A cross-sectional study on the periodontal status and prevalence of red complex periodontal pathogens in a Japanese population

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Abstract: This large-scale study cross-sectionally examined the periodontal status and prevalence of “red complex” bacteria (Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia) in Japanese adults. A total of 977 participants were enrolled in the study. Probing depth (PD), bleeding on probing (BOP), and bone crest level (BCL) were recorded, and the presence of red complex bacteria in the saliva was examined using polymerase chain reaction. The mean BCL value and the percentage of sites with a PD ≥4 mm or the presence of BOP were significantly higher in older participants. The detection rates of P. gingivalis, T. denticola, and T. forsythia were 46.3%, 76.4%, and 61.1%, respectively. The P. gingivalis detection rate significantly increased with age, while those of T. denticola and T. forsythia were comparably high for all age groups. A close correlation between P. gingivalis and the percentage of sites with PD ≥4 mm was indicated by nonlinear canonical correlation analysis. Current smokers exhibited a more advanced disease condition and a significantly higher P. gingivalis detection rate than non-smokers. In conclusion, periodontal condition worsens with age, and P. gingivalis appears to be the red complex bacterium most closely associated with periodontitis.

Keywords: periodontal diseases; epidemiology; saliva; Porphyromonas gingivalis.

Introduction
Periodontitis is a major oral disease that can lead to tooth loss, and bacterial plaque is the principal etiological factor in most forms of periodontitis. Many clinical and in vitro studies have demonstrated that several species of gram-negative anaerobic bacteria are associated with the onset and progression of periodontal diseases. In particular, “red complex” bacteria (Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia) frequently occur at the same site and are associated with
the severity of periodontal disease (1). Porphyromonas gingivalis is known to have many virulence factors such as gingipain, vesicles, and fimbriae (2) and is considered a prime pathogenic bacterium in periodontitis. Although the pathogenicities of T. forsythia and T. denticola are still under research, knowledge of their association with periodontal disease has greatly improved in recent years (3,4).

Clinically, microbiological testing using molecular-based techniques has been performed to determine the presence/absence of these pathogenic bacteria as therapeutic targets. However, red complex bacteria have been found even in the absence of disease (5,6). Umeda et al. (7) reported that many children with a healthy periodontium tested positive for T. forsythia. The role of these three periodontopathic bacteria in the progression of periodontal diseases is still unclear and the host impact seems to differ depending on the bacterial species involved. Periodontitis is a polymicrobial infectious disease; therefore, an understanding of the core bacteria in periodontopathic populations may be helpful for planning preventive/therapeutic strategies for periodontal disease.

Previous epidemiological studies on the prevalence of periodontopathic bacteria suggest that the distribution varies among geographically or ethnically distinct populations (8,9). While large-scale studies examining the prevalence of periodontopathic bacteria have been performed in Western countries (10,11), there is limited information regarding Japanese populations; only case-control studies with relatively small sample sizes are available.

The main aim of this study was to determine the prevalence of red complex bacteria in a Japanese population. The periodontal status and detection rate of these bacteria in patients at a private practice in Tsukuba city, Japan, as well as their association with age, were determined using cross-sectional data.

Materials and Methods

Study population

Periodontal examination data from initial patient visits were obtained from clinical records at a private dental clinic in Tsukuba city (Tsukuba Healthcare Dental Clinic, Ibaraki, Japan) between March 2003 and March 2006. These included the clinical parameters, radiographic analysis results, and confirmation of the presence/absence of P. gingivalis, T. denticola, and T. forsythia. Tsukuba is a suburban city famous for science and culture with an estimated 200,000 residents, more than 300 research facilities, and one national university. The clinic practices general dentistry and is also proactive in preventive care.

Exclusion criteria were as follows: age under 17 years, intake of antibiotics within the last 3 months, history of periodontal treatment in the past 6 months, and lack of clinical data from periodontal examinations. Clinical data recorded as part of the dental treatments were used. Study-related information was posted in the clinic and we obtained comprehensive informed consent from the patients after confirming they had read the notice. The study protocol was approved by the Ethics Committee of the Faculty of Dentistry, Tokyo Medical and Dental University (#635), and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013, and the ethical guidelines for epidemiology research of 2002, as revised in 2008 (Ministry of Health, Labor and Welfare, Japan).

