ELSEVIER

FEMS Immunology and Medical Microbiology 39 (2003) 81–86

FEMS Immunology and Medical Microbiology

www.fems-microbiology.org

Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tetQ* gene and total bacteria

Hiroshi Maeda [a], Chiyo Fujimoto [a], Yasuhiro Haruki [a], Takemasa Maeda [a], Susumu Kokeguchi [b], Millan Petelin [c], Hideo Arai [a], Ichiro Tanimoto [a], Fusanori Nishimura [a], Shogo Takashiba [a,*]

[a] *Department of Patho-Physiology, Division of Periodontal Science, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8525, Japan*

[b] *Department of International Environmental Science, Division of Oral Microbiology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8525, Japan*

[c] *Department of Oral Medicine and Periodontology, University of Ljubljana, Ljubljana, Slovenia*

Received 29 April 2003; received in revised form 18 June 2003; accepted 25 June 2003

First published online 12 August 2003

Abstract

Accurate quantification of bacterial species in dental plaque is needed for microbiological diagnosis of periodontal diseases. The present study was designed to assess the sensitivity, specificity and quantitativity of the real-time PCR using the GeneAmp^{R} Sequence Detection System with two fluorescence chemistries. TaqMan probe with reporter and quencher dye, and SYBR Green dye were used for sources of the fluorescence. Primers and probes were designed for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia* and total bacteria based on the nucleotide sequences of the respective 16S ribosomal RNA genes. Since spread of antibiotic resistance genes is one of the crucial problems in periodontal therapy, quantitative detection of *tetQ* gene, which confers resistance to tetracycline, was included in the examination. The detection of *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* was linear over a range of 10–10^7 cells (10–10^7 copies for *tetQ* gene), while the quantitative range for total bacteria was 10^2–10^7 cells. Species-specific amplifications were observed for the three periodontal bacteria, and there was no significant difference between the TaqMan and SYBR Green chemistry in their specificity, quantitativity and sensitivity. The SYBR Green assay, which was simpler than TaqMan assay in its manipulations, was applied to the clinical plaque samples. The plaque samples were obtained from eight patients (eight periodontal pockets) before and 1 week after the local drug delivery of minocycline. Although the number of *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* markedly decreased after the antibiotic therapy in most cases, higher copy numbers of the *tetQ* gene were detectable. The real-time PCR demonstrated sufficient sensitivity, specificity and quantitativity to be a powerful tool for microbiological examination in periodontal disease, and the quantitative monitoring of antibiotic resistance gene accompanied with the antibiotic therapy should be included in the examination.

Keywords: Real-time PCR; Periodontal bacterium; 16S rRNA gene; *tetQ* gene; TaqMan; SYBR Green

1. Introduction

For microbiological examinations, techniques such as immunoassays, biochemical tests and hybridization using nucleotide probes have been developed and used [1]. However, since these methods required bacterial culture, they are mostly laborious and time-consuming. Recently, advances of novel molecular biology techniques enabled culture-independent methods. PCR is a typical example and is widely spread for the microbiological diagnosis of periodontal diseases. The gene encoding the small subunit of 16S ribosomal RNA (16SrDNA) has been frequently used as a target of the PCR examination because of its structural characteristics [2,3]. Nucleotide sequences of some portions of the 16SrDNA are highly conserved through

* Corresponding author. Tel.: +81 (86) 235-6677; Fax: +81 (86) 235-6679.
E-mail address: stakashi@cc.okayama-u.ac.jp (S. Takashiba).

0928-8244/03/$22.00
doi:10.1016/S0928-8244(03)00224-4

evolution, while other regions have species-specific variable sequences. The conserved sequences can provide PCR primers for amplification of 16SrDNA from all of the bacterial species and the species-specific regions can be used for identifications of unique bacterial species. The 16SrDNA-based PCR is a rapid, sensitive and specific molecular technique, which can be used for various purposes. However, the method still has a problem in quantification.

Antibiotic treatment is indispensable for periodontal therapy. For evaluation of the treatment, accurate quantification of periodontal bacteria is needed. In addition, we have to be alert to the increase of antibiotic resistance. It has recently been reported that a tetracycline-resistant gene, *tetQ*, has spread intensely through conjugal gene transfer [4,5]. Tetracycline-resistant *Prevotella* and *Bacteroides* strains isolated from periodontal pockets have been reported to possess either or both of the *tetQ* and *tetM* genes frequently [6–8]. The nucleotide sequence of the *tetQ* gene was almost identical among the clinical isolates, supporting the horizontal transfer of the gene [9]. Although PCR methods have already been used for the detection of *tetQ* gene in clinical plaque samples [7,10], quantitative monitoring of gene presence is required for analysis of its dynamics in periodontal pockets.

