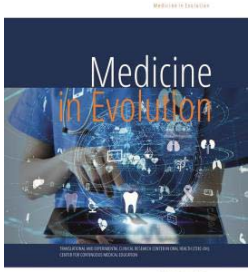
The aesthetic impact of black stains in paediatric patients - a study of chemical and microbiological composition



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Abstract

The present study aims to present the clinical implications of black stains but also their microbiological and chemical components. The study involved three paediatric patients divided into two groups on which samples were taken and through specific tests the presence of extrinsic chromogenic bacteria as well as metals and sulfuric components was demonstrated. The microbiological and chemical evaluation provides additional information that can be used by clinicians to improve the treatment protocol by knowing the bacteria involved and their specifics.

Keywords: chromogenic bacteria, paediatric dentistry, microbiology, chemical composition

INTRODUCTION

By way of life, the vast majority of chromogenic bacteria are saprophytic, but some have the ability to cause putrefaction and fermentation, being therefore not only chromogenic, but at the same time saprogenic.

It is known that chromogenic bacteria cells are completely colorless, but pigments appear and accumulate outside the cells, alongside them, deposited in the form of small droplets, grains or even crystals that dissolve and diffuse into the environment, coloring it for a considerable length [1].

BS (black stain) are caused by anaerobic chromogenic bacteria. The species predominantly responsible for these are actinomyces spp. (Gram-positive: Actynomyces israelii, Actynomyces naeslundii, which produce hydrogen), very rarely, Porphyromonas (Gram-negative) and Prévotella melaninogénica (Gram-negative).

After years of observation, epidemiological studies in the 1950s and subsequent decades showed an association between black spots (BS) on permanent teeth and a decrease in dental caries [2], as this staining is thought to unbalance the microbial flora, thus reducing the number of cariogenic bacteria, and increases the number of chromogenic bacteria responsible for tooth pigmentation. They affect both temporary and permanent dentition. These discolorations often begin in early childhood and peak by puberty or adolescence, and can be seen even in young adults [2].

From an aesthetic point of view, dental staining caused by chromogenic bacteria belongs to an extrinsic staining, which is deposited on the surface of the teeth and does not involve the internal structure of the tooth, found predominantly at the cervical level of the enamel and follows the gingival contour and can also form on all surfaces of the tooth (vestibular, oral, proximal and incisal/occlusal). BS may appear either as dark lines with 1 mm margins parallel to the gingival margin, as incomplete coalescence, or in the form of dark spots that rarely extend above the cervical third, and appear especially in the primary dentition. Sometimes it can also appear in the grooves or pits of the teeth. This brownish-black coloration is very adherent to the tooth and has a high chance of recurrence [3].

Aim and objectives

This study aims to identify the chromogenic bacteria potentially involved in tooth staining and to stop their growth and biofilm formation capacity, with the help of different non-toxic and easily available compounds, without unwanted side effects and financial constraints, to find both preventive methods as well as curative, and to apprehend the consequences of their presence in the oral cavity.

The microbiological and chemical evaluation provides additional information that can be used by clinicians to improve the treatment protocol by knowing the bacteria involved and their specifics.

MATERIAL AND METHODS

The research protocol provides a sample of two subjects that present the colourings (study group) and one subject that does not present the colourings (control group). Three children between the ages of 2 and a half and 12 years were selected, of which, two female subjects and one male subject, all living in Timiş county, of Romanian nationality, patients from the Department of Paediatric Dentistry, suffering from BS.

The criteria for including patients in the study are:

✓ Patients with oral manifestations of chromogenic staining

✓ Patients aged between 2.5 and 18 years

✓ Patients who have not benefited from professional dental cleaning in the last 6 months

✓ Clinically healthy patients

✓ Patients who do not have dental anomalies of number, shape and volume, MIH, amelogenesis imperfecta, dentinogenesis imperfecta, as well as other oro-dental anomalies.

✓ Patients regardless of their background.

Three patients were selected:

1. Male patient aged 9 years, part of the study group.
2. Female patient aged 12 years, part of the study group.
3. Female patient aged 6 years, in the control group, the last two being sisters.

1. BACTERIOLOGICAL TEST

Two of the three subjects were selected for bacteriological testing, patient number 2, aged 12 years, BS positive (+), and patient number 3, aged 6 years, BS negative (-). The analysis was performed by approaching the traditional method of growth and identification of oral microorganisms, by growing a bacterial culture.

Samples were collected with a sterile ESwab™ swab from COPAN, which is a patented liquid-based collection and transport system for microbiology samples.

