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**Isolation and Detection of Some Molecular Characterization
of *Streptococcus mutans* from Dental Caries and Dental Biofilm
patients at Thi-Qar Province/Iraq**

A thesis

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/Microbiology

By

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

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Certification

We certify that this thesis entitled (**Isolation and detection of some molecular characterization of *Streptococcus mutans* from dental caries and dental biofilm patients at Thi-Qar province/Iraq**) was prepared under our supervision at Department of Biology/College of science/Thi-Qar University as a partial fulfillment of requirements for the degree of Master of Science in Biology/Microbiology.

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Dedication

To.....

The spirit of my cousin..... Maysam

To.....

The compassionate parents My father..... My mother

To

My sisters.....My brothers

To

My life My wife My son My daughters

I dedicate this modest effort

Hamza

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In the name of **ALLAH**, most gracious, most merciful.

Praise be to **ALLAH**, the lord of the worlds and blessing be upon all his prophets and upon the last and messengers. **Mohammed**, and his family.

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To all; please accepts my truthful thanks

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Summary

Dental caries is one of an important common human infectious disease that can lead to loss of tooth structure the result is absent of oral functions. It is occur due to the metabolic activation of the plague microorganisms, especially *Streptococcus mutans* bacterium.

The aim of this study is Isolation and identification of *Streptococcus mutans* bacterium from the dental caries and dental biofilm patients at Thi-Qar province, and investigation the role of some molecular characterizations of *Streptococcus mutans*, glucosyltransferase B (*gtfB*) gene and surface protein antigen P (*spaP*) gene, and ability of this bacteria to form the dental plaque.

In the present study, eighty samples were collected from saliva and dental plaque from patients suffering from dental caries and dental plaque (age range between 7-25 years) from both sexes at Specialized Dental Hospital and Dental Clinics at Thi-Qar province during the period from November to December 2016. The samples were cultured on Mitis Salivarius Bacitracin agar (MSB agar), then the positive samples were purified and diagnosed according to morphological characteristics, biochemical tests, Api 20 Sterp system. Where the initial results were revealed that; only 33/80 (41.25%) gave positive growth for *S.mutans*. The final diagnosis of isolates depended on present the 16S rRNA gene showed that 28/33(84.84%) isolates considered as *S. mutans*. In the analysis of the nucleotides sequence of the partial 16S rRNA gene, the results were showed that these bacteria were actually related to *S.mutans*, basing on this, four of these isolates were registered globally in the NCBI bank, and the numbers of these isolates are (MF314454, MF314455, MF314456 and MF314457).

The isolates were investigated for detection the presence of two virulence genes (*gtfB* and *spaP* genes), the results appeared that 23/28 (82.14%) of isolates were gave positive results for *gtfB* gene and the positive results of *spaP* gene were 20/28 (71.42%).

Streptococcus mutans bacteria were tested for detected its ability to formation of biofilm (by spectrophotometric method), and the results showed that 24/28 (85.71%) of isolates able to formation of biofilm, whereas, the results showed that 23/23(100%) of isolates which possess *gtfB* gene are able to form a biofilm, and 19/ 20 (95%) of isolates which possess *spaP* gene are able to form a biofilm. During the study of the relationship between the presence of the genes (*gtfB* and *spaP* genes) and ability of bacteria to formation of biofilm, the results showed that the pathogenicity of *S.mutans* to form the dental plaque increase with present of *gtfB* gene compared with *spaP* gene.

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List of Abbreviations

Abbreviation	Meaning
Ag	Antigen
API	Analytic profile Index
ATR	Acid Tolerance Response
BHI-B	Brain Heart infusion broth
Bp	base pair
CFA	Colonization factor Antigen
DNA	Deoxyribose Nucleic Acid
ELIZA	Enzyme-Linked Immunosorbent Assay
EPS	Extracellular polymeric Substance
<i>et al</i>	and other
Gbp	Glucan-binding protein
Gp	Glycoprotein
Gtf B	Glucosyltransferase B
<i>gtfB</i>	glucosyltransferase B gene
IU	International Unite
mg	Milligram
M.S.	Mutans Streptococci
MSA	Mitis Salivarius Agar
NCBI	National Center Biotechnology Information

NO.	Number
OD	Optical Density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
SpaP	Surface protein antigen P
<i>SpaP</i>	surface protein antigen P gene
VP	Voges Proskauer
WHO	World Health Organization

Chapter One

Introduction

INTRODUCTION

1.1 Introduction

Oral cavity of human is considered proper sterile at birth, but after 2-5 days a microbes begin to enter the mouth through feeding until it is reach during few months to more than a million bacterial cell(Ion, 2013). Most of them are harmless, but and under certain condition, about 12-17 different bacterial species have been associated with dental plaque (Metwalli *et al.*, 2013).

Dental plaque (dental Biofilm), a thin and sticky layer of microorganisms(with greater than 10^{10} bacterial cell per milligram) which embedded in a matrix composed mainly of insoluble polysaccharides and colonize of teeth surface, is a common etiological factor of dental caries and other oral diseases such as; gingivitis and periodontal disease (Moreira *et al.*, 2015).

Dental caries is one of an important common human infectious disease that can lead to loss of tooth structure the result is absent of oral functions (Schwendicke *et al.*, 2015). It is occur due to the metabolic activation of the plague microorganisms, especially *Streptococcus mutans* bacterium, whereas these bacteria are ability to ferment the dietary carbohydrates(e.g. sucrose and glucose), which are remains between the teeth, for production the organic acids that lead to breakdown the hard tissue(enamel, dentin and cement) due to remove minerals of tooth surface demineralization process (Veale *et al.*, 2016). Dental caries is considered a highly prevalent chronic disease and it is described as a pandemic disease that prevails in all age categories, especially in children, and in developed and developing countries (Jakubovics, 2014).

Worldwide, dental caries is the most common childhood illness. It was reported to be occur five times more common than asthma. The World Health Organization(WHO) also published a report revealed that about 60-90% of school children and nearly 95-100% of adults have dental caries(Samara, 2015), usually lead to pain, difficult in chewing, also effected on speech, smile and cause discomfort (Kanasi, 2008).

The primary colonizers of microorganisms that can attach to the teeth surface and formed dental plaque are mostly gram positive cocci, gram positive rods, filaments and few from gram negative cocci (Peterson *et al.*, 2013). The gram positive cocci species which are amidst the first to colonize include different species of Streptococci such as *Streptococcus mitis*, *S.salivarius*, *S. mutans*, *S.oralis*, *S.sanguis*, *S.sobrinus*, other gram positive rod like *Lactobacillus* spp., filaments such as *Antinomyces* spp., (Singh *et al.*, 2015), *Veillonella* spp. and *Bifidobacterium* spp.(Karpiński and Szkaradkiewicz, 2013).

Several previous clinical studies have shown that *S. mutans* is considered as a primary etiological factor for dental caries (Saravia *et al.*, 2011). Also detection and identification the *S.mutans* among other pathogens of dental caries may be important for not only predicting and prevention of tooth decay, but also this bacterium is associated with infective endocarditis (Nomura *et al.*, 2006; Nobbs *et al.*,2009), mouth , skin, muscles, and central nerve system (Forssten *et al.*, 2010).

Moreover, because of the high importance and prevalence of *S.mutans* (74-94%) among the oral pathogens that cause dental caries(Duque *et al.*, 2009), we deals with study this bacterium molecularly and detect the most important genes (*spaP* and *gtfB* genes) which are responsible for the formation of decay and biofilm on teeth surface.

In the present study, diagnosis of *S.mutans* not only relying on traditional methods in identification which are usually determine from phenotypic characteristics such as colony morphology, hemolysis, carbohydrates fermentation, and analytic profile index (API) 20 Sterp testing, but we also used the PCR technique to confirm the presence of the 16S rRNA genes in all isolates.

Polymerase chain reactions (PCR) technique is high specificity and sensitivity, therefore it is currently being applied in a wide range of medical diagnostics and research. The occurrence of several genes copies of 16S rRNA in the cell and the key role of this genetic target for the PCR detection of bacteria in all different field of microbiology (Petti *et al.*, 2005; Al-Ahmad *et al.*, 2006 ; Seghatoleslami *et al.*, 2013).

There are two important characteristics of the *S.mutans* in promoting the dental caries development, one of these characteristics is ability of this bacteria to colonize the tooth surface and initiate biofilm formation via synthesize extracellular glucan from sucrose, mainly water-insoluble polysaccharides, using the glucosyltranceferase (GTF) enzyme that encoded by the *gtfB* gene (Nishimura *et al.*, 2012) , other important of *S.mutans* virulence factor is found the wall-associated protein antigen(*spaP*), that encoded by the *spaP* gene, whereas this protein interact with host or bacterial products adsorbed on the tooth surface so it play an important role in the formation of dental plaque and thereby a cavities (Ahn *et al.*, 2008).

1.2 Aim of the study

The aim of this study is investigate the molecular characterization of *S.mutans* isolated from dental caries and dental plaque patients at Thi-Qar province by:

1. Isolation and identification of *S. mutans* bacterium from the dental caries patient's at Thi-Qar province by using the cultural methods, biochemical tests, API 20 Sterp testing, and molecular methods (16S rRNA gene).
2. Detect of the nucleotides sequence of partial 16S rRNA gene of *S.mutans* bacteria.
3. Study the virulence genes (*gtfB* and *spaP* genes) of *Streptococcus mutans* isolated from dental caries and dental biofilm patients by using PCR technique.
4. Study the ability of *S.mutans* to biofilm formation.

Chapter Two

Literature Review

Literature Review

2.1 Dental plaque and its role in dental caries

Normally, the composition of human oral micro-flora is in a dynamic balance. When this balance is disturbed, the normal oral flora becomes disordered, lead to the development of dental plaque(Singh *et al.*, 2015). Dental plaque is a biofilm or mass of bacteria that found between or behind the teeth(Societies & Facklam, 2017), on the front of teeth or on chewing surfaces, at first it is a sticky colorless deposit, but if it does not remove will evolve and become as a white greenish or pale yellow film on teeth surfaces (El-sherbiny, 2014;Abduloma, 2015). High carbohydrate intake causes the accumulation of acidic metabolites within mature plaque, leading to a low pH environment. In addition, the number of acid-tolerant bacteria with cariogenic potential will increase significantly or bacterial virulence will be enhanced. In this circumstance, non-cariogenic plaque will change into cariogenic plaque producing a large number of pathogenic substance that can cause the development of dental caries (Koo *et al.*, 2013).

The first event in the formation of dental plaque is the initial attachment of *S. mutans* to the tooth surfaces as in figure (2-1). Whereas, the tooth surface is covered with a proteins and glycoproteins of saliva which interacts with *S. mutans* adhesin (known as antigen I/II) to constituents of the tooth pellicle. Other components at the surface of *S. mutans* include glucan-binding protein (Gbp) and glucosyltransferase B (gtfB) (Philip and Michael , 2009). In the presence of sucrose, gtfB

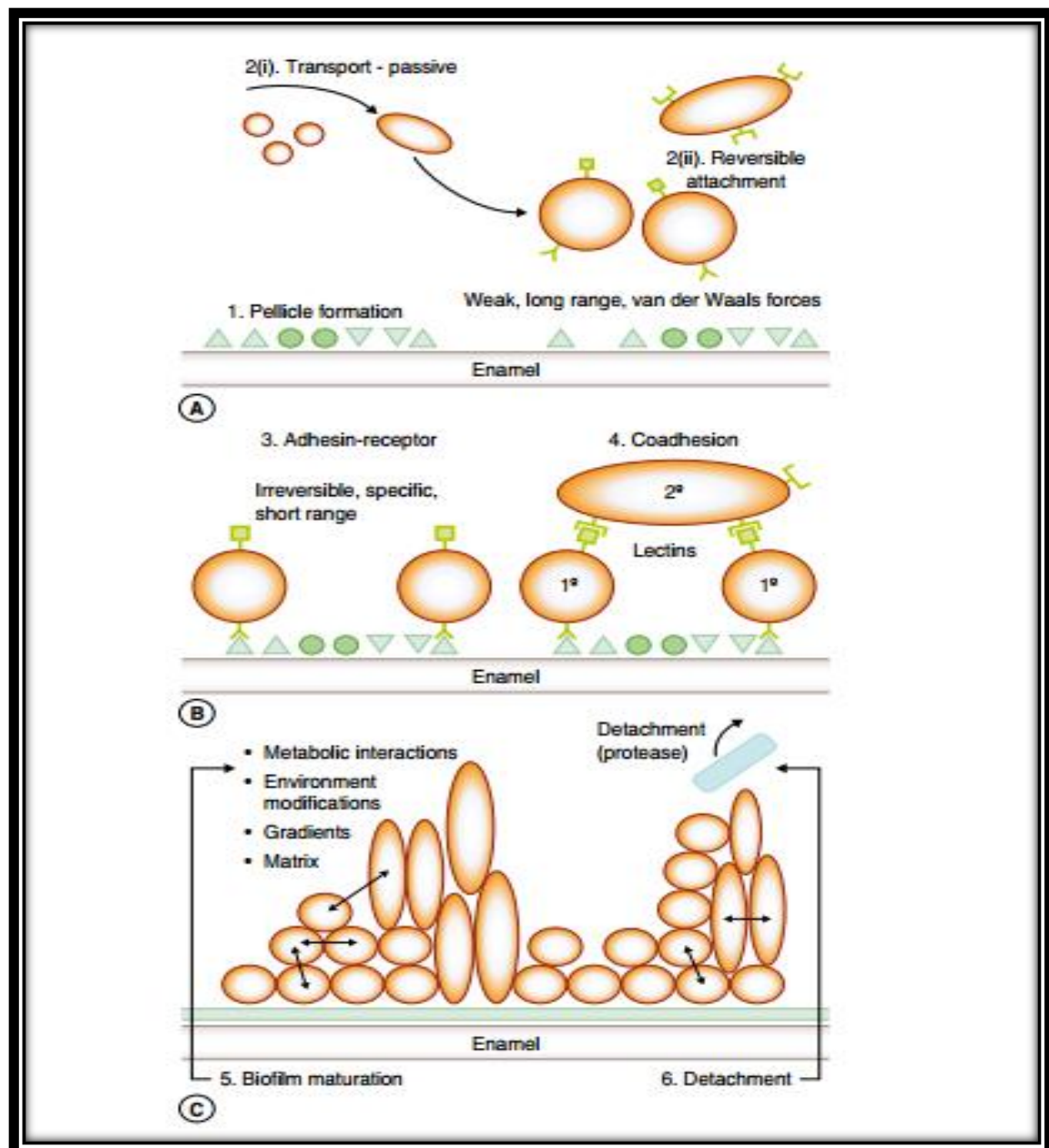


Figure (2-1): Schematic representation of the different stages in the formation of dental plaque:

(A) 1. Pellicle forms on a clean tooth surface. 2(i) Bacteria are transported passively to the tooth surface where they 2(ii) may be held reversibly by weak electrostatic forces of attraction. (B) 3. Attachment becomes irreversible by specific stereo chemical molecular interactions between adhesins on the bacterium and receptors in the acquired pellicle, and 4. Secondary colonizers attach to primary colonizers, often by lectin-like interactions (cohesion). (C) 5. Growth results in biofilm maturation, facilitating interbacterial interactions. 6. Eventually, detachment can occur, sometimes as a result of the degradation by bacteria of their adhesins. Adapted from (Philip and Michael, 2009).

synthesize extracellular glucan (Exopolysaccharides) from glucose (after the breakdown of sucrose into glucose and fructose), and this is thought to be the second even in the formation of dental plaque(Zhao *et al.*, 2014). Exopolysaccharides produced by gtfB enzymes from *S. mutans* are the main constituents in the matrix of cariogenic plaque-biofilms and are recognized as essential virulence factors associated with dental caries (Koo *et al.*, 2013). Gtfs themselves also have a glucan-binding domain and can therefore also function as receptors for glucans. So, *S. mutans* bind pre-formed glucans through Gbp and Gtfs, and this gives rise to aggregates of *S. mutans*. During the third event in the formation of dental plaque, acid formation (as end product of metabolic activation) by cariogenic bacteria such as *S. mutans* drives the dissolution of calcium and phosphate (decalcification) in the hydroxyapatite crystal structure(Guo *et al.*, 2015). Decalcification process is depended on several factors including; the amount of plaque microbes and its activity ,the concentration of the carbon source, the flow rate of saliva and the nature of the tooth surface (Forssten *et al.*, 2010; Nishimura *et al.*, 2012).

As plaque levels increase, and species diversity evolves, the plaque becomes harder to remove and more pathogenic in nature(Leung *et al.*, 2006). This biofilm, which was once reversible, under the right conditions, quickly becomes more established. This enhanced communication between bacteria is now considered irreversible(Rouabhia and Chmielewski, 2012). Co-adherence and co-aggregation between bacterial cells regulate a firm community, which with time becomes more cohesive and the environmental conditions become anaerobic and these conditions are a good environment for growth these micro-organisms(especially *S.mutans* cells) , therefor, leading to dissolution of enamel (Pfepper, 2011).

These different types of bacteria which found on the teeth surface increase the tolerance to antimicrobial and inhibitory agents and increase the resistance to the host defense system (Houry *et al.*, 2012). Also, metabolism is improved and becomes more diverse and efficient. The metabolic waste products of one species could become nutrients for another species (syntrophism). Causing a disease requires a wide range of virulence feature by an organism, and this becomes more feasible when different microbes interact together (pathogenic synergism)(Marsh *et al.*, 2011). Microbes gain many benefits when they all together work in synergy and constitute such complicated communities as dental plaque which is yellowish colored film on the surface of the teeth .(Mounika & Jagannathan, 2015). A variety of carbohydrates provide substrates for these microbes to grow on tooth surface and the waste products of their metabolism, acids, initiate the tooth decay by process dissolving tooth enamel (Amir, 2013; Mounika & Jagannathan, 2015).

