Oral Microbiology Immunology 2005: 20: 216–220
Printed in Singapore. All rights reserved

Oral Microbiology and Immunology

Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients

Y. Sawamoto[1], N. Sugano[1,2], H. Tanaka[3], K. Ito[1,2]
[1]Department of Periodontology, Division of Advanced Dental Treatment, Dental Research Center, [2]Department of Bacteriology, [3]Nihon University School of Dentistry, Tokyo, Japan

Sawamoto Y, Sugano N, Tanaka H, Ito K. Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients.
Oral Microbiol Immunol 2005: 20: 216–220. © Blackwell Munksgaard, 2005.

We assessed the salivary levels of periodontopathic bacteria and 8-hydroxydeoxyguanosine (8-OHdG) in patients with periodontitis. The salivary levels of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* (formerly *Bacteroides forsythus*) were assessed using real-time polymerase chain reaction. The 8-OHdG levels were determined using an enzyme-linked immunosorbent assay. The salivary levels of 8-OHdG, *P. gingivalis*, and *T. forsythia* in the periodontitis patients were significantly higher than those in healthy subjects. By contrast, the *A. actinomycetemcomitans* level in healthy subjects was higher than that in periodontitis patients. 8-OHdG was significantly correlated with *P. gingivalis*. Statistically significant decreases in the levels of *P. gingivalis*, probing depth, bleeding on probing, and 8-OHdG were observed after initial periodontal treatment. These results suggest that the 8-OHdG levels in saliva reflect the load of periodontal pathogens. 8-OHdG could be a useful biomarker for assessing periodontal status accurately, and for evaluating the efficacy of periodontal treatment.

Key words: 8-OHdG; *Actinobacillus actinomycetemcomitans*; oxidative damage; *Porphyromonas gingivalis*; real-time polymerase chain reaction; saliva; *Tannerella forsythia*

Naoyuki Sugano, Department of Periodontology, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101–8310, Japan
Tel.: +81 3 3219 8087;
fax: +81 3 3219 8349;
e-mail: sugano-n@dent.nihon-u.ac.jp
Accepted for publication January 23, 2005

Oxidative stress is involved in many pathologic conditions, such as cancer, atherosclerosis, and inflammatory diseases (26, 35). Oxidative stress can attack nucleic acids in living cells. In the nucleus, 8-hydroxydeoxyguanosine (8-OHdG), an oxidized nucleoside of DNA, is frequently detected with DNA damage. With DNA repair, 8-OHdG is excreted in the body fluids (43). Several studies have indicated that the 8-OHdG levels in body fluids are a biomarker of oxidative stress (9). For example, elevated urinary 8-OHdG has been detected in patients with cancer, atherosclerosis, and diabetes. Previously, we found that the 8-OHdG levels in saliva from periodontitis patients were significantly higher than those of clinically healthy subjects (36). We also observed a significant decrease in salivary 8-OHdG after periodontal treatment. Oxidative stress during the destruction of periodontal tissues results from polymorphonuclear leukocyte infiltration as a key event in the host defense mechanisms against bacterial invasion (1, 4, 7, 42).

A preponderance of several oral bacterial species has been found in subjects with periodontitis. Of these, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* (*Bacteroides forsythus*) are the most convincing etiologic agents of periodontitis (34, 37, 39, 40, 47). *A. actinomycetemcomitans* and *P. gingivalis* lead to, and increase, the oxidative stress in periodontal tissue (21, 24, 25, 31, 38). However, no study has made a direct investigation of the correlation between the 8-OHdG levels and periodontopathic bacteria.

This investigation assessed the salivary levels of 8-OHdG, *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* in patients with periodontitis.

Material and methods

Patients and saliva samples

The study group consisted of 29 systemically healthy subjects with periodontitis, with a mean age of 55.0 years (range 36–68 years). The clinical criteria of zperiodontitis were judged from standard measurements of clinical pocket depths.

The periodontitis patients had at least two sites with probing depths greater than 4 mm. Twenty systemically healthy subjects with clinically healthy periodontium served as controls (mean age 35.2 years, range 25–65 years). The clinical examination recorded sites with bleeding on probing (mean 44.8%, range 22.2–100%) and probing depth (mean 3.49 mm, range 2.48–6.70 mm). Clinical records at the first appointment and after an initial periodontal treatment were examined in 17 periodontitis patients (8 women and 9 men, age range 36–68 years). The 2- to 4-month-long initial periodontal treatment consisted mainly of oral hygiene instruction and scaling and root planing. At clinical examination, paraffin wax-stimulated whole saliva was collected, and the samples were stored at − 80°C until analyzed.