Recently, real-time PCR has been developed for the purpose of DNA quantification. Real-time PCR using the GeneAmp^{R} Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) or LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany) is accomplished by the continuous measurement of the PCR products. The method allows easy, rapid and quantitative detection of microorganisms in clinical samples. Some attempts have already been made for periodontal bacteria [11,12]. In the current study, we used the GeneAmp Sequence Detection System with TaqMan and SYBR Green chemistry for the quantification and identification of periodontopathic bacteria. We compared the TaqMan and SYBR Green chemistry in their specificity and sensitivity for further improvement of the real-time PCR assays in microbiological diagnosis of periodontal diseases. In addition, quantitative monitoring of *tetQ* gene was included in the examination.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study were *Actinobacillus actinomycetemcomitans* ATCC 43718 (Y4), *Actinomyces viscosus* ATCC 15987^{T}, *Escherichia coli* K-12, *Porphyromonas gingivalis* ATCC 33277^{T}, *Treponema denticola* ATCC 35405^{T}, *Fusobacterium nucleatum* ATCC 25586^{T}, *Prevotella intermedia* ATCC 25611^{T}, *Prevotella nigrescens* ATCC 33563^{T}. They were obtained from the American type culture collection (ATCC, Rockville, MD, USA). Each *P. gingivalis*, *P. endodontalis*, *P. intermedia* and *P. nigrescens* strain was cultured anaerobically in GAM broth (Nissui Seiyaku, Tokyo, Japan) supplemented with 0.0005% hemin and 0.0001% vitamin K_3. *A. actinomycetemcomitans*, *A. viscosus* and *T. denticola* were grown as previously described [13,14]. *E. coli* was cultured aerobically in Luria–Bertani medium. In addition to the type strains, a tetracycline-resistant *P. nigrescens* was selected from laboratory stock of clinical strains by using the medium containing 4 μg ml^{-1} tetracycline-hydrochloride.

2.2. DNA extraction

Total bacterial DNAs were extracted from cultivated strains and clinical plaque samples by using InstaGene Matrix (Bio-Rad Lab., CA, USA) according to the instructions. Plaque samples and cultivated bacteria suspended in 1 ml PBS (–) (Gibco BRL, MD, USA) were pelleted and resuspended in 200 μl of InstaGene Matrix. The suspension was incubated at 56°C for 30 min and then 100°C for 8 min. After the incubation, the suspension was centrifuged and 5 μl of the resulting supernatant was used for real-time PCR. To determine the quantitative range of real-time PCR, DNAs were prepared from 10–10^{7} cells of cultured bacteria.

2.3. tetQ gene

A cloned version of the *tetQ* gene was kindly provided from Dr. Progulske-Fox A. and Dr. Burks J. (Department of Oral Biology and Periodontal Disease Research Center, University of Florida). The optical density (260 and 280 nm absorbance) of the purified recombinant plasmid was measured to estimate the amount and copy number of the plasmid.

2.4. Plaque samples

Eight patients with adult type periodontitis, during the supportive periodontal treatment in Okayama University Dental Hospital, were selected for sample collection. All patients had not received any periodontal therapy in the previous 3 months and not received any systemic or local antibiotics within the preceding 6 months. Plaque sample was obtained from one periodontal pocket of 4–5 mm in each patient by using the paper points as described previously [15]. After the initial sampling, 2% minocycline–HCl paste (Periocline, Sunstar Inc., Osaka, Japan) was injected into the pocket without any other periodontal therapy. Plaque samples were also obtained from the minocycline-treated periodontal pockets after 1 week.

2.5. Primers and probes

The sequences of primers and probes used in this study

are listed in Table 1. For identification of unique bacterial species, primers and probes were designed from the species-specific region on the 16SrDNA, while the conserved sequences were used for amplification of total bacteria. For the design, Primer Express (version 1.0) and OLIGO (version 4.0) software were used. TaqMan probes were labeled with the reporter dye 6-carboxyfluorescein (FAM) at the 5′ end and with the quencher dye 6-carboxytetramethylrodamine (TAMRA) at the 3′ end.

