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Multiplex amplification of target genes of periodontal pathogens in continuous flow PCR microfluidic chip†

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Porphyromonas gingivalis (*P.g*), *Treponema denticola* (*T.d*), and *Tannerella forsythia* (*T.f*) are believed to be the major periodontal pathogens that cause gingivitis, which affects 50–90% of adults worldwide. Microfluidic chips based on continuous flow PCR (*CF*-PCR) are an ideal alternative to a traditional thermal cycler, because it can effectively reduce the time needed for temperature transformation. Herein, we explored multi-PCR of *P.g*, *T.d* and *T.f* using a *CF*-PCR microfluidic chip for the first time. Through a series of experiments, we obtained two optimal combinations of primers that are suitable for performing multi-PCR on these three periodontal pathogens, with amplicon sizes of (197 bp, 316 bp, 226 bp) and (197 bp, 316 bp, 641 bp), respectively. The results also demonstrated that by using multi-PCR, the amplification time can be reduced to as short as 3′48″ for the short-sized amplicons, while for *T.f* (641 bp), the minimum time required was 8′25″. This work provides an effective way to simultaneously amplify the target genes of *P.g*, *T.d* and *T.f* within a short time, and may promote *CF*-PCR as a practical tool for point-of-care testing of gingivitis.

Introduction

Periodontitis is a chronic inflammatory disease that occurs in the gums.[1] It not only can induce tooth loss, but may also affect a patient's systemic health and increase their risk of atherosclerosis, adverse pregnancy outcomes, rheumatoid arthritis, aspiration pneumonia and cancer.[2,3] Research has revealed that periodontal pathogens (*e.g.*, *Porphyromonas gingivalis* (*P.g*), *Treponema denticola* (*T.d*), and *Tannerella forsythia* (*T.f*)) are the main causative agents of periodontitis. Therefore, early diagnosis of those periodontal pathogens is quite important for periodontal disease control.

Compared with colony morphology, polymerase chain reaction (PCR) is more effective for the determination of periodontal pathogens,[4] because the former requires many hours for cell culture. Moreover, colony morphology may be unsuitable for bacterial identification if the morphologies of different species of bacteria are almost the same. PCR involves three steps for amplifying the target genes: 90–95 °C for denaturation, 50–60 °C for annealing and 72–77 °C for extension. However, traditional PCR thermocyclers are not only bulky, but also require about 1 hour for 40 cycles, as the temperature of the same aluminum holder must be repetitively changed. Liu *et al.* performed multiplex PCR for identifying periodontal pathogens using a traditional PCR thermocycler, which took at least 41 min. Each of the 30 thermal cycles consisted of preheating for 1 min at 94 °C; denaturation for 20 seconds at 94 °C, annealing for 20 seconds and extension for 20 seconds at 72 °C; and the final extension for 10 min at 72 °C.[5] Therefore, various methods have been proposed to reduce the time needed or simplify the thermal recycling process.

Notomi's group first proposed the loop-mediated isothermal amplification (LAMP) of DNA.[6] This method only requires the heating of solutions under isothermal conditions, and thus the time for DNA amplification can be reduced. Although four specially designed primers for LAMP can amplify the target sequence with high selectivity, the primer design is complicated and it can easily induce false positives. Wittwer and coworkers developed a prototype instrument that heats the PCR solution between two water baths, and consequently, they successfully amplified targets of 45–102 bp in 15–60 seconds.[7] This is the fastest PCR

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method reported so far, but the instrument is quite massive and there have been no further reports about this technology to date. By partitioning individual analyte molecules into many replicate reactions, Tewari's team applied nanoliter-sized droplet technology paired with digital PCR for the analysis of serum microRNA.[8] Although it can precisely determine the concentration of the analyte, the process of dispersing a diluted nucleic acid solution into millions of reactors is quite complicated, and the imaging system needs to be very sensitive to detect the fluorescence of one molecule. Krishnan *et al.* first proposed natural convection PCR, which involves heating the bottom and top of a cell.[9] The steady circulatory flow based on Rayleigh–Bénard convection can bring the DNA template solution to the right temperature zone for denaturing, annealing and extension. Qiu's lab developed a method for the rapid diagnosis of influenza A (H1N1) virus.[10] However, we also found that the geometry of the cell should be optimized if the temperature of its bottom and top is certain; otherwise the circulatory flow field will be fixed in a certain temperature zone.[11]

