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Isolation and Molecular Detection of Virulence Factor*(Gtfb)* and *(Gtfc)* to *Streptococcus Mutans* Associated with Dental Caries in Kut City of Iraq

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Abstract

Dental caries is perhaps the most prevalent chronic disease. The outcome of the disease is dental decay. The aim of this study detection of virulence factor of *S. mutans* glucosyltransferases *(gtfB) and (gtfC).* One hundred and twenty-four samples were collected from patients by taking swabs from mouth cavity. Those patients were attended at Al-Kut city, in dental specialist center and Teiba specialized medical center for the purpose of isolation and diagnosis of *Streptococcus* then examined under microscope and private clinics in Waist province during the period from August 2020 to December 2020. The result of PCR showed that 90(72.5%) isolates were PCR positive for *gtfB* gene and 34(27.4%) were PCR negative. The *gtfC* gene in sixty-seven isolates of *Streotococcus mutans* gives PCR positive results in 90(72.5%) while 34(27.4%) isolates were PCR negative.

Keywords: *Streptocuccus mutans*; Virulence genes; gtfB; gtfC; Dental caries

Introduction

Dental caries is one of the most prevalent disorders in humans (Islam *et al*., 2007). Tooth caries is a slowly developing chronic disease characterized by partial and irreversible removal of teeth (Rouabhia and Chmielewski, 2012; Zero *et al*., 2009). Dental caries is still a major problem worldwide, particularly in children who encounter dental cavities as their principal cause of tooth loss, considering the clinical advancement of dental caries in the last 150 years. In the United States, 42% of adolescents between the ages of 2 and 11 suffer from tooth decay in their main teeth. In the adult community, periodontal disorders and dental caries impact 60-90 percent of people worldwide (Rouabhia and Chmielewski, 2012). Dental caries is caused by complex interactions between types of microorganisms adhering to the surface of teeth, and are affected by genetic factors such as saliva and diet. Metabolic microbial interactions in tooth biofilms lead to the formation of extracellular glucan and acid production, thereby enhancing the correlation between microbes and teeth (Kidd and Fejerskov, 2004; Islam *et al*., 2007; Rouabhia and Chmielewski, 2012). *Streptococcus mutans* is generally considered to be one of the main pathogens of ECC (early childhood caries) (Berkowitz *et al*., 1984; Milnes and Bowden, 1985; Karp and Berkowitz, 2008; Palmer *et al*., 2010), although other organisms may also have its pathogenesis (Palmer *et al*., 2010; Gross *et al*., 2010).

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Materials and Methods

Sample collection

174 samples were collected by taking swabs from mouth cavity from patient with different dental caries (pit, fissure and dental roots), These samples were put into peptone water and then streaked on blood agar and then subcultured on mitis sulivarius bacitracin (MSB) agar for the period between November 2020 and January 2020. Those patients were attended at Al-Kut city, in Dental specialist center and Teiba specialized medical center for the purpose of isolation and diagnosis of *Streptococcus*, then examined under microscope. Data collection for these cases, name, age, diabetic and clinical manifestation by physician was listed on survey paper.

Bacterial Isolation and Identification

Isolation of *S. mutans*

Bacterial isolates obtained from dental caries samples were streaked on selective medium (Mitis Salivaris Bacitracin Agar) for isolation of S. mutans, then plates were incubated at 37°C for 48 h under anaerobic conditions in candle jar. This step was repeated until pure culture was obtained (Nolte, 1982).

Identification of *Streptococcus* isolates

Bacterial isolates were identified according to their morphological (microscopic) and cultural characteristics, biochemical tests and VITIK-II system (Carlsson, 1967; Emilson, 1983).

Morphological and cultural characteristics

Colony size, shape and color of the bacterial isolates were studied on Mitis Salivaris Bacitracin agar after incubation at 37°C for 24 h under anaerobic conditions in candle jar. Shape, clump and arrangement of cells were studied under microscope.

Biochemical tests:

Catalase test

Clump of growth from pure culture of each bacterial isolate was transferred onto a microscopical slide using a wooden stick applicator, then two drops of 3% hydrogen peroxide solution were added on bacterial cells. Presence of gaseous bubbles indicates a positive result production of catalase (Brown, 2005).

