Quantitative Analysis of *Selenomonas noxia* in Periodontitis Patients via Two Different Sample Collection Methods

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Abstract

Selenomonas noxia is a bacterium that is a putative periodontal pathogen found in the gingival crevice and periodontal pocket and is suspected to have a significant role in the initiation and progression of periodontitis. This study aimed to analyse the relationship between S. noxia bacterium quantity in healthy and periodontitis patients, based on clinical findings marked by periodontal pocket depth (PD), periodontal bleeding index (PBI), and clinical attachment loss (CAL) via two sample collection methods using paper points (PP) and Gracey curettes (GC). The samples with PD, PBI, and CAL measured, were collected using PP and GC in each site, resulting in 35 samples in total for each collection method. The S. noxia strain was obtained from the American Type Culture Collection (ATCC 51893) and used to create a standard curve in real-time polymerase chain reaction (qPCR).

This study shows that the quantity of S. noxia numbers were higher (PP, P=0,01; GC, P=0,03) in periodontitis subjects than in healthy subjects. The difference in S. noxia numbers also observed in CAL 0 mm and <5 mm, and 0 and CAL ≥5 mm, also in sulcus (1–3 mm) and PD <6 mm groups. These results show that patients with periodontitis had higher S. noxia levels than healthy subjects did. However, there was no positive correlation in the PBI category and S. noxia amount, and in bacterium load in PP and GC samples. Thus, S. noxia can be detected with either PP or GC samples.

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Introduction

Periodontitis is an bacteria-associated disease caused by a variety of microorganisms and characterized by bone loss, presence of gingival recession and/or periodontal pocket formation, which eventually leads to clinical attachment loss (CAL).[1] Periodontal pocket is defined as pathologic deepening of the gingival sulcus around the tooth at the gingival margin, forming a space between the pathologically detached gingiva and tooth.[2] Bleeding on probing also is a trusted indicator in the clinical diagnosis of periodontitis.[3] For more accurate diagnosis, Saxer and Muehlemann[4] constructed the papillary bleeding index (PBI): Score 0, no bleeding; Score 1, a single discreet bleeding point; Score 2, several isolated bleeding points or a single line of blood appears; Score 3, the interdental triangle fills with blood shortly after probing; and Score 4, profuse bleeding after probing, with blood flowing immediately into the marginal sulcus. The more periodontitis progresses, the more CAL will be seen. Clinical periodontal tissue attachment loss is characterized by periodontal pocket formation and apical migration of the gingival margin (gingival recession) around the tooth.[3]

Every person has periodontal pathogenic bacteria in their supragingival and subgingival areas that remain for some time but in little quantity and variety.[5–7] As a disease caused by bacterial infections, few of the several hundred species of microorganisms have been identified as a putative pathogenic bacterium with a role in periodontitis development. *Selenomonas noxia* is

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a newly discovered bacterium that is a putative periodontal pathogen found in the gingival crevice and periodontal pocket and is suspected to have a significant role in initiation and progression of periodontitis.[8]

Although the pathogenesis of periodontitis is multifactorial, including genetic and epigenetic factors, the development of periodontitis is modulated by microbial biofilm that forms on and around the teeth, where free-soluble components from live planktonic and biofilm bacteria from periodontal pockets also exist.[9,10] According to Noiri et al.[11], subgingival plaque is divided horizontally into three regions: tooth-attached, unattached (loosely attached), or epithelium-associated plaque; and vertically into shallow, middle, and deep pocket zones. Some bacteria, such as *Prevotella nigrescens*, *Fusobacterium nucleatum*, *Treponema denticola*, *Eikenella corrodens*, and *Aggregatibacter Actinomycetemcomitans*, were detected at a specific location and definite area in periodontal pockets. A bacterium that attaches loosely to superficial layers could migrate easily from the biofilm, and cause acute periodontitis.[11] Hartroth et al.[12] used a curette/scaler to obtain samples from the flowing and loosely attached plaque, and the more tightly tooth or epithelium-attached plaque, and while using paper points, found that bacteria of the more tightly attached plaque could be underestimated. As far as they knew, where *S. noxia* was located and how it was attached in the subgingival area remained unknown.

Studies about the role of *S. noxia* in periodontal disease began to emerge since its discovery in 1987 by Antonie van Leeuwenhoek. Later, Kollenbrander et al. and Moore et al.[13,14] suspected it to be a newly found potentially pathogenic anaerobic bacterium with a role in periodontal disease progression. However, based on studies by Tanner et al.,[15] *S. noxia* was suspected to initiate periodontitis from healthy condition. The numbers of studies done especially about the quantitative level of *S. noxia* in periodontitis patients still show controversies; some studies report a significant difference in the levels of *S. noxia* in healthy patients compared to those with periodontitis, and some report otherwise.[5,16,17]

In Indonesia, a study about this bacterium and its clinical relevance has not yet been established in the periodontology field. The aim of this study is to analyse the relationship between *S. noxia* bacterium amount in periodontitis and healthy patients with clinical findings marked by PD, PBI, and CAL from two sample collection methods using paper points (PP) and Gracey curettes (GC).

