Identification Quantity of Actinomyces in Children Saliva with Black Stain in Tooth Enamel Surface

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Objective: The aim of this study is to differentiate the quantity of Actinomyces on saliva of children with and without black stain at the surface of tooth enamel.

Methods: The subject is chosen from children aged 4-11 years with black stain more than 8 surfaces of tooth enamel and without black stain. The saliva is taken by instructing subject to expectorate into a sterile container and inserted into a sterile plastic with Oxoid Anaerobic Gas pack to keep the anaerobic condition when transported to laboratory. In the laboratory, serial dilution was done and sample was inserted into a plate which contains Difco Actinomycetes Isolate agar. Put the plate into an anaerobic jar and incubated in incubator. From the plate, subculture identification was done to identify the morphology of Actinomyces. The colony of Actinomyces on the plate was count with colony counter using the colony forming unit method.

Results: The result was analyzed with t-test two groups unpaired and concluded that the quantity of Actinomyces on children's saliva with and without black stain of the enamel surface is not different.

Conclusion: The results of this study indicate the quantity of Actinomyces bacteria in saliva of children with black stain is higher than in children without black stain saliva. But the results of the statistical test using t-test, it was found that the quantity of actinomyces in saliva of children with black stain and without black stain did not differ significantly.

Keywords: actinomyces, black stain, saliva

Introduction

Tooth discoloration or commonly called staining is deposit pigmentation found on the surface of the teeth that can cause discoloration on tooth (1-3). Discoloration of the teeth caused by extrinsic and intrinsic factors, is quite often found associated with the clinical state of dentition involving aesthetic issues (4,5). Discoloration resulting from acquired pellicle pigmentation by chromogenic bacteria, food or other chemicals (2,5,6). Stain will give different colors, based on the etiology, clinical features, composition, location, severity rate and level of adherence to the surface of tooth (4,7). Stain is often found in children and nonsmokers from dental plaque colored because of the activities of chromogenetic bacteria. A rough enamel surface is often caused rapidly stain (8). This stain usually categorized as black stain with the main factor is gram-positive chromogenetic bacteria such as Actinomyces and Bacteroides melaninogenicus (9).

Sutcliffe reported prevalence of black staining or black stain on population of 1000 children in age group 11 to 13 years reached 21% cases. Research done by Koch on children age group 7 to 15 years in Switzerland, children with black stain achieved as much as 19.9% of prevalence rate. Research in Brazil, the prevalence of black stain on children age group 6 to 13 years is at 9.3% and 2.5% in children age group 3 to 5 years (7).
Research at the State University of Iowa to 355 children, the prevalence of black stain was found at 11-14% of all samples10. Research in India to 1472 children with a mean age of 9.3 years, found that 18% of the samples suffered black stain and the relationship between black stain and dental caries severity rates (10).

Incidence of black stain on the surface of tooth enamel of children in Indonesia, especially Jakarta is increasingly encountered in daily practice. Stain also causes children and parents of sufferers are having a problem of aesthetics. Research devoted about factors that cause black stain on the surface of the tooth enamel of children in Indonesia, especially Jakarta is still rare. Research on etiology factors of black stain by examining plaque from children's tooth had been done before and found that the quantity of actinomyces on plaque of children’s tooth with black stain had significantly different amounts than on plaque of children’s tooth without black stain.

In this study, the researchers will identify actinomyces in the saliva of children with and without black stain and differentiate the quantity of actinomyces in the saliva of children with and without black stain. This research is important to do as soon as possible in order to know what etiological factors from black stain so it can be investigated further on its countermeasures and prevention.

1. Black stain

Black stain is a thin black line on the labial enamel surface of the tooth and near the lingual gingival margin and spread to the proximal surface (Figure 1). This stain attaches strongly, has a high recurrence rate, is more common in women and occurs in patients with good intra oral condition (5,11). This type of stain is very difficult to be eliminated, particularly those inherent in the deep niche (5).

The results of the research in Denmark on 11 children aged 3-5 years who have black stain at least 10 teeth, there are significant differences in plaque microflora of normal dental plaque and plaque with black stain. In dental plaque with black stain, gram-positive rod bacterium (Actinomyces) found in 90% of the organisms in acquired pellicle, which when compared to the number of gram-positive rod bacteria in normal dental plaque is only 35-42%.

A study is conducted on 100 children plaque samples with black stain and 100 children plaque without black stain in 2006. By using inspection of Polymerase Chain Reaction, it was found that the Porphyromonas gingivalis bacterium and Prevotella melaninogenica have no role in causing black stain, but Actinomyces assessed role in the pigmentation process.

