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Relationship between halitosis and periodontal disease – associated oral bacteria in tongue coatings

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Abstract: *Aim:* The objective of our study was to investigate the relationship between halitosis and oral bacteria in tongue coating (TC) and saliva samples from patients with halitosis, and to evaluate the effect of tongue cleaning on halitosis. *Methods:* Ninety-four participants complaining of oral malodour were included in the study. Organoleptic (OR) values, volatile sulphur compound (VSC) concentrations determined by gas chromatography and TC scores were used as clinical parameters of halitosis. Quantitative real-time polymerase chain reactions were used to determine the numbers of periodontal disease–associated oral bacteria. *Results:* There was a significant correlation between TC scores and OR values, methylmercaptan (CH_3SH) concentrations and VSC concentrations (Spearman's rank-correlation coefficient test, $P < 0.01$). There was also a positive correlation between the clinical parameters of halitosis and total bacterial numbers and *Prevotella intermedia, Fusobacterium nucleatum* and *Campylobacter rectus* concentrations in the TC samples. However, there was no similar correlation with respect to the saliva samples. The participants were sub-divided into two groups based on whether they had the habit of tongue cleaning or not. The participants with the habit of tongue cleaning had significantly lower OR scores, VSC concentrations and *P. intermedia, F. nucleatum* and *C. rectus* levels than the other participants (Mann–Whitney *U*-test, $P < 0.05$). *Conclusion:* These results suggested that periodontal disease–associated oral bacteria in TCs are closely related to halitosis and that tongue cleaning may be an effective method for improving halitosis.

Key words: halitosis; oral bacteria; periodontal disease; tongue cleaning; tongue coating

Introduction

Halitosis can be a serious problem for individuals because it may interfere with their daily activities and interpersonal relations. It has been reported that volatile sulphur compounds (VSCs), volatile nitrogen compounds and short-chain fatty acids are mostly responsible for unpleasant or foul smelling mouth air (1, 2). The intensity of the unpleasant smell is correlated with the level of VSCs, which have an offensive putrid odour (3, 4). As such, high VSC concentrations in mouth air are indicative of a problem with halitosis. The main VSCs in mouth air are hydrogen sulphide [H_2S], methyl mercaptan [CH_3SH] and dimethyl sulphide [$(CH_3)_2S$)], which are produced by various oral bacterial species that degrade and metabolize sulphur-containing amino acids such as cysteine and methionine (1).

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Periodontal disease–associated bacteria are capable of producing large amounts of VSCs (2, 5).

The tongue dorsum is the largest surface in the mouth, and its papillary structure is highly colonized by bacteria (5–7). The tongue coating (TC) is a biofilm formed on the dorsum and consists of epithelial cell debris, blood cells and food debris as well as oral bacteria that metabolize these substrates. The TC is thus a rich source of VSCs because of the large bacterial population (5). It has also been reported that approximately 60% of VSCs originate from the tongue surface in patients with periodontitis (8). This finding suggests that assessing TC deposition may be a good indicator of oral malodour.

The aim of this study is to investigate the relationship between bacterial numbers, periodontal conditions and VSC concentrations in mouth air from the participants. In addition, the effect of tongue cleaning on halitosis was evaluated.

Study population and methods

Study population and clinical parameters of periodontal conditions

The study population consisted of 94 patients (50.9 ± 13.1 years; 27 men and 67 women) who complained of oral malodour and who were referred to the Clinic for Breath Odor at the Tokushima University Hospital, Japan. The tongue cleaning habits of each participant was investigated using an interview and a questionnaire. The participants recruited for the study were completely dentulous men and women 20 years of age or older. Current smokers and participants who had received antibiotic treatment within the previous 3 months or who showed evidence of a systemic disease that may have influenced the degree of oral malodour were excluded from the study. The examination of halitosis were performed against all participants; however, the periodontal examination were performed only on participants willing to receive some treatments at the University Hospital instead of private dental clinic. Forty participants were examined to measure full-mouth periodontal probing depths (PPD) and the prevalence of bleeding-on-probing (BOP) positive. Participants belonging to the periodontitis group were selected based on the following criteria: the presence of at least two sites with a PPD ≥5 mm on separate teeth and BOP-positive deep pocket sites determined using the method of Hinode *et al.* (9). Participants excluded of these criteria were defined as control group in this study.

