, absorption and scattering and they also show heating effect**.** (Mettler, 2005)

1.2.8.1.2 Chemical Properties:

X-Ray induces color changes of several substances **or** their solutions Ex:methylene blue gets bleached, X-Rays bring about chemical changes in solution because X-Rays produce highly active radical OH ions in water, which react with the solutes and X-Rays cause destruction of the fermenting powers of enzymes . (Mettler, 2005)

1.2.8.1.3Biological Properties

The excitation property of X-Rays are used in treatment of malignant lesions, X-Rays also have a germicidal or bactericidal effect and the somatic effect of X-Ray ranges from simple sun burn to severe dermatitis or to change in blood supply to malignancy. (Mettler, 2005)

1.2.8.1.4 Physiochemical Property:

X-Rays are capable of producing an image on a photographic film. (Mettler, 2005)

1.2.9- Biological Effects of Radiation on Other Cellular Constituent

Radiation is a process in which energy is transmitted in the form of waves or particles through a medium or through space. There are several sources of radiation. The most common ones are the sun, X-ray machines, microwave ovens and nuclear reactors. Radiation is categorized into ionizing and non-ionizing. Non-ionizing radiation is named so because the energy level is low. Ionizing radiation has a high-energy level as in UV rays and X-rays to name a few, the biological effects of radiation which suggest that radiation may cause cancers if the human body is exposed to high doses of it. Even low doses are supposed to have genetic and somatic effects on the body. Radiation can damage cells, but then the impaired cells are at times capable of repairing themselves. At other times, the cells die, but they would soon be replaced through normal biological process. And there are occasions when the cells can't repair themselves, resulting in pronounced biological effects (Internet 4)

1.2.9.1 Direct Effect of Radiation

Direct effect of radiation means that if a cell is exposed to radiation , the radiation interacts "directly" with the atoms in a DNA molecule, or any other constituent of a cell that is pivotal for survival. This direct effect can even lead to destruction of the life-sustaining system. (Internet 4)

1.2.9.2 Indirect Effect of Radiation

Indirect effect of radiation takes place when a cell is exposed to radiation. The radiation interaction with the atoms in the DNA molecule is negligent. However, the cell is still not immune to destruction. This is because the main constituent of the cell is water. The radiation interacts with water, which breaks the bond of the water to H and OH. These elements recombine with other elements that may form toxic components such as hydrogen peroxide (H_2O_2). A direct interaction of radiation with an active cell could result in the death or mutation of the cell, because dividing cells require correct DNA information in order for the cell's offspring to survive whereas a direct interaction with the DNA of a dormant cell would have less of an effect (Internet 4)

1.2.10-The Laser

In 1917, Albert Einstein made the proposal that particles of light with energy of a particular frequency could stimulate electrons to emit radiation of light of the same frequency. This phenomenon is the key to the operation of the laser .The word laser is an acronym for (**L**ight **A**mplification by **S**timulation **E**mission of **R**adiation) (Saunders, *et al*. 1980)

Laser brought a revolution in optical technology and spectroscopy and had a far-reaching influence in various fields of science and technology.

In the life science, medical science and even in nuclear fusion, many research projects are being carried out using laser. There are natural phenomena such as self-focusing of light and optical bistability. This could only have been discovered through the use of laser (Shimoda, 1984).

1.2.10.1-properties of laser

Some lasers generate visible light but others generate infrared or ultraviolet rays, which are invisible. Although the wavelength of visible light extend from about (0.37_0.75) μm. Laser with a wavelength between0.1μm in vacuum ultraviolet and 1mm in the microwave region are known. There are various types of lasers, ranging in size from semiconductor lasers, which are smaller than 1mm.to laser used in nuclear fusion experiment, which can be as large as 100m. However, their basic properties are more or less the same. (Shimoda, 1984).

1.2.10.2 Components of the laser system

Laser system contains four primary components regardless of the style, the size or the application: (Kandela, 1991)

a-Ative medium

The active medium may be solid crystals such as ruby or Nd: YAG, liquid dyes , gases like CO_2 or He-Ne, or semiconductors such as GaAs. Active media contain atoms whose electron may be excited to a metastable energy level by an energy source.

b-Excitation mechanism

Excitation mechanism pumps energy into the active medium by one or more of three basic methods: optical, eclectic or chemical.

c-High reflectance Mirror

A mirror, which reflects essentially 100% of the laser light (Kandela, 1991).

D-Partial transmissive Mirror

A mirror which reflects less than 100% roughly 80% of the light and transmits the remainder.

1.2.10.3 Application of laser

When lasers were invented in 1960, they were called "a solution looking for a problem".Since then, they have become ubiquitous, finding utility in thousands of highly varied applications in every section of modern society, including consumer electronics, information technology, science, medicine, industry, law enforcement, entertainment, and military application.(Charles,2003)

The first use of lasers in the daily lives of the general population was the supermarket barcode scanner, introduced in 1974. The laserdisc player, introduced in 1978, was the first successful consumer product to include a laser but the compact disc player was the first laser-equipped device to become common, beginning in 1982 followed shortly by laser printers.Some other uses are :(Dalrymple *et al*.,1977;Dalrymple,1983)

- Medicine: Bloodless surgery, laser healing, surgical treatment, kidney stone treatment, eye treatment, dentistry
- Industry: Cutting, welding, material heat treatment, marking parts, non-contact measurement of parts.

- Military: Marking targets, guiding Imunitions, missile defence, electro-optical countermeasures (EOCM), alternative to radar, blinding troops.
- Law enforcement: used for latent fingerprint detection in the forensic identification field
- Research: Spectroscopy, laser ablation, laser annealing, laser scattering, laser interferometry, LIDAR, laser capture microdissection, fluorescence microscopy
- Product development/commercial: laser printers, optical discs (e.g. CDs and the like), barcode scanners, thermometers, laser pointers, holograms, bubblegrams.
- Laser lighting displays: Laser light shows
- Cosmetic skin treatments: acne treatment, cellulite and striae reduction, and hair removal. In 2004, excluding diode lasers, approximately 131,000 lasers were sold with a value of US$2.19 billion. In the same year, approximately 733 million diode lasers, valued at $3.20 billion, were sold. (Steele,2005).

1.2.11-The plasma

1.2.11.1-Introduction to Plasma in physics:

In physics and chemistry, **plasma** is a state of matter similar to gas in which a certain portion of the particles are ionized. Heating a gas may ionize (reduce the number of electrons in its molecules or atoms), thus turning it into plasma, which contains charged particles: positive ions and negative electrons. Ionization can be induced by other means, such as strong electromagnetic field applied with a laser or microwave generator, and is accompanied by the dissociation of molecular bonds, if present (Sturrock,1994).

In physics plasma is considered the fourth state of matter next to solids, liquids and gases. In 1879 the British chemist and physicist William Crookes first described it as “radiant matter “. (Crookes, 2009).

The presence of a non-negligible number of charge carriers makes the plasma electrically conductive so that it responds strongly to electromagnetic fields. Plasma, therefore, has properties quite unlike those of solids, liquids, or gases and is considered a distinct state of matter. Like gas, plasma does not have a definite shape or a definite volume unless enclosed in a container; unlike gas, under the influence of a magnetic field, it may form structures such as filaments, fig. (1-6), beams and double layer. Some common plasmas are stars and neon signs. (Daniel and Henry, 2000)

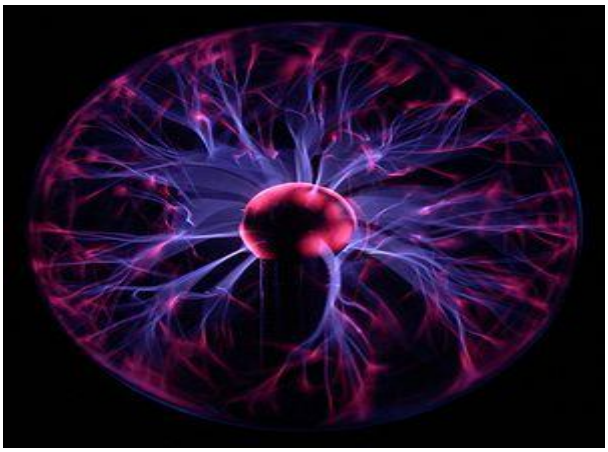


Figure (1-6): Plasma lamp, illustrating some of the more complex phenomena of plasma, including filamentation. (Daniel andHenry, 2000)

1.2.11.2-Definition of a plasma

Plasma is loosely described as an electrically neutral medium of positive and negative particles (i.e. the overall charge of plasma is roughly zero). It is important to note that although they are unbound, these particles are not ‘free’. When the charges move they generate electrical currents with magnetic fields, and as a result, they are affected

by each other's fields. This governs their collective behavior with many degrees of freedom (Sturrock, 1994; Hazeltine and Waelbroeck, 2004). A definition can have three criteria (Dendy, 1990; Daniel and Henry, 2001):

a. The plasma approximation: Charged particles must be close enough together that each particle influences many nearby charged particles, rather than just interacting with the closest particle (these collective effects are a distinguishing feature of a plasma). The plasma approximation is valid when the number of charge carriers within the sphere of influence (called the *Debye sphere* whose radius is the Debye screening length) of a particular particle is higher than unity to provide collective behavior of the charged particles. The average number of particles in the Debye sphere is given by the plasma parameter, "Λ" (the Greek letter Lambda).

b. Bulk interactions: The Debye screening length (defined above) is short compared to the physical size of the plasma. This criterion means that interactions in the bulk of the plasma are more important than those at its edges, where boundary effects may take place. When this criterion is satisfied, the plasma is quasineutral.

c. Plasma frequency: The electron plasma frequency (measuring plasma oscillations of the electrons) is large compared to the electron-neutral collision frequency (measuring frequency of collisions between electrons and neutral particles). When this condition is valid, electrostatic interactions dominate over the processes of ordinary gas kinetics. A plasma is an ionized gas that contains ions, radicals, and electrons, It usually emits UV light, figure (1-7),A well known example of a plasma is the sun. (Raizer *et al*., 1995).

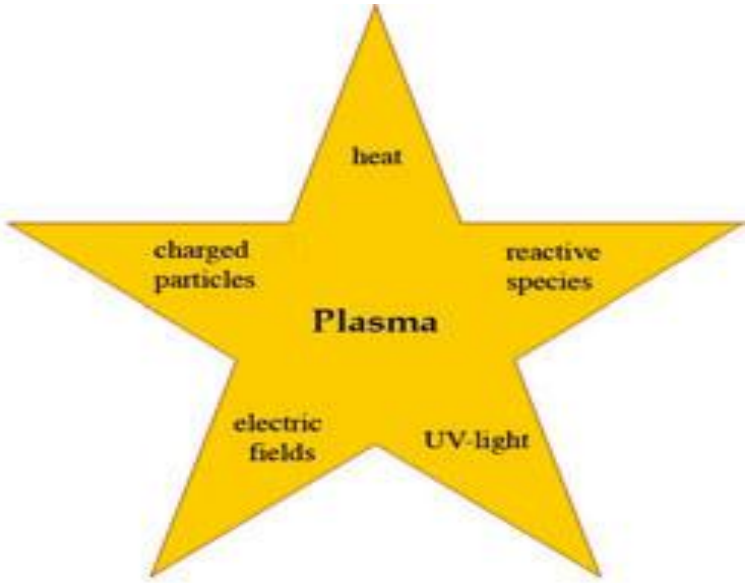


Figure (1-7): Relevant components of plasma. Charged particles and density of free radicals(reactive oxygen and nitrogen species) are mainly responsible for the effects of plasma(Raizer *et al*., 1995).