2.2 Dental caries

Oral health is a substantial component of general health and well-being and as such dental caries has a significant impact on an individual's quality of life. Dental caries is remain a serious issue worldwide, particularly in children where it is the primary source of tooth loss and it is associated with a substantial economic burden(Casamassimo *et al.*, 2009). Dental caries (also called tooth decay or cavities) is remain one of the most common chronic infectious human diseases, and individuals remain susceptible to the disease throughout their lifetime (Marthaler, 2004; ;Edelstein & Chinn, 2009).This disease progresses slowly and is characterized by localized and (if left untreated)irreversible destruction of the tooth by micro-organisms action (Richard & Howard , 2010 ; Rouabhia & Chmielewski,2012) as shown in Figure (2-2).

Dental caries is a preventable and reversible infection that end result is loss of tooth. Dental caries of the primary dentition can have additional complications for young children, including impairment of growth and development due to decreased nutritional intake associated with the pain of tooth decay and speech disorders arising due to missing teeth can lead to subsequent development of psychological disorders, such as poor self-respect(Jennifer , 2013; Sena *et al.*, 2013).

The initial lesion that is detectable clinically is known as a white spot and can be reversed by remineralization and regrowth of hydroxyapatite crystals, a process enhanced by fluoride(Takeuchi *et al.*, 2012; Somaraj *et al.*, 2017).

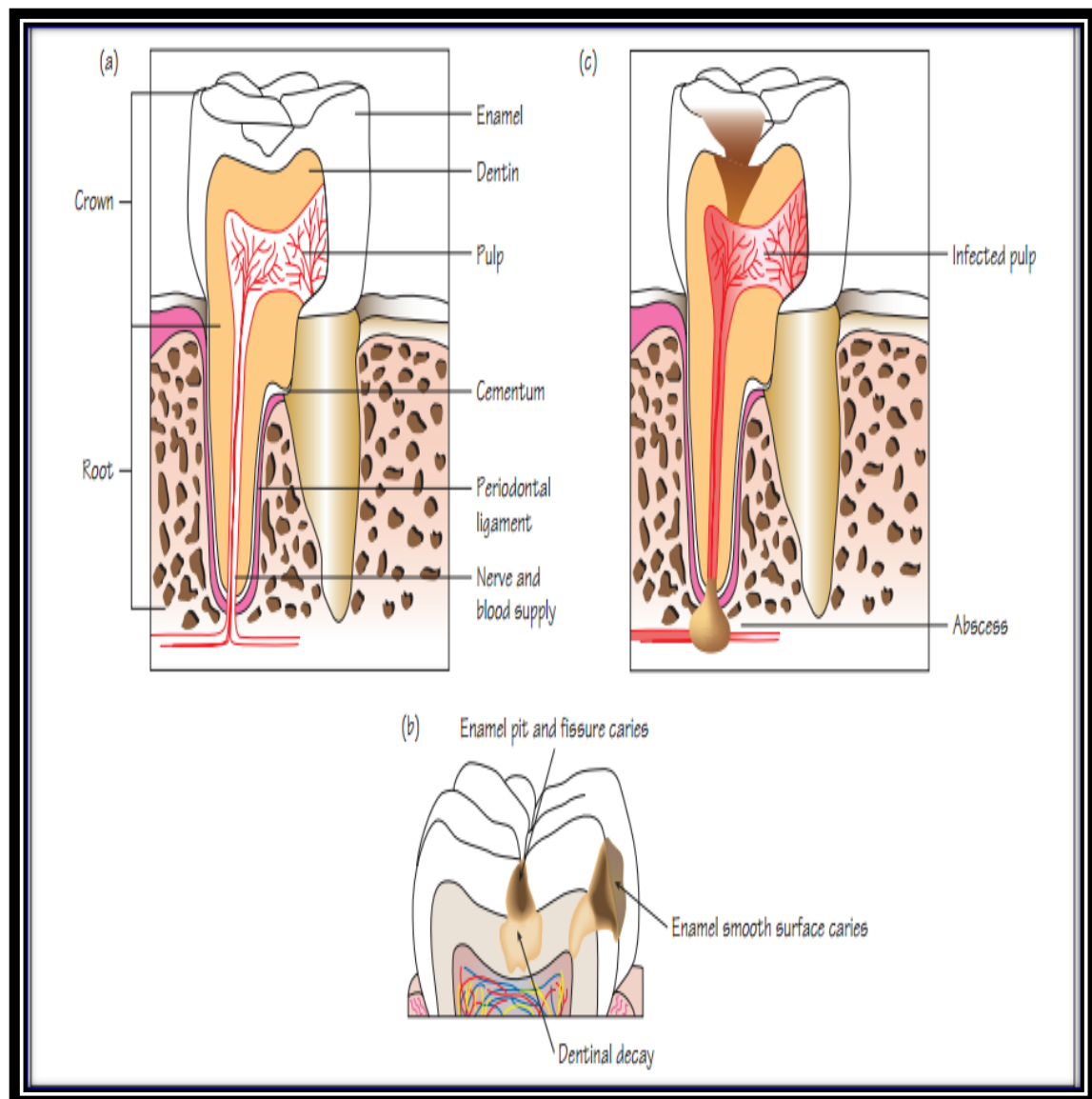


Figure (2-2): Dental caries stages.

(a) Vertical section showing major components of tooth. Caries begins as reversible demineralization (white spot). (b) If demineralization continues, the enamel decays and destruction eventually spreads through the dentin to the pulp chamber, which can lead to a periapical abscess (c). Adapted from (Richard and Howard, 2010).

The carious process is initiated by bacterial fermentation of dietary carbohydrates (mostly sucrose), leading to the formation of a variety of organic acids (especially lactic acid) which leads to remove the minerals (demineralization) from enamel and cement and a fall in PH (Strange *et al.*, 2016). Initially H^+ will be taken up by buffers in plaque and saliva, but

when the pH continues to fall (H^+ increases), the fluid medium will be depleted of OH^- and PO_3^+ , which react with H^+ to form H_2O and HPO_3^+ . On total depletion, the PH can fall below the critical value of 5.5, where the aqueous phase becomes undersaturated with respect to hydroxyapatite, therefore, whenever surface enamel is covered by a microbial deposit, the ongoing metabolic processes within this biomass result in pH fluctuation, and occasional steep drops in pH, which may result in dissolution of the mineralized surface(Lr *et al.*, 2017).

Advanced caries results in cavitation, and can progress to the dentin and into the pulp hole at the latest causing necrosis and periapical abscesses (Goldberg & Hirata, 2017).

In general, there are four main factors that cause dental caries including ;host (such as teeth, saliva and moist of mouth), bacteria, cariogenic diet and time), when these factors interaction between them tooth decay will be formed .In addition to above main factors, there are predisposing factors that also have an impact on the occurrence of dental caries (Fisher-owens *et al.*, 2007; Hadi, 2011; Krol, 2014) as shown in Figure(2-3).

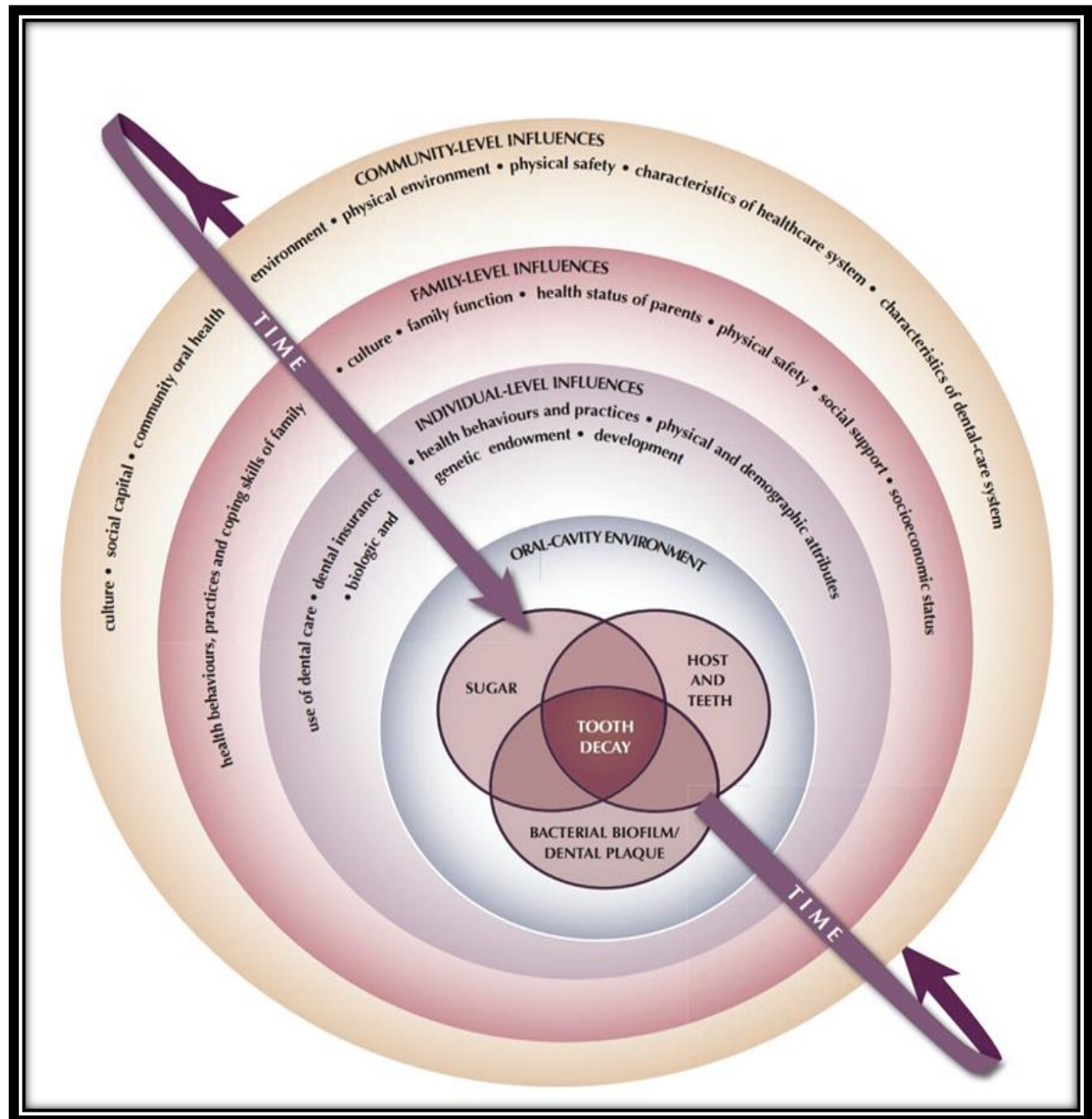


Figure (2-3): Dental caries is a multifactorial disease. Adapted from (Fish-Owens *et al.*, 2007).

2.3 Development of caries on tooth

Teeth are composed of a thin layer (1-2mm) of dental enamel which forms the hard protective coating over the tooth. This consists mainly of phosphate, calcium, and other ions in a structure known as "hydroxyapatite"(Leeuw & Naili, 2017). Dental enamel is porous and is

susceptible to acid dissolution during the process of demineralization. This demineralization process is stabilizer by the repair process known as remineralization (Strange *et al.*, 2016). The balance between demineralization and remineralization of the enamel determines whether caries occurs or not(Bharti *et al.*, 2017). Tooth susceptibility varies among individuals and the reasons are not fully known, but influences include: shape, size and order of the teeth which affect the washing effects of saliva. Salivary components which can be critical in controlling dental caries since they affect bacteria, immune status, plaque formation, and enamel structure and can neutralize acids(Fejerskov & Kidd, 2008 ;Takahashi, 2015). Enamel structure can be altered by a selection of mineral ions and fluoride, aswell as by acid. Availability of fluoride favors remineralization(Badjatia, 2017). The earliest sign of a new carious lesion is the appearance of a chalky white spot on the surface of the tooth, indicating an area of demineralization of enamel, this is referred to as an incipient carious lesion(Roopa *et al.*, 2015). As the lesion continues to demineralize, it can turn brown but will eventually turn into a cavitation. Before the cavity forms, the process is reversible, but once a cavity is formed, the lost tooth structure cannot be regenerated. A lesion that appears brown and shiny suggests dental caries were once present but the demineralization process has stopped, leaving a stain(Süntar *et al.*, 2017). A brown spot that is dull in appearance is probably a sign of active caries(Kawala *et al.*, 2015). The affected areas of the tooth change color and become soft to the touch and when this areas (enamel and dentin) are destroyed, the cavity becomes more noticeable. In highly progressed cases, infection can spread from the tooth to the surrounding soft tissues. Once the decay passes through enamel, the dentinal tubules, which have passages to the tooth nerve, become exposed and causes a toothache (Colak *et al.*, 2013). The pain may worsen with exposure to heat, cold, or sweet foods and drinks (Marsh,

2003; Dinesh *et al.*, 2015). The list of signs and symptoms mentioned in various sources for dental caries includes the 8 symptoms listed at Tooth pain, Bad breath, Fever, Chills, Foul taste, Abscess, Cervical adenopathy and Trismus (Kenneth & Ray, 2004; Philip & Michael, 2009; Salyers *et al.*, 2010).

2.4 Microbial etiology of dental caries

Dental caries will not occur if the oral cavity is free of bacteria. These bacteria can be transferred from mother to child and are present at varying levels in all human mouths. These bacteria are organized into a material known as dental plaque which is yellowish colored film on the surface of the teeth. (Colak *et al.*, 2013).

Miller and Black were two of the earliest educated dentists in the world. Miller might have been the first dentist who had thorough training in natural sciences and who was interested in understanding the biological basis of the dental caries process in 1879. He performed extensive studies of the oral micro-flora and its relationship to dental caries. The key discovery by Pasteur that microorganisms convert sucrose to organic acid helped Miller to develop a “catchy” term – the chemo-parasitic theory. The juncture of Miller’s chemo-parasitic theory with the description of dental plaque by Black provided key elements for the modern concept of etiology of dental caries (Lacin, 2008). This concept has stimulated considerable scientific investigation and as a result it has become one of the essential paradigms of oral biology. Even though the link between the microbial acid production from dietary substrates and etiology of dental decay was provided in the 19th century, researchers were not able to associate any single acidogenic species with decay. In 1960’s, Loesche claimed that the mouth is colonized by 200 to 300 bacterial species, but only a limited

number of these species participate in dental caries(Mounika & Jagannathan, 2015).

Oral streptococci constitute the most prevalent genus of the dental micro-biota and are also present at other various sites in the mouth. Oral streptococci are commensal bacteria; however, some streptococci species are opportunists and are known for causing dental caries, periodontitis and occasional systemic infections such as infective endocarditis or brain abscesses (Knirsch & Nadal, 2011; Jakubovicset *et al.*, 2014). The pioneer colonizers of the tooth surface are members of the mutans group (in particular *S. mutans* and *S. sobrinus*) (Mounika & Jagannathan, 2015). In a previous study,(Becker *et al.*, 2002) compared the bacterial species found in early childhood caries to those found in caries free children. Some species, such as *Streptococcus sanguinis*, were associated with health, while others, such as *S. mutans*, other *Streptococcus* spp., *Veillonella* spp., *Actinomyces* spp., *Bifidobacterium* spp., and *Lactobacillus fermentum*, were associated with caries (Becker *et al.*, 2002). These data also suggested that *Actinomyces gerencseriae* and other *Actinomyces* spp. play an important role in caries initiation(Wade, 2013). These colonizers play a critical role in the initiation and development of dental plaque(Karpiński & Szkaradkiewicz, 2013). In 1924 *Streptococcus mutans* was isolated from human carious lesions by Clark, but was not thoroughly studied until the 1960s. Loesche identified that *S. mutans* was the main cause of dental decay (Lacin, 2008). Thus, *S. mutans* can be present in this initial colonizing community on the tooth pellicle, albeit not as one of the most abundant organisms. However, when dietary sugars, particularly sucrose, become available, *S. mutans* can become the numerically dominant organism, thereby orchestrating the formation of cariogenic biofilms (Lemos *et al.*, 2013).

2.5 Mutans Streptococci (M.S.)

Oral Streptococci constitute a major population in the oral cavity, with several different species colonizing the various ecological niches of the mouth. The oral group has sometimes been named the viridans streptococci, referring to the partial clearing of the erythrocytes around the colony. The current classification of the oral streptococci places the bacteria into four species groups (Moreira *et al.*, 2015), as shown in table (2-1).

Table (2-1):Classification of Oral Streptococci(Kreth & Herzberg, 2015)

Group	Species	Comments
Anginosus	<i>S. anginosus</i> <i>S. constellatus</i> <i>S. intermedius</i>	Frequently isolated from abscesses
Mitis	<i>S.mitis</i> <i>S. oralis</i> <i>S. crista</i> <i>S. infantis</i> <i>S.peroris</i> <i>S. orisrattis</i>	Pioneer species in plaque formation and a common cause of infectious endocarditis
Salivarius	<i>S.salivarius</i> <i>S. vestibularis</i>	Found mainly on mucosal surface and rarely pathogenic
Mutans	<i>S. mutans</i> <i>S. sobrinus</i>	Increased number associated with caries

Streptococci consist a large proportion of the resident oral flora, it is known that roughly one-quarter of the total cultivable flora from gingival and supragingival plaque and half of the isolates from the tongue and saliva are Streptococci, they are vertically transmitted from mother to child, infective endocarditis caused by these bacteria is generally a result of their entry into the blood stream during intraoral surgical procedures (e.g. tooth extraction), and sometimes even during tooth brushing (Krol, 2014).

