Análise Genético-Molecular dos Genes para Sorotipo e Mutacina em *Streptococcus Mutans* em uma População Adulta

Genetic-Molecular Analysis of Serotype and Mutacin Genes in *Streptococcus Mutans* in Adults

Natalia Valarini[a]*; Augusta Piovezan[b]; Miula Portelinha Braga[c]; Sandra Mara Maciel[d]; Flaviana Bombarda de Andrade Ferreira[e]; Regina Célia Poli-Frederico[f]

Resumo

O objetivo do estudo foi avaliar a freqüência dos genes para mutacina (I, II, III e IV) e sorotipos de antigenicidade *c, e, f*, em isolados de *Streptococcus mutans* em uma população adulta com diferentes níveis de cárie. Foram avaliados 280 isolados de S. mutans em indivíduos entre 18 a 34 anos de idade pela reação em cadeia da polimerase (PCR) utilizando-se *primers* específicos para as mutacinas I/III e II/IV e sorotipos *c, e* e *f*. Os amplicons foram separados por eletroforese em gel de agarose 1% corado por brometo de etídio. A severidade de cárie foi classificada de acordo com a Organização Mundial da Saúde. Encontrou-se apenas um caso de cárie moderada, onde não foi observada a amplificação para o gene mutacina. Os demais se enquadraram na categoria de alta severidade da doença, tendo sido detectado, a amplificação negativa pela PCR para mutacina em 36% dos isolados e 20% mostraram genótipos positivos para mutacina IV. Vale ressaltar que, em alguns casos, os isolados apresentaram mais de uma mutacina, sendo a maior proporção (12%) para a combinação I/III. A taxa de sorotipo registrada foi de 84,2% para o tipo *c* e 15,8% para *c* e *f*. Não foi identificada a presença do sorotipo *e*. Os achados do presente estudo apontam para a maior freqüência do sorotipo *c* e do gene para mutacina IV na população estudada.

Palavras-chave: Biologia molecular. Cárie dentária. Bacteriocinas. Polissacarídeos bacterianos.

Abstract

The aim of this study was to access the frequency of mutacin genes (I, II, III and IV) and serotypes c, e and f antigenicity in Streptococcus mutans isolates from an adult population with different levels of caries. A total of 280 S. mutans isolates from individuals aged 18 to 34 years were evaluated by polymerase chain reaction (PCR), using specific primers for mutacin I/III and II/IV and serotypes c, e and f. Amplicons were separated by electrophoresis in 1% agarose gel stained by ethidium bromide. The levels of dental caries were measured by the DMFT index, according to the World Health Organization criteria. Among the population studied, only one case of moderate dental caries was registered and it presented no amplified product for mutacin gene. In the remainder, who showed high levels of the disease, the negative amplification for mutacin by PCR was detected in 36% of the isolates and 20% showed positive genotypes for mutacin IV. It must be highlighted that in some cases, the isolates presented more than one mutacin, being the higher proportion (12%) for the combination I/III. The results showed that serotype c it was most frequently found in the oral cavities (84%).The mixed infection (c and f) it was observed in 16% of the preschool children in the caries group.The presence of serotype e was not identified. The findings of this study point out to the greater frequency of serotype c and the mutacin IV in the studied population.

Keywords: *Molecular biology. Dental caries. Bacteriocins. Polysaccharides Bacterial.*

[a] Mestranda em Odontologia - Universidade Norte do Paraná (UNOPAR). E-mail: naty.valarini@gmail.com.
[b] Graduada em Odontologia - Universidade Norte do Paraná (UNOPAR). E-mail: gutapiovezan@hotmail.com.
[c] Graduanda do Curso de Odontologia - Universidade Norte do Paraná (UNOPAR). E-mail: miumiula@hotmail.com.
[d] Doutora em Saúde Pública - Universidade de São Paulo (USP). Docente - Universidade Norte do Paraná (UNOPAR). E-mail: sanmaciel@sercomtel.com.br.
[e] Doutora em Endodontia - Universidade de São Paulo (USP) Docente da Universidade Norte do Paraná (UNOPAR). E-mail: flavianaferreira@uol.com.br.
[f] Doutora em Genética - Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP). Docente da Universidade Norte do Paraná (UNOPAR). E-mail: reginafrederico@yahoo.com.br.

* Endereço para correspondência: Rua Paranaguá, 450, apt 60, Centro. CEP. 86.020-030, Londrina – PR.

1 Introduction

Streptococcus mutans has been strongly implicated as one of the causative organisms of dental caries. The dental biofilm consists of a complex bacterial community and the ability of specific strains of *S. mutans* to compete with other strains may be essential for colonization.

