

A.actinomycescomitans Adhesi Protein Increasing IL-8 in Heart of Wistar Rat Aggressive Periodontitis.

by Retno Indrawati.,drg.,msi

Submission date: 18-Jun-2018 09:48AM (UTC+0800)

Submission ID: 976507866

File name: easing_IL-8_in_Heart_of_Wistar_Rat_Aggressive_Periodontitis..pdf (272.11K)

Word count: 4365

Character count: 25381

A. actinomycetemcomitans adhesin protein increasing IL-8 titre in heart of wistar rat with aggressive periodontitis (Protein adhesin dari *A. actinomycetemcomitans* meningkatkan titer IL-8 di dalam jantung tikus wistar dengan periodontitis agresif)

Rini Devijanti Ridwan, Retno Indrawati

Department of Oral Biology
Faculty of Dentistry Airlangga University
Surabaya, Indonesia.

ABSTRAK

Penyakit kardiovaskular dan periodontal adalah kondisi peradangan yang umum dalam populasi manusia. Limfosit T berpartisipasi dalam patogenesis dan inflamasi pada aterosklerosis. Sel-sel kekebalan ini memasuki dinding arteri sehingga terjadi peradangan dan bergabung dengan makrofag melalui sejumlah interferon-c-inducible kemokin. Faktor kemokin (IL-8), sitokin, dan pertumbuhan juga berpartisipasi dalam proses ini. Interaksi antara IL-8 dan reseptornya, CXCR2, juga dapat berkontribusi untuk pembentukan lesi pada tikus. Penyebab utama periodontitis agresif adalah *Actinobacillus actinomycetemcomitans*. Pada penelitian sebelumnya, terungkap bahwa protein adhesin dengan berat molekul 24 kDa dari *A. actinomycetemcomitans* adalah adhesin spesifik, yang berperan dalam proses adesi pada host. Adesi protein adhesin ini dalam perlekatan pada sel epitel menyebabkan kolonisasi dan invasi *A. actinomycetemcomitans* yang merangsang respons kekebalan tubuh host. Penelitian ini dimaksudkan untuk menganalisis pengaruh induksi protein adhesin *A. actinomycetemcomitans* dengan berat molekul 24 kDa terhadap kadar IL-8 dalam jantung tikus Wistar dengan periodontitis agresif menggunakan metoda Elisa untuk mengukur dan menganalisis kadar IL-8. Setelah dianalisis dengan analisis of variance, menunjukkan perbedaan yang signifikan kadar IL-8 pada kelompok kontrol dan kelompok induksi *A. actinomycetemcomitans*, *A. actinomycetemcomitans*+protein adhesin *A. actinomycetemcomitans* 24 kDa dan hanya dengan protein adhesin *A. actinomycetemcomitans* 24 kDa saja. Disimpulkan bahwa protein adhesin *A. actinomycetemcomitans* dengan berat molekul 24 kDa memiliki peran dalam peningkatan kadar IL-8 dalam jantung tikus Wistar dengan periodontitis agresif.

Kata kunci: *A. actinomycetemcomitans*, protein adhesin, kadar IL-8, jantung, periodontitis agresif

ABSTRACT

Cardiovascular and periodontal diseases are common inflammatory conditions in the human population. T-lymphocytes participate in the pathogenesis and inflammatory events of atherosclerosis. These immune cells enter the inflamed artery wall and join macrophages via a number of interferon-c-inducible chemokines. Chemokines (IL-8), cytokines, and growth factors also participate in this process. The interaction between interleukin- 8 and its receptor, CXCR2, can also contribute to lesion formation in mice. The main causes of aggressive periodontitis is *Actinobacillus actinomycetemcomitans*. Previous studies have proven that adhesin protein with 24 kDa molecular weight from *A. actinomycetemcomitans* is a specific adhesin, this adhesin proteins play a role in the adhesion process on host. this kind adhesion in the epithelial attachment would lead to colonization and invasion of *A. actinomycetemcomitans* that will stimulate the host immune response. This study aimed to analyze the influence of induction 24 kDa *A. actinomycetemcomitans* adhesin protein to the titre of IL-8 in heart of Wistar rat with aggressive periodontitis using Elisa method to measure and analyze the titre of IL-8. After analyzed with analysis of variance, showed significant differences of IL-8 titre in the control group and the group with the induction by *A. actinomycetemcomitans*, *A. actinomycetemcomitans* plus 24 kDa *A. actinomycetemcomitans* adhesin protein, and only with 24 kDa *A. actinomycetemcomitans* adhesin protein. It can be concluded that *A. actinomycetemcomitans* adhesin protein with 24 kDa molecular weight has a role in increasing of IL-8 titre in heart wistar rat with aggressive periodontitis.

