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النسق الجزيئي لجينات الضراوة في المكورات العقدية الطافرة المعزولة من تسوس الاسنان

رسالة مقدمة الى

مجلس كلية العلوم /جامعة بابل كجزء من متطلبات نيل درجة الماجستير

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١٤٤٤هـ **٢٠٢٣ م**

الخلاصة

شملت هذه الدراسة جمع 100 عينة تسوس أسنان من الاشخاص البالغين الذين تراوحت أعمـــارهم بين 18-67 سنة من كلا الجنسين. جمعت العينات من المرضى الذين حضروا إلى عيادات التدريس في كـــلية طب الأسنان في جامعة بابل ومن المركز التخصصي لطب الأسنان في بابل خلال الفترة من تشرين الثاني 2021 إلى كانون الثاني 2022.

زرعت جميع العينات على اوساط مختلفة مثل أكار الدم واكار الماكونكي بالإضافة إلى الزرع على الاوساط الانتقائية (Mitis-Salivarius agar , Mitis-Salivarius bacitracin agar) . اجريت الاختبارات الكيموحيوية واختبارات الصفات الشكلية الخارجية ونظام VITEK-Π لعزل وتوصيف الـعقدية الطـــافرة المرتبطــة بتسوس الأسنان في الإنسان وتوكيدها باستخدام تقنية تفاعل سلسلة انزيم البلمرة.

تم تشخيص 88 عزلة من أصل 100 عينة لتسوس الأسنان على أنها زرع موجب لعزلات بكتيرية. كانت 70 عزلة تتعلق بجنس المكورات العقدية وبالخصوص المكورات العقدية المخضرة من مختلف المجموعات؛ وشملت العقدية الطافرة *S. mutans* النسبة المئوية العليا 67.14% (47/70) بالمقارنة مع أنواع المكورات العقدية الأخرى *S. sobrinus* 15.71% (11/70) ، *S. mitis* 11.42 % (8/70) و *S. salivarius* 5.71% (4/70) . في حين أن العزلات الأخرى شملت 18% من النمو الكلي وهي المكورات المعوية البرازية (14% من العزلات) والمكورات العنقودية الذهبية (4% من العزلات).

أظهر الكشف الجزيئي عن العقدية الطافرة باستخدام بادئات خاصة تستـــــهدف الموروثة الخاصـــة بالنوع gene *Sm479* ان 42 عزلة فقط من اصــل 47 عزلة كانت تنتمي للعقدية الطافرة.

أظهر الكشف الجزيئي عن مورثات الضراوة في عزلات العقدية الطافرة أن النسب المئوية للمورثات كانت :

gbpA 80.95% (34/42), *gbpB* 85.71% (36/42), *spaP* 76.19% (32/42), *relA* 66.66% (28/42), *comD* 54.76% (23/42) and *comE* 71.42% (30/42).

فحصت قابلية عزلات العقدية الطافرة على تكوين الاغشية الحيوية مظهريا باستخدام طريقة صفيحة الزرع النسيجي وصبغة البنفسج البلوري (microtiter plate crystal violet method). كانت النسب المئوية لتكوين الأغشية الحيوية بواسطة عزلات العقدية الطافرة 14.28% (6/42) منتجة لغشاء حيوي قوي ، 35.71% (15/42) منتجة لغشاء حيوي معتدل، 28.57% (12/42) منتجة لغشاء حيوي

ضعيف و 21.42% (9/42) غير منتجة للغشاء الحيوي. كانت هناك فروقات كبيرة ذات دلالة إحصائية في القدرة على تكوين الأغشية الحيوية بين عزلات العقدية الطافرة اعتمادا على ($p < 0.05$).

اختبرت الحساسية الدوائية لعزلات العقدية الطافرة ضد (17) نوعا من المضادات الحيوية المختلفة باستخدام طريقة انتشار القرص ل Kirby-Bauer وطريقة تحديد التركيز المثبط الادنى. كانت جميع عزلات العقدية الطافرة حساسة ل (ليفوفلوكساسين ، أوفلوكساسين ، دوريبينيم وإرتابينيم). كان هناك اختلاف في حساسيتها ايضا للفانكومايسين 92.85% (39/42) ، الاريثروميسين 80.95% (34/42) ، أزيثروميسين 95.23% (40/42) ، كلاريثروميسين 95.23% (40/42) ، الكلورامفينيكول 30.95% (13/42) ، الكليندامايسين 88.09% (37/42) ، لينزوليد 95.23% (40/42) ، البنسلين 90.47% (38/42) والأمبيسلين 88.09% (37/42). في حين كانت جميع عزلات العقدية الطافرة مقاومة للسيفبيم ، ومقاومة لسيفوتاكسيم بنسبة 97.61% (41/42) ، سيفترياكسون 95.23% (40/42) والتتراسيكلين 97.61% (41/42).

أظهر التحليل الإحصائي وجود فروقات كبيرة بين المضادات الحيوية المختبرة اعتمادا على ($p < 0.05$)، باستثناء الكلورامفينيكول ($p = 0.98$) لم تكن هناك فروقات كبيرة بين العزلات المقاومة والحساسة والمعتدلة. كانت 35.72% (15/42) من عزلات العقدية الطافرة مقاومة للعديد من الأدوية (MDR) و 64.28% (27/42) من العزلات كانت غير مقاومة للأدوية المتعددة (Non MDR).

فحصت عزلات العقدية الطافرة للكشف عن إنتاج الهيموليسين. أظهرت النتائج أن 30.95% (13/42) من عزلات العقدية الطافرة كانت محللة للدم من نوع الفا (α) و 19.04% (8/42) كانت محللة للدم من نوع بيتا (β) في حين كانت 50٪ (21/42) من العزلات غير محللة للدم.

كانت جميع عزلات العقدية الطافرة قادرة على تخمر المانيتول والسوربيتول والسكروز والرافينوز والميليبيوس وغيرت لون الاوساط من الأحمر إلى الأصفر.

كان هناك ارتباط كبير ذو دلالة إحصائية بين درجة تكوين الأغشية الحيوية مع المقاومة للأدوية العديدة ومع المورثات (*gbpA, gbpB, spaP*) لبكتيريا العقدية الطافرة المعزولة من تسوس الأسنان.

Ministry of Higher Education and

Scientific Research

University of Babylon

College of Science

Department of Biology

Molecular Profile of Virulence Genes for *Streptococcus mutans* Isolated from Dental Caries

A thesis

Submitted to the Council of College of Science at University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master in Biology

By

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B.Sc./ Biology / Microbiology / University of Babylon /2004

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2023 A.D **1444 A .H**

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

﴿ وَلَقَدْ آتَيْنَا دَاوُودَ وَسُلَيْمَانَ عِلْمًا وَقَالَا الْحَمْدُ لِلَّهِ

الَّذِي فَضَّلَنَا عَلَىٰ كَثِيرٍ مِّنْ عِبَادِهِ الْمُؤْمِنِينَ ﴾

صَدَقَ اللَّهُ الْعَلِيُّ الْعَظِيمِ

سورة النمل (الآية: ١٥)

Certification

I certify that the preparation of this thesis was performed by **Ahmed Turki Abdul Hassan Hassoun** under my supervision at University of Babylon, College of Sciences, Department of Biology, as a partial fulfillment of the requirement for the Degree of Master of science in Biology –Microbiology. Accordingly ,I recommend this study for discussion.

Signature

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In view of available recommendations, I forward this thesis for debate by the examining committee.

Head of Department

Assist Prof. Dr.Adi Jassim Abd Al-Razzaq

Department of Biology- College of science

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Date: / / 2023

Dedication

To the prophet of peace "Mohammed" Peace and prayer be up on him and his purified family.

To the eyes that have always been awake to help me move forward with my dreams, to the heart and lips that have continued to pray until my dreams come true, to my source of strength and my first teacher. To my father may Allah have mercy on him.

To the heart that beats to give me hope, to the cuddle that gave me assurance, to the hands that suffered for my comfort, to the candle that lights my way, to the spring of my life and my paradise … my precious mother.

To My Superviser.. **Assist. Prof. Farah Tariq Abdul-Ridha.**

Ask Allah to protect them**.**

Ahmed .2023

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Ahmed. 2023

Summary

This study included collection of 100 samples of adults with dental caries aged 18-67 years of both sex. The study samples were recruited from patients attended to the teaching dental clinics at College of Dentistry of Babylon University and Specialized Center for Dentistry in Babylon within the period of November 2021 to January 2022.

All samples were cultured onto different media such as blood agar, and MacConkey agar, as well as selective cultivation on Mitis-Salivarius agar and Mitis-Salivarius bacitracin agar. Morphological, biochemical tests, VITEK-П compact system and conventional PCR assay were carried out to isolate and identify *Streptococcus mutans* associated with dental caries in human.

Eighty eight isolates out of 100 dental caries samples were diagnosed as positive culture of bacterial isolates. Seventy isolates were considered to be related to the genus *Streptococcus* and specially to the viridans Streptococci of various group; The *S. mutans* was comprised the high percentage 67.14% (47/70) in comparison with the other *Streptococcus* species (*S. sobrinus* 15.71% (11/70), *S. mitis* 11.42% (8/70) and *S. salivarius* 5.71% (4/70). While other isolates were 18% of total growth included *E. faecalis* (14% of isolates) and *S. aureus* (4% of isolates).

Molecular detection of *S. mutans* using specific primers targeting the species-specific *Sm479* gene of *S. mutans* revealed that, out of 47 isolates, only 42 isolates were *S. mutans.* Molecular detection of virulence genes in *S. mutans* isolates showed that the percentages of genes were: *gbpA* 80.95% (34/42*), gbpB* 85.71% (36/42), *spaP* 76.19% (32/42), *relA* 66.66% (28/42), *comD* 54.76% (23/42) and *comE* 71.42% (30/42).

Biofilm formation by *S. mutans* isolates was estimated phenotypically by the microtiter plate crystal violet method. Percentages of biofilm formation by *S. mutans* isolates were 14.28% (6/42) strong biofilm producer, 35.71% (15/42) moderate biofilm producer,

28.57% (12/42) weak biofilm producer and 21.42% (9/42) non biofilm producer. There was a statistically significant difference in the biofilm formation ability among the isolates of *S. mutans* based on ($p < 0.05$).

Antibiotic susceptibility testing of *S. mutans* isolates against (17) types of different antibiotics had been carried out by using modified Kirby-Bauer disc diffusion method and minimum inhibitory concentration method. All *S. mutans* isolates were susceptible to levofloxacin, ofloxacin, doripenem and ertapenem. Also there was a variation in its sensitivity to vancomycin 92.85% (39/42), erythromycin 80.95% (34/42), azithromycin 95.23% (40/42), clarithromycin 95.23% (40/42), chloramphenicol 30.95% (13/42), clindamycin 88.09% (37/42), linezolid 95.23% (40/42), penicillin 90.47% (38/42) and ampicillin 88.09% (37/42). While all *S. mutans* isolates were resistant to cefepime, and resistant to cefotaxime 97.61% (41/42), ceftriaxone 95.23% (40/42) and tetracycline 97.61% (41/42).

The statistical analysis showed a significant differences among tested antibiotics based on ($p < 0.05$), with the exception of chloramphenicol ($p = 0.98$). There was no significant differences among resistance, sensitive and intermediate isolates. 35.72% (15/42) of *S. mutans* isolates were multi-drug resistant and 64.28%(27/42) non- multi-drug resistant.

S. mutans isolates were investigated to detect hemolysin production. The result showed that 30.95% (13/42) of *S. mutans* isolates produced α-hemolysis and 19.04% (8/42) produced β-hemolysis, but 50% (21/42) of isolates non-hemolysis. All *S. mutans* isolates were able to ferment mannitol, sorbitol sucrose, raffinose, melibiose, and changing the color of media from red to yellow.

There was a statistically significant correlation between biofilm formation grade with [multi-drug resistant and (*gbpA, gbpB and spaP*) genes] of *S. mutans* bacteria isolated from dental caries.

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4-11 E	Agarose gel electrophoresis 1% at 80 volt for 1 hour to detection of PCR product of *comE* gene (127 bp) in *S. mutans* visualized under U.V light at 280 nm after staining with safe red stain. Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18) isolates give positive result for this gene.	82
4-10 F	Agarose gel electrophoresis 1% at 80 volt for 1 hour to detection of PCR product of *spaP* gene (101 bp) in *S. mutans* visualized under U.V light at 280 nm after staining with safe red stain. Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17) isolates give positive result for this gene; Lane(18) isolate give negative result.	82

List of Abbreviations

No.	Abbreviation	Terms
1.	Ag I/II	Antigen I/II
2.	AgDS	agmatine deiminase system
3.	ATR	Acid tolerance response
4.	BHI	Brain heart infusion
5.	bp	base pair
6.	CFU	colony forming unit
7.	CLSI	Clinical and Laboratory Standards Institute
8.	D.W	Distilled water
9.	Da	Dalton
10.	DDAG	Dextran (glucan)-dependent aggregation
11.	DDM	Disk diffusion method
12.	DNA	Deoxyribose nucleic acid
13.	*E. faecalis*	*Enterococcus faecalis*
14.	EDTA	Ethylene diamine-tetra- acetic acid
15.	EPH	Ecological plaque hypothesis
16.	EPS	Extracellular polysaccharides
17.	FTase, FTF	Fructosyltransferase
18.	G -ve, G + ve	Gram negative, Gram positive bacteria
19.	Gbps	Glucan-binding proteins
20.	Gtfs, GTase	Glycosyltransferases
21.	IPS	Intracellular polysaccharides
22.	IU	International unite
23.	MDR	Multi-drug resistant

24.	mg	Milligram
25.	MHA	Muller Hinton Agar
26.	MIC	Minimum inhibitory concentration
27.	MR-VP	Methyl red-Voges Proskauer broth
28.	MS	Mutans Streptococci
29.	MSA	Mitis-Salivarius agar
30.	MSBA	Mitis-Salivarius bacitracin agar
31.	OD	Optical density
32.	PAc	Cell surface protein antigen c
33.	PAc, AgB, Pl, Sr, SpaA, PAg, SspA, SspB, B, P1, and MSL-1	Other terms of SpaP analogous protein are established in most mouth Streptococci
34.	PCR	Polymerase chain reaction
35.	PDLs	periodontal ligaments
36.	Ph	Power of hydrogen
37.	PTS	phosphotransferase system
38.	*S. aureus*	*Staphylococcus aureus*
39.	*S. mitis*	*Streptococcus mitis*
40.	*S. mutans*	*Streptococcus mutans*
41.	*S. salivarius*	*Streptococcus salivarius*
42.	*S. sobrinus*	*Streptococcus sobrinus*
43.	sIgA	Secretory Immunoglobulin A
44.	SpaP	Surface protein antigen P
45.	SPSS	Statistical Package for the Social Science
46.	STT	Salt tolerance test
47.	TBE	Tris-Borate EDTA buffer

48.	TSB	Tryptic soy broth
49.	UV	Ultraviolet

CHAPTER

ONE

INTRODUCTION

1.1 Introduction

Dental caries, the most common infection affecting the oral cavity and one of the most prevalent infectious diseases worldwide, is characterized by the enamel demineralization of the tooth (Souissi *et al*., 2021). Dental caries and periodontal disease are the two most prevalent forms of chronic oral diseases, and not only affect oral physiological function and physical appearance but are also associated with systemic diseases, including diabetes, cardiovascular diseases, osteoporosis and respiratory diseases (Zhang *et al*., 2020).

Oral microbiota, one of the most complex microbial communities in the human body, contains about 700 kinds of microorganisms that inhabit the human mouth (Lu *et al*., 2019). Oral Streptococci are primarily members of the viridans group Streptococci, a group of 20 species that are commensal occupants of the oropharyngeal cavity, as well as the gastrointestinal and genital tracts of mammals, and are thought to be the principal cause of dental caries in mammals (Abo Bakr *et al*., 2021).

Mutans Streptococci (MS) species that have been implicated in dental caries are *Streptococcus mutans* (serotypes c, e, f) and *Streptococcus sobrinus* (serotypes d, g), and are facultative anaerobes, non-motile and catalase-negative Gram-positive cocci (Nomura *et al*., 2020).

The formation of biofilms on tooth surfaces is a predominant factor in the etiology of dental caries and periodontal diseases. *S. mutans* is a key contributor to the formation of the pathogenic dental biofilms, mainly due to its ability to synthesize extracellular polysaccharides (EPS) such as water insoluble glucans or fructans by the action of glucosyltransferases (GTFs) and fructosyltransferase (FTF) (Aqawi *et al*., 2021).

The glucans are the primary keys that comprise the matrix in cariogenic biofilms (Swedan *et al*., 2018). Non enzymatic glucan-binding proteins (Gbps) can bind to glucan and are assumed to take part in the sucrose dependent adhesion and the cohesive nature of the dental plaque biofilm (Wang *et al*., 2020).

Cell surface adhesins of *S. mutans*, such as Gbps and Surface protein antigen P (SpaP), are important for bacterial adhesion to tooth surface. *S. mutans* carry *gbpA*, *gbpB*, and *gbpC* genes related with the adhesion. The *gbpA*, *gbpB*, and *gbpC* genes encode GbpA, B, and C, which are known to play an important role in the adhesion of *S. mutans* to glucan molecules, a kind of extracellular polysaccharide of plaque matrix (You, 2019).

GbpA contributes to the development of optimal plaque biofilm, which minimizes stress on the bacterial population. A deficiency of GbpA results in loose binding to the EPS matrix, resulting in a weak non-uniform biofilm structure. Thus, GbpA has important roles as a protein for formation of firm and stable biofilm (Matsumoto-Nakano, 2018). The *gbpB* gene expresses an adhesion protein named glucan-binding protein B which facilitates the binding of glucan to the physical and biological surfaces (Rouabhia and Semlali, 2021).

The cell surface protein antigen c (PAc) is one of the major surface proteins of *S. mutans* and known by a number of other names, including SpaP , antigen I/II and B, P1, and MSL-1. PAc is known to be correlated with virulence of the organism for development of dental caries and participates in bacterial adherence to teeth via interaction with the salivary pellicle, which is termed sucrose-independent adhesion (Ancuceanu *et al*., 2019).

Break down of sugars and production of lactic acids (acidogenicity) is one of the important virulence traits of *S. mutans* , thus causing tooth decay . In addition, *S. mutans*

bacteria have an acid tolerance response (ATR) (acidouricity) to combat the harmful acidic environment when exposed to sub lethal pH values (Abo Bakr *et al*., 2021).

The *relA* gene carried by *S. mutans* encodes RelA, which is known to regulate the formation of biofilm and to contribute to quorum-sensing (the regulation of phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS), the glucose uptake system) (You, 2019). Among the genes carried by the *S. mutans*, The *comD* gene which encodes a histidine kinase receptor and the *comE* gene which encodes a cognate response regulator of the competence-stimulating peptide, which are part of the quorum-sensing cascade of *S. mutans* (Mull and Tal-Gan, 2021).

Following an extensive research, they realized that there is more than one primer for PCR test that was done well for pure *S. mutans* cultures. A lot of the specific primers were directed to specific genes that are related with virulence in *S. mutans*, *Sm479* gene which used for identification of *S. mutans* from all *Streptococcus* species that found in environment of oral cavity (Al-mohammadawy *et al*., 2018).

1.2 Aim of the Study

The study aims to determine some causes that lead to caries and treated of them to reduce tooth loosing in human and detection of key virulence factors in *Streptococcus mutans* bacteria and the achievement of this aim by the following objective:

1. Isolation and identification of Streptococci by routine methods and confirmed by VITEK-П compact system and PCR method.

2. Antibiotics susceptibility test.

3. Detection of biofilm formation.

4. Carbohydrates fermentation test.

5. Molecular detection of virulence genes by PCR using specific primers.

6. Correlation between antibiotics resistance, biofilm formation and virulence genes.

CHAPTER TWO LITERATURES REVIEW

2. Literature Review

2.1 Oral Microbiota

The mouth of human is accessible open growth system and considered as a complex ecosystem in body of human represented in microbiota. Different surfaces such as (Teeth, Gingival, Tongue, Throat, and Buccal mucosa) provide microbial colonization (Kamaluddin, 2021). The oral cavity of individuals contains hundreds of different bacterial, viral, and fungal species which are resistant to mechanical stress or antibiotic treatment (Karkowska-Kuleta *et al*., 2022).

Change in the normal flora microbiota may result in the beginning of oral disease such as periodontitis and dental caries (Belibasakis *et al*., 2019). The initiation and successful development of dental caries is caused by multiple bacterial and host factors, such as the composition and biochemical activity of the biofilm organisms, dietary habit, genetic constitution and behavior of the host, tooth architecture and exposure to fluoride (Hossain *et al*., 2021).

The formation of biofilms on tooth surfaces is a predominant factor in the etiology of dental caries and periodontal diseases. A biofilm is an architectural colony of microorganisms wrapped in a matrix of extracellular polymeric substances that are produced by them. The biofilm protects the bacteria from environmental stress stimuli. Sessile cells embedded in the biofilm are up to 1000 times more resistant to antibiotics than cells in their planktonic state. These structures also enable communication between the microorganisms in a process termed quorum sensing (Aqawi *et al*., 2021).

Number of Gram negative and Gram positive microbes habitat in oral cavity, but few microbes which have resident on mouth can adhere to the surface of teeth and colonized

the dental surfaces such as ; *S. mutans*, *S. salivarius*, *S. sanguinis.* Other strains of Streptococci adhere strongly to the cheeks and gums but not to teeth (Sowmya, 2016).

According to recent phylogenomic studies,the genus *Streptococcus* includes at many as eight monophyletic groups, and the oral Streptococci are mainly distributed in six of these groups: Mitis, Sanguinis, Anginosus, Mutans, Salivarius, and Downei, each of which is named with a representative species. Among these, the mutans and downei groups are known to contain many caries-promoting species, which are normally minor in oral microbiome under healthy condition but can grow to markedly higher proportions under disease condition (Twetman, 2018).

Among the mutans Streptococci, *S. mutans* which is serotype c, e and f and *S. sobrinus* serotype d, g and h are the most commonly isolated from human carious lesions and cause caries in various animal model. These species are the quintessential tooth organisms, moreover, these species appear to be the most cariogenic of the oral Streptococci (Gupta, 2018). Although a number of bacterial species of the dental plaque community have been associated with the cariogenic process, *S. mutans* is considered the principal etiological agent (Belibasakis *et al*., 2019).

This Gram-positive facultative anaerobic bacterium is acid tolerant and metabolizes dietary carbohydrates to produce high amounts of acids, including lactic acid, which has a strong demineralizing action. The initial adhesion of *S. mutans* to dental surfaces is a prerequisite for biofilm formation, which also involves the synthesis of an extracellular polysaccharide matrix, known as glycans, by glycosyltransferases. Biofilm formation allows *S. mutans* to be firmly attached and to trap acids close to the tooth surface, thus promoting the development of carious lesions (Souissi *et al*., 2021).

The ability to form biofilm on tooth surface, production of organic acid from various carbohydrates (acidogenicity), ability to survive at low pH (acidurance), outstanding ability to outcompete other bacteria by the production of bacteriocin and the adaptation to rapidly changing environment can be attributed as the major virulence factors of *S. mutans* (Hossain *et al*., 2021).

2.2 Teeth Structure

The two anatomical parts of tooth are: a root and a crown each of them are composed of three layers the enamel, dentin and bone-like tissue layer called the cementum and one component of soft tissue, the pulp (Houg, 2021), as shown in figure (2-1).