The collection was carried out by circular tamping movements, strictly on the hard surfaces of the teeth (vestibular, palatal, lingual), without touching other surfaces or objects, in order not to compromise the results obtained (Fig.1). After collecting the samples, the sterile swab was immediately immersed in the plastic tube, and kept at room temperature at 21°, for ~12 hours from the time of collection, until they were handed over to the accredited laboratory for processing the microbiological samples.

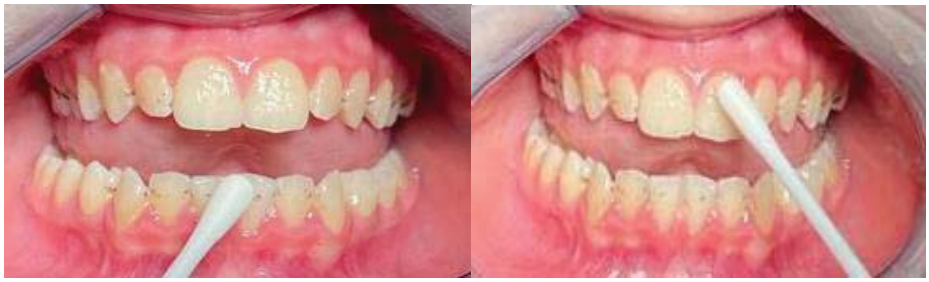


Figure 1. Sample collection from the BS positive (+) patient with the sterile swab from the eSwab™ system on the vestibular surface of the lower and upper teeth

2. COLLECTION OF SAMPLES FOR QUANTITATIVE AND QUALITATIVE CHEMICAL ANALYSIS OF COLORED DEPOSITS

Black spot samples were scraped using a sterile scalpel port and a 12D type blade. After scraping, from the areas where the coloured deposits were more abundant, and more precisely from the level of the lingual surface of the lower incisors, of the lingual surface of the lower premolars, and from the level of the mesial and vestibular surfaces of the upper first permanent molars (1.6 and 2.6), an amount of about 1-2 mg of coloured deposit was obtained (Fig. 2). Before scraping, the tooth surface was first cleaned and then wiped with a sterile gauze soaked in physiological solution.

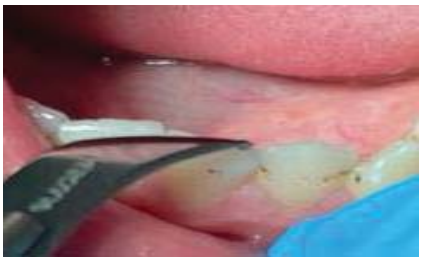


Figure 2. The colored deposits that can be seen on the tip of the 12D blade, resulting from their scraping

Dental plaque was collected along with black stain (BS) and then the blade was detached and placed it in a suitable 12x100mm transparent glass transport medium, from the GLASSCO company.

The collected sample was treated with concentrated HCl (concentration 37%) in order to solubilize the salts of the species of interest. When adding the reagent, the obtained solution turned yellow, indicating the possible presence of ferric ion salts (Fe3+) which in aqueous solution causes the formation of a yellow coloration (Fig. 3).

Figure 3. Solubilization of the collected sample in concentrated HCl (37%)

All ionic species identification reactions were performed from a qualitative perspective, using approximate reagent volumes ranging from 0.5 to 10 mL. In order to be able to confirm with certainty the presence of the ferric cation, two identification reactions were carried out, both of which were positive.

✓ First reaction: in the presence of potassium ferrocyanide (potassium hexacyanoferrite) K4[Fe(CN)6] of 0.1M concentration, the Fe3+ ion precipitates in acidic medium, ferric ferrocyanide or ferric hexacyanoferrite, also called Berlin blue or Prussian blue (reaction 1, Fig. 4).

$$4\ Fe3{+}{+}\ 3\ \boldsymbol{K}{+}\mathbf{14}[\boldsymbol{Fe}{+}\mathbf{2}(\boldsymbol{CN}{-}\mathbf{1})\mathbf{6}] \rightarrow Fe{+}\mathbf{3}4[Fe{+}\mathbf{2}(CN{-}1)6]3\downarrow{+}\ 12\ K{+}\ \text{(Reaction 1)}$$

Figure 4. Identification reaction of Fe3+ ion in the presence of potassium ferrocyanide on the well plate

✓ Second reaction: identification involved the use of ammonium sulfocyanide NH4SCN. In the presence of the sulfocyanide anion, the ferric cation forms a reddish color

due to the formation of the complex ion hexasulfocyanoferrate (III) (reaction 2, Fig. 5). The reaction shows a high sensitivity, and the intensity of the color is influenced by the concentration of the analytical species, the orange-reddish coloration obtained upon the addition of ammonium sulfocyanide of concentration 0.1 M indicating the presence of Fe3+ ions in relatively low concentration. At high concentrations, the colour of the sample becomes blood-red.