Various descriptions were used to identify plasma.It could be described as an ionic gas or a gaseous complex comprised of electrons, ions of either polarity, gas atoms, molecules in ground and excited state, and light quanta 10 occurring due to the application of an electric field.

Plasmas may also be classified based on the method of generation as .cold. or Non-Thermal Plasma (NTP), if the gas temperature is at ordinary temperature and the electrons are at a higher temperature and .hot., or Thermal Plasmas (TP), in which there is Complete Thermal Equilibrium (CTE). In other words, the electron and gas temperatures are in equilibrium. Thus plasmas can be categorized as thermal plasma (high temperature plasma or equilibrium plasma) and non-thermal plasma (non-equilibrium plasma or cold plasma) based on their energy level, temperature and ionic density. (Eliasson and Kogelschatz,1991).

Non-equilibrium plasmas are divided into five groups depending on the mechanism used for generation, pressure range and electrode geometry : (Eliasson and Kogelschatz,1991):

a) The glow discharge

b) The corona discharge

c) The silent discharge

d) The radio frequency (RF) discharge

e) The microwave discharge.

Plasmas can also be created on earth, in a laboratory: in most cases this is done inside a vacuum vessel at low pressure. This type of plasmas is used e.g., for activating the surface of polymers, growing solar cells and etching materials. (Hippler *et al*., 2001; Reece Roth, 2001).

1.2.11.3- Plasma in Medicine and Biology

A-Plasma-Based Sterilization

Sterilization is an act or process, physical or chemical, that destroys or eliminates all forms of life, especially microorganisms.

Conventional sterilization techniques, such as those using autoclaves, ovens, and chemicals like ethylene oxide (EtO), rely on irreversible metabolic inactivation or on breakdown of vital structural components of the microorganism. Plasma sterilization operates differently because of its specific active agents, which are ultraviolet (UV) photons and radicals (atoms or assembly of atoms with unpaired electrons, therefore chemically reactive, e.g., O and OH, respectively). An advantage of the plasma method is the possibility, under appropriate conditions, of achieving such a process at relatively low temperatures (≤50 ˚C), preserving the integrity of polymer-based instruments, which cannot be subjected to autoclaves and ovens . Furthermore, plasma sterilization is

safe, both for the operator and the patient, in contrast to EtO. (Moisan *et al*., 2001)

B-Plasma in biomedical field

On the basis of their high bactericidal effectiveness plasmas were also used to sterilize medical devices and in packaging of food stuffs. The development of diverse, usually non-thermal atmospheric-pressure plasma sources makes its use also in the (bio)medical field possible. While in the past only the thermal properties of plasmas (> 80 °C) were utilized – cauterization, sterilization of heat-resistant instruments , cold atmospheric-pressure plasmas at room temperature lead to diverse reactions in tissue.(Morfill *et al*.,2009)

C- Plasma in Wound Treatment: Living Tissue Sterilization

Already in 1970 Robson and coworkers recognized that more than 105 colonyforming units of _-hemolytic streptococci, *Staphylococcus aureus* and *Pseudomonas aeruginosa* on a wound suffice to disturb wound healing . Wound healing is also delayed if more than four different bacteria species are found on a wound. (Kalghatgi *et al.,* 2009). Studies on cell cultures prove that a plasma treatment can influence wound healing not only by a reduction of bacterial colonization but also by direct effects on epidermal and dermal cells .(Wende *et al*.,2009;Kalghatgi *et al*.,2009)

D- Promoting Blood Coagulation with FE-DBD Plasma

Local application of high-temperature plasma for hemostasis and for sclerosing angiodysplasias and ablation of tumors has been used since about 30 years, e. g. in the form of the argon plasma coagulator (APC) in

many surgical disciplines including endoscopic procedures (40–75 W in the MHz range, 1–10 l gas flow rate, ~ 120 °C, ~ 3 eV).(Morrison, 1977; Ginsberg *et al.,* 2002)

The effect of ionized gas applied without tissue contact is thermal and is based on protein denaturation and desiccation. In dermatology the technique was first employed in 1997 by Katsch and his workers for the treatment of intraepithelial disorders (e. g. actinic keratoses, condylomata acuminata etc). The depth of penetration is only 2–3 mm, making the method relatively free of side effects. Nevertheless, depending on site of application diverse complications such as pain, bleeding, perforation, strictures, disturbances in swallowing, the development of gas emphysema of the mucosa, neuromuscular stimulation and even gas explosions have been reported .(Manner *et al.,* 2008)

E- Skin Flora Sterilization by DBD

In quantitative evaluation of the ability of DBD plasma to sterilize the treated tissue from bacteria they observed roughly a 6 to 7 log reduction in 5 seconds of treatment. This supports initial results where they indicated that this plasma treatment achieves complete sterilization in 1 to 3 seconds of treatment. This could also be a promising result for wound healing oriented applications where it could be desirable to sterilize the wound tissue without simultaneously coagulating the blood (Gregory, 2006).

F-Cosmetic applications

In 2005 the Food and Drug Administration (FDA) licensed plasma skin regeneration technology (PSR) for skin rejuvenation and for

treatment of wrinkles. Here a hot, but rapidly cooling plasma, that is produced by a radiofrequency plasma jet with nitrogen as carrier gas, is employed. The heat application induces controlled thermal damage in the skin which results in the new production of collagen, a reduction of elastic fibers and a restructuring of dermal architecture that can be confirmed histologically .(Bogle *et al*.,2007;Foster *et al*.,2008)

In addition to wrinkle treatment the method can also be used in the therapy of, among others, actinic keratoses, seborrheic keratoses, viral papillomas, scars and sun-damaged skin including pigmentary disturbance or in combination with aesthetic-surgical procedures. During treatment local or systemic anesthesia is required. (Kilmer *et al*., 2005; Kilmer *et al*., 2007)

G- Further Applications of Direct Plasma Treatment

A further possible indication in dermatology is the treatment of cutaneous leishmaniasis. Fridman and colleagues demonstrated in a cell culture a successful 100 % inactivation of *Leishmania major* promastigotes within 20 s, while for inactivation of human macrophages in contrast a two-minute treatment was required to inactivate 20–30 %. (Gregory, 2006).

Many bacterial skin diseases such as impetigo contagiosa, folliculitis or ecthyma, fungal infections such as tinea pedis (because ability to penetrate textiles) or even viral diseases might profit from a treatment with cold plasma in the future. A further revolution might also take place in (hospital) hygiene: The Hand PlaSter®, a low temperature plasma device using barrier coronal discharge (BCD) technology (_ 0.5 W/cm², 18 kVpp, 12.5 kHz) that was developed for potential use for hand disinfection allows for *in vitro* a reduction of the bacterial load by over 5

log steps within few seconds (< 10 s). This new form of (hand) disinfection has some advantages over conventional liquid disinfectants, which must be applied Plasma medicine ,therapy was also performed daily over 2 min. for several minutes and irritate the skin.(Morfill *et al*.,2009)

Cold plasmas may also find applications in dentistry in the future. Due to the ability to enter microscopically small openings, theoretically therapeutic measures against periodontitis, chronic gingivitis and in the treatment of infected root canals are possible. Clinical evidence for this must still be gathered. Further, *in vivo* the combination with H_2O_2 showed an increase in the bleaching effect as well as protein removal on the surface of plasma-treated teeth (Lee *et al*.,2009;Sun *et al*.,2009).

Chapter two

Materials &Methods

Chapter Two

2.1 Materials and Method

2.1.1 Apparatus and Equipment:

Apparatus or Equipment	Company(Origin)
Autoclave	Tomy(Japan)
Camera	Sanyo(Japan)
Compound light microscope	Olympus(Japan)
Distillator	GFL(Germany)
Incubator	Memmert(Germany)
Laser system	MILTA(Russia)
Micropipettes	SLAMED(Germany)
Mini API apparatus	BioMerieux(France
Oven	Sanyo
Plasma jet system	Locally designed and Manufactured
Power supply	Desaga(Germany)
Refrigerator	Ishtar(Iraq)
Refrigerated centrifuge	Gallenkamp(UK)
Sensitive Balance	Sartorius(Germany)
Spectrophotometer	Miltonroy(USA)
UV-transilluminator	VilberLourmat(France)
Vortex	Stuart(UK)
Water bath	GFL

2.1.2 Chemicals

Chemical	Company(Origin)
Gram stain	Fluka(Switzerland)
rapid ID 32 strep	BioMerieux(France)
Hydrogen peroxide(H_2O_2)	Local market(IRAQ)
Agarose	Sigma(USA)
Glycerol	BDH(UK)
Agar-agar	Himedia(India)

2.1.3 Antibiotic discs (Bioanalysis (UK))

Antibiotic	Symbol	Conc.(µg/disc)
Bacitracin	B	10
Ampicillin	AM	10
Cephalothin	KF	30
Cefixim	CFM	5
Cephalexin	CL	30
Erythromycin	E	15
Tetracycline	TE	30
Gentamycin	CN	30

2.1.4 Culture Media

2.1.4.1 Ready-to-use media:

Media	Company(Orogin)
Blood agar base	Himedia(India)
Brian heart infusion broth	Himedia
Brian heart infusion agar	Himedia
Mitis salvarius agar base	Himedia
Transport medium swabs	Local market(Iraq)

They were prepared according to the instruction of the manufacturer company before autoclaving at 121˚C for 15mintus.

2.1.4.2 Blood agar (Atlas *et al*., 1995)

The blood agar base was prepared according to Manufacturer Company instruction, after sterilization by autoclave at 121˚C for 15 minutes. The agar was cooled to 50˚C and (5-10%) of human blood were added.

2.1.5 Buffers,Reagents and Solutions:

2.1.5.1 Agarose gel electrophoresis (Maniatis *et al.*, 1982)

Tris-Acetate (50X), pH 8.0

Tris base (2M)	242 g
Glacial Acetic Acid	57, 1 ml
EDTA (0.5)	100 ml
D.W	1000 ml

2.1.5.2 Phosphate buffer solution. (Atlas *et al*., 1995)

NaH_2PO_4	6.00g/L
Na_2HPO_4	9.52g/L

In 1000ml of distilled water and sterilized by autoclave at 121˚C for 15 minutes.

2.1.5.3 Catalase reagent

This reagent composed of 3% of hydrogen peroxide.