Keywords: *Actinobacillus actinomycetemcomitans*, adhesin proteins, IL-8 titre, heart, aggressive periodontitis

Correspondence: Rini Devijanti Ridwan, c/o: Department of Oral Biology, Faculty of Dentistry Airlangga University, Jl. Mayjen Prof. Dr. Moestopo 47 Surabaya 60132, Indonesia. E-mail: devirini@yahoo.co.id

INTRODUCTION

Periodontal disease is by far the most common oral infection, and is the subject of most studies concerning the relationship between systemic disease and oral health. Periodontal disease caused by bacteria that found in dental plaque, that causes inflammation in the tissues of the gums and mouth. This inflammation can cause destruction of the

tissues, periodontal ligaments, and even bone. Many researchers have found that periodontitis is associated with other health problems such as cardiovascular disease (CVD), stroke, and bacterial pneumonia.¹ The colonization and invasion of *A. actinomycetemcomitans* role in the stimulation of proinflammatory cytokine IL-8 is secreted by monocytes, keratinocytes, fibroblasts and endothelial

cells and. This spending will stimulate MMP-8 by neutrophils. The periodontal bacteria can enter the bloodstream and travel to major organs and begin new infections. Evidence suggests that this process may contribute to the development of heart disease, increase the risk of stroke, increase a woman's risk of delivering a preterm low-birth-weight baby, and pose a serious threat to people whose health is compromised by diabetes mellitus, respiratory disease or osteoporosis.² Chemokines and their receptors are considered promising targets for the regulation of leukocyte infiltration in inflammatory and immune diseases, and many pharmaceutical companies are currently conducting clinical development programs targeting the chemokine system. Acute inflammation such as that is caused by bacterial infection, ischemia-reperfusion injury and acute glomerulonephritis, predominantly neutrophils infiltrate into the tissue. The critical role of CXCL8, related molecules, and their receptors CXCR1/2 has been established in various disease models. The contribution of each ligand and the role of the receptors in different tissues and circumstances varies. Thus, the dual specific antagonists for CXCR1/2 would be an ideal way to block neutrophil infiltration. If acute inflammation is unable to be resolved by neutrophils, the inflammation perpetuates and develops in chronic inflammation in which macrophages and lymphocytes predominate are the infiltrating cells.³

Based on Pussinen et al's study on 2005, an infection by *A. actinomycetemcomitans* may be associated with the incidence of coronary heart diseases (CHD). Consistent with their findings, high serum levels of Immunoglobulin A (IgA) antibodies to *A. actinomycetemcomitans* have been found to predispose to cerebrovascular stroke in subjects free from CHD at baseline. Their study suggests that high IgA antibodies levels to both *P. gingivalis* and *A. actinomycetemcomitans* are associated with CHD. These results also indicate that serum antibodies to periodontal pathogens might be worth screening when mapping individual risk factors for CVD.⁴ Research using animals model in 1985 managed to find any biochemistry substrate that depresses the function of heart in a state of sepsis such as IL-1, IL-8, and C3a. Endotoxins derived from gram-negative bacteria lipopolysaccharide form will initiate the onset of TNF- α that will interact with toll-like receptors-4 and cause interference with cell function cardiac muscle. The production of nitric oxide produced by induce nitric oxide synthetase (iNOS) in the state sepsis also brings adverse effects to the function heart through the onset of substance-the substance is a strong oxidant.^{5,6} The data indicated that 70% of endocarditis cases

caused by *A. actinomycetemcomitans* have underlying heart diseases, such as valvopathy and prosthetic valves. The compromised condition of the host cardiac valves, together with a transient bacteremia induced through daily activities, allows this endogenous oral bacterium to colonize, invade, and replicate at the extraoral sites.

