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Genetic polymorphism of *Streptococcus mutans* strains associated with incomplete caries removal

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Abstract

Aim: Despite the antibacterial properties of dental materials, the survival of residual bacteria under restorations has been demonstrated after incomplete caries removal. The aim of this study was to evaluate the genetic polymorphism of *Streptococcus mutans* strains isolated from deep dentinal lesions before and three months after incomplete caries removal. **Methods:** Samples of carious dentin were collected from 33 primary and/or permanent molars before and after indirect pulp treatment and processed for microbiological isolation of mutans *streptococci* (MS). After three months of the dental treatment, positive cultures for MS were detected in only ten of these teeth. DNA of MS isolates were obtained and subjected to polymerase chain reaction (PCR) for identification of *S mutans*. The arbitrary primed-PCR method (primer OPA-13) was used to detect the genetic polymorphism of *S. mutans* strains. **Results:** Identical or highly related *S. mutans* genotypes were observed in each tooth, regardless of the collect. Considering each tooth separately, a maximum of nine genotypic patterns were found in each tooth from all the collects. In addition, at least one genotypic pattern was repeated in the three collects. Genetic diversity was observed among the *S. mutans* isolates, obtained from different teeth after three months of the dental treatment. **Conclusions:** The persistence of identical genotypic patterns and the genetic similarity among the isolates, from the same tooth in distinct collects, showed the resistance of some *S. mutans* strains after incomplete caries removal treatment.

Keywords: *Streptococcus mutans,* polymerase chain reaction, polymorphism, genetic, dental caries.

Introduction

The wide distribution and variety of oral bacteria demonstrate their ability to survive among their human hosts, as a result of the efficient transmission of strains and their persistence in the oral cavity[1]. There are several microenvironments within the mouth that harbor communities of pathogenic or non-pathogenic bacteria, called biofilms, which are mainly found in the hard surfaces of the teeth[2]. The biochemical instability between tooth substance and the overlying biofilm, determined by intense production of acids derived from bacterial metabolism, and may lead to dissolution of the dental hard tissues and consequently to the development of a carious lesion[3].

Among the oral pathogens, mutans *streptococci* (MS) comprise a group of seven bacterial species of which only *Streptococcus mutans, S. sobrinus, S. rattus* and *S. cricetus* can be found on the human oral cavity[4]. The highest prevalence of *S. mutans* (74 to 94%) among oral *streptococci,*

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isolated from carious lesions demonstrates their association with caries development[5]. *S. mutans* has the ability of tolerating continuous cycles of acid shock, produced in the oral environment by mechanisms of proton extrusion from the cell via membrane-associated processes and by acid end product efflux, protecting important cellular components, especially DNA, from aggressive effects of acidification[6]. In addition, in adverse environments with nutrient restriction, some bacteria, such as *S. mutans,* are able to obtain carbohydrates from host-derived saliva or serum glycoproteins[7-8].

Genetic diversity among strains of the same species may reflect their ability to survive extreme environmental changes. Therefore, selection of strains best fitted to a given environment can be related to the generation of genetic variants, representing clonal populations, which conserve DNA into single cell lines, or non-clonal populations, which incorporate DNA from other cells[9]. Several studies have shown genetic heterogeneity, among *S. mutans* strains, using modern molecular typing techniques, such as arbitrary primed polymerase chain reaction (AP-PCR), multilocus enzyme electrophoresis (MLEE), restriction fragment length polymorphisms (RFLP) and repetitive extragenic palindromic PCR (REP-PCR)[9-15].

AP-PCR is a valuable DNA analysis tool that have been used mainly in streptococcal epidemiology and transmission studies, by virtue of its rapidity, efficiency and reproducibility in generating genetic fingerprints of bacterial isolates[10,16]. Several studies using AP-PCR have demonstrated that caries-active children have greater genotypic diversity of *S. mutans*, compared to caries-free children[14,17]. In addition, a site-specific colonization pattern of *S. mutans* genotypes in coronal and root caries lesions has been reported[12,18].

The survival of oral *streptococci* has been observed even months or years after incomplete caries removal and cavity sealing[19-21], suggesting the generation of treatment-resistant strains. Paddick et al.[15] have detected different genotypes of *Streptococcus oralis* and *Actinomyces naeslundii,* five months after incomplete carious dentin removal, with reduced phenotypic and genotypic diversity compared to strains isolated from the initial samples before treatment. Therefore, the aim of this study was to evaluate the genetic polymorphism of *S. mutans* strains isolated from deep dentinal lesions before and three months after incomplete caries removal.

