

Correlations between Hydrogen Sulfide and Methyl Mercaptan Levels and the Proportion of *Porphyromonas Gingivalis* in Patients with Periodontitis

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Abstract

The mixture of hydrogen sulfide and methyl in volatile sulfur compounds (VSCs) are known to cause oral malodor along with the action of microorganisms, particularly the periodontal pathogen *Porphyromonas gingivalis* (*P. gingivalis*).

This study aimed to determine the correlation between hydrogen sulfide and methyl mercaptan levels and the proportion of *P. gingivalis* in the gingival crevicular fluid (GCF) and tongue coatings of patients with periodontitis.

Clinical samples were collected from the GCF and tongues of 32 subjects, including periodontitis group and healthy individuals. Hydrogen sulfide and methyl mercaptan levels were measured using a gas chromatograph. Measurements of the probing pocket depth, bleeding on probing, and tongue coating scores were included as the diagnostic criteria. A pocket depth of ≥ 3 mm was taken into consideration. The quantities of *P. gingivalis* in the GCF and tongue coatings were evaluated by quantitative real-time polymerase chain reaction. Correlations between the levels of the two gases and the clinical parameters were analyzed using Spearman's correlation test.

Thirty-two samples collected were divided into three groups: healthy/control group ($n = 6$), periodontitis group A with a pocket depth 3–4 mm ($n = 12$), and periodontitis group B with a pocket depth of ≥ 5 mm ($n = 14$). Moderate positive correlations were found between both gases, hydrogen sulfide ($r = 0,55$; $p < 0,05$) and methyl mercaptan ($r = 0,432$; $p < 0,05$), and the proportion of *P. gingivalis* in the GCF. In the tongue coatings, hydrogen sulfide ($r = 0,455$, $p < 0,05$), but not methyl mercaptan ($r = 0,256$; $p > 0,05$), was correlated to *P. gingivalis*.

Weak- to moderately-positive correlations between hydrogen sulfide and methyl mercaptan levels and the proportion of *P. gingivalis* were seen in the GCF and tongue coatings of the patients with periodontitis, which may be related to halitosis.

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Introduction

Volatile sulfur compounds (VSCs) comprise of a mixture of hydrogen sulfide and methyl mercaptan, dimethyl sulfide, organic acids, odor substances, and diamines (putrescine and cadaverin); among them, hydrogen sulfide and methyl mercaptan are two dominant gases.¹

VSCs, also known as protein substances, are produced by proteolytic, anaerobic, gram-

negative bacteria that cause persistent or endogenous malodor. They are produced by chemical reactions from amino acids that consist of sulfur, L-cysteine, and L-methionine.² The bacteria digest the proteins from food, cells, and salivary debris and convert them into amino acids. This reaction forms amino acid chains that emit unpleasant odor resulting in oral malodor or halitosis.³ Oral malodor is a common complaint of patients in Indonesia and can lead to diminished levels of self-confidence.⁴

Most of the microorganisms related to periodontal health are involved in the development of halitosis. A positive correlation between oral malodor and periodontitis has been reported in the literature. Similarly, periodontal

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pocket depth has been positively correlated with the concentration of VSCs in the oral cavity. Nevertheless, patients with healthy periodontal tissues may experience halitosis from the thick coating on the tongue, which may be caused due to food impaction, bacteria, leukocytes, and desquamated epithelial cells on the dorsum of the tongue. The wide surface and the papillary structure may facilitate the retention of bacteria and other debris on the tongue.⁵

VSCs are toxic to the periodontal tissue; they can cause extensive periodontal tissue damage even at low concentrations. Exposure of the oral mucosa to hydrogen sulfide and methyl mercaptan may increase its permeability leading to inflammation and tissue destruction.⁶ VSCs may cause other bacterial enzymes and antigens, such as lipopolysaccharides (endotoxin), to enter the underlying lamina propria owing to the increase in permeability.⁷ One of the common bacteria responsible for oral malodor is *P. gingivalis*, which play a role in periodontal tissue destruction.^{3,7} Morita and Wang reported a correlation between the sulfide levels in the oral cavity and periodontal bone destruction, thus indicating that sulfide level can be an indicator of the severity of periodontal disease.⁸ In another study, the prevalence of periodontal pathogens in the saliva was found to affect the periodontal health and the level of VSCs.⁹ An increase in the periodontal health status may reduce the prevalence of *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, and also reduce the VSC level in the oral cavity.⁹ The use of a mouthwash, such as chlorine dioxide, to reduce the microbial activity was found to decrease halitosis.¹⁰