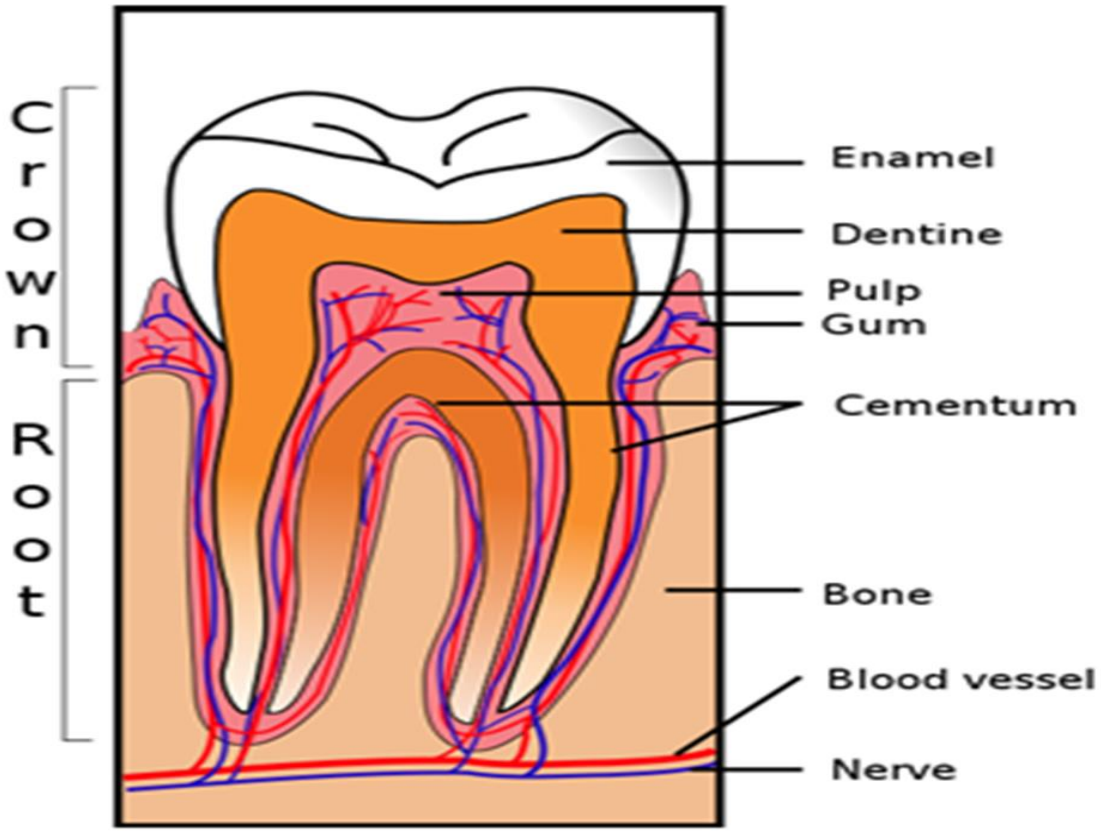


Figure (2-1): Anatomy of the tooth according to (Loesche, 2000).

The crown outer layer is the enamel which considered the hardest structure in the body, not containing any nerves or blood vessels and their function as a resistant outer structure (Talal *et al*., 2020). The middle layer of the tooth is dentin, softer than enamel which act as a protective layer and support the crown of the tooth (Farci and Soni, 2021).

The central layer of the tooth are the pulp, which have nerves and blood vessels, dental pulp maintain the tooth nutrition and vitality. There are two types of pulp: coronal pulp in the central pulp chamber of the crown and radicular pulp which located in the pulp canals of the root (Shabbir *et al*., 2021).

Cementocytes are the cellular components of cementum. The cementum, which is the thin mineral layer lining the dental root and connecting the periodontal ligaments (PDLs), functions as the anchor of the teeth to the PDLs and as a regulator of tooth position. Loss of cementum leads to periodontal disorder and tooth displacement and loss. It has been previously reported that 48-66% of roots suffer resorption of cementum after orthodontic treatment, indicating the susceptibility of the cementum to orthodontic force . Although the root's outermost layer, the cellular cementum, which is also known as cellular intrinsic fiber cementum, is considered to exhibit a general protective function against resorption, the response and function of cementocytes are largely unknown (Wei *et al*., 2020).

2.3 Dental Caries

Dental caries is a common chronic infectious disease that occurs in the dental hard tissues. Dental caries and its complications can exacerbate or induce systemic diseases, which seriously reduce the quality of human life and cause a great economic burden. According to current investigations, there are still great challenges in dental caries prevention and treatment. The prevalence of dental caries is very high. The results of the global burden of disease study released by Lancet in 2017 showed that among 328

diseases, the prevalence of permanent dental caries ranked first, and the incidence ranked second (Cheng *et al*., 2022).

Dental caries is a microbial-mediated oral disease initiated by the presence of stagnant plaque biofilms, where pathogens such as *S. mutans* metabolise dietary sugars and produce acids. This results in dysbiosis, which sustains *S. mutans* biofilm growth, creating acidic microenvironments that demineralise the dental hard tissues, leading to dental caries (Vijayakumar *et al*., 2021).

Acid production and the glucan-rich matrix in *S. mutans* biofilms enable the spatial orientation of other cariogenic microbes constituting an elaborate microbial network leading to the development of caries (D. Kim *et al*., 2020). In addition, multiple virulence factors, including acid and stress tolerance, cell persistence, genetic competence and bacteriocin production collectively contribute to the progression of dental caries (Shanmugam *et al*., 2020).

Common aspects of the lifestyle, including antibiotics, high-fat diets, alterations in saliva and even actions and experiences can persistently change commensal microbial communities (David *et al*., 2014). Some microorganisms that colonize the oral cavity can form a dental plaque. Plaque formation can lead to two of the most common oral diseases; dental caries and periodontal diseases. The absence of oral hygiene, one of the essential reasons, causes the plaque to accumulate (Abo Bakr *et al*., 2021).

Over the years, considerable effort has been invested in explaining and understanding the etiology of the major dental diseases, caries and periodontal diseases (Nyvad and Takahashi, 2020). There are three microbial hypotheses regarding the etiology of dental caries, namely the specific plaque hypothesis, the non-specific plaque hypothesis, and the ecological plaque hypothesis (EPH). The first hypothesis has proposed that only a small

number of species of the all microflora are actively involved in disease (Rosier *et al*., 2014).

The non-specific plaque hypothesis suggests that caries is the result of the overall interaction of all the groups of bacteria within plaque (Xuedong, 2015). While the EPH suggests that dental caries is the result of an imbalance in the microflora due to ecological stress, resulting in an enrichment of certain disease-related micro-organisms (Rosier *et al*., 2014).

According to the ecological hypothesis, changes in the environment can predispose a site to disease because of modifications in the composition of the resident microflora. This ecological concept is now the prevailing hypothesis to understanding the circumstances associated with the development of caries and periodontal diseases (Nyvad and Takahashi, 2020).

Microorganisms are the main agent responsible for causing dental caries. A number of aerobic and anaerobic bacteria dominate the microbial community of dental caries (Khushbu Yadav and Prakash, 2017). The two microbial species which are predominantly associated with dental caries are *S. mutans* and *S. sobrinus* which are frequently isolated from dental plaque. These species are found to produce large amounts of acids and extracellular polysaccharides and this, in turn, promotes dental caries (Bottner *et al*., 2020). *S. mutans* in addition to exhibiting the property of acidogenicity and acidurity also consume sucrose to produce insoluble glucans and this plays a vital role in the caries development (Poorni *et al*., 2022).

The involvement of *S. mutans* in caries development includes three phases. First, the bacteria can efficiently adhere to the surfaces of teeth and promote the formation of an extracellular, multi-dimensional structure known as a dental biofilm. Under health

condition, a wide diversity of oral microbes dwells in the dental biofilm and the number of caries promoting bacteria like *S. mutans* is maintained at low level. Second, when frequently exposed to fermentable carbohydrates in a diet, a large quantity of acidic products would be generated and released into the extracellular matrix and bacteria like *S. mutans* would be favored by lower pH environment in the biofilm and the growth of other oral bacteria would be inhibited. Third, once a dominant status of acid-producing and acid-tolerating bacteria like *S. mutans* is established within the biofilm, the acidic products would accumulate and promote the demineralization of tooth enamel, and eventually form dental caries (Kilian *et al*., 2016; Manji *et al*., 2018).

There are almost 85 species of fungi in the human mouth. The most prevalent fungus organism found on human mucosal surfaces, frequently contributes to producing polymicrobial biofilms on soft tissue and acrylic surfaces is *Candida species* (Nyvad and Takahashi, 2020).

The presence of *C. albicans* increased the production of exopolysaccharides (EPS), such that cospecies biofilms form more biomass and contain more viable *S. mutans* cells than single-species biofilms. The biofilm architecture showed that *S. mutans* microcolonies surround fungal cells (Khoury *et al*., 2020).

Several studies have revealed that the level of *S. mutans* is not necessarily high in caries-associated biofilms, suggesting that the microbial basis of this disease is subtler, and yet more complicated at the same time. Indeed, the increasing acid environment facilitates increased proportions of different *S. mutans* genotypes and other acidogenic species (Zhou *et al*., 2018).

Gram-positive bacteria (G + ve) including Streptococci of the mitis and mutans species (which are considered as initial colonizers of the biofilm) then form EPS, which enhance

the adherence of other organisms. Emerging evidence shows that acid-producing bacterial species of the genera *Veillonella*, *Scardovia*, *Lactobacillus*, and *Propionibacterium* could be present in the dental biofilm as colonizers and may induce in cariogenic conditions in the mouth (Obata *et al*., 2019; Widyarman and Theodorea, 2019).

The major EPS components in cariogenic biofilms are polysaccharides, particularly *S. mutans* -derived glucans as well as soluble glucans and fructans produced by other species (e.g., *Actinomyces*, *Streptococcus salivarius*, and *Streptococcus gordonii*) (Fakhruddin *et al*., 2019).

2.4 Causes of Dental Caries

Dental caries is an infectious disease that is characterized by a progressive demineralization process in hard tooth tissue. The pathogenesis of this disease involves various interacting and synergizing factors, namely hosts (teeth and saliva), agents (microorganisms), substrate (diet containing sugar), and time. Saliva, substrate, and bacteria form a layer of biofilm on the tooth surface which called dental plaque (Chismirina *et al*., 2021).

2.4.1 Host Factors (Tooth and Saliva)

The structure of tooth is very vital, several areas of the similar tooth are greatly at risk to get the infection than the others, probably due to differences in mineral composition (chiefly fluoride) (Men *et al*., 2016). An interaction between the salivary parameters such as calcium, inorganic salivary phosphate, salivary pH, and the dental caries has been established (Nicolae *et al*., 2016).

Saliva plays an important role in *S. mutans* biofilm formation. Oral Streptococci interact with the enamel salivary pellicle to form biofilm on tooth surfaces. Streptococcal cell wall components such as adhesins mediate adherence to various salivary molecules, especially sugars or oligosaccharides. Following initial adherence, bacteria grow and survive only if the physical and chemical environment such as pH, oxygen levels, and redox potential is conducive (Aqawi *et al*., 2021).

2.4.2 Agents (Microorganisms)

Mutans Streptococci (MS) have been commonly associated as major cariogenic bacteria. *S. mutans* is present in oral flora and has been demonstrated to be a causative specialist for dental caries because of its capacity to metabolize fermentable carbohydrate into organic acids. These acids can cause a fall in pH, which can lead to an increase of enamel solubility that is dental caries (Al-Shami *et al*., 2019).

2.4.3 Substrate

The role of the substrate in the pathogenesis of dental caries is very important because in the presence of the substrate, then the nutrients needed by bacteria to ferment through the process of glycolysis in which the byproducts in the form of acid are available. Acid produced from the fermentation process causes the plaque pH to drop below the critical point within 1-3 minutes. The decrease in pH over time will result in the dissolution of minerals in tooth enamel known as tooth demineralization. The process of demineralization that occurs continuously will result in the formation of white spots on the tooth surface which ultimately results in the formation of holes in the teeth. This condition is called dental caries (Khushbu Yadav *et al*., 2020).

2.5 Streptococci

Streptococci is considered as a large proportion of the resident oral flora. It is known that roughly one-quarter of the total cultivable flora from gingival and supragnigival plaque and half of the isolates from the tongue and saliva are Streptococci. They are vertically transmitted from mother to child, infective endocarditic 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 (Health *et al*., 2014).

Streptococci are G + ve with spherical or ovoid cells with a diameter of 0.5-2.0 µm that grow in pairs or chains. They are catalase-negative and most commonly facultative anaerobes that require a rich medium and occasionally 5% CO2 for growth. The temperature optimum for the bacteria is around 37 ºC, although the minimum and maximum temperature varies within the genus (Hulting, 2016) .

They are divided into three groups by the type of hemolysis on blood agar: beta-hemolytic (complete lysis of red cells), α-hemolysis (green hemolysis), and gamma-hemolytic (no hemolysis). Beta-hemolytic Streptococci are characterized as group A Streptococci (*Streptococcus pyogenes*) and group B Streptococci (*Streptococcus agalactiae*) (Kanwal and Vaitla, 2022).

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 are Mutans, Salivarius, Anginosus and Mitis group (Moreira *et al*., 2015).

Presently, oral Streptococci are found in all groups except the pyogenic and bovis groups. The mitis group is the largest of the groups found in the oral cavity, currently with 20 species. Species within the mitis group have been challenging to differentiate based on 16S RNA sequence alone, particularly *S. oralis* and *S. mitis*. Recent efforts to re-classify many of the species within this group indicate there are many strains that have been misclassified and new species descriptions may soon emerge (Jensen *et al*., 2016).

In particular, communities collected from dentin carious lesions contained notorious acidogenic (acid producing) and aciduric (acid tolerant) species, including *S. mutans*, *Scardovia wiggsiae*, *Parascardovia denticolens*, and *Lactobacillus salivarius* (Richards *et al*., 2017).

Mutans Streptococci (MS) are subdivided into seven different species and eight serotypes based on the DNA homology. Four serotypes of *S. mutans* have been reported, namely, serotype c, e, f, and k to be responsible for dental caries (Kavitha *et al*., 2019).

MS are prevalent in the oral cavities of humans and other animals and are accepted as the main causative bacteria of dental caries. Species of the mutans group that have been isolated from the human oral cavity are *S. mutans* (serotypes c, e, f, and k) and *S. sobrinus* (serotypes d and g). The MS isolated from animals are *Streptococcus criceti* (serotype a), *Streptococcus ratti* (serotype b), *Streptococcus macacae* (serotype c), *Streptococcus downei* (serotype h) and *Streptococcus ferus* (serotype c) (Okamoto *et al*., 2016).

About 70-80% of strains found in the oral cavity classified as serotype c, followed by e (approximately 20%), and f and k (less than 5% each) (Bedoya-Correa *et al*., 2019). A common biological feature of k serotype is its low cariogenicity due to the lack of important antigens related to the onset and development of dental caries. In contrast, it survives in blood for a longer duration due to its low antigenicity, which enables virulence

over a longer period of time and explains its association with the pathogenesis of certain cardiovascular diseases (Nomura *et al*., 2014).

There is a 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 MS (Yoshida *et al*., 2015).

Although culture-independent methods have been widely used to identify the presence and measure relative abundance of *S. mutans* in oral samples, the critical quantitative caries risk indicator remains to be that individual with more than 10^5 colony forming unit (CFU) /ml of salivary *S. mutans* are considered as high risk for caries (Fan *et al*., 2019). Therefore, isolation, identification and quantification of the *S. mutans* from oral samples (saliva, plaque and swab) using culture-dependent methods still present as essential components for clinical and epidemiological studies, in addition to other culture independent assays (Xiao *et al*., 2016).

Mitis-Salivarius with Bacitracin medium (MSBA), is usually used in identification of Streptococci. This media is capable for inhibiting the development of a large amount of other microorganism except Streptococci, since it has Trypan blue and crystal violet.These compounds could restrain the existence of Gram–negative bacteria, Trypan blue is captivated via colony of Streptococci resulting a navy dye in appearance. Modification of MS-agar via addition 0.2 U/ml bacitracin and mounting attentiveness of sucrose to 20 % leading to suppress increase of *S. sobrinus* and *S .cricetus* (Abeas *et al*., 2020).

On MS agar, *S. mutans* colonies are small, raised, irregularly margined and adherent, while *S. sobrinus* colonies are surrounded by a zooglea with a gelatinous consistency. On MSBA, most strains of *S. mutans* produce colonies of about 1 mm in diameter with beads, droplets or puddles containing soluble extracellular polysaccharide. On blood agar *S.*

mutans colonies are white or gray, circular or irregular, 0.5-1.0 mm in diameter, sometimes tending to adhere to the surface of the agar (Al-kazirragy, 2010).

S. salivarius digest sucrose and give abundant colonies that have shape like a "gum-drop" looks. *S. mitis* and Enterococcus haven't ability to take sucrose and, at the same time as, figureless significant results di-potassium phosphate be a buffer for preserving medium (MacFaddin, 2000).

S. mutans are mesophilic and grow at temperatures between 18-40 C (Javed *et al*., 2012). They are able to metabolize number of sugars and glycosides such as glucose, sucrose, fructose, lactose, galactose, mannose, cellobiose, glycosides, maltose and group of sugar alcohols, synthesizes intracellular glycogen like polysaccharides in the presence of extracellular sucrose and glucose (Khushbu Yadav, 2016).

2.6 *Streptococcus mutans*

S. 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). *S. mutans* was first described In 1924 by J. Kilian Clarke who found in deep dentin caries lesions small, chained coccobacillus, more oval than spherical in shape. He suggested that these microorganisms were mutant Streptococci and called them *Streptococcus mutans* (Grönroos, 2000). However, *S. mutans* received greater attention from the scientific community only in the late 1950s, and by the mid-1960s, it was recognized as a main aetiological agent in dental caries (Lemos *et al*., 2013).

All strains of *S. mutans* ferment glucose, lactose, raffinose, mannitol, inulin and salicin with rapid production of acid. The fact that the colonies of *S. mutans* closely adhere to the surface of the teeth appears to be of great importance (Gharajalar and Hassanzade, 2017).

S. mutans bacteria are facultatively anaerobic Gram-positive cocci that belong to the lactic acid-producing bacteria. Break down of sugars and production of lactic acids (acidogenicity) is one of the important virulence traits of *S. mutans*, thus causing tooth decay. In addition, *S. mutans* bacteria have an ATR (acidouricity) to combat the harmful acidic environment when exposed to sub lethal pH values (Abo Bakr *et al*., 2021).

S. mutans is a key contributor to the formation of the pathogenic dental biofilms, mainly due to its ability to synthesize extracellular polysaccharides such as water insoluble glucans or fructans by the action of GTFs and FTF (Aqawi *et al*., 2021). *S. mutans* also produces multiple Gbp, which are thought to promote adhesion to matrix glucans and to shape the overall architecture of the biofilm (Jakubovics *et al*., 2021). The natural habitat of *S. mutans* is the human mouth. It is mostly α or γ- hemolytic on sheep blood, but a few β- hemolytic strains (Zhou and Li, 2021).

2.7 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 (Health *et al*., 2014).

The most important virulence features associated with cariogenicity and distinguished of *S. mutans* strains from the other oral Streptococci isolated from the human oral cavity are: (1) capability of the bacteria to produce enormous amounts of organic acids from carbohydrate metabolism; (2) the potency of the bacteria to reside at lower pH

(aciduricity); and (3) the excellency to synthesis extracellular glucan homopolymers using sucrose and all these characters perform the acts like early adherence, colonization and buildup of biofilms on tooth surfaces (Lemos *et al*., 2013).

2.7.1 Bacterial Adhesion

Formation of the dental plaque occurs via complex multistage processes. The first step is the acquired pellicle formation Figure (2-2 A). The enamel surface surrounded by the hydration layer has negative charge because it has a high concentration of phosphate group. Cations such as calcium ions bind to the negative charge of the enamel surface, eventually changing the enamel surface to positive charge. In saliva, there are acidic proteins such as phosphoproteins and sulfate glycoproteins, which are negatively charged. Acidic proteins bind to the enamel surface via calcium ions to form acquired pellicle. Calcium ion acts as a bridge between the negative charge on the enamel surface and the negative charge of acid proteins. Oral bacteria are captured on the acquired pellicle of tooth surface Figure (2-2 B). Bacterial capture in the early stage is reversible attachment which is caused by ionic bond or van der Waals interaction (Huang *et al*., 2013).

The captured oral bacteria form irreversible adhesions to the tooth surface covered by the acquired pellicle Figure (2-2 B). Calcium bridge, hydrophobic interaction, polymer bridge, or covalent bond between the tooth surface covered with the acquired pellicle and bacterial surface are the main mechanisms of irreversible adhesion, and also cell surface adhesins of *S. mutans*, such as GBPs and SpaP, are important for bacterial adhesion to tooth surface. *S. mutans* carry *gbpA*, *gbpB* , and *gbpC* genes related to the adhesion. The *gbpA*, *gbpB*, and *gbpC* genes encode GBPA, B, and C, respectively, which are known to play an important role in the adhesion of *S. mutans* to glucan molecules, a kind of extracellular polysaccharide of plaque matrix (You, 2019).

In particular, the GBPC is involved in dextran (glucan)-dependent aggregation (DDAG) of oral bacteria (Kim *et al*., 2015). *S. mutans* also carry the *spaP* gene, which encodes the cell surface antigen, SpaP. The SpaP is also known as Ag I/II, PAc, AgB, Pl, Sr, SpaA, PAg, SspA, SspB and SoaA, which adheres to salivary agglutinin glycoprotein and proline-rich protein of the acquired pellicle on the tooth surface as a kind of surface fibrillar adhesion. The irreversible adhesion is followed by oral bacterial growth, division and then colonization (Jeong *et al*., 2013).

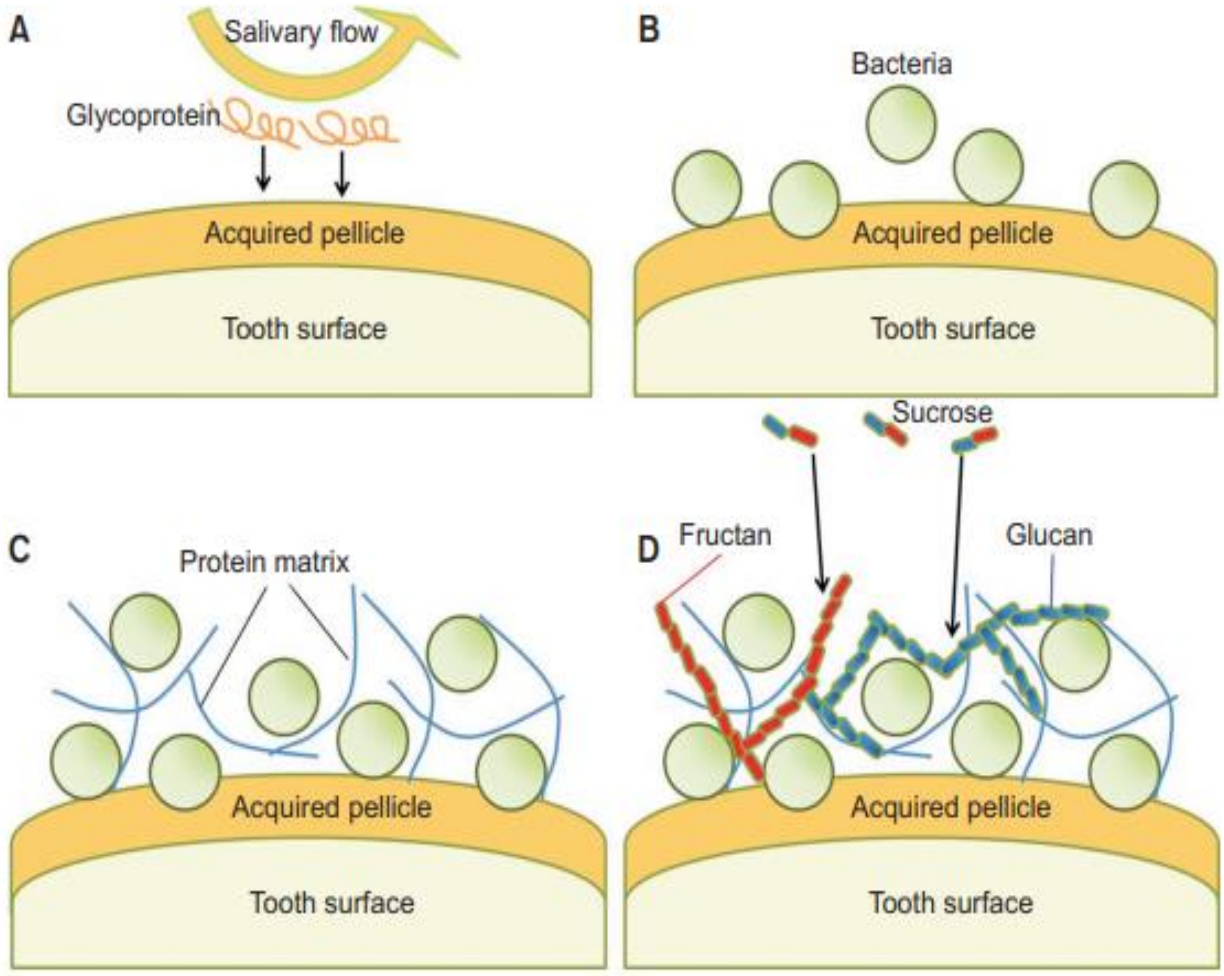


Figure (2-2): Dental biofilm formation. (A) Acquired pellicle formation. (B) Bacterial adhesion. (C) Protein matrix formation. (D) Extracellular polysaccharide formation (You, 2019).

