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DETECTION OF *STREPTOCOCCUS MUTANS* ASSOCIATED WITH DENTAL CARIES AMONG ADULTS IN JEDDAH PROVINCE

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ABSTRACT: Dental caries is one of the serious bacterial diseases of the oral cavity caused by diverse Streptococcus species. *Streptococcus mutans* strains displayed remarkable genotypic and phenotypic diversity. Glucosyl transferase genes are recognized as essential enzymes leading to biofilm production, the destruction of the ecosystem of normal bacterial communities, and the development of dental caries. The purpose of this study is to detect *S. mutans* associated with Dental Caries among adults. In our studies, a total of 50 plaque specimens, 23 from male and 27 from female from dental caries patient's samples, were collected from dental clinics in Jeddah province. All bacterial isolates were identified by culture on Tryptone Yeast Extract Cystine w/Sucrose (TYCSB), Bile esculin slant agar, and Blood agar (BA) media. The results of the current study revealed that only four (8.0%) bacterial isolates out of 50 plaque specimens were identified as *S. mutans*. These four bacterial isolates named RHA1, RHA2, RHA3, and RHA4 were identified by biochemical and molecular tests. Partial DNA fragment of Glucosyl transferase (GtfB) gene was successfully amplified from the four bacterial isolates using PCR serotype-specific primer for *S. mutans* serotype (e). Partial nucleotide sequence analysis of the GtfB gene showed 98 to 99% similarities with other *S. mutans* strains on NCBI.

INTRODUCTION: Tooth decay is one of the most prevalent diseases of the oral cavity caused by oral bacteria that thrive in the habitation of teeth and live-in symbiosis with the immune system ¹. Multiple diverse microbial phylotypes are found in the oral cavity. The humid, sticky nature of the mouth provides optimal environmental conditions and a reasonable degree of openness for micro-organisms.

If there is low oxygen and low pH, only organisms that can live in the human mouth can survive. However, only a small number of these organisms engage in oral diseases such as cavities or tooth decay. *Streptococcus mutans*, *Streptococcus gordonii*, and *Streptococcus sanguinis* are the primary bacteria responsible for dental decay, although there are also certain Lactobacillus bacteria ².

Streptococcus mutans is very significant to study, not only because it is almost found in everyone in the world, but it shows various symptoms. That affects our daily lives. As the bacteria develop in the mouth, they can cause tooth destruction, impaired speech, difficulty chewing, multiple infections, Psychological problems such as poor

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social interaction, concentration problem, etc³. As a primary etiology of dental caries, *S. mutans* poses a strong adhesive ability to attach to the tooth surface, which is the decisive initial step in colonization, biofilm formation, and caries development^{4,5}. Since, the occurrence of *S. mutans* can thrive and produce lactic acid, promoting the formation of dental caries, the study aimed to identify these bacteria and to examine the presence of virulence gene GtfB by PCR. Strains of *Streptococcus mutans* bind to the teeth surface by hydrophobic attachments and ferment dietary carbohydrates like sucrose. Sucrose metabolism results in increased adherence to bacteria to tooth enamel, and acidic compounds produced are crucial in the production of dental caries, periodontal diseases, and gum recession. In addition to the involvement of bacterial communities associated with fermentative metabolism, an increase in dietary carbohydrates, particularly sucrose results in more acid production that may exceed both the capacity of the saliva to extract acid end-products and the neutralizing power of the salivary/plaque buffer system, causing additional acidification of the biofilm⁷.

The role of *Streptococcus mutans* in the initiation of caries was due to the fact that it is acidogenic and aciduric⁸. *S. mutans* also produce an extracellular layer of polysaccharides in dental biofilms which are the glucosyltransferases products synthesized by gtf B and gtf C genes^{9,10,11}. The aim of the present study is to detect *S. mutans* isolates in clinical isolates among adults with dental caries from different dental clinics in Jeddah province.

