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Clinical evaluation of salivary periodontal pathogen levels by real-time polymerase chain reaction in patients before dental implant treatment

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Abstract

Objective: Periodontal pathogens in dental plaque are the main causative agents of periodontitis and peri-implantitis. Detection of the presence of such periodontal pathogens early would serve as a useful tool in the diagnosis and treatment of this disease. Therefore, the purpose of this study was to investigate whether the periodontal pathogen levels in saliva were correlated with the periodontal status of patients receiving implant treatment.

Materials and Methods: A total of 291 patients visiting Tokyo Dental College Chiba Hospital were divided into four groups: a no-periodontitis (np) group, a mild-periodontitis (mip) group, a moderate-periodontitis (mop) group, and a severe-periodontitis (sp) group. The levels of the following five periodontal pathogens in saliva were evaluated using real-time polymerase chain reaction: *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, and *Prevotella intermedia*.

Results: The levels of *P. gingivalis* and *T. forsythia* were significantly higher in mop group than in np group ($P < 0.05$). The levels of all periodontal pathogens tested except

A. actinomycetemcomitans were significantly higher in sp group than in np group ($P < 0.05$).

Conclusion: The detection levels of the periodontal pathogens targeted in saliva samples were correlated with the periodontal status. This suggests that using saliva to screen for periodontopathic bacteria offers an easier-to-use clinical tool than the paper point method in the diagnosis and treatment of periodontitis and peri-implantitis.

Periodontitis is a multibacterial infection that affects the periodontal tissues and eventually leads to loss of teeth. Periodontitis is a chronic inflammatory disease, and its bacterial etiology has been confirmed by numerous reports (Listgarten & Hellden 1978; Tanner et al. 1979; Moore 1987; Paster et al. 2001). It appears essential, however, to screen for periodontal pathogens that could directly destroy the periodontal tissue. Currently, microbial tests are used to identify pathogenic bacteria, identify patients with high risk of periodontal disease, motivate and educate patients, and provide an indication in the choice of antibiotics. However, whether bacterial tests should be conducted in the treatment of periodontal disease remains open to debate. Although periodontitis is a bacterial infection, there are no established microbiological tests that can be easily applied clinically, and

periodontitis is often treated without identifying the responsible bacteria. In recent years, various chair-side microbial tests have been developed aiming at the detection of periodontal disease, including enzyme assay, DNA probe method, and polymerase chain reaction. None of these tests, however, is widely employed due to issues related to cost and convenience (Socransky & Haffajee 1994; Ashimoto et al. 1996; Takahashi et al. 1998; Komiya et al. 2000). At present, it is possible to quantify periodontal pathogens by real-time polymerase chain reaction (RT-PCR), and clinical bacterial tests are being performed much more frequently (Boutaga et al. 2003, 2006, 2007; Kuboniwa et al. 2004; Hyvarinen et al. 2009). Sampling of microbes is usually performed by inserting paper points in periodontal pockets or obtaining samples of saliva, with the latter method being more

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convenient and suitable for screening of bacteria in the entire oral cavity. Recently, periodontal pathogens have been detected at high frequency in the foci of peri-implantitis, implicating them in the development of this disease (Mombelli & Lang 1998; van Winkelhoff et al. 2000; Quirynen et al. 2002). Periodontal bacteriological examination is expected to be effective as a tool to determine the risk of peri-implantitis.

Therefore, the aim of this study was to determine whether periodontal pathogens in saliva showed a correlation with periodontal status. A periodontal pathogen test that could easily be applied in a clinical setting would serve as an effective tool in identifying the cause of and risk of periodontitis and peri-implantitis.