2.6. Real-time PCR

The real-time PCR was performed using GeneAmp^{R} 5700 Sequence Detection System (PE Applied Biosystems). The reaction mixture (50 μl) for TaqMan assay contained 2× TaqMan Universal PCR Master Mix (PE Applied Biosystems), 20 pmol of forward and reverse primers, 10 pmol of TaqMan probe and 5 μl of extracted DNA. While, the reaction mixture for SYBR Green assay contained 2× SYBR Green PCR Master Mix (PE Applied Biosystems), 20 pmol of forward and reverse primer and 5 μl of extracted DNA. Thermocycling program was 40 cycles of 95°C for 15 s and 60°C for 1 min with an initial cycle of 95°C for 10 min. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate with optical caps (PE Applied Biosystems). At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye from the TaqMan probes or dsDNA-binding SYBR Green. After the PCR, a dissociation curve (melting curve) was constructed in the range of 60°C to 95°C. All data were analyzed using the GeneAmp 5700 SDS software.

Table 1
Primers and probes

targets	chemistry	sequence of primer and TaqMan probe
Aa	SYBR Green	5′-cttacctactcttgacatccgaa-3′ 5′-atgcagcacctgtctcaaagc-3′
	TaqMan (probe)	5′-cttacctactcttgacatccgaa-3′ 5′-atgcagcacctgtctcaaagc-3′ (5′-agaactcagagatgggtttgtgccttag-3′)
Pg	SYBR Green	5′-cttgacttcagtggcggcag-3′ 5′-agggaagacggttttcacca-3′
	TaqMan (probe)	5′-tagcttgctaaggtcgatgg-3′ 5′-caagtgtatgcggttttagt-3′ (5′-tgcgtaacgcgtatgcaacttgcc-3′)
Pi	SYBR Green	5′-aatacccgatgttgtccaca-3′ 5′-ttagccggtccttattcgaa-3′
	TaqMan (probe)	5′-aatacccgatgttgtccaca-3′ 5′-ttagccggtccttattcgaa-3′ (5′-tgacgtggaccaaagattcatcggtgga-3′)
total bacteria	SYBR Green	5′-gtgStgcaYggYtgtcgtca-3′ 5′-acgtcRtccMcaccttcctc-3′
tetQ	SYBR Green	5′-aattactgttcgggcttcta-3′ 5′-gcttgtatgccttcctttgc-3′
	TaqMan (probe)	5′-aattactgttcgggcttcta-3′ 5′-gcttgtatgccttcctttgc-3′ (5′-atctattatctggaatggagtgaaatgcaa-3′)

Aa: *A. actinomycetemcomitans*, Pg: *P. gingivalis*, Pi: *P. intermedia*.

3. Results and discussion

3.1. Specificity of primers and probes

For specificity testing, extracted DNAs from 10^7 cells of periodontal bacteria listed in Section 2 were examined as templates with the primers and probes prepared for *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*, respectively. PCR amplifications were observed correctly when the target strains were used as templates. The primers and probes did not increase the fluorescence with the mismatched strains (data not shown). Dissociation curves of all PCR products showed a sharp peak at the expected T_m of the products (data not shown). These results indicate that each real-time PCR specifically amplified the target DNA. Since additional hybridization of the probe is required for generation of the fluorescence, TaqMan assay is thought to be more specific than SYBR Green assay in general. However, SYBR Green chemistry demonstrated sufficient specificity for the three periodontal bacteria.

Primers and probe for *tetQ* gene did not increase the fluorescence with any type strains used in this study. PCR amplification was observed with the tetracycline-resistant *P. nigrescens* and the recombinant plasmid carrying the *tetQ* gene (data not shown). For total bacteria, only the SYBR Green method was employed because enhanced specificity was not required.