MATERIALS AND METHODS:

Sample Collections: Fifty oral samples were collected from different age and sex groups of patients with dental caries. Tryptone Yeast Extract Cystine w/Sucrose (TYCSB) agar media containing bacitracin antibiotic was inoculated with toothpicks from dental caries samples and incubated at CO₂ incubator at 37 °C for 48 h.

Further, a single bacterial colony from each plate was streaked on Blood Agar (BA) media and incubated at 37 °C for 48 h in anaerobic conditions. The patients who had taken antibiotic therapy for the last two weeks were excluded from the study.

Bacterial Culture and Growth: Fresh (24-h) bacteria were inoculated on Bile esculin slant agar (Merck, Germany) selective and differential medium which is employed to presumptively identify enterococci and group D streptococci based on the ability of an organism to hydrolyze esculin. Bile esculin agar contains oxgall (bile salts), nutrients, esculin, and ferric citrate that promote the growth enterococci and group D streptococci and inhibit the growth of the other gram-positive bacteria. *S. mutans* can hydrolyze the glycoside esculin and produces esculetin and dextrose; the esculetin can then react with the ferric citrate to form a dark brown or black phenolic iron complex.

When the bacteria hydrolyze esculin, the color of the media will change to dark brown or black. The slants were incubated at 35 °C in ambient air 12 with loose caps for 48 h. Readings were taken at 24 and 48 h. A reaction was considered positive when one-half or more of the medium was blackened¹³.

Acidogenesis of *S. mutans* Isolates: *Streptococcus mutans* was inoculated in 10 mL of TYCSB broth (pH 8.32) to evaluate the potential acid production as described by¹⁴. The pH of the media was measured at different time intervals (0 h, 24 h, 48 h and 72 h). The tests were conducted twice.

Genomic DNA Isolation from Bacterial Isolates: Bacterial genomic DNAs were isolated using QIAamp DNA Mini Kit according to QIAGEN kit handbook. A single colony was picked from the TYCSB agar plate and the bacterial cells were suspended directly into TYCSB broth media and incubated in 5% CO₂ at 37 °C O/N.

One milliliter bacterial suspension containing 0.5 McFarland was centrifuged at 14000 xg for 10 min and the pellets were resuspended in 180 µl Buffer AL and 20 µl Proteinase K. After incubation at 56°C for 10 min, 200 µl ethanol (96 to 100%) was added to each sample and then transferred to QIAamp Spin Columns. The spin columns were centrifuged at 6000 xg for 1 min, and the filtrates were discarded, and 500 µl of Buffer AW1 was added to Spin Column. After centrifugation at 6000 xg for 1 min, 500 µl of AW2 Buffer was added to the Spin Column again, centrifuged at the previous speed and the filtrate was discarded.

The Spin Columns were placed in a clean 1.5 ml microcentrifuge tube, and 150 µl of AE Buffer was added to silica filter to elute the DNA. The eluted in DNAs were kept at -20 °C until use.

PCR Amplification of Gtfb Gene: The gDNAs of *S. mutans* were confirmed by PCR using gtf Bgene specific primer set GTFB-F 5'- ACTA CACT TTCG GGTG GCTT GG-3' and GTFB-R 5'- CAGT ATAA GCGC CAGT TTCA TC-3'¹⁵ using Qiagen® Taq PCR Master Mix Kit.

The PCR mixture (10 µl) consisted of 0.2 mM each deoxyribo nucleoside triphosphate, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 1 U of Ex Taq DNA polymerase (Qiagen® Taq PCR), a 0.5 µM concentration of each primer, and 10 ng of template DNA.

After denaturation at 96 °C for 2 min, a total of 25 PCR cycles were performed; each cycle consisted of 15 s of denaturation at 96 °C, 30 s of annealing at 61 °C and 1 min of extension at 72 °C, followed by final extension cycle at 72 °C for 10 min and a 10 °C soak.

An aliquot of 2 µL of each PCR product was analysed by electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide and visualized on UV trans illuminator to confirm the presence of a 517 bp band.

