Iraqi Journal of Biotechnology, 2026, Vol. 25, No. 1, 119-127.

Rapid molecular diagnosis of streptococcus mutans causing dental caries using *16SrRNA* and *gtfB*

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Received: October 2, 2024 Accepted: November 28, 2024 Published March 30, 2026

Abstract

Background. Dental caries, or "tooth decay," is the most prevalent chronic infectious disease in the oral cavity. Among both younger and older generations, dental caries is the leading cause of tooth loss and root decay. One of the microorganisms that is frequently connected to dental caries is *Streptococcus mutans*. **Aim.** To isolate and identify *S. mutans* from dental caries, **Methods.** To achieve these goals, a total of one hundred specimens were obtained from patients clinically diagnosed by dental physicians with dental caries. All specimens were screened to detect the presence of *S. mutans* on different culture media using morphological and biochemical tests, specimens were further processed for molecular characterization method, bacterial DNA was isolated to be identified by polymerase chain reactions for specific primers pairs of *16S rRNA* and *gtfB genes*. **Results.** The results of morphological and biochemical tests revealed that 34 isolates *S. mutans* isolates were isolated from a total of 100 dental caries samples with a percentage of 34% which confirmed by molecular diagnosis. **Conclusion.** This study demonstrates findings imply that PCR analysis, employing particular primers (*gtfB;16SRNA*), is appropriate for the straight forward, quick, and accurate identification of *S. mutans*.

Keywords: *S. mutans,* Dental caries, *16S rRNA*, *gtfB* genes.

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Introduction

Dental caries, commonly referred to as tooth decay, has become one of the most prevalent conditions affecting both children and adults. Dental caries, a persistent illness, originates in the normal microbiome of the mouth. Some bacteria in the mouth may cause periodontal disease and dental caries, but that's only a small fraction of the total germs in the mouth. *S. mutans*, *Actinomycetes*, and *Lactobacillus* are the three most often associated bacteria with dental caries. When planning a course of therapy, it is important to consider the patient's medical history, microbiological virulence factors, and the likelihood of etiological variables maintaining or decreasing (1). The process of dental caries involves the balance of mineral loss in the teeth and their gradual replacement in response to the acid attack brought on by food consumption. Most parts of life change with time, and diet is no exception. According to (2), the sugar level of all these foods and drinks is extremely high. The three main conditions that can negatively affect oral health are gum disease, tooth decay, and oral

cancers. The majority of these ailments are preventable. Not necessarily as causes of poor dental health, but rather as factors that may exacerbate it, diabetes, heart disease, cognitive decline, and dietary deficits have all been related to poor oral health (3, 4).

As per the WHO's 2022 Global Oral Health Status report, about 3.5 billion people globally have oral health issues, with three quarters of those affected living in middle-income countries. Dental caries is a common condition in Iraq, as it is in other developed and developing nations. Due mostly to a lack of knowledge about oral healthcare, the number of patients with dental caries has increased. Dental caries is caused by plaque buildup. The production of acid by *S. mutans* increases the risk of tooth demineralization when it is present on the tooth surface. Oral cancer, tooth discoloration, and toxicity have been recorded with commercial antiseptics, antibacterial agents, and antioxidants used in the treatment and prevention of plaque formation. Dental caries infections are the most significant dental illnesses (5).

Moreover, *S. mutans* has been shown to be resistant to antibiotics including penicillin, amoxicillin, tetracycline, (6) fluoroquinolones, and chloramphenicol because these drugs can cause genetic alteration in their target, or mutation, which lowers the drug's affinity for its substrate (7) As a result, natural chemicals might be recommended for preventing dental caries instead of manufactured antimicrobials, as they may have less side effects (8). Streptococcus mutans possesses several virulence factors. In order to create a dental biofilm, *GTFs* are useful in absorbing more oral bacteria. The *gtfB, gtfC, GtfD*, and *SpaP* genes are examples of virulence factors that may be linked to the target genes for the PCR (9) (10) (11). According to (12), biofilm can cause bacteria to become more resistant to antibiotics and make it difficult for host inflammatory cells to phagocytose biofilm cells.

