The Relationship between the Quantitative Measurement of *Streptococcus sanguinis* on Dental Plaque with the Periodontal Status of Patients with Coronary Heart Disease

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Abstract

Streptococcus sanguinis (*S. sanguinis*) is a common bacteria found in periodontal disease and coronary heart disease (CHD). This bacteria is suspected to have an important role in the relationship between both diseases through the blood stream. Objectives: To analyze the difference in thequantity of *S. sanguinis* on dental plaque between CHD and non-CHD patients. Methods: 66 CHD and 40 non-CHD patients were examined for periodontal status, and supragingival dental plaque was collected. A quantitative measurement of *S. sanguinis* was done with a real-time polymerase chain reaction. As the result showed that statistical analysis using the Mann-Whitney test was no significant difference between the number of *S. sanguinis* in CHD and non-CHD patients ($p > 0.05$). The Spearman test showed there was no correlation between the quantity of *S. sanguinis* with plaque accumulation, gingival bleeding, and pocket depth in CHD and non-CHD patients ($p > 0.05$). It was concluded that there is no difference between the quantity of *S. sanguinis* in CHD and non-CHD patients. There is no correlation between the quantity of *S. Sanguinis* and periodontal status in CHD and non-CHD patients.

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Introduction

Coronary heart disease (CHD) is one of the main forms of cardiovascular disease (heart and blood vessel disease), which is one of the main and first causes of death in developed and developing countries.[1] CHD is a disease that is not realized by most people and does not provide significant complaints because of the mild symptoms, such as chest pain on the left side that lasted briefly, thus making early stage sufferers less alert.[2]

Heart and blood vessel diseases are one of the major health problems in developed and developing countries. CHD is the number one cause of death in the world every year. Based on the results of the survey Mozaffarian et al. conducted in the United States from 2009 to 2012 on the prevalence of CHD by age and sex, men aged 60 years and over have 19.9% chance and women have a 9.7% chanceof suffering from CHD.[3]

According to a meta-analysis, there are several risk factors for CHD, such as systemic disease, traditional cardiovascular risks (habits, diet, lifestyle, and family history), and risk of infection.[4] The process of atherosclerosis in the coronary arteries is triggered by various risk factors such as smoking, hypertension, high cholesterol, diabetes mellitus, and obesity. Other circumstances that are thought to contribute to CHD are age, sex, and family history, but it is suspected that there are additional factors that contribute more to CHD, i.e., inflammation and infection.[5,6]

Inflammation plays an important role in atherothrombogenesis, and strong evidence suggests that infection by specific pathogenic bacteria is an additional risk factor for atherosclerosis.[5] Gram-positive and Gram-negative bacteria have been frequently

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identified in bacteremia and are said to have a role in vascular disease.[7]

Periodontitis is a chronic inflammatory disease caused by bacteria, which damages connective tissue and bone supporting the teeth. Signs and symptoms of this disease include gingival swelling, gingival discoloration, gingival bleeding during brushing, migration, tooth agitation, abscesses, poor mastication, and halitosis.[8] Periodontitis can be seen clinically from a loss of connective tissue around the teeth and a deep sulcus (gingival pocket). In radiological pictures, periodontitis presents a loss of alveolar bone.[9,10]

Streptococcus sanguinis (*S. sanguinis*) is the first bacterial colonization on the tooth surface and acts as a plaque-forming pioneer that can cause periodontal disease. *S. sanguinis* has an important role in the plaque maturation process on the tooth surface because of its ability to aggregate with other bacteria. *S. sanguinis* invade the epithelium of the gingival sulcus through the mechanism of releasing exotoxins, endotoxins, and proteolytic enzymes, subsequently affecting the immunologic response. Epithelial activation leads to the release of inflammatory mediators.[11] This process is one of the risk factors for atherosclerosis, which, in turn, leads to cardiovascular system disease. The bacteria of the oral cavity and the toxins they produce (endotoxins/exotoxins) can spread through the bloodstream. *S.sanguinis* dental plaque bacteria (Gram-positive bacteria) has been shown to cause platelet activation and aggregation through collagen-like expression and the platelet aggregation–associated protein (PAAP). Platelet aggregation may contribute to hypercoagulability and thrombosis, leading to thromboembolic events.[12]

