

Detection of oral bacteria on the tongue dorsum using PCR amplification of 16S ribosomal RNA and its association with systemic disease in middle-aged and elderly patients

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Abstract. The association between oral health and systemic disease is recognized in the literature. The present study aimed to clarify the association between oral bacteria on the tongue dorsum and factors associated with oral health and systemic disease in middle-aged and elderly patients. The association between bacterial numbers, oral health status and systemic disease was preliminarily investigated in 70 patients (mean age, 69.5 years; range, 45-92 years) who visited the Department of Oral Health, Hiroshima University Hospital (Hiroshima, Japan). The bacterial 16S ribosomal RNA gene was employed to quantitate bacterial numbers using real-time polymerase chain reaction (PCR). PCR was also performed to detect the DNA of periodontal disease-related bacteria. Oral bacterial numbers were marginally negatively correlated with moisture levels on the tongue surface [Spearman's rank correlation coefficient (R)=-0.131, P=0.28]. Subjects with bleeding on probing (BOP) or a ≥ 4 mm probing depth (PD) exhibited higher *Porphyromonas gingivalis* (*P. gingivalis*)-positive rates (50.0 and 51.1%, respectively) than those without BOP or a < 4 mm PD (39.5 and 30.4%, respectively). Subjects with medical histories of hypertension, diabetes, stroke and heart

disease exhibited a trend toward higher *P. gingivalis*-positive rates than those without such disorders. These findings indicated that the tongue moisture level may be associated with bacterial numbers on the tongue surface, while *P. gingivalis* on the tongue surface may be associated with systemic and periodontal diseases. Further investigation in a larger number of participants is necessary to clarify the correlation between bacterial numbers and systemic disease.

Introduction

A relationship between oral pathogenic bacteria and systemic and oral diseases has been recognized in elderly and hospitalized patients (1,2). In particular, elderly patients exhibit high incidence rates of aspiration pneumonia, which is considered to be associated with poor oral hygiene and impaired oral function (1). Additionally, dry mouth is common among the elderly, and it may lead to oral diseases including dental caries, oral candidiasis and swallowing disorders (3). Importantly, oral health care can aid to remove dental plaques, moisturize the oral cavity, relieve mucosal inflammation, and promote oral hygiene by reducing oral bacterial numbers (2,4). Thus, regular oral care serves a vital role in the maintenance of oral health, which may result in a decreased risk of systemic disease in elderly subjects.

In a previous study, our group prospectively investigated the changes in oral bacterial numbers in colorectal cancer patients during the perioperative period (4). Notably, bacterial numbers on the tongue dorsum changed less markedly than those of the gingiva of the upper anterior teeth and palatoglossal arch, even when perioperative oral health care had been performed (5). Bacteria of the tongue dorsum may be less susceptible to oral health care practices such as tongue cleaning with a sponge or tongue brushes (5,6). This may be because the anatomical features of the tongue dorsum, namely the papillae, creates reservoirs for pathogenic bacteria, which may be associated with oral and systemic disease (7). To date, the association of between bacteria on the tongue surface with oral health status and systemic disease remains unclear. Thus, the correlation between bacteria on the tongue surface and oral and general health status should be clarified.

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Abbreviations: 16S rRNA, 16S ribosomal RNA; BOP, bleeding on probing; PD, probing depth; CFU, colony forming units; *P. gingivalis*, *Porphyromonas gingivalis*; *T. forsythia*, *Tannerella forsythia*; *T. denticola*, *Treponema denticola*; PCR, polymerase chain reaction

Key words: 16S ribosomal RNA gene, bacterial number, periodontal disease-related bacteria, real-time polymerase chain reaction, tongue dorsum

In taxonomic studies, sequence analysis of the 16S ribosomal RNA (rRNA) gene is widely used to identify bacterial species (8-10). This method enables the measurement of numerous bacterial strains without specialized culturing conditions. Bacterial 16S rRNA genes generally contain 9 hypervariable regions that demonstrate considerable sequence diversity among bacterial species and can be used for species identification (9,10). Conserved regions of the gene can be used to design universal primers to amplify various bacteria (9,10), thus allowing total bacterial numbers to be estimated using a bacterial DNA sample. Herein, the present study performed real-time polymerase chain reaction (PCR) using universal primers for the bacterial 16S rRNA gene to calculate oral bacterial numbers.

