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Research Report

Analysis of anti-*Streptococcus sanguinis* IgY ability to inhibit *Streptococcus sanguinis* adherence

Suryani Hutomo,[1] Heni Susilowati,[2] Dewi Agustina,[3] and **Widya Asmara[4]**
[1] Department of Microbiology, Faculty of Medicine, Universitas Kristen Duta Wacana
[2] Department of Oral Biology, Faculty of Dentistry, Universitas Gadjah Mada
[3] Department of Oral Medicine, Faculty of Dentistry, Universitas Gadjah Mada
[4] Department of Microbiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada
Yogyakarta - Indonesia

ABSTRACT

Background: *Streptococcus sanguinis (S. sanguinis), an oral commensal bacterium, is often implicated in infective endocarditis. Its adherence to the tooth surface is the initial step in dental plaque formation. In addition to the important role of S. sanguinis in systemic disease and antimicrobial resistance, it is necessary to develop methods to control dental plaque formation. Immunoglobulin Y (IgY) has been used to prevent bacterial infection.* ***Purpose:*** *The purpose of this study is to analyze the ability of anti-S. sanguinis IgY antibodies to inhibit S. sanguinis adherence to hydroxyapatite (HA) discs as a model of the tooth surface.* ***Methods:*** *Antibodies were produced by immunizing hens with S. sanguinis suspension. Boosters were given three times following the first injection. An agar gel precipitation test (AGPT) was used to detect the presence of anti-S. sanguinis IgY. A bacterial adherence assay was performed twice to analyze the ability of IgY and the optimal concentration required to inhibit bacterial adherence.* ***Results:*** *The formation of a precipitation line using AGPT confirmed the presence of the antibody. In addition, it was shown that the anti-S. sanguinis IgY antibody could inhibit bacterial adherence to HA. Statistical analysis using One-way ANOVA revealed a significant difference in the optical density (OD) value between the groups ($p<0.05$). The results of electron microscopy scanning confirmed the quantitative analysis by means of a bacterial adherence test.* ***Conclusion:*** *Anti-S. sanguinis IgY has the ability to inhibit adherence of S. sanguinis to HA discs at an optimal concentration of 30%. The inhibitive effect was stronger in the presence of saliva.*

Keywords: *Streptococcus sanguinis; IgY; bacterial adherence*

Correspondence: Suryani Hutomo, Department of Microbiology, Faculty of Medicine, Universitas Kristen Duta Wacana, Jl. Dr. Wahidin Sudirohusodo No. 5-25, Kotabaru, Gondokusuman, Yogyakarta 55224, Indonesia. E-mail: suryanihutomo_drg@yahoo.com

INTRODUCTION

Streptococcus sanguinis (*S. sanguinis*) is known as a primary colonizer of the tooth surface. These bacteria can attach to the tooth surface and the oral epithelium by means of adhesin, a cell wall protein component.[1] It attaches to the hydroxyapatite (HA) surface which is the main component of tooth enamel through interaction with salivary glycoprotein in the acquired pellicle.[2] Maturation of dental plaque occurs in the presence of Gram negative bacteria such as *Veillonella*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis.*[3]

S. sanguinis is often implicated in infective endocarditis and among the viridans group of Streptococci it is, in fact, the most commonly involved.[4] It becomes attached to the fibrin and platelet vegetation that occurs in the heart valve, forming a biofilm that may cause bacteremia.[5] Poor quality of dental care is a predisposing factor in infective endocarditis, although the bacteria can also enter the body through the daily diet.[6] Besides its important role in systemic infection, control of dental plaque is necessary to obtain healthy gingival tissue and, thus, prevent systemic bacterial invasion. Due to the possibility of bacteremia as the result of dental care procedure, the prescribing of prophylactic antibiotics is sometimes necessary, especially in high risk patients. However, such prophylactic measures are not always effective. Previous studies reported the occurrence of amoxicillin-resistant *Streptococcus*

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viridans (*S. viridans*),[7] and also fluoroquinolone-resistant *S. viridans* which are responsible as the cause of bacteremia in neutropenic cancer patients.[8] Therefore, development of effective prophylaxis measures is necessary.

