Using Absorbent Paper Strips for the Collection of Cell-Free DNA in Patients with Periodontal Diseases

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Abstract. The cell-free DNA in gingival crevicular fluid (GCF) is a recent interesting diagnostic marker in patients with periodontal disease. The methods used to collect GCF are very important to get the proper specimen. There are several techniques used for sample collection, such as gingival washing or using a capillary tube, however, these methods are not practical to perform in the clinic since they need well-trained examiner. This study aimed to use absorbent paper strips to collect cell-free DNA fragment in GCF of patients as a diagnostic measure for periodontal diseases comparing to conventional washing technique. Thirty-nine periodontitis and 26 gingivitis teeth were selected from 26 patients according to clinical parameters. The GCF sample was randomly collected from each tooth by either paper strips or washing technique. Then, after one week, the collection of samples from the same tooth was repeated with another technique. The samples were centrifuged to get cell-free DNA in the supernatant and extracted by InstaGene Matrix. The concentration and purity of extracted DNA were determined by NanoDrop spectrophotometer. Three sets of specific primers to the human β-globin gene were used to evaluate the DNA fragment lengths, by amplifying 110 base pair (bp), 536 bp, and 2000 bp products in the polymerase chain reaction (PCR). The results showed no significant differences in the cell-free DNA fragment concentrations and the prevalence of PCR products between using absorbent paper strips and washing technique in either gingivitis or periodontitis groups. Therefore, with the ease and rapidity of the technique, using the absorbent paper strips for the quantitative and qualitative investigation of cell-free DNA fragment in patients with periodontal diseases instead of washing technique is recommended. Further studies are still required to confirm this finding in a large group of patients as well as in patients with other systemic health problems.

1. Introduction

Periodontal diseases are common oral infectious diseases found in people worldwide. The diseases are characterized by an inflammation of periodontal tissue or supporting tissue of the teeth (gingiva, bone, and periodontal ligament) [1]. They are the major causes of tooth loss in an adult that have affected to function of chewing, talking, smiling and social appearance. Gingivitis is the initial stage of periodontal...
diseases which is a reversible condition and can usually be eliminated by professional prophylaxis. In contrast, the advanced form is called periodontitis of which the patients’ tissues undergo irreversible destruction during the inflammatory process.

Gingival crevicular fluid (GCF) is regarded as a promising mean in investigations associated with the diagnosis, pathogenesis, and prognosis of periodontal diseases. The amount of GCF and flow rate is an indicator of changes in vascular permeability which depends on the degree of inflammation. GCF is composed of inflammatory cells and mediators, bacteria, enzymatic and non-enzymatic components as well as cell-free DNA fragments from the damaged cells which are biomarkers for the diagnosis of periodontal diseases [2-5]. It can be collected from the gingival sulcus or periodontal pocket of the patients with periodontal diseases. Thaweboon et al has proposed using different polymerase chain reaction (PCR) products of cell-free DNA i.e. 110 base pairs (bp), 536 bp, and 2000 bp, specific to the human β-globin gene in GCF to classify the periodontal conditions of the patients [6] The 2000 bp and 536 bp products were found mostly from periodontitis teeth while only the 536 bp product was from gingivitis teeth.

There are several techniques used for GCF collection, such as gingival washing technique and the use of capillary tube [7]. However, these methods are not feasible to perform in the clinic since they need well-practiced examiner. In the gingival washing method, an isotonic solution such as a phosphate buffer solution (PBS) is pipetted to the gingival sulcus then all the contents and fluid from the sulcus will be aspirated back. This method is highly sensitive but requires the participation of a trained, experienced investigator to collect samples. For the capillary method, the small capillary tube is placed at the entrance of gingival sulcus until an adequate amount of fluid is obtained. Even though this method seems to be simple but it depends on crucial skill and takes time especially in healthy teeth. Using absorbent paper to collect GCF can be an alternative, especially for the detection of cell-free DNA fragment from the patients since it is fast, easy and can be applied to a particular site.

The aim of this study was to use absorbent paper strips to collect cell-free DNA fragment in GCF of patients as a diagnostic measure for periodontal diseases compared to conventional washing technique.

2. Materials and Methods

The study was conducted in the Oral Medicine and Periodontology Clinic, Faculty of Dentistry, Mahidol University. Twenty-six patients, who had at least 4 chronic periodontitis teeth, were recruited in this study. Patients who had any history of smoking, a systemic disease affecting periodontal disease progression (e.g. diabetes, immune deficiency disease), pregnancy or lactation at the time of the study, and had taken the antibiotic medication within 6 weeks prior to the study were excluded. The study protocol was approved by the committee of Institutional Review Board of the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Thailand (MU-DT/PY-IRB 2013/033.1009).

In the 1st visit, clinical parameter measurements were performed on each patient. The GCF sample was randomly collected from the selected tooth by washing technique or using an absorbent paper strip in the 2nd visit. Then, another technique was done during the 3rd visit. The time interval between visits was 7 to 10 days.

2.1. GCF Collection

Twenty-six patients were recruited in the study (13 men and 13 women, aged 53.10±5.28 years). One gingivitis tooth that was not adjacent to the periodontitis tooth was selected for the gingivitis group and one or two periodontitis teeth in the different quadrant from each patient were selected for the periodontitis group.

