Black stains in the mixed dentition: A PCR microbiological study of the etiopathogenic bacteria.

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The aim of this work is to emphasize that particular stains on the third cervical of the buccal and lingual surfaces in mixed dentition, called "black stain." Previous research showed the microbiological etiology of this discoloration by chromogen bacterias. Our study shows bacteria spp involved in stains by means of PCR process and electrophoresis gel on the agarose medium. Sample was formed by 100 subject with black stain and 100 control subjects stain-free. A statistical analysis (SPSS 10.0) using X2 was performed in this study. Porphyromonas gingivalis and Prevotella melaninogenica, were not involved in both in black stain subjects and in the control. On the contrary, Actinomyces could be involved in the pigmentation process.

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INTRODUCTION

Black stain is a peculiar and characteristic dental extrinsic discoloration, which occurs sometimes along the third cervical line of the buccal and/or lingual surfaces of teeth, both in the primary and permanent dentition.[15-16-23-24] (Figure 1)

The objective of the present study is to determine the agent involved in the formation of black stains using modern and sophisticated techniques, to propose preventive and therapeutic protocols, which, along with the solution of the esthetical problem, does not alter the delicate equilibrium of the oral microbiota.

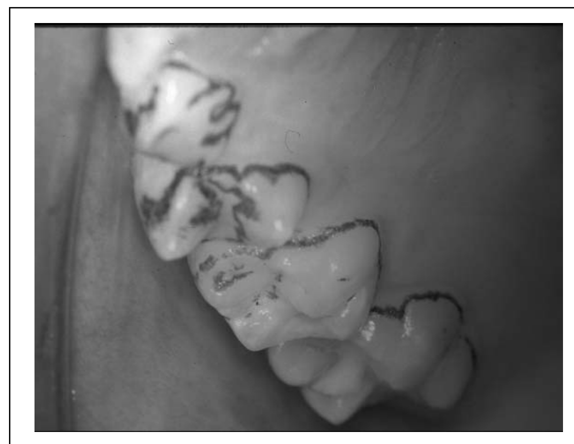

Figure 1. Black stain

This entity has been described in literature especially for the low caries-experiences that these patients show compared with those that do not show any kind of extrinsic pigmentation.[1,2-6-10,16,22,26,27] From the etiopathogenic point of view only suppositions have been advanced. The etiology of black stain has been attributed in different ways to bacteria able to form black colonies on blood agar.[3,14,22,28]

Melanogenic bacteria belonging to the genus *Prevotella* and *Porphyromonas*, could form *in vivo* such pigmentations in the presence of gingivitis or in any case of periodontitis which creates bleeding and thus, the availability of iron, in whose absence microorganisms would not be able to form pigments.[3,13,18,19,21]

Since there is no clinical evidence of patients with

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periodontitis which show minor caries incidence as compared to patients without pigmentation,[1,2,8-11,14-17] the supposition has been advanced that, in such a particular clinical picture, a member of species of *Actinomyces*[4,12,13,23] is present and that, in the genesis of chromatic modification, the peculiar quali-quantitative composition of saliva of the subjects which show black stain is involved.[1-25]

In order to determine the possible presence of bacteria known as etiological agents found in periodontitis, PCR has been adjusted to highlight the presence of genetic material of *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans*,[20,28] anaerobic or facultative anaerobic bacteria, whose natural habitat is found in the gingival margin where the available oxygen is reduced to the minimum. Besides PCR has been adjusted to highlight *Actinomycetes* spp. and *Prevotella melaninogenica* (*ex Bacteroides melaninogenicus)* that has been mentioned in literature as likely etiological agents of pigmentation.[1,3,4,11-13,17-19,21,22,26,27]

MATERIALS AND METHODS

A consecutive series of 850 subjects aged 6 to 12 yrs. (mean 8.3 years) were evaluated from the Department of Pediatric Dentistry of the University of Rome "La Sapienza." Our inclusion criterion was directed to patients showing black stains and negative to other extrinsic discoloration (eg. Brown, green or orange staining). 100 patients met these criteria (case group).

We also considered 100 subjects, which did not show any kind of extrinsic pigmentation, meeting the same criteria for age and dental condition (control group). The ratio case:contol was 1:1 due to the high costs of curettes and also to the impossibility to remove the DNA of the previous patient by chemiclave cycle. Parents of recruited patients were previously informed and consented to a thorough "informed consent."

