Journal of Chromatography A, 1361 (2014) 286–290

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Quantification of Periodontal Pathogens Cell Counts by Capillary Electrophoresis

Zhenqing Li[a,*], Shaoxiong Chen[a], Chenchen Liu[a], Dawei Zhang[a], Xiaoming Dou[b], Yoshinori Yamaguchi[b,c,a,*]

[a] Engineering Research Center of Optical Instrument and System, Ministry of Education, Shanghai Key Lab of Modern Optical System, University of Shanghai for Science and Technology, No. 516 JunGong Road, Shanghai 200093, China
[b] Photonics and Bio-medical Research Institute, Department of Physics Faculty of Science, East China University of Science and Technology, No. 130 Meilong Road, Shanghai 200237, China
[c] Department of Applied Physics, Graduate School of Engineering, Osaka University, Yamadaoka Suita-city, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 9 May 2014
Received in revised form 31 July 2014
Accepted 31 July 2014
Available online 7 August 2014

Keywords:
Capillary electrophoresis
Polymerase chain reaction
Periodontal pathogens
Analytical methods
Fluorescence intensity

ABSTRACT

Gingivitis is a highly prevalent periodontal disease around the worldwide. *Porphyromonas gingivalis* (*P.g*), *Treponema denticola* (*T.d*) and *Tannerela forsythia* (*T.f*) were considered to be three important periodontal pathogens related to gingivitis, and research shows that the counts of periodontal pathogen cells in the patients before, during, and after fixed orthodontic appliance therapy were quite different. We proposed a simple method to extract the periodontal pathogens from the periodontal pocket in this work and demonstrated a new approach to determine periodontal pathogen level based on capillary electrophoresis (CE). After polymerase chain reaction amplification of *P.g* (197 bp), *T.d* (311 bp), and *T.f* (641 bp), it shows that they can rapidly identified by CE within 5 min. The peak area in the eletropherogram is linearly related to the concentration of *P.g*, *T.d*, and *T.f*, and the correlation coefficients R^2 corresponding to them are 0.993, 0.993, and 0.956, respectively. According to this linearly relationship, the estimated concentration of *P.g*, *T.d*, and *T.f* in gingival crevicular fluid from one volunteer was inferred to be about 9.90×10^2, 1.48×10^3, and 9.01×10^2 cells/μl, respectively.

1. Introduction

Rapid diagnosis and better quantification of periodontal pathogens remain a challenge for analysts. Gingivitis, the mildest form of periodontal disease, is highly prevalent and has affected 50–90% of adults worldwide [1]. Traditionally, the clinical diagnosis of periodontal disease is based on visual and radiographic assessment of the periodontal tissues and on the measurement of the space between the tooth and gingiva [2]. However, chronic periodontitis is usually asymptomatic until the disease is so severe that teeth are shift, loosen, or even lost. Furthermore, experiments also demonstrated that the counts of periodontal pathogen cells in adolescents before, during, and after fixed orthodontic appliance therapy were quite different [3]. Thus timely discovery of the periodontal pathogen level is crucial for planning strategies for periodontal disease control.

Research shows that the establishment and progression of the periodontal disease result from the presence of high levels of periodontal pathogens in the sulcular fluid [4], and chronic periodontitis is basically caused by mixed infections with the subgingival microbiota being organized as a biofilm and characterized by a continual flux [5]. Currently over 400 different bacterial species have been found in periodontal pockets, but only a few (e.g., *Porphyromonas gingivalis* (*P.g*), *Tannerela forsythia* (*T.f*), and *Treponema denticola* (*T.d*)) were conceived to be the major periodontal pathogens strongest related to periodontal tissue destruction [6–8]. For example, *P.g*, which is a fimbriae, hair-like appendages on the bacterial surface, is considered to be critical virulence factors to mediate bacterial interactions with and invasion of host tissues [9]. Furthermore, *P.g*, *T.f*, and *T.d* were also confirmed in the periodontal pockets by commercially available microbiological tests [10].