DNA Sequencing: The PCR products were purified using Multiscreen filter plate (Millipore Corp.) The purified PCR products of GtfB gene were sequenced using an automated DNA sequencer ABI PRISM 3730XL Analyzer (96 capillary type) using Big Dye (R) Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions. Sequences were submitted to Gen Bank on the NCBI website (<http://www.ncbi.nlm.nih.gov>).

Sequences obtained in this study were compared with the Gen Bank database using the BLAST software on the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>). Gtf Bgene sequences were aligned using the BLAST program, and Phylogenetic trees were constructed after multiple sequence alignments using CLC Main Workbench V8.1.3 (Qiagen, Bioinformatics) to construct a neighbor-joining tree using Jukes-Cantor model.

RESULTS: Fifty clinical specimens were collected from various Dental Clinics in Jeddah city, 46% were males, and 54% were females.

The numbers of clinical samples and the age groups of the patients with dental carries are presented in **Table 1** & **Fig. 1**. The median age of patients with dental caries was 33 years old in males and 35 years old in females.

The majority of patients with dental caries were females: 54% as compared to 46% among males.

TABLE 1: DESCRIPTION OF THE STUDY SUBJECTS WITH DENTAL CARRIES

Characteristics	Male	Female
Number	23 (46%)	27 (54%)
Age group		
15 to 25	12	13
25 to 40	8	10
>40	3	4
Mean Age	33.1	35

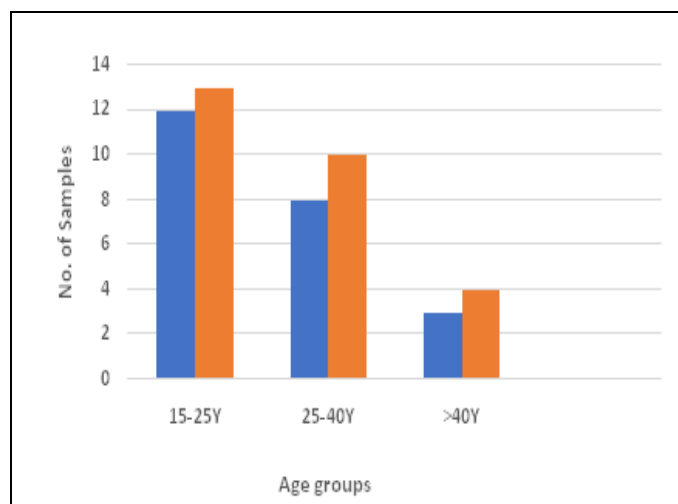


FIG. 1: THE NUMBERS OF CLINICAL ISOLATES COLLECTED FROM DENTAL CLINICS SHOWING AGE GROUPS, DISTRIBUTION AMONG MALE AND FEMALE PATIENTS

Bacterial Culture and Growth: Four out of 50 clinical isolates named RHA1, RHA2, RHA3, and RHA4 were positive for *S. mutans* on blood agar.

The morphology of bacterial colonies appeared white smooth surface colonies, 1-2 mm in diameter, with alpha (α) haemolysis **Fig. 2A**.

Biochemical test of the bacterial isolates cultured on Bile esculin slant agar showed brown-black color **Fig. 2B**. The four isolates were identified as *S. mutans* based upon biochemical and molecular assays.