P. gingivalis is one of the red-complex bacteria, which are known as risk factors for periodontal diseases. Studies suggest that periodontitis is dominated by the presence of gram-negative anaerobic bacteria, especially *P. gingivalis*. Furthermore, *P. gingivalis* can be used as a marker of periodontitis because they are found in the deep pockets. These microorganisms can pollute the soft tissues and escape during the surgical debridement of the periodontal tissue.^{11,12}

P. gingivalis is known as one of the dominant microorganisms that produce VSCs in the serum.¹³

However, the correlation between hydrogen sulfide and methyl mercaptan levels

and the quantity of *P. gingivalis* has not been evaluated so far.

Objectives: This study aimed to determine the correlation between the proportion of this microorganism in the GCF and tongue coating and the levels of hydrogen sulfide and methyl mercaptan in patients with periodontitis.

Materials and methods

Study Design

This cross-sectional study comprised clinical samples that were collected from the Dental Teaching Hospital, Faculty of Dentistry, Universitas Indonesia (RSKGM FKG UI) in June–July 2019 using the simplified randomized sampling technique. The laboratory work was conducted in the Laboratory of Oral Biology at the Faculty of Dentistry, University of Indonesia. This study was approved by the Ethical Committee of Dental Research (KEPKG), Faculty of Dentistry, Universitas Indonesia (protocol number 090460419).

Subject Collection

A total of 32 patients (23 women and 9 men) aged 17–50 years old with neither systemic disease nor smoking habits were included in this study. The samples were divided into three groups as follows: healthy subjects (n = 6) with normal sulcus or no periodontal pocket; periodontitis group A comprising samples from patients with a pocket depth of 3–4 mm (n = 12), and periodontitis group B comprising samples from patients with a pocket depth of ≥ 5 mm (n = 14). Informed consent was obtained from all the patients. The anamnesis was initiated by asking the patient about their chief complaint, presence of oral malodor, self-perception about oral malodor (subjective/objective complaint), tooth brushing habits, presence of systemic disease, and intake of medications.

Gas analysis

Hydrogen sulfide and methyl mercaptan levels were measured initially using a gas chromatograph (OralChroma, Morita, Japan) that was calibrated in 2014. The patients were asked to refrain from talking for 3 min, and the gas was collected by inserting a 1cc syringe into the oral cavity. They were instructed to avoid blowing into or sipping from the syringe while the gas was being collected from the oral cavity. A needle (27 G) was inserted into the 1cc syringe and put the needle into a small hole in the OralChroma.

Intraoral examinations were conducted while the levels of the two gases were being measured. The pocket depth and extent of coating on the tongue were evaluated. The depth of the absolute pocket with no gingival recession was measured from the gingival margin to the base of the sulcus.

Quantification of *P. gingivalis*

P. gingivalis were collected from the GCF using sterilized paper points (no 30), which were inserted inside the deepest pocket among all teeth and placed there for 30 s. Tongue coating was collected using a sterilized cotton swab. The samples were inserted into sterilized 1.5 mL microcentrifuge tubes containing Ringer's Solution as the transfer medium. They were refrigerated and transferred to the Oral Biology Laboratory for further analysis. Total DNA extraction was performed using GENEzol™ reagent following the protocol provided by the company. The DNA concentration was calculated using the Qubit® 3.0 Fluorometer (ThermoFisher Scientific, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the ABI StepOnePlus Real-Time PCR System with the PCR master SYBR Green (Applied Biosystems, USA) according to the manufacturer's instructions. The DNA samples were amplified with the *P. gingivalis* primers: forward 5'- TAC CCA TCG TCG CCT TGG T- 3' and reverse 5'- CGG ACT AAA ACC GCA TAC ACT GTG - 3' [14]. As a relative proportion, the Universal 16S rRNA was used. Primer sequences for the Universal 16S rRNA gene were: forward 5'- ACT CCT ACG GGA GGC AGC AGT- 3', and reverse 5'- ATT ACC GCG GCT GCT GGC- 3' [15]. The PCR conditions were set as follows: pre-denaturation at 95°C for 5 min; followed by 40 amplification cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The melt curve profile was set at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s.

Statistical analysis

Data analysis was performed using the SPSS software program (SPSS, Chicago, USA). The collected data on the proportion of *P. gingivalis* in the shallow and deep pockets were compared using the Kruskal-Wallis. Correlations between the levels of the two gases (hydrogen sulfide and methyl mercaptan) and the levels of *P. gingivalis* in the GCF and tongue coating were measured using Spearman's rank correlation test. $P < 0.05$ was considered significant.

