Quantitative Measurement of Porphyromonas Gingivalis and Treponema Denticola Levels on Dental Plaque and its Relationship with Periodontal Status and Coronary Heart Disease

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Abstract

Coronary heart disease (CHD) is a narrowing of coronary artery due to plaque build-up, a process called atherosclerosis. Chronic infection such as chronic periodontitis is a risk factor for atherosclerosis. Porphyromonas gingivalis and Treponema denticola are linked to chronic periodontitis.

To analyse quantitative difference of P.gingivalis and T.denticola on dental plaque and its relationship with periodontal status of CHD patients and control. Methods: Periodontal status of 66 CHD patients and 40 controls was obtained. Subgingival plaque was isolated. P.gingivalis and T.denticola level were measured using real-time PCR.

P.gingivalis level of CHD patients (6.95(1.77) log10 CFU/ml) was significantly different from control (6.12(1.32) log10 CFU/ml). T.denticola level of CHD patients (5.26(2.27) log10 CFU/ml) was significantly different from control (3.59(1.93) log10 CFU/ml). P.gingivalis was linked to the pocket depth (PD) of CHD patient ($p=0.03$). T.denticola was not significntly associated with any periodontal status ($p<0.05$).

P.gingivalis and T.denticola levels of CHD patients were higher than control. P.gingivalis level is not linked to any periodontal status, except for pocket depth of CHD patient. T.denticola was not associated with any periodontal status.

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Introduction

Coronary heart disease (CHD) is a medical condition characterized by a narrowing of coronary artery due to the build up of fatty material inside the artery.[1] Coronary heart disease is the main cause of death and disability in most developed countries. Although mortility rate of coronary heart disease has decreased in the past four decades, coronary heart disease still remains the leading cause of death in a third or more of individuals aged 35 or above.[2] According to Basic Research of Health 2013, the prevalence of the emergence of symptoms and diagnosed coronary heart disease in Indonesia is 1.5%.[3] At present, many studies showed that the chronic inflammation disease might increase the chance of contracting cardiovascular disease. This has been the main reason for the growing numbers of researches pertinent to the effect of

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chronic infection on the pathogenesis of cardiovascular disease.[4]

Periodontitis is an inflammation of tooth's supporting structure, caused by specific microorganisms. This leads to progressive destruction of periodontal ligament and alveolar bone, along with an increase in pocket depth, recession, or both. Plaque build-up, calculus and gingival bleeding on probing are commonly found in periodontitis patient. Chronic periodontitis is the type of periodontitis commonly found in adult. The main etiology of this condition is plaque, intercellular matrix consisting of proliferating microorganisms, epithelial cells, leucocytes, and macrophage. Microorganisms frequently encountered in subgingival plaque of patient with chronic periodontitis are Porphyromonas gingivalis and Treponema denticola.[5]

According to American Academy of Periodontology, present studies have not yet provide any support to the causal relationship between the cardiovascular and periodontal disease. Some studies also demonstrated conflicting results.[6] At the present, there has never been any study in Indonesia that compares the quantitative measurement of Porphyromonas gingivalis and Treponema denticola in dental plaque of patients with coronary heart disease (CHD) and healthy patients linked periodontal status.

Objectives: To compare the level of P.gingivalis and T.denticola level on dental plaque between CHD and control. This study also aims to find the relationship between P.gingivalis and T denticola level on dental plaque and periodontal status in CHD and control patients.

Materials and methods

Collecting Samples

This study is a cross sectional study involving two clinical centers between November 2015-February 2016. Data for coronary heart disease patients was collected from Harapan Kita National Cardiovascular Center Hospital, while data for control was obtained from Periodontology Specialist Clinic, Dental Teaching Hospital, Faculty of Dentistry, Universitas Indonesia. This study was approved by ethic committee of Faculty of Dentistry, Universitas Indonesia and Harapan Kita National Cardiovascular Center Hospital.

Inclusion critetia for CHD are male or female, age 40–74 years old, who were diagnosed with stable angina and will undergo bypass surgery. Control group consisted of chronic periodontitis patient without angina (confirmed with negative treadmill test and normal ECG). Edentulous patients, pregnant, or with other systemic diseases were excluded from this study. Total 106 patients (66 CHD patients and 40 controls) were participated.

