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Biofilms of Black Tooth Stains: PCR Analysis Reveals Presence of *Streptococcus mutans*

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This study investigated the presence of the black-pigmented bacteria *Prevotella nigrescens* and *Prevotella intermedia*, the non-black-pigmented bacteria *Actinomyces* spp and particularly the cariogenic pathogen *Streptococcus mutans* in the dental biofilms of patients with or without black extrinsic tooth stains, using the multiplex polymerase chain reaction (PCR) technique. Analysis of the dental biofilms of patients with (n=26) or without (n=26) black tooth stains was performed using duplex PCR for the 16S ribosomal RNA gene (*P. nigrescens, P. intermedia, Actinomyces spp*) and glucosyltransferase-I gene for *S. mutans*. *P. nigrescens* and *S. mutans* were the most frequent bacteria detected in both groups. The least frequently detected were *P. intermedia* and *Actinomyces spp*. The similar bacterial composition of dental biofilms of black tooth stains and healthy tooth surfaces indicates that black tooth stains are not free of cariogenic bacteria.

Key Words: black tooth stain, dental caries, mutans *streptococci*, black-pigmented bacteria, PCR.

INTRODUCTION

Black extrinsic stains are biofilms formed on the smooth surfaces of teeth and are characterized by distinct dark spots comprised of insoluble iron salt and high concentrations of calcium and phosphate (1-4).

Black-pigmented bacteria (BPN) are present in the oral cavity (5) and are associated with black extrinsic tooth stains (6,7). Among the BPNs, *Prevotella intermedia* and *Prevotella nigrescens* are dependent on the heme portion of hemoglobin as a required iron source for bacterial growth (8). *P. nigrescens* has specific surface proteins to bind to hemoglobin (9) and *P. intermedia* degrades hemoglobin to release the heme portion to the surface of the hemoglobin molecule and subsequently sequester iron for its growth (10). *P. nigrescens* and *P. intermedia* degrade oxyhaemoglobin to form (Fe(III) $PPIX)_2O$ as an intermediate, which is converted into Fe(III)PPIX.OH by a depression in pH. The low pH encourages cell-surface deposition of insoluble Fe(III) PPIX.OH which would act as a barrier against oxygen and reactive oxygen species, and also protect against H_2O_2 through its inherent catalase activity (3).

The presence of black extrinsic tooth stains has been associated with low occurrence of caries in patients with these stains when compared to patients presenting healthy tooth surfaces (6,11,12), but this finding could not be confirmed by all authors (13,14). Reasons for these results are not clear but it has been speculated that they are related to the specific oral microflora described in black stain-affected individuals (15). However, in studies about the microbiota of black extrinsic tooth stains, the presence of *Streptococcus mutans*, which is one of the main pathogens associated with dental caries (16,17), has been little investigated.

The purpose of this study was to investigate the presence of the BPN *P. nigrescens* and *P. intermedia*, the non-black-pigmented bacteria *Actinomyces* spp, and particularly the cariogenic bacterium *S. mutans*, in the dental biofilms of patients with or without black extrinsic tooth stains, using the multiplex polymerase chain reaction (PCR) technique.

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MATERIAL AND METHODS

Sample Collection

This study involved 52 volunteer patients (25 females and 27 males) aged 9 to 50 years. The individuals were divided into two groups (n=26) according to the presence or absence of black extrinsic tooth stains (groups BS and NBS, respectively). The investigation protocol was approved by the Ethics Committee for Human and Animal Medical Research of the Clinics Hospital at the Federal University of Goiás, Brazil. The samples were collected after the patients signed the informed consent form. Using sterile curettes, samples of the biofilms were collected from the vestibular and lingual surfaces of the teeth of each patient and transferred to 1.5 mL plastic tubes containing 500 µL of saline solution. The samples were stored frozen at -20ºC.

Identification of the Bacteria by Multiplex PCR

Genomic DNA was extracted using the Illustra blood genomic Prep Mini Spin Kit (GE Healthcare UK Limited, Buckinghamshire, UK). The 16S ribosomal RNA gene was used to detect *P. nigrescens* (5'ATGAAACAAAGGTTTTCCGGTAAG3' 5'CCCACGTCTCTGTGGGGCTGCGA3'(804bp)) and *P. intermedia* (5'TTTGTTGGGGAGTAAAGCGGG3' and 5'TCAACATCTCTGTATCCTGCGT3' (575bp)) according to Tomazinho and Ávila-Campos (18). The target gene to detect *S. mutans* was the glucosyltransferase-I (5'ACTACACTTTCGGGTGGCTTGG3' and 5'CAGT ATAAGCGCCAGTTTCATC3'(517bp)) according to Franco and Franco et al. (19) and the 16S ribosomal RNA gene was used to detect *Actinomyces* spp 5'CTTAGCTTGCTAAGTATGCCGTTTAG3' and 5'CAGCTGACTTATACTCCCGAAATC3' (889bp) according to Saba et al. (7). Positive controls used in each experiment were the genomic DNA of *S. mutans*, *P. nigrescens* (ATCC 35406), *P. intermedia* (ATCC 25611) and *Actinomyces* spp (B19SC strain).