Results

P. gingivalis proportion in the GCF and tongue coatings

In order to know the distribution of *P. Gingivalis* in the oral cavity, we quantified the proportion of *P. gingivalis* both in the GCF and tongue coatings. Table 1 shows comparison between *P. gingivalis* proportion taken from the GCF between healthy subjects, periodontitis A and periodontitis B. Kruskal-Wallis comparison test shows there was significant difference between three groups. As we expected, healthy group had no or less *P. gingivalis* compared to the groups of periodontitis A and B. Further, periodontitis B, which has deeper pocket depth (≥ 5 mm), had a higher proportion of *P. gingivalis* than periodontitis A. In contrast, there was no significant difference in *P. gingivalis* proportion in the tongue coating were observed between the healthy subjects, periodontitis A and periodontitis B (Table 2). Collectively, our findings here are consistent with the fact that *P. gingivalis*, an anaerobic Gram Negative bacteria, grows better in the appropriate environment in the pockets, not the tongue coatings.

	Median [Min-Max]	P-value
Healthy subjects, [n = 6]	0 [0-0.0004]	0.004*
Periodontitis A, PD 3-4 mm [n=12]	0.015 [0-0.08]	
Periodontitis B, PD ≥ 5 mm [n = 14]	0.0406 [0-0.3]	

Table 1. Comparison of *P. gingivalis* proportions in the GCF between healthy subjects, periodontitis A and periodontitis B groups.

*Kruskal-Wallis Test, $p < 0.05$; PD, pocket depth. Universal 16S rRNA was used to get the *P. gingivalis* proportion.

	Median [Min-Max]	P-value
Healthy subjects, [n = 6]	0 [0-0.0007]	0.095
Periodontitis A, PD 3-4 mm [n=12]	0.0004 [0-0.003]	
Periodontitis B, PD ≥ 5 mm [n = 14]	0.0007 [0-0.01]	

Table 2. Comparison of *P. gingivalis* proportion in Tongue coating between healthy subjects, periodontitis A and periodontitis B groups.

*Kruskal-Wallis Test, $p < 0.05$; PD, pocket depth. Universal 16S rRNA was used to get the *P. gingivalis* proportion.

The level of hydrogen sulfide and methylmercaptan in periodontitis

Despite the proportion of *P. gingivalis*, we examined the gases production in all groups. As

shown in the table 3, the levels of hydrogen sulfide in the healthy subjects, periodontitis A, and periodontitis B groups were significantly observed. Similarly, there was significant differences in methylmercaptan level between the healthy subjects, periodontitis A, and periodontitis B groups (Table 4). Our data suggest that the periodontitis subjects with deeper pockets may have higher level of hydrogen sulfide and methylmercaptan.

	Median [Min-Max] (ppm)	P-value
Healthy subjects, [n = 6]	0 [0-7]	0.01*
Periodontitis A, PD 3-4 mm [n=12]	62.5 [4-478]	
Periodontitis B, PD ≥ 5 mm [n = 14]	88 [4-1727]	

Table 3. Comparison of Hydrogen Sulfide level between healthy subjects, periodontitis A and periodontitis B groups.

*Kruskal-Wallis Test, $p < 0.05$; PD, pocket depth.

	Median [Min-Max] (ppm)	P-value
Healthy subjects, [n = 6]	0 [0-0.0007]	0.002*
Periodontitis A, PD 3-4 mm [n=12]	14.5 [4-211]	
Periodontitis B, PD ≥ 5 mm [n = 14]	22 [1-663]	

Table 4. Comparison of Methylmercaptan level between the periodontitis A and periodontitis B groups.

*Kruskal-Wallis Test, $p < 0.05$; PD, pocket depth.

Parameter	r of <i>P. gingivalis</i> in GCF	r of <i>P. gingivalis</i> in TC
Hydrogen sulfide (p value)	0.55 (0.01)*	0.455 (0.009)*
Methyl Mercaptan (p value)	0.432 (0.01)*	0.256 (0.157)

Table 5. Correlations [r] between the proportion of *P. gingivalis* and the levels of both hydrogen sulfide and methyl mercaptan in the GCF and tongue coating.

*Spearman's Correlation was significant at $p < 0.05$; sGCF, gingival crevicular fluid; TC, tongue coating.