Assessment of oral malodour

VSC and OR measurements were taken at least one hour after oral activities such as eating, drinking and oral hygiene procedures. VSC concentrations in mouth air samples were measured using a GC-8APF gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame photometric detector and a β, β-ODPN 25% Chromosorb W-HP60/80 column (3.1 m × 3.2 mm; Shimadzu). The participants were asked to close their mouths for 60 s. Air samples (5 ml) collected from their mouths using a Hamilton Samplelock™ syringe (Hamilton Co., Reno, NV, USA) were injected on the column. The concentrations of H_2S, CH_3SH and $(CH_3)_2S$ were determined using standard gases prepared with a PD-1B Permeater (Gastec Co., Ayase, Japan). For the organoleptic (OR) assessments, mouth air from the participants breathing out gently was collected in plastic bags (GL Science Co., Tokyo, Japan). Examiners immediately assessed the odours. Standardization of OR of the sense of examiner were performed by the method of Murata *et al.* (10) using T&T Olfactometer™ (Daiichi Yakuhin Sangyo Co., Tokyo, Japan). OR scores were estimated based on the following 0–5 scale: 0, no odour; 1, barely noticeable; 2, slight but clearly noticeable; 3, moderate; 4, strong; and 5, extremely strong. OR values were assigned to the participants based on identical scores recorded by at least two examiners. The examiners of OR were five dentists; a further examiner was assigned when the value did not correlate between the two examiners.

Assessment of tongue coating

The accumulation of TC was assessed by a visual examination based on the following scoring criteria of Kojima (7): 0, not visible; 1, less than one-third of the tongue dorsum covered with a thin coating; 2, less than two-thirds covered with a thin coating or less than one-third with a thick coating; 3, more than two-thirds covered with a thin coating or less than two-thirds with a thick coating; and 4, more than two-thirds covered with a thick coating. The scores were assigned by comparison with standard colour photographs of TC only by a dentist.

Determination of bacterial concentrations in TC and saliva samples by real-time PCR

Campylobacter rectus ATCC 33238 (American Type Culture Collection, Rockville, MD, USA), *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 23726 and *P. intermedia* 163 were grown under the appropriate conditions as described previously (11, 12), and bacterial suspensions were prepared for real-time PCR analysis. The bacteria were washed in phosphate-buffered saline (PBS) and were counted using a Petroff-Hausser chamber. DNA was extracted using InstaGene Matrix kits (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Tenfold serial dilutions (10^2–10^8 cells per 200 µl) of bacterial standards were prepared, incubated at 56°C for 30 min, vortexed for 30 s, incubated at 100°C for 8 min and then stored at −80°C until used for the real-time PCR analysis.

TC samples were collected from the middle surface of the tongue dorsum using sterile 4-mm-wide plastic spatulas (Asone Co., Osaka, Japan) by swabbing five times from back to front (approx. 1-cm-long swabbing motions). The samples were

suspended in 1 ml of PBS. Unstimulated whole saliva (resting saliva) samples were collected in sterile plastic tubes prior to assessing PPD and BOP. The TC samples or saliva samples (200 µl) were centrifuged at 10 000 *g* for 10 min at 4°C. Following centrifugation, the pellets were washed in PBS and were suspended in 200 µl of InstaGene Matrix. The suspensions were incubated at 56°C for 30 min, vortexed for 30 s, incubated at 100°C for 8 min and then stored at −20°C until used for the real-time PCR analysis.

The real-time PCR analyses were performed using a MiniOpticon system (Bio-Rad Laboratories) with SYBR Green I dye and species-specific primers designed based on bacterial 16S rRNA gene sequences registered in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). The oral bacterial species–specific primers were generated using the method of Yokoyama *et al.* (12). The primer pairs were prepared by Hokkaido System Science Co. (Sapporo, Japan). The primers were verified for primer-dimer formation, melting temperature and the guanine–cytosine content. A standard curve prepared using the bacterial standards was used to determine the number of bacteria in the TC and saliva samples.