Blood hemolysis test

This test was used to detect the ability of bacterial isolates to produce hemolysin and determine the type of hemolysis, and was achieved by streaking each bacterial isolate on blood agar medium, then incubated at 37°C for 48 h under anaerobic conditions (Collee *et al*., 1996).

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Carbohydrate fermentation test

This test was used to detect the ability of bacterial isolates to utilize different carbon sources (mannitol, sorbitol and raffinose) as a sole source of carbon and energy. This was achieved by inoculating test tubes of brain heart infusion broth containing each carbon source (3%) with each bacterial isolate. All tubes were then incubated at 37°C for 24hrs under anaerobic conditions. The change in color of indicator from red to yellow indicates a positive result (Yoo *et al*., 2005).

Bacterial genomic DNA Extraction

Fungal genomic DNA from of *Streptococcus mutans* isolates were extracted according to company instructions as following steps:

Preparations:

- Absolute ethanol was added (100 ml) to wash Buffer then mixed by shaking for a few seconds, and bottle of wash buffer was closed tightly after each use to avoid ethanol evaporation.
- ddH2O was (1 ml) to Proteinase K then vortexed to ensure Proteinase K is completely dissolved. Once it is dissolved completely, centrifugation was performed for a few second to spin down the mixture, and stored at 4°C till use.

Procedure:

1. **Sample Preparation:***S. mutans* isolates have been grown on nutrient broth for 24h in 37°C were transferred to a 1.5 ml eppinedorf tube, and centrifuged for 1 minute at 14-16,000 × g then supernatant was discarded. Add Lysozyme (0.8 mg\200 µl) to Gram+Buffer (in the 15 ml centrifuge tube) then vortex to completely dissolve the Lysozyme. Transfer 200 µl of Gram+Buffer (make sure Lysozyme was added) to the sample in the 1.5 ml microcentrifuge tube then re-suspend the pellet by vortex or pipette. Incubate at 37°C for 30 minutes. During incubation, invert the tube every 10 minutes. Add 20 µl of Proteinase K (make sure ddH2O was added) then mix by vortex. Incubate at 60°C for at least 10 minutes. During incubation, invert the tube every 3 minutes.
2. **Lysis:** 200µl GB Buffer were added to the sample and mixed by vortexing for 10 seconds. Incubate at 70°C for at least 10 minutes to ensure the sample lysate is clear. During incubation, tubes were inverted every 3 minutes. At this time, pre-heat the required Elution Buffer (200µl per sample) to 70°C

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3. **DNA Binding:** 200µl of absolute ethanol was add to the sample lysate and mixed immediately by shaking vigorously. If precipitate appears, break it up as much as possible with a pipette. GD Column has been in a 2 ml collection tube. Mixture was transferred to the GD column then centrifuged at 14-16,000 × g for 2 minutes. Discard the 2 ml collection tube containing the flow-through been discarded and then place the GD column in a new 2 ml collection tube.
4. **Wash:** 400µl of W1 Buffer was added to the GD column and centrifuge at 14-16,000 × g for 30 seconds and the flow-through was discarded GD column placed been back in the 2 ml collection tube. 600µl of Wash Buffer was added to the GD column and centrifuge at 14-16,000 × g for 30 second then the flow-through. GD column placed back in the 2 ml collection tube and centrifuge again for 3 minutes at 14-16,000 × g to dry the column matrix.
5. **Elution:** The dried GD column was transferred to a new 1.5 ml microcentrifuge tube. 100µl of pre-heated Elution Buffer was add, preheated TE Buffer was added into the center of the column matrix and left for 3 minutes and Centrifuged at 14-16,000 × g for 30 seconds to elute the purified DNA.

Polymerase chain reaction (PCR)

PCR technique was performed for detection *Streptococcus mutans* based on 18S rRNA genes, as well as detection virulence factor genes (*gtfB* and *SAP* genes). The method was carried out according to method described by (NEXpro e PCR 2X8 Strip tube Mix) as following steps:

Preparation of PCR master mix reaction

PCR master mix reaction was prepared using AccuPower PCR PreMix Kit, and this master mix was done according to the company instructions as showing in tables (1, 2).