The research protocol provided:

- Sample of pigmented (study-group) and not pigmented plaque (control-group)
- Total DNA extraction (genomic and plasmidic)
- Sample analysis by PCR technique
- Electrophoresis in agarose gel
- Statistical analysis

Plaque sampling

Considering the characteristic adhesiveness of pigmentation to enamel surface, black stain were obtained from the dental surface by "scraping" using new metallic scalers. The scaler could not be used again after sterilization as it was conjectured that bacteria could remain adhered to the surfaces of the scaler and that, after sterilization, could present some genetic material on the instrument which would alter the results. The model protocol sampled the same amount from a tooth of a more intense pigmentation and which as a rule corresponded to the maxillary first or second primary molar. The surface of tooth was first cleaned and then wiped with a sterile gauze soaked in physiological solution.

Total DNA extraction (genomic and plasmidic)

Sample analysis by Polymerase Chain Reaction (PCR) needed first its total DNA extraction. In this phase substances or treatments that lyse bacterial cell are used, destroying the cell wall, removing cytoplasmic content with the exception of its DNA.

The procedure uses the predetermined amount of lysing solution containing:

- TRIS HCl 1M (pH 8.5) (3-hydroximethylaminomethane hydrochloric acid) is an alkaline buffer that creates the right environment for the enzyme to be operational.
- EDTA 2 mM (ethylendiaminotetraacetic acid) has the purpose to inhibit some bacterial metalloenzymes, as DNA-ase.
- PROTEINASE-K acts digesting bacterial proteins. Its function is to inhibit the DNA-ase activity and to change the structural integrity of bacterial wall.
- SDS 2% (sodium dodecylsulphate) is a surfactant that shows a marked tropism towards membrane lipids, and is able to change the structural integrity of bacterial wall aiding in such a way the proteinase-k action.
- WATER (H_2O)

To 100 µl of each sample 100 µl of lysing solution were added and the test tubes were immersed in a thermo stated bath at 55°C for 18 to 24 hours. Successively the samples were centrifuged at 1200 gravity (g) for 5 minutes, and the supernatant directly used as DNA source for PCR.

With the aim to concentrate DNA, two alternative methods were used.

The first provide for the transfer of a sample amount three times the previous one, in test tubes which are frozen to –80°C for one hour. Then the sample is freeze-dried and suspended in 50µl of above solution (TRIS HCl; EDTA; proteinase-k; SDS): the test tubes are dipped in a thermo stated bath at 55°C for 18 to 24 hours and then frozen to –80°C. The sample is then concentrated six times.

The second DNA extraction method permits to concentrate it 10 times. At this purpose 150µl of sample were taken and transferred in test tubes, whose content is heated to boiling temperature, which is maintained for 10 minutes. In this way DNA is denaturized and then precipitated using solutions, in fixed amounts on the basis of DNA amount.

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To the sample are added:
- 75 μl of 7.5 M ammonium acetate
- 450 μl of absolute ethanol

This solution is brought to –80°C, centrifuged after 24 hours at the temperature of 2°C at 20000 g for 30 minutes.

The formed pellet, once separated from supernatant and freed from ethanol, which evaporates at room temperature, was suspended again with 1.5 μl of TE pH 8.5. DNA so diluted was directly used as PCR template.

Sample analysis by PCR

Polymerase Chain Reaction (PCR) is a technique permitting the *in vitro* amplification of a DNA segment. The reaction is based on the specific paioning and the extension by polymerization of a region within a double stranded DNA sequence (template), starting from two oligonucleotide primers flanking the region to be amplified (Figure 2). The reaction occurs in an appropriate mixture (final volume 50 or 100μl) in which besides template to be amplified, polymerase reaction buffer (1X) is present, added with 2.5mM MgCl2; the four deoxynucleotides, each at a 0.2mM final concentration; the two primers at a 0.5pmol/μl concentration and 2 Taq DNA polymerase units (New England Biolabs®). This enzyme, isolated from microorganism *Thermus Aquaticus*, is thermo-stable and has an optimum temperature activity of 72°C. This property is very important as PCR has a number of cycles comprising a DNA denaturizing step at 96°C, followed by paioning of primers with template (which occurs at a temperature depending on the properties of the primers), and then extension (polymerization) which occurs at the Taq optimum activity temperature.

