



**UNIVERSIDADE ESTADUAL DE CAMPINAS  
FACULDADE DE ODONTOLOGIA DE PIRACICABA**



# **CURSO DE GRADUAÇÃO EM ODONTOLOGIA**

**Monografia de Final de Curso**

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**Orientadora: Profa. Dra. Cínthia Pereira Machado Tabchoury**

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**TCC 485**

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Maria Clara Sayuri Kajiki de Sousa

**“AVALIAÇÃO DA DIVERSIDADE GENOTÍPICA  
DE *STREPTOCOCCUS MUTANS* EVIDENCIADOS  
POR DIFERENTES PRIMERS ARBITRÁRIOS”**

Monografia apresentada ao  
Curso de Odontologia da  
Faculdade de Odontologia de  
Piracicaba – UNICAMP, para  
obtenção do diploma de  
Cirurgião-Dentista.

Orientadora: Profa. Dra. Cíntia Pereira Machado Tabchoury

Piracicaba  
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Aos meus pais pelo,  
amor e incentivo. Ao  
meu namorado Marco  
Antonio, companheiro  
eterno.

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## Lista de abreviaturas e siglas

AP-PCR= arbitrarily primed polymerase chain reaction

BHI= brain heart infusion

CFU= colony-forming units

et al. = e outros (abreviatura de "et alii")

MSA= mitis salivarius agar

MSB= mitis salivarius-bacitracin agar

OPA= arbitrary primers

## RESUMO

*Streptococcus mutans* têm sido considerados os principais agentes etiológicos da cárie dental e a sua diversidade genotípica poderia ser considerada como fator de virulência para estes microrganismos. Para a genotipagem com reação em cadeia da polimerase com primer arbitrário (AP-PCR), vários primers têm sido utilizados para melhorar a complexidade e a especificidade dos padrões de amplicons. Deste modo, o objetivo deste trabalho foi avaliar o grau de concordância na identificação genotípica de *S. mutans* da saliva, por meio de AP-PCR utilizando 5 diferentes primers arbitrários. Para isso, saliva estimulada de 11 voluntários adultos foi coletada para isolamento de *S. mutans* e um total de 88 isolados (8 por voluntário) foram genotipados com os primers arbitrários OPA 02, 03, 05, 13 e 18. Quatorze diferentes genótipos foram identificados nas amostras salivares, com a maioria dos voluntários, 9 dos 11, apresentando apenas um genótipo na saliva. O primer OPA 18 identificou apenas um genótipo de um dos voluntários, ao contrário dos outros primers que identificaram dois genótipos para os isolados deste mesmo voluntário. Deste modo, os resultados sugerem que os primers OPA 02, 03, 05 e 13 foram adequados para avaliar a diversidade genotípica de *S. mutans* isolados da saliva de voluntários adultos.

## CAPITULO

O presente artigo intitulado: **“Evaluation of genotypic diversity of *Streptococcus mutans* using distinct arbitrary primers”**, foi aceito para publicação no periódico “Journal of Applied Oral Science”, ISSN -1678-7757.

## 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 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, with most of the volunteers, 9 out of 11, presenting just one genotype. Our results suggest that primers OPA 02, 03, 05 and 13 were suitable for genotypic identification of *S. mutans* isolates of saliva from adult volunteers.

## INTRODUCTION

Dental caries is a multifactorial infectious disease, related to biofilm accumulation on dental surface<sup>16</sup> and frequent consumption of fermentable carbohydrates<sup>2</sup>. Through 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 demineralization<sup>3,4</sup>. Additionally, the acid environment selects cariogenic bacteria, such as mutans streptococci<sup>16</sup>. Among them, *Streptococcus mutans* is known to be one of the most important cariogenic microorganism<sup>15,16</sup>, since, besides being acidogenic and acid-tolerant, it uses sucrose to produce insoluble glucans in biofilm matrix<sup>3</sup>, which may play an important role in the development of caries<sup>3,5,17,22</sup>. Different genotypes of *S. mutans* may present different expression levels of glucosyltransferases<sup>19</sup> and higher production of insoluble polysaccharides has been reported by genotypes from caries-active individuals<sup>8,20</sup>.

It has been reported that different genotypes of *S. mutans* are found in saliva and dental biofilm and AP-PCR technique has been widely used to discriminate this genotypic diversity<sup>1,6,7,9,11,12,18,23,26</sup>. This technique has discriminatory potential comparable to other techniques for genotypic identification of *S. mutans*<sup>13,14,28</sup>.

