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DNA profiling of *Streptococcus mutans* in children with and without black tooth stains: A polymerase chain reaction analysis

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ABSTRACT

Background: The purpose of this study was to identify the genetic patterns of *Streptococcus mutans* by DNA finger printing among caries-active, caries-free, and in children with black tooth stains.

Materials and Methods: In this in vitro study Ten children with black tooth stains and ten caries-free and caries-active children between 3 and 6 years of age were recruited. Saliva samples were collected using a sterile tube, followed by microbial culture of *S. mutans*, DNA isolation, and polymerase chain reaction amplification. The molecular weights of each band were converted into binary data, and data were entered into SPSS to generate similarity dendrograms.

Results: Dendrogram interpretation of black tooth stain and caries-free samples revealed an identical genetic pattern in 15 samples with high genetic similarity. Dendrogram interpretation of black tooth stain and caries-active samples exhibited a greater genetic diversity.

Conclusion: This study concludes that identical genotypes and high genetic similarity among isolates of the caries-free and black tooth stains. Polymorphism and genetic diversity exist between the black tooth stain and caries-active samples.

Key Words: DNA profiling, stains, *Streptococcus mutans*

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INTRODUCTION

Stains in the teeth are a frequent dental finding, and these strains differ in etiology, appearance, location, composition, and degree of severity and adherence.[1] Black stains (BSs) are formed on the smooth surfaces of teeth parallel to the gingiva and it is characterized by black dots, lines with incomplete coalescence of dots, or continuous lines.[2] These BS formations are repetitive even after an efficient tooth brushing or prophylaxis.[3] Several studies had elucidated the etiology of BSs.[4-7] Dietary chromogens found in beverages and tea, iron supplements, and the usage of chlorhexidine mouth rinses are considered as an etiological agent due to absorption of these agents onto the tooth surface through plaque or the acquired pellicle.[4,5]

Studies of chemical composition of saliva demonstrated that lower glucose and higher calcium and phosphate content was present in the saliva of people with BSs.[6] Higher calcium levels and buffering capacity of saliva were also considered as an

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important factor for low caries experience in children with BSs than in children without BSs.[7] Few research has addressed the association between the dietary iron intake and development of BSs. Pushpanjali *et al*. documented a positive correlation between black extrinsic stains and the iron concentration in water sources.[8] Garcia Martin *et al*. studied the relationship between iron supplements and the development of BS among 5-year-old Spanish children and the findings suggest a positive significant relationship exist in children consuming iron and their mother during pregnancy.[9]

Studies have reported that the presence of these BS is associated with low level of caries experience[2,10,11] and França-Pinto *et al*. suggested that these BSs are protective indicators against dental caries.[12] Theilade *et al*. and Saba *et al*. had reported that the reduction of caries prevalence in individuals with BSs was due to the presence of low cariogenic microbiota.[13,14]

Since *Streptococcus mutans* is a main culprit in the development of dental caries, there still remains a controversy regarding the activity of *S. mutans* in children with BSs. Akyuz *et al*. had reported that there was no relation between black tooth stains and caries activity,[15] and few studies positively support the low caries prevalence in children with BSs.[10,11,16] Since there is no complete absence of *S. mutans* in saliva or plaque of children with BSs,[7,17] it is imperative to elicit the reason for their low caries activity in children with these BSs. Studies have suggested that the bacteria associated with the BSs could establish a competitive environment for bacteria related to caries development and prevent the adhesion of these bacteria to dental surfaces, thereby reducing the potential for caries lesion.[13,14]

Due to the existence of several conflicting results regarding the presence of BSs and its association with low caries activity, there is a need to identify the genetic pattern of the *S. mutans*. Hence, the purpose of this research was to determine the genetic patterns of *S. mutans* by DNA finger printing to elicit the genetic pattern of *S. mutans* among caries-active, caries-free, and in children with BSs.

