Assessing the use of Quantitative Light-induced Fluorescence-Digital as a clinical plaque assessment

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ABSTRACT

Background: The aims of this study were to compare the relationship between red fluorescent plaque (RF plaque) area by Quantitative Light-induced Fluorescence-Digital (QLF-D) and disclosed plaque area by two-tone disclosure, and to assess the bacterial composition of the RF plaque by real-time PCR.

Methods: Fifty healthy subjects were included and 600 facial surfaces of their anterior teeth were examined. QLF-D was taken on two separate occasions (before and after disclosing), and the RF plaque area was calculated based on Plaque Percent Index (PPI). After disclosing, the stained plaque area was analyzed to investigate the relationship with the RF plaque area. The relationship was evaluated using Pearson correlation and paired t-test. Then, the RF and non-red fluorescent (non-RF) plaque samples were obtained from the same subject for real-time PCR test. Total 10 plaque samples were compared the ratio of the 6 of bacteria using Wilcoxon signed rank test.

Results: Regarding the paired t-test, the blue-staining plaque area (9.3 ± 9.2) showed significantly similarity with the RF plaque area (9.1 ± 14.9, p = 0.80) at ΔR20, however, the red-staining plaque area (31.6 ± 20.9) presented difference from the RF plaque area (p < 0.0001). In addition, bacterial composition of Prevotella intermedia and Streptococcus anginosus was associated with substantially more the RF plaque than the non-RF plaque (p < 0.05).

Conclusions: The plaque assessment method using QLF-D has potential to detect mature plaque, and the plaque area was associated with the blue-staining area using two-tone disclosure.

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1. Introduction

Detection of old plaque in oral cavity has distinct advantages to caution against oral disease. Old and mature plaque cause oral disease and it could be a sign to notify risk of oral disease. However, young plaque do not immediately affect patients' oral disease. Two-tone disclosing agent was developed to distinguish old and young plaque due to the result of diffusion phenomenon of active ingredient [1,2]. It has been frequently used in dental clinic for old plaque assessment. However, plaque disclosures have some limitations of which they cannot selectively disclose only plaque, but dye soft debris and pellicle as well [3]. Also, it needs time to remove the plaque at the chair side. Another method of measurement for old plaque is Silness & Löe plaque index [4]. This index has been developed for grading of plaque thickness. However, it is also relatively time consuming and the result may be influenced by the examiner's subjective decision [5].

Quantitative Light-induced Fluorescence-Digital (QLF-D BilluminatorIII, Inspektor Research Systems BV, Amsterdam, The Netherlands) is a novel dental diagnostic tool which is based on the autofluorescence of teeth. It is the updated version of the first product, the QLF device (InspektorTM Pro, Inspektor Research Systems BV, Amsterdam, The Netherlands), and it is able to get more clear plaque image in red using improved filter set (D007; Inspektor Research Systems BV, Amsterdam, The Netherlands). When a tooth with plaque is excited by a visible light of 405 nm from the QLF, red fluorescence were shown on the plaque accumulation area [6,7], and the QLF was able to detect and quantify the area. Previous studies have shown that mature plaque may produce red auto-fluorescence and it is associated with products of microbe metabolism which are called porphyrins [5,6]. The porphyrins are known to be produced from late colonizing oral bacteria, such
as *Porphyromonas gingivalis* and *Prevotella intermedia* which are usually found in heavily accumulated plaque [8]. A recent study reported that the intensity of red fluorescence of plaque which aged in different concentrations of sucrose had a relationship with low pH and cariogenic plaque [7]. Nevertheless, there is still lack of clinical studies on the characteristics of the RF plaque and its potential pathogenicity.

The prevalence of microorganisms can be investigated using the 16S-rRNA-based polymerase chain reaction (PCR) method. The PCR method has been known that it is the most sensitive and rapid method [9], and real-time PCR using the LightCycler™ system is useful to detect and quantify bacteria in clinical samples [10].

