Association of Salivary Count of *Streptococcus sanguinis* with the Periodontal Status of Coronary Heart Disease Patients: A Quantitative Study

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Abstract

Background: Periodontal disease is a risk factor for coronary artery disease. *Streptococcus sanguinis* is a pioneer bacterium, which colonizes the tooth surface and causes periodontal disease. *S. sanguinis* is found in atherosclerotic plaques. Periodontitis increases risk of cardiovascular disease. *S. sanguinis* is linked to both diseases. Objective: To analyze the relationship between the salivary count of *S. sanguinis* and the periodontal status of patients with and without coronary heart disease (CHD). Material and Methods: The periodontal status (plaque index, papilla bleeding index, pocket depth) of 40 non-CHD patients (controls) and 66 CHD patients was evaluated. Saliva samples were collected, and a quantitative analysis of *S. sanguinis* was conducted using the real-time polymerase chain reaction (RT-PCR). Results: The *S. sanguinis* count differed in CHD patients and non-CHD patients. The *S. sanguinis* count of CHD patients was not associated with the plaque index, papilla bleeding index, or pocket depth. Conclusion: The *S. sanguinis* count of CHD patients was lower than that of non-CHD patients. The *S. sanguinis* count was not associated with the periodontal status of CHD patients.

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Introduction

Coronary heart disease (CHD) is one of the leading causes of death in developed countries.[1] Atherosclerosis plays an important role in CHD-related mortality.[1] Atherosclerosis is a progressive disease, which results in inflammation of blood vessel walls and local accumulation of fat (cholesterol), especially low-density lipoprotein.[1,2] In recent years, understanding of the pathogenesis of atherosclerosis in cardiovascular disease has expanded from a primarily etiological view to one that takes account of inflammatory processes, including periodontal disease.[3] According to the current literature, infection can act as an inflammatory stimulus and lead to atherogenesis.[3]

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Periodontitis refers to chronic inflammation caused by a bacterial infection. Periodontitis is a destructive, non reversible condition resulting in loss of tooth connective-tissue attachment to bone, which ultimately leads to loss of the involved teeth.[4] This disease might be caused by over growth of one or more species in the resident population or by colonization by exogenous pathogens. Periodontitis can be seen clinically from a deep pocket, loss of connective tissue. In radiograph, periodontitis presents a loss of alveolar bone.[4]

Many studies have demonstrated the association of periodontitis with heart disease. Factors that play a role in the association of periodontitis with heart disease and atherosclerosis include the host's response to infection, which alters blood coagulability, endothelial, vascular wall strength, and platelet function.[5] Previous research reported that periodontal disease and CHD shared similar risk factors, such as age, sex, diabetes, blood pressure, obesity, smoking, diet, and low

economic status.[6] In a study of the association of CHD with periodontal disease, Holmlund reported, the mortality caused by CHD was predictable from the number of remaining teeth in which people with ten or fewer teeth were seven times at more risk of death caused by CHD than the ones with twenty five or more teeth.[7] Kumaralso reported, a prevalence of coronary heart disease is found to be significantly increased in patients with periodontitis.[8]Blaizot et al states that the risk of developing cardiovascular diseases is 34% higher in patients with periodontitis when compared with patients without periodontitis.[9] Epidemiological evidence of 12 studies compiled by Dietrich et al supports that the incidence of CHD is higher in patients with worsened periodontal status.[10]

Streptococcus sanguinis, a pioneer bacterium on the tooth surface, plays an important role in the plaque maturation process because of its ability to aggregate with other bacteria.[11] Biofilms develop via bacterial attachment, leading to coaggregation and the formation of supragingival plaque.[11] *S. sanguinis* is a normal part of the bacterial flora of saliva and dental plaque. Previous studies reported increased numbers of *S. sanguinis* in saliva and dental plaque of CHD patients.[6]