Material and methods

Patients and clinical evaluation

A total of 291 adult Japanese patients requesting dental implant treatment visiting the Department of Maxillofacial and Oral Implantology at Tokyo Dental College Chiba Hospital between December 2007 and May 2010 were evaluated. The health status and background of each patient were recorded, including age, sex, and probing pocket depth (195 women and 96 men with a mean age ± SD of 53.3 ± 11.3 years). The patients were classified into the following four groups based on probing pocket depth: a no-periodontitis (np) group (all remaining teeth with ≤3 mm probing pocket depth); a mild-periodontitis (mip) group (less than four teeth with ≥4 mm probing pocket depth); a moderate-periodontitis (mop) group (at least four teeth with ≥4 mm probing pocket depth); and a severe-periodontitis (sp) group (at least 30% of the remaining teeth with ≥6 mm probing pocket depth) (Table 1). Pocket probing was carried out with a Williams probe and recorded at six sites (mesiofacial, midfacial, distofacial, mesiolingual, midlingual, and distolingual) in each tooth. A modified version of the classification system proposed by the American Academy of Periodontology was used in this study (Armitage 1999). All patients were systemically healthy, had normal salivary flow, and had received no periodontal treatment or antibiotics for at least 6 months prior to participating in this study.

Informed consent was obtained from each patient. The present study was conducted with approval from the Ethics Review Board of Tokyo Dental College.

Table 1. Classification of experimental groups

Classification	Number (Male/Female)	Age (mean ± SD)	Evaluation basis of periodontal status
No-periodontitis (np) group	53 (22/31)	42.6 ± 12.0	Probing Pocket Depth (PPD) among all residual teeth <3 mm
Mild-periodontitis (mip) group	103 (49/54)	51.1 ± 9.6	Less than four teeth showing PPD 4–6 mm among all residual teeth
Moderate-periodontitis (mop) group	113 (56/57)	55.3 ± 13.4	More than four teeth showing PPD 4–6 mm among all residual teeth
Severe-periodontitis (sp) group	22 (10/12)	54.3 ± 10.3	More than 9 teeth > PPD 6 mm or Rate of teeth > PPD 6 mm is over 30% among all residual teeth

Subjects were divided into four groups based on severity of periodontal disease. Indicate the total number of people, the gender split in each group, mean age.



Fig. 1. Sampling of saliva. Patients were required to chew gum for 5 min, after which 3–5 ml saliva was obtained.

① DNA extraction

- DNA was extracted from saliva obtained from each subject.



② TaqMan® probe method

Periodontal pathogens	Primer/probe	Sequence (5' – 3')
<i>Aggregatibacter actinomycetemcomitans</i>	Primer F	GAACCTTACCTACTCTTGAC
	Primer R	TGCAGCACCTGTCTCAAAGC
	Probe	FAM-AGAACTCAGAGATGGGTTTGTGCCTTAGGG-TAMRA
<i>Tannerella forsythia</i>	Primer F	GGGTGAGTAACGCGTATGTAACCT
	Primer R	ACCCATCCGCAACCAATAAA
	Probe	FAM-CCGCGAACAGAGGGATAACCCGG-TAMRA
<i>Prevotella intermedia</i>	Primer F	CGGCTGTTAAGCGTGTGT
	Primer R	CACCATGAATCCGCATACG
	Probe	FAM-TGGCGAATTGAGTGCACGC-TAMRA
<i>Treponema denticola</i>	Primer F	CGAATGTGCTCATTACATAAAGGT
	Primer R	GATACCCATCGTTGCCTTGGT
	Probe	FAM-ATGGGCCCGGCTCCATTAGC-TAMRA
<i>Porphyromonas gingivalis</i>	Primer F	GGCTCAACGTTACGCC
	Primer R	CACGAATCCGCCTGC
	Probe	FAM-CACTGAACCTAAGCCCGGCAGTTTCAA-TAMRA
Universal	Primer F	TCCTACGGGAGGACGAGT
	Primer R	GGACTACCAGGGTATCTAATCTGT
	Probe	FAM-OGTATTACCGGCTGCTGGCAC-TAMRA

- 20 µl of each periodontal pathogen reaction mix + 5 µl of sample + 5 µl of cDNA (or negative control)

- Thermal cycler conditions

50°C (2 min) × 1, 95°C (10 min) × 1, 95°C (15 s) × 45, 60°C (1 min) × 45



③ Data analysis

Fig. 2. TaqMan® RT-PCR assay was employed to determine count of infectious agents targeted using primers and techniques indicated.