There is substantial evidence indicating a causative relationship between dental caries and the Mutans streptococci, with many studies demonstrating that the development of caries is preceded by increased colonization with the Mutans streptococci (Yoshida *et al.*, 2015). Mutans streptococci are routinely isolated from the mouths of children and adults, suggesting that dental caries is the most widespread infectious disease of humans. *S. mutans* (serotypes c, e and f) and *S. sobrinus* (serotypes d and g) are implicated as the primary etiological agents associated with the initiation and progression of dental caries. These strains are gram positive, facultative anaerobic cocci, non-motile and non-spore forming, they commonly found in the human oral cavity and are a significant contributor to tooth decay, when they grow on blood agar will give alpha hemolysis after 24-48 hrs. incubation in 37°C anaerobically, few strains will produce beta hemolysis and non-hemolysis (Baggio *et al.*, 2015).

Mutans group are divided into five biotypes according to carbohydrate fermentation, whereas they can ferment sorbitol and mannitol, while other biotypes can ferment raffinose and melibiose as shown in table (2-2).

Table (2-2): Biotypes and serotypes of M.S. according to carbohydrates fermentation(Mohart and Fitzgerald, 1980).

Species name	Biotype	Serotype	Carbohydrates			
			Mannitol	sorbitol	raffinose	meilibiose
<i>S.mutans</i>	I	C, e, f	+	+	+	+
<i>S.rattus</i>	II	B	+	+	+	+
<i>S.cricetus</i>	III	A	+	+	+	—
<i>S.sobrinus</i>	IV	D, g	+	+	—	—
<i>S.downei</i>	V	H	+	+	—	—

S.mutans and *S.sobrinus* are found mostly in the dental plaque and they isolated from carious lesion in human, but *S. mutans* is more prevalent from *S.sobrinus* further it is detected together with *S.sobrinus*(Salli, 2016).

2.6 *Streptococcus mutans* and dental caries

Streptococcus mutans is a Gram-positive, non-motile, non-spore forming, catalase negative facultative anaerobic cocci bacterium and grow optimally at 37°C, that typically occur in pairs or chains under microscopic examination (El-sherbiny, 2014). It is found to be the most common streptococcal species isolated from human oral cavity(Lemos *et al.*, 2017).

Previous studies shown that the level of *S. mutans* from clinical samples, such as dental plaque and saliva, was highly associated with dental caries prevalence(Chanika, 2012). *S. mutans* is a major cariogenic organism—the result of their ability to produce large quantities of glucans as well as acid, exceeding the salivary buffering capacities, which gives the bacteria an advantage to outcompete noncariogenic commensal species at low pH environments(Falsetta *et al.*, 2012; Lemos *et al.*, 2017). In particular, sucrose, the substrate for glucosyltransferase-mediated sucrose-dependent glucan production, promotes the adhesion of *S. mutans* to tooth surfaces. Sucrose is a fermentable disaccharide and may act as a substrate for extracellular polysaccharide synthesis. Changes in the local environment at the tooth surface induced by sucrose exposure disturb the microbial balance and may promote the growth of acidogenic populations including *S. mutans* and other bacteria(Decker, 2014) .

This ability to survive in an acid environment (aciduric) by modulating sugar metabolic pathways coupled with irreversible binding to teeth is a key component to *S. mutans* pathogenesis, in the second stage of invasion, *S. mutans* coadhere or coaggregate with other microbial species, followed by proliferation and spread into other sites in the oral mucosa modulated by concerted action of genes and signaling molecules, in the final stage, the biofilm reaches a steady state which changes the equilibrium balance of the oral ecology; as a result, bacteria gain access into the deeper tissues and recesses in the gingival areas, ultimately causing dissolution of hydroxyapatite crystals in enamel and dentin which results in cavitation within the tooth(Metwalli *et al.*, 2013).

2.7 Taxonomy of *S.mutans*

The scientific classification of *S.mutans* is :(Jawetz, *et al.*, 2013)

Kingdome: Bacteria

Phylum: Firmicutes

Class: bacilli

Order: Lactobacillales

Family: Streptococcaceae

Genus: Streptococcus

Species: *Streptococcus mutans*

S. mutans was first isolated from carious lesions by Clarke as early as 1924, but it was not until the 1960's that taxonomical advances allowed researchers to match strains of oral streptococci recovered from human carious lesions (and similar strains recovered from rodent caries) with the original *S. mutans* isolate from 1924. But even at that point, the taxonomy of the oral streptococci remained difficult and has remained in a state of flux ever since. *S. mutans* was the name given to all oral streptococci that were isolated from carious lesions that could ferment mannitol and sorbitol, that produced extracellular glucans and were cariogenic in rodent models of caries. They were called “mutans” due to their appearance on Gram stains where they resembled mutant versions of streptococci, possessing a smaller and more oval appearance. *S. mutans* is classified into serotypes c, e and f with approximately 70-80% of strains as serotype c, followed by e (~20%) and f (less than 5%). The most common human species remains *S. mutans*; *S. sobrinus* is also common in humans, but less

so than *S. mutans*. *S. criceti* and *S. rattii* are only rarely recovered from humans.(David , 2010).

2.8 Virulence factors of *S. mutans*

Virulence factors of *S.mutans* help to protect the bacteria against possible host defenses and maintain its ecological niche in the oral cavity, while contributing to its ability to cause host damage (Krol, 2014).

S. mutans possess several attributes that contribute to its success as a cariogenic organism: (1) ability to adhere to the tooth surface and develop plaque communities; (2) production of glucans and other polysaccharides from excess carbohydrate (often sucrose) in the diet, leading to plaque accumulation; (3) production of acids (principally lactic acid), that generate a low pH environment and enrich for aciduric organisms.(Richard and Howard , 2010).

2.8.1 Attachment to tooth surfaces

Adhesions of *S.mutans* are virulence factors with functions critical to viability. These adhesions are found as components of cell wall or are associated with cell structure, such as fibrils, capsules or fimbria. The receptors may be salivary components (amylase, glycoproteins, lysozyme, mucins, IgG, IgA, statherins, and proline –rich proteins) or components of bacteria (glucan and glucosyltransferase) which are bound to oral surface (Brandtzaeg, 2013).

A major surface protein produced by *S. mutans* is the AgI/II family protein called P1 (or SpaP) that encoded by *SpaP* gene(Bt *et al.*, 2011). This protein contributes to the fibrillar layer that is on the outside of *S. mutans* cells, made up of teichoic acids, proteins and polysaccharides. The AgI/II polypeptide of *S. mutans* mediates attachment to salivary pellicle,

principally through binding to human salivary agglutinin (also called gp340)(Haukioja *et al.*, 2008). AgI/II contains approximately 1500 amino acid residues, and several different regions that bind to salivary glycoproteins, collagen and fibronectin. *S. mutans* can also adhere to earlier colonizing streptococci (such as *S.gordonii*), a process that can be enhanced by bridging gp340 molecules.(Yoshida *et al.*, 2015).

Acidogenic bacterium is require the hard surface furnished by teeth for sustained colonization and accumulation. The initial binding to the teeth is achieved via the interaction between the proteins of bacteria (P1) with lectin in the dental pellicle covering the surface of tooth. Antigens of *S.mutans* cell have been demonstrated to attach to salivary components in experimental tooth pellicles.

Immunological approaches support the adhesins-related functions of the antigen of proteins and their repeating regions. Numerous in vivo and in vitro evidence indicates that antibody with specificity for *S.mutans* antigen SpaP can interfere with bacterial adherence and subsequent tooth decay (Angela *et al.*, 2009). Antibody directed to the intact antigen P1 molecule or to its salivary-attach domain blocked adherence of *S.mutans* to saliva-coated hydroxyapatite(Cai *et al.*, 2016). Furthermore, abundant immunization approaches have shown that active with intact antigen or passive immunization with monoclonal or transgenic antibody to putative salivary-binding epitopes domain within this component can protect primates, human and rodents from dental caries that caused by *S.mutans*(Brandtzaeg, 2013).

2.8.2 Glucosyltransferase (GTF)

Glucosyltransferases (GTF) catalyze the synthesis of water-insoluble glucan polymers (gluey substances) from sucrose that is necessary for the formation of dental plaque by *S. mutans*(Ren *et al.*, 2016) . Glucans (that produced outside the cells) can be broken down later into glucose residues a few hours after dietary intake has ceased. The *gtfB* gene (that encoded to this enzyme) is randomly arranged on *S. mutans* chromosomal DNA(Al-jumaily *et al.*, 2014). The nucleotide sequences of *gtfB* gene from different oral streptococci comply with the same basic pattern, and the GTFs are approximately 1300- 1700 amino acids long(Bowen & Koo, 2011; Al-jumaily *et al.*, 2014). Streptococcal GTFs have two common functional domains. The amino- terminal portion, the catalytic domain, is responsible for the cleavage of sucrose, and the carboxyl terminal portion, the glucan binding domain, is responsible for glucan binding(Salyers *et al.*, 2010). Glucans, synthesized from dietary sucrose (and possibly in combination with starch) by glucosyltransferases, enhance the pathogenic potential of dental plaque by promoting the adherence and accumulation of cariogenic streptococci on the tooth surface, and by contributing to the bulk and structural integrity of plaque. (Amir, 2013; Krzy *et al.*, 2014). The synthesized glucans provide the possibility of both bacterial adhesion to the tooth enamel and microorganisms to each other. GTF B and C have been shown to be essential virulence factors of mutans streptococci associated with the pathogenesis of dental caries(Krzy *et al.*, 2014). These polysaccharides provide the organisms with a unique micro-environment for their growth, metabolism and survival. Therefore, inhibition of formation and accumulation of cariogenic biofilm communities by affecting the synthesis of these polysaccharides is an attractive route for preventing both dental caries and plaque formation(Williams *et al.*, 2014).

2.8.3 Acidogenicity and Aciduricity

Carbohydrate metabolism by *S. mutans* via the glycolytic pathway is of considerable importance to the ecology of the dental plaque biofilm, since the resulting acid formation lowers the pH of plaque (Welin-neilands, 2007) as in figure (2-4).

S. mutans bacterium is considered more rapidly than other oral micro-organisms which ferment many different sugars and produce the large quantities of organic acids (acidogenicity) (Marsh *et al.*, 2015). The acids produced not only are responsible for tooth demineralization but also rapidly acidify the dental plaque environment around the bacterium. While *S. mutans* can adapt to the surrounding pH as low as pH 4.5, this vastly acidic condition is toxic to most other bacteria (Dhawan, 2013; Dinesh *et al.*, 2016). Additional virulence properties of *S. mutans* include its ability to tolerate environmental changes, such as low pH (aciduricity) (Prabu *et al.*, 2006; Cohen-berneron, 2016). The degree and rate of the pH fall increase with the concentration of carbohydrate in the food such that the plaque pH can reach values of less than 5.0 and remain at low levels for some time (Mcneill and Hamilton, 2003). These metabolic reactions make the dental plaque acidic in the presence of a fermentable carbon source, and the acid tolerance of the *S. mutans* bacteria enables them to continue metabolisms even at low pH (Gross *et al.*, 2012). It has been demonstrated that *S. mutans* is more acid tolerant than all other bacteria examined, with the exception of lactobacilli bacteria (Arul, 2011; Lemos *et al.* 2017).

An inducible property exists in *S. mutans* and *S. sobrinus* which permits adaptation to acidic environments (Welin-neilands, 2007). Under acidic conditions, *S. mutans* alters its physiology in a variety of ways in order to survive, including the synthesis of stress-responsive proteins,

exhibiting increased glycolytic activity and increased activity of the proton-trans locating ATPase regulating intracellular pH (Welin-neilands *et al.*, 2007). These changes are now known to be associated with an adaptive response that has been termed the acid tolerance response (ATR) (Kenneth & Ray, 2004; Salyers *et al.*, 2010; Jiang *et al.*, 2017).

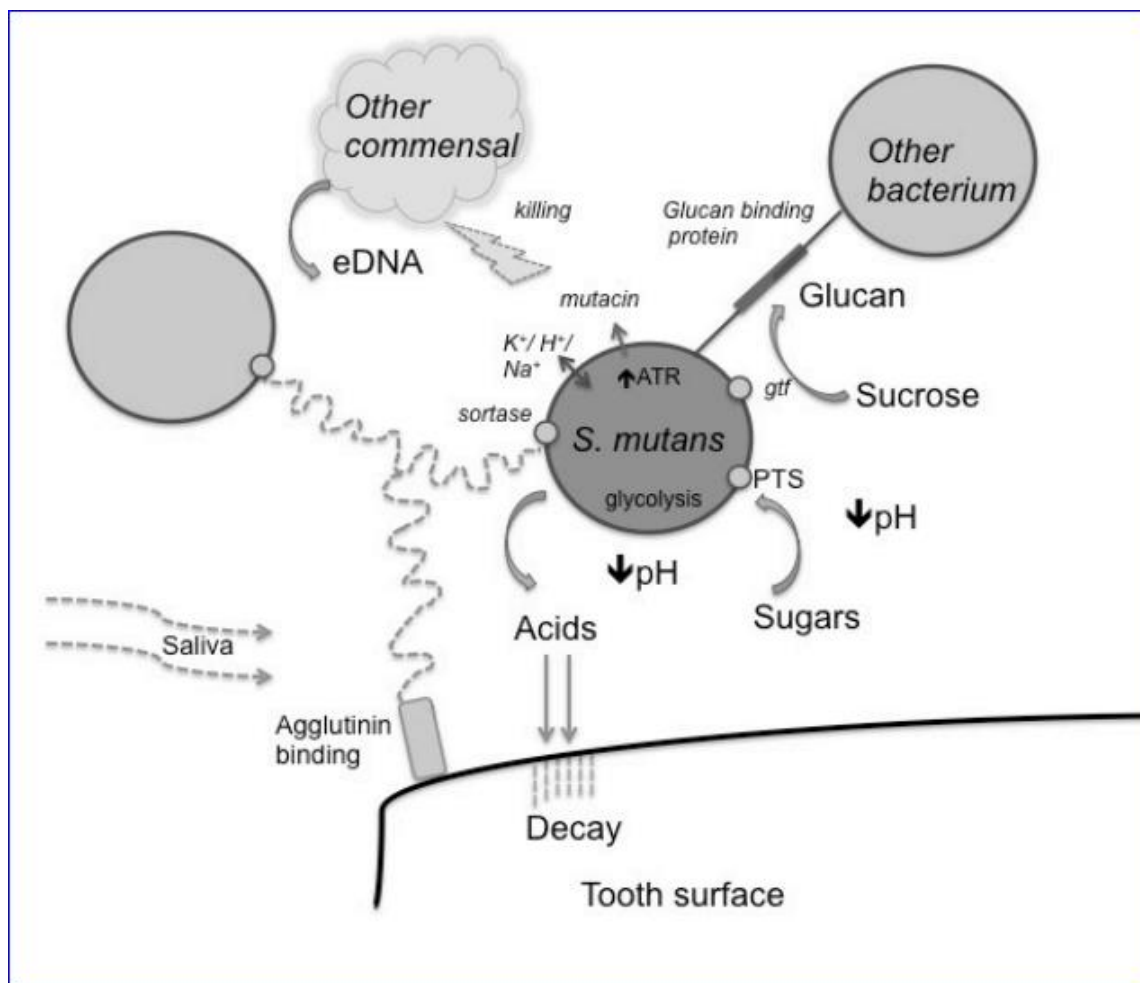


Figure (2-4): Summary of *S. mutans* contributory factors to cariogenicity in dental plaque. Adapted from (Gursonika, 2016).

S. mutans adheres to the tooth surface and other bacteria with the help of glucan and glucan binding proteins in the presence of sucrose. In the absence of sucrose, adhesion occurs via SpaP, also known as antigen I/II, which binds to salivary agglutinin glycoprotein. *S. mutans* can metabolize

various dietary sugars through multiple sugar transport systems and a robust glycolytic pathway, to produce acid end products, which leads to dissolution of the tooth enamel and killing of other commensal bacteria. A reduction in environmental pH can activate a specialized acid tolerance response. During stress tolerance adaptation, *S.mutans* secretes antimicrobial and signaling molecules. The function of these antimicrobial molecules is to cause cell death of other competing bacteria, while *S. mutans* alters its community phenotype dramatically by the secretion and detection of signaling peptides (Gursonika , 2016).

2.8.4 Biofilm formation

The pathogenicity of certain micro-organisms, such as *S.mutans* bacterium, is inseparably associated with their ability to form biofilms on solid surfaces((Pfepper, 2011 ; Wu *et al.*, 2014). Biofilm formation is an important virulence factor for *S. mutans*. This feature allows microorganisms to form three-dimensional structures in which cells become more resistant to antibiotics and changing environmental conditions, among others, through changes occurring as a result of interbacterial interactions and the presence of an exopolysaccharide matrix protecting the entire structure (Koo *et al.*, 2013).

S. mutans is the biofilm-forming bacterium that is primarily associated with dental caries (Arruda *et al.*, 2013).

The expression of genes associated with biofilm development, such as *SpaP* gene and those involved in the formation of extracellular glucans (*gtfB*), plays an important role in the regulation of biofilm development by *S. mutans* (Shemesh *et al.*, 2010; Guo *et al.*, 2015). In addition to the above, among the genes were selected for this study are *gtfB* gene that encoding to polysaccharide synthesizing enzyme (glucosyltransferase B), also we

studied *SpaP* gene, which contributes to the adhesion of *S.mutans* to tooth surface.