Following inflammation and deepening of the sulcus, the biofilm can extend into the subgingiva, where it produces a mature subgingval biofilm.[6] *S. sanguinis* can activate platelets, resulting in platelet aggregation.[12] Platelet aggregation aids coagulation (blood clotting) and the repair of damaged tissue.[12] Tilley and Kerrigan reported, Streptococcus sanguis expresses a platelet aggregation-associated protein (PAAP), which contributes little to adhesion to platelets.[13]

Jingyuan reported in experiments on rabit, *Streptococcus sanguinis* induced platelet aggregation and contributes to the virulence of streptococci in infective endocarditis.[14]In a study of rabbits, the authors found an increase in blood vessel pressure, heart rate, and heart contraction, mediated by platelet aggregation-associated protein expression.[15]

Saliva is an important source of nutrients for the growth of micro organisms.The largest volume of saliva is produced before, during, and after meals. Saliva production reaches a peak at 12 noon and is minimal at night during sleep.[16] Various physiological and pathological conditions can modify the production of saliva.[16] These include odor, arousal, chewing, psychological status, hormones, drugs, age, heredity, oral hygiene, and physical exercise.[16] The pH is an important parameter in oral microbial ecology, microorganisms generally cannot survive under extreme pH conditions.[16] The oral cavity has a neutral pH (6.7–7.3). Saliva eliminates the metabolism of carbohydrates and acids produced by bacteria. Sugar intake can lower the pH of dental plaque to less than 5.[16,17]

The real-time polymerase chain reaction (RT-PCR) method can be used to determine the number of *S. sanguinis* bacteria in saliva. The RT-PCR measures the amount of DNA that has been previously propagated through enzymatics without the use of organisms. This method is highly accurate and has been used extensively in microbiological diagnoses in cases of periodontal disease.[18] The aim of the study was to analyze the relationship between the salivary count of *S. sanguinis* and the periodontal status of patients with and without CHD.

Material and Methods

Sample collection

This clinical and laboratory study used a cross-sectional design. The study consisted of patients aged 40–74 years. The patient group comprised 66 CHD patients diagnosed with stable angina who were scheduled to undergo coronary artery bypass graft surgery at Harapan Kita Cardiovascular Hospital. The control group consisted of 40 patients without CHD who attended the periodontology clinic in the dental hospital at the University of Indonesia. The absence of CHD was confirmed by a cardiovascular doctor based on a negative treadmill test and normal electrocardiograms. The exclusion criteria were edentulous patients, pregnancy, and the presence of systemic diseases. This study was conducted between November 2015 and February 2016.

Clinical periodontal examination

The periodontal status of the CHD and non-CHD patients was clinically examined after the patients had signed informed consent forms agreeing to participate in the research. The periodontal status was assessed according to

the Silness and Loe plaque index, Saxer and Muhlemann papillary bleeding index, and pocket depth. During the periodontal examination, the examiner measured the periodontal probing depth and clinical attachment loss at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual/palatal, mid-lingual/palatal, and disto-lingual/palatal).

Saliva collection

Each patient spat into a sterile centrifuge tube through a funnel. A minimum of 1 ml was collected, without stimulation. The centrifuge tube was then sealed, labeled, coded, and stored in a cooler containing ice cubes.

DNA extraction protocol

The DNA extraction proceeded as follows: First, the sample was thawed at room temperature, followed by the addition of 50–150 µL of phosphate buffer saline (PBS). hen, the sample (1000µL) was transferred to a new micro tube, and the plaque was weighed using an analytical balance. The sample was centrifuged at 13000×g for 10 min. The supernatant was then discarded, and 1000 µL of PBS were added, the sample resuspended, and centrifuged at 13000×g for 10 min.

Following the removal of the supernatant, 200 µL of nuclease-free water were added, and the samples were incubated in a thermo block at 100°C for 30 min. Subsequently, the samples were transferred to an ice tray for 10 min, followed by centrifugation at 10000×g for 2 min. The supernatant (180–200µL) was then transferred to a new micro tube, taking care not to include any pellets.

Spectrophotometry was performed to determine the concentration and purity of the DNA. The procedure was performed as follows: First, 5µL of DNA sample and495µL aquabidest were mixed in a cuvette. In each cycle, there were five samples and one blank cuvette (500 µL aquabidest). The cuvette was inserted in the spectrophotometer and scanned at multi wave lengths of 260 and 280 nm.