Further clinical investigation is required to clarify the relationship between oral bacteria and general health status. Therefore, this study preliminarily investigated bacteria on the tongue surface using PCR amplification of the bacterial 16S rRNA gene, and examined the correlation between tongue bacteria and systemic disease in middle-aged and elderly patients.

Materials and methods

Subjects. The current study investigated 70 patients (23 males, 47 females; mean age, 69.5 years; range, 45-92 years) who visited the Department of Oral Health of Hiroshima University Hospital, Hiroshima, Japan from March 2018 to April 2018. A 0-3 mm probing pocket depth was considered to indicate mild or no periodontitis (11). Moderate periodontal disease was defined as the presence of ≥ 4 and < 6 mm probing pocket depth (11). Severe periodontitis was defined as the presence of ≥ 6 mm pocket (11). All patients exhibited a mild to moderate periodontal status. No patients exhibited symptoms of acute periodontal disease. Regarding toothbrushing frequency, all participants brushed their teeth more than twice daily. None used a tongue scraper to clean the surface area of the tongue. The study design was approved by the Ethics Committee of Hiroshima University, and all participants signed an informed consent agreement. Clinical data were obtained regarding age, sex, smoking status, alcohol consumption and medical history.

Oral sample processing and DNA extraction. Samples were collected from the tongue dorsum using a Orcellex® Brush (Rovers Medical Devices, Oss, The Netherlands) which allows collection of cells or bacteria from oral epithelial layers. The tongue surface was swabbed prior to tooth brushing using the Orcellex® 10 times. DNA was extracted and purified using a PureLink™ Microbiome DNA Purification kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). For PCR analysis, 1 μ l was used from a total of 50 μ l of oral sample DNA.

Quantification of bacterial numbers. The bacterial 16S rRNA gene was used to quantify bacterial numbers using real-time PCR. Since 16S rRNA gene copy numbers differ among bacteria, it is difficult to use them in the accurate quantification of bacteria. Many bacteria have 1 to 10 16S rRNA gene copies, while some have >10 (12,13). Here, 16S rRNA

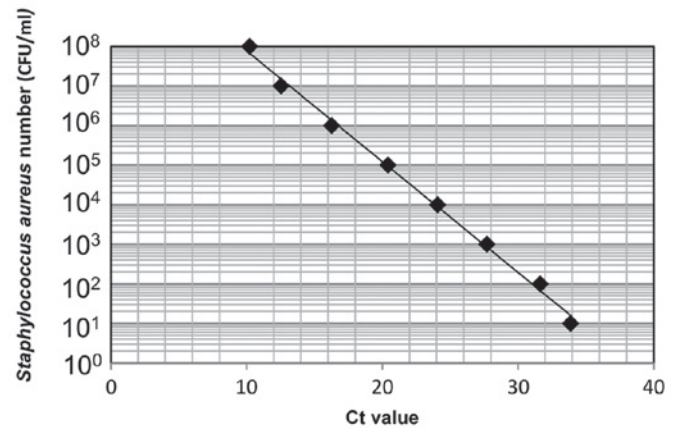


Figure 1. Standard curve indicating Ct values vs. *Staphylococcus aureus* numbers (CFU/ml). Serial 10-fold dilutions of *Staphylococcus aureus* DNA (ranging from 10^1 to 10^8 CFU/ml) were used to make a standard curve. CFU, colony forming units.

gene copy numbers were used from *Staphylococcus aureus* (5 copies per cell) to quantify approximate oral bacterial numbers.

First, DNA was extracted from *Staphylococcus aureus* [1.0×10^8 colony forming units (CFU)/ml] and used for real-time PCR. Serial 10-fold dilutions of the DNA sample (ranging from 10^1 to 10^8 CFU/ml) were prepared to make a standard curve (Fig. 1). DNA levels were quantified using a CFX connect real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and SYBR-Green PCR Master Mix (Toyobo Life Science, Osaka, Japan), with a reaction mixture containing 1 μ l DNA, 9 μ l SYBR-Green Mix, and 10 μ mol of each oligonucleotide primer pair. Amplifications were performed with initial melting at 95°C for 5 min, followed by 40 cycles of denaturing at 95°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 1 min. The 16S rRNA primer sequences were forward, 5'-CGTTAGTAATCGTGGATCAGAATG-3' and reverse, 5'-TGTGACGGGCGGTGTGTA-3' (14). A standard curve indicating the cycle threshold value versus the 16S rRNA gene was obtained to estimate the bacterial number per sample (15).

