GENOTYPIC PROFILES BY AP-PCR OF STREPTOCOCCUS MUTANS IN CARIES-ACTIVE AND CARIES-FREE PRESCHOOLERS

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ABSTRACT

Streptococcus mutans, an acidogenic and aciduric microorganism that colonizes the oral cavity is recognized as the main causal agent of dental caries. Epidemiological studies have shown a strong correlation between the number of S. mutans in the oral cavity and prevalence and incidence of caries. At present, different genotypic and phenotypic methods are known to determine the profiles of settling and epidemiological distribution of S. mutans. The aim of this study was to investigate the profiles of S. mutans isolated from children with and without dental caries by using the AP-PCR (arbitrarily primed polymerase chain reaction) and api-Zym methods. In the AP-PCR method, random DNA segments of the target bacterium are amplified with single primers of arbitrary sequence. The api-Zym system (bioMérieux, Marcy-létoile, France) is a phenotypic micro-method that allows simultaneous detection of 19 enzymatic activities from bacterial inoculum. A transversal observational study was conducted, which finally included 120 3- to 5- year-old children (75 with and 45 without dental caries), who attended a preschool institution in Bogota (Colombia). S. mutans was isolated from 15 of the 45 children without dental caries (33.3%) and from 31 of the 75 children with caries (41.33%). In the 46 children, 69 S. mutans isolates were identified: 24 isolates in the 15 children without dental caries and 45 isolates in 31 children with dental caries. With api-Zym system, 36 different phenotypes were detected: 22 in the caries group and 15 in the caries-free group. The phenotype XX was present in both groups. With the AP-PCR method, 27 different fingerprinting profiles were identified: 22 for the caries group and 9 of the healthy group; the two groups of patients shared four of these genomic profiles. In conclusion, the information shows a great diversity in S. mutans genotypes and phenotypes in the population studied.

Key words: dental caries, S. mutans, genotypic profiles, AP-PCR.

PERFILES GENOTÍPICOS POR AP-PCR DE *STREPTOCOCCUS MUTANS* EN NIÑOS PREESCOLARES CON Y SIN CARIES DENTAL

RESUMEN

La caries dental es considerada una enfermedad infecciosa multifactorial que conlleva a la destrucción del tejido dental duro. Streptococcus mutans, un microorganismo acidogénico y aciduro que normalmente se encuentra colonizando la cavidad oral, es considerado el principal microorganismo asociado al desarrollo de esta enfermedad. Estudios epidemiológicos han mostrado una fuerte correlación entre el número de unidades formadoras de colonias de S. mutans en la cavidad oral y la prevalencia e incidencia de caries dental. El hecho de reconocer a S. mutans como el microorganismo cariogénico más importante, ha conducido al diseño de medidas preventivas y de control tendientes a eliminarlo o reducir su presencia en la cavidad oral. En la actualidad se utilizan diferentes métodos fenotípicos y genotípicos para demostrar la heterogeneidad y variabilidad genética de cepas S. mutans presentes en la cavidad oral. El objetivo de este estudio fue explorar la utilidad de la técnica AP-PCR en el: 1. conocimiento del genotipo en aislamientos clínicos de S. mutans provenientes de niños con y sin caries, y 2. en el establecimiento de diferencias en los perfiles de tipificación en comparación con la técnica fenotípica Api-ZYM. En el método AP-PCR fragmentos del DNA de la bacteria son amplificados con primers simples que se anidan al azar. El sistema api-Zym es un micro-método semicuantitativo de investigación que permite detectar rápida y simultáneamente 19 actividades enzimáticas a partir de pequeñas cantidades de inóculo de la bacteria. En este estudio observacional descriptivo se incluyeron finalmente 120 niños de 3 a 5 años de un preescolar en Bogotá (Colombia). Se encontró S. mutans en 15 de los 45 niños sin caries dental (33.3%) y en 31 de los 75 niños con caries (41.33%). En total se identificaron 69 aislamientos de S. mutans en los 46 niños: 24 en los 15 niños sin caries dental y 45 en los 31 niños con caries dental. Con el sistema Api-Zym se determinaron 36 fenotipos: 22 en el grupo de caries y 15 en el grupo sin caries. Los dos grupos solamente presentaron en común el fenotipo XX. Con el método AP-PCR se identificaron 27 perfiles, 22 en el grupo con caries y 9 en el grupo sin caries; ambos grupos de pacientes compartieron 4 perfiles genómicos. En conclusión, la información muestra una gran diversidad en perfiles de genotipos y fenotipos de S. mutans en la población objeto de estudio, los cuales en algunos casos se complementan para establecer con claridad diferencias intra e inter-individuo.

