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MOLECULAR DETECTION OF BACTERIA ASSOCIATED WITH DENTAL LESIONS

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By Adian Amer Dhannoon DHANNOON

July 2022

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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Adian Amer Dhannoon DHANNOON

ABSTRACT

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Master of Science in Biology

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The present work aimed to isolate and identify *S. mutans* thawere late from saliva by using PCR technique. Saliva was collected from forty healthy subjects in Baghdad city; ages were between 19-21 years old. Before beginning the research, approval of the protocol was acquired from the relevant Ethical Committee, and each subject gave their written permission after receiving appropriate information. The results showed that 100 healthy patients of Dentistry College from Baghdad University were included in this study, 65% of them were male and 35% were female, 87% were residents of Baghdad and 13% were non-Baghdad residents. In order to verify the identity of the bacteria, an amplification of the 16S rRNA was carried out using 12 separate samples. After using primers targeting a conserved area of 16S rRNA to amplify the DNA of *S. mutans* isolates using PCR, the resulting DNA fragments were separated on an agarose gel. *S. mutans* contained a 16S rRNA gene band of 332 bp in 12 (100%) of the samples. 16S rRNA is more accurate than bacterial and biochemical tests in identifying *S. mutans* isolates. Due to the over 100 identified species in the genus Streptococcus, traditional microbiological culturing techniques restrict investigations regarding the identification of distinct populations of *S. mutans*. As a result, 16S rRNA is more accurate than bacterial and biochemical testing for identifying *S. mutans* isolates.

2022, 52 pages

Keywords: Streptococcus mutans, Saliva, PCR technique, DNA extraction,

ÖZET

DENTAL LEZYONLARLA İLİŞKİLİ BAKTERİLERİN MOLEKÜLER TESPİTİ

Adian Amer Dhannoon DHANNOON
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Temmuz 2022

Bu çalışmada, PCR tekniği kullanılarak tükürükten izole edilen *S. mutans*'ın izole edilmesi ve tanımlanması amaçlanmıştır. Bağdat şehrinde kırk sağlıklı denekten tükürük toplandı; yaşları 19-21 arasındaydı. Çalışmaya başlamadan önce protokol için Etik Kurul Onayı alındı ve tüm katılımcılardan yazılı bilgilendirilmiş onam alındı. Sonuçlar, Bağdat Üniversitesi Diş Hekimliği Fakültesi'nden 100 sağlıklı hastanın bu çalışmaya dahil edildiğini, bunların %65'inin erkek ve %35'inin kadın, %87'sinin Bağdat'ta ikamet ettiğini ve %13'ünün Bağdat'ta ikamet etmediğini gösterdi. Bakteriyel tanımlamayı doğrulamak için 12 izolattan 16S rRNA amplifikasyonu yapıldı. 16S rRNA'nın korunmuş bölgesi için primerler tasarlanmış ve *S. mutans* izolatlarının DNA'sının PCR ile amplifikasyonu için kullanılmış, ardından PCR ürünleri agaroz jel üzerinde ayrılmıştır. Sonuç, 12 (%100) *S. mutans*'ın 332 bp ile 16S rRNA gen bandına sahip olduğunu gösterdi. *S. mutans* izolatlarının 16S rRNA kullanılarak tanımlanması, bakteriyolojik ve biyokimyasal analizlerden daha doğrudur. Klasik mikrobiyolojik kültür yöntemleri, Streptococcus cinsinde 100'den fazla tanınan tür nedeniyle *S. mutans*'ın spesifik popülasyonlarının tanımlanmasına yönelik çalışmaları sınırlandırmaktadır. Bu nedenle, *S. mutans* izolatlarının 16S rRNA kullanılarak tanımlanması, bakteriyolojik ve biyokimyasal testlerden daha doğrudur.

2022, 52 sayfa

Anahtar Kelimeler: Streptococcus mutans, Tükürük, PCR tekniği, DNA ekstraksiyonu

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Adian Amer Dhannoon DHANNOON
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CONTENTS

LIST OF SYMBOLS

-	Minus
%	Percent
+	Plus
°C	Degrees Celsius
µL	Microliter
g	Gram
L	Liter
m2	Square-meters
mg	Milligram
min	Minutes
mL	Milliliters
mM	Millimoles
ng	Nanogram
nm	Nanometre
pmol	Picomole
U	Unit

LIST OF ABBREVIATIONS

CAMBRA	Caries management by risk assessment approach
DIFOTI	Digital imaging fiber-optic translllumination
ECM	Electronic caries monitor
ECM	Electronic caries monitor
LSI	Laser speckle imaging
MetaHIT	Metagenomics of the human intestinal tract
NGS	Next generation sequencing
PCR	Polymerase chain reaction
QLF	Quantitative light fluorescence
THC	Tetrahydrocannabinol

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

Humans, like with all other complex multicellular eukaryotes, are not autonomous creatures but rather biological units that integrate a vast range of microbial symbionts and the genomes of those symbionts. This is true for all complex multicellular eukaryotes (Bordenstein and Theis 2015). The bacteria that are found both within and on the outside of our bodies come together to produce a functioning organ that is essential to both our physiology and our health. We are a "superorganism," sometimes known as a holobiont, because of the symbiotic relationships we have with other microorganisms. The overall number of cells that make up our bodies is at least equal to the number of bacteria that live in the human holobiont, and it is quite likely to be considerably more (Sender *et al.* 2016, Lederberg and McCray 2001). The development of innovative genomic technologies, such as bioinformatics tools and (NGS), has resulted in the availability of powerful new methods for comprehending the role that the human microbiome plays in the maintenance of good health. The Metagenomics of the Human Intestinal Tract (MetaHIT) and Human Microbiome Project (Turnbaugh *et al.* 2007) are only two examples of recent initiatives that have created the resources and skills necessary to characterize and comprehend the human microbiome (Kilian *et al.*2016).

Oral cavity is home to hundreds of different bacterial species, making it one of the most complex microbial ecosystems. Because of this, oral cavity is regarded to be one of the most difficult places to study microbes. These bacteria play an important role in both the preservation of oral homeostasis and the development of a wide variety of oral diseases, most notably periodontal disease and dental caries. People of all ages and demographics around the globe are impacted by dental caries, which is regarded to be one of the most critical issues affecting global health today (Strużycka *et al.* 2014).

This disorder is a multifactorial disease that emerges from interactions between a susceptible host, cariogenic bacteria, and cariogenic diets. Cariogenic foods are foods that may cause carcinogenesis in humans. Cariogenic diets are those that promote the development of caries. The development of dental caries begins with bacterial changes

inside the supragingival biofilm, continues with the formation of a polymicrobial biofilm on the surface of the tooth, and concludes with the aggregation of several bacterial species in a complexly organized fashion (Marsh 2006). Both Streptococcus mutans and S. sobrinus, which have been believed to be the predominant cariogenic pathogens for many decades, are the primary oral pathogens that are related with dental caries in humans. Other pathogens that are related with dental caries include Actinomyces, Lactobacillus, non-mutans streptococci, Veillonella, and many species of Bifidobacterium (Xu *et al.* 2014). It is now well acknowledged that a change in the oral bacterial populations, often known as dysbiosis, is a significant role in the development of dental caries (Palmer *et al.* 2014). In spite of the substantial impact it has on human health, the diversity and make-up of the oral bacterial population, and the prevalence of oral diseases, as well as the changes that occur in these factors when a person moves between healthy and ill states, are not yet well understood (Lamont *et al.* 2018). In addition, oral microbes may be significant pathogens that have a role in the development of systemic disorders such as cardiovascular disease, cancers of the digestive tract and colorectum, respiratory tract infections, diabetes, and unfavorable outcomes during pregnancy (Nagao and Tanigawa 2019). It is possible that the transmission of germs into the circulation and an increase in inflammation throughout the body are both responsible for the correlation between severe systemic illness and periodontal disease (Hajishengallis 2015). Even though traditional culture-dependent methods have been used to isolating and identifying over 300 different species of oral bacteria, the bulk of the oral microbiome is unable to be cultured in vitro (Aas *et al.* 2005). In recent years, molecular methods have seen a significant amount of application in research that investigates the composition of the oral microbial population. This has included the identification and characterisation of culturable and non-culturable bacteria at a greater resolution than was previously attainable using culture-based approaches (Ahn *et al.* 2011).

In order to investigate the connections between microbial diversity and oral disorders, researchers have used metagenomics to sequence the 16S ribosomal RNA (rRNA) genes of microbes. This has resulted in the production of bacterial profiles as well as genomic profiles (Xu and Gunsolley 2014). Taking use of the heterogeneity in the (16S rRNA) gene sequence has been regarded as a method for high-throughput characterization that

is both efficient and cost-effective in human oral microbiome research (Chen *et al.* 2010). The (16S rRNA) gene is exclusive to bacteria and is regarded to be a barcode that can be used to detect particular bacterial species. This allows for the identification of a wide variety of bacteria, even those that cannot be cultured in a lab (Nossa *et al.* 2010). Recent research has shown that the oral microbiota of people of different geographic locations (Nasidze *et al.* 2011) and ethnicities are distinct from one another (Mason *et al.* 2013). In light of the fact that the variety of the oral microbiota is thought to be the primary causative component in the development of dental caries, a number of further investigations have to be carried out in order to get a deeper comprehension of this dental issue.

1.1 Objectives of the Study

- Detection of Moleculargene of bacteria associated with dental lesions which causes Ditterent Diseases infection in children and adolescents and adults of Male and Female in Baghdad province.
- As molecular changes of bacteria associated with dental lesions with 16SRNA gene.