MATERIALS AND METHODS

Subject recruitment

In this *in vitro* study The protocol received ethical approval from the Institutional Review Board of Saveetha Dental College, Chennai (SRB/STPG-4a). The parents were given a clear explanation about the objective of the study, and informed consent was obtained along with the child's approval to participate. The subject recruitment began in June 2016 and ended in December 2016. Children between 3 and 6 years of age, who visited the Department of Pedodontics and Preventive Dentistry during this time period, were screened and recruited for the study. A total of 1161 children were screened and 10 children were diagnosed with BSs during the study period. On recruitment of each child with BSs, one caries-free and one caries-active child was recruited in the same week.

Inclusion criteria

- Children between 3 and 6 years of age
- Children with no systemic illness
- No intake of antibiotics for past 3 months
- No intake of iron supplements for past 6 months.

Intraoral examination

Intraoral examination was carried out by a single, qualified pediatric dentist. The oral screening was done using an artificial light and a nonmagnifying sterilized mouth mirror and probe. Dental caries was examined using the WHO criteria and scored according to decayed, missed, and filled teeth (dmft) index.[18] In caries-active children, the dmft score should be >1, and in caries-free children, the dmft score should be 0. Clinical diagnosis for BS was done based on the presence of dark adherent pigmented lines parallel to the gingival margin or covering up to one-third or more of the clinical tooth crown.[10] Intraexaminer reliability was assessed for both BS evaluation and dmft index. High reproducibility was observed and the Cohen's kappa statics value was >0.8.

Saliva collection

Unstimulated whole saliva was collected in a sterile tube. The participants were instructed not to eat anything 45 min before saliva collection. Saliva was collected based on the technique explained by Wu *et al*.[19] The child was seated in a well-ventilated and in a calm atmosphere. The head was kept at 45° flexion with one hand holding the sterile tube for 2 min. The saliva was allowed to drip into the sterile tube held to the lower lip to the required marking without measuring froth and was asked to provide a 5 ml sample.

Streptococcus mutans cultivation and isolation

The samples were transferred to the Microbiology Laboratory of the Saveetha Dental college

immediately for bacterial culture. The samples were diluted in 1:40 and 10 µl of the sample was transferred to Mitis-sanguis agar (Difco). The samples were spread evenly all over the plate and incubated at 37°C for 48 h in an atmosphere of 10% CO_2. Isolation of *S. mutans* was done by observing its colony morphology and the number of colony-forming units in each plate. The tests were done in triplicate to minimize the test error.

Bacterial genomic DNA Isolation of *Streptococcus mutans* and purification

Genomic DNA preparation, isolation, and purification were done in Biozone Research Technology laboratory according to the technique described by Chassy and Giuffrida.[20] A volume of 1.5 ml of bacterial culture was transferred to a microcentrifuge tube and spun at 10,000 rpm for 2 min at 4°C. The supernatant was decanted and the pellet was resuspended in 467 µl of Tris-EDTA (TE) buffer by repeated pipetting. A volume of 30 µl of 10% sodium lauryl sulfate and 3 µl of 20 mg/ml of proteinase K were added to the sample and incubated for 1 h at 37°C. Equal volumes of phenol: chloroform (24:1) were added and mixed gently by inverting the tubes until the phase was completely mixed. The tubes were spun at 12,000 rpm for 10 min at 4°C. The cellular RNA was removed. The upper aqueous layer was transferred to a new tube and an equal volume of chloroform was added. The samples were mixed by gently inverting the tubes and spun at 12,000 rpm for 10 min at 4°C. The upper aqueous phase was transferred to a new tube and one-tenth volume of 3M sodium acetate was added. Double the volume of 95% ice cold ethanol was added and mixed by inversion until the DNA was precipitated. The tube was spun for 10 min at 12,000 rpm at 4°C and the supernatant was discarded. The pellet was washed with 0.2 ml of 70% ethanol and tube was spun as before. Nearly 70% ethanol was discarded and the pellet was air dried. The DNA was then suspended in TE buffer and run on 0.8% agarose gel to confirm the quality of DNA by agarose gel electrophoresis. Purification of DNA was calculated by the ratio of absorbance at 260 and 280 nm ultraviolet light and the value was found to be 1.8.