The PCR mixtures contained diethylpyrocarbonate-treated water, SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories), forward and reverse primers and DNA from a bacterial standard, TC sample or saliva sample. The amplification conditions were as follows: an initial denaturation step (3 min at 95°C) followed by denaturation (5 s at 95°C), annealing (10 s at 60°C) and extension (10 s at 60°C) steps. The number of cycles for *P. gingivalis*, *F. nucleatum*, *P. intermedia* and *C. rectus* were 45, 38, 33 and 38, respectively. Fluorescent products were analysed before and after each denaturation step. A melting curve spanning 60–95°C, with 0.2°C-intervals, was constructed. Melting peaks were used to confirm the specificity of the PCR. The results were analysed using CFX MANAGER software, version 2.1 (Bio-Rad Laboratories), and the concentrations of each bacterial species in the TC and saliva samples were calculated from the number of copies of the target sequence designed from the regions of the bacterial 16S rRNA gene sequence (12).

Statistical analysis

The results were expressed as means ± standard deviations (SD). The analyses were performed using SPSS, version 17.0J (SPSS Japan Inc., Tokyo, Japan). Spearman's correlation coefficients were calculated to determine the correlation of each clinical parameter with VSC concentrations, OR values and the number of bacteria. The Mann–Whitney *U*-test was used to compare the results from separate TC groups. Differences were considered statistically significant at $P < 0.05$.

Ethics

The Ethics Committee of Tokushima University Hospital approved the study (protocol approval number 218). The methods and objectives of the study were explained to the participants, who provided written informed consent prior to their participation in the study.

Results

There were significant positive correlations between the TC scores and the OR values and VSC concentrations (Table 1); between the TC scores and the numbers of total bacteria, *P. intermedia*, *F. nucleatum* and *C. rectus* in the TC samples; between the number of *P. gingivalis* and the OR values and CH_3SH concentrations; between the numbers of total bacteria; *P. intermedia*, *F. nucleatum*, *C. rectus* and the OR values and VSC concentrations; and between the percentage of *P. intermedia* and OR values and VSC concentrations.

The correlation between the TC scores, the OR values and the VSC concentrations, and the number of oral bacteria using resting saliva from 63 participants (50.8 ± 13.2 years, 22 men and 41 women) were investigated. There was no positive correlation between the number or percentage of bacteria and the halitosis parameters (data not shown).

The participants were sub-divided into two groups based on whether they had the habit of tongue cleaning or not. There were significant differences between the two groups in terms of TC scores, OR values, H_2S and total VSC concentrations, total number of bacteria and numbers of *P. intermedia*, *F. nucleatum* and *C. rectus* (Table 2).

The clinical characteristics related to periodontitis of selected participants are presented in Table 3. On the one hand, there were no significant differences between the periodontitis and control groups with respect to age, number of teeth, TC scores, numbers of total bacteria and concentrations of individual bacterial species. On the other hand, there were significant differences between the two groups in terms of OR scores and CH_3SH concentrations. While there were no significant correlations within the periodontitis group, significant correlations were observed between the VSCs values and bacterial numbers (except *P. gingivalis*) in the control group (Table 4).

Discussion

Real-time PCR has been shown to be a more sensitive and faster method for detecting and quantifying individual microbial species than conventional cultural procedures (13, 14). The high sensitivity and specificity of quantitative PCR justify its use in epidemiological studies and, as an adjunct, in the clinical diagnosis and follow-up of periodontitis patients (14). It has previously been used to quantitatively measure VSC-producing oral bacteria in TC samples and periodontopathogenic bacteria in subgingival plaque samples (15). In the present study, we used this procedure to study the relationship between halitosis and periodontal disease–associated oral bacteria.