To increase the utility of the QLF-D into dental clinic, more studies are required. Therefore, the aims of this study were to evaluate the quantification method of the RF plaque area by QLF-D can replace existing old plaque assessment method by two-tone disclosure, and to compare of bacterial composition ratio between the RF and non-fluorescent plaque (non-RF plaque) by real-time PCR test in vivo.

2. Materials and methods

2.1. Subjects

Ethical approval was obtained from the Yonsei University Dental Hospital (IRB No: 2-2012-0045). This study was performed from December 2012 to June 2013. This study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. Total 50 participants through clinical trial recruitment were included, with a mean age of 34.6 years (±11.3). Inclusion criteria were that the participants have sound anterior teeth with good general health. Volunteer who had stained teeth or dental caries region were excluded. Informed consent was given when the participants visited for this study. They were asked to refrain from any oral hygiene behavior and food intake for at least 4 h before visiting.

2.2. Quantitative Light-induced Fluorescence-digital examination

Intra-oral photographs with QLF-D were taken on two occasions before and after disclosing procedure with disclosing solution (2-Tone™, Young Dental, Earth City, USA). Facial surface of upper and lower anterior teeth were taken with edge to edge bite. The tooth surfaces were dried before photographing. Two different images, a QLF image and a white light image, were captured at one shooting with a digital SLR camera (model 550D, Canon, Tokyo, Japan) using following condition: shutter speed of 1/30 s (QLF image) and 1/50 s (white light image), aperture value of 5.6 (QLF image) and 8.0 (white light image), focal length of 0.32 mm, and ISO speed of 1600. The camera was vertically placed on the facial surface. The images were automatically stored by default as a bitmap image (BMP). To reduce ambient light, we covered the cone of the QLF-D with a blackout fabric.

2.3. Image analysis of the plaque area

Among 600 anterior teeth, 170 teeth (28.3%) showing the stained plaque in blue (blue plaque) with the disclosing agent when an examiner observed with naked eyes, were selected to investigate the relationship of the plaque area between the RF plaque and the disclosed plaque. Plaque area from the plaque images was revealed as Plaque Percent Index (PPI). The index was calculated by the pixel number of tooth and covered plaque area based on planimetric method [11,12]. The QLF image of the RF plaque was analyzed using proprietary software (QA2 v1.21, Inspektor Research Systems BV, Amsterdam, The Netherlands) (Fig. 2(E)). The software provides pixel numbers of whole tooth area and intensities of red fluorescence as the thirteen threshold levels (from ΔR0 to ΔR120). As increasing the threshold level, it means that fluorescence intensity is getting stronger. For example, ΔR30 means that at least 30% of redness difference with respect to that of sound teeth is exist between the plaque and the tooth [13], and ΔR120 is the strongest red intensity of the plaque. And white light image of the disclosed plaque was analyzed using image analysis software (Image-Pro PLIS, Media Cybernetics, MD, USA). An outline was drawn using an irregular AOI options (Fig. 2(A)–(D)). Then the tap called ‘count & measure object’ and ‘select colors’ on manual options were used to adjust color-range within histogram base. The images were generated using a function which displays the value of the red, green, and blue channel (RGB). The red and blue values in the histogram were fixed as 255 and the value of green was adjusted to find thresholds of a border line of the red- and blue-staining plaque. When the red-staining plaque area was selected, the blue-staining area was included. Forty teeth were randomly selected and analyzed to decide the optimum thresholds which could be determined as acceptable on visual assessment by a single examiner. The final threshold was decided as 49 for a border line of the stained plaque in red (red plaque) and 29 for that of the blue plaque. Examiner then transferred it to an Excel spreadsheet to calculate the PPI (PPI<sub>rg</sub>, PPI<sub>red</sub>, and PPI<sub>blue</sub>). All analysis was performed by a single examiner.