The steps of biofilm formation by *S.mutans* includes(Kreth & Herzberg, 2016):

2.8.4.1 Salivary Pellicle Adhesion

S.mutans adhere to macromolecular complexes found on the mucosal cells or saliva-coated tooth, this salivary film is commonly called pellicle(Haukioja *et al.*, 2008). Although *S. mutans* bacteria (and some of oral streptococci) are able to adhere directly to hydroxyapatite (Salli *et al.*, 2016), the major mineral found in enamel of teeth, the initial attachment process involves the pellicle since the teeth and oral surfaces are consistently bathed with saliva and naked enamel occurs rarely in the oral cavity. All evidence indicates that the streptococcal surface interacts with salivary components including salivary proteins(Blanca *et al.*, 2013).

Salivary proteins solubilized in the liquid or gel fractions of saliva are able to attach avidly to the tooth surface via negatively charged residues and electrostatic interactions through the hydrophilic regions; when tightly bound to hydroxyapatite, hydrophobic domains buried within the three-dimensional structure of the salivary proteins can become exposed and facilitate additional interactions(Kreth & Herzberg, 2016).

Glycoprotein (gp) 340, is a one components of saliva and in the pellicle, interact with surface protein antigen P(SpaP) of *S.mutans*, this type of the binding called sucrose-independent attachment (Nobbs, 2011). Interestingly, *S. mutans* AgI/II protein exhibits significantly higher levels of adhesion to gp-340-I compared with glycoforms II and III, and this correlates with increased caries susceptibility in individuals producing gp-340-(Jakubovics, 2014). Thus, the specificity of bacterial adhesin-receptor

interactions can influence both oral colonization and disease susceptibility (Nobbs *et al.*, 2011).

2.8.4.2 Binding to Cellular Components

Surface proteins of Streptococci also bind the bacteria to host cellular components and salivary proteins that form a pellicle on mucosal epithelial cells. About 80% of the oral cavity surface consists of soft tissue, providing a large area for bacterial attachment (Kanasi, 2008). Oral microbes employ other host components as binding receptors including the integrin family of cell surface receptors responsible for cell–cell attachment (Kreth & Herzberg, 2016). For example, the *S. mutans* multi-ligand antigen I/II family adhesin SpaP mediates interaction with $\alpha 5\beta 1$ integrins (Engels-deutsch *et al.*, 2011) other host components, and bacterial cells of other species. Antigen I/II also mediates binding to fibronectin, collagen, salivary glycoproteins and gp 340 (Brady *et al.*, 2011).

Therefore, surface adhesin proteins on oral bacteria do not only ensure binding to oral surfaces during biofilm development but also contribute to the virulence of the respective species and influence the capacity of the host to respond to the presence of the biofilm communities.

2.8.4.3 Production of Exopolysaccharide (EPS)

After initial attachment of *S. mutans*, the next step in biofilm formation is defined by the production of EPS by this bacteria via (GtfB) enzyme. If dietary sucrose is available in excess, glucosyltransferase activity is intimately linked to caries development. (Klein *et al.*, 2015). The insoluble EPS (glucan) will play a role in adhesion of *S. mutans* to teeth which then contribute to the formation of dental plaque (dental biofilm) (Bt *et al.*, 2011). EPS synthesis makes *S. mutans* a formidable opponent to oral

health, since the glucans produced by secreted Gtfs that are incorporated into the pellicle provide bacterial binding sites in situ to promote tight adherence and microbial accumulation on tooth surfaces Gtfs also bind many oral bacteria, such that glucans are formed on the surfaces of *S. mutans* and other oral microorganisms, which progressively accumulate to enmesh these microorganisms in the matrix (Koo *et al.*, 2010)as in figure(2-4).

When cells are growing in an established biofilms the antimicrobial effect is limited to the superficial layers(Singh *et al.*, 2015). Sucrose is extremely important in *S. mutans* biofilm formation. The presence of sucrose decreases the antimicrobial susceptibility of *S. mutans*, probably due to the subsequent biofilm formation initially, or in the absence of sucrose, *S. mutans* adheres using ionic and lectin-like interactions. Streptococcal protein antigen P binds to salivary agglutinin glycoprotein (SAG) and SAG coated surfaces. In the presence of sucrose, glucans are synthesized by glucosyltransferases (GTFs). They form an extracellular layer of EPS that promote adhesion and biofilm formation(Rostami *et al.*, 2016).

Chapter Three

Materials and Methods

Materials and Methods

3.1 Materials:

3.1.1 Instruments and equipments

The instruments and equipment have been used in this study are listed in table (3-1).

Table (3-1): Instruments and equipments and their company.

No.	Equipment & instrument	Company
1	Autoclave	Monarch MSI/Germany
2	High Speed Cold centrifuge	Hettich /Germany
3	Incubator	Memmert/Germany
4	Oven	Memmert/Germany
5	Light Microscope	Olympus/Japan
6	Sensitive Balance	Sartorius/Germany
7	Water Bath	Memmert/Germany
8	Vortex	CYAN / Belgium
9	Micropipettes 5-50, 0.5-10, 100-1000µl	Gelson/ Franc

10	Refrigerator	Concord /Lebanon
11	PCR Thermo-cycler	Invertogen/ U.S.A.
12	Electrophoresis	MD-300N/ UK
13	Eliza Instrument	Gelson/ Japan
14	UV transilluminator	Wised/Korea
15	Camera	Samsung/China
16	Hot plat stirrer	Labtech /Korea
17	Eppendorf tubes	Sigma/ England
18	Disposable Petri dishes	Al-Hani company / Lebanon
19	Nanodrop	Memmert/Germany
20	Millipore filter	Sartorius/ Germany
21	Anaerobic jar	BBL/ France
22	Dissecting Microscope	Leitz-Wetzlar/Germany

3.1.2 Chemical and Reagents

The chemical and reagents with their company and countries of origin which have been used in this study are listed in the table (3-2).

Table (3-2): The chemicals with their companies and countries of origin used in this study.

No.	Chemical	Company and Origin
1	Absolute Ethanol	BDH /England
3	10X TBE (Tris- Boric acid- EDTA)	Bio Basic/U.S.A.
4	DNA ladder (2000-100bp)	Bioneer /Korea
5	Agarose gel	Bio Basic /U.S.A.
6	Ethidium Bromide	Bio Basic /U.S.A.
7	Free nuclease water	Bioneer /Korea
8	Lysozyme	Bio Basic /U.S.A.
9	Cider oil	Syrbio/ Switzerland
10	Sodium chloride (NaCl)	Himedia/ India
11	Glycerol	Fluka / England
12	Hydrogen peroxide	Fluka/ England
13	Sucrose, Mannitol , Sorbitol, Raffinose, Meilibiose	BHD/UK
14	Methyl red	BHD/ UK
15	Potassium tolerate	Himedia/ India

16	Arabic chewing gum	Iraq
17	Bacitracin	AppliChem, Germany
18	Phosphate buffer saline (PBS)	Himedia /India

3.1.3 Culture Media

The media have been used for culture in this study are listed in table (3 -3).

Table (3-3): Cultures media and their manufacture.

No.	Culture Media	Manufacture / Origin
1	Brain Heart infusion Broth	BBL/France
2	Tryptic soy broth	Difco
3	Thioglycolate broth	Oxoid
4	Blood agar base	Oxoid
5	Mitis – Salivarius agar	Hi-Media

3.1.4 Materials and Kits used for biochemical test and molecular methods

The kits were used in this study are listed in table (3-4).

Table (3-4): Types of kits used in the present study and their company.

No.	Kit	Company /Origin
1	Gram's stain	SyBio/ Syria
2	API system Identification	BioMerieux/France
3	Genomic DNA extraction kit	Geneaid/UK
4	Ladder	Bioneer/ South Korea
5	AccuPower PCR PreMix	Bioneer/ South Korea
	Taq DNA polymerase	
	dNTPs (dATP, dCTP, dGTP, dTTP)	
	Tris-HCl pH 9.0, KCl, & MgCl ₂	
	Stabilizer and Tracking dye	

3.1.4.1 The components of Gram's stain are:-

- Crystal violet.
- Lugols iodine.
- Ethanol alcohol 95%
- Safranin stain

3.1.4.2 The components of API 20 strep system are:

- 1) Mineral oil dropper.
- 2) Incubation Box.
- 3) Chemical reagents.
- 4) Saline suspension medium (NaCl 0.85% suspension).
- 5) GB medium.

3.1.4.3 Genomic DNA extraction kit

Table (3-5): The kits used in this study with their companies and country.

Kit	Company	Country
Genomic DNA Extraction Kit	Geneaid	UK
GT buffer		
GB buffer		
W1 buffer		
Wash buffer		
Elution buffer		
GD column		
Collection tube 2ml		

3.1.4.4 Molecular Weight Marker (Ladder)

The molecular weight markers have been used in this study was from Bioneer / South Korea (100-2000bp).

3.1.5. Primers

The primers, in this study, were designed by using NCBI Gene-Bank and Primer 3 online and provided by (Bioneer Company, Korea) as in table (3- 6).

Table (3-6): Primers sequences used for genes amplification.

Gene name	Primer Sequence(5'-3')		Product Size
16S rRNA gene <i>S.mutans</i>	F	CCACACTGGGACTGAGACAC	507 bp
	R	GTTTACGGCGTGGACTACCA	
<i>gtfB</i> gene	F	CCAACAAATCCAGTGTGCCG	315 bp
	R	TGACAACCACGCGATCAGAA	
<i>SpaP</i> gene	F	CCGACACCAACACCAGATCA	696 bp
	R	CGTTGAACCGGCAAGAACTG	

3.2 Methods

3.2.1 Sterilization Methods

A- Sterilization of the culture media by autoclave at 121 °C for and pressure 15 psi for 15 min.

B- Sterilization of the glasses by dry heat in electric oven at 180 °C for 2 h.

C- Sterilization of Bacitracin by Millipore filtration.

3.2.2 Preparation of Reagents and solutions

The following reagents and solutions were used in this study:

3.2.2.1 Catalase reagent

Hydrogen peroxide (3%) was prepared from stock solution in a dark bottle and it has been used for detection of the ability of the isolates to produce catalase enzyme (MacFaddin, 2000).

3.2.2.2 Bacitracin solution

Bacitracin was prepared in concentration of (200 IU) to be used in the preparation of Mitis- Salivarius Bacitracin Agar (MSB Agar), 0.364gm of Bacitracin antibiotic (AppliChem, Germany) was dissolved in 100ml of sterilized distilled water mixed well by using magnetic stirrer to ensure dissolution of the whole quantity of the antibiotic, the solution was sterilized by Millipore filter (0.20 μ m) then kept in refrigerator until use. A new fresh solution was prepared every 2-3 weeks (Al-Mizraqchi, 1998; Hadi, 2011).

3.2.2.3 Normal Saline

Normal saline prepared by dissolving (9g) of NaCl in one liter of distilled water; the final pH was adjusted at 7.3, and then distributed into screw capped bottles, 9.9 ml. in each one then the bottles were sterilized by autoclave and stored in refrigerator until used (Brinboin, 1997).

3.2.2.4 Potassium Tellurite 1%

The buffer is prepared by dissolving 1g of potassium Tellurite at 90 ml sterile distilled water then the volume is completed to 100 ml and sterilized by Millipore filter and preserved at 4 °C.

3.2.2.5 McFarland Standard (4)

McFarland standard solution tube number (4) has been prepared as the standard guidelines by mixing 0.4 ml of 1.175% (wt/vol) of barium chloride dehydrate ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) solution with 9.6 ml of a 1% (vole/vole) sulfuric acid . To checking the accuracy of the density of the solution must be use spectrophotometer and must be stored at room temperature. Number (4) McFarland standard can be compared usually to a suspension of bacteria in sterile saline or nutrient broth. If the bacterial suspension is very turbid, it can be diluted with more diluent but if the turbidity to bacterial suspension is not enough, more bacteria can be added.

3.2.3 Preparation of culture media

3.2.3.1 Brain Heart infusion Broth (BHI-B)

The medium was prepared and sterilized according to Hi-Media Company directions; 37 gm. were suspended in 1000 ml distilled water day it was and dispensed into screw capped bottles. Sterilization was done by autoclave. For best results, the manufacturer recommended that the medium should be used on the day it was prepared.

3.2.3.2 Tryptic soy broth (TSB)

Preparation of made TSB was according to the instruction of bioMerieux Company by suspending 30 gm. powder in 1000 ml water, mixed well using magnetic stirrer to ensure dissolution of the whole quantity of the powder.

3.2.3.3 Thioglycolate broth

Preparation of made Thioglycolate broth was according to the instruction of bioMerieux Company by suspending 28 gm. powder in 1000

ml water, mixed well using magnetic stirrer to ensure dissolution of the whole quantity of the powder.

3.2.3.4 Thioglycolate –carbohydrates broth

After the preparation of thioglycolate broth as described in 3.2.3.3 the Carbohydrate (mannitol, sorbitol, sucrose, raffinose and inulin) were added (1%) to the thioglycolate media and heated to insure dissolution of the whole quantity of the sugar in the medium.(Al-jumaily *et al.*, 2014).

3. 2.3.5 Blood Agar

Preparation of this medium done by dissolved 37g of blood agar base in 1 liter distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclave cool to 45-50 °C and aseptically add 5% v/v sterile defibrinated blood. Mix well and pour into sterile petri plates.

3. 2.3.6 Mitis- Salivarius Bacitracin Agar (MSB Agar)

This agar is the selective medium for the cultivation of *S. mutans*. It was prepared from mitis salivarius agar (MSA) according to Hi-Media Company instructions. With 20% (w/v) sucrose and 200 Units/L bacitracin. Medium was prepared according to the instructions labeled on the package by suspending 90gm of the powder in 1000 ml distilled water, mixed well using magnetic stirrer to ensure dissolution of the whole quantity of the powder. To increase the specialty of MSA to the isolation of MS, the addition of sucrose in a concentration of 150 g/l (before sterilization) was performed. After autoclaving, the medium was allowed to cool to about 45 °C and one ml of bacitracin solution (Sec. 3.2.2.2) was then added for each one liter of the agar, then it was poured in petri dishes and permitted to cool and set and then stored in the refrigerator until used (Dhawan & Dhawan, 2013).

3.2.4 Isolation and identification of *S.mutans*

3.2.4.1 Samples Collection

3.2.4.1.1 Saliva samples

Eighty dental patients (age range between 7-25 years) from both sexes suffering from dental caries in Specialized Dental Hospital and Dental Clinics in Thi-Qar province during the period from November to December 2016.

Each individual was asked to chew a piece of Arabic chewing gum (0.4-0.5g) for five minutes to stimulate salivary collection as much as possible then saliva a proximally (2-3ml) was collected in sterilized tube .

3.2.4.1.2 Plaque sample

Dental plaque samples were collected by using sterile curettes (Gracey Curettes). Supragingival plaque was taken first from the crown site, and then was taken from the sub gingival plaque. The same procedure was applied to the plaque samples obtained from the natural tooth. The plaque samples were suspended in 1 ml of sterile Phosphate buffer saline PBS (pH =7.4).

The two type of samples were transported on icebox to the laboratory.

3.2.4.2 Isolation of *S.mutans*

Saliva and plaque samples were collected under standard conditions according to Hadi, (2011).Whereas, each types of samples were homogenized by vortex mixer for two minutes. Tenfold serial dilutions were prepared by using normal saline. Two dilutions were selected for each microbial type and inoculated on the following culture media:

MSB Agar [the selective media for Mutans Streptococci (MS)] 0.1ml. was withdrawn from dilutions (10^3 - 10^5) and then spread in duplicate by using sterile micro biological spreader on the plates of MSB agar then the plates were incubated anaerobically by using a gas pack supplied in an anaerobic jar for 48 hrs. at 37 °C followed by aerobic incubation for 24 hrs. at 37 °C.

3.2.4.3 Identification of *S.mutans*

The identification of *S.mutans* based on:

3.2.4.3.1 Morphology of colony

The colonies on MSB agar was examined directly under dissecting microscope (magnification X 20). The identification of *S.mutans* isolates were according to (Hadi, 2011).

3.2.4.3.2 Gram stain

A colony was picked up from MSB agar plates separately under sterilized conditions and subjected to Gram's stain according to (Jawetz *et al.*,2007) ; a small inoculum was taken from a discrete, singly isolated colony, it was emulsified in a drop of normal saline on glass slide to form suspension which was then spreaded, dried and heat-fixed according to Gram's method starting with crystal violet for one minute, and then washed with water, stained with Lugols iodine for one minute , and then washed with water, decolorized with ethanol alcohol for 30 seconds and washed. Counter stained with Safranin for one minute then washed and dried. The slide was examined under light microscope with 100x magnification for cells morphology, arrangement, spores formation and staining characteristics.

3.2.4.3.3 Biochemical Tests

Small colony was picked up from MSB agar under sterilized conditions using inoculating loop and then spread on blood agar plates (to see type of hemolysis) and incubated anaerobically for two days, subcultures were repeated several time in order to obtain pure culture. After this the following tests were conducted:

A-Catalase Production Test:

This test effected by Hydrogen peroxide 3% (H₂O₂) used to detect the activity of catalase enzyme production. A small amount of pure isolates of *S.mutans* cultures were transferred separately using a sterile loop to the surface of clean dry glass slide. Drops of hydrogen peroxide 3% immediately placed onto a portion of bacterial culture on the slide, absence of gas bubbles indicate the absence of catalase enzyme. This test had been carried out also on the colonies of MSB agar plates directly.