Palabras clave: caries dental, S. mutans, perfil genotípico, AP-PCR.

INTRODUCTION

Dental caries is considered a multifactorial, chronic, localized, post-eruptive transmissible infectious disease that leads to the destruction of hard dental tissue[1,2]. According to Keyes' etiological trilogy, modified by Newbrum, three factors sustained over time are needed for dental caries to develop: a susceptible host, cariogenic microbiota in the bacterial plaque and an adequate substrate[1]. *Streptococcus mutans*, an acidogenic and aciduric microorganism

normally found in the oral cavity, is considered to be the main microorganism associated to the development of dental caries[1-3].

Different findings have shown a strong correlation between the number of *S. mutans* colony-forming units in the oral cavity and the prevalence and incidence of dental caries[1,4,5]. It is also important to consider that as a result of dental treatments, *S. mutans* may produce bacteremia, systemic infection and subacute endocarditis[6].

Recognizing *S. mutans* as the most important cariogenic microorganism has led to the design of preventive and control measures that tend to eliminate it or reduce its presence in the oral cavity[7].

One of the aims of molecular typing is to identify specific virulent clones within a bacterial species and study the bacterial clones involved in an epidemiological event. Several molecular methods have enabled the study of oral streptococci[8, 9]; of which restriction endonuclease analysis and ribotyping have revealed genetic heterogeneity within *S. mutans*[10,11]. The AP-PCR technique (arbitrarily primed PCR) has been widely applied for genotyping of many bacterial species, including oral pathogens [12, 13]. Genotyping by means of the AP-PCR technique is currently being used to show different DNA profiles in clinical isolates of *S. mutans*[14-18].

Phenotyping with the Api-ZYM system, based on the enzymatic action of microorganisms on different substrates has been of great value for typing and relating *S. mutans* isolates from different sources[19,20].

A consensus has begun to arise in recent years regarding the use of the AP-PCR technique for studying the genetic variability of *S. mutans* clinical isolates. The main aim of this study was to explore the usefulness of the AP-PCR technique for: 1) obtaining knowledge of the genotype in *S. mutans* isolates from children with and without caries and 2) establishing differences in typing profiles compared to the Api-ZYM phenotyping technique.

MATERIALS AND METHODS

1. *S. mutans* isolation, count and identification

Study Population

Alter receiving informed consent from parents or guardians, and in compliance with the bioethical requirements for sampling and handling samples, 120 3- to 5-year-old preschoolers from Bogotá, Colombia, were included in the study. Each child's caries experience was determined by means of a clinical revision performed by an examiner, who determined the dmft index (decayed, missing and filled teeth) according to World Health Organization criteria[21]. No X-ray was taken of any of the children. Out of the 120 children, 45 had no dental caries and the other 75 had caries with an average dmft index of 2.9 (range 2-5). The 120 children in the study had no systemic infectious diseases and had not been under anti-microbial treatment for at least 7 days prior to sampling. Saliva samples were collected between 8 and 10 a.m., the children having previously agreed not to eat in the morning and not to brush their teeth before the sampling. A spontaneous saliva sample (0.2-1 ml) was obtained from each child by gentle suction with a plastic pipette[22], and kept on ice until it was sent to the bacteriological laboratory.

Sample processing

Saliva samples were dispersed for 15 seconds in a vortex and serially diluted (1/10, 1/100 and 1/1000) with 0.05M phosphate buffer. In order to perform selective isolation and count of *S. mutans,* 50 ul of the serial dilutions were plated in duplicate on Mitis Salivarius-Bacitracin agar (MSB; Difco Laboratories, Detroit, MI) and Sheep Blood agar. MSB agar contains digested pancreatic casein, proteose peptone No. 3, proteose peptone, dextrose, 20% sucrose, dipotassium phosphate, Trypan blue, crystal blue, agar, Chapman tellurite and 0.2 U/ml bacitracin. The MSB and Sheep Blood agar plates were incubated under anaerobic conditions (H_2:CO_2:N_2 10:10:80) for 2 days at 37°C. After bacterial growth on MSB agar, the colonies with morphological characteristics of *S. mutans* were counted and expressed as colony-forming units (CFU) per ml of non-stimulated saliva [23]. Five colonies per sample with *S. mutans* characteristics were examined using Gram stain, catalase test and subject to the following biochemical tests: raffinose, mannitol, mellobiose, trehalose and inulin fermentation; esculin hydrolysis in presence and absence of bile; urease; arginine hydrolysis; and bacitracin resistance. The biochemical profile for *S. mutans* is: raffinose, mannitol, mellobiose, trehalose and inulin fermentation positive; esculin hydrolysis in presence of bile negative and esculin hydrolysis in absence of bile positive; urease negative; arginine hydrolysis negative; and resistance to 2 U of bacitracin. The commercial Api 20S system (bioMerieux, Marcy-létoile, France) was also used for identifying strains. The Chi-square test was used to establish differences in *S. mutans* counts in the groups with and without caries.