2.4. Real-time PCR test

To investigate the characteristics of the bacterial composition of the RF plaque, real-time PCR test was performed. Among 50 participants, the plaque samples from 10 subjects (20%) were collected. Plaque emitting red fluorescence was collected as the RF plaque sample. And if the plaque was dyed and did not show red fluorescence, it was collected as the non-RF plaque sample to compare the bacterial composition. The RF and the non-RF plaque samples were obtained from different teeth of the same subject.

The test was performed according to the manufacturer’s instructions. Plaque samples were collected from subject’s anterior teeth using sterilized dental probe. The samples were put into a 1.5 ml tube containing 1 ml sterilized distilled water then they were stored in a freezer at –70 °C as soon as possible until their use. Whole genomic DNA was extracted using DNeasy Blood & Tissue kit (Qugen, Chatsworth, CA, USA). Then isolated DNA was quantified by Spectrophotometer (Nanodrop ND-1000; NanoDrop Technologies, DE, USA). Real-time PCR amplification reactions were carried out using Master mixture of 1 μl DNA. The Master mixture used in this study was Light Cycler 480 SYBR Green (Roche Diagnostics, Basel, Switzerland) with LC480II(Roche, Basel, Switzerland). The following bacteria were studied: Streptococcus mutans [14], Lactobacillus casei [15], Actinomyces israelii [16], Streptococcus anginosus [17], P. gingivalis [18], and *P. intermedia* [19].

The condition for initial denaturation of six bacteria was at 95 °C for 10 min. 50 polymerase chain reaction method cycles were as follows: *P. gingivalis*, *S. mutans*: 50 cycles of 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 20 s, and *A. israelii*, *L. casei*, *P. intermedia*, *S. anginosus* and total bacteria: 50 cycles of 95 °C for 20 s, 50 °C for 20 s, and 72 °C for 20 s. After the amplification, melting curve analysis was performed to identify whether the real-time PCR reaction ordinarily was done.

To compare bacterial compositions between the RF and the non-RF plaque, relative quantification was performed. The result expressed as C<sub>T</sub> which is the number of cycle passed threshold to detect the mRNA. To normalize the value, we carried out ΔC<sub>T</sub> (target mean C-reference mean C<sub>T</sub>). Higher ΔC<sub>T</sub> means lower expression of mRNA. Each ΔC<sub>T</sub> of the samples was used to compare the
mRNA amount. For this analysis, LightCycler 480 software (LC480 1.5.0.39, Roche Applied Science, Mannheim, Germany) was used.

2.5. Data analysis

Pearson correlation coefficient and paired $t$-test were tested to compare plaque area between the RF plaque and the disclosed plaque (blue and red plaque). In comparison of the mRNA amount of the 6 bacteria between the RF and the non-RF plaque, data was tested using Wilcoxon-signed rank test using PASW Statistics ver.18.0 (SPSS, Chicago, IL, USA) ($\alpha = 0.05$).

3. Results

3.1. Comparison of plaque area between RF plaque and disclosed plaque

As a result of the correlation analysis, the PPI$_{RF}$ shows that higher correlation with the blue plaque area (PPI$_{blue}$) than that with the red plaque area (PPI$_{red}$) at every $\Delta R$ levels (Fig. 1). The correlation coefficient of the PPI$_{red}$, on the other hand, shows gradually decreasing tendency. Scatterplot matrix demonstrated that the PPI$_{blue}$ was observed slightly linear distribution, on the other hand, there was no linear association in the PPI$_{red}$ (Fig. 3). Regarding the paired $t$-test, the PPI$_{red}$ was significantly different from the PPI$_{RF}$ at $\Delta R20$ ($p < 0.0001$), however, the PPI$_{blue}$ presented similarity with the PPI$_{RF}$ at $\Delta R20$ ($p = 0.80$) (Table 1).