2.7.2 Extracellular and Intracellular Polysaccharides Formation

Matrix of dental plaque is formed after colonization of the oral microorganisms figure (2-2 C). The plaque matrix can be formed in the absence of food. The plaque matrix is classified into protein matrix and extracellular polysaccharide matrix. *S. mutans* has *gtfB, gtfC, gtfD,* and *ftf* genes, each of which encode GTFs B, C, D, and FTF, respectively. After the protein matrix of the dental plaque is formed, *S. mutans* decomposes sucrose in the oral cavity into glucose and fructose using bacterial invertase, and then synthesizes glucan by polymerizing glucose using GTF. FTF synthesizes polysaccharides such as fructan by polymerizing fructose. The extracellular polysaccharides thicken and harden the dental plaque and lower the oxygen permeability, and play a decisive role in dental plaque maturation figure (2-2 D) (Shemesh *et al*., 2007).

Another virulence property which is shown by half the MS species (*S. mutans*, but not *S. sobrinus*) is the ability to synthesize intracellular polysaccharides (IPS). The storage carbohydrates are metabolized by *S. mutans* to produce acid, which enhances cariogenicity by maintaining an acidic pH (<5.5) in the environment (Bao *et al*., 2015). This property of *S. mutans* permits continual acid production after dietary carbohydrates have been depleted or during periods of low concentration of exogenous substrate. This activity maintains acidogenicity and fosters enamel demineralization during periods of low salivary secretion during sleep (Costa Oliveira *et al*., 2017).

2.7.3 Biofilm Formation

One of the most important virulence properties in *S.mutans* is an ability to form biofilm on tooth surfaces known as dental plaque (Matsumoto-Nakano, 2018). The dental plaque formed in oral cavity is a mixed population of biofilms made by various bacteria combining themselves. Oral Streptococci species such as *Streptococcus sanguinis*, *S.*

mutans, *Streptococcus gordonii*, *Streptococcus mitis*, *and Streptococcus oralis* play the important role in the formation of supragingival plaque and dental caries. *Actinomyces* species such as *Actinomyces viscosus* are closely related to the development of dental plaque and dental caries on the root surface (Larsen and Fiehn, 2017).

These bacteria combine with the protein matrix formed by the oral bacteria and the extracellular polysaccharides such as glucan and fructan to form the dental plaque on the tooth surface. The *relA* gene carried by *S. mutans* encodes RelA, which is known to regulate the formation of biofilm and to contribute to quorum-sensing (Lemos *et al*., 2004).

Among the genes carried by the *S. mutans*, *smu630* is reported to be important for sucrose dependent and sucrose-independent dental biofilm formation (Shemesh *et al*., 2007). The *brpA* gene in *S. mutans* is also known to regulate biofilm formation (Steinberg *et al*., 2008). The *comD* gene encodes a histidine kinase receptor and the *comE* gene encodes a cognate response regulator of the competence-stimulating peptide, which are part of the quorum-sensing cascade of *S. mutans* (Steinberg *et al*., 2008).

2.7.4 Carbohydrates Fermentation (Sugar Uptake and Metabolism)

S. mutans bacterium is considered more rapid than other oral microorganisms which ferment many different sugars and produce the large quantities of organic acids (acidogenicity) (Marsh *et al*., 2015). Oral bacteria uptake sugars that are consumed in food and metabolize them with glucose, then use glucose to get the necessary energy via glycolysis and fermentation, and produce organic acids as metabolic products (Yang *et al*., 2016).

S. mutans are capable of maintaining their glycolytic activity under this condition, which provides them a competitive advantage over other oral Streptococci and assists them to dominate in the dental plaque resulting in initiation and progression of dental caries (Matsui and Cvitkovitch, 2010).

The *relA* gene of *S. mutans* encodes RelA, which is known to contribute to the regulation of phosphoenolpyruvate: carbohydrate PTS, the glucose uptake system (Lemos *et al*., 2004). The *eno* gene of *S. mutans* encodes the bacterial enolase, which is a major component of the PTS in the bacteria, and is known to contribute to bacterial sugar uptake. In addition, the *ldh* gene of *S. mutans* encodes lactic acid dehydrogenase, which contributes to lactic acid formation (Xu *et al*., 2011).

2.7.5 Acid Tolerance

Additional virulence properties of *S. mutans* include its ability to tolerate environmental changes, such as low pH (aciduricity) (Cohen-Berneron *et al*., 2016). 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 (Dinesh *et al*., 2016).

Oral bacteria metabolize the carbohydrates in the food to produce organic acids. Then the surrounding environment becomes acidic because the proton concentration is increased. *S. mutans* can survive and grow in the acidic environment because of its several genes enabling to overcome acidic environment. The *atpD* gene in *S. mutans* encodes the F1F0-ATPase. F1F0-ATPase is a proton pump that discharges H+ from within the bacteria to the outside, to overcome acid stress and maintain acid tolerance (Lemos and Burne, 2008).

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 (Chakraborty and Burne, 2017). In addition, *aguD* encodes the agmatine deiminase system (AgDS), which produces alkali, enabling to overcome acid stress and maintain acid tolerance. The *brpA* gene and *relA* also contribute to acid tolerance (Xu *et al*., 2011).

2.7.6 Production of Mutacin

One of the principal virulence factors of *S. mutans* is production of bacteriocins (peptide antibiotics), referred to as mutacins (Matsumoto-Nakano, 2018). The increase in bacterial resistance to antibiotics impels the development of new anti-bacterial substances. Mutacins (bacteriocins) are small antibacterial peptides produced by *S. mutans* showing activity against bacterial pathogens (Salh, 2014).

Bacteriocins are important factors allowing these oral bacteria to outcompete others in the acquisition and/or maintenance of particular niches (Le *et al*., 2022). Bacteriocins are synthesized by ribosomes as precursor peptides with a signal peptide at the N-terminus, typical of secreted proteins ,which is cleaved concomitantly with the export of the mature peptide across the membrane. The production of bacteriocins by G + ve is generally associated with the transition from log phase to stationary phase of bacterial growth or the cell density in the culture medium (Tajbakhsh *et al*., 2017).

Kamiya *et al*., (2011) divided the bacteriocins from *S. mutans* into two groups according to their molecular weight and sensitivity to heat. The first group was inactivated with heating at 80C° for 20 minute and possessed a molecular mass less than 10,000 Da. The second group was composed of small molecules (more than 10,000 Da) and heat stable, but the spectrum of activity, sensitivity to either, chloroform and the trypsin varied considerably within the same group.

Bacteriocins produced by G+ve are peptide antibiotics classified as class I (lantibiotics) or class II (non-lantibiotics), based on their posttranslational modifications, in *S. mutans*, both classes of bacteriocins are produced. The lantibiotic mutacins have a wide spectrum of activity against G + ve, including multipledrug-resistant pathogens, while the non-lantibiotic mutacins are more specific to closely related streptococcal species such as the mitis group Streptococci and group A Streptococci (El Issaoui *et al*., 2020).

The mutacin activity of *S. mutans* may facilitate the transmission of the species between mother and child and increase the ratio of this species in the dental biofilm, contributing to increased risk of caries (Carletto-Körber *et al*., 2010). Mutacins could play an important biological role in the regulation and composition of dental biofilm due to their synergistic or antagonistic activity, suggesting that wide spectrum mutacins may be more important in the colonization and stabilization of this cariogenic species, mainly in the stable niche of highly complex microbial activity (Napimoga *et al*., 2005).

2.7.7 Proteases

Streptococci have an array of virulence factors, which include surface proteins for adhesion, invasion/internalization, extracellular enzymatic proteases, and toxins delivered to cell surface as well as extracellular environments, which are associated with their colonization at various sites in the human body, dissemination, evasion from immune

system for survival, destruction of host tissues, and modulation of the host immune function (Yumoto et al., 2019).

S. mutans proteases contribute to virulence and are involved in the breakdown of host proteins for bacterial nutrition and the direct degradation of host structural proteins (Karygianni *et al*., 2020). *S. mutans* has been shown to produce two extracellular proteases, capable of degrading both gelatin and collagen-like substrates (Jotshi and Kanekar, 2019).

Streptococcal proteases may also be involved in the destruction of some components of the host immune system (Lindsay *et al*., 2017). Many oral Streptococci produce sIgA protease, which impairs the host defence by cleaving the secretory IgA (Mulks, 2018).

2.8 Antibiotics Sensitivity of *S. mutans*

Expanding resistance of bacterial pathogens to regularly utilize antibiotics has turned into general human concern. The spread of antibiotic resistance is causing fatalities, as well as a high financial inconvenience. In low economic nations, antibiotic resistance is considered to be more prevalent than in the developed countries (Kapi, 2014).

In the present clinical scenario globally, there is a great interest in the use of antimicrobial agents for prevention and treatment of dental caries due to the spread of antibiotic resistance (Mulks, 2018). The biofilm protects the bacteria from environmental stress stimuli. Sessile cells embedded in the biofilm are up to 1000 times more resistant to antibiotics than cells in their planktonic state (Aqawi *et al*., 2021).

This striking difference is due to the different resistance mechanisms of biofilms. Microbial resistance is mainly of two types, intrinsic and acquired resistance. Intrinsic or

innate resistance is the natural chromosomally determined resistance and physiological adaptation which is specific for a particular microorganism. Acquired resistance refers to the resistance resulting from mutations and the selection of resistant mutants from a population exposed to antimicrobial agents, or due to the incorporation of plasmids or tansposons, which results in resistance to antimicrobials (Mangalea and Duerkop, 2020).

Salman and Senthikumar, (2015) showed that all *S. mutans* and *S. sobrinus* isolates were susceptible to *β*-Lactam antibiotics (penicillin, ampicillin, cefotaxime, cephalothin, cefazolin and methicillin) and non *β*-Lactam antibiotic (erythromycin and chloramphenicol).

Penicillin is effective against strains of the gram-positive Streptococci and Staphylococci, as well as some gram-negative bacteria. The first use of penicillin to treat dental caries dated from 1946, when McClure and Hewitt reported that penicillin inhibited caries in rats (Qiu *et al*., 2020).

Marron *et al*. (2001) studied the susceptibility of *S. mutans* strains, were resistant to erythromycin, imipenem, ciprofloxacin, ceftriaxone, ceftazidime, cefpirome and cefepime. Ready *et al*. 2002 indicated that *S. mutans* showed different levels of resistance to erythromycin, vancomycin, ampicillin and tetracycline.

The emergence of resistance against newly developed antibiotics further supports the require for monitoring of antibiotic consumption, prevention, diagnosis and rapid reduction in the misuse of these drugs (Cassir et al., 2014).

2.9 Molecular Detection of *S. mutans*

Dental caries has a polymicrobial etiology with *S. mutans* being the major pathogen. Conventionally, studies of *S. mutans* have relied heavily upon cultivation to identify and characterize *S. mutans* in the oral cavity (Sorensen ,*et al*., 2010). The major limitations of culture methods include a finite threshold of detection of *S. mutans* in clinical samples; an inconsistent morphology depending on the culture medium used; and its high cost and labor intensiveness. Moreover, cultivation requires viable samples, making its application in field epidemiological studies and high-throughput research impractical (Poster *et al.*, 2010).

Several molecular study has recently developed for promoting identification of target bacterial species isolates. PCR amplification methods were widely used for detection of specificity of these species and considered as a reliable diagnostic tool for investigation bacterial isolates (Al-mohammadawy *et al*., 2018). A lot of the specific primers were directed to specific genes that are related with virulence in *S. mutans*, *Sm479* is species – specific gene has widely used, showed elevated rank of effectiveness for identification and evaluation of *S. mutans* in dental caries specimen (Salehizadeh, 2016).

In a study conducted by (Class, 2009), it was demonstrated that primer set(s) directed against Sm479F/Sm479R can accurately and rapidly identify and measure *S. mutans* in clinical samples (either purified or mixed DNA samples) or in subjects. These species-specific primers and probes can be used to conduct high-throughput epidemiological studies, monitor, and evaluate *S. mutans* infection and *S. mutans* response to prevention and treatment. The targeted segment comprises a portion of the htrA locus and a part of an intergenic locus of the *S. mutans* genome. The final size of the PCR amplicon is 479 base pairs.

CHAPTER

THREE

MATERIALS

AND

METHODS

3. Materials and Methods

3.1 Materials

3.1.1 Laboratory Apparatus and Equipments

The Laboratory apparatus and equipments have been used in this study are listed in table (3-1).

Table (3-1): Laboratory Apparatus and Equipments

NO	Apparatus and Equipments	Company	Company
1.	Autoclave	Gemmy	Taiwan
2.	Burner	Amal	Turkey
3.	Candle – jar	BBL	France
4.	Centrifuge	MSE	England
5.	Cooling box	Shanghai Blopak Co. Limited	China
6.	Cotton	Medical ject	Syria
7.	Disposable Petri dishes	AFCO	Jordan
8.	Dissecting Microscope	Leitz-Wetzlar	Germany
9.	Distillator	Ogawa	Japan
10.	Electric sensitive balance	Sartorius	Germany
11.	Electrophoresis Device	Bio-Red	U.S.A.

12.	Eppendorf Centrifuge	Eppendorf	Germany
13.	Eppendorf tube	Fisher Scientific	U.S.A.
14.	Excavator	Supreme	China
15.	Finn tips with different sizes(20µl, 100 µl, 500 µl, 1000 µl)	Eppendorf	Germany
16.	Gel documentation system	UVP	U.S.A.
17.	Glass tubes	Fisher	U.S.A.
18.	Hood	Bio LAB	Korea
19.	Hotplate / Stirrer	Biocote	England
20.	Incubator	Binder	Germany
21.	Light microscope	Olympus	Japan
22.	Magnetic stirrer	Gallenkamp	England
23.	Micropipettes (Automatic) (0.5-10 µl , 20-200 µl , 100-1000 µl)	Dragonlab	China
24.	Microtiter plate reader	Memmert	Germany
25.	Microwave	Sanyo electric	Japan
26.	Millipore filter unit 0.80 µm, 0.45 µm, 0.22 µm	Chm	U.S.A.
27.	Camera	Samsung	Vietnnam
28.	Nanodrop	Optizen	Korea

29.	Oven	Memmert	Germany
30.	Para film	BDH	England
31.	PCR tube	Eppendorf	Germany
32.	pH meter	Sartorius	Japan
33.	Plain tube	AFCO	Jordan
34.	Platinum Wire Loop	Himedia	Indian
35.	Power supply	Bio-Red	U.S.A.
36.	Refrigerator	Marubeni	Japan
37.	Rucks	Shanghai Blopak Co. Limited	China
38.	Screw capped test tubes	BBL	U.S.A.
39.	Shaker-incubator	Sartorius	Japan
40.	Slides and Cover slide	Sail Brand	China
41.	Thermal cycler	Bioneer	Germany
42.	Transport swabs	AFCO	Jordan
43.	UV transilluminator	Wise	U.S.A.
44.	Vitek 2 system	Biomerieux	France
45.	Volumetric flasks	Jlassco	India

46.	Vortex	Eppendorf	Germany
47.	Water bath	Memmert	Germany
48.	Wooden sticks	Supreme	China

3.1.2 Chemical and Biological Materials

The chemical and biological materials used in this study are listed in table (3-2).

Table (3-2): Chemical and Biological Materials

NO	Chemical and biological materials	**Company**	**Origin**
1.	Absolute ethanol (99%)	Fisher	England
2.	Acetone	BDH	England
3.	Agarose powder	Bio Basic	Canada
4.	Bacitracin	AppliChem	Germany
5.	Barium chloride	Hopkins and Williams Limited	England
6.	Bromophenol blue	Fisher	U.S.A.
7.	Deionized water	Sigma	U.S.A.
8.	EDTA (Ethylene Diamine-Tetra- Acetic acid)	BDH	England
9.	Ethyl ether	BDH	England

10.	Glucose	Fluka	Switzerland
11.	Glycerol	Riedeel-deHáen	Germany
12.	Gram stain	HIMEDIA	India
13.	Human Blood	Imam Sadiq Teaching Hospital	Iraq
14.	Hydrogen peroxide H2O2 30%	BDH	England
15.	Mannitol, sorbitol, raffinose, meilibiose, sucrose	BHD	UK
16.	Methyl red	BDH	England
17.	Normal Saline solution	S.D.I	Iraq
18.	Nuclease free water	Promega	U.S.A.
19.	Oxidase indicator (N,N,N,N-tetramethyl-ρ-phenylenediamine	HIMEDIA	India
20.	Potassium tellurite	Himedia	India
21.	Safe red stain	Fisher	U.S.A.
22.	Sodium chloride	Sigma-Aldrich	Switzerland
23.	Tris-base	Merck	Germany
24.	Tris-Borate EDTA buffer (TBE)	Promega	U.S.A.
25.	Urea Solution	SD-Fine	India

3.1.3 Culture Media

The bacterial culture media used in this study were listed in table (3-3).

Table (3-3): Culture Media

NO	Materials	Company	Origin
1.	Agar agar powder	HIMEDIA	India
2.	Blood agar		
3.	Brain heart infusion agar(BHIA)		
4.	Brain heart infusion broth(BHIB)		
5.	Carbohydrate fermentation media		
6.	MacConkey agar		
7.	Methyl Red-Voges Proskauer(MR-VP) broth		
8.	Mitis – Salivarius agar		
9.	Muller Hinton agar		
10.	Nutrient agar		
11.	Nutrient Broth		
12.	Simmon’s citrate agar		
13.	Tryptic Soy broth (TSB)		
14.	Urea agar base		

3.1.4 Materials and Kits used for Molecular Diagnostic

The materials and kits used in this study for molecular diagnostic were listed in table (3-4).

Table (3-4): Molecular Diagnostic Materials and Kits

NO	Kit	Company	Origin
1.	DNA ladder marker (100-10000) bp	Cleaver	UK
2.	Genomic DNA Extraction Kit	Geneaid	U.S.A.
3.	Green Master Mix 2X	Promega	U.S.A.
4.	Primers for Sm479, gbpA, gbpB, comD, comE, relA and spaP genes in bacteria	Macrogen	Korea

3.1.5 Contents of Genomic DNA Extraction Kit

The contents of Genomic DNA Extraction Kit (Geneaid,USA) used in this study were listed in table (3-5)

Table (3-5): Contents of Genomic DNA Extraction Kit

NO	Contents
1.	Collection tube 2ml
2.	Elution buffer
3.	GB buffer
4.	GD column
5.	GT buffer
6.	Lysozyme
7.	proteinase k
8.	W1 buffer
9.	Wash buffer

3.1.6 Contents of Green Master Mix

The master Mix contents (Promega, USA) used in the study were listed in table (3-6).

Table (3-6): Contents of Master Mix

NO	Materials
1.	dNTPs (400µm dATP, 400µm d GTP, 400µm dCTP and 400µm dTTP)
2.	$MgCl_2$ (3mM)
3.	Reaction buffer (pH 8.3)
4.	Taq DNA polymerase

3.1.7 Primers

The Primers sequences used in the this study for genes amplification were listed in table (3-7).

Table (3-7): Primers Sequences Used for Genes Amplification

Genes	Primer sequence 5 to 3		Product	References
Sm479	F	TCGCGAAAAAGATAAACAAACA	479 bp	Al-mohammadawy *et al*., 2018
	R	GCCCCTTCACAGTTGGTTAG		
gbpA	F	GGTGGTTCTGTGCCTGATGA	162 bp	Aqawi *et al*., 2021
	R	TTGCCAGCCTGATACACGTT		
gbpB	F	AGCAACAGAAGCACAACCATCAG	150 bp	Abo Bakr *et al*., 2021
	R	CCACCATTACCCCAGTAGTTTCC		
comD	F	TGAAAATAGCATAGGTGAGTCAAAG	268 bp	Aqawi *et al*., 2021
	R	ATTTAGGTTAGCTGATTAACACTATACAC		

comE	F	CACAACAACTTATTGACGCTATCCC	127 bp	Aqawi *et al*., 2021
	R	TGATTGGCTACTTCCAGTCCTTTC		
relA	F	ACAAAAAGGGTATCGTCCGTACAT	101 bp	
	R	AATCACGCTTGGTATTGCTAATTG		
spaP	F	GACTTTGGTAATGGTTATGCATCAA	101 bp	
	R	TTTGTATCAGCCGGATCAAGTG		

3.1.8 Antibiotics

The following antibiotic used in the this study were listed in table (3-8) and table (3-9).

Table (3-8): Antibiotic Used by Disk Diffusion Method (DDM)

NO	**Antimicrobial Agent**	**Abbreviation**	**Disk Content µg**	**Inhibition zone diameter (mm)**			**Company/ Origin**
				S	**I**	**R**	
1.	Azithromycin	AZM	15	≥ 18	14–17	≤ 13	Bioanalyse/ Turkey
2.	Cefepime	CPM	30	≥ 24	22–23	≤ 21	Himedia/ India
3.	Cefotaxime	CTX	30	≥ 28	26–27	≤ 25	Bioanalyse/ Turkey

4.	Ceftriaxone	CTR	30	≥ 27	25–26	≤ 24	Himedia/ India
5.	Chloramphenicol	C	30	≥ 21	18–20	≤ 17	Himedia/ India
6.	Clarithromycin	CLR	15	≥ 21	17–20	≤ 16	Himedia/ India
7.	Clindamycin	CD	2	≥ 19	16–18	≤ 15	Himedia/ India
8.	Erythromycin	E	15	≥ 21	16–20	≤ 15	Condalab/ Spain
9.	Levofloxacin	LEV	5	≥ 17	14–16	≤ 13	Bioanalyse/ Turkey
10.	Linezolid	LZ	30	≥ 21	–	–	Himedia/ India
11.	Ofloxacin	OF	5	≥ 16	13–15	≤ 12	Himedia/ India
12.	Tetracycline	TE	30	≥ 23	19–22	≤ 18	Roseto /Italy
13.	Vancomycin	VA	30	≥ 17	–	–	Bioanalyse/ Turkey

Table (3-9): Antibiotic Used by Minimum Inhibitory Concentration (MIC)

NO	Antimicrobial Agent	Abbreviation	MIC Breakpoints, µg/mL			Company/ Origin
			S	I	R	
1.	Ampicillin	AMP	≤ 0.25	0.5–4	≥ 8	Himedia/ India
2.	Doripenem	DOR	≤ 1	–	–	
3.	Ertapenem	ETP	≤ 1	–	–	
4.	Penicillin	P	≤ 0.12	0.25–2	≥ 4	

3.2. Methods

3.2.1 Preparation of Culture Media

All media have been prepared according to the manufacturer instructions (Hemedia, India). After preparation and sterilization by autoclave at 15 lbs pressure (121ºC) for 15 minutes, each medium poured in sterile plates and incubated overnight at 37 ºC. Then, these plates stored at 4 ºC until use (Abeas *et al*., 2020).

3.2.1.1 Blood Agar Medium

This medium has been prepared according to the manufacturer instructions by dissolving 40 gm of blood agar base in 1000 ml D.W. Afterward sterilized by autoclave, cold to 45ºC and 5% of fresh human Blood was added. It was used as enrichment medium for the bacterial isolates and to determine their ability to hemolysis RBCs (Forbes *et al*., 2007).

3.2.1.2 Brain Heart Infusion (BHI) Broth

This medium has been prepared according to the manufacturer instructions by dissolving 37gm in 1 liter of distilled water (D.W) and sterilized by autoclave. It was used for developing and activating bacteria as well as for preserving bacterial isolates by adding 15% glycerol to 85% of the liquid medium after sterilization by autoclave. Also this medium was used as transport media to transfer the samples to the laboratory (Forbes *et al*., 2007).

3.2.1.3 Brain Heart Infusion Agar

Brain-heart infusion agar was prepared according to the manufacturing company by dissolving 52 gm in 1000 ml of D.W. The medium was sterilized by autoclave and used for detection of different biochemical tests (MacFaddin, 2000).

3.2.1.4 Nutrient Broth

This medium was prepared according to the manufacture company by dissolving 13 gm in 1000 ml of D.W and sterilized by autoclave. It is used for activation the isolates (MacFaddin, 2000).

3.2.1.5 Nutrient Agar Medium

Nutrient agar medium was prepared according to the manufacturer instructions by dissolving 28 gm of nutrient agar in a liter of D.W and sterilizing by autoclave. It has been used to preserve and sustain bacterial isolates for a short period of 2-3 weeks and in DNA extraction, by culturing the bacteria onto the medium for 24 hours, and then extracted from this medium (MacFaddin, 2000).