Clinical evaluation

Probing depth (PD) and bleeding on probing (BOP) were recorded at six locations (buccal-mesial, mid-buccal, buccal-distal, lingual-mesial, mid-lingual, and lingual-distal) on all residual teeth using a calibrated standard probe (15 UNC Color-Coded Probe; Hu-Friedy Mfg. Co., Chicago, IL, USA). Measurements of PD and BOP were performed by three dental hygienists and then calibrated by a well-trained periodontist (O.C.). Full mouth introral radiographs were taken and standardized with the parallel technique using a specially designed film holder (CID-III positioning indicator; Hanshin Technical Laboratory Ltd., Nishinomiya, Japan). Periapical radiographs were scanned into the computer and magnified approximately five times on a monitor. Vertical linear distances from the cement-enamel junction to the point of the bone-root contact (bone crest level; BCL) were measured at the mesial and distal sites in all teeth on the radiographs and were averaged for each patient. The BCL measurements were taken by eleven examiners (C.O., Y.T., A.A., Y.S., K.M., N.A., Y.I., M.G., M.U., N.K., T.S.) using the measuring ruler as reported by Schei et al. (12). Smoking status was confirmed by questioning the participants and recorded as current smoker (CS; ≥1 cigarette per day) or non-smoker (NS; former smoker or never smoked).

Sample collection and processing

Stimulated whole saliva was obtained after chewing paraffin gum for 5 min. An aliquot of saliva (500 µL) was washed with 200 µL of phosphate buffered saline, mixed for 1 min, and then centrifuged (6,000 ×g, 5 min). Bacterial DNA was extracted from the pellet using a commercial kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s
instructions, and was stored at −30°C until analysis.

**Bacterial identification**

Real-time polymerase chain reaction (RT-PCR) was used to detect the three periodontopathic bacteria in the saliva. Specific primers for each bacterium (P. gingivalis, T. denticola, and T. forsythia) were used according to the methods described by Ashimoto et al. (13). Real-time PCR was performed with the LightCycler system (Roche Molecular Biochemicals) and the double-stranded DNA binding dye SYBR Green I (Roche Molecular Biochemicals) (14).

Briefly, amplification was performed in a 20-µL final volume containing 2 µL of template DNA, 2 µL of LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals), 1 µM of each primer, and 4 mM MgCl₂. The protocol for P. gingivalis included an initial denaturation step at 95°C for 10 min, followed by 45 cycles in four steps, comprising heating at 20°C/s to 95°C with a 0-s hold, cooling at 20°C/s to 6°C with a 5-s hold, heating at 20°C/s to 72°C with a 16-s hold, and heating at 20°C/s to 84°C with a 1-s hold. The protocol for T. denticola included an initial denaturation step at 95°C for 10 min, followed by 45 cycles in four steps, comprising heating at 20°C/s to 95°C with a 0-s hold, cooling at 20°C/s to 59°C with a 5-s hold, heating at 20°C/s to 72°C with a 13-s hold, and heating at 20°C/s to 85°C with a 1-s hold. The protocol for T. forsythia included an initial denaturation step at 95°C for 10 min, followed by 45 cycles in four steps, comprising heating at 20°C/s to 95°C with a 0-s hold, cooling at 20°C/s to 62°C with a 5-s hold, heating at 20°C/s to 72°C with a 26-s hold, and heating at 20°C/s to 83°C with a 1-s hold.

Fluorescent products were detected in the last step of each cycle. After amplification, a melting curve was obtained by keeping the temperature at 20°C/s to 95°C, cooling at 20°C/s to 70°C, and slowly heating at 0.2°C/s to 95°C with fluorescence collection at 0.2°C intervals. Melting peaks were used to determine the specificity of the PCR. Data was analyzed using the LightCycler analysis software (Roche Molecular Biochemicals). A subject was designated as positive for a specific bacterium when the amount exceeded 1,000/mL in a sample.

