Journal of Advanced Clinical & Research Insights (2018), 1, 59–64

ORIGINAL ARTICLE

Association of dental caries in children with black stain and non-discolored dental plaque: A microbiological study

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Keywords:
Black stain, dental caries, non-discolored plaque

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Received 02 March 2018;
Accepted 04 April 2018

doi: 10.15713/ins.jcri.212

Abstract

Background: Dental caries is one of the most common oral diseases. Low caries has been observed in children with the presence of black stain (BS). Studies have shown that the interaction between microbiota related to extrinsic stain, cariogenic pathogens, and caries remains obscure. Very few studies have shown the prevalence rate of BS and dental caries among Indian population. Thus, the aim of the study is to estimate and compare the composition of bacteria in plaque with BS compared to the non-discolored plaque and to compare the caries experience in children with and without stains in plaque.
Materials and Methods: A total of 30 healthy children with non-discolored plaque and 30 children with BS aged between 3 and 11 years were included in the study.
Results: BS was present in 7–11 years age group. Children with BS revealed a lower overall caries experience than the children with non-discolored plaque with this correlation microbial culture showed significant lower number of cariogenic organisms *Streptococcus mutans* and *Lactobacillus* species in the BS as compared to non-discolored plaque. Higher number of *Actinomyces naeslundii, Aggregatibacter actinomycetemcomitans,* and *Fusobacterium nucleatum* indicating the good correlation between BS plaque and low caries index in Indian population.
Conclusion: The caries prevalence and microorganisms differ in BS and non-discolored plaque in children.

Introduction

Dental caries is polarizing on a worldwide basis, and the prevalence of caries is increasing in an alarming rate in developed countries.[1] The WHO records a global decay-missing-filled teeth (DMFT) of 1.61 for 12 years old in 2004, a reduction of 0.13 as compared to a DMFT of 1.74 in 2001. In India, data from the National Oral Health Survey (2002–2003) state that in children aged 12 years, the caries prevalence is 53.8% and the mean DMFT is 1.8.[2] The data suggested that dental caries is the single most common chronic childhood disease.

Although effective methods are known for prevention and management of the disease, the unmet need for treatment, especially in children, does not seem to be diminishing. Thus, the investigations of potential caries protective factors are an essential key to reduce dental caries inequalities.

"Caries-resistant" persons develop very few caries lesions in spite of exposure to cariogenic diet not because the plaque as less cariogenic microorganisms but probably due to maintenance of neutral pH as hypothesized by PD Marsh.[3] Microbiologic investigations of this sort of dental plaque might further elucidate the relationship between different bacteria and dental caries. Similarly, it has been observed that person with black stain (BS) shows decrease in incidence of dental caries.[4]

BS is a extrinsic tooth discoloration of non-metallic origin.[5] The etiology for BS may be due to dietary chromogens found in tea, coffee, and other beverages, also produced by tobacco, iron supplements, and chlorhexidine mouth rinses which contains medicines and some metal salts. Apart from this industrial exposure to iron, manganese and silver can also produce extrinsic stains on tooth surface.[6] BS is clinically diagnosed as pigmented, dark lines parallel to the gingival margin or as incomplete coalescence of dark dots rarely extending beyond the cervical third of the crown of the tooth and can be easily distinguished from intrinsic stains.[7]

The components of BS are insoluble ferric salt, probably ferrous sulfide, with a high content of calcium and phosphate.

The saliva of BS children consists of higher content of calcium, inorganic phosphates, copper, sodium, and total protein but less glucose compared to children without staining.[7]

The microorganism examination of BS reveals Gram-positive rods like *Actinomycetes* as the predominant microorganisms and some pigmented Gram-negative rods.[7] However, the correlation between the microorganisms and the BS is not completely understood. There are many studies which investigated the presence of BS and its potential caries protective effect and concluded that the presence of BS and its association with lower level of caries.[8-11]

Studies have shown that BS and dental caries being influenced by demographic, social, and behavioral factors.[12] There is only one study which has investigated the relationship between the BS and its microbiota in prevention of dental caries and such studies among the Indian population are lacking.[8] Hence, the present study aimed to fill the lacunae of caries experience and microbiota in children with BS and non-discolored plaque among Indian population.

The study was designed as follows: (1) To investigate microbiota in children with and without BS, (2) to compare the microbiota in children with and without BS, and (3) to compare the caries index and microbiota in children with and without BS.

Materials and Methods

Subject selection

The study was commenced after obtaining approval from the institutional review board and ethical committee (Reference No 131).