However, different arbitrary primers have been used for *S. mutans* genotyping. The application of more than one arbitrary primer was suggested to increase discriminatory power of AP-PCR genotyping<sup>13,29</sup>. Saarela, et al.<sup>28</sup> (1996) reported that primers OPA 05 and OPA 13 were efficient to identify *S. mutans* genotypes. Truong, et al.<sup>29</sup> (2000), investigating genotypic diversity among mutans streptococci, verified that primers OPA 03 and OPA 18 could differentiate *S. mutans* from other oral microorganisms. In addition, Li and Caufield<sup>13</sup> (1998) after the evaluation of various arbitrary primers in the genotyping of reference strains of *S. mutans*, suggested that the primer OPA 02 showed the best power of discrimination of genotypes, giving the highest number of amplicons when compared to other primers. OPA 02 has been recently widely adopted in AP-PCR protocols<sup>1,9,12,18,20,23</sup>. Nevertheless, there is always the question whether other primers should be used to ascertain this

diversity and, in addition, the evaluation of different primers was performed with only reference strains<sup>13</sup>.

Thus, the aim of the present study was to evaluate the degree of agreement of genotypic identification between AP-PCR reactions performed with distinct arbitrary primers (OPA 02, OPA 03, OPA 05, OPA 13 and OPA 18) of *Streptococcus mutans* isolated from saliva of adult volunteers.

## MATERIALS & METHODS

### ***Streptococcus mutans* isolates**

This research was approved by the Research and Ethics Committee of FOP-UNICAMP (Protocol n<sup>o</sup> 078/2007), the volunteers were informed about the procedures and written consent was obtained prior to the commencement of the study. Stimulated saliva from 11 volunteers, who participated in a previous study<sup>1</sup>, 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<sup>3</sup> to 10<sup>6</sup> colony-forming units (CFU) per mL) and exclusion criteria (antibiotic use for the last 2 months before starting the study, use of any form of medication that modifies salivary secretion, periodontal disease, general/systemic illness) took part in this study. *S. mutans* morphological types were isolated, and DNA from these colonies was extracted<sup>1,18</sup>. After this, PCR with specific primers (*gtfB* and *gbpB*) was conducted<sup>18,24</sup> 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 within fasting condition and without previous teeth brushing. Saliva samples were diluted in sterile 0.9% NaCl and plated on mitis salivarius-bacitracin agar (MSB). After incubation for 48 h at 37°C in 10% CO<sub>2</sub>, eight representative morphological types of *S. mutans* colonies were collected from each sample, subcultured on mitis salivarius agar (MSA) and pure cultures

stored at -70°C in 10% skim milk medium<sup>10</sup>. 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), which was incubated for 24 h at 37°C in 10% CO<sub>2</sub>. The colonies from BHI agar were inoculated into Todd Hewitt Broth and incubated for 18 h at 37°C and 10% CO<sub>2</sub>. Cultures were then centrifuged at 4°C for 15 min, genomic DNA was extracted and purified from cell pellet<sup>18</sup>, 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'-ACTACACTTTCGGGTGGCTTGG-3' and 5'-CAGTATAAGCGCCAGTTTCATC-3')<sup>24</sup>, and specific to *gbpB*, encoding glucan-binding protein B (5'-CAACAGAAGCACAACCATCA-3' and 5'-TGTCCACCATTACCCCAGT-3')<sup>18</sup>. Although *GtfB* primers amplify *S. mutans* *gtfB* gene, previous study revealed that *gtfB* primers yield some cross-amplification with several clinical isolates of *S. sobrinus* (defined as *S. sobrinus* species by sequencing of 16SrRNA)<sup>10</sup>. To circumvent this problem, we also tested the strains with *GbpB* primers, because these primers yield amplicons of predicted size in all *S. mutans* genotypes tested in a previous study<sup>18</sup>, and do not amplify *S. sobrinus* sequences<sup>18</sup>. Each reaction consisted of 1 µl template DNA, 10 µM of each primer, 10 mM of DNTP, 2.5 µl 1x PCR buffer, 50 mM MgCl<sub>2</sub> and 5U/µl *Taq* DNA polymerase in a total volume of 25 µl. The amplification reaction was performed in a thermocycler (Techne TC-412) 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), *S. sobrinus* and distilled and 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, Japan).