2. Phenotyping

All *S. mutans* isolates were phenotyped using the api-ZYM system (bioMérieux, Marcy- létoile, France) according to the manufacturer's instructions. The api-Zym system is a semi-quantitative investigation micro-method by means of which 19 enzymatic activities can be detected quickly and simultaneously from small quantities of bacterial inoculum. The system consists of a strip with 20 cupules (1 control and 19 tests), the bottoms of which contain the enzyme substrates and buffer, allowing contact between the microorganism's enzyme and the usually insoluble substrate. The substrates are inoculated with a dense suspension of bacteria (McFarland turbidity 5-6) which rehydrates and produces enzymatic action on the substrates contained in the cupules. The end products generated during 4-hours' incubation are detected by means of the color reactions that take place after adding reagents. Phenotyping was duplicated and phenotypes were assigned according to the action of the *S. mutans* isolates on the 19 substrates in the system[24]. After the isolates were identified and phenotyped, they were preserved at -85^0 C in trypticase soy broth with 3% glycerol until the molecular techniques were performed.

3. Genotyping *S. mutans* by AP-PCR

In order to obtain bacteria and extract DNA, aliquots of the preservation medium with bacteria were taken and re-inoculated in trypticase soy (TS) broth for 4 hours at 37°C in an anaerobic atmosphere (H_2:CO_2:N_2, 10:10:80). Then the bacterial growth in TS broth was re-plated on TS agar and incubated for 16 hours at 37°C in an anaerobic atmosphere (H_2:CO_2:N_2, 10:10:80). The pure, viable *S. mutans* colonies from the TS agar were inoculated in TS broth and incubated for 16 hours at 37°C in an anaerobic atmosphere (H_2:CO_2:N_2, 10:10:80). Finally, the bacterial growth was collected by centrifuging at 4°C for 15 minutes. DNA was extracted from the cell pellet using the commercial Wizard Genomic DNA Purification Kit (Promega; Madison, WI, USA), according to the manufacturer's instructions. DNA integrity was assessed by electrophoresis in 1% agarose gel stained with ethidium bromide. AP-PCR amplification was carried out using the primer OPA-05 (5´ AGGGGTCTTG 3´, Invitrogen; Carlsbad, CA, USA). Amplification was done in 25 ul reaction mixture containing: 2.5 ul PCR buffer, 2.0 ul magnesium chloride, 1 ul of each dNTP, 2 ul of the OPA primer – 5, 2 ul target DNA, 0.5 ul *Taq* polymerase and 12 ul pure water. Positive and negative controls were set up at each amplification. Amplification was carried out in the iCycler Thermal Cycler (Bio-Rad Laboratories Inc; Hércules, CA, USA), with the following protocol: initial denaturing cycle at 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute (denaturing), 36°C for 2 minutes (annealing) and 72°C for 2 minutes (extension), and a final 5-minute extension at 72°C. The amplification products were analyzed on 1.5% agarose gel, stained with ethidium bromide (1 ug/ml) and viewed with ultraviolet light in a Mini-Transilluminator (Bio-Rad Laboratories Inc; Hércules, CA, USA). The genotypes obtained (amplitypes) were analyzed by means of visual comparison of the band patterns, taking as a reference a pattern with molecular-size marcker 500 bp (500 bp DNA ladder; Invitrogen, Carlsbad, CA, USA).

RESULTS

Detection and identification of *S. mutans*.