Materials and methods

Samples were collected using PP and GC from 2 healthy and 5 periodontitis subjects, resulting in total 35 periodontal pockets (site) as sample, recruited from the Dental Teaching Hospital at the Faculty of Dentistry, Universitas Indonesia in Jakarta, Indonesia from February to June 2018. Medical and dental histories were recorded, and a full mouth periodontal examination was performed. Based on these data and radiographs, the periodontal diagnosis was made, and subjects who fulfilled the inclusion/exclusion criteria provided informed consent for participation. Inclusion criteria were periodontitis and healthy subjects based on the clinical examination and confirmed with a radiographic examination, age range 18–55 years, presence of at least 20 teeth in the mouth, a periodontal pocket (≥4 mm), CAL (≥ 4 mm), and systemically healthy.[16,18] Exclusion criteria were prior periodontal treatment for at least 6 months,[18] prior antibiotic treatment for at least 3 months,[17] alcoholism, smoking, pregnancy, and lactation.[19–21] The study was approved by the ethical committee of Dental Research, Faculty of Dentistry, Universitas of Indonesia: 20/Ethical Approval/FKGUI/III/2018 (protocol number 090220218).

PD, PBI, and CAL were examined at 6 sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual, mesiolingual) in all teeth and the 5 deepest periodontal pocket sites were chosen for sample collection. PD and CAL were measured using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA).[10,16]

S. noxia strain was obtained from the American Type Culture Collection (ATCC 51893) and used to create a standard curve in real-time polymerase chain reaction (qPCR). Clinical parameters from samples were taken, and supragingival plaque and calculus were removed. The 5 deepest periodontal pocket site samples were obtained from healthy and chronic periodontitis subjects using three sterile PPs #30 at each site for 20 s each and using Gracey curettes, subgingival plaque from periodontitis

subject were collected and slightly from the healthy subjects so it doesn't damage surrounding periodontium too much.[20,22] These samples were immediately immersed in 200 µL TE buffer in 1.5 mL microtubes per site and kept at −80°C until DNA extraction.

DNA from samples was extracted from all samples using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.[23] DNA extracts were kept at −80°C until ready to use.

A spectrometer was used to measure the purity and amount of DNA to obtain 10 ng/mL of each sample. The assay was performed in 100 µL consisting of 5 µL DNA extract and 95 µL Aquabides. DNA/protein concentration mode (Absorbance at 260 and 280 nm with 320 nm correction) was used for measurement.

The standard curve of *S. noxia* bacterium was performed with dilution of the bacterium from 10^5 to 10^0 based on total platelet count of *S. noxia* bacterium cultured in blood agar (previously 6 × 10^6 colony-forming units [CFU]/mL). The standard curve was used in every test run to obtain absolute quantification from the quantitative PCR (qPCR) assay.[24]

According to Cruz et al.,[8] *S. noxia* primers were: forward primer–SNF1, TCTGG GCTACACACGTACTACAATG (25 base pairs [bp]) and reverse primer–SNF1, GCCTGCAATCCGAACTGAGA (20 bp). The PCR cycling parameters were as follows: initial incubation step of 50°C for 2 min, denaturation of the template DNA at 9°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Real-time PCR conditions were: 2×SYBRgreen Master Mix 250 µL, SNF1 forward primer 0.9 µM for 25 µL, SNF1 reverse primer 0.9 µM for 25 µL, diluted in 50 µL Nuclease-free water (NFW).[25] Then, 7 µL from this solution was inserted into each well added to 3 µL template DNA so that we had 10 µL in each well. The analyses were performed in 48-well plates using a Step-One real-time PCR system instrument (Applied Biosystems, Foster City, CA, USA) on standard mode. Negative controls contained 10 µL NFW. DNA extracted from *S. noxia* served as positive controls in each qPCR run.

The clinical parameters and the bacterium absolute quantification results in CFU were compared and categorized by the PD, PBI, and CAL. The results then were compared using the Mann-Whitney *U* test and correlated using Spearman's test between healthy and periodontitis groups.

Results

The mean age of periodontitis subjects, 41.5 years and healthy subjects, 31.5 years. In periodontitis subjects, PD was 4–7 mm, CAL 4–12 mm, and PBI 0.40–2.20. Sulcus depth was 1–3 mm in healthy subjects.

S. noxia was detected in healthy and periodontitis subjects in PP and/or GC samples. Absolute bacterium quantification was measured with the *S. noxia* standard curve generated in every test run. The absolute amount of *S. noxia* bacteria then was investigated to evaluate its association with the clinical parameters.