3.2.1.6 Muller-Hinton Agar (MHA)

Muller-Hinton agar was prepared according to the manufacturing company (38gm/L). It was used in anti-bacterial susceptibility testing (MacFaddin, 2000).

3.2.1.7 MacConkey Agar Medium

MacConkey agar medium has been prepared according to the manufacturer instructions by dissolving 49.53 gm of MacConkey agar in 1000 ml D.W and sterilizing by autoclave. It is used for primary isolation of most G-ve bacteria and differentiation of lactose fermentation from the non-lactose fermentative (Collee *et al*., 1996).

3.2.1.8 Sugar Fermentation Medium

This medium involves :

1- Medium base: 100 ml of BHI broth was mixed with 0,0082 gm of phenol red as indicator, the pH was adjusted to7.4, then this media sterilized by autoclave.

2- Sugar solution: 1gm of each of the following sugars (mannitol, sorbitol, raffinose, melibiose, and sucrose), were added to the broth separately and sterilized by filtration with Millipore filter, later poured into sterile plain tubes (Forbes *et al*., 2007).

3.2.1.9 Urea Agar Medium

This medium had been prepared by adding 5ml of urea solution (40% sterilized by Millipore filter) in a volume of 95 ml autoclaved urea agar base after cooling to 45ºC. The pH was adjusted to 7.1 and the medium was distributed into sterilized test tubes and allowed to solidify in a slant position. It was used to test the ability of bacteria to produce urease enzyme (MacFaddin, 2000).

3.2.1.10 Simmon's Citrate Medium

This medium was prepared by dissolving 24.28 gm of medium in 1000 ml D.W, and then sterilized by autoclave. Simmon's Citrate medium was used for determining the ability of bacteria to utilize citrate as the sole carbon source (MacFaddin, 2000).

3.2.1.11 Motility Medium (Semi-solid Medium)

This medium was prepared by adding 4 gm of agar to 100 ml of brain-heart infusion broth and completed to1000 ml with D.W. It was then sterilized by autoclave. It was distributed in tubes. This medium was used to detect bacterial motility (MacFaddin, 2000).

3.2.1.12 Tryptic Soy Broth (TSB)

This medium was prepared according to the manufacturer's instructions by dissolving 8 gm in 1000 ml of D.W. TSB with 1% glucose has been used to detection of the bacterial ability to produce biofilm by tissue culture plate (TCP) method (biofilm assay) based on the methods described by (Christensen *et al*., 1985; Mathur *et al*., 2006).

3.2.1.13 Mitis-Salivarius Agar (MSA) Medium

This medium was prepared according to the manufacturer's instruction by dissolving 90.07 g of MSA in 1000 ml purified D.W, and heat to dissolve the medium completely. Afterwards sterilized by autoclave, and left to cool until 45-50 ºC and aseptically add 1ml of sterile 1% Potassium Tellurite solution. Mix well and pour into sterile petri plates. This medium is the main components of the prepared media that suppress the growth of most microorganisms but allows the growth of *Streptococcus* Spp.

3.2.1.14 Mitis-Salivarius Bacitracin Agar (MSBA) Medium

This medium is the selective medium for the cultivation of *S. mutans*. It was prepared by addition of selective agents: bacitracin antibiotic (200 IU/L) and sucrose 20% (w/v) to the Mitis–Salivarius agar (MSA) medium. After the sterilization of the MSA medium till cooling, bacitracin antibiotic solution and sucrose solution were added (Geigy, 1962; Abeas *et al*., 2020).

3.2.2 Preparation of Reagents and Solutions

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 (Forbes *et al*., 2007).

3.2.2.2 Oxidase Reagent

It has been prepared by dissolving 1 mg of N, N, N, N-tetramethyl-ρ phenylenediamine dihydrochloride in 100 ml of D.W. Then it was stored in a dark bottle and used immediately (Forbes *et al*., 2007).

3.2.2.3 Methyl Red Indicator

Methyl red indicator was prepared by dissolving 0.1gm of methyl red powder in 300 ml of 95% ethanol and then the volume is completed to 500 ml D.W. It was use to indicate the complete glucose hydrolysis (MacFaddin, 2000).

3.2.2.4 Vogas-Proskauer (VP) Reagent

This reagent was composed of two solutions:

VP1: α-naphthol solution that is prepared by dissolving 5 gm of α-naphthol in 100 ml of (95%) ethanol, kept in dark bottle, and the solution mixed before use.

VP2: 40% Potassium Hydroxide solution that is prepared by dissolving 40 gm of KOH in 100 ml of D.W and the solution mixed before use (MacFaddin, 2000).

3.2.2.5 Bacitracin Solution (200 I.U/ liter)

A bacitracin stock solution is prepared by dissolving 0.2661gm of bacitracin powder in 100 ml of de-ionized water. This will supply concentration of 200 IU /L (1 unit of bacitracin = 0. 0133 mg). Millipore filter (0.4μm) was used to sterilize bacitracin solution. Then it was stored in a dark bottle in a refrigerator. A new fresh solution was prepared every 2-3 weeks (Abeas *et al*., 2020).

3.2.2.6 Potassium Tellurite 1%

The buffer is prepared by dissolving 1gm of Potassium Tellurite at 90 ml sterile D.W, then the volume is completed to 100 ml and sterilized by millipore filter and preserved at 4 ºC.

3.2.2.7 Turbidity Standard (McFarland)

Turbidity standard [McFarland solution (No. 0.5)] was prepared according to (Citron *et al*.,1990). 0.5 ml of 1.175% (w/v) barium chloride dehydrate (BaCl2.H2O) was added to 99.5 ml of 1% sulfuric acid in a graduated cylinder, 10 ml of the mixture was put in a sterile test tube and was stored in the dark place at room temperature. The absorbance was measured by a spectrophotometer at a wavelength of 600 nm. The acceptable absorbance range for the standard is 0.08 - 0.13. This solution was used to determine the number of bacterial cells before using an antibiotic susceptibility test using 10 isolates.

3.2.3 Preparation of Molecular Materials

3.2.3.1 Preparation of 1X TBE Buffer

The preparation of 1X TBE buffer was performed by dilution of a concentrated 10X TBE buffer, this dilution was accomplished as 1:10 (v/v); 1 volume of 10X TBE: 9 volumes of D.W. This solution was used to prepare agarose gel and as a transmission buffer in electrophoresis process. Thus each 100ml of 10X TBE added to 900 ml of sterile distal water to produce final concentration,1X TBE (Green and Sambrook, 2012).

3.2.3.2 Preparation of Agarose Gel

The agarose gel was prepared by dissolving 1gm agarose to 100ml of 1X TBE buffer (10 ml completed with 90 ml distal water). The solution was heated to boiling (using a microwave) until all the gel particles dissolved, the solution was allowed to cool down within 50-60 ºC, and mixed with 3µl of safe red stain (Green and Sambrook, 2012).

3.2.3.3 Lysozyme Solution

It was prepared according to the manufacturer's instructions (Geneaid,USA) by adding 0.8 mg weight of lyophilized lysozyme in G + buffer in a volume of 200 µL and it is stored at -20°C. Lysozyme solution is used for extract DNA.

3.2.3.4 Proteinase K

According to the manufacturer's instructions (Geneaid,USA) 11 mg of lyophilized proteinase K was dissolved in 1.1 mL of free deionized D.W, use as a stock solution and store at 4°C. Proteinase K is commonly used in molecular biology to digest protein and remove contamination from preparations of nucleic acid.

3.2.3.5 Solutions of Primers

3.2.3.5.1 Stock Solution (100 picomole/microliter)

All lyophilized primers were dissolved in 300µl nuclease free deionized D.W except comD-R. It was dissolved in 290µl nuclease free deionized D.W, depending on the information of primers manufacture (Macrogen/Korea), then vortex and stored at -20°C until use.

3.2.3.5.2 Working Solution

This solution was prepared by mixing of 10 µl of stock solution (100 pmol.µl-1) with 90 µl of nuclease free deionized D.W and stored at-20°C until use.

3.2.4. Specimens Collection

The study included collection of 100 samples of adults with dental caries aged 18-67 years of both sex . The study samples were recruited from patients attended to the teaching dental clinics at the College of Dentistry of Babylon University and Specialized Center for Dentistry in Babylon within the period of November 2021 to January 2022. The samples were obtained from the outermost layer of carious dentin and removed with a sharp, sterile excavator (Aas *et al*., 2008). Then the samples were transferred to the laboratory and were cultured on different media and were subjected to biochemical tests, microscopic examination and molecular diagnosis to isolate and diagnose *S. mutans* bacteria as shown in study design [figure (3-1)]. Subjects with a history of diabetes, malignant diseases, immunodeficiency, pregnant women and subjects who took antibiotics were excluded. The number of subjects who smoked was 46 and all were men. Informed consent was obtained from all persons.

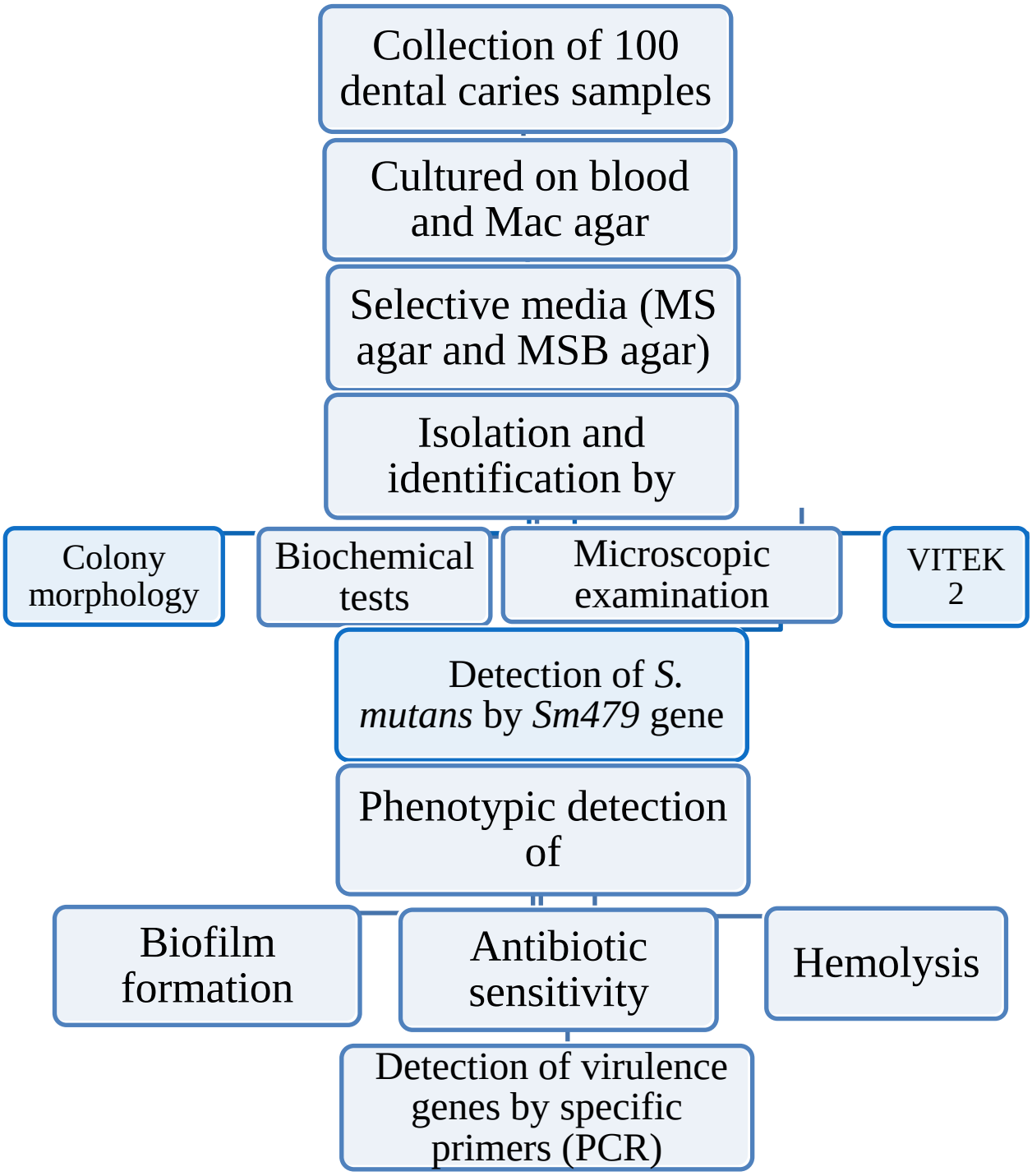


Figure (3-1): Study Design

3.2.5 : Laboratory Diagnosis of Streptococci

3.2.5.1: Isolation of Streptococci

After collection of dental caries samples, the samples were placed in plain tube containing 5ml of BHI broth, then transferred to the microbiology laboratory within 10-15 min, and incubated at 37°C for 24 h., the growth was inoculated onto the surface of blood agar plates by streaking, then the plates were incubated at 37°C for 24-48 h under anaerobic conditions in candle jar. Single colony was taken from each primary positive culture on blood agar, then cultured onto MSA and MacConkey agar (to exclude gram-negative bacteria), and incubated at the same conditions. The pure bacterial isolates obtained from MS agar were cultured onto MSB agar, and incubated at the same conditions for isolation of *S. mutans* (Al-Jumaily *et al*., 2014).

3.2.5.2 Identification of Streptococci

After estimation of positive sample on the surface of MSA and MSBA. Its identification depends on the morphology properties (colony size, shape, color, edge, pigment and texture). The colony was then investigated by Gram stain to observe bacterial shape and reaction. Specific biochemical tests were done to reach to the final identification (Forbes *et al*., 2007).

3.2.5.3 Biochemical Tests

The following biochemical tests were performed for identification of *S. mutans* isolates from other isolates.

3.2.5.3.1 Catalase Test

A colony of organisms was transferred by sterile wooden stick to a clean, dry slide and mixed with few drops of 3% H2O2. The evolution of bubbles of gas indicates for positive test (Forbes *et al*., 2007, Balay *et al*., 2017; Cappuccino and Welsh, 2018).

3.2.5.3.2 Oxidase Test

A filter paper was moistured with few drops of prepared oxidase reagent 1%, and then a small portion of the colony to be tested was picked up and rubbed on the moistened filter paper, changing the color to blue or purple within 30 seconds indicated for a positive test. (Baron *et al*., 2003, Balay *et al*., 2017; Cappuccino and Welsh, 2018).

3.2.5.3.3 Voges-Proskauer Test

Methyl Red-Voges Proskuer medium was inoculated with the colony of the tested bacteria and incubated anaerobically at 37^{o}C for 48 hours, 0.06ml of vp1(α-naphthol reagent) and 0.2ml of 40% vp2 (KOH solution) were added. After 15 minutes, the formation of red color is indicative for the presence of acetoin (acetyl methyl carbinol) as positive result (Cappuccino and Welsh, 2018).

3.2.5.3.4 Citrate Utilization Test

The surface of Simmon's citrate medium was inoculated with colony of the tested bacteria and incubated anaerobically at 37°C for 1-3 days. Conversion of the medium color from green to blue indicates the ability to utilize citrate as a sole carbon source and indicate positive result (Cappuccino and Welsh, 2018)**.**

3.2.5.3.5 Urease Test

The surface of urea agar slant was streaked with a young bacterial culture and incubated at 37˚C. Results were read after 6 hr. - 24 hr.. Changing the color of medium to purple-pink indicate a positive result (Cappuccino and Welsh, 2018).

3.2.5.3.6 Motility Test

Tubes containing motility medium were inoculated with a young bacterial culture by stabbing the center of the medium and incubated at 37˚C for 24-48 hr.. Cloudy growth formation out of the line of stab indicates a bacterial motility (MacFaddin, 2000).

3.2.5.3.7 Carbohydrate Fermentation Test

The media for carbohydrate fermentation were inoculated with a bacterial suspension at 37C for 24hr.. This test is positive if the indicator is changing the color of media from red to yellow indicating the ability of bacteria to ferment the sugar (Yoo *et al*., 2005).

3.2.5.3.8 Salt Tolerance Test (STT)

Three colonies have been inoculated into tube of a nutrient broth supplement with 4%, 6.5% NaCl at 35C for three days. The appearance of the turbidity has indicated the ability of isolates to grow in the presence of these NaCl concentration (MacFaddin, 2000).

3.2.5.4 Hemolysin Production (Blood Hemolysis Test)

Hemolysis production was carried out by inoculating of blood agar medium with bacterial isolates at 37°C for 24-48 hrs.. An appearance of a clear zone around the colonies referred to complete hemolysis (β-hemolysis) or greenish zone around the colonies referred to partial hemolysis (α-hemolysis), while the no changing around the colonies referred to non-hemolytic (γ-hemolytic) (MacFaddin, 2000).

3.2.5.5 Detection of Biofilm Formation

Biofilm formation by *S. mutans* isolates was estimated using the microtiter plate crystal violet method according to (Stepanović *et al*., 2000; Zhou *et al*., 2018). Then the isolates of *S. mutans* from the glycerol stock were cultured on brain heart agar media and incubated at 37ºC in a candle jar for 48 h. A sterile plastic loop transferred a loopful of bacterial colonies into a tube containing 5 ml of isotonic saline and adjusted to match the 0.5 McFarland turbidity standard. Then, 100 µl from the standardized saline was transferred into 10 ml brain heart broth and 200 µl of each of the diluted solutions was transferred to a sterile flat-bottom 96-well plate containing 100 µl of fresh media per well (TSB medium supplemented with 1% glucose) in triplicates and incubated at 37ºC in a candle jar for 24 h. The negative control wells contained all components except the bacteria. Following incubation, the broth was removed, and the wells were gently washed three times with saline. The plates were left to dry, then followed by biofilm quantified using 200 µl of 0.1% crystal violet for 15 min. The excess stain was washed off by saline and inverted on tissues and left to dry, after that resolubilized by 100 µl of 98% ethanol for 15 min. The optical density (OD) was measured at 570 nm by Stat Fax-2600 microplate reader (Memmert, USA). The results obtained by the microtiter plate were classified according to (Stepanović *et al*., 2000). The cut-off OD (ODc) of the negative control was defined as: $OD \leq ODc$: Non-adherent, $ODc < OD \leq 2 \times ODc$: Weakly adherent, $2 \times ODc < OD \leq 4 \times ODc$: Moderately adherent, $4\times ODc < OD$: Strongly adherent.

3.2.5.6 Antimicrobial Susceptibility Test

3.2.5.6.1 Disk Diffusion Method (DDM)

Antimicrobial susceptibility testing of *S. mutans* isolates against group of antimicrobial agents had been carried out according to (Jubair, 2015) using the modified Kirby-Bauer disc diffusion method (DDM) on Muller-Hinton agar medium.

A pure culture previously identified bacteria being prepared by adding a growth from an isolate colony to 5 ml o sterile normal saline in a cell density equivalent turbidity of stander McFarland tube No (0.5) which about equal to bacterial density of 1.5 x 10^8 cells/ml. A sterile cotton swab was used to obtain inoculums to be streaked on MHA with 5% human Blood. Then antibiotic discs were being laid on the surface of the medium at equally spaced intervals with flamed sterile forceps to prevent contamination of the culture. The plate was incubated at 37°C for 20 to 24 hrs. under 5% CO2. Antibiotic inhibition zone were measured by using a ruler. Zone diameter was compared to standard values being recommended by clinical laboratory standards institute documentation (CLSI, 2022).

3.2.5.6.2 Determination Method of Minimum Inhibitory Concentration (MIC)

To determine the MIC of the antibiotics, stock antibiotic solutions of penicillin G, ampicillin, doripenem and ertapenem were prepared. The MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2022) by a macrodilution method in BHI broth. The inoculum was prepared by suspending several colonies from an overnight blood agar culture, in sterile 0.9% saline solution and adjusting the turbidity to 0.5 McFarland standard. The bacteria were inoculated into serially diluted antibiotic solutions in test tubes in macrodilution method for final concentrations of 5×10^5 CFU/ml. The final volumes were 2 ml. The macrodilution tubes were incubated in an ambient air incubator at 37°C for 24 h. MIC was defined as the lowest concentration of antibiotic which inhibits visible bacterial growth. Strains were classified for their susceptibility or resistance in accordance with the breakpoints recommended by the CLSI.

3.2.6 Identification by Using VITEK-II System

The innovative VITEK-II microbial identification system includes an expanded identification database, the most automated platform available, rapid results, improved confidence, with minimal training time. Bacterial isolates suspected to be Streptococci were completely identification by using VITEK-II identification system.

3.2.7 Molecular Diagnostic Methods

Polymerase chain reaction (PCR) technique was performed to diagnose of *S. mutans* by amplification of specific gene (*Sm479*) of *S. mutans* for the isolates which given positive results for the above tests. Also this technique has been used to determine virulence genes used in this study, as following stages:

3.2.7.1 Genomic DNA Extraction

Genomic DNA was extracted from *S.mutans* bacterial isolates by using Genomic DNA Bacterial Kit (Geneaid,USA) according to manufacturer's instructions as following steps:

1. Pure bacterial colonies cultured on nutrient agar plate for 24 hours were transferred to a 1.5 ml Eppendorf tube containing 1ml normal saline solution then centrifuged in high speed centrifuge at 14000 rpm for 1 minute then the supernatant discarded.
2. Lysozyme buffer (200 µl) [Gram+ Buffer contains 0.8 mg of lysozyme (0.8 mg/200 µl)] were added to the tube and re-suspended the cell pellet by shaking vigorously by vortex, then incubated for 30 min at 37 °C, and the tubes were inverted every 10 minutes through incubation periods.
3. Then 20 µl of proteinase k was added to the tube and mixed by vortex, then incubated at 60° C for at least 10 min, and the tube was inverted every 3 min.

4. GB buffer (200 µl) were added to each tube and mixed by shaking vigorously for 10 seconds. Then the tubes were incubated at 70ºC for 10 minutes and inverted every 3 minutes through the incubation periods. This step was done to complete the bacterial cell lysis. The elution buffer, in this step was placed in the oven at 70°C to be warm for the final step.
5. Absolute ethanol (200 µl) were added to the clear lysate and immediately mixed by shaking vigorously to prevent precipitation.
6. 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 14,000 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.
7. W1 buffer (400µl) were added to the GD column, then centrifuge at 14,000 rpm for 30 seconds. The flow-through was discarded and placed the GD column back in the 2 ml collection tube.
8. Wash Buffer(ethanol) 600 µl were added to the GD column. Then centrifuged at 14,000 rpm for 30 seconds. The flow-through was discarded and the GD column is placed back in the 2 ml collection tube.
9. All the tubes were centrifuged again for 3 minutes at 14,000 rpm to dry the column matrix.
10. The dried GD column was transferred to a clean 1.5 ml micro centrifuge tube and 100 µl of pre-heated elution buffer were added to the center of the column matrix.
11. The tubes were let stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 14,000 rpm for 30 seconds to elute the purified DNA and stored at −20 °C.

3.2.7.2 Estimation of Extracted DNA

The extracted DNA was checked by using Nanodrop that measured DNA concentration (ng/µL) 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 2µl 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 1µl of DNA was added to measurement.

3.2.7.3 PCR Master Mix Preparation

A PCR mixture was prepared by using master mix according to the manufacturer's instructions (Promega, USA). with a total volume of 25 µl as shown in table (3-10).

Table (3-10): PCR Reaction Mixture

NO.	Contents of reaction mixture	Volume
1.	Forward primer (10 pmol/µl)	1.5 µl
2.	Master Mix	12µl
3.	Nuclease free water	5 µl
4.	Reverse primer (10 pmol/µl)	1.5 µl
5.	Template DNA	5 µl

3.2.7.4 PCR Thermo Cycler Conditions

PCR thermo cycler conditions were done by using optimal protocol of conventional PCR thermo cycler system as shown in table (3-11).