By running the PCR solution in the serpentine channel of a microfluidic chip, Kopp *et al.* fabricated the first continuous flow PCR (*CF*-PCR) chip and realized the 20-cycle PCR amplification of a 176 bp fragment from the DNA gyrase gene of *Neisseria gonorrhoeae*.[12] Since then, there have been many other studies, *e.g.*, improving the sample injection method,[13] achieving PCR product detection,[14] and developing easy ways to fabricate *CF*-PCR microfluidic chips.[15] Our group also investigated factors relating to the design of a *CF*-PCR chip.[16] We then developed a portable all-in-one microfluidic device based on an integrated *CF*-PCR-electrophoresis microfluidic chip, and realized the amplification of periodontal pathogens and on-line detection of PCR products in succession.[17] However, most research focuses on amplifying one target gene in the *CF*-PCR microfluidic chip, which may reduce the PCR efficiency. To our knowledge, there is still a lack of research on amplifying the target gene of several bacteria in one *CF*-PCR microfluidic chip.

Herein, we designed a system based on a *CF*-PCR microfluidic chip for the multi-PCR of periodontal pathogens, and systematically investigated the factors that may affect the PCR efficiency. This device can reduce the time for the detection of periodontal pathogens, and will be of great value for the diagnosis of periodontitis.

Materials and methods

Materials

Hydroxyethyl cellulose (HEC, 1300 k) was bought from Polysciences (Warrington, PA, USA). SpeedSTAR HS DNA Polymerase and 100 bp DNA ladder were purchased from Takara (Shiga, Japan). Polyvinyl pyrrolidone and Tween 20 were obtained from Aladdin (Shanghai, China). 10 000× SYBR Green I and 10× TBE (1× TBE = 89 mM Tris/89 mM boric acid/2 mM EDTA, pH = 8.4) buffer were from Solarbio (Beijing, China). Bacterial strains of *P.g* (ATCC 33277), *T.d* (ATCC 35405) and *T.f* (ATCC 43037) were from Microbiologics Inc.

Periodontal pathogens preparation

The detailed protocol for the preparation of periodontal pathogens has been described in a previous study[18] and is illustrated in Fig. 1A. In brief, the volunteers first needed to rinse their mouths for routine cleaning. Next, a sterile paper point was carefully inserted into the volunteers' gums for 1 min. Then the sterile paper tip was placed in a centrifuge tube containing 100 μl phosphate-buffered saline (Fluka, Switzerland), and the solution was centrifuged at 10 000 rpm for 10 min. Finally, 2 μl of the supernatant was used as the sample for PCR.

Amplification of periodontal pathogens in thermocycler

The PCR solution contained 5.0 μl 10× Fast Buffer I, 4.0 μl dNTP mixture (2.5 μM), 0.25 μl SpeedSTAR HS DNA Polymerase (Takara, Japan), 4.0 μl polyvinyl pyrrolidone (PVP), 2.0 μl Tween 20, 1.0 μl template and 200 nM primers (Generay Biotech, Shanghai, China). Ultrapure water was added to 50.0 μl. The primers for *P.g*, *T.d* and *T.f* are listed in Table 1.

Fabrication of *CF*-PCR microfluidic chip

Fig. 2 shows the fabrication of the replica mold and the chip. First, SU-8 photoresist was spin-deposited on a silicon wafer. Next, it was prebaked at 65 °C for 5 min and soft baked at 95 °C for 15 min. Using a photolithography mask, a pattern was engraved onto the photoresist by a photolithography machine (MJB4, SUSS MicroTec, Germany). The wafer was then kept at 65 °C for 3 min, followed by 95 °C for 7 min. The silicon wafer was cleaned in developer solution (Alfa Aesar, USA) and was shaken gently until the channel could be seen. Finally, the wafer was cleaned in isopropanol (Aladdin, Shanghai,