Children of age 3–11 years were selected from the schools of Belagavi, Karnataka, India. We considered schoolchildren of Belagavi because we get diverse population of Indian community and will have mixed population from all over India. The school selected for the study was those who teach Central Board of Secondary Educational syllabus. These schools will contain children from all over India who are currently residing in Belagavi. Their parents or caretakers were interviewed whether the children may participate in the clinical and microbiological study to determine the microbiota in plaque samples. An informed consent was obtained from the parents or the caretakers of the participants in the study. The children involved in the study were systemically healthy, and they had not undergone any antibiotic treatment within 6 months before the onset of the study. Children under treatment of iron medicine were excluded from the study. There was no diet restriction for the children who were participating in the study.

The BS group and non-discolored group were compared for the dental caries status by dmft, and dmfs. The children having dmft as zero (caries free) were segregated from those having more than 1 dmft (caries prone).

Sample collection

Following aseptic procedure, the BS or non-discolored plaque was collected from the buccal or lingual surfaces of the primary molars and incisors by scraping with sterile metal scalers. The samples were transferred on reduced transport fluid and samples were subjected for microbiological analysis.

The organisms selected for the culture were based on caries invasion and caries progression. *Streptococcus mutans* are the early invaders for caries while *Lactobacillus* helps for caries progression.[13] *Actinomyces naeslundii* is a Gram-positive organism and is associated with good oral health.[14] *Aggregatibacter actinomycetemcomitans* are associated with BS production.[7] *F. nucleatum* is the bridging organism between early and late colonizers, and it also helps in caries formation.[14] *Porphyromonas gingivalis* and *Prevotella intermedia* are species of *Bacteroides* family. These are chromogenic bacteria and etiological factor for the stain (pigment) production.[15] Thus, based on these criteria, we selected *S. mutans, Lactobacillus, A. naeslundii, A. actinomycetemcomitans, F. nucleatum, P. gingivalis*, and *P. intermedia* organisms for culture.

The selected culture media for the organisms were Mitis Salivaris Agar (MSA) for *S. mutans*, Rogosa for *Lactobacillus*, nalidixic acid colistin blood agar (NACB) for *A. naeslundii,* dentoide for *A. actinomycetemcomitans*, crystal violet erythromycin for *F. nucleatum*, Kanamycin blood agar for *P. gingivalis*, and Kanamycin blood agar for *P. intermedia*. The organisms were incubated for 48–72 h. These organisms were confirmed based on their colony morphology. *S. mutans* produces change in color to dark blue mucoid colonies on MSA media and identified based on colony morphology. *Lactobacilli* produce white mucoid colonies on Rogosa media. *F. nucleatum* produces smooth violet color colonies crystal violet erythromycin media. NACB media is for Gram-positive organisms and it inhibits Gram-negative organisms. *A. naeslundii* produces brown moist colonies. *P. gingivalis* produces brick red, and older colonies develop black to brown pigment. *P. intermedia* produces pinpoint black colonies.[16,17] Further, confirmation was done through preliminary biochemical analysis indole catalase test, nitrate reduction test, and sugar fermentation test was done for *Lactobacillus*, *A. naeslundii, A. actinomycetemcomitans, F. nucleatum, P. gingivalis,* and *P. intermedia.*

Biochemical analysis[16,17]

a. *A. naeslundii* - positive nitrate reductase test, turns to pink,
b. *A. actinomycetemcomitans* - positive catalase test, nitrate reductase test positive, turned to pink color,
c. *F. nucleatum* - positive indole test, violet color to green,
d. *P gingivalis* - positive indole test, black color to green,
e. *P. intermedia* - glucose fermentation test positive,[18] violet color to yellow color,
f. *Lactobacillus* sp. - positive catalase test, produces effervescence, positive nitrate reductase test, turned to pink color observed.

Results

The percentage of microbial analysis of the organisms and the descriptive analysis of the colony count of the BS plaque in

comparison with the organisms with the non-discolored plaque is depicted in Tables 1 and 2.

The range of age of children in this study was between 7 and 11 years. Children with BS showed lower caries experience than the children with non-discolored plaque and the difference was significant (dft and dfs $P < 0.001$) [Table 3].

The microbial analysis of the plaque samples of BS compared to non-discolored plaque showed higher number of *A. naeslundii, A. actinomycetemcomitans*, and *F. nucleatum,* but these differences were not statistically significant. In contrast, there was significantly lower number of *S. mutans* and *Lactobacillus* in BS group compared to non-discolored plaque samples [Table 4].