B-The fermentation of different carbohydrates sources

The bacterial isolates can fermented different carbohydrates sources, were determined by the following method:

Thioglycolate broth supplemented with 10% of each (mannitol, sorbitol, sucrose, raffinose and inulin) Thioglycolate broth medium used as negative control, while Sucrose as positive control, all carbohydrate solutions were sterilized by filtration 0.45um and added aseptically to the autoclaved Thioglycolate broth which contain a 0.02% of phenol red, the suspended media inoculated with the tested isolates and incubated anaerobically at 37 °C for 72 hours, the color of media has changed from red to yellow as compared with the controls indicated the ability of these bacteria to ferment these carbohydrates sources (Fingold and Barone, 1986; Al-Jumaily *et al.*, 2014).

C- API 20 strep. System

This test has been done according to (Leboffe and Piercr, 2005); it is used clinically for the rapid identification of the bacterial isolates. This test has 25 plastic strips and each strip contains 20 small tubes with upper orifice (Cupule) and lower orifice (tube) which contain dried material and represent the biochemical test, color changes happened in the tubes either during incubation or after addition of the reagents. These colors reveal the presence or absence of chemical active and thus, a positive or negative result.

The instructions for use are:

1- Preparation the strip:

To formation humid condition, 5 ml distill water is adding to the strip container.

2- Preparation bacterial suspension:

Pure colonies were transferred from culture with suspected *Streptococcus* isolates by a loop into a sterile 5 ml saline suspension medium and mixed well to form a suspension.

3- Inoculation of strip:

The tube of each strip has been filled with a bacterial suspension by a sterile pasture pipette, except the tubes.

4- To formation anaerobic condition, the upper part of tube ADH, RIB, ARA, MAN, LAC, TRE, INU, RAF, AMD and GLYG are covered with a sterile mineral oil.

5- The strip container is covered and incubated at 37 °C for 18-24 hrs.

6- Reading the result:

After 4-4:30 hours from incubation, the reagents are adding as: a one drop of VP1 reagent then a one drop of VP2 directly into VP tube. A one drop of NIN into HIP. A one drop of ZYM A followed by one drop of ZYM into PYRA to LAP. After that wait 10 minutes and reading the result. After incubation (24 hrs.) all result were converted into numerical profile and compared with the company index. Each strip was divided into seven parts and each part has three tests numbered as (1, 2 and 4), and each positive test has been given a specific number, but the negative test has been given the number (0) then all three numbers of each group are about 0-7 (7 digit profile), therefore, there were seven numbers which compared with the index to give the genus and species of the bacterial isolates.

3.2.5 Molecular study

Polymerase chain reaction (PCR) technique was used to confirm the presence or absence of the 16S rRNA genes in the 33 isolates (which are given positive results for the above tests). As well as, this technique has been used to determine the some biofilm formation genes (*gtfB* & *spaP* genes) as following stages:

3.2.5.1 Genomic DNA extraction

Genomic DNA was extracted from *S.mutans* bacterial isolates by using Genomic DNA Mini Bacteria Kit, company of Geneaid, as following steps:

- 1- One ml of (24 hours) incubated cultured bacterial cells was transferred to a 1.5 ml micro centrifuge tube then centrifuged in high speed centrifuge at 15000 rpm for 1 minute then the supernatant discarded.
- 2- Lysozyme buffer(200µl) were added to the tube and re-suspended the cell pellet by shaking vigorously by vortex, then incubated at room

- temperature for 10 minutes, and the tubes inverted every 3 minutes through incubation periods.
- 3- GB buffer (200 μ l) were added to each tube and mixed by shaking vigorously for 5 seconds. Then the tubes were incubated at 60°C for 10 minutes and inverted every 3 minutes through incubation periods.
 - 4- Absolute ethanol (200 μ l) were added to the clear lysate and immediately mixed by shaking vigorously, then precipitates broke it up by pipetting.
 - 5- A GD column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to the GD column. Then centrifuged at 15,000 rpm for 2 minutes. And the 2 ml collection tube containing the flow-through were discarded and placed the GD column in a new 2 ml collection tube.
 - 6- W1 buffer (400 μ l) were added to the GD column, then centrifuge at 15,000 rpm for 30 seconds. The flow-through was discarded and placed the GD column back in the 2 ml collection tube.
 - 7-Wash Buffer 600 μ l were added to the GD column. Then centrifuged at 15,000 rpm for 30 seconds. The flow-through was discarded and placed the GD column back in the 2 ml collection tube, and the tubes were centrifuged again for 3 minutes at 15,000 rpm to dry the column matrix.
 - 9- The dried GD column was transferred to a clean 1.5 ml micro centrifuge tube and 100 μ l of pre-heated elution buffer were added to the center of the column matrix.

10- The tubes were let stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 15,000 rpm for 30 seconds to elute the purified DNA.

3.2.5.2 Estimation of extracted DNA

The extracted DNA was checked by using Nanodrop that measured DNA concentration (ng/ μ L) and checked the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2 μ l of free nuclease water onto the surface of the lower measurement pedestals for blank the system.
3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1 μ l of DNA was added to measurement.

3.2.5.3 PCR master mix preparation

PCR master mix was prepared by using (AccuPower® PCR PreMix Kit) and this master mix done according to company instructions as table (3-7).

Table (3-7): The components and volume of PCR.

No.	PCR component	Volume
1	DNA template	5 μ L
2	Forward primer (10pmol)	1.5 μ L
3	Reveres primer (10pmol)	1.5 μ L
4	PCR water	12 μ L
5	Total volume	20 μ L

After that, the PCR components that mentioned in table above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction(PCR master mix) such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl₂,and loading dye). Then, all the PCR tubes transferred into Exispin centrifuge at 3000rpm for 3 minutes, and then placed in PCR Thermo cycler (MyGene, Bioneer. Korea).

3.2.5.4 PCR Thermo cycler Conditions

PCR thermo cycler conditions were done by using conventional PCR thermo cycler system for each gene as table (3-8).

Table (3-8): PCR conditions of 16S rRNA, *gtfB* and *spaP* genes

PCR steps	Temp.	Time	Repeat
Initial Denaturation	95 °C	5min	1

Denaturation	95 °C	30sec.	30 cycle
Annealing	58 °C	30sec.	
Extension	72 °C	1 min.	
Final extension	72 °C	5min.	1
Hold	4 °C	-	-

3.2.5.5 PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis following steps:

- 1- One percent agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.
- 2- Then 3µL of ethidium bromide stain (0.5 µg/ml) were added into agarose gel solution.
- 3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 30 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp DNA Ladder) in one well.

- 4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 Ampere for 1 hour.
- 5- PCR products were visualized by using UV Transilluminator.

3.2.7 Biofilm Formation(Ardin *et al.*, 2014).

Biofilm formation was detected for 28 isolates of *S.mutans* as follow:

- 1- Isolates were inoculated in test tube contain 3 ml of Tryptic soy broth (TSB), and incubated for 24 hours at 37°C.
- 2- Culture was centrifuged at 3000 round / min. the precipitate was washed three time TSB and centrifuged after each one.
- 3- Suspensions were compared with MacFland standard 0.5 to obtain concentration 1.5×10^8 CFU/ml.
- 4- Added 200ul from suspension to wells of micro plate round bottom and incubate at 25 °C for 24 hours and use TSB for control.
- 5- Remove culture from wells by washing with normal saline three times.
- 6- Biofilm was measured by spectrophotometric method. Micro plate was incubated in 60 °C 1 hours for fixation membrane then micro plate stained with crystal violet for 5 min then washed with tap water to remove surplus stain. ELISA reader was used for measurement of biofilm at 490 nm.

3.2. Gene Sequencing

The PCR product of four isolates was sent to macrogen company at south koreia for sequencing of partial 16S rRNA gene. Sequences of nucleotides were edited by using (MEGA6.0, multiple alignment analysis tool), and compared with sequences of standard strain of

S.mutans (KM052315.1), as well as against standard strains of different *Streptococcus* species reported in GeneBank in National Center Biotechnology Information (NCBI).

Chapter Four

Results

RESULTS

4-1 Isolation and Identification

In the present study, a total eighty samples of saliva and dental plaque from patients suffering from dental caries and dental plaque have been collected and tested during period from November to December 2016. Only thirty-three samples are given growth for *S.mutans* on MSB agar, this is about 41.25% of patients as in figure (4-1).

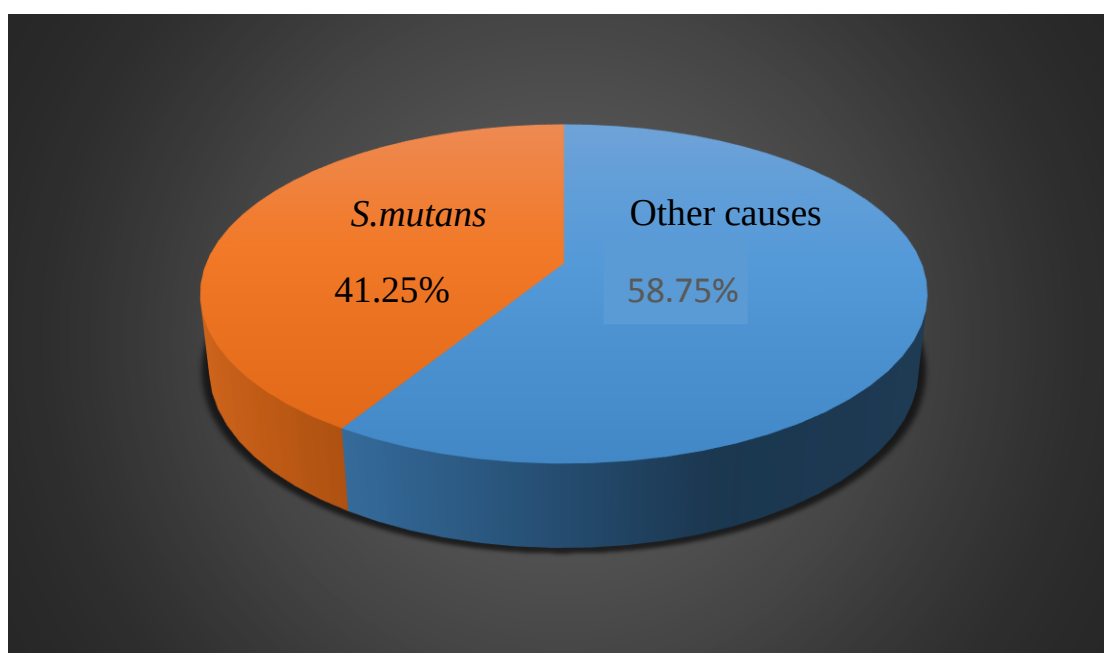


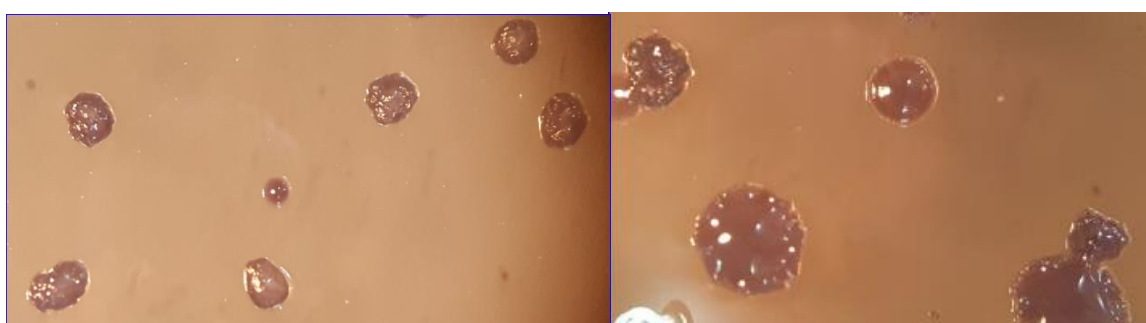
Figure (4-1): The occurrence of *S.mutans* isolated from eighteen saliva and dental plaque samples.

Further identification of *S.mutans* was carried out by 5 stages:

4.1.1 Morphology of Colony

On the selective MSB agar plates, *S.mutans* colonies appeared pale-blue in color about 2-3mm in diameter as avoid or spherical in morphology with

raised or convex surface adhered well to the medium surface. Some colonies appeared as irregular colonies with rough or frosted-glass surface appearance (rough colonies), while others appeared with smooth surfaces colonies (smooth colonies). Most of *S.mutans* colonies had a depression at the middle of the colony containing a drop of polysaccharide, or sometimes the whole colony submerged in a pool of polysaccharide as in figure (4-2).



Figure(4-2): *S.mutans* colonies growth on MSB agar.

4.1.2 Microscopic Examination

This results of this test have been showed that all *S.mutans* (33 positive sample were gram positive, avoid or spherical in their shape, arranged in short or medium length chain as in figure(4-3).

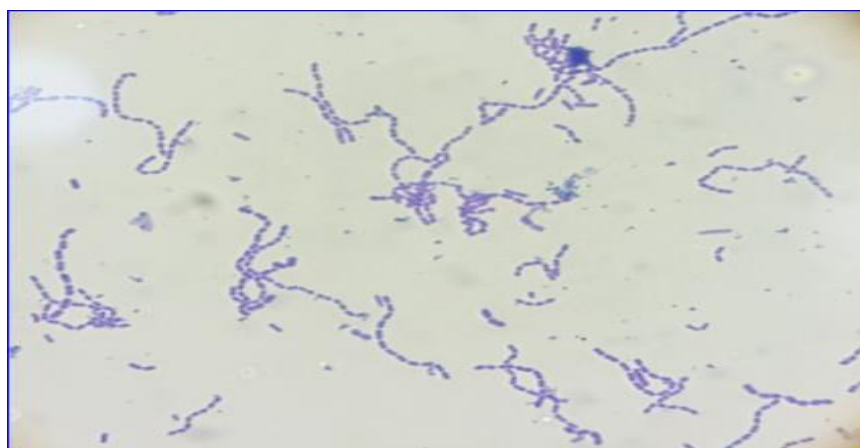


Figure (4-3): Gram's stain of *S.mutans* cells showing the gram positive stain (1000x magnification).

4.1.3 Conventional Biochemical Results

The results of biochemical tests showed that all isolates were negative for catalase test and had the ability to ferment inulin, while it gave positive results for hydrolysis of bile esculin and fermentation of Mannitol, Sorbitol, Sacrose, and Raffinose as shown in table (4-1).

Table (4-1): Biochemical tests of *S.mutans*

NO.	Biochemical Test		Result
1	Catalase test		-
2	Hydrolysis of bile isculin		+
3	A bility to ferment carbohydrates	Mannitol	+
		Sorbitol	+
		Raffinose	+
		Sucrose	+
		Innulin	-

4.1.4 API 20 Strep. system

The result of Api-20 strep. test has revealed that only 33 isolates from 80 samples were identified as *S.mutans* as in table (4-2) and figure(4-4).

Table (4-2): API 20 Strep. tests of *S.mutans*.

No.	Biochemical Tests	Results
1	Vogas-Proskauer (VP)	+
2	Hippurate test (HIP)	-

3	Esculin hydrolysis (ESC)	+
4	Proglutamate-aminopeptidase (PYRA)	-
5	Alpha-Galactosidase (α GAL)	+
6	Beta-Glucaronidase (β GUR)	-
7	Beta-Galactosidase (β GAL)	-
8	Phenylalanine ammonia-lyase (PAL)	-
9	Leucine aminopeptidase (LAP)	+
10	Arginine dihydrolase (ADH)	-
11	Ribose (RIB)	-
12	Arabinose (ARA)	-
13	Mannitol (MAN)	+
14	Sorbitol (SOR)	-
15	Lactose (LAC)	+
16	Trehalose (TRE)	+
17	Inuline (INU)	-
18	Raffinose (RAF)	+
19	Amidone (AMD)	-
20	Glycogene (GLYG)	-

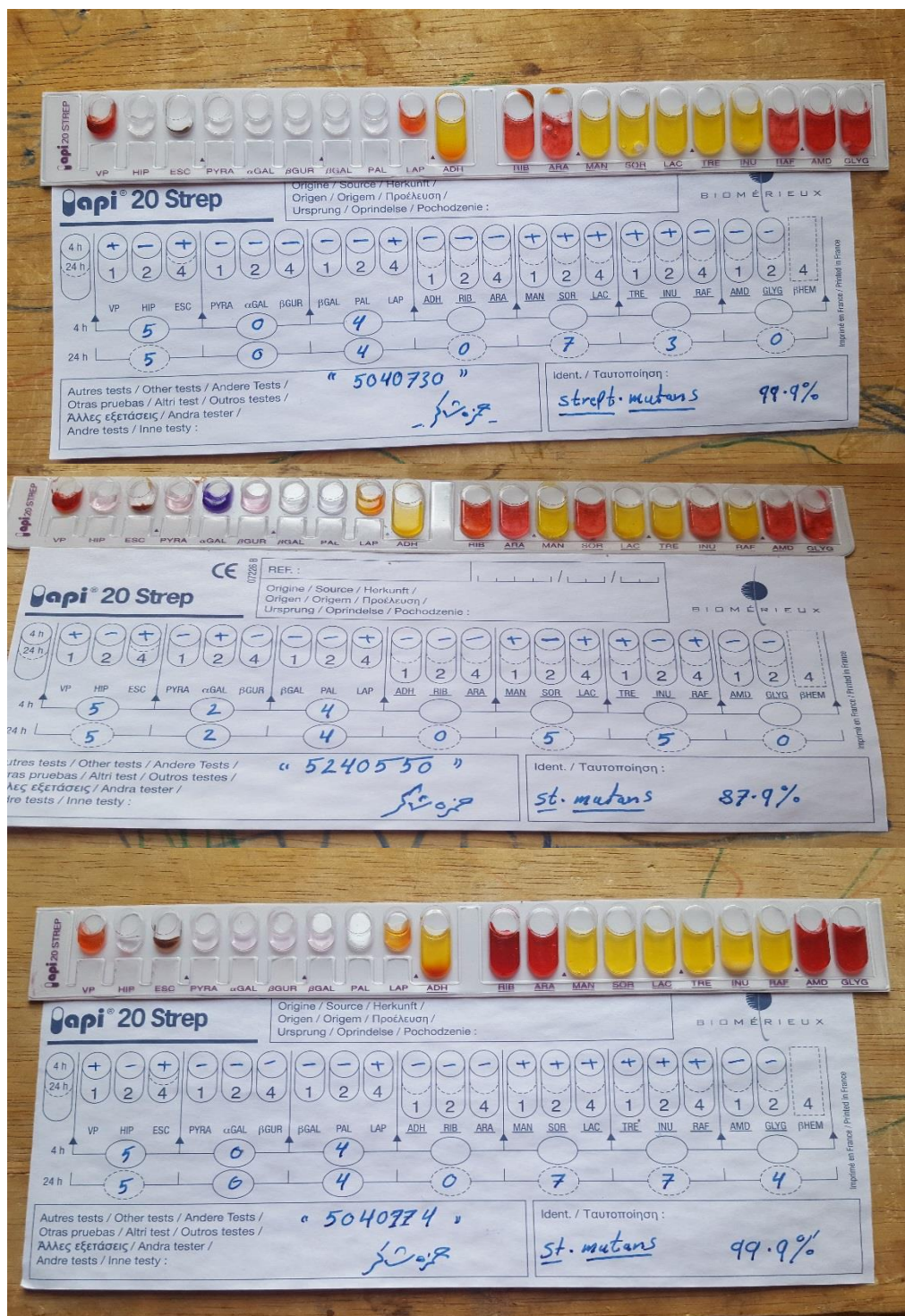


Figure (4-4): Calculate the numerical profile in Api-20 Strep. System(+): The test positive; (-):The test negative; (5240550),(5040730), and (540774) are the numerical profile.