Coincidental with advances in the understanding of inflammation mechanisms, including in part the discovery of the cell adhesion molecules described above, studies of atherosclerosis progressed beyond light microscopy, histopathology and into molecular biology. It is in this realm that the inflammatory hypothesis has garnered significant support. Another candidate trigger of both autoimmune responses and inflammatory that leads to the initiation and or acceleration of atherosclerosis are infection. Evidence in humans suggest that infection predisposes to atherosclerosis is derived from studies demonstrating that the infectious agents reside in the wall of atherosclerotic vessels, and the seroepidemiological studies demonstrate an association between the pathogen-specific IgG antibodies and atherosclerosis. Recently, it has been proposed that multiple infectious agents contribute to atherosclerosis, and that the risk of cerebrovascular disease (CVD) posed by infection is related to the number of pathogens to which an individual has been exposed.⁷ There is an evidence to support the relationship between human periodontal disease and an increased risk for acute myocardial infarction.^{8,9} Periodontal disease is characterized by generalized alveolar bone resorption and, in severe cases, early loss of dentition.¹⁰ Despite rigorous clinical intervention strategies, recent reports suggest that up to 30% of adults in the USA over the age of 40 have measurable periodontal bone loss.¹¹ Case control studies have demonstrated a significant correlation between CVD and periodontal disease after adjusting to cholesterol, smoking, hypertension, social class, and body mass index.^{8,12} Following binding and invasion by the pathogenic microbes, endothelial cells respond by producing cell adhesion molecules (CAM) such as E-selectin, ICAM-1 and VCAM-1, as well as a vast array of cytokines including TNF- α , interferon-gamma (IFN- γ), and chemokines including IL-8, monocyte chemoattractant protein-1 (MCP-1). The expressed CAM plays a role in arresting inflammatory cells such as monocytes that are honed to the site of the bacterially infected endothelium. Emerging data support that sICAM and sVCAM are released from endothelium, and that these molecules are associated with CVD. As the host vascular endothelium becomes activated in the response to infection, endothelial cells produce a variety of soluble

mediators, including IL-8, MCP-1, and IL-6, which collectively support atherogenesis.¹ Adhesins are important in the initial recognition of receptors distributed on the surface of the host tissue. Both fimbrial and nonfimbrial adhesins have been identified on the surface of *A. actinomycetemcomitans*.¹³

The aim of this study was to analyze the influence of induction 24 kDa adhesin protein of *A. actinomycetemcomitans* to the titre of IL-8 in heart of wistar rat with aggressive periodontitis because of the role of *A. actinomycetemcomitans* adhesion protein in the pathogenesis of aggressive periodontitis, specially the influence of this adhesin protein on heart was not yet known.

MATERIALS AND METHOD

Culture and isolation of *A. actinomycetemcomitans* clinical isolate

The sample was *A. actinomycetemcomitans* clinical isolate that is isolated from patients with aggressive periodontitis in Periodonsia Clinic Faculty of Dentistry Airlangga University Surabaya. Bacteria were grown in *A. actinomycetemcomitans* growth medium (AAGM) or in Luria Bertani (LB) medium and incubated at 37°C anaerobically for 24 hours. Identification of *A. actinomycetemcomitans* on AAGM plates based on gross morphology such as adherence to the medium surface, a starlike inner structure and positive catalase. The identification of bacteria *A. actinomycetemcomitans* confirmed the use of Microbact system and PCR.

Isolation of adhesin protein of *Actinobacillus actinomycetemcomitans* clinical isolate

A. actinomycetemcomitans was cultured in 250 ml AAGM or LB medium, added with 10 ml of 3% trichloro acetic acid (TCA), and allowed to stand for 30-60 minutes. After that they were centrifuged at 6.000 rpm at 4°C for 15 minutes. Supernatant was discarded and the sediment suspended in 50 ml PBS on pH 7.4 then fimbriae was cut using Omnimixer as a cutting tool and then centrifuged at 12.000 rpm for 15 minutes and the supernatant (fimbriae pieces) were stored.

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Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight monitoring was done by means of SDS-PAGE following Laemmli method.¹⁴ Protein sample was heated 100°C for 5 minutes in a buffer solution containing 5 mM Tris HCl pH 6.8, 2-mercapto ethanol 5%, w/v sodium dodecyl sulfate 2.5%, v/v glycerol 10%, with bromophenol blue as color tracker. The gel was 12.5% mini slab gels (4% gel tracking);

using 120 mV voltage. Coomassie brilliant blue was used for staining and pre stained, protein ladder was used as the protein marker.