2 LITERATURE REVIEW

2.1 Dental Caries

Caries in the teeth is a condition that affects many people and is considered a serious problem for public health. It prevents people of all ages from achieving and maintaining good oral health (Thean *et al.* 2007, Yadav and Prakash 2017). The prevalence pattern and severity of dental caries varies with race, sex, economic status, age, food practice, geographical location, socio-demographic characteristics, and practices about oral hygiene in different countries or regions of the same nation in different areas of the globe. This is true even when comparing countries or regions that are geographically close to one another (Petersen *et al.* 2005).

The origin of the term "caries" may be traced back to the Latin word meaning "rotten." According to Bader, dental caries is a disease that lasts for a long time and may be passed from one person to another. This condition is brought on by the complex interplay of oral microorganisms found in tooth plaque, a person's diet, and a broad range of host variables. Host variables include anything from cultural and environmental influences to genetics and biochemical/immunologic responses. Bader says that dental caries is caused by an oral microorganism in dental plaque (Bader *et al.* 2001). When cariogenic microorganisms colonize a susceptible tooth surface and there is a supply of sucrose or refined sugar accessible in the diet, cavities may develop, dental caries may develop, the result is the development of tooth decay. Carbohydrate fermentation, caused by the activity of bacteria, results in the creation of lactic acid. This acid, in turn, liquefies the hydroxyapatite crystal structure of the tooth, which is the fundamental cause of dental caries (De Marchi *et al.* 2011, Yadav *et al.* 2015).

2.1.1 Types of dental caries

Dental caries of the primary teeth: It is possible for it to happen on the various surfaces of the teeth. On a surface that is approximal, the lesion begins and appears below the

contact zone that is formed between the teeth. This is where the teeth come into touch with one another. This occurs when the teeth are in close proximity to one another. Pit and fissure lesions may also include caries on an occlusal surface, however this is a restricted aspect of these lesions. Caries of the enamel is a three-dimensional subsurface demineralization that spreads along with the enamel prisms and may take place on either the occlusal or the approximal surfaces of the tooth (Waldman 1989, Yadav and Prakash 2016).

Secondary caries is a lesion that may be seen near the margin of a dental repair. Secondary caries can be painful. The presence of evidence of demineralization alongside the cavity wall, which may be the consequence of micro leaks, distinguishes it from primary caries. Primary caries does not have this evidence. Nevertheless, microbiological and clinical investigations imply that there is no active demineralization occurring below the repair caused by this leakage (Waldman 1989, Yadav and Prakash 2016).

2.1.2 Etiology

Dental caries is a disease that is caused by several variables, including those related to the host, the agent, and the environment. The bacteria known as mutans streptococci are the primary factor in dental caries being present in a patient's mouth (MS). MS attaches itself to the dental pellicle by adhesion and then begins to break down glucose for energy, which results in the production of lactic acid and an acidic environment surrounding the tooth. (Çolak *et al.* 2013) found that as a consequence of this, the demineralization of the enamel and, ultimately, the dentin takes place. The tooth itself, germs that are present in the form of dental plaque, and a diet that is high in sugar are the three main contributors to the development of dental caries. There is much data suggesting that the quantity of sugar consumed, the quality of that sugar, and the frequency with which it is consumed all play a part in the occurrence and prevalence of dental caries.

2.1.3 Caries diagnosis

In order to quantify bacterial products in caries lesions, DIAGNOdent makes use of laser fluorescence technology; this method has the potential to be sensitive enough to identify early demineralization (Lussi *et al.* 2004). A numerical number ranging from 0 to 99 indicates the intensity of the fluorescence that is being measured. The Digital Imaging Fiber-Optic Transllllumination (DIFOTI) technique makes use of fiber-optic light to generate an image, which may be helpful for spotting beginning regions of demineralization, fissures, or fractures, and also gives a quantitative analysis of the caries process (Schneiderman *et al.* 1997). The technique known as quantitative light-induced fluorescence (QLF) takes use of the fluorescence that may be produced by human enamel under certain environments. Due to scattering, demineralized enamel has a lower fluorescence, which may be related to the fact that the fluorescence is caused by cross-links between structural proteins (Angmar-Månsson and Ten Bosch 2001).

The Electronic Caries Monitor (ECM) analyzes the differences in the electrical impedance of sound enamel and demineralized tooth tissue. This is done because demineralized tooth tissue has a greater electrical conductivity than normal tooth tissue. This is done so that normal teeth may be compared to demineralized teeth (Ashley *et al.* 1998). A technology that can reliably identify whether the caries lesion is active and requires intervention is not yet commercially available, thus a dentist cannot depend only on such equipment when planning treatment. As a result, the dentist is to additionally take into consideration the general risk of caries as well as the susceptibility of specific patients.

2.1.4 Diagnosis of dental lesions

The symptoms of caries might vary greatly from patient to patient. On the other hand, the phases of development and risk factors are comparable. It is possible that at first it may seem like a little chalky region (smooth surface caries), but with time it will most likely grow into a huge cavitation. Sometimes caries will be plainly obvious to the patient. On the other hand, additional methods of detection, such as X-rays, are applied for less

obvious areas of teeth in order to establish the level of tooth destruction that has occurred. Caries may now be detected without the need of ionizing radiation thanks to lasers, it may also be used for the analysis of interproximal decline (between the teeth).

The first step in diagnosing a dental problem is to examine all of the tooth surfaces that are visible by utilizing a quality light source, an explorer, and a dental mirror. It is possible for dental radiographs (X-rays) to detect dental caries (also known as cavities) before they are apparent to the naked eye, particularly caries that have developed between the teeth. It is common for large regions of dental caries to be visible to the naked eye, while smaller lesions of dental caries might be more difficult to see. Visual and tactile examinations, in addition to radiography, are common diagnostic tools used by dentists, particularly for the purpose of identifying pit and fissure caries (Pitts 2001). Caries that have not yet reached the cavity stage may often be identified by passing dry air over the area in question. This drives moisture away from the surface and alters the optical characteristics of the enamel that has not yet become calcified.

The use of dental explorers, particularly those with sharp ends, has been warned against by a number of dental researchers (Schwartz and Robbins 2004), particularly because of the risk of caries. It is possible for a cavity to form as a result of the pressure applied by the dental explorer in situations when just a very little amount of The demineralization process has started, but the cavity in the tooth has not yet formed. Due to the fact that the cariogenic process may be reversed before a cavity becomes visible, it is conceivable that caries may be arrested using fluoride and the tooth surface can be remineralized in the process. In the event that there is an existing cavity, a restoration will be necessary in order to rebuild the portion of the tooth's structure that was destroyed due to the cavity.

It might be difficult to identify caries in the pits and fissures of teeth at times. It is possible for bacteria to break through the dentin layer of the enamel, but once they do, the surface may remineralize, particularly in the presence of fluoride (Zadik and Bechor 2008). In spite of the fact that these cavities, which are frequently referred to as "hidden caries," will still be evident on (X-ray) radiographs, a visual inspection of the tooth will reveal that the enamel is either unharmed or just mildly perforated.

Dental fluorosis and tooth developmental anomalies such as hypomineralization and hypoplasia are two disorders that might be considered alternative diagnoses to dental caries. The demineralization of the tooth surface, which results in a change in the optical characteristics of the tooth, is a characteristic of the early carious lesion. Diagnostic assistance for the early identification of carious lesions may be provided by technology that makes use of laser speckle imaging (LSI) methods (Deana *et al.* 2013).

2.2 Molecular Analysis of Dental Caries

The treatment of dental caries has many primary goals, restoring the structure and function of the tooth is among the most important of these treatments. offer a tooth surface that can be cleaned, and stop the development of the illness. Eliminating the highly contaminated biomass of dentine that is located inside the lesion is one strategy for accomplishing these goals (Acs *et al.* 1999, Hasegawa *et al.* 1991). Because of this, it is essential to have an understanding of how bacteria are distributed throughout a lesion and, more particularly, whether or not bacterial invasion extends into the dentine beyond the clinical excavation limit of a lesion is something that must be determined. It is believed that germs that are left behind after the cavity preparation process may be made harmless if they are encased in a repair that is completely watertight (Maidak *et al.* 1997). The make-up of the bacterial population that is contained inside the tooth will, however, play a role in determining the extent to which one may rely on the previous assumption.

Odontopathic bacteria, such as those that populate carious dentine, are part of a dynamic and intricate ecology that is always shifting. The surface zone is soft, necrotic, severely infected, and permanently demineralized, while the deeper tissue is less diseased and more reversibly damaged. This feature may be thought of as two separate habitats that are separated by a barrier: the soft, necrotic, and permanently demineralized zone, and the deeper tissue. Streptococcus mutans, Veillonella species, Actinomyces species, and Lactobacillus casei, were found to be present in coronal lesions of carious dentine in decreasing order of abundance, according to the findings of studies that evaluated the primary cultivable flora in carious dentine. Proteolytic bacteria, the majority of which are obligate anaerobes, may be detected in dentine near the front of lesions that are

progressing (Caufield *et al.* 2000, Zhang *et al.* 2000). It is quite unlikely that bacteria such as streptococci, which normally use salivary glycoproteins as a source of energy via the process of breakdown, could produce a disease, would be able to thrive underneath a "sealed" repair since saliva is required for the survival of these bacteria. On the other hand, other bacteria with proteolytic activity, such as numerous slow-growing anaerobes, may very well be able to continue to live and develop on serum-like nutrients that infiltrate from the pulp chamber via tubular gaps. This is something that has been hypothesized to be the case. This is one of the many theories that have been proposed to explain this phenomenon. This is the case even though it is unlikely that bacteria This theory is supported by the presence of a disproportionately high number of proteolytic organisms near the leading edge of carious lesions (Zhang *et al.* 2000).