Detecting periodontal disease-related bacteria by PCR. For PCR, 1 μ l DNA samples (bacterial numbers: 1.0×10^3 - 1.0×10^6 CFU/ml) were used. Each mixture was amplified with 10X PCR buffer, dNTPs, Taq DNA polymerase (both from Toyobo Life Science) and primers. The following previously reported PCR primer sets were used: For *Porphyromonas gingivalis* (*P. gingivalis*) forward, 5'-AGGCAGCTTGCCAT ACTGCG-3' and reverse, 5'-ACTGTTAGCAACTACCGA TGT-3'; For *Tannerella forsythia* (*T. forsythia*) forward, 5'-GCGTATGTAACTGCCCCGCA-3' and reverse, 5'-TGC TTCAGTGTCACTTATACCT-3'; and for *Treponema denticola* (*T. denticola*) forward, 5'-TAATACCGAATGTGCTCAT TTACAT-3' and reverse, 5'-TCAAAGAAGCATTCCCTCTTC TTCTTA-3' (16). The PCR program included initial melting at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 56°C for 1 min and 72°C for 30 sec. Following the reaction, 10 μ l of the PCR product was electrophoresed on 2% agarose gels with ethidium bromide staining.

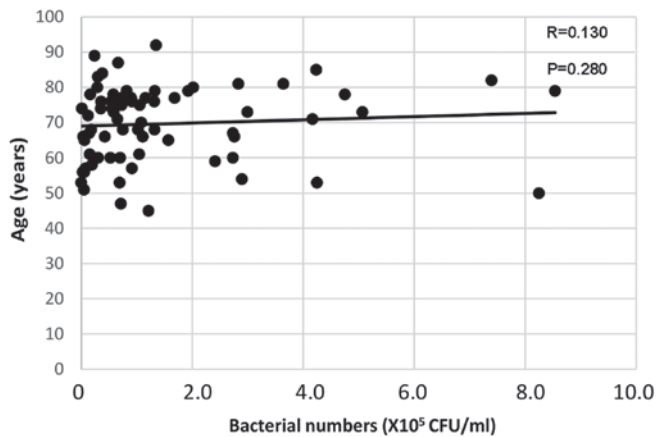


Figure 2. Correlation between age and bacterial numbers on the tongue surface. Age was weakly positively correlated with bacterial numbers on the tongue surface.

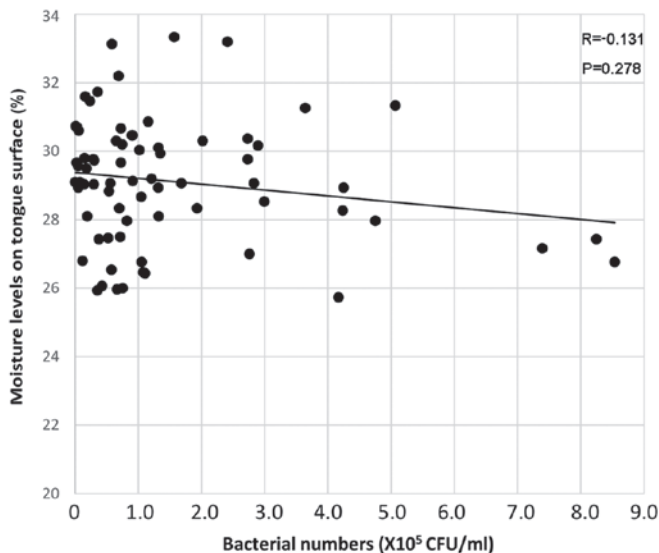


Figure 3. Correlation between moisture levels and bacterial numbers on the tongue surface. A weak negative relationship was indicated between oral bacterial numbers and moisture levels.

Oral examination. Oral examination including periodontal examination [to assess probing depth (PD) and bleeding on probing (BOP)] was performed by dentists at Hiroshima University Hospital. Remaining teeth and denture use were recorded. Oral wetness was evaluated using an oral moisture-checking device (Moisture Checker Mucus®; Scalar Corporation, Tokyo, Japan) as previously described (17). The oral moisture level was measured at the tongue dorsum 10 mm from the apex linguae and expressed as a percentage, with mean values calculated from three independent measurements; a value of >30% was defined as high moisture level and a value of ≤30% as low moisture level as in previous study (17). The tongue coating was classified as none, light-thin (visibly pink underneath) or heavy-thick (no pink observed). The presence of a light-thin and/or heavy-thick coating was considered positive (18).

Statistical analysis. Statistical analysis was performed using SPSS software, version 24.0 (IBM Corp, Armonk, NY, USA).