Protein purification of *A. actinomycetemcomitans* adhesin protein hemagglutinin

The protein profile from the adhesin collections was performed by SDS-PAGE. The gels were cut straight at the desired molecular weight, collected, and put into a dialysis membrane tube containing electrophoresis running buffer liquid. Electrophoresis used an electrophoresis apparatus horizontal (voltage of 125 mV for 25 minutes). Then the result of electrophoresis was dialyzed. In the first 24 hours the dialysis used dH₂O and continued with PBS pH 7.4 for the next 2x24 hours. Dialysate fluid of the SDS-PAGE protein band was precipitated using cold absolute ethanol. The precipitate was assessed using hemagglutination test.

Hemagglutination assay

The hemagglutination test was done following Hanne and Finkelstein.¹⁵ The sample was diluted into concentration of ½ in the microplate wells for each 50 µl volume. Each well was added with suspension of red blood cell of mice at concentration of 0.5%, and then shaken in rotator plate for one minute and resided at room temperature for an hour. The titer was determined by the presence of red blood agglutination on the lowest dilution. The samples were whole-cell *A. actinomycetemcomitans* lysat, which were intact bacterial cells, and adhesin protein was already prepared. The type of red blood cells was taken from healthy mice.

Elisa method

Indirect Elisa was used to count the IL-8 titre in wistar rat heart, ELISA Kit, BG-RAT11336 (Novateinbio). First, heart tissue from wistar rat was prepared, add heart tissue with PBST (5X Volume)+ PMSF 4mM, crushed in mortal cold, then performed sonication about 10 minutes and centrifugation in 6000 rpm during 15 minutes in room temperature. Supernatant was added with cold absolute ethanol, centrifuge in 1000 rpm. In the next 10 minutes add pellets with Buffer Tris-Cl 20 mM.

Prepare all reagents before starting stages of the procedure. It is recommended that all standards are included in the duplicate samples microelisa strip plate. Diluting 20x wash solution becoming 1x wash solution with ddH₂O. First, set the standard. Next, samples were tested at the plate measurements and then added the standard 50 µL and added 50 µL sample that was diluted in the sample well (10 µL

sample+40 μ L sample solvent). In well standar add the solvent blank added 50 μ L of HRP-antibody conjugate each well except the blank well. Then slow shaker was uses to homogenize this solution and incubate it for 60 minutes at 37°C. Then discard the solution as much as possible, removing fluid as much as possible the contents well with washing solution. Homogenization was performed with a shaker for 1 minute, remove the washing solution and the excess liquid with filter paper. Repeat this procedure 4 times for a total 5 times washing. Add substrates A and B, each 50 μ L in each well and make it homogenized then incubated for 15 min at 37°C. Add 50 μ L stop solution to stop the reaction marked by the color change from blue to yellow. Measure the optical density at a wavelength of 450 nm for 15 minutes. Create a standard curve with optical density as the Y-axis and concentration on the X-axis and calculate the linear regression equation. Thus, the concentration of the sample can be determined.

Statistical analysis.

One-way Anova and Tukey's test was used to detect differences between the titre of IL-8 in heart on the control group and the one on the group with adhesin, adhesin plus *A. actinomycetemcomitans* and only *A. actinomycetemcomitans* induction.

RESULTS

Adhesin protein was isolated from *Actinobacillus actinomycetemcomitans* by using NOG in fifth time separation. Marker proteins used was protein marker ladder (Sigma). Protein molecular weight was in the unit of kDa. There were five major proteins of the proteins with their molecular mass of 60 kDa, 53 kDa and 42 kDa, 28 kDa and 24 kDa. Furthermore,

each of the collection protein passed through hemagglutinations test (fig.1) using mice erythrocytes after purification by using electroelution. Aglutination of the fifth adhesin protein collection was at the highest titer (protein dilution 1/128) compared with other collections. The five collection proteins 24 kDa of adhesin protein would be used as a sample for subsequent study.