After clinical cavity preparation, more than 90 % of the bacterial load has been shown to be eliminated by the use of conventional culture techniques as a demonstrative tool (Maidak *et al.* 2000). On the other hand, a study that was conducted not too long ago used quantitative fluorescence microscopy on bacteria that were isolated from various depths in carious lesions and marked with universal rhodamine-labeled oligonucleotide probes. The findings of this investigation demonstrated that there were more than one hundred times as many bacteria at the leading edge of the lesions as had been shown by culture in the preceding study (Bowden 1990). One of the most major drawbacks of the cultural studies that have been done in the past is the fact that about half of the oral microflora does not grow on typical artificial culture media in the laboratory (Sakamoto *et al.* 2000). Molecular solutions to this issue have been created, and they are based on the tried-and-true methodologies of (PCR), sequencing of (16S rRNA) and gene cloning, (Quivey *et al.* 2000). The microflora that is associated with dentoalveolar abscesses, endodontic infections, and periodontitis have all been characterized using this method, and in each instance, it has been shown that a significant portion of the microflora that is present is composed of lineages that have not been characterized as of yet (Newacheck *et al.* 2000, Dymock *et al.* 1996).

It is now conceivable, as a result of the development of these innovative molecular approaches, to rethink the etiology of oral infections. Determine whatever organisms are

already present at the location of the infection. This is the initial step in the study of any illness.

2.3 Bacteria

The most frequent types of bacteria that are associated with dental cavities are streptococci mutans, namely Streptococcus sobrinus and Streptococcus mutans, as well as lactobacilli. Cariogenic bacteria, which are the ones that are capable of causing the illness, are found in dental plaque; however, they are often present in quantities that are insufficient to produce issues unless there is a disruption in the natural order of things (Marsh *et al.* 2015). This is caused by changes in the local environment, such as an excessive consumption of sugar or an insufficient amount of biofilm cleaning (toothbrushing) (Marsh *et al*. 1994). If the condition is not addressed, it may result in discomfort, the loss of teeth, and infection (Ege *et al.* 2008).

Streptococcus mutans and various species of Lactobacillus are two of the many oral bacteria that may be found in the mouth; nevertheless, it is thought that only a select few kinds of bacteria are responsible for the development of dental caries. Gram-positive bacteria known as streptococcus mutans are responsible for the formation of biofilms on the teeth's surfaces. These organisms are capable of producing significant quantities of lactic acid as a result of the fermentation of dietary carbohydrates as well as are resistant to the deleterious effects of low pH, both of which are qualities that are required for cariogenic bacteria (Hardie *et al.* 1992). Because the cementum that covers root surfaces might lose its minerals more quickly than the cementum that covers enamel surfaces, root surfaces are more likely to have cavities, root caries have the potential to be caused by a wider variety of bacteria than enamel caries do. These bacteria include Streptococcus mutans, Nocardia spp., Actinomyces spp., and Lactobacillus acidophilus. Plaque is a sticky, creamy-colored substance that forms as a result of the accumulation of bacteria around gums and the teeth. Plaque functions as a biofilm. Plaque tends to accumulate more often in some areas than in others, such in areas with a lower rate of salivary flow. Plaque bacteria may get trapped in the grooves that are seen on the occlusal surfaces of molar and premolar teeth, as well as in the interproximal spaces between the teeth. Plaque

may also form above or below the gingiva, in which case it is referred to as supra-gingival plaque or respectively, sub-gingival plaque. Plaque can also collect in between the teeth, which is referred to as sub-gingival plaque. Plaque can also accumulate in between the teeth and the gums. Plaque can also collect in between the teeth and under the gingiva. These strains of bacteria, most notably *S. mutans*, have the potential to be handed on, from a caregiver to a kid either via the act of kissing or by the consumption of food that has already been pre-masticated (Douglass *et al.* 2008).

2.4 Risk Factors

There is an association between lower salivary flow increased caries and rate because the buffering function of saliva is not available to counteract the acidic environment that is caused by specific diets. This causes caries to become more prevalent in patients. Dry mouth and, as a consequence of this, widespread tooth decay are likely to come from medical diseases that diminish the quantity of saliva generated by salivary glands, in particular the submandibular gland and the parotid gland. The two salivary glands that are most negatively impacted by this are the submandibular gland and the parotid gland. Examples include Sjogren syndrome, diabetes mellitus, diabetes insipidus, and sarcoidosis (Ali *et al.* 2014). Medications, such antihistamines and antidepressants, have the potential to decrease the quantity of saliva that is produced as well. In addition to obstructing the passage of saliva to an extraordinary degree, stimulants, most notably methylamphetamine, are infamous for their addictive properties. This condition is often known as "meth mouth". THC or Tetrahydrocannabinol, is the psychoactive component of cannabis. It is also the molecule that produces "cotton mouth," which refers to a virtually total cessation of saliva production. In addition, dry mouth is listed as a potential adverse reaction for six thirds of the top ten most widely prescribed drugs in the United States (Ali *et al.* 2014). Radiation treatment to the head and neck may potentially cause cell damage in the salivary glands, which can lead to an increased risk of dental caries (Lawrence and Wiebe 2017, Sison *et al.* 2017).

It is probable that a modified metabolism in the tooth, namely fluid flow in the dentin, is to blame for a tooth's vulnerability to dental caries. This is due to the fact that bacteria

that are present in the mouth are the root cause of dental caries. The rate of fluid mobility in dentin was revealed to be "significantly inhibited" when it was treated to a high-sucrose, cariogenic diet, as proven by studies carried out on rats. This was proved by the findings of the study (Steinman and Leonora 1971).

Smoking cigarettes may also increase the likelihood of developing dental caries (commonly known as "dental cavities"). Some forms of smokeless tobacco include a significant amount of sugar, which increases the likelihood of developing dental caries (Ali *et al*. 2014). Smoking tobacco poses a significant risk for developing periodontal disease, which may result in a receding of the gingiva (the tissue that lines the gums). When gingival recession causes the gingiva to lose its attachment to the teeth, the root surface of the tooth will become more obvious in the mouth. This may happen when gingival recession is caused by periodontal disease. Root caries is a potential risk in the event that this does take place due to the fact that cementum, which is the part of the tooth that covers the roots, is demineralized by acids at a faster rate than enamel is (Banting 2001). However, there is evidence to support the hypothesis that root-surface caries are related to cigarette smoking. Even though there is not enough data to demonstrate a causal association between smoking and coronal caries, there is evidence that suggests a relationship between smoking and root-surface caries at this time (US Department of Health and Human Services 2014). If children are exposed to secondhand smoke, their risk of developing cavities in their teeth is significantly increased (Zhou *et al.* 2014). The development of cavities in adult teeth has been linked to lead poisoning in gestation and in neonates (Campbell *et al.* 200, Gemmel *et al.* 2002, Billings *et al.* 2004).

In addition to lead, any other atoms that have a charge on them and an ionic radius that is equivalent to that of bivalent calcium (Leroy *et al.* 2001), such as cadmium, simulate the calcium ion. Examples of these atoms include lead and cadmium. As a consequence, exposure to these atoms may result in tooth decay. One example of such an atom is lead. Cadmium is an example of such an element (Arora *et al.* 2008). The individual's degree of poverty is likely to be one of the most significant socioeconomic variables that will have an impact on their oral health (Dye and Thornton-Evans 2010). It has been established that caries in the teeth is connected with a lower socioeconomic position; as

a result, it is possible to think of caries in the teeth as a disease of poverty (Pitts *et al.* 2017). While dental patients are being treated, caries risk assessment forms may be collected; this procedure utilizes the evidence-based Caries Management by Risk Assessment approach (CAMBRA) (American Dental Association Council on Scientific Affairs 2012). At this time, it is unknown whether or not the identification of high-risk patients could result in therapy that is more successful over the long term for patients and that or turns back the clock on the evolution of lesions, prevents the beginning of caries, and halts new caries from forming. This information is needed in order to determine whether or not the identification of high-risk patients could result in more effective long-term patient treatment. Moreover, it is unknown whether or not this may result in fewer patients developing caries in the future (Tellez *et al.* 2013).

Iodine and epidermal growth factor are two other components that may be discovered in saliva. It has been shown that the effects of EGF are useful in the processes of cellular proliferation, differentiation, and survival (Herbst 2004). Salivary EGF has a very significant physiological function in the preservation of oral and gastric tissue integrity. This role seems to be controlled in part by the intake of inorganic iodine from diet, which also seems to play a role in the regulation of salivary EGF. This is due to the fact that it seems that dietary inorganic iodine is able to modulate salivary EGF. Iodine, on the other hand, has been shown to be useful in the prevention of dental cavities as well as in the maintenance of oral health (Venturi and Venturi 2009).