One important virulence element in the pathophysiology of dental caries in humans and animals is the capacity of *S. mutans* to synthesis extracellular glucans (13, 14, 15). Glucan production can be impacted by changes in the expression level of the *gtfs* genes, which in turn impact bacterial adherence and biofilm formation (16).*16SrRNA* sequencing has been used to examine the features of the oral microbiome in patients with and without caries. In studies on the human oral microbiome, taking advantage of heterogeneity in the *16SrRNA* gene sequence has been seen as a low-cost high-throughput characterisation strategy. Based on *16SrRNA* gene sequencing, this method has been well verified and shown to be accurate and practical for oral microbiome studies (17, 18).

This study's main objective is to Isolate and characterize *S. mutans*, which is commonly present on the surface of dental caries infections using morphological and biochemical tests and molecular diagnosis of *S. mutans* by detection of *16SrRNA* and *gtfB* genes using conventional PCR.

Material and methods

A total of one hundred specimens were obtained from patients clinically diagnosed by dental physicians with dental caries, that

visited Specialized Dental Center Hay Al Hussein Specialized Center at Mysan city-Iraq, between May 2023 to July 2023, which obtained via taking swabs from mouth cavities of patients suffered from various dental caries (dental roots, fissure and pit). Specimens are put into peptone water and transported to the laboratory immediately.

Morphological and Biochemical Tests

Upon receipt and processing the very same day. In preparation for inoculation, the sample is vortexed for fifteen seconds and then diluted one thousand times in an isotonic saline solution. One loop (1/1000 ml of sample) is inoculated on the Mutans Sanguis Agar (Himdia., India), for 48 h under anaerobic conditions in an anaerobic environment (anaerobic container, and sterile transparent adhesive tape was used to seal the container cover) created by an anaerogen gas pack (Oxoid Ltd., England). Researchers use Mutans Sanguis agar plate to examine the size, shape, and colour of bacterial colonies that have been isolated. The agar plates were incubated in an anaerobic environment at 37°C for 48 hours. After gram staining, the shape, clump size, and cell arrangement may be examined under a microscope (19). Bacteria are diagnosed based on their biochemical efficacy and their ability toenzymes production. The isolates' biochemical characteristics are examined in accordance with Bergey's Manual of Systematic Bacteriology (20).

Molecular Diagnosis

Genomic DNA Extraction Mini Kit, iNtron, Korea, a commercial purification method developed to separate DNA from Gram-positive and Gram-negative bacteria, was used to extract DNA from the studied isolates. DNA was extracted by this kit according to the manufacturer’s directions using the bacterial protocol (for Gram-positive bacteria). A fragment of Chromosomal DNA was amplified via PCR technique using a thermocycler (PCR thermal cycler, Bioneer, Korea). The oligonucleotides used as primers to amplify *16SrRNA* and *gtfb genes* are shown in (Table 1). The PCR mixture (25 µl) consisted of 12.5µl of master Mix(2X),1µl of each of primers (forward and reverse), 5µl of nuclease free water,and 5µlof template DNA. The amplification conditions began with a 5-minute denaturation at 95°C, followed by 40 cycles of denaturation, annealing, extension, and final extension at 60°C, 72°C, and 5 minutes, as shown in (Table 2) respectively. After PCR, the products were electrophoresed in a 1.5% agarose gel.

Table (1): Sequence of universal Primer that Used in this Study

Primer		Sequence	Product Size (bp)	References
16SrRNA	F	CCTACGGGAGGCAGCAGTAG	101	(21)
16S rRNA	R	CAACAGAGCTTTACGATCCGAAA		
GtfB	F	AGCAATGCAGCCAATCTACAAAT	96	
GtfB	R	ACGAACTTTGCCGTTATTGTCA		

Table (2): PCR Program for the amplification of studied *S. mutans* genes.

Step	Temperature (°C)	Time (min)	cycles
Initial Denaturation	95	5	1
Denaturation	95	15	40
Annealing	60	1	
Extension	60	1	
Final extension	72	5	1

Results

Morphological and Biochemical Tests

Traditional bacteriological techniques, such as phenotypic identification by micro-scopical analysis after Gram staining and culture on selective medium, were first used to achieve the identification. A total of 100 clinical specimens were purified by sub culturing on blood agar media in anaerobic conditions at 37 °C for 48 h. Bacterial isolates grown on selective Mutans Sanguis Agar medium, the growth of isolates on the MSA medium after incubation in anaerobic conditions at 37 °C for 48 h showed small, high, irregular colonies were attached to each other (Figure 1). Gram staining was used for the final identification of *S. mutans* in addition to the colony characteristics mentioned above, Gram-positive were observed. There were also round or spherical form appear in medium chains, raised or convex elevation, with yellow colonies color, frosted glass appearance with smooth surface under alight microscope after gram staining.

