Antigenic Protein Profile of *Streptococcus mutans* Biofilm For Developing of Dental Caries and Periodontal Disease Risk Biomarker

To cite this article: M Ni'mah et al 2019 *IOP Conf. Ser.: Earth Environ. Sci.* 217 012050

View the article online for updates and enhancements.
Antigenic Protein Profile of *Streptococcus mutans* Biofilm For Developing of Dental Caries and Periodontal Disease Risk Biomarker

M Ni’mah¹, I L Kriswandini², A Baktir³*

Department of Microbiology Faculty of Sains and Technology¹, Department of Microbiology Faculty of Dentistry² - Department of Chemistry Faculty of Sains and Technology¹ Universitas Airlangga, Surabaya, Indonesia

* afaf-b@fst.unair.ac.id

**Abstract.** *Streptococcus mutans* is a virulent and biofilm forming bacteria causing dental caries. Dental caries lead to several diseases, including mediastinitis, sepsis, facial cellulitis, osteomyelitis, endocarditis and pneumonia. Alternative effort to suppress dental caries prefalency is early diagnostic of dental caries risk using protein biomarker. The aim of the research is to determine the antigenic protein profile of *S. mutans* biofilm as biomarker of dental caries and periodontal disease risk. *S. mutans* biofilm was formed on glass slide submerging in BHIBS media for 24 hours. Crude proteins of biofilm were obtained by lysing it using ultrasonication 7 x 30s, 40 Hz. Protein profiles of *S. mutans* biofilm was done by using SDS-PAGE with separation gel of 12%. Antigenic analysis of the proteins biofilm were done by Western blot method using sIgA contained in the dental caries free saliva, compared with dental caries saliva as control. Protein profile of *S. mutans* biofilm consist of seventeen bands of MW 181, 176, 172, 154, 105, 90, 70, 58, 52, 40, 29, 22, 19, 16, 14, and 13 kDa, while the antigenic reaction towards sIGA were the protein with MW of 105, 52, and 29 kDa.

**Key Words:** *S. mutans*, Biofilm, Antigenic protein profile, Caries risk biomarker.

1. Introduction

About 80% of infections in the human body are caused by biofilms, such as respiratory tract infections, urinary tract infections, intestinal infections, periodontal disease and dental caries (Neilands, 2007). The term of biofilm is used to describe a set of microorganisms adhering on the surface of supported material and covered by extracellular matrix of the cell and environment (Marsh, 1999). Composition of the biofilm are microbial cells and EPS which are the main content of the biofilm, about 50 to 90%, and water (Donlan, 2002). *Streptococcus mutans* are virulent bacteria on the formation of an exopolysaccharide matrix in the dental biofilm. The formation of dental caries are started by biofilm of glucan matrix which is synthesized by *S. mutans*.

Early diagnose a person susceptible to dental caries or periodontal disease, followed by special dental maintenance by a dentist, will suppress the incidence of dental caries. Agent for dental caries diagnostic can be developed from proteomics study, which includes the determination of antigenic proteins that compose *S. mutans* biofilm.

When the surface of supported materials coated with proteins, for example the proteins from saliva, then the microbes will stick on the surface and multiply themselves to build microcolonies. Some of the microbes will leave biofilm and colonize on the new surface. Subsequently, the microbe produces extracellular matrix consisting of polysaccharides, then the biofilm grows and develops.

Important factor in determining the prevalence of caries is salivary substances (Petersen, 2005). Salivary secretory IgA (sIgA) is the important main factor, beside pH and flow rates of saliva in determining the oral mucosal immunity (Benderli *et al*, 2000). sIgA is the main Ig in saliva and is considered to be the prominent specific defence mechanism in the oral cavity. sIgA have role in controlling the oral cavity against disease, including dental caries and periodontal disease, by inhibiting microbial adherence to epithelial and tooth surfaces. Salivary immunoglobulin levels were
determined by genetic factors, and may also change depending on the salivary flow rate, hormonal factors, emotional states, physical activity and environmental factors (Russell et al., 1999). A lower concentration of slgA is considered to be risk factor for periodontal disease and dental caries. A lower concentration of slgA is also susceptible for upper respiratory infection in children and the elderly (Koga-Ito et al., 2004). Numerous studies have reported the protective role of salivary slgA against dental caries in both children and adults (Bratthall et al., 1997). The aim of the present study was to investigate the protein antigenic biofilm \textit{S} \textit{mutans} as marker for the level of salivary slgA which correlate to dental caries and periodontal risk.