2.5 An Unbalanced Oral Microbiome and General Health

Oral and overall health are both influenced by the make-up of the oral microbiota, and the lack of this microbial community in the mouth may have a detrimental effect on a person's general state of health. The influence of nitrate-reductase-expressing oral bacteria offers a good illustration of this process since it has been shown that these bacteria speed up the conversion of dietary nitrates to nitrite. Following ingestion, salivary nitrite is subjected to a second conversion process that results in the production of nitric oxide. Nitric oxide is a potent vasodilator and antibacterial agent that plays a significant part in the upkeep of good cardiovascular function (Kapil *et al.* 2010). In

addition to this, nitrite is known to stimulate the production of mucus in the stomach (Lundberg *et al.* 2009). It has been discovered that ingesting even a little amount of nitrate may result in a lowering of blood pressure, an inhibition of platelet activity, and a decrease in endothelial dysfunction. This is the case even when the amount of nitrate consumed is quite small. These effects can be seen even in moderate amounts of nitrate consumption (Lundberg *et al.* 2011). Individuals who had hypercholesterolemia were shown to benefit from enhanced vascular function when they ingested dietary nitrates in a regular manner, as was the outcome of a recent piece of study (Velmurugan *et al.* 2016).

It has been shown that changes in the makeup of the oral microbiome, in favor of organisms that are capable of nitrite reduction, are associated to these advantages (Velmurugan *et al.* 2016). It is true that dietary nitrates have the ability to stimulate the formation of nitrites; however, It is also true that the superoxide radicals that have been generated by immune cells may combine with the nitric oxide that is present in tissues to produce peroxynitrite anions. This is the case despite the fact that dietary nitrates have the ability to stimulate nitrite formation. It is believed that these peroxynitrite anions, which are produced when nitric oxide in tissues interacts with superoxide radicals, might have negative effects on cells, such as causing damage to DNA. These peroxynitrite anions are produced when. As a result of the fact that the effects that nitrate, nitrite, and nitric oxide have on a person's physical well-being are still the subject of great controversy (Chapple and Matthews 2007). However, a number of studies with a more constrained scope have demonstrated that using mouthwashes that contain chlorhexidine may lower the content of nitrites in both plasma and saliva, in addition to causing a little increase in the user's blood pressure. This is in addition to the fact that these mouthwashes cause a slight increase in the user's blood pressure (Kapil *et al.* 2013, Woessner *et al.* 2016, Govoni *et al.* 2008).

2.6 16S Ribosomal RNA

The expensive expense of Sanger sequencing of 16S rRNA clones and the lengthy laboratory work that is needed are two of the disadvantages of this method. Because of these considerations, the total number of samples and clones that can be researched in a

practical setting is limited (Siqueira *et al.* 2012). The development of "next generation sequencing" (NGS) has, thankfully, made it possible to circumvent this constraint (NGS). The use of NGS makes it possible to analyze microbial communities in an unprecedented manner, both in terms of depth and breadth, and at a cost that is far lower. As a result, it is a great resource for analyzing the state of the oral microbiome in both illness and health (Siqueira *et al.* 2012).

Investigations that use NGS to define microbial communities often focus on one or more regions of the (16S rRNA) gene. These areas, which are strong indicators of bacterial taxa in samples owing to their hypervariability, are targeted in most of these studies. In a recent set of studies that looked at the microbiomes of dental caries, this technique was one of the methods that was applied.(Jagathrakshakan *et al.* 2015). The findings of these investigations provide a deeper understanding of the complex microbial ecology that is associated with tooth caries. And in spite of the fact that these studies all used different methodologies in terms of sampling, areas of hypervariability that were chosen for sequencing, as well as the bioinformatics analysis workflow that was implemented. There were a few different taxonomic groups that persistently exhibited relationship with dental caries. These taxa include *S. mutans*, Veillonella spp, Propionibacterium spp, Lactobacillus spp, and Atopobium s.

However, targeted (16SrRNA) gene sequencing requires gene amplification by PCR, which is recognized to introduce errors such as insertion, nucleotide substitution, and deletion, as well as chimera formation. These errors can lead to the detection of spurious species and an overestimation of the diversity of microorganisms. Chimera formation can lead to the detection of spurious species as well (Kunin *et al.* 2010). Furthermore, The polymerase chain reaction (PCR) has a variety of flaws, one of which is restricted primer coverage, which may lead to the inability to amplify some taxa, especially new ones (Hong *et al.* 2009), as well as differential amplification of templates, that can alter the The initial microbial community structure is distorted as a result of the relative abundance of species. Both of these biases may result in the inability to amplify certain taxa, especially new ones (Pinto and Raskin 2012).

WMS does not need PCR amplification and allows for a more resolution identification of microbial species in a community than 16S rRNA-based sequencing. It also makes it possible to investigate other types of microbes, such as viruses and fungi, in addition to bacteria. Shotgun whole metagenome sequencing was developed by Craig Venter and colleagues at the Broad Institute of MIT and Harvard (Ranjan *et al.* 2016). In addition, The information that was acquired from the WMS may be looked at in order to evaluate the potential for the community of microbes to perform their functions (pathway analysis and gene).

2.7 Polymerase Chain Reaction

Due to a method known as polymerase chain reaction, researchers are able to take a very little sample of DNA and amplify it (or a portion of it) to a big enough quantity to investigate in depth despite the fact that the original sample was extremely small (PCR), it is a technique that is often used to produce millions to billions of copies (full or partial) of a certain (DNA) sample in a short amount of time. PCR was first developed in the year 1983.

The polymerase chain reaction is an essential step in a wide variety of genetic testing and research operations, involves the analysis of samples of ancient DNA and the identification of pathogenic diseases. PCR is a technique for the exponential amplification of extremely tiny quantities of DNA sequences by subjecting them to a number of cycles in which the temperature is varied. In today's modern medical laboratories, PCR is a widespread and frequently crucial method utilized for a wide range of purposes, including biomedical research and criminal forensics (Saiki *et al.* 1985, Hamer *et al.* 1988).

The great majority of PCR methods include the use of thermal cycling. In thermal cycling, In order to speed up a variety of reactions that are temperature-dependent, the reactants are put through a series of heating and cooling cycles on a recurring basis. More specifically, thermal cycling makes it possible for DNA to melt and for enzymes to drive replication of DNA. In the process of polymerase chain reaction (PCR), the two most important chemicals are called primers and DNA polymerase. Primers are short stretches

of DNA that are single-stranded and contain a sequence that is complementary to a section of the DNA that is the target of the experiment. Primers are also known as oligonucleotides.

2.7.1 Applications for infectious diseases

Through the use of PCR, infectious disorders, such as those caused by bacteria or viruses, may be diagnosed quickly and with a high degree of specificity (Cai 2014). PCR also enables the identification of microorganisms that cannot be cultured or grow very slowly, such as anaerobic bacteria, mycobacteria, or viruses, using animal models or tissue culture assays. PCR diagnostic applications in the area of microbiology are based on the basic idea that the identification of infectious agents and the distinction of non-pathogenic strains from pathogenic strains may be accomplished via the use of specific genes (Cai *et al.* 2014, Khyade 2018).

2.8 Real-Time and Traditional PCR

When real-time chemistry is used, it is possible to identify an amplified PCR product at the first phase of the reaction. This was not previously possible. Even when employing more traditional methods of chemistry, this is still the case. In comparison to other, more conventional approaches to PCR detection, the monitoring of the rate at which the reaction is occurring during the early stages of the PCR process presents the opportunity for a significant improvement to be made. Electrophoresis carried out on an agarose gel is often used in conventional methods in order to determine whether or not the PCR amplification process has entered its final phase. The purpose of this analysis is to determine whether or not the process has been completed. The process of determining where the end points are takes a considerable amount of time, and the length of time that passes until the result is revealed may typically be measured in several hours. On the other hand, the selection method included the application of size bias, which brought in a different set of outcomes. In addition, the results of the experiment concerning the end point do not seem to be repeatable from one sample to the next. Real-time PCR has a sensitivity that is adequate to detect this shift, in contrast to the possibility that gels will

not be able to resolve the variation in yield. Gels may not be able to resolve the difference in yield. End point detection has a number of drawbacks, including low sensitivity, poor precision, a short dynamic range (2 log), low resolution, a procedure that is not automated, size-based discrimination as the only method used, post-PCR processing that results in carry-over contamination, and results that are not expressed as numbers. These drawbacks are compounded by the fact that the end point detection procedure is not numerically expressed. A technique that is not automated, size-based discrimination being the sole approach employed, post-PCR processing that leads in carry-over contamination, and findings that are not accurate are some of the additional downsides (Niesters 2001)

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

All equipments used in this study are listed, as shown in Table 3.1

Table 3.1 The instruments used in the current study

NO	APPARATUS	ORIGIN (COMPANY)
1	Autoclave	SAKURA (Japan)
2	Auto vortex mixer	Stuart (England)
3	Balance	Shimdzu (Japan)
4	Centrifuge	Eppendrpf (Germany)
5	Digital Camera	SONY (Japan)
6	Distillator	Gallenkamp (England)
7	Electric balance	Mettler (Switzerland)
8	Incubator	Yamato (Japan)
9	Light microscope	Olympus (Japan)
10	VITEK® 2	Biomerieux (USA)
11	Magnetic stirrer hotplate	Gallenkamp (England)
12	Medical Deep Freezer	SANYO (Japan)
13	Semi autoanalyzer	Biochemistry analyzer (china)
14	pH-meter	Gallenkamp (England)
15	Concord (France)	Refrigerator
16	Memert (Germany)	Oven
17	Gallenkamp (England)	Water bath