Determination of salivary 8-OHdG using ELISA

Saliva samples were centrifuged at 10,000 × *g* for 10 min and the supernatant was used to determine the 8-OHdG levels with a competitive ELISA kit (8-OHdG Check, Highly Sensitive 8-OHdG Check, Japan Institute for the Control of Aging, Shizuoka, Japan). Levels ranged from 0.125 to 200 ng/ml.

Real-time polymerase chain reaction (PCR)

The samples were boiled for 10 min and then centrifuged at 10,000 × g for 5 min, and 5 μl of the supernatant was used as a template for PCR. Real-time PCR was performed using an ABI PRISM 7700 Sequence Detection System (ABI, Foster City, CA). Each reaction tube contained 50 μl of reaction mixture, including 5 μl of sample, 1 × Universal PCR Master Mix (ABI), 900 nM of each primer, and a 250 nM probe.

The primer and probe sets for the three bacterial species are listed in Table 1 (22, 32, 46). To quantify total bacteria, conserved sequences in reported 16S genes were selected (22). Total bacterial rRNA was amplified in a separate reaction, at the same time, under the same conditions as those used for specific amplification of the three bacteria. The probes were labeled at the 5′-end with the reporter dye 6-carboxyfluorescein (6-FAM) and at the 3′-end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA). Data were analyzed using the SEQUENCE DETECTION SYSTEM software from ABI. The number of bacterial cells was determined using DNA from known amounts of bacterial strains *A. actinomycetemcomitans* Y4, *P. gingivalis* ATCC33277, and *T. forsythia* ATCC43037. These control bacteria were grown under appropriate culture conditions. Bacterial levels were expressed as a percentage of the total bacteria.

Table 1. Sequences of oligonucleotide primers and probes

	Sequence (5′, 3′)
Total bacteria	
Forward	GGATTAGATACCCTGGTAGTC
Reverse	TACCTTGTTACGACTT
Probe	TGACGGGCGGTGTGTACAAGGC
A. actinomycetemcomitans	
Forward	CAGCATCTGCGATCCCTGTA
Reverse	GCCCTTTGTCTTTCCTAGGT
Probe	TCGAGTATTCCTCAAGCATTCTCGCACG
P. gingivalis	
Forward	TACCCATCGTCGCCTTGGT
Reverse	CGGACTAAAACCGCATACACTTG
Probe	GCTAATGGGACGCATGCCTATCTTACAGCT
T. forsythia	
Forward	GGGTGAGTAACGCGTATGTAACCT
Reverse	ACCCATCCGCAACCAATAAA
Probe	CCCGCAACAGAGGGATAACCCGG

Statistical analyses

Differences in clinical parameters and bacterial levels between patients were analyzed using the Mann–Whitney *U*-test. The Spearman rank correlation test was used to determine possible associations between the clinical parameters and bacterial levels. *P*-values were considered significant when $P < 0.05$. Differences in the salivary levels of bacteria, probing depth, and bleeding on probing before and after the initial periodontal treatment were analyzed using Student's *t*-test. Statistical analyses were performed using SPSS® software (SPSS Inc., Chicago, IL).

Results

The salivary levels of *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* were assessed using real-time PCR. *A. actinomycetemcomitans* was detected in 36.6% of the periodontitis patients and 85.0% of the healthy subjects. *P. gingivalis* was detected in 84.4% and 55.0%, respectively, and *T. forsythia* in 93.1% and 55.0%, respectively (Table 2). The salivary levels of 8-OHdG, *P. gingivalis*, and *T. forsythia* in periodontitis patients were significantly higher than those in healthy subjects. By contrast, the *A. actinomycetemcomitans* level in periodontitis patients was significantly lower than that in healthy subjects.

There were significant relationships in periodontitis patients between *P. gingivalis* and 8-OHdG ($r = 0.55$, $P < 0.01$), probing depth ($r = 0.49$, $P < 0.01$), *T. forsythia* ($r = 0.45$, $P < 0.05$), and bleeding on probing ($r = 0.41$, $P < 0.05$). In addition, there was a significant relationship between probing depth and bleeding on probing ($r = 0.70$, $P < 0.01$) (Table 3).