**Statistical analysis**

Numerical data are presented herein as means ± standard deviations for the four age groups (≤29, 30-39, 40-49, and ≥50 years). The chi-squared test and one-way analysis of variance with Tukey-Kramer HSD test for multiple comparisons were used to assess the periodontal status of the participants. The chi-squared test, Fisher’s exact test (extended), and Cochran-Armitage trend test were carried out to assess the effects of smoking on clinical parameters and periodontopathic bacteria prevalence. Nonlinear canonical correlation analysis was used to assess the relationship between periodontal condition and candidate risk factors for periodontitis. The first variable set consisted of age, smoking status, and the presence or absence of P. gingivalis, T. denticola, and T. forsythia. The second variable set consisted of the percentage of sites with a PD ≥4 mm, the percentage of sites with BOP, and the mean BCL. The age, percentage of sites with a PD ≥4 mm, the percentage of sites with BOP, and mean BCL were separated into four categories and treated as ordinals. Statistical analyses other than Fisher’s exact test and nonlinear canonical correlation analysis were performed using JMP 9.0.3 (SAS institute Inc. Cary, NC, USA). Fisher’s exact test was carried out using a computer program from the website http://aoki2.si.gunma-u.ac.jp/exact/exact.html, which was based on the method reported by Mehta et al. (15). The nonlinear canonical correlation analysis was performed using SPSS Statistics V22.0 (IBM Corp. Armonk, NY, USA). P values less than 0.05 were considered significant.

**Results**

**Subject characteristics**

A total of 1,124 of 4,722 outpatients agreed to pay for a test to check the presence of periodontopathic bacteria. Of these, 147 patients were excluded from the study based on the exclusion criteria. Therefore, the data for 977 participants (324 men and 653 women) were ultimately included in the analyses. Table 1 shows the clinical characteristics of the four age groups (≤29, 30-39, 40-49, and ≥50 years). The mean age of the participants was 38.0 ± 10.3 years (range: 18-74 years). The mean number of residual teeth was 26.7 ± 2.3, which decreased slightly with age. The mean BCL and the percentage of sites with a PD ≥4 mm or the presence of BOP significantly increased with age. The prevalence of CS was 16.6%, and smoking rates were similar among all age groups. The total detection rates of P. gingivalis, T. denticola, and T. forsythia were 46.3%, 76.4%, and 61.1%, respectively. The detection rate of P. gingivalis significantly increased with age from 32.5% in the youngest age group to 65.5% in the oldest. Treponema denticola and T. forsythia were detected at elevated levels even in the ≤29 age group (75.3% and 63.9%, respectively) and showed no significant difference with age (range: 73.6-82.1% and 57.2-63.9%, respectively).
Evaluation of clinical parameters by age group and smoking habit

Clinical parameters in CS and NS were evaluated for each age group (Figs. 1-3). The mean BCL per subject and the percentage of sites with a PD ≥4 mm or the presence of BOP were significantly different among the age groups in both NS and CS. In general, these clinical parameters tended to show elevated values at older ages (Figs. 1a, 2a, 3a). An increase in BCL in CS in the 40-49 and ≥50 age groups was noticeable (Fig. 3a). The participants were further classified into four groups based on the prevalence of PD sites ≥4 mm (<5%, ≥5 and <25%, ≥25 and <50%, and ≥50%), the prevalence of BOP (<5%, ≥5 and <25%, ≥25 and <50%, and ≥50%), and the severity of mean BCL (<1.5 mm, ≥1.5 and <2.0 mm, ≥2.0 and <3.0 mm, and ≥3.0 mm) (Figs. 1b, 2b, 3b). In CS, the ratio for the number of participants with more than 50% deep pockets increased with age, and the ratios were 2.8, 3.0, and 5.3 times higher in the 30-39, 40-49, and ≥50 age groups, respectively, compared with those in NS (Fig. 1b). Smoking also influenced the severity of BCL. Although the ratio of participants with BCL ≥3.0 mm clearly increased with age in both NS and CS groups, it increased more rapidly in CS (from 2.4% to 63.2%)
compared with NS (from 2.0% to 39.7%), and was 3.0, 2.9, and 1.6 times higher in CS compared with NS in the 30-39, 40-49, and ≥50 age groups, respectively (Fig. 3b).