3.1.2 Chemicals

All chemicals used in this study are listed, as shown in Table 3.2

Table 3.2 The chemicals used in the current study

NO	CHEMICALS	ORIGIN (COMPANY)
1	Agarose	BDH (UK)
2	Glycerol	BDH (UK)
3	Methyl red	BDH (UK)
4	Phenol red	BDH (UK)
5	Sodium hydroxide	BDH (UK)
6	α – naphthol (C_{10} H_8 O)	BDH (UK)
7	$BaCl_2$. H_2O	BDH (UK)
8	Chloroform	α-Alpha (India)
9	Starch	BDH (UK)
10	bromide Ethidium	BDH (UK)
11	Xylene	BDH (UK)
12	Tetra methyl – P – Phenylene diamine dihydrochloride	BDH(UK)
13	H_2O_2	Oxoid (UK)
14	Agar – Agar	Himedia (India)
15	Congo red	Oxoid (UK)
16	Iodine crystal	BDH (England)
17	Iodine	Sigma(Germany)
18	Sodium dodecyl-sulfate SDS)(	Sigma(Germany)
19	Absolute Ethanol	Fluka(Switzerland)
20	Peptone	Fluka(Switzerland)
21	Urea	Ajax (Australia)
22	KH_2Po_4	Ajax (Australia)
23	Gram stain	Himedia (India)
24	Kovac's reagent	Vac.& Sera Ins.(Iraq)
25	NaCl	BDH (England)
26	HCL	BDH (England)
27	Glucose	Oxoid (UK)
28	Isoamyl alcohol	Difco (U.S.A)
29	Yeast extract	Difco (U.S.A)
30	Mineral Oil	Promega (U.S.A)

3.1.3 Requirements used

All requirements used in this study are listed, as shown in Table 3.3 and Table 3.4

Table 3.3 The requirements used in the current study

CHEMICALS	ORIGIN (COMPANY)
API 20E Kit	BioMerieux (France)
RapID One System	Remel (USA)
Eppendorf tubes	Promega (USA)
Millipore filters (0.22 µL- 0.45 µL)	Sigma (Germany)
Micropipettes (50 - 100 - 1000) µL	Gillson (Germany)

Table 3.4 Materials that used in PCR study

NO	MATERIALS	COMPANY
1.	6X loading dye	Intron/ Korea
2.	Red safe staining solution	Intron/ Korea
3.	Agarose	Conda/ USA
4.	TBE buffer 10X	Conda/ USA
5.	Pre mix pcr	Intron/ Korea
6.	Ladder 100 bp	Intron/ Korea
7.	i-genomic BYE DNA extraction Mini kit	Intron biotechnology/ Korea
8.	Primer	Intrgrated DNA technology/ USA

3.2 Protocol

1. Prepare bacteria sample
2. Transfer 1-2 mL cultured cell into 2 tube.
3. Pellet bacteria by centrifugation for 1 min at 13000 rpm, then discard supernatant expect 20 µL of supernatant.
4. Resuspend completely the bacteria pellet into remnant supernatant by tapping or vigorously votexing.
5. Add 100 Al Buffer MP and 3 L Lysozyme Solution into Sample Tube, and resuspend by Vortex for 30 See or Pipetting Vigorously.
6. Incubate The Lysicate for 15 min at 37 °C.
7. Centrifuge The Pre-Lysicate for 1 min at 13.000 rpm at room temperature. Discard Supernatant, Ensuring That All Liquid Is Completely Removed. And then resuspend by vortexing or tapping of Cell Pellet to Pre-Lysis Cell Perfetly.

8. Add 200 µL buffer mg, 20 µL protinase k and 5 L rnase a Solution into Sample Tube, and Resuspend by Vortex Vigorously.
9. Incubate the lysate for 15 min at 65 °C.
10. After Lysis Completely, add 250 µL Buffer MB To The Lysate, and Mix By Pipetting or Gently Inverting 5 to 6 times. Do Not Vortex. After Mixing, Spin Down to Remove Drops from Inside the Lid.
11. Add 250 µL 80% ethanol to the Lysate, and Mix by Pipetting or Gently Inverting 5 to 6 times. Do Not Vortex. After Mixing, Spin Down to Remove Drops from Inside the Lid.
12. Pipette 750 L of the Mixture from Step 11 Into The Spin Column.
13. Place The Spin Column into A New 2.0 mL Collection Tube (Additionally Supplied), Add 700 µL Buffer MW to The Spin Column, and Centrifuge for 1 min at 13.000 rpm Centrifuge For Additionally 1 Min to Dry The Membrane (Reuse The collection). Discard The Flow-Through, and Again
14. Place The Spin Column INTO A NEW 1.5 mL Tube (Not Supplied), and 50~100 µL Buffer Me Directly onto The Membrane. Incubate for 1 min at Room Temperature, and Then Centrifuge for 1 min at 13,000 rpm to elute.

3.3 Methods

3.3.1 Catalase test

After immediately adding one to two drops of 3 % v/v hydrogen peroxide (H_2O_2) to the part of broth on the slide (PeroxyChem, USA), repeat the procedure, an inoculum was taken from a pure culture of *S. mutans* that had been grown on MSBA and transferred to the surface of a clean, dry glass slide using a sterile loop. A positive result on a test for catalase may be determined when gas bubbles begin to form.

3.3.2 Methyl red reagent

In order to produce it, 0.1 g of methyl red were first dissolved in 300 mL of ethanol (95 %), and then DW was added to bring the total volume of the solution up to 500 mL. To fully reveal the use of polysaccharides and the production of acetyl methyl carbinol (butnidol).

3.3.3 Oxidase reagent

It was made by dissolving 0.1 g of tetra methyl-p-phenyl diamine dihydrochloride in 10 mL of DW in a dark container. This was done in order to determine whether or not the isolates had the capacity to create the enzyme oxidase.

3.3.4 Voges-proskauer detector

This reagent is made up of two different solutions, namely:

- Solution A (α-naphthol) is made by dissolving five Klg of alpha-naphthol into 100 mL of pure alcohol (ethanol 96 %).
- The solution containing 40% KOH was made by dissolving 40g of potassium hydroxide in 100 mL of distilled water. It was used for the detection of the development of mixed acid fermentation as well as the partial utilization of sugars.

3.3.5 Kovac's reagent

It was prepared by dissolving 10 g of P-dimethyl amino benzaldehyde in 150 mL of Isoamylalcohol in a water bath at 55°C, then adding 50 mL of concentrated hydrochloric acid HCL and keeping the reagent in an opaque and sterilized vial at a temperature of 4°C until Usage: The reagent was used in the indole assay (Benson *et al.* 2002).

3.4 Preparatory Culture Media

3.4.1 MSBA preparation

The mitis salivarius bacitracin agar (MSBA) that is used as the selective medium for Streptococcus mutans was made in accordance with the instructions provided by the manufacturer. In 500 mL of distilled water, 45 g of the powdered mitis salivarius agar (MSA) medium that was manufactured by Himedia in India was dissolved. The combination was then given an additional 75 mg. After that, the mixture was autoclaved for 15 min at 121 C, and then it was allowed to cool at 45°C. Lastly, 0.5 mL of sterile bacitracin solution (200 U/L) was added to the medium (Oxoid Inc. Canada), which was followed by the pouring of the solution into numerous Petri plates and the waiting for the solution to cool.

3.4.2 Media macconkey agar

Suspend 49.53 g of the dried medium in 1000 mL of distilled water. Heat to boiling to completely dissolve the medium. Then the medium was sterilized by autoclave for 15 min. Then cool the medium to 45°C – 50°C. Then mix well before pouring into sterile Petri dishes.

3.5 Saliva Collection

Between November 2021 and February 2022, saliva was collected from forty healthy participants living in the city of Baghdad. The subjects' ages ranged from 19 to 21 years old. Before beginning the research, approval of the protocol was acquired from the relevant Ethical Committee, and each subject gave their written permission after receiving appropriate information. After confirming that each student had not consumed anything for at least one hour prior to having their saliva collected, we collected between two and 5 mL of saliva from each of them. All of the collected saliva samples were analyzed to determine their pH and flow rate, both of which were determined in the first five min of

the saliva collecting process. The salivary flow rate was calculated as the amount of saliva that was collected in a certain amount of time. The pH of the saliva was estimated using a pH strip manufactured in Belgium by the company Consort (Animireddy *et al.* 2014).

3.6 Culturing of *S. Mutans*

Salivary samples were used to create a stepwise dilution of concentrations, going from 10-1 to 10-5. From each dilution, 50 µL was removed and cultivated on MSBA before being placed in an anaerobic jar and heated to 37°C for 24 hours. The jar was then transferred to an aerobic incubator for another 24 hours. A colony, cell morphology, and a few biochemical tests were ultimately used to identify the *S. mutans* colonies that were found later (Beighton 1986).

3.7 Morphological Characteristics

With the use of the dissecting microscope, directly on the MSBA plates (magnification of X 15), the morphology of the separated *S. mutans* colonies was analyzed and compared (Olympus, Japan). The determination of *S. mutans* colonies is dependent on the morphological characterisation of the colonies on the selective agar as reported by Edwardson. After that, the total number of colonies was counted, the dilution factor was taken into account, and the results were reported as the number of colony-forming units per milliliter of saliva, or CPU/mL (Al-Mizrakchi 1998).

3.8 Gram Stain Cell Morphology

According to (Koneman *et al.* 1997), a colony was taken from the MSBA plates while they were being sterilized. The colony was then stained with Gram's stain and studied using a dissecting microscope.

3.9 Microscopic Examination

Bacterial colonies were diagnosed based on the morphological characteristics of the germ cells under the microscope through the nature of their interaction with the gram stain, which shows the type of interaction and the shape and arrangement of the germ cell.

3.10 Agarose Gel Electrophoresis of DNA

Electrophoresis was used to identify dna pieces after the extraction procedure or to detect the result of the Pcr interaction in the presence of standard DNA in order to differentiate the bundle size of the Pcr interaction on the agarose gel. Both of these tasks were carried out in order to determine whether or not the standard DNA was present.