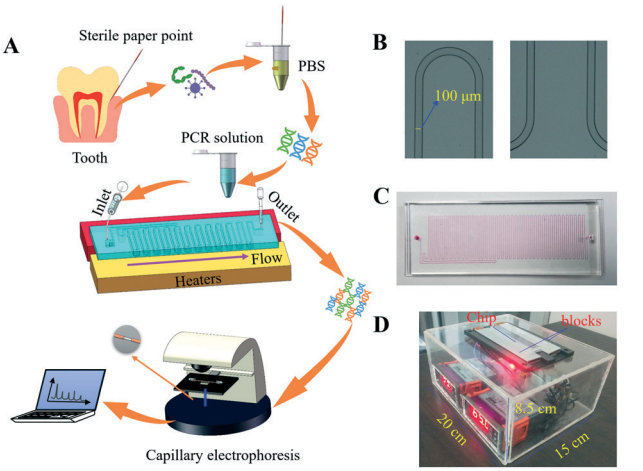

Fig. 1 (A) Schematic showing multi-PCR of periodontal pathogens by continuous flow PCR microfluidic chip. (B) Microchannels viewed by microscope. (C) Photograph of *CF*-PCR microfluidic chip. (D) The *CF*-PCR system.

Table 1 The primers employed for the periodontal pathogens

Target	Sequence 5′……3′	Amplicon (bp)
P.g	Fw TGTAGATGACTGATGGTGAAAACC	197
	Rw ACGTCATCCCCACCTTCCTC	
P.g-2	Fw GCGCTCAACGTTCAGCC	67
	Rw CACGAATTCCGCCTGC	
T.d	Fw AAGGCGGTAGAGCCGCTCA	311
	Rw AGCCGCTGTCGAAAAGCCCA	
T.d-2	Fw TAATACCGAATGTGCTCATTTACAT	316
	Rw TCAAAGAAGCATTCCCTCTTCTTCTTA	
T.f	Fw GCGTATGTAACCTGCCCGCA	641
	Rw TGCTTCAGTGTCAGTTATACCT	
T.f-2	Fw ATCCTGGCTCAGGATGAACG	226
	Rw TACGCATACCCATCCGCAA	

China) to remove surface impurities, dried at room temperature, and baked at 200 °C for 15 min.

Next, PDMS was obtained by mixing the precursor and curing agent in a ratio of 10 : 1. Then the mixture was poured into the replica mold after degassing. By solidifying it at 80 °C for 1 h, the patterned PDMS was carefully peeled from the mold. Then the surface of the replica and a slide were bonded after they were activated by a plasma cleaner. Finally, they were baked at 80 °C for 30 min.

Construction of *CF*-PCR system

The *CF*-PCR system (Fig. 1D) is composed of a syringe pump (PHD2000, Harvard Apparatus), a microfluidic chip (Fig. 1C), and two PTC ceramic heaters inside aluminium blocks. The heaters were controlled by two PID temperature controllers (XH-W2023, Shanghai, China), which were employed for maintaining the aluminium blocks (10 cm × 2 cm × 0.4 cm, length–width–height) at different temperatures. The sensitivity of the temperature controllers was 0.1 °C. The syringe was applied for pushing the PCR solution into the channels of the microfluidic chip. The outer dimensions of the chip are 3 cm × 8 cm. There are 40 serpentine cycles (100 μm × 1.46 m × 100 μm, width–length–depth) in the microfluidic chip (Fig. 1B). The microfluidic chip is placed above two aluminium heating blocks. There is a 12 mm air gap between the two heating blocks.

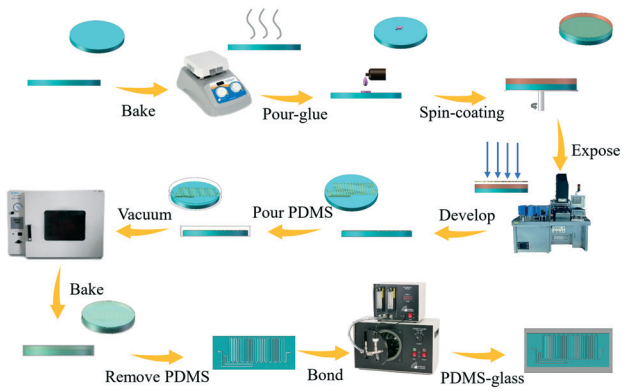

Fig. 2 The fabrication process of the *CF*-PCR microfluidic chip.

