QUANTIFICATION OF STREPTOCOCCUS SANGUINIS ISOLATED FROM DENTAL PLAQUE AND SALIVA OF SUBJECTS WITH AND WITHOUT CORONARY HEART DISEASE – ANALYSIS USING REAL-TIME PCR

(KUANTIFIKASI *STREPTOCOCCUS SANGUINIS* YANG DIISOLASI DARI PLAK DAN SALIVA GIGI SUBYEK DENGAN DAN TANPA PENYAKIT JANTUNG KORONER DENGAN MENGGUNAKAN PCR *REAL-TIME*)

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Abstract

Coronary Heart Disease (CHD) is the major cause of death in most countries in the world. Gram-positive and Gram-negative bacteria have been identified in bacteremia cases and known to have a role in various vascular diseases, including *Streptococcus sanguinis* which is most frequently isolated from endocarditis patients and often associated with CHD. The purpose of this study was to analyze the number of *Streptococcus sanguinis* isolated from dental plaque and saliva of subjects with and without CHD. Bacterial colonies isolated from the dental plaque and saliva of 16 subjects without CHD and 8 subjects with CHD were planted in Mitis salivarius agar, and then the DNA was extracted and quantified with a Real-Time PCR technique using 16S rRNA specific primers. The quantification of Real-Time PCR showed that there was a difference in the number of *S. sanguinis* between the two groups of subjects, but an unpaired T-test showed that the difference was not statistically significant. Furthermore, the number of *S. sanguinis* from dental plaque in CHD subjects tends to be higher than that of non-CHD subjects whereas the number of *S. sanguinis* from saliva in non-CHD subjects tends to be higher than that of CHD subjects.

Key words: coronary heart disease, real-time PCR, *Streptococcus sanguinis*

Abstrak

Penyakit Jantung Koroner merupakan penyebab utama kematian di dunia. Bakteri positif Gram dan negatif Gram telah sering diidentifikasi pada bakteremia dan disebut memiliki peran dalam penyakit vascular, termasuk *Streptococcus sanguinis* yang paling sering diisolasi dari pasien endokarditis dan sering dikaitkan dengan PJK. Tujuan penelitian ini adalah untuk menganalisis jumlah *Streptococcus sanguinis* yang diisolasi dari plak gigi dan saliva subjek non-PJK dan PJK. Koloni bakteri dari plak gigi dan saliva 16 subjek non-PJK dan 8 subjek PJK ditanam pada agar Mitis salivarius, diekstraksi DNA kemudian dikuantifikasi dengan teknik Real-Time PCR menggunakan primers spesifik 16S rRNA. Kuantifikasi Real-Time PCR menunjukkan perbedaan jumlah *S. sanguinis* antara subjek kelompok non-PJK dan PJK namun uji t tidak berpasangan menunjukkan perbedaannya tidak signifikan. Pada subjek yang menjadi sampel penelitian ditemukan kecenderungan jumlah *S. sanguinis* asal plak gigi subjek PJK lebih tinggi dibandingkan subjek non-PJK dan jumlah *S. sanguinis* asal saliva subjek non-PJK cenderung lebih tinggi dibanding subjek PJK.

Kata kunci: penyakit jantung koroner, *real-time PCR, Streptococcus sanguinis*

INTRODUCTION

Coronary Heart Disease (CHD) is still the leading cause of death in the world.[1-5] Myocardial infarction, the first sequence of death causes, is commonly caused by CHD.[6] Gram-positive and Gram-negative bacteria have been frequently identified in bacteriaemia and are said to have a role in vascular diseases.[7]

Streptococcus sanguinis is a Gram-positive bacteria which is a major component in the normal flora

of the mouth.[8,9] Streptococcus *sanguinis* has been reported to be closely related to the occurrence of infective endocarditis which is often caused by the entry of bacteria into the bloodstream due to trauma.[10] Moreover, *Streptococcus sanguinis* is one of the most isolated organisms from dental plaque, one of the most frequently isolated organisms from patients with sub-acute bacterial endocarditis[11], and often associated with coronary heart disease.[10,11,12]