S. mutans was found in 15 of the 45 caries-free children (33.3% frequency) and 31 of the 75 caries- active children (41.33% frequency). A total 69 *S. mutans* isolates were identified in the 46 children: 24 isolates from the 15 children without caries and 45 isolates from the 31 children with caries. The *S. mutans* count in the general population ranged from 10^3 to over 10^7 CFU/ml. Caries-active children had a higher *S. mutans* count than caries-free children, and the differences between counts were statistically significant ($p<0.05$).

***S. mutans* phenotypes**

Tables 1 and 2 show the phenotypes found in the 69 *S. mutans* isolates from children with and without caries. Phenotypes were assigned according to prior standardization and considering the enzymatic action of the bacteria on the 19 substrates of the Api-Zym system[20]. The broad activity of these is reflected by the 36 phenotypes found: 22 in the caries-active group and 15 in the caries-free group. The only phenotype that the two groups had in common was XX, with 1 isolate from the caries-active group and 3 isolates from the caries-free group. In the caries-active group, the most frequent phenotypes were XV, XI, XVII, XII, XIV, XVI, XVIII and VII, with 9, 4, 4, 3, 3, 3, 3 and 2 isolates respectively, the rest of the phenotypes appeared at a frequency of 1 isolate. In the caries-free group, the most frequent isolates were XXVI, XX and XXXVII, with 7, 3 and 2 isolates, respectively; with the rest of the phenotypes appearing at a frequency of 1 isolate.

Genotyping with the AP – PCR technique

The genotypic profiles were established according to the number of bands in each isolate and their molecular weight, taking the molecular-size marker as a reference. The lowest number of bands was 3 and the highest was 7. Tables 1 and 2 show the 27 profiles found in the caries-free and caries-active groups. Figure 1 is an example showing 9 genotypic profiles (Samples 1-9) from isolates, with number 10 showing the molecular-size marker. There were 22 genotypes in the caries-active group and 9 in the caries-free group. The two groups shared genotypes 7, 10, 22 and 25. In the caries-active group, the most frequent genotypes were 4, 7, 11 and 5, with 8, 6, 4

Table 1: Genotype-phenotype relationship in *S. mutans* isolates from caries-free children

Clinical isolates	Genotype	Phenotype
1FS1	21	XXVI
5FS1	23	XX
5FS2	23	XX
6FS	21	XXVI
6FS1	25	XXVI
6FS2	24	XXVI
6FS3	25	XX
8FS	25	XXVI
11FS1	26	XXXVII
11FS2	21	XXV
15FS	10	XXXVI
16FS	7	XXXV
18FS	21	XLIII
23FS1	22	XXIX
23FS2	22	XXXIX
25FS1	21	XXVII
26FS1	24	XXVI
26FS2	27	XXXIII
27FS	10	XXXIV
29FS	7	XXX
30FS	24	XXXII
30FS1	24	XXVI
30FS2	26	XXXI
30FS3	26	XXXVII

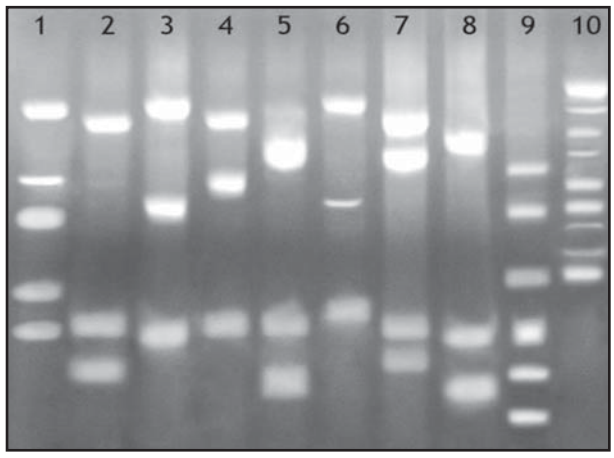

Fig. 1: Genotypic profiles obtained by AP-PCR from 9 Streptococcus mutans clinical isolates (samples 1 to 9) and the molecular-size marker (number 10).

