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

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Resumo
O objetivo do estudo foi avaliar a frequência 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 etidio. 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 frequência do sorotipo c e do gene para mutacina IV na população estudada.


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 sorotype 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 absence of sorotype 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.

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 bacteria. 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.

Mutacins have been classified in two families: the lantibiotics (containing lanthionine and/or β-methyllanthionine 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. The structural genes of the prepropeptides of mutacins I, II, III and IV (mutA) have been sequenced, and their biosynthetic loci is formed by several genes, including those involved in regulation, cleavage, transport and immunity to the produced mutacin.
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 β-linked glucosidic residues. The serotype c S. mutans strains are predominant in the human oral cavity among the serotype c, e and f strains. The serotype c RGP structure may have advantages for S. mutans colonization of the oral cavity.

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°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). The debris were pelleted and the supernatants were stored in a freezer at -20°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 GCC TTGG3’ and 5’ CAG TAT AAG CGC CAG TTT CAT C3’ (Invitrogen, São Paulo, Brazil), yielding an amplicon of 517 pb 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), yielding an amplicon of 517 pb 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 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. The PCR mixture for mutacins consisted of 1X PCR buffer 10x, 2.5mM MgCl₂, 0.5µg/ml 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 94°C for 5 min, a total of 30 PCR cycles were performed; each cycle consisted of 30s of denaturation at 92°C, 30s of annealing at 55°C, 1 min of extension at 72°C, and the final extension 5 min at 72°C.

For the detection of serotypes c, e and f, a multiplex PCR was done. The PCR mixture (10µl) consisted of 0.2mM each deoxyribonucleotide triphosphatase, 1X PCR buffer, 2mM MgCl₂, 1U Taq DNA polymerase (Invitrogen, São Paulo, Brazil), 0.5µM concentration of each primer, and 2µl of template DNA. After denaturation at 96°C for 2 min, a total of 25 PCR cycles were performed; each cycle consisted of 15s of denaturation at 96°C, 30s of annealing at 55°C, 1 min of extension at 72°C.

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 χ² test and the Spearman’s coefficient of correlation. Statistical significance was considered to be at α<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 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.
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. 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.

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. Clinically, mutacins have been considered important for the establishment and equilibrium of bacteria in dental biofilms. Supporting this hypothesis, the antimicrobial spectrum of mutacin IV is specifically against members of the mitis group of oral streptococci. Nevertheless, our study suggests that given the increasing complexity of the oral microbiota, as found in caries-active individuals, 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 which S. mutans were recovered were more diverse, probably because production of organic acids and mutacins with the biofilm results in a more complex community compared to caries-free individuals. 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. Mutacin IV is produced by planktonic cells while mutacin I is produced by biofilm-like cells. 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.

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). 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 suggest 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

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