Materials and methods

Thirty-three molars (27 primary and 6 young permanent) with deep occlusal caries lesions were obtained from 20 children of both genders, aged four to ten years and subjected to indirect pulp treatment. The absence of irreversible pulpal or periapical diseases was determined through clinical and radiographic examinations of all selected teeth. This study was conducted under a protocol approved by the Research Ethics Committee of Faculdade de Odontologia de Araraquara of Universidade Estadual Paulista, in Brazil, "Júlio de Mesquita Filho" (Unesp) and a signed informed consent was obtained from the legal tutors for the the children's participation.

Clinical procedures and microbiological sampling

Indirect pulp treatment was divided in two sessions. At the first session, anesthesia was delivered and a rubber dam applied to isolate the tooth and prevent contamination by saliva. Rubber cup pumice prophylaxis and antisepsis of operative area, using 0.2% chlorhexidine digluconate, were performed. If necessary, access to infected dentin was gained using a sterile # 245 carbide bur (KG Sorensen, Barueri, SP, Brazil) at high speed.

After removing the superficial necrotic dentin, an initial collect (A) of carious dentin was sampled with a sterile excavator, immediately immersed in 1 mL of saline and maintained in refrigerated boxes (4 ºC). A sterile round steel bur at low speed was used to clean all carious tissue from the dentinoenamel junction, leaving a layer of soft dentin on the cavity floor to avoid pulp exposure. After washing and air-drying the cavities to remove debris, a second collect (B) of carious dentin was obtained from the mesial portion of the cavity floor for microbiological analysis, as previously described. In order to standardize the amount of collected dentin, a small cavity was created at the flat end of an amalgam plugger, which was completely filled with dentin. The remaining carious dentin was covered with resin-modified glass ionomer cements (Vitrebond; 3M/ESPE, St. Paul, MN, USA or Fuji Lining LC; GC Corp., Tokyo, Japan) or a calcium hydroxide-based cement (Dycal; Dentsply, Milford, DE, USA). The cavities were restored with temporary zinc oxide-eugenol cement (IRM; Dentsply). After three months, the second session of the indirect pulp treatment was undertaken. Under the same initial conditions of anesthesia and isolation, teeth were reopened and the liner materials, carefully and completely removed. A third collect (C) of carious dentin was sampled from the distal portion of the cavity floor, as already described. Later, the teeth were lined with glass ionomer cement and restored definitively with silver amalgam (Dispersalloy, Dentsply International, USA).

Microbiological procedures

The tubes containing the carious dentin samples were vortexed for two minutes and the suspension was serially diluted with saline. For each dilution, 25 µL of the samples were placed duplicated in agar sacarose bacitracin (SB) – SB-20[22] and incubated at 37 ºC for 24 hours. After incubation, six to eight representative colonies of *streptococci* were collected and inoculated individually in 5 mL of brain heart infusion (BHI) (Brain Heart Infusion, Difco Laboratories, BD, Sparks, MD, USA) broth for 24 hours. The purity of the cultures were confirmed using Gram technique and aliquots of the subculture frozen at -20 ºC, in 10% glycerol BHI for posterior molecular analysis of bacterial isolates.

Polymerase chain reaction

The extraction of chromosomal DNA was described by Nociti et al.[23] and modified by Nascimento et al.[18]. Briefly, overnight cultures in

BHI broth were centrifuged, followed by washing twice with TRIS-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0) and boiled for ten minutes. After centrifugation, 60 µL of supernatant was collected and used as templates for the PCR. In order to confirm *S. mutans* molecular identity, DNA from MS isolates obtained from carious dentin collects was submitted to PCR method, using specific primers for portions of the glucosyltransferase B gene (gtfB) following the bases sequences': 5' – ACT ACA CTT TCG GGT GGC TTG G – 3' e 5' – CAG TAT AAG CGC CAG TTT CAT C – 3', to amplify a517 bp DNA fragment[24].

