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Research Report

Differences of *Streptococcus mutans* adhesion between artificial mouth systems: a dinamic and static methods

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ABSTRACT

Background: *Various materials have been used for treating dental caries. Dental caries is a disease that attacks hard tissues of the teeth. The initial phase of caries is a formation of bacterial biofilm, called as dental plaque. Dental restorative materials are expected for preventing secondary caries formation initiated by dental plaque. Initial bacterial adhesion is assumed to be an important stage of dental plaque formation. Bacteria that recognize the receptor for binding to the pellicle on tooth surface are known as initial bacterial colonies. One of the bacteria that plays a role in the early stage of dental plaque formation is Streptococcus mutans (S. mutans). Artificial mouth system (AMS) used in bacterial biofilm research on the oral cavity provides the real condition of oral cavity and continous and intermittent supply of nutrients for bacteria.* ***Purpose:*** *This study aimed to compare the profile of S. mutans bacterial adhesion as the primary etiologic agent for dental caries between using static method and using artificial mouth system, a dinamic method (AMS).* ***Method:*** *The study was conducted at Faculty of Dentistry and Integrated Research and testing laboratory (LPPT) in Universitas Gadjah Mada from April to August 2015. Composite resin was used as the subject of this research. Twelve composite resins with a diameter of 5 mm and a width of 2 mm were divided into two groups, namely group using static method and group using dynamic method. Static method was performed by submerging the samples into a 100µl suspension of 1.5 x 10^8 CFU/ml S. mutans and 200µl BHI broth. Meanwhile AMS method was carried out by placing the samples at the AMS tube drained with 20 drops/minute of bacterial suspension and sterile aquadest. After 72 hours, five samples from each group were calculated for their biofilm mass using 1% crystal violet and read by a spectrofotometer with a wavelength of 570 nm. Meanwhile, one sample from each group was taken for its surface image using Scanning Electron Microscope (SEM).* ***Result:*** *The results showed that S. mutans biofilm mass in the group using static method was 0.34, while in the group using AMS method was 0.09. The results of the statistical analysis then showed that there was a significant difference (p=0.02) in the formation of bacterial biofilm mass between those groups. SEM image in the group using static method also showed that the attachment of S. mutans was more numerous and had a longer chain than in the group using AMS method.*
Conclusion: *There is a difference in the profile of S. mutans bacterial adhesion between using AMS method and static method.*

Key words: *biofilm; S. mutans; artificial mouth system*

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INTRODUCTION

Secondary caries could lead to a failure in restoration if occurred between the tissue of teeth and the edge of restoration. The incidence of dental caries process begins with the formation of biofilm, called as plaque. Based on data from Basic Health Research (Riset Kesehatan Dasar) in 2013, 93.998, 727 people in Indonesia suffered from dental caries.[1] *Streptococcus mutans* (*S. mutans)* are bacteria playing a role in the formation of dental caries. *S. mutans* have an ability to produce acids that play a role in the process of tooth demineralization.[2] Thus, a colony of *S. mutans* can indicate the early formation of dental plaque. These bacteria also have an ability to co-aggregate with

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other bacterial species in the early colonization stage of dental plaque formation, as a result, they are able to bind to different hosts of several types of molecules.[3]

Biofilms, moreover, are colonies of bacteria protected by a matrix against anti-bacterial agents. The process of formation of biofilm begins with an attachment (adhesion) on the surface of the objects. The adhesion of bacteria, therefore, is influenced by the surface structure of the bacteria as well as the structures of the microorganisms adhering to. Extracellular polymeric substances (EPS) located on the surface of the bacteria in the biofilm provide mechanical stability, mediate adhesion to the material, as well as establish cohesiveness and 3-dimensional polymer bonding on biofilm.[4] In the formation of biofilm using static method, the bacterial adhesions resulted tend to be higher and also have stronger interaction between the cells.[5] On the other hand, artificial mouth system (AMS) method is used simulate the formation of biofilms in the oral cavity as well as to evaluate the adherence of bacteria to surfaces through the dynamic conditions created.[6] This study aimed to compare the profile of S. mutans bacterial adhesion as the primary etiologic agent for dental caries between using static method and using artificial mouth (AMS), a dynamic method system.

MATERIALS AND METHOD

This study was conducted at the Faculty of Dentistry and Integrated Research and testing laboratory (LPPT) in Universitas Gadjah Mada from April to August 2015. RK disc-shaped samples were made using molds made from PVC and plastic-coated on the inside with a diameter of 5 mm and a thickness of 2 mm. Plate glasses were placed on the surface of the molds that had been filled with RK, and the irradiation was performed using the LED light curing unit with a wavelength of 460 nm of more than 20 seconds to enable the polymerization reaction. The surface of RK was polished using finishing and polishing dics with different levels of roughness. Next, the samples were removed from the molds using the tweezers and put in a microtube wrapped in aluminum foil and stored at 37° C. The samples then were avoided from any form of contamination on the surface of RK.