Table 1. PCR master Mix for *S. mutans* and *gtfC*

PCR Master mix	Volume
DNA template	7 µL
F. primer 20pmol	1 µL
R. primer 20pmol	1 µL
PCR water	11 µL
Total volume	20 µL

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Table 2. PCR master Mix for *gtfB*

PCR Master mix	Volume
DNA template	5 µL
F. primer 20pmol	1 µL
R. primer 20pmol	1 µL
PCR water	5.5 µL
Total volume	12.5 µL

PCR thermocycler conditions

PCR thermocycler conditions for each gene were done by using convential PCR thermocycler system as following tables (3, 4, 5, 6).

Table 3. PCR thermocycler system of *S. mutans*

PCR cycle	repeat	Temp.	Time
Initial denaturation	1	95 ˚C	5 min
Denaturation	35	94˚C	30 sec
Annealing		55˚C	30 sec
Extension		72˚C	30 sec
Final extension	1	72˚C	5 min

Table 4. PCR thermocycler system of *gtfB*

PCR cycle	repeat	Temp.	Time
Initial denaturation	1	94˚C	5 min
Denaturation	37	94˚C	30 sec
Annealing		57˚C	40 min
Extension		72˚C	5 min
Final extension	1	72˚C	5 min

Table 5. PCR thermocycler system of *gtfC*

PCR cycle	repeat	Temp.	Time
Initial denaturation	1	94˚C	10 min
Denaturation	40	95˚C	15 sec
Annealing		60˚C	1 min
Extension		60˚C	1 min
Final extension	1	72˚C	5 min

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Table 6. PCR thermocycler system of PLB1

PCR cycle	repeat	Temp.	Time
Initial denaturation	1	94˚C	3 min
Denaturation	30	94˚C	30 sec
Annealing		53˚C	30sec
Extension		72˚C	30sec
Final extension	1	72˚C	10 min

Gel electrophoresis of PCR product

PCR products of each genes were analyzed by using agarose gel electrophoresis method as following steps:

1. 2% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100˚C for 15 minutes, after that, left to cool 45˚C.
2. Then 3µL of ethidium bromide stain were added into agarose gel solution.
3. Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.
4. The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.
5. PCR products were visualized by using ultraviolet trans illuminator.

Statistical analysis

All results obtained from the present study were entered and analyzed statistically by the statistical package for social science (SPSS) version 26 for Windows Software and Microsoft Excel 2019. Chi-square test and one-way analysis of variance (ANOVA) with least significant differences were used for the assessment of association between the variables studied. The level of statistical significance was set at alpha equal to 0.05 (a = 0.05). A value of $P < 0.05$ was considered statistically significant (Field, 2005).

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Results and discussion

Bacterial and Fungal isolation and identification

Isolation of *Streptococcus mutans*

The oral cavity samples were streaked to the semi-selective Mitis Salivaris agar medium (MSA). This medium promotes the growth of streptococci and inhibits the growth of other bacterial species because it contains crystal violet and potassium tellurite which inhibits most Gram-negative bacilli and Gram-positive bacteria except streptococci (Zimbro *et al*., 1998). Bacterial isolates grown on MSA were then re-streaked on selective Mitis Salivarius Bacitracin agar (MSBA) which composed of MSA, 20% sucrose and 0.2 U/ml of Bacitracin to inhibit growth of most bacteria except *S. mutans* and *S. sorbinus*. The inclusion of sucrose in this medium leads to the formation of glucan and distinctive colony appearance that aids identification of *S. mutans* (Yoo *et al*., 2007). Bacterial isolates then streaked onto brain heart infusion agar plates and incubated at 37°C under anaerobic conditions. Colonies of these isolates appeared as pin points into the culture medium and surrounded by a white opaque halo.

