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Molecular characterization of *Streptococcus mutans gtfB* gene isolated from families

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ABSTRACT

OBJECTIVE: Caries is a multifactorial infectious disease and the main etiological agent is the bacteria *Streptococcus mutans* due to its virulence factors, which enable the adherence to dental enamel and favours the formation of dental biofilm through the production of extracellular polysaccharides. The transmission of *S. mutans* can occur between people, often within families. The present study aimed to evaluate the intrafamily genetic pattern of *S. mutans* through partial sequencing of the gene that encodes the glucosyltransferase *β* (*gtfB*) enzyme.

METHODS: We previously analyzed saliva samples from 40 individuals from nine families, and it was obtained 64 isolates biochemically characterized as *S. mutans.* The isolates were evaluated by random amplification of polymorphic DNA (RAPD). Those with greater similarity were characterized by partial sequence *16S* rRNA gene and the *gtfB* gene sequencing.

RESULTS: It was observed genetic similarity among strains isolated from individuals with caries activity; while isolates from individual without caries showed that they are genetically distinct, suggesting a different virulence pattern.

CONCLUSION: The present results demonstrated that partial sequencing of the *gtfB* gene showed to be a potential marker to investigate genetic pattern and virulence of *S. mutans*, deserving further investigation in order to identify families at risk of caries.

Keywords: *Streptococcus mutans*; glucosyltransferase *β*, virulence.

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Caracterização molecular do gene *gtfB* em *Streptococcus mutans* isolados de famílias

RESUMO

OBJETIVO: A cárie é uma doença infecciosa multifatorial e o principal agente etiológico é a bactéria *Streptococcus mutans* devido a seus fatores de virulência, que permitem a adesão ao esmalte dental e favorecem a formação de biofilme dental através da produção de polissacarídeos extracelulares. A transmissão de *S. mutans* pode ocorrer entre as pessoas, muitas vezes na família. O presente estudo teve como objetivo avaliar o padrão genético intrafamiliar de *S. mutans* por meio de sequenciamento parcial do gene que codifica a enzima glicosiltransferase *β* (gtfB).

METODOLOGIA: Foram previamente avaliadas amostras de saliva de 40 indivíduos de nove famílias, e obteve-se 64 isolados bioquimicamente caracterizados como *S. mutans*. Os isolados foram avaliados por amplificação aleatória de DNA polimórfico (RAPD). Aqueles com maior similaridade foram caracterizados pela sequência parcial do gene *16S* rRNA e sequenciamento do gene *gtfB*.

RESULTADOS: Observou-se similaridade genética entre as linhagens isoladas de indivíduos com atividade de cárie, enquanto isolados de indivíduo sem cárie mostraram-se geneticamente distintos, sugerindo-lhes um padrão de virulência diferente.

CONCLUSÃO: Os resultados demonstraram que o sequenciamento parcial do gene *gtfB* mostrou ser um marcador potencial para investigar o padrão genético e a virulência de *S. Mutans,* merecendo mais investigação para identificar famílias em risco de cárie.

Palavras-chave: *Streptococcus mutans*; glucosyltransferase *β*; virulência.

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INTRODUCTION

Dental caries forms through a complex interaction over time between acid producing bacteria and fermentable carbohydrate, and many host factors including teeth and saliva [1]. This disease, that is prevalent around the globe, causes the teeth's mineral base to dissolve through the action of organic acids, such as lactic acid. These acids are final metabolic products. They are excreted by certain microbiota present in dental biofilm, especially by *Streptococcus mutans*, which is present in the mouth and is associated with dental caries in humans [2].

The *S. mutans* is the key element for the formation of the extracellular polysaccharide matrix in dental biofilm, mainly glucans synthesized by streptococcal glycosyltransferases (Gtfs). Accumulated glucan molecules provide sites on the dental surface where microorganisms can accumulate and then establish pathogenic biofilms [3,4]. The *S. mutans* bacterium produces at least three glucosyltransferases, which are products of the *gtfB*, *gtfC*, and *gtfD* genes. GtfB synthesizes the majority of insoluble glucan, GtfC synthesizes a mixture of soluble and insoluble glucans, and GtfD synthesizes mainly soluble glucans. Thus, the glucosyltransferase produced by *S. mutans* is a pro-virulence enzyme that is involved in the pathogen colonization in the mouth. Moreover, it could be used as a marker for biotypes with different colonization patterns [3], as well as to investigate *S. mutans* transmissibility. According to Nakai et al. [5], it is important that strategies to prevent childhood caries involve measures that interfere in the *S. mutans* transmission among family members.