Prevalence of periodontopathic bacteria in saliva by age group and smoking habit

The detection rate of *P. gingivalis* was significantly higher in CS than NS (Fig. 4a). The detection rate of *P. gingivalis* increased with age in both NS and CS, although a significant difference was observed only in the NS group (Fig. 4b). Although the detection rates of *P. gingivalis* in the CS were higher than those in the NS in the ≤29, 30-39, and 40-49 age groups, a statistically significant difference was only observed in the 40-49 age group (Fig. 4c). Detection rates of *T. denticola* and *T. forsythia* were always high regardless of age in both CS and NS, and there was no significant difference between CS and NS in each age group (Fig. 4d, e).

The prevalence of *P. gingivalis*, *T. denticola*, and *T. forsythia*, and the combination of all three in NS and CS is shown in Fig. 5. The rate of no detection for all three bacteria in the ≤29, 30-39, 40-49, and ≥50 age groups, respectively. All CS aged ≥50 years were carriers of at
Fig. 4  a) Detection rate of bacteria in current smokers (CS) and non-smokers (NS). b) Detection rate of bacteria in the four age groups in NS and CS, respectively. c) Detection rate of Porphyromonas gingivalis in CS and NS. d) Detection rate of Tannerella forsythia in CS and NS. e) Detection rate of Treponema denticola in CS and NS. Pg; P. gingivalis, Td; T. denticola, Tf; T. forsythia.

Fig. 5  Detection rate of co-occurrence between red complex bacteria. Pg; P. gingivalis, Td; T. denticola, Tf; T. forsythia.
least one of the tested bacteria. When the prevalence was analyzed in terms of only solo species detected from the target bacteria, *T. denticola* was most frequently detected (15.0–20.9%), followed by *T. forsythia* (2.1–9.3%) and then *P. gingivalis* (0.5–3.0%). When two species were detected from the target bacteria, co-occurrences of *T. denticola* and *T. forsythia* (13.8–33.0%), and *P. gingivalis* and *T. denticola* (9.3–23.5%) were frequently observed, while the occurrence of *P. gingivalis* with *T. forsythia* was observed in relatively few cases (1.0–3.6%). The detection rates of all three species were generally higher in older age groups (18.0, 24.6, 32.3, and 39.3% in the ≤29, 30-39, 40-49, and ≥50 age groups, respectively). In the 40-49 and ≥50 age groups, co-occurrence of the three species was the primary pattern of bacterial detection. In the ≤29, 30-39, and 40-49 age groups, co-occurrence of the three red complex bacteria was observed more frequently in CS compared with NS.

Relationship between periodontal condition and candidate risk factors of periodontitis

The relationship between periodontal condition and candidate risk factors for periodontitis was evaluated using nonlinear canonical correlation analysis (Fig. 6). Compared to *T. denticola* and *T. forsythia*, the percentage of sites with a PD ≥4 mm was closely correlated with the presence of *P. gingivalis*. High correlation was also observed between age and the severity of mean BCL.

Discussion

This large-scale study was performed to examine the periodontal status and prevalence of red complex bacteria in an urban Japanese adult group. The smoking rate in the study population (16.6%) was similar with previously reported data from Japan, North America, and Europe (Health at a glance 2015: OECD indicators, 68-69, 2015). Although participants were selected without regard to periodontal condition, the percentage of sites with deep periodontal pockets and mean bone loss clearly increased with age. These findings are in accordance with previous epidemiological studies that confirmed an increase in the severity of periodontal parameters with age (16,17). It should be noted that the proportion of participants with severe periodontal destruction did not increase evenly with age. For example, the number of participants with advanced periodontal destruction (more than 50% PD sites ≥4 mm) was limited in ≤49-year-olds, while the prevalence increased in participants aged ≥50 years. A similar trend was also observed for BOP detection.