Clinical Periodontal Examination

The periodontal status was assessed by measuring Plaque Index (PI) (according to Silness and Loe Index), Calculus Index (CI) (according to Bjorby and Loe), Papillary Bleeding Index (PBI) (according to Saxer and Muhlemann), Pocket Depth (PD), and Clinical Attachment Loss (CAL). Both PD and CAL was measured using periodontal probe (colorvue probe, Hu-Friedy, USA). During periodontal examination, examiners measured periodontal probing depth (PD) and clinical attachment loss (CAL) at 6 sites per tooth (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 subgingival plaque

Subgingival microbial samples were collected from 4-6 mm pocket depth. The tooth was isolated using cotton roll, its supragingival plaque removed, and its root surface dried by air. Subgingival sample was obtained using excavator (Crown, Japan) and transferred into microtube containing 1000 µl Phosphate Buffer Saline (PBS). Sample was stored in -4ºC until RT-PCR will be performed.

DNA Extraction Protocol

Each microtube was thawed in room temperature using vortexer (BR-2000 Vortexer, Bio-Rad Laboratories, USA). Phosphate Buffer Saline of 50-150 µL (*tera volume* to ±1000 µL) was added to the *microtube* dan sample was mixed with pipette. Sample volume of 1000 µL was then added into new microtube, its weight measured using scale (Ohaus Explorer, USA), and centrifugation was done at 13000xG for 10 minutes (Sorvall Legend Micro 17 Microcentrifuge, Thermo Scientific, USA) Supernatant was removed carefully and 1000 µL new PBS was added. Sample was again mixed and centrifugated at 13000xG for 10 minutes.

Supernatant was removed and 200 µL Nuclease Free Water (NFW) was added into the microtube. Sample was incubated in thermoblock at 100 ºC for 30 minutes (Thermo-block NB-305TB, N-Biotek, Korea). Cooling was done by putting sample into ice tray for 10 minutes. Sample was centrifugated at 10000xG for 2 minutes. Supernatants with a volume of ±180-200 µL was placed into new microtube. DNA was stored at -20°C until PCR reaction was ready to be performed.[7]

DNA Quantification and Standardization

Spectrophotometry was performed to determine DNA concentration and purification. Each spectrophotometry cycle consisted of 5 sample cuvette and 1 reference cuvette (500 µL air aquabidest). Each sample cuvette consisted of 5 µL DNA sample dan 495 µL aquabidest. Cuvette was put into spectrophotometer (*Ultrospec 4000 Pro,* Amersham Pharmacia Biotech, UK) with wavelength of 260 dan 280 nm (*multi-wavelength*). DNA concentration was obtained from absorbance value at 260 nm and multiplied by 50 ng/µL. DNA purity was measured by comparing absorbance value of 260:280 nm. Concentration of DNA was standardised to100 ng/µL in 100 µL by using NFW.[7]

Real-Time Polymerase Chain Reaction

All reactions were performed using Step One Plus Real-Time PCR Systems (Applied Biosystem, USA) and iTaq Univer SYBR Green (Bio-Rad USA). Primers of P.gingivalis and T.denticola used for RT-PCR are displayed at table 1.

Primers	Sequence (5'-3')	Bacteria
PG-Forward	TACCCATCGTCGCCT TGGT	*P.gingivalis*
PG-Reverse	CGGACTAAAACCGCATACACTTG	
TD-Forward	AGAGCAAGCTCTCCCTTACCGT	*T. denticola*
TD-Reverse	TAAGGGCGGCTTGAAATAATG	

Table 1. Primers used in Real Time PCR[8].

Real-time PCR was performed in a final volume of 10 µl, consisting of 5 µl SYBR Green, 0.5 µl forward primers, 0.5 µl reverse primers, 3 µl DNA template, and 1 µl H_2O. Thermal profile consisted of initial denaturation at 95 °C for 10 minutes, followed by 80 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 minute (for PG), or at 65 °C for 1 minute (for TD). Each cycle-threshold (CT) of every sample was obtained at the end of RT-PCR. CT value was inserted into standard curve equation which was determined previously. The standard curve for PG is y= -0.25x+12.284, TD is y = -0.2096x + 14.213.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 .Univariate analysis was performed to obtain the value of Mean, Standard Deviation (SD), Minimum and Maximum (Min-Max) of all parameters. Quantitative differences of P.gingivalis and T.denticola between CHD and control patients were assessed using Mann Whitney test. Correlation between quantitative of P.gingivalis, T.denticola and periodontal status were assessed using Spearman's correlation test.

Results

Distribution of distribution of the results are shown in Table 2.