Capillary electrophoresis

The self-built CE system has been described in detail in a previous study.[17] It consists of a high-voltage power supply (MODEL 610E, TREK, Medina, NY, USA), a BX51 epi-illumination microscope (Olympus, Japan), and a R928 photomultiplier tube (PMT) (Hamamatsu Photonics, Japan). An instrument control software based on LabVIEW (National Instrument, USA) was employed for data control and acquisition. The DNA sample was electrokinetically (100 V cm^{-1}, 2.0 s) introduced into the capillary (75 μm/365 μm, Polymicro Technologies, Phoenix, USA). The light emitted from a mercury lamp was filtered for 460–495 nm excitation light. The fluorescence from a mixture of DNA and SYBR Green I was collected by PMT. The user-interface (Fig. S1†), program (Fig. S2†) and hardware (Fig. S3†) for data collection are illustrated in the ESI.† The experiment was performed in a dark room. After each run, the capillary was flushed with sterilized water for 1 min. All separations were performed at room temperature.

Results and discussion

Thermal uniformity of the *CF*-PCR system

For *CF*-PCR systems, the temperature distribution of the heater is the key factor that determines the PCR efficiency, because the stored heat will be directly transferred to the PCR solution in the microchannel. We evaluated the temperature distribution of the aluminium block and slide using an IR camera (Testo 865, Testo, Inc., and Germany). We first set the temperature of the two heating blocks to 68 °C and 107 °C, and it took about 3 min and 4 min for them to reach the required temperature, respectively. The results showed that there were obviously two temperature zones corresponding to the two aluminium blocks (see Fig. S4 in the ESI†). We further measured the temperature profile of the aluminium heating blocks using TCM-M207 temperature sensors (EasyShining Technology, Chengdu, China). A temperature drop was observed at the interface between the heating block and the air. The temperature was dropped

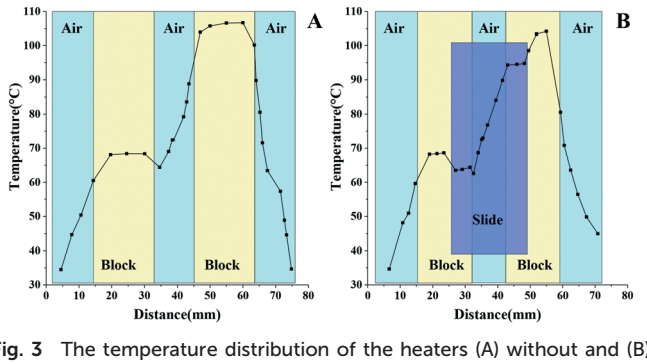

Fig. 3 The temperature distribution of the heaters (A) without and (B) with slide. Block means aluminium block.

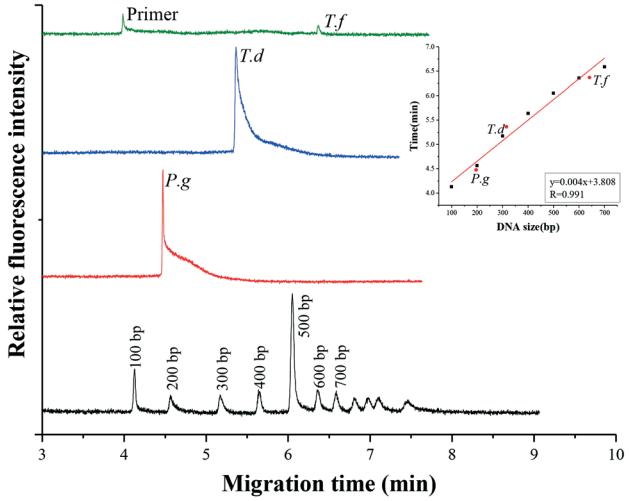

Fig. 4 The electropherogram of periodontal pathogens after amplification by the *CF*-PCR microfluidic chip. Capillary electrophoretic conditions: 0.5% HEC (1300 k) in 0.5× TBE solution; electric field strength: 100 V cm^{-1}; sample injection: 1500 V, 2.0 s; total length and effective length of the capillary: 15.0 cm/8.0 cm.