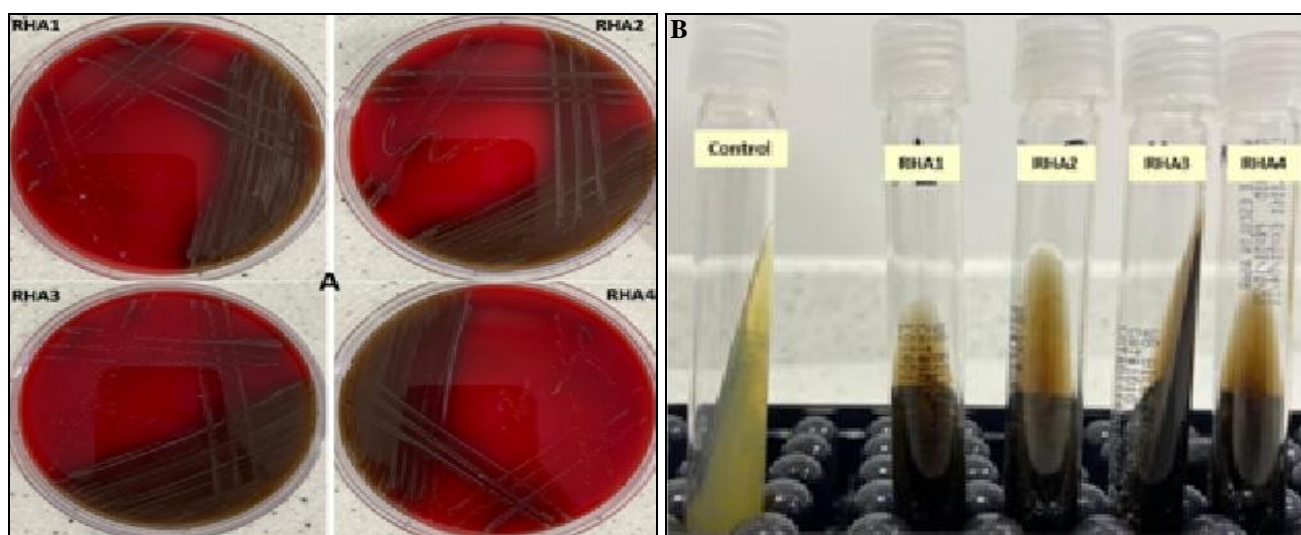


FIG. 2: MORPHOLOGICAL AND BIOCHEMICAL IDENTIFICATION OF *S. MUTANS* ISOLATED FROM DENTAL CARRIES OF ADULTS. A. THE MORPHOLOGY OF THE COLONIES OF *S. MUTANS* ON BLOOD AGAR (BA) SHOWING SMALL WHITE COLONIES WITH A HEMOLYSIS. B: BIOCHEMICAL ASSAY OF *S. MUTANS* CULTURED ON BILE ESCULIN SLANT AGAR SHOWING BROWN-BLACK COLOR

Acido Genesis of *Streptococcus Mutans*: To examine the capability of the *S. mutans* isolates for acid production since the acido genesis is the most important virulence factor for dental caries. TYCSB broth media was inoculated with the *S. mutans* and grown overnight and the pH value was measured at different time intervals.

The results showed that the four clinical isolates had a noticeable ability to grow on acid medium while growing in TYCSB broth **Fig. 3** and converted the initial pH of the media from 8.34 to more acidic pH (up to pH 5.5). Most of the clinical isolates showed high acid production.

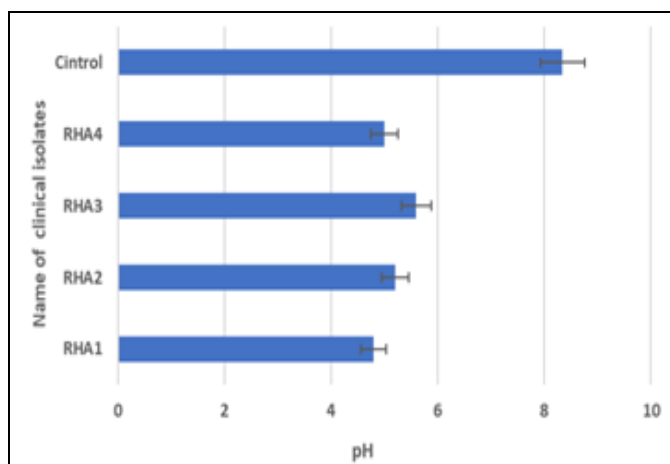


FIG. 3: ACID PRODUCTION BY THE FOUR CLINICAL ISOLATES UNDER STUDY. BACTERIAL ISOLATES WERE INCUBATED IN TYCSB BROTH AND THE PH WAS MEASURED EVERY 24-HOUR INTERVAL WITH A PH METER. ERROR BAR REPRESENTS THE STANDARD DEVIATION