Traditional identification of target microorganisms involves light and electron microscopy and cultural techniques. However, few microorganisms have sufficiently distinctive morphology to be recognized by microscopy. Culture-dependent methods are

* Corresponding authors at: Engineering Research Center of Optical Instrument and System, Ministry of Education, Shanghai Key Lab of Modern Optical System, University of Shanghai for Science and Technology, No. 516 JunGong Road, Shanghai 200093, China. Tel.: +86 21 55276023; fax: +86 21 55276023.
E-mail addresses: zhenqingli@163.com (Z. Li), yoshi.yamaguchi@ap.eng.osaka-u.ac.jp (Y. Yamaguchi).

http://dx.doi.org/10.1016/j.chroma.2014.07.100
0021-9673/

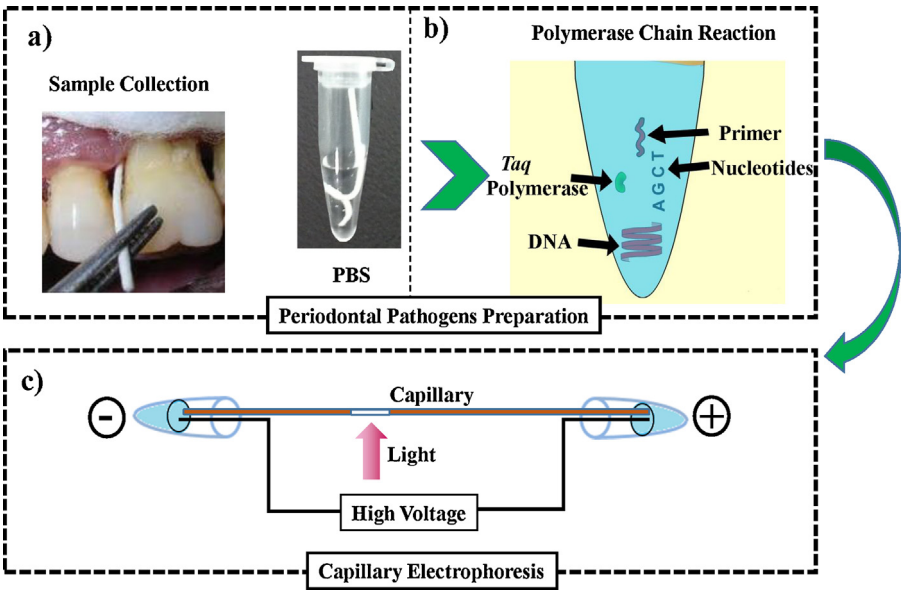

Fig. 1. Detection process of periodontal pathogens by CE. (a) Sample extraction schematic by PBS solution. (b) PCR amplification of the target periodontal pathogens. (c) Separation of the PCR products by self-build capillary electrophoresis.

restricted, because a microorganism can be cultivated only after its physiological niche is perceived and duplicated experimentally [11,12]. It is said that 80% or more of microbes remain undiscovered [13]. Polymerase chain reaction (PCR) is a common method for the microbiological diagnosis. The gene coding the small subunit of 16S ribosomal RNA (16S rRNA) has been frequently used as a target of the PCR examination because of its structural characteristics [14]. Because nucleotide sequences of some portions of the 16S rRNA are highly conserved through evolution, while other regions contain more variable sequences [15]. Facilitated though technical advances, 16S rDNA-based PCR coupled with electrophoresis has been developed a specific molecular technique for detecting the target genes [16].

Capillary electrophoresis (CE) was widely applied in the analysis of nucleic acids because of its time and sample efficient characteristics. In particular, CE coupled with laser induced fluorescence detection (LIF) method is highly sensitive [17–19]. However, the instrument for CE-LIF was complicated and expensive. Herein, we report a simple method to estimate the quantity of the cells of *P.g*, *T.d*, and *T.f* based on CE-PCR. The schematic of the process was shown in Fig. 1. Such a method is easy to operate, low cost, and address a critical need for the rapid diagnose of periodontal pathogen levels in patients with periodontal disease.