2. Actinomyces

Actinomyces is a fertile organisms, potential pathogens from this species lived together in the mouth of humans and animals. Actinomyces is a major component of dental plaque, especially on the part of the approximal tooth. Actinomyces were isolated to produce a black pigment and occur calcification. In vitro studies showed that the formation of black pigment in dentin is caused by Actinomyces (5).

Actinomyces gram positive characteristics are small, thin, straight, filamentous branching rods, non-motile, non Sporing, non acid fast (12). Actinomyces also have a general no acid resistance, live in anaerobic environments, the tissue can branch and then change into a rod shape (12,13). A colony of these organisms will look like a yellowish sulfur granules. Actinomyces grow under anaerobic conditions on blood or serum glucose at 35-37°C (12), within one week will visible form of small, white, colonized on blood agar.

3. Saliva

The chemical compositions of saliva is composed of several ions such as sodium, potassium, calcium, chloride, bicarbonate and phosphate with different concentrations. Saliva also contains a variety of enzymes such as amylase in large numbers, and also lysozyme and hyaluronidase (14). According to Cruickshank (1931), the baby's mouth flora after birth still in a sterile condition. Within 6-10 hours after birth, the baby’s

![Figure 1. (A, B) Clinical features black stain on teeth is contained in the third cervical (Braz Dent j. 2003; 14 (3): 157-61).](image-url)
mouth flora will be contaminated by staphylococci bacteria and some other bacterias. The number of bacteria on the mouth will increase rapidly on the second day. After 12 days, Streptococcus salivarius was found as a single species, and is characterized by reduction of microorganism (15).

Lammers (1952) stated that the balance of oral flora can be seen from the development of bacteria in the oral cavity. This is something that fixed and distinctive in each individual, but in contrast to other individuals. Biological balance will maintain antibacterial reaction in the oral cavity. Antibacterial substances from saliva is a product from metabolic of Streptococcus viridians and Lactobacillus (15). Along with the development of dental plaque, oral flora change with circumstances, in which micro-organisms that dominate is a gram-positive rod bacteria and filamentous organisms such as Corynebacteria and Actinomyces (16).

**Material and Methods**

Type of the research is Laboratory and design of this study is an observational laboratory.

**Working definition:**
1. Saliva is a liquid in oral cavity as the result of parotid gland secretion, submandibular and sublingual. How to take saliva sampling is with instructions to spit without given stimulants. Nominal scale.
2. The quantity of gram-positive, rod shaped bacteria (Actinomyces) represents the number of gram-positive, facultatively anaerobic, microaerophilic, branched, catalase-negative rod shaped bacterial colonies. Be calculated by Colony Forming Units per milli liter of diluted saliva. Nominal scale.

There are differences in the quantity of gram-positive rod shaped bacteria (Actinomyces) in saliva of children with and without black stain on the enamel surface (Table 1). Saliva sample was collected from the study subjects according criteria. Number of sample 15. Techniques of data collection is consecutive sampling. Techniques of data analysis were analyzed by t-test is not paired with the device SPSS with significance limit of $p \leq 0.05$.

**Results**

The research was conducted at the IPEKA International Christian School, Meruya and Laboratory of Microbiology, Faculty of Medicine, University of Indonesia on October 5 - December 23, 2011. Under this method, consecutively, of the study population, amounting to 615 children, found subjects who met the inclusion criteria as many as 30 children.

Here is the calculation of the number of colonies of Actinomyces in saliva of children with black stain and children without black stain (Figure 2).

From Table 2. It can be said that there is no significant difference in the quantity of Actinomyces in saliva of children with black stain and children without black stain ($p > 0.05$).

**Discussion**

This research is a study about the differences in the quantity of Actinomyces in saliva of children with black stain and a child without a black stain on the enamel surface, which is a preliminary study to identify the quantity of Actinomyces in saliva as well as seeking the etiology of the black stain on the enamel surface of the child. Research in Denmark for 11 children aged 3-5

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**Table 1. Distribution of the number of colonies of Actinomyces in saliva of children with and without black stain**