One-way Anova test results showed the value of the IL-8 in titre in the control group and the group with adhesin, adhesin plus *Actinobacillus actinomycetemcomitans*, and the *Actinobacillus actinomycetemcomitans* induction were significantly different (table 1), $p=0.000$ ($p<0.005$) and then analyzed by the LSD test that found a significant difference from the IL-8 titre in the control group and the group treated with induction adhesin, adhesin plus *Actinobacillus actinomycetemcomitans*, and *A. actinomycetemcomitans* and between the treatment groups. The highest IL-8 titre in wistar rat heart was in group with adhesin plus *Actinobacillus actinomycetemcomitans*, then in group only induction with *A. actinomycetemcomitans*, after that in group with adhesin protein induction, and the lowest IL-8 titre was in control group. This condition is shown in fig.2.

DISCUSSION

This study found the highest IL-8 titre in wistar rat heart was in group with adhesin plus *Actinobacillus actinomycetemcomitans*, then in group of only induction with *A. actinomycetemcomitans*, followed by group with adhesin protein induction, and the lowest was in control group. The previous studies have found an increasing expression and levels of IL-8 in gingival wistar rata after induction with adhesin

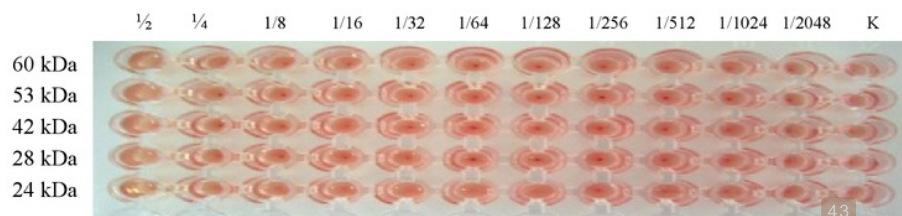


Fig.1 Hemagglutination profile of five major adhesin proteins of *A. actinomycetemcomitans* (60 kDa, 53 kDa, 42 kDa, 28 kDa, and 24 kDa)

Table 1 The mean and standart deviation number of IL-8 titre in wistar rat heart

Group	X	SD	Min	Max	Anova
Control	2.93 ^a	1.07	2.16	3.69	
Adhesin	6.06 ^b	0.51	5.69	6.43	F=98.146
Adhesin+ <i>A. actinomycetemcomitans</i>	10.66 ^d	1.18	9.82	11.51	P=0.000
<i>A. actinomycetemcomitans</i>	8.02 ^c	1.23	7.14	8.90	

There were different superscripts indicate that there are significantly differences between groups ($p<0.05$)

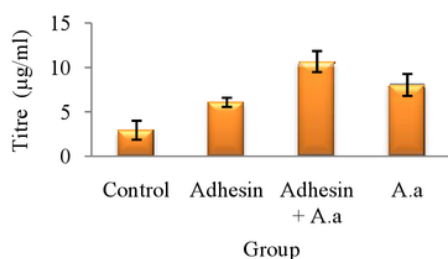


Figure 2 IL-8 titre in Wistar rat heart

protein of *A.actinomycetemcomitans*.¹⁶ This result showed that the *A.actinomycetemcomitans* adhesin protein influence the IL-8 expression and titre in host. IL-8 is an inflammatory chemokine which functions mainly as a neutrophil chemoattractant and activating factor. IL-8 is found at higher levels in gingival crevicular fluid prior to clinical signs of inflammation. As periodontal disease seems to be related to the progression of the inflammatory process to deeper periodontal tissue, chemokines found in both gingival tissue and crevicular fluid may play an important role on its pathogenesis. In this regard, subjects with a history of periodontitis have high levels of CXCL8 in gingival tissue and crevicular fluid; these levels are correlated with disease severity. Chemokines are a family of potent chemotactic cytokines that regulates the trafficking and recruitment of leukocytes to distant sites of inflammation. The fine tuning of the regulation of the chemokine system is essential for the host homeostasis and defense, and its abnormal expression is often associated with pathological processes. The first cytokine identified to have chemotactic activity was IL-8, which is proven to be a selective neutrophil chemoattractant. Chemokines are found in gingival tissue and crevicular fluid and are produced by a number of cell types in the periodontium, such as fibroblasts, polymorphonuclear leukocytes, epithelial cells, endothelial cells, osteoclasts, macrophages, monocytes, lymphocytes, and mast cells and exert their effects locally in paracrine or autocrine fashion. Some chemokines have important proinflammatory effects and are related to periodontal tissue destruction that involves the stimulation of bone resorption and induction of tissue damage. Chemokines can also affect the recruitment, differentiation, or fusion of precursor cells to form osteoclasts or enhance osteoclast survival. They could also interfere with periodontal disease by recruiting cells, such as neutrophils, which protect host against bacterial invasion.¹⁷

