Relationship between the Quantitative Measurement of *Tannerella Forsythia* on Dental Plaque and Its Relationship with the Periodontal Status of Patients with Coronary Heart Disease

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Abstract

The pathogenesis of the development of atherosclerosis in subjects with coronary heart disease (CHD) has evolved to the extent where abnormal fat accumulation is no longer the culprit; rather, a certain inflammatory process, including periodontitis, has been deemed a major concern. *Tannerella forsythia* is a gram-negative anaerobic bacteria that has a fusiform rod shape and has played a role in inducing the development of CHD and periodontal diseases. The aim of this study was to analyze the difference in the quantitative measurement of *Tannerella forsythia* accumulated on plaque and the periodontal status of subjects with and without coronary heart disease. *Tannerella forsythia* was counted by utilizing real-time polymerase chain reaction (RT-PCR). The periodontal status of 66 CHD patients and 40 controls was obtained. Subgingival plaque was isolated. *Tannerella forsythia* levels were measured using real-time PCR. *Tannerella forsythia* levels of CHD patients (-6.29 log10 CFU/ml) was significantly different than the control (-19.63 log10 CFU/ml). *Tannerella forsythia* was not significantly associated with any periodontal status ($p<0.05$). *Tannerella forsythia* levels of CHD patients were higher than the control patients. *Tannerella forsythia* was not associated with any periodontal status.

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Introduction

Statistics derived from the World Health Organization showed that in 1995, cardiovascular diseases had caused 20% of all deaths worldwide. In developed countries, cardiovascular diseases have been the cause of 50% of the deaths.[1] Coronary heart disease (CHD) is the main cause of death and disability in most developed countries and CHD is the leading cause of death in a third or more of individuals aged 35 or older.[2] According to Basic Research of Health 2013, the prevalence of people with emerging symptoms and diagnosed CHD in Indonesia is 1.5%.[3]

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Currently, many studies have shown that chronic inflammation disease might increase the chance of contracting cardiovascular disease. This has been the main reason for the growing number of research studies on the effect of chronic infection on the pathogenesis of cardiovascular disease.[4]

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

Porphyromonas gingivalis (*P.gingivalis*) and *Treponema denticola* (*T.denticola*).[5]

According to the American Academy of Periodontology, the current literature has not yet provided any support regarding the causal relationship between cardiovascular and periodontal disease. However, some studies have shown conflicting results.[6] Currently, there has never been a study in Indonesia that compares the quantitative measurement of *Tannerella forsythia* in the dental plaque of patients with CHD and their periodontal health status. The aim of the current study was to compare the level of *Tannerella forsythia* levels on dental plaque between CHD and control patients. This study also aimed to find the relationship between *P.gingivalis* and *T. denticola* levels on dental plaque and periodontal status in CHD and control patients.

Materials and methods

Collecting Samples

This study was a cross-sectional study involving two clinical centers; the study took place between November 2015 and February 2016. Data for CHD patients were collected from Harapan Kita National Cardiovascular Center Hospital while data for the control were obtained from Periodontology Specialist Clinic, Dental Teaching Hospital, Faculty of Dentistry, Universitas Indonesia. This study was approved by the ethic committee of the Faculty of Dentistry, Universitas Indonesia and Harapan Kita National Cardiovascular Center Hospital. Inclusion criteria for the CHD patients were male or female, age 40–74 years old, diagnosed with stable angina, and would be undergoing bypass surgery. The control group consisted of chronic periodontitis patient without angina (confirmed by a negative treadmill test and normal ECG). Edentulous patients, who were pregnant, or who had other systemic diseases were excluded. A total of 106 patients (66 CHD patients and 40 controls) participated in the study.

Clinical Periodontal Examination

Periodontal status was assessed by measuring the plaque index (PI) (according to Silness and Loe's 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 were measured using a periodontal probe (colorvue probe, Hu-Friedy, USA). During periodontal examination, examiners measured periodontal probing depth (PD) and clinical attachment loss (CAL) at six 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 because of their large variation in anatomy and position in the oral cavity.

