ISSN: 0067-2904
GIF: 0.851

Detection of Biofilm Genes (*gtf*) in *Streptococcus mutans* Isolated from Human Dental Caries

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Abstract:
Sixty samples from saliva and dental plaque were selected from patients with caries active at ages from 4-65years. 22 isolates belong to *Streptococcus mutans*. All isolates pronounced adhesion and biofilm formation in various degrees. By using Polymerase Chain Reaction (PCR) Techniques, it was found that these isolates had *gtfB* encode GtfB with 80 bp, *gtfC* encode GtfC with 81 bp, and *gtfD* with 324 bp which explain their potential of biofilm formation.

Keywords: PCR, *gtf*, *Streptococcus mutans*.

التحري عن جينات (*gtf*) المكونة للغشاء الحيوي في بكتريا *Streptococcus mutans* المعزولة من عينات تسوس الاسنان

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الخلاصة
في هذه الدراسة جمعت ستون عينة من اللعاب و الاسنان المتسوسة من مرضى يعانون من تسوس الاسنان باعمار مختلفة تراوحت بين 4–65 سنة واختبرت قابلية عزلات *S. mutans* على الالتصاق الحيوي ووجد بانها تمتلك القدرة على تكوين الاغشية الحياتية بدرجات متفاوتة. تم تشخيص امتلاك هذه العزلات لجينات *gtf* التي تساهم في تكوين الاغشية الحيوية باستخدام تقنية ال PCR (Polymerase Chain Reaction) ووجد ان 80 زوج قاعدة لجين *gtfB* والمشفر لانزيم GtfB و81 زوج قاعدة لجين *gtfC* والمشفر لانزيم GtfC و324 زوج قاعدة لجين *gtfD* والمشفر لانزيم GtfD مما يفسر قدرتها على تكوين الاغشية الحيوية المسببة لتسوس الاسنان .

Introduction:

Streptococcus mutans, a primary etiologic agent of human dental caries, is particularly effective at forming biofilms on the hard tissues of the human oral cavity. Adherence of *S. mutans* to dental surfaces is the first step in the formation of biofilms by this organism and is mediated by sucrose-dependent and sucrose-independent mechanisms [1-2]. *S. mutans* expresses several surface adhesins that can bind to salivary pellicles formed on the teeth [3], whereas sucrose-dependent adherence is mediated by glucan binding proteins and water-insoluble glucans produced from sucrose by glucosyl - transferase(GTF) enzymes [2].

Biofilms are sessile bacterial communities adherent to a surface, and their formation occurs in response to a variety of environmental cues [4-5]. Biofilm bacteria undergo a developmental program in response to environmental signals that leads to the expression of new phenotypes that distinguish

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these sessile cells from planktonic cells (4-6). Of importance with respect to medicine, biofilm cells have been shown to be up to 1,000-fold more tolerant of antibiotics than are planktonic cells and genes [7] and protein expression profiles are altered in planktonic cells relative to those in biofilm-grown cells [6-4].

The ability of *Streptococcus mutans* to synthesize extracellular glucans is a critical virulence factor involved in the pathogenesis of dental caries in animals and humans [8, 9]. This bacterium harbors three distinct *gtf* genes expressing glucosyltransferase (GTF) activity [10]. The *gtfB* and *gtfC* genes are in an operon-like arrangement and encode enzymes that produce mostly water-insoluble α-(1-3)-linked glucans, whereas the *gtfD* gene, which is not linked to the *gtfBC* locus, encodes an enzyme that synthesizes water-soluble α-(1- 6)-linked glucans. Glucans provide binding sites for, and promote accumulation of, cariogenic streptococci (and other oral microorganisms) on the tooth surface, and they contribute to the establishment of the extracellular polysaccharide matrix, which provides bulk and structural integrity to dental biofilms (known as dental plaque). The goal of this study is to detect *gtf* gent at different regiones (gtfB,gtfC,gtfD) in *S. mutans* isolates from dental plaque and caries that had efficient biofilm formation.