$Fe3+ + 6\ \boldsymbol{NH4SCN} \rightarrow K+13[Fe+3(SCN-1)6] + 3\ NH4+$ (Reaction 2)

Figure 5. Identification reaction of Fe3+ ion in the presence of ammonium sulfocyanide on the well plate

The analysis of the ferrous cation (Fe2+) also confirmed its presence, two distinct identification methods being used during the experiment.

✓ The first involves the use of potassium ferricyanide (potassium hexacyanoferrate) K3[Fe(CN)6] of 0.1 M concentration as a specific reagent. In its presence, the ferrous cations in the sample precipitate, in an acidic medium, the blue-green ferrous ferricyanide, the ferrous hexacyanoferrate, also known as Turnbull blue (Reaction 3, Fig. 6).

$3\ Fe2++ 2\ \boldsymbol{K+13[Fe+3(CN-1)6]} \rightarrow Fe+23[Fe+3(CN-1)6]2\downarrow + 6\ K+$ (Reaction 3)

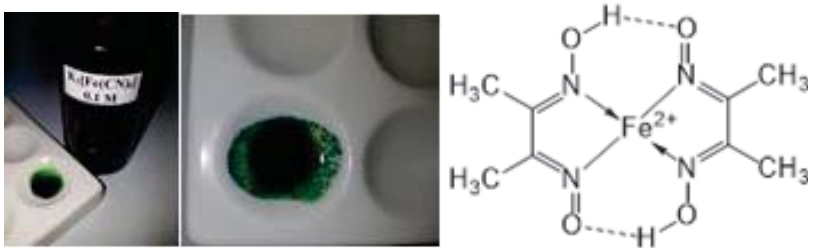
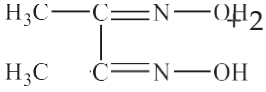


Figure 6. Identification reaction of Fe2+ ion in the presence of potassium ferricyanide on the well plate

✓ The second reaction to identify the ferrous ion was carried out by using a 1% ethanolic solution of dimethylglyoxime (DMG) which, in a basic environment provided with a NH3 2N ammonia solution, causes the formation of a red-carmine color, due to the formation of a complex combination, ferrous dimethylglyoximate (Reaction 4, Fig. 7)

$Fe_{2+} + 2$  $2\ NH_3 \rightarrow$ $\downarrow + 2\ NH_{4+}$

(Reaction 4)

Figure 7. Fe2+ ion identification reaction in the presence of dimethylglyoxime on the well plate

The presence of calcium ions (Ca2+) was identified using ammonium oxalate as a specific reagent (concentration 0.1 M) after neutralizing the sample with 2 N NaOH and bringing it to pH 7 (value checked using pH indicator paper). In the presence of ammonium oxalate ((NH4)2C2O4), Ca2+ ions form a white, crystalline precipitate, calcium oxalate (Reaction 5) which in the yellow sample is visible with a yellowish coloration (Fig. 8).

$Ca2+ + (NH4)2C2O4 \rightarrow CaC2O4 \downarrow +2\ NH+$ (Reaction 5)

Figure 8. Ca2+ ion identification reaction in the presence of ammonium oxalate

The presence of Cu2+ ions (cupric cation) could not be identified in the sample solubilized with either of the two tested reagents (ammonia NH3 and ammonium tetrasulfocyanomercurate (NH4)2[Hg(SCN)4]), most likely due to the pH- of the strong acid of the sample obtained during solubilization, even after attempts to neutralize it.

The presence of the phosphate anion (PO43-) was also impossible to detect (silver nitrate AgNO3, barium chloride BaCl2 and ferric chloride FeCl3 were tested as reagents) due to the same pH considerations, since all potential reaction products are solubilized with ease in the acidic medium generated by the HCl used to solubilize the sample.

Instead, the presence of sulfur in the form of sulfate ion (SO42-) and sulfite (SO 2-) could be detected. Thus, in the presence of silver nitrate AgNO (0.1 M), sulfite ions precipitate in neutral solution, with the formation of silver sulfite, a white, crystalline precipitate (Reaction 6, Fig. 9). The neutral solution was obtained after adding 2 N NaOH until reaching pH 7 checked with pH indicator paper.