PCR Amplification of *Gtfb* Gene: PCR successfully identified the four *S. mutans* isolates using specific primers for glucosyltransferase gene (*GtfB*). The primer set synthesized for this study allowed the PCR amplification of a gene fragment of *S. mutans* isolates after 20 cycles. The amplified products of a size 517 bp DNA fragment exclusively detected in the four *S. mutans* isolates **Fig. 4**. These results showed the expected size of *Gtfb* gene in *S. mutans* serotype (e) and hence support the species specificity of the primers used.

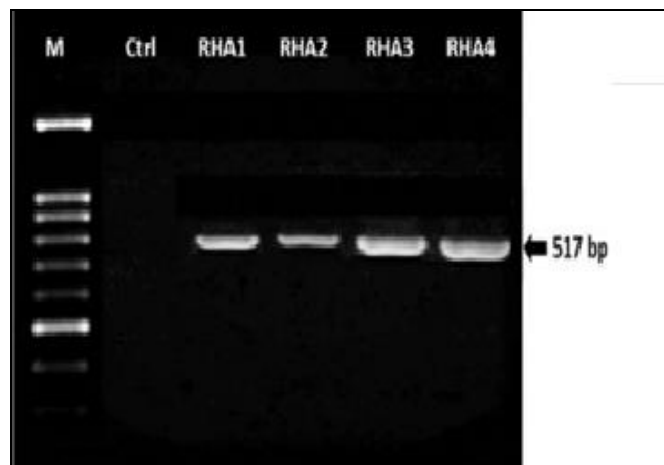


FIG. 4: DETECTION OF *S. MUTANS* ISOLATES BY PCR AMPLIFICATION OF *GTFB* GENE. M: MOLECULAR WEIGHT DNA MARKER. LANE CTRL: NEGATIVE CONTROL, RHA1, RHA2, RHA3, AND RHA4: PCR PRODUCTS OF *S. MUTANS* SEROTYPE (E), THE ARROW INDICATES THE SIZE OF PCR PRODUCTS (517 BP). THERMO SCIENTIFIC GENERULER DNA LADDER MIX, 2 UL OF DNA LOADED

DNA Sequencing: Partial nucleotide sequence analysis of the identified bacterial isolates aligned with other *S. mutans* strains and their sources from NCBI showed 98 to 99% similarities are given in

Table 2. The Gtf Bpartial gene sequence analysis was performed with CLC Main Workbench V8.1.3 (Qiagen, Bioinformatics) to construct a neighbor-joining tree using the Jukes-Cantor model **Fig. 5**.

TABLE 2: INFORMATION AND IDENTIFICATION FOR GTFB GENE SEQUENCES OF S. MUTANS RHA1, RHA2, RHA3 AND RHA4

S. no.	Strain	Identification	GtfB Gene Sequences				Reference accession Number
			Identity (%)				
			RHA1	RHA2	RHA3	RHA4	
1	UA140	<i>S. mutans</i>	98%	99%	98%	98%	JX072983.1
			436/447	453/459	455/464	443/452	
2	T8	<i>S. mutans</i>	98%	99%	99%	98%	CP044492.1
			443/452	457/459	459/464	450/457	
3	NCH105	<i>S. mutans</i>	98%	99%	99%	98%	CP044221.1
			443/452	457/459	459/464	450/457	
4	S1	<i>S. mutans</i>	98%	99%	99%	98%	CP050271.1
			443/452	457/459	459/464	450/457	
5	FDAARGOS_685	<i>S. mutans</i>	98%	99%	99%	98%	CP050962.1
			443/452	457/459	459/464	450/457	
6	UA96	<i>S. mutans</i>	98%	99%	99%	98%	JX072987.1
			443/452	457/459	459/464	450/457	
7	NG8	<i>S. mutans</i>	98%	99%	99%	98%	CP013237.1
			443/452	457/459	459/464	450/457	
8	BM31	<i>S. mutans</i>	98%	99%	99%	98%	MG766902.1
			443/452	457/459	459/464	450/457	
9	BM35	<i>S. mutans</i>	98%	99%	99%	98%	MG766903.1
			443/452	457/459	459/464	450/457	