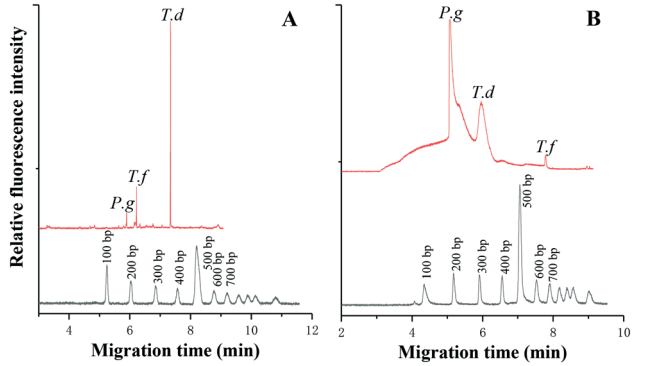

Fig. 5 The electropherogram of multi-PCR of periodontal pathogens with different primers. The size of the amplicons were (A) 197 bp, 316 bp, 226 bp and (B) 197 bp, 316 bp, 641 bp. The electrophoretic conditions were the same as those in Fig. 4.

from 65.82 °C to 64.38 °C, and then it was increased to 106.61 °C because of the natural convection (Fig. 3A). We also measured the temperature profile for the top surface of the slide when it was placed on the heaters (Fig. 3B). This revealed that the temperatures of the top surfaces of the slides were about 63.87 ± 0.45 °C and 94.50 ± 0.23 °C, respectively, and there were temperature zones of about 72 °C in the air gaps. Because the PCR microchannel inside PDMS was oscillating on the slide, and the isothermal zones matched well with the temperature required for the PCR process, it is reasonable to assume that the DNA in the *CF*-PCR microfluidic chip maintained the temperature needed for denaturing, annealing and extension.

Amplification of single periodontal pathogens in *CF*-PCR chip

We first tested the system for the amplification of the target genes of *P.g*, *T.d* and *T.f* in the *CF*-PCR microfluidic chip. The PCR solution for these bacteria flowed through the microchannel at a rate of 90.05 μl h^{-1}, 96.31 μl h^{-1} and 83.62 μl h^{-1}, and it took about 9′45″, 9′07″ and 10′30″ for them to go through the channel from the inlet to the outlet of the chip, respectively. The sizes of the amplicons corresponding to *P.g*, *T.d* and *T.f* were 197 bp, 316 bp and 641 bp, respectively. Slab gel electropherograms demonstrated the species-specific PCR products for *P.g*, *T.d* and *T.f* (see Fig. S5 in the ESI†). Furthermore, we carried out electrophoresis of the 100 bp DNA ladder and amplicons at the same electrophoretic conditions. The background electrolyte was 0.5× TBE containing 0.5% HEC (1300 k) and 1× SYBR Green I. The electric field strength was 100 V cm^{-1}, and the effective length and total length of the capillary was 8 cm and 15 cm, respectively. The data in Fig. 4 show that the DNA ladder was resolved within 8 min. Peaks were also observed in the electropherogram for the PCR products, although the peak of *T.f* was relatively low, which is possibly because the amplicon was long. The sizes of the PCR products were further evaluated based on calibration plots of DNA size and migration time (see insert in Fig. 4). These results demonstrated that the DNA fragments ranging in size from 100 bp to 700 bp were linearly related to the migration time (R = 0.991), and thus the size of the amplicons could be determined for *P.g*, *T.d* and *T.f*. We also performed negative control tests by running the PCR solution without a template in the microchannels. The results showed that only the peaks of the primers were observed (data not shown). Moreover, the repeatability of the CE system was validated by run-to-run experiments, which demonstrated that the variations in the peak height and the migration time corresponding to the peak were ±0.56% and ±0.20%, respectively.