The DNA concentration obtained from the absorbance value at 260 nm was multiplied by 50 ng/µL. The DNA purity obtained from the ratio of absorbance at 260:280 nm was 1.8–2.0. The DNA concentration was standardized to 100 ng/µL in a 100 µL volume using nuclease-free water.

Spin down the DNA located on the tube cap to the bottom of the tube, a 1x TE 1X primary buffer for 10 sec. Primary *S. sanguinis* consisted of two types, forward primers and reverse primers . the primer was dissolved in TE 1X buffer with 10 times the amount (nmol) of oligonucleotide in accordance with the instructions of the manufacturer to produce a concentration of 100 µM. The primary was vortexed for 20 sec. A primary stock of 10 µM (diluted 10×) was produced by dissolving 20 µL of each primer in 180 µL of TE 1X buffer. The primary stock of 100 µM and stock of 10 µM were stored at -20°C.

Amplification of bacteria with RT-PCR reaction

A master mix was prepared for real-time PCR samples using primary *S. sanguinis*. The following was added to each well: 5 µL of SYBR Green, 0.5 µL of the forward primer (10 µM SS), 0.5 µL of the reverse primer (10 µM SS), and 1 µL of H2O. The final volume of each well after the addition of 3 µL of DNA template was 10 µL. Each sample was duplicated. In the negative control wells, 3 µL of nuclease-free water were added in place of the DNA template.

Detection and amplification were done using the RT-PCR method. The pre-denaturation stage was carried out at 95°C for 10 min, followed by 80 cycles of denaturation at 95°C for 15 sec and an annealing-elongation stage at 60°C for 1 min. The cycle-threshold value of each sample was obtained at the end stage of the RT-PCR.

Statistical Analysis

Statistical analyses were performed using SPSS 20.0. Univariate analysis was performed to obtain the values for the mean, standard deviation (SD), minimum and maximum of all parameters. The Kolmogorov–Smirnov test (n = > 50) was conducted to determine the normal data of periodontal status and *S. sanguinis* count of the CHD patients. The Shapiro–Wilk test (n = < 50) was conducted to determine normal data of periodontal status and *S. sanguinis* count of the non-CHD patients.

As the distribution of the data was not normal, the Mann–Whitney test was conducted to detect significant differences between the *S. sanguinis* count in the CHD and non-CHD groups.

The relationship between the *S. sanguinis* count and periodontal status of the CHD patients was tested using Spearman's test.

Results

Table 1 shows the mean periodontal status (plaque accumulation, gingival bleeding, and pocket depth) of the CHD and non-CHD patients. As shown in Table 1, the mean count of *S. sanguinis* in the CHD patients was 7.20 (3.42) log10 Colony Forming Unit (CFU)/ml, with a range of 0.27–14.12 CFU/ml. In the non-CHD patients, the *S. sanguinis* count was 12.84 (4.18) log10 CFU/ml, with a range of 5.66–24.5 CFU/ml.

Table 1. Average distribution, standard deviation, and minimum and maximum scores for the plaque index, gingival bleeding, pocket depth, and *S. sanguinis* count in patients with and without CHD

Variable	CHD (*N* = 66)		Non-CHD (*N* = 40)	
	Mean (SD)	Min–Max	Mean (SD)	Min–Max
Plaque index	1.37 (0.63)	0.08–3	1.42 (0.57)	0.2–2.8
Gingival bleeding	0.8 (0.60)	0–2.3	1.09(0.72)	0–3.14
Pocket depth (mm)	4.74 (0.75)	4–6	5.1 (0.84)	4–6
S. sanguinis count (log10 CFU/ml)	7.20 (3.42)	0.27–14.12	12.84 (4.18)	5.66–24.5

Table 2. Periodontal status and *S. sanguinis* count in CHD and non-CHD patients

Variable	*P* value	
	CHD	Non-CHD
Periodontal status		
Plaque index	0.200*	0.787*
Gingival bleeding	0.001	0.038
Pocket depth	0.000	0.000
S. sanguinis count	0.200*	0.000

Table 2 shows the normal distribution test between the periodontal status and *S. sanguinis* count in saliva in CHD and non-CHD patients.