### AP-PCR reactions

AP-PCR assays were performed with the arbitrary primers: OPA 02 (5'-TGCCGAGCTG-3')<sup>13</sup>, OPA 03 (5'-AGTCAGCCAC-3')<sup>29</sup>, OPA 05 (5'-

AGGGGTCTTG-3')<sup>28</sup>, OPA 13 (5'-CAGCACCCAC-3')<sup>28</sup> and OPA 18 (5'-AGGTGACCGT-3')<sup>29</sup>. 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 MgCl<sub>2</sub> 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 sec (denaturation), 36°C for 30 sec (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, 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 comparisons<sup>1,11,25</sup>. 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 descriptively analyzed 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 of the isolates were identified as *S. mutans* in the PCR reactions with specific primers. A total of fourteen distinct genotypes were identified among the 88 isolates tested. Most of the volunteers, 9 out of 11, carried just one genotype (Table 1). The exceptions were volunteer 8, who harboured three genotypes identified with all the arbitrary primers tested (Figure 1), and volunteer 11, who presented two genotypes when evaluated by the following primers: OPA 02, 03, 05 and 13. Using primer OPA 18, volunteer 11 presented just one genotype in saliva. Considering the proportions of genotypes in relation to the total number of isolates, in volunteer 8, the predominant genotype was "H" (75%) and the others, "I" and "J", represented 12.5% each one of the 8 isolates of this volunteer. For volunteer 11, the predominant genotype was "M" (87.5%) and the genotype "N" represented 12.5%. In addition, the genotypes were distinct among all volunteers. Table 1 also presents the number of bands produced after the AP-PCR reaction with each one of the arbitrary primers tested. The amplification with OPA 02 primer resulted in a higher number of bands (mean of 11.9), in relation to the other primers. OPA 03 primer presented around 9.7 bands and the other primers between 8.6 and 8 bands.

## DISCUSSION

It is well known that the oral cavity harbor distinct genotypes of *S. mutans*<sup>9,12,18,20,27,28</sup>. In the present study, one genotype was found in the saliva of nine volunteers, out of eleven (Table 1). This agrees with previous studies in saliva or dental biofilm samples of children<sup>9,12,18,20,27,28</sup> and adult subjects<sup>1,21</sup>. This low genotypic diversity could be related to the fact that other genotypes might be present in saliva in a proportion below to the detection limit of the microbiological method used<sup>26</sup>. 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<sup>21</sup>. Despite this low genotypic diversity found in our study, saliva samples harbored those genotypes present at higher proportions in teeth biofilms<sup>1,12</sup>. In the

present study, although distinct genotypes had been identified by all primers tested, the OPA 02 showed the best results, considering the number of bands produced through the reaction (Table 1). These data are in agreement with the study of Li and Caufield<sup>13</sup> (1998).

Despite the lower number of bands yielded in reactions with OPA 03, when compared with primer OPA 02, primer OPA 03 allowed an efficient differentiation of genotypes. This result is in contrast with Li and Caufield<sup>13</sup> (1998) study, in that OPA 03 showed lower discriminatory capacity than OPA 02. In addition, OPA 05, 13 and 18 showed lower number of bands than OPA 02 and OPA 03 (Table 1). The decreased 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 less bands than OPA 02 and 03, which might 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, 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 higher number of volunteers and different samples, such as biofilm, which present higher diversity with different primers.