2.1.6 Kits:

2.1.6.1 Plasmid DNA isolation Kits (Fermanats Kit,Germany)

Buffer S1 (Bacterial Lysis buffer): 3M guanidine HCL; 20%Tween 20.

Buffer S2 (Equilibration Buffer):750 Mm NaCl; 50 Mm MOPS; 15% isopropanol; 0.15% triton x-100, pH7.0.

(Washing buffer):1.0 M NaCl; 50 mM MOPS; 15% isopropanol, pH 7.

(Elution buffer):1.25 mM NaCl; 50 mM tris-Cl; 15% isopropanol, pH 8.5.

2.1.6.2 Rapid ID 32 kit (BioMerieux,France)

Rapid ID 32 strep kit is a standardized system for the identification of *Streptococci* and *Enterococci*, and those most common related organisms, in 4 hours. Are shown in Appendix.

2.2 Methods

2.2.1 Collection of samples:

One hundred twenty four oral samples (swabs) were collected from patients and x-ray technicians of AL-Yarmook hospital and private X-ray Clinic in Baghdad, Twenty other samples were taken from healthy persons as control during the period from October /2010 to June/2011.

2.2.2 Isolation of *Streptococcus*:

All samples were taken by sterile cotton swabs befor streaking on the surface of blood agar or on the selective media Mitis salvarious agar and incubated 24 hrs at 37˚C in anaerobic jar.

2.2.3 Identification of bacterial isolates (Holt *et al.*, 1996; Atlas *et al.*, 1995)

2.2.3.1 Cultural Examination:

Morphology of colonies was studied on brain heart infusion agar. Color, size and edge of colonies were recorded after 24 hrs of incubation at 37˚C.

2.2.3.2 Microscopic examination (Atlas *et al.*, 1995):

Gram staining method was used to describe gram reaction, shape and arrangement of the bacterial cell.

It was done by placing loopful of water in the ringed area of the slide. Small amount of bacteria was mixed in the water and spreaded it out, the smear was air dried then heat –fix by passing the slide rapidly through the burner flames three times.After that, the smear stained with Gram stained with crystal violet, treated with iodine, decolorized with 95% ethanol ,and counterstained with safranin ,then the smear was examined under oil immersion objective.

2.2.4 Sterilization Methods (Atlas *et al.*, 1995)

Three methods of sterilization were used:

2.2.4.1 Moist Heat Sterilization (Autoclaving)

Media and solutions were sterilized by the autoclave at121 ˚C (15Ib/in^2) for 15 min.

2.2.4.2 Dry Heat Sterilization

Electric oven was used to sterilize glasswares and others at 160-180 ˚C for 2-3 hrs.

2.2.4.3 Membrane Sterilization (Filtration)

It was used to sterilize heat sensitive solutions by using millipore filters (0.22µm) in diameter.

2.2.5 Maintenance of bacterial isolates (Atlas *et al*., 1995)

2.2.5.1 Short term storage

Colonies of bacteria were maintained for period of few weeks on the surface of Mitis salvarius agar medium, the plates were tightly wrapped in the parafilm and stored at 4˚C.

2.2.5.2 Medium term storage

Bacterial isolates were maintained by streaking on slant of brain heart infusion agar for period of few months .This medium was prepared in screw-capped vials containing 10-15ml of the medium. The isolate was streaked on slant medium and incubated at 37˚C for 24hrs, after that, the slants were taken and wrapped with parafilm and stored at 4˚C for a period of few months.

2.2.5.3 Long term storage

Bacteria were stored for one year and more in brain heart infusion broth medium containing 50% glycerol. This was done by adding 5ml of sterilized glycerol to the mid growth of bacteria in a small screw capped vials with final volume of 10ml and stored at -20˚C.

2.2.6 Biochemical test:

2.2.6.1 Catalase production (Maza *et al*, 1997)

This test was performed by adding (2-3) drops of hydrogen peroxide (H_2O_2)3% on a single colony grown on brain heart infusion agar plate.Appearance of bobble was regarded as positive result.

2.2.6.2 Tolerance to NaCl (6.5%) (Atlas *et al*., 1995)

A loop full of an overnight culture of bacterial isolate was inoculated in brain heart infusion broth supplemented with 6.5% NaCl before incubated at 37˚C for 24 hrs.Bacterial growth was regarded as positive result.

2.2.6.3 Hemolysis patterns on blood agar (Sneath *et al*., 1986)

A single colony of overnight growth culture was streaked on surface of blood agar.The type of hemolytic produced by growing colonies was observed and recorded.

2.2.7 Identification of bacteria by rapid ID32 system

2.2.7.1 Preparation of the strip:

. The strip was removed from its packaging.

. The desiccant was discarded.

. The lid was placed on the strip.

. The strain reference was record on the elongated flap of the strip.

2.2.7.2 Preperation of the inoculums:

. The anampulla of API suspension medium (2 ml) was opened.

. Sterile swab was used to harvest the growth appeared on the surface of Blood agar plate.

.bacterial suspension was prepared with a turbidity adjusted to (O.D. 0.4) by Densitometer.

2.2.7.3 Inoculation of the strip:

The ampule of inoculated API suspension medium was homogenized and dispense (55 µl) of the suspension into each cupule of the strip using the electronic Pipette.

2.2.7.4 Incubation of the strip:

. The lid was placed on the strip before incubated at 37 ^{0}C for 4 hours in aerobic conditions.

2.2.7.5 Reading of the strip

. After 4 hours of incubation, the following reagent were added to the cupules:

a- VP test (test 0.0): add 1 drop of VP A and VP B reagents.

b- Tests APPA to GTA (tests 0.1 , 0.2 , 0.3 , 0.4 and 0.5): add 1 drop of FB reagent.

c- HIP test (test 0.6): add 1 drop of NIN reagent.

. Read after 5 minutes by using the MINI API instrument , The reader records the color for each cupule and transmits the information to the computer.

2.2.8 Isolation of plasmid DNA with (Fermantas kit,Germany)

A single colony of suspected *Streptococcus* spp. was grown in 5ml of BHIB with cephalothin (30µg/ml) overnight. The cell pellet from 3 to 5ml of the culture was collected in a microfuge tube. The plasmid fermantas protocol for the kit was applied as followed: Pellets were resuspended in 250µl of buffer S1 resuspension solution (2.1.6.1), then 250µl of buffer S2 lysis solution(2.1.6.1), incubated at a room temperature(25˚C) for 5 min, and 300µl of neutralization buffer(2.1.6.1) was added respectively. Mixed solution was centrifuged at maximum speed for 10 min. Supernatant was taken out and transferred into plasmid quick pure column which was placed in 2ml collection tube. Centrifuged at high speed for 1min, the flow through was discarded, then 600µl of washing buffer (2.1.6.1) was added, centrifuged and the flow through was discarded. Plasmid DNA was purified

by absorption by resin and eluted with 50µl of elution buffer (2.1.6.1) after centrifugation at high speed (16000rpm) for 1min.

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

Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus 0.8% agarose gel was prepared in 1X TAE buffer, and safe stain was added to give a 0.5 µg/ml final concentration in melted agaros gel. Electrophoresis was performed at 60 Volts for 45-60 min. The DNA bands visualized on a short wave UV transilluminator and photographed by using Vilber Lourmat Gel Imaging System. Gene Ruler DNA marker (1Kb) (Fermantas/Germany) was used to determine the molecular weight of DNA bands.

2.2.10 Detection the ability of bacteria for biofilm formation (test tube method) (Christensen *et al.*, 1982).

The ability of bacterial isolates for biofilm formation were detected by inoculation 5ml of Trypton soya broth(Tsb)with particular isoletes and incubated for48hrs at37˚C, after that, the contents of the tubes were removed carefully and added the crystal violet stain 1% to each tubes for 15 mintus then rinsed the tubes and let tubes to dry at room temperature (20-25) ˚C.The result was read by notice the formation of biofilm as a layer at the internal wall of tubes by naked eye and comprise with negative control (tube contain Tsb without inoculation), thickness and color of layer consider a parameter of bacterial ability for biofilm formation.

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

A sterile cotton swab was dipped into inoculums of freshly culture 18hrs.($1.5x10^8$) CFU of *Streptococcus spp.* Isolates and the entire surface of the Mitis Salvarius agar plates was swabbed three times by rotating the plate approximately 60mm between streaking to ensure even distribution ,then the discs of antibiotics were applied and incubated at 37˚C for 24hrs. The diameter of the zone of inhibition (clear area around disc) indicates the sensitivity of bacteria to that antibiotic, and the results were compared with (NCCLs, 2007).

2.2.12 Irradiation the bacteria with Diode laser (AL-Aamirry, 2003)

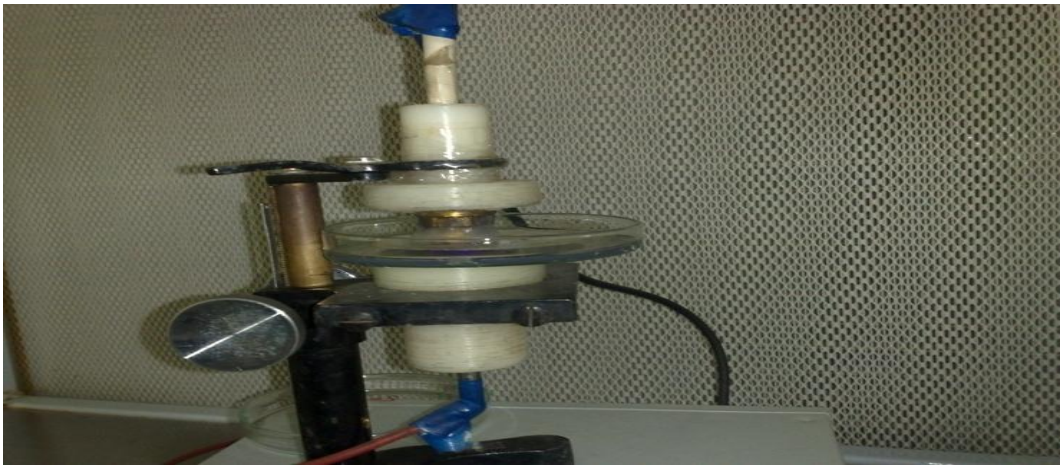
It is considred as pulsed laser fig. (2.1) that has the following parameters: It emits the light at a wavelength gamma corresponding to 890nm, the spot size is about 2cm, peak power (0.5 and 1) Watt and pulse repetition rate, (P.R.R), was maintained at 1000Hz. The exposure time in this study varied with each peak power where the following times were used in this study for each peak power (1, 2, 5, and 10) minutes to get different energy densities.