Saliva sample

Each patient was required to chew gum for at least 5 min prior to collection of saliva samples. A total of 3–5 ml stimulated whole saliva was then collected in an empty, sterile, 50-ml

test tube (Fig. 1). The patients were instructed not to brush their teeth or eat for up to 1 h prior to sampling, which was performed between 9:30 and 11:30 AM. The whole saliva was stored at –20°C until processing.

Real-time polymerase chain reaction

One hundred microliter diluted saliva samples were used for automated DNA extraction and purification with the Puregene Core kit A (QIAGEN, Tokyo, Japan).

The RT-PCR analysis was performed by the TaqMan® probe method (MIROKU MEDICAL LABORATORY Inc., Nagano, Japan) and the following five periodontopathic bacteria quantified: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Prevotella intermedia*. The primer and probe sets for the five periodontal pathogens targeted and experimental conditions are shown in Fig. 2 (Shelburne et al. 2000; Martin et al. 2002;

Kuboniwa et al. 2004; Boutaga et al. 2005). The bacterial copy count of each pathogen and proportion of each bacterium to total copy count were determined.

Statistical analysis

The statistical analysis was performed using the SPSS 15.0 statistical package for Windows (SPSS Inc., Chicago, IL, USA). The bacterial copy count of each periodontal pathogen and proportion of each bacterium to the total copy count were compared using the Kruskal–Wallis tests. Tukey's HSD test was used to assess the difference in each group as post hoc tests. *P*-values of <0.05 were considered significant.

Results

Figure 3 show the average bacterial counts of the five periodontal pathogens targeted. No significant difference was detected in the salivary *A. actinomycetemcomitans* level among the four groups. Salivary levels of *P. gingivalis* and *T. forsythia* were significantly higher in both the mop and sp groups (*P* < 0.05) than in the np group. Salivary levels of *T. denticola* and *P. intermedia* were significantly higher in the sp group than in the np group (*P* < 0.05). Figure 4 show the average proportion of each bacterium to total salivary count in each patient. The average proportion for *P. gingivalis* in the mop and sp groups was markedly high