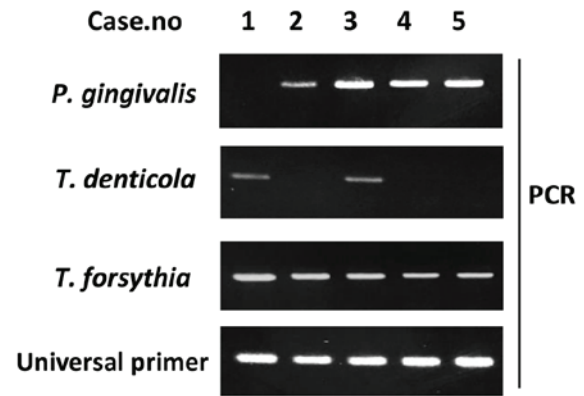


Figure 4. PCR detection of periodontal disease-related bacteria on the tongue surface. PCR was performed to detect *T. denticola*, *T. forsythia* and *P. gingivalis* DNA. 10 μ l of each 20- μ l PCR product was separated on a 2% agarose gel. *P. gingivalis*, *Porphyromonas gingivalis*; *T. forsythia*, *Tannerella forsythia*; *T. denticola*, *Treponema denticola*; PCR, polymerase chain reaction.

Determination of Spearman's rank correlation coefficient was performed to examine the correlation of bacterial number with age and moisture level. The Mann-Whitney U test with Bonferroni correction or the Kruskal-Wallis test was used to evaluate significant differences in bacterial numbers and clinical factors. All Kruskal-Wallis tests indicated no significant difference and therefore post-hoc tests were not applicable. The χ^2 test or Fisher's exact test was used to evaluate significant differences between positive rates of periodontal bacteria and clinical factors. $P < 0.05$ was considered to indicate statistical significance.

Results

Correlation between bacterial numbers on the tongue surface and clinical factors. Age was weakly correlated with bacterial numbers on the tongue surface [Spearman's rank correlation coefficient (R)=0.130, P =0.280; Fig. 2]. Meanwhile, the oral bacterial numbers were weakly negatively correlated with moisture levels (R =-0.131, P =0.278; Fig. 3). Table I summarizes the associations between bacterial counts detected by real-time PCR and clinical factors. Oral bacterial numbers appeared to be increased in individuals with fewer than 10 remaining teeth and in denture users; however, bacterial numbers did not differ significantly according to remaining teeth or denture use (Table I). Furthermore, no significant difference was identified between bacterial numbers on the tongue surface and oral hygiene status [BOP and PD (≥ 4 mm)]. Individuals with a medical history of stroke exhibited increased bacteria compared with those without, but the difference was not significant (Table I).

Detecting periodontal disease-related bacteria by PCR. Red complex bacteria (e.g., *T. denticola*, *T. forsythia* and *P. gingivalis*) serve major roles in periodontal disease etiology (19). Therefore, the present study performed PCR to detect DNA from these bacteria (Fig. 4), to examine the relationship between periodontal bacteria of the tongue dorsum and clinical factors. A total of 31 out of the 70 participants (57.1%) were *P. gingivalis*-positive (Table II), with the rate appearing

Table I. Associations between bacterial numbers on the tongue surface and clinical factors.