A study by Johansson et al. found that periodontal diseases were higher in prevalence in CHD patients than in healthy subjects.[1] Another study conducted by Nakajima et al. showed that periodontitis was a risk factor for the occurrence of Coronary Heart Disease.[2] Moreover, Hujoel et al. also conducted a study to evaluate the risk of CHD in subjects with periodontitis, gingivitis, and healthy periodontium. The result of this study did not show a causal relationship between periodontal diseases and the risk of CHD.[13]

Spahr et al. investigated the relationship between periodontitis and CHD which was seen from microbiological characteristics of periodontitis. This study found a strong and significant relationship between the number of periodontal bacteria and CHD.[4] Another study by Nakano et al. found *S. sanguinis* on a tissue taken from the heart valve and atheromatous plaque specimens taken from patients undergoing cardiac surgery.[14] In addition, Deng et al. and Chen et al. proved an increase in the number of S. sanguinis in saliva and dental plaque of CHD patients.[15,16]

The present study was conducted to determine the number of *Streptococcus sanguinis* isolated from dental plaque and saliva of non-CHD subjects compared with CHD subjects using a Real-Time PCR method. This was a preliminary study for a study by Kemal et al. about *S. sanguinis* in CHD patients up to the number of genotypes.

MATERIALS AND METHODS

The subjects of this research were patients with and without CHD, and the materials used were dental plaque and saliva collected from the research subjects. Ethical approval was obtained from the Research Ethics Committee of Dentistry, University of Indonesia. The type of research was laboratory observation.

The location of the research sampling was at Cipto Mangunkusumo Hospital and Periodontics Specialist Clinic at the Faculty of Dentistry, University of Indonesia. The measurement of the number of *S. sanguinis* was conducted at the Oral Biology Laboratory in the Faculty of Dentistry, University of Indonesia. This study was conducted from April to June 2012. The determination of subjects whether they suffered from CHD or not was done with an ECG examination performed at Cipto Mangunkusumo Hospital, Jakarta.

Subjects examined in the present study were 24 subjects consisting of men and women with an age range of 50-60 years. Based on their smoking status, there were former smokers, smokers, and non-smokers.

The research used a consecutive sampling to determine the research subjects in which a minimum number of samples were 16 samples. The sample criteria were as follows: (1) CHD or non-CHD subjects determined by an ECG examination; (2) an accumulation of plaque on the teeth and production of saliva; (3) aged of 50-60 years; and (4) willing to participate in the research and signed informed consent. The exclusion criteria were pregnant women and uncommunicative patients.

The work procedure started by making mitis Salivarius agar. The measurement of the plaque score, calculus, periodontal index, and OHIS score was done according to their respective measurement criteria. Afterward, the dental plaque of the subjects was collected on the lingual side of the lower anterior teeth by swabbing. Then, the samples were inserted into a 1.5 ml Eppendorf tube filled with sterile PBS fluid, sealed, and labelled with a name and code. Saliva samples were obtainnned after holding it for 5 minutes in the mouth without stimulation.

The bacteria were bred on a selective medium of mitis salivarius using a glass inoculating loop and were inserted into an anaerobic tube for incubation at the temperature of 37ºC for 3 days. Then a dilution series and calculation of *S. sanguinis* control bacteria was done to determine the CFU/ml value used as the standard value in the PCR Real-Time amplification process.

The DNA extraction of the bacteria used GeneJETTM genomic DNA Purification Kit (Fermentas®). A 5 µl of the extracted DNA was collected and inserted in a cuvette for spectrophotometry. After that, DNA amplification was conducted by using Real-Time PCR. The calculation of the number of bacteria was obtained from the dilution of *S. sanguinis* culture planted on mitis salivarius agar base.

RESULTS

Primary data was collected through clinical examination in the form of plaque accumulation, calculus accumulation, electrocardiogram examination, and laboratory work to determine the number of *S. sanguinis* isolated from dental plaque and saliva.

The number of subjects examined was 25 subjects. Based on an ECG examination, 16 subjects were healthy (non-CHD), 8 subjects suffered CHD, and 1 subject was eliminated for not having an ECG examination.