Studies that examine the relationship between periodontal and cardiovascular disease are common. Soeroso et al. evaluated the relationship between periodontitis and CHD in Indonesia. The results of his research indicate that there is a relationship between the two diseases, where there is a significant difference in the calculus index between periodontitis patients with CHD and without CHD.[13] This is in line with research conducted by Bahekar et al., which stated that the prevalence and incidence of CHD significantly increased with periodontitis.[14] Dave et al. also stated that patients who had recently had a myocardial infarction had poorer oral hygiene and a worsening periodontal status.[15]

There is a debate about the relationship between CHD and periodontal disease. Kebschull et al. stated that no data suggests that the prevention of periodontal infections would result in a decrease in theincidenceof cardiovascular disease.[16]

Previous research has proven the relationship between periodontitis and CHD, where periodontitis is one of the risk factors of CHD. Periodontitis is an infectious disease caused by bacteria. *S. sanguinis* are the first bacteria that attach to the tooth surface, and they can aggregate with other bacteria to cause periodontitis.[17]

Materials and Methods

Samples Collection

This study was a cross-sectional study involving two clinical centers from November 2015–February 2016. Data for CHD patients was collected from Harapan Kita National Cardiovascular Center Hospital while data for the control was obtained from the Periodontology Specialist Clinic, Dental Teaching Hospital, Faculty of Dentistry, University of Indonesia. This study was approved by ethic committee of Faculty of Dentistry, University of Indonesia and Harapan Kita National Cardiovascular Center Hospital. The inclusion criteria for CHD patients was that they were male or female, aged 40–74 years old, were diagnosed with stable angina, and would undergo bypass surgery. The control group consisted of chronic periodontitis patient without angina (confirmed with negative treadmill tests and normal electrocardiograms). Patients who were edentulous, pregnant, or with other systemic diseases were excluded from this study. A total of 106 patients (66 CHD patients and 40 control patients) participated.

Clinical Periodontal Examination

Periodontal status was assessed by measuring the plaque index (according to the Silness and Löe Index), the papillary bleeding index (according to Saxer and Mühlemann), and pocket depth (PD). Both PDs were measured using a periodontal probe (colorvue probe, Hu-Friedy, USA). During the periodontal

examination, examiners measured the periodontal probing depth (PD) and clinical attachment loss at six sites per tooth (i.e., mesio-buccal, mid-buccal, disto-buccal, mesio-lingual/palatal, mid-lingual/palatal, and disto-lingual/palatal). Third molars were not included in the clinical assessment due to their large variation in anatomy and position in the oral cavity.

Collection of Supragingival Plaque

Supragingival microbial samples were collected from thegingival margin. The tooth was isolated using a cotton roll, and its root surface was dried by air. The supragingival sampleswere obtained using an excavator (Crown, Japan) and transferred into a microtube containing 1000 µlphosphate buffer saline (PBS). The samples were stored in -4^{0}C until real-time polymerase chain reaction (RT-PCR) was performed.

DNA Extraction Protocol

Each microtube was thawed at room temperature using a vortexer (BR-2000 Vortexer, Bio-Rad Laboratories, USA). A 50–150 µL solution of PBS (*tera volume* to ±1000 µL) was added to the microtube, and the sample was mixed with pipette. A 1000-µL volume of the sample was then added into a new microtube, its weight was measured using a scale (Ohaus Explorer, USA), and it was centrifuged at 13000 × *g*for 10 minutes (Sorvall Legend Micro 17 Microcentrifuge, Thermo Scientific, USA).

