The relationship between the presence of periodontopathogenic bacteria in saliva and halitosis

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Objective: To evaluate the association between the presence of periodontal pathogenic bacteria in saliva and halitosis in mouth air. **Design:** Cross-sectional microbiological and clinical oral examination of adult patients. **Subjects:** 101 adult patients (25 males, 76 females) who attended the Preventive Dentistry and Breath Odour Clinic of Kyushu Dental College. Their average age was 50.0 ± 13.5 years old (mean ± SD). **Setting:** The subjects were classified into three groups: halitosis subjects with a probing depth (PD) ≥ 4mm (P group), halitosis subjects without PD ≥ 4mm (H group), and non-halitosis subjects without PD ≥ 4mm (C group). **Methods:** All subjects received a periodontal examination. Volatile sulphur compounds (VSC: hydrogen sulphide and methyl mercaptan) were measured using gas chromatography. The presence of *Bacteroides forsythus*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia* in the saliva was detected by polymerase chain reaction. **Results and conclusion:** The presence of *B. forsythus*, *P. gingivalis* and *P. intermedia* influenced the production of VSC. Specifically, the presence of *B. forsythus* in subjects with periodontitis was strongly correlated to the concentration of VSC in mouth air.

Key words: Halitosis, polymerase chain reaction, periodontopathogenic bacteria, Bacteroides forsythus, *volatile sulphur compounds*

Specific gram-negative bacteria that colonise the subgingival area are the main pathogenic factors in various types of human periodontal conditions. *Porphyromonas gingivalis* has been implicated as a key organism in adult periodontitis[1-4], while *Actinobacillus actinomycetemcomitans* is generally recognised as the main infectious agent in localised juvenile periodontitis[3-5]. *Bacteroides forsythus* is frequently found in adult periodontal patients, and is regarded as one of the important risk factors for attachment level loss or alveolar crestal bone height loss[6-8]. *Prevotella intermedia* might be an important factor in periodontal diseases accompanied by a higher gingival index[9,10].

Volatile sulphur compounds (VSC) such as hydrogen sulphide (H_2S) and methyl mercaptan (CH_3SH) play an important role in halitosis[11]. The concentration of VSC in mouth air was significantly correlated with the intensity of oral malodour that was evaluated by organoleptic measurement[12]. VSC in mouth air are produced by the bacterial metabolism of proteins with sulphur-containing amino acids[13]. The oral gram-negative anaerobic bacteria such as *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus*, and *P. intermedia* found in periodontal pockets produce a certain amount of VSC *in vitro*[14], but the different microorganisms yield varying amounts and ratios of VSC[14].

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The detection of periodontal pathogenic microorganisms in the oral cavity may predict the risk of developing halitosis as well as periodontal conditions. Polymerase chain reaction (PCR)-based methods, which can detect low numbers of bacteria, have proven to be valuable in the detection of periodontal pathogenic bacteria in subgingival plaque or saliva[15-18]. Hence, the objective of this study was to examine the relationship between the presence of pathogenic bacteria, detected in human saliva by PCR, and oral malodour.

Table1 Species-specific and ubiquitous primers for PCR

Primer pairs	Base position (amplicon length in bp)
B. forsythus (16s rRNA) f primer 5'-GCGTATGTAACCTGCCCGCA-3' r primer 5'-TGCTTCAGTGTCAGTTATACCT-3'	120-760 (641 bp)
P. gingivalis (16s rRNA) f primer 5'-AGGCAGCTTGCCATACTGCG-3' r primer 5'-ACTGTTAGCAACTACCGATGT-3'	729-1132 (404 bp)
A. actinomycetemcomitans (16s rRNA) f primer 5'-GCTAATACCGCGTAGAGTCGG-3' r primer 5'-ATTTCACACCTCACTTAAAGGT-3'	166-608 (443 bp)
P. intermedia (16s rRNA) f primer 5'-CGTGGACCAAAGATTCATCG-3' r primer 5'-CCGCTTTACTCCCCAACAAA-3'	209-467 (259 bp)

Subjects and methods

Subjects

The subjects comprised 101 adult patients (25 males, 76 females) at the Preventive Dentistry and Breath-Odour Clinics of Kyushu Dental College Hospital. Their average age was 50.0 ± 13.5 (mean ± SD). None of the patients were undergoing treatment for systemic diseases. The procedures were explained to the patients and their informed consent was obtained prior to the investigation.