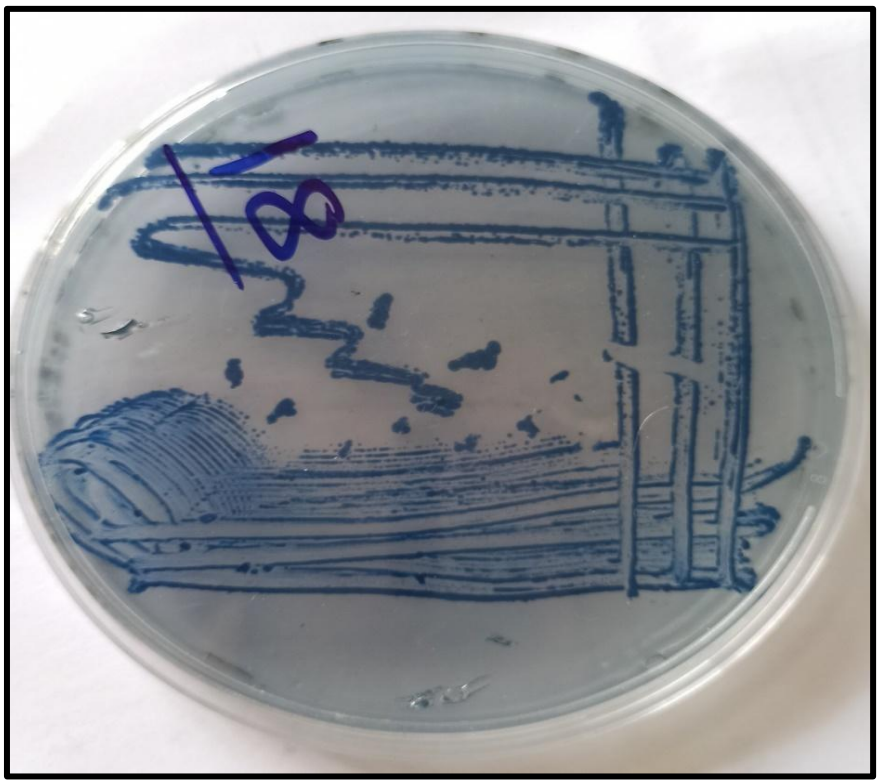

Figure 1. *S. mutans* on Mitis Salivaris agar after incubation at 37°C for 48 h under anaerobic conditions.

Identification of bacterial isolates

Bacterial isolates suspected to be *S.mutans* were grown on selective medium (MSBA) and identified according to their morphological and cultural characteristics and biochemical test:

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Morphological and cultural characteristics

Bacterial isolates grown on Mitis Salivaris Bacitracin agar medium were the first identification according to their morphological and cultural characteristics. Results showed that these isolates were Gram positive, spherical cells and appear in medium chains. Colonies of these isolates are highly convex, raised, light – blue, frosted glass appearance with smooth surface as shown in figure (1). These colonies were also highly adherent to the agar surface if it picked up by loop, polysaccharide formatters were observed as a glistening drop on top of the colony or as a pool besides the colony. This result was agreed with (Koneman *et al*., 1998).

Biochemical tests

When bacterial isolates suspected to be belonged to the genus *Streptococcus* were subjected to examined for their biochemical characteristics, results showed that these isolates were Gram positive, negative for catalase and able to ferment mannitol, sorbitol and raffinose sugars. These isolates were unable to produce hemolysin exhibiting gamma hemolysis on blood agar medium as shown in figure (2). According to the such results, ten isolates were regarded as *S. mutans* (Yoo *et al*., 2005).

Figure 2. *S. mutans* on blood agar

VITEK-II system

To confirm the identification of the ten bacterial isolates as *S. mutans*, they were examined also by the VITEK-II system. Result showed that *S. mutans* were negative to hydrolysis of urease

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(URE) and arginine dihydrolase1(ADH1) and positive to alphaglucosidase (AGAL) and fermentation of Lactose (LAC).

Streptococcus mutans Diagnosed by PCR

Conventional PCR Screening for 16S rRNA Gene of *S. mutans*

Molecular identification of *S. mutans* in this study, relied conventional PCR for the amplification of a partial gene of 18S rRNA by specific primer sequences. This gene was present in 102/102 (100%) samples with a PCR product size of 500 bp, as shown in the figure (3).