PCR reaction is done using DNA Thermal Cycler 2400 (Perkin-Elmer®, Foster City, CA) apparatus, in the following conditions: 60 seconds at 94°C, to promote DNA denaturizing; one minute at optimum temperature (varying), to allow paioning of primers with template specific target sequences; and 120 seconds at 72°C, to allow Taq polymerase to carry out the extension reaction, 35 cycles being repeated.

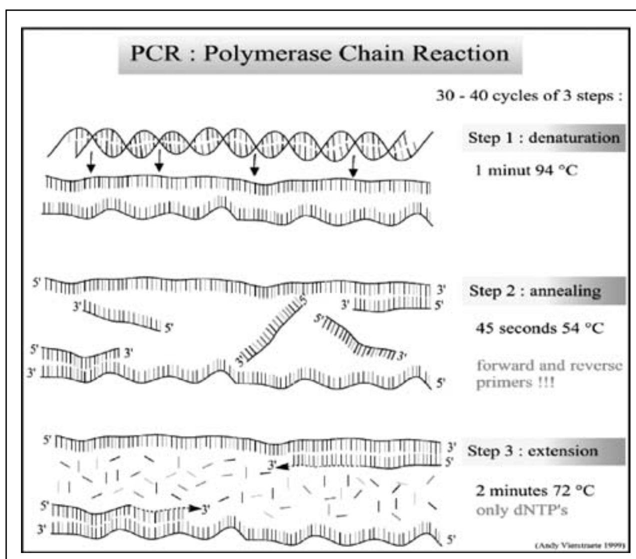

Figure 2. PCR subsequent steps

Electrophoresis in agar gel

Electrophoresis is a technique used to separate mixtures of ionic solutes by difference in migration speed, due to the different molecular weight they present when an electric field is applied.

This technique allows for determination, separation and assessment of molecular weight of linear DNA fragments based on comparison between the electrophoretic mobility of DNA sample under examination, and that of linear DNA of known molecular weight. In this experiment 0.7% agar gel has been used.

Electrophoresis has been done at 85 volts for about one hour to allow an optimum separation of DNA fragments. The buffer used was TAE (0.04 M tris-acetate; 0.02 M EDTA).

At the end of electrophoresis step, gel is dipped in etidium bromide, a colorless and odorless liquid, which has to be handled very carefully as it is cancerous and teratogenic. Etidium bromide has the ability to bind itself to DNA and emit fluorescence if lightened with ultraviolet rays.

Statistical analysis

Data for this study were analyzed with SPSS 10.0 (SPSS, Chicago, USA). Differences in proportions were tested using X2 test. We calculate odds ratio (OR) and confidence intervals (95% CI) for the bacterial species: 889 base pairs (bp) for *Actinomyces* spp. and 978 bp for *A. Actinomycetemcomitans* using CIA program (CIA 2.0 2000, University of Southampton).

To determine if bacterial species differed significantly between subjects with and without black stain, we performed a stepwise forward logistic regression analysis. The dependent variable is presence or absence of black stain.

RESULTS

In this research, four different primers couples have been used, specific to four different bacterial species: *Actinomyces* spp., *Actinobacillus actinomycetetemcomitans (A. Actinomycetemcomitans), Porphyromonas gingivalis, Prevotella melaninogenica.*

Amplymers length

On the basis of primers selected, the possible presence of single-stranded DNA under examination, should be demonstrated by bands having height of:

- 889 base pairs (bp) for *Actinomyces* spp.
- 978 bp for *A. Actinomycetemcomitans*

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• 1614 bp for *Porphyromonas gingivalis*
• 870 bp for *Prevotella melaninogenica*

In the following photos, samples from 1 to 10 belong to patients with black stain. Samples from 11 to 20, belong to control patients.