Streptococcus spp. *w*as cultivated in BHIB at 37˚*C* for 18hrs, followed by centrifuging 10ml at 4000 rpm for 10min. The cell pellet was diluted in sterile phosphate buffer PH7, and adjusted to concentration of 10^6 CFU/ml.A diluted bacterial suspension was put in tubes and exposed to laser light at different times (1, 2, 5, 10) minutes to peak power of(0.5, 1)W except one of these tubes which contain bacterial suspension was not exposed to laser light so as to act as a control. An irradiated suspension and none irradiated were grown on MSA for 24hrs at 37˚C.

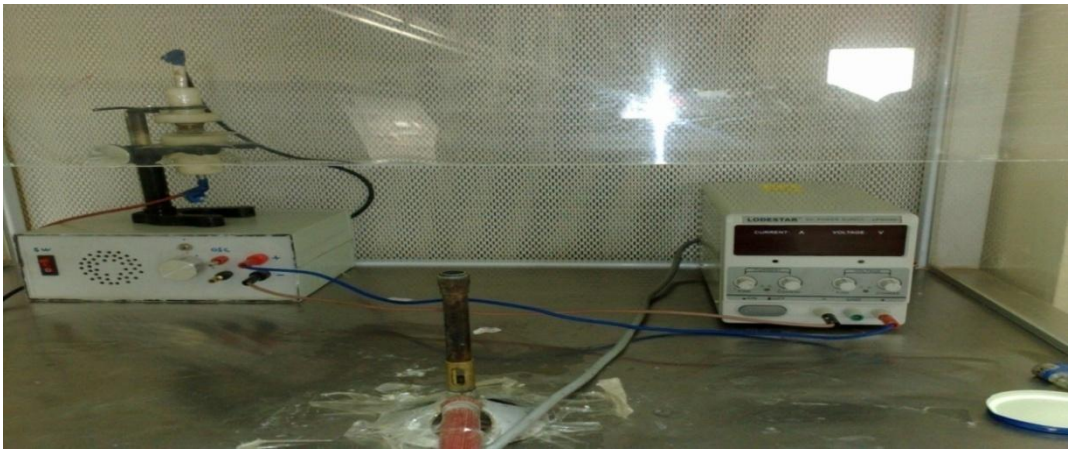
Figure (2.1): Diode laser 890.

2.2.13 Plasma irradiation (Gregory, 2007)

Streptococcus spp. *w*as cultivated in BHIB at 37˚*C* for 18hrs, followed by centrifuging 10ml at 4000rpm for 10min. The cell pellet was diluted in sterile phosphate buffer PH7, and adjusted to concentration of 10^6 CFU/ml.A diluted bacterial suspension of 20µl was put in petri dish contained 10ml from Mitis salivarius agar medium and exposed to plasma ray(as shown in fig.(2.2) at different times(6,8,10,12,14,16,18,20,22) minutes ,except one of these petri dish which contain bacterial suspension was not exposed as a control. An irradiated suspension and irradiated were grown on MSA for 24 hrs at 37˚C.



A



B

Figure (2.2): Plasma system designed by Dr.Hamed Hafid at Physics Department, College of Women, Baghdad University.

Chapter Three

Results & Discussion

Chapter three

Results and Discussion:

3-1 Isolation and identification of *Streptococcus* spp.

After performing microscopic examination on bacterial isolates from healthy persons, results showed that 9 (45%) isolates were identified as *Streptococcus* spp., *3(*15%) *Staphylococcus spp., 2(*10%*) Bacillus spp*., and 6(30%) isolates belonged to other bacteria and yeast, Fig. (3-1)

After performing microscopic examination on bacterial isolates from patients exposed to x-ray, results showed that 44 (35, 4%) isolates were identified as *Streptococcus* spp.,34*(*27,4%)*Staphylococcus* spp., 15 *(*12,2%*) Bacillus spp*., and 31(25%) isolates belonged to other bacteria and yeast, Fig. (3-2).

Higher percentage were reported by AL-Fatlawi (2000) who found the percentage of *Streptococcus* was 47.3%, While AL-Mudallel(2002) who found it as53.3%.

The cultural characterization showed that viridans *Streptococcus* was non hemolytic when grew on blood agar ,Grows on mitis salvarius agar produced small, blue, smooth colonies, (1 - 5 mm) in diameter depending on the number of colonies on the plate. . These colonies may become easier to distinguish with longer incubation. .moreover microscopic examination demonstrated Gram positive rod aggregates in pairs or chains, non motile, non spore forming. Often Arranged in Chains, They prefer anaerobic environment, temperature of growth 37˚C, range of pH (6.5-7.5).

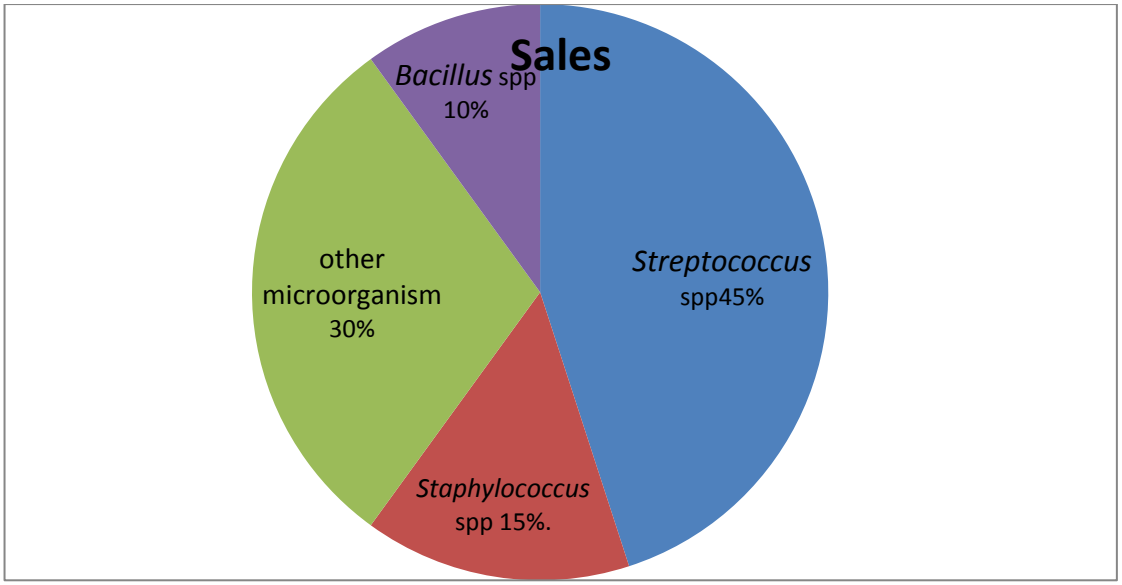


Figure (3-1): Percentage of bacterial and Candida isolates obtained from oral cavity of healthy persons.

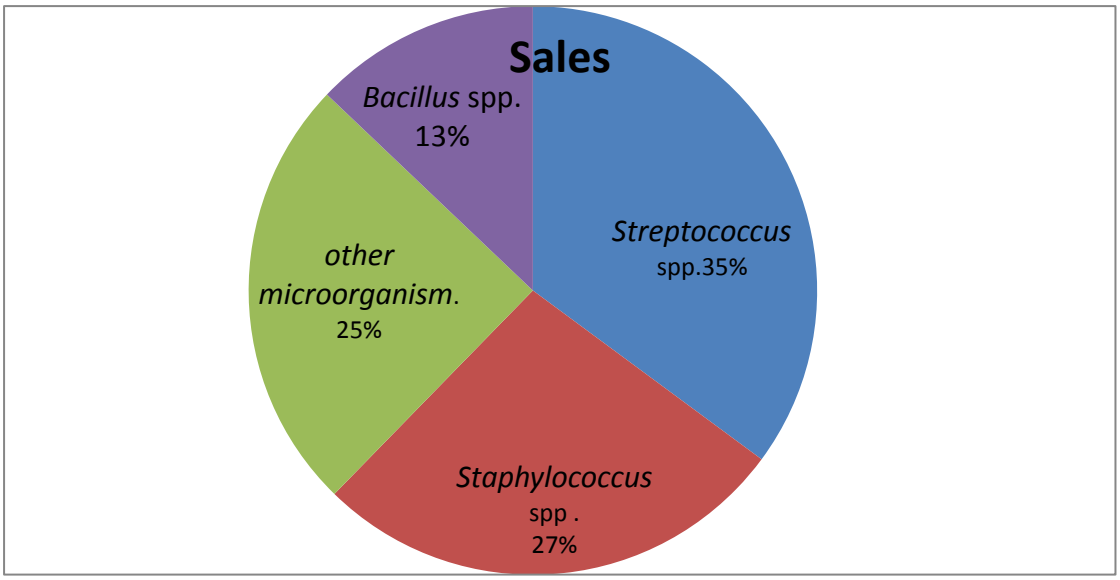


Figure (3-2): Percentage of bacterial and Candida isolates obtained from oral cavity of X-Ray exposed patients.

Morphological and biochemical charectrization agreed with the data stated by Holt *et al*., 1996 and Atlas *et al*., 1995.

Table (3-1) showed the biochemical characterization of seven isolates of *Streptococcus* spp. from healthy persons and seven isolates of *Streptococcus* spp. from X-Ray exposed patients. Results indicated that all isolates were negative to catalase, resisted optochin, unable to grow at 6.5% NaCl and unable to produce hemolycin.

Table (3-1): Biochemical characterization of seven isolates of *Streptococcus* spp. from healthy persons and seven isolates of *Streptococcus* spp. from X-Ray exposed patients.

Bacterial isolates	Test	Results
Streptococcus spp.(14 isolates ,7 from healthy and 7 from X-ray exposed patients)	Catalase	-ve
	Hemolysis	Non hemolysis
	Optochin sensitivity	Resistant
	Growth at 6.5%NaCl	-ve

After performing Rapid ID 32 Strep on seven isolates of *Streptococcus* from patients exposed to X-Ray, results showed that 5(71.4%) isolates identified as *Streptococcus oralis*.1(14.2%) isolates identified as *Streptococcus salvaris* and 1(14.2%) isolates identified as *Streptococcus viridians*.

Result of Rapid ID 32Strep of seven isolates of *Streptococcus* from healthy persons, decleared that 4(57.1%) isolates were identified as *Streptococcus oralis*.,2(28.5%) as *Streptococcus feacalis*.and 1(14.2%) as *Streptococcus mutans* .