Each PCR mixture contained 5 µL of the DNA template, 5 µL of 10x PCR amplification buffer (100 mM Tris-HCl, 500 mM KCl, pH = 8.3), 0.2 mM of dNTPs (DNA polymerization mix), 3.0 mM $MgCl_2$, 1 µM of each primer, 2.5 U of Taq DNA polymerase and sterile distilled water, in order to make a final volume of 25 µL. All PCR reagents were obtained from Invitrogen, Life Technologies, São Paulo, Brazil. Positive and negative controls of PCR were purified in genomic DNA of *S. mutans* (ATCC 25175) and sterile water, respectively. The amplification of DNA was performed in a thermocycler (GeneAmp PCR System 2400, Perkin-Elmer's Applied Biosystems Division, Foster City, CA, USA) with initial denaturation at 95 °C for five minutes, followed by 30 cycles of denaturation at 95° C for 30 seconds, annealing at 59 °C for 30 seconds, extension at 72 °C for one minute, ending with final extension at 72 °C for seven minutes[24]. The PCR amplification products were separated by electrophoresis in 1% agarose gels in Tris-borato-EDTA (TBE), running buffer (pH = 8.0) at 75 V for two hours. Gels were stained with 0.5 µg of ethidium bromide/mL and visualized under ultraviolet light illumination (UltraLum; Labtrade do Brasil, São Paulo, Brazil). A 100 bp DNA ladder was included as a molecular-size marker in each gel.

AP-PCR

Strains identified as *S. mutans* by PCR method were used for genotyping. AP-PCR amplification was performed with primer OPA-13 (5'- CAGCACCCAC – 3')[10,14,18]. All reactions were processed in a volume of 25 µL, containing 2.5 µL of 10x PCR amplification buffer (100 mM Tris-HCl, 500 mM KCl, pH = 8.3), 7 mM $MgCl_2$, 0.2 µM of dNTP, 1 µM of primer, 2.5 U of Taq DNA polymerase, 50 ηg of DNA and distilled water[18]. The amplification was performed in the same thermocycler with an initial denaturation at 94 °C for five minutes and 45 cycles of denaturation at 94 °C for 30 seconds, annealing at 36 °C for 30 seconds, extension at 72 °C for one minute, ending with final extension at 72 °C for three minutes.

Amplicons generated by AP-PCR were analyzed electrophoretically in 1.4% agarose gel, in TBE running buffer and stained in 0.5 µg/mL ethidium bromide. A 1 Kb DNA ladder was used as molecular-size marker. The gels were photographed and their images, captured with a digital imaging system (Kodak Digital Science 1D; Eastman Kodak Company, Rochester, NY, USA). The molecular weights for each band or amplicon were computed and analyzed using the Sigma Gel software (Jandel Scientific, San Rafael, CA, USA). The amplicons were converted in binary data and submitted to NTSYS-pc software (Applied Biostatistics, Inc., Setauket, NY, USA), using the simple matching coefficient (S_{SM}), and the unweighted pair group method with mathematic average (UPGMA) cluster analysis to generate similarity dendrograms.

Results

Only ten deciduous teeth were included in the study that showed positive culture in all collects (A, B and C). From the 429 selected MS colonies, 377 (87.9%) were identified as *S. mutans*. **Table 1** shows the number of MS isolates and *S. mutans* strains, according to the collected period and material group. One hundred and seventy-three *S. mutans* isolates were chosen for AP-PCR analysis considering the three collects (A, B and C). Because the distribution of the teeth was not similar among the material groups, data analysis was performed for all teeth, independent of the lining material. All of them were subjected to AP-PCR. Each isolate received a specific code according to the following sequence: number of the tooth (from 1 to 10), collect (A, B and C) and number of the bacterial isolate (from one to eight), for example: 5A2.

The amplification of genomic DNA of this species resulted in fragments (amplicons or eletrophoretic bands), ranging from 0.3 to 2.2 Kb in size. Some of them were species-specific, whereas others were found in only one or few *S. mutans* strains. The AP-PCR fingerprinting profile analysis with primer OPA-13 showed distinct genotypes patterns of *S. mutans* obtained from caries samples.

In this study, the same genotypic pattern was considered for identical or highly related samples with genetic similarity (S_{SM}) ≥ 0.869 (threshold). Considering each tooth separately, a maximum of nine genotypic patterns were found in each tooth from all the collects (A, B and C). In addition, at least one genotypic pattern was repeated in the three collects. **Table 2** shows the total number of genotypic patterns detected in each tooth, without repetitions among the collects (genetic similarity S_{SM} ≥ 0.869).