## CONCLUSIONS

In conclusion, our results 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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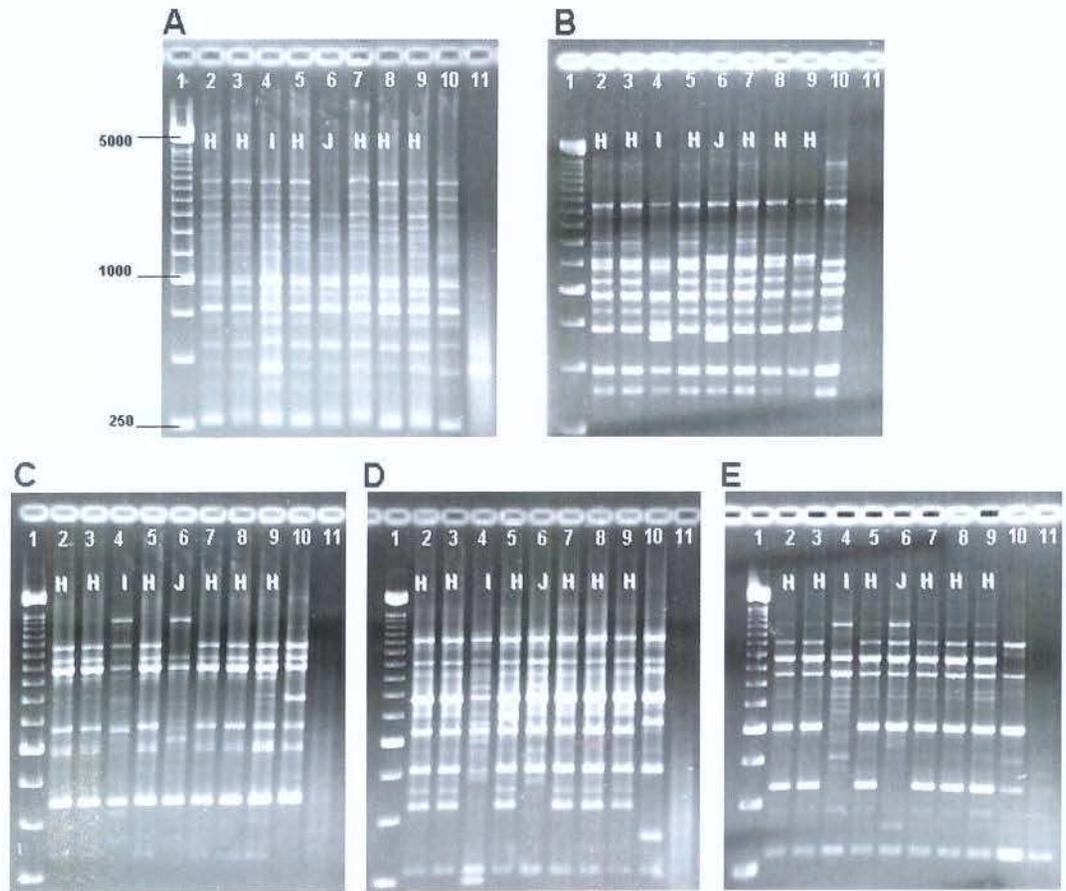
## **ANEXOS**

**Table 1.** Genotypic diversity of *S. mutans* (%) in stimulated saliva and number of bands produced by each primer

<b>Volunteer</b>	<b>Genotypes (%)<sup>*</sup></b>	<b>Number of bands</b>				
		<b>OPA 02</b>	<b>OPA 03</b>	<b>OPA 05</b>	<b>OPA 13</b>	<b>OPA 18</b>
<b>1</b>	A (100)	9	11	8	8	7
<b>2</b>	B (100)	10	9	7	8	7
<b>3</b>	C (100)	11	11	6	9	9
<b>4</b>	D (100)	14	8	11	11	9
<b>5</b>	E (100)	13	10	8	10	9
<b>6</b>	F (100)	15	10	10	11	8
<b>7</b>	G (100)	13	9	8	9	8
<b>8</b>	H (75)					
	I (12.5)	11	11	7	8	7
	J (12.5)					
<b>9</b>	K (100)	13	10	7	7	9
<b>10</b>	L (100)	11	10	8	7	9
<b>11<sup>**</sup></b>	M (87.5)	11	8	8	7	10
	N (12.5)					
<b>Mean</b>						
<b>number of bands</b>		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 by OPA 18.



**Figure 1.** Representative AP-PCR profiles (amplitypes) identified among *S. mutans* strains isolated from volunteer 8, with OPA 02 (A), OPA 03 (B), OPA 05 (C), OPA 13 (D) e OPA 18 (E). Lane 1: 250-bp DNA ladder; lanes 2 to 9: *S. mutans* amplitypes; Lane 10: AP-PCR profile of the control *S. mutans* strain UA 130; lane 12: negative control (water).

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Dear Dr. Cinthia Pereira Machado Tabchoury,

I am delighted to inform you that the revised version of your manuscript "JAOS-217 - Evaluation of genotypic diversity of Streptococcus mutans using distinct arbitrary primers" has been accepted for publication in the Journal of Applied Oral Science.

Soon you will receive the galley proofs for your corrections.

Thank you for choosing our journal to publish your work.

Please, do not hesitate in contacting me for any further information.

Yours sincerely,

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Carlos F. Santos, DDS, PhD  
Associate Professor, Editor-in-Chief  
Journal of Applied Oral Science  
<http://submission.scielo.br/index.php/jaos>