3.2. Quantification of cultured bacteria and tetQ gene

DNA extracts from 10–10^7 cells of three periodontal bacteria, *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*, were subjected to both TaqMan and SYBR Green assays. In both TaqMan and SYBR Green assay, detection and quantification for each strain was linear over the examined range. A typical result of *A. actinomycetemcomitans* is shown in Fig. 1. Lyons et al. reported the quantitative range of *P. gingivalis* as 10^2–10^8 cells using nested PCR with a TaqMan probe [11], and Sakamoto et al. demonstrated quantitative detection of five periodontal bacteria in the range of 10^3–10^8 cells using the LightCycler™ system [12]. Our study demonstrated a wider quantitative range of real-time PCR. However, since the previous studies did not examine the smaller amount, their actual limits of the quantitative detection may be smaller than the reported data. A copy number of the 16S ribosomal operon in each strain could possibly contribute to the differential sensitivity for the detections. The number of 16S ribosomal operons in *P. gingivalis* revealed to be four through the TIGR (The Institute for Genomic Research) database. Since genome projects for *A. actinomycetemcomitans* and *P. intermedia* are still under progress, the 16S ribosomal numbers were analyzed through genomic blast search and were estimated to be six and five, respectively. Remarkable differences of the copy number

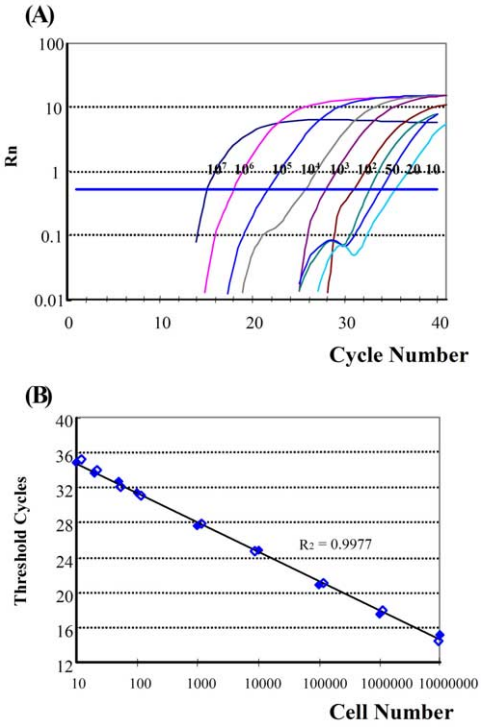

Fig. 1. Amplification and standard curve of the real-time PCR for *A. actinomycetemcomitans*. Panel A demonstrates the amplification curve constructed by SYBR Green assay. The TaqMan assay constructed a similar amplification curve (data not shown). Panel B demonstrates the standard curves constructed by SYBR Green assay (♦) and TaqMan assay (◇). The horizontal bar in panel (A) indicates the threshold line. The vertical axis of panels (A) and (B) indicates the monitored fluorescence and threshold cycles (cycle number when the fluorescence reached threshold line), respectively. The horizontal axis of panel (B) indicates cell number of *A. actinomycetemcomitans*. The amplification was linear in the range of 10–10^7 (correlation coefficient = 0.99) in both TaqMan and SYBR Green assays. The data were analyzed by GeneAmp 5700 SDS software.

were not seen, and therefore, we estimate that the detection limit is similar among these three species.

In order to examine if the quantification was influenced with contamination of another bacterial species, 10^5 cells of seven periodontal bacteria, other than the target, were added to each serial dilution (10^2–10^7) of the target strain. Fig. 2A shows the influence of other bacterial contamination to the quantification of *A. actinomycetemcomitans*, which was examined by the SYBR Green assay. The standard curve of the contaminated samples was almost the same with the normal standard, demonstrating limited influence of the contamination, if any at all. In the same way, no influence was observed to the quantification of *P. gingivalis* and *P. intermedia* in both TaqMan and SYBR Green assay (data not shown).

Quantification of total bacteria was examined using the DNAs from eight periodontal bacteria prepared in this study. The different strains showed similar results of linear detection in the range of 10^2–10^7. A typical result for *A. actinomycetemcomitans* is shown in Fig. 2B. The similar results reflect the similar copy numbers of the 16S ribosomal RNA operon in the strain tested as has been discussed above. An average of the copy number in both the *Pasteurellaceae* family (including genus *Actinobacillus*) and the *Bacteroidaceae* family is similar (5.8), and *E. coli* possesses seven copies [16]. However, since the copy number of the 16S ribosomal operon can vary from one to as many as 15 copies [16], total bacterial counts evaluated by the number of 16SrDNA may be slightly fluctuated according to the differences of microflora especially in case of polymicrobial communities such as dental plaque. The limit of quantitative detection for the total bacteria was 100 cells, while the quantitative detection of unique bacteria was less than 10. This was caused by a high level of background (Fig. 2B). Since the primers can hybridize to any bacterial species, even small contamination of bacteria in the experimental manipulation or the contamination of *E. coli* DNA in the recombinant Taq polymerase might be a cause of the conspicuous background.