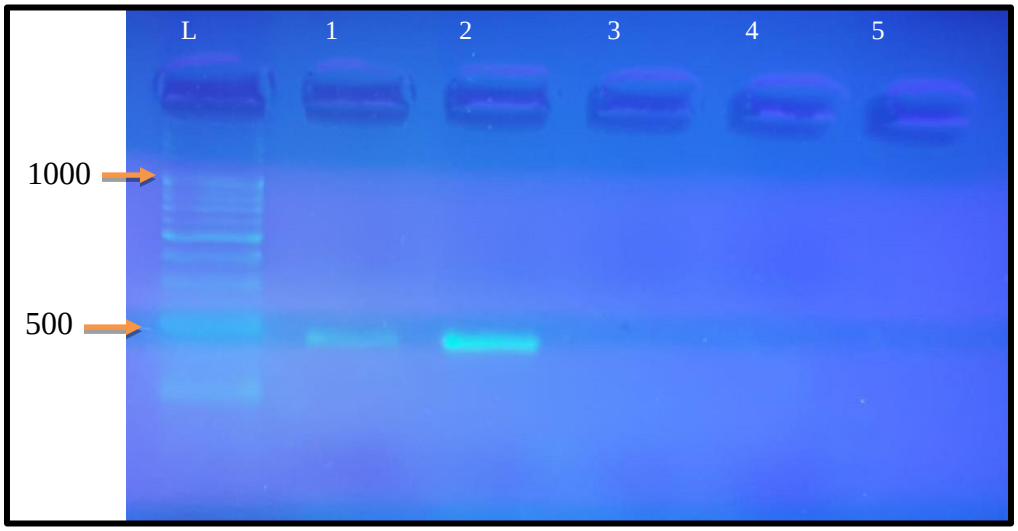

Figure 3. Agarose gel electrophoresis image shows PCR product analysis of pathogenic *S. mutans*. Lane M Marker ladder (1000 bp), lanes (1-5): 16S rRNA gene of *S. mutans* isolate with 500 bp.

Conventional PCR Screening for glycosyltransferases (*gtfB*)

Ninety-five isolates belong to *S. mutans* were positive for *gtfB* gene 95/102 (93.1%), PCR product size of this gene was 98 bp, figure (4).

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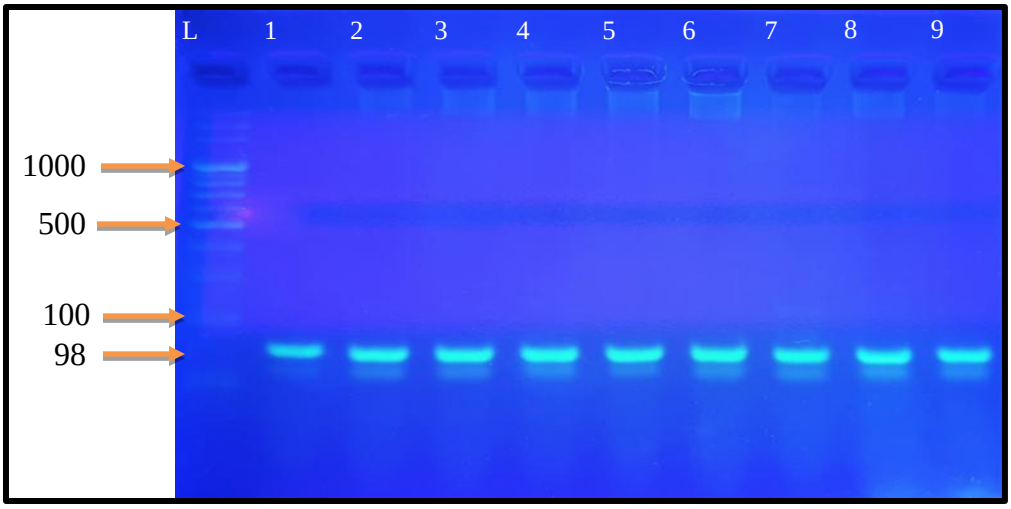

Figure 4. Agarose gel electrophoresis image shows PCR product analysis of pathogenic *S. mutans*. Lane M Marker ladder (1000 bp), lanes (1-9): glycosyltransferases (*gtfB*) gene of *S. mutans* isolate with 98 bp.

Conventional PCR Screening for glycosyltransferases (*gtfC*)

Ninety-two isolates belong *S. mutans* were positive for *gtfC* gene 92/102 (90.1%), PCR product size of this gene was 88 bp, figure (5).