Table 1. Distribution of Demographic Data for Gender and Smoking Status

Variable		Group					
		Non-CHD		CHD		Total	
		n	%	n	%	N	%
Gender	Male	12	75.0%	6	75.0%	18	75.0%
	Female	4	25.0%	2	25.0%	6	25.0%
Smoking Status	Former smoker	3	18.8%	4	50.0%	7	29.2%
	Smoker	7	43.8%	3	37.5%	10	41.7%
	Non-smoker	6	37.5%	1	12.5%	7	29.2%

The results of a univariate analysis in Table 1 showed that most of the subjects were male (75%) with the number of smokers reached 42%.

In the non-CHD group, the number of subjects where the quantification of *S. sanguinis* from dental plaque samples were successful with the Real-Time PCR technique was 15, whereas in the CHD group there was only 7 subjects that were successfully quantified. Quantification of *S. sanguinis* from saliva samples were all successful in 24 subjects.

Table 2. The Number of *Streptococcus sanguinis* from Samples of Dental Plaque and Saliva

	Mean	Standard Deviation	Median	Minimum	Maximum	Distribution
The number of S.s from plaque* (CFU/ml)	50.32	167.37	0.01	-	806.50	Non-normal
The number of S.s from saliva* (CFU/ml)	501.63	2.323.89	0.02	0.00	11,407.50	Non-normal
logCFUplaque	11.36	1.86	10.45	9.28	14.91	Normal
logCFUsaliva	11.39	2.03	10.23	9.10	16.06	Normal

*) in trillion units (10^{12})

Note: For the normal distribution, valid parameters were mean and standard deviation whereas for non-normal distribution data, valid parameters were median, minimum values, and maximum values.

Table 2 shows the total number of *Streptococcus sanguinis* bacteria from dental plaque and saliva samples of the non-CHD and CHD groups based on calculations using the Real-Time PCR method. In the case of normal distribution, the data were transformed by Kolmogorov-Smirnov test in the form of log values of the total number of *S. sanguinis* from dental plaque and saliva samples.

Table 3. Transformation values comparison in the number of *S. sanguinis* from samples of dental plaque and Saliva between non-CHD and CHD subjects.

Group	Log CFU Plaque		Log CFU Saliva		p-value
	Average	SD	Average	SD	
Non-CHD	11.13	1.62	11.60	2.01	0.413
CHD	11.84	2.35	10.98	2.13	0.493

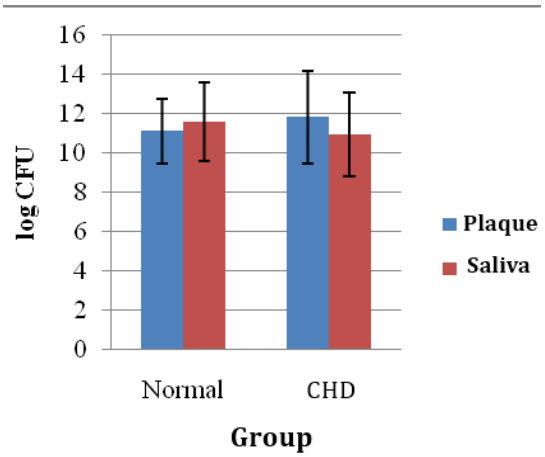

Figure 1. Comparison of *S. sanguinis* bacterial counts from dental plaque and saliva samples in the non-CHD and CHD groups

Table 3 and Figure 1 show a difference in the log-CFU/ml value of *S. sanguinis* from dental plaque and the log CFU/ml of *S. sanguinis* from Saliva between subjects in the non-CHD and CHD groups. As shown, the log CFU/ml value of *S. sanguinis* from dental plaque of the CHD subjects was higher than the non-CHD subjects.

However, an unpaired T-test result found no significant difference between log CFU/ml value of *S. sanguinis* from plaque in both groups (p=0.413).

Furthermore, Table 3 and Figure 1 show that log CFU/ml value of *S. sanguinis* from saliva for non-CHD subjects was slightly higher than that of the CHD subjects although the unpaired T-test result found no significant difference between log CFU/ml of *S. sanguinis* values from saliva in both groups (p=0.493).