Moser et al. [6] used repetitive extragenic palindromic PCR to study the diversity and transmission of *S. mutans* but the results did not support the maternal transmission of *S. mutans* in children with severe caries, indicating the necessity to establish robust markers in order to demonstrate it.

In this study, we analysed nine families that had received care in the SUS (Unified Health System, Sistema Único de Saúde) of Curitiba [7]. The selection of the families for the study was based on their social and biological risk of developing caries. In addition, isolates of *S. mutans* from saliva samples from each nuclear family were subjected to molecular characterization by RAPD (random amplification of polymorphic DNA) markers [8] and evaluated by partial sequencing of the gene encoding glucosyltransferase B (*gtfB*) in order to demonstrate the genetic profile of *S. mutans* within the family and to detect specific serotypes concerning to the virulence.

METHODS

Subjects

Nine low-income families treated in the SUS (Unified Health System) of Curitiba (40 individuals in total) with similar social conditions, who had high dental caries activity were assessed to identify *S. mutans* serotypes. This study protocol was approved by the Research Ethics Committee of the Federal University of Paraná (Universidade Federal do Paraná – UFPR), number 098.SI 061/04-08. Informed consent was provided by the children's legal caretakers.

Strains studied

We previously evaluated saliva samples from the 40 individuals, and a total of 157 isolates were obtained. The 64 isolates biochemical characterized as *S. mutans* had the genetic similarity evaluated by RAPD markers [8]. We identified in this way, sixteen intra-familial isolates that had genetic similarity and came from members of two families: family A consisted of a mother and son, and family B consisted of a mother, father, and five children (**Table 1**). The reference strains are listed in **Table 2**.

Table 1. Case history based on epidemiological and microbiological analysis, and accession number in the European Nucleotide Archive of partial sequences of the region that encodes for the glucosyltransferase *β* enzyme from *S. mutans* intra-familial isolates

Species	Nomenclature in this study- origin	DMF-T added to deft	Presence of biofilm	CFU/mL	*Accession n. gtfB
S. mutans	SM1 (family A-mother)	32	–	2.2×10^4	HE962158
S. mutans	SM2 (family A-mother)	32	–	2.2×10^4	HE962159
S. mutans	SM3 (family A-only child)	9	++	9×10^4	HE962160
S. mutans	SM4 (family A-only child)	9	++	9×10^4	HE962161
S. mutans	SM5 (family B-mother)	5	+++	6×10^4	HE962162
S. mutans	SM6 (family B-mother)	5	+++	6×10^4	HE962163
S. mutans	SM7 (family B-father)	3	+++	1.3×10^5	HE962164
S. mutans	SM8 (family B-eldest child)	0	+	1×10^5	HE962165
S. mutans	SM9 (family B-eldest child)	0	+	1×10^5	HE962166
S. mutans	SM10 (family B-second eldest child)	2	+	1.5×10^5	HE962167
S. mutans	SM11 (family B-middle child)	9	++	1.7×10^5	HE962168
S. mutans	SM12 (family B-middle child)	9	++	1.7×10^5	HE962169
S. mutans	SM13 (family B-second youngest child)	6	++	2×10^4	HE962170
S. mutans	SM14 (family B-second youngest child)	6	++	2×10^4	HE962171
S. mutans	SM15 (family B-second youngest child)	6	++	2×10^4	HE962172
S. mutans	SM16 (family B- youngest child)	-	–	2×10^2	HE962173

DMF-T (decayed, missing and filled permanent teeth), deft (decayed, extracted and filled deciduous teeth). Presence of dental biofilm visible in the cervical third (+), in the middle third (++) and presence of mineralized dental biofilm (+++). CFU/mL (colony forming unit, per mL of,

Table 2. The different serotypes of *S. mutans* and *Streptococcus troglodytae* selected by GenBank that were used in this study

Lineage	GenBank number	Source	Reference
MT4239	D88657	Human	Fujiwara et al.[9]
MT4251	D88660	Human	Fujiwara et al.[9]
pTH1	D89977	Human	Fujiwara et al.[9]
pSK6	D88651	Human	Fujiwara et al.[9]
pYT216	D88654	Human	Fujiwara et al.[9]
Ua318	JX072985	Human	Argemon et al.[10]
Ua113	JX072981	Human	Argemon et al.[10]
UA114	JX072982	Human	Argemon et al.[10]
Ua96	JX072987	Human	Argemon et al.[10]
Bv24	JX072974	Human	Argemon et al.[10]
UAB G71	KM889586	Human	Momeni et al.[11]
BAHAT	JX072980	Human	Argemon et al.[10]
MARK	JX072980	Human	Argemon et al.[10]