3.11 Prepare of the Agarose Gel

The agarose gel was created in 1.5 % condensation, as described by (Sambrook *et al.* 1989), by melting 1.5 g of agarose in 100 mL of TBE Buffer that had been prepared in advance. After bringing the agarose to a boil, it was allowed to cool at a temperature between 45 and 50°C. After preparing the plate of agarose support and adjusting the comb so that it can generate holes large enough to hold the samples, the gel was poured into the pour plate. This allowed the holes in the plate to be used to hold the samples. After being carefully poured so as to avoid creating air bubbles, the gel was then let to cool for a period of thirty minutes. The comb was carefully extracted from the solid agarose and set aside. In the horizontal unit of the electrophoresis technique, the plate has been firmly connected to its support, which is represented by the tank that is being utilized. The buffer TBE has been poured into the tank until it is completely full, and it now covers the gel surface.

3.12 Preparation of Sample

After the process of mixing, the process of loading is now being carried out to the holes in the gel. The processor loading buffer, which was manufactured by Intron and located in Korea, was given a volume of 3 μL, and 5 μL was devoted to the loading dye, which was a representation of the DNA that was expected to be separated by electrophoresis. After having been subjected to an electric current of 70 V cm^2 for one to two hours, the tincture was transferred to the other side of the gel. After putting the gel in a pool that included 30 μL of a red safe nucleic acid staining solution and 500 mL of distilled water, the gel was examined using a source of UV radiation with a wavelength of 336 nm.

3.13 Red Safe Nucleic Acid Staining Solution

For the detection of nucleic acid in agarose gels, the new and safe nucleic acid stain known as Red safe Nucleic Acid Staining Solution (20.000x) is an alternative to the traditional stain known as Ethidium Bromide (ETBR). When it is attached to DNA or RNA, the fluorescence that it produces is green. This novel stain displays two fluorescence excitation maxima when attached to nucleic acid; the first one is located at 309 nm, while the second one is centered at 419 nm. In Addition, There Is Only One Visible Excitation at The 514nm Wavelength.

At a wavelength of 537 nm, the fluorescence emission of red safe that is bound to DNA is centered. ETBR and redsafe nucleic acid staining solution (20.000x) have the same level of sensitivity. The methodology for staining nucleic acids using red safe nucleic acid staining solution (20.000x) is quite similar to the process for ETBR. red safe nucleic acid staining solution (20.000x) induces much less mutations in the AMES test when compared to TOETBR, which is known to be a potent mutagen. Furthermore, a negative result was obtained using the res safe nucleic acid staining solution (20.000x), when subjected to the mouse marrow chromophilous erythrocyte micronucleus test as well as the mouse spermary spermatocyte chromosomal aberration test. Therefore, when detecting nucleic acid on agarose gels, it is best to use a red safe nucleic acid staining solution (20.000x) instead of ETBR (Cat. No. 2141).

3.14 The Primers Used in the Interaction

The primers were lyophilized and then dissolved in free ddh20 to provide a stock solution with a final concentration of 100 pmol/µL. After that, they were stored at a temperature of -20°C in order to prepare 10 pmol/µL concentration as work primer. Finally, an investigation using IDT was performed on a volume of 100 µL that contained 10 µL of the stock solution suspended in 90 µL of the free ddH20 (Table 3.5).

Table 3.5 The specific primer 16s rRNA of gene

PRIMER	SEQUENCE	Tm (0C)	GC (%)	PRODUCT SIZE
Reverse	5-GGTTACCTTGTTACGACTT-3	49.4	42.1	1250-1500 base pair
Forward	5-AGAGTTTGATCCTGGCTCAG-3	54.3	50.0	

3.15 Maxime Pcr Premix Kit (I-Taq) 20ulrxn (Cat. No. 25025)

Not only does the ineron maxime pcr premix kit come with a variety of different premix kits that are tailored to the user's level of expertise, but it also comes with a 2X Master Mix solution. The Maxime Pcr Pre Mix Kit (I-TAQ) is a product that combines all of the necessary components for a single round of RXN PCR into a single tube. These components include Taq DNA Polymerase, DNTP Mixture, and Reaction Buffer. This is the product that can get the greatest possible outcome using the system that is the easiest to use.

The first advantage is that it already has all of the necessary components for doing PCR; hence, all that is required to carry out PCR is the addition of a template DNA, primer set, and D.W. The second reason is that it provides a gel loading buffer for electrophoresis, which allows us to conduct gel loading without having to first treat the sample in any way. It has a quick and easy application process, making it suited for a wide range of sample types (Table 3.6).

Table 3.6 The components of the maxime PCR PreMix kit (i-Taq)

MATERIALS	VOLUME
Reaction buffer (10X)	1X
DNTPs	2.5 mM
Gel loading buffer	1X
i-Taq DNA polymerase	5U/µL

3.16 Diagnosis of Gene

Table 3.7 shows the diagnosis of the gene in this study

Table 3.7 Combination of the particular gene interactions necessary for diagnosis

COMPONENTS	CONCENTRATION
Forward primer	10 pmol/µL
DNA	1.5 µL
Reverse primer	10 pmol/µL
Taq PCR PreMix	5 µL
Distill water	16.5 µL
Final volume	25 µL

After several experiments to determine the optimal condition for (annealing and initial denaturation), the temperature was changed for all samples using (Gradient Pcr), and the concentration of DNA template was changed between (1.5-2 µL), where these two factors are considered important factors in primer annealing with complement. The optimal condition has been identified for (initial denaturation and annealing) (Table 3.8).

Table 3.8 The optimum condition of detection

PHASE	Tm (0C)	TIME	NO. OF CYCLE
Initial denaturation	94	3 min	1 cycle
Denaturation-2	94	45 sec	35 cycle
Annealing	56	1 min	
Extension-1	72	45 sec	
Extension-2	72	7 min	1 cycle

3.17 Statistical Analysis

The statistical program known as Statistical Package for the Social Sciences was used in order to do the analysis on the data (SPSS, Version 20, IBM Analytics). The graphs and tables were created by using both Microsoft Word and Microsoft Excel. It has also been determined, via the use of the t-test and the one-way analysis of variance (ANOVA), which variables had the most significant influence on the research parameters.

4 RESULTS AND DISCUSSION

4.1 Demographic Data

This research comprised one hundred healthy patients from the Dentistry College at Baghdad University. 65% of the patients were male and 35% were female. 87% of the patients were inhabitants of Baghdad, and 13% were residents of other cities (Table 4.1).

Table 4.1 Demographic data

VARIABLES		NUMBER	FREQUENCY	P VALUE
Sex	Male	65	65%	0.1833
	Female	35	35%	
Occupation	Baghdad resident	87	87%	0.0837
	Non Baghdad resident	13	13%	

The shape and diameter of *S. Mutans* isolates on Mitis salivarius bacitracin agar are shown in Figure 4.1. Where, bacterial isolates of the genus were diagnosed according to the microscopic characteristics such as the interaction of Gram stain. In addition, colony characteristics such as colony color, texture, metallic luster and pigment production, all of these parameters were used to determine isolates and genera.

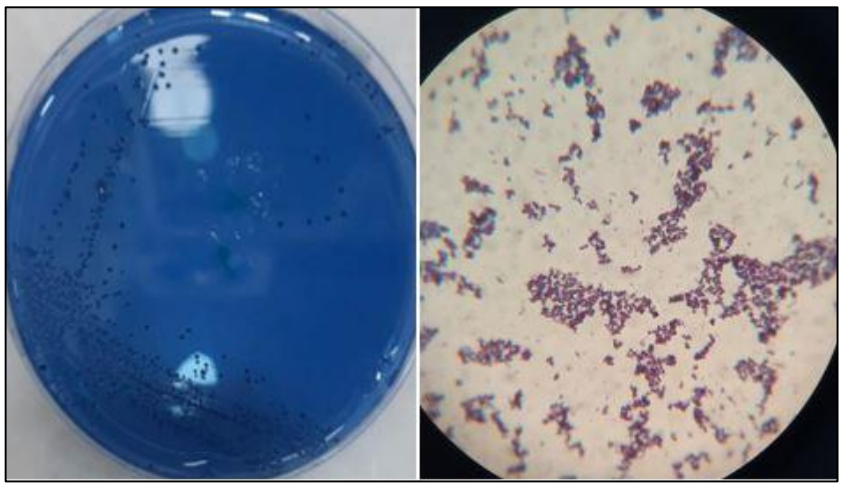

Figure 4.1 Mutans isolates on Mitis salivarius bacitracin agar

Then, biochemical tests were performed for *S. mutans* as shown in the Table 4.2 and the diagnosis was confirmed using API 20E kit and Vitek system.