2. Experimental Methods

2.1 Bacteria strain, samples and media.
\textit{S. mutans} was obtained from the culture collection of Microbiology Laboratory, Faculty of Dentistry, Universitas Airlangga. The growth media were BHIB (Brain Heart Infusion Broth), BHIIS and TYC (Tripticase Yeast Cystine). Sample of saliva from individual with dental caries and non caries were obtained from Ittaqu boarding school (Jl. Moris Menanggal Surabaya) using a suction method. Materials for SDS-PAGE consist od APS (Bio-Rad, cat 161-0700), Broad Marker Protein (Bio-Rad) and Coomassie blue R-250 (Bio-Rad), Mini Protean II electrophoresis device (Biorad, USA), and Western blot device (Biorad, USA).

2.2 Rejuvenation of \textit{S. mutans} in TYC solid media.
The solid media for \textit{S. mutans} was made by weighing tryptone 15g / L, yeast extract 5g / L, L-cystine 0.2g / L, Na2SO3 0.1g / L, NaCl 1 g / L, Na2HPO4 0.8 g / L, Na2CO3 2g / L, CH3COONa 12g / L, sucrose 50g / L, Agar 12g / L, then dissolved with distilled water and sterilized by autoclaving. After the lukewarm, then the media was poured in a petri dish. After the media become cold, \textit{S. mutans} from the old stock was etched with a wire loop.

2.3 Growth of \textit{S. mutans} in the BHIB media.
The liquid media for the growth of \textit{S. mutans} were BHIB 37g dissolved in 1 L destilled water and sterilized in an autoclave. Single \textit{S. mutans} colony on TYC solid media were transferred into liquid medium BHIB 10ml, then incubated at 37°C for 24 hours in candle jar. Furthermore, \textit{S. mutans} were put into BHIB media by taking a single colony of \textit{S. mutans} from the solid media TYC with a sterile wire loop. Then, the media were incubated 37 ° C for 24 hours in a candle jar.

2.4 Formation of \textit{S. mutans} biofilm.
BHIBS media for the biofilm formation was made by weighing 37g LBHIB and 2% sucrose. \textit{S. mutans} breeding were centrifuged at 8000rpm for 10 minutes, then the cell pellets were dropped on a slide glass of 7.5 cm x 1.25cm. After the incubation for 2 hours, biofilm were removed from erlenmeyer by solution of chloramphenicol 0.8mg / ml for five times. Subsequently, the sterile slide glass will be put into BHIBS media and incubated at 37 ° C for 22 hours with a jar candle.

2.5 Harvesting of \textit{S. mutans} biofilm.
After 22 hours incubation, the biofilm were put into a sterile eppendorf tube 1.5ml by using a sterile wire loop, and was added with 1ml TEM buffer pH 6.8, containing 10 mM Tris-HCl, 1 mM EDTA and 5mM MgSO4.

2.6 Lysis of \textit{S. mutans} biofilm.
The harvested biofilm were lysed by using ultrasonication method 7 x 30s, 40Hz. Then, the lysate were stored in -20 ° C, and can be used at any time for analysis of \textit{S. mutans} biofilm profile using SDS-PAGE.