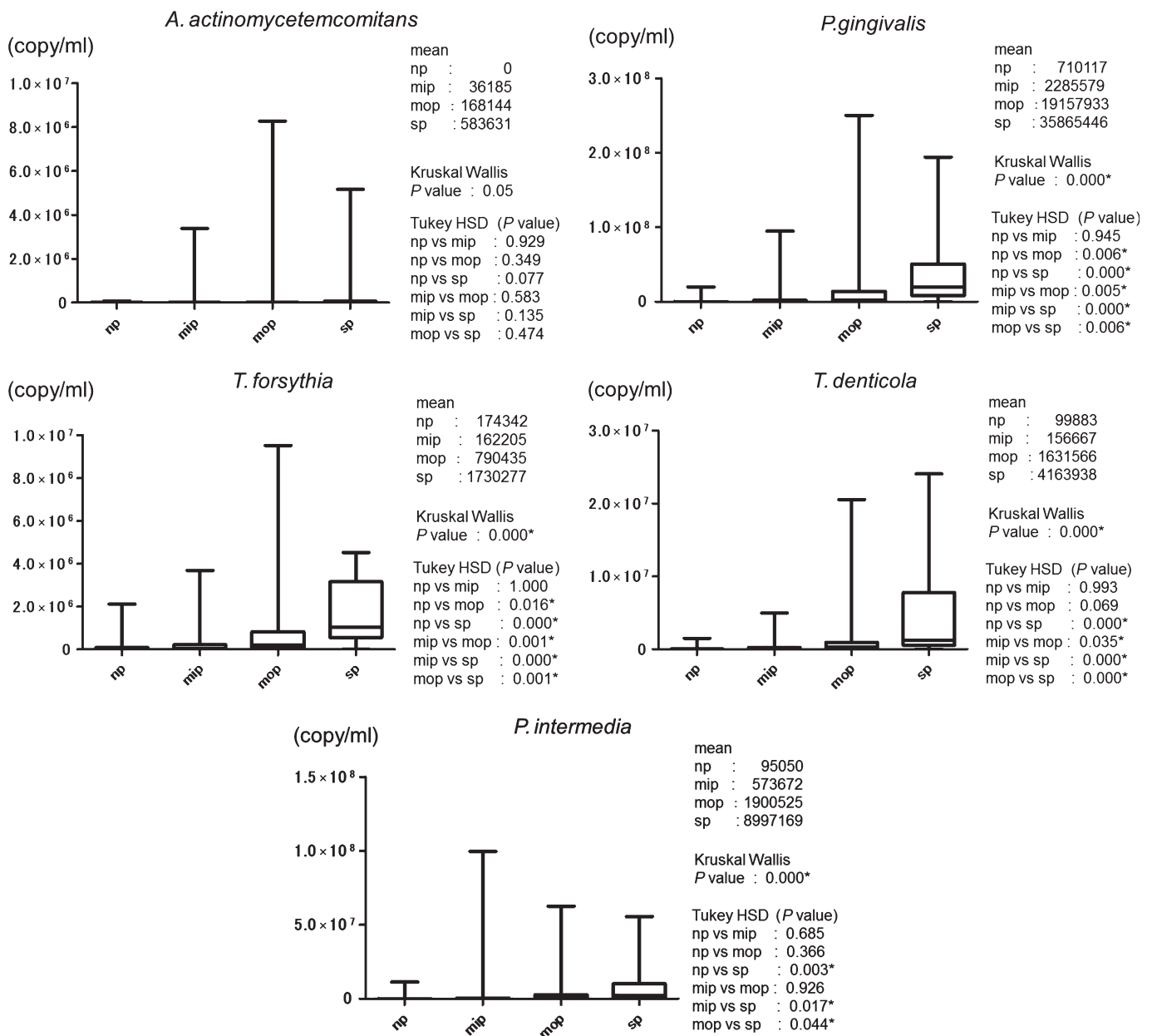


Fig. 3. Box plot of copy counts number (copy/ml) of five periodontal pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Prevotella intermedia*) in saliva sample.

