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Research Report

The activity of polyclonal IgY derived from *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* in inhibiting colonization of *Fusobacterium nucleatum* and *Streptococcus sanguinis*

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ABSTRACT

Background: *Fusobacterium nucleatum (F. nucleatum) and Streptococcus sanguinis (S. sanguinis) play a role in dental plaque formation which leads to periodontitis. Immunoglobulin Y (IgY) is present in both serum and egg yolk and can bind to the surface components of bacteria. F. nucleatum and S. sanguinis feature the same type of IV pili as Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans). Saliva binding protein (SsaB) in S. sanguinis is a FimA homolog. FimA constitutes a surface element of Porphyromonas gingivalis (P. gingivalis). F. nucleatum and P. gingivalis possess the same outer membrane protein (OMP) molecular mass.* ***Purpose:*** *The study aimed to determine the activity of A. actinomycetemcomitans and P. gingivalis polyclonal IgY present in serum and egg yolk that can inhibit colonization of F. nucleatum and S. sanguinis.* ***Methods:*** *IgY samples were diluted with phosphate buffer saline (PBS). Several holes were made in the nutrient medium with 10 µl antigen (F. nucleatum/S. sanguinis) being inserted into the center hole. 10 µl PBS, 1:1, 1:2, 1:4, 1:8, 1:16 A. actinomycetemcomitans or P. gingivalis polyclonal IgY were subsequently introduced into the surrounding holes. The results of incubation at 37°C were observed after 24-48 hours. Kruskal Wallis and Mann-Whitney tests were administered to analyse the data.* ***Results:*** *A. actinomycetemcomitans and P. gingivalis polyclonal IgY groups in serum showed a precipitation line at dilution ratios of 1:1 and 1:2, whereas in egg yolk this occurred only at a 1:1 dilution ratio with F. nucleatum and S. sanguinis bacteria in this study. No significant differences were evident between each dilution ($p>0.05$) and none existed between serum and egg yolk ($p>0.05$).* ***Conclusion:*** *IgY polyclonal of A. actinomycetemcomitans and P. gingivalis in both serum and egg yolk initiate activities that can inhibit colonization of F. nucleatum and S. sanguinis.*

Keywords: *Aggregatibacter actinomycetemcomitans; Fusobacterium nucleatum; IgY; Porphyromonas gingivalis; Streptococcus sanguinis*

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INTRODUCTION

Gingivitis generally occurs between 10 and 21 days after dental plaque formation under poor and untreated oral hygiene conditions and can develop into periodontitis.[1] The latter condition results from ecological imbalances between microbial communities in dental biofilms that support the growth of *Streptococcus* pathogenic bacteria.[2] *Streptococcus sanguinis* (*S. sanguinis*) constitutes a pioneer bacterium on the tooth surface which plays an important role in plaque maturation due to its ability to aggregate with other bacteria resulting in periodontal disease.[3] The initiation and development of periodontitis is caused by *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*), *Treponema denticola* (*T. denticola*), *Prevotella intermedia* (*P. intermedia*) and *Fusobacterium nucleatum* (*F. nucleatum*) bacteria.[4] Inhibition of plaque matrix formation and initiation of bacterial aggregation can prevent initial colonization with the result that final

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colonization does not occur.[5] *F. nucleatum* and *S. sanguinis* bacteria promote the formation of dental plaque that can cause periodontitis.

Over the past two decades, dentists have applied antibiotic therapy to periodontal disease treatment.[6] In addition, mouthwash, the main component of which is chlorhexidine, can prevent plaque formation.[7] To reduce antibiotic resistance and the side effects of chlorhexidine gluconate mouthwash such as taste disorders, oral irritation and local allergy symptoms, an unprecedented therapeutic approach is required. One potential strategy is to explore passive oral immunotherapy using Immunoglobulin Y (IgY) as a control method in inhibiting dental plaque.[7,8] IgY is one class of antibody contained in the blood serum and egg yolk of amphibian, reptilian and poultry groups.[9] IgY constitutes a polyclonal antibody that has been shown to be effective in the prevention and treatment of several diseases.[10] Moreover, its low cost and simple production process renders this antibody suitable for research and diagnosis.[11]