2. Experimental

2.1. Reagents

SpeedSTAR HS DNA Polymerase and 100 bp DNA ladders were purchased from Takara (Shiga, Japan). Hydroxyethyl cellulose (HEC, 1300 k) was bought from Polysciences (Warrington, PA, USA). Bacterial strains of *P.g* (ATCC 33277), *T.f* (ATCC 43037) and *T.d* (ATCC 35405) strains were from Microbiologics Inc (217 Osseo Avenue North, St. Cloud, MN 56303, USA). 10,000× SYBR Green I was got from Invitrogen (Carlsbad, CA, USA). 10× TBE (1× TBE = 89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.4) buffer was bought from Bio-Rad (Hercules, CA, USA). 0.5× TBE was prepared by mixing 10× TBE and distilled water with a ratio 1:19, 1× SYBR Green I for DNA separation. 1× SYBR Green I was obtained by diluting the 10,000× SYBR Green I to a final concentration of 1/10,000.

2.2. Capillary electrophoresis

The CE system designed and build in our lab has previously been described in detail [20,21]. High-voltage power supply (MODEL 610E, TREK, Medina, NY, USA) was used to drive electrophoresis. The excitation wavelength from a mercury lamp was filtered to be 460–495 nm, which was the wavelength of the excitation maximum of the conjugate of SYBR Green I and the nucleic acid by the optical filter (U-MWIB-3, Olympus, Tokyo, Japan). The fluorescence emission was collected by a 60× objective (PlanApo/IR, Olympus), and then was detected by a photomultiplier tube (R928, Hamamatsu Photonics, Japan). The applied voltage and data collection were controlled by LabVIEW software (National Instrument, Austin, TX, USA). A certain length fused-silica capillary with ID/OD = 75/365 (μm/μm) was covalently coated with polyacrylamide [22,23]. The total capillary length (l_t) was 11 cm and effective length (l_e) was 6 cm. The entire detection system was enclosed in a black box. DNA sample was electrokinetically introduced into the capillary at 100 V/cm for 1.0 s. After each electro-separation, the injection side of the capillary was flushed with sterilized water by pump for 1.0 min. All separations were performed at 26 °C in the clean room controlled by air-conditioner.

2.3. Periodontal pathogens sample preparation

Earlier research demonstrated that the prevalence and severity of periodontitis, including missing teeth increased significantly with age for nonhuman primates [24]. We also found that it was very hard to found *P.g*, *T.d* and *T.f* by PCR-CE from younger people in our previous experiments, and thus we recruited 75 adult male aged 30–60 years old as volunteers for bacteria analysis. The gingival crevicular fluid was collected from periodontal pocket of upper central incisor (Fig. 1a). Two hours after meal, volunteers were required to rinse their mouth with a gulp of water. Then a sterile paper point was carefully inserted into the target site and held in place for 1.0 min. At last, the paper point was transferred in 100 μl phosphate-buffered saline (Fluka, Switzerland) for 3.0 min, and then was centrifuged in Chibitan-II personal centrifuge (Hitachi, Tokyo, Japan) (at 10,000 rpm) for 10 min.

Table 1
Primer sequence for periodontal pathogens detection.

Pathogen/gene target	Sequence 5′---------3′	PCR product (bp)
Porphyromonas gingivalis	Fw TGTAGATGACTGATGGTGAAAACC Rv ACGTCATCCCCACCTTCCTC	197
Treponema denticola	Fw AAGGCGGTAGAGCCGCTCA Rv AGCCGCTGTCGAAAAGCCCA	311
Tannerella forsythia	Fw GCGTATGTAACCTGCCCGCA Rv TGCTTCAGTGTCAGTTATACCT	641

2.4. PCR amplification of periodontal pathogens

PCR was performed with T-100 thermal cycler (Bio-Rad, USA). The primer designed for 16S rRNA gene and the target sequences are listed in Table 1 [25–27]. For all bacteria, the reaction was performed with 1.0 μl sample and 49 μl reaction volumes containing 5.0 μl 10× Fast Buffer I, 4.0 μl dNTP mixture (2.5 μM), 200 nM primers (FASMAC Co., Ltd., Kanagawa, Japan), and 0.25 μl SpeedSTAR HS DNA Polymerase. The thermo cycling program was 40 cycles of 95 °C for 10 s (denaturation) and 64 °C for 30 s (annealing and extension) with an initial cycle of 95 °C for 2 min, and the sizes of the corresponding amplified products were 197, 311, and 641 bp for *P.g*, *T.d*, and *T.f*, respectively. In order to confirm the quality, specificity and selectivity of the PCR products, genomic DNA of strains of *P.g* (ATCC 33277), *T.d* (ATCC 35405), and *T.f* (ATCC 43037) were used as positive controls, and distilled water was used as a negative control. The templates of *P.g*, *T.d*, and *T.f* were extracted from 1×10^3 cells per microliter (μl), which was counted by C-Chip (Biochrom, Berlin, Germany). The PCR products were directly analyzed for CE without any further sample preparation.