Table (3-11): PCR Thermo Cycler Conditions

Gene	Temperature °C/ Time				
	Initial denaturation for 1 Cycle	Cycling condition for 40 Cycle			Final extension for 1 Cycle
		Denaturation	Annealing	Extension	
comD	94/3 min	95/30 sec	49/30 sec	72/59 sec.	72/5 min
comE	94/3 min	95/30 sec	53/30 sec	72/59 sec.	72/5 min
gbpA	94/3 min	95/30 sec	55/30 sec	72/59 sec.	72/5 min
gbpB	94/3 min	95/30 sec	55/30 sec	72/59 sec.	72/5 min
relA	94/3 min	95/30 sec	51/30 sec	72/59 sec.	72/5 min
Sm479	94/3 min	95/30 sec	55/30sec	72/59 sec.	72/5 min
spaP	94/3 min	95/30 sec	51/30 sec	72/59 sec.	72/5 min

3.2.7.5 PCR Product Analysis in Gel Electrophoresis

1. The PCR products were analyzed by agarose gel electrophoresis as the following steps:
2. Agarose sheet was prepared by dissolving 1gm of agarose powder according to the manufacturer's instructions of DNA ladder (Cleaver, UK) in 100ml 1X TBE buffer (10ml of TBE buffer completed with 90ml distal water) and melted in the microwave until the solution becomes clear, after that, left to cool 50°C.
3. A 3µl of safe red stain were added into agarose gel solution and have been mixd well by wooden sticks.

4. 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 5µL of PCR product were added in to each comb well and 5µL of (100bp DNA Ladder) in the first well.
5. The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 80 volt and 70 Ampere for 1 hour.
6. PCR products were visualized by using UV Transilluminator.

3.3 Statistical Analysis

Statistical analyses were performed by using statistical analysis software (Statistical Package for the Social Science) (SPSS) version 20. Chi-square test ($\chi 2$) was employed to assess the association between qualitative variables and the study groups (disease outcome). Correlation coefficient between two quantitative variables was also computed to know the strength of the relationship between them. For all tests, a probability (P) of <0.05 has been considered as significant.

CHAPTER FOUR

RESULTS and DISCUSSION

4. Results and Discussion

4.1 The Results of Laboratory Isolation and Identification of Bacteria

A total of 100 samples were obtained from dental caries patients who were admitted to the teaching dental clinics at College of Dentistry of Babylon University and Specialized Center for Dentistry in Babylon within the period of November 2021 to January 2022. Among 100 clinical samples, 47 *Streptococcus mutans* isolates which recovered by using selective media (MS agar and MSB agar) and non-selective media (blood agar and MacConkey agar).

Out of 100 dental caries samples 88 samples gave positive bacterial culture. While (12) samples gave no growth, as shown in figure (4-1). 70 isolates belonged to genus *Streptococcus*. While 18 isolates belonged to *Enterococcus faecalis* (14% of isolates) and *Staphylococcus aureus* (4% of isolates).

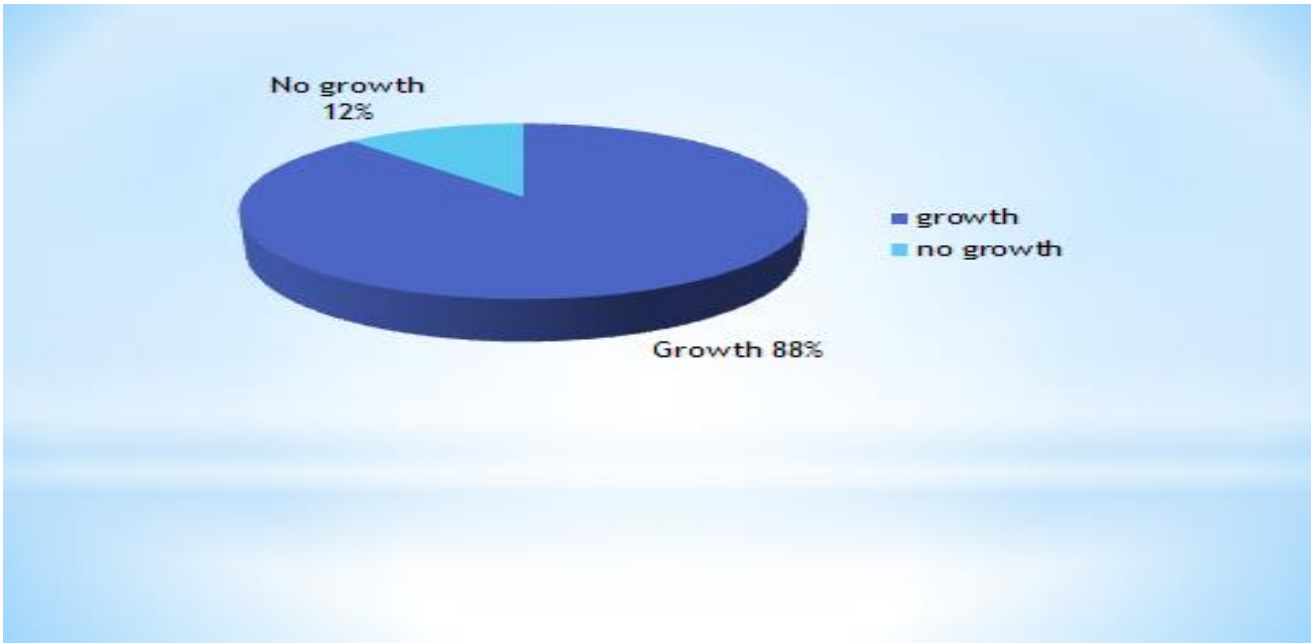


Figure (4-1) : Percentage of the specimens according to growth

Seventy isolates were considered to be related to the genus *Streptococcus* and specially to the viridans Streptococci of various group; The *S. mutans* was comprised the high percentage 67.14% (47/70) in comparison with the other *Streptococcus* species (*S. sobrinus* 15.71% (11/70), *S. mitis* 11.42% (8/70) and *S. salivarius* 5.71% (4/70), as shown in figure (4-2).

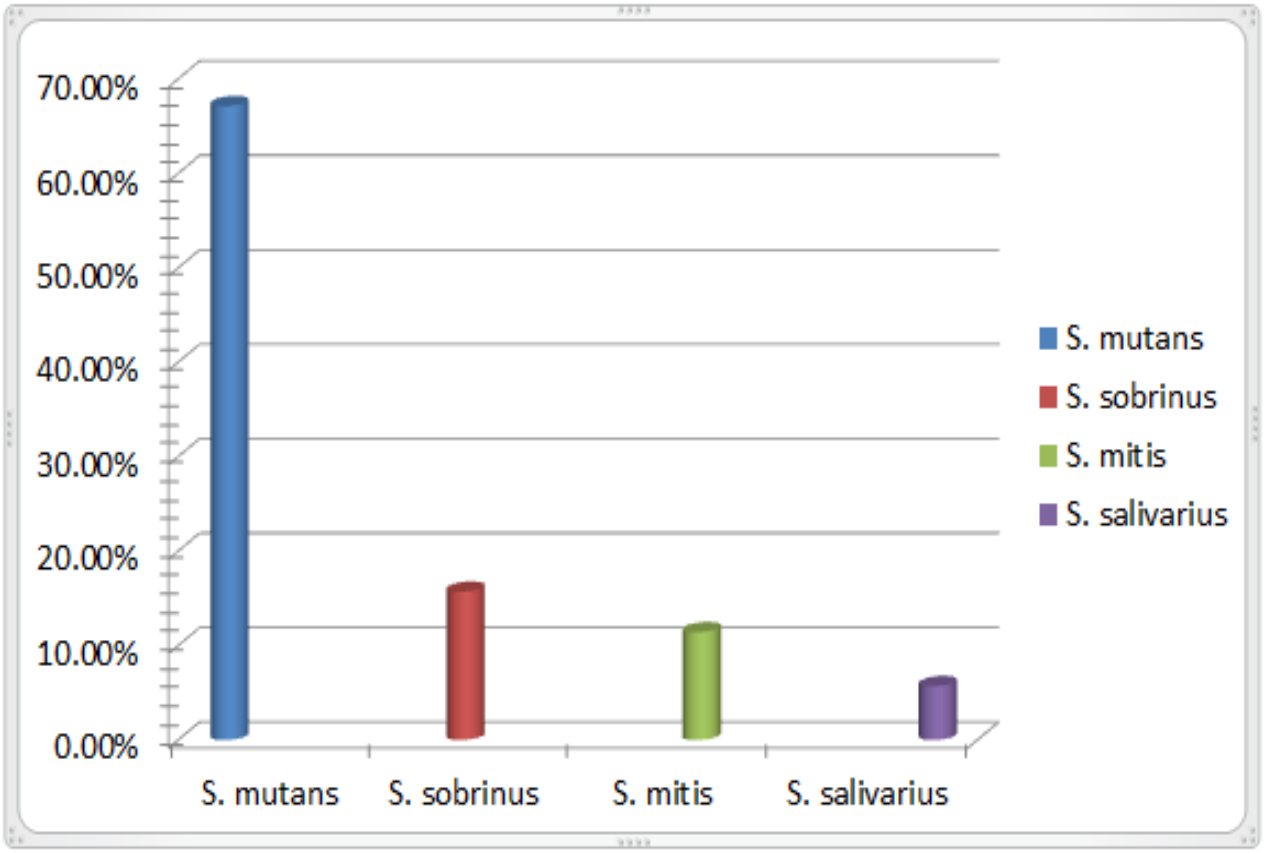


Figure (4-2): Percentage of *S. mutans* in comparison with the other *Streptococcus* species

Streptococci colonies were examined and diagnosed according to their morphological characteristics on MS and MSB agar plates. They appeared as circular or ovoid in shape with raised or convex surface. They were light blue or violet in colour and about 1mm in diameter, as shown the figure (A, B, C, D) in the Appendix (1).

S. mutans and *S. sobrinus* shared similar colony characteristics; both of them had unique morphology of frosted glass, irregular margin, black blue, rooted into the agar colonies. The colonies of *S. sobrinus* were surrounded by more distinct polysaccharide. *S. mitis* colonies were small, flat, hard, blue in color with a domed center. While *S. salivarius* colonies were large, pale-blue, mucoid that are glistening ("gum-drop") in appearance.

After 18–24 h of incubation at 37 °C on blood agar, Streptococci had white, smooth, glossy, and translucent colonies and appeared with zones of *α*, *β*-hemolysis or no hemolysis. Under microscopic examination, Streptococci were gram-positive spherical or ovoid cells arranged in short or medium in length chains.

S. mutans was the most frequent species of *Streptococcus*, as well as *S. mutans* is thought to be a crucial pathogen involved in the formation of dental caries, also it was found the presence of *S. mutans* is 70 times higher in caries-affected subjects than in caries-free subjects (Peterson *et al*., 2013).

The results of this study are in agreement with the results obtained by (Al-mohammadawy *et al*., 2018) who found that total growth of *Streptococcus* species (*mutans*, *mitis* and *salivarius*) was 82/120 (68.3%) specimen isolated on MSB agar and *S. mutans* recorded high frequency among recovered isolates about 48 isolates.

Yadav *et al*., 2015 showed that *S. mutans* was the most predominant to cause dental caries and play an important role in the development of disease.

The results of this study are in disagreement with the results obtained by (Moayad *et al*., 2015) who found that the prevalence of *S. mutans* isolates was (30%) from the culture. Also the results of this study are in disagreement with the results obtained by (Bibi *et al*.,

2018) who showed that only (4.34%) of total isolates was identified as *S. mutans* on the basis of microscopy and biochemical tests.

On the blood agar, *S. aureus* displayed light to golden yellow pigment or white pigment, while *E. faecalis* appeared pointed white shape. Colony morphology of *S. aureus* on MSA was dark black, perfect round, regular margin, flat and shiny, while *E. faecalis* was dark black or gray, round and small colony, figure (A-B) in the Appendix (2).

Using Gram's stain, *S. aureus* was gram-positive coccus and appeared in clusters while *E. faecalis* was gram-positive cocci in single or in pairs, as shown in figure (4-3).

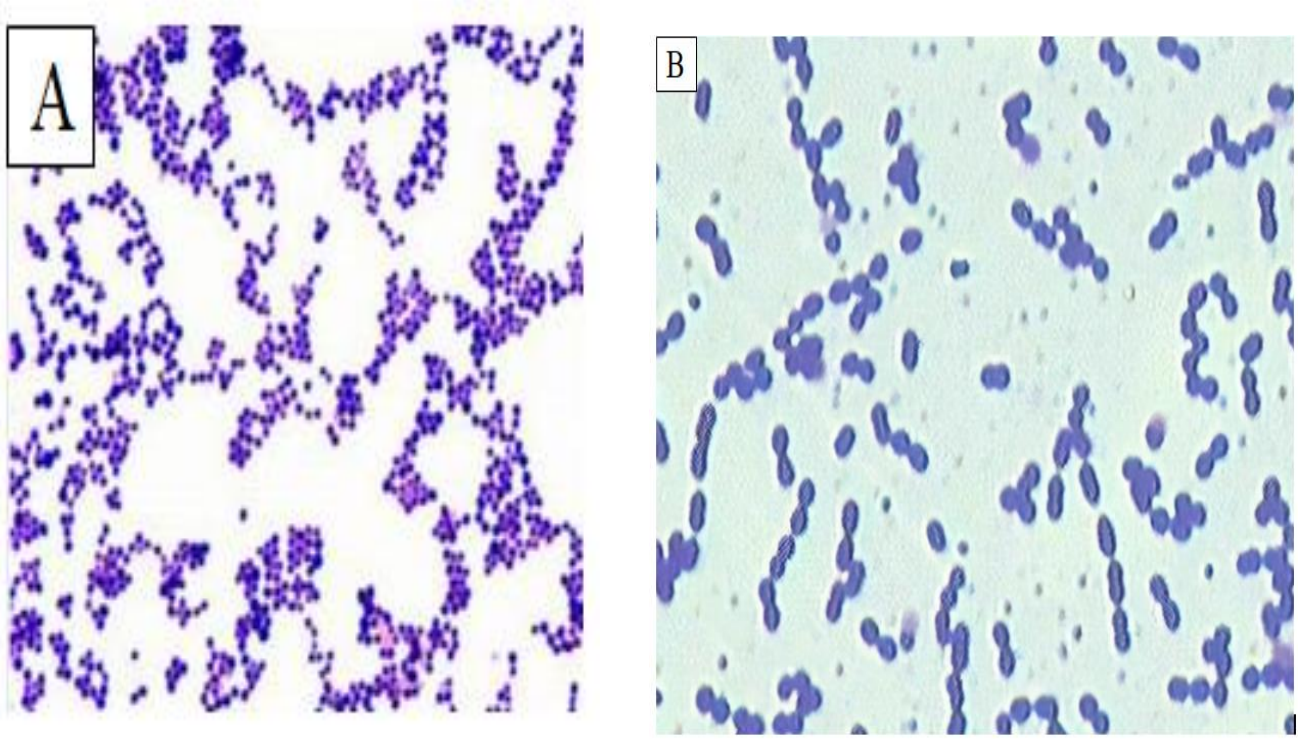


Figure (4-3) : Microscopic examination of (A) *S. aureus*, (B) *E. faecalis* using 100X oil immersed lens

The results of biochemical test of the bacterial isolates that gave positive culture were listed in the table (4-1).

Table (4-1): Biochemical Test of the Isolates Clinically Isolated from Patients with Dental Caries

Characteristics	*S. mutans*	*S. sobrinus*	*S. mitis*	*S. salivarius*	*Enterococcus faecalis*	*Staphylococcus aureus*
H2O2(catalase)	−	−	−	−	−	+
Oxidase	−	−	−	−	−	−
Urease	−	−	−	d	−	+
Growth in 4% NaC	+	+	−	d	+	+
Growth in 6.5% NaCl	−	d	−	−	+	+
Hemolysis	α, β, γ	α, γ	α	α, β, γ	α, γ	β
Carbohydrates fermentation test						
Mannitol	+	d	−	−	+	+
Melibiose	+	d	d	−	−	d
Raffinose	+	d	d	d	−	d
Sorbitol	+	d	d	−	+	d

Symbols: +, 90% or more of strains are positive; −, 90% or more of strains are negative; d, 11–89% of strains are positive; α, alpha hemolysis; β, beta Hemolysis; γ, gamma hemolisis

4.2 Confirmatory of Bacterial Isolates by VITEK-Π Compact System

The results of VITEK-Π compact system showed that *S. mutans* was the most frequent species of *Streptococcus*. *S. mutans* 47 isolates, *S. sobrinus* 11 isolates, *S. mitis* 8 isolates and *S. salivarius* 4 isolates and these results were exactly identical to the morphological

diagnosis. All the results of the VITEK-Π compact system were mentioned in the Appendix (3).

4.3 Identification of *S. mutans*

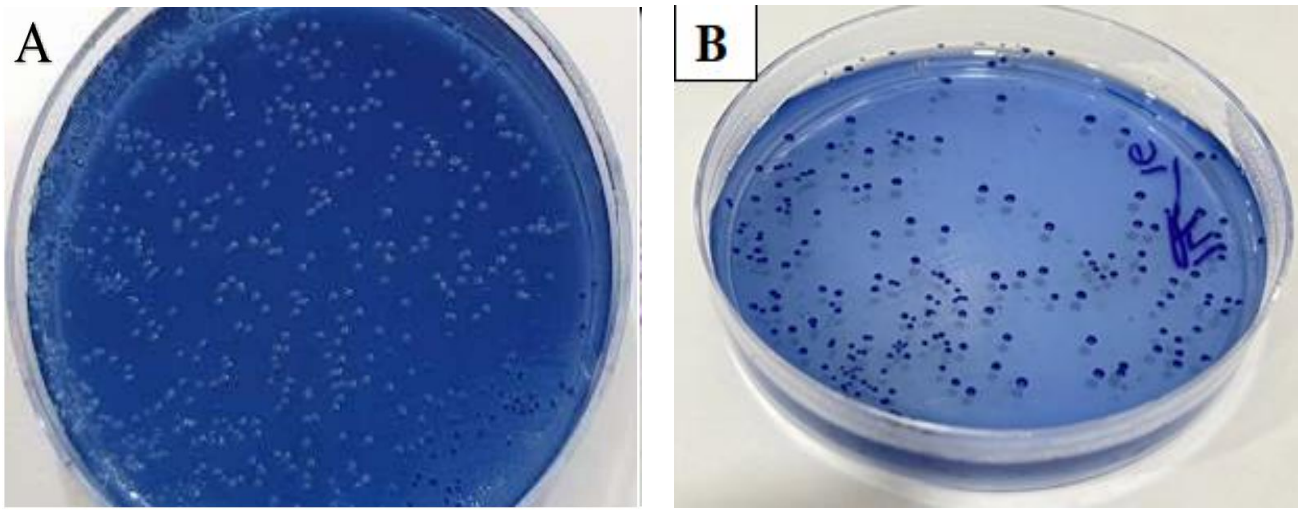
Identification of *S. mutans* was depending on the morphological diagnosis, VITEK-Π compact system and molecular detection.

4.3.1 Morphological Diagnosis

Identification of *S. mutans* by morphological characteristics was depended on distinctive colonial morphology, microscopic examination and biochemical tests.

4.3.1.1 Colony Morphology

On the selective MS and MSB agar plates, *S.mutans* colonies appeared pale-blue in color about 1-2 mm in diameter, avoid or circular 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 (smooth colonies), as shown in figure (4-4 A, B, C, D).



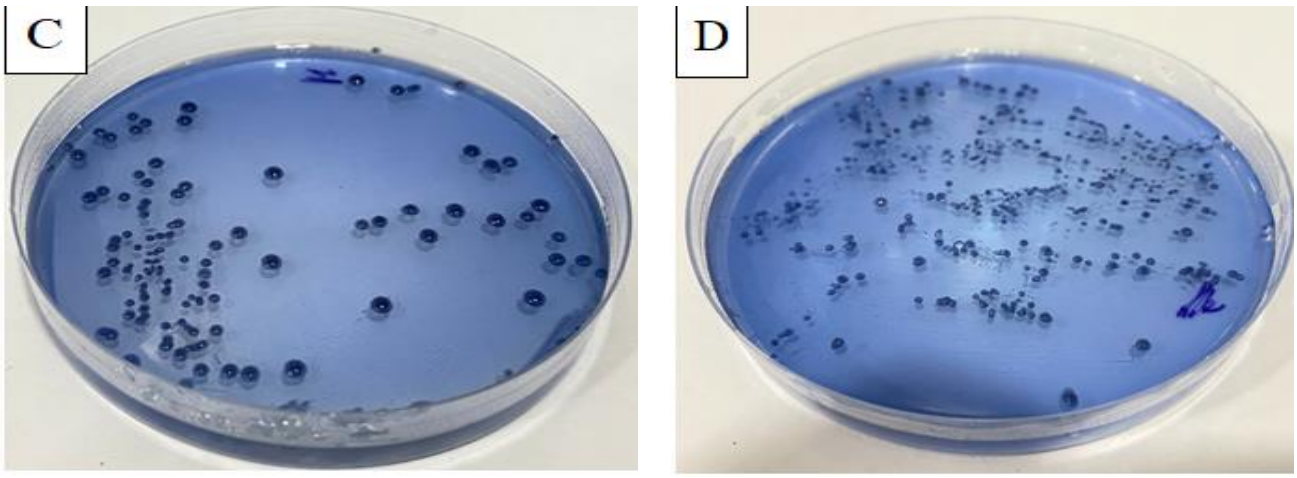


Figure (4-4): *S. mutans* on selective media A, on MSB agar; B, C, D on MS agar

The differences in colonial morphologies for *S. mutans* are due to the differences in DNA composition and DNA base sequence similarities between strains (Burns *et al*., 1995). *S. mutans* strains also display phenotypic variability in accordance with the variation in their genetic repertoire (Hossain *et al*., 2021).

While their morphology when grow on non-selective media such as blood agar after incubation anaerobic condition for 48 hrs. seemed gray or white, circular, 0.5-1mm in diameter and tending to be sticky to the surface of the medium as shown in figure (4-5).

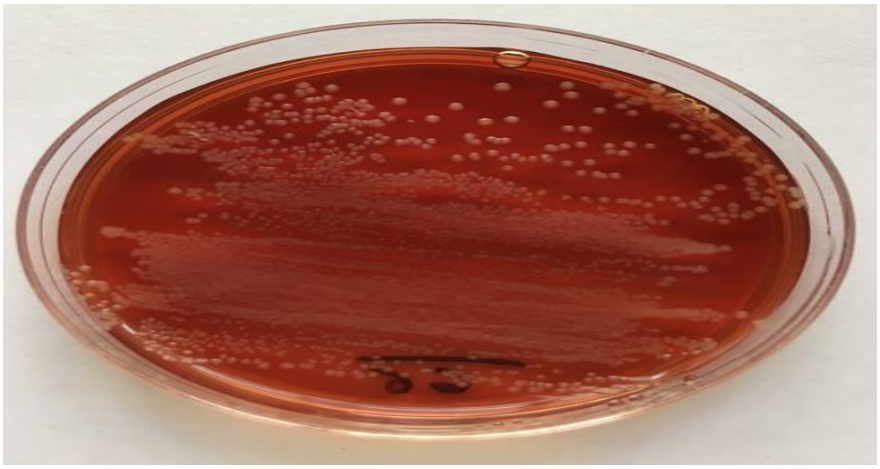


Figure (4-5): Colonies of *S. mutans* on blood agar

4.3.1.2 Microscopic Examination

Microscopic examination revealed that *S. mutans* was a Gram positive cocci occurs in pairs or in short- or medium-length chain, non-spore forming, as shown in figure (4-6).

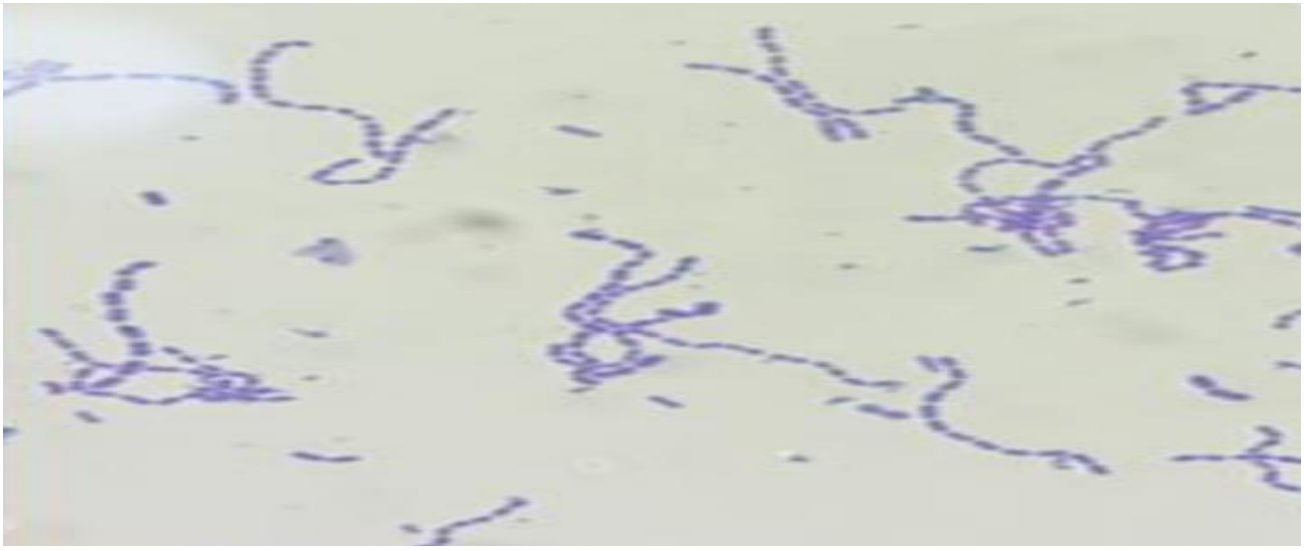


Figure (4-6): Gram's stain of *S.mutans* cells (1000x magnification).