Table 1. MS colonies isolated from SB-20 medium and *S. mutans* strains identified by PCR

Lining material	MS colonies			Total	*S. mutans strains*			Total (%)
	Baseline		Reentry		Baseline		Reentry	
	A	B	C		A (%)	B (%)	C (%)	
Vitrebond	70	51	14	136	57 (81.5)	44 (86.3)	14 (100)	115 (84.6)
Fuji lining LC	57	55	47	154	55 (96.5)	55 (100)	37 (78.7)	142 (92.2)
Dycal	59	51	25	135	49 (83.1)	47 (92.3)	19 (76)	115 (85.2)
Total	186	157	86	429	161 (86.6)	146 (93)	70 (81.4)	377 (87.9)

Table 2 - Total of *S. mutans* strains and genotypic patterns found in each tooth from all collects (A, B and C)

Teeth	1	2	3	4	5	6	7	8	9	10
Isolates	15	12	15	16	21	15	18	20	21	20
Genotypic patterns[a]	2	3	4	3	7	5	2	9	6	3

[a] genotypic patterns (genetic similarity $S_{SM} \geq 0.869$) among the collects were considered only once.

Based in the matrices generated by the UPGMA analysis using coefficient S_{SM}, the genetic similarity levels obtained among the *S. mutans* strains are illustrated in Figure 1, which shows representative dendrograms of *S. mutans* isolates obtained for each tooth (1 to 10). The genetic similarity values ranged from $0.555 \leq S_{SM} \leq 1$. Two to four *S. mutans* groups (clusters) containing identical or highly related isolates ($S_{SM} \geq 0.869$) were found in each tooth. These groups can be identified by tonalities of gray in the dendrograms. Identical or highly related isolates were found in all collects in the same tooth.

Comparing the genotypic patterns in Collect C, which were obtained three months after dental treatment, a dendrogram with the SM isolates is shown in Figure 2 and the genetic similarity index observed was $0.621 \leq S_{SM} \leq 1$. UPGMA analysis revealed 11 groups containing identical or highly related isolates ($0.869 \leq S_{SM} \leq 1$). Seven of these groups had isolates corresponding to a specific tooth (Groups 1, 3, 7, 8, 9, 10 and 11). Other three groups had isolates from two distinct teeth (Groups 4, 5 and 6) and one group had isolates from four teeth (Group 2), demonstrating genetic similarity among *S. mutans* strains, obtained from the same tooth and greater polymorphism among isolates from different teeth.

Discussion

S. mutans is the major pathogen associated with dental caries in humans. Several studies have demonstrated its capacity to tolerate extreme pH changes, varying from alkalinity[25] to high levels of acidity[6]. Furthermore, in situations of environmental nutrient stress, such as after cavity sealing, *S. mutans* is capable of producing glycosidic enzymes that release carbohydrates from serum glycoproteins[7-8] present in the dentinal tubules[15]. Therefore, it is plausible to suggest that specific phenotypic characteristics could be expressed by genotypes best fitted to survive and/or grow in adverse environments[1].

In the present study, three months after incomplete caries removal, genetic diversity of *S. mutans* strains was observed in samples of deep caries lesions even after the contact with antibacterial materials, such as glass-ionomer cements[26]. Analyzing each tooth individually, a maximum of nine different genotypic patterns was observed from all the collects. Several studies have demonstrated genetic heterogeneity among *S. mutans* strains, obtained from saliva or dental biofilm samples[10,13], especially in caries-active individuals[14,17]. Some investigators have suggested that the genotype frequency can even vary among the oral sites, but a site-specific colonization of genotypes seems to exist in dental caries lesions[12,18].

Some identical or highly genotypic patterns were isolated in more than one collect, in a same tooth. Although few clones ($S_{SM} = 1$) have been detected, the great genetic similarity could indicate resistance of *S. mutans* strains. Genetic groups containing highly related *S. mutans* isolates are thought to have derived from a single ancestral cell[9], which could have originated new strains with genetic variations. It is commonly accepted that genetic polymorphism between close species is determined by modifications in base pairing, by deletion or insertion of new genetic sequences[27], in addition to clone transmission to external sources[28]. However, the frequency of these events *in vivo* is not known yet[8].