Variabel	CHD Patient		Non-CHD Patient	
	Mean (SD)	Min – Max	Mean (SD)	Min – Max
Age (years)	58.14(8.37)	40.00-74.00	52.37(8.99)	41.00-73.00
Plaque Index	1.37(0.64)	0.08-3.00	1.42(0.57)	0.20-2.89
Calculus Index	1.62(0.86)	0-3.00	1.57(0.79)	0-3.00
Papilla Bleeding Index	0.80(0.59)	0-2.38	1.10(0.72)	0-3.14
Pocket Depth (mm)	4.74(0.75)	4.00-6.00	5.10(0.84)	4.00-6.00
Clinical Attachment Loss (mm)	6.03(1.73)	4.00-12.00	5.58(1.24)	4.00-10.00
Quantitative of Porphyromonas gingivalis (log10 CFU/ml)	6.95(1.77)	3.03-11.04	6.12(1.32)	2.33-8.27
Quantitative of Treponema denticola (log10 CFU/ml)	5.26(2.27)	-0.70-8.44	3.59(1.93)	-1.72-5.81

Table 2. Mean Distribution, Standard Deviation, Minimum and Maximum Value of Plaque Index, Calculus Index, Papilla Bleeding Index, Pocket Depth, Clinical Attachment Loss, and Quantitative Measurement of P. gingivalis and T denticola.

Normality test on clinical parameter, periodontal status, and quantitative of microorganisms on CHD patients was done using Kolmogorov-Smirnov test (table 3). Normality test showed that distribution of plaque index, calculus index, and quantitative of P.gingivalis were normal. The distribution quantitative of T.denticola was abnormal.

Normality test on clinical parameter, periodontal status and quantitative of microorganisms on CHD patients was done using Shapiro-Wilk test (table 4). Normality test showed that distribution of plaque index and calculus index was normal. The distribution quantitative of P.gingivalis and T.denticola were abnormal.

Variable	p value
Clinical Parameter	
Age	0,006
Periodontal Status	
Plaque Index	0.20*
Calculus Index	0.08*
Papilla Bleeding Index	0.001
Pocket Depth (mm)	0.000
Clinical Attachment Loss (mm)	0.000
Quantitative of Microorganism	
Quantitative of Porphyromonas gingivalis	0.20*
Quantitative of Treponema denticola	0.002

Kolmogorov-Smirnov Test; p> 0.05 = normal distribution

Table 3. Result for Normal Distribution Test on Clinical Parameters, Periodontal status, and Quantitative of P.gingivalis and T denticola on CHD Patients.

Variable	p value
Clinical Parameter	
Age	0.008
Periodontal Status	
Plaque Index	0.79*
Calculus Index	0.23*
Papilla Bleeding Index	0.04
Pocket Depth (mm)	0.000
Clinical Attachment Loss (mm)	0.000
Quantitative of Microorganism	
Quantitative of Porphyromonas gingivalis	0.02
Quantitative of Treponema denticola	0.000

Shapiro-Wilk Test; p > 0.05 = normal distribution

Table 4. Result for Normal Distribution Test on Clinical Parameters, Periodontal status, and Quantitative of P.gingivalis and T.denticola, on Control Patients.

P.gingivalis Level	N	Mean (SD)	p value
CHD	66	6.95(1.77)	0.004*
Non CHD	40	6.12(1.32)	

Mann-Whitney test;*p<0.05 = significant.

Table 5. Comparative Analysis of P.gingivalis Level in Dental Plaque between CHD and Control Patients.

T.denticola Level	N	Mean (SD)	p value
CHD	66	5.26(2.27)	0.000*
Non CHD	40	3.59(1.93)	

Mann-Whitney test;*p<0.05 = significant.

Table 6. Comparative Analysis of T.denticola Level in Dental Plaque between CHD and Control Patients.

This table shows that there is significant difference of *P.gingivalis* count between CHD patients and Control patients (table 5).

This table shows that there is significant difference of T.denticola level between CHD patients and Control patients (table 6).

This table shows that there are no significant correlation between P.gingivalis level on dental plaque with gingival bleeding and clinical attachment loss on CHD patients, while there is a significant correlation between P.gingivalis level on dental plauqe with pocket depth of CHD patients (table 7).

CHD (N=66)		Gingival Bleeding	Pocket Depth	Clinical Attachment Loss
P.gingivalis Level	r	0.19	0.27	0.12
	p	0.13	0.03*	0.33

Spearman test, p< 0.05 = hypothesis accepted, r = correlation coefficient.

Table 7. Correlation Analysis between P.gingivalis Level in Dental Plaque and Gingival Bleeding, Pocket Depth, Clinical Attachment Loss of CHD Patients.

This table shows that there are no significant correlation between P.gingivalis level on dental plaque with gingival bleeding, pocket depth, and clinical attachment loss on Control patients (table 8).