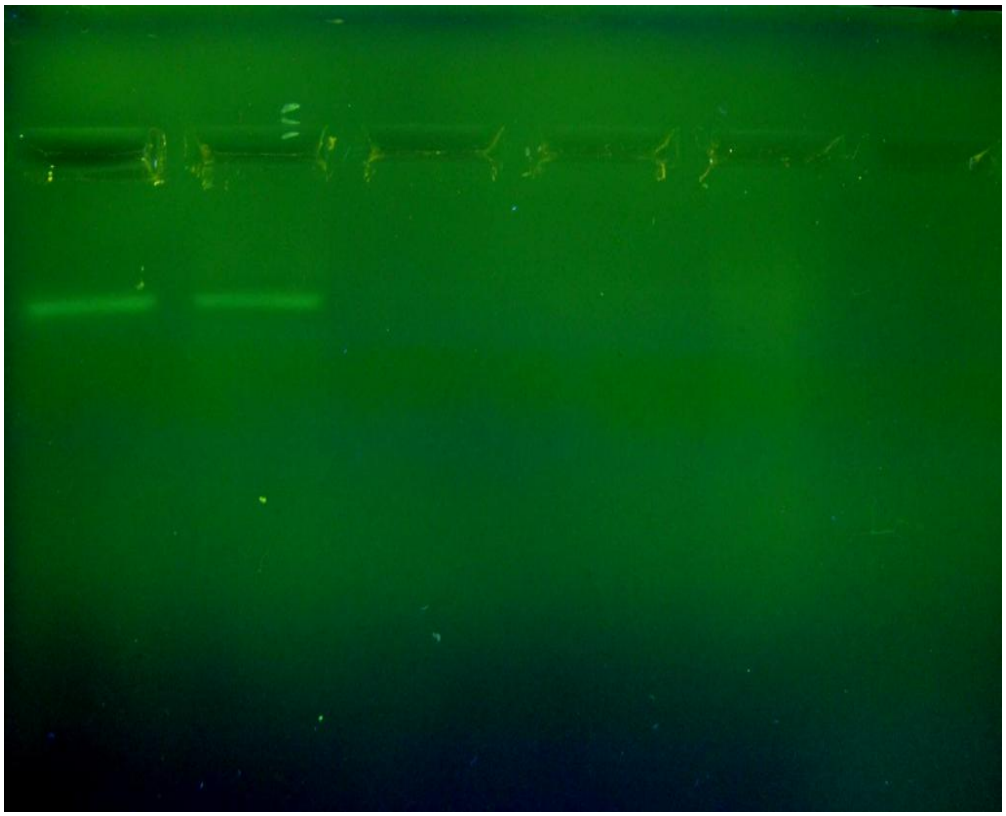
A simillar result was reported by Salako *et al*.(2007) who found that the percentage of *Streptococcus mutans* was12%,While AL-Fatlawi(2000) found that the percentage of *Streptococcus mutans* was 44.4%.
While agreed with AL-Fatlawi(2000)who found that the percentage of *Streptococcus salvaris* 16.6% ,While Salako *et al.(*2007) found 27%.

3-2 Isolation of plasmid from *Streptococcus*:

The Kit was used to isolate plasmid from seven isolates from healthy persons and seven isolates from X-Ray exposed patients of *Streptococcus spp.* Results in fig.(3-3) indicated that the *Streptococcus* spp isolated from X-Ray exposed patients (R1(*Streptococcus salvaris*) ,R2 (*Streptococcus oralis*), R3(*Streptococcus viridians*) lost their plasmids ,while the two isolates of *Streptococcus* spp isolated from healthy persons (N5(*Streptococcus oralis*),N6(*Streptococcus oralis*) contained a small plasmid bands.

Results in fig. (3-4) showed that two isolates N5(*Streptococcus oralis*),N6(*Streptococcus oralis*) isolated from healthy persons possessed a single plasmid band .
The cell wall of Gram-positive bacteria can be broken by the use of enzymes that degrade peptidoglycans such as the lysozyme and mutanolysin present in the commercial kits which cause the method to be expensive. The use of cheaper alternative methods is not always appropriated given the difficulty in breaking the cell wall when some reagents are used, such as phenol-ether (Rantakokko-Jalava and Jalava, 2002).

1 2 3 4 5



Figure(3-3):Gel electrophoresis pattern of isolated plasmid profile from Streptococcus spp. migration on agarose gel(0.8%)in TBE buffer at 60v for 2hrs.

(1) Plasmid contained of *Streptococcus oralis* isolated from healthy persons
(2) Plasmid contained of *Streptococcus oralis* isolated from healthy persons
(3) Plasmid contained of *Streptococcus salvaris* isolated from x-Ray exposed patients
(4) Plasmid contained of *Streptococcus oralis* isolated from X-Ray exposed patients
(5) Plasmid contained of *Streptococcus viridans* isolated from X-Ray exposed patients.

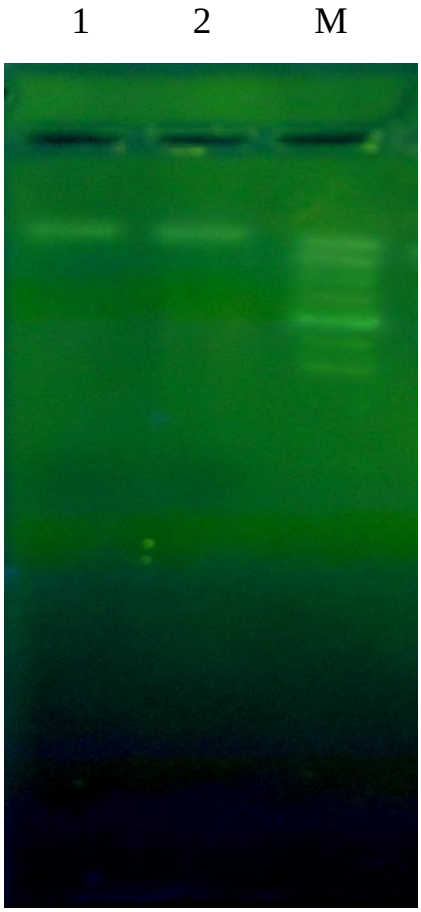


Figure (3-4): Gel electrophoresis pattern of isolated plasmid profile from Streptococcus spp. migration on agarose gel(0.8%)in TBE buffer at 60v for 2hrs.

(1) Plasmid contained of *Streptococcus oralis* isolated from healthy persons.
(2) Plasmid contained of *Streptococcus oralis* isolated from healthy persons.
(M)1kb –DNA-Ladder Marker

In this sense, bacterial DNA can be extracted from sera by boiling without using any toxic reagent, overcoming the use of expensive commercial kits for DNA purification (Mayoral *et al.*, 2005).

Some researchers have already used cetyltrimethylammonium bromide (CTAB) with good results and considerable cost reduction (Doyle and

Doyle, 1987; Petersen and Scheie, 2000), At times being combined with sodium dodecyl sulfate (Dellaporta *et al*., 1983).

Several factors complicate plasmid DNA isolation from Gram-positive bacterium such as rigid cell wall composition for which most plasmid extraction protocols were established. However, this can easily be overcome by performing a 30- to 60-minute pretreatment with lysozyme to degrade the thick peptidoglycan layer of the cell wall. Secondly, Gram-positive bacterium is grown in de Man, Rogosa and Sharpe (MRS) broth with Tween® 80 at low pH rather than in Luria-Bertaini broth, which also could influence DNA isolation. (Vriesema *et al*.,1996).

A significant problem that exists with current methods for isolating large plasmids from dairy streptococci is that the yield of large plasmids is very low. This observation was initially reported in studies linking the ability to metabolize lactose to a 30-megadalton (Mdal) plasmid in *Streptococcus lactis* C2 and to a 36-Mdal plasmid in *Streptococcus cremoris* B1 (McKay and Baldwin, 1975;McKay *et al* 1976;Anderson and McKay, 1977)

Plasmid DNA was prepared as a cleared lysate by the method of Cords *et al*.,1974 purified by cesium chloride-ethidium bromide density gradient centrifugation, and examined by electron microscopy. The isolation of plasmid DNA from viridans group streptococci and various other gram-positive bacteria is hampered by the rigidity of the streptococci and lactococci often yield plasmid DNA contaminated with large amounts of chromosomal DNA. To obtain pure plasmid DNA, such preparations need to be purified by CsCl-ethidium bromide buoyant density gradient centrifugation, which is a time-consuming and laborious step. The

availability of a rapid plasmid isolation protocol yielding pure plasmids

NO.of isolates	Ability for biofilm formation
R1(*Streptococcu salvaris*	++ve

will facilitate selection and recovery of specific genes using plasmid-based cloning systems, abolishing the need for chromosomal integration of selected DNA fragments. (LeBlanc and Lee, 1979;Macrina *et al*.,1980;Anderson and McKay, 1983; Klaenhammer, 1984; Vriesema *et al*.,1996)

3-3Biofilm formation:

Test tube method used to detect ability of *Streptococcus spp.* isolates which isolated from healthy persons and X-Ray exposed patients for biofilm formation.Table (3-2)showed the ability of *Streptococcus spp* for biofilm formation (The isolates chosen randomly).

Table(3-2): Biofilm formation by *Streptococcus* spp.

R2(*Streptococcus oralis)*	++ve
R3(*Streptococcus viridians)*	++ve
R4(*Streptococcus viridians)*	-ve
R5(*Streptococcus oralis*)	-ve
R6(*Streptococcus oralis*)	-ve
N3(*Streptococcus feacales)*	++Ve
N4(*Streptococcus mutans)*	++Ve
N5(*Streptococcus oralis*)	++ve
N6(*Streptococcus oralis*)	++ve

R1,R2,R3: *Streptococcus* isolated from patients.

N3.N4.,N5,N6: *Streptococcu*s isolated healthy persons.

++ve: high biofilm formation , +ve: low biofilm formation,-ve: no biofilm formation

Biofilm production by bacteria and ther microorganisms is genetically controlled property,which represent one of the most important virulence factor in productive organisms(Mireles *et al.*,2001;Santo *et al*.,2006) Bacterial adhesion and subsequent conolization of surfaces are the first step toward biofilm formation.Biofilm consisted of micricolonies encased in extracellular polysaccharide which formed under selected conditions(Olson *et al*.,2002;Anderson *et al*.,2007)

Biofilm has an active role in bacterial pathogenesity because bacteria embedded in a matrix of host proteins and microbial slime ,which provided a home for organism and promote increased drug resistance thus antibiotic less effective in biofilm cells than in plank tonic cell(Mah and Toole;Soto *et al* .,2007).Table (3-3) showed decrease in biofilm formation after exoposure to diod laser 890nm.

Table (3-3): Biofilm formation of *Streptococcus* after exoposure to diod laser 890nm

No. of isolates	Biofilm formation					
	Dose of laser 890 nm(w)					
	0.5(w)			1(w)		
	Time of exposure (min)					
	1	5	10	1	5	10
R1	++ve	+ve	+ve	++ve	+ve	+ve
R2	++ve	+ve	+ve	++ve	+ve	+ve
R3	++ve	+ve	+ve	++ve	+ve	+ve
N5	++ve	++ve	+ve	++ve	++ve	+ve
N6	++ve	++ve	+ve	++ve	++ve	+ve

++ve: a thick biofilm formation , +ve: thin biofilm formation

R1, R2, and R3: *Streptococcus* spp isolated from patients.