As a part of the evolution process, bacteria have the ability to gain genetic material from other cells using the mechanism of transformation. For recombination to occur, the foreign DNA must share between 70 and 100% identity with the sequence in the recipient strain's chromosome[29]. Some *S. mutans* strains may acquire several cariogenic properties[30], fluoride[31] and antibiotic resistance[32] by transformation. It is possible that *S. mutans* could act as a donor of DNA to another species, such as *S. sanguis* and *S. milleri*[33]. Cvitkovitch[2] suggested that bacterial transformations can occur in environments which experience extreme changes and fluctuations in population dynamics, such as the oral cavity. Li et al.[34] have demonstrated that *S. mutans* cells are hypertransformable when grown in biofilms *in vitro*. Bacteria in these environments are frequently exposed to various stress conditions, such as nutrient excess or shortage, low pH, high osmolarity and the consumption of antimicrobial agents by the host[9,34]. Therefore, natural genetic transformation could be considered an important mechanism of cell's adaptation to environmental changes, providing microbial resistance, genetic variation and rapid evolution of the virulence factors[2,29,34].

Comparing *S. mutans* genotypic patterns isolated from carious samples after three months of the dental treatment, genetic diversity was observed among the teeth and high intra-tooth similarity was detected. These results may suggest that different resistant *S. mutans* strains have tooth-specific colonization, because it was not possible to verify a common genetic pattern within the sample subjected to the treatment proposed in this study. Some investigators have shown genetic similarity among *S. mutans* genotypes obtained from members of the same family[11,13,35-36] or individuals that cohabit in environments, such as nursery schools, denoting horizontal transmission[37]. Generally, non-related subjects rarely share identical *S. mutans* genotypes[10]. Nascimento et al.[18] have compared 40 genetic types determined by AP-PCR, isolated from nine different patients and it was observed a great diversity among them, demonstrating that the maximum value of the similarity indices ($S_{SM} = 0.960$) was observed only in isolates from the same individual.

Despite some deficiencies, such as the difficulty to visualize low-intensity bands and the need for more than one primer to increase the technique accuracy[14], studies have shown the efficacy of AP-PCR in the detection of genetic polymorphism of various bacte-