Table 4.2 Biochemical tests for *S. mutans* using the usual method

Isolate	Catalasc	Oxidase	I	M	VI	C	TSI		Urease	Motility	Manitol Fermentation
			Indole	MR	VP	Citrate	TSI Reaction	H_2S Productio			
S1	+	+	-	+	+	-	K/K gas	+	-	-	+
S2	+	+	-	+	+	-	K/K gas	+	-	-	+
S3	+	+	-	+	+	-	K/K gas	+	-	-	+
S4	+	+	-	+	+	-	K/K gas	+	-	-	+
S5	+	+	-	+	+	-	K/K gas	+	-	-	+
S6	+	+	-	+	+	-	K/K gas	+	-	-	+
S7	+	+	-	+	+	-	K/K gas	+	-	-	+
S8	+	+	-	+	+	-	K/K gas	+	-	-	+
S9	+	+	-	+	+	-	K/K gas	+	-	-	+

4.2 Genetic Study

4.2.1 DNA extraction and amplification

The surface of a single agar plate was covered with fungal growth, which was then put into a sterile ceramic mortar that had been pre-cooled to -208°C, covered with liquid nitrogen, and pounded into a fine powder using a sterile ceramic pestle. To suspend the powder, 2 mL of buffer G-2 from the Genomic DNA buffer set sold by Qiagen in Valencia, California, were added. The RNase concentration was set at 200 gm per milliliter by the Sigma Chemical Company in St. Louis, Missouri, and the mixture was then poured into a clean test tube. After adding 45 µL of a proteinase K solution that

included 20 mg/mL of stock solution (provided by Sigma), the suspension was heated to 558°C and incubated for three hours while undergoing intermittent shaking.

After centrifuging the solution at 21.500 g for ten minutes, the supernatant was transferred into a clean test tube, and the DNA was extracted and purified using Genomictip 20/G columns (Qiagen) in accordance with the instructions provided by the manufacturer. After the DNA was eluted, 2.5 µL of a glycogen solution containing 20 mg/mL (provided by Gentra Systems in Minneapolis, Minnesota) was added to it. After this, the DNA was precipitated by following normal procedures using isopropanol and ethanol. After being resuspended, the DNA was placed in 60 mL of DNA rehydration buffer (from the PureGene kit sold by Gentra Systems) and frozen at -208°C until it was needed.

The results of the extraction of *S. mutans* using the special equipment for this purpose and the streptococcus by agarose gel and its detection using the safe red dye and examination under ultraviolet rays showed during which one molecular size as in the Figure 4.2

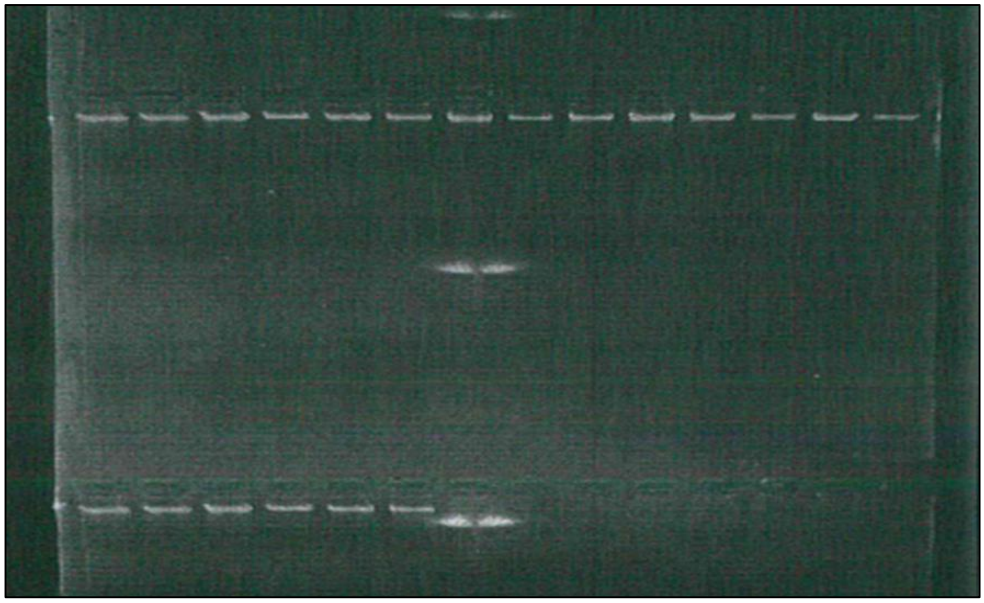

Figure 4.2 Genomic DNA isolated from bacteria was electrophoresed on a gel, run at vol/cm for 45 min at a concentration of 1% agarose in the gel.

4.2.2 Polymerase chain reaction (PCR) techniques

For the purpose of diagnosing DNA purified from local isolates of *S. mutans*, global primers were adopted to amplify the 16S RNA region anterior (5-AGAGTTTGATCCTGGCTCAG-3) and posterior (5-GGTTACCTTGTTACGACTT-3) as an approved taxonomic and genetic index to study genetic data between different bacterial isolates in this region. The polymerization products were of one molecular size (332 bp) for all bundles as in the Figure 4.3.

In order to verify the identity of the bacteria, an amplification of the 16S rRNA was done on 12 different isolates. After the amplification of DNA from *S. mutans* isolates using PCR and subsequent separation of PCR products on agarose gel (Figure 4.3), primers were generated for a conserved area of 16S rRNA. Following that, these primers were used. The investigation revealed that all 12 samples of *S. mutans* had a 16S rRNA gene band of 332 base pairs (bp). The use of 16S rRNA for the identification of *S. mutans* isolates is more accurate than the use of bacteriological and biochemical techniques. It was demonstrated by (Rampini *et al.* 2011) that the 16S rRNA gene PCR was sensitive, specific, and useful for the diagnosis of culture-negative bacterial infections. Additionally, it was demonstrated that it was useful for the identification of bacterial pathogens in patients who had previously been treated with antibiotics.

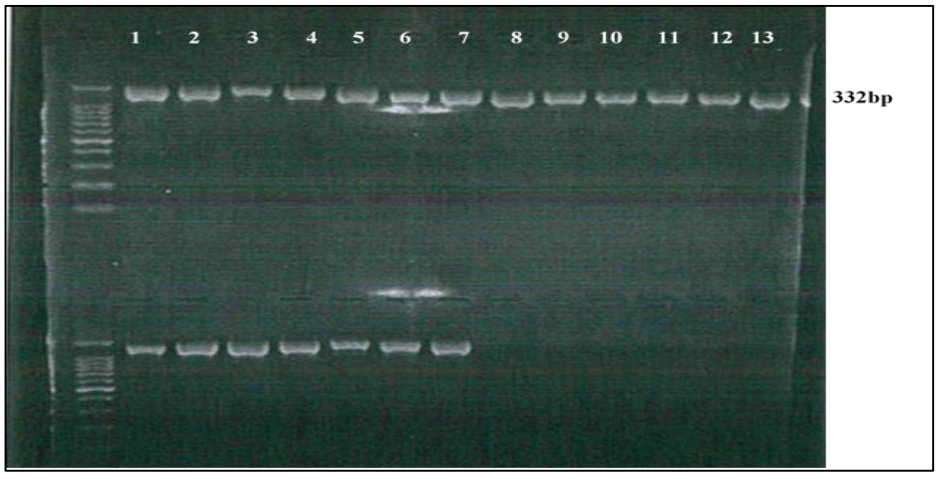

Figure 4.3 PCR products the band size 125-1500bp. The product was electrophoresis on 2% agarose at volt/cm2. 1x TBE buffer for 1 hour. N: DNA ladder (100)

In order to solve the issues that arise from culture, many molecular biology techniques have been created. As a result, the PCR was put to use in the process of identifying microorganisms found in environmental and clinical materials. In comparison to culture-based traditional techniques of bacterial determination, this one was much more sensitive, specific, and expedient. It enables the identification of living as well as non-viable bacteria, and as a result, it directly detects DNA. In addition, it requires much less time and labor than traditional approaches.

The sequence was compared in NCBI's database against the whole genome of the reference strain of *S. mutans*. The identification result was 100 % accurate. The sequence was compared in NCBI's database against the whole genome of the reference strain of *S. mutans*. The identification result was 100 % accurate.

(Al-Ahmad *et al.* 2006) the *S. mutans*-specific primers employed in nested (16S rRNA) gene PCR were shown to not be specific for *S. mutans*, but instead identified 12 other streptococcal strains, including major oral streptococci. In this work, there was no need to use a different primer for another gene in order to detect cariogenic *S. mutans* since the *S. mutans*-specific primers used in the 16S rRNA gene PCR were specific for *S. mutans*. Also, (Al-Ahmad *et al.* 2006) have mentioned that 22 (100%) isolates are identified as *S. mutans* by 16S rRNA gene.

4.2.3 Analysis and determination results of the nitrogenous bases of replication reaction products

Due to the fact that there are over 100 known species in the genus Streptococcus, the traditional techniques of cultivating microorganisms in microbiological cultures place restrictions on the research that can be conducted about the identification of particular populations of *S. mutans*. Therefore, when compared to bacteriological and biochemical testing, the use of 16S rRNA in the process of identifying *S. mutans* isolates yields more accurate results.

(Rampini *et al.* 2011) demonstrates that the 16S rRNA gene PCR was sensitive and specific, and that it was used for the identification of diseases caused by culture-negative bacteria. In patients who had been treated with antibiotics in the past, it was also helpful in identifying the bacteria that caused the infection that led to the treatment. However, the conventional methods for identification of microorganisms are sometimes inaccurate. The use of our existing PCR technology, which is less complicated, speedier, species-specific, and accurate for the identification of cariogenic species, might be used to tackle issues of this kind. Therefore, our PCR approach would make the process of identifying isolates from clinical samples easier and would be more beneficial than the traditional methods that were employed in the prior research.