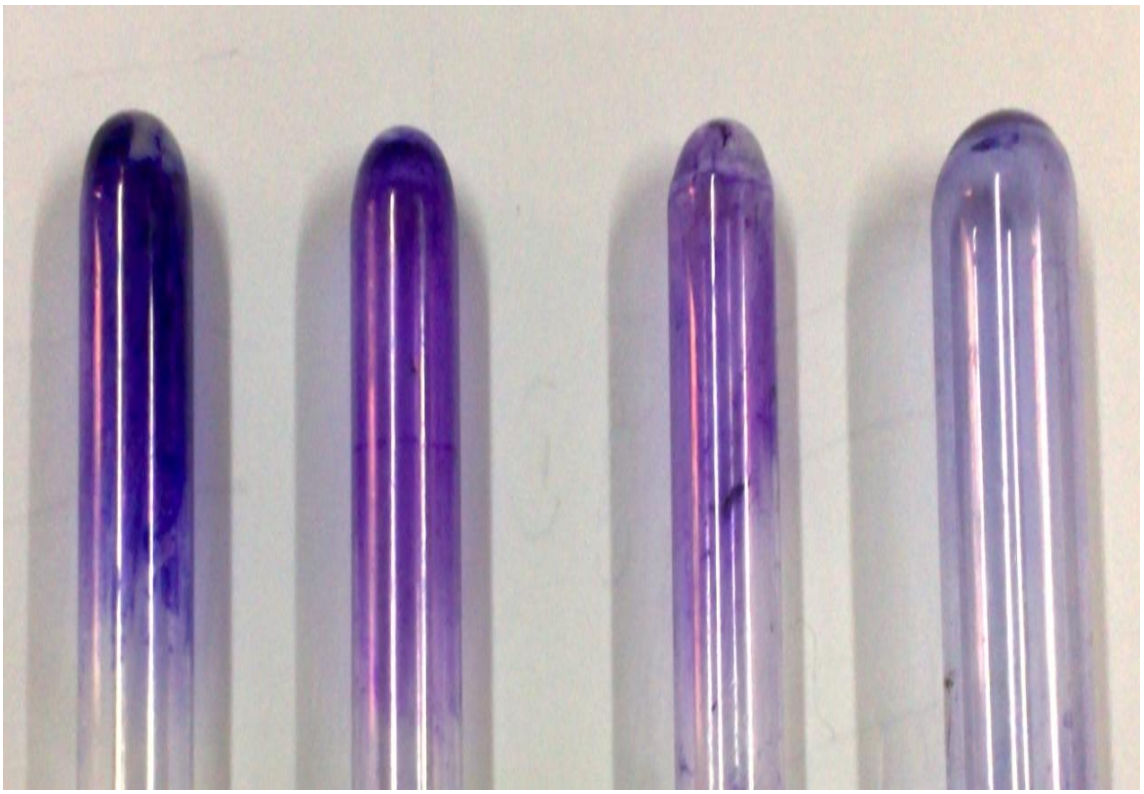
N5, N6: *Streptococcus* spp isolated from healthy persons.

Because of the matrix of biofilm modify the environment of adherent cells by concentrating nutrients and protecting the cells from surfactant, biocides and phagocytic cells. Antibiotic are generally on very effective against organism embedded in bioflm.Cells from biofilm express properties distinct from planktonic cells which increased resistance to antimicrobial agents.(Taylor *et al*.,1987;Anderson *et al*., 2004;Garofol *et al*.,2007)

These results agreed with Hauser *et al*. (2010) (Internet 5) who found the diode laser effectively reduced the viability of adhered Streptococcus by inactivation biofilm

Figure (3-5) showed the biofilm formation of *Streptococcu*s spp. isolated from healthy persons after exposed to diod laser, while figure (3-6) showed biofilm formation of *Streptococcu*s spp. isolated from patients after exposed to diod laser.

A B C Control



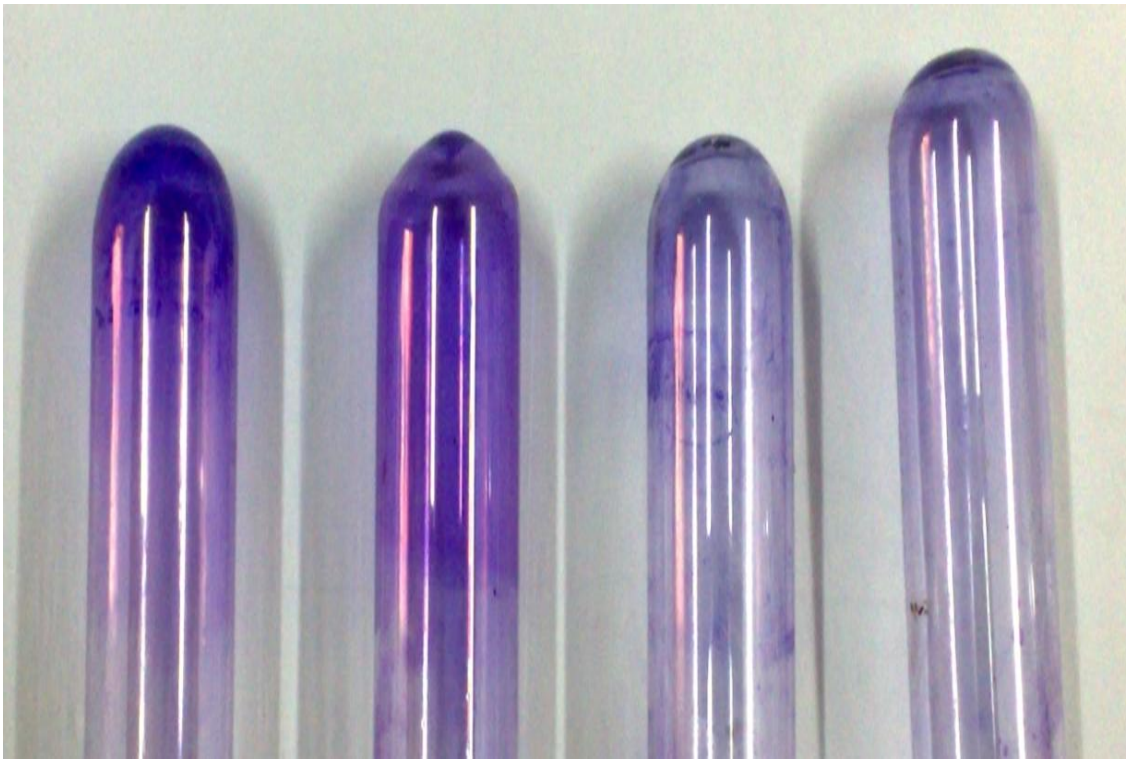
Figure(3-5): Biofilm formation of *Streptococcu*s spp. isolated from healthy persons after exposed to diod laser.

A: biofilm formation after (1 min) from exposure to diod laser 890nm.

B: biofilm formation after(5 min) from exposure to diod laser 890nm

C: biofilm formation after(10 min) from exposure to diod laser 890nm

A B C Control



Figure(3-6): biofilm formation of *Streptococcu*s spp isolated from patients after exposed to diod laser.

A: biofilm formation after (1 min) from exposure to diod laser 890nm.

B: biofilm formation after(5 min) from exposure to diod laser 890nm

C: biofilm formation after(10 min) from exposure to diod laser 890nm.

Table(3-4): Biofilm formation of *Streptococci* after exposed to non thermal plasma .

Symbol of isolates	Time of exposure(second)				
	6	10	14	18	22
N3	++ve	++ ve	+ ve	- ve	- ve
N4	++ ve	++ ve	+ ve	- ve	- ve
R1	- ve	+ve	++ ve	++ ve	++ ve
R2	- ve	+ ve	++ ve	++ ve	++ ve
R3	- ve	+ ve	++ ve	++ ve	++ ve

++ve: Thick biofilm formation.

+ve: Thin biofilm formation.

-ve:no biofilm formation.

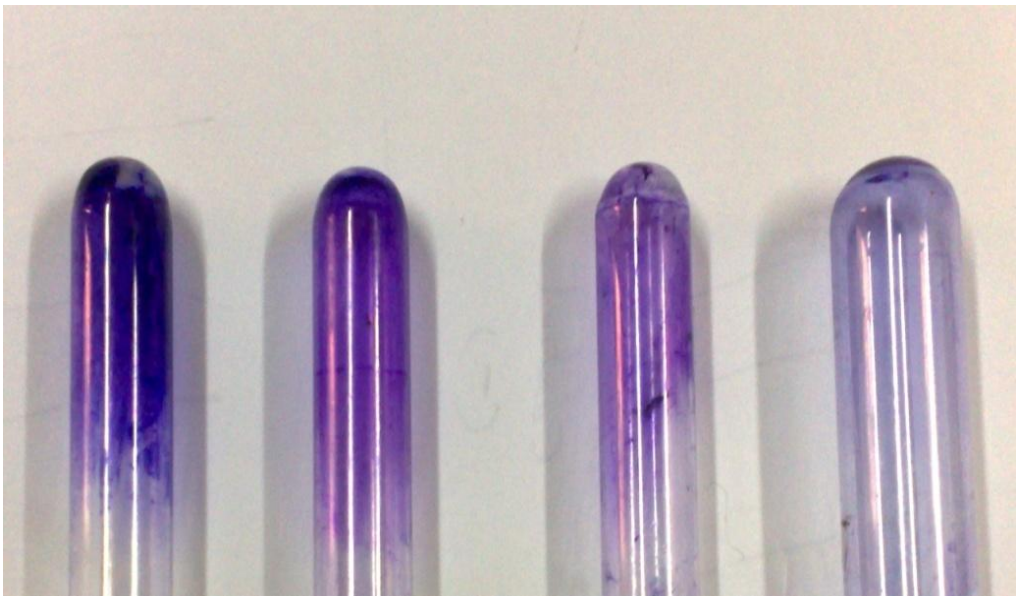
R1,R2,R3: *Streptococcus* spp isolated from patients.

N3,N4: *Streptococcus* spp isolated from healthy persons.

Fig (3-7) showed decrease in biofilm formation of of *streptococcus* spp. isolated from healthy persons after exposed to non –thermal plasma with increased time of exposure.

These results agreed with Jonathan *et al.,* (2008) who found the potential of plasma for biofilm inactivation and suggest that cells go through a sequential set of physiological and morphological changes before inactivation. and because the plasmas contain a mixture of charged particles, chemically reactive species and UV radiation that inactivation biofilm formation.

A **B** **C** **D**



Figure(3-7): Biofilm formation of *Streptococcus* spp. isolated from healthy persons after exposed to non thermal plasma with increased times of exposure.