4.2 Molecular study

4.2.1 Detection of *S.mutans* by 16S rRNA gene

A total of thirty-three isolates of *S.mutans* which identified by conventional methods such as; morphology of coloine, microscopic examination, biochemical tests and API 20 strep. were subjected to DNA extraction and PCR assay for presence of 16S rRNA gene. The results demonstarted that 28 (84.8%) of isolates had 16S rRNA gene with band 507 pb. as in figure (4-5).

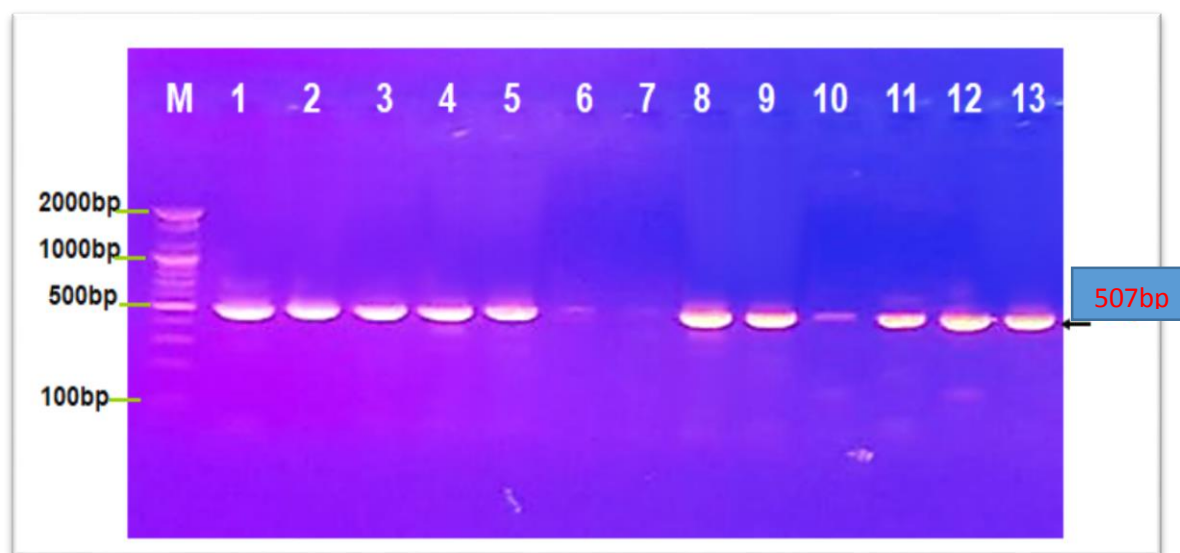


Figure (4-5): Conventional PCR for detection of 16S rRNA gene (507 bp), in *Streptococcus mutans* isolates, M: Marker DNA Ladder (2000-100bp), lanes (6, 7, 15, 24 and 25) only negative amplification for 16S rRNA gene.

4.2.2 Detection of virulence genes (*gtfB* and *spaP* genes) in *S.mutans*

In the present study , PCR techique was used to detect the presence of virulence genes (*gtfB* and *spaP* genes) in *S.mutans* isolated from patients with dental carries and dental pluqae. These genes encode the virulence

associated factors of *S.mutans* *gtfB* gene (315 pb) and *spaP* gene (696 pb) each gene represented by a single band in the corresponding region of the DNA ladder. All *S.mutans* isolates are subjected to standardized PCR for detection of these two virulence associated genes; not all isolates were positive , whereas 23 (82.14%) isolates gave positive for *gtfB* gene as in figure (5-3) , while 20 (71.43%) isolates gave positive results for *spaP* gene as in table (4-3) and figures (4-6), (4-7).

Table (4-3): The presence of *spaP* & *gtfB* genes in *S.mutans* isolates by PCR.

Type of gene	Isolates No. which contain gene (%)
<i>spaP</i> gene	20/28 (71.43)
<i>gtfB</i> gene	23/28 (82.41)

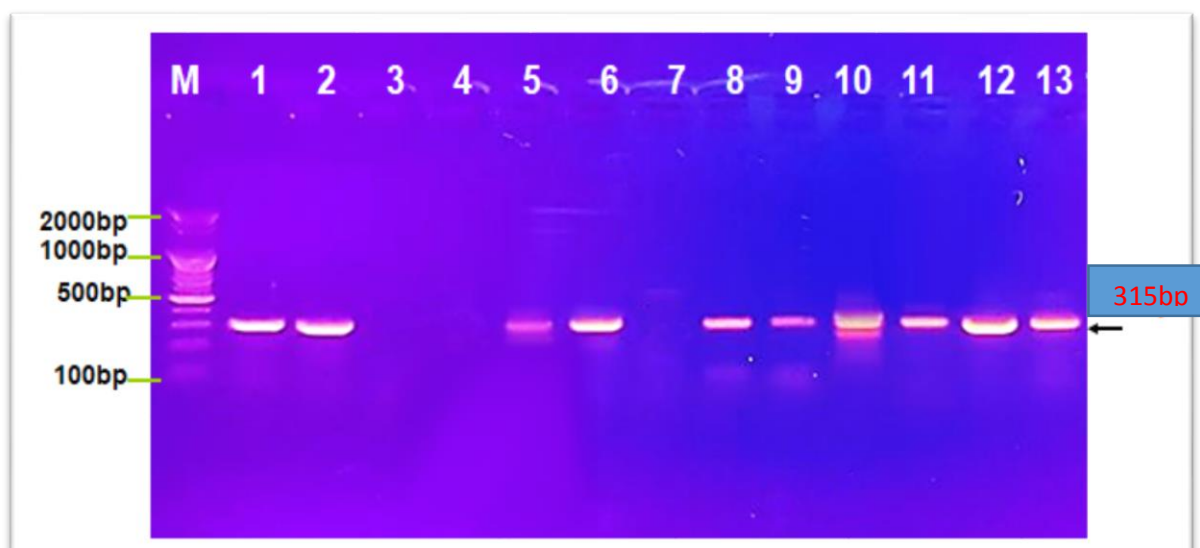


Figure (4-6): Conventional PCR for detection of (*gtfB*) gene (315 bp), in *Streptococcus mutans* isolates, M: Marker DNA Ladder (2000-100bp), lanes (3, 4, 7 and 26) negative amplification for (*gtfB*) gene.



Figure (4-7): Conventional PCR for detection of (*spaP*) gene (696 bp) in *Streptococcus mutans* isolates, M: Marker DNA Ladder (2000-100bp), lanes (3, 4, 7, 9, 10, 17, 21 and 24) negative amplification for (*spaP*) gene.

4.3 Sequencing analysis

The PCR product of four isolates was sent to Macrogen company in south koreia for sequencing of partial 16S rRNA gene was blasted in NCBI against standard strains of *S.mutans*, the accession number (KM052315.1), as well as against standard strains of different streptococcus species as in figure(4-8).

DNA Sequences	Translated Protein Sequences
Species/Abbrv	***** **
1. Streptococcus mutans IQ-SM.1 isolate	TCCCGAGCGTTGTCGGATTATTTGGGCGTAAAGGAGCGCAGGCGGTCAGGAAAGTCTGGAGTAAAGGCTAT(
2. Streptococcus mutans IQ-SM.2 isolate	TCCCGAGCGTTGTCGGATTATTTGGGCGTAAAGGAGCGCAGGCGGTCAGGAAAGTCTGGAGTAAAGGCTAT(
3. Streptococcus mutans IQ-SM.3 isolate	TCCCGAGCGTTGTCGGATTATTTGGGCGTAAAGGAGCGCAGGCGGTCAGGAAAGTCTGGAGTAAAGGCTAT(
4. Streptococcus mutans IQ-SM.4 isolate	TCCCGAGCGTTGTCGGATTATTTGGGCGTAAAGGAGCGCAGGCGGTCAGGAAAGTCTGGAGTAAAGGCTAT(
5. Streptococcus mutans 16S ribosomal RNA gene (KM052315.1)	TCCCGAGCGTTGTCGGATTATTTGGGCGTAAAGGAGCGCAGGCGGTCAGGAAAGTCTGGAGTAAAGGCTAT(
6. Streptococcus agalactiae 16S ribosomal RNA gene (AF015927.1)	TCCCGAGCGTTGTCGGATTATTTGGGCGTAAAGCGAGCGCAGGCGGTTCTTTAAGTCTGAAGTTAAAGGCAGT(
7. Streptococcus dysgalactiae 16S ribosomal RNA (AF433167.1)	TCCCGAGCGTTGTCGGATTATTTGGGCGTAAAGCGAGCGCAGGCGGTTCTTTAAGTCTGAAGTTAAAGGCAGT(
8. Streptococcus pharyngis 16S ribosomal RNA (LN651170.1)	TCCCGAGCGTTGTCGGATTATTTGGGCGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTTAAAGGCAGT(
9. Streptococcus pneumoniae 16S ribosomal RNA (AJ617796.1)	TCCCGAGCGTTGTCGGATTATTTGGGCGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTTAAAGGCAGT(
10. Streptococcus pyogenes 16S ribosomal RNA (LC071824.1)	TCCCGAGCGTTGTCGGATTATTTGGGCGTAAAGCGAGCGCAGGCGGTTTTTAAGTCTGAAGTTAAAGGCATT(

Figure (4-8):Multiple sequence alignment analysis(MEGA6.0).

Multiple sequence alignment analysis of the partial 16S rRNA gene sequence for local *Streptococcus mutans* isolates and *Streptococcus* spp. of NCBI Genbank by using (MEGA6.0, multiple alignment analysis tool). The multiple alignment analysis showed similarity(*) and difference in 16S rRNA gene nucleotide sequence as shown in figure (4-8).

The nucleotide sequence of the partial 16S rRNA of local isolated standard *S.mutans* and other species of streptococcus species was analysed by using the phylogenetic tree analysis shown in figure (4-9).

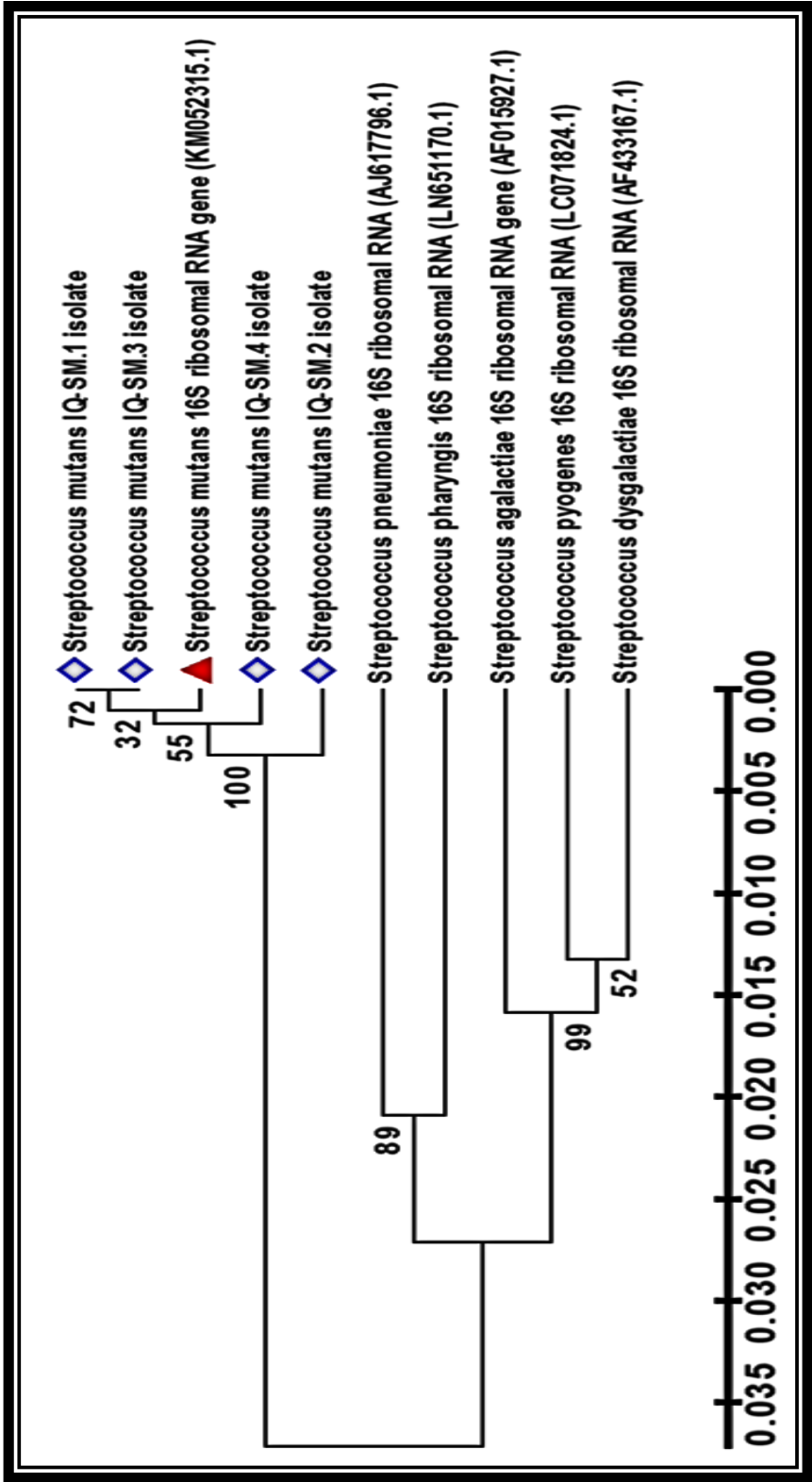


Figure (4-9): Phylogenetic tree analysis.

Phylogenetic tree analysis based on the partial 16S ribosomal RNA gene sequence that used for confirmative detection of *Streptococcus mutans* isolates. The phylogenetic tree was constructed using UPGMA tree

method (MEGA 6.0 version). The local *Streptococcus mutans* (IQ-SM.1-IQ-SM.4) were show closed related to NCBI-Blast *Streptococcus mutans* (KM052315.1), whereas, other NCBI-Blast *Streptococcus* species were show different and out of tree.

4.3.1 Nucleotide sequence accession numbers

The sequences were submitted to the NCBI gene bank and were assigned the accession numbers listed in table (4-3). Also the identifiction restuls showed that 100% for one isolate and 99% of three isolates are *S.mutans* as in table (4-4), and this isolates were recorded in NCBI site as in appendix.

Table (4-4): NCBI-Blast alignment identify of local *S.mutans* (IQ-SM.1-4) isolates with NCBI-Blast *S.mutans* isolate and accession numbers

Isolates	NCBI-Genebank accession number	NCBI-Blast identfy to <i>S.mutans</i> (KM052315.1)
IQ-SM.1	MF314454	99%
IQ-SM.2	MF314455	100%
IQ-SM.3	MF314456	99%
IQ-SM.4	MF314457	99%

4.4 Biofilm Formation Test

Biofilm was estimated by spectrophotometric method optical density for control (TSB without growth) 0.05Nm. The 28 isolates were tested and it appeared that 24(85.7%) isolates were produced biofilm as in table (4-5).

Table (4-5): Optical density for biofilm producers by *S. mutans* isolates.

No. Isolates	Optical Density(Nm)	Capacity of biofilm formation
1	0.377	Strong
2	0.492	Strong
3	0.099	Note
4	0.091	Note
5	0.101	Weak
6	0.449	Strong
7	0.081	Note
8	0.301	Strong
9	0.210	Moderate
10	0.503	Strong
11	0.315	Strong
12	0.273	Strong
13	0.206	Moderate
14	0.202	Moderate
15	0.399	Strong
16	0.429	Strong
17	0.242	Strong

18	0.567	Strong
19	0.107	Weak
20	0.240	Strong
21	0.208	Moderate
22	0.543	Strong
23	0.505	Strong
24	0.450	Strong
25	0.211	Moderate
26	0.099	Not
27	0.561	Strong
28	0.425	Strong

* Strong (Optical Density values more than 0.225 nm).

