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Isolation and Identification of *Streptococcus Mutans* and Study of Biofilm Formation

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Abstract: The samples were collected from soft deep caries lesion, from patients who were visited to Medical Educational Clinics of the College of Dentistry / University of Al-Kufa, during a period of five months from November 2022 to end of March 2023, provided the study's 100 participants with soft deep caries lesion specimens. The samples were grown in a number of different media. After that, they spent 18-24 hours in a (37ºC) incubator. Colony morphology, microscopy, and biochemical testing were first used by scientists to identify *Streptococcus mutans*. Only 20% of the isolates were confirmed to be *St. mutans* from the 100 positive clinical samples, the automated Vitek 2 system deployed GP-ID cards including 64 biochemical assays to guarantee the isolates were indeed *St. mutans*. The approach promptly and accurately identified 20 distinct bacterial isolates with probabilities ranging from 94% to 99.7%. In the present study, it was used ELISA to distinguish between *St. mutans* isolates that produced biofilms and those that did not, by using the median values of optical density (OD) at 570 nm (> 0.240, 0.120, and 0.120), bacterial biofilms cause chronic diseases that are difficult to treat. Twenty distinct isolates were tested for their ability to form biofilm, 9(45%) were found to be strong biofilm producers, while 11(55%) were rated as moderate biofilm producers.

Keyword: *Streptococcus mutans*, Biofilm formation, TCP method, oral cavity.

Introduction: Biofilms, or surface-attached clumps of microbes contained in a matrix of extracellular polymeric material, are common in most environments, including the human body (1). Polysaccharides, structural proteins, enzymes, DNA, lipids, and water all make up the biofilm matrix. Long-term colonization and spatial organization are made possible by the biofilm's protection from environmental threats including phagocytosis. Microorganisms can thrive in a wide variety of habitats thanks to physical and chemical gradients (2).
Synergistic interactions, such as co-aggregation, benefit the members of mixed biofilms by facilitating colonization, the exchange of extracellular enzymes, mutual feeding, and mutual

protection (3). Ecological succession is governed by competition between community members, and segregation is triggered by competition. Microbial communication (quorum sensing), or the coordinated, population-wide response to an altered environment, is facilitated by the close proximity of cells in a biofilm (4).

The majority of multibacterial infections are caused by oral biofilms. The human oral microbiome is one of the most well investigated human-associated ecosystems. Saliva flow, the host immune defense, and regular oral hygiene routines generally keep biofilms of oral bacteria, archaea, viruses, fungus, and protozoa in check. Carbohydrate-rich diet, smoking, and chemo- or radiotherapy are all environmental variables that contribute to the disease (5).

Oral diseases like dental cavities, periodontal disease, and peri-implantitis can all be traced back to biofilm-forming bacteria. Streptococci and other acidogenic bacteria that adhere to enamel cause tooth caries by converting sugars and starches in the diet into acids (6). Regular acidification promotes enamel demineralization and favors aciduric species like *Streptococcus mutans* that further reduce pH. Cavities occur and grow as a result of enamel demineralization and the breakdown of dentin's biological matrix (7).

Periodontopathogens, such as *Porphyromonas gingivalis*, work together to weaken the body's defenses, allowing them to unleash damaging inflammation in the gum tissue surrounding a tooth. Gingivitis, the mild form of the disease, can proceed to periodontitis, the more severe form of the disease, which is characterized by the loss of alveolar bone supporting the tooth and, in its most severe form, can lead to tooth loss. Due to the abundance of proteins and peptides in inflamed gum, proteolytic and immunogenic species increase as the disease advances (8). Dysbiotic biofilms and the autoimmune response reinforce one another. Periodontopathogens can quickly colonize dental implants used to replace teeth, causing harm to the peri-implant tissue and, eventually, implant failure (9).

Aim of study: This study aimed to Isolation and Identification of *Streptococcus mutans*, and Study the Biofilm formation among the isolates.

Materials and methods: This study were included 100 infected patients, with age range (25-60 years old). The samples were collected from soft deep caries lesion, from patients who were visited to Medical Educational Clinics of the College of Dentistry / University of Al-Kufa, during a period of five months from November 2022 to end of March 2023. The soft deep caries lesion samples were generally collected by twice rolating a sterile cotton swab for culture should be placed in transport media until taken to laboratory. The swab was inoculated on culture media and incubated aerobically at 37°C for 24h. The samples were transferred by means of a cooled box to the Dentistry Laboratory College / Al-Kufa University for the purpose of identifying the bacteria and performing laboratory analyzes.

Ethical approval : The necessary ethical approval from ethical committee of the Medical educational clinics and patients and their followers must obtained. Moreover, all subjects involved in this work are verbal informed and the agreement required for doing the experiments and publication of this work are obtained from each one prior the collection of samples.