3.2. Comparison of bacterial composition ratio between RF plaque and non-RF plaque

The sequences of primers were shown in Table 2. Regarding the Wilcoxon-signed rank test, P. intermedia and S. anginosus in the RF plaque shows significantly more amount than that in the non-RF plaque (Table 3, $p < 0.05$). Although there was no statistical difference, A. israelii and P. gingivalis showed more amount in the RF plaque than that in the non-RF plaque. S. mutants and L. casei, which are known not emitting red fluorescence, were also observed in the RF plaque with similar amount to the non-RF plaque.

4. Discussion

In this study, the characteristics of the RF plaque were investigated with two experimental approaches. The one was the clinical approach by comparing with a representative disclosing method. The other one was molecular biological analysis of dental biofilm. First, it was observed that the RF plaque area was significantly similar with blue plaque area by two-tone agent. The area of the RF plaque demonstrated a higher correlation with the area of blue plaque than that of red plaque (Fig. 1). Second, the RF plaque was compared with non-RF plaque in real-time PCR test. At the results of assessing the composition ratio of each 6 bacterial species associated with dental caries and/or periodontal disease, the $\Delta Ct$ value in the RF plaque was lower than that in the non-RF plaque except for in S. mutans. In particular, P. intermedia and S. anginosus showed a significant difference between two plaque samples (Table 3, $p < 0.05$).

Red auto-fluorescence from endogenous porphyrins emitted with a wavelength of 405 nm has been observed in old and mature plaque [5,6,8,20]. Kim et al. reported that the red fluorescence of dental microcosm biofilm was observed from the 3rd day, and the intensity of the RF was increased over time [20]. The authors demonstrated that the aciduric bacterial CFUs and the severity of demineralization were increased with maturation of biofilm. Red auto-fluorescence is also observed in carious lesions even though cariogenic species have been known that they might do not fluoresce red [7,21,22]. Coulthwaite et al. [6] explained the reason that mature plaque may play role as harbors of plaque. Hence, the bacteria which do not fluoresce red could be observed in the RF plaque. The QLF-D, therefore, can be used as a detecting device to distinguish between pathological region and healthy tooth surface by assessing the red fluorescence. With this context, QLF system can substitute for conventional staining procedure with two-tone disclosure to find old plaque. The QLF-D device can capture two images (QLF image and white light image), and it can compare between before and after oral hygiene care. Furthermore, it will be leading to not only reduction of time to remove disclosing parts but also patient’s compliance for oral health instruction.

In comparison on the plaque area, the RF plaque demonstrated a higher correlation with the blue plaque area than the RF plaque, red fluorescent plaque.
red plaque area. As redness intensity was rising, the correlation coefficient with blue plaque was improved. The highest correlation with blue plaque was shown at the PPI of $\Delta R_{110}$ ($r = 0.62, p < 0.01$). This result was in accordance with previous studies which reported a relation between the RF and maturation of plaque [5,6]. However, the PPI of the RF plaque at $\Delta R_{20}$ was only 4.4% respect to the tooth surface area, and it was not similar with blue plaque area in the paired t-test. On the other hands, the PPI of the RF plaque at $\Delta R_{20}$ showed the similarity as 9.1% with the blue plaque area. Regarding the paired t-test results, we chose the $\Delta R_{20}$ as criteria for detecting old plaque assessment then the PPI at the $\Delta R_{20}$ was presented in Table 1.

There is lack of study comparing the RF plaque and disclosed plaque by two-tone agent. Early study which used a previous version of QLF system demonstrated that the QLF plaque analysis was a reliable technique but auto-fluorescing plaque volume is not related to total plaque volume [5]. In the study, however, authors compared between the RF plaque and disclosed plaque using a single type of disclosing agent. The other study was conducted to assess the relationship between the RF plaque and dark-blue stained plaque [23]. The result showed that width of red-fluorescing plaque had high correlation with that of dark-blue stained plaque ($\rho = 0.61, p < 0.001$). This study, however, measured plaque using an ordinal scale based on Quigley–Hein plaque index and did not consider relationship with red stained plaque area.