CHD (N=66)		Gingival Bleeding	Pocket Depth	Clinical Attachment Loss
P.gingivalis Level	r	-0.08	-0.13	-0.20
	p	0.62	0.44	0.22

Spearman test, p< 0.05 = hypothesis accepted, r = correlation coefficient.

Table 8. Correlation Analysis between P.gingivalis level in Dental Plaque and Gingival Bleeding, Pocket Depth, Clinical Attachment Loss of Non-CHD Patients.

CHD (N=66)		Gingival Bleeding	Pocket Depth	Clinical Attachment Loss
T.denticola level	r	0.23	0.07	0.05
	p	0.06	0.56	0.69

Spearman test, p< 0.05 = hypothesis accepted, r = correlation coefficient.

Table 9. Correlation Analysis between T.denticola level in Dental Plaque and Gingival Bleeding, Pocket Depth, Clinical Attachment Loss of CHD Patients.

This table 9 shows that there are no significant correlation between T.denticola level

on dental plaque with gingival bleeding, pocket depth, and clinical attachment loss on CHD patients.

CHD (N=66)		Gingival Bleeding	Pocket Depth	Clinical Attachment Loss
T.denticola Level	r	-0.02	-0.03	0.01
	p	0.89	0.85	0.94

Table 10. Correlation Analysis between T.denticola level in Dental Plaque and Gingival Bleeding, Pocket Depth, Clinical Attachment Loss of Control Patients.

This table 10 shows that there are no significant correlation between T.denticola level on dental plaque with gingival bleeding, pocket depth, and clinical attachment loss on Control patients.

Discussion

Samples were taken from 106 subjects (66 CHD subjects and 40 non CHD subjects), age 40 – 74 years old. This age range is chosen according to Riset Kesehatan Dasar 2013 that states that CHD, heart failure, and stroke patients are more frequent in 45-54 years old, 55-64 years old, and 65-74 years old age group.[3] Distribution of demographic data in this study shows that there are more male CHD patients compared to female. This findings is in line with the study done by Mosca et al that shows prevalence of male CHD patients is higher for every age goups up to 75 years old. One of the protection factor against CHD in female is estrogen hormone. Estrogen cam regulate several metabolic factor such as lipid, inflammation marker, and coagulation system. Estrogen also has vasodilation against α and β receptor in blood vessel walls.[9]

The result of this research showed that there is significant difference between P.gingivalis count of CHD and control patients (p=0.004). P.gingivalis level of CHD (6.95±1.77 log10 CFU/ml) is higher than control (6.12±1.32 log10 CFU/ml). This findings is similar with study by Jaideep et al that shows that P.gingivalis proportion on subgingival plaque in patients that will undergo CABG (coronary artery bypass graft) has significant difference compared to non-CHD patient. P.gingivalis proportion of subgingival plaque on CHD patients is 64.7% vs 33% in non-CHD patients.[10] Significant relation is also found for bacterial count in subgingival plaque and in atherosclerotic plaque of CHD patients. Ghizoni et al found that P gingivalis prevalence in stroke patient is 60% much higher than in control patient (10%).[11]

The result also showed that there was a significant difference between T.denticola level of CHD and control patients (p=0.000). T.denticola level of CHD (5.26 log10 CFU/ml) is higher than control (3.59 log10 CFU/ml). Mahendra et al. shows that the presence of periodontal bacteria was significantly increased in patients with CHD. T.denticola was increased from 41.2% to 66.7% in patients with CHD. Ishihara et al. also found an increase T.denticola level in 7 from 10 subgingival plaque of CHD patients.[10]

There are no significant association between P.gingivalis level on dental plaque with gingival bleeding and clinical attachment loss on CHD patients, while there is a significant correlation between P.gingivalis level on dental plaque with pocket depth of CHD patients (p=0.03). This finding is supported by study done by Kumar et al. and Abdulaziz et al., where these 2 studies conclude that there is a positive correlation between P. gingivalis and pocket depth.[12,13] Vajawat et al. And Kawada et al. states that for every mm increase of pocket depth, P.gingivalis count will increase by ten times.[12,14] Spearman correlation test for P.gingivalis and pocket depth in non-CHD patient shows that there is no significant correlation between the two (p=0.44). These findings are different from other studies.

There is a significant no correlation between T.denticola level on dental plaque with pocket depth of CHD and Non-CHD patients. Metraux et al. reported that T.denticola is actually found in deep pockets. T.denticola motility increase when the pressure decreased oxygen in anaerobic condition deep pockets. Popova et al. showed that clinical and microbiological data evaluated in patients with chronic periodontitis linked T.denticola. The number of bacteria found in some other kind of pocket depths between the shallow pockets (<4mm), medium (4-6mm) and deep (> 6mm). The amount of T.denticola was significantly found to increasing from the group of shallow pockets (5,9x104) against the deep pockets (7,4x109), whereas in the group pockets were not found significant improvement.