Moderate (Optical Density values between 0.225-0.150 nm).

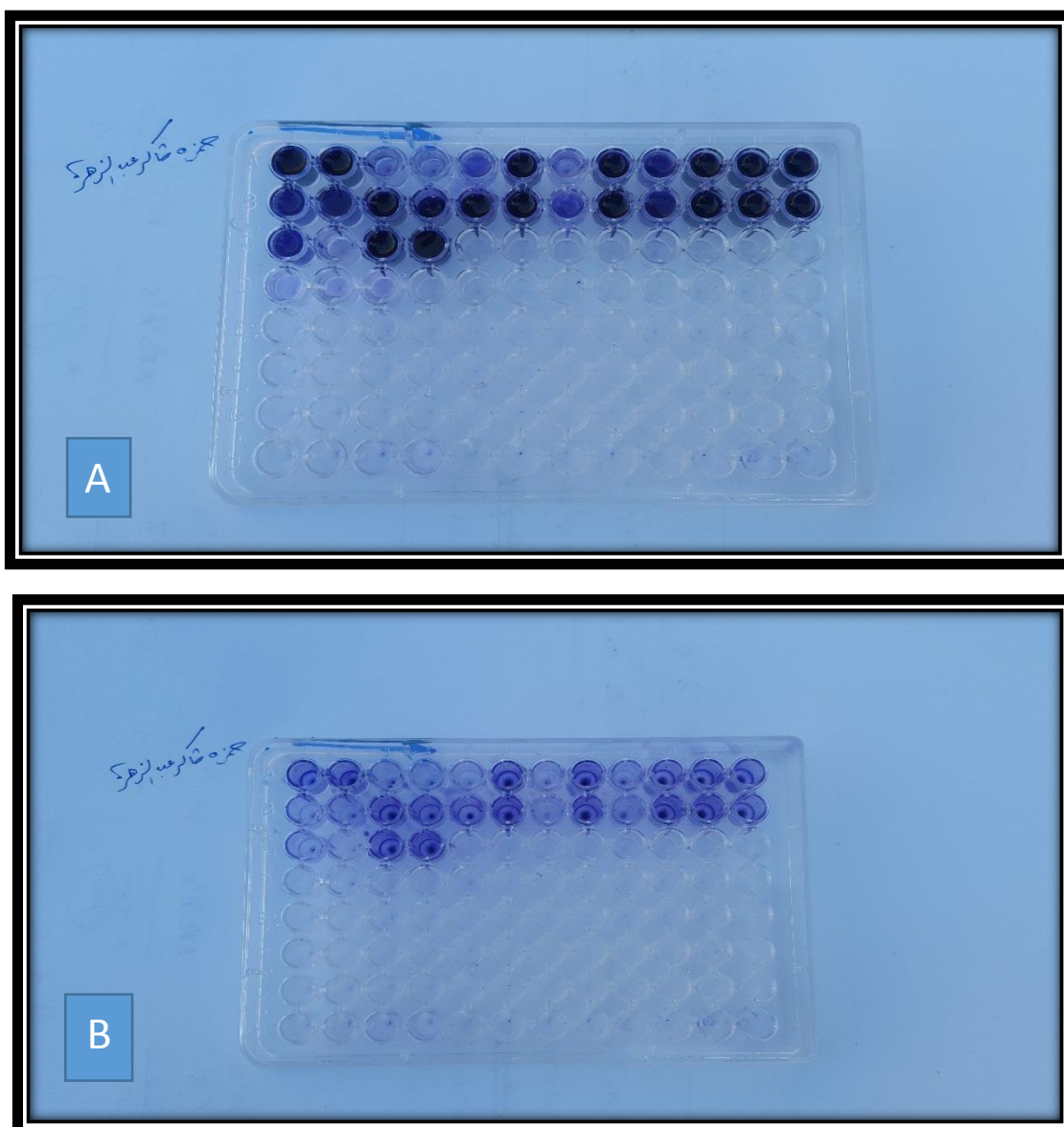
Weak (Optical Density values between 0.149-0.1 nm).

Not (Optical Density values less than 0.1 nm).

The results showed a different potential capacity to biofilm forming, whereas, 17(60.7%) isolates were considered as a strong or high biofilm producer. Five (17.9%) isolates were considered as a moderate biofilm forming. Only 2(7.1%) isolate were considered as a weak bifilm producer, while 4(14.3%) isolates were not formed biofilm as in table (4-6) and figure (4-10).

Table (4-6): Percentage of *S.mutans* to biofilm formation by measuring the OD.

Degree Of Biofilm Formation	No. Of <i>S.mutans</i> Isolates (%)
Strong	17(60.7)
Moderate	5(17.9)
Weak	2(7.1)
Not	4(14.3)
Total	28(100)



Figure(4-10):Biofilm formation by *S.mutans*, (A):befor the washing; (B):after the washing.

When studing the relationship between the presence of these genes (*gtfB* and *spaP* genes) and the ability of *S. mutans* to biofilm formation, the results showed a slight discrepancy between the presence of each gene alone and the ability *S. mutans* bacteria to formation of biofilm. whereas, 23/23(100%) isolates were contain *gtfB* gene and formed biofilm.while, 19/20 (95%) isolates were contain *spaP* gene and formed biofilm, as in table (4-7).

Table (4-7): Relationship between biofilm formation with presence of *gtfB* & *spaP* genes in *S.mutans* isolates.

Type Of Gene Present	No. of Isolates Forming Biofilm (%)
<i>spaP</i> gene	19/20(95)
<i>gtfB</i> gene	23/23(100)
Neither <i>spaP</i> or <i>gtfB</i> genes	1/5(20)

Chapter Five

Discussion

DISCUSSION

Dental caries remains the most prevalent disease worldwide, burdening billions of people, especially children, with pain and subsequently poorer quality of life and general health (Schwendicke *et al.*, 2015).

S.mutans a primary etiologic agent of human dental caries, is especially pathogen at forming biofilms on the hard tissues of the human oral cavity. The ability of *S.mutans* to biofilm formation on teeth surface, dental caries to synthesize extracellular glucans is a critical virulence factor involved in the pathogenesis of dental caries in humans and animals. The present study, is considered important study for detecting some of molecular characterization of *S.mutans* isolated from dental caries and dental plaque patients.

5-1 Isolation and characterization of *S.mutans*

The present study deals with *S.mutans* isolation and identification, these were done according to cultural features, microscopic examination, biochemical tests and using the API 20 Strep system which is specific for identification of Streptococcaceae from other bacteria. In addition, the final identification of *S.mutans* was based on presence of the 16S rRNA gene.

MSB agar is used for the isolation of *S.mutans*. MSB agar induce growth of *S.mutans* by provisioning bacteria with sucrose as a unique source for carbon, as well 0.2 unit/ ml bacitracin which is added to medium making it highly selective for very small numbers of *S.mutans* from clinical samples. Colonies of *S.mutans* appear small pale-blue granular colonies on MSB medium.

Data demonstrated that 33 (41.25%) *S.mutans* isolates were isolated from 80 dental plaque individuals age (7-25 years), this result is consistent with the study by Zainb, (2005) at AL-Hilla city, whereas this study mentioned that *S.mutans* recorded (40%) from dental caries. Also these results correspond to those previously by Israa and Mahdi (2015) in Kufa city, whereas they find that the ratio of *S.mutans* in dental caries patients is (40%).

AL-Jumaily *et al.*, (2014) reported that *S.mutans* ration in dental caries cases is (41%) 12 isolates of 29 samples.

EL-Sherbiny, (2014) studied dental caries in Egypt country, whereas he mentioned that *S.mutans* account at (40%) from this disease

It is clear that there is a convergence between the percentages of *S.mutans* in the current study and its proportion in previous studies. This may be due to similarity in the method and conditions of isolation of these bacteria the previous studies which referred to above used the same medium (MSB agar), as well as the method of sampling did not differ significantly from our study.

Oral health is not only an important factor for dental heath, it is an important component of an individual's overall health because it affects general health by causing pain, suffering and by changing people of , speech and their quality of life and well-being (Suad and Amira, 2015)

The quantity, quality, and frequency of sugar intake (e.g dessert and sugar contain drinks) have a definitive influence on the incidence and prevalence of caries.(Lee, 2013). Also of the other reason leading to the accurse of decay lack of oral hygiene, where the remain the diet in the moth or between the teeth, as well as, not to remove plaque from teeth surface, all of these factors assist in the events the dental caries.

Napimoga *et al.*, (2005) they defined a dental caries as a transmissible infections disease, therefore due to transmission bacteria (*S.mutans*) from mother or people relevant to children will give chance to these bacteria for cause early decay and clear necrosis in your teeth, for this, these teeth are sensitive and weak at aging. Whereas, this phenomenon occurs frequently in families with a low income (Li *et al.*, 2007; Folayan *et al.*, 2015).

5.2 Molecular study

5.2.1 Detection of 16S rRNA gene

Depending on molecular diagnostic methods, an amplification of 16S rRNA from 33 isolates was performed to confirm bacterial identification. The result showed that 28/33 (84.8) of *S.mutans* had 16S rRNA gene with band 507 bp.

Adhraa *et al.*, (2016) have mentioned that 22 (100%) isolates are identified as *S.mutans* by 16S rRNA gene.

The classical microbiological cultures methods limit the studies about the identification of specific populations of *S.mutans*, due to over 100 recognized species in the genus *Streptococcus*. Therefore, identification of *S.mutans* isolates by using 16S rRNA is more accurate than bacteriological and biochemical assays. Rampini *et al.*, (2011) demonstrate that 16S rRNA gene PCR was sensitivity, specific, and used for diagnosis of culture negative bacterial infections also useful for identification of bacterial pathogens in patients pretreated with antibiotics.

5.2.2 Molecular detection of virulence genes in *S.mutans*

PCR methodology provides a more sensitive mean of detection of putative bacterial species even non cultivable bacteria if compared with

conventional culture techniques. Also it is able to detect low numbers of bacterial species, being quick and relatively simple to perform. Moreover, a PCR assay has been found to be suitable for the specific detection and identification of human cariogenic bacteria like *S.mutans* (Sato *et al.*, 2003)

Traditional PCR assay, in the present study, is used to detect the presence of virulence factor genes; the virulence genes which used in our study were *gtfB* and *spaP* genes.

The present study showed that 23 (82.1%) of isolates were positive to *gtfB* gene. Recently, the application of the PCR assay to detected the *gtfB* gene in *S.mutans* isolated from human dental caries was (55%) (Israa and Mahdi, 2015).

Al jumaily *et al*; (2014), indicated that the *gtfB* gene was present in 29 isolates out of 42 isolate (69%).

From all above, we found that the *gtfB* gene is responsible for oral disease occurrence such as caries. The glucosyltransferase B is an enzyme produced by *streptococcus mutans*, which catalyzes synthesis of insoluble glucans from sucrose that provide support to the biofilm. It is one of the main virulence factors the generation of dental caries.

Salivary agglutinin is glycoprotein in human saliva that mediates the adhesion and aggregation of *S. mutans* via the cell wall-associated adhesin P1 (a member of the AgI/II family of cell surface proteins), encoded by *spaP* gene and this may facilitate their ability to colonize tooth surface in absence of sucrose.(Jakubovics *et al.*, 2005).

Our study appeared that *spaP* gene present in 20/28 (71.42%) of isolates.

Previous study by Duran *et al.* (2011) recorded that the *spaP* gene of *S.mutans* in dental caries samples was (91.3%). Do *et al.* (2010) have reported that 122/135 (90.2%) isolates gave positive results for *spaP* gene. In another studies, Israa and Mahdi, (2015) have showed that the *spaP* gene was present in 50/100 (50%) of isolates from dental caries infections.

The *spaP* gene encoded important adhesins, which bind to surface receptors and is important adhesins for the successful establishment of *S.mutans* biofilm(Banas and Vickerman, 2003 ; Arruda *et al.*, 2013).

However, the *gtfB* gene that encode the glucosyltransferase enzyme was more present from *spaP* gene. This may be due to many factors; dietary factors, whereas the carbohydrates(sucrose abundance) have a major influence on biofilm-associated gene expression(Shemesh *et al.*, 2008), also the environment condition of mouth such as; oral hygiene, pH, flow of saliva and take the antibiotic, all of these factors may be influence on detect the type of virulence gene of *S.mutans*.

5.3 Biofilm Formation

When *S.mutans* bacterium adhere to surface of teeth, its develop biofilm, therefore, it is altered phenotype through the repression or activation of specific genes. In fact, Biofilm formation by *S.mutans* is followed by differential appearance to several genes compared to planktonic cells. (Arruda *et al.*, 2013)

Our results from biofilm test demonstrate that 24/28 (85.7%) of isolates are able to produce a biofilm, whereas 17(70.8%) isolates were considered as a high biofilm formation. Five (20%) isolates were considered as a

moderate biofilm producer. Only 2(8%) isolates were considered as a weak biofilm producer.

Israa and Mahdi, (2015) recorded that (95%) of isolates were able to form a biofilm. Adhraa *et al*, (2016) mentioned that 22 isolates formed biofilm, whereas 16/22 (72%) isolates considered as a strong biofilm producers, while 6/22 (27%) isolates were moderate biofilm producer.

Al-Kazirragy , (2010) reported that 99% isolates from a biofilm, (53%) from it was considered as strong biofilm producers, while (46%) isolates were a weak producers to biofilm formation.

The results of our study demonstrated that 23(82.1%) isolates had *gtfB* gene and 20/28 (71.42%) isolates had *spaP* gene with different ability to form biofilm on teeth surface, we found that the isolates which more expression to *gtfB* gene were more pathogenicity to form a biofilm. When we studied the relationship between biofilm formation with presence of *gtfB* & *spaP* genes in *S.mutans* isolates, the results demonstrated that 23(100%) of isolates was contain *gtfB* gene and able to form of biofilm, while the percentage of *spaP* genes was 19 (95%).

This differential pattern of gene appearance may be influenced by the amount of carbohydrates in the mouth or due to the source of isolates (dental plaque or saliva).

Conclusions & Recommendations

Conclusions

The results of the present study yield the following conclusions:

1. *S.mutans* bacteria associated with dental disease that appear in high rate of occurrence (41.25%).
2. The percentage of accurate identification of *S.mutans* bacterium by traditional methods was (84.8%).
3. Molecular diagnostic methods were more accurate than conventional methods.
4. Nucleotides sequence analysis of 16S rRNA gene of local *S.mutans* showed that 99% of identify with subjected *S.mutans* in NCBI-Blast.
5. *spaP* and *gtfB* genes play important role in formation of dental Biofilm.
6. The present study showed that the *gtfB* gene is more prevalence than *spaP* gene in *S.mutans*.
7. Most of *S.mutans* produce strong biofilm.

Recommendations

According to the results obtained in the present study, we can recommend the following:-

1. Execution of epidemiological survey for *S.mutans*.
2. Further investigation to study the role of other factors, non *gtfB* and *spaP* genes, such as; Glucan-binding protein (Gbp), glucosyltransferase D and glucosyltransferase C (*gtfD* and *gtfC*) in dental disease.
3. Prevention of long term use of antibiotics which effect on normal flora and then facilitate the colonization of resistant bacteria.
4. Further studies are required on the use of natural extract in the treatment of oral disease.
5. Investigation of the role of other organisms in dental disease.
6. Further investigations to study the role of plasmids profile of *S.mutans* in dental caries.

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Appendix

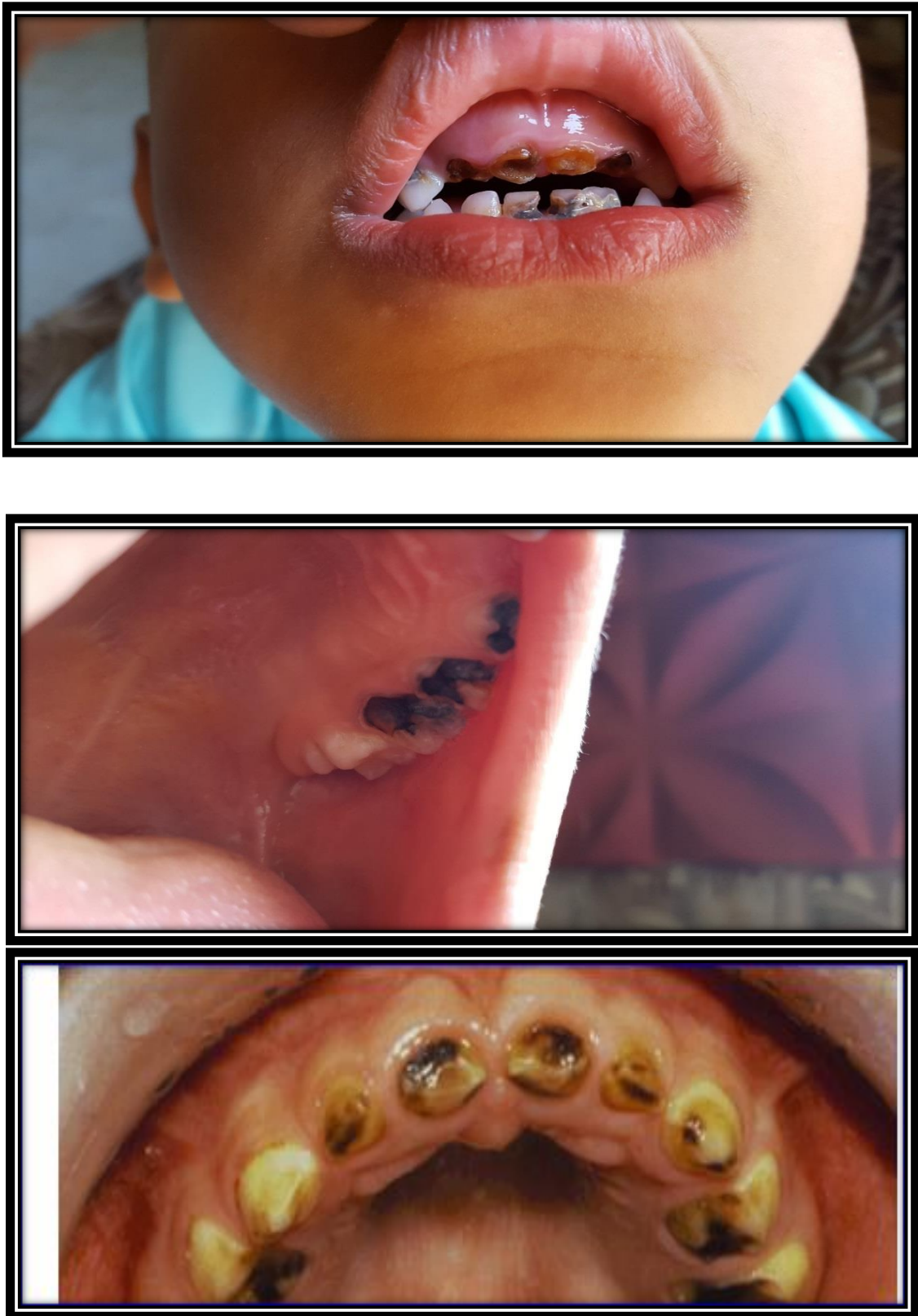


Figure (1): Infections of Dental Caries and Dental Plaque.