The supernatant was carefully removed, and 1000 µL of new PBS was added. The sample was again mixed and centrifuged at 13000 × *g* for 10 minutes. The supernatant was removed, and 200µL of nuclease-free water (NFW) was added into the microtube.

The sample was incubated in a thermoblock at 100^{0}C for 30 minutes (Thermo-block NB-305TB, N-Biotek, Korea). Cooling was done by putting the sample into an ice tray for 10 minutes. The sample was centrifugated at 10000 × *g* for 2 minutes.

Supernatants with a volume of ±180–200 µL were placed into new microtube. DNA was stored at -20°C until Real-Time Polymerase Chain Reaction (RT-PCR) was ready to be performed.

DNA Quantification and Standardization

Spectrophotometry was performed to determine DNA concentration and purification. Each spectrophotometric cycle consisted of 5 sample cuvettes and 1 reference cuvette (500µL air Aqua Bidest). Each sample cuvette consisted of a 5-µL DNA sample and 495µL of Aqua Bidest. Each cuvette was put into a spectrophotometer (Ultrospec 4000 Pro, Amersham Pharmacia Biotech, UK) with a wave length of 260 and 280nm (multi-wave length). The DNA concentration was obtained from the absorbance value at 260nm and multiplied by 50ng/µL. DNA purity was measured by comparing the absorbance value at 260:280nm. The concentration of DNA was standardized to100ng/µL in 100µL by using NFW.

Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed in a final volume of 10 µl, consisting of 5µl of SYBR Green, 0.5µl of forward primers, 0.5 µl of reverse primers, 3µl of DNA template, and 1µl of H_2O. The thermal profile consisted of initial denaturation at 95°C for 10 minutes, followed by 80 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. Each cycle-threshold (CT) of every sample was obtained at the end of the RT-PCR. The CT value was inserted into a standard curve equation that had been determined previously.

Statistical Analysis

All statistical analyses were performed using SPSS 20.0. Univariate analysis was performed to obtain the valuesfor the mean, standard deviation (SD), minimum and maximum (min-max) of all parameters. The quantities of *S.sanguinis*in CHD and control patients were assessed using the Mann-Whitney test. Correlations between the quantities of *S.sanguinis* and periodontal status were assessed using Spearman's correlation test.

Results

Table 1 shows the mean periodontal status (plaque index, papilla bleeding index, pocket depth) of CHD and non-CHD patients. This table also shows the quantitative measurements of *S.sanguinis*in CHD patients,

which was 13.01 (5.38) log 10 CFU/ml with a range of 5.48–24.53 CFU/ml, and in non-CHD patients there were 15.50 (6.69) log10 CFU/ml of *S. Sanguinis* with a range of 5.53–24.93 CFU/ml.

Table 1. Mean Distribution, Standard Deviation, Minimum and Maximum Value of Plaque Index, Papilla Bleeding Index, Pocket Depth, and Quantitative Measurements of *S.sanguinis* in Patients with CHD and without CHD

	CHD Patients		Non-CHD Patients	
Variable	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
Papilla Bleeding Index	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
Quantity of *S. sanguinis*(log10 CFU/ml)	13.01±5.38	5.48–24.53	15.50±6.69	5.53–24.93

A normality test on the periodontal status and quantity of *S.sanguinis* in patients with CHD was done using the Kolmogorov-Smirnov test (*n*> 50). It was seen that the distribution of the plaque index, papilla bleeding index, pocket depth, and quantityof *S.sanguinis* from this data was abnormal (Table 2).

Table 2. Result for the Normal Distribution Test on Periodontal Status and Quantity of *S. sanguinis* in CHD Patients

Variable	*p* value
Periodontal Status	
Plaque Index	0.200
Papilla Bleeding Index	0.001*
Pocket Depth	0.000*
Quantity of *Streptococcus sanguinis* (log10 CFU/ml)	0.000*

Kolmogorov-Smirnov Test: *p*> 0.05 = normal distribution

A normality test on the periodontal status and quantity of *S. sanguinis* in non-CHD patients was done using the Shapiro-Wilk test (*n*<50).