2.7 Analysis of the protein profile of \textit{S. mutans} biofilm.
Producing a separation gel (12%) with reagents include (Acrylamide 2.5 mL, Tris HCl (pH 8.8) 1.2 mL, 0.5% SDS 1.2 mL, 1 mL of distilled water, 50 µL TEMED, APS 10% 30 mL). Then, it is put a 12% running gel through walls up to a limit of 1 cm from the top. After that, it is added with butanol ± 1 mL and allowed 25 minutes.
Producing stacking gel with reagents include (Acrylamide 0.66 mL, Tris-HCl (pH 8.8) 0.8 mL, SDS 0.5% 0.8 mL, TEMED 4μL, APS 10% 20 µL). After the gel solidifies, the butanol was discarded and put through the wall until full. Comb was put and left to stand for 25 minutes, and taken. Furthermore, the gel mold was taken and put into the Bio-Rad (Elektroforesa). Before the protein sample was inserted to wells gel, the first action was to do a sample preparation by adding a solution of 5x sample buffer (1M tris-HCl, 50% glycerol, 10% SDS, 2-mercaptoethanol, 1% Bromophenol blue, and aquadest).

Inserting protein sample in wells gel. Then, electricity was operated with \( V = 125, \ mA = 60 \). After the blue color is go down, the tool is switched off and the gel was taken. Then, it was put on petridisk. The coloration with staining solution (Comassie blue, methanol, acetic acid, H2O), and shaken for 5-10 minutes. After that, the gel was washed with a destaining solution (methanol, acetic acid, H2O), and shaken for 5 minutes. Washing with destaining solution was done for 3x, and then the gel was shaken for 30 minutes in the third washing process. Furthermore, the gel was soaked with sterile distilled water for 5 minutes.

2.8 Analysis of antigenic protein of *S. mutans*.

The Western blot method was used to determine the antigenic protein of *S. mutans* biofilm. We used sIgA contained in caries free human saliva. There were 4 samples saliva from from free dental caries and 4 saliva sampels from dental caries. Protein antigenic related to dental caries will be bound by salivary sIgA.

The process was started by soaking the SDS-PAGE gel with distilled water for 5 minutes. Furthermore, the gel was soaked with transfer buffer solution for 30 minutes. Nitrocellulose membrane (NC) was soaked first for 5 minutes before used, and then soaked with transfer buffer solution for 10 minutes. Next, the filter paper and sponge was soaked with transfer buffer solution. Then, the materials were arranged in the following order: a sponge, 3 filter paper sheets, NC, gel, two sheets of filter paper, and sponge.

The protein transfer was performed at 100V for 2 hours at 4 °C. The membrane was ponceau for 5 minutes, after which the membrane NC was blocked with TBS-BSA 5% at 4 °C overnight. Then, the membrane was washed with TBS-tween 0.2% 3x5 minutes and primary antibody in TBS was added and left for 2 hours, then washed with TBS-tween 0.2% 3x5 min. Secondary antibody was then added and allowed to stand for 1 hour, then washed with TBS-tween 0.2% 3x5 min. SA-HRP was poured and incubated for 1 hour at room temperature, then the membrane was washed with TBS-tween 0.2% 3x5 min. The TMB substrate was inserted and left for 15 minutes in a dark room, and the reaction was stopped with distilled water.

3. Result and Discussion

3.1 Growth of *S. mutans* in the BHIB media

The growth of *S. mutans* cells in BHIB media was characterized by yellowish white color. *S. mutans* grew well when planted in fortified medium such as Brain Heart Infusion Broth (BHIB) in anaerobic condition. *S. mutans* cells formed long chain coccus. *S. mutans* grew well in the BHIB media as planctonic free cells. However, at the end of growing phase, after 24 hours incubation time, *S. mutans* cells attached to the base of erlenmeyer as biofilm (Figure 1). At the end of growing phase, the availability of nutrients especially carbon source began to decline. This condition induced the formation of biofilm. Generally, biofilm microorganism can be induced by (1) using minimal medium like spider medium (Baktir *et al.*, 2014), or (2) by extending incubation time to achieve stationary phase where carbon source drop dramatically (Baktir *et al.*, 2014), or by using specific carbon source that will be used in the present study to synthesize extrapolymeric substance “glucan” (Figure 2). Masfufatun *et al* (2017) report that a lack of nutrients induces biofilm formation in vitro, while in vivo biofilm formation is induced by treatment with antibiotics and corticosteroids. Suspension of *S. mutans* after growing on BHIB media are shown in Figure 1.
3.2 Growth of *S. mutans* Biofilm.