**Fig. 2.** Image analysis procedure of the stained plaque on the tooth surface (A) outlining (B) selected tooth surface area (C) selected red stained plaque area (D) selected blue stained plaque area (E) RF plaque area with QLF-D.

**Fig. 3.** Scatterplot matrix of red (A) and blue (B) stained plaque area with the RF plaque area at the highest correlation threshold. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
(young plaque). On the other hand, the present study compared the previous studies was found more information according to comparing the RF plaque at 13 threshold levels of red intensity with young and old plaque due to continuous scale and image analysis.

In comparison of composition of bacteria, relative quantity of P. intermedia and S. anginosus in the RF plaque was significantly larger than that in the non-RF plaque (Table 3, p < 0.05). More L. casei, A. israelii, P. gingivalis were existed in the RF plaque (p > 0.05), however the p-values of A. israelii and P. gingivalis were much smaller (p = 0.114 and 0.093, respectively) than that of L. casei (p = 0.103). On the other hand, relative quantity of S. mutans showed that there was no difference between in the non-RF plaque and in the RF plaque (p = 0.575). As results, there were substantially more bacterial species except for S. mutans in the RF plaque than in the non-RF plaque, including the caries pathogens. The common caries pathogens, Lactobacilli and Streptococci, are known that they do not show red fluorescence [24]. As a pilot study, the result of real-time PCR test in this study was investigated a potential of the QLF system for assessing old plaque. Therefore, further studies are required with increasing the number of bacteria species.

The RF plaque has been known to be composed of bacteria which are usually found in mature plaque, such as anaerobic bacteria [6,8]. P. gingivalis, P. intermedia and Fusobacterium nucleatum was mainly found in mature plaque and they are considered as a pathogenic microbiota related in periodontal disease [23]. A. israelii concerned in early periodontitis and root caries has been frequently found in heavy and mature plaque [6]. These species have been detected on supragingival plaque even if it was not associated with the presence of active dental caries [26]. In one of the early studies, the authors concluded that when the thickness of biofilm increases with aging, increased intensity of the red fluorescence was observed by QLF [8]. They reported that Actinomyces odontolyticus and P. intermedia showed a strong red fluorescence, and aerobic bacteria however did not show red fluorescence. Also, P. gingivalis displayed obligate red fluorescence when it existed with Peptostreptococcus micros. And the red fluorescent plaque was comprised 62% of total plaque.

In previous studies, differences in red fluorescence intensity in vitro were examined due to different cariogenic characteristics [8,27]. Some cariogenic bacteria did not reveal red auto-fluorescence when they exist as a single species [27]. The results however revealed that with increasing cariogenicity, intensity of red fluorescence was higher. The authors explained that this occurred because the cariogenic biofilm is associated with the production of porphyrin complex. The other study was conducted revealing higher red fluorescence with increasing maturity [28]. The researchers evaluated the effect of time and biofilm thickness on red fluorescence using in vitro biofilm model. After 7 days, red fluorescence was observed and the intensity was higher with increasing thickness of the biofilm (r² = 0.47, p < 0.001). Thus, diversity of bacteria would be more associated with the intensity of the red fluorescence rather than the presence of a single bacteria species. It can be explained that why caries pathogen were detected in the RF plaque in present study. To enhance the utilization of QLF-D in research and clinical environment, further studies are needed to investigate bacterial species in the RF plaque and its role. From our results, it was expected that the QLF technology can be used as a novel method for detecting mature plaque based on microbial characteristics. In order to do this, further studies using more various bacterial species are needed.

5. Conclusions

The plaque assessment method using the QLF-D was a clinical acceptable tool to detect mature plaque. The consists of bacteria was more associated with the maturity of plaque rather than the presence of a certain single species, and using two-tone plaque was associated with the blue stained area using two-tone disclosure.

Conflict of interest

The authors declare that they have no conflict of interest.

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