| Factor (n) | Bacterial numbers | |
|---------------------|---------------------------------|---------|
| | Mean \pm SD ($\times 10^5$) | P-value |
| Sex | | |
| Male (23) | 1.53 \pm 1.84 | 0.66 |
| Female (47) | 1.62 \pm 2.17 | |
| Age (years) | | |
| ≥ 65 (50) | 1.58 \pm 1.39 | 0.52 |
| < 65 (20) | 1.63 \pm 2.01 | |
| Alcohol consumption | | |
| Non-drinker (42) | 1.54 \pm 1.93 | 0.06 |
| Social drinker (15) | 1.00 \pm 1.13 | |
| Every day (13) | 2.45 \pm 2.39 | |
| Smoking status | | |
| Non-smoker (62) | 1.66 \pm 2.10 | 0.93 |
| Smoker (8) | 1.06 \pm 0.85 | |
| Stroke | | |
| No (66) | 1.54 \pm 1.91 | 0.14 |
| Yes (4) | 2.54 \pm 2.14 | |
| Heart disease | | |
| No (64) | 1.61 \pm 1.95 | 0.69 |
| Yes (6) | 1.54 \pm 1.65 | |
| Hypertension | | |
| No (50) | 1.53 \pm 1.85 | 0.39 |
| Yes (20) | 1.74 \pm 2.13 | |
| Diabetes | | |
| No (59) | 1.70 \pm 2.03 | 0.51 |
| Yes (11) | 1.00 \pm 0.99 | |
| Hyperlipidemia | | |
| No (60) | 1.65 \pm 1.99 | 0.77 |
| Yes (10) | 1.27 \pm 1.44 | |
| Remaining teeth | | |
| 0-9 (3) | 2.23 \pm 2.46 | 0.73 |
| 10-19 (13) | 1.31 \pm 1.39 | |
| ≥ 20 (54) | 1.63 \pm 2.02 | |
| Denture use | | |
| Non-user (54) | 0.88 \pm 1.72 | 0.12 |
| User (16) | 1.70 \pm 1.70 | |
| Oral wetness | | |
| Low (47) | 1.74 \pm 2.16 | 0.11 |
| High (23) | 1.30 \pm 1.29 | |
| Tongue coating | | |
| Negative (66) | 1.57 \pm 1.92 | 0.53 |
| Positive (4) | 1.92 \pm 2.12 | |
| Probing depth | | |
| < 4 mm (23) | 1.55 \pm 1.83 | 0.93 |
| ≥ 4 mm (47) | 1.61 \pm 1.98 | |
| Bleeding on probing | | |
| No (38) | 1.72 \pm 2.07 | 0.98 |
| Yes (32) | 1.44 \pm 1.74 | |

SD, standard deviation.

Table II. Associations between positive rate of *P. gingivalis* and clinical factors.

| Factor (n) | P. gingivalis | | P-value |
|---------------------|---------------|------------|---------|
| | (-) | (+) | |
| Sex | | | |
| Male (23) | 11 (47.8%) | 12 (52.2%) | 0.44 |
| Female (47) | 28 (59.6%) | 19 (40.4%) | |
| Age (years) | | | |
| ≥ 65 (50) | 25 (50.0%) | 25 (50.0%) | 0.18 |
| < 65 (20) | 14 (70.0%) | 6 (30.0%) | |
| Alcohol consumption | | | |
| Non-drinker (42) | 26 (61.9%) | 16 (38.1%) | 0.14 |
| Social drinker (15) | 9 (60.0%) | 6 (40.0%) | |
| Every day (13) | 4 (30.8%) | 9 (69.2%) | |
| Smoking status | | | |
| Non-Smoker (62) | 37 (59.7%) | 25 (40.3%) | 0.13 |
| Smoker (8) | 2 (25.0%) | 6 (75.0%) | |
| Stroke | | | |
| No (66) | 38 (57.6%) | 28 (42.4%) | 0.32 |
| Yes (4) | 1 (25.0%) | 3 (75.0%) | |
| Heart disease | | | |
| No (64) | 37 (57.8%) | 27 (42.2%) | 0.40 |
| Yes (6) | 2 (33.3%) | 4 (66.7%) | |
| Hypertension | | | |
| No (50) | 31 (62.0%) | 19 (38.0%) | 0.12 |
| Yes (20) | 8 (40.0%) | 12 (60.0%) | |
| Diabetes | | | |
| No (59) | 36 (61.0%) | 23 (39.0%) | 0.05 |
| Yes (11) | 3 (27.3%) | 8 (72.7%) | |
| Hyperlipidemia | | | |
| No (60) | 35 (58.3%) | 25 (41.7%) | 0.30 |
| Yes (10) | 4 (40.0%) | 6 (60.0%) | |
| Remaining teeth | | | |
| 0-9 (3) | 1 (33.3%) | 2 (66.7%) | 0.26 |
| 10-19 (13) | 5 (38.5%) | 8 (61.5%) | |
| ≥ 20 (54) | 33 (61.1%) | 21 (38.9%) | |
| Denture user | | | |
| Non-user (54) | 32 (59.3%) | 22 (40.7%) | 0.41 |
| User (16) | 7 (43.8%) | 9 (56.2%) | |
| Oral wetness | | | |
| Low (47) | 25 (53.2%) | 22 (46.8%) | 0.73 |
| High (23) | 14 (60.9%) | 9 (39.1%) | |
| Tongue coating | | | |
| Negative (66) | 37 (56.0%) | 29 (44.0%) | 1.00 |
| Positive (4) | 2 (50.0%) | 2 (50.0%) | |
| Probing depth | | | |
| < 4 mm (23) | 16 (69.6%) | 7 (30.4%) | 0.17 |
| ≥ 4 mm (47) | 23 (48.9%) | 24 (51.1%) | |
| Bleeding on probing | | | |
| No (38) | 23 (60.5%) | 15 (39.5%) | 0.47 |
| Yes (32) | 16 (50.0%) | 16 (50.0%) | |

