EVALUATION OF GENOTYPIC DIVERSITY OF 
*Streptococcus mutans* USING DISTINCT ARBITRARY 
PRIMERS

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ABSTRACT

*Streptococcus mutans* has been considered one of the main etiological agents of dental caries and the genotypic diversity rather than its salivary counts may be considered as a virulence factor of this bacterium. For genotyping with polymerase chain reaction (PCR) with arbitrary primers, several primers have been used in order to improve complexity and specificity of amplicon patterns. Thus, the aim of this study was to evaluate the degree of agreement of genotypic identification among AP-PCR reactions performed with 5 distinct arbitrary primers of *S. mutans* isolated from saliva. Stimulated saliva was collected from 11 adult volunteers for isolation of *S. mutans*, and a total of 88 isolates were genotyped with arbitrary primers OPA 02, 03, 05, 13 and 18. Fourteen distinct genotypes were identified in the saliva samples. Most volunteers (9 out of 11) presented only one genotype. The results of the present study suggest that primers OPA 02, 03, 05 and 13 were suitable for genotypic identification of *S. mutans* isolates of saliva from adult volunteers.

Key words: AP-PCR. Arbitrary primers. Genotypes. Saliva. *Streptococcus mutans*.

INTRODUCTION

Dental caries is a multifactorial infectious disease, related to biofilm accumulation on dental surface¹⁶ and frequent consumption of fermentable carbohydrates². By the fermentation of dietary carbohydrates, the bacteria in the dental biofilm produce acids that decrease the pH and increase the biofilm potential in promoting dental demineralization¹³. Additionally, the acid environment selects cariogenic bacteria, such as mutants streptococci¹⁶. Among them, *Streptococcus mutans* is known to be one of the most important cariogenic microorganisms¹⁵,¹⁶ because, in addition to being acidogenic and acid-tolerant, it uses sucrose to produce insoluble glucans in biofilm matrix¹, which may play an important role in the development of caries¹³,¹⁷,²². Different genotypes of *S. mutans* may present different expression levels of glucosyltransferases¹⁹ and higher production of insoluble polysaccharides has been reported by genotypes from caries-active individuals⁸,²⁰.

Different genotypes of *S. mutans* have been found in saliva, and dental biofilm and AP-PCR technique has been widely used to discriminate this genotypic diversity¹,³,⁷,⁹,¹¹,¹²,¹³,¹⁸,²³,²⁶. This technique has a discriminatory potential comparable to other techniques for genotypic identification of *S. mutans*¹³,¹⁴,²⁸. However, different arbitrary primers have been used for *S. mutans* genotyping. The application of more than one arbitrary primer was suggested to increase the discriminatory power of AP-PCR genotyping¹³,²⁹. Saarela, et al.²⁸ (1996) have reported that primers OPA 05 and OPA 13 were efficient to identify *S. mutans* genotypes. Truong, et al.²⁹ (2000), investigating genotypic diversity among mutants streptococci, verified that
Streptococcus mutans 

MATERIAL AND METHODS 

Evaluation of Genotypic Diversity of Streptococcus mutans Using Distinct Arbitrary Primers 

This research was approved by the Research Ethics Committee of the Dental School of Piracicaba, State University of Campinas (Protocol #078/2007), the volunteers were fully informed about the procedures, and written consent was obtained prior to the beginning of the study. Stimulated saliva from 11 volunteers, who participated in a previous study evaluating the effect of sucrose on genotypic diversity, was collected for isolation of mutans streptococci. Healthy volunteers (18 to 28 years old), who fulfilled inclusion criteria (counts of mutans streptococci in saliva from 10^3 to 10^6 colony-forming units (cfu) per mL) and exclusion criteria (antibiotic use for the last 2 months, use of any form of medication that modifies salivary secretion, periodontal disease, general/systemic diseases) took part in this study. S. mutans morphological types were isolated, and DNA from these colonies was extracted. Thereafter, PCR with specific primers (gtfB and gfbB) was conducted for identification of S. mutans, and then, these isolates were submitted to genotyping protocols by arbitrarily primed polymerase chain reaction (AP-PCR) with 5 different arbitrary primers: OPA 02, OPA 03, OPA 05, OPA 13 and OPA 18.