Statistically significant difference was not observed when caries-free children BS and non-discolored plaque samples when analyzed separately for bacterial colony count [Table 5].

Discussion

This study was conducted to investigate the caries experience and microbiota in children with BS and non-discolored plaque among Indian population. In the present study, samples were collected from same age group children, so as to exclude the influence of age on dental caries. Permanent teeth were not included in our study because of their short exposure time to the oral environment, and the time from eruption to the onset of caries[6] is very less in the age group considered for the present study.

The caries experience in children with BS and non-discolored plaque was significantly lower in BS plaque group and non-discolored plaque group [Table 3]. These results were in concordance with the other studies.[8,9,11,12] Thus, the caries

Table 1: The microbial analysis of the organisms of the black stain plaque in comparison with the organisms of the non-discolored plaque

Organisms	Present in BS plaque	% 30	Present in non-discolored plaque	% 30
A. naeslundii	22	0.73%	17	0.56%
P. gingivalis	4	0.13%	3	0.1%
S. mutans	21	0.7%	28	0.93%
Lactobacillus	NG	0	6	0.2%
A. actinomycetemcomitans	13	0.43%	9	0.3%
F. nucleatum	25	0.83%	20	0.66%
P. intermedia	2	0.06%	NG	0

Each sample of plaque consists of multiple microorganisms. Hence, the numbers do not add to 100%. *A. naeslundii: Actinomyces naeslundii, P. gingivalis: Porphyromonas gingivalis, S. mutans: Streptococcus mutans, A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans, F. nucleatum: Fusobacterium nucleatum, P. intermedia: Prevotella intermedia,* BS: Black stain

Table 2: The comparison of maximum and minimum colony count of the organisms in BS plaque group and non-discolored plaque group

Group	Organisms	N	Minimum	Maximum	Mean±SD
Study group	*A. naeslundii*	30	0.00	60000.00	10500.0000±16323.77154
	P. gingivalis	30	0.00	30000.00	1833.3333±6136.84932
	S. mutans	30	0.00	100000.0	18433.3333±25702.11763
	Lactobacillus	30	0.00	0.00	0.0000±0.00000
	A. actinomycetemcomitans	29	0.00	115000.0	12965.5172±23261.68333
	F. nucleatum	30	0.00	50000.00	12600.0000±14537.94085
	P. intermedia	30	0.00	35000.00	1166.6667±6390.09650
	Valid *N* (listwise)	29			
Control Group	*A. naeslundii*	30	0.00	80000.00	15700.0000±21001.06730
	P. gingivalis	27	0.00	0.00	0.0000±0.00000
	S. mutans	30	0.00	150000.0	43466.6667±42396.10696
	Lactobacillus	24	0.00	0.00	0.0000±0.00000
	A. actinomycetemcomitans	30	0.00	30000.00	5800.0000±9749.97790
	F. nucleatum	30	0.00	40000.00	12500.0000±13418.33537
	P. intermedia	30	0.00	2000.00	66.6667±365.14837
	Valid *N* (listwise)	22			

A. naeslundii: Actinomyces naeslundii, P. gingivalis: Porphyromonas gingivalis, S. mutans: Streptococcus mutans, A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans, F. nucleatum: Fusobacterium nucleatum, P. intermedia: Prevotella intermedia, BS: Black stain, SD: Standard deviation

prevalence and experience differ in children with BS and non-discolored plaque could be confirmed for caries experience in primary teeth.

The microbial composition findings demonstrate difference in individual species between the BS plaque and non-discolored plaque. Studies have shown that there is higher number of Gram-positive rods, especially *Actinomyces* species and lower number of *Streptococci*.[7] Similar to this, the present study has shown significant lower number of cariogenic organisms *S. mutans* and *Lactobacillus* species in the BS as compared to non-discolored plaque, higher number of *A. naeslundii* and *A. actinomycetemcomitans* though the difference was not statistically significant [Table 4].