It appears that the distribution of the gingival bleeding index, pocket depth, and quantity of *S. sanguinis* from this data wasabnormal (Table 3).

Table 3. Result for Normal Distribution Test on Periodontal Status and Quantity of *S. sanguinis* in non-CHD Patients

Variable	p value
PeriodontalStatus	
Plaque Index	0.787
Papilla Bleeding Index	0.038*
Pocket Depth	0.000*
Quantitative of *Streptococcus sanguinis* (log10 CFU/ml)	0.000*

Shapiro-Wilk Test: *p*> 0.05 = normal distribution

The Mann-Whitney test in both subject groups showed a *p* value = 0.069. This table shows that there is no quantitative difference of *S. sanguinis* counts on dental plaque between CHD and non-CHD patients (Table 4).

Table 4. Comparative Analysis of *S. sanguinis* Level in Dental Plaque between CHD and Control Patients

Variable	*n*	Mean (SD)	*p* value
Quantity of *S. sanguinis* (log 10 CFU/ml)			
CHD	66	13.01±5.38	0.069
Non-CHD	40	15.50±6.69	

Mann-Whitney Test: $p<0.05$ = significant

The table below shows no significant correlation between *S. sanguinis* level on dental plaque with the plaque index, papilla bleeding index, and pocket depth of CHD patients (Table 5).

Table 5. Correlation Analysis between *S. sanguinis* Level on Dental Plaque and Plaque Index, Papilla Bleeding Index, and Pocket Depth of CHD Patients.

		Plaque Index	Papilla Bleeding Index	Pocket Depth
Quantity of *S. sanguinis*	*r*	-0.86	-0.02	-0.25
	p	0.49	0.99	0.84
	n	66	66	66

*Spearman test. $p< 0.05$ → hypothesis accepted, $p> 0.05$ → hypothesisrejected

The table below shows no significant correlation between S. sanguinis level on dental plaque with plaque index, papilla bleeding index, and pocket depth of non-CHD patients (Table 6).

Table 6. Correlation Analysis between *S. sanguinis* Level on Dental Plaque and Plaque Index, Papilla Bleeding Index, and Pocket Depth of Non-CHD Patients.

		PlaqueIndex	Papilla Bleeding Index	Pocket Depth
Quantity of *S. sanguinis*	*r*	0.06	-0.02	0.15
	p	0.69	0.99	0.35
	n	40	40	40

*Spearman test, $p< 0.05$ → hypothesis accepted, $p> 0.05$ → hypothesisrejected

Discussion

The samples were taken from 106 subjects (66 CHD subjects and 40 non-CHD subjects) with ages that ranged from 40–74 years old. Riset Kesehatan Dasar (2013) stated that CHD, heart failure, and stroke are more frequent in individuals who are 45–54 years old, 55–64 years old, and 65–74 years old.[18]

A periodontal examination was performed on all study subjects. *S. sanguinis* is one of the normal species found at the beginning of colonization on the tooth surface and is a pioneering bacterium. *S. sanguinis* has an important role in the process of plaque maturation on tooth surfaces because of its ability to aggregate with other bacteria.[19]

All subjects received an intake of supra gingival dental plaque and were then examined in the Oral Biology Laboratory Faculty of Dentistry, University of Indonesia. Quantitative calculations of *S. sanguinis* bacteria were performed with RT-PCR.

Quantitative Differences of *S. sanguinis* on Dental Plaque between CHD and Non-CHD Patients.