4.3.1.3 Biochemical Test

The results of biochemical tests were listed in the table (4-1), as a complementary characteristics of the initial diagnosis of *S. mutans*. *S. mutans* isolates are characterized by their ability to ferment a number of sugars such as (Mannitol, Sorbitol, Sucrose, Raffinose, Melibiose). It gave negative result to catalase, oxidase, urease, Simmon's citrate, and motility. While it gave positive result to VP test and it has been shown that *S.mutans* can grow in 4% Nacl but do not grow in 6.5% Nacl concentration.

4.3.2 Molecular Detection of *S. mutans*

DNA extraction were done for 47 isolates which were identified by morphological diagnosis and VITEK-Π compact system. The purity of extracted DNA which checked by using Nanodrop was 1.80-2.0. Conventional PCR was carried out using specific primers targeting the *Sm479* gene, according to the sequences and programs listed in table (3-7) and (3-11) respectively. After that gel electrophoresis showed that, out of the 47 isolates, only 42 isolates produced the specific 479 bp DNA fragment when compared with DNA ladder; as shown in figure (4-7).

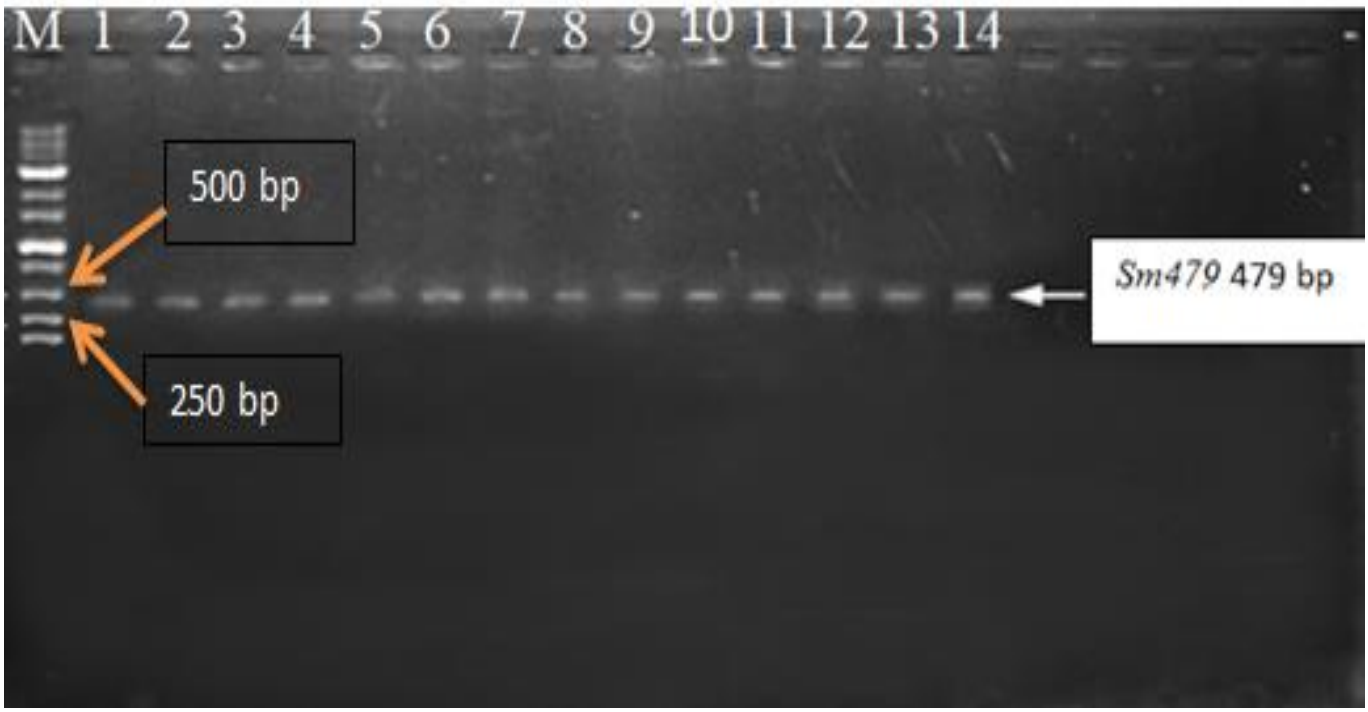


Figure (4-7): Agarose gel electrophoresis 1% at 80 volt for 1 hour for PCR-based detection of specific (*Sm479*) gene of *S. mutans* visualized under U.V light at 280 nm after staining with safe red stain. Lane M: DNA marker (100-10000)bp; Lane (1,2,3,4,5,6,7,8,9,10,11,12,13,14) isolates give positive result for this gene, the size of product is 479 bp.

PCR assay showed that the percentages of *S. mutans* isolates 60% (42/70) instead of 67.14% (47/70). Other isolates that were not diagnosed by PCR as *S. mutans* they were considered as other *Streptococcus* spp. because they possessed all the characteristics of *Streptococcus*.

Microbial investigation of the clinically derived dental caries is mainly done by Gram staining, biochemical tests, and colony morphology identification; but these features of bacteria may change under stress or with evolution. A common bacteria under stress may show an unusual phenotype, thus identifying the bacterial phenotype based on these characteristics or inexperience in unusual colonial morphology may lead to false identification of the bacteria (Ochman *et al*., 2005).

PCR methods to be more sensitive for detection than conventional culture techniques which able to detect slow-growing, unusual, and fastidious bacteria as well as for bacteria that are poorly differentiated by conventional methods (Patel, 2001).

The result of current study had confirmed and demonstrated that the Sm479F/R primer set is highly sensitive and species-specific for PCR based detection of *S. mutans*, which refer to unique sequence for identification of *S. mutans* and distinguishes it from the rest types of Streptococci. This result was agree with the result by (Li *et al*., 2001).

The results of this study are in agreement with the results obtained by (Al-hamadani and Al-Yasiri, 2016) who found that *S. mutans* were detected in 40% of samples collected from 100 patients with dental caries.

While the results of present study are in disagreement with the results obtained by (Salman *et al*., 2017) who showed that 55.38% of clinical isolates were identified as *S. mutans* by molecular methods.

4.4 Detection of *S. mutans* Rate According to the Patient's Sex

The results showed that the rate of *S. mutans* infection in male was (48.529%) while in the female, it was (28.125%), as shown in table (4-2).

Table (4-2): Distribution of *S. mutans* According to the Patient's Sex

Sex	**No of Samples**	***S. mutans* isolates**	
	No. of samples	**No(%) +ve**	**No(%) –ve**
Male	68	33(48.529%)	35(51.47%)
Female	32	9(28.125%)	23(71.875%)
Total	100	42(42%)	58(58%)

The present study showed that the prevalence of *S. mutans* was higher in males than female. The results in this study nearly similar to the results of (Wu *et al*., 2003) who found (26.5%) of females and (53.5%) of males were positive for *S. mutans*.

The results of this study are in disagreement with the results obtained by (Umar *et al*., 2015) who showed that females having the highest number of caries cases caused by the *S. mutans* with percentage (70%) caries compared to (60%) in males patients.

The results showed that the rate of *S. mutans* infection in smokers male was (81.818), while in Non-smoking males (18.181). These results may explain the direct effect of smoking in dental plaque and therefore dental caries. Accompanying flora and its effect in producing vasoconstriction in blood vessels in periodontium, which may lead to reduce availability of serum- derived protective factors such as antibodies and polymorphnuclear leukocytes in periodontal tissue, this was due to the effect of smoking on healing process

after infection because during smoking the nicotine stores in fibroblast cells and impairs their function (Anderson, 2004) .

4.5 Morphological Detection of Virulence Factors in *S. mutans*

4.5.1 Hemolysin Production

In present study, *S. mutans* isolates were investigated to detect hemolysin production. The result showed that 30.95% (13/42) of *S. mutans* isolates produced α-hemolysis and 19.04% (8/42) produced β-hemolysis but 50% (21/42) of isolates non-hemolysis, as shown the figure in the Appendix (4).

The results of this study were accepted with (Zhou and Li, 2021) who explained that α- or γ- hemolysis can be around colonies of mainly strains, whereas β-hemolytic zones can also be observed with the colonies of a few strains.

Mohammed, 2012 showed that (25%) of *S. mutans* isolates have the ability to produce hemolysin enzyme, which explain the flexibility of such bacteria to have different mechanisms for acquisition of iron from its environments.

The results of this study were in disagreement with the results of (Nizar, 2014) who showed that all *S. mutans* isolates showed α-hemolysis (green coloration on blood agar plates)

4.5.2 Biofilm Formation of *S. mutans*

Biofilm formation on polymeric surfaces by *S. mutans* isolates was estimated using the microtiter plate crystal violet method according to (Stepanović *et al*., 2000; Zhou *et al*., 2018). Crystal violet is a basic dye known to bind to negatively charged molecules on the

cell surface as well as nucleic acids and polysaccharides, and therefore, gives an overall measure of the whole biofilm. It has been used as a standard technique for rapidly accessing cell attachment and biofilm formation in a range of Gram positive (Djordjevic *et al*., 2002).

In this study, grades of biofilm formation were 14.28% (6/42) strong biofilm producer, 35.71% (15/42) moderate biofilm producer, 28.57% (12/42) weak biofilm producer and 21.42% (9/42) non biofilm producer, as shown in figure (4-8).

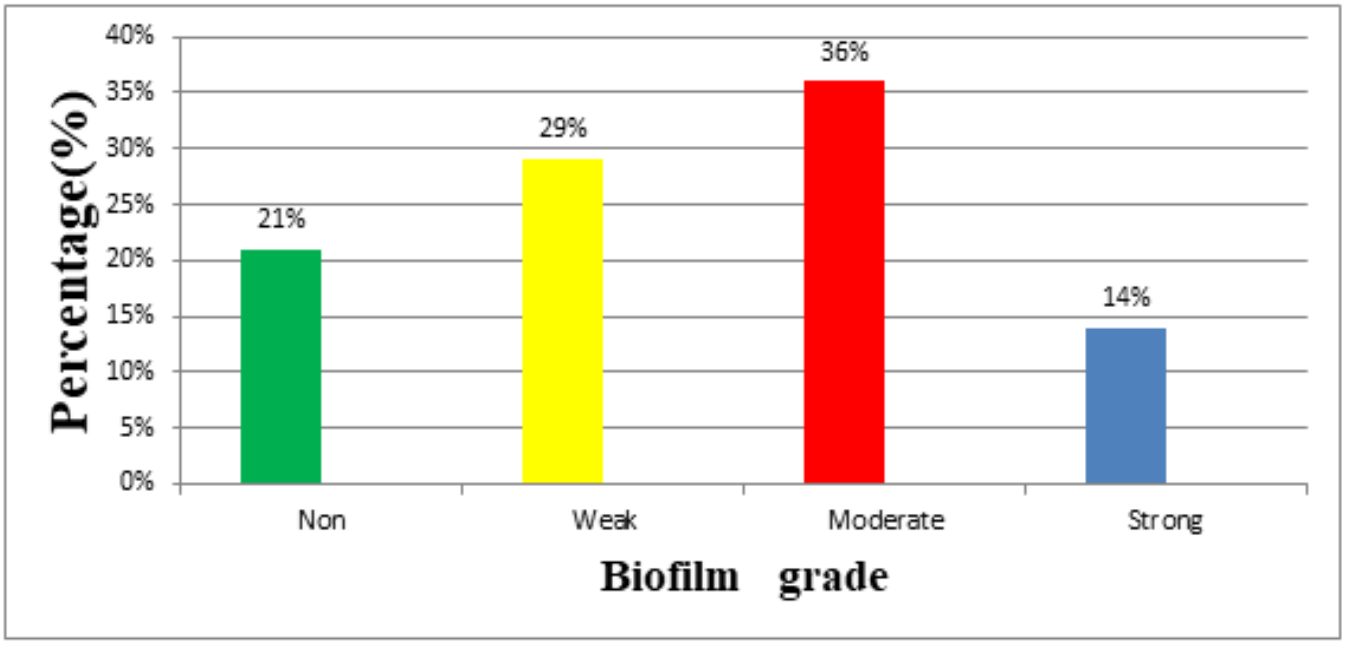


Figure (4-8): Biofilm formation grade by *S. mutans*

According to mean of OD value at 570 nm, the results were interpreted as non-production, weak, moderate and strong biofilm former when the mean of OD value were ($\leq$ 0.102, 0.102< – < 0.218, 0.218 – < 0.458 and $\geq$ 0.458) respectively. The results of biofilm formation grade by OD value were mentioned in the Appendix (5).

Many studies from the literature revealed that *S. mutans* bacteria could produce biofilm both in vivo and in vitro conditions (Kulshrestha *et al*., 2016; Ahn *et al*., 2018; Senpuku *et al*., 2019).

The results in this study nearly similar to the results obtained by (Jubair, 2015) who demonstrated (90%) of *S. mutans* isolates were isolated from dental caries have the ability to biofilm forming.

The results of this study were in disagreement with the results of (Patidar *et al*., 2012) who showed that out of 100 clinical isolates (92%) showed strong biofilm forming potential and (8%) clinical isolates showed moderate biofilm formation capability.

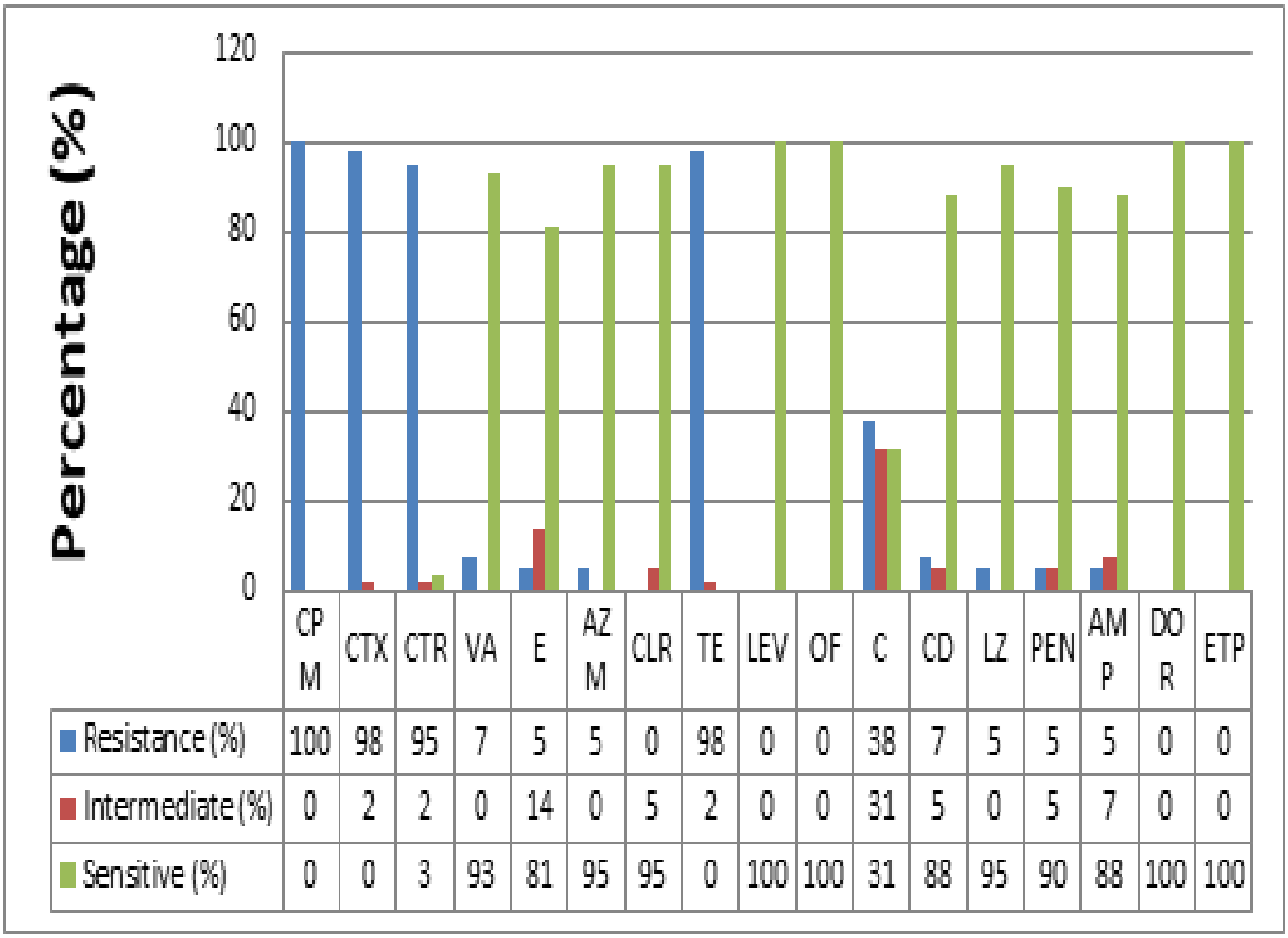
According to data obtained by this study, the emergence of strong, moderate, weak and non-production of biofilm refer to there was a significant difference in the ability to biofilm formation among the recovered isolates. Thus, there will become a difference in the ability of bacteria to adhere to the oral cavity.

These difference in the ability to biofilm formation can be explained by differences in growth condition such as ionic forces, pH and the number of subculture (Grivet *et al*., 2000).

4.5.3 Antibiotic Susceptibility Pattern of *S. mutans*

Antimicrobial susceptibility testing of *S. mutans* isolates against (17) types of different antibiotics had been carried by modified Kirby-Bauer disc diffusion method (DDM) and determination method of MIC, 13 antibiotics by DDM and 4 antibiotics by MIC were used to show their effect on *S. mutans* isolates according to the (CLSI) guidelines (CLSI, 2022).

The results of antibiotic susceptibility testing were mentioned in the Appendix (6), and *S. mutans* ' susceptibility patterns to different antibiotics used in this study are illustrated in the figure (4-9).



	CPM	CTX	CTR	VA	E	AZM	CLR	TE	LEV	OF	C	CD	LZ	PEN	AMP	DOR	ETP
Resistance (%)	100	98	95	7	5	5	0	98	0	0	38	7	5	5	5	0	0
Intermediate (%)	0	2	2	0	14	0	5	2	0	0	31	5	0	5	7	0	0
Sensitive (%)	0	0	3	93	81	95	95	0	100	100	31	88	95	90	88	100	100

Figure (4-9): Susceptibility patterns of *S. mutans* to different antibiotics used in this study

Abbreviations: CPM, Cefepime; CTX, Cefotaxime; CTR, Ceftriaxone; VA, Vancomycin; E, Erythromycin; AZM, Azithromycin; CLR, Clarithromycin; TE, Tetracycline, LEV, Levofloxacin; OF, Ofloxacin; C, Chloramphenicol; CD, Clindamycin; LZ, Linezolid; P, Penicillin; AMP, Ampicillin; DOR, Doripenem; ETP, Ertapenem.

According to data obtained in this study, all *S. mutans* isolates were susceptible to (levofloxacin, ofloxacin, doripenem and ertapenem), and there was a variation in its sensitivity to vancomycin 92.85% (39/42), erythromycin 80.95% (34/42), azithromycin 95.23% (40/42), clarithromycin 95.23% (40/42), chloramphenicol 30.95% (13/42), clindamycin 88.09% (37/42), linezolid 95.23% (40/42), penicillin 90.47% (38/42) and ampicillin 88.09% (37/42). While all *S. mutans* isolates were resistant to cefepime, and highly resistant to cefotaxime 97.61% (41/42), ceftriaxone 95.23% (40/42) and tetracycline 97.615% (41/42).The statistical analysis showed a significant differences among tested antibiotics based on ($p < 0.05$), with the exception of chloramphenicol ($p = 0.98$), there was no significant differences, as shown in table in the Appendix (7).

In this study, the number of multi-drug resistant (MDR) isolates was 15/42 (35.72%) while Non- multi-drug resistant (Non-MDR) isolates of *S. mutans* of was 27/42 (64.28%) as shown in table (4-3).

Table (4-3): Percentages of MDR and Non-MDR Isolates of *S. mutans*

MDR		**Non-MDR**		**P value**
No.	%	No.	%	
15	35.72	27	64.28	**0.00**

The results of this study showed that the percentages of MDR *S. mutans* isolates increased with the increase in percentages of biofilm grade. Thus, there was a significant correlation between biofilm formation grade and MDR isolates of *S. mutans* as shown in table (4-4).

Table (4-4): Correlation Between Biofilm Formation Grade and MDR Isolates of *S. mutans*

Biofilm	*S. mutans* isolates (biofilm former)		MDR		P value
	No	%	No	%	
non	9	21.43	2	22.22	**0.00**
weak	12	28.57	4	33.33	
moderate	15	35.71	6	40	
strong	6	14.29	3	50	

Biofilm facilitates the adherence of bacteria to biomedical surfaces and protect them from antimicrobial therapy and host immune response, also the production of biofilm may promote the colonization and lead to increase the infection rate of causative agent, then may be difficult to be treated as they exhibit multidrug resistance (Anderson et al., 2006).

One of the recent investigation showed the MDR species of *S.mutans* can grow at any fluctuating conditions and different concentrations of growth parameters like pH, temperature and NaCl tolerance in oral cavity (Karikalan and Mohankumar, 2016).

The results of recent study showed that all *S. mutans* isolates were resistant to cefepime, and highly resistant to ceftriaxone 95.23% (40/42). Marron *et al*. studied the susceptibility of *S. mutans* strains which were resistant to erythromycin, imipenem, ciprofloxacin, ceftriaxone, ceftazidime, cefpirome and cefepime (Marron *et al*., 2001).

Also the results of current study showed that 97.61% of *S. mutans* isolates were resistant to cefotaxime, The results are nearly similar to the results obtained by (Gharajalar and Hassanzade, 2017) who reported that about 90% of *S. mutans* isolates were resistant to cefotaxime.

While this result was contrasted with the results of (Pasquantonio *et al*., 2012) who showed that 89% of *S. mutans* isolates were susceptible to ceftriaxone and cefotaxime.

Also the results of this study were in disagreement with the results of (Karikalan and Mohankumar, 2016) showed that 50% of *S. mutans* isolates were resistant to cefotaxime.

The high bacterial resistance to *β*-lactam antibiotics due to a number of mechanisms, most notably the ability to produce enzymes (*β*-lactamase) which broken bind β-lactam, change the permeability barrier intimacy between the antibiotic and locations of the target penicillin-binding protein (Cherian *et al*., 2003).

The results of this study had been shown that 92.85% of *S. mutans* were susceptible to vancomycin.The results were nearly agreed with the results obtained by (El Sherbiny, 2014) who reported that susceptibility of *S. mutans* isolate to ten antibiotics revealed highly sensitive to vancomycin with percentage 95%.

while this result was contrasted with the results of (Chun *et al*., 2015) who showed all *S. mutans* isolates are resistance to vancomycin antibiotics.

The results of recent study showed that 80.95% of *S. mutans* isolates were susceptible to erythromycin antibiotic. The results of this study were in agreement with the results of (El Sherbiny, 2014) who found that the resistance rate to erythromycin was 27% from all *S. mutans* isolates. This difference in resistance may be due to the influence of different

factors like how much this antibiotics was used in community and the age of the patient and this is different from person to person.

The results of this study displayed that 97.615% of *S. mutans* isolates were resistant to tetracycline. The results of this study is in agreement with the results obtained by (Al-mohammadawy *et al*., 2018) who found that all *S. mutans* isolates were resistance to tetracycline.

The results of this study were in disagreement with the results of (Jain and Pundir, 2009) who found that tetracycline was moderately effective against three strains of *S. mutans*.