Polymerase chain reaction amplification

DNA from the isolates of *S. mutans* was proceeded for polymerase chain reaction (PCR) amplification using primer OPA 13 (Operon Technologies Inc., California) as described in previous studies.[21-23] The sequence of OPA 13 primer is 5'-CAGTATAAGCGCCAAGTTTCATC3'. The PCR amplification was carried out using an Eppendorf Mastercycler Personnel Thermal Cycler (Mastercycler® nexus gradient) using the following program: initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 45 s, annealing at 59°C for 1 min, followed by extension at 72°C for 1 min, and final extension at 72°C for 7 min. The PCR amplified products were separated on 2% agarose gels and visualized under ultraviolet light illumination by staining with 0.5 µg of ethidium bromide/ml. A 1Kb DNA ladder was loaded along with the samples as a molecular marker. The PCR amplification was also confirmed using arbitrary primer OPN6. The gels were picturized using a digital imaging system (Kodak Digital Science). The molecular weights of each band were converted into binary data and data were entered into SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA) to generate similarity dendrograms.

RESULTS

A total of thirty saliva samples were collected from all enrolled participants. All samples identified *S. mutans* in their oral cavities by microbial sampling. All the isolated strains of *S. mutans* were selected for DNA isolation and PCR analysis

The amplification of genomic DNA of *S. mutans* resulted in electrophoretic bands in 1 Kb in size. The arbitrarily primed-PCR fingerprinting profile analysis with primer OPA 13 showed distinct genotypes patterns of *S. mutans* obtained from all the thirty saliva samples. A qualitative estimation of testing *S. mutans* DNA with a reference strain by electrophoresis was depicted in Figure 1a and b.

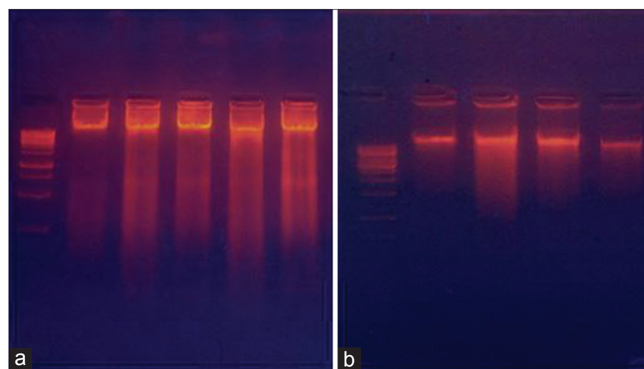

Figure 1: (a and b) Qualitative estimation of DNA by gel electrophoresis.

Figure 2a and b demonstrates the overall fingerprints of PCR amplification with primer OPA 13 and random primer OPN6. The 1 kb ladder markers are in base pairs and the marked size amplicons indicate a distinct characteristic of *S. mutans*. Based on the similarity matrices generated by the dendrogram analysis, the genetic similarity levels obtained between the *S. mutans* in black tooth stain and caries-free individuals and illustrated in Figure 3. This illustrates the similarity of *S. mutans* strains, which shows representative dendrograms of *S. mutans* isolates obtained from the caries-free and black tooth stains. Identical isolates were found in 15 samples.

Figure 4 interprets the genetic polymorphism in DNA patterns of *S. mutan*s between black tooth stain and caries-active children. There exists a genetic polymorphism, where the *S. mutans* of caries-active children exhibited more clonal or genotypes types than the children with black tooth stains.

DISCUSSION

The purpose of the study was to determine the genetic pattern and their similarity matrix of *S. mutans* in children with and without BSs. This is one of the first studies that addressed the investigation of genetic patterns of *S. mutans* in children with BS and compared it with caries-free and caries-active children. The BSs have been considered to be a special form of dental plaque that differs from other types of plaque because of its composition.[24] This dental biofilm should have an ability to compete with other strains of *S. mutans* for its colonization. Since there exists conflicting results regarding the prevalence of caries in children with BSs, this study aimed to determine whether *S. mutans* present in the BS individuals represent any genetic similarity with the caries-active and caries-free individuals.