The subjects were classified into three groups: halitosis patients with probing depth (PD) of more than 4mm (P group, n = 56), halitosis patients without PD ≥ 4mm (H group, n = 31), and non-halitosis subjects without PD ≥ 4mm (C group, n = 14).

Clinical examination

The PD and bleeding on probing (BOP) were evaluated using a Williams probe. Probing was performed at three points on both buccal and lingual sides of each tooth. The sites with PD of more than 4mm and sites with BOP were counted and reported.

The amount of coating on the tongue's dorsal surface was estimated by visual examination as heavy (3), medium (2), light (1), or none (0).

Halitosis assessment

Gas chromatography is the gold standard for the evaluation of halitosis, particularly for research purposes[19]. In this study, hydrogen sulphide (H_2S) and methyl mercaptan (CH_3SH) concentrations in mouth air were determined with a gas chromatograph (G2800 gas chromatograph, Yanaco, Kyoto, Japan) equipped with a flame photometric detector and with a 3.4mm × 3m glass column packed with 1,2,3-tris (2-cyanoethoxy) propane 25 per cent in chromosorb W (AW-DMCS, 60/80 mesh). The column conditions were as follows: column temperature, 60°C; injection port temperature, 120°C; flame photometric detector temperature, 120°C; nitrogen gas flow pressure, 1.2kg/cm^2; hydrogen gas flow pressure, 1.0kg/cm^2; and air flow pressure, 1.0kg/cm^2.

Sampling of saliva

While the subjects were chewing on paraffin wax, whole saliva samples were collected into a sterile plastic tube over a period of five minutes, and their salivary flow rate was measured. The saliva samples were immediately stored at –80°C until use.

PCR detection

The four putative periodontal pathogenic bacteria (*B. forsythus, P. gingivalis, A. actinomycetemcomitans* and *P. intermedia*) were detected in saliva samples. The DNA templates for PCR amplification were obtained from the stored saliva using the EASY-DNA KIT (Invitrogen, CA) according to the manufacturer's instructions. PCR procedures for detecting microorganisms have been previously described[15,16]. Each PCR reaction mixture (100μl) contained 5μl sample, 10μl 10 x PCR buffer (Promega, WI), 0.2mM of each deoxyribonucleotide (Promega, WI), 2.5mM $MgCl_2$ (Promega, WI), 0.4μM of each primer and 1.25 unit *Taq*Bead™ Hot Start Polymerase (Promega, WI). The sequences of the specific primers for each of the study species are shown in *Table 1*. The temperature profile included an initial step of 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute, and a final step of 72°C for 7 minutes.

Data analysis

The comparisons of the parameters among the P group, H group and C group were analysed using the Kruskal-Wallis test and Tukey's WSD. The prevalence of each target bacterial species detected by PCR in each group was determined using the Chi-square test. The comparisons of the parameters based on the differences in the prevalence of each bacterial species in the P group or H group were analysed using the Mann-Whitney

Table 2 Profiles of P group, H group and C group

	Mean (SD)		
	P group	H group	C group
Age (year)	52.8 (12.1)	46.5 (15.0)	46.4 (13.6)
Number of teeth	25.4 (4.0)	26.1 (3.2)	27.1 (3.1)
H_2S (ng / 10 ml)**	7.0 (13.1)	5.1 (7.7)	0.0 (0.0)
CH_3SH (ng / 10 ml)**	11.2 (19.1)	6.0 (12.3)	0.0 (0.0)
PD (number)**	16.3 (17.4)	0.0 (0.0)	0.0 (0.0)
BOP (number)**	21.2 (17.6)	8.5 (9.7)	5.3 (6.5)
Tongue coating (score)*	1.2 (0.5)	1.2 (0.6)	0.8 (0.6)
Whole saliva (ml / 5 min)	7.0 (3.3)	7.8 (4.9)	6.1 (2.5)

*Kruskal Wallis test; P <0.05.
**Kruskal Wallis test; P <0.001.
Lines indicate significant differences between groups (Tukey's WSD; P<0.01)

U test. The odds ratio by the method of Mantel and Haenszel was determined to show the degree of association between each detected bacterial species and the VSC level in the P group or H group. The parameter for the VSC ($H_2S + CH_3SH$) level was dichotomised using a cut-off point of 10ng/10ml. Statistical analyses were performed using SPSS software (SPSS Inc., IL).