A: Biofilm formation after 10 sec. from exposure to non thermal plasma**.**

B: Biofilm formation after 14 sec. from exposure to non thermal plasma**.**

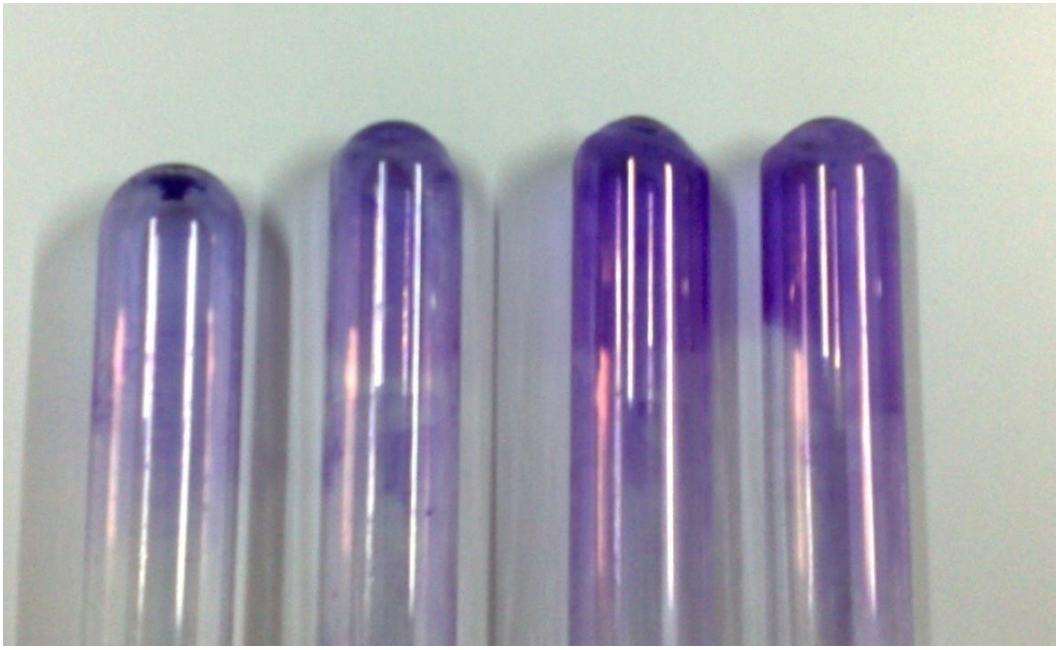
C: Biofilm formation after 18 sec. from exposure to non thermal plasma**.**

D: Biofilm formation after 22 sec. from exposure to non thermal plasma**.**

The result are also agreement with Ermolaeva *et al*.(2010) who found potential for non-thermal plasma in eliminating pathogenic bacteria from biofilms and wound surfaces.

Results in Fig.(3-8) showed increase in biofilm formation of *Streptococcus* spp . isolated from patients after exposure to non thermal plasma .

A B C D



Figure(3-8): Biofilm formation of *Streptococcus* spp. isolated from healthy persons after exposed to non thermal plasma with increased times of exposure.

A: Biofilm formation after 10 sec. from exposure to non thermal plasma**.**

B: Biofilm formation after 14 sec. from exposure to non thermal plasma**.**

C: Biofilm formation after 18 sec. from exposure to non thermal plasma**.**

D: Biofilm formation after 22 sec. from exposure to non thermal plasma**.**

Results in fig.(3-8) indicated a large and thick biofilm formation with increasing time of exposure to non thermal plasma in previously X-ray exposed bacteria, and such results may be due to that X-ray increases the resistance of the bacteria toward the non thermal plasma.

3-4Antibiotic sensitivity:

The disc diffusion method was used to determine susceptibility of *Streptococcus* spp isolates to several antibiotic, resistance among *Streptococcus* isolates varied according to nature of isolates or antibiotics.

Results illustrated in table (3-5) indicated the antibiotic sensitivity of isolates of *Streptococcu*s spp. isolated from healthy persons and isolates of *Streptococcus* spp. from patients against 8 antibiotics.
The determination of resistance or sensitivity of bacterial isolates depends on the measurement of the diameter of the inhibition zone.

Results shows low resistance to gentamycin (14.2%), This result was agreed with Potgieter *et al*. (1992)who found that viridians group *Streptococcus* none had high level resistance to gentamicin.

Results showed that resistance of *Streptococcus* to tetracycline (28.5%) but Streptococcus spp. isolated from patients higher resistant (42.8%) than *Streptococcus* spp isolated from healthy persons *(*14.2%).

Table (3-5): Antibiotics sensitivity of *Streptococcus* spp. isolated from healthy persons and *Streptococcus* spp. from patients.

NO. Of Isolates	**Antibiotic disc(µg/disc)**							
	KF(30)	**B(10)**	**AM(10)**	**CFM(5)**	**CL(30)**	**E(15)**	**TE(30)**	**CN(30)**
*R1	-	9 mm	19mm	-	-	13mm	18mm	16mm
*R2	-	9mm	12 mm	-	-	13mm	18mm	16mm
*R3	-	8mm	15 mm	-	-	-	12 mm	-
*R4	-	8 mm	13 mm	-	-	12mm	23mm	15mm
*R5	-	9mm	13mm	-	-	12mm	22mm	16mm

*R6	-	7mm	13mm	-	-	12mm	23mm	18mm
*R7	-	8mm	14mm	-	-	12mm	23mm	18mm
*N1	-	9mm	13mm	-	-	18mm	21mm	14mm
*N2	-	9mm	13mm	-	11mm	17mm	20mm	10mm
*N3	21mm	7mm	22mm	29mm	21mm	-	18mm	30mm
*N4	8mm	11mm	18mm	-	12mm	23mm	22mm	18mm
*N5	23mm	-	16mm	27mm	16mm	-	19mm	30mm
*N6	-	12mm	14mm	-	-	19mm	24mm	16mm
*N7	-	12mm	14mm	-	-	19mm	24mm	16mm

-:resistant

KF:cephalothin, B:bacitracin, AM:ampicillin,CFM:cefixime, CL:cephalexine, E:erythromycin, TE:tetracycline, CN:gentamycin.

*R(1-7): *Streptococcus* spp. from patients.

*N(1-7): *Streptococcus* spp. isolated from healthy persons.

Results of this study agreed with Brown *et al.*,(2008) who found that tetracycline resistance in *Streptococcus* (29-34%),While Wisplinghoff *et al.,(*1999) found(39).

Results of this study shows that high resistance of *Streptococcus* against erythromycin (64.2%), the all Streptococcus *spp.* exposed to X-Rays resist erythromycin(100%)while healthy *Streptococcus spp(*28 %).
Results of this study agreed with Brown *et al.*,(2008)who found that Erythromycin resistance(40-51%).

The increased incidence erthromycin resistance has been correlated with the emergence of a new mechanism of erythromycin resistance—efflux. This is devoted to the mechanisms responsible for resistance to macrolides and related antibiotics in *Streptococcus spp*. (Brown *et al.*, 2008)

Additionally Wisplinghoff *et al., (*1999) found that (32%) of *Streptococcus* spp. resist erythromycin, that notic increased in erythromycin resistanc , The authors emphasized that a substantial increase in erythromycin resistance was associated with the increase in the consumption of macrolide antibiotics.

Syrogiannopoulo *et al. (*2001*)*found that The current high (38%) prevalence of erythromycin-resistant *Streptococcus* in Central and Southern Greece requires continuous surveillance and careful antibiotic policy.

These results are also shows high resistance of *Streptococcus* against cefixime and cephalxine and ampicillin(92.8%), the all Streptococcus spp. exposed to X-Rays resist these antibiotic(100%)while healthy *Streptococcus* spp*(*85.7 %).

These results agreed with Ellen *et al.(*2010)(Internet 6) who fond that Microbial resistance to ampicillin, amoxicillin/clavulanic acid, cefoxitin, cephalothin, amikacin, chloramphenicol and nalidixic acid was particularly high..Additionally these results shows high resistance of *Streptococcus* against cephalothin (85.7%), the all *Streptococcus* spp. exposed to X-Rays resist cephalothin (100%)while healthy *Streptococcus* spp*(*71 %).

The cephalosporins have greatly varied in susceptibility to β-lactamases. Cephalothin has been more resistant to hydrolysis by β-lactamases of *staphylococci*, whereas cefoxitin has been more resistant to β-lactamases produced by aerobic gram-negative rods. but also shows a disseminated resistance to both cefoxitin and cephalothin among aerobic gram-positive

cocci and gram-negative bacilli. (Das *et al.,* 2006; Khosravi *et al*., 2007;British Society for Antimicrobial Chemoterapy. BSAC, 2008)

The results are also shows high resistance of *Streptococcus* against Bacitracin (50%), the high *Streptococcus* spp. exposed to X-Rays resist Bacitracin (71.4%) while healthy *Streptococcus* spp*(*28.5 %),and agreed

with Hiromasa *et al*.(2002) who found that *Streptococcus* spp resist Bacitracin and have two gen locus ivolves resistant, Although the bacitracin sensitivities of *Streptococcus* spp that had defects in flanking genes.

Some researchers have reported that the resistant strains to various antibiotics were located in a high copy number and transferable plasmid DNAs and the plasmids of these strains can be developed as cloning vectors for use in Gram-positive bacteria used in recombinant DNA technology because of their traits .(Levy.1987; Swenson *et al*.,1990)

Bacterial resistance to an antimicrobial agent may be due to some innate property of the organism or it may due to acquisition of some genetic trait, The combined effects of fast growth rates, high populations of cells, genetic processes of mutation and selection, and the ability to exchange genes, account for the extraordinary rates of adaptation and evolution that can be observed in the bacteria. For these reasons bacterial adaptation (resistance) to the antibiotic environment seems to take place very rapidly in evolutionary time. (Toder, 2004)

Table (3-6)showed the antibiotic sensitivity by using 8 antibiotic discs against *Streptococcus* spp. isolated from healthy persons and

Streptococcus spp. Isolated from patients after exposed to diod laser890nm.

Table (3-6):Antibiotic sensitivity of healthy *Streptococcus* spp. and *Streptococcus* spp. exposed to X-Ray and after exposed to diod laser.

No. of isolates	Dose of diod laser 890nm(w)	Time of exposure (min)	Antibiotic disc(µg/disc)							
			KF (30)	B (10)	AM (10)	CFM (5)	CL (30)	E (15)	TE (30)	CN (30)
*R3	0.5	5	-	13mm	-	-	-	-	9mm	11mm
*R3	0.5	10	-	13mm	-	-	-	-	10mm	10mm
*R3	1	5	-	14mm	-	-	-	-	11mm	-
*R3	1	10	-	14mm	-	-	-	-	7mm	10mm
*N3	0.5	5	-	13mm	-	-	-	-	11mm	11mm
*N3	0.5	10	-	14mm	-	-	-	-	10mm	-
*N3	1	5	-	12mm	-	-	-	-	9mm	10mm
*N3	1	10	-	13mm	-	-	-	-	7mm	11mm

-:resistant

R3: *Streptococcus* spp. isolated from patients.*

N3: *Streptococcus* spp. isolated from healthy persons. *

KF:cephalothin, B:bacitracin, AM:ampicillin,CFM:cefixime, CL:cephalexine, E:erythromycin, TE:tetracycline, CN:gentamycin

Results showed that change in susceptibility to antibiotic of *Streptococcus spp*. after exposure to diod laser 890nm. These result agreed with Al- Rassam(2010)who found that effect of diode laser that reduced the MIC values and increase the susceptibility

of bacteria to antibiotics.