The Kolmogorov–Smirnov test (n = > 50) was conducted to determine the normality distribution of periodontal status and *S. sanguinis*count in the CHD patients. The Shapiro–Wilk test (n = < 50) was conducted to determine the normality distribution of periodontal status and *S. sanguinis* count in the non-CHD patients. The results suggested that the papilla bleeding index, pocket depth, and *S. sanguinis* count showed a non-normal distribution.

As the distribution of the data was not normal, the Mann–Whitney test was conducted to detect significant differences between the *S. sanguinis* count in the CHD and non-CHD groups The Mann–Whitney nonparametric test revealed a significant difference between the *S. sanguinis* count of CHD and non-CHD patients. (Table 3)

Table 4 shows the Spearman's test of the relationship beetween the *S. sanguinis* count was not significantly associated with plaque accumulation, gingival bleeding, or the pocket depth.

Table 5 shows the Spearman's test of the relationship between the *S. sanguinis* count and periodontal status of the non-CHD patients revealed no significant association between the numbers of *S. sanguinis* and plaque accumulation, gingival bleeding, and pocket depth.

Table 3.*S. sanguinis* count in CHD and non-CHD patients

S. sanguinis count	N	Mean (log 10 CFU/ml) (min–max)	*P* value
CHD	66	7.20 (0.27–14.12)	0.000*
Non-CHD	40	12.84 (5.66–24.5)	

Table 4. Relationship between the *S. sanguinis* count and periodontal status (plaque accumulation, gingival bleeding, and pocket depth) of CHD patients

CHD (*N* = 40)		Plaque index	Gingival bleeding	Pocket depth
S. sanguinis count	*r*	0.161	-0.149	-0.001
	p	0.198	0.231	0.991

Spearman's test; *p< 0.05 → hypothesis accepted, p> 0.05 → hypothesis rejected

Table 5. Relationship between the *S. sanguinis* count and periodontal status (plaque accumulation, gingival bleeding, and pocket depth) of non-CHD patients

Non-CHD (*N* = 40)		Plaque index	Gingival bleeding	Pocket depth
S. sanguinis count	*r*	-0.249	-0.187	0.010
	p	0.122	0.249	0.949

Spearman's test, p< 0.05 → hypothesis accepted, p> 0.05 → hypothesis rejected

Discussion

This study involved 66 CHD patients and 40 non-CHD patients, with ages that ranged from 40-74 years old. Based on research conducted by the Ministry of Health of the Republic Indonesia in 2007, CHD, heart failure, and stroke are particularly common among those aged 45–54 years, 55–64 years, and 65–74 years, respectively.[19] Data obtained from Harapan Kita Cardiovascular Hospital showed that CHD was more common among males.[20] Risk factors are higher in men because of higher levels of estrogen, which is cardio protective, in women as compared to those in men.[20] Physiologically, in addition to having vasodilatory effects, estrogen increases high-density lipoprotein and decreases low-density lipoprotein levels.[20]

There can be as many as 1–100 million bacteria in 1 ml of saliva, depending on oral hygiene, frequency of meal consumption and diet, and the rate of saliva flow. The attachment of *S. sanguinis*, which is a naturally occurring bacterium on the surface of clean teeth, was strongly influenced by the presence of salivary secretions.[16] The results of the present study pointed to a significant difference between *S. sanguinis* numbers in saliva of CHD and non-CHD patients, with fewer numbers in the saliva of CHD patients as compared to that of the controls. The latter is likely because patients pay more attention to oral hygiene because they already know the relationship of periodontitis and CHD. Poor oral hygiene leads to increased plaque accumulation, which increases *S. sanguinis* proliferation and subsequently the presence of various other bacterial species, such as *Spirochetes* and *Actinomycesviscosus*, that play a role in the pathogenesis of periodontal disease.[21]