Most clones of *Streptococcus mutans* produce bacteriocins, named mutacins. Bacteriocins are by definition proteinaceous antibacterial substances that some bacteria produces to interfere with the growth of other, generally closely related bacterias[1]. Clinically, mutacins have been considered important for the establishment and equilibrium of bacteria in dental plaque: the mutacin-producing strains might colonize more easily and suppress nonproducing strains[2].

Mutacins have been classified in two families: the lantibiotics (containing lanthionine and/or ß-methylanthionine residues) and the non-lantibiotics. Classification of mutacin-producer strains based on their bactericidal activity, sensitivity to other self-produced mutacins and presence of plasmids divides mutacins in four types: I, II, III and IV[3,6]. The structural genes of the prepropeptides of mutacins I, II, III and IV (mutA) have been sequenced[4,6], and their biosynthetic locus is formed by several genes, including those involved in regulation, cleavage, transport and immunity to the produced mutacin[4, 5, 7].

S. mutans organisms have been classified into *c*, *e* and *f* serotypes based on the chemical composition of their cell surface polysaccharides. The serotype-specific polysaccharide of *S. mutans* is known to consist of rhamnose-glucose polymers, with a backbone of rhamnose and side chains of α- or ß-linked glucosidic residues. The serotype *c S. mutans* strains are predominant in the human oral cavity among the serotype *c, e* and *f* strains[8]. The serotype *c* RGP structure may have advantages for *S. mutans* colonization of the oral cavity[9].

In the present study, the objective was to analyze the frequency of serotype *c, e* and *f* and of mutacins I, II, III and IV from *S. mutans* isolates in caries-free and caries-active individuals.

2 Material and Method

2.1 Subjects

The group consisted of 28 individuals aged between 18 and 34 years. The aim and details of the experiments were explained, and the informed consent forms were obtained prior to the beginning of the experimental procedures. The research was approved by the Ethics Committee of the University of North of Parana and by the local Health and Education Authorities (PP/034/06).

Caries experience was measured by the DMFT (decayed, missing and filled teeth) index, according to the World Health Organization criteria. The clinical examination was performed by the same examiner (FJSP). The intra-examiner agreement was high (κ= 0.92).

2.2 Bacterial strains and DNA extraction

Streptococcus mutans clinical isolates were obtained from Mitis-Salivarius Agar with bacitracin and potassium telurite. About 10 colonies resembling *S. mutans* from each child were transferred to brain heart infusion broth – BHI (Difco, Detroit, USA) and incubated at 37^{0}C for 48h in an anaerobic jar. DNA from 280 isolates were extracted by using a simple DNA preparation in which the cells were washed and boiled for 10 minutes with TE buffer (10mM Tris/HCl, 1mM EDTA, pH 8.0). The debris were pelleted and the supernatants were stored in a freezer at -20^{0}C until use.

2.3 PCR analyses

Isolates were confirmed for species identity in PCR reactions with primers specific for gtfB, encoding glucosyltransferase 5'ACT ACA CTT TCG GGT GGC TTGG3' and 5' CAG TAT AAG CGC CAG TTT CATC3'[10] (Invitrogen, São Paulo, Brazil), yielding an amplicon of 517pb for *S. mutans gtfB* gene. Each reaction consisted of 5 µl template DNA, 1 µM of each primer, 200 µM of each dNTP, 5 µl 1x PCR buffer, 1.5 mM MgCl2 and 1 U Taq DNA polymerase (Invitrogen, São Paulo, Brazil) in a total volume of 25 µl. The amplification reaction was performed in 30 cycles as follows: denaturation 95^{0}C for 30s, annealing at 59^{o}C for 30s, and extension at 72^{o}C for 1 min. One reference strain (ATCC 25175) was used as a positive control of *S. mutans* and distilled water was used as a negative control. Amplification products were analysed electrophoretically in 1% agarose gels using TBE buffer (89 mmol l^{-1} Tris borate, 89 mmol l^{-1} boric acid, 2 mmol l^{-1} EDTA; pH 8), stained with ethidium bromide and observed under UV light. A 100 bp DNA ladder served as molecular-size marker in each gel. All reactions were repeated at least twice.

2.4 PCR Screening of mutacin and serotype genes

The detection of genes encoding mutacin types I, II, III and IV was performed by PCR using specific primers [4, 6, 11]. The PCR mixture for mutacins consisted of 1X PCR buffer 10x, 2.5mM $MgCl_2$, 200µl of each deoxynucleotide, 0.3µM of each oligonucleotide primer, 1.25U of Taq DNA polymerase (Invitrogen, São Paulo, Brazil) and 50ng of template DNA.