Identification of *St. mutans* by GP-ID with VITEK-2 Compact

All strains of *Streptococcus mutans* were detected using the BioMerieux Compact VITEK-2 System. This phenotypic identification uses biochemical processes to distinguish between isolates. For biochemical research using fluorescence, the Vitek-2 card comes with 64 wells. Phosphatase, urea, nitrate, and actidione were evaluated in 20 out of 64 assays for carbohydrate absorption. Cards were filled, sealed, and delivered to the incubator at 35°C by means of the Vitek-2 machine. The output reports are deciphered using algorithms. The findings were recognized using the ID-GP database. Results are suggested by ID by the software of these systems. In cases where the first results indicated "poor discrimination" or "no ID," further tests were administered and the results were analyzed. Each strain was incubated with the inoculated culture medium overnight at 37°C. One colony was identified using the phenotypic VITEK-2 Systems (BioMerieux).

Biofilm production

According to (10), the conventional biofilm detection technique was the tissue culture plate method (TCP) assay, which is also called the semi quantitative micro titer plate test (biofilm assay).

1. Fresh agar plate isolates were cultured in 1% glucose tryptcase soya broth (TSB) for 72 hours in anaerobic conditions at 37°C. After that, the culture was diluted 1:100 with TSB.
2. The diluted cultures were placed into 150 µl wells of sterile, polystyrene, 96-well, flat-bottom tissue culture plates. To ensure that the binding was not media-specific, broth alone was used as a control. Every single isolate was injected three time
3. After 24 hours, the tissue culture plates were placed in an incubator set at 37°C. After incubation, drain the contents of the well gently using tap water. Floating bacteria were eliminated from the wells after four rounds of treatment with phosphate buffer saline (pH7.2).
4. After 30 minutes in an oven set at 37°C, the plate biofilms were set.
5. All of the wells were stained by crystal violet (0.1% v/v). After the excess discolouration was wiped off with deionized water, the plates were allowed to dry.
6. Dissolved bound crystal violet in 150µl of a mixture of acetone and ethanol (20:80, v/v). The 570 nm optical density (O.D.) was deciphered using Table (1).

Table (1): Differentiating biofilm types using the TCB approach

Mean of O.D. Value At 630 Nm	Biofilm Formation
<0.120	Non
0.120-0.240	Moderate
>0.240	High

Results and Discussion

The samples were collected from soft deep caries lesion, from patients who were visited to Medical educational clinics of the College of Dentistry / University of Al-Kufa, during a period of five months from November 2022 to end of March 2023, provided the study's 100 participants with soft deep caries lesion specimens. The samples were grown in a number of different media. After that, they spent 18-24 hours in a (37ºC) incubator. Colony morphology, microscopy, and biochemical testing were first used by scientists to identify *Streptococcus mutans*. Only 20% of the isolates were confirmed to be *St. mutans* from the 100 positive clinical samples, the automated Vitek 2 system deployed GP-ID cards including 64 biochemical assays to guarantee the isolates were indeed *St. mutans*. The approach promptly and accurately identified 20 distinct bacterial isolates with probabilities ranging from 94% to 99.7%. The outcomes were summarized in Table (2).

Table (2) Isolation and identification of *Streptococcus mutans* by automated Vitek 2 system

No. Of Samples	Automated Vitek 2 System	
	St. Mutans	**Other Types of Bacteria**
100	**20(20%)**	**80(80%)**

Involvement of plaque, bacteria, and sugar intake in the genesis of dental caries led to its classification as a chronic illness. While the hard dental tissues may show symptoms of carious demineralization, the disease process really begins in the bacterial biofilm (dental plaque) that coats the tooth surface (11). The amount of sugar in the diet, the makeup of saliva, how often one brushes one's teeth, and whether or not one is exposed to fluoride are all determinants (12).

The results in this study were identical with results of (13) who found that, *St. mutans* was often found in soft deep caries lesions due to its specific characteristics and role in the development of dental caries (tooth decay), and it found in 25%. (13) found dental caries was a multifactorial disease influenced by various factors such as diet, oral hygiene, and the presence of specific bacteria in the oral cavity.

A research by (14) found that, *St. mutans* was known for its ability to metabolize dietary sugars and produce acids as byproducts. These acids can demineralize the tooth enamel, leading to the formation of cavities. In deep caries lesions, the acidic environment can create conditions favorable for the survival and proliferation of *St. mutans* (15). Also, *St. mutans* has adaptations that allow it to thrive in acidic conditions. It can survive and grow in an environment with a low pH, which is often present in caries lesions due to the acid produced during the fermentation of sugars according to study of (16). (17,18) found *St. mutans* was considered one of the primary cariogenic (cavity-causing) bacteria. Its presence and activities contribute significantly to the initiation and progression of dental caries. The bacteria can adhere to tooth surfaces, produce

acids, and contribute to the breakdown of tooth. In addition, (19) found *St. mutans* has specific mechanisms for utilizing sucrose, a common dietary sugar. The metabolism of sucrose by *S. mutans* results in the production of extracellular polysaccharides, which contribute to the formation and stability of dental plaque. The presence of *St. mutans* in soft deep caries lesions was associated with its ability to metabolize sugars, produce acids, form biofilms, tolerate acidic conditions, and contribute to the overall cariogenic process (20).