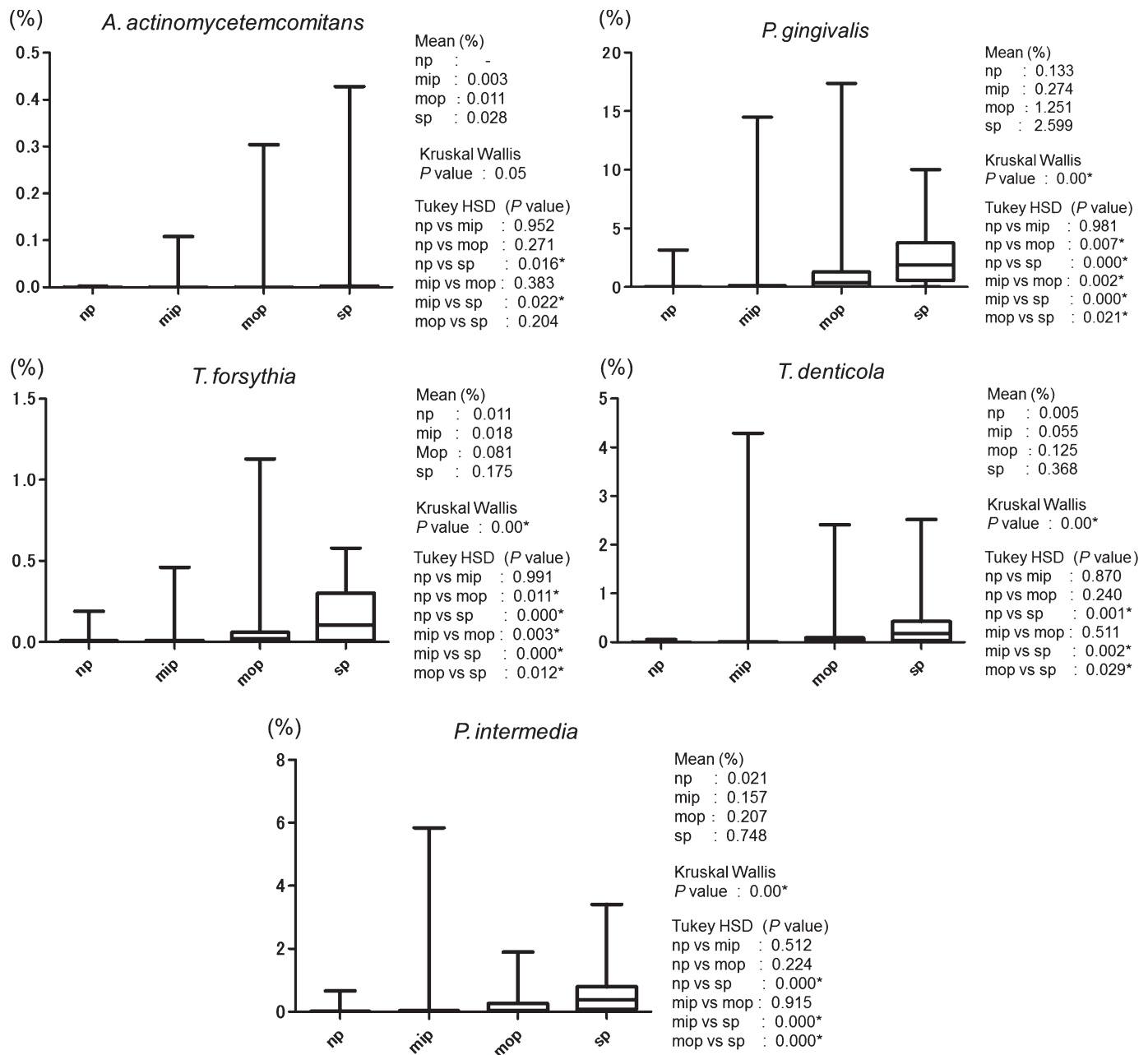


Fig. 4. Box plot of ratio (%) of each bacterium to total copy count of five periodontal pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Prevotella intermedia*) in saliva sample.

at 1.25% and 2.60%, respectively. In the mop group, the average proportions were 0.08% for *T. forsythia*, 0.13% for *T. denticola*, and 0.21% for *P. intermedia*. In terms of significant differences, these results showed a similar trend to those of previously reported bacterial copy counts of periodontal pathogens ($P < 0.05$).

Discussion

In the present study, patients with periodontitis were classified based on severity of disease

(a no-periodontitis group, a mild-periodontitis group, a moderate-periodontitis group, and a severe-periodontitis group), and five periodontal pathogens (*A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *T. denticola*, and *P. intermedia*) from saliva samples were quantified and compared. *Porphyromonas gingivalis*, *T. forsythia*, and *T. denticola* are referred to as the red complex bacteria, and along with *A. actinomycetemcomitans*, the element of exogenous infection is believed to be strong (Socransky & Haffajee 2002). As these four bacteria and *P. intermedia* are considered to be the main agents of periodontitis, these

were the ones targeted in the present study. Both the number and level of *P. gingivalis*, *T. forsythia*, *T. denticola*, and *P. intermedia* from the saliva samples were significantly higher in the severe-periodontitis group than in the no-periodontitis group. The number and proportions of *P. gingivalis* were significantly different between the no-periodontitis and moderate-periodontitis groups. Periodontal pathogens are found in increased numbers in severe periodontal disease, and all of the periodontal pathogens targeted in the present study were detected in elevated numbers in the severe-periodontitis group. Large numbers