P. gingivalis, *Porphyromonas gingivalis*.

higher in people aged 65 years and over (50.0%) compared with in those aged under 65 (30.0%). Additionally, smokers exhibited higher *P. gingivalis*-positive rates than nonsmokers. Subjects with medical histories of hypertension, diabetes, stroke and heart disease exhibited higher *P. gingivalis*-positive rates than those without these disorders. Subjects with BOP and ≥ 4 mm PD exhibited higher *P. gingivalis*-positive rates (50.0 and 51.1%, respectively) than those with no BOP and < 4 mm PD (39.5 and 30.4%, respectively), but these differences were not significant.

In contrast to the results for *P. gingivalis*, *T. forsythia* exhibited no apparent differences in positive rates between those with and without factors associated with systemic and oral disease (Table III). All smokers were *T. forsythia*-positive, but no significant difference was identified in positive rate between smokers and never-smokers.

For *T. denticola*, men were indicated to exhibit significantly higher positive rates than women (Table IV). *T. denticola* positivity did not differ between those with and without a medical history of hypertension, diabetes, stroke or heart disease. Interestingly, there appeared to be a significant association between the *T. denticola*-positive rate and the number of remaining teeth. All individuals with fewer than nine teeth exhibited *T. denticola*-positivity, while the positive rates in those with 10 or more teeth were $< 40\%$. These results indicate that *T. denticola* on the tongue surface may be associated with tooth loss.

Discussion

Real-time PCR has advantages in calculating total bacterial numbers in terms of sensitivity and rapidity compared with other methods (e.g., DNA hybridization and flow cytometry) (20,21). Therefore, in the present study real-time PCR methods were employed using universal primers for the 16S rRNA gene to quantitate approximate oral bacterial numbers. Bacterial counters use a dielectrophoretic impedance measurement method via a microelectrode chip on which bacteria from liquids are captured by dielectrophoresis (22). Initially, the current study compared oral bacteria numbers obtained from real-time PCR using 16S rRNA gene copy numbers with those obtained from a bacterial counter (Panasonic Healthcare Co., Ltd., Tokyo, Japan) and identified a significant positive association between these methods (data not shown). This confirmed that real-time PCR targeting the 16S rRNA gene can be used to estimate approximate oral bacterial numbers.

Bacterial numbers were weakly negatively correlated with moisture levels on the tongue surface. This indicated that low moisture levels of the tongue may be associated with bacterial growth on the tongue surface. Kobayashi *et al* (6) previously demonstrated that moisturizing the tongue is important for reducing bacterial numbers on the tongue surface. They determined that combining tongue cleaning with a mouthwash and moisturizing gel was most effective for reducing bacterial numbers on the tongue (6). Thus, moisturizing the tongue surface may serve an important role in inhibiting oral bacterial growth.

Regarding associations between systemic disease and oral health, all diabetic subjects exhibited ≥ 4 -mm periodontal

pockets (not shown). Although no significant association was observed between diabetes and oral health status, periodontitis likely serves a role in developing diabetes (23). Periodontal disease-associated cytokines may induce systemic inflammation leading to insulin resistance in type 2 diabetic patients (24). Indeed, the host inflammatory response may be involved in the mechanisms of diabetes (23,24).

In the present study, *P. gingivalis* infection was more common among elderly (≥ 65 years old) subjects. Previously, the *P. gingivalis* detection rate was observed to significantly increase with age, while that of *T. denticola* and *T. forsythia* was comparably high across all age groups (25). In the current study, participants with ≥ 4 -mm pockets exhibited higher positive rates of the red complex bacteria *P. gingivalis* and *T. denticola* compared with those with < 4 -mm pockets. Favari *et al* (26) reported that the tongue surface is an important reservoir for periodontal pathogens including *P. gingivalis*. These results indicate that periodontal disease-associated bacteria (i.e., *P. gingivalis* and *T. denticola*) on the tongue surface may be associated with deep periodontal pockets and periodontal tissue inflammation.