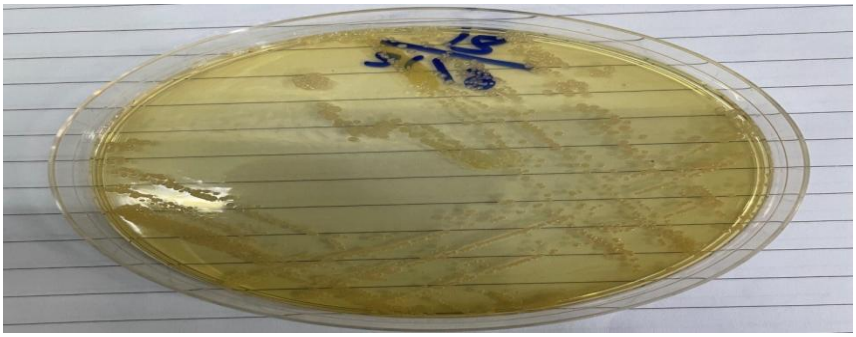

Figure (1): *Streptococcus mutans* on Mutans Sanguis Agar incubated at 37°C for 48 h under anaerobic conditions.

For biochemical behaviour, the findings demonstrated that the *S. mutans* isolates were non-motile, negative for oxidase, and did not create the typical foam when H_2O_2 was added, indicating that the bacteria were catalase-negative. The isolates fermented the tested sugars, including mannitol and they produced acids during carbohydrate fermentation, which caused the phenol red indicator's colour to shift from red to yellow. The alpha-haemolytic pattern was seen after inoculating the isolates on blood agar. Bacterial isolates grown on Bile esculin slant agar revealed a brownish-black colour in biochemical test.

Molecular study

The DNA of (34) 34 % isolates of *S. mutans* was successfully extracted from colonies identified as *S. mutans* using genome DNA Extraction Mini Kit (INtron, Korea). PCR-based molecular identification was conducted on all *S. mutans* isolates using pairs of primers targeting the *S. mutans*. The amplicon sizes of the primers used for the *16S rRNA* gene were 101bp and 96 bp for *gtfB* gene. The PCR reactions were carried out in order to amplification of the DNA template for the PCR assay. Each of the primer pairs amplified single-target genes. The target gene was amplified from all *S. mutans* isolates identified by phenotypic microbiological and biochemical methods. Figures 2 and 3 shows representative agarose gel electrophoresis results of PCR amplicon size of 101bp for16S rRNA gene and 96 bp for *gtfB* gene. Depending of these results, indicated that 34% of *S. mutans* isolates possessed the *gtfB* gene (Figure 3).

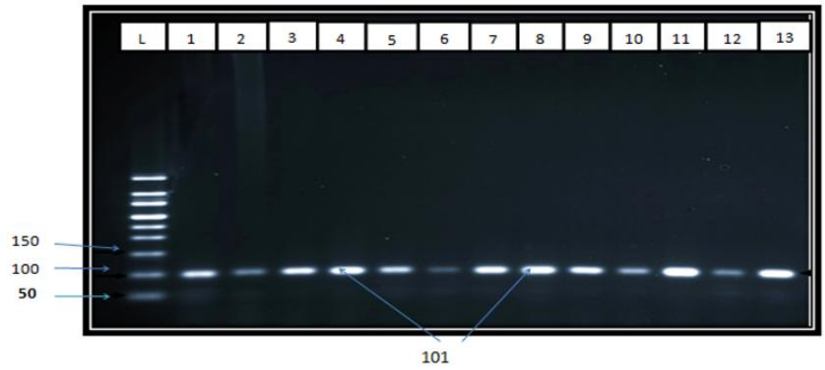

Figure (2): The amplification of *16SrRNA* gene fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 50bp ladder marker. Lanes 1-10 resemble 101bp PCR products.