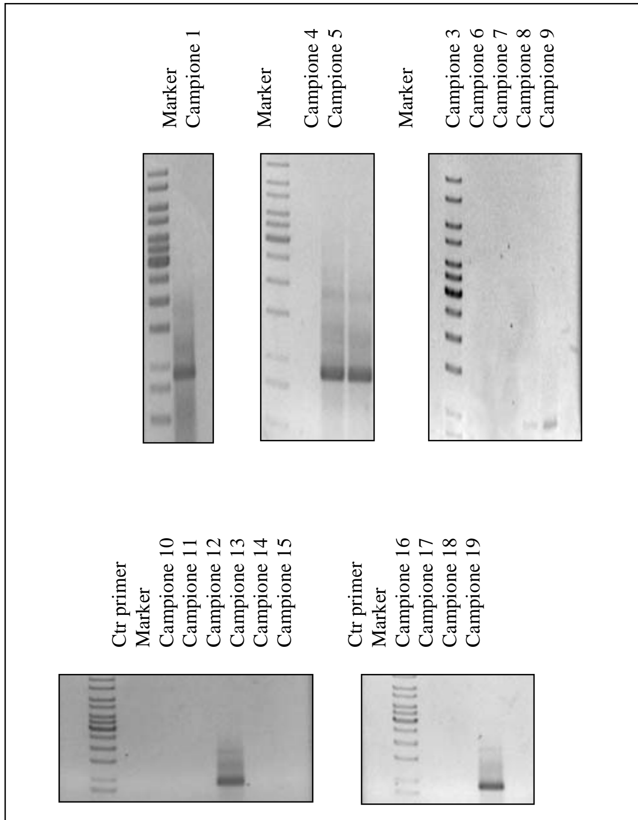

Figure 3. Agar gel to display DNA amplymers of *Actinomyces* spp.

Bands 889 bp high which display the presence of *Actinomyces* DNA, are evident in samples 1, 4, 5, 7 e 8, of patients with black stain, and in the samples 13 and 18 of patients which do not show black pigmentation.

By revealing these results as percent we can say that, 50% of samples with black stain are positive to *Actinomyces*, while only 20% of DNA of same bacterium has been observed in samples used as control (figure 3).

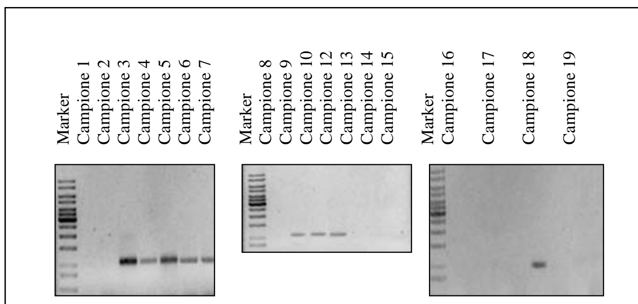

Figure 4. Agar gel to display DNA amplymers of *Actinobacillus actinomycetemcomitans*

As to the determination of bands of height compatible to that expected for the presence of *A. actinomycetemcomitans* DNA, bands 978 bp high are evident in samples 3, 4, 5, 6, 7, 9, 10 of patients with black stain, and in sample 12 and 18 of patients which do not show pigmentation (figure 4).

By revealing these results as percent we can say that, 70% of samples with black stain are positive to *A. actinomycetemcomitans*, while only 20% of DNA of same bacterium has been observed in samples used as control.

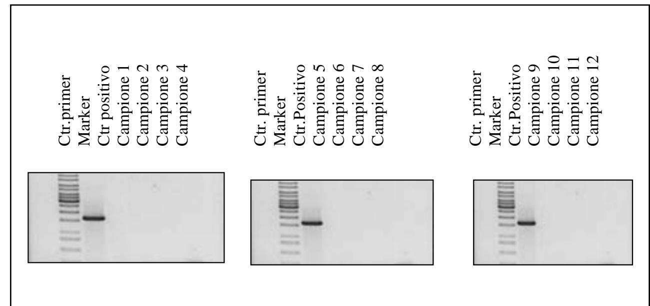

Figure 5. Agar gel to display DNA amplymers of *Porphyromonas gingivalis*

Bands 1614 bp high that indicate the presence of *Porphyromonas gingivalis* are evident only in a sample used as positive control. In the samples of this experiment, indeed, no band is evident (Figure 5).

The bacterial species is therefore absent both in the samples of patients with black pigmentation, and in samples of patients not showing this clinical evidence.