Hens produce polyclonal antibodies which play an important medical role. Immunoglobulin Y (IgY) is the predominant immunoglobulin in the serum of hens, amphibians and reptiles that is transferred from serum to egg yolks. Its function is to provide passive immunity to the embryo and the neonate. Immunoglobulin Y is similar in function to immunoglobulin G (IgG) in mammals.[9] In general, the molecular structure of IgY is the same as IgG, but the molecular weight of IgY is 180 kDa, heavier than that of IgG at 150 kDa.[10] In veterinary medicine, IgY is also used in therapeutic technology targeting enteric bacterial infection.[11] Specific IgY antibodies are obtained by immunizing the hen with the antigen of interest. In this study, hens were immunized with *S. sanguinis* to produce the anti-*S. sanguinis* IgY antibody. The aim of this study is to analyze the potential of the anti-*S. sanguinis* IgY antibody to inhibit *S. sanguinis* adherence to the HA discs as a model of the tooth surface. The final goal of the anti-*S. sanguinis* IgY antibody in this research is the control of dental plaque formation.

MATERIALS AND METHODS

Initially, *S. sanguinis* ATCC 10556 was cultured in brain-hearth infusion broth (BHI; Oxoid, Hampshire, UK). A 100 µl heat-killed suspension of *S. sanguinis* in Freund complete adjuvant (Sigma - Aldrich, Saint Louis USA) was then subcutaneously injected into hens in order to produce an antibody response. Three booster immunizations with heat-killed suspension of *S. sanguinis* in Freund incomplete adjuvant (Sigma-Aldrich, Saint Louis, USA) were administered over a time span of two weeks. Purification of IgY was completed according to the chicken IgY purification kit protocol (Sigma-Aldrich, Saint Louis, USA). Finally, the presence of antibodies was detected by performing an agar gel precipitation test (AGPT).

Human saliva was collected from three healthy volunteers who, before pooling their saliva, rinsed their mouths with water to decrease bacterial contamination. The saliva provided was then centrifuged for 15 minutes at 3000 g and 4° C. The supernatant was stored at -80° C prior to use.[12] The HA discs (10mm in diameter, 1.2 mm in thickness) were created by placing 500 mg of hydroxyapatite powder in a mould and pressed at 120 Mpa. Finally, discs were sintered for two hours at 1300° C. The sterilization of the hydroxyapatite discs was achieved by keeping the discs in the autoclave for 15 minutes at 100° C.[13]

Bacterial adherence assay was performed by modification of the Johansen method.[14] Saliva-coated HA discs were incubated with various concentrations of anti-*S. sanguinis* IgY antibody for 30 minutes at 37° C, then stimulated with 100µl of 1.5×10^8 colony forming unit (CFU) *S. sanguinis* suspension and incubated at 37° C overnight. After incubation, HA discs were washed with phosphate buffer saline (PBS) solution and fixed with 250µl absolute methanol for 15 minutes. Adherent bacteria were stained with 0.1% crystal violet and washed twice with PBS. Stained adherent bacteria were extracted from the disks using 96% ethanol and transferred to a fresh 96-well plate. The absorbance of the extract from stained adherent bacteria was measured at 595 nm using a microplate reader (Thermo Scientific, Rockford, Illionis, USA).[14,15]

To further analyze the ability of anti-*S. sanguinis* IgY to inhibit bacterial adherence in the presence and absence of saliva, additional bacterial adherence assays were performed using optimum IgY concentration in the four different treatment groups. The HA discs in groups I and II were coated with saliva, while in groups III and IV they were left uncoated. In group I, anti-*S. sanguinis* IgY antibodies were incubated with *S. sanguinis* before HA disks were added, whereas in group II HA disks were cultured with anti-*S. sanguinis* IgY antibodies before bacterial inoculation. The treatment for group III was the same as group II, while in group IV HA disks were cultured with *S. sanguinis* without antibodies. Observation by means of a scanning electron microscope (JEOL JED-2300; JEOL, Tokyo, Japan) was performed to provide a general overview of bacterial adherence to the HA disks.[16]

RESULTS

Anti-*S. sanguinis* IgY antibodies were detected using AGPT. The *S. sanguinis* antigens in the center well and the antibodies in the outer wells each diffused outward and became bound to each other, forming an antigen-antibody complex. This complex precipitated in the gel, forming a white precipitation line, while there was no such formation between the *S. sanguinis* and IgY from non-immunized hens (Figure 1).