Biofilm *S. mutans* grow attached to the glass slide after 24 hours incubation. Biofilm *S. mutans* was yellowish white colored. The biofilm composition consists of microorganisms, extracellular product and extrapolymeric substance, eg. glucan as adhesive agent (Baktir, et al., 2014). *S. mutans* biofilm with a yellowish color grew adhere to the slide glass is submerged in the media after incubation for 22 hours.

Sucrose is specific substrate for glucosyltransferase and fructosyltransferases, enzyme produced by *S. mutans* to synthesize polymer of glucose with a high molecular weight consist of glycoside bond alpha (1-6) and alpha (1-3). Communication between cells can be done such as: by releasing chemicals that will send a signal to the other cells far apart, establishing direct contacts through specific molecules in the membrane. The addition of chloramphenicol provides a pressure to *S. mutans* to form biofilms as a defense mechanism. In addition, chloramphenicol was bound to the 50s ribosomal subunit and inhibit peptidyl transferase enzyme as a catalyst to form a peptide bond in protein synthesis.
3.3 Lysis of S. mutans Biofilm
Biofilm lysis was done by sonication of 30s, 40Hz which was repeated seven times. During sonication process buffer provide protection against protein denaturation due to heat that was generated by ultrasonic vibrations.

3.4 Profile of biofilm proteins
The protein profile of S. mutans biofilm with dilution variations as shown in Figure 3.

![Figure 3. SDS PAGE profiles of S. mutans biofilm proteins. S1, S2 and S3: S. mutans biofilm proteins with 3x, without and 2x dilutions respectively. M: Size marker protein](image)

Rf of each band (Figure 3) were directly measured by using eraser (Table 1). The molecular weight of each protein biofilm was determined by inserting the Rf data to the following formula. \( Y = 0.359X^2 - 1.422X + 2.294 \).

| RF (X) | Log MW (Y) |
|--------|------------|
| 0.01   | 2.301      |
| 0.17   | 2.066      |
| 0.20   | 1.988      |
| 0.36   | 1.822      |
| 0.41   | 1.744      |
| 0.62   | 1.562      |
| 0.71   | 1.491      |
| 0.91   | 1.332      |
| 0.94   | 1.158      |
| 0.98   | 0.984      |

The molecular weight of each biofilm protein bands were 181, 176, 172, 154, 108, 90, 70, 58, 53, 43, 41, 28, 22, 19, 16, 14, and 13 kDa. The profile of S. mutans biofilm shows all its biofilm S. mutans protein composer. SDS system used in this research is a discontinuous system (Laemmli). In this system, the protein migrates quickly on stacking gel and separating gel, so that the protein is
concentrated in a thin line in the form of thin tape or band. In addition, the optimization of the concentration of polyacrylamide previously has been made at concentrations of 12%. The increase of SDS on electrophoresis process serves to make protein become denatured, so it shaped primary structure (elongated) by binding the hydrophobic residues from each amino acid.

3.5 Profile of antigenic biofilm proteins of S. mutans
Analysis of antigenic biofilm proteins of S. mutans was conducted by using Western blot method, using caries free saliva containing high sIgA and caries saliva (low sIgA). Saliva sample that contained low sIgA (lanes 1, 2, 3 and 4) profide protein bands thinner than the samples that contained high sIgA (lanes 5, 6, 7 and 8). Data from the analysis of antigenic proteins as shown in Figure 4.