Materials and methods:

1) Sample collection and Identification

Sixty plaque and saliva samples were collected from individuals aged from 4 to 65 years old. Twenty two mutans streptococci local isolates were isolated from human dental plaque and dental caries by growing on : Mitis Salivarius Agar with 200 I.U/L bacitracin (MSB)and trypticase, yeast, cystine, sucrose, bacitracin agar (TYCSB) and identified according to biochemical test by growing blood agar medium and incubated anaerobically at 37^{o}C for 48 hrs and tested for their ability to ferment sucrose , mannitol , sorbitol and raffinose

2) Sucrose-induced biofilm formation

Crystal violet staining was used to measure biofilm formation as reported by Motegi *et al*. [11]. One hundred and sixty microliters of TSB (Tryptic soya broth with 1% sucrose) with 40 mL of an overnight culture of bacteria were incubated in selected wells of sterile 96-well microtiter plates, control wells contain TSB without the addition of bacterial suspention .After incubation for 24 h, the planktonic bacteria were removed and the wells were washed with 0.9% saline, then the biofilm was fixed with 2% sodium acetate, washed again and 0.1% crystal violet was added to stain the biofilm for 15 min. The wells were rinsed again with 0.9% saline and 200 mL of ethanol was added to the wells to solubilize the dye, at last the ethanol was transferred to a new plate and the absorbance at 490 nm was recorded.

Polymerase chain reaction

Polymerase chain reaction (PCR) was used to confirm the presence or absence of the *gtf* BCD genes in the 22 isolates. One colony of each bacterium from an agar plate was used as the template. The DNA was extracted by using ExiPrep^{TM}Plus Bacteria Genomic DNA Kit (BIONEER, Korea) according to protocol for DNA extraction using ExiPrep$^{TM\ 16}$ Plus machine. All primers used in detection *gtf* genes were designed according to Bioedit program and NCBI BLAST http://www.ncbi.nlm.nih.gov. Website with conserved region listed in Table-1. PCR was performed with 2 µl (100 ng) of template DNA in a total reaction volume of 20 µl consisting of 10 µl of GoTaq Green Master Mix (promega), 2 µl of Forword Primer (10 µM), 2 µl Reverse Primer (10 µM), 4 µl Nuclease free water. The PCR program consisted of 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 1 min) to amplified *gtfB*, while 30 cycles of denaturation (94°C for 1 min), annealing (62°C for 1 min), and extension (72°C for 1 min) and a final extension step at 72°C for 3 min to amplified *gtfC* and *gtfD*. A Master thermocycler gradient (Eppendorf, Germany) was used for PCR. The positive result of *gtf* genes was confirmed by 2% agarose gel electrophoresis stained with ethidium bromide, electrophoresed in 75 volt for 1 hr, and photographed under ultraviolet (UV) transilluminator.

Table 1- Primers of *gtf* genes used in qRT-PCR

Primers Name	Primer sequence (5′ – 3′)	Conserved region	Origin
gtf B	**Forward** TGGAAAAACTTCCCAATGTAAAA	**(457-589)**	Promega /USA
	Reverse ACGAACTTTGCCGTTATTGTC		
gtf C	**Forward** AGCAGATTCAACTGACGACC	**(1-233)**	
	Reverse TCTTTTGCTGCTTCACTTGTCG		
gtf D	**Forward** GTTGACTTAGCGGCCATTCC	**(482-908)**	
	Reverse TGAAGCTGTCCACGTTTTGC		

Results and Discussion

Biofilm formation by *S. mutans* isolates

Twenty two isolates 36% were identified depending on the morphological and biochemical properties, and then confirmed by vitek 2 compact systems.Microtiter plates were used detecting the ability of *S. mutans* isolates to forming biofilm indicated that each isolate showed a different potential capacity to form biofilm,16 (72%) isolates were considered as a high or strong biofilm producers since their optical density were higher than 0.5 nm with the absorbance values ranged between 0.512nm- 2.252nm. Six (27%) isolates were considered as a mild or good biofilm producer since their optical density was higher than 0.1 nm Figure-1. The obtained results were higher than results of another local study by Al-kazirragy [12] found out 53% of the tested isolates were high producers while (46%) of isolates were good or mild producers.