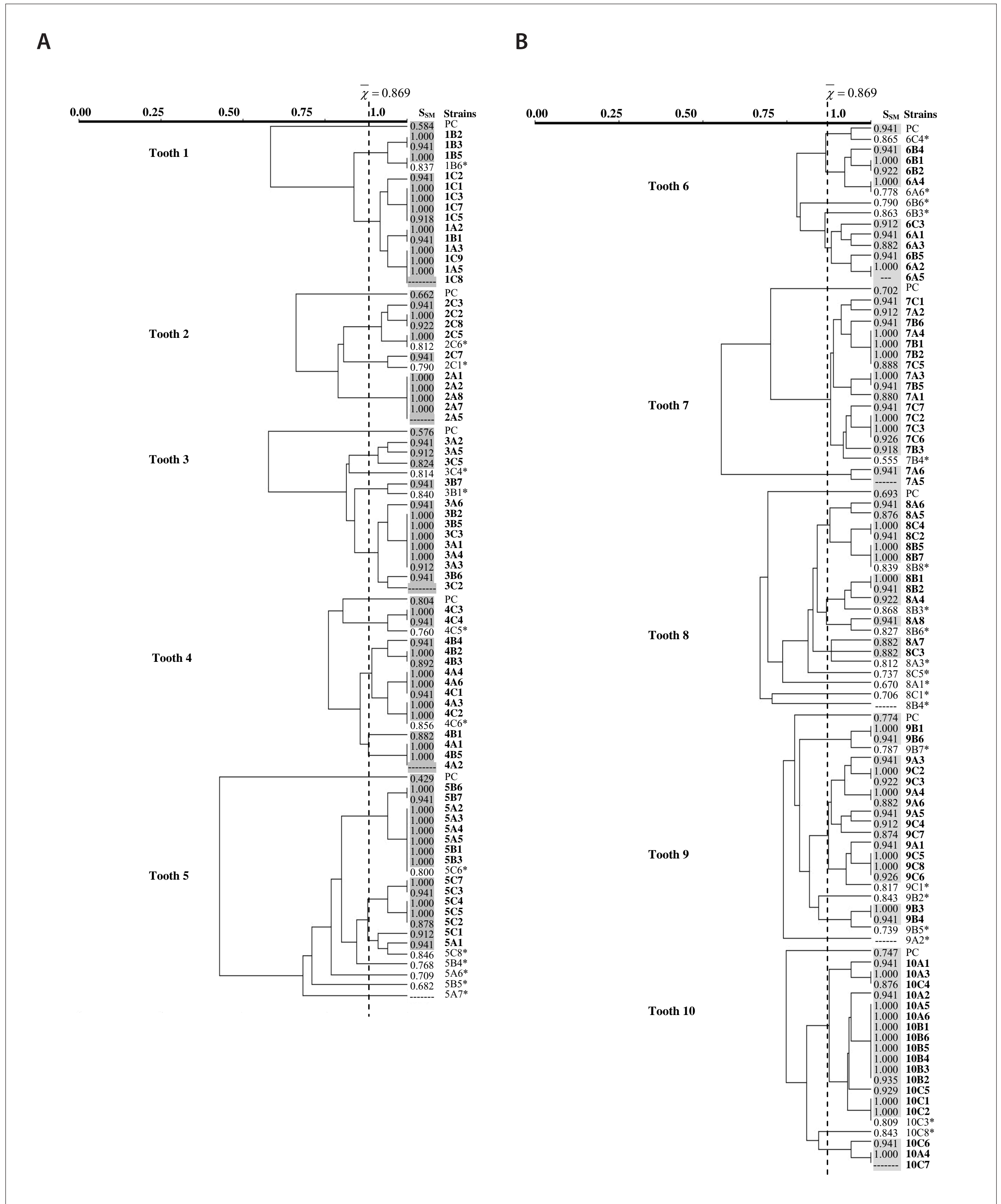

Figure 1. Genetic similarity indices (AP-PCR method, primer OPA-13) verified among *S. mutans* strains sampled from caries lesions of each teeth, submitted to indirect pulp treatment. A: dendrograms obtained to isolates of teeth 1 to 5. B: dendrograms obtained to isolates of teeth 6 to 10. Individual bands were analyzed by matrices generated by UPGMA analysis using coefficient SSM (simple matching). Tonalities of gray in the dendrograms illustrate identical or highly related isolates (S_{SM} ≥ 0,869) found in each tooth. * Different genotypic patterns obtained in each tooth (S_{SM} < 0,869).

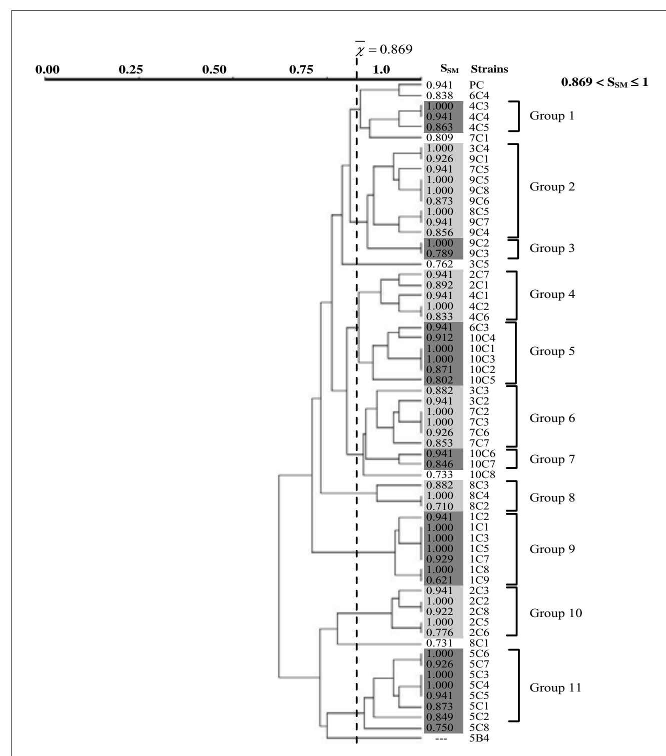

Figure 2. Genetic similarity indices (AP-PCR method, primer OPA-13) verified among *S. mutans* strains sampled from caries lesions three months after dental treatment – Collect C. Dendrogram generated from UPGMA analysis, using coefficient S_{SM} (simple matching). Different tonalities of gray in the dendrograms illustrate identical or highly related isolates ($S_{SM} \geq 0,869$).

rial species[10,16], obtaining similar results to more sophisticated techniques like MLEE[14].

In this study, genetic heterogeneity was verified among *S. mutans* strains isolated form caries lesions, even three months after incomplete caries removal. The persistence of some identical genotypes and high genetic similarity among isolates of the same tooth, in distinct collects, denoted resistance of some *S. mutans* strains to dental treatment. The polymorphism observed in different teeth may suggest that resistant strains are specific to each tooth, since a common genetic pattern among individuals was not found. However, further studies are necessary to evaluate the phenotypic characteristics of different *S. mutans* genotypes resistant to indirect pulp treatment or another incomplete caries removal technique, observing possible similarities in the production of enzymes that participate of several virulence mechanisms of this species.

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