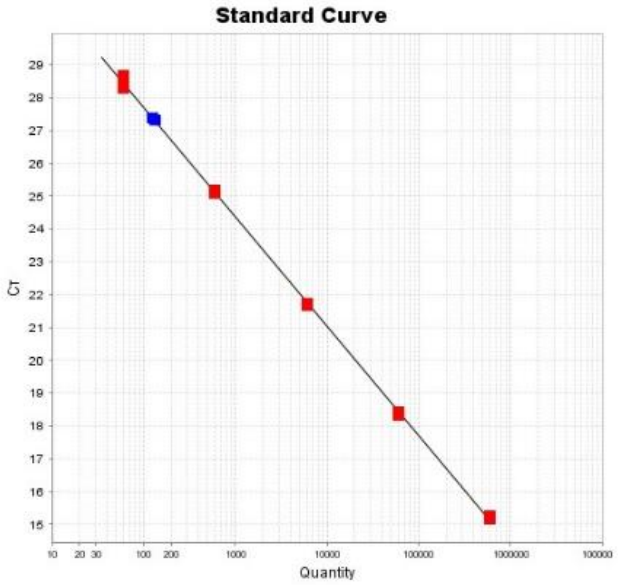

Figure 1. Standard curve of *Selenomonas noxia* (Slope = -3.331, Y-intercept = 34.381; R2 = 0.999; eff%= 99.606%). The red dot shows *S. noxia* bacterium diluted from ATCC, meanwhile the blue dot shows DNA of the sample.

Mean quantity of *S. noxia* extracted from GC and PP samples was 3.6 × 10^3 and 1.7 × 10^3 CFU/mL, respectively, in periodontitis subjects and 1.9 × 10^2 and 2 × 10^2 CFU/mL in healthy subjects.

The Shapiro-Wilk statistical test showed that the data were not normally distributed (P <.05). Therefore, the quantity of *S. noxia* in periodontitis and healthy subjects was compared with the Mann–Whitney *U* test, and data were correlated with the Spearman test.

Levels of *S. noxia* bacteria were significantly more elevated in periodontitis compared to healthy subjects (PP, *P*=0,01 ; GC, *P*=0,03) in the PP and GC samples (Table 1). Significant results also were detected for CAL 0 mm compared to CAL <5 and ≥5 mm, and sulcus (1–3 mm) compared to PD <6 mm. A higher level of *S. noxia* bacteria was detected in CAL <5 and ≥5 mm, and PD <6 mm.

	S. noxia (CFU/mL)		
	Healthy (N = 2; n = 10)	Periodontitis (N = 5; n = 25)	*P value
PP (mean ± SD)	200.70 ± 119.04	1796.70 ± 1778.85	.01*
GC (mean ± SD)	193.15 ± 85.80	3605.06 ± 7138.23	.03*

Table 1. Comparison of *S. noxia* amount between healthy and periodontitis subjects from PP and GC samples.
*N = subject count, †n = periodontal pocket (site) count.

The correlation test for clinical parameters showed that both sample collection methods provided a positive correlation between the amount of *S. noxia* bacteria in healthy and periodontitis subjects, also CAL. However, the correlation between CAL and level of *S. noxia* was detected only in the PP sample. PBI and PD, however, showed no correlation with the amount of *S. noxia* in both samples tested (Tables 2, 3).
Periodontal bacteria, especially anaerobic bacteria, often have a causative role in periodontal disease progression. Detection of pathogenic bacteria in periodontal and healthy patients depends on the techniques used. In this study, we used qPCR assay for quantification of *S. noxia* in PP and GC samples[26]. Based on our findings we assumed that the amount of *S. noxia* might have a role in development of periodontal disease, as the bacteria also was detected in healthy periodontal sulcus, but in lower quantity.

	S. noxia (mean ± SD; CFU/mL)			
	Healthy (N = 2; n = 10)	Periodontitis (N = 5; n = 25)	Correlation coefficient (*r*)	*P value
PP	200.70 ± 119.04	1796.70 ± 1778.85	.55	.01*
GC	193.15 ± 85.80	3605.06 ± 7138.23	.49	.02*

Table 2. Correlation between periodontal condition and *S. noxia* amount in test subjects.
*N = Subject count
†n = periodontal pocket (site) count
‡Spearman rank correlation coefficient: $r < 0.2$, very low correlation; $r < 0.5$, low correlation; $r < 0.7$, moderate correlation; $r < 0.9$, high correlation; $r > 0.9$, very high correlation.[24]