3. Results and discussion

3.1. Specificity and selectivity of PCR target sequence and primers

The template of *P.g*, *T.d*, and *T.f* were amplified with their corresponding specific species primers yielding PCR products between 197 and 641 bp sizes. Slab-gel electropherograms show species-specific PCR products for *P.g*, *T.d*, and *T.f* (Fig. 2). Then we have performed CE of the PCR products and 100 bp DNA ladder (Takara, Shiga, Japan) to check out the size of amplification products. The DNA ladder markers contain 11 double stranded fragments ranging in size from 100 to 1500 bp, and PCR reaction mixtures were diluted 20 times prior to CE. They were resolved in 0.5× TBE buffer containing 0.2% HEC (1300 k) polymer and 2× SYBR Green I. Data

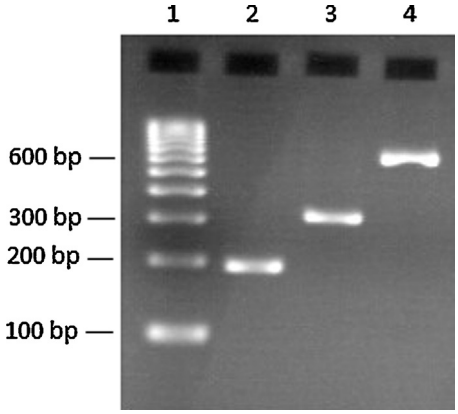

Fig. 2. Slab gel electrophoresis of 100 bp DNA ladders (lane 1) and PCR products of *P.g* (lane 2), *T.d* (lane 3), and *T.f* (lane 4). Electrophoretic conditions: 2.0% agarose, 10 V/cm, 26 °C.

Table 2
The relationship between peak area and concentration of periodontal pathogen (cells/μl). *a*: intercept, *b*: slope, R^2: correlation coefficient.

Periodontal pathogen	$F = a + b^*C$		
	a	*b*	R^2
P.g	0.001	0.021	0.993
T.d	0.009	0.010	0.993
T.f	0.036	0.034	0.956

in the electrophergoram (Fig. 3) showed that the PCR products and DNA ladder were baseline resolved within 5 min. Through CE of PCR negative control (containing no DNA template) (data not shown), we found that only the peaks corresponding to the primers could be observed in the electropherogram. Size determination of PCR products was based on the production of calibration plot. Regression line of DNA ladder size versus its migration time was plotted in the insert of Fig. 3. Result shows that the relationship between migration time and length of DNA fragments has a good linearity (correlation coefficient R^2, 0.9928) in the size ranged from 100 to 700 bp, and thus *P.g*, *T.d*, and *T.f* were determined and marked in Fig. 3.

3.2. Quantification of periodontal pathogens cells based on fluorescence intensity

In CE, the SYBR Green I dye binds to the DNA in multiple locations along the entire length of the molecule. The fluorescence intensity is a function of the length and the concentration of the DNA molecule. When the length of the DNA fragments was certain, the higher the DNA concentration was, the greater its fluorescence intensity became. Thus the estimated quantity of PCR products can be calculated by plotting the peak area in the electropherogram with the concentration of the target gene. We have carried out the PCR amplification of *P.g*, *T.d*, and *T.f* with 40 cycles. Each PCR product was diluted to a desired concentration gradient: 1%, 2%, 3%, 4%, and 5%, and then we performed CE in 0.2% HEC (1300 k) at 150 V/cm. In order to test the fluorescence intensity stability, each sample ran 5 times in CE. The peak area was obtained by integration with migration time to estimate the fluorescence intensity of the sample. Then we got the relationship between fluorescence intensity and concentration of periodontal pathogens by regression fitting. Result shows that for *P.g*, *T.d*, and *T.f*, the peak area seems linearly related to the concentration of the PCR products, and the correlation coefficients R^2 were 0.993, 0.993 and 0.956, respectively. A detailed description of the relationship between fluorescence intensity and diluted concentration of *P.g*, *T.d*, and *T.f* was tabulated in Table 2.