DNA extraction and PCR

In order to extract genomic DNA, CTAB (hexadecyl trimethyl ammonium bromide) buffer was used, together with a 2:1 mixture of silica gel and celite. The solutions were sonicated with 3 pulses per 30 s [12]. Partial amplification of 16S gene was performed with PCR reaction as described by Nakano et al. [13], using the primer pairs 536f (5'-CAG CAG CCG CGG TAA TAC-3') and 1050r (5'-CAC GAG CTG ACG ACA-3') (16S rRNA) to confirm species identity. GTFB amplicons were generated with primers GTFB-F (5'-ACT TAC ACA CTT TTC GGG TGG CTT GG-3') and GTFB-R (5'-CAG TAT AAG CGC CAG TTT CAT C-3') (Invitrogen Life Technologies), according to the protocol described by Oho et al. [14].

DNA sequencing

DNA marking for subsequent sequencing was performed with a BigDye™ Kit, according to manufacturer's recommendations. The sequence reaction was done with 30 ng of previously purified DNA. Amplification reactions consisted of 1 min at 96ºC followed by 35 cycles at 96ºC for 10 s, 50ºC for 5 s, and 60ºC for 4min for the final extension. The marked product was purified in 75% isopropanol, followed by 70% ethanol. After drying in a SpeedVac at 60ºC for 40 min, the samples sequencing was performed on an ABI 3130 automatic sequencer (Applied Biosystems).

Alignment, phylogenetic reconstruction of DNA, and molecular analysis

Sequences were analyzed and corrected using STADEN package [15] and the MEGA, version 6.0 [16] was used to align them, obtaining the nucleotide and amino acid composition and calculations of the variable nucleotide distances and positions, and to perform phylogenetic analyses. We compared the sequences of the intra-familial isolates with sequences of different serotypes of *S. mutans* that had been previously deposited in GenBank (**Table 2**).

RESULTS

We analysed sixteen intra-familial strains isolated from two families (A and B). The epidemiological and microbiological analyses of the families evaluated were presented in the **Table 1**. The individuals evaluated presented dental biofilm visible with salivary variation on *Streptococcus mutans* concentration (**Table 1**). The identity of the isolates was confirmed based on the biochemical markers and partial gene 16S rRNA sequencing.

The *gtfB* sequences from the sixteen intra-familial *S. mutans* isolates were compared with *gtfB* sequences from reference strains. The tree was built with Maximum Likelihood implemented in RaxML v. 7.0.4 using the substitution model General Time Reversible.The empirical base frequencies were pi (A): 0.389512, pi(C): 0.169736, pi(G): 0.165714, pi(T): 0.275037, with 1,000 boot-strap inferences. The strains from family B consisting isolate SM 5, obtained from mother and the isolate SM 16 from the youngest child were clustered in the Clade I supported by a 94% bootstrap value. Similarly, the strains from family A consisting isolate SM 1, obtained from mother and the isolate SM 4 from the youngest child were clustered in the Clade I. The remaining strains were grouped in three separate groups: Clade II clustered two isolates (SM 8 and SM 9) from eldest child from family B, Clade III formed by isolates collected from members of family B (isolates SM 6, SM 7, SM 10 and SM 15), and the Clade IV consisted of isolates from both families obtained from individuals with a high incidence of the disease.

The nucleotide variations on partial sequences of the *gtfB* gene revealed amino acid changes (**Table 3**). Clustering based on a comparison of the amino acids obtained through 16 partial sequences o *gtfB* gene generated two groups showed in the JTT Model matrix [14], RaxML likelihood method, version 7.0.4 [15] (**Figure 2**).