The multiplex PCR reaction was prepared with Invitrogen reagents (Invitrogen, Carlsbad, CA, USA): 2.5 µL PCR 10X buffer solution, 1.75 µL 50 mM magnesium chloride solution, 0.5 µL 10 mM dNTP (DNA Polymerization Mix), 1.0 µL each specific primer, 0.2 µL Taqpolymerase and 13.55 µL ultra-pure water. The total volume of each reaction was 25.0 µL (22.5 µL of the PCR solution and 2.5 µL of extracted DNA).

The following cycle was used: initial denaturation at 95°C for 5 min; and denaturation at 95°C for 30 s, annealing at 56°C for 1 min, extension at 72°C for 1 min (40 cycles), and extension at 72°C for 5 min. PCR products were then resolved on 2% agarose gel electrophoresis (Invitrogen), diluted in TBE buffer solution (tris-borate-EDTA buffer, pH=8.5). The electrophoresis was performed in a TBE buffer solution at 100 V, for 60 min. The gel was stained with 15 µL of GelRed™ (Biotium, Hayward, CA, USA) diluted in 100 mL 0.1 M NaCl, under shaking for 30 min and the gel was visualized in a photo documentation system (Bio Rad Laboratories, Hercules, CA, USA).

Statistical Analysis

The data are presented as absolute numbers and frequencies (%), which were compared between groups with Fisher's exact test, using GraphPad Prism software (GraphPad Inc., San Diego, CA, USA). The level of significance was set at $p<0.05$.

RESULTS

At least one of the analyzed bacteria was detected in 61.5% (16/26) of the samples collected from patients with or without black tooth stains. Table 1 presents the four bacteria detected in the two groups. In group BS, *P. nigrescens* was the most frequently detected bacterium (30.7%, 8/26). This frequency was similar (p=0.27) to that detected in the control patients (46.1%, 12/26). *P. intermedia* was detected in 3.8% (1/26) of the patients from the group BS, showing no significant difference (p=0.59) of that detected in the group NBS (11.5%, 3/26). Among the non-pigment-producing bacteria, *S. mutans* was similarly detected in the two groups (23.1%

Table 1. Comparison of the prevalence of bacteria detected between patients with (group BS) and without (group NBS) black extrinsic tooth stains.

Bacteria	Group BS (n=26)		Group NBS (n=26)		p
	n	%	n	%	
P. nigrescens	8	30.7	12	46.1	0.27
P. intermedia	1	3.8	3	11.5	0.59
S. mutans	6	23.0	8	30.7	0.72
Actinomyces spp	5	19.2	3	11.5	0.42

vs. 30.7%, p=0.72) as was *Actinomyces* spp (19.2% *vs.* 11.5%, p=0.42).

Considering only positive samples for presence of bacteria (16 samples in each group), among the bacteria detected in group BS, *P. nigrescens* was the most frequent (50.0%, 8/16) while *P. intermedia* was detected in only one sample (6.2%, 1/16). The non-pigmented bacteria *Actinomyces* spp and *S. mutans* were detected in five and six samples, respectively (31.2% and 37.5%, n=16, Fig. 1A). Similar results were detected in the patients who did not exhibit black extrinsic stains, with *P. nigrescens* being the most frequently detected pigmented bacterium (75.0%, 12/16), while *P. intermedia* was the least detected (18.7%, 3/16). The non-pigmented bacteria *Actinomyces* spp and *S. mutans* were detected in 3 and 8 samples, respectively (18.7% and 50.0%, n=16, Fig. 1B).

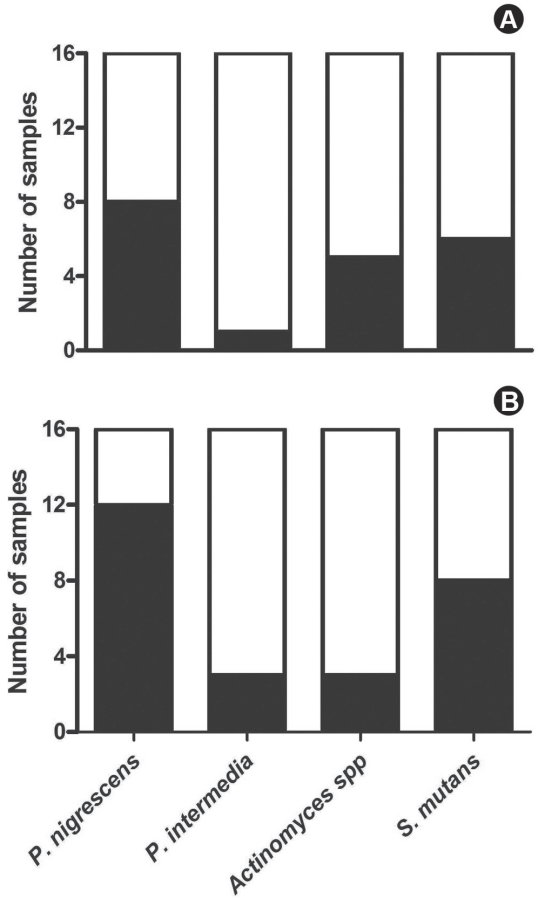

Figure 1. Bacteria-positive samples of patients with (A) or without (B) black tooth extrinsic stains were identified by the multiplex PCR technique. The data represent the number of samples analyzed (n=16) and the number of samples testing positive (filled columns) or negative (empty columns) for the above-mentioned bacteria.