The isolates of *Streptococcus mutans* 1 had the sequence of nitrogenous bases of the gene in the studied regions by 95% with that of *Streptococcus mutans* isolates in India recorded in the gene bank with the number: KP975192.1, as shown in Table 4.3. The isolates of *Streptococcus mutans* 5 had the sequence of nitrogenous bases of the gene in the studied regions by 90% with that of *Streptococcus mutans* isolates recorded in the gene bank with the number: GU424134.1. The isolates of *Streptococcus mutans* 7 had the sequence of nitrogenous bases of the gene in the studied regions by 90% with that of *Streptococcus mutans* isolates recorded in the gene bank with the number: GU424134.1. The isolates of *Streptococcus mutans* 8 had the sequence of nitrogenous bases of the gene in the studied regions by 90% with that of *Streptococcus mutans* isolates recorded in the gene bank with the number: GU424134.1. The isolates of *Streptococcus mutans* 10 had the sequence of nitrogenous bases of the gene in the studied regions by 90% with that of *Streptococcus mutans* isolates recorded in the gene bank with the number: GU424134.1. The isolates of *Streptococcus mutans* 13 had the sequence of nitrogenous bases of the gene in the studied regions by 90% with that of *Streptococcus mutans* isolates recorded in the gene bank with the number: GU424134.1.

The isolates of *Streptococcus mutans* 16 had the sequence of nitrogenous bases of the gene in the studied regions by 97% with that of *Streptococcus mutans* isolates recorded in the gene bank with the number: GU424134.1 (Table 4.4) (Table 4.5).

Table 4.3 Comparison of sequence similarity ratio of nitrogenous bases of Streptococcus mutans isolates isolated in this study with other isolates

NO	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
1	Transversion	459	T/G	ID: KP975192.1	Streptococcus mutans strain H32 16S ribosomal RNA gene	95%
	Transversion	460	T/A			
	Transition	462	C/T			
	Transversion	474	C/G			
	Transversion	475	C/G			
	Transversion	495	G/T			
	Gaps/insersion	516	A			
	Transition	518	G/A			
	Transversion	547	T/G			
	Transversion	559	T/G			
1	Error					
2						
3						
4						
5		320	T/C	ID: GU424134.1	Streptococcus mutans clone RD031 16S ribosomal RNA gene	90%
		384	A/C			
		351	G/C			
		353	A/G			
		360	G/A			
		372	C/T			
		377	T/C			
		379	C/G			
		383	C/A			
		385	T/C			
		394	T/A			
		405	C/T			
6	Error					
7		357	C/G	ID: GU424134.1	Streptococcus mutans clone RD031 16S ribosomal RNA gene	90%
		367	A/G			
		378	G/C			
		399	T/A			
		402	G/A			
		420	A/G			
		427	G/T			
		430	T/A			
		433	G/T			
		434	G/C			

Table 4.4 Comparison of sequence similarity ratio of nitrogenous bases of Streptococcus mutans isolates isolated in this study with other isolates

NO	STREPTOCOCCUS MUTANS					
	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
8		265	A/G	ID: GU424134.1	Streptococcus mutans clone RD031 16S ribosomal RNA gene	95%
		271	G/A			
		273	G/A			
		346	A/G			
		357	C/G			
		378	G/C			
		399	T/A			
		430	T/A			
		433	G/T			
		434	G/C			
9	Error					
10		290	A/G	ID: GU424134.1	Streptococcus mutans clone RD031 16S ribosomal RNA gene	90%
		296	A/G			
		297	C/G			
		303	T/A			
		309	A/G			
		315	C/G			
		322	T/C			
11	Error					
12	Error					
13		307	A/G	ID: GU424134.1	Streptococcus mutans clone RD031 16S ribosomal RNA gene	90%
		320	T/C			
		353	A/C			
		356	G/C			
		358	A/G			
		365	G/A			
		372	C/T			
		377	T/C			
		379	C/G			
		383	C/A			
		385	T/C			
		394	T/A			
		406	C/T			
14	Error					
15						

Table 4.5 Comparison of sequence similarity ratio of nitrogenous bases of Streptococcus mutans isolates isolated in this study with other isolates

NO	STREPTOCOCCUS MUTANS					
	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
16		346	A/G	ID: GU424134.1	Streptococcus mutans clone RD031 16S ribosomal RNA gene	97%
		357	C/G			
		378	G/C			
		399	T/A			
17	Error					
18						
19						
20						

Also, through the Table 4.3, Table 4.4 and Table 4.5, two types of mutations (transition and transvertion) were found for the species included in the current study. These mutations occurred in different locations in which a nitrogenous hall transvertion another nitrogen base and these types are:

As for *Streptococcus mutans*1, 10 mutations occurred, two of which were transition, one at position 462 where cytosine was replaced by the nitrogenous base thymine, another one at position 518 where guanine was replaced by the nitrogenous base adenine. The third mutation was insersion type. As for the rest of the mutations, they were transvertion at position 459 where thymine was replaced by the nitrogenous base guanine, at site 460 where thymine was replaced by the nitrogenous base adenine, at site 474 where cytosine was replaced by the nitrogenous base guanine, at site 475 where cytosine was replaced by the nitrogenous base guanine, at site 547 where thymine was replaced by the nitrogenous base guanine and at site 455 where thymine was replaced by the nitrogenous base guanine.

As for *Streptococcus mutans*5, It showed 12 mutations, at position 320 where thymine was replaced by the nitrogenous base cytosine, at site 384 where adenine was replaced by the nitrogenous base cytosine, at site 351 where guanine was replaced by the nitrogenous

base cytosine, at site 353 where adenine was replaced by the nitrogenous base guanine, at site 360 where guanine was replaced by the nitrogenous base adenine and at site 372 where cytosine was replaced by the nitrogenous base thymine, at position 377 where thymine was replaced by the nitrogenous base cytosine, at site 379 where cytosine was replaced by the nitrogenous base guanine, at site 383 where cytosine was replaced by the nitrogenous base adenine, at site 385 where thymine was replaced by the nitrogenous base cytosine, at site 394 where thymine was replaced by the nitrogenous base adenine and at site 405 where cytosine was replaced by the nitrogenous base thymine.

As for *Streptococcus mutans*7, It showed 10 mutations, at position 357 where cytosine was replaced by the nitrogenous base guanine, at site 367 where adenine was replaced by the nitrogenous base guanine, at site 378 where guanine was replaced by the nitrogenous base cytosine, at site 399 where thymine was replaced by the nitrogenous base adenine, at site 402 where guanine was replaced by the nitrogenous base adenine, at site 420 where adenine was replaced by the nitrogenous base guanine, at position 427 where guanine was replaced by the nitrogenous base thymine, at site 430 where thymine was replaced by the nitrogenous base adenine, at site 433 where guanine was replaced by the nitrogenous base thymine, at site 434 where guanine was replaced by the nitrogenous base cytosine.

As for *Streptococcus mutans*8, It showed 10 mutations, at position 265 where adenine was replaced by the nitrogenous base guanine, at site 271 where guanine was replaced by the nitrogenous base adenine, at site 273 where guanine was replaced by the nitrogenous base adenine, at site 346 where adenine was replaced by the nitrogenous base guanine, at site 357 where cytosine was replaced by the nitrogenous base guanine, at site 378 where guanine was replaced by the nitrogenous base cytosine, at position 399 where thymine was replaced by the nitrogenous base adenine, at site 430 where thymine was replaced by the nitrogenous base adenine, at site 433 where guanine was replaced by the nitrogenous base thymine, at site 434 where guanine was replaced by the nitrogenous base cytosine.

As for *Streptococcus mutans*10, It showed 7mutations, at position 290 where adenine was replaced by the nitrogenous base guanine, at site 296 where adenine was replaced by the nitrogenous base guanine, at site 297 where cytosine was replaced by the nitrogenous

base guanine, at site 303 where thymine was replaced by the nitrogenous base adenine, at site 309 where adenine was replaced by the nitrogenous base guanine, at site 315 where cytosine was replaced by the nitrogenous base guanine, at position 322 where thymine was replaced by the nitrogenous base cytosine.

As for *Streptococcus mutans*13, It showed 13 mutations, at position 307 where adenine was replaced by the nitrogenous base guanine, at site 320 where thymine was replaced by the nitrogenous base cytosine, at site 353 where adenine was replaced by the nitrogenous base cytosine, at site 356 where guanine was replaced by the nitrogenous base cytosine, at site 358 where adenine was replaced by the nitrogenous base guanine, and at site 365 where guanine was replaced by the nitrogenous base adenine, at position 372 where cytosine was replaced by the nitrogenous base thymine, at site 377 where thymine was replaced by the nitrogenous base cytosine, at site 379 where cytosine was replaced by the nitrogenous base guanine, at site 383 where cytosine was replaced by the nitrogenous base adenine, at site 385 where thymine was replaced by the nitrogenous base cytosine, at site 394 where thymine was replaced by the nitrogenous base adenine, at site 406 where cytosine was replaced by the nitrogenous base thymine.

As for *Streptococcus mutans*16, it showed 4 mutations, at position 346 where adenine was replaced by the nitrogenous base guanine, at site 357 where cytosine was replaced by the nitrogenous base guanine, at site 378 where guanine was replaced by the nitrogenous base cytosine, at site 399 where thymine was replaced by the nitrogenous base adenine.

5 CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

- According to the findings of the research, there were a total of one hundred healthy patients from the Dentistry College at Baghdad University who participated in this study. Of those patients, sixty-five % were male and thirty-five % were female.
- The investigation revealed that all 12 samples of *S. mutans* had a 16S rRNA gene band of 332 base pairs (bp).
- The use of 16S rRNA, rather than bacteriological and biochemical tests, is the most reliable method for identifying *S. mutans* isolates.

5.2 Recommendation

- Study different types of genes that responsible of virulence factors.
- Estimate some types of virulence factors that responsible about the pathological of bacteria.
- Study the histological effects of *S. mutans* on gum of patients.

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