The system we used to detect *S. mutans* in unstimulated saliva by means of a polymerase chain reaction has been used in other studies and has shown itself to be valid and reliable.[25] Since studies have suggested that both plaque and saliva samples can be used successfully for DNA isolation,[26-28] in the present study, saliva was used for DNA isolation.[27]

A similar pattern of *S. mutans* was evident between the samples of caries-free and black tooth stains and only five strains exhibit genetic diversity [Figure 4]. The presence of this identical genetic pattern of *S. mutans* in the BSs and in the caries-free individuals confirm that the oral microbiota of these patients is sharing a similar genetic pattern. This could be one of the reasons for the lack of caries development in children with BS. Costa *et al*. investigated the biofilms in patients' black tooth stains and compared with non-BS individuals by PCR analysis in a group of Brazilian individuals. The author had concluded that the black tooth stains are not free of *S. mutans*[17]

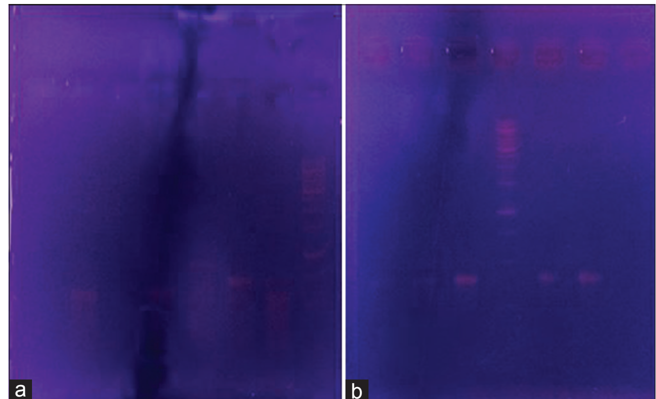

Figure 2: (a) DNA finger patterns of *Streptococcus mutans* strains obtained with primer OPA 13. (b) DNA finger patterns of *Streptococcus mutans* strains obtained with primer OPN 6.

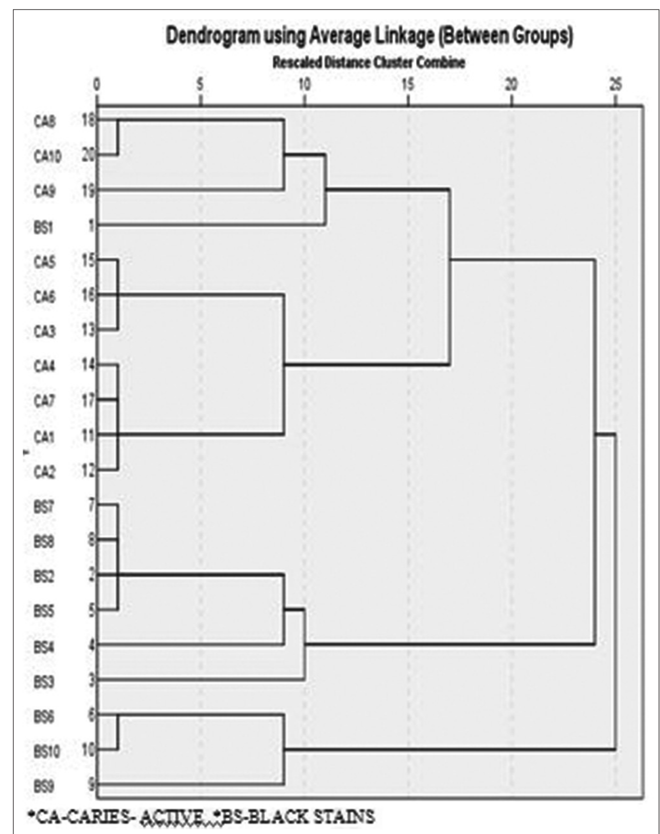

Figure 3: Genetic similarity (polymerase chain reaction method, primer OPA 13) among *Streptococcus mutans* in caries-active children and in children with black stains.