Significant decreases in the *P. gingivalis* level, 8-OHdG, probing depth, and bleeding on probing were observed after periodontal treatment (Table 4).

Discussion

In periodontitis caused by infection with a periodontal pathogen, neutrophils comprise

Table 2. Detection of salivary bacterial levels in periodontitis patients and clinically healthy subjects

Variable	Periodontitis patients	Healthy subjects
No. of subjects	29	20
No. of total bacteria/ml	$1.02 \pm 0.46 \times 10^9$	$2.16 \pm 0.14 \times 10^8$
8-OHdG (ng/ml)	4.36 ± 0.18	1.48 ± 0.08**
A. actinomycetemcomitans levels (%)	0.009 ± 0.001	0.141 ± 0.014*
A. actinomycetemcomitans-positive subjects (%)	36.6	85.0
P. gingivalis levels (%)	0.123 ± 0.008	0.012 ± 0.002*
P. gingivalis-positive subjects (%)	84.4	55.0
T. forsythia levels (%)	0.049 ± 0.002	0.008 ± 0.001*
T. forsythia-positive subjects (%)	93.1	55.0

All values are expressed as the mean ± SE.
Mann–Whitney *U*-test, statistically significant difference between periodontitis patients and healthy subjects; **$P < 0.01$, *$P < 0.05$.

Table 3. Correlation between salivary bacterial levels and clinical measurements in periodontitis patients (n = 29)

Parameter	Bleeding on probing	Probing depth	*T. forsythia*	*P. gingivalis*	*A. actinomycetemcomitans*
8-OHdG (ng/ml)	0.32	0.21	0.36	0.55**	0.21
A. actinomycetemcomitans levels (%)	0.37	0.26	0.01	0.03	
P. gingivalis levels (%)	0.41*	0.49**	0.45*		
T. forsythia levels (%)	0.33	0.37*			
Probing depth (mm)	0.70**				

**$P < 0.01$, *$P < 0.05$.

Table 4. Changes in the clinical measurements and salivary bacterial levels in periodontitis patients (n = 17) before and after treatment

Variable	Before	After
Probing depth (mm)	3.26 ± 0.03	2.60 ± 0.03**
Range (mm)	2.48–5.66	1.77–3.80
Bleeding on probing (%)	46.2 ± 1.1	19.8 ± 0.9**
Range (%)	22.2–86.1	0–30.0
8-OHdG (ng/ml)	4.05 ± 0.17	1.75 ± 0.09**
No. of total bacterial/ml	$1.33 \pm 0.10 \times 10^9$	$1.65 \pm 0.06 \times 10^9$
A. actinomycetemcomitans levels (%)	0.009 ± 0.001	0.002 ± 0.000
A. actinomycetemcomitans-positive subjects (%)	35.3	5.9
P. gingivalis levels (%)	0.143 ± 0.016	0.020 ± 0.002**
P. gingivalis-positive subjects (%)	100	70.6
T. forsythia levels (%)	0.053 ± 0.005	0.020 ± 0.003
T. forsythia-positive subjects (%)	94.1	70.6

All values are expressed as the mean ± SE.
Student's *t*-test, statistically significant difference before, and after periodontal treatment; **$P<0.01$.

the initial inflammatory component of the response to the pathogen (1). Oxidative stress is enhanced in periodontitis (1, 4, 7, 36, 42). However, the association between oxidative stress and the infective load of periodontal pathogens remains unclear. This study examined the relationship between the levels of periodontopathic bacteria and oxidative stress. The amount of periodontal pathogens present in plaque and saliva samples has previously been studied using culture, immunoassay, DNA hybridization, and PCR (8, 10, 12, 17, 20, 23, 28, 32). However, all of these methods suffer from a lack of specificity, sensitivity, or quantity. Therefore, it is difficult to clarify the causality of specific pathogens in periodontitis. A method facilitating the precise detection of these pathogens would be of major benefit for periodontal assessment. Real-time PCR with species-specific primers is precise and sensitive, and can be used to quantify periodontal pathogens and total bacteria counts more accurately (2, 5, 18, 22, 30, 45).