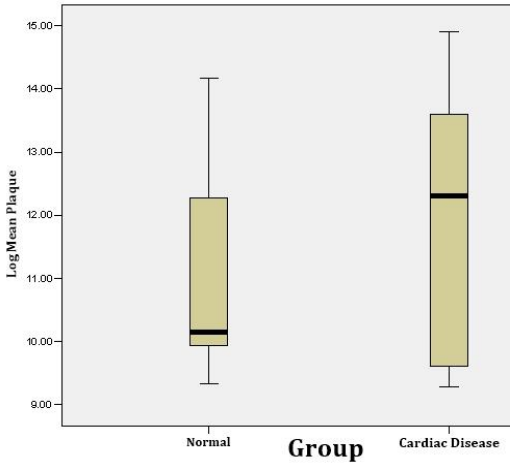

Figure 2. The number of *S. sanguinis* bacteria from plaque samples in the non-CHD and CHD groups

Figure 2 shows a boxplot diagram depicting the log CFU/ml value of *S. sanguinis* from dental plaque between the non-CHD and CHD subjects. As shown, the log CFU/ml value of *S. sanguinis* from dental plaque of the CHD subjects was higher than that of the non-CHD subjects although the difference was not statistically significant.

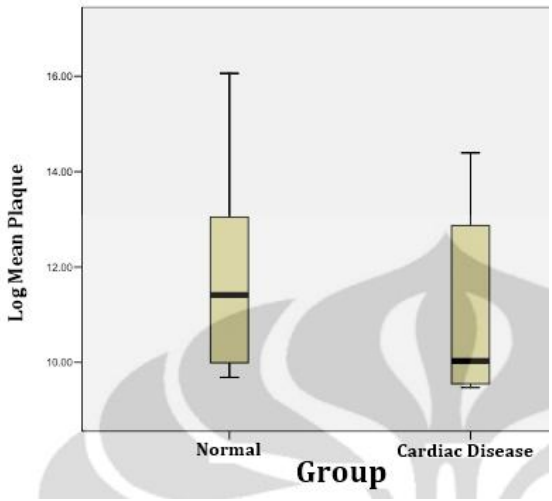

Figure 3. The number of *S. sanguinis* bacteria from saliva samples of the non-CHD and CHD groups

Figure 3 shows a boxplot diagram depicting the comparison of log CFU/ml of *S. sanguinis* from saliva between subjects in the non-CHD and CHD

groups. It is seen that the log CFU/ml value of *S. sanguinis* from the saliva of the non-CHD subjects was higher than that of the CHD subjects, but the difference was not significant statistically.

The data obtained were then analyzed by ROC to see if the number of *S. sanguinis* bacteria in the dental plaque and saliva samples could be used to distinguish non-CHD and CHD subjects.

Table 4. ROC analysis to see the number of *Streptococcus sanguinis* from the samples of dental plaque and saliva in distinguishing non-CHD and CHD subjects

Test results variable	Area	Std. Error (a)	Asymptotic Sig.(b)	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
logCFUplaque	.486	.165	**.916**	.162	.809
logCFUsaliva	.629	.152	**.341**	.331	.926

a Under the nonparametric assumption
b Null hypothesis: true area = 0.5

Table 4 shows the analysis results of ROC showing that the number of *S. sanguinis* from dental plaque and saliva samples was not sensitive to distinguish non-CHD and CHD subjects. This result was obtained from an insignificant p-value in log CFU/ml of *S. sanguinis* from dental plaque (p= 0.916) and log CFU/ml of *S. sanguinis* from saliva (p=0.341).

DISCUSSION

The purpose of this study was to analyze the number of *S. sanguinis* isolated from dental plaque and saliva samples of the non-CHD and CHD subjects. The sample collection from the research subjects was conducted at the Periodontics Clinic of Dental and Mouth Hospital, Faculty of Dentistry, University of Indonesia.

The number of subjects recruited was 24 subjects in whom most of the subjects were male (75%) and the number of smokers reached 42%. Moreover, the age range of the subjects was a minimum 50 to a maximum 60 years old. This age range was selected because cholesterol level during this age range is the highest, so it also has a high risk of CHD.[21]

Primary data was collected through a clinical examination of Plaque Index, Calculus Index, Periodontal Index, OHIS, and laboratory examination in the form of *S. sanguinis* quantification examined from dental plaque and saliva. The laboratory examination was conducted at the Oral Biology Laboratory, Faculty of Dentistry, University of Indonesia.