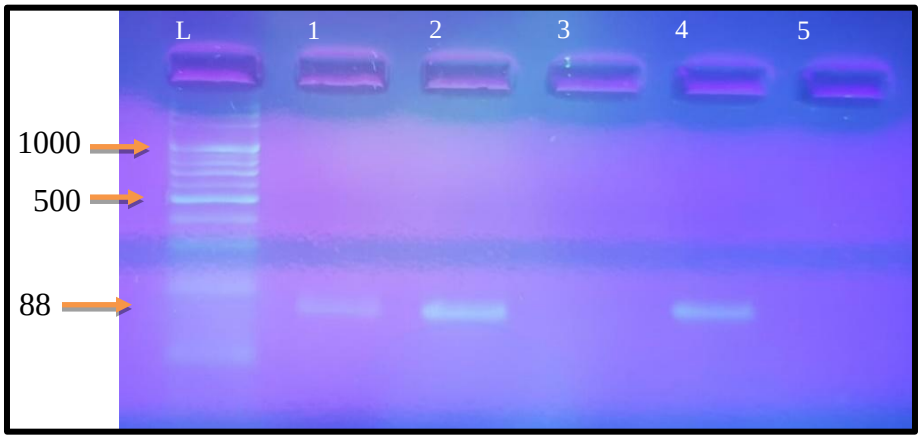

Figure 5. Agarose gel electrophoresis image shows PCR product analysis of pathogenic *S. mutans*. Lane M Marker ladder (1000 bp), lanes (1-5): glycosyltransferases (*gtfC*) gene of *S. mutans* isolate with 88 bp.

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Molecular identification of *S. mutans* in this study, done by conventional PCR by amplification of a part of the mitochondrial gene encoding for the large subunit of 18S rRNA gene by specific primer sequences. The yield of the detected 102/102 (100%). The result was concordant with those of Vitek 2 system.

In the present study, the percentage result of *S. mutans* was higher than these obtained in Jabn by (Oho *et al*., 2000) 18 (40.3%) who applied PCR using a species-specific primer of the 26S rRNA gene *S. mutans* involved in dential caries. These differences in the proportions of each study may come from the difference in the primers used for PCR techniques, the discrepancy in the number of isolates enrolled in each study and the skills of laboratory investigators. PCR has been increasingly used to diagnose *S. mutans*, as it is fast, simple, specific, sensitive and reliable (Mannarelli and Kurtzman, 1998).

In the present study the percentage of obtained *S. mutans* by PCR is agree with a study done in Jaban (100%) by employing molecular technique in bacteria identification PCR and sequencing of the 26S rRNA genes (Nur Dianawati *et al*., 2020).

Rapid identification and molecular biology-based tests have begun to be used more easily and effectively than conventional tests in the identification of fungal pathogens. Diagnostic polymerase chain reaction (PCR) method has been widely applied in laboratories for identification of many fungal species due to their speed, high sensitivity, and specificity (Kano *et al*., 2002).

In this study, Ninety- five isolates out of 102 (93.1%) belong to *S. mutans* were positive for *gtfB* gene. While 92/102 isolates belong *S. mutans* were positive for *gtfC* gene as (90.1%).

The present results and regarding *gtfB* gene and *gtfC* genes for *S. mutans* were seen higher than those obtained in a study done by (Yoshida *et al*., 2002) (60%) for *gtfB* and (30%) for *gtfC* gene respectively which isolated from dental caries.

The higher colonization rate of *S. mutans*, as demonstrated in this study would be ruled by antigen I/II protein that strengthens the adherence to the tooth surface. It was also facilitated by glycoprotein receptors present in saliva, called salivary agglutinin. (Lamont *et al*., 2013). The other factors are cell-to-cell adherence and development of cohesive and pathogenic biofilms via the expression of GTFs. These enzymes (140 to 160 kDa) produce extracellular adhesive glucans that vary in chain length, contain α-1,3 and α-1,6 glucosyl linkages, and have a degree of branching and solubility. *S. mutans* comprises three genes for GTF: GTF-B, responsible for insoluble glucan synthesis; GTF-C, for soluble and insoluble glucan synthesis; and GTF-D, for soluble glucan synthesis. *S. sobrinus* expresses GTF-I and GTF-S, encoding enzymes that produce insoluble and soluble glucans, respectively (Lamont *et al*., 2013).

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Conclusions

Diabetes and smoking are major factors for tooth decay. Consuming excess sugars activates the bacteria that cause cavities.

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