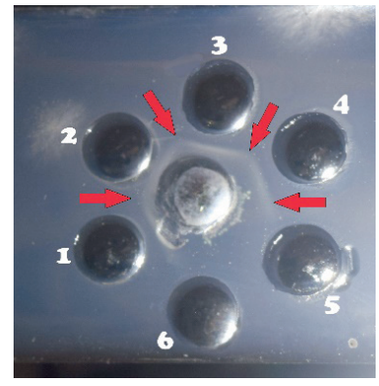

Figure 1. The result of AGPT. Arrows indicate the precipitation lines which formed between the *S. sanguinis* (center well) and the anti-*S. sanguinis* antibodies (wells 1, 2, 3, 4) while there was no such formation between *S. sanguinis* and IgY from non-immunized hens (wells 5 and 6).

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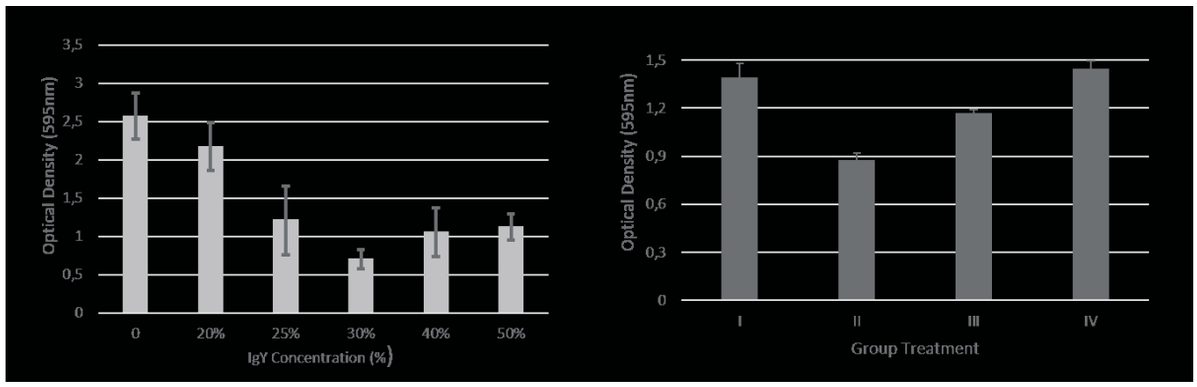

Figure 2. A) Optical density value of *S. sanguinis* adherence at various concentrations; B) optical density value of *S. sanguinis* adherence in four different treatment groups.

The bacterial adherence assay demonstrated that inhibition occurred at concentrations of 20%, 25% and 30%, characterized by a decreased optical density (OD) value. The inhibition of bacterial adherence can be observed in the treatment of 40% and 50% antibodies, but not to the same degree as the inhibition effect of 30% antibodies as seen in Figure 2A. Based on the results of the first bacterial adherence assay, the optimum concentration of antibodies for effective bacterial inhibition was 30%. Consequently, this concentration was employed for the second assay.

The result of the second bacterial adherence assay demonstrated that the lowest OD, and hence the greatest bacterial inhibition, occurred in group II, followed by groups III, I and IV respectively (Figure 2B). The data was analyzed using One-way ANOVA at a significance level of 0.05 and there was a significant difference between groups (p=0.000).

Observation through SEM confirmed these results. Numerous bacteria and matrix plaque were both present in group I, while HA crystals were not visible. In group II, bacteria and matrix plaque were present and HA crystals were also visible. Bacteria and matrix plaque could be observed but less so than in group I or group III. Considerable numbers of bacteria and thick matrix plaque were found in group IV (Figure 3). This confirms that anti-*S. sanguinis* IgY could more effectively inhibit *S. sanguinis* adherence to HA disks in the presence of saliva.