There are several possibilites for the difference in P.gingivalis and T.denticola count in CHD and non CHD patients, namely atherosclerosis, medication, and immune system. Higher level in CHD patient may be caused by atherosclerosis which worsen periodontitis. Atherosclerosis increases cytokine local reactions. Proinflammatory cytokine such as interleukin-1β, IL-6, dan TNF-α can induce liver to produce C-reactive protein and fibrinogen, which cause acute phase reactions and promote periodontitis.[15]

Medications which are used to treat cardiovascular disease tends to reduce salivary flow rate. Study conducted by Araujo et al. showed that reduction of saliva flow rate found in 17.5% of beta blocker user. Salivary flow rate is closely related to its consistency. The lower the salivary flow rate, the lower concentration, dilution capacity, self cleansing, and pH of saliva, severely affecting its oral hygiene.[16,17] Baliga et al. explain that pH of generalized chronic periodontitis patient is lower than control patient. Majority of subgingival microorganisms use or produce acidic product.[18] The difference in microbial environment in both quality and quantity of saliva and pH may cause the difference of P.gingivalis and T.denticola count in CHD and control patients.

The mean age of patient in CHD group is 58.14 (8.37) years, which is older compared to mean age of patient in control group 52.37(8.99) years old. The difference in age can also affect immune system response to bacterial infections. Castello-Branco et.al states that a decrease in immune system will increase vunerabiliy and prevalence to infections. Age affects adaptive respons, more than innate respons of immune system.[19]

Higher P.gingivalis and T.denticola level in CHD patients compared to non CHD patients indicates that this bacteria is involved in the pathogenesis of atherosclerosis. There are several mechanisms in which P.gingivalis causes atherosclerosis: endothelial dysfunction, smooth muscle proliferation, platelet activation, and inflammatory markers production.[20–26] T.denticola role in coronary heart disease through several mechanisms, including direct arterial infections, endothelial dysfunction, activation of the inflammatory response, as well as causing changes in the lipid profile as well as modify the expression of certain genes.

Majority of subgingival plaque sample in CHD patients were taken from 4 mm pocket (43.9%), yet P.gingivalis and T.denticola levels of CHD is greater than control. Correlation of P.gingivalis count and pocket depth only exists in CHD patients. This indicate that there could be certain P.gingivalis serotype that easily grows and survives in oral environment of CHD patients compared to non CHD patients. There is no correlation between quantity of T.denticola and periodontal status of CHD and control.

This research showed no significant correlation between P.gingivalis and T.denticola level and PBI of CHD and control patients. This result is supported by Cortelli et al. that detected periodontal pathogen species in biofilm. In their research, P.gingivalis and oral spirochetes group (T.denticola) are almost always found in mild, moderate, and severe bleeding group with detection frequency of 96.7%, 100%, and 91.1% respectively. Meanwhile, detection frequency of P.intermedia is higher in severe bleeding group (66.7%), compared to moderate bleeding (57.6%) and mild bleeding group (56.5%). The study concluded that P.intermedia is more related to gingival bleeding, not P.gingivalis and T.denticola.[27]

There is no significant correlation between P.gingivalis and T.denticola levels and clinical attachment loss of CHD (p=0.33) and non-CHD patients (p=0.22).This result is supported by a study done by Abdulaziz et al. that observed effect of P.gingivalis to periodontal health. Abdulaziz et al. found that there is no significant correlation betweeen P.gingivalis and clinical attachment loss.[13] This may happen due to the existance of multiple of pathogenic bacteria in periodontal lesion, not only P.gingivalis.[29] Clinical attachment loss is measured from the CEJ to the bottom of the pocket, and it is a combination of pocket depth and gingival recession.[30] Deep pocket with mild recession will have similar clinical attachment loss with shallow pocket with severe recession. P.gingivalis and T.denticola levels on both cases will be different due to the difference of oxygen pressure in periodontal sulcus.

Conclusions

There is a significant difference of P.gingivalis level on dental plaque between CHD and non CHD patients. There is a significant

correlation between P.gingivalis count on dental plaque and pocket depth of CHD patients. There is no significant correlation between P.gingivalis level and gingival bleeding and clinical attachment loss of CHD and control.

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Declaration of Interest

The authors report no conflict of interest.

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