Regarding the association between systemic disease and red complex bacteria, subjects with medical histories of hypertension, diabetes, stroke and heart disease exhibited higher *P. gingivalis*-positive rates than those without such diseases. *P. gingivalis* may be associated with heart disease by inducing endothelial cell dysfunction and atherosclerosis via increased cytokine production, direct bacterial infection and induction of an immune response to bacterial heat-shock proteins (27,28). Additionally, *P. gingivalis* is likely involved in both diabetes and oral health conditions (29). Among participants with systemic disease, diabetic patients exhibited increased *P. gingivalis*-positive rates (72.7%) than those without (39.0%), indicating that there is an important association between *P. gingivalis* and diabetes. These findings and other previous results (29) suggest that *P. gingivalis* on the tongue surface serves a crucial role in the development of diabetes. However, it remains unclear whether there is a significant association between other red complex bacteria including *T. forsythia* and *T. denticola* and systemic disease.

Interestingly, males exhibited a greater *T. denticola*-positive rate than females, although the reason for this is unknown. Additionally, people with fewer than 10 teeth exhibited a higher *T. denticola*-positive rate than those with 10 teeth or more. *T. denticola* has been detected more frequently and in higher counts in people with recurring periodontal disease (30). Thus, *T. denticola* may serve an important role in deteriorating periodontal tissue, which may be associated with greater tooth loss.

In conclusion, 16S rRNA gene-targeted PCR using swab samples from the tongue dorsum may be useful for counting approximate oral bacterial numbers and detecting periodontal disease-associated bacteria to predict the condition of periodontal tissue. The number of participants in the current study was relatively small; therefore, further investigation in a large-scale study is necessary to clarify the correlations between oral bacterial numbers and systemic disease and factors associated with oral health, particularly in elderly subjects.

Table III. Associations between positive rate of *T. forsythia* and clinical factors.

| Factor (n) | <i>T. forsythia</i> | | P-value |
|---------------------|---------------------|------------|---------|
| | (-) | (+) | |
| Sex | | | |
| Male (23) | 4 (17.4%) | 19 (82.6%) | 0.17 |
| Female (47) | 16 (34.0%) | 31 (66.0%) | |
| Age (years) | | | |
| ≥65 (50) | 12 (24.0%) | 38 (76.0%) | 0.24 |
| <65 (20) | 8 (40.0%) | 12 (60.0%) | |
| Alcohol consumption | | | |
| Non-drinker (42) | 15 (26.3%) | 42 (73.7%) | 0.57 |
| Social drinker (15) | 4 (40.0%) | 6 (60.0%) | |
| Every day (13) | 1 (33.3%) | 2 (66.7%) | |
| Smoking status | | | |
| Non-smoker (62) | 20 (32.3%) | 42 (67.7%) | 0.05 |
| Smoker (8) | 0 (0.0%) | 8 (100.0%) | |
| Stroke | | | |
| No (66) | 19 (28.8%) | 47 (71.2%) | 1.00 |
| Yes (4) | 1 (25.0%) | 3 (75.0%) | |
| Heart disease | | | |
| No (64) | 37 (57.8%) | 27 (42.2%) | 0.40 |
| Yes (6) | 2 (33.3%) | 4 (66.7%) | |
| Hypertension | | | |
| No (50) | 16 (32.0%) | 34 (68.0%) | 0.39 |
| Yes (20) | 4 (20.0%) | 16 (80%) | |
| Diabetes | | | |
| No (59) | 17 (32.2%) | 42 (67.8%) | 1.00 |
| Yes (11) | 3 (27.3%) | 8 (72.7%) | |
| Hyperlipidemia | | | |
| No (60) | 17 (28.3%) | 43 (71.7%) | 1.00 |
| Yes (10) | 3 (30.0%) | 7 (70.0%) | |
| Remaining teeth | | | |
| 0-9 (3) | 0 (0.0%) | 3 (100.0%) | 0.68 |
| 10-19 (13) | 3 (23.1%) | 10 (76.9%) | |
| ≥20 (54) | 17 (31.5%) | 37 (68.5%) | |
| Denture use | | | |
| Non-user (54) | 17 (31.5%) | 37 (68.5%) | 0.36 |
| User (16) | 3 (18.8%) | 14 (81.2%) | |
| Oral wetness | | | |
| Low (47) | 13 (27.7%) | 34 (72.3%) | 1.00 |
| High (23) | 7 (30.4%) | 16 (69.6%) | |
| Tongue coating | | | |
| Negative (66) | 20 (30.3%) | 46 (69.7%) | 0.32 |
| Positive (4) | 0 (0.0%) | 4 (100.0%) | |
| Probing depth | | | |
| <4 mm (23) | 9 (39.1%) | 14 (60.9%) | 0.26 |
| ≥4 mm (47) | 23 (48.9%) | 24 (51.1%) | |
| Bleeding on probing | | | |
| No (38) | 13 (34.2%) | 25 (65.8%) | 0.30 |
| Yes (32) | 7 (21.9%) | 25 (78.1%) | |