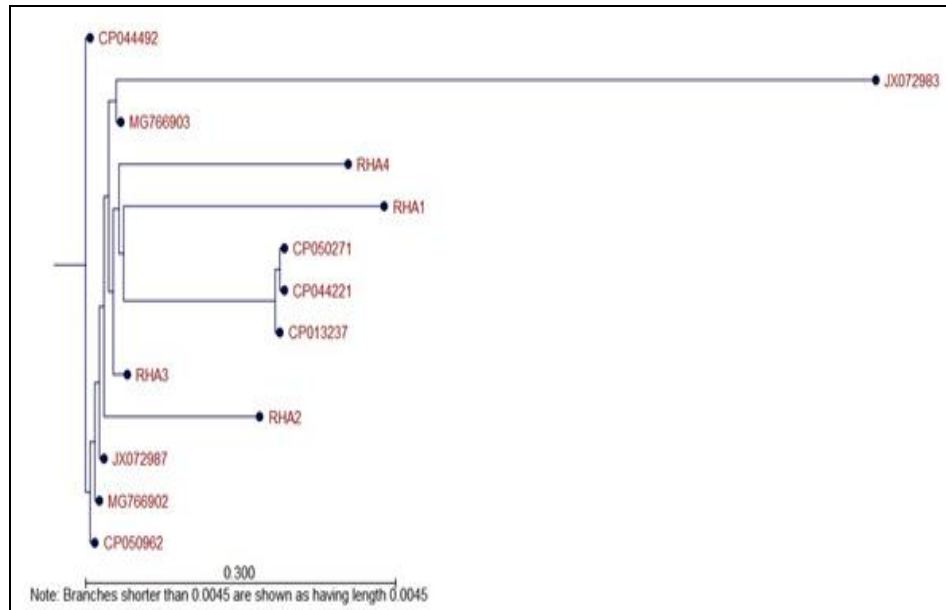


FIG. 5: PHYLOGENETIC RELATIONSHIPS BASED ON THE GTFB GENE SEQUENCE ALIGNMENT OF S. MUTANS ISOLATES RHA1, RHA2, RHA3 AND, RHA4 (CURRENT STUDY) WITH OTHER S. MUTANS STRAINS. THE DENDROGRAMS WERE OBTAINED BY NEIGHBOUR-JOINING (NJ) USING CLC MAIN WORKBENCH V8.1.3 (QIAGEN, BIOINFORMATICS). THE BRANCH NODES SHOWED NUMBERS THAT REPRESENT THE BOOTSTRAP VALUES

DISCUSSION: Dental caries is a multifaceted condition closely linked to the quality of life and social environment ¹⁶. There is also little knowledge about the association between dental

caries and the existence of *S. mutans* in Saudi Arabia. There are large numbers of microorganisms capable of producing dental caries. *Streptococcus mutans*, *Streptococcus sanguis*, *Lactobacillus casei*,

and *Actinomyces viscosus* are the organisms that are the most responsible bacteria that meeting all the requirements needed¹⁷. The mutans species of streptococci play a central role in the initiation of caries on the smooth surfaces and fissures of the crowns of the teeth and suggest that they have a potent etiologic role in the induction of root surface caries too^{18, 19}. *S. mutans* is one of the best characterized Gram-positive pathogens categorized into four serotypes (c, e, f and k) and is able to use sucrose as a nutritional source, partially for intracellular storage components and for the development of extracellular glucans through the glucosyltransferases GtfB, GtfC and GtfD²⁰. *S. mutans* has evolved with many cariogenic properties, such as the ability to bind to the tooth surface, thrive in low pH, and develop acids and biofilm^{21, 22}. Around 75% of strains isolated from dental plaque belong to serotype c, 20% to serotype "e", and the remaining 5% classified as serotypes "f" or "k"^{23, 8}. In this study, fifty clinical specimens were collected from dental clinics in Jeddah, KSA. The prevalence of *S. mutans* associated with dental caries (8.0%) was lower than previously reported in other studies^{24, 19}. The median age of males and females was 33 years and 35 years respectively. According to estimates, most patients with dental decay were women (54%).