Simultaneous amplification of *P.g*, *T.d* and *T.f* in the microfluidic chip

Primers play a crucial role in the multiplex amplification of target genes for periodontal pathogens. Without appropriate primers, the process may suffer from cross-reactions or false-positive PCR products. To realize the multiplex amplification of *P.g*, *T.d* and *T.f* in the microfluidic chip, we used typical primers reported in the literature (Table 1). We selected three of those primers corresponding to *P.g*, *T.d* and *T.f*. To increase the PCR efficiency, we chose primers with a GC content below 60%, because a high GC content may induce the formation of stable DNA secondary structures in the template. Such secondary structures can inhibit enzymatic extension of the primers. Thus, we selected two groups of primers for the multiplex amplification of these three periodontal pathogens. The sizes of the amplicons correspond to (197 bp, 316 bp, 226 bp) and (197 bp, 316 bp, 641 bp). The PCR solution containing each group of primers ran through the microchannel at a flow rate of 108.62 μl h^{-1} and 91.30 μl h^{-1}, and it took about 8′05″ and 9′37″,

respectively. Afterwards, we performed electrophoresis of PCR products in 0.5% HEC (1300 k) and 100 V cm^{-1} of electric field strength. The results in Fig. 5 demonstrate that they were well resolved within 8 min and the products corresponding to *P.g*, *T.d* and *T.f* were obviously observed. However, we also noted that for the former group of primers, there were some unexpected electropherogram peaks (Fig. 5A), which may correspond to the false positives, because these peaks were still observed after we processed them using a DNA Purification Kit (Solarbio, Beijing, China). We also performed multiplex PCR of *P.g*, *T.d* and *T.f* with other groups of primers. The results showed that they were prone to unspecified PCR products (see Fig. S6 in the ESI†).

Minimum time required for multiplex PCR of periodontal pathogens

In order to evaluate the minimum time needed for multiplex PCR, we conducted a series of experiments with flow rates ranging from 77.47 μl h^{-1} to 478.91 μl h^{-1}, and each experiment was performed three times. Because of the inherent properties of the fluid, there are slight deviations of the time taken for the PCR solution to run through the microfluidic chip. The sizes of the amplicons for *P.g*, *T.d* and *T.f* were 197 bp, 316 bp and 641 bp, respectively. Through an electropherogram of the PCR products (see Fig. S7 in the ESI†), we found that no peak was observed when the run time from inlet to outlet was lower than 1′50″. This indicates that the time was not enough for denaturing, annealing and extension to occur, and that there were no PCR products. When the run time was between 3′48″ and 6′12″, only *P.g* and *T.d* were successfully amplified, while the PCR products of *T.f* appeared when the running time was longer than 8′25″. This is possibly because the size of amplicon for *T.f* is so long that it needs more time on the heaters to finish the PCR process. Furthermore, we evaluated the volume of PCR products by plotting the peak area (Fig. 6A) and the peak height (Fig. 6B) with the PCR solution run time, respectively. This revealed that both were positively correlated with the run time,

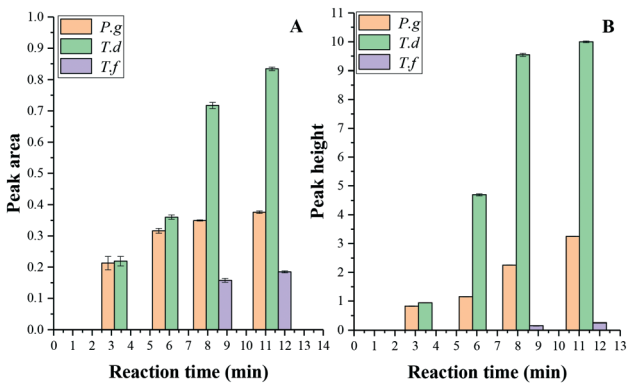

Fig. 6 The volume of the multi-PCR products with different reaction times calculated by (A) peak area and (B) peak height in the electropherogram.