Collection of subgingival plaque

Subgingival microbial samples were collected from a 4–6 mm pocket depth. The tooth was isolated using a cotton roll, its supragingival plaque removed, and its root surface air dried. The subgingival sample was obtained using an excavator (Crown, Japan) and transferred into a microtube containing 1000 µl phosphate buffer saline (PBS). The sample was stored in -4°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 phosphate buffer saline of 50–150 µL (*tera volume* to ±1000 µL) was added to the *microtube*, and the sample was mixed with a pipette. A sample volume of 1000 µL was then added into the new microtube, its weight measured using a scale (Ohaus Explorer, USA), and centrifugation was performed at 13000xG for 10 minutes (Sorvall Legend Micro 17 Microcentrifuge, Thermo Scientific, USA). The supernatant was removed carefully, and 1000 µL of new PBS was added. The sample was again mixed and centrifugated at 13000xG for 10 minutes. The supernatant was removed, and 200 µL nuclease free water (NFW) was added into the microtube. The sample was incubated in a thermos-block at 100 °C for 30 minutes (Thermo-block NB-305TB, N-Biotek, Korea). Cooling was carried out by putting the sample into an ice tray for 10 minutes. The sample was centrifugated at 10000xG for 2 minutes. Supernatants with a volume of ±180–200 µL were placed into a 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 the DNA's concentration and purification. Each spectrophotometry cycle consisted of five sample cuvettes and one reference cuvette (500 µL air aquabidest). Each sample cuvette consisted of 5 µL DNA sample and 495 µL aquabidest. The cuvette was put into a spectrophotometer (*Ultrospec 4000 Pro,* Amersham Pharmacia Biotech, UK) with a wavelength of 260 and 280 nm (*multi-wavelength*). DNA concentration was obtained from an absorbance value of 260 nm and multiplied by 50 ng/µL. DNA purity were measured by comparing the absorbance value of 260:280 nm. The concentration of DNA was standardized to 100 ng/µL in 100 µL in order for each sample to contain the same amount of DNA.[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). The primers of *Tannerella forsythia* that were used for RT-PCR are displayed in Table 1.[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. The 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 a standard curve equation that was previously determined. The standard curve for PG is y = -0.25x + 12.284, TD is y = -0.2096x + 14.213.

Primers	Sequence (5'-3')	Bacteria
PG-Forward	ATCCTGGCTCAGGAT	*T. forsythia*
PG-Reverse	TACGCATACCCATCCGCAA	

Table 1. Primers used in Real Time PCR.

Statistical analysis

All statistical analyses were performed using SPSS 20.0. Univariate analysis was performed to obtain the mean, standard deviation (SD), and minimum and maximum (Min-Max) values of all the parameters. The quantitative differences of *Tannerella forsythia* between CHD and control patients were assessed using the Mann–Whitney test. The correlation between quantitative *Tannerella forsythia* and periodontal status was assessed using Spearman's correlation test.

Results

This study involved 106 subjects, 66 of whom were suffering from coronary heart disease, while the remaining 40 subjects did not have this condition. The age of the subjects ranged from 40–74 years old, and this range was chosen following the inclusion and exclusion criteria. Samples of subgingival plaque were collected at a pocket depth of 4–6 mm. Primary data collection was completed using a questionnaire and demographic data, signed informed consent, examination of periodontal status, and subgingival plaque sample collection. Laboratory analysis (using real-time PCR) was completed at the Oral Biology Laboratory at the Faculty of Dentistry, Universitas Indonesia. The result of this analysis was further analyzed using a univariate and bivariate analysis.

Table 2 summarizes the distribution of average value, standard deviation, minimum and maximum age of subjects, periodontal status, and quantitative measurement of *Tannerella forsythia* of patients with and without coronary heart disease. The average quantity of *Tannerella forsythia* of patients with CHD was -6.29 log10 CFU/mL; in patients without coronary heart disease, the average quantity of *Tannerella forsythia* was -19.63 log10 CFU/mL.

The normality test of the data obtained from the periodontal status and the quantity of *Tannerella forsythia* in subjects with CHD was performed with a Kolmogorov Smirnov test (test subjects > 50). And the result showed that the distribution of this data were not normal (Table 3). The normality test of periodontal status and the quantitative measurement of *Tannerella forsythia* of subjects without CHD were completed using a

Shapiro–Wilk test, mostly because of the number of subjects involved (less than 50). Table 4 demonstrates that the distributions of papilla bleeding index, pocket depth, loss of clinical attachment, and quantity of *Tannerella forsythia* were not normal.

To further analyze or not there was a significant difference between the quantity of *Tannerella forsythia* in subjects with and without coronary heart disease, several tests were conducted. The test used for the paired numerical variables was the Mann–Whitney test. The distribution of the results is shown in Table 2. The normality test for the data obtained from the periodontal status and the quantity of *Tannerella forsythia* in subjects with CHDwas performed with a Kolmogorov Smirnov test (test subjects > 50). And the result showed that the distribution of this data was not normal (Table 3). The normality test for the periodontal status and the quantitative measurement of *Tannerella forsythia* of subjects without CHDwas completed using a Shapiro–Wilk test because of the number of subjects involved (less than 50). Table 3 demonstrates that the distributions of the papilla bleeding index, pocket depth, loss of clinical attachment, and quantity of *Tannerella forsythia* were not normal.

To further analyze whether there was a significant difference between the quantity of *Tannerella forsythia* in subjects with and without coronary heart disease, several tests were conducted. The test used for the paired numerical variables with abnormal distributions was the Mann–Whitney test.