S. mutans Isolation and Identification 

Stimulated saliva samples were collected by parafilm chewing in the morning, under fasting condition, and without previous toothbrushing. Saliva samples were diluted in sterile 0.9% NaCl and plated on mitis salivarius-bacitracin agar (MSB) (Difco, Sparks, MD, USA). After incubation for 48 h at 37°C in 10% CO2, 8 representative morphological types of S. mutans colonies were collected from each sample, subcultured on mitis salivarius agar (MSA) (Difco, Sparks, MD, USA) and pure cultures stored at -70°C in 10% skim milk medium. The purity and identity of the isolates were checked by Gram’s stain and colonial morphology on MSA.

Then, aliquots were collected from skim milk and plated on Brain Heart Infusion (BHI) (Difco, Sparks, MD, USA), which was incubated for 24 h at 37°C in 10% CO2. The colonies from BHI agar were inoculated into Todd Hewitt Broth (Difco, Sparks, MD, USA) and incubated for 18 h at 37°C and 10% CO2. Cultures were then centrifuged at 4°C for 15 min, genomic DNA was extracted and purified from cell pellet, and stored at -20°C. Integrities of the genomic DNA samples were checked in samples electrophoretically resolved in 1% agarose gel and stained with ethidium bromide (5 µg/mL). Isolates were confirmed for species identity in PCR reactions with primers specific for gtfB, encoding glucosyltransferase B (5’-ACTACACTTTTCGGGTGGCTTGG-3’ and 5’-CAGTATAACGCCAGTTTTCATC-3’)4, and specific to gfbB, encoding glucan-binding protein B (5’-CAACAGAGCAAACACCATA-3’ and 5’-TGTCACCATTTACCCAGT-3’)18. Although GtfB primers amplify S. mutans gtfB gene, a previous study revealed that gfbB primers yield some cross-amplification with several clinical isolates of S. sobrinus (defined as S. sobrinus species by sequencing of 16S rRNA)10. To overcome this problem, the strains were also tested with GfbB primers because these primers yield amplicons of predicted size in all S. mutans genotypes tested in a previous study18, and do not amplify S. sobrinus sequences18. Each reaction consisted of 1 µL template DNA, 10 µM of each primer, 10 µM of DNTP, 2.5 µL 1x PCR buffer, 50 mM MgCl2 and 5U/µL Taq DNA polymerase in a total volume of 25 µL. The amplification reaction was performed in a thermocycler (TC-412, Techne, Duxford, Cambridge, UK) in 30 cycles as follows: denaturation 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min, using S. mutans UA130 (kindly provided by Dr. Page W. Caufield, New York University, NY, USA) and S. sobrinus, and distilled/deionized water as positive and negative controls, respectively. The resulting amplicons were submitted to electrophoresis in 2% agarose gels and the images were captured by a digital imaging system (Gel logic 100 Imaging System, Kodak, Tokyo, Japan).

AP-PCR Reactions 

AP-PCR assays were performed with the arbitrary primers: OPA 02 (5’-TGCCGAGCTG-3’13, OPA 03 (5’-AGTCAGCCAC-3’)29, OPA 05 (5’-AGGGGTCTTG-3’)28, OPA 13 (5’-CAGCACCCAC-3’)24 and OPA 18 (5’-AGGTGACCGT-3’)29. The reactions were processed in 50 µL mixtures containing 1x PCR buffer, 5 U/µL of Taq DNA polymerase, 10 mM DNTP, 20 µM primer, 50 mM MgCl2 and 2 µL template DNA. Reactions were performed with the following conditions: 

- OPA 02: one initial cycle of denaturation at 95°C for 2 min, followed by 45 cycles of 94°C for 30 s (denaturation), 36°C for 30 s (annealing) and 72°C for 1 min (extension) and a final extension at 72°C for 5 min; 
- OPA 03 and 18: one initial cycle of denaturation at 95°C for 2 min, followed by 30 cycles of 94°C for 1 min (denaturation), 32°C for 1 min (annealing) and 72°C for 2
min (extension) and a final extension at 72°C for 5 min;
- OPA 05 and 13: one initial cycle of denaturation at
95°C for 2 min, followed by 35 cycles of 95°C for 1 min
(denaturation), 36°C for 2 min (annealing) and 72°C for 2
min (extension) and a final extension at 72°C for 5 min.