$SO23- + 2\ AgNO3 \rightarrow Ag2SO3 \downarrow +2\ NO3-$ (Reaction 6)

Figure 9. Identification reaction of the SO32- anion in the presence of silver nitrate

The sulfate anion (SO42-) was identified following the reaction with barium chloride (BaCl2) of 0.5 M concentration. In the presence of the reagent, the sulfate anion reacts with the formation of a blue-white, crystalline precipitate, barium sulfate, visible at the optical microscope in the form of prismatic crystals (reaction 7, Fig. 10).

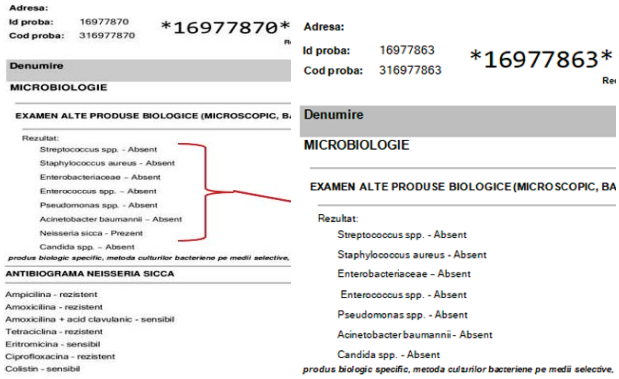
$SO2- + BaCl2 \rightarrow BaSO4 \downarrow +2\ Cl-$ (Reaction 7)

Figure 10. Crystallographic identification reaction of the SO42- anion in the presence of barium chloride performed on a glass slide and evaluated with a Nikon E100 optical microscope (10x objective)

RESULTS

1. BACTERIOLOGIC RESULTS

Following the bacteriological examinations, the analysis bulletin was drawn up, in which Neisseria sicca, having as its natural habitat the upper respiratory tract, considered a commensal species of the oral cavity, was found to be present in the processed samples from patient number 2, aged 12 years, BS positive (+) patient. The sample taken from patient number 1 aged 6 years BS negative (-) confirmed the absence of Neisseria sicca (Fig.11).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MICROBIOLOGIE
EXAMEN ALTE PRODUSE BIOLOGICE (MICROSCOPIC, B
Rezultat:
Streptococcus spp. - Absent
Staphylococcus aureus - Absent
Enterobacteriaceae – Absent
Enterococcus spp. – Absent
Pseudomonas spp. - Absent
Acinetobacter baumannii – Absent
Neisseria sicca - Prezent
Candida spp. – Absent
produs biologic specific, metoda culturilor bacteriene pe medii selective,
ANTIBIOGRAMA NEISSERIA SICCA
Ampicilina - rezistent
Amoxicilina - rezistent
Amoxicilina + acid clavulanic - sensibil
Tetraciclina - rezistent
Eritromicina - sensibil
Ciprofloxacina - rezistent
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Rezultat:
Streptococcus spp. - Absent
Staphylococcus aureus - Absent
Enterobacteriaceae – Absent
Enterococcus spp. - Absent
Pseudomonas spp. - Absent
Acinetobacter baumannii - Absent
Candida spp. - Absent
produs biologic specific, metoda culturilor bacteriene pe medii selective,

Figure 11. The analysis report

2. CHEMICAL COMPOSITION RESULTS

Following chemical analysis, the deposits proved to be strongly acidic and difficult to dissolve. However, the presence of ferric ion (Fe3+), ferrous cation (Fe2+), calcium ions (Ca2+), sulphur in the form of sulphate ion (SO42-) and sulphite (SO32-) was identified in the deposits.

Clinical implications of the identified chemical substances:

- their strong stability and increased insolubility explain the very high adhesion to dental surfaces and the persistence of black stains including airflow procedures

- the inorganic chemical composition indicates the presence of numerous strongly acidic ions

- the presence of ferric ion (Fe3+) and ferrous cation (Fe2+) was proven in the sampled deposits, which suggests a clear involvement of the gingival bleeding index and the ferric component in the blood with the deposition and extension of the stains, also the characteristic colour of the stains, may be directly related to the presence of ferric ion and ferrous cation in the blood and in the collected samples.

- the presence of calcium ions (Ca2+) is attributed to explaining the fact of adhesion to the dental structures and the impermeable structure of the stains

- sulphur, which is an essential ingredient in bone minerals, favouring balance between them, again being found considerably in all the mineralized structures in the body as well as in the deposits taken from the teeth.

DISCUSSIONS

The results of some studies suggest that black deposits are iron deposits formed as a result of the chemical interaction on the surface of the teeth between the hydrogen sulphide formed by the action of anaerobic bacteria and the iron contained in saliva. The proportions of hydrogen can vary from one individual to another. Iron is contained in the saliva of people with a normal diet or is released from red blood cells during gingivorrhea [2]. Epidemiological studies by REID et al. in 1976 and 1977 compared to those of THEILADE et al. since 1973, confirmed that BS is a form of dental plaque, characterized by flora with a tendency to calcify. It contains an insoluble iron salt, and a high content of inorganic phosphorus and calcium [2].