of periodontal pathogens were detected in patients with deep periodontal pockets (Listgarten & Hellden 1978; Edwardsson et al. 1999). Our findings are mostly in agreement with the results of previous papers (Griffen et al. 1998; van Winkelhoff et al. 2002; Sanz et al. 2004). *Aggregatibacter actinomycetemcomitans* is involved in the pathogenesis of aggressive periodontitis and has been found in younger patients aged between 10 and 30 years (Armitage 1999). The relatively high age of the subjects (mean age, 53.3 ± 9.0 years) may explain the extremely low level of detection of this bacterium in the present study. The bacterial levels observed in the present study were higher than the risk assessment criteria reported in previous studies. Rams et al. (1996) have reported that the possibility of recurrence of periodontitis increased in patients with a threshold proportion of $> \text{ or } = 0.01\%$ for *A. actinomycetemcomitans*, $> \text{ or } = 0.1\%$ for *P. gingivalis*, and $> \text{ or } = 2.5\%$ for *P. intermedia*. Moreover, Brown et al. (1994) noted that a ratio of $> \text{ or } = 2\%$ for *P. gingivalis* or *P. intermedia* under adult gingival margin was effective in predicting periodontal progression. The RT-PCR method employed in the present study quantifies bacteria by amplifying the DNA of the sample bacteria. This approach offers greater sensitivity than conventional bacterial tests such as culture and has been used a lot recently for the study of periodontal pathogens (Boutaga et al. 2003, 2006, 2007; Kuboniwa et al. 2004; Hyvarinen et al. 2009). The cutoff point for determining the risk of periodontal disease, however, remains to be established. The present results were little different from the risk assessment criteria employed in conventional culture tests, suggesting that it may be necessary to establish new risk assessment criteria.

The components of saliva are related to those found in blood. Therefore, clinically, saliva is useful in investigating and monitoring the metabolism. It aids not only in the diagnosis of dental caries or periodontitis, but

also in diseases affecting other areas of the body, and many studies have been published in this field since 1983 (Ericksson 1983; Rigas & Levine 1983; Vining et al. 1983). The National Institute of Health (NIH) of the United States has shown interest in research on "whether saliva can be used for the medical checkup of the whole body like blood" and earmarked a budget of 5 million dollars for this in 2002 (Wong 2006). All the indications are that saliva testing is destined to become a familiar clinical tool in the future (Ito et al. 2008). Collection of saliva samples is convenient, is painless, and can be performed in a short period of time. Umeda et al. (1998) reported that whole saliva was superior to pooled periodontal pocket samples in the detection of *P. gingivalis*, *P. intermedia*, *P. nigrescens*, and *T. denticola* in the oral cavity. Studies on periodontal pathogens using saliva are still few (Tamura et al. 2006; Saygun et al. 2011). However, the present results indicate that saliva testing would offer a useful tool in the diagnosis of periodontal disease. With tests in which paper points are placed in periodontal pockets, it may not be possible to identify isolated diseased sites or bacterial flora in the subgingival biofilm. A saliva test, however, reflects the microbial status of the entire oral cavity and may serve as a useful screening technique in the reduction of risk of oral diseases.

In patients requesting implant treatment in the present study, microbiological tests were carried out to screen for periodontal disease before implant treatment. The results of this study indicated that large numbers of periodontal pathogens were detected in more severe periodontitis. Ito et al. (2011) reported that most of the patients who requested dental implant treatment had been suffering from periodontal disease. It has been reported that patients with periodontal disease are more susceptible to peri-implantitis (Hardt et al. 2002; Schou et al. 2006). Moreover,

microbiological studies documented a correlation between failed implant therapy and periodontal pathogens (Mombelli & Lang 1998; Quirynen et al. 2002, 2006; Shibli et al. 2008). The onset mechanism for peri-implantitis is believed to be the same as that for periodontitis, as the same bacteria present in periodontal pockets are involved (Takanashi et al. 2004; Aoki et al. 2012). Furthermore, it has been reported that implant therapy in patients with untreated periodontitis increases the risk of peri-implantitis (Renvert & Persson 2009). Because periodontal pathogens are likely to be associated with peri-implantitis, it appears valid to test for periodontal disease-causing bacteria before implant therapy and ascertain the risk of peri-implantitis. Finding a correlation between specific oral bacteria and the onset of peri-implantitis would be of considerable clinical use.

The results of the present study confirmed a correlation between the bacterial count of each periodontal pathogen targeted or ratio of each bacterium to total count and periodontitis, suggesting that the present saliva test could be used to assess risk of not only this disease, but also implant failure and peri-implantitis. Further studies are needed, however, to clarify how specific levels of each bacterium correlate with the development of periodontitis and peri-implantitis.

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