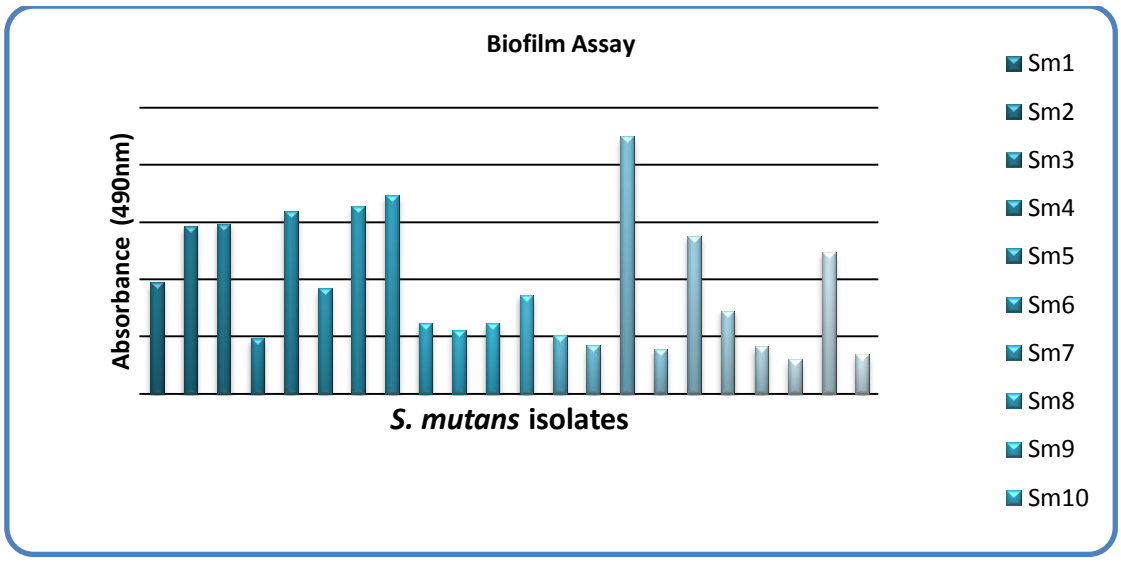

Figure 1- Biofilm formation for *S. mutans* isolates.

Amplification of *gtf* genes from *S. mutans* isolates

Glucosyltransferases (GTFs) are the primary virulence factors of *S. mutans* responsible for formation of dental biofilms and development of dental caries, therefore 3 genes *gtfB*, *gtfC*, and *gtfD* encoded GTFs were detected using monoplex PCR technique.

The DNA of 22 100% isolates of *S. mutans* were amplified for the detection *gtf* genes using monoplex pattern. Figure-2 showed that all *S. mutans* isolates have *gtfB* gene with 80bp that encoded a glucosyltransferase, GtfB, synthesized mostly insoluble glucans containing elevated amounts of α-1,3-linked glucose .After using designed primer for conserved region in *gtfC* gene, the result showed that all *S. mutans* isolates had *gtfC* gene appeared with band of 81bp, Figure-3 which encoded a glucosyltransferase GtfC that synthesized a mixture of insoluble and soluble glucans. In addition, Figure-4 depicts that all *S. mutans* isolates had bands with 324 bp that represented *gtfD* gene which encoded a glucosyltransferase GtfD that synthesized predominantly soluble glucans.

The results showed that all 22 100% of *S. mutans* isolates had 3 *gtf* with different capacity to form biofilm due to the source of isolation (dental plaque, dental caries) also Shemesh *et al* [13] mentioned that accumulation of bacteria to the different types of dental surfaces such as implant, restorative material, or enamel, can be associated with the formation of different type of a biofilm due to different patterns of gene expression, especially those genes associated with biofilm regulation, formation and bacterial physiology.