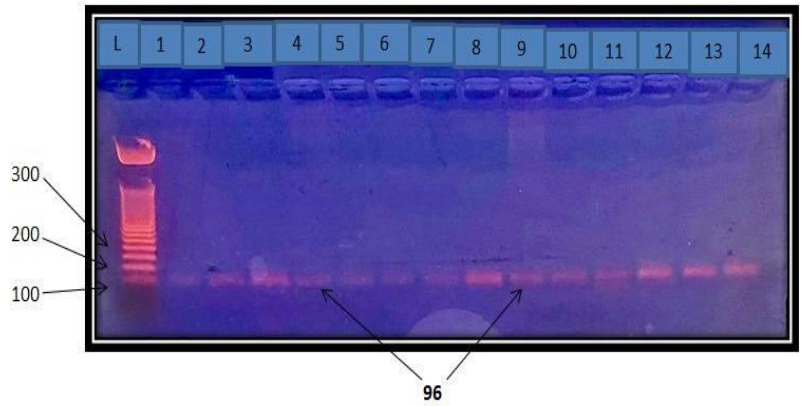

Figure (3): The amplification of *gtfB* gene was fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1-10 resemble 96bp PCR products.

Discussion

Dental caries is a common condition in Iraq, as it is in other developed and developing nations. The bacterial isolates were grown on selective Mutans Sanguis Agar medium (Figure1). All bacteria except *S. mutans* and *S. sorbinus* are inhibited from growing by this combination (22). Because sucrose is added to this medium, *S. mutans* will produce more glucan and have a distinctive colony appearance, which will aid in identification (23).Gram stain was also used for final identification. The biochemical behaviour that is indicative of *S. mutans* was seen in bacterial isolates that were tested for the biochemical traits. In this study, 100 patients with dental caries infections of different ages and sex had 100 randomly selected samples. 34 isolates (34% of the samples) had positive bacterial cultures, while 66% of the samples had negative bacterial cultures. According to a study by (24), samples randomly selected from patients of different ages and sex produced 120 isolates with positive bacterial cultures (80%), while 30 samples (20%) with negative bacterial cultures.

Few studies have compared the virulence genes of the two main caries pathogens and linked them to markers of oral health, despite decades of research on caries and its causes. Among these genes, *gtfB* and *gtfC*, which can

mediate the synthesis of water-insoluble glucan, and *gtfD*, which encodes the glucosyltransferase enzyme, hence can synthesis water-soluble glucan. Genetic identification also lent credence to the bacterial morphology test, and new possibilities for faster bacterial detection have opened up thanks to technological advancements. The challenges associated with culture have led to the development of molecular biology approaches.

Consequently, PCR was used for the purpose of identifying bacteria in both environmental and clinical samples. In terms of bacterial determination, it outperformed traditional culture-based approaches in terms of sensitivity, specificity, and speed (25) (26) The visualization of *16SrRNA* gene amplification results for 34 S.mutans isolates showed one band of DNA with size about 101bp (Figure 2). According to several researchers' findings such as (27) using *16SrRNA* sequencing to detect dental caries, S. mutans can be found in the caries. Previous studies utilising *16SrRNA* sequencing discussed how variations in oral circumstances affect the types of microorganisms present in the mouth (28)(29) .Glucosyl transferases (*GtfB*) gene partial DNA sequencing and PCR amplification were used for molecular identification. In order to verify the identification of S. mutans isolates, a PCR analysis was conducted using particular pairs of oligonucleotide primers that were specifically targeted to the *S. mutans gtfB* gene. The findings indicated that 34% of *S. mutans* isolates possessed the *gtfB* gene (Figure 3), indicating that these primers can be regarded as specific for *S. mutans* identification (30). The *S. mutans* isolates were effectively identified by PCR using particular primers for the glucosyl transferase gene (*GtfB*)(31) (10) The *gtfB* virulence gene of the isolated S. mutans was effectively found using PCR (Figure 3). The results of the PCR analysis of the *S. mutans* isolates aligned with the findings of previous studies (9);(33) (34) .that show the *gtfB* primer sets are highly specific and sensitive for PCR-based detection and assessment of *S. mutans* colonisation in the oral cavity.

Conclusions

In conclusion, our investigation demonstrates findings imply that *S. mutans* isolated from tooth decay can be identified using the identification techniques used here. Additionally, they show that PCR analysis, employing particular primers (*gtfB*; *16SrRNA*), is appropriate for the straight forward, quick, and accurate identification of *S. mutans.*

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