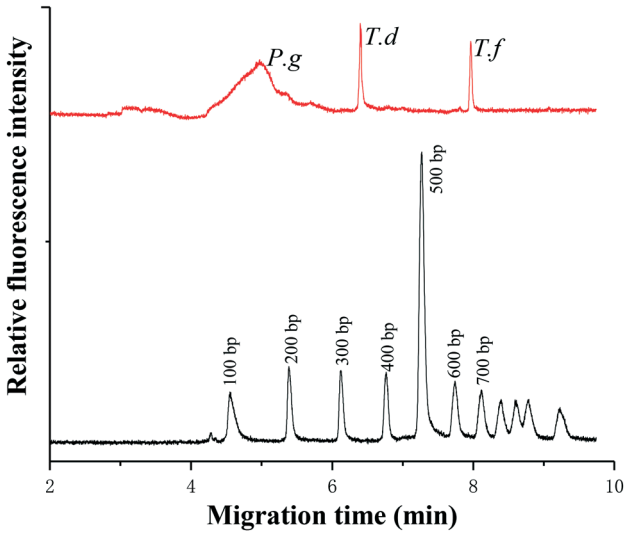

Fig. 7 Electropherogram of multi-PCR of periodontal pathogens from a real sample, which was performed by the *CF*-PCR microfluidic chip. The electrophoretic conditions were the same as those in Fig. 4.

although the peak height for *P.g* reached the maximum when the run time exceeded 8′25″. Therefore, we supposed that evaluating the volume of PCR products using the peak area in the electropherogram was more reasonable. Moreover, we also performed multiplex PCR on the T100 thermal cycler. The results demonstrated that only the PCR products of *P.g* were observed after 11′20″ (see Fig. S8 in the ESI†).

Examination of periodontal pathogens cells from a real sample

Finally, we applied this *CF*-PCR microfluidic chip to the amplification of *P.g*, *T.d* and *T.f* from a real sample. The gingival crevicular fluid was from a volunteer who was a 40-year-old man. It took about 12′33″ for the PCR solution to run from the inlet to the outlet of the microfluidic chip. Through electrophoresis of the PCR products and 100 bp DNA ladders under the same electrophoretic conditions, we found that there were PCR products corresponding to *P.g*, *T.d* and *T.f* (Fig. 7). We also carried out the PCR process for the same sample in the thermal cycler, and found that the volumes of the PCR products obtained by microfluidic chip (which had taken about 12′33″) were nearly equivalent to those obtained by the conventional PCR thermal cycler, which took about 47′52″ (see Fig. S9 in the ESI†).

Conclusions

In summary, we designed a *CF*-PCR microfluidic chip and fabricated a portable system for the multiplex amplification of periodontal pathogens. We analyzed the thermal distribution of the heaters for stable PCR, and then investigated typical primers applied for *P.g*, *T.d* and *T.f* for multi-PCR. Finally, we obtained optimal primers suitable for simultaneous amplification of *P.g*, *T.d* and *T.f* in the microchannels of the chip. The PCR products were validated

by a self-built CE system, and run-to-run experiments demonstrated that the variations in the peak height and migration time corresponding to the peak were ±0.56% and ±0.20%, respectively. The results showed that the target genes of *P.g* and *T.d* can be amplified in a short time of 3′48″, while those of *T.f* (641 bp) required at least 8′25″. We also found that when multi-PCR of these bacteria was performed in a traditional thermal cycler, only *P.g* was observed after 11′20″. Thus, *CF*-PCR demonstrates an overwhelming advantage in speed. However, it should be noted that this *CF*-PCR microfluidic chip can be reused 3 or 4 times after rinsing with water, and the solution will leak from the microchannels if they are rinsed more times, because the bonding between PDMS and the slide will be destroyed.

Author contributions

Zhenqing Li: project administration, supervision, funding acquisition, writing; Jiahui Liu and Lulu Zheng: methodology, data acquisition; Shinichi Sekine and Ping Wang: methodology, periodontal pathogen extraction; Chunxian Tao and Dawei Zhang: project administration, supervision, review & editing; Yoshinori Yamaguchi: data analysis, review & editing, conceptualization and supervising the research.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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