T. forsythia, *Tannerella forsythia*.Table IV. Associations between positive rate of *T. denticola* and clinical factors.

| Factor (n) | <i>T. denticola</i> | | P-value |
|---------------------|---------------------|------------|---------|
| | (-) | (+) | |
| Sex | | | |
| Male (23) | 11 (47.8%) | 12 (52.2%) | 0.03 |
| Female (47) | 36 (76.6%) | 11 (23.4%) | |
| Age (years) | | | |
| ≥65 (50) | 32 (64.0%) | 18 (36.0%) | 0.42 |
| <65 (20) | 15 (75.0%) | 5 (25.0%) | |
| Alcohol consumption | | | |
| Non-drinker (42) | 32 (56.1%) | 10 (43.9%) | 0.05 |
| Social drinker (15) | 10 (66.7%) | 5 (33.3%) | |
| Every day (13) | 5 (38.5%) | 8 (61.5%) | |
| Smoking status | | | |
| Non-smoker (62) | 43 (69.4%) | 19 (30.6%) | 0.43 |
| Smoker (8) | 4 (50.0%) | 4 (50.0%) | |
| Stroke | | | |
| No (66) | 44 (66.7%) | 22 (33.3%) | 1.00 |
| Yes (4) | 3 (75.0%) | 1 (25.0%) | |
| Heart disease | | | |
| No (64) | 42 (65.6%) | 22 (34.4%) | 0.66 |
| Yes (6) | 5 (83.3%) | 1 (16.7%) | |
| Hypertension | | | |
| No (50) | 35 (70.0%) | 15 (30.0%) | 0.58 |
| Yes (20) | 12 (60.0%) | 8 (40.0%) | |
| Diabetes | | | |
| No (59) | 41 (69.5%) | 18 (30.5%) | 0.49 |
| Yes (11) | 6 (54.5%) | 5 (45.5%) | |
| Hyperlipidemia | | | |
| No (60) | 41 (68.3%) | 19 (31.7%) | 0.72 |
| Yes (10) | 6 (60.0%) | 4 (40.0%) | |
| Remaining teeth | | | |
| 0-9 (3) | 0 (0.0%) | 3 (100.0%) | 0.04 |
| 10-19 (13) | 8 (61.6%) | 5 (38.4%) | |
| ≥20 (54) | 39 (72.2%) | 15 (27.8%) | |
| Denture use | | | |
| Non-user (54) | 37 (68.5%) | 17 (31.5%) | 0.88 |
| User (16) | 10 (62.5%) | 6 (37.5%) | |
| Oral wetness | | | |
| Low (47) | 30 (63.8%) | 17 (36.2%) | 0.43 |
| High (23) | 17 (73.9%) | 6 (26.1%) | |
| Tongue coating | | | |
| Negative (66) | 45 (68.2%) | 21 (31.8%) | 0.59 |
| Positive (4) | 2 (50.0%) | 2 (50.0%) | |
| Probing depth | | | |
| <4 mm (23) | 17 (73.9%) | 6 (26.1%) | 0.43 |
| ≥4 mm (47) | 30 (63.8%) | 17 (36.2%) | |
| Bleeding on probing | | | |
| No (38) | 25 (65.8%) | 13 (34.2%) | 1.00 |
| Yes (32) | 22 (68.8%) | 10 (31.2%) | |

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Availability of data and materials

All data generated or analyzed in this study are included in this article.

Authors' contributions

CYS designed the study and performed experiments, analyzed and interpreted the data and was involved in manuscript writing. HS performed experiments, analyzed and interpreted the data and aided to write the paper. RN performed experiments. KO analyzed and interpreted the data. MS designed the study, analyzed and interpreted the data and aided to write the paper.

Ethics approval and consent to participate

The study design was approved by the Ethics Committee of Hiroshima University and all participants signed an informed consent agreement prior to participation.

Patient consent for publication

All participants consented to the publication of relevant data following anonymization of personal information.

Competing interests

The authors declare that they have no competing interests.

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