We did not find any significant differences between male and female participants in terms of oral malodour values, which is in agreement with the findings reported by Miyazaki

Table 1. **Correlation coefficients between parameters of halitosis, TC and oral bacteria in TC samples**

	Age	TC score	OR	H_2S	CH_3SH	VSC	Total bacteria	*P. g.*	*P. i.*	*F. n.*	*C. r.*	% *P. g.*	% *P. i.*	% *F. n.*	% *C. r.*
Age	1														
TC score	0.027	1													
OR	0.203	0.33**	1												
H_2S	0.078	0.199	0.651***	1											
CH_3SH	0.143	0.392***	0.714***	0.737***	1										
VSC	0.072	0.274**	0.704***	0.911***	0.873***	1									
Total bacteria	0.255*	0.383***	0.324**	0.278**	0.355***	0.354***	1								
P. gingivalis (P. g.)	0.472***	0.057	0.253*	0.069	0.231*	0.173	0.388***	1							
P. intermedia (P. i.)	0.266**	0.403***	0.403***	0.347**	0.436***	0.423***	0.931***	0.432***	1						
F. nucleatum (F. n.)	0.169	0.251*	0.314**	0.328**	0.325**	0.388***	0.847***	0.330**	0.726***	1					
C. rectus (C. r.)	0.173	0.337**	0.250*	0.253*	0.313**	0.321**	0.936***	0.369***	0.893***	0.821***	1				
% *P. g.*	0.433***	−0.036	0.186	0.000	0.139	0.095	0.133	0.935***	0.189	0.119	0.126	1			
% *P. i.*	0.092	0.314**	0.375***	0.308**	0.411***	0.364***	0.390***	0.290**	0.682***	0.154	0.416***	0.164	1		
% *F. n.*	−0.097	−0.063	0.039	0.191	0.023	0.187	0.160	0.020	0.017	0.605***	0.228*	−0.013	−0.284**	1	
% *C. r.*	−0.082	0.061	−0.085	0.038	0.021	0.050	0.295**	0.098	0.294**	0.353***	0.579***	−0.002	0.174	0.387***	1

Correlation coefficients were determined using Spearman's rank-correlation coefficient test, $*P < 0.05$, $** P < 0.01$, $***P < 0.001$.
OR, organoleptic measurement; TC, tongue coating.

et al. (16). TC scores, number of oral bacteria and assessment values related to oral malodour were significantly higher in participants with genuine halitosis than in participants with pseudo-halitosis, suggesting that oral malodour is related to TC deposition and oral bacteria.

Several studies have shown that TC is related to VSC production (9, 16–18). Miyazaki *et al.* (16) reported high correlation coefficients (≥0.44 in all ages). Our study confirmed this relationship; however, the value of correlation coefficient of TC (0.274) and the others in Table 1 overall were relatively low (less than 4.0), which means weak relationship. Tanaka *et al.* (19) used real-time PCR to show that five periodontal pathogens (*P. gingivalis*, *Tannerella forsythia*, *P. intermedia*, *Prevotella nigrescens and Treponema denticola*) in TC samples are major contributors to VSC production. Washio *et al.* (20) reported that an increase in the number of H_2S-producing bacteria such as *Prevotella* species as well as *Veillonella* and *Actinomyces* in TCs is responsible for oral malodour. This study also showed that there is a significant correlation between VSC concentrations and the percentage of *P. intermedia*, which produces H_2S. This result suggested that specific periodontopathogenic bacteria (presence and proportion) in TCs are closely associated with VSC concentrations in mouth air. Regarding *P. gingivalis*, a significant correlation was only observed between the bacterial numbers and CH_3SH concentrations, which is in agreement with a previous study (19).

It has been reported that *F. nucleatum*, while present in healthy sites, is found in much higher numbers in diseased periodontal sites and plays a role in biofilm formation through coaggregation with other bacteria (21). This Gram-negative bacterial species possesses the ability to produce both H_2S and CH_3SH (2). Significant correlations were observed between the number of *F. nucleatum* and TC scores and VSC concentrations, which is in agreement with a previous study (22). *C. rectus* is also known to produce H_2S and CH_3SH (2). We found significant positive correlations between the number of *C. rectus* and TC scores and VSC concentrations. This is the first report to associate *C. rectus* with halitosis.