IgY can inhibit the attachment of bacteria to host cells[12] since IgY antibacterium binds to certain components on the target bacteria surface such as outer membrane protein (OMP), lipopolysaccharide (LPS), flagella and fimbriae.[13] It has been reported that *A. actinomycetemcomitans* contains type IV pili.[14] Type IV pili are also expressed by other Gram-positive pathogens such as *Clostridium perfringens* (*C. perfringens*) and *S. sanguinis.*[15] Phenotyping screening also confirms type IV pili to be present on the cell surface of *F. nucleatum*.[16] *P. gingivalis* pili contains FimA.[17] *S. sanguinis* bacteria carry SsaB, a FimA homolog, on their surface. Bacterial adhesion molecules play an important role in the adhesion between bacteria and host cell.[18] *P. gingivalis* and *F. nucleatum* have an outer membrane protein with a molecular mass of 40-kDa.[19] Against this background, the research aim was to determine whether IgY anti *A. actinomycetemcomitans* and *P. gingivalis* can be employed to inhibit colonization of *F. nucleatum* and *S. sanguinis* bacteria.

MATERIALS AND METHODS

The type of research employed an experimental laboratory methodology. The samples analyzed comprised polyclonal IgY anti-*A. actinomycetemcomitans* and polyclonal IgY anti-*P. gingivalis* in serum and egg yolk. More than three replications were produced, their number being determined by the Federer formula. The polyclonal IgY contained in the serum and egg yolk produced by hens previously injected four times (booster) with *A. actinomycetemcomitans* serotype b strain Y4 ATCC 4371 and P. gingivalis ATCC 3327 was subsequently analyzed using Elisa.[11] The research method employed was granted ethical clearance (certificate number: 294/HRECC.FODM/XI/2018) by the Faculty of Dental Medicine, Universitas Airlangga.

Serum was diluted with phosphate buffer saline (PBS) at a ratio of 1:16. Each vial/bottle contained 20 µl of assay buffer. The contents of the first vial were mixed with 20 µl serum samples at a dilution level of 1:1. 20 µl of the 1:1 sample dilution were subsequently transferred to a second vial where they were further diluted at a ratio of 1:2. 20 µl of the sample were transferred to a third vial where the dilution rate was one of 1:4. 20 µl of the sample was transferred to a fourth vial at a dilution rate of 1:8. 20 µl of the sample were transferred to the fifth vial and diluted at a rate of 1:16. This procedure was repeated for the IgY samples in egg yolk.[20]

The nutrient medium solution was cooled to 50-60°C, placed in 15 ml petri dishes on a horizontal surface and

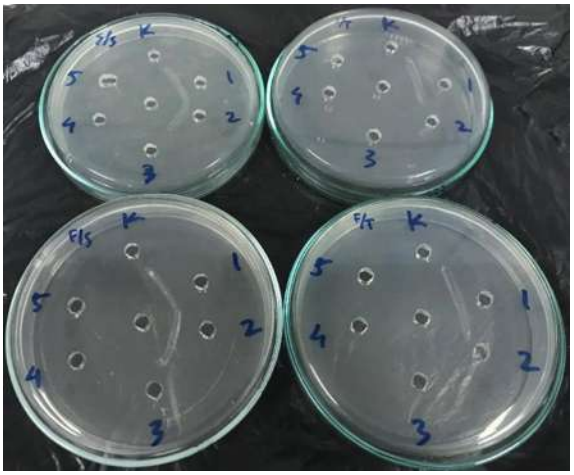

Figure 1. The precipitation line of *A. actinomycetemcomitans* polyclonal IgY groups in serum and egg yolk in *F. nucleatum* and *S. sanguinis*. (S/S: *S. sanguinis*/IgY in Serum; S/T: *S. sanguinis*/IgY in egg yolk; F/S: *F. nucletum*/IgY in serum; F/T: *F. nucleatum*/IgY in egg yolk).

