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Association between the cariogenicity of a dental microcosm biofilm and its red fluorescence detected by Quantitative Light-induced Fluorescence-Digital (QLF-D)

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ABSTRACT

Objective: This study evaluated whether Quantitative Light-induced Fluorescence-Digital (QLF-D) can detect the levels of cariogenicity of dental microcosm biofilms by assessing the red fluorescence intensity.

Methods: Dental microcosm biofilms were initiated from human saliva on bovine enamel discs. Biofilms with various levels of cariogenicity were then grown in artificial saliva supplemented with sucrose at different concentrations (0.05%, 0.1%, 0.2%, and 0.5%) in 24-well microplates. After 10 days, fluorescence images of the biofilms were captured by the QLF-D to analyse the red fluorescence intensity, which was quantified as the red/green ratio (R/G value). The supernatant pH was also measured, as well as the total and aciduric bacteria counts of the collected biofilms. Mineral loss in enamel was also evaluated by calculating the percentage of surface microhardness changes (%SHC).

Results: The R/G values of the biofilms differed significantly with the sucrose concentration ($p < 0.0001$), increasing consistently as the sucrose concentration increased from 0.05% (=0.91) to 0.5% (=2.56). Strong correlation was identified between the R/G value and the number of aciduric bacteria ($r = 0.83$, $p < 0.0001$), supernatant pH ($r = -0.95$, $p < 0.0001$), and %SHC ($r = 0.90$, $p < 0.0001$).

Conclusions: The red fluorescence as observed by the QLF-D was correlated with the cariogenic properties of dental microcosm biofilms *in vitro*, which indicates that this device can be used to detect the levels of cariogenicity of a dental biofilm.

Clinical significance: The QLF-D is able to assess the cariogenic levels of dental plaque based on the intensity of red fluorescence.

1. Introduction

Dental plaque represents a complex ecosystem composed of numerous species of bacteria inhabiting a tooth surface as a biofilm.[1] In response to changing environmental factors that induce the caries process, the microbial composition of dental biofilms changes from a balanced state to an imbalanced state, in which cariogenic bacteria, such as acidogenic and aciduric species, become dominant. The imbalances in the resident

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microflora result in the formation of cariogenic biofilms, which further induces demineralisation of dental hard tissue.[1–3] It would be helpful to be able to detect cariogenic dental biofilms and assess the metabolic activities of the resident microflora in these dental biofilms in order to successfully control and manage the development of dental caries.[4–6] Also, because the cariogenicity of the dental plaque is directly associated with the initiation and severity of dental caries,[5] knowledge of cariogenic biofilms based on scientific evidence and objective standards would facilitate the ability to predict the risk of dental caries.[4] In addition, the cariogenicity of dental plaque can vary between individuals and even at sites within the same oral cavity due to caries-related factors such as oral hygiene, dentition, diet, and saliva, which makes an understanding of the qualitative properties of the biofilms essential.[6]

Since plaque properties are determined by interactions between internal bacteria, it is vital to reproduce the natural microbial ecology in order to evaluate and research these properties. At the same time, the ethical issues and limited access of *in vivo* studies leads to a need for a laboratory model to allow investigations under controlled conditions.[7,8] However, dental biofilms composed of a single or only a few species do not represent the diversity, complexity, and heterogeneity of *in vivo* plaque. For these reasons, the dental microcosm biofilm formed from saliva inoculum, which is a resource of natural oral microflora has been used in various laboratory studies.[9–11]

A new type of Quantitative Light-induced Fluorescence (QLF) device called the QLF-D Biluminator™ (Inspektor Research Systems BV, Amsterdam, The Netherlands) has been newly developed to enable the detection and quantification of dental plaque by representing endogenous porphyrins produced by oral bacteria species as red fluorescence.[12–14] The auto fluorescence phenomenon of this equipment is known to be caused by certain oral bacteria that can synthesize high concentrations of endogenous metal-free fluorescent porphyrin.[15] The QLF-D Biluminator™ (henceforth referred to simply as the QLF-D) is an upgraded version of QLF devices that examines plaque more clearly as red fluorescence by strengthening these principles, making quantification of the plaque possible. This new device uses a narrow-band blue light source (centred at 405 nm) obtained by modifying the filter set (D007; Inspektor Research Systems BV, Amsterdam, The Netherlands) and consists of a Biluminator™ mounted on a high-specification digital single-lens reflex (SLR) camera fitted with a 60-mm macro lens, which is equipped with an illumination tube with white and blue light-emitting diodes positioned in a ring around the lens opening (Fig. 1). This device can produce high-quality photographs without any requirement for ambient light, visualize plaque more clearly, and detect subtle changes in plaque at a high resolution.[14] When viewed under its lighting conditions, regions where it is difficult to detect the presence of plaque with the naked eye are shown as red fluorescence, while the teeth themselves appear in their white natural state. These characteristics may make the new device useful as a diagnostic clinical tool for plaque control, as well as in the laboratory for the analysis of plaque.[14]