3.3. Examination of periodontal pathogens cells from real sample

Moreover, we have carried out the PCR amplification of periodontal pathogens from volunteers, and separated the PCR products in CE. The PCR thermal cycling was repeated 40 cycles and then PCR products were diluted 20 times prior to CE. For one of them, the peak area in the electropherogram calculated for *P.g*, *T.d*, and *T.f* was 0.105, 0.0829, and 0.189, respectively. According to the linearly relationship in Table 2, the concentration of *P.g*, *T.d*, and *T.f* in gingival crevicular fluid was inferred to be 9.90×10^2, 1.478×10^3, and 9.01×10^2 cells/μl, respectively.

In order to compare the fluorescence signal of *P.g*, *T.d*, and *T.f* from the same individual, we have also mixed the PCR products equally, and then analyzed the mixture in 0.5% HEC (1300 k) at 100 V/cm by CE (l_t/l_e = 14 cm/6 cm). The PCR products were diluted 10 times prior to CE. Most of time, *P.g* and *T.d* could be observed in the electropherogram, but *T.f* was rarely found. Fig. 4 was one example of the electropherogram for 3 volunteers. The high peak

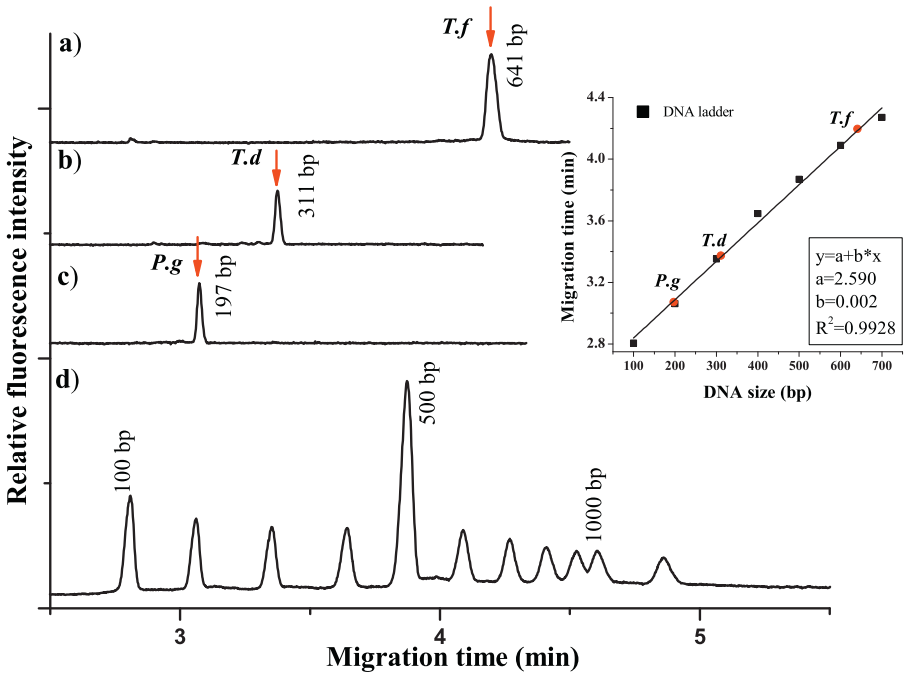

Fig. 3. Separation of PCR products of (a) *T.f*, (b) *T.d*, (c) *P.g*, and (d) 100 bp DNA ladder markers by CE. CE was performed at 150 V/cm. DNA was separated in 0.2% HEC polymer. The total length and the effective length of the capillary were 11 and 6.0 cm, respectively. Sample loadings: 100 V/cm (1.0 s).