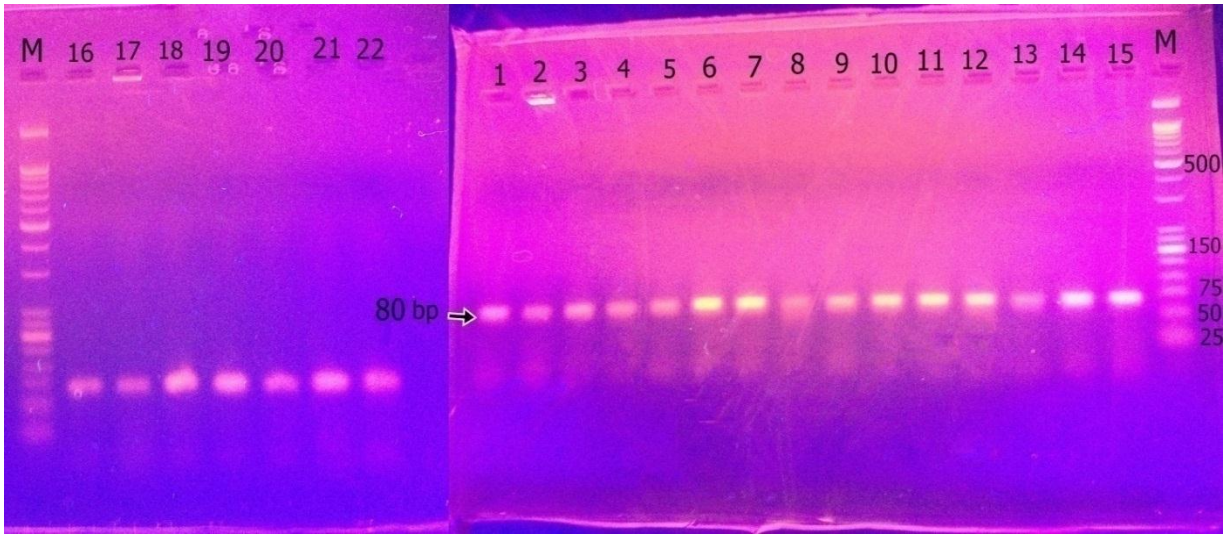

Figure 2- Amlification of a 80-bp *gtB* gene of *S.mutans* isolates on agarose gel (2%) electrophoresed in 75 volt for 1 hr, M: molecular marker (25bp DNA Ladder), lanes 1 - 22 refer to (Sm1-m12)