Previous studies found that the QLF could be used for the evaluation of dental plaque by presenting differences in fluorescence based on characteristic changes therein. When used under optimal conditions the QLF presented heavy dental plaque deposits on teeth as orange or deep red fluorescence, which indicated the presence of black-pigmented obligate anaerobes and secondary plaque colonizers.[16,17] Also, it was observed that the microbial composition of the plaque changed from Gram-positive to Gram-negative as well as from facultative to obligate anaerobes as it matured and also that the colour of the plaque fluorescence changed from being predominantly green to red or that the intensity of red fluorescence increased.[15,18] However, these findings were confirmed only in the previous QLF studies, and not in QLF-D studies; moreover, no previous study has investigated the ability of the new device to diagnose dental plaque. The intensity of red fluorescence seen during clinical investigations designed to observe dental plaque on tooth surfaces under the QLF-D has been found to vary between individuals and also between sites in the same oral cavity. Moreover, no study has identified any correlation of this red fluorescence intensity detected by the QLF-D with the cariogenicity of the dental biofilms. Thus, if the ability of the QLF-D to distinguish

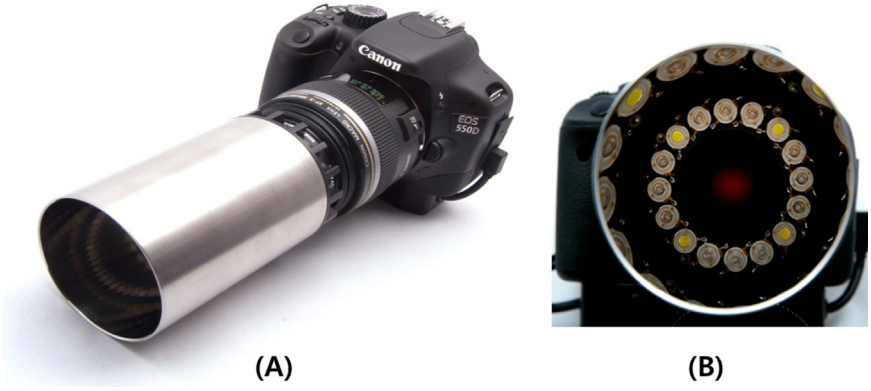

Fig. 1 – The QLF-D Biluminator™ used in this study (image courtesy of Inspektor Research Systems). This device (A) is based on a full-sensor SLR camera (Canon 550D) equipped with an illumination tube with white and blue light-emitting diodes positioned in a ring around the lens opening (B).

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between qualitative differences of dental biofilms could be verified, the cariogenicity of the biofilms could be predicted noninvasively by simply observing and analyzing the red fluorescence intensity of dental plaque.

Therefore, the aim of this study was to determine whether the QLF-D can detect various levels of cariogenicity of dental biofilms by assessing the red fluorescence intensity detected from the dental biofilms.

2. Materials and methods

Biofilms were formed in a caries-related microcosm biofilm model as described in detail by Filoche et al.,[8] but modified for this study, in which 24-well cell culture plates and bovine enamel specimens were used. To allow the growth of biofilms with different cariogenicity, the growth conditions were varied by supplementing Basal Medium Mucin (BMM)[7] – on which biofilms grow – with sucrose at four concentrations: 0.05%, 0.1%, 0.2%, and 0.5%. The experiments involved four groups, with each comprising three enamel specimens. Each experiment was repeated 3 times, resulting in a total of 36 specimens.

Human saliva was used as an inoculum, and the biofilms were grown in the BMM supplemented with sucrose at different concentrations for 10 days. The growth medium was replaced daily. After 10 days, fluorescence images were captured by the QLF-D to assess the red fluorescence intensity of the biofilms. The biofilms were then collected to determine the total and aciduric bacteria counts, and the supernatant pH was measured to analyse their cariogenic properties. Mineral loss in the enamel discs was also evaluated by calculating the percentage of surface microhardness change.

2.1. Preparation of enamel discs

Enamel specimens were obtained from sound bovine incisors without defects such as cracks and white spots. The labial surfaces of the bovine incisors were sectioned (8 mm × 3 mm) using a low-speed diamond saw, and the sections were serially ground flat with water-cooled abrasive sand papers (600, 800, 1000, and 1200 grit). All specimens were then embedded in circular acrylic moulds at 1 mm below its surface, thereby leaving a 1-mm space for the biofilms to accumulate. Before the enamel blocks received any treatment, the baseline surface microhardness [quantified as the Vickers microhardness number ($VHN_{baseline}$)] of each enamel block was measured by making four indentations (200 g, 10 s; JT Toshi, Tokyo, Japan) on the enamel surface, with the measurement points separated by 100 μm. The mean value of the four measurements was calculated for each specimen.

2.2. Inoculation

Human saliva was used as an inoculum to form dental microcosm biofilms consisting of the bacteria found in the oral cavity. Stimulated saliva was obtained from a healthy male donor without active caries or periodontal disease and no history of antibiotic use within the previous 3 months. The donor had not performed any oral hygiene practices for 24 h prior to the collection of paraffin wax-stimulated saliva samples.[8] The collected saliva samples were filtered through sterilized glass wool (Duksan Chemicals, Ansan, Korea) to remove any debris, and diluted in sterile glycerol to achieve a final concentration of 30% glycerol stock. The prepared saliva was frozen for the experiment, and the same batch of frozen saliva was used as an inoculum for each set of experiments.