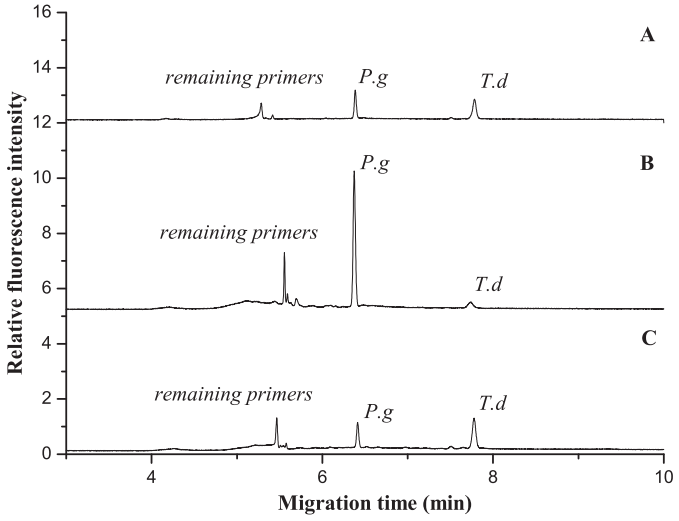

Fig. 4. Electropherogram of periodontal pathogens from volunteers with (A) healthy teeth; (B) teeth after therapy; (C) healthy teeth. The sample was resolved in 0.5% HEC polymer. The total length and the effective length of the capillary were 14 and 8.0 cm, respectively. Sample loadings: 100 V/cm (1.0 s).

in Fig. 4b corresponds to the volunteer with tooth decay, indicating that there may be great counts of *P.g* in the member with periodontal disease, the volume of *P.g* and *T.d* was quite similar in the people with healthy teeth (Fig. 4A and C).

4. Conclusion

In summary, we have proposed a simple method to extract periodontal pathogens from the periodontal pocket, and attempted to determine the periodontal pathogens cell counts in gingival crevicular fluid by CE. Results demonstrate that the fluorescence intensity, which was related to the peak area in the electropherogram, shows high linear relationship with the concentration of periodontal pathogens. This work may potentially allow one to quantify the pathogen cell counts in clinical trial.

The authors have declared no conflict of interest.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (No. 21205078 and No. 61378060), Research Fund for the Doctoral Program of Higher Education of China (No. 20123120110002). We acknowledged on partly financial support by Grand-in-Aid for Scientific Research (Houga) (No. 25600049 (Y. Y.), JSPS, Japan.

References

[1] B.L. Pihlstrom, B.S. Michalowicz, N.W. Johnson, Periodontal diseases, Lancet 366 (2005) 1809–1820.
[2] G.C. Armitage, The complete periodontal examination, Periodontol. 2000 34 (2004) 22–33.
[3] M.J. Thornberg, C.S. Riolo, B. Bayirli, M.L. Riolo, E.A. Van Tubergen, R. Kulbersh, Periodontal pathogen levels in adolescents before, during, and after fixed orthodontic appliance therapy, Am. J. Orthod. Dentofac. 135 (2009) 95–98.
[4] S. Socransky, A. Haffajee, Microbial mechanisms in the pathogenesis of destructive periodontal diseases: A critical assessment, J. Periodontal Res. 26 (1991) 195–212.
[5] F.V. Derdilopoulou, J. Nonhoff, K. Neumann, A.M. Kielbassa, Microbiological findings after periodontal therapy using curettes, Er: YAG laser, sonic, and ultrasonic scalers, J. Clin. Periodontol. 34 (2007) 588–598.
[6] J.J. Zambon, Periodontal diseases: Microbial factors, Ann. Periodontol. 1 (1996) 879–925.
[7] P.J. Ezzo, C.W. Cutler, Microorganisms as risk indicators for periodontal disease, Periodontol. 2000 32 (2003) 24–35.
[8] P.M. Loomer, Microbiological diagnostic testing in the treatment of periodontal diseases, Periodontology 2000. 34 (2004) 49–56.
[9] A. Amano, Molecular interaction of *Porphyromonas gingivalis* with host cells: Implication for the microbial pathogenesis of periodontal disease, J. Periodontol. 74 (2003) 90–96.
[10] R. Cosgarea, A. Bäumer, B. Pretzl, S. Zehaczek, T.S. Kim, Comparison of two different microbiological test kits for detection of periodontal pathogens, Acta Odontol. Scand. 68 (2010) 115–121.
[11] E. Ercan, K. Eratalay, O. Deren, D. Gur, O. Ozyuncu, B. Altun, C. Kanli, P. Ozdemir, H. Akincibay, Evaluation of periodontal pathogens in amniotic fluid and the role of periodontal disease in pre-term birth and low birth weight, Acta Odontol. Scand. 71 (2013) 553–559.