IL-1, IL-6, IL-8, and TNF- α C3a are inflammatory factors that arise in response to systemic infection

in the state of sepsis. TNF- α is an early mediator in response to onset of endotoxins that produced by activated macrophages and cardiac muscle cells. It can induce septic shock and in a state of advanced instrumental in the early stages of a decline in pump function heart through the induction of prostanoid and nitric oxide. The exact mechanism underlying the TNF- α resulted in impaired heart function until is not yet clear. There is a hypothesis that explains the disruption of calcium balance which plays a role essential to cardiac muscle contraction at the level of and increased cellular production of peroxy nitrite produced by iNOS pathway in sepsis state.^{5,18}

Although most of the bacteraemias are transient, it has long been recognized that bacteria in the blood stream may cause distant site infections. Several studies have demonstrated the presence of certain oral bacteria in atherosclerotic plaques and abdominal aortal aneurysms, in particular species implicated in the pathogenesis of periodontitis. Recently, many prospective studies have also provided serological evidence that infections caused by major periodontal pathogens like *P.gingivalis* and *Aggregatibacter actinomycetemcomitans* are associated with future stroke, increased risk of myocardial infarction, and acute coronary syndrome (ACS). These bacteria may not only colonize distant sites; their components will also elicit a host-tissue response characterized locally by a dense infiltrate of neutrophils, macrophages, and different lymphoid cells. These cells together with the adjacent host-tissue cells will subsequently elicit an immune-inflammatory response with the release of different cytokines and prostanoids, such as IL-1, IL-6, IL-8, TNF- α , prostaglandin E2, and different matrix metalloproteinases, which play a pivotal role in the connective tissue and bone destruction occurring in the periodontal lesion. These bacteria and released metabolites beyond this potential local pathogenicity may disseminate systemically and influence directly or indirectly the atheroma pathophysiology.¹⁹

After stimulation by bacteria and their components (LPS, and peptidoglycans), the periodontal tissue produces inflammatory cytokines (IL-1, TNF-, IL-6, INF-, IL-12, and IL-10), chemokines (MCP-5, IL-8, MIP-1), PGE2, and NO. LPS from *Aggregatibacter actinomycetemcomitans* will significantly enhance the expression of α 2 integrins and L-selectins. Peptidoglycans are components of the bacterial walls and, like LPS, they contribute to activation of immune cells via binding to TLR-2 receptor. In addition, peptidoglycans are recognized by the complement system and specific receptors resulting in production of TNF-, IL-1, IL-6, IL-8, and MIP-1 and NO in macrophages. Furthermore, in comparison with

LPS, peptidoglycans are not so strong stimulators of immune reaction. The presence of circulating oral bacteria or bacterial components may stimulate blood cells to produce cytokines. IL-6 levels significantly increased eight hours after scaling, while the IL-8 decreased. The cases of infectious endocarditis caused by bacteria of dental plaque. The following bacteria were proven as etiological factors: *Aggregatibacter actinomycetemcomitans*, *Capnocytophaga*, *Eikenella corrodens*, *Streptococcus* species, *Neisseria*, and *Lactobacillus*. Bacteria of dental plaque and their components in the periodontal tissues may penetrate into the circulation and exhibit pathogenic potential.²⁰

From the discussion, it can be concluded that *A. actinomycetemcomitans* adhesin protein with 24 kDa

molecular weight has a role in increasing of IL-8 titre in heart Wistar rat with aggressive periodontitis. Currently, adhesin protein-based vaccine development to control infectious diseases thrive. Barriers to the first stage of infection, namely bacterial attachment to host cell receptors and colonization can prevent infection. Vaccine based on adhesion material is very interesting because this vaccine will provide two advantages, besides the formation of antibodies to the adhesin, which causes adhesion barrier in the entry of bacteria, as well as the antibodies produced will improve the process of elimination of bacteria by the immune system. Adhesin protein is a one of the material that can be used as a vaccine candidate to manage aggressive periodontitis.

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