Saliva, as a sample for detecting periodontopathic bacteria, is easy to collect, and its bacterial component shows a high correlation with a pooled subgingival plaque sample (18,19). Therefore, we considered saliva suitable for examining bacterial prevalence accurately and employed it for bacterial detection in the present study. Unexpectedly, the results revealed that only 4.0% of the 977 participants were free of all red complex bacteria, although a considerable number of participants with healthy periodontium or gingivitis were included in the study. Of note, *T. denticola* and *T. forsythia* were found in approximately two-thirds of the participants irrespective of age, while the carriage rate of *P. gingivalis* was much lower in younger participants and significantly increased with age.

Only a few studies have reported the prevalence of periodontopathic bacteria at the general population level. Könönen et al. (20) examined salivary carriage of periodontopathic bacteria in 1,294 Finnish adults. The detection rates of *P. gingivalis*, *T. denticola*, and *T. forsythia* were lower (35.4%, 38.2%, and 56.9%, respectively) compared with our results (45.9%, 76.4%, and 62.2%, respectively), while a relatively similar trend was observed regarding the relationship between the detection rates of bacteria and age. There was a considerable increase in the carriage of *P. gingivalis* with age, but not with *T. denticola* and *T. forsythia*. In addition, a study of 299 outpatients at a Japanese university hospital reported a higher detection of *P. gingivalis* in subgingival plaque in older age groups (21).

Large-scale studies examining the prevalence of
periodontopathic bacteria have been performed in Asian countries. In a study of Sri Lankan tea workers, a total of 536 plaque-sampling sites in 268 subjects were examined to identify major periodontopathic bacteria (22). That study found a slightly lower prevalence of P. gingivalis (39.9%), even though 75.5% of plaque-sampling sites showed moderate or advanced periodontitis. The prevalence of P. gingivalis in an Indonesian population (23) was higher (67%) than that in the present study. In addition, high carrier rates of P. gingivalis (100%), T. denticola (97.9%), and T. forsythia (98.6%) were observed in Chinese adults (24). Several studies have suggested that the prevalence of periodontopathic bacteria varies in different geographic locations and/or ethnic groups (25-27). Although these previous studies used different types of samples, sampling methods, and bacterial detection techniques, it seems reasonable to conclude that ethnic and/or geographical differences can affect the prevalence of periodontopathic bacteria among the population. Many cross-sectional studies have shown that the detection frequencies of P. gingivalis, T. denticola, and T. forsythia in periodontitis patients are significantly higher than those in healthy subjects in various Asian countries (28). We have also reported the significantly higher prevalence of these three bacteria in Japanese chronic and aggressive periodontitis patients (29). Overall, the subgingival prevalence of P. gingivalis and T. forsythia ranges from 60 to 100%, and that of T. denticola ranges from 70% to almost 100% among middle-aged chronic periodontitis patients (30). These bacteria were confirmed to be established pathogens for periodontitis in Asian populations.

Cortelli et al. (31) showed that T. denticola and T. forsythia occurred in the subgingival sulcus of children and in the tongue and/or cheek mucosa of edentulous newborns, whereas P. gingivalis was not detected at any sites. Treponema denticola and T. forsythia are known to evade the host immune system (32,33) and may be harbored more easily than P. gingivalis in the oral cavity. A coaggregate reaction of T. denticola and T. forsythia has been reported (34), leading to the colocalization of these bacteria. Co-occurrence of T. denticola and T. forsythia was frequently observed in all age categories in the present study. Treponema denticola and T. forsythia can interact with each other and promote adhesion and colonization of other bacteria including P. gingivalis. In particular, not only has an aggregation of P. gingivalis and T. denticola been reported (35), their symbiotic and synergistic associations, such as in nutrient utilization or growth promotion, have also been demonstrated (36-38). Therefore, it can be inferred that P. gingivalis colonization generally occurs following the localization of T. denticola and T. forsythia in a biofilm, and the establishment of the red complex can manifest their pathogenicity.