[12] Z. Yu, R.M. Schmaltz, T.C. Bozeman, R. Paul, M.J. Rishel, K.S. Tsosie, S.M. Hecht, Selective tumor cell targeting by the disaccharide moiety of bleomycin, J. Am. Chem. Soc. 135 (2013) 2883–2886.
[13] D.M. Ward, R. Weller, M.M. Bateson, 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community, Nature 345 (1990) 63–65.
[14] H. Maeda, C. Fujimoto, Y. Haruki, T. Maeda, S. Kokeguchi, M. Petelin, H. Arai, I. Tanimoto, F. Nishimura, S. Takashiba, Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, tetQ gene and total bacteria, FEMS Immunol. Med. Microbiol. 39 (2003) 81–86.
[15] U.B. Göbel, A. Geiser, E.J. Stanbridge, Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between Mycoplasma species, J. Gen. Microbiol. 133 (1987) 1969–1974.
[16] A. Ashimoto, C. Chen, I. Bakker, J. Slots, Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions, Oral Microbiol. Immunol. 11 (1996) 266–273.
[17] Y. Kim, E.S. Yeung, Separation of DNA sequencing fragments up to 1000 bases by using poly (ethylene oxide)-filled capillary electrophoresis, J. Chromatogr. A 781 (1997) 315–325.
[18] K. Altria, D. Elder, Overview of the status and applications of capillary electrophoresis to the analysis of small molecules, J. Chromatogr. A 1023 (2004) 1–14.
[19] H.-C. Chen, Y.-S. Chang, S.-J. Chen, P.-L. Chang, Determination of the heterogeneity of DNA methylation by combined bisulfite restriction analysis and capillary electrophoresis with laser-induced fluorescence, J. Chromatogr. A 1230 (2012) 123–129.
[20] Z. Li, X. Dou, Y. Ni, Y. Yamaguchi, Separation of long DNA fragments by inversion field capillary electrophoresis, Anal. Bioanal. Chem. 401 (2011) 1665–1671.
[21] Z. Li, C. Liu, X. Dou, Y. Ni, J. Wang, Y. Yamaguchi, Electromigration behavior of nucleic acids in capillary electrophoresis under pulsed-field conditions, J. Chromatogr. A 1331 (2014) 100–107.
[22] S. Hjertén, High-performance electrophoresis: elimination of electroendosmosis and solute adsorption, J. Chromatogr 347 (1985) 191–198.
[23] D. Schmalzing, C.A. Piggee, F. Foret, E. Carrilho, B.L. Karger, Characterization and performance of a neutral hydrophilic coating for the capillary electrophoretic separation of biopolymers, J. Chromatogr. A 652 (1993) 149–159.
[24] J. Ebersole, M. Steffen, J. Gonzalez-Martinez, M. Novak, Effects of age and oral disease on systemic inflammatory and immune parameters in nonhuman primates, Clin. Vaccine Immunol. 15 (2008) 1067–1075.
[25] K. Watanabe, T.O. Frommel, *Porphyromonas gingivaiis*, *Actinobacillus actinomycetemeomitans* and *Treponema denticola* detection in oral plaque samples using the polymerase chain reaction, J. Clin. Periodontol. 23 (1996) 212–219.
[26] S.D. Tran, J.D. Rudney, 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. 37 (1999) 3504–3508.
[27] C. Padilla, O. Lobos, E. Hubert, C. Gonzalez, S. Matus, M. Pereira, S. Hasbun, C. Descouvieres, Periodontal pathogens in atheromatous plaques isolated from patients with chronic periodontitis, J. Periodontal Res. 41 (2006) 350–353.