A previous report suggested that *P. gingivalis* and *P. intermedia* in saliva contribute to oral malodour (23). However, we found no positive correlation between specific oral bacterial species in resting saliva and VSC concentrations. This may be due to the fact that we used real-time PCR, while the other study used a semi-quantitative PCR procedure.

There were significant positive correlations between VSC concentrations, bacterial numbers and TC scores in participants not affected by periodontitis, whereas no such correlations were observed among the periodontitis patients. There was no difference in TC deposition between two groups, while the CH_3SH concentrations in the periodontitis group were higher than those in the non-periodontitis group. VSCs might thus not only be produced by bacteria in the TC but also by bacteria in other niches such as periodontal pockets. Patients with periodontal disease are at higher risk of developing

Table 2. **Comparison of parameters of halitosis and oral bacteria between participants with or without tongue cleaning habits**

	Tongue cleaning habits		
	Yes	No	*P*-value
Number of participants (male/female)	46 (12/34)	48 (15/33)	
Age (years)	49.1 ± 13.0	52.7 ± 13.0	0.213
OR	1.8 ± 1.0	2.3 ± 1.1	*
Gas chromatograph (ppb)			
H_2S	29.7 ± 72.0	101.1 ± 265.7	*
CH_3SH	8.6 ± 12.5	77.6 ± 263.5	0.083
Total VSC	39.5 ± 81.1	181.1 ± 447.0	*
TC score	1.9 ± 0.9	2.6 ± 1.0	**
Oral bacteria (cells/ml)			
Total bacteria	$4.47 \times 10^7 \pm 6.42 \times 10^7$	$9.49 \times 10^7 \pm 1.61 \times 10^8$	*
P. gingivalis	$1.14 \times 10^4 \pm 3.88 \times 10^4$	$2.27 \times 10^4 \pm 5.37 \times 10^4$	0.131
P. intermedia	$9.42 \times 10^5 \pm 2.00 \times 10^6$	$3.35 \times 10^6 \pm 9.52 \times 10^6$	**
F. nucleatum	$1.99 \times 10^5 \pm 3.35 \times 10^5$	$6.44 \times 10^5 \pm 1.72 \times 10^6$	*
C. rectus	$8.11 \times 10^4 \pm 2.41 \times 10^5$	$1.41 \times 10^5 \pm 3.18 \times 10^5$	*

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, by Mann–Whitney *U*-test.
Each value represents the mean ± SD.
OR, organoleptic measurement; TC, tongue coating.

Table 3. **Characteristics of periodontitis group and control group in regard to clinical parameters, VSC levels and oral bacteria**

	Periodontitis group	Control group	*P*-value
Number of participants (male/female)	13 (5/8)	27 (6/21)	
Age (years)	55.1 ± 10.8	53.9 ± 11.2	0.795
Number of present teeth	26.0 ± 4.5	25.0 ± 4.5	0.521
Number of teeth with PPD ≥5mm	6.6 ± 6.3	0.2 ± 0.4	***
Percent teeth with BOP	65.2 ± 29.8	36.0 ± 22.9	**
OR	2.8 ± 1.0	2.0 ± 0.9	*
Gas chromatograph (ppb)			
H_2S	83.5 ± 98.6	64.6 ± 103.7	0.274
CH_3SH	145.4 ± 428.8	15.9 ± 36.4	**
Total VSC	235.2 ± 436.8	81.4 ± 123.3	0.057
TC score	2.4 ± 1.1	2.4 ± 1.0	0.869
Oral bacteria (cells/ml)			
Total bacteria	$1.27 \times 10^8 \pm 2.39 \times 10^8$	$4.59 \times 10^7 \pm 5.22 \times 10^7$	0.170
P. gingivalis	$6.45 \times 10^4 \pm 1.03 \times 10^5$	$7.85 \times 10^3 \pm 1.71 \times 10^4$	0.131
P. intermedia	$6.30 \times 10^6 \pm 1.69 \times 10^7$	$1.21 \times 10^6 \pm 2.27 \times 10^6$	0.220
F. nucleatum	$3.74 \times 10^5 \pm 4.97 \times 10^5$	$3.65 \times 10^5 \pm 7.08 \times 10^5$	0.122
C. rectus	$1.13 \times 10^5 \pm 1.57 \times 10^5$	$1.15 \times 10^5 \pm 3.08 \times 10^5$	0.116