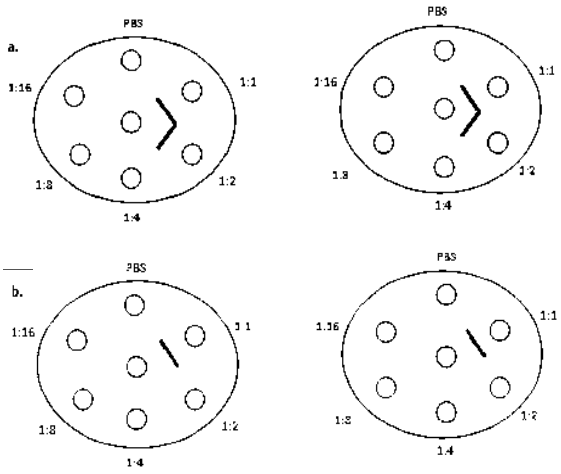

Figure 2. Result diagrams of the *A. actinomycetemcomitans* polyclonal IgY group in bacteria: a) *F. nucleatum*; b) *S. sanguinis*; a) Serum preparation; b) Egg yolk preparation.

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left to stand for 30 minutes. The nutrient medium was perforated inappropriate sites in the marked section in order to accommodate this sample. 10 µl of the PBS and serum samples at dilutions of 1:1, 1:2, 1:4, 1:8, 1:16 were deposited in each of the holes on the outer rim of the medium. 10 µl of the antigen (*F.nucleatum/S.sanguinis*) were inserted into the center hole of each petri dish and incubated at 37°C for 24 hours. After incubation, the line of precipitation between the antigen hole and the IgY serum holes was observed. This procedure was repeated for the IgY samples in egg yolk.[20,21]

RESULTS

This study confirmed inhibition of the colonization of *F. nucleatum* and *S. sanguinis* bacteria in the *A. actinomycetemcomitans* polyclonal IgY group present in serum and egg yolk (Figure 1). Based on these results, the IgY anti-*A. actinomycetemcomitan* group in the serum showed a precipitation line against *F. nucleatum* and *S. sanguinis* at 1:1 and 1:2 dilutions of the treatment groups. In contrast, the IgY anti-*A. actinomycetemcomitan* group in the egg yolk showed the presence of precipitation lines

Table 1. Data analysis of *A. actinomycetemcomitans* polyclonal IgY group and *F. nucleatum*

Antigen	AA1 (1:1)	AA2 (1:2)	AA3 (1:4)	AA4 (1:8)	AA5 (1:16)	AA6 (PBS)	*p* value
F/S	+	+	-	-	-	-	0.051
	+	+	-	-	-	-	
F/T	+	-	-	-	-	-	0.051
	+	-	-	-	-	-	

Table 2. Data analysis of *A. actinomycetemcomitans* polyclonal IgY group and *S. sanguinis*

Antigen	AA1 (1:1)	AA2 (1:2)	AA3 (1:4)	AA4 (1:8)	AA5 (1:16)	AA6 (PBS)	*p* value
S/S	+	+	-	-	-	-	0.051
	+	+	-	-	-	-	
S/T	+	-	-	-	-	-	0.051
	+	-	-	-	-	-	

Note: +: there is a precipitation line; -: there is no precipitation line; *: significance (p value <0.05)

Table 3. Comparison of the results of *A.actinomycetemcomitans* polyclonal IgY group in the serum and egg yolk and *F.nucleatum* and *S.sanguinis*

Antigen	*p* value
F/S F/T	0.514
S/S S/T	0.514

Note: *significance (*p* value <0.05)

Table 6. Comparison of the result of IgY *P.gingivalis* group in the serum and egg yolk and *F.nucleatum* and *S.sanguinis*

Antigen	*p* value
F/S F/T	0.514
S/S S/T	0.514

Note: *: significance (*p* value <0.05)

Table 4. Data analysis of *P. gingivalis* polyclonal IgY and *F. nucleatum*

Antigen	PG1 (1:1)	PG2 (1:2)	PG3 (1:4)	PG4 (1:8)	PG5 (1:16)	PG6 (PBS)	*p* value
F/S	+	+	-	-	-	-	0.051
	+	+	-	-	-	-	
F/T	+	-	-	-	-	-	0.051
	+	-	-	-	-	-	

Table 5. Data analysis of *P. gingivalis* polyclonal IgY and *S. sanguinis*

Antigen	PG1 (1:1)	PG2 (1:2)	PG3 (1:4)	PG4 (1:8)	PG5 (1:16)	PG6 (PBS)	*p* value
S/S	+	+	-	-	-	-	0.051
	+	+	-	-	-	-	
S/T	+	-	-	-	-	-	0.051
	+	-	-	-	-	-	

Note: +: there is a precipitation line; -: there is no precipitation line; *: significance (p value <0.05)

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against *F. nucleatum* and *S. sanguinis* during 1:1 dilution treatment. Data analysis was performed with a Kruskal Wallis test, the results of which are contained in Tables 1 and 2, and a Mann-Whitney test whose results are shown in Table 3.