The results of this study showed that all *S. mutans* isolates were susceptible to levofloxacin. This results were fully consistent with the results obtained by (Pasquantonio *et al*., 2012) who found that all *S. mutans* isolates were susceptible to levofloxacin.

The present results showed that all *S. mutans* isolates were susceptible to ofloxacin. This results were fully consistent with the results obtained by (Khushbu Yadav, 2016) who found that all *S. mutans* isolates were susceptible to ofloxacin.

Also (Roberts, 2002) explained the ofloxacin are effective for inhibiting the growth of *S. mutans* bacteria and hence should be recommended for use.

while (Jain and Pundir, 2009) found that ofloxacin was moderately effective against three strains of *S. mutans*.

The results in this study showed that *S. mutans* isolates were (38.09% resistant, 30.95% susceptible and 30.95% intermediate) to chloramphenicol, which mean that *S. mutans* isolates have various sensitivity patterns for chloramphenicol.

The results in this study nearly similar to the results obtained by (Al-Saadi, 2006) in University of Tikrit who found that 52.17 % (36/69) of *S. mutans* isolates were resistant to chloramphenicol.

While the results of this study were in disagreement with the results of (Salman and Senthikumar, 2015) who showed all *S. mutans* isolates were sensetive to chloramphenicol.

Also the results of this study were in disagreement with the results of (Karikalan and Mohankumar, 2016) who showed the least (7%) of *S. mutans* isolates were resistant to Chloramphenicol antibiotic.

The present results showed 88.09% of *S. mutans* isolates were susceptible to Clindamycin. This study was in agreement with the results of (Pasquantonio *et al*., 2012) who found that 82% *S. mutans* isolates were susceptible to Clindamycin. Also (79.3%) of *S. mutans* isolates were susceptible to Clindamycin according to data obtained by (Al-Shami *et al*., 2019).

In addition to a direct antibacterial influence on protein synthesis (ribosomal units), clindamycin has a number of unique pharmacologic features that enhance its clinical efficacy. Clindamycin is the only proven antibiotic that reduces the adherence of bacteria to the epithelial cells of mucosal surfaces and inhibits the expression of virulence factors, it inhibiting capsule formation by facultative gram-positive *Streptococcus* species. Clindamycin induces morphologic changes on the surface of bacteria that render them more susceptible to being killed and also stimulates chemotaxis, thus promoting mobilization of polymorphonuclear leukocytes to the site of infection and resulting in ingestion of bacteria (Brook *et al*., 2005).

The results of recent study showed that 95.23% of *S. mutans* isolates were susceptible to linezolid. This results were in agreement with the results of (De *et al*., 2016) who showed that all *S. mutans* isolates were susceptible to linezolid.

The study results displayed that 90.47% of *S. mutans* isolates were susceptible to penicillin. The current results nearly similar to the results of (Pasquantonio *et al* ., 2012) who showed that 86.6% % of *S. mutans* isolates were susceptible to penicillin.

While the results of this study were in disagreement with the results of (Khushbu Yadav, 2016) who displayed that *S. mutans* isolates were highly resistant to penicillin.

Different antibiotics are routinely described for prophylaxis to the patients before massive dental procedures. It has been reported that the introduction of penicillin in the prophylactic treatment reduced the infection, but the long-term use of penicillin results in evolution resistant strains (Fani *et al*., 2007).

Also this study showed that 88.09% of *S. mutans* isolates were susceptible to ampicillin. The results of this study were agreed with the results obtained by (Pasquantonio *et al* ., 2012) who showed that 87% of *S. mutans* isolates were susceptible to ampicillin.

In this study, all *S. mutans* isolates were susceptible to doripenem and ertapenem. Carbapenems occupy a very important place in our fight against bacterial infections. This is because they are able to resist the hydrolytic action of beta-lactamase enzyme. Among the several hundreds of known beta- lactams, carbapenems possess the broadest spectrum of activity and greatest potency against Gram-positive and Gram-negative bacteria. As a result, they are often called “antibiotics of last resort” and are administered when patients with infections become gravely ill or are suspected of harboring resistant bacteria (Torres *et al*., 2007).

4.6 Molecular Detection of Virulence Genes in *S. mutans*

Conventional PCR technique has been used to amplify *gbpA*, *gbpB*, *relA*, *comD*, *comE* and *spaP* genes. Conventional PCR was carried out using specific primers targeting these genes, according to the sequences and programs listed in table (3-7) and (3-11) respectively. After that gel electrophoresis detected that not all isolates gave positive results for these genes table (4-5), and each gene represented by a single band in the corresponding region of the DNA ladder, as shown in figure (4-10A-F).

Table (4-5): Distribution of Virulence Genes in *S. mutans* Isolates

Genes	**Positive isolates**		**Negative isolates**		**Total**	**P value**
	No	**%**	**No**	**%**		
gbpA	34	80.95	8	19.05	42(100%)	**0.000**
gbpB	36	85.71	6	14.29	42(100%)	**0.000**
relA	28	66.66	14	33.34	42(100%)	**0.000**
comD	23	54.76	19	45.24	42(100%)	**0.008**
comE	30	71.42	12	28.58	42(100%)	**0.000**
spaP	32	76.19	10	23.81	42(100%)	**0.000**
P value	**0.000**					

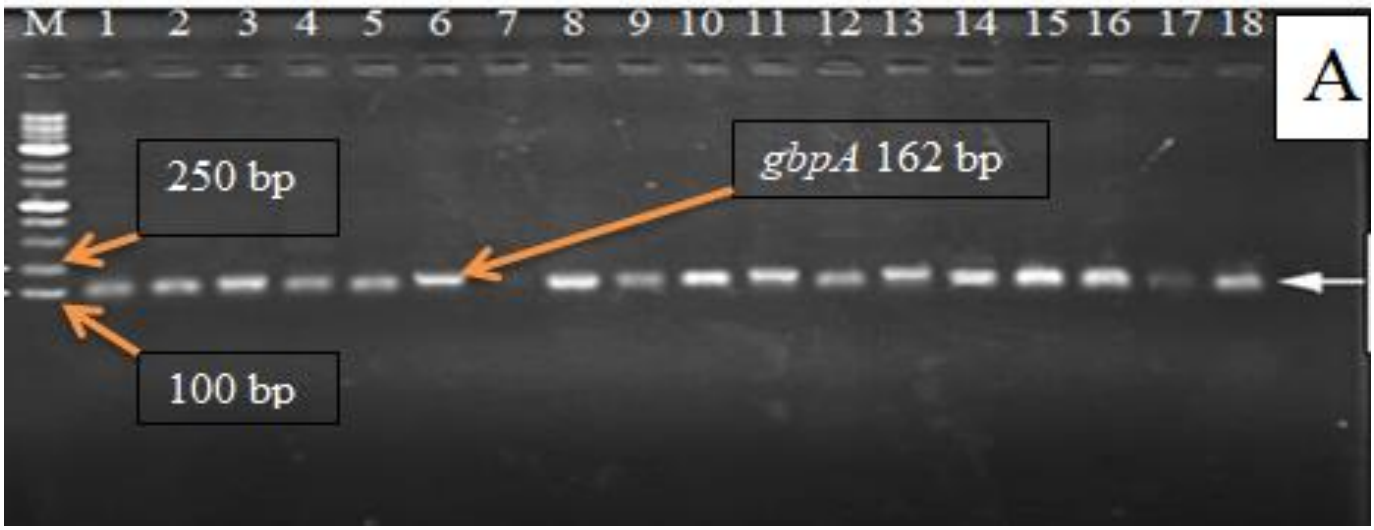


Figure (4-10 A): Agarose gel electrophoresis 1% at 80 volt for 1 hour to detection of PCR product of *gbpA* gene (162 bp) in *S. mutans* visualized under U.V light at 280 nm after staining with safe red stain. Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,8,9,10,11,12,13,14,15,16,17,18) isolates give positive result for this gene ;Lane(7) isolate give negative result.

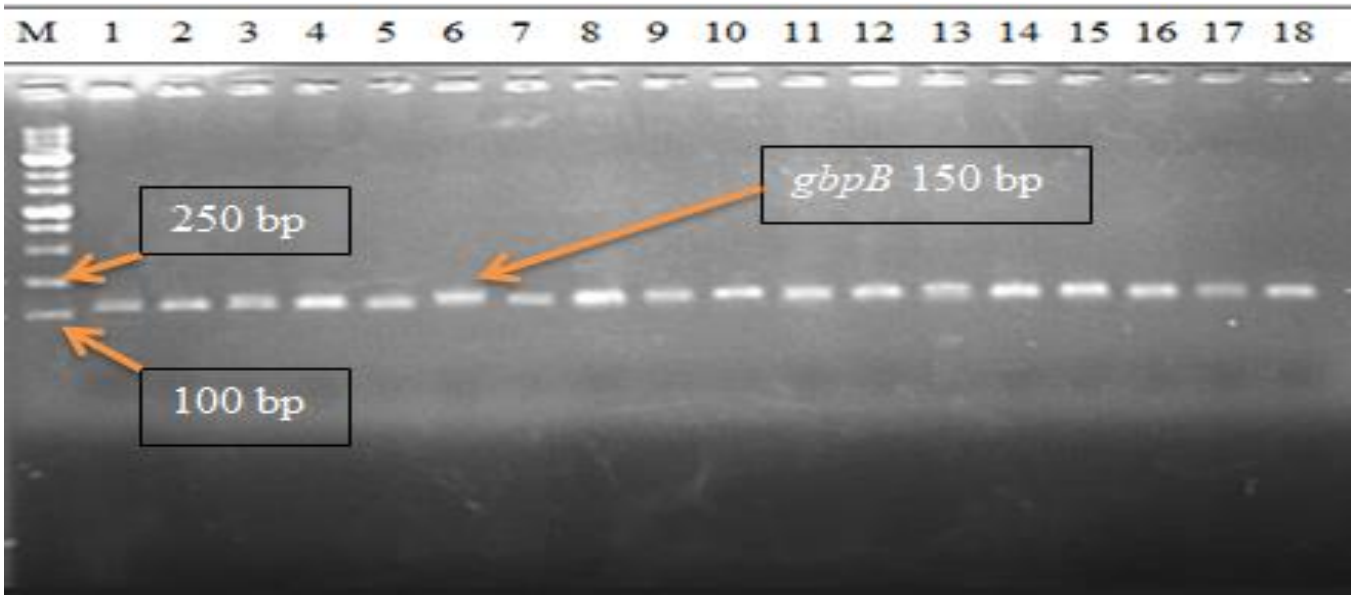


Figure (4-10 B): Agarose gel electrophoresis 1% at 80 volt for 1 hour to detection of PCR product of *gbpB* gene (150 bp) in *S. mutans* visualized under U.V light at 280 nm after staining with safe red stain. Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18) isolates give positive result for this gene.

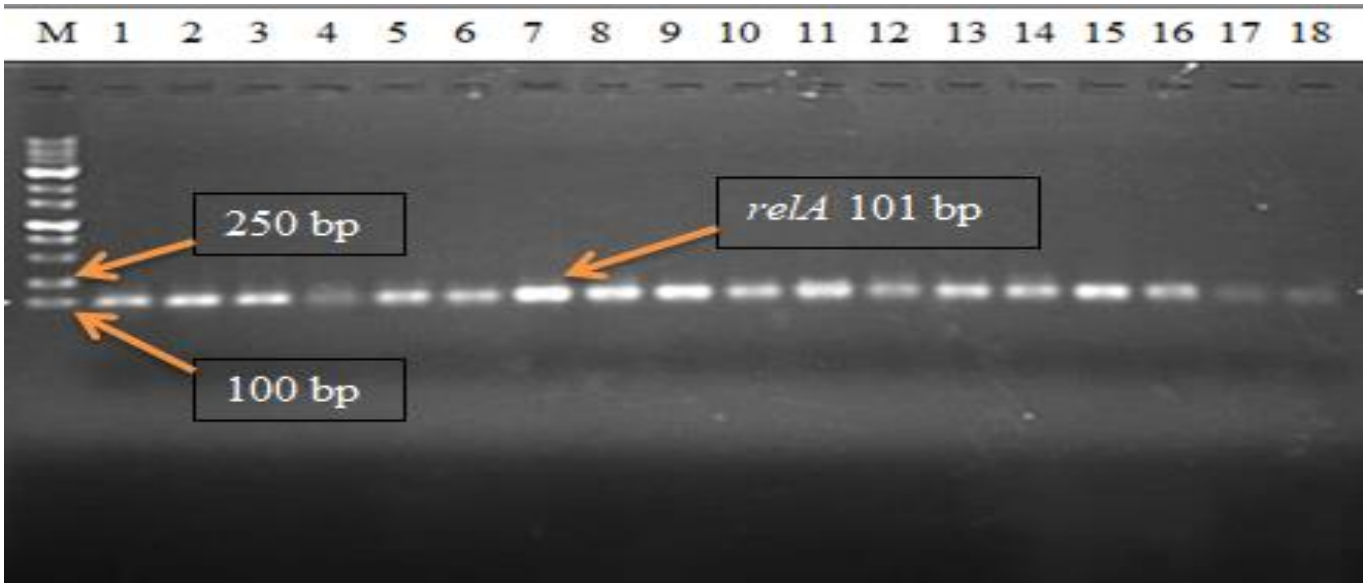


Figure (4-10 C): Agarose gel electrophoresis 1% at 80 volt for 1 hour to detection of PCR product of *relA* gene (101 bp) in *S. mutans* visualized under U.V light at 280 nm after staining with safe red stain. Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18) isolates give positive result for this gene.

Figure (4-10 D): Agarose gel electrophoresis 1% at 80 volt for 1 hour to detection of PCR product of *comD* gene (268 bp) in *S. mutans* visualized under U.V light at 280 nm after staining with safe red stain. Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,7,8,9) isolates give positive result for this gene.

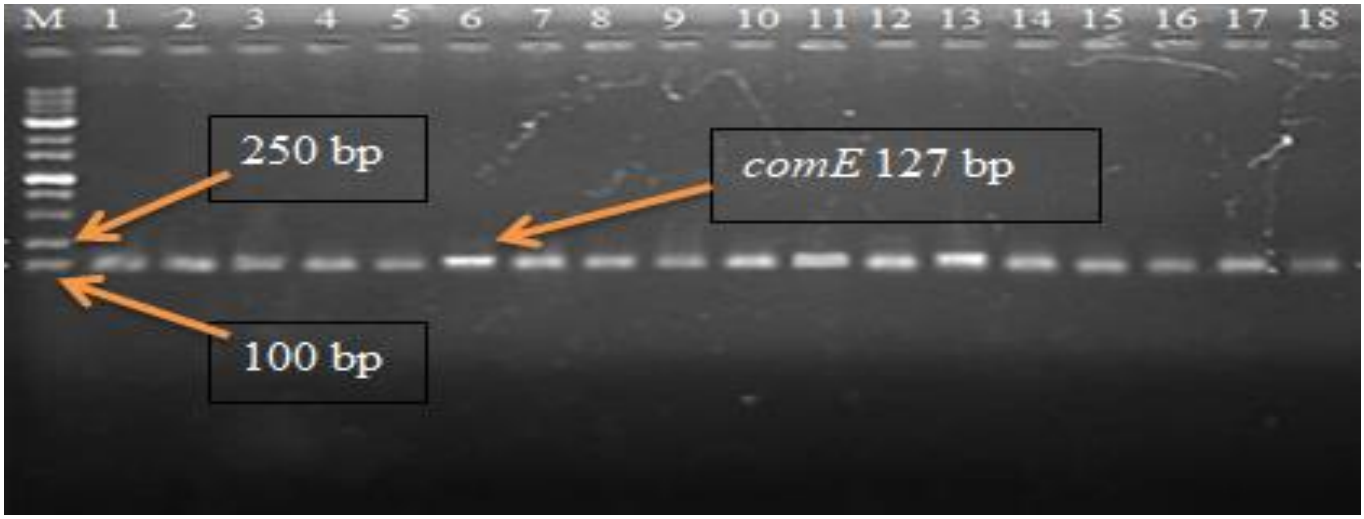


Figure (4-10 E): Agarose gel electrophoresis 1% at 80 volt for 1 hour to detection of PCR product of *comE* gene (127 bp) in *S. mutans* visualized under U.V light at 280 nm after staining with safe red stain. Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18) isolates give positive result for this gene.

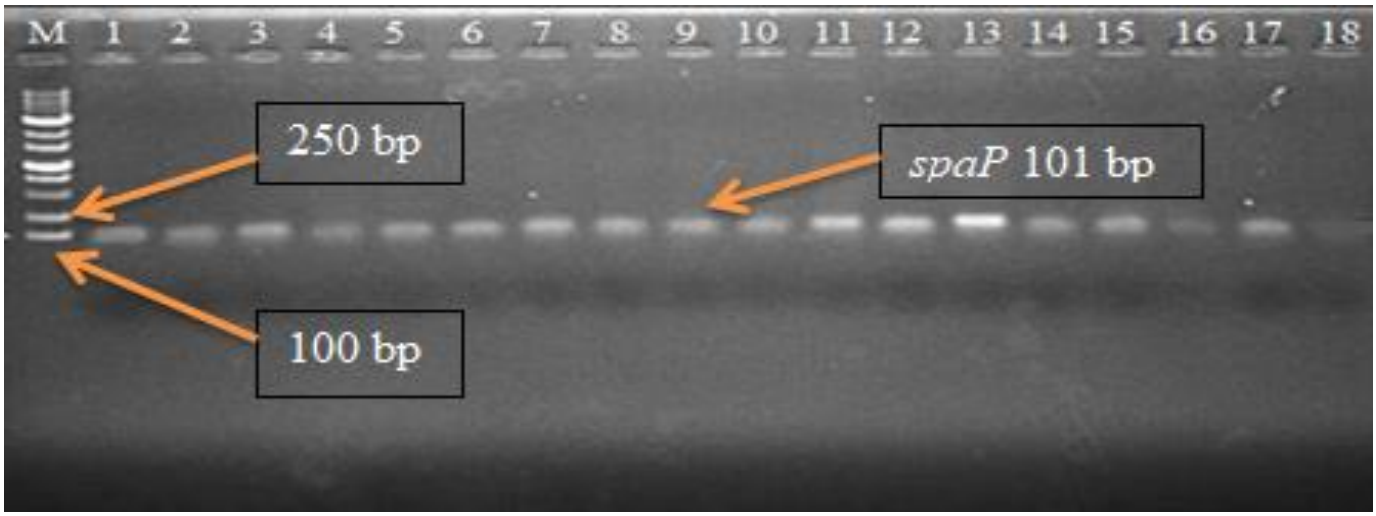


Figure (4-10 F): Agarose gel electrophoresis 1% at 80 volt for 1 hour to detection of PCR product of *spaP* gene (101 bp) in *S. mutans* visualized under U.V light at 280 nm after staining with safe red stain. Lane M: DNA marker (100-10000 bp); Lane (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17) isolates give positive result for this gene; Lane(18) isolate give negative result.

S. mutans isolates were examined by PCR for the presence of *S. mutans gbpA* gene. Thirty four (80.95%) of isolates harbored this gene and produced a detectable DNA band at the expected 162 bp PCR product. The results in this study nearly similar to the results obtained by (Hossain *et al*., 2021) who revealed that *gbpA* gene was present in all clinical isolates with expected size PCR product.

The *gbpB* gene also was detected in 36 (85.71%) of *S. mutans* isolates and gave the expected PCR product of 150 bp. The results in this study nearly similar to the results obtained by (Abo Bakr *et al*., 2021) who found that the *gbpB* gene was detected in all *S. mutans* isolates and produced the expected PCR product of 150 bp.

The results of this study nearly similar to the results obtained by (Conrads *et al*., 2014) who observed that all these genes (*relA, comD, comE*) are conservative for at least seven out of eight strains. While the results of this study are in disagreement with the results obtained by (Hossain *et al*., 2021) who showed that the ratio of *comD* and *comE* 100% of clinical isolates with expected size PCR product.

The result of this study was in consistence with the results of (Abd Al- Zahra, 2018) who found that *spaP* gene present in (71.42%) of isolates. Previous study by (Durán-Contreras *et al*., 2011) recorded that the *spaP* gene of *S. mutans* in dental caries samples was (91.3%). While the results of this study are in disagreement with the results obtained by (Israa and Mahdi, 2015) who showed that the *spaP* gene was present in (50%) of isolates from dental caries infections.

4.7 Role of Virulence Genes in *S. mutans* for Antibiotics Resistance

The pattern of virulence genes and antibiotics resistance of *S. mutans* is shown in the table (4-6).

Table (4-6): Pattern of Virulence Genes and Antibiotics Resistance of *S. mutans*

Genes	*gbpA* (34)		*gbpB* (36)		*relA* (28)		*comD* (23)		*comE* (30)		*spaP* (32)	
Antibiotics	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Cefepime	34	100	36	100	28	100	23	100	30	100	32	100
Cefotaxime	34	100	36	100	28	100	22	96	30	100	32	100
Ceftriaxone	34	100	36	100	28	100	22	96	30	100	31	97
Vancomycin	2	6	3	8	2	7.14	3	13	2	7	3	9
Erythromycin	2	6	2	6	1	3.57	2	9	1	3	2	6
Azithromycin	2	6	2	6	1	3.57	1	5	1	3	2	6
Clarithromycin	0	0	0	0	0	0	0	0	0	0	0	0
Tetracycline	34	100	36	100	28	100	23	100	30	100	32	100
Levofloxacin	0	0	0	0	0	0	0	0	0	0	0	0
Ofloxacin	0	0	0	0	0	0	0	0	0	0	0	0
Chloramphenicol	15	44	15	42	14	48	10	43	12	40	14	44
Clindamycin	3	9	3	8	2	7	2	9	0	0	3	9
Linezolid	2	6	2	6	2	7	1	5	1	3	2	6
Penicillin	2	6	2	6	1	3	1	5	0	0	2	6
Ampicillin	2	6	2	6	1	3	2	9	1	3	2	6
Doripenem	0	0	0	0	0	0	0	0	0	0	0	0
Ertapenem.	0	0	0	0	0	0	0	0	0	0	0	0

The results of recent study showed that the virulence genes associated with the resistance of some antibiotic such as (cefepime, cefotaxime, ceftriaxone, tetracycline), while it have not impact to the resistance of other antibiotic such as (clarithromycin, levofloxacin, ofloxacin, doripenem, ertapenem).

The genetic analysis of *S. mutans* had shown a high diversity of virulence-related genes, transmission of antibiotic-resistance bacteria genes and metabolic pathways, all this together provides to the *S. mutans* a high resistance to antibiotics and other chemical agents (Song *et al*., 2013).

Liu *et al*., (2017) showed that (45.9%) of *S. mutans* was considered one of more the bacterial species that presented antibiotic-resistance bacteria may be due to its high genetic diversity that allowed it to produce different substances and contains different genes that contribute to spreading the infection through facilitating the escape from neutrophil killing through degradation.

Some *S. mutans* isolates have begun to show extensive resistance to commonly used antibiotics. The characterization of these drug resistant isolates and their heterogeneity studies may provide more information on their genetic makeup and also be useful in the management and control of drug resistance to at least that region (Prakash *et al*., 2014).

4.8 Role of *gbpA, gbpB* and *spaP* Genes for Biofilm Formation

In the current study, the influence of *gbpA, gbpB and spaP* genes to biofilm formation was studied because of the importance of these genes in bacterial adhesion to tooth surface.

The results of biofilm formation assay showed that 14.28% strong, 35.71% moderate, 28.57% weak and 21.42% non-production biofilm formers. This results nearly similar to

the results obtained by (Jubair, 2015) who demonstrated (90%) of *S. mutans* isolated from dental caries have the ability to biofilm forming.

According to these results, there is a significant difference in the biofilm formation ability among the recovered isolates, this is due to the influence of these genes to biofilm formation.

The results of this study proved that in the absence of any one of these genes, biofilm formation often will be affected. The statistical analysis showed a significant correlation between biofilm formation grade and these genes based on (p = 0.05), as shown in the table (4-7).

Table (4-7): Correlation Between (*gbpA*, *gbpB* and *spaP*) Genes and Biofilm Formation Grade of *S. mutans*

Gene	**Biofilm Grade**								
Gene									**P value**
	None (9)		**Weak (12)**		**Moderate (15)**		**Strong (6)**		
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**	
gbpA	6	66.67	10	83.33	13	86.67	6	100	**0.00**
gbpB	6	66.67	11	91.67	13	86.67	6	100	**0.00**
spaP	2	22.22	10	83.33	14	93.33	6	100	**0.00**
P value	**0.011**								

The result of this study were consistent with the results of (Lynch et al., 2007) who revealed that the reason for the decrease in biofilm thickness varied based on the particular Gbp lost. GbpA has important role as a protein for formation of firm and stable biofilm, its deficiency results in loose binding to the EPS matrix, resulting in a weak non-uniform biofilm structure (Matsumi *et al*., 2015).