	Amount of *S. noxia* (Mean ± SD; CFU/mL)			
	Healthy (N = 2; n = 10)	Periodontitis (N = 5; n = 25)	Correlation coefficient (*r*)	*P value
PBI:				
PP			.19	.40
0.0	200.72 ±			
0.4	119.04	2848.44 ± 1879.76		
1.56		1407.75 ± 587.24		
1.7		559.55 ± 531.07		
2.18		3958.42 ± 1731.11		
2.20		209.33 ± 148.06		
GC			.13	.57
0.0				
0.4	193.15 ±	3822.67 ±1616.08		
1.56	85.80	2640.03 ± 3252.87		
1.7		1089.52 ± 1344.49		
2.18		10159 ± 15811.20		
2.20		314.11 ± 222.95		
CAL:				
PP			.46	.04*
0 mm	200.72 ±			
< 5 mm	119.04	2648.38		
≥ 5 mm		1735.86 ± 1829.73		
GC			.40	.07
0 mm		4574.98		
< 5 mm	193.15 ±	3535.78 ± 7402.46		
≥ 5 mm	85.80			
PD:				
PP			.26	.27
1–3 mm	200.72			
< 6 mm	±119.04	1714.16 ± 1436.03		
≥ 6 mm		1920.50 ± 2351.59		
GC			.24	.29
1–3 mm				
< 6 mm	193.15 ±	2428.48 ± 2163.7		

Table 3. Correlation between clinical parameter and *S. noxia* amount in test subjects.
*N = subject count
†n = periodontal pocket (site) count
‡Spearman rank correlation coefficient: $r < 0.2$, very low correlation; $r < 0.5$, low correlation; $r < 0.7$, moderate correlation; $r < 0.9$, high correlation; $r > 0.9$, very high correlation.[24]

Discussion

PP and GC samples were taken from the same periodontal pocket site. Mean *S. noxia* quantity was higher in GC compared to PP samples (3.6×10^3 and 1.7×10^3 CFU/mL, respectively). These higher numbers were achieved because GC samples not only taking the flowing and loosely attached plaque, but also the epithelium-attached plaque. This difference, however, was not significant. From these data, we assumed that *S. noxia* largely exists as flowing and loosely attached plaque, leaving only a small portion attached on the tooth and/or epithelium. In our study, the PP method was suitable and better for collecting samples as it was a more non-traumatic and non-invasive technique, more convenient, and easier to transfer specimens into the microtubes, although

it has higher potential to contamination and difficulties with volume determination. [12,27]

S. noxia levels were significantly higher in periodontitis compared to healthy patients. The same result was found when GC and PP samples were compared. Comparison of *S. noxia* levels and clinical parameters showed significant results in some categories: no CAL and CAL <5 mm, no CAL and CAL ≥5 mm, and sulcus (1–3 mm) and PD <6 mm, with higher *S. noxia* levels detected in CAL <5 and ≥5 mm, and PD <6 mm. Further, a positive correlation between *S. noxia* quantity and periodontal condition was found in the GC (low) and PP (moderate) samples. A low positive correlation on CAL also was found but only in the PP sample. Further study is needed to verify this result. PBI and PD, however, showed no correlation with *S. noxia* amount in both samples tested.

These results were similar to those of several previous studies,[5,16,18,28] where *Selenomonas sp.*, including *S. noxia*, were found at higher levels in periodontitis patients. At the 1996 World Workshop in Periodontics, a consensus list of periodontal pathogens was made using an evidence-based approach, and *Selenomonas sp.* were included as a probable periodontal pathogen group, and in 2010, *S. noxia* was included as one of them.[5] In a bacterial culture study done by Faveri et al.,[28] *Selenomonas* and *Streptococcus* were the most prevalent bacteria in periodontitis patients, and in his other study using 16S RNA sequencing analysis, high levels of *Selenomonas sp.*, including *S. noxia*, were observed at the diseased sites of subjects with periodontitis. Our result is in contrast with that of Dahlén et al.,[17] who used a checkerboard methodology and found that *S. noxia* level was not significantly different between healthy and periodontitis patients. Our study also supported a study by Galimanas et al.,[9] stating that *S. noxia* can be used as a healthy periodontal tissue indicator to differentiate between healthy and periodontitis conditions, but not the severity of periodontitis.

Conclusions

Patients with periodontitis identified by clinical parameters (CAL, PD, PBI) had higher *S. noxia* levels compared to healthy patients in samples taken with PP or GC. *S. noxia* could be a health indicator for differentiating between healthy and periodontitis conditions in patients, but not the severity of the disease. Either collection method can obtain a reliable sample with qPCR assay, but as PP is more non-invasive, it could be favoured more for sample collection, since there were no significant differences between these two methods. *S. noxia* number correlated with CAL and PD, but not with PBI.

From this study, we assumed that *S. noxia* was largely distributed unattached/loosely attached in periodontal pockets. Further studies evaluating this bacterium in other non-invasive sample sources, such as saliva and buccal mucosa, in periodontitis patients and their location in subgingival pockets can be considered for future study.

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Declaration of Interest

The authors report no conflict of interest.

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