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, by Mann–Whitney *U*-test.
Each value represents the mean ± SD.
BOP, bleeding on probing; PPD, periodontal probing depth; OR, organoleptic measurement; TC, tongue coating.

halitosis than healthy individuals (17). Their malodour is primarily associated with gingival inflammation and the TC (24). Yaegaki *et al.* (25) reported that the CH_3SH/H_2S ratio is significantly higher in patients with periodontal disease. The same trend was observed in the present study, where the CH_3SH/H_2S ratios in participants with periodontal disease and without periodontal disease were 1.74 and 0.25, respectively. Furthermore, there was a positive correlation between CH_3SH concentrations and the number of teeth with PPD ≥5 mm ($r = 0.435$, $P < 0.01$, data not shown). In Table 4, no relationship was observed between TC score and VSC parameters in periodontal group. These results suggested that VSC production in participants with periodontal disease mainly originates from periodontal pockets. On the other hand, VSC production in participants without periodontal disease mainly originated from the TC.

Ohmori *et al.* (26) reported that breath odour and TC status improve after instructions on tongue brushing are provided by a dental hygienist or dentist at least once a day. They concluded that tongue cleaning is recommended for reducing breath odour. In the present study, the participants who had the habit of tongue cleaning had significantly lower TC scores and lower concentrations of CH_3SH and total VSCs in their mouth air than those in the group who had no habit of tongue cleaning. Based on the results of a questionnaire completed by 107 participants in our clinic, the ratio of patients with no habit of daily tongue cleaning was 52.3%; furthermore, the ratio of no cognition of TC in the TC3 or TC4 group of

Table 4. **Correlation coefficients between halitosis and bacteria from periodontitis group and control group**

	VSCs in mouth air		
	H_2S	CH_3SH	Total VSC
Periodontitis group (n = 13)			
TC score	−0.295	0.301	0.100
Total bacteria	−0.355	0.088	−0.011
P. gingivalis	−0.396	0.110	0.017
P. intermedia	−0.264	0.159	0.005
F. nucleatum	−0.124	0.363	0.313
C. rectus	−0.160	0.236	0.126
Control group (n = 27)			
TC score	0.452*	0.637***	0.488**
Total bacteria	0.527**	0.505**	0.531**
P. gingivalis	0.098	0.195	0.136
P. intermedia	0.512**	0.535**	0.532**
F. nucleatum	0.529**	0.408*	0.499**
C. rectus	0.445*	0.394*	0.442*

Correlation coefficients were determined using Spearman's rank-correlation coefficient test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. TC, tongue coating.

participants was 70.4% (D. Hinode, unpublished data). As tongue cleaning may be an effective method for improving oral malodour, patients should be more assertively informed that this simple procedure is an effective method for preventing or improving genuine halitosis.

Conclusion

Our results suggested that periodontopathogens in the TC are closely associated with halitosis and that VSC production in participants without periodontal disease is mainly associated with the TC. In addition, tongue cleaning may be an effective method for preventing or improving halitosis.

Clinical relevance

Scientific rationale for study

To assess tongue coating (TC) deposition may be a good indicator of oral malodour, and real-time PCR has shown to be a useful and sensitive method for measuring oral bacteria in TC.

Principal findings

Periodontal disease–associated oral bacteria in TC contribute to oral malodour. VSC production in participants without periodontal disease is mainly associated with TC. Tongue cleaning habit shows effects for reducing oral malodour.

Practical implications

As tongue cleaning may be an effective method for improving oral malodour, patients should be more assertively informed that this procedure is an effective method for preventing or improving halitosis.

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