Initially, we found that the salivary levels of 8-OHdG, *P. gingivalis*, and *T. forsythia* in periodontitis patients were significantly higher than those in healthy subjects. This confirms the findings of previous studies, which suggested the importance of *P. gingivalis* and *T. forsythia* as true pathogens in adult patients with periodontitis (14, 15, 22). By contrast, the *A. actinomycetemcomitans* level in patients with periodontitis was significantly lower than that in healthy subjects. Correlation analysis also showed no significant correlation between the level of *A. actinomycetemcomitans* and 8-OHdG or *P. gingivalis* or *T. forsythia*. Previous studies have also reported that *A. actinomycetemcomitans* is not associated with adult periodontitis (13, 27, 33). The distribution of *A. actinomycetemcomitans* serotypes may be more specific and indicative of *A. actinomycetemcomitans* as a high-risk pathogen. Serotype c strains are more commonly found in periodontal health (3, 48). In contrast, serotype b strains, highly leukotoxic strains, are most often associated with periodontal disease (11). However, the prevalence of serotype b in Japanese patients is low (29, 44). Further studies will be needed prove the relationship between *A. actinomycetemcomitans* and periodontitis.

Correlation analysis showed a significant positive correlation between the level of *P. gingivalis* and 8-OHdG in saliva from periodontitis patients. In our previous study, 8-OHdG did not correlate with clinical data, age, and smoking status (36). A significant decrease in the *P. gingivalis* level and 8-OHdG was observed after periodontal treatment. A number of studies have shown that successful treatment significantly reduces *P. gingivalis* and *T. forsythia* levels (6, 16, 19, 41). Eliminating the periodontal pathogens, and a subsequent reduction of host inflammation of the periodontium, should logically decrease 8-OHdG levels. Our results suggest that the 8-OHdG levels in saliva reflect the load of periodontal pathogens.

In conclusion, we found a relationship between periodontal pathogens and the oxidative stress marker 8-OHdG. 8-OHdG could be a useful biomarker for assessing periodontal status accurately and evaluating the efficacy of periodontal treatment. Recently, we examined 8-OHdG levels in gingival crevicular fluid of teeth with advanced periodontal destruction (mean probing depth 7.2 mm). 8-OHdG levels were detected in eight of 18 crevicular fluid samples from periodontally hopeless teeth but not in 18 crevicular fluid samples from predictable teeth with advanced periodontal destruction (Takane M *et al.*, submitted paper). These data may indicate that periodontally involved hopeless teeth are a major source of salivary 8-OHdG. We plan to examine 8-OHdG in a longitudinal study to determine its potential as a predictor of progressing disease. Early detection and continuous monitoring of 8-OHdG may be useful in periodontal treatment to prevent periodontal disease development.

Acknowledgments

This work was supported by a Grant-in-Aid for Technology to Promote Multidisciplinary Research Projects from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. American Academy of Periodontology. The pathogenesis of periodontal diseases. J Periodontol 1999: **70**: 457–470.
2. Asai Y, Jinno T, Igarashi H, Ohyama Y, Ogawa T. Detection and quantification of oral treponemes in subgingival plaque by real-time PCR. J Clin Microbiol 2002: **40**: 3334–3340.
3. Asikainen S, Lai CH, Alaluusua S, Slots J. Distribution of *Actinobacillus actinomycetemcomitans* serotypes in periodontal health and disease. Oral Microbiol Immunol 1991: **6**: 115–118.