The quantitative range for *tetQ* gene was also examined by both TaqMan and SYBR Green assay. Serial dilutions of the recombinant plasmid, which contained the *tetQ* gene, were prepared and used as templates. The results were almost the same between the two methods and the

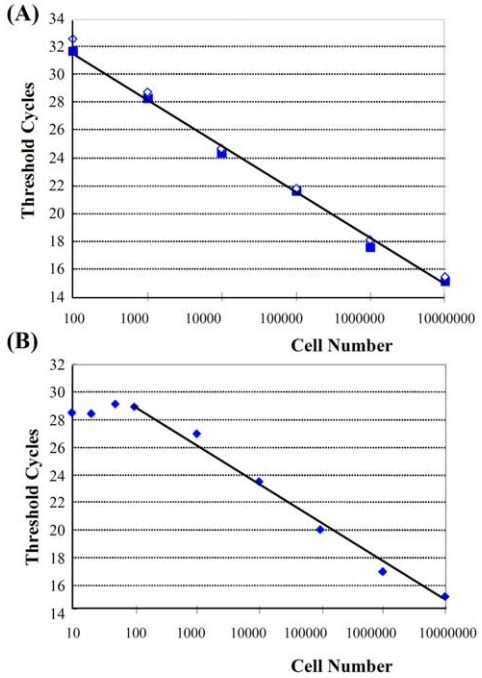

Fig. 2. (A) Quantification of *A. actinomycetemcomitans* in crude DNA samples (SYBR Green assay). The standard curve of *A. actinomycetemcomitans* was constructed using the serial dilutions of *A. actinomycetemcomitans* DNA containing other bacterial DNA (◇). The extracted DNAs from 10^5 cells of *E. coli*, *P. gingivalis*, *P. intermedia*, *F. nucleatum*, *T. denticola*, *A. viscosus* and *P. nigrescens* were added to the serial diluted templates. The data were dotted with the normal standard of *A. actinomycetemcomitans* (■). B: Standard curve for total bacteria (SYBR Green assay). Universal primers designed from the conserved regions on 16SrDNA were used with the *A. actinomycetemcomitans* DNA. The detection was linear in the range of 10^2–10^7 cells. The strength of the fluorescence did not change between 10 and 10^2 cells of the template. The vertical axis indicates the threshold cycles.

detection of *tetQ* was linear in the range of 10–10^7 copies of the gene (data not shown).

3.3. Examination of the plaque sample

The real-time PCR using the SYBR Green chemistry was applied to the clinical plaque samples as has been described above. The original 5 μl of extracted DNA samples and 5 μl of diluted samples (10^{-1}) were run in duplicate. Total bacterial numbers in the periodontal pockets did not consistently decrease after the local application of minocycline (Fig. 3A). The number increased in three patients and did not change in one patient, while the number decreased in four patients. On the contrary, the number of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* decreased remarkably. Fig. 3B shows the percentage of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* among total bacterial counts before and after the antibiotic therapy. Local delivery of tetracycline has been effectively used for periodontal therapy [17,18]. The qualitative change of the microbial community may account for the effect.

The *tetQ* gene in the plaque samples was evaluated by the SYBR Green assay using the standard of the recombinant plasmid. The number of *tetQ* genes increased after the minocycline therapy in four patients, and the gene