In the present study, it was used ELISA to distinguish between *St. mutans* isolates that produced biofilms and those that did not, by using the median values of optical density (OD) at 570 nm (> 0.240, 0.120, and 0.120), as previous research has shown that, bacterial biofilms cause chronic diseases that are difficult to treat. Twenty distinct isolates were tested for their ability to form biofilm, and as shown in Table (3-2), 9(45%) were found to be strong biofilm producers, while 11(55%) were rated as moderate biofilm producers as shown in Table (3).

Table (3): Biofilm formation of *St. mutans* isolates

Bacterial Isolates (No.)	Biofilm Formation	
	Strong	Moderate
***St. Mutans* (20)**	**9(45%)**	**11(55%)**

(21) found that half of *St. mutans* isolates may attach to any community of microorganisms in which cells cling to each other and commonly stick to a surface, therefore our results were consistent with theirs. It is usual for adherent cells to produce their own extracellular polymeric matrix.(22) Noting that, 35% of *St. mutans* clinical isolates generated a strong biofilm, emphasized the need of creating an efficient treatment. (23) looked at 44% *St. mutans* samples of dental caries have strong biofilm and (24) found that 26.5% of *St. mutans* had moderate biofilm development. *St. mutans* was proficient in forming biofilms (dental plaque) on tooth surfaces. Biofilms provide a protective environment for bacteria, allowing them to adhere to the tooth structure and resist removal by mechanical means, such as brushing. This biofilm formation contributes to the persistence of *St. mutans* in caries lesions (25). However, *St. mutans* was highly proficient in biofilm formation, and this ability plays a significant role in the development and progression of dental caries, especially in soft deep caries lesions because *St. mutans* possesses specific surface structures, such as adhesins and fimbriae, that enable it to adhere to the tooth surfaces (26). These adhesion mechanisms allow the bacteria to establish a stable attachment to the enamel and dentin, initiating the formation of a biofilm according to study of (27). *St. mutans* has a unique ability to metabolize sucrose, a common dietary sugar. When sucrose was available, *St. mutans* converts it into extracellular polysaccharides through glucan synthesis enzymes. These extracellular polysaccharides contribute to the formation of a sticky matrix that helps bind bacterial cells together, facilitating the development of a robust biofilm (19). A study of (28) found *S. mutans* can engage in cooperative behavior with other bacteria

present in the oral microbiota. The interactions between different bacterial species can enhance the overall stability and structure of the biofilm. So, *St. mutans* was well-adapted to acidic environments, and it can thrive in conditions with low pH. The production of acids during the metabolism of sugars by *St. mutans* can create an acidic microenvironment within the biofilm. This acidic environment can be detrimental to competing bacteria that are less tolerant to low pH, allowing *St. mutans* to dominate and persist in the biofilm (29). Biofilms, often known as slime, were aggregated polymers such as extracellular DNA, proteins, and polysaccharides. Biofilms may form on both living and nonliving surfaces. They were found in a wide variety of environments, including the natural, industrial, and healthcare settings (30). Microbes that live in biofilms are physiologically distinct from their planktonic cell counterparts. The majority of the extracellular matrix surrounding biofilms consists of polysaccharides, proteins, nucleic acids, lipids, and other macromolecules and substances (31). Many factors may stimulate microbial biofilm development, including the discovery of attachment sites on a surface by individual cells, the availability of nutrients, and even the exposure of planktonic cells to sub-inhibitory doses of antibiotics. Cells undergo phenotypic alterations and the differential regulation of large sets of genes when switching to the biofilm formation mode (32). Biofilm production consists of four stages: bacterial attachment to a surface, the formation of microcolonies, biofilm maturity, and bacterial removal (called dispersion) to possibly colonize new places. Sessile bacteria, those present in biofilms, are distinguishable from planktonic bacteria by their dormant growth condition and distinctive morphologies (33).

Conclusion:

Colony morphology, microscopy, and biochemistry found *Streptococcus mutans*. GP-ID cards and 64 biochemical assays indicated 20% of clinical sample isolates were *St. mutans* using automated Vitek 2. Biofilm-caused disorders are difficult to cure.55% of isolates generated moderate biofilms, whereas 45% formed robust ones.

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