After denaturation at 94ºC for 5 min, a total of 30 PCR cycles were performed; each cycle consisted of 30s of denaturation at 92ºC, 30s of annealing at 55ºC, 1 min of extension at 72ºC, and the final extension 5 min at 72ºC.

For the detection of serotypes *c, e* and *f*, a multiplex PCR was done[9]. The PCR mixture (10µl) consisted of 0.2mM each deoxyribonucleotide triphosphate, 1X PCR buffer, 2mM $MgCl_2$, 1U Taq DNA polymerase (Invitrogen, São Paulo, Brazil), 0.5µM concentration of each primer, and 2µl of template DNA. After denaturation at 96ºC for 2 min, a total of 25 PCR cycles were performed; each cycle consisted of 15s of denaturation at 96ºC, 30s of annealing at 61ºC, and 1 min of extension at 72ºC.

The PCR products were analyzed by electrophoresis in 1% agarose gel using Tris/borate/EDTA buffer (pH 8.0). A 250bp DNA ladder was included in each gel. The DNA was stained with 0.5µg ml^{-1} ethidium bromide and visualizes under UV illumination.

2.5 Statistical analysis

The differences between the frequency of mutacin and serotype genes and dmft/ caries experience were evaluated by $\chi 2$ test and the Spearman's coefficient of correlation. Statistical significance was considered to be at $\alpha<0.05$. The Software Statistical Package for Social Science, v. 11.5 (SSPS, Chicago, IL, USA) was used for the data analysis.

3 Results

The *S. mutans* were isolated from individuals of 18–43 years-old (28.6±6.6). The prevalence of dental caries (DMFT>0) was found to be 92.9%, with a mean DMFT score of 11.64±6.33.

The results showed that serotype *c* was the most common found in the oral cavity (84%). The mixed infection (*c* and *f*) was observed in 16% of the preschool children in the caries group.

The PCR screening showed positive with primers of

mutacin I, II and IV for S. mutans isolates in the caries-free subjects. PCR for the mutacin III did not yield amplicon in any *S. mutans* isolates in this group. The PCR with primers of mutacin IV showed that 9 out of 28 (32.1%) *S. mutans* isolates were positive in the caries-active group; on the other hand, the amplicons I/III genes revealed that 6 out of 28 (21.4%) isolates carried these genes.

A positive correlation was found between age and caries severity (r=0.612; P = 0.01) and between caries experience and severity of the illness (r = 0.480; P = 0.010). A significant association between the presence of mutacins and dental caries was not found.

4 Discussion

Inside the oral ecosystem, the development of the bacterial community generally involves a succession of populations and competition for receivers of adhesion, foods and the production of inhibitory substances such as the bacteriocins[12] .The mutacins and serotypes have been implicated as virulence factors in dental caries. The relationship between caries activity and the higher synthesis of some virulence factors by different genotypes of *S. mutans* has been demonstrated in the literature[13].

In this study it was found a higher proportion of mutacin I/III and mutacin IV in *S. mutans* isolates from caries-active individuals. A previous study found that isolates recovered from caries-active individuals showed a higher frequency of detection of mutacins IV and I/III[14]. Clinically, mutacins have been considered important for the establishment and equilibrium of bacteria in dental biofilms[2]. Supporting this hypothesis, the antimicrobial spectrum of mutacin IV is specifically against members of the mitis group of oral streptococci[6]. Nevertheless, our study suggests that given the increasing complexity of the oral microbiota, as found in caries-active individuals[15,] the *S. mutans* strains producing a wide spectrum of mutacins, including mutacins I, II and III, could become prevalent in most oral sites.

In the caries-active individuals the sites from wich *S. mutans* were recovered were more diverse, probably because production of organic acids and mutacins with the biofilm resultes in a more complex community compared to caries-free individuals[16]. Almost certainly due to this complexity, *Streptococcus mutans* genotypes recovered from caries-active individuals presented higher frequencies of mutacin IV and a wide spectrum of mutacins, such as I/III, and presented greater mutacin activity in vitro compared to mutacin I/III, but did not reveal inhibitory activity against any of the indicator strains.

Clinical isolates of *Streptococcus mutans* were searched for the presence of mutacin IV genes by PCR and found > 50% positive results.[17] Mutacin IV is produced by planktonic cells while mutacin I is produced by biofilm-like cells. [6]. Different mutacins may serve different purposes during the process of colonization by *S. mutans*. For instance, production of mutacin IV by planktonic cells in saliva may help *S. mutans* kill the primary colonizers on the tooth surface to make room for its own population. Supporting this hypothesis, the antimicrobial spectrum of mutacin IV is specifically against members of the mitis group of oral streptococci[6].