The results of this study indicate that there is no significant difference in the quantity of *S. sanguinis* on dental plaque between CHD and non-CHD patients ($p= 0.069$). The decrease in the number of *S. sanguinis* in CHD patients

was suspected because CHD patients who receive bypass surgery fast during salivary sampling. The decrease in salivary flow rate causes a decrease in salivary pH, resulting in an acidic condition of the oral cavity. A cause that can decrease saliva flow rate is the consumption of medication to treat heart disease. Araujo et al. stated that a decrease in saliva flow rate was found in 17.5% of all beta-blocker users. A decrease in the salivary flow rate can also cause changes in the oral microflora by altering the bacterial dominance into anaerobic bacteria.[20] Genetic factors seem to play an important role in determining whether bacteria can colonize the host. Infect genomics are an approach used to explain the relationship between the host's genetic profiles to the colonization of pathogenic microbes. It is based on the fact that the antibody response of each individual to infection varies.[21]

The association between periodontitis and cardiovascular disease is difficult to prove because both diseases have multifactorial risk factors. Risk factors such as smoking, genetics, stress, other systemic diseases, and age can independently cause periodontal disease and heart disease. This study did not distinguish between CHD and non-CHD patients who smoked and who did not smoke. The smoking status of patients needs to be explored more deeply, including whether the patient has never smoked for all of their life and the number of cigarettes smoked per day.[22]

Relationships between the Quantity of *S. sanguinis* on Dental Plaque with the Periodontal Status of CHD and Non-CHD Patients

The results of this study indicate that there is no significant relationship between the quantity of *S. sanguinis* in CHD and non-CHD patients with periodontal status based on the plaque index, papilla bleeding index, and pocket depth. Dental plaque quality factors such as specific pathogenic microorganisms found in dental plaque can affect the development and severity of periodontal disease. The early formation of dental plaque, bacteria is dominated by Gram-positive bacteria, and, if the plaque is not cleared, it will experience a maturation process dominated by Gram-negative bacteria. Their research used colors to divide the bacteria based on their relationship to the health and severity of periodontal disease.[23] *S. sanguinis* is a normal flora in the oral cavity. This bacterium first colonizes the tooth surface and initiates the formation of dental plaque. *S. sanguinis* has so far been known to have no direct role in causing periodontal disease. This species only serves to facilitate the colonization of other bacteria, including anaerobic bacteria, which play an important role in the occurrence of periodontal disease. Previous research has suggested that *S. sanguinis,* through their colonization on tooth surfaces, have a high aggregation ability with other bacteria, and thus have an important role in the maturation process of plaque and the process of gingivitis.[6] In chronic periodontitis, the number of *S. sanguinis* bacteria in dental plaque is very little.

This finding is thought to explain the reason for the absence of a relationship between *S. sanguinis* on dental plaque and plaque accumulation of CHD and non-CHD patients.[19] The bacteria involved as pathogens in periodontal disease dominated Gram-negative and anaerobic bacterial species while in the early stages by the Gram-positive aerobic bacterial species. The groups of bacteria are differentiated by frequency into five complexes. *S. sanguinis* are grouped in members of the yellow complex, which is a group of early bacteria (initiators).[24] Marsh and Martin undertook research on the distribution of bacteria on the plaque surface during the initial three-week period.

The results of the study suggest that from six hours to seven days, there is an increase in *Streptococcus* bacteria, followed by a significant decrease in the number by week 3. The decrease in the number of *S. sanguinis* is followed by an increase in other groups of Gram-negative anaerobic bacteria. This study stated that there is no relationship between the quantity of *S. sanguinis* on dental plaque with pocket depth in patients with CHD or without CHD.

This result is supported by research by Socransky et al. about microbial complexes in dental plaque and suggests that yellow complex species have a close relationship with shallow pockets (<3mm). The CHD and non-CHD subjects who were studied diagnosed with chronic periodontitis. Thus, no significant association was found between the periodontal status (gingival bleeding and pocket depth) and

the role of *S. sanguinis* in dental plaque. The number of *S. sanguinis* decreases with increasing pocket depth.[25]

Conclusions

There is no difference between the quantity of *S. sanguinis* in CHD and non-CHD patients. There is no correlation between the quantity of *S. sanguinis* and periodontal status (plaque index, bleeding on probing, and pocket depth) in CHD and non-CHD patients.

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