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Quantification of Periodontal Pathogens Cell Counts by Capillary Electrophoresis

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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

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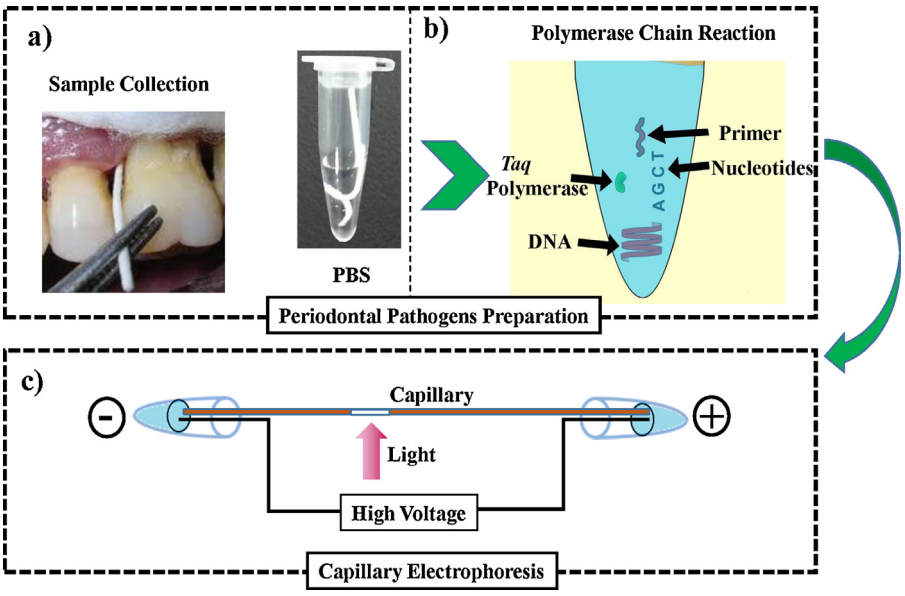

Fig. 1. Detection process of periodontal pathogens by CE. (a) Sample extraction schematic by PBS solution. (b) PCR amplification of the target periodontal pathogens. (c) Separation of the PCR products by self-build capillary electrophoresis.

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.