S. mutans is a cariogenic organism; it has ability to ferment even if there is the absence of food substance. *Lactobacillus* helps in caries progression.[13,19] The present study got lower number of *S. mutans* and *Lactobacillus* which is in good agreement with caries reduction in BS children involved when compared to non-discolored plaque. *A. naeslundii* is a Gram-positive organism, and it is the first bacteria to occupy the oral cavity and colonize the tooth surface and it is associated with good oral health.[14] Increase in *A. naeslundii* organism indicates maintenance of good oral health. Hence, *A. naeslundii* and *S. mutans* adhesion shows less number of caries which is in good agreement with other studies.[7] As *A. actinomycetemcomitans* is a periodontopathogenic organism and the colony count was high in BS plaque, these children who were positive for the organisms may in future suffer for periodontal problems. *Actinomyces* belong to the resident oral microbiota of supragingival plaque.[14] Some *Actinomyces* strains produce hydrogen sulfide, which can result in ferric sulfide formation in the presence of iron in saliva or gingival exudates.[5]

Actinomyces species is a periodontopathogenic organism[7,14] and helps in adhesion of periodontopathogenic organism. Hence, it helps in aggregation of *P. gingivalis* and *Porphyromonas sp.* These organisms help in pigment production. Studies have shown *Prevotella melaninogenica*, *P. gingivalis,* and *P. intermedia* as pigment-producing organisms in BS plaque.[15] We observed

Table 3: Comparison of different components of dft index in study and control group by independent samples *t*-test

	Group	*N*	Mean±SD	*P* value and significance	Mean difference	95% confidence interval for difference	
						Upper bound	Lower bound
dt	Study group	30	0.70±1.09	<0.001	−2.90	−4.09	−1.71
	Control group	30	3.60±3.07	Significant			
ft	Study group	30	0.10±0.40	0.084 Not significant	−0.23	−0.50	0.03
	Control group	30	0.33±0.61				
dft	Study group	30	0.80±1.32	<0.001	−3.53	−4.92	−2.14
	Control group	30	4.33±3.57	Significant			
ds	Study group	30	0.97±1.71	<0.001	−4.67	−6.68	−2.65
	Control group	30	5.63±5.25	Significant			
fs	Study group	30	0.17±0.59	0.308	−0.17	−0.49	0.16
	Control group	30	0.33±0.66	Not significant			
dfs	Study group	30	1.30±2.45	<0.001	−6.43	−9.21	−3.66
	Control group	30	7.73±7.19	Significant			

Inference: Dental caries (dt, ds) was significantly lower in study group compared to control group. SD: Standard deviation

Table 4: Comparison of the prevalence of selected bacterial species (>10^2 CFU) between the study (black stained plaque) compared to non-colored group

Microorganism	Study group (%)	Control (%)	Total (%)	Fisher's Exact test: *P* value and significance
A. naeslundii	22 (55.0)	18 (45.0)	40 (100.0)	0.206 Not significant
P. gingivalis	4 (57.1)	3 (42.9)	7 (100.0)	0.500 Not significant
S. mutans	21 (42.9)	28 (57.1)	49 (100.0)	0.021 Significant
Lactobacillus	0 (0.0)	6 (100.0)	6 (100.0)	0.012 Significant
A. actinomycetemcomitans	15 (60.0)	10 (40.0)	25 (100.0)	0.147 Not significant
F. nucleatum	24 (54.5)	20 (45.5)	44 (100.0)	0.191 Not significant
P. intermedia	1 (50.0)	1 (50.0)	2 (100.0)	0.754 Not significant
Number of caries-free children	18 (78.3)	5 (21.7)	23 (100.0)	<0.001 Significant

A. naeslundii: Actinomyces naeslundii, P. gingivalis: Porphyromonas gingivalis, S. mutans: Streptococcus mutans, A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans, F. nucleatum: Fusobacterium nucleatum, P. intermedia: Prevotella intermedia

Table 5: Comparison of the prevalence of selected bacterial species (>10^2 CFU) between the study (Black stained plaque) compared to non-colored group separately for caries-free and caries-affected persons (Fisher's exact test)

Microorganism	Caries-free children				Caries-prone children			
	Study group (%)	Control (%)	Total (%)	*P* value and sig.	Study group (%)	Control (%)	Total (%)	*P* value and sig.
A. naeslundii	15 (83.3)	3 (16.7)	18 (100.0)	0.291 Not significant	7 (31.8)	15 (68.2)	22 (100.0)	0.599 Not significant
P. gingivalis	4 (80.0)	1 (20.0)	5 (100.0)	0.709 Not significant	0 (0.0)	2 (100.0)	2 (100.0)	0.450 Not significant
S. mutans	12 (70.6)	5 (29.4)	17 (100.0)	0.184 Not significant	9 (28.1)	23 (71.9)	32 (100.0)	0.182 Not significant
Lactobacillus	0 (0.0)	1 (100.0)	1 (100.0)	0.217 NS	0 (0.0)	5 (100.0)	5 (100.0)	0.122 Not significant
A. actinomycetemcomitans	9 (69.2)	4 (30.8)	13 (100.0)	0.251 Not significant	6 (50.0)	6 (50.0)	12 (100.0)	0.115 Not significant
F. nucleatum	15 (83.3)	3 (16.7)	18 (100.0)	0.291 Not significant	9 (34.6)	17 (65.4)	26 (100.0)	0.487 Not significant
P. intermedia	1 (100.0)	0 (0.0)	1 (100.0)	0.783 Not significant	12 (33.3)	24 (66.7)	36 (100.0)	0.676 Not significant