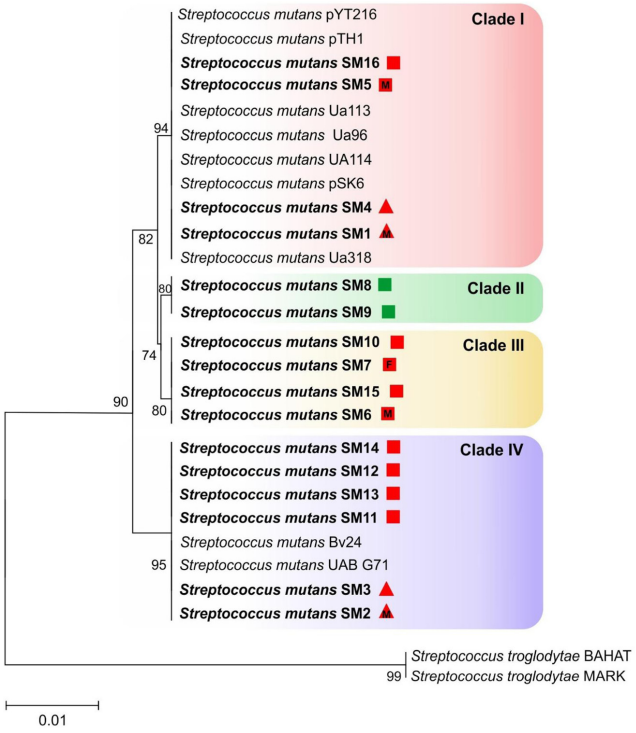

Figure 1. Phylogenetic tree of *S. mutans* strains based on partial sequences of the *gtfB* gene, with Maximum Likelihood implemented in RaxML v. 7.0.4 using the substitution model General Time Reversible. Bootstrap values >70% from 100 resample datasets are shown with branches in bold. *Streptococcus troglodytae* was taken as outgroup. In bold the *S. mutans* strains evaluated, squares and triangles correspond to families A and B, respectively, in red: individual with caries and in green: individual without caries. M (Mother) and F (Father).

Table 3. Amino acid changes observed in the intra-familial isolates used in this study

Position	Type of substitutions	Alteration	Isolates
28*	Transversion	Proline → Leucine	1, 4, 5, 6, 7, 10, 15, 16
61*	Transversion	Alanine → Serine	2, 3, 11, 12, 13, 14
66*	Transversion	Arginine → Glutamine	2, 3, 11, 12, 13, 14

* Location of amino acid changes among a group of 16 parcial sequences of the *gtfB* gene, analysed by serotypes selected from GenBank.

DISCUSSION

Previous data based on RAPD markers [8] yielded evidence of transmissibility pattern wherein isolates from mother and child, spouse and sons, were grouped together with high similarity. In this study, we used the partial sequencing analyses of *gtfB* gene to demonstrate the *S. mutans* intrafamily genetic pattern. According to molecular analysis, the strains from different individuals of the same family belonged to the same group (Clades I, II and IV) (**Fig. 1**), which could confirm the intrafamily transmissibility previously verified [8].

A systematic review showed scientific evidence about the transmission of *S. mutans* from mother to child [18], based on studies including serotype classification, bacteriocin activity profiles, chromosomal DNA fingerprinting, ribotyping, arbitrarily primed PCR method and multilocus sequence typing. The results of most studies using these techniques have demonstrated mother to child transmission. In addition, they also revealed an improvement in the methods applied throughout the years, but evidence of transmission was independent of the sample size and genetic analyses. Slayton [19], based on 46 studies about *S. mutans* transmission reported potential transmissibility within the family. According to this review, the mother with high salivary concentration of bacteria seemed to be the most frequent source of transmission. Domejean et al. [20], using arbitrary primers, also demonstrated horizontal transmission of *S. mutans* between unrelated children 5-6 years of age, thereby indicating the circulation of serotypes between people who are close to each other. According to our results, based on *gtfB* gene sequenced, the isolates from mother and child belonged to the same cluster (family A and B) (Clade I) grouping with low variability among the intrafamily isolates, what might be related with vertical transmissibility (**Fig. 1**).

The glucosyltransferases play a critical role in virulent dental plaque development and are responsible for glucans formation from sucrose, considering that the synthesized glucans provide the possibility of both bacterial adhesion to the tooth enamel and microorganisms to each other.

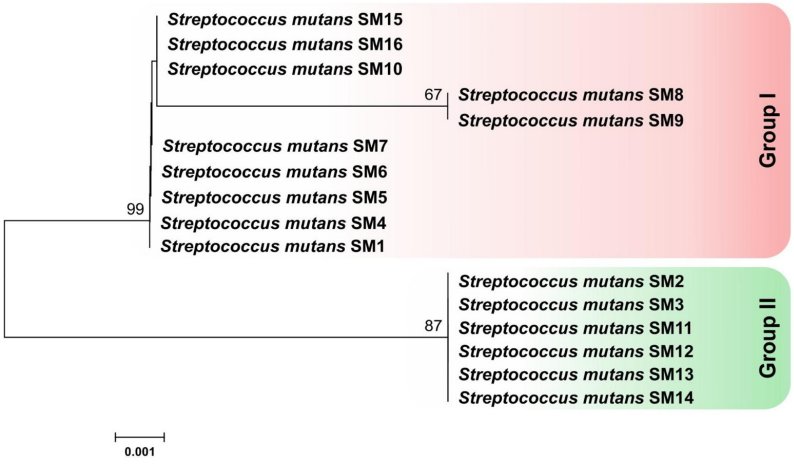

Figure 2. Maximum likelihood tree resulting from amino acid sequences of *gtfB* region of intra-familial isolates of *S. mutans*, based on the JTT Model matrix, RaxML likelihood method, version 7.0.4 [17]. The numbers at each fork node indicate the bootstrap values (based on 1,000 permutations) (shown only when >60%).