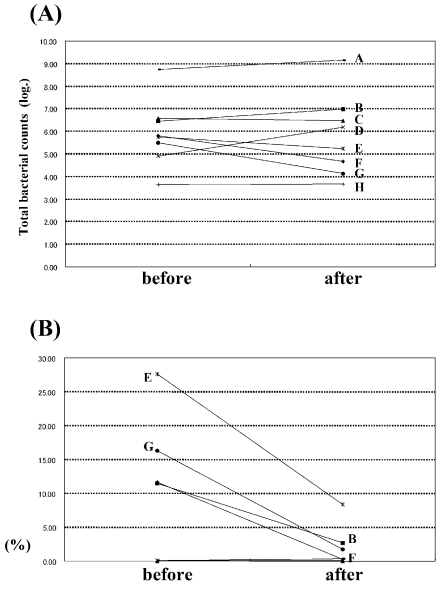

Fig. 3. A: Total bacterial number in subgingival plaque samples. Total bacterial number was determined before and after the local administration of minocycline. The data of the identical pocket were connected to each other. Samples (A–H) were collected from eight periodontal pockets (eight patients). The vertical axis indicates the cell number in logarithm. B: The percentage of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* among total bacterial counts. The cell numbers of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* were added and the percentage in the total bacterial number was calculated. The data of the identical pocket (A–H) at different sampling times (before and after the antibiotic therapy) were connected. The samples A, C, D and H contained very few numbers of tested strains.

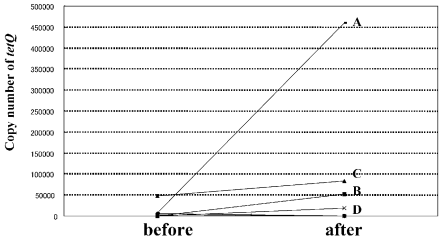

Fig. 4. Copy numbers of the *tetQ* gene in subgingival plaque. The data of the identical pocket (A–H) were connected. The *tetQ* gene could not be detected from four patients (E–H).

could not be detected in the remaining four patients at the both sampling times (Fig. 4). Preus et al. [19] and Wade et al. [20] reported the similar results that locally delivered tetracycline selected for the resistant bacteria. Although several PCR methods had already been reported for the monitoring of *tetQ* gene [7,10], real-time PCR seems to be useful as well.

The current study demonstrated the potential use of real-time PCR for the analysis of clinical plaque samples. However, besides the method itself, sampling procedures would be another issue. Since the real-time PCR is very sensitive, the sampling procedure could greatly affect the results. Although bacterial sampling using paper point employed in this study is a well-established method [15,21], technical difficulties have also been reported [21–24]. Sample collection methods appropriate for the real-time PCR need to be investigated.

Both TaqMan and SYBR Green assays showed sufficient sensitivity, specificity and quantitativity. Since the TaqMan assay required additional manipulation and cost for the probe, the SYBR Green assay may be suitable for routine clinical examinations. In addition, quantitative monitoring of the antibiotic resistance genes should be included in the microbiological diagnosis.

Acknowledgements

We thank Dr. Progulske-Fox A. and Dr. Burks J. (Department of Oral Biology and Periodontal Disease Research Center, University of Florida) for providing the *tetQ* gene. This study was supported by Grants-in-Aid for Scientific Research (B12557192, C13672186 and C13671901) from the Japan Society for the Promotion of Science.

References

[1] Zambon, J.J. and Haraszthy, V.I. (1995) The laboratory diagnosis of periodontal infections. Periodontology 2000, 69–82.
[2] Slots, J., Ashimoto, A., Flynn, M.J., Li, G. and Chen, C. (1995) Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. Clin. Infect. Dis. 20, 304–307.