According to articles published between 2001 and 2014, the prevalence of BS varies from 2.4% to 18% with equal gender distribution. Most confirm the correlation between the presence of BS and the lower cariogenic activity in the oral cavity, and at the same time mention Actinomyces spp., as being directly involved in the mechanism of the appearance of BS. [4].

Theliade et al. demonstrated that BS is a dental deposit, formed by microorganisms embedded in an intermicrobial substance, with a tendency to calcify [5]. Thus, it can be considered a type of dental plaque, although it is composed of other types of bacteria. Comparing unstained plaque and BS, the former contains a lower number of potentially cariogenic bacteria [4].

At the BS level, the presence of gram-positive, anaerobic and facultative anaerobic bacteria is mainly reported [4].

Different studies mainly mark the direct involvement of actinomyces spp. [5,6] - gram positive, anaerobic bacteria, with a diameter of 0.2 - 1.0 micron, being the largest morphological group found at the level of black spots, the latter being different from other bacteria by its ability to form a well-developed mycelium. For example, in the study by C. SABA et al. [7] Actinomyces spp. could be involved because its presence was demonstrated in

5 out of 10 patients with specific black deposits (50%) and in only 2 out of 10 control patients (20%).

Another study carried out by SLOTS, found that the predominant microorganisms in the formation of BS are actinomyces [8]. Also, the research done by Reid et al. is consistent with that of Theilade et al., which confirmed the involvement of actinomyces species in the formation of BS [5].

The most recent PCR study found, confirmed this, Actinomyces naeslundii is more prevalent in patients with BS, Instead Lactobacillus spp. and Fusobacterium nucleatum can be found in greater numbers in subjects without dark spots [9].

A relevant study on the chemical composition of BS was carried out by Parnas et al. He took samples from two groups of patients: from study group A, samples were taken with metallic instruments, while from study group B, samples were taken with non-metallic instruments and the chemical composition was assessed using energy dispersive spectrometry (EDS). No differences were found between the amounts of carbon, oxygen, sodium, magnesium, silicon, sulphur, chloride and potassium present in group A compared to group B. Instead iron, copper, titanium, aluminum and zirconium were detected in the samples scraped with the metal instrument. This fact suggests that the results obtained may be influenced by the instrumentation used in sampling [4].

To circumvent this problem, Tantbirojn et al. performed a study on extracted teeth that had naturally deposited black deposits. Chemical analysis of the deposits showed the presence of areas of high concentration of iron and copper, which correspond to areas of high concentration of sulphur [4]. This finding suggests that the metal ions and the sulphur complex are responsible for the black colour of the spots [10].

An analysis of the black material that forms this colouring found that it consists of iron salts (Fe+) and an increased content of calcium (Ca) and inorganic phosphorus (F). The stains represent deposits of ferrous sulphates, they appear as a result of chemical interactions on the tooth surface between the hydrogen sulphide produced by the anaerobic bacteria present in the saliva that produce hydrogen (including actinomyces, lactobacillus sp, phorphyromonas, prevotella melaninogenica [7]) and existing salivary or gingival exudate iron, resulting in chromogenic insoluble ferrous deposition and blackening of the teeth, but the exact mechanism is not yet known (this includes electrostatic forces, van der Waals, hydration, hydrophobic interactions and hydrogen bonds) [3].

CONCLUSIONS

Although black stains are present in a large number of paediatric patients, the present study revealed, in accordance with the international literature, the following conclusions:

✓ correct information on the problem plays the main role, avoiding the confusion between BS and carious lesions

✓ chromogenic staining affects all teeth and all their surfaces

✓ gingival inflammation caused by plaque, in association with frequent gingival bleeding, are a basic nutritional substrate in the evolution of the amount and speed of staining deposition

✓ the BS manifest clinically identically in both sexes

✓ dietary pattern rich in iron associated with unsatisfactory hygiene, directly influences the speed of deposition of black stains

✓ the presence of ferric ion (Fe3+), ferrous cation (Fe2+), calcium ions (Ca2+), sulphur in the form of sulphate ion (SO 2-) and sulphite (SO 2-) was identified in the deposits, which explains the adhesion and the increased insolubility of the dyes

✓ metal ions and sulphur complex are responsible for the black colour of the stains
✓ transmission of BS between family members has not been proven
✓ Neisseria sicca is found to be present in the processed samples

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