In our study, serotype *c* predominated and only three isolates presented multiple serotypes (*c* and *f*). *S. mutans* were isolates from 198 of 432 preschool children (3 to 4 years old)[9]. The data revealed that serotype *c* predominated, serotype *e* was the next most common and serotype *f* occurred rarely in Japanese preschool children. Furthermore, in this study we found that serotype *f* was the next most common and that the presence of serotype *e* was not identified.

This study evaluated the frequency of mutacins I, II, III and IV and the presence of serotypes *c,e* and *f* in caries-active and caries-free individuals. Our results suggests that the production of mutacins can play an important role in colonization by *S. mutans* strains in a complex bacterial community and that the PCR method developed will be a powerful technique for clarifying the clinical importance of serotyping *Streptococcus mutans.*

References

1. Gronroos L, Saarela M, Matto J, Tanner-Salo U, Vuorela A, Alaluusua S. Mutacin production by Streptococcus mutans may promote transmission of bacteria from mother to child. Infect Immun. 1998;66(6):2595-600.

2. Hillman JD, Dzuback AL, Andrews SW. Colonization of the human oral cavity by a Streptococcus mutans mutant producing increased bacteriocin. J Dent Res. 1987;66(6):1092-4.

3. Caufield PW, Childers NK, Allen DN, Hansen JB. Distinct bacteriocin groups correlate with different groups of Streptococcus mutans plasmids. Infect Immun. 1985;48(1):51-6.

4. Qi F, Chen P, Caufield PW. Purification of mutacin III from group III Streptococcus mutans UA787 and genetic analyses of mutacin III biosynthesis genes. Appl Environ Microbiol. 1999;65(9):3880-7.

5. Qi F, Chen P, Caufield PW. Functional analyses of the promoters in the lantibiotic mutacin II biosynthetic locus in Streptococcus mutans. Appl Environ Microbiol. 1999;65(2):652-8.

6. Qi F, Chen P, Caufield PW. The group I strain of Streptococcus mutans, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. Appl Environ Microbiol. 2001;67(1):15-21.

7. Chen P, Qi F, Novak J, Caufield PW. The specific genes for lantibiotic mutacin II biosynthesis in Streptococcus mutans T8 are clustered and can be transferred en bloc. Appl Environ Microbiol. 1999;65(3):1356-60.

8. Gronroos L, Matto J, Saarela M, Luoma AR, Luoma H, Jousimies-Somer H, et al. Chlorhexidine susceptibilities of mutans streptococcal serotypes and ribotypes. Antimicrob Agents Chemother. 1995;39(4):894-8.

9. Shibata Y, Ozaki K, Seki M, Kawato T, Tanaka H, Nakano Y, et al. Analysis of loci required for determination of serotype antigenicity in Streptococcus mutans and its clinical utilization. J Clin Microbiol 2003;41(9):4107-12.

10. Oho T, Yamashita Y, Shimazaki Y, Kushiyama M, Koga T. Simple and rapid detection of Streptococcus mutans and Streptococcus sobrinus in human saliva by polymerase chain reaction. Oral Microbiol Immunol. 2000;15(4):258-62.

11. Novak J, Caufield PW, Miller EJ. Isolation and biochemical characterization of a novel lantibiotic mutacin from Streptococcus mutans. J Bacteriol. 1994;176(14):4316-20.

12. Marcotte H, Lavoie MC. Oral microbial ecology and the role of salivary immunoglobulin A. Microbiol Mol Biol Rev. 1998;62(1):71-109.

13. Napimoga MH, Hofling JF, Klein MI, Kamiya RU, Goncalves RB. Tansmission, diversity and virulence factors of Sreptococcus mutans genotypes. J Oral Sci. 2005;47(2):59-64.

14. Kamiya RU, Napimoga MH, Hofling JF, Goncalves RB. Frequency of four different mutacin genes in Streptococcus mutans genotypes isolated from caries-free and caries-active individuals. J Med Microbiol. 2005;54(Pt 6):599-604.

15. Napimoga MH, Kamiya RU, Rosa RT, Rosa EA, Hofling JF, Mattos-Graner RO, et al. Genotypic diversity and virulence traits of Streptococcus mutans in caries-free and caries-active individuals. J Med Microbiol. 2004;53(Pt 7):697-703.

16. Paddick JS, Brailsford SR, Kidd EA, Gilbert SC, Clark DT, Alam S, et al. Effect of the environment on genotypic diversity of Actinomyces naeslundii and Streptococcus oralis in the oral biofilm. Appl Environ Microbiol. 2003;69(11):6475-80.

17. Qi F, Chen P, Caufield PW. Purification and biochemical characterization of mutacin I from the group I strain of Streptococcus mutans, CH43, and genetic analysis of mutacin I biosynthesis genes. Appl Environ Microbiol. 2000;66(8):3221-9.