Variable	CHD Patient		Non-CHD Patient	
	Mean (SD)	Min – Max	Mean (SD)	Min – Max
Papilla Bleeding Index	0.80±0.59	0.00-2.38	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
Quantity of *T.forsythia* (log10 CFU/ml)	-6.29±8.12	-16.81-13.53	-19.63±2.3	-23.16-(-13.04)

Table 2. Distribution of average value, standard deviation, minimum and maximum value of papilla bleeding index, pocket depth, clinical attachment loss, and quantitative measurement of *Tannerella forsythia* in subjects with and without coronary heart disease.

Variable	p value
Periodontal Status	
Papilla Bleeding Index	0.001
Pocket Depth (mm)	0.000
Clinical Attachment Loss (mm)	0.000
Quantity of *Tannerella forsythia* (log10 CFU/ml)	0.000

Table 3. Result for Normal Distribution Test on Clinical Parameters, Periodontal status, andQuantitative of *Tannerella forsythia* on CHD Patients.

Variable	p value
Periodontal Status	
Papilla Bleeding Index	0.038
Pocket Depth (mm)	0.000
Clinical Attachment Loss (mm)	0.000
Quantity of *Tannerella forsythia* (log10 CFU/ml)	0.024

Table 4. Result for Normal Distribution Test on Clinical Parameters, Periodontal status, and Quantitative of *Tannerella forsythia* on Control Patients.

Variable	n	Average ± SD	Nilai p
Quantity *T.forsythia* (log 10 CFU/ml)			
Subjects with coronary heart disease	66	-6.29±8,12	0.000*
Subjects without coronary heart disease	40	-23.16-(-13.04)	

Table 5. Comparative Test of the Quantity of *Tannerella forsythia* in Subjects with and without Coronary Heart Disease. Mann-Whitney test;*p<0.05 = significant.

The Mann–Whitney test performed for the two experimental groups (subjects with and without coronary heart disease) showed a value of $P = 0.0000$; thus, there was a quantitative difference between the amount of *Tannerella forsythia* found in plaque collected from patients with and without coronary heart disease. The relationship between the quantity of *Tannerella forsythia* and the periodontal status of subjects

with and without CHD was tested with the Spearman correlation test (Table 6 and 7).

		Papilla Bleeding Index	Pocket Depth	Clinical Attachment Loss
Quantity of *T.forsythia*	r	-0.87	-0.39	-0.12
	p	0.49	0.21	0.34
	n	66	66	66

Table 6. Relationship between Quantity of *T.forsythia* and Gingival Bleeding, Pocket Depth, and Clinical Attachment Loss in Subjects with Coronary Heart Disease. Spearman Test, $p < 0,05$ → Hypothesis accepted, $p > 0,05$ → hypothesis rejected

		Papilla Bleeding Index	Pocket Depth	Clinical Attachment Loss
Quantity of *T.forsythia*	r	0.17	-0.16	-0.73
	p	0.27	0.81	0.65
	n	40	40	40

Table 7. Relationship between Quantity of T. forsythia and the Gingival Bleeding, Pocket Depth, Clinical Attachment Loss in Subjects Without Coronary Heart Disease. Spearman Test, $p < 0,05$ → Hypothesis Accepted, $p > 0,05$ → Hypothesis Rejected.

The result of the correlation test between the quantity of *Tannerella forsythia* and the papilla bleeding index, pocket depth, and clinical attachment loss in subjects with CHD yielded a p-value of 0.49; 0.21; and 0.34, respectively (p-value > 0.05). There was no relationship between the quantity of *Tannerella forsythia* collected from dental plaque and gingival bleeding, pocket depth, and clinical attachment loss in patients with CHD can be rejected. The correlation test between the quantity of *Tannerella forsythia* and the papilla bleeding index, pocket depth, and clinical attachment loss in patients without CHD yielded a p-value of 0.27; 0.81; and 0.65, respectively (p-value>0.05). Thus, the minor hypothesis stating that there was a relationship between the quantity of *Tannerella forsythia* and the gingival bleeding, pocket depth, and clinical attachment loss of subjects without CHDcan be rejected.