Modified washing technique [6]: a 5 µL aliquot of sterile phosphate buffered saline (PBS) solution (PBS, pH 7.2) was used to gently flush at the entrance of the pocket or sulcus by micropipette. Afterward, the aspirated contents and fluid were collected in a 1.5 ml sterile plastic microtube. The procedure was repeated 5 times at buccal aspect and another 5 times at lingual aspect of the selected teeth.

Absorbent paper strip technique: the top three depths of pocket from each tooth were selected for this collection. An absorbent paper strip was gently placed into each selected site until light resistance is felt.
It was left for 30 seconds [8]. All 3 paper strips were put in a 1.5 ml microtube containing 100 µl PBS at 4°C. The samples were eluted from the paper strips by a vortex mixer for 1 minute.

To separate the host cells, the samples from both techniques were centrifuged at 3,000 g for 10 minutes at 4°C. The supernatant, a cell-free fraction, was kept in 80% of ethanol at -20°C until DNA extraction was performed.

2.2. DNA Extraction and Polymerase Chain Reaction (PCR)
The cell-free fraction in ethanol was centrifuged at 13,000 g for 10 minutes at 4°C. The supernatant was removed and the tube was placed upside-down on a paper towel to remove excess ethanol from the pellet. The dried pellet was dissolved in 30 µL of sterile deionized water and mixed with 200 µL of DNA extraction liquid (InstaGene Matrix; Bio-Rad, Hercules, CA, USA) according to the manufacturing protocol. The concentration and purity of DNA sample were measured by NanoDrop2000c spectrophotometer (Thermo Scientific, USA).

Conventional PCR using 3-primer set specific to the human β-globin gene was done to amplify PCR products (110 bp, 536 bp, and 2000 bp) using forward and reverse primers as mentioned previously by Doan et al [6, 9]. DNA extracted from buccal cells of a healthy volunteer and deionized water was used as positive and negative controls, respectively. Afterward, all PCR products were observed on 1.5% agarose gel electrophoresis and ethidium bromide stain.

2.3. Statistical Analysis
DNA concentration from the two different techniques in the gingivitis or periodontitis groups were compared and analyzed by Pair T-test. Comparison of the positive number of each PCR product between washing technique and paper strips technique was evaluated by McNemar’s test. The significance level was set at a p-value less than 0.05.

3. Results
Cell-free DNA concentrations obtained by the paper strips and washing techniques in gingivitis or periodontitis groups were not significant differences between the two techniques (table 1). In addition, using paper strips seemed to yield a little more concentration of cell-free DNA than washing technique in the gingivitis group. Regarding the PCR product yield, there were no significances between the two techniques (table 2).

| Table 1. Cell-free DNA concentrations between paper strips and washing techniques in gingivitis and periodontitis groups. |
|----------------|----------------|----------------|-----------|
|                | Paper strips   | Washing technique | p-value  |
| Gingivitis (N=26) | 10.44±7.61    | 8.81±7.76       | >0.05     |
| Periodontitis (N=39) | 17.83±14.21  | 20.50±15.93     | >0.05     |

| Table 2. The number of samples with positive PCR products in the gingivitis and periodontitis groups. |
|----------------|----------------|----------------|-----------|
| PCR Product (bp) | Number of samples with positive PCR products |
| Paper strips | Washing technique | p-value |
| Gingivitis (N=26) | 536 | 19/26 | 18/26 | > 0.05 |
| Periodontitis (N=39) | 536 | 35/39 | 39/39 | > 0.05 |
| 2000 | 30/39 | 31/39 | > 0.05 |
4. Discussion
The cell-free DNA fragment found in serum, plasma, or other body fluids is a current interesting diagnostic tool in various medical fields [10-12] and dentistry [6, 9]. The cell-free DNA fragment released by dead cells in the fluid can directly represent the severity of inflammation and cell destruction (17, 18) as appeared in periodontal diseases. The present study showed that both techniques were suitable to collect the human cell-free DNA fragment in GCF because all samples could be amplified the 110 bp PCR product of the human β-globin gene. Although no significant difference in cell-free DNA concentration was noticed between the two techniques, the paper strips seemed to provide a higher yield, especially in the gingivitis group. This might be from the fact that paper strips could easily absorb all GCF in the shallow pocket of gingivitis condition while the washing technique was quite difficult to aspirate all the fluid back since it usually leaked out from the pocket. On the other hand, with the presence of deep pocket in the periodontitis group, the flushing and aspirating by washing technique could be done without any escape of the fluid.

With regard to the quality of cell-free DNA, the present study has shown the consistent result to that of our previous study [6] which stated that the 2000 bp PCR product could be as a biomarker to differentiate the periodontitis from gingivitis conditions. Furthermore, there was no significant difference in the prevalence of 536 bp and 2000 bp PCR products between using paper strips and washing technique. Several studies described the benefit of using absorbent paper strips for the quantitation of various inflammatory cytokines (IL-2,-4,-6,-8,-10, TNF-α,), enzymes (MMP-1,-2,-8,-13, myeloperoxidase, elastin) and proteins (osteocalcin, calprotectin, melatonin) in GCF to support clinical periodontal diagnosis in both pediatric and adult patients [7, 8, 13-16]. Therefore, it might imply that paper strips are efficiently used to collect GCF for the detection of cell-free DNA fragments which can be adequately recovered from the paper for further disease diagnosis in patients with periodontal diseases equivalent to the conventional washing technique.

5. Summary
With regard to the quantity and quality of cell-free DNA fragment, the absorbent paper strips are useful means for GCF collection in patients with periodontitis instead of the washing technique.

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