[3] Harper-Owen, R., Dymock, D., Booth, V., Weightman, A.J. and Wade, W.G. (1999) Detection of unculturable bacteria in periodontal health and disease by PCR. J. Clin. Microbiol. 37, 1469–1473.
[4] Guiney, D.G. and Bouic, K. (1990) Detection of conjugal transfer system in oral, black-pigmented *Bacteroides* spp. J. Bacteriol. 172, 495–497.
[5] Shoemaker, N.B., Vlamakis, H., Hayes, K. and Salyers, A.A. (2001) Evidence that extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. Appl. Environ. Microbiol. 67, 561–568.
[6] Nikolich, N.P., Hong, G., Shoemaker, N.B. and Salyers, A.A. (1994) Evidence that conjugal transfer of a tetracycline resistance gene (*tetQ*) had occurred very recently in nature between the normal microflora of animals and the normal microflora of humans. Appl. Envion. Microbiol. 60, 3255–3260.
[7] Lacroix, J.M. and Walker, C.B. (1996) Detection and prevalence of the tetracycline resistance determinant Tet Q in the microbiota associated with adult periodontitis. Oral Microbiol. Immunol. 11, 282–288.
[8] Arzese, A.R., Tomasetig, L. and Botta, G.A. (2000) Detection of *tetQ* and *ermF* antibiotic resistance genes in *Prevotella* and *Porphyromonas* isolates from clinical specimens and resident microbiota of humans. J. Antimicrob. Chemother. 43, 1523–1524.
[9] Chung, W.O., Young, K., Leng, Z. and Roberts, M.C. (1999) Mobile elements carrying *ermF* and *tetQ* genes in gram-positive and gram-negative bacteria. J. Antimicrob. Chemother. 44, 329–335.
[10] Manch-Citron, J.M., Lopes, G.H., Dey, A., Rapley, J.W., Mac Neill, S.R. and Cobb, C.M. (2000) PCR monitoring for tetracycline resistance genes in subgingival plaque following site-specific periodontal therapy. A preliminary report. J. Clin. Periodontol. 27, 437–446.
[11] Lyons, S.R., Griffen, A.L. and Leys, E.J. (2000) Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. J. Clin. Microbiol. 38, 2362–2365.
[12] Sakamoto, M., Takeuchi, Y., Umeda, M., Ishikawa, I. and Benno, Y. (2001) Rapid detection and quantification of five periodontopathic bacteria by real-time PCR. Microbiol. Immunol. 45, 39–44.
[13] Kokeguchi, S., Kato, K., Nishimura, F., Kurihara, H. and Murayama, Y. (1991) Isolation and partial characterization of a 39-kDa major outer membrane protein of *Actinobacillus actinomycetemcomitans* Y4. FEMS Microbiol. Lett. 61, 85–89.
[14] Miyamoto, M., Noji, S., Kokeguchi, S., Kato, K., Kurihara, H., Murayama, Y. and Taniguchi, S. (1991) Molecular cloning and sequence analysis of antigen gene *tdpA* of *Treponema denticola*. Infect. Immun. 59, 1941–1947.
[15] Nishimura, F., Nagai, A., Kurimoto, K., Isoshima, O., Takashiba, S., Kobayashi, M., Akutsu, I., Kurihara, H., Nomura, Y., Murayama, Y., Ohta, H. and Kato, K. (1990) A family study of a mother and daughter with increased susceptibility to early-onset periodontitis: Microbiological, immunological, host defensive, and genetic analysis. J. Periodontol. 61, 755–765.
[16] Klappenbach, J.A., Saxman, P.R., Cole, J.R. and Schmidt, T.M. (2001) rrndb: the Ribosomal RNA operon copy number database. Nucleic Acids Res. 29, 181–184.
[17] Newman, M.G., Korman, K.S. and Doherty, F.M. (1994) A 6-month multi-center evaluation of adjunctive tetracycline fiber therapy used in conjunction with scaling and root planing in maintenance patients. Clinical results. J. Periodontol. 65, 685–691.
[18] Lowenguth, R.A., Chin, I., Caton, J.G., Cobb, C.M., Drisko, C.L., Killoy, W.J., Michalowicz, B.S., Pihlstrom, B.L. and Goodson, J.M. (1995) Evaluation of periodontal treatment using controlled-release tetracycline fibers: Microbiological response. J. Periodontol. 66, 700–707.
[19] Preus, H.R., Lassen, J., Aass, A.M. and Ciancio, S.G. (1995) Bacterial resistance following subgingival and systemic administration of minocycline. J. Clin. Periodontol. 22, 380–384.
[20] Wade, W.G., Moran, J., Morgan, J.R., Newcombe, R. and Addy, M. (1992) The effects of antimicrobial acrylic strips on the subgingival microflora in chronic periodontitis. J. Clin. Periodontol. 19, 127–134.
[21] Hartroth, B., Seyfahrt, I. and Conrads, G. (1999) Sampling of periodontal pathogens by paper points: evaluation of basic parameters. Oral Microbiol. Immunol. 14, 326–330.
[22] Dahlen, G., Renvert, S., Wikstrom, M. and Egelberg, J. (1990) Reproducibility of microbiological samples from periodontal pockets. J. Clin. Periodontol. 17, 73–77.
[23] Baker, P.J., Butler, R. and Wikesjo, U.M. (1991) Bacterial sampling by absorbent paper points. An in vitro study. J. Periodontol. 62, 142–146.
[24] Smola, S.F., Rettenberger, G., Simmet, Th. and Burysek, L. (2003) Comparison of sample collection methods for the PCR detection of oral anaerobic pathogens. Lett. Appl. Microbiol. 36, 101–105.