Discussion

Samples were taken from 106 subjects (66 CHD subjects and 40 non-CHD subjects), age 40–74 years old. This age range was chosen according to Indonesian health survey (2013); it that CHD, heart failure, and stroke patients are more frequently found in the 45–54, 55–64, and 65–74 age groups.[3] The distribution of demographic data in this study showed that there are more male CHD patients compared to female. This finding is in line with the study done by Mosca et al., which shows the prevalence of male CHD patients is higher for every age group up to 75 years old. One of the protection factors against CHD in females is the estrogen hormone. Estrogen can regulate several metabolic factors, such as lipids, inflammation markers, and the coagulation system. Estrogen also has vasodilation against α and β receptors in blood vessel walls.[8]

The examination of periodontal status was done thoroughly in all subjects. The pocket depth was measured in six different areas (distal, middle, and mesial of the buccal and the lingual surfaces) using a periodontal probe. The selection of a 4–6 mm pocket depth as the site of sample collection came from previous studies that noted the amount of *Tannerella forsythia* collected is significantly higher in deeper depth of periodontal pockets compared to the shallow depth.[9] The collection of the subgingival plaque sample was done using an excavator to replace curettage so as to reduce the risk of bleeding during sample collection in patients with CHD.[10]

The results of this study demonstrated that there was a significant difference between the quantity of *Tannerella forsythia* taken from the dental plaque of patients with and without CHD (P=0.000). The quantity of *Tannerella forsythia* in patients with CHD (-6.29 $\pm$ 8.12 log10 CFU/ml) was higher than that of patients without CHD (-19.63 $\pm$ 2.3 log 10 CFU/ml). The negative value showed that the bacteria were found in very small amounts. This finding is supported a study conducted by Mahendra et al., where patients who were about to undergo coronary artery bypass grafts (CABGs) had eight periodontal pathogens that were at significantly increased levels. The amount of *Tannerella forsythia* increase in patients without CHD to 39.2% and the amount of this bacteria reached 43.1% in patients with CHD.[10] *Tannerella forsythia* was also found in a sample of atheroma plaque in patients undergoing CABG. The increased quantity of *Tannerella forsythia* in patients with CHD indicated that these bacteria may play a role in the development of

atherosclerosis. Chukkapalli et al. conducted research on mice infected by *Tannerella forsythia*. The damage in their hearts, aortas, and lungs were found after 12 weeks of exposure. This showed that *Tannerella forsythia* has the potential to get into the systemic circulation and aorta through the oral cavity.[11]

Several virulence factors possessed by these bacteria are the trypsin—such as protease, PrtH protease, sialidases SiaH, nanH and leucine—rich repeat cell—surface-associated protein BspA; they are capable of taking nutrients available in the periodontal pocket and protecting *Tannerella forsythia* against the immune system. These virulence factors also contributed to the degradation of gingival tissue, activation of degrading enzymes such as collagenase, modification of the protein of host cells to help in the colonization of bacteria, and the number of antigens in the immune system. Yan et al. reported that exposing tissue to *Tannerella forsythia* would induce the production of TNF-α, which canh cause a decrease in the expression of Nitric oxide synthase. The decreased production of Nitric oxide is one of the symptoms of endothel dysfunction, a marker for the development of atherosclerosis. TNF-α also can cause changes in the oxygen compound that elicits the oxidative stress reaction. Recently, Lee et al. reported that the BspA protein found in the surface of the membrane of *Tannerella forsythia* can induce the formation of foam cells and accelerates the development of atherosclerosis in vivo.

The current study demonstrated that there was no relationship between the quantity of *Tannerella forsythia* in the dental plaque and gingival bleeding, pocket depth, and clinical attachment loss in patients with and without coronary heart disease. This was not what previous studies had discovered. Ardilla et al. found that there were some difficulties in finding a relationship and the prevalence of certain bacteria because of technical issues.[12] Homma et al. revealed that *Tannerella forsythia* is the least studied because of problems in genetically manipulating this bacterium. Some studies showed that the increase in quantity of *Tannerella forsythia* might be related to periodontal status, including gingival bleeding, pocket depth, and clinical attachment loss. This study showed a very small amount of *Tannerella forsythia*. This might contribute to the lack of significant relationship between the quantity of *Tannerella forsythia* and the three variables of periodontal status studied.[13]

Tannerella forsythia in one of the red complex bacteria often isolated from periodontal pocket; many studies have classified these bacteria into the anaerobic obligate group of bacteria. Farias et al. reported that this group of bacteria is commonly found in deep pockets where the pressure of oxygen is lower.[14] *Tannerella forsythia* has an aerotolerance system for adjusting to air changes, oxidative stress, and reactive oxygen compounds constantly found in oral cavities.[14] Farias et al. evaluated the clinical and microbiological data of patients with chronic periodontitis and found that the amount of *Tannerella forsythia* was elevated in pockets with a depth exceeding 8 mm, the quantity of *Tannerella forsythia* was elevated following increased interactions with several other bacteria, such as *Porphyromonas gingivalis* and *Treponema denticola*.[14]

Conclusions

There is a significant difference in *Tannerella forsythia* levels found on dental plaque between CHD and non-CHD patients. There is no significant correlation between *Tannerella forsythia* levels and gingival bleeding and clinical attachment loss of CHD.

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