Based on the contents of Tables 1 and 2, it is evident that none of the F/S, F/T, S/S and S/T groups showed significant value because the p value equaled 0.051 which was greater than 0.05 (p> 0.05). This figure indicated that the concentration had no effect on the results. The data in Table 3 indicates that the value of F/S with F/T was 0.514, while that of S/S with S/T was 0.514. These results, arrived at through statistical calculation, indicated no difference between the polyclonal IgY group of *A. actinomycetemcomitans* in serum and egg yolk and *F. nucleatum* and *S. sanguinis*. They were considered to be insignificant with a p value >0.05. This study confirmed colonization inhibition of *F. nucleatum* and *S. sanguinis* bacteria of the IgY anti-*P. gingivalis* group in serum and egg yolk which can be characterized by the precipitation line shown in Figure 3.

These results of the IgY anti *P. gingivalis* group in serum showed a precipitation line for the *F. nucleatum* and *S. sanguinis* bacteria occurring at 1:1 and 1:2 dilutions in the treatment groups. By contrast, in the *P. gingivalis* polyclonal IgY group, egg yolk showed the presence of precipitation lines in the *F. nucleatum* and *S. sanguinis* bacteria in the 1:1 dilution treatment group. Data analysis was performed by means of a Kruskal Wallis test, the results of which are shown in Table 4 and 5, and a Mann-Whitney test whose results appear in Table 6.

Based on contents of Tables 4 and 5, it is known that none of the F/S, F/T, S/S and S/T group showed significant values because the p value equaled 0.051, indicating that it was higher than 0.05 (p>0.05). This indicated that concentration had no effect on the results. The contents of Table 6 show that the value of F/S with F/T was 0.514, while that of S/S with S/T was 0.514. These statistical calculation-based results showed no difference between the polyclonal IgY group of *P. gingivalis* in serum and egg yolk in the *F. nucleatum* and *S.sanguinis* bacteria due to insignificant results since the p value >0.05.

DISCUSSION

This study used IgY anti-*A. actinomycetemcomitans* and IgY anti-*P. gingivalis* in serum and egg yolk to be tested against two other bacteria, i.e. *F. nucleatum* and *S. sanguinis*, using a double immunodiffusion method. *A. actinomycetemcomitans* polyclonal IgY and *P. gingivalis* polyclonal IgY were obtained from chickens previously immunized with specific antigens i.e. *A. actinomycetemcomitans* or *P. gingivalis* prior to serum being taken. Testing of the two polyclonal IgY was conducted using a double immunodifusion method.

The results of the data from the IgY anti-*A. actinomycetemcomitans* group for serum in the *F. nucleatum* and *S. sanguinis* bacteria, found a formation precipitation line at 1:1 and 1:2 dilutions. In contrast, for the IgY anti-*A. actinomycetemcomitans* group in egg yolk formation of a precipitation line occurred at 1:1 dilution in the *F. nucleatum* and *S. sanguinis* bacteria.

Similar results were found in the IgY anti *P. gingivalis* group. The results of the IgY anti-*P. gingivalis* group in serum for *F. nucleatum* and *S. sanguinis* bacteria found the precipitation line at 1:1 and 1:2 dilutions. Contrastingly, in the IgY anti-*P. gingivalis* group in egg yolk, a precipitation line formed at 1:1 dilution in *F. nucleatum* and *S. sanguinis* bacteria. These results show that precipitation lines are formed at low dilutions and that the concentration of antibodies in dilution is higher than that of other dilution groups, meaning that the group with low dilution levels is of an appropriate concentration to be able to interact with