Appendix

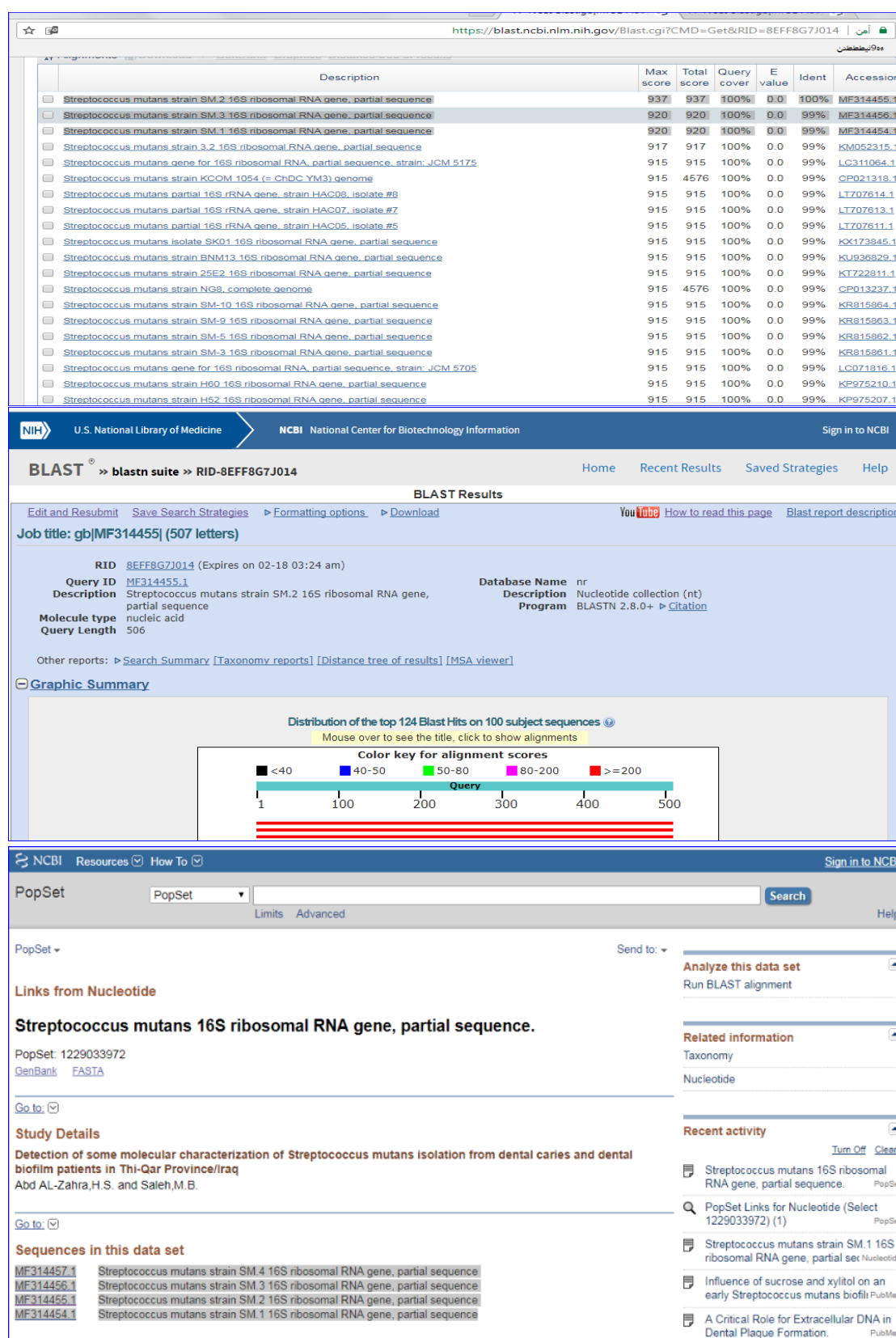


Figure (2): Sequencing of 16SrRNA gene, the sequence was blasted in NCBI against standard strain of *S. mutans*.

NCBI
Resources
How To
Sign in to NCBI

Nucleotide
Nucleotide
Search
Advanced
Help

GenBank
Send to:
Change region shown
Customize view

Streptococcus mutans strain SM.2 16S ribosomal RNA gene, partial sequence

GenBank: MF314455.1

[FASTA](#) [Graphics](#) [PopSet](#)

[Go to:](#)

LOCUS	MF314455	507 bp	DNA	linear	BCT 13-AUG-2017
DEFINITION	Streptococcus mutans strain SM.2 16S ribosomal RNA gene, partial sequence.				
ACCESSION	MF314455				
VERSION	MF314455.1				
KEYWORDS	.				
SOURCE	Streptococcus mutans				
ORGANISM	Streptococcus mutans Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus.				
REFERENCE	1 (bases 1 to 507)				
AUTHORS	Abd AL-Zahra,H.S. and Saleh,M.B.				
TITLE	Detection of some molecular characterization of Streptococcus mutans isolation from dental caries and dental biofilm patients in Thi-Qar Province/Iraq				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 507)				
AUTHORS	Abd AL-Zahra,H.S. and Saleh,M.B.				

Analyze this sequence

Run BLAST

Pick Primers

Highlight Sequence Features

Find in this Sequence

Related information

Taxonomy

PopSet

Recent activity

[Turn Off](#) [Clear](#)

Streptococcus mutans strain SM.2 16S ribosomal RNA gene, partial sequence

Figure (3): NCBI litter with names of others.

Appendix

Appendix (4): NCBI-BLAST sequence alignment identity to four isolates.

Streptococcus mutans IQ-SM.1 isolate

Sequence ID: Query_140240 Length: 507 Number of Matches: 1

Range 1: 1 to 507 [Graphics](#)

▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
924 bits(500)	0.0	504/507(99%)	0/507(0%)	Plus/Plus
Query 230	CCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCG			289
Sbjct 1	CCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCG			60
Query 290	GCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTA			349
Sbjct 61	GCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCTTA			120
Query 350	AAGCTCTGTTGTAAAGTCAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAGC			409
Sbjct 121	AAGCTCTGTTGTAAAGTCAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAGC			180
Query 410	TTRCCAGAAAGGRACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGC			469
Sbjct 181	TTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGC			240
Query 470	GTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTCAGGAAAGTCTGGAGTAAAA			529
Sbjct 241	GTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTCAGGAAAGTCTGGAGTAAAA			300
Query 530	GGCTATGGCTCAACCATAGTGTGCTCTGGAAACTGTCTGACTTGAGTGCAGAAGGGGAGA			589
Sbjct 301	GGCTATGGCTCAACCATAGTGTGCTCTGGAAACTGTCTGACTTGAGTGCAGAAGGGGAGA			360
Query 590	GTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAAGTGGCGAAA			649
Sbjct 361	GTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAAGTGGCGAAA			420
Query 650	GCGGCTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGATTA			709
Sbjct 421	GCGGCTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGATTA			480
Query 710	GATACCCTGGTAGTCCACGCCGTAAC	736		
Sbjct 481	GATACCCTGGTAGTCCACGCCGTAAC	507		

Appendix

Streptococcus mutans IQ-SM.2 isolate

Sequence ID: Query_140241 Length: 507 Number of Matches: 1

Range 1: 1 to 507 [Graphics](#)

▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
917 bits(496)	0.0	503/507(99%)	0/507(0%)	Plus/Plus
Query 230	CCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCG			289
Sbjct 1	CCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCG			60
Query 290	GCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTA			349
Sbjct 61	GCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCTTA			120
Query 350	AAGCTCTGTTGTAAGTCAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAGC			409
Sbjct 121	AAGCTCTGTTGTAAGTCAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAGC			180
Query 410	TTRCCAGAAAGGRACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCAGAC			469
Sbjct 181	TTTCCAGAAAGGAACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCAGAC			240
Query 470	GTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTGAGGAAAGTCTGGAGTAAAA			529
Sbjct 241	GTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTGAGGAAAGTCTGGAGTAAAA			300
Query 530	GGCTATGGCTCAACCATAGTGTGCTCTGGAAACTGTCTGACTTGAGTGCAGAAGGGGAGA			589
Sbjct 301	GGCTATGGCTCAACCATAGTGTGCTCTGGAAACTGTCTGACTTGAGTGCAGAAGGGGAGA			360
Query 590	GTGGAATTCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAA			649
Sbjct 361	GTGGAATTCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAA			420
Query 650	GCGGCTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGATTA			709
Sbjct 421	GCGGCTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGATTA			480
Query 710	GATACCCTGGTAGTCCACGCCGTAAAC	736		
Sbjct 481	GATACCCTGGTAGTCCACGCCGTAAAC	507		

Appendix

Streptococcus mutans IQ-SM.3 isolate

Sequence ID: Query_140242 Length: 507 Number of Matches: 1

Range 1: 1 to 507 [Graphics](#)

▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
924 bits(500)	0.0	504/507(99%)	0/507(0%)	Plus/Plus
Query 230	CCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCG			289
Sbjct 1	CCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCG			60
Query 290	GCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTA			349
Sbjct 61	GCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCTTA			120
Query 350	AAGCTCTGTTGTAAGTCAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAGC			409
Sbjct 121	AAGCTCTGTTGTAAGTCAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAGC			180
Query 410	TTRCCAGAAAGGRACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTCCCGAGC			469
Sbjct 181	TTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTCCCGAGC			240
Query 470	GTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTGAGGAAAGTCTGGAGTAAAA			529
Sbjct 241	GTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTGAGGAAAGTCTGGAGTAAAA			300
Query 530	GGCTATGGCTCAACCATAGTGTGCTCTGGAAACTGTCTGACTTGAGTGCAGAGGGGAGA			589
Sbjct 301	GGCTATGGCTCAACCATAGTGTGCTCTGGAAACTGTCTGACTTGAGTGCAGAGGGGAGA			360
Query 590	GTGGAATTCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAAGTGGCGAAA			649
Sbjct 361	GTGGAATTCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAAGTGGCGAAA			420
Query 650	GCGGCTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGATTA			709
Sbjct 421	GCGGCTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGATTA			480
Query 710	GATACCTGGTAGTCCACGCCGTAAAC	736		
Sbjct 481	GATACCTGGTAGTCCACGCCGTAAAC	507		

Appendix

Streptococcus mutans IQ-SM.4 isolate

Sequence ID: Query_140243 Length: 507 Number of Matches: 1

Range 1: 1 to 507 [Graphics](#)

▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
924 bits(500)	0.0	504/507(99%)	0/507(0%)	Plus/Plus
Query 230	CCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCG			289
Sbjct 1	CCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCG			60
Query 290	GCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTA			349
Sbjct 61	GCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTA			120
Query 350	AAGCTCTGTTGTAAGTCAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAGC			409
Sbjct 121	AAGCTCTGTTGTAAGTCAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAGC			180
Query 410	TTRCCAGAAAGGRACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTCCCAGC			469
Sbjct 181	TTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTCCCAGC			240
Query 470	GTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTGAGGAAAGTCTGGAGTAAAA			529
Sbjct 241	GTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTGAGGAAAGTCTGGAGTAAAA			300
Query 530	GGCTATGGCTCAACCATAGTGTGCTCTGGAACTGTCTGACTTGAGTGCAGAAGGGGAGA			589
Sbjct 301	GGCTATGGCTCAACCATAGTGTGCTCTGGAACTGTCTGACTTGAGTGCAGAAGGGGAGA			360
Query 590	GTGGAATTCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAAGTGGCGAAA			649
Sbjct 361	GTGGAATTCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAAGTGGCGAAA			420
Query 650	GCGGCTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGATTA			709
Sbjct 421	GCGGCTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGATTA			480
Query 710	GATACCCTGGTAGTCCACGCCGTAAAC			736
Sbjct 481	GATACCCTGGTAGTCCACGCCGTAAAC			507

الخلاصة

يعد تسوس الأسنان واحد من أهم الأمراض المعدية والشائعة التي تصيب الإنسان والذي يؤدي إلى فقدان هيكل الأسنان، وبالنتيجة غياب بعض وظائف الفم. ويحدث ذلك بسبب الفعاليات الأيضية للاحياء المجهرية، وخاصة بكتيريا *Streptococcus mutans*.

هدفت هذه الدراسة الى عزل وتشخيص بكتيريا *Streptococcus mutans* من المرضى الذين يعانون من تسوس الأسنان و المرضى الذين تتكون على اسنانهم ما يسمى بالغشاء الحيوي في محافظة ذي قار، و توضيح دور بعض الخصائص الجزيئية (الجين *gtfB* والجين *spaP*) لهذه البكتيريا والتحري فيما اذا كانت هذه البكتيريا قادرة على تكوين اللويحة السنية Dental Plaque على سطح الاسنان.

حيث تم عزل البكتيريا *Streptococcus mutans* من اللعاب واللويحة السنية من الاشخاص الذين يعانون من هذا المرض (حيث تراوحت أعمارهم بين 7-25 سنة) ومن كلا الجنسين خلال شهري كانون الاول وتشرين الثاني لعام 2016. حيث زرعت العينات التي تم جمعها على وسط (Mitis Salivarius Bacitracin agar (MSB agar ، ثم تم تنقية العزلات الموجبة وتشخيصها وفقا للخصائص الشكلية، والاختبارات البايوكيميائية واختبار Api 20 Sterp system , حيث اظهرت النتائج الاولية بأن(41.25%) 33/80 من العزلات أعطت نموا إيجابيا لبكتيريا *Streptococcus mutans*.

اعتمد التشخيص النهائي للعزلات الموجبة على وجود الجين 16S rRNA gene حيث تبين بأن (84.8%) 28 /33 عزلة تعود الى البكتيريا *Streptococcus mutans* .

وعند تحليل تتابع النيوكليوتيدات لجين 16S rRNA gene اظهرت النتائج بأن هذه العزلات تعود فعلا لبكتيريا *Streptococcus mutans*، وعليه تم تسجيل اربعة من هذه العزلات في البنك العالمي NCBI Bank وبأرقام الانضمام التالية (MF 314454), (MF 314455), (MF 314456), و (MF 314457).

كما تم التحري عن جينين من جينات الضراوة لهذه البكتيريا وهما الجين *gtfB* والجين *spaP*، حيث اظهرت النتائج بأن (82.14%) 23/28 عزلة اعطت نتيجة موجبة للجين (*gtfB*)، بينما كانت النتائج الموجبة للجين الاخر (*spaP*) هي (71.42%) 20/28 عزلة.

أيضاً، في هذه الدراسة اختبرنا قابلية هذه البكتيريا لتكوين الغشاء الحيوي (Biofilm) على الأسنان بالطريقة الطيفية، وأظهرت النتائج بأن (85.7%) 24/28 عزلة قادرة على تكوين الغشاء الحيوي. حيث أظهرت النتائج بأن (100%) 23/23 عزلة تمتلك الجين (*gtfB*) تكون قادرة على تكوين البايوفلم ، بينما كانت نسبة العزلات التي تمتلك الجين (*spaP*) وتكون قادرة على تكوين البايوفلم هي (95%) 19/20 عزلة.

ومن خلال دراسة العلاقة بين وجود هذه الجينات وقدرة هذه البكتيريا على تكوين الصفيحة السنية، وجد بأن امراضية هذه البكتيريا وقدرتها على تكوين الغشاء الحيوي (Biofilm) تزداد عندما يزداد التعبير الجيني للجين (*gtfB*) مقارنة بالجين (*spaP*) .



وزارة التعليم العالي والبحث العلمي
جامعة ذي قار
كلية العلوم
قسم علوم الحياة



عزل وتحديد بعض الصفات الجزيئية لبكتريا *Streptococcus mutans* من
مرضى تسوس الاسنان والصفیحة السنیة فی محافظة ذي قار/العراق

رسالة مقدمة الى

عمادة كلية العلوم – جامعة ذي قار

وهي جزءا من متطلبات نيل شهادة الماجستير في علوم الحياة - الاحياء المجهرية

من قبل

حمزة شاكر عبد الزهرة

بكلوريوس في علوم الحياة

2008

بأشراف

أ.د منال بادي صالح