The AP-PCR products were electrophoretically resolved
in 1.5% agarose gels, using S. mutans UA130 and distilled
and deionized water as positive and negative control,
respectively. The gel was stained with a 5 µg/mL of ethidium
bromide solution for 10 min, and their images were captured
by a digital imaging system (Gel logic 100 Imaging System,
Kodak, Tokyo, Japan). A 1-Kbp DNA ladder served as a
molecular-size marker in the gel. The amplicon profiles
(amplitypes) of the same volunteer were always resolved
side-by-side in the same gel for visual comparisons1,11,25.
Isolates were considered as having the same genotypic
identity when presented identical AP-PCR product-size
profiles. Any repeatable difference regarding the strong
bands was considered discriminatory. The genotypes found
were analyzed descriptively and their proportion, in relation
to the number of colonies isolated in each sample and
condition, was calculated. Also, the number of bands from
each genotype amplified by each of the arbitrary primers
was counted and the mean value was calculated.

RESULTS

A total of 88 representative colonies of S. mutans were
isolated from saliva, being 8 from each volunteer. All isolates

| Volunteer | Genotype (%) | Number of bands |
|-----------|--------------|----------------|
|           | OPA 02       | OPA 03 | OPA 05 | OPA 13 | OPA 18 |
| 1 A (100) | 9            | 11     | 8      | 8      | 7      |
| 2 B (100) | 10           | 9      | 7      | 8      | 7      |
| 3 C (100) | 11           | 11     | 6      | 9      | 9      |
| 4 D (100) | 14           | 8      | 11     | 11     | 9      |
| 5 E (100) | 13           | 10     | 8      | 10     | 9      |
| 6 F (100) | 15           | 10     | 10     | 11     | 8      |
| 7 G (100) | 13           | 9      | 8      | 9      | 8      |
| 8 H (75)  | 11           | 11     | 7      | 8      | 7      |
|           | J (12.5)     | 11     | 11     | 11     | 11     |
| 9 K (100) | 13           | 10     | 7      | 7      | 9      |
| 10 L (100)| 11           | 10     | 8      | 7      | 9      |
| 11 M (87.5)| 11          | 8      | 8      | 7      | 10     |
|           | N (12.5)     | 11     | 8      | 8      | 7      |

Mean number of bands

11.9 | 9.7 | 8.0 | 8.6 | 8.6

* The proportion (%) of the genotypes in relation to the number of colonies isolated in each condition is represented within the parenthesis. ** The genotypes M and N were identified by all primers, except for OPA 18.
DISCUSSION

It is well known that the oral cavity harbors distinct genotypes of S. mutans. In the present study, only one genotype was found in the saliva of 9 out of 11 volunteers (Table 1). This agrees with the findings of previous studies in saliva or dental biofilm samples of children and adult subjects. This low genotypic diversity could be related to the fact that other genotypes might be present in saliva in a proportion below the detection limit of the microbiological method used. Also, certain genotypes present in the oral cavity could become permanently established, while other genotypes, due to their reduced ability of interacting with the host, form a transient population. Despite this low genotypic diversity found in the present study, saliva samples harbor those genotypes present at higher proportions in dental biofilms. In the present study, although distinct genotypes were identified by all primers tested, the OPA 02 showed the best results, considering the number of bands produced by reaction (Table 1). These data are in agreement with the study of Li and Caufield (1998).

Despite the lower number of bands yielded in reactions with OPA 03, when compared to primer OPA 02, primer OPA 03 allowed an efficient differentiation of genotypes. This result is in contrast with those of Li and Caufield (1998), who found that OPA 03 presented a lower discriminatory capacity than OPA 02. In addition, OPA 05, 13 and 18 showed a smaller number of bands than OPA 02 and OPA 03 (Table 1).

The reduced number of bands might decrease the differentiation among genotypes, since just one genotype was identified by OPA 18 in volunteer 11, compared to the other two genotypes identified with the other primers (Table 1). In addition, although OPA 05 produced fewer bands than OPA 02 and 03, which might make difficult the differentiation of the genotypes, it seems that this characteristic did not impair the identification and differentiation of the genotypes in the samples analyzed. Because of this finding, considering the results of the present study, an association between OPA 02 and OPA 03 or OPA 02 and OPA 05 may be useful in the identification of genotypic diversity. Nevertheless, further studies should evaluate a larger number of volunteers and different samples, such as biofilm, which present a higher diversity, using different primers.

CONCLUSIONS

In conclusion, the results of the present study suggest that primers OPA 02, 03, 05 and 13 were suitable for genotypic identification of S. mutans isolates of saliva from adult volunteers.
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