Each gene makes a unique contribution to the development of a mature and optimal dental biofilm, which minimizes stress on the bacterial population through the important role of Gbps in cellular adherence to tooth surfaces, binding proteins and exopolysaccharides for construction of biofilm, and maintenance of a balanced environment. Thus, *gbpA*, *gbpB* and *spaP* genes play important role in formation of firm and stable dental biofilm.

CONCLUSIONS

AND

RECOMMENDATIOS

Conclusions

The study reaches the following conclusions:

1. dental caries is caused by large number of bacteria. *S. mutans* considers principle etiological agent of dental caries that isolated in high ratio (42%), followed by *Enterococcus faecalis* (14 %).

2. There are a significant correlation between biofilm formation grade and MDR of *S. mutans* isolates .

3. The *gbpA*, *gbpB* and *spaP* genes play important role in formation of dental biofilm, because in the absence of any of one of these genes, biofilm formation grade often will be affected.

4. The *gbpB* gene is more prevalence than other genes in *S. mutans*, which explains that this gene contributes to pathogenicity.

5. *S. mutans* isolates showed high level of resistance to cefepime, cefotaxime, ceftriaxone and tetracycline, and low level of resistance to levofloxacin, ofloxacin, doripenem, ertapenem, azithromycin, clarithromycin, linezolid, vancomycin, penicillin, ampicillin and clindamycin.

6. Molecular diagnostic methods were more accurate than conventional methods.

Recommendations

1. Further investigation to study the role of other virulence genes for *S. mutans* that involved in extracellular polysaccharide formation,sugar uptake and metabolism, acid tolerance, and regulation

2. More studies on genotyping of microorganisms that are involved in the formation of cohesive biofilm that protects the main causes of dental caries.

3. Avoid using antibiotics continuously and randomly for their negative effect on the balance of bacterial diversity within the body in general and mouth particularly.

4. Enhancing the role of the immune system against species that cause tooth decay by coming up with a safe vaccine instead of traditional methods of preventing caries.

5. Conduct additional genetic studies in different populations in order to diagnose and treat tooth decay from a more molecular or genetic basis.

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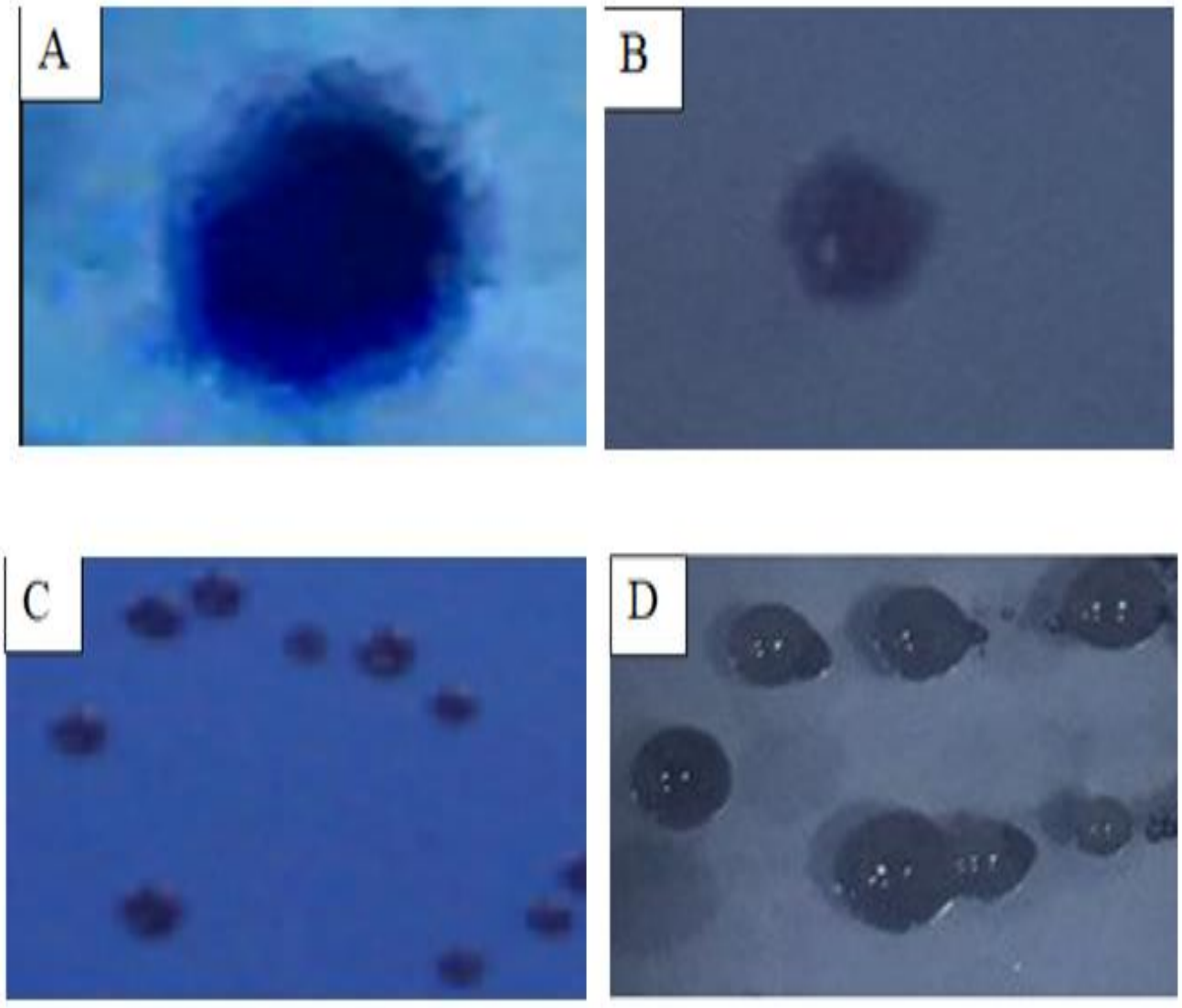
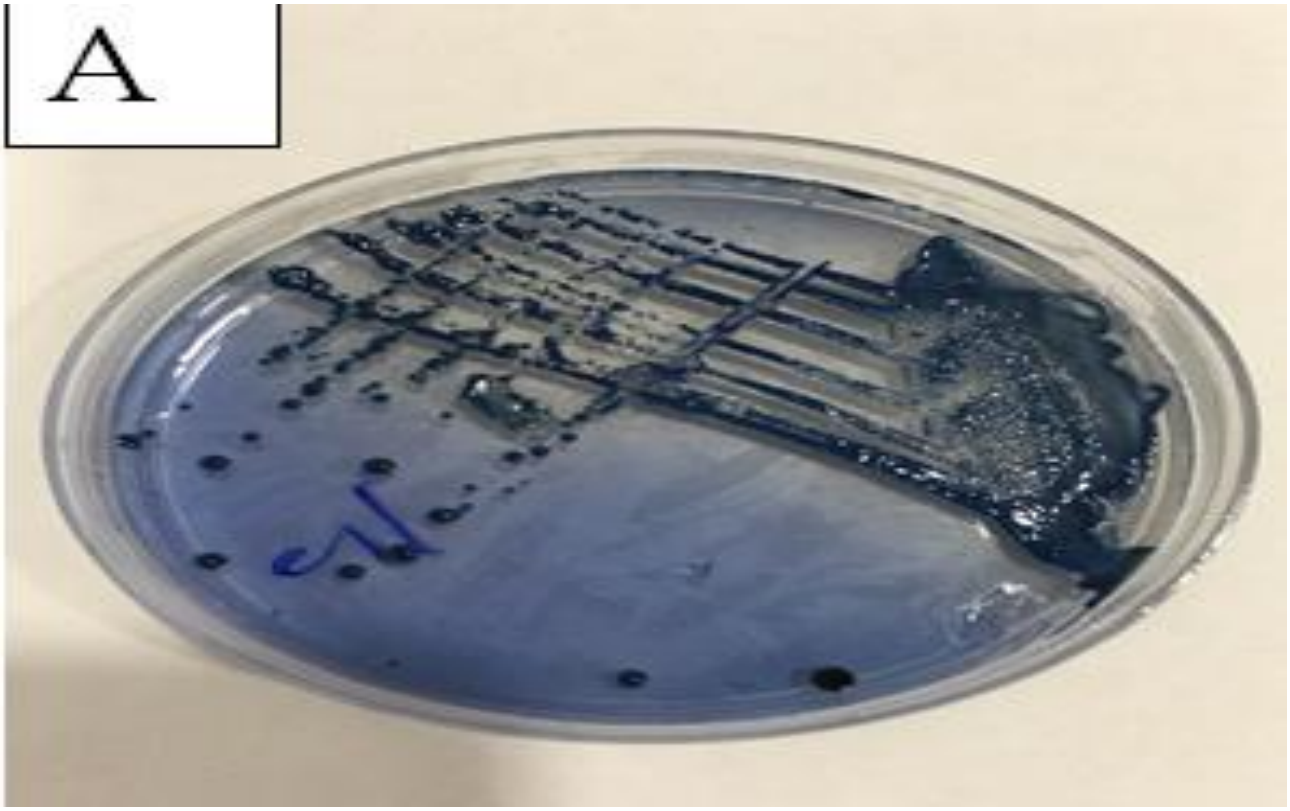
Appendix

Appendix (1): Colony Morphology on MSA of (A) *S. mutans*, (B) *S. sobrinus*, (C) *S. mitis*, (D) *S. salivarius*



Appendix (2): (A) *S. aureus* on MSA, (B) *E. faecalis* on MSA



Appendix (3) : The Results of VITEK-II Compact System

bioMérieux Customer:

Microbiology Chart Report

Printed February 22, 2022 10:27:48 PM CST

Patient Name: 9 turky , ahmed

Patient ID: 220227

Location:

Physician:

Lab ID: 220227

Isolate Number: 1

Organism Quantity:

Selected Organism : Streptococcus mutans

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	4.79 hours	**Status:** Final
Selected Organism	99% Probability	**Streptococcus mutans**	
	Bionumber:	566012765777771	
ID Analysis Messages			

Biochemical Details																	
2	AMY	-	4	PIPLC	-	5	dXYL	+	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	+	23	ProA	-	24	BGURr	-	25	AGAL	+	26	PyrA	-	27	BGUR	-
28	AlaA	+	29	TyrA	-	30	dSOR	+	31	URE	-	32	POLYB	-	37	dGAL	+
38	dRIB	+	39	lLATk	+	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	-	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	+	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (3) : The Results of VITEK-II Compact System

bioMérieux Customer:

Microbiology Chart Report

Printed February 22, 2022 10:27:48 PM CST

Patient Name: 5 turky , ahmed

Patient ID: 220227

Location:

Physician:

Lab ID: 220227

Isolate Number: 1

Organism Quantity:

Selected Organism : Streptococcus sorbinus

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:** 4.92 **hours**	**Status:** Final
Selected Organism	98% Probability **Streptococcus sorbinus** **Bionumber:** 566012765777782	
ID Analysis Messages		

Biochemical Details																	
2	AMY	-	4	PIPLC	-	5	dXYL	+	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	+	23	ProA	-	24	BGURr	-	25	AGAL	+	26	PyrA	-	27	BGUR	-
28	AlaA	+	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	-	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	+	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (3) : The Results of VITEK-II Compact System

bioMérieux Customer:

Microbiology Chart Report

Printed February 22, 2022 10:27:48 PM CST

Patient Name: 6 turky , ahmed

Patient ID: 220227

Location:

Physician:

Lab ID: 220227

Isolate Number: 1

Organism Quantity:

Selected Organism : Streptococcus mitis

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	**5.74 hours**	**Status:**	Final
Selected Organism	97% Probability	**Streptococcus mitis**		
	Bionumber:	566012765777793		
ID Analysis Messages				

Biochemical Details																	
2	AMY	-	4	PIPLC	-	5	dXYL	+	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	+	23	ProA	-	24	BGURr	-	25	AGAL	+	26	PyrA	-	27	BGUR	-
28	AlaA	+	29	TyrA	-	30	dSOR	+	31	URE	+	32	POLYB	-	37	dGAL	+
38	dRIB	+	39	lLATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	+	58	O129R	+	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (3) : The Results of VITEK-II Compact System

bioMérieux Customer:

Microbiology Chart Report

Printed February 22, 2022 10:27:48 PM CST

Patient Name: 7 turky , ahmed

Patient ID: 220227

Location:

Physician:

Lab ID: 220227

Isolate Number: 1

Organism Quantity:

Selected Organism : Streptococcus salivarius

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	**5.22 hours**	**Status:** Final
Selected Organism	95% Probability	**Streptococcus salivarius**	
	Bionumber:	566012765777764	
ID Analysis Messages			

Biochemical Details																
2	AMY	-	4	PIPLC	-	5	dXYL	+	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	+	23	ProA	-	24	BGURr	-	25	AGAL	+	26	PyrA	-	27	BGUR	-
28	AlaA	+	29	TyrA	-	30	dSOR	+	31	URE	-	32	POLYB	-	37	dGAL	+
38	dRIB	+	39	lLATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	-	54	MBdG	+	56	PUL	-
57	dRAF	+	58	O129R	+	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (3) : The Results of VITEK-II Compact System

bioMérieux Customer:
System #:

Laboratory Report

Printed by: Labadmin
Patient ID: 8/3/22/2

Patient Name: ahmad, 2
Isolate: 8/3/22/2-1 (Approved)
Card Type: GP Bar Code: 2421850503689964 Testing Instrument: 00000B4E1D71 (1830)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 050402033763331
Organism Quantity:

Selected Organism: Staphylococcus aureus

Comments:	

Identification Information	**Card:**	GP	**Lot Number:**	2421850503	**Expires:**	Dec 20, 2022 12:00 GMT-06:00
	Status:	Final	**Analysis Time:**	4.88 hours	**Completed:**	Jan 3, 2022 21:57 GMT-06:00
Organism Origin	VITEK 2					
Selected Organism	92% Probability **Staphylococcus aureus** Bionumber: 050402033763331 Confidence: Good identification					
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s) Staphylococcus aureus dRAF(1),URE(2),						

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	+
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	+	32	POLYB	+	37	dGAL	-
38	dRIB	+	39	ILATk	+	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	+	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (3) : The Results of VITEK-II Compact System

bioMérieux Customer:
System #:
Patient Name: ahmad , 4
Isolate: 8/3/22/4-1 (Approved)
Card Type: GP Bar Code: 2421850503689967 Testing Instrument: 00000B4E1D71 (1830)
Setup Technologist: Laboratory Administrator(Labadmin)

Laboratory Report

Printed by: Labadmin
Patient ID: 8/3/22/4

Bionumber: 114002721773461
Organism Quantity:

Selected Organism: Enterococcus faecalis

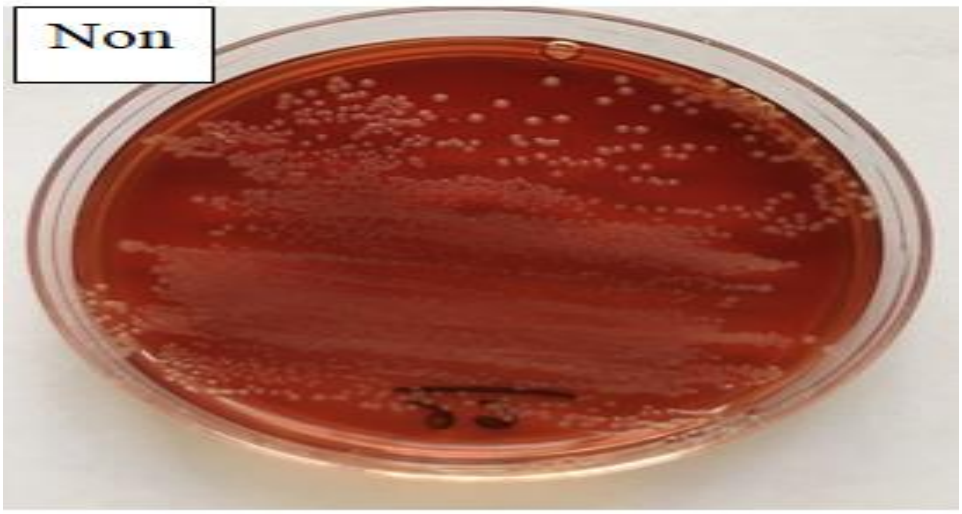
Comments:	

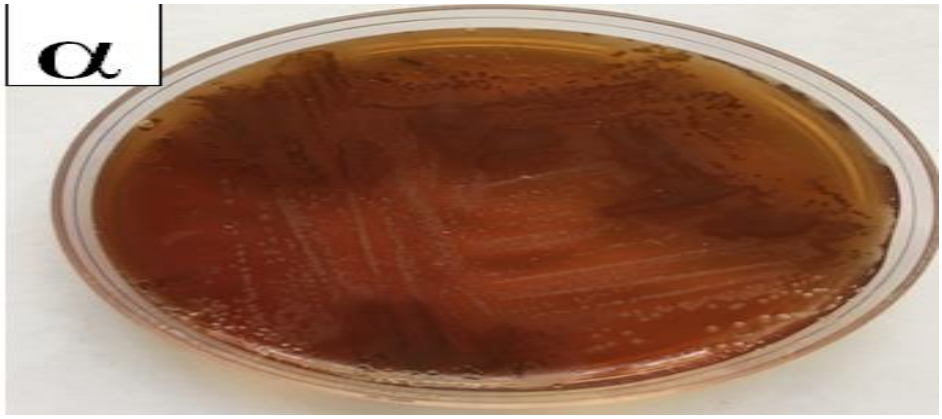
Identification Information	**Card:** GP	**Lot Number:** 2421850503	**Expires:** Dec 20, 2022 12:00 GMT-06:00
	Status: Final	**Analysis Time:** 2.67 hours	**Completed:** Jan 3, 2022 19:44 GMT-06:00
Organism Origin	VITEK 2		
Selected Organism	99% Probability **Enterococcus faecalis** Bionumber: 114002721773461 Confidence: Excellent identification		
Analysis Organisms and Tests to Separate:			
Analysis Messages:			
Contraindicating Typical Biopattern(s)			

Biochemical Details

2	AMY	+	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	+	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	+	31	URE	-	32	POLYB	+	37	dGAL	-
38	dRIB	+	39	lLATk	-	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	-	59	SAL	+	60	SAC	-	62	dTRE	+	63	ADH2s	+
64	OPTO	+															

Appendix (4): Types of Hemolysis by *S. mutans*





Appendix (5): The Results of Biofilm Formation Grade by OD Value

Isolate No	OD (nm)	Grade
Sm1	0.458	Strong
Sm2	0.424	Moderate
Sm3	0.111	Weak
Sm4	0.113	Weak
Sm5	0.097	Non
Sm6	0.326	Moderate
Sm7	0.473	Strong
Sm8	0.184	Weak
Sm9	0.075	Non
Sm10	0.513	Strong
Sm11	0.396	Moderate
Sm12	0.114	Weak
Sm13	0.148	Weak
Sm14	0.137	Weak
Sm15	0.377	Moderate
Sm16	0.326	Moderate
Sm17	0.251	Moderate
Sm18	0.102	Non
Sm19	0.124	Weak
Sm20	0.079	Non
Sm21	0.141	Weak
Sm22	0.382	Moderate

Sm23	0.092	Non
Sm24	0.29	Moderate
Sm25	0.218	Moderate
Sm26	0.116	Weak
Sm27	0.508	Strong
Sm28	0.274	Moderate
Sm29	0.307	Moderate
Sm30	0.28	Moderate
Sm31	0.094	Non
Sm32	0.493	Strong
Sm33	0.531	Strong
Sm34	0.078	Non
Sm35	0.211	Weak
Sm36	0.209	Weak
Sm37	0.083	Non
Sm38	0.186	Weak
Sm39	0.222	Moderate
Sm40	0.081	Non
Sm41	0.416	Moderate
Sm42	0.417	Moderate

Appendix (6): The Results of Antibiotic Susceptibility Testing

Isolate No	CPM	CTX	CTR	VA	E	AZM	CLR	TE	LEV	OF	C	CD	LZ	P	AMP	DOR	ETP
Sm1	R	R	R	S	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm2	R	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Sm3	R	R	R	S	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm4	R	R	R	R	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm5	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Sm6	R	R	R	S	S	S	S	R	S	S	R	S	S	S	S	S	S
Sm7	R	R	R	R	R	S	I	R	S	S	R	S	R	I	R	S	S
Sm8	R	R	R	S	I	S	S	R	S	S	R	R	S	S	I	S	S
Sm9	R	R	R	S	S	S	S	R	S	S	R	S	S	S	S	S	S
Sm10	R	R	R	S	I	R	S	R	S	S	R	I	S	I	S	S	S
Sm11	R	R	R	S	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm12	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Sm13	R	I	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Sm14	R	R	R	S	I	S	S	R	S	S	I	S	S	S	S	S	S
Sm15	R	R	R	S	S	S	S	I	S	S	S	S	S	S	S	S	S
Sm16	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Sm17	R	R	R	S	S	S	S	R	S	S	R	S	S	S	S	S	S
Sm18	R	R	I	S	S	S	S	R	S	S	R	S	S	S	S	S	S
Sm19	R	R	R	S	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm20	R	R	R	S	I	S	S	R	S	S	I	S	S	S	S	S	S
Sm21	R	R	R	S	S	S	S	I	S	S	S	S	S	S	S	S	S
Sm22	R	R	R	S	S	S	S	R	S	S	R	I	S	S	S	S	S
Sm23	R	R	R	S	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm24	R	R	R	S	S	S	S	R	S	S	R	S	S	S	S	S	S
Sm25	R	R	R	S	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm26	R	R	R	R	R	R	S	R	S	S	R	R	S	R	R	S	S
Sm27	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Sm28	R	R	R	S	S	S	S	I	S	S	S	S	S	S	S	S	S
Sm29	R	R	R	S	S	S	S	R	S	S	R	S	S	S	S	S	S
Sm30	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Sm31	R	R	R	S	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm32	R	R	R	S	I	S	S	R	S	S	R	R	R	R	I	S	S
Sm33	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Sm34	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Sm35	R	R	R	S	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm36	R	R	R	S	S	S	S	R	S	S	R	S	S	S	S	S	S

Sm37	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Sm38	R	R	R	S	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm39	R	R	R	S	I	S	I	I	S	S	R	S	S	S	I	S	S
Sm40	R	R	R	S	S	S	S	R	S	S	I	S	S	S	S	S	S
Sm41	R	R	R	S	S	S	S	R	S	S	R	S	S	S	S	S	S
Sm42	R	R	R	S	S	S	S	R	S	S	R	S	S	S	S	S	S

Abbreviations : R, resistance; S, sensitive; I, intermediate; CPM, Cefepime; CTX, Cefotaxime; CTR, Ceftriaxone; VA, Vancomycin; E, Erythromycin; AZM, Azithromycin; CLR, Clarithromycin; TE, Tetracycline, LEV, Levofloxacin; OF, Ofloxacin; C, Chloramphenicol; CD, Clindamycin; LZ, Linezolid; P, Penicillin; AMP, Ampicillin; DOR, Doripenem; ETP, Ertapenem.

Appendix (7): The Results of Statistical Analysis of Antibiotic Susceptibility Pattern

No.	Antibiotic	Percentage of *S. mutans* isolates			P value
		Sensitive	Intermediate	Resistance	
1	Cefepime	0	0	100	**0.00**
2	Cefotaxime	0	2	98	**0.00**
3	Ceftriaxone	2	2	95	**0.00**
4	Vancomycin	93	0	7	**0.00**
5	Erythromycin	81	14	5	**0.00**
6	Azithromycin	95	0	5	**0.00**
7	Clarithromycin	95	5	0	**0.00**
8	Tetracycline	0	3	98	**0.00**
9	Levofloxacin	100	0	0	**0.00**
10	Ofloxacin	100	0	0	**0.00**
11	Chloramphenicol	31	31	38	**0.98**
12	Clindamycin	88	5	7	**0.00**
13	Linezolid	95	0	5	**0.00**
14	Penicillin	90	5	5	**0.00**
15	Ampicillin	88	7	5	**0.00**
16	Doripenem	100	0	0	**0.00**
17	Ertapenem.	100	0	0	**0.00**
P value		**0.00**			