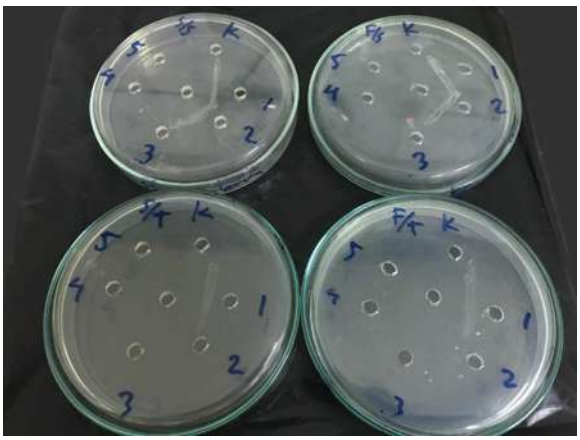

Figure 3. The precipitation line of IgY anti *P. gingivalis* group in the serum and egg yolk in *F. nucleatum* and *S. sanguinis*. (S/S: *S. sanguinis*/IgY in serum; S/T: *S. sanguinis*/IgY in egg yolk; F/S: *F. nucletum*/IgY in serum: *F. nucleatum*/IgY in egg yolk).

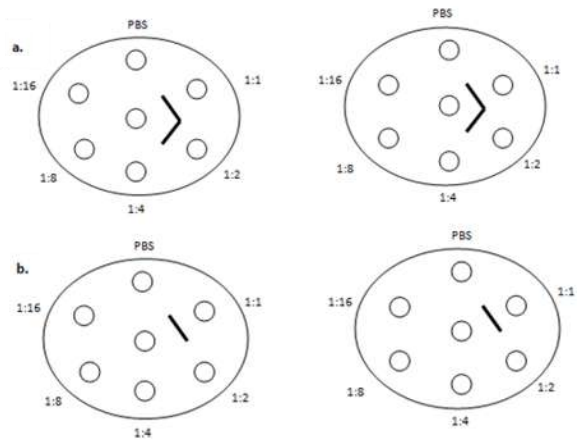

Figure 4. Result diagrams of IgY anti *P. gingivalis* group in *F. nucleatum* and *S. sanguinis*; a) serum preparation; b) Egg yolks preparation.

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the antigen. Excessively low antibody concentration will produce negative results[22] similar to those of previous research conducted by Sharon *et al*.[20] in which the precipitation line occurred at 1:2 dilution.

The precipitation lines formed between antigens and antibodies, both in the IgY anti-*A. actinomycetemcomitans* group in serum and egg yolk for *F. nucleatum* and *S. sanguinis* bacteria, and IgY anti-*P. gingivalis* group in serum and egg yolk for *F. nucleatum* and *S. sanguinis* bacteria, indicated some form of activity between the IgY anti-*A. actinomycetemcomitans* with *F. nucleatum* and *S. sanguinis*, as well as between the IgY anti-*P. gingivalis* with *F. nucleatum* and *S. sanguinis*. The activity took the form of binding between antibodies and antigens, possibly because IgY is a polyclonal antibody that can bind to various epitope antigens. IgY anti-*A. actinomycetemcomitans* can bind to pili from bacteria, such as *F. nucleatum* and *S. sanguinis*, because all have type IV pili. Polyclonal IgY *P. gingivalis* can also bind to the homologous element found on the surface of *S. sanguinis*, namely SsaB, and can bind to OMP *F. nucleatum* because it possesses the same molecular mass. This binding can inhibit the colonization of *F. nucleatum* and *S. sanguinis* bacteria due to the disruption of the function of the surface components of these bacteria which can be useful for their adhesion.

Previous research conducted by Lee *et al*.[23] showed that *Salmonella* specific IgY binds to *Salmonella* surface molecules with the result that it can inhibit homologous *Salmonella* growth. IgY can affect colonization of *Salmonella enteritidis* and *Salmonella typhimurium* by binding to OMP. OMP *Salmonella* is useful for adhesion and mucosal invasion. The binding causes disruption of OMP biological function, with the result that invasive *Salmonella* is reduced due to the loss of ability to colonize the digestive tract.[24]

The results of the statistical analysis indicated no difference between the inhibitory colonization in the polyclonal IgY *A. actinomycetemcomitans* dilution group and the polyclonal IgY *P. gingivalis* group. This is consistent with the theory that this test is less sensitive because the formation of precipitation lines depends on the equivalent concentration of antigen antibodies and specific antibodies.[25] It can be concluded that the activities of *A. actinomycetemcomitans* and *P. gingivalis* polyclonal IgY in serum and egg yolk can inhibit colonization of *F. nucleatum* and *S. sanguinis*.

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