Table 2: Genotype-phenotype relationship in *S. mutans* isolates from caries-active children

Clinical isolates	Genotype	Phenotype
10FC	18	XI
11FC	3	XV
14FC	7	XII
85FC	19	XI
100FC1	5	XVII
101FC1	4	XXI
101FC2	11	XIV
140FC	4	IX
143FC1	4	XV
143FC2	5	XIX
147FC1	6	XVIII
147FC2	17	XVIII
147FC3	20	XVIII
148FC	7	XX
152FC1	4	III
152FC2	10	VIII
154FC1	4	XVI
154FC2	5	XV
154FC3	12	XV
163FC	11	XII
163FC1	7	XV
167FC	13	XXII
168FC	11	XV
171FC	7	I
171FC1	4	XIV
173FC	9	II
178FC	7	XII
182FC	8	XI
185FC	15	IV
189FC	1	XIII
190FC1	2	XXIII
190FC2	19	XVI
194FC	16	XXIV
194FC1	2	XV
195FC1	11	XVII
195FC2	17	XVII
197FC1	9	XV
197FC2	7	XVII
205FC	12	XV
214FC	14	XI
216FC	4	XIV
217FC1	18	VII
217FC2	25	VII
218FC1	22	VI
219FC	4	XVI

and 3 isolates, respectively, and genotypes 2, 9, 12, 17, 18 and 19, with 2 isolates each. The rest of the genotypes had a frequency of 1 isolate. In the caries-free group the most frequent genotypes were 21, 24, 25 and 26, with 5, 4, 3 and 3 isolates, respectively; only genotype 27 appeared in a single isolate, and the rest of the genotypes (7, 10, 22 and 23) had a frequency of 2 isolates.

Relationship between genotypes and phenotypes in the caries-free group

In the children without caries, the number of types found by genotyping was lower than the number found by phenotyping (9 genotypes versus 15 phenotypes). In the isolates from patients 6 (6FS, 6FS1, 6FS2 and 6FS3), 11 (11FS1 and 11FS2), 26 (26FS1 and 26FS2) and 30 (30FS, 30FS1, 30FS2 and 30FS3), intra-individual differences were found by both typing methods (Table 1). However, neither typing method determined intra-individual differences in the isolates from patient 5 (5FS1 and 5FS2); while in patient 23 (23FS1 and 23FS2), the genotyping method did not determine any intra-individual differences, although they were determined using the phenotypic method.

Relationship between genotypes and phenotypes in the caries-active group

In the children with caries the two typing techniques showed the same number of types (22 genotypes versus 22 phenotypes). In the isolates from patients 101 (101FC1 and 101FC2), 143 (143FC1 and 143FC2), 152 (152FC1 and 152FC2), 154 (154FC1, 154FC2 and 154FC3), 163 (163FC and 163FC1), 171 (171FC and 171FC1), 190 (190FC1 and 190FC2), 194 (194FC and 194FC1) intra-individual differences were shown by the two typing methods (Table 2). However, in isolates from patients 147 (147FC1, 147FC2 and 147FC3) 195 (195FC1 and 195FC2) and 217 (217FC1 and 217FC2) the genotypic method determined intra-individual differences that were not determined by the phenotypic method (Table 2).

DISCUSSION

The establishment and subsequent multiplying of *S. mutans* in the oral cavity is influenced by various factors[25]. Its metabolic capacity for synthesizing insoluble glucanes from the sucrose provided in the diet and producing acids that lower the pH and lead to demineralization of the tooth is very important in the process of initiating and development of dental caries[25]. The fact that *S. mutans* has been clearly recognized as the main bacterial species involved in dental caries has led to the implementation of prevention and control measures aimed at eliminating or reducing it in the oral cavity[26].

Different studies have shown that there is a direct relationship between the quantity of *S. mutans* in the oral cavity and the incidence and prevalence of dental caries[1, 5, 27]. In this study, in addition to the fact that the frequency of *S. mutans* in children with caries was higher than in children without caries (41.33% vs. 33%), statistically significant differences ($p<0.05$) were found between counts in the two populations, indicating a high rate of *S. mutans* colonization in children with caries.

In the past, phenotypic and genotypic methods were used to demonstrate the genetic heterogeneity and variability of clinical isolates of *S. mutans* in the oral cavity[14,15,20,25,28-30]. It has been well established that there are different *S. mutans* genotypes in children with dental caries and that genotyping with AP-PCR is useful for demonstrating the diversity of genotypes[14-18]. In this study, a greater number of genotypes was found in the caries-active group (22 genotypes), compared to only 9 found in the caries-free group. The 27 genotypic profiles enabled inter- and intra-individual differences to be established (Tables 1 and 2).