Some species of *Streptococcus* in the oral cavity are able to produce acids and grow

in an environment with an acidic pH (acidophilic), whereas the growth of other streptococcal species and that of other bacteria is inhibited at low pH.[22] According to a study by Palmer of the growth of *Streptococcus* in the oral cavity under an acidic pH, *S. sanguinis* was unable to grow under such conditions.[22] The same study showed that although some *Streptococcus* species had the ability to rapidly adapt to fluctuations in pH values and re-colonize the environment, *S. sanguinis* were unable to adapt and regenerate in response to pH changes.[22]

In the present study, the decrease in the *S. sanguinis* count in saliva of CHD patients was likely attributed to fasting prior to undergoing bypass surgery. During fasting, a decrease in the salivary flow rate leads to a decline in the salivary pH, resulting in acidic conditions in the oral cavity. Heart disease drugs can also decrease the saliva flow rate.[23] Araujo and Souza reported a decrease of 17.5% in the saliva flow rate of beta-blocker users.[23] The authors noted that such a increase the dominance of anaerobic bacteria, thereby changing the oral microflora.[23] Other variables that inhibit salivary secretion include older age and psychological factors, such as stress.[17] Saliva production is very important to ensure the microflora balance in the oral cavity and protect the mouth from infection.[17]

The results of this study indicated that there was no significant relationship between the *S. sanguinis* count and periodontal status (plaque accumulation, gingival bleeding, and pocket depth) of CHD and non-CHD patients. *S. sanguinisis* the first bacterium to colonize the tooth surface and functions as a pioneer in the formation of dental plaque, which results in caries and periodontal disease.[11]Huang et al, stated that early bacterial colonization consisted of *Streptococcus* spp.[11] Previous studies showed that saliva stimulated biofilm formation through *S. sanguinis* binding of salivary proteins.[24] Research also suggested that *S. sanguinis* played an important role in the process of plaque maturation and gingivitis via the colonization of tooth surfaces and its ability to aggregate with other bacteria.[24]

Streptococcus is divided into four major groups of species: *S. mutans*, *S. salivarius*, *S. anginosus*, and *S. mitis. S. sanguinis* is grouped with *S. mitis*.[25] The percentage of adherent *S. sanguinis* was highest on the enamel.[25] The a fore mentioned finding likely explains the absence of a relationship between *S. sanguinis* in saliva and plaque accumulation in the CHD and non-CHD patients in the present study.

More than 700 bacterial species have been identified in dental plaque.[26] In periodontal disease, gram-negative and anaerobic bacterial species are the dominant pathogens, whereas gram-positive aerobes predominate in the early stages of the disease.[11]*S. sanguinis* is a member of the yellow complex (i.e., an early initiator bacterium).[11] Previous study suggested that numbers of *Streptococcus* increased after 6 h to seven days, followed by a significant decrease in numbers up to week three. In their study, a decrease in numbers of *S. sanguinis* was followed by an increase in numbers of bacteria belonging to groups of anaerobic gram-negative bacteria. Both the CHD and non-CHD patients had chronic periodontitis. Thus, no significant association was found between the periodontal status (gingival bleeding and pocket depth) and salivary count of *S. sanguinis*.[24]

The present study found no relationship between the *S. sanguinis* count in saliva and the pocket depth in patients with or without CHD. This result is supported by the findings of a study by Ge et al. of the mikobra complex in dental plaque that pointed to a close relationship between yellow complex species and shallow (< 3 mm) pockets. In their study, the prevalence of *S. sanguinis* decreased in accordance with an increase in the pocket depth.[26,27]

Conclusions

The *S. sanguinis* count in saliva of CHD patients was lower than that of non-CHD patients. There was no relationship between the *S. sanguinis* count in saliva and plaque accumulation in CHD and non-CHD patients. There was also no association between the salivary *S. sanguinis* count and gingival bleeding in CHD and non-CHD patients. Furthermore, there was no relationship between the numbers of *S. sanguinis* in saliva and pocket depths in CHD and non-CHD patients.

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