DISCUSSION

This study showed that at least four bacteria can be detected in dental biofilms of black tooth stains, including black-pigmented and non-black pigmented cariogenic bacteria. The four analyzed bacteria (*P. nigrescens*, *P. intermedia*, *S. mutans* and *Actinomyces* spp) were detected in a similar prevalence in samples from patients with or without black tooth stains, suggesting a similar bacterial colonization of dental biofilms of these patients. According to the literature (5,20,21), among the 13 species of *Prevotella*, *P. nigrescens* and *P. intermedia* are the ones most frequently found in the oral cavity. *P. nigrescens* is indigenous microbiota of oral cavity whereas *P. intermedia* is related to periodontal disease. The high prevalence of *P. nigrescens* in the samples of group BS (30.7%) and group NBS (46.1%) is consistent with indigenous microbiota, confirming the prevalence of *P. nigrescens* over *P. intermedia. Actinomyces* spp non-pigmented bacterium was present in similar frequencies in groups BS and NBS (19.2% *vs.* 11.5%, respectively). These results do not support those reported by Saba et al. (7), who found that individuals with *Actinomyces* spp have a four-fold higher probability of presenting black stains than do individuals without these bacteria.

A similar prevalence of *S. mutans* was detected in the two groups (23.0% *vs* 30.7%). The high frequency of *S. mutans* found in samples of group BS differs from the results of Slots (6), who found that S. *mutans* was only detected in 5% of samples of biofilm from black stains. Slots (6) detected a correlation between the low number of *S. mutans* and the low rate of caries associated to the presence of black stains. However, Reid and Beeley (11) suggested that the reduction of the incidence of caries in individuals with black extrinsic tooth stains is due to the calcium and phosphate content of the biofilm of the stain. Analyzing the biofilm of black extrinsic tooth stains, some authors found a positive correlation between the presence of stains and the low incidence of caries (2,12). However, other studies (13,14) did not find such a correlation. The discordant findings can be related to the multifactorial nature of caries disease and the hypothesis that the positive correlation between the presence of black stains and the low incidence of caries is a simplistic and unifactorial evaluation. In a review of the fundamentals about black stains, its diagnosis and possible differential diagnoses, microbiology and therapy, Ronay and Attin (15) concluded that the reasons why the results are not clear about the black

stains are related to the specific oral microflora of these stains. Another study (22) reinforced that chlorhexidine solution could cause some stains on teeth surface and confirms the great potential of using copaiba oil and chlorhexidine solution against the growth of *S. mutans*. The present study demonstrated that black pigmented bacteria (*P. nigrescens* and *P. intermedia*) as well as cariogenic non-black pigmented bacteria (*S. mutans* and *Actinomyces* spp) can be detected in dental biofilms of patients carrying black tooth stains or not. The presence of *S. mutans* in the biofilm of black extrinsic stains confirms that the oral microbiota of these patients is not free of cariogenic bacteria. These findings indicate that analyzing the presence of different bacteria in dental biofilms is only the first step to understand the etiology of black tooth stains and its relation to caries status; other factors related to diet, hygiene techniques, caries status, and even other microorganisms in the oral cavity should also be examined.

RESUMO

O objetivo deste estudo foi investigar a presença das bactérias pigmentadoras de negro *Prevotella nigrescens* e *Prevotella intermedia*, da não pigmentadora de negro *Actinomyces* spp e particularmente a bactéria cariogênica *Streptococcus mutans,* no biofilme dentário de pacientes com ou sem manchas dentárias extrínsecas negras, utilizando a técnica multiplex PCR (reação em cadeia da polimerase). Análises do biofilme dentário de pacientes com manchas (n=26) e sem manchas (n=26) foram realizadas utilizando a multiplex PCR para o gene 16S RNA ribosomal (*P. nigrescens, P. intermedia, Actinomyces spp*) e o gene glucosiltransferase-I para *S. mutans. P. nigrescens* e *S. mutans* foram as bactérias mais frequentemente detectadas em ambos os grupos. As menos frequentemente detectadas foram *P. intermedia* e *Actinomyces spp*. A similaridade entre a composição bacteriana dos biofilmes dentários das manchas dentárias extrínsecas negras e das superfícies dentárias sem manchas indicam que as manchas dentárias extrínsecas negras não estão livres de bactérias cariogênicas.

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