The *Streptococcus* spp. exposed to X-Ray showed increase suscepibility in only one antibiotic Bacitracin,While healthy *Streptococcus spp* shows increase resistant to three antibiotics cephalothin,cefixime and gentamycin and increase suscepibility to Bacitracin.

The *Streptococcus* spp. isolated from patients exposed to X-Ray and produce thick biofilm showed a less change in antibiotic sensitivity in comparison with *Streptococcus spp*. from healthy persons.

Changes in sensitivity of bacterial isolates to the antimicrobial agents after treatment with diode laser is may be due to the combination effect of laser and antimicrobial agent making the bacterial cell more sensitive to the antimicrobial agents. The sensitivity of bacteria to antibiotic may be due to the changing in bacterial pumping system (efflux pump) that mainly responsible on bacterial resistance to antibiotics such as (B-lactams, aminoglycoside). Failure of bacteria to produce specific enzymes that chemically modify specific antibiotic also may be increased the bacterial sensitivity to the antibiotics. (Karu, 1993)

Table (3-7) shows antibiotic sensitivity of *Streptococcus spp.* isolated from healthy persons and *Streptococcus* spp. Isolated from patients after exposed to non thermal plasma.

These result shows increase suscebtibility of *Streptococcus spp.* exposed to X-Ray to bacitracin , While healthy *Streptococcus spp* shows increase resistant to three antibiotics cephalothin,cefixime and gentamycin and increase suscepibility to bacitracin .

Table(3-7): Antibiotic sensitivity of healthy *Streptococcus* spp. and Streptococcus spp. exposed to X-Ray after exposed to non thermal plasma.

NO. of isolate	Time of exposure (second)	Antibiotic disc**(µg/disc)**							
		KF (30)	B (10)	AM (10)	CFM (5)	CL (30)	E (15)	TE (30)	CN (30)
*R3	16	-	13mm	-	-	-	-	11mm	-
*R3	18	-	14mm	-	-	-	-	12mm	-
*R3	20	-	15mm	-	-	-	-	11mm	-
*R3	22	-	16mm	-	-	-	-	11mm	-
*N3	16	-	13mm	-	-	-	-	12mm	-
*N3	18	-	14mm	-	-	-	-	12mm	-
*N3	20	-	16mm	-	-	-	-	12mm	-
*N3	22	-	16mm	-	-	-	-	12mm	-

- :resistant

R3: *Streptococcus* spp. isolated from patients. *

N3: *Streptococcus* spp. isolated from healthy persons.*

KF:cephalothin, B:bacitracin, AM:ampicillin,CFM:cefixime, CL:cephalexine,E:erythromycin, TE:tetracycline, CN:gentamycin.

These change in antibiotic suscebtibility due to the plasma is ionize gas and Ionizing radiation causes atoms and molecules to become ionized or excited. These excitations and ionizations can: Produce free radicals, Break chemical bonds,Produce new chemical bonds and cross-linkage between macromolecules,Damage molecules that regulate vital cell processes (e.g. DNA, RNA, proteins),fig(3-9).

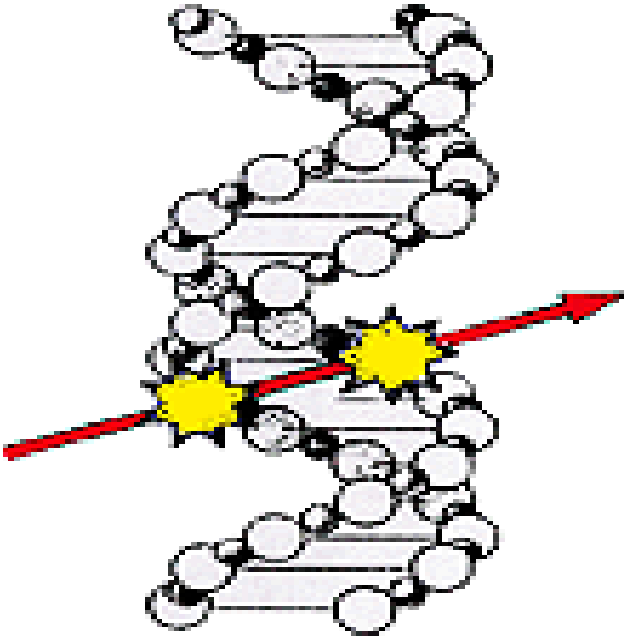


Figure (3-9): Damage molecules (DNA) by Ionizing radiation. (Internet 7)

The cell can repaire certain levels of cell damage. At low dose, such as that received every day from background radiation, cellular damage is rapidly repaired. (Internet 7).

Chapter four

Conclusion &Recommendation

Conclusions:

1-Diod laser 890nm had slightly effect on viability of bacteria, while non thermal plasma had effect on the viability of bacteria with increased time of exposure.

2-*Streptococci* isolated from patients exposed to X-Ray was highly resistant to antibiotic than *Streptococci* isolated from healthy persons.

3 *Streptococcus spp* very high resistance against cefixime and cephalxine , ampicillin ,cephalothin, bacitracin, erythromycin and tetracycline but low resistance to gentamycin.

4- *Streptococci* isolated from patients exposed X-Ray lost their plasmid and only two isolates from healthy persons have small plasmid bands.

5-Diod laser 890nm effected antibiotic sensitivity of *Streptococcus* spp isolated from healthy persons more than- *Streptococci* isolated from patients exposed to X-Ray,while exposure to diod laser 890nm lead to decrease in biofilm formation .

6- Non thermal plasma lead to change the antibiotic sensitivity of *Streptococcus* spp. isolated from healthy persons more than those isolates from patients exposed to x-ray, While the exposure to non thermal plasma lead to decrease in the biofilm formation of *Streptococcus* spp. isolated from healthy persons,While exposure to non thermal plasma lead to increase in the biofilm formation of *Streptococcus* spp. isolated from patients exposed to x-ray.

Recommendations:

1-More studies on effect of X-ray,diod laser 890nm and non thermal plasma on other species of bacteria ,in addition medical fungi and yeast.

2- Studies the mutagenic effect of X-ray, diod laser 890nm and non thermal plasma on other species of bacteria and for the detection of the resultant mutation on the molecular levels.

3-Performing futural studies on effect of the X-Ray, diod laser 890nm and non thermal plasma on virulence factors of *Streptococcus* spp.

4-Using accurate methods to determine the plasmid encoding antibiotic resistance or virulence factor like PCR with specific primer for these genes.

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الخلاصة

تضمنت هذه الدراسة عزل وتشخيص عزلات بكتريا *Streptococcus* spp. من مسحات البلعوم لمرضى معرضين للاشعة السينية ومن اشخاص طبيعيين حيث تم جمع (144)عينة بواقع (122)عينة من المرضى المعرضين للاشعة السينية و(20)عينة من الاشخاص الاصحاء جمعت من بعض المستشفيات و عيادات الاشعة في بغداد.

اظهرت نتائج التشخيص لهذه العزلات بواسطة الاختبارات الكيموحيوية ان 44(35,4%)عزلة تعود للجنس *Streptococcus* spp. 34,(27,4%) عزلة للجنس *Staphylococcus* spp 15 (12,2%) عزلة للجنس *Bacillus* spp. 31,(35%) عزلة تعود لاجناس اخرى من البكتريا والخمائر من مجموع عينات المرضى المعرضين للاشعة السينية.

في حين ان نتائج تشخيص عينات الاشخاص الاصحاء اظهرت ان 9(45%)عزلة تعود لجنس *Streptococus* spp. 3,(15%) عزلة لجنس *Staphylococcus* spp,2(10%) عزلة لجنس *Bacillus* spp 6,(30%) عزلة تعود لاجناس اخرى من البكتريا والخمائر.

بينت نتائج تشخيص عزلات بكتريا *Streptococus* spp بواسطة نظام **Rapid ID 32** ان (71,4%)عزلة كانت تعود للنوع *Streptococcus oralis* (14,2%), عزلة للنوع *Streptococcus viridians*,(14,2%)عزلة للنوع *Streptococcus salvairus*.في حين كانت نتائج التشخيص بهذا النظام لعزلات *Streptococcus* spp المعزولة من الاشخاص الاصحاء ان (57,1%) عزلة للنوع *Streptococcus oralis* ,(28,5%)عزلة للنوع *Streptococcus feacalis*, (14,2%)عزلة للنوع *Streptococcus mutans* .

اظهرت نتائج عزل الدنا البلازميدي ان *Streptococcus* spp المعزولة من المرضى المعرضين للاشعة السينية انها فاقدة للبلازميد, في حين احتوت كل من عزلتي *Streptococus* spp المعزولة من الاشخاص الاصحاء وجود حزمة صغيرة من البلازميد.

وفيما يخص المقاومة للمضادات الحيوية فقد كانت النتائج 92,8% ,85,7% , 50% , 28,5% و14,2%. من عزلات بكتريا *Streptococus* spp المعزولة من الاشخاص الاصحاء مقاومة الى الامبسيللين ,السيفالوثين ,الباستراسين ,التتراسايكلين و الجنتامايسين على التوالي. في حين ان اظهرت تلك المعزولة من المرضى المعرضين للاشعة السينية اظهرت مقاومة اكثر تجاه نفس المضادات.

لوحظ من نتائج التعرض لاشعة الليزر الدايود 890 نانوميتر تغيرفي مقاومة بكتريا *Streptococus* spp للمضادات الحياتية ,حيث ازدادت حساسية العزلات المعزولة من المرضى المعرضين للاشعة السينية زيادة في حساسيتها لمضاد الباستراسين,بينما اظهرت عزلات الاشخاص الاصحاء زيادة في المقاومة تجاه السيفالوثين والسيفوتاكسيم و الجنتامايسين وزيادة في الحساسية تجاه الباستراسين كما لوحظ ان انتاج الغشاء الحيوي (biofilm) لجميع العزلات قد انخفض بعد التعرض لهذه الاشعة.

وتم الحصول على نتائج مشابهةعند تعرض عزلات *Streptococus* spp لاشعة البلازما مع زيادة وقت التعرض لهذه الاشعة ما عدا زيادة تكوين الغشاء الحيوي للعزلات المعزولة من المرضى المعرضين للاشعة السينية بازدياد وقت التعرض لاشعة البلازما..

الاهداء

الى وطني الغالي.....العراق

الى من اوصلني الى هنا.....اساتذتي الافاضل

الى روح والدي(رحمه الله)

الى فخري واعتزازي.....نبع الحنان.....الى من حثتني على الجد

والمثابرة ....والدتي العزيزة

الى سندي في الحياة .....الى الذي استمد منه عزيمتي.....زوجي

الى ذخري في الحياة..... اخوتي

الى بناة المستقبل..... اولادي

الى كل من مد لي يد العون والمساعدة

اهدي ثمرة جهدي المتواضع

ريم