Only four isolates represented a percentage of about (8.0%) from the whole fifty isolates were identified as *S. mutans* serotype (e) based on biochemical and molecular identification. *Streptococcus mutans* was first isolated using selective enrichment technique including culturing of cells on TYCSB agar, Blood Agar (BA) and Bile esculin slant agar, which promotes growth of *Streptococcus mutans* and suppress other bacterial species^{25, 26, 27}.

For further identifications of the *S. mutans* isolates for acid production since the acidogenesis is the most important virulence factor for dental caries. The results showed that the pH value varied between 5.5 and 6.0, which indicates that the bacterial isolates had the ability to grow on acidic medium. These results were in agreement with that obtained by⁸. In order to confirm the biochemical tests and their results that had been done and recorded for bacterial isolates, molecular identification was made finally by PCR

amplification and partial DNA sequencing of Glucosyl transferases (GtfB) gene. Glucosyl transferases (Gtfs) genes found in *S. mutans* are the main enzymes involved in glucan synthesis, which cause biofilm formation. These are encoded by gtf b, gtf c, and gtf d genes, respectively. Gtf B tends to synthesize a majority of the water-insoluble glucans (WIG), GtfC synthesizes both soluble and insoluble glucans, and GtfD is primarily for soluble glucans^{28, 29}.

Glucans formed by GtfB and GtfCact as the key structure of the extracellular polysaccharides (EPS) matrix and provide adhesive sites for *Streptococcus mutans* to a tooth surface. Water-insoluble glucans (WIG) synthesized by GtfB and GtfC form the main scaffold of the EPS matrix and provide adhesive sites for *S. mutans* to a tooth surface as well as to other microbes^{30, 31}.

In the current study, DNA-PCR was successfully used to detect the presence of gtf B virulence gene of the isolated *S. mutans* (RHA1, RHA2, RHA3 and RHA4). The PCR findings of the four *Streptococcus mutans* isolates supported the detection of the serotype (e) and were compatible with the results obtained by^{32, 33}.

S. mutans strains isolated from humans harbor two genes encoding enzymes capable of synthesizing water-insoluble glucans (WIG), gtf B and gtf C. There is a strong homology between these two genes and they might be tandemly arranged in the bacterial chromosome, which indicates that they might originate from gene duplication.

A chimeric gtfB gene, thought to have arisen from recombination between gtf B and gtf C genes, has been detected in UA101 strain^{34, 35, 36}. In the current study, the partial nucleotide sequencing data indicated that only one open reading frame (ORF) be identified in the gtf B gene and sharing homology 98 to 99% with the gtfb genes of other *S. mutans* **Table 2**.

The phylogenetic tree would suggest that the *streptococcus mutans* isolates gtf B gene showed high similarity with other accession numbers in NCBI. The phylogenetic tree was constructed using neighbour-joining (NJ) using CLC Main Workbench V8.1.3 method. The value on each branch is the expressed as a percentage for the

position of the branches, as determined by bootstrap analysis. Only values exceeding 90% are shown. In conclusion, the results suggest that the identification methods employed here are useful for the identification of *Streptococcus mutans* isolated from decayed teeth.

And also indicate that PCR analysis is suitable for simple, rapid, and reliable identification of *S. mutans* using species-specific primers. Gtf B DNA sequencing was highly sensitive for the identification of *S. mutans* compared to the conventional methods. Serotype “e” was found to be the predominant among the study subjects.

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CONFLICTS OF INTEREST: The authors declare that they have no conflict of interest.

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