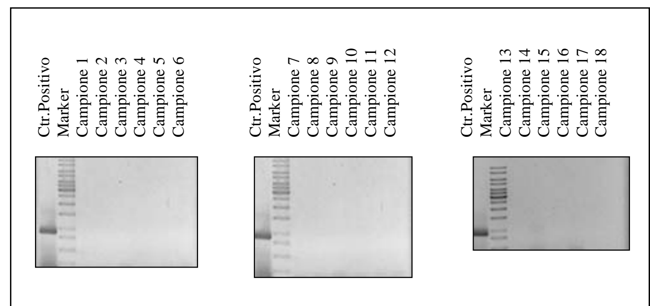

Figure 6. Agar gel to display DNA amplymers of *Prevotella melaninogenica*

Bands 870 bp high that indicate the presence of *Prevotella melaninogenica* are evident only in a sample used as positive control with the purpose to compare it with the samples of this experiment (figure 6).

It is clear that, the absence of this kind of bands, demonstrates the lack of bacterial DNA of species under examination.

Statistical results

For the 889 base pairs (bp) for *Actinomyces* spp. we calculate OR= 4.0, 95% CI (2.1 to 7.5) this means that subjects with *Actinomyces* spp. have four times the

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odds of black stain compared with the subjects without this bacterial species.

For the 978 bp for *A. Actinomycetemcomitans* OR= 9.3, 95% CI (4.9 to 17.9). This means that subjects with *A. Actinomycetemcomitans* have nine times the odds of black stain compared with the subjects without this bacterial species.

The logistic regression analysis confirm this finding OR= 3.5, 95% CI (1.7 to 7.2) for *Actinomyces* spp. and OR= 8.7, 95% CI (4.4 to 17.1) for *A. Actinomycetemcomitans*. If we consider subjects with both bacterial species the OR are the same: this means that the two bacterial species are independent.

Table 1. Bacterial species by group

Actinomyces spp	cases	Controls	total
present	50	20	7
absent	50	80	130
total	100	100	200

Table 2. Bacterial species by group

A.Actinomycetemcomitans	cases	Controls	total
Present	70	20	90
Absent	30	80	120
Total	100	100	200

DISCUSSION

The results obtained demonstrate the lack of *Porphyromonas gingivalis* and *Prevotella melaninogenica*, both in black stain subjects and in control. Hence it is possible to say that these species are not involved in the genesis of such nosological entity.

On the contrary *Actinomyces* could be involved in the growth of pigmentation because its presence was demonstrated in 5 of 10 patients with black stain (50 percent) and in 2 of 10 control patients (20 percent).

A. actinomycetemcomitans similarly could be involved in the emergence of pigmentation, because 7 of 10 patients with pigmentation are positive to the presence of this bacterium (70 percent), versus 2 of 10 control patients (20 percent).

Pronounced presence of this species in the pigmented plaque samples, whatever the role, is rather surprising considering that these samples deal with healthy children from the periodontal point of view, especially taking into account that literature reports only occasional presence of the species in healthy subjects, and that the percentage of isolation is of 5% on both primary and permanent teeth.[5] On the basis of results obtained by this experiment it should be necessary, increasing the patients number to confirm the results of this research.

Data obtained during this work could also be object of further study, by monitoring the patients through periodical checks, with the purpose to evaluate over the time the role of bacterial species detected, with particular attention to *A. actinomycetemcomitans* to establish if, along with a lower caries-experiences,[1,2,8-11,14-17] these patients instead do risk to develop forms of local and/or general juvenile periodontitis.

CONCLUSIONS

As the patients and parents become more interested in esthetics, the origin of extrinsic discoloration, becomes more important. This study examined some of the origins of "black stain" in the mixed dentition. Previously it was thought to occur by chromogenic bacteria. This study proposed the possible bacteria spp involved in stains by means of PCR process and electrophoresis gel on the agarose medium. 100 subjects with black stain and 100 control subjects stain-free formed the sample. A statistical analysis (SPSS 10.0) using X2 was performed in this study. *Porphyromonas gingivalis* and *Prevotella melaninogenica*, were not involved both in black stain subjects and in the control group. However, *Actinomyces* could be involved because its presence was demonstrated in 5 of 10 patients with black stain (50 percent) and in 2 of 10 control patients (20 percent).

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