<table>
<thead>
<tr>
<th>Subject</th>
<th>CFU ($\times 10^7$ kol/ml)</th>
<th>With black stain</th>
<th>Subject</th>
<th>CFU ($\times 10^7$ kol/ml)</th>
<th>With black stain</th>
</tr>
</thead>
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<tr>
<td>1.1</td>
<td>48.20</td>
<td>2.1</td>
<td>44.60</td>
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<td></td>
</tr>
<tr>
<td>1.2</td>
<td>48.30</td>
<td>2.2</td>
<td>22.40</td>
<td></td>
<td></td>
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<tr>
<td>1.3</td>
<td>35.50</td>
<td>2.3</td>
<td>14.80</td>
<td></td>
<td></td>
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<tr>
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<td>10.90</td>
<td>2.4</td>
<td>51.60</td>
<td></td>
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<tr>
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<td>2.5</td>
<td>10.10</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>15.10</td>
<td>2.6</td>
<td>49.80</td>
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</tr>
<tr>
<td>1.7</td>
<td>60.30</td>
<td>2.7</td>
<td>8.50</td>
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<tr>
<td>1.8</td>
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<td>2.8</td>
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<td>2.12</td>
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<td>21.50</td>
<td>2.13</td>
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</tr>
<tr>
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<td>42.10</td>
<td>2.15</td>
<td>24.20</td>
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</tr>
</tbody>
</table>

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**Figure 2. Clinical features black stain that found in patient.**
years who had a black stain, there are significant differences in
the micro flora of plaque from teeth without black stain and with
black stain. In dental plaque with black stain, gram-positive
rod-shaped bacteria in 90% of the organisms in the acquired pel-
cicle, this is a very high percentage compared to the number of
gram-positive rod-shaped bacteria in normal dental plaque
which is 35-42% of the organisms in the acquired pellicle. Gram-positive rod-shaped bacteria are the most prevalent and
found in all samples. Most of the isolated microorganisms are
facultative anaerobic, microaerophilic, branched, catalase neg-
ative that are characteristic of Actinomyces and Arachnia (9).
This research will study the quantity of Actinomyces in saliva
of children with black stain.

This study is an observational laboratory research, by using
the method of consecutive sampling, all subjects are available
and meet the criteria for inclusion in the study subjects to
the number of subjects required to be fulfilled. Under this method,
consecutively, of 619 children were examined, obtained 30 sub-
jects consisting of 15 children with black stain more than 8 sur-
faces of tooth enamel and 15 children without black stain. Along
with research at the State University of Iowa to 355 children,
the prevalence of black stain found in 11-14% of all samples
(10). Research in India to 1472 children with an average age of
9.3 years, found 18% of the sample suffered from black stain
(11). Homogeneity subjects done by choosing the subject of a
specific community.

Black stain more common in school-age children who are in
the period of baby teeth or teeth mixed phases. According to the
nature of primary teeth enamel surface that has a high perme-
ability and porosity levels higher than permanent teeth and en-
amel thickness that is thinner than the first-born permanent
teeth. Results of analysis of tooth enamel surface using SEM
found that the dimensions of the enamel prisms born slightly
smaller than the enamel permanent (17).

This study used a sample of saliva because there have been pre-
vious studies that prove that the quantity of Actinomyces in den-
tal plaque of children with significant black stain plaque number
compared to children without the black stain. While no studies
have concentrated on the assessment of the quantity of
Actinomyces in saliva of children with black stain. Therefore,
researchers are interested in studying and identifying the quant-
tity of Actinomyces in saliva of children with black stain. In se-
veral studies have shown that microorganisms in dental plaque
live in constant touch with the microorganisms in the saliva and
saliva have a role for the attachment of bacteria or antibacterial
to the surface of tooth enamel (18), thus expected quantity of
Actinomyces in saliva of children with black stains can also de-
scribe the quantity of Actinomyces in child plaque with black
stain.

Actinomyces are fertile and potentially pathogens organisms
in the mouth of humans and animals. Actinomyces is a major
component of dental plaque, especially in the approximal of the
teeth and is known to increase in some types of gingivitis
(16,19). In this study does not use samples from dental plaque
as it is known on the literature that Actinomyces will dominate
microorganisms on early colonization stages of plaque for-
mation, i.e. within 24 hours after cleaning and cleaning is not
done anymore (20). Other literature said that the plaque is domi-
nated by gram-positive, rod-shaped bacteria facultative anae-
robic within 2 days without cleaning (21). Along with the devel-
opment of dental plaque, oral flora change within circum-
stances, with the dominating microorganisms are gram-pos-
itive, rod-shaped bacteria and filamentous organisms such as
Corynebacteria and Actinomyces (16). Because one way or an-
other, it is difficult to conduct the homogeneity of the whole sub-
ject to not do the cleaning of the oral cavity within 24-48 hours.