Hajishengallis et al. (39) showed that a low-abundance of P. gingivalis in mice can trigger a change in the amount and composition of microbiota via modulation of the complement function and lead to subsequent inflammatory periodontal bone loss. In addition, Endo et al. (40) performed a comparative genome analysis of the red complex species and revealed that deficiencies of the butyric acid metabolism pathway genes were mutually compensated when the genes of all three species were taken into account. Butyric acid is known to be strongly implicated in inflammatory responses and/or apoptosis of periodontal tissues (41,42). Therefore, although each of the three species have their own virulence factors, synergistic activation of their pathogenicity is required for the progression of periodontitis formed as a polymicrobial disease. In the present study, the severity of the periodontal condition increased with age, alongside an increasing frequency of red complex species. In particular, there was a positive correlation between the prevalence of P. gingivalis and increasing age in the present study. Further, nonlinear canonical correlation analysis revealed a strong correlation between the presence of P. gingivalis and the severity of the PD. This bacterium may play an important role in the activation of pathogenicity.

Smoking is known to increase the susceptibility of periodontitis, and current smokers generally have more severe periodontal disease than non-smokers (43). In the present study, deeper pockets and greater bone loss were confirmed in CS compared with NS in the Japanese population. Moreover, the number of CS with periodontal destruction was higher in younger age groups compared with NS; these results are in accordance with previous reports (44,45).

Tobacco products depress the metabolic ability of periodontal tissue and also induce an impaired immune response in the host (46). Although smoking would also influence the oral microbial community (47,48), there are conflicting findings on the prevalence of periodontopathic bacteria in smokers. Several studies have claimed an increased risk of infection with periodontopathic bacteria in smokers (49,50). Zambon et al. collected subgingival plaque from 1,426 participants (798 current/former smokers included) and showed a high prevalence of P. gingivalis, T. forsythia, and Aggregatibacter actinomycetemcomitans in smokers (51). An in vitro study by Bagaitkar et al. (52) showed that cigarette smoke extract accelerated the formation of P. gingivalis biofilms. In contrast, several researchers reported no difference
in subgingival microbiota between smokers and non-smokers (53, 54). Moreover, Mager et al. (55) failed to find a statistical difference between smokers and non-smokers in the proportion of periodontopathic bacteria in the saliva. However, in the present study, the carriage rates of \textit{P. gingivalis} in CS were significantly higher than those in NS. Generally, cumulative smoking exposure and duration of smoking cessation seem to be associated with periodontitis (56). However, since the smoking history was not recorded, such associations were not clarified in the present study. Furthermore, variations in sampling and bacterial identification methods and/or the number of participants may potentially mask the effects of smoking on the microbiota, leading to conflicting reports.

Reportedly, the salivary levels of some periodontopathic bacteria including \textit{P. gingivalis} and \textit{T. forsythia} may reflect the periodontal status of periodontitis patients (57). In the present study, bacteria were identified using real-time PCR. The quantitative evaluation of bacteria and its relationship with clinical periodontal condition will be presented in a future publication.

The limitation of this study was that only three established periodontopathic bacteria were identified. Over 700 species have been identified in the oral cavities of different individuals, and at least 30 to 100 species may be recovered from a single site (58). These bacteria comprise a community and reside as a biofilm. Red complex bacteria are considered key species for periodontitis (40), and other groups, whose roles are not well established, may also be involved with the disease. We have attempted a comprehensive analysis of microbiota in polymicrobial diseases including periodontitis (59, 60), and will evaluate broadly based dysbiotic microbiota and the associated pathogenicity in a future study.

In summary, to the best of our knowledge, this is the first large-scale study to analyze the prevalence of red complex periodontal bacteria in Japanese adults. \textit{T. denticola} and \textit{T. forsythia} were highly detected in participants from a young age and the detection rate was constant. In contrast, the detection rate of \textit{P. gingivalis} was much lower in young participants compared with \textit{T. denticola} and \textit{T. forsythia} and increased significantly with age. \textit{P. gingivalis} had a significantly high detection frequency in CS than in NS. Co-occurrence of red complex bacteria was observed more frequently with advancing age and clinical periodontal parameters indicated disease progression with age. These findings therefore indicate that \textit{P. gingivalis} is the red complex bacterium most closely associated with the progression of periodontal disease, highlighting it as a potential etiologic agent.

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Conflict of interest
The authors have no conflict of interest to declare.

References


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