The examination of dental plaque and saliva samples collected from the subjects was performed with Real-Time PCR using universal primers. The advantages of using the Real-Time PCR technique are including high sensitivity and specificity, also quick and effective calculations.[17] In addition, the bacterial samples which will be counted with the Real-Time PCR should not necessarily be alive, and the samples remain stable for long-term storage if frozen.

Real-Time PCR with specific primers will generate accurate and sensitive methods for the identification and quantification of species and bacterial populations (i.e. total bacteria) obtained from the study samples. Primers used in this study were 16S rRNAs because these primers were particularly useful for the detection and quantification of bacteria in various environments and health-related situations in which the sample was a multi-species and impure population.[19]

The quantification method used to calculate the number of bacteria in this study was the absolute quantification which was a quantification of bacteria in a sample based on the relative comparison to the 16S rRNA amplification results of the standard bacteria and by using the pure strain bacterial standard of the American Type Culture Collection. The absolute quantification produces the exact number of targeted DNA molecules by comparison with standard bacterial DNA. The total number of *S. sanguinis* bacteria was obtained from the calculation based on the conversion value of the C_T value.[20]

Spahr et al. measured directly the number of periodontal pathogens and found that an increase in the number of periodontal pathogens was associated with an increase in the incidence of CHD.[4] A study by Chen et al. who took plaque samples from subgingival plaques showed that 89he number of *S. sanguinis* in plaque and saliva of CHD patients was significantly higher than that of healthy patients.[15] In the present study, the number of *S. sanguinis* from dental plaque samples of CHD subjects was higher than that of the non-CHD subjects. However, the difference was not statistically significant due to the small number of samples. This is in accordance with the results obtained by Chen and colleagues. This result also corresponds to an increase in the number of periodontal pathogens in CHD subjects found by Spahr and colleagues.

In this study, *S. sanguinis* from the saliva samples of the non-CHD subjects was slightly higher than that of the CHD subjects, but the difference was not statistically significant. This raises the assumption that the difference occurs because of the different strains of *S. sanguinis* in plaque and saliva. It is possible that the strains of *S. sanguinis* which have a ro-

le in forming biofilms in adhesions on the teeth surfaces are more prevalent in CHD subjects. To prove this assumption, further research is needed.

McNicol et al. conducted a study to determine the role of immunoglobulin G in relation to cardiovascular diseases caused by bacteria, especially *S. sanguinis*. This study concluded that platelet activation depended on IgG and its bonding with the strains of *S. sanguinis*. Among four strains studied, three strains showed a significant number of IgG while the other strain had a low bond, so platelet aggregation was regulated by the extent to which the IgG bond responded to the presence of *S. sanguinis*.[22] The results of this study showed that strains present in non-CHD and CHD might differ.

According to the literature, there is a similarity in the composition of bacteria in saliva and the tongue. The tongue is a considerable reservoir of *S. sanguinis*, and this explains the high number of *S. sanguinis* in saliva.[23] Therefore, an increase in the number of *S. sanguinis* in dental plaque does not reflect the number of *S. sanguinis* in saliva. Oral streptococcal colonization on the teeth surfaces depends on the adhesion of bacteria to the components of saliva adsorbed onto the teeth surfaces.[8] Studies showed that some of the saliva components namely proline-rich protein, agglutinin, and α-amylase were proven to bind to oral streptococci. Okahashi et al. found that the structure of *S. sanguinis* named pili binds to salivary α-amylase and plays a role in biofilm formation on saliva-coated surfaces. It is also argued that *S. sanguinis* strains without pili are unable to form biofilms.[8] This supports the assumption that *S. sanguinis* strains in plaque and saliva may be different because the structure is different between *S. sanguinis* which can and cannot form biofilms.

The ROC analysis showed that the number of *S. sanguinis* from dental plaque and saliva samples found in the subjects was not sufficiently sensitive to distinguish between CHD and non-CHD subjects because the number of samples in this study was relatively small. Therefore, this requires further research with larger samples. In conclusion, there was a difference in the number of *S. sanguinis* isolated from dental plaque and saliva of the non-CHD and CHD subjects in which the number of *S. sanguinis* from the dental plaque samples of the CHD subjects tends to be higher than that of the non-CHD subjects. In addition, the number of *S. sanguinis* from saliva samples of the non-CHD subjects tends to be higher than that of the CHD subject.

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