Results

Profiles of the study subjects

There were no significant differences between the clinical parameters of males and females in each of the P, H and C groups. No significant differences were observed among age, the number of teeth, and the rate of salivary flow in each group. There were no significant differences between P and H groups in concentrations of H_2S and CH_3SH and in the tongue-coating scores by visual examination (*Table 2*).

Prevalence of target bacterial species

The prevalence of the bacterial species in groups P, H and C respectively are given below. *B. forsythus* was detected as 44.6 per cent, 29.0 per cent and 7.1 per cent. *P. gingivalis* was detected as 87.5 per cent, 83.9 per cent and 64.3 per cent. *A. actinomycetemcomitans* was detected as 30.4 per cent, 25.8 per cent and 21.4 per cent, and finally, *P. intermedia* was detected as 85.7 per cent, 67.7 per cent and 42.9 per cent. The distributions of *B. forsythus* and *P. intermedia* among the groups were significantly different (P <0.05) (*Figure 1*). The prevalence of *B. forsythus* and *P. intermedia* were higher in the P group than in the H group or C group. The prevalence of *P. gingivalis* and *A. actinomycetemcomitans* were not significantly different among P, H and C groups (*Figure 1*).

Comparisons of parameters based on the differences in the prevalence of detected bacterial species

The means of age, number of teeth, tongue coating and salivary flow were not significantly different between the positive and negative groups of bacteria. However, significant differences were found between the positive and negative groups of *B. forsythus* in their means of H_2S, CH_3SH, PD and BOP (P <0.01). Significant differences were also found in CH_3SH of *P. gingivalis* groups (P <0.05), as well as in CH_3SH, PD, and BOP of *P. intermedia* groups (P <0.05) (*Table 3*). There were no significant differences between the groups of *A. actinomycetemcomitans* (*Table 3*).

The concentration of CH_3SH was significantly different between the positive and negative groups of *B. forsythus* in the P group (P <0.01). However, there was no significant difference in the clinical

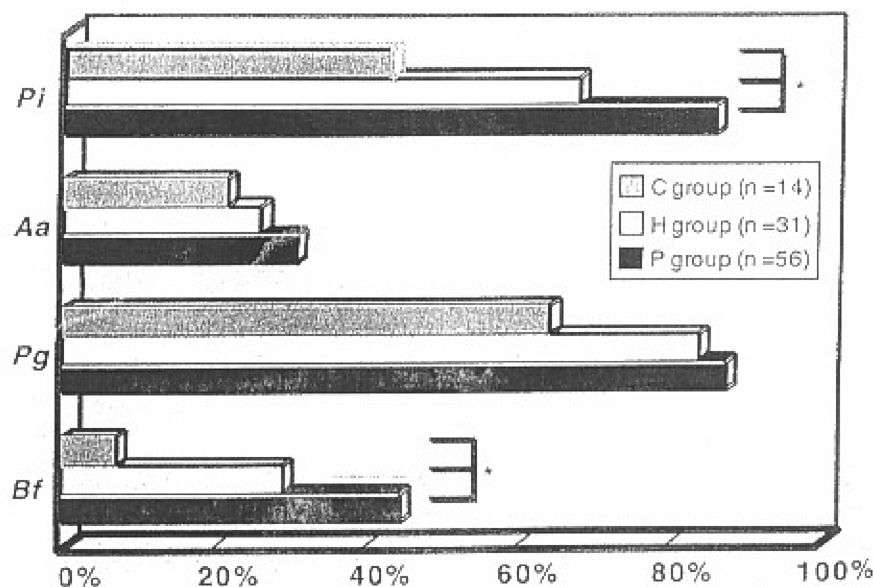

Figure 1. *Prevalence of each target bacterial species detected by PCR in each group.* *Chi-square test; P<0.05. (*Bf*: *B. forsythus*, *Pg*: *P. gingivalis*, *Aa*: *A. actinomycetemcomitans*, *Pi*: *P. intermedia*.)

Table 3 Comparisons of H_2S, CH_3SH, PD and BOP based on the differences in the prevalence of each baterium in all subjects

	Mean rank							
	Bf		*Pg*		*Aa*		*Pi*	
	+	−	+	−	+	−	+	−
H_2S	62.8	44.1**	52.9	38.6	52.6	49.7	53.5	42.0
CH_3SH	64.3	43.9**	54.2	35.4*	53.7	50.0	54.9	39.8*
PD	62.0	45.1**	53.0	41.2	55.5	49.3	55.5	38.0**
BOP	62.2	45.0	51.6	47.9	57.0	48.7	55.5	38.1**

Analysis by Mann-Whitney U-test, *$P<0.05$ and **$P<0.001$

Bf: B. forsythus; Pg: P. gingivalis; Aa: A. actinomycetemcomitans; Pi: P. intermedia

Table 4 Association between detected bacteria and VSC level in P group and H group.