Microcolonies are formed, contributing to the formation of biofilm and each of the three types of Gtfs plays a different, though the similar role in biofilm formation and, therefore, the loss or mutation of one of them impairs the role process [21]. Argimon et al. [10] based on phylogenetic analysis of glucosyltransferases has reported that acquisition and diversification of *S. mutans* Gtfs predates modern humans and is unrelated to dietary sucrose consumption increase. The authors suggested that although the elevated quantities of extracellular polysaccharides can be involved in the regulation of *gtf* genes, there are evolutionary steps of agents and hosts involved in the clinical picture of the disease.

The evaluated families in our study reported high sucrose consumption. When dietary sucrose is consumed frequently, *S. mutans*, as a member of the oral biofilm community, continues to synthesize polysaccharides and metabolize this sugar to form organic acids. The elevated amounts of extracellular polysaccharides, which may involve upregulation of *gtf* genes in response to pH and carbohydrate availability, increase the virulence of biofilm [4]. According to the authors, under these conditions, *S. mutans*' competitiveness in relation to other species present in the biofilm on the dental surface is reinforced. Moreover, the mouth's ecological system is extremely sensitive to physical and chemical fluctuations, as a result of eating foods associated with oral hygiene measures [22], where microbial activity and imbalances in the resident microflora may be the ultimate mechanisms favouring the development of dental caries [23].

We also observed strains isolated from individuals with a high incidence of the disease belonging to the same clusters (**Fig 1**, Clades I, III e IV). Likewise, the isolates from the individual without caries grouped separate in Clade II, demonstrating they are genetically distinct, and probably with a different virulence potential. Additionally, microbiological studies have reported that most humans harbour *S. mutans*, but not all manifest disease, so it has been proposed that strains of these bacteria associated with severe early caries in children are genetically different from those found in caries-free children [24].

We observed in this study, that the mother from the family A wore a total dental prosthesis, having lost all her teeth to caries. Her son had active caries associated with biofilm accumulation on the dental surface and a significant history of caries, with nine permanent teeth compromised by cavities at the age of 13 (**Table 1**). He yielded a DMF-T index higher than expected for his age group, showing unbalance in the oral ecological system, although he had a moderate salivary concentration of *S. mutans*. The results showed (**Fig. 2**) that this individual was multi colonized by distinctly grouped biotypes (isolates SM 3 and SM 4), with different potential virulence, which may explain the disease aggressiveness.

Studies on biofilm formed by *S. mutans* clearly show that the virulence of strains is dependent on environmental conditions, and, thus, on in vivo model host-dependent characteristics. On the other hand, to form biofilm structure, it is not sufficient to ensure appropriate environmental conditions. Microorganisms alone must possess characteristics which, in terms of the above-mentioned favourable conditions, will allow the adhesion and formation of micro colonies [21].

However, our data showed that nucleotide changes in the sequence of the *gtfB* gene may be related with observed caries patterns and consequently variability in the virulence of these isolates, as demonstrated the isolates in Group II (**Fig. 2**) obtained from individuals with high caries activity. In addition, **Figure 2** shows the Group I, whith isolates from families A and B, demonstrating similarity among isolates from individuals with different disease histories, that grouped the two isolates proceeding of the individual without caries with high similarity (SM 8 and SM 9).

The variation in the clinical pattern of the disease and the similarities in the amino acid sequences of the isolates suggest that, in addition to transmissibility, there are passive carriers of virulent biotypes, pointing to their intra-familial circulation and indicating that the presence of internal factors to the host also influences the disease. The amino acid modifications observed in this study (**Table 3**) may implicate in the configuration and subsequent activity of the enzyme, thus influencing the pattern of virulence. Therefore, Bannas et al. [25] proposed a model indicating the consequence of changes in biofilm structure. According to the authors the intergenic region between GtfB and GtfC are known to be unstable and to undergo homologous recombination during caries developments. In this way, the knowledge of the *S. mutans* strains is important because the virulence of the microorganisms is varied and the virulence affects the dental caries evolution rat [18].

CONCLUSION

Cumulatively, the results demonstrated that partial sequencing of the *gtfB* gene, with the identification of nucleotides sequences in common to the strains, showed to be a potential marker to investigate genetic pattern and virulence of *S. mutans*, deserving further investigation in order to identify families at risk of caries.

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