P value: Probability value, Sig.: Significance. Inference: There was no significant difference between the study and the control groups when analyzed separately for the caries-free and caries-prone individuals. *A. naeslundii: Actinomyces naeslundii, P. gingivalis: Porphyromonas gingivalis, S. mutans: Streptococcus mutans, A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans, F. nucleatum: Fusobacterium nucleatum, P. intermedia: Prevotella intermedia*

higher number of *P. gingivalis* in BS plaque compared to non-discolored plaque which is in accordance with previous studies. However, these results are not significant as children with lower age group might not be susceptible for the growth of the organism.

Previous studies have shown that there is decrease in *F. nucleatum,* but in contrast, our study has got higher number of *F. nucleatum* in BS plaque as compared to non-discolored plaque.[7]

F. nucleatum is the bridging organism between early and late colonizers in the dental plaque.[14] This species was detected in carious lesions, but its role in caries progress is not cleared. *F. nucleatum* can be present as colonizer in plaque formation. The geographic area and the environment can also influence the microbial composition. Different geographic area may have different microbial compositions, and this may influence the caries prevalence and the stain formation.[20]

Lower caries experience in BS may also be due to dietary habits such as consumption of vegetables, fruits, dairy products, eggs, and soy sauce and who had never been fed with nursing bottle. Using of fluoride toothpaste and mouth rinse containing fluoride encourages stain formation, but in the present study, these parameters were not analyzed which is one of the limitations. Biochemical studies on BS composition showed higher calcium content in BS patients, and this may be responsible for the black color of the stain. The black compound is ferric sulfide formed by the reaction between hydrogen sulfide produced by bacteria and iron in saliva or gingival fluid.[5] Salivary parameters such as pH, buffering capacity, calcium, and phosphate ion concentrations are well-known caries protective factors. Studies have shown that significantly higher levels of calcium, inorganic phosphates, copper, sodium, and total protein were found in patients with BS and glucose levels were significantly lower in BS group.[5] Probably, this might be the reason for less number of caries in BS plaque.

Even though there was good correlation established between BS plaque and low caries index in Indian population, the present study has certain limitations such as small sample size and certain confounding factors like diet, and salivary pH was not assessed as this was not the main objective of our study. The children examined in the study were not investigated for gingival and periodontal status which might be one more limitation, as the association between periodontopathogenic bacteria and periodontal health in children with BS could not be assessed. Our study has taken restricted organisms for culture, but other organisms might be present which can establish the relationship between the BS and low caries. The factors such as demographic, social, and behavioral-like diet and drinking water content can also influence the BS and its caries protective effect.

Conclusion

Bacterial composition of BS showed significantly lower number of *S. mutans* and *Lactobacillus sp*. as compared to non-discolored plaque indicating less cariogenic alteration of plaque ecology. The other aims of the study were to investigate the caries experience in BS, and non-discolored plaque was confirmed by statistical analysis, and there is significantly lower number of caries in BS plaque and non-discolored plaque. To eliminate the role of periodontopathogenic bacteria in BS in the development of periodontal disease in children, longitudinal studies are necessary. We also found cariogenic microorganisms to be less prevalent in the BS plaque indicating less cariogenic alterations of plaque etiology. The dietary habits like high intake of iron content food and beverages such as coffee and tea may also produce BS on the teeth, but studies are necessary to establish the correlation of dietary habit and the microbiota in BS.

Further, scope of the study includes analyzing various other oral microorganisms involved in BS plaque through molecular technique and long-term follow-up of the children with BS plaque to see whether the permanent teeth also are caries free in these groups.

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How to cite this article: Mutsaddi S, Kotrashetti VS, Nayak R, Pattanshetty S, Hosmani JV, Babji D. Association of dental caries in children with black stain and non-discolored dental plaque: A microbiological study. J Adv Clin Res Insights 2018; 5: 59-64.