The Api-Zym system has been valuable for phenotyping *S. mutans* by demonstrating its enzyme action on the system's 19 substrates[19,20]. It is important to highlight the large number of phenotypes obtained with the Api-Zym technique in this study: 36 phenotypes, 22 from the caries-active group and 15 from the caries-free group. The phenotypic profiles determined, which show that *S. mutans* strains have high, widely ranging enzyme activity, as well as the genotypic profiles, allowed inter- and intra-individual differences to be determined (Tables 1 and 2).

One of the aims of this study was to assess the usefulness of each typing system for determining intra- and inter-individual differences, and how they might complement each other. In children without caries, intra-individual differences were found in isolates from patients 6, 11, 26 and 30 with both typing methods. However, in the isolates from patient 5 neither typing method determined intra-individual differences and in patient 23 the genotypic method did not determine intra-individual differences, although the phenotypic method did.

In children with caries, both typing methods showed intra-individual differences in the isolates from patients 101, 143, 152, 154, 163, 171, 190 and 194. However, in the isolates from patients 147, 195 and 217 the genotypic method showed intra-individual differences which were not found with the phenotypic method. These findings show the high capacity for determining differences and that the two typing methods are complementary, enabling inter- and intra-individual differences to be determined, and that consequently they may be valuable tools for epidemiological studies of intra-family transmission and for prevention strategies[31].

In agreement with other research, our study found a greater number of genotypes in patients with caries than in patients without caries[18-30]. Napigoma MH[18] evaluated the relationship between clone diversity and some virulence factors of *S. mutans* isolated from 8 caries-active and 8 caries-free individuals; and found 44 different genotypes, with a maximum number of 8 genotypes in one individual. They also found a large number of *S. mutans* genotypes with increased capacity for synthesizing insoluble glucanes. Emanuelsson IM et al.[30] analyzed the distribution and persistence of *S. mutans* group in different sites of the tooth in the oral cavity, and found a maximum 7 different genotypes in one individual.

Pieralisi FJS et al.[32] evaluated genotypic diversity of *S. mutans* in preschoolers with and without caries in Brazil; finding 62 different genotypes in the 28 children included in the study. All these results clearly illustrate the complexity and heterogeneity of *S. mutans* colonization and persistence in the oral cavity of individuals with dental caries, as well as the possible influence of fermentable sugars in the diet.

The AP-PCR technique is being used successfully in the study of *S. mutans* genotypes in children with and without caries[17,18,27,32]. In this technique, bacterial DNA fragments are amplified with single primers of arbitrary sequence[17-18]. Different primers have been used in order to increase the discriminatory power of the AP-PCR technique[17,18,27,32]. There are two trends: one in which the primers OPA 02 and OPA 13 are used[17,18,27,32,33] and another in which the primer OPA 05 is used[17,34-36]. In order to determine the discriminatory potential of the primers, Machado et al.[17] evaluate genotypic diversity in *S. mutans* using the primers OPA 02, OPA 03, OPA 05 and OPA 13; the genotypic variability and heterogeneity found in the results make it clear that any of the 4 primers are useful for genotyping *S. mutans*.

Our study used the primer OPA 05, obtaining 27 genotypic profiles from the 69 isolates. This result closely matches Saarela et al.[34], who used the primer OPA 05 on 81 *S. mutans* strains, obtaining 33 genotypes. Genotypic variability in the selected population depends upon the random nesting of single primers on the DNA of the bacterium under study [17, 34].

Like Truong et al.[37], who evaluated 45 reference strains and 49 isolates of *S. mutans* from saliva and plaque, we evaluated the reproducibility in our study of the AP-PCR technique for typing *S. mutans* by duplicating all samples, finding identical AP-PCR profiles in all cases. Truong et al. [37] conclude that because AP-PCR technique is quick and reproducible, it is of great value for distinguishing *S. mutans* and *S. sobrinus* species and other oral streptococci strains.

CONCLUSION

1. This study phenotyped and genotyped 69 *S. mutans* isolates from patients with and without dental caries; 2. The 69 isolates were grouped into 36 phenotypes, 22 from the caries-active group and 15 from the caries-free group; and 27 genotypes, 22 from the caries-active group and 9 from the caries-free group; 3. Both typing techniques presented a wide range of types, complementing each other in some cases, thus enabling intra- and inter-individual differences to be established clearly.

ACKNOWLEDGMENTS

This study was funded by Dental Research Centre (Faculty of Dentistry-Javeriana University) within the projects academia units under the title "Genotyping by AP-PCR of *Streptococcus mutans* isolates from caries-active and caries-free patients" and register number 1912.

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