AMS model was made as a modification of Ikeda's and Rahim's procedures.[7,8] AMS model consisted of two transparent tubes made of glass with a diameter of 10 cm. The first tube was used to accommodate BHI broth as a nutrient medium for bacteria, while the second tube kept RK samples in anaerobic state and also avoided RK samples from contamination. The base of the first tube was closed with a rubber stop connected with a hypodermal needle to regulate the amount of media droplets on the second tube.

The bottom surface of the second tube then was given valve to remove the rest of the media in the bottom of the tube. Falcon tube that had been cut and closed its upper surface with a wire was laid on the second tube. The second tube then was placed in an incubator with a thermostat set at 37° C. Container of sterile distilled water was used as a rinse of the samples connected with a hipodermal needle to set the number of droplets in the second tube. The first tube and sterile distilled water were placed on a pillar with a height of approximately 50 cm from the second tube. Droplets of the first tube and sterile distilled water were centered in the middle of the sample RK7. AMS scheme is showed in Figure 1.

In the static method, RK samples were put into a sterile tube with a polished surface facing up. Bacterial suspensions were 100 mL and 200 mL of BHI broth put into a sterile tube. The samples then were incubated at 37° C for 72 hours. Every 24 hours media replacement was conducted as much as 100 µl.[8] Meanwhile, in the AMS method, the samples were placed at the bottom of both RK tubes of AMS with polished surface position facing upwards. The suspension of bacteria then was inserted into the tubes using a hypodermic needle. BHI broth media was inserted into the tubes with the speed of 20 drops/ minute. Samples in the AMS then were put in an incubator at 37° C.

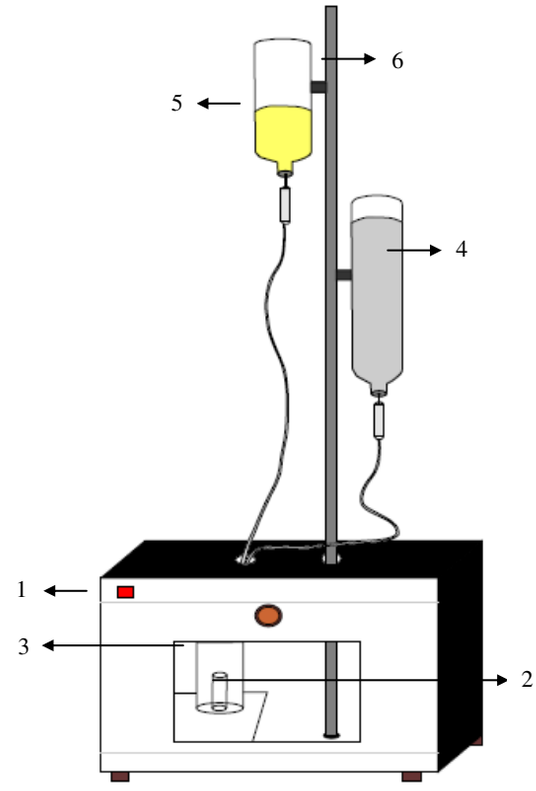

Figure 1. Schema of AMS model.
Note: 1) Incubator with a thermostat of 37° C; 2) falcon tube covered with strimin as a place to put RK samples; 3) tubes used for maintaining anaerobic conditions and avoiding contamination from RK samples; 4) sterile distilled water placed 50cm from the second tube and set at 20 drops/minute; 5) the first tube placed 50cm from the second tube and set at 20 drops/minute; 6) pillar.

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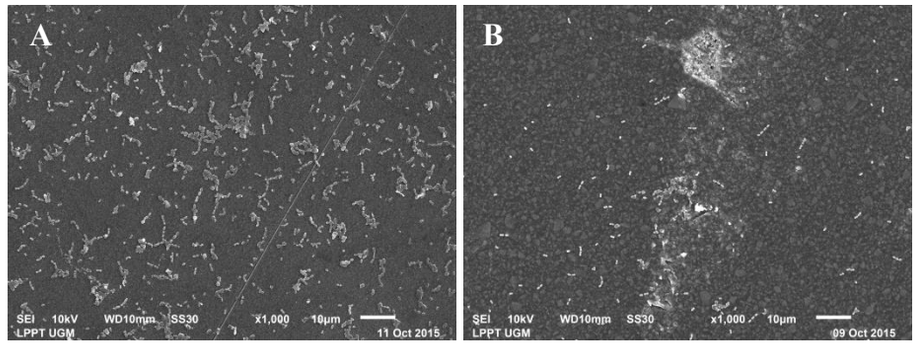

Figure 2. SEM images of the group using static method (A) and the group using AMS method (B).