![Figure 4](image)

Figure 4. Western blot analysis of antigenic proteins biofilm of S. mutans against caries saliva (low level sIgA) and caries free saliva (high level sIgA). Lanes 1, 2, 3 and 4: low level sIgA; Lanes 5, 6, 7 and 8: high level sIgA; M: size marker.

Analysis of crude protein using Western blot method aim to determine the antigenic proteins of S. mutans biofilm. The principle work of the Western blot is the epitope introduction of certain proteins by the primary antibody. Hence, the primary antibody are recognized by the secondary antibody which is a contra for the primary antibody. Secondary antibody binds to an enzyme that can bring color after reacting with the substrate. The enzyme used is SA-HRP (Streptavidin-horseradish peroxidase) and biotin conjugate, then it reacts with the enzyme substrate TMB (3,3′,5,5′-Tetramethylbenzidine). The labeling is intended to bring up the color so that the interaction between the epitope of the target protein with paratop can be observed.

We have found 4 types of antigenic protein that has a molecular weight of 105, 52, 40, and 29 kDa (Figure 4). The antigenic proteins showed the ability to distinguish individu with low (samples 1,2,3, and 4) dan high secretory IgA (samples 5,6,7, and 8) in their saliva. The band thickness differeny (Figure 4) indicate the difference in the concentration of sIgA in saliva samples. Individu with caries or caries risk showed thin or no band. While caries free and low risk individu will show thick bands. These antigenic proteins are capable of recognizing human specific epitopes of the four proteins of S. mutans biofilm which cause dental caries.

4. Conclusion
The protein profile of S. mutans biofilm on SDS-PAGE 12% as many as 17 bands with a molecular weight of 181, 176, 172, 154, 105, 90, 70, 58, 52, 40, 29, 22 , 19, 16, 14, and 13 kDa. While the antigenic protein profile of S. mutans biofilm as a biomarker of risk of dental caries has a molecular
weight of respectively 105, 52, and 29 kDa. We will develop biomarker caries risk from the antigenic proteins.

References
[1] Baktir A., Masufatun, Hanum G.R., Amalia K.R and Purkan, 2014, Construction and Characterization of the Intestinal Biofilm Model of *Candida* spp., RJPBCS 5(1), p 204-211
[2] Donlan M.R., 2002, Biofilms: Microbial Life on Surfaces, Centers for Disease Control and Prevention, Vol. 8, No. 9
[3] Marsh P., Martin V., 1999, Oral Microbiology fourth edition, Reed Educational and Publishing Ltd, England, p.58-82
[4] Masufatun, Bayasud S.L., Yasinta M.S., Ni’matuzahro, Baktir A., 2017, Serum Acetaldehyde as a Potential Biomarker for The Detection of Pathogenic Biofilm Formation by *Candida albicans*, Journal of Chemical Technology & Metallurgy. Vol. 52 Issue 6, p1032-1038.
[5] Neiland J., 2007, Acid Tolerance of Streptococcus mutans Biofilm, Malmö university, p.16-24
[6] Petersen PE., 2005, Sociobehavioural risk factors in dental caries –international perspectives. Community Dent Oral Epidemiol;33:274–279.
[7] Benderli Y, Erdilek D, Koray F, Telci A, Turan N., 2000, The relation between salivary IgA and caries in renal transplant patients. Oral Surg Oral Med Oral Pathol Oral Radiol Endod;89:588–593.
[8] Russell MW, Hajishengallis G, Childers NK, Michalek SM., 1999, Secretory immunity in defense against cariogenic mutants streptococci. Caries Res ;33:4–15.
[9] Koga-Ito CY, Martins CA, Balducci I, Jorge AO. Correlation among mutans streptococci counts, dental caries, and IgA to Streptococcus mutans in saliva. Braz Oral Res 2004;18:350–355.
[10] Bratthall D, Serirarch R, Hamberg K, Widerstrom L., 1997, Immunoglobulin A reaction to oral streptococci in saliva of subjects with different combinations of caries and levels of mutans streptococci. Oral Microbiol Immunol;12:212–218.