	VSC level odds ratio	
	P group	H group
B. forsythus	5.1*	1.3
P. gingivalis	4.8	1.8
A. actinomycetemcomitans	0.3	0.3
P. intermedia	2.3	0.5

*Analysis by Mantel-Haenszel; $P<0.01$

parameters between the positive and negative groups of other microorganisms in both P group and H group.

Relationship between bacterial species and VSC levels in the P and H groups

The Odds ratios were calculated by the Mantel-Haenszel method for the evaluation of the relationship between the presence of the four target bacterial species in the saliva and high VSC level (≥ 10ng/10ml) in the P and H groups. The presence of *B. forsythus* in the P group was significantly associated with the VSC level ($P<0.01$). *P. gingivalis, A. actinomycetemcomitans* and *P. intermedia* showed no statistically-significant associations with the VSC level in the P and H groups (*Table 4*).

Discussion

In this study the role of periodontal pathogenic bacteria in malodour production was examined. *P. gingivalis, Treponema denticola* and *B. forsythus* produce VSC *in vitro* and are periodontally pathogenic. These microorganisms are detected through their ability to hydrolyse the synthetic trypsin substrate N-benzoyl-DL-arginine-2-naphthylamide (BANA test)[20,21]. As a consequence, the BANA test has also been used as a potential diagnostic tool for periodontal disease. Previous studies have reported the associations between VSC levels in mouth air and periodontal pathogenic bacteria detected by the BANA test at various oral sites[22,23]. However, the BANA test cannot determine the specific role of the bacterial species, *P. gingivalis, T. denticola*, or *B. forsythus* in the production of oral malodour. In contrast, PCR-based diagnostics using saliva are capable of determining the distribution of the pathogenic species in the oral cavity in a non-quantitative way[15]. PCR has proven to be a suitable and useful procedure for evaluating the relationship between the presence of bacterial species and halitosis.

Relationship between the presence of target species and VSC

Subjects with *B. forsythus* in the saliva had a higher level of VSC in the mouth air and more severe periodontal conditions compared to the subjects without these bacteria. It is suggested that the presence of *B. forsythus* in the saliva is correlated with halitosis and the pathology of periodontitis. The level of CH_3SH in the *B. forsythus*-positive P group was significantly higher than in the *B. forsythus*-negative P group. Also, the odds ratio of the *B. forsythus*-positive P group was 5.1 times higher than in the *B. forsythus*-negative P group for the high level generation of VSC in mouth air. However, the H group had as high a VSC level as the P group, although the prevalence of *B. forsythus* in the H group was significantly lower than that in the P group. Therefore, one might suggest that the presence of *B. forsythus* in subjects with periodontitis could be a risk factor for halitosis.

P. gingivalis is the most active microorganism in producing CH_3SH *in vitro*[14]. In this study, subjects with *P. gingivalis* demonstrated a higher level of CH_3SH than the subjects without *P. gingivalis*. However, there was no significant difference between the VSC levels of the P and the H groups, as mentioned above. Furthermore, there was no difference in the prevalence of *P. gingivalis* among all P, H and C groups. Therefore, although *P. gingivalis* contributes to VSC production, one may suggest that other factors played more important roles in VSC production than the presence of *P. gingivalis*.

The prevalence of *P. intermedia* was significantly different among the P, H and C groups. Although it is apparent that *P. intermedia* produces VSC *in vitro*[14], there have been no reports that reveal a relationship between *P. intermedia* and halitosis *in vivo*. However, this study demonstrated that the presence of *P. intermedia* as well as *B. forsythus* in the saliva was associated with both halitosis and periodontitis.

A. actinomycetemcomitans is seemingly less able to produce VSC *in vitro* than any of *B. forsythus, P. gingivalis* or *P. intermedia*[14]. In this study, a relationship between the presence of *A. actinomycetemcomitans* and VSC production could not be found.

Conclusion

In this study, it was shown that among the four periodontopathogenic bacteria, the presence of *B. forsythus*, *P. gingivalis* and *P. intermedia*, but not *A. actinomycetemcomitans* influenced the production of VSC. Also the presence of *B. forsythus* in patients with periodontitis was strongly correlated with the intensity of VSC.

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