Collecting saliva was done by asking subjects to spit into a ster-
ile container that has been prepared. Spitting implemented
without the use of stimulants and performed every 1 minute for
3 minutes. This is consistent with research in 2008 in Spain in
calculating Actinomyces in saliva, samples were also taken
from the saliva that is not being stimulated (22).

Dilution of samples by using liquid medium Brain Heart
Infusion medium that is enriched with nutrients, is used for cul-
turing certain types of bacteria, fungi and yeast. Breeding with
Actinomyces Isolate Agar in a sterile petri dish, then the petri
dish was put in an anaerobic jar and incubated at 37°C for 7 days
and observed up to 14 days. In accordance with research in Spain
that the samples were cultured, incubated at 37°C in an anaero-
bic atmosphere for 7 days to obtain maximum growth of
microorganisms. The number of bacteria in the samples was cal-
culated by the calculation of colony forming units (CFU) in mil-
illiters sample solution (22).

In order for this study, researchers using Difco Actinomyces
Isolate Agar which has a specific composition that can stimulate
the growth of Actinomyces. In this case, Actinomyces Isolate
Agar that were used to contain sodium casein which serves as
a source of nitrogen, asparagines as amino acids and organic ni-
trogen sources, sodium propionate which is a fermentation sub-
strate, dipotassium phosphate has the ability to maintain pH balance; magnesium sulphate is the source of sulfate and metal ions (23).

Results are seen in a petri dish preparation provides an overview of in the form of white sulphur granules from Actinomyces agar culture. As noted in the literature that the collection of this organism will look like yellowish sulphur granules. Also an anaerobic Actinomyces species are normal flora of the mouth. Actinomyces grow under anaerobic conditions in the blood or serum glucose that at 35-37°C (24). Seen a small white form, colonies on blood agar within one week. Due to the relatively slow growth, isolating this organism from specimens is difficult because other organisms develop rapidly and are likely to complicate the growing visibility of slow Actinomyces. Set of sulphur granules lesions are clues to a picture of the organism (16).

In some circumstances, these granules can be destroyed, do gram staining, was observed for gram positive branching filaments and cultures taken on the media selected (16,25). In accordance with the statement that four important criteria for the characterization and classification of bacteria include: morphology, cultural characteristics, physiological characteristics and pathogenicity (26). Characteristics of bacterial cultures can be obtained from the macroscopic and microscopic examination of bacterial colonies. Important taxonomic characteristics of microorganisms are their response to gram staining. So that in this study, the identification is also continued to perform gram staining of bacteria growing in a petri dish. Obtained the suitable picture of Actinomyces morphology. After that, the colonies were counted directly using a Colony Counter and Colony Forming Unit methods.

The results of calculations by the method of Colony Forming Unit found that the quantity of Actinomyces in saliva of children with black stain is higher when compared to the quantity of Actinomyces in saliva children without black stain. As argued that chromogenic bacteria cause staining on the tooth enamel and the most common bacteria found in the black stain is a species of Actinomyces. Black stain consist ferum sulfate which is the result of the formation of the reaction between hydrogen sulfide produced by bacteria and ferum in saliva (27). Actinomyces is also a normal flora in the oral cavity so that in this study Actinomyces in saliva children without black stain was also found albeit in lower quantities.

The results of data analysis to t-test of the two unpaired groups can be said that there is no significant difference between the quantity of Actinomyces in saliva of children with black stains and saliva children without black stain. The reason is that the process of black stain was also influenced by other factors. Quantity of Actinomyces is not a major etiologic of black stain process but there are other things that also intervene in the black stain on the enamel surface. As it is said that the black stain is a special form of dental plaque, in accordance with previous studies which reported that the pigment found in black stain is a collection of black insoluble ferric, sulfate ferum possibility. Set of iron ions usually leads to teeth having black stain. These findings indicate that ferum sulfate can cause black stains on plaque (4). One of the main requirements for the formation of metal sulfide is the denaturation of the protein pellicle. Denaturation occurs at extrinsic discoloration of the teeth. Increasing the amount of Fe and S ion occur in brown stain, while simultaneously increasing ferrum sulfide and stannic sulfide gives a strong black coloring (28).

Conclusion

The results of this study indicate the quantity of Actinomyces bacteria in saliva of children with black stain is higher than in children without black stain saliva. But the results of the statistical test using t-test, it was found that the quantity of actinomyces in saliva of children with black stain and without black stain did not differ significantly.

References