Tabel 1. Optical density values on the use of static method and AMS method

No. Sample	Static Method	AMS Method
1	0.25	0.11
2	0.33	0.07
3	0.37	0.12
4	0.48	0.07
5	0.28	0.08
Mean ± SD	0.34 ± 0.09	0.09 ± 0.02

The images of the restoration material surface of RK were analyzed using SEM, a modification of procedures conducted by Fu.[9] Media in *microtube* and AMS were discarded, and the surface of RK was washed using PBS to clean up the remaining BHI broth attached to the surface. One of RK samples from each group was fixed with 4% paraformaldehid solution with a pH of 7.4 for 30 minutes. RK samples that had been fixed were inserted into alcohol 70%, 80%, 95%, and absolute alcohol to the dehydration process. RK samples then were dried with aerated, and polishing was conducted using gold to be observed with SEM at 10kV.

Afterwards, five samples of each group were washed three times using sterile distilled water. Those samples then were immersed in a solution of 1% gentian violet and incubated for 20 minutes. After 20 minutes, the solution was discarded, and the samples were washed using alcohol-acetone mixture with a ratio of 80:20 v/ v for 3 times. The determination of biofilms then was performed using a spectrophotometer with a wavelength of 570nm.[10]

Data analysis were conducted in two stages. The first stage was a qualitative analysis of the results of SEM imaging, while the second stage was a quantitative analysis of bacterial biofilm mass in the form of optical density (OD) using spectrophotometer. In the second stage, statistical analysis were also performed using Independent Sample t-test.

RESULTS

The results of this research on the biofilm attachment of *S. mutans* bacteria showed the value of OD as presented in Table 1.Table 1 shows that the mean value of OD in the group using AMS method was smaller than in the group using static method. The results of these calculations then were analyzed using Independent Sample t-test. P value obtained was 0.02 ($p<0.05$) indicating that there were significant differences in OD between the group using AMS method and the group using static method.

The results of SEM image in each treatment group can be seen in Figure 2. A number of the colonies on the surface of the samples using AMS method were less than using static method. Morphology of *S. mutans* bacteria in the group using static method showed a longer chain than in the group using AMS method (Figure 2).

DISCUSSION

Biofilm is a collection of bacteria attaching to surfaces, and its formation occurs in response to environmental changes.[11] Biofilm is composed of micro-colony of bacterial cells (15-20% of volume) dispersed in a matrix or glycocaly*x* (75-80% of the volume).[12] Based on DVLO theory, total interaction between the surface and the particles is a combination of Van der Waals bonds and Coulomb interactions. The existence of charged particles in liquid environment even will cause the formation of a double electric layer because of the withdrawal of ions on the surface of the particles. The majority of the bacteria in a liquid environment have negative particles.[13]

Consequently, based on the results of this research, OD values in the group using static method (immersion) were higher than in the group using AMS. It means that the biofilm mass of *S. mutans* bacteria in the group using static method was heavier than in the group using AMS. The existence of a nutrient in a liquid environment BHI broth provides an opportunity for the adhesion of the bacteria to the surface of objects.[14] In the use of AMS method, the

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flow of nutrients and distilled water on the surface of a material actually is capable of lowering the ionic strength so that when the bacteria are trying to identify the surface, then energy barrier will be generated causing the bacteria to move away from the surface.[15]

SEM images, moreover, showed that there were differences in the attachment of *S. mutans* bacterial colonies to the surface of RK between the groups. The adhesion in the group using static method was higher than in the group using AMS method. In addition to the change of ionic charge on the surface, the process of the bacterial adhesions is also affected by EPS in the form of mucus layer on the surface of the bacteria.[4] EPS matrix consists of polysaccharides and other macromolecules, such as proteins, DNA, lipids, and some other substances often found in the attachment of bacteria on the surface of objects. Interaction between EPS and the surface is actually caused by non-covalent bonds that have weak connective power than covalent bonds.[16]

As a result, dynamic environment at AMS can cause bonding between EPS and the surface becomes weak, and its attachments are reversible so that co-aggregation of the bacteria can be prevented. Thus, the use of AMS on bacterial biofilm researches can evaluate both the oral cavity microbe interactions in dental plaque that are stimulated and the same biofilm, as well as monitor the physical, chemical, biological, and molecular aspects with high accuracy.[6] Artificial mouth system is even capable of supplying nutrients continuously interspersed with the cleaning done by sterile distilled water as the substitute of saliva. It can be concluded that there are differences in profiles of biofilm attachment of *S. mutans* bacteria on the use of AMS method and static method.

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