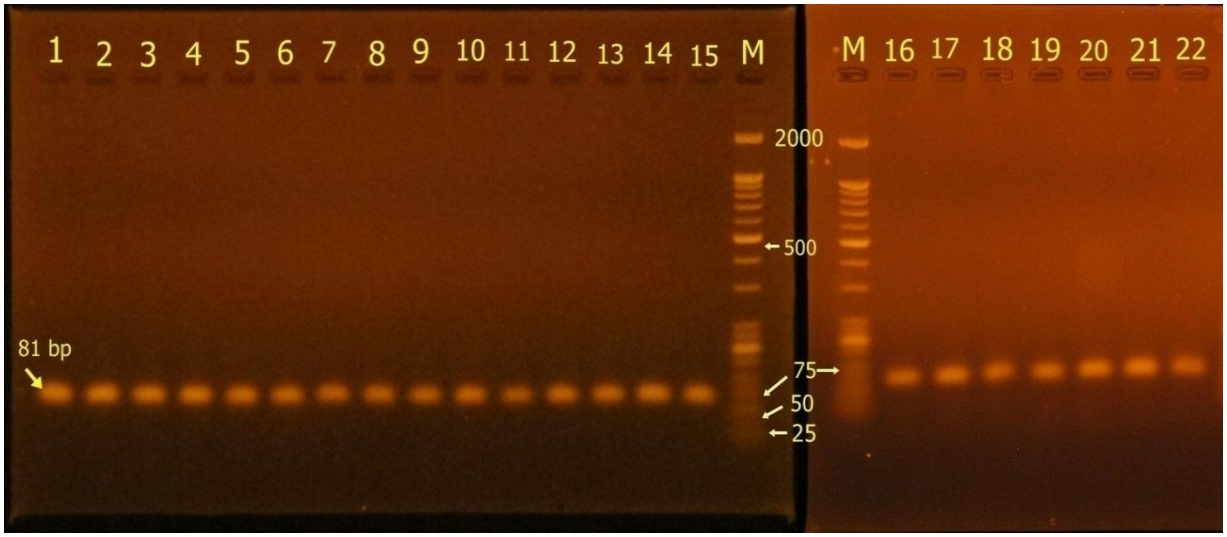

Figure 3- Amlification of a 81-bp *gtC1* gene of *S.mutans* isolates on agarose gel (2%) electrophoresed in 75 volt for 1 hr, M: molecular marker (25bp DNA Ladder), lanes 1 - 22 refer to (Sm1-m12).

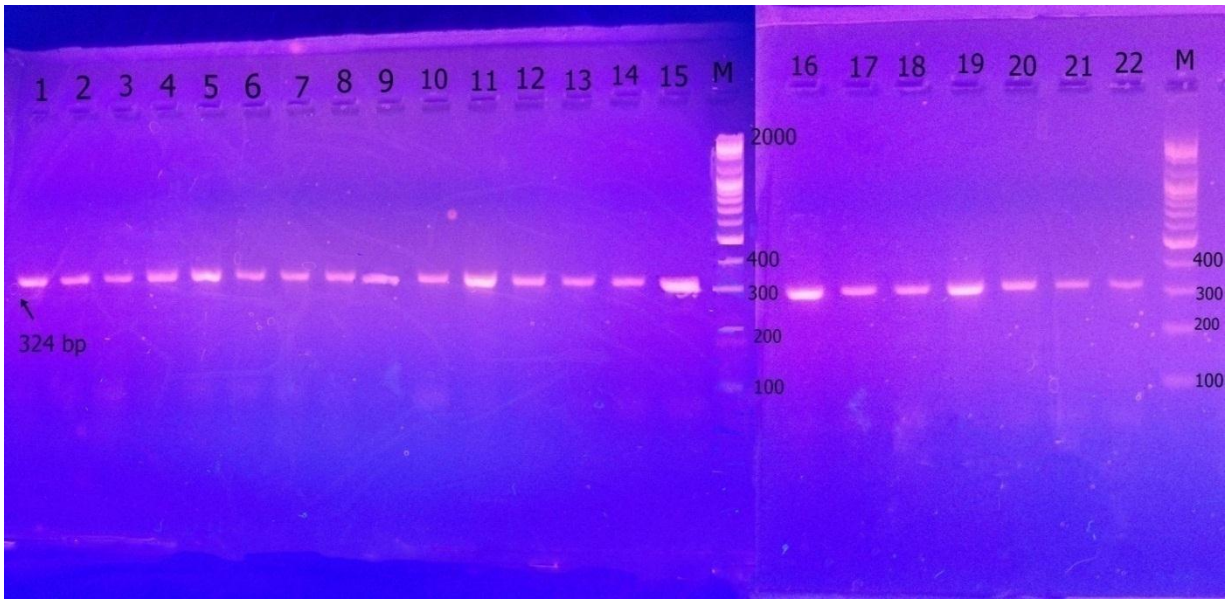

Figure 4- Amlification of a 324-bp *gtD*1 gene of *S.mutans* isolates on agarose gel (2%) electrophoresed in 75 volt for 1 hr, M: molecular marker (100 bp DNA Ladder), lanes 1 - 22 refer to (Sm1-m12).

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