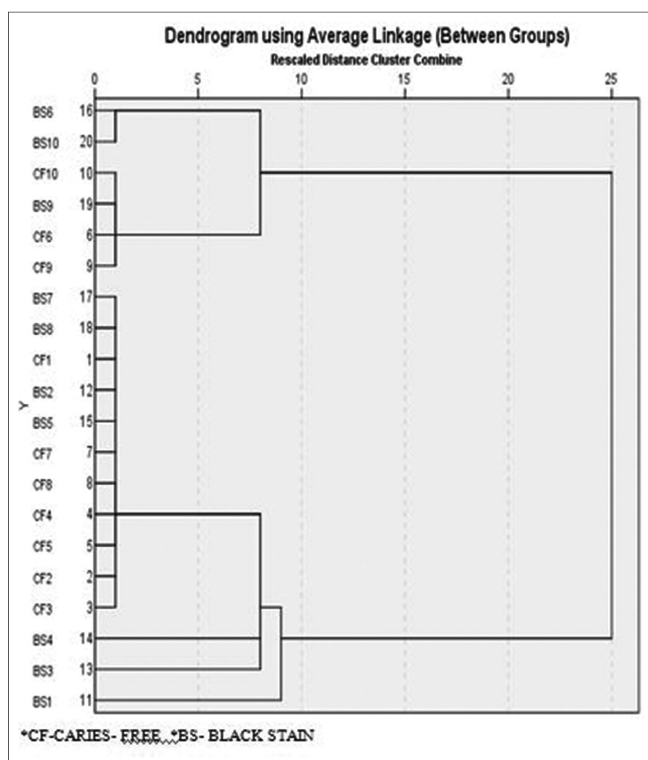

Figure 4: Genetic similarity (polymerase chain reaction method, primer OPA 13) verified among *Streptococcus mutans* in caries-free children and in children with black stains.

and this result is in agreement with the current study, where the results of the current study revealed the presence of *S. mutans* in black tooth stain. This study supports the findings of França-Pinto *et al.*, who suggested that these BSs are protective indicators against dental caries.[12]

Greater diversity exists in the *S. mutans* genetic pattern between the caries-active and black tooth stains. This finding is contradictory to the prevalence study done by Akyuz *et al.*, who had stated that there was no association between the BSs and low caries prevalence.[15] The current study strongly suggests that there exists a greater diversity in the *S. mutans* between the caries-active and black tooth stains. The knowledge of this genotypic diversity of *S. mutans* in BSs and caries-active individuals may help in the development of new treatment strategies for the prevention of these BSs.

The results of this study also suggest that caries-susceptible children bear more genotypes than caries-free children. This is in accordance with the earlier reports published by Napimoga *et al.*, Alaluusua *et al.*, and Redmo Emanuelsson and Wang.[22,29,30] Since studies have stated that *S. mutans* along with *Streptococcus sobrinus* increases the risk of caries development,[31,32] in the present study, we had isolated only *S. mutans* strains for PCR analysis. This may be a limitation of this study, where the isolation of *S. Sobrinus* would have added further diagnostic values.

The presence of identical genotypes and high genetic similarity among isolates of the caries-free and black tooth stains indicates that these *S. mutans* are sharing same genetic characteristics with low cariogenicity. The polymorphism observed between the black tooth stain and caries-active samples may suggest a genetic diversity exist in these samples. However, further studies are necessary to evaluate the phenotypic characteristics of different *S. mutans* genotypes to observe possible similarities.

CONCLUSION

With the light of the available evidence, this study concludes that:

- Identical genotypes and high genetic similarity among isolates of the caries-free and black tooth stains
- Polymorphism and genetic diversity exist between the black tooth stain and caries-active samples.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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