Hydrogen sulfide and methyl mercaptan levels correlate to *P. gingivalis* proportions in the GCF and tongue coating

As shown in the Table 5, a positive correlation between the quantities proportion of *P. gingivalis* in the tongue coating and the level of methyl mercaptan was noted, but statistical significance notwithstanding. A moderate positive correlation was found between hydrogen sulfide levels and *P. gingivalis* proportions in the GCF and tongue coating (statistically significant). Likewise, a moderate positive correlation

between methyl mercaptan and *P. gingivalis* proportions were noted in the GCF.

Discussion

In the present study, we elaborated 32 subjects with pockets of varying depths, including normal sulcus. The subjects were divided into three groups: one control group and two periodontitis groups (periodontitis A, wherein the presented a pocket depth of 3–4 mm and periodontitis B, where the patients presented a pocket depth of ≥ 5 mm). Patients with a pocket depth of < 3 mm were considered as normal sulcus or healthy. This classify based on the assumption that *P. gingivalis* levels vary with the depth of the pocket. A pilot study on the microbiome in pockets of different depths showed that the proportion of the bacteria increased with the increase in the depth of the pocket. As the pocket depth increases, the environment becomes more anaerobic, thus creating an ideal ecosystem for the growth of the anaerobic bacteria.¹⁶ It is a Gram-Negative anaerobic bacteria and a part of the red-complex bacteria.^{11,17} This condition also explains the higher proportion of *P. gingivalis* in Periodontitis A and B, and statistically significant.

The proportion of *P. gingivalis* in the tongue coating also higher in Periodontitis A and B, but not statistically significant. *P. gingivalis* is found in small amounts in the tongue coating, despite the aerobic environment on the dorsum of the tongue. A previous study reported that this bacterium is found on the dorsum of the tongue and may cause oral malodor or halitosis. A higher percentage of *P. gingivalis* on the dorsum of the tongue was observed in the patients with periodontitis. Periodontal disease may induce tongue biofilm formation; as a result, the thick coating on the tongue can create anaerobic conditions and become a reservoir for anaerobic bacteria.^{18,19} The dorsum of the tongue consists of fissures and papillae, which offer a unique environmental niche for biofilm coating and adhesion. Several factors such as age, sex, salivary secretion, immunological defense, and gastrointestinal conditions may be associated with the degrees of change on the surface of the tongue.²⁰

The periodontal bacteria have been shown to be strongly related to VSC production and may be found not only in the deep pockets

but also on the dorsum of the tongue. However, *P. gingivalis* was not detected in patients who presented with halitosis but not periodontitis¹⁸. This explain how the two main gases of VSC, hydrogen sulfide and methyl mercaptan, both increase in Periodontitis A and B, and also statistically significant.

This study aimed to analyze the correlation between the specific gases that cause oral malodor and the periodontal bacteria, *P. gingivalis*. Moderate correlations were observed between this bacterium and both the gases (statistical significance) except the correlation between *P. gingivalis* proportion in the tongue coating with methyl mercaptan. These findings similar to previous studies, *P. gingivalis* produce hydrogen sulfide from the degradation of cysteine, and increase the Interleukin-8 productions (IL-8) to stimulate inflammation and periodontal destruction, thus giving a better environment for periodontal pathogen such as *P. gingivalis*,^{11,17,21} meanwhile methyl mercaptan were produced from L-methionine through L-methionine-a-deamino-g-mercaptomethane-lyase (METase) reaction. METase may cause the production of methyl mercaptan in periodontal pocket and elsewhere in the oral cavity.^{22,23} However, weak correlation and statistically not significant was found between *P. gingivalis* proportion in the tongue coating with methyl mercaptan. This indicates that *P. gingivalis* alone cannot produce this gas. Its interaction with other bacteria may create food chains or specific reactions that produce.^{18,24,25}

One limitation of this study is that the measurements of oral malodor were merely evaluated based on pocket depth and tongue coating. The presence of local factors such as deep caries and tooth radix, were not recorded.

Conclusions

Weak- to moderately-positive correlations between hydrogen sulfide and methyl mercaptan levels and the proportion of *P. gingivalis* were seen in the GCF and tongue coatings of the patients with periodontitis, which may be related to halitosis. However, further studies which minimize the local factors contribute to oral malodor, such as deep caries, tooth radix, etc, is needed to empahsize the role of *P. gingivalis* as periodontal pathogenic bacteria in producing oral malodor gases.

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Declaration of Interest

The authors report no conflict of interest.

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