4. Battino M, Bullon P, Wilson M, Newman H. Oxidative injury and inflammatory periodontal diseases. the challenge of antioxidants to free radicals and reactive oxygen species. Crit Rev Oral Biol Med 1999: **10**: 458–476.
5. Boutaga K, Van Winkelhoff AJ, Vandenbroucke-Grauls CM, Savelkoul PH. Comparison of Real-Time PCR and culture for detection of *Porphyromonas gingivalis* in subgingival plaque samples. J Clin Microbiol 2003: **41**: 4950–4954.
6. Bragd L, Dahlen G, Wikstrom M, Slots J. The capability of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* to indicate progressive periodontitis; a retrospective study. J Clin Periodontol 1987: **14**: 95–99.
7. Chapple IL. Reactive oxygen species and antioxidants in inflammatory diseases. J Clin Periodontol 1997: **24**: 287–296.
8. Chaves ES, Jeffcoat MK, Ryerson CC, Snyder B. Persistent bacterial colonization of *Porphyromonas gingivalis. Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans* in periodontitis and its association with alveolar bone loss after 6 months of therapy. J Clin Periodontol 2000: **27**: 897–903.
9. Chiou CC, Chang PY, Chan EC, Wu TL, Tsao KC, Wu JT. Urinary 8-hydroxydeoxyguanosine and its analogs as DNA marker of oxidative stress. development of an ELISA and measurement in both bladder and prostate cancers. Clin Chim Acta 2003: **334**: 87–94.
10. Conrads G, Flemmig TF, Seyfarth I, Lampert F, Lutticken R. Simultaneous detection of *Bacteroides forsythus* and *Prevotella intermedia* by 16S rRNA gene-directed multiplex PCR. J Clin Microbiol 1999: **37**: 1621–1624.
11. DiRienzo JM, Slots J, Sixou M, Sol MA, Harmon R, McKay TL. Specific genetic variants of *Actinobacillus actinomycetemcomitans* correlate with disease and health in a regional population of families with localized juvenile periodontitis. Infect Immun 1994: **62**: 3058–3065.
12. Doungudomdacha S, Rawlinson A, Douglas CW. Enumeration of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* in subgingival plaque samples by a quantitative-competitive PCR method. J Med Microbiol 2000: **49**: 861–874.
13. Ezzo PJ, Cutler CW. Microorganisms as risk indicators for periodontal disease. Periodontol 2000 2003: **32**: 24–35.
14. Grossi SG, Genco RJ, Machtei EE, Ho AW, Koch G, Dunford R, et al. Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. J Periodontol 1995: **66**: 23–29.
15. Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, et al. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. J Periodontol 1994: **65**: 260–267.
16. Haffajee AD, Dzink JL, Socransky SS. Effect of modified Widman flap surgery and systemic tetracycline on the subgingival microbiota of periodontal lesions. J Clin Periodontol 1988: **15**: 255–262.
17. Kasuga Y, Ishihara K, Okuda K. Significance of detection of *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Treponema denticola* in periodontal pockets. Bull Tokyo Dent Coll 2000: **41**: 109–117.
18. Kumagai K, Sugano N, Takane M, Iwasaki H, Tanaka H, Yoshinuma N, et al. Detection of *Streptococcus anginosus* from saliva by real-time polymerase chain reaction. Lett Appl Microbiol 2003: **37**: 370–373.
19. Lai CH, Listgarten MA, Shirakawa M, Slots J. *Bacteroides forsythus* in adult gingivitis and periodontitis. Oral Microbiol Immunol 1987: **2**: 152–157.
20. Loesche WJ, Lopatin DE, Stoll J, van Poperin N, Hujoel PP. Comparison of various detection methods for periodontopathic bacteria: can culture be considered the primary reference standard? J Clin Microbiol 1992: **30**: 418–426.
21. Lynch MC, Kuramitsu HK. Role of superoxide dismutase activity in the physiology of *Porphyromonas gingivalis*. Infect Immun 1999: **67**: 3367–3375.
22. Lyons SR, Griffen AL, Leys EJ. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. J Clin Microbiol 2000: **38**: 2362–2365.
23. Matto J, Saarela M, Alaluusua S, Oja V, Jousimies-Somer H, Asikainen S. Detection of *Porphyromonas gingivalis* from saliva by PCR by using a simple sample-processing method. J Clin Microbiol 1998: **36**: 157–160.
24. Miyasaki KT, Wilson ME, Brunetti AJ, Genco RJ. Oxidative and nonoxidative killing of *Actinobacillus actinomycetemcomitans* by human neutrophils. Infect Immun 1986: **53**: 154–160.
25. Miyasaki KT, Wilson ME, Genco RJ. Killing of *Actinobacillus actinomycetemcomitans* by the human neutrophil myeloperoxidase-hydrogen peroxide-chloride system. Infect Immun 1986: **53**: 161–165.