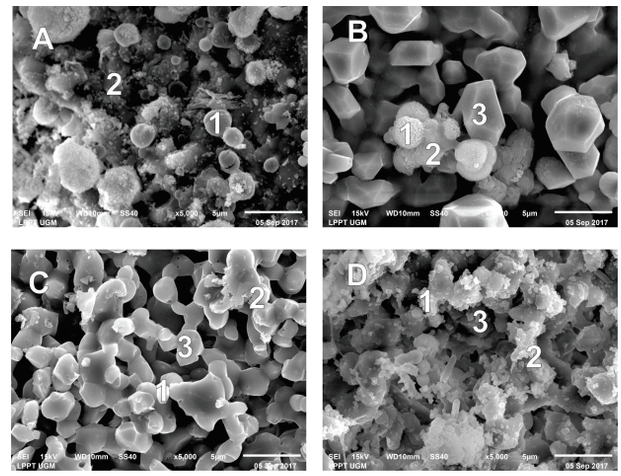

Figure 3. Scanning electron microscopy of HA surface colonized by bacteria. Group I (A): lots of bacteria were present (1-round shapes) as well as matrix plaque (2- dense mass, irregular shape), HA crystals were not visible. Group II (B): some bacteria (1) and matrix plaque (2), HA crystals were visible (3- regular solid mass with sharp borders). Group III (C): bacteria and matrix plaque were seen but fewer than in A. Group IV (D) Lots of bacteria and thick matrix plaque.

DISCUSSION

The bacterial adherence assay demonstrated that inhibition began at concentrations of 20% and increased at those of 25% and 30%. The inhibition of bacterial adherence still occurred through the administering of 40% and 50% antibody, but not as much as at a concentration of 30%. This phenomenon might occur if antibody concentrations above 40% result in a saturation of antigen-antibody bonds, rendering further adherence inhibition impossible. A similar dose response was observed in a study conducted by Fujibayashi *et al.* using anti-*Candida spp* IgY.[17]

The result of the second bacterial adherence assay demonstrated that the I *S. sanguinis* group that was exposed to the IgY anti-*S. sanguinis* antibody subsequently bound to each other to form an antigen-antibody complex. Proteases produced by the bacteria broke down the antibodies so that they were unable to bind to the bacteria.[18] In this group, free bacteria adhered to saliva-coated HA discs initiating the formation of a biofilm.

The lowest extent of bacterial adherence to disks occurred in group II. This was due to a strong affinity between the saliva and HA disks, and also between the saliva and antibodies. The tooth surface is negatively charged which means that positive salivary ions such as Ca^{2+}, Na^{+} and K^{+} are able to form strong bonds.[19] Saliva-tooth surface binding also occurred due to salivary glycoprotein on the tooth surface which was absorbed and

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rearranged by covalent hydrogen and ionic bonds.[20] The primary mechanism through which IgY limits the pathogen is by inhibition of adhesion such as blocking the surface epitopes required for binding and interfering with binding to mucin.[10]

Antibodies in group III were able to block surface epitopes on the HA disc, but not as effectively as group II because, while the IgY was able to cover the HA discs, it was unable to bond. Bacteria were, therefore, able to penetrate and adhere to the HA discs. In group IV, bacteria could adhere and form a biofilm on the HA discs in the absence of saliva and antibodies. In order to be able to colonize the tooth surface, the bacteria must adhere directly to it or other cells that can bind to the teeth.[21] The results of this study demonstrate that anti-*S. sanguinis* IgY can effectively inhibit bacterial adherence to saliva coated HA discs as a model of the tooth surface, thus limiting dental plaque formation. In conclusion, this study confirms that anti-*S. sanguinis* IgY antibody can inhibit *S. sanguinis* adherence to HA discs at an optimum concentration of 30%. The inhibition effect was stronger in the presence of saliva. This antibody can be recommended as a potential topical application in the oral cavity to control dental plaque formation.

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