26. Rall LC, Roubenoff R, Meydani SN, Han SN, Meydani M. Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) as a marker of oxidative stress in rheumatoid arthritis and aging: effect of progressive resistance training. J Nutr Biochem 2000: **11**: 581–584.
27. Rodenburg JP, van Winkelhoff AJ, Winkel EG, Goene RJ, Abbas F, de Graff J. Occurrence of *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in severe periodontitis in relation to age and treatment history. J Clin Periodontol 1990: **17**: 392–399.
28. Rudney JD, Chen R, Pan Y. Endpoint quantitative PCR assays for *Bacteroides forsythus*, *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans*. J Periodontal Res 2003: **38**: 465–470.
29. Saito A, Hosaka Y, Nakagawa T, Seida K, Yamada S, Takazoe I, et al. Significance of serum antibody against surface antigens of *Actinobacillus actinomycetemcomitans* in patients with adult periodontitis. Oral Microbiol Immunol 1993: **8**: 146–153.
30. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y. Rapid detection and quantification of five periodontopathic bacteria by real-time PCR. Microbiol Immunol 2001: **45**: 39–44.
31. Shapira L, Gordon B, Warbington M, Van Dyke TE. Priming effect of *Porphyromonas gingivalis* lipopolysaccharide on superoxide production by neutrophils from healthy and rapidly progressive periodontitis subjects. J Periodontol 1994: **65**: 129–133.
32. Shelburne CE, Prabhu A, Gleason RM, Mullally BH, Coulter WA. Quantitation of *Bacteroides forsythus* in subgingival plaque comparison of immunoassay and quantitative polymerase chain reaction. J Microbiol Meth 2000: **39**: 97–107.
33. Slots J, Feik D, Rams TE. *Actinobacillus actinomycetemcomitans* and *Bacteroides intermedius* in human periodontitis: age relationship and mutual association. J Clin Periodontol 1990: **17**: 659–662.
34. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998: **25**: 134–144.
35. Stenvinkel P. Interactions between inflammation, oxidative stress, and endothelial dysfunction in end-stage renal disease. J Ren Nutr 2003: **13**: 144–148.
36. Takane M, Sugano N, Iwasaki H, Iwano Y, Shimizu N, Ito K. New biomarker evidence of oxidative DNA damage in whole saliva from clinically healthy and periodontally diseased individuals. J Periodontol 2002: **73**: 551–554.
37. Tanner A, Maiden MF, Macuch PJ, Murray LL, Kent RL Jr. Microbiota of health, gingivitis, and initial periodontitis. J Clin Periodontol 1998: **25**: 85–98.
38. Thompson HL, Wilton JM. Effects of anaerobiosis and aerobiosis on interactions of human polymorphonuclear leukocytes with the dental plaque bacteria *Streptococcus mutans*, *Capnocytophaga ochracea*, and *Bacteroides gingivalis*. Infect Immun 1991: **59**: 932–940.
39. Tran SD, Rudney JD. Multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. J Clin Microbiol 1996: **34**: 2674–2678.
40. Tran SD, Rudney JD. Improved multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, and *Porphyromonas gingivalis*. J Clin Microbiol 1999: **37**: 3504–3508.
41. van Winkelhoff AJ, van der Velden U, de Graaff J. Microbial succession in recolonizing deep periodontal pockets after a single course of supra- and subgingival debridement. J Clin Periodontol 1988: **15**: 116–122.
42. Waddington RJ, Moseley R, Embery G. Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. Oral Dis 2000: **6**: 138–151.
43. Wu LL, Chiou CC, Chang PY, Wu JT. Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin Chim Acta 2004: **339**: 1–9.
44. Yamamoto M, Nishihara T, Koseki T, He T, Yamato K, Zhang YJ, et al. Prevalence of

Actinobacillus actinomycetemcomitans serotypes in Japanese patients with periodontitis. J Periodontal Res 1997: **32**: 676–681.
45. Yano A, Kaneko N, Ida H, Yamaguchi T, Hanada N. Real-time PCR for quantification of *Streptococcus mutans*. FEMS Microbiol Lett 2002: **217**: 23–30.
46. Yoshida A, Suzuki N, Nakano Y, Oho T, Kawada M, Koga T. Development of a 5′ fluorogenic nuclease-based real-time PCR assay for quantitative detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. J Clin Microbiol 2003: **41**: 863–866.
47. Zambon JJ. Periodontal diseases: microbial factors. Ann Periodontol 1996: **1**: 879–925.
48. Zambon JJ, Umemoto T, De Nardin E, Nakazawa F, Christersson LA, Genco RJ. *Actinobacillus actinomycetemcomitans* in the pathogenesis of human periodontal disease. Adv Dent Res 1988: **2**: 269–274.