2.3. Microcosm dental biofilm formation

Approximately 1.5 ml of the prepared saliva was inoculated onto each enamel block in each well of the 24-well cell culture plates, and the plates were incubated anaerobically at 37 °C for 4 h. The saliva was gently aspirated from the base of the wells, and 1.5 ml of growth medium that contained BMM and sucrose was added to each well. Sucrose concentrations of 0.05%, 0.1%, 0.2%, and 0.5% were used. The BMM artificial saliva medium contained 2.5 g/l porcine mucin (type III, Sigma Chemicals, MO, USA), 10.0 g/l proteose peptone, 5.0 g/l trypticase peptone, 5.0 g/l yeast extract, 1 mmol/l urea, 1 mmol/l arginine, 2.5 g/l KCl, and 1 mg/l menadione (pH 7.0).[7] The plates were then incubated under an anaerobic hood with in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 at 37 °C for 10 days. The growth medium of each well was replaced daily by transporting the enamel block to a new well containing 1.5 ml of fresh growth medium.

2.4. Analysis of biofilm red fluorescence intensity using the QLF-D

The red fluorescence of the 10 day-matured biofilms was evaluated using the QLF-D (QLF-D Biluminator™, Inspektor Research systems BV, Amsterdam, The Netherlands). Fluorescence images of all enamel specimens were captured with a 'Live View'-enabled digital full-sensor SLR camera (model 550D, Canon, Tokyo, Japan) at the following setting: shutter speed of 1/45 s, aperture value of 3.2, and ISO speed of 1600. Proprietary software (C3 v1.16, Inspektor Research Systems BV) was used to capture and store all digital images on a PC automatically. All fluorescence images were analysed using a computer programme (Image-Pro PLUS 6.0, Media Cybernetics, Washington, USA) by measuring red and green values in the same area of biofilms on each block. The red and green values were measured for every pixel within selected areas, and the average red/green ratio (R/G value) was calculated for each image to compare the red fluorescence intensities of the biofilms with various levels of cariogenicity. All analyses were performed by a single trained examiner.

2.5. Analysis of biofilm microbial composition

On the 10th day of the experiment, the enamel blocks with biofilms were rinsed with 1 ml of cysteine peptone water (CPW) three times to remove loose bacteria. The blocks with biofilms were then transferred into conical tubes containing 2 ml of CPW, and the biofilms were dispersed by vigorous vortexing for 1 min and sonication for 1 min. The bacterial suspensions were then serially diluted (10^0–10^{-8}) in CPW and plated in duplicate onto a 5% tryptic soy blood agar plate in order to determine total bacteria counts. The suspensions

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Table 1 – Mean ± SD values of cariogenicity variables and the red fluorescence of dental microcosm biofilms (*R/G* value) according to different sucrose concentrations.

Sucrose concentration (%)	*R/G* value (ratio)	Total bacteria CFUs (log_{10} CFUs)	Aciduric bacteria CFUs (log_{10} CFUs)	Supernatant pH (pH)	%SHC (%)
0.05	0.91 ± 0.16a	8.44 ± 0.11a	7.37 ± 0.38a	7.68 ± 0.27a	39.78 ± 6.04a
0.1	1.02 ± 0.19a	8.51 ± 0.09a	7.65 ± 0.20b	7.34 ± 0.19b	59.28 ± 3.66b
0.2	1.55 ± 0.16b	8.81 ± 0.03b	8.16 ± 0.09c	6.85 ± 0.13c	77.25 ± 1.01c
0.5	2.56 ± 0.05c	8.57 ± 0.24a	8.43 ± 0.20d	4.91 ± 0.11d	95.51 ± 2.68d

Different letters within the same column indicate significant differences between groups by Tukey's *post hoc* analysis at $\alpha = 0.05$.
R/G values mean the ratios of red pixels to green pixels in biofilm red-fluorescence images captured by the QLF-D.
CFUs means colony-forming units.
%SHC is the percentage of surface microhardness change calculated as $\%SHC = 100(VHN_{baseline} - VHN_{after})/VHN_{baseline}$.

were also plated onto a brain heart infusion agar plate adjusted to pH 4.8 for aciduric bacteria.[9] All plates were incubated in an anaerobic hood as described above for 72 h. The numbers of colony-forming units (CFUs) of total and aciduric bacteria were measured and calculated by a single trained examiner.

2.6. *Measurement of biofilm acidogenicity*

Before the biofilms were harvested following the 10 days of growth, the supernatant (in which biofilms were matured) was collected from each well, and the pH of the supernatant was measured using a pH electrode (Orion 4-Star, Thermo Scientific, Waltham, USA) to assess the acidogenicity of the biofilms.

2.7. *Measurements of microhardness changes*

After the biofilms were collected from the enamel blocks, the surface microhardness of each enamel block was again measured (*i.e.*, the VHN_{after} value) by making four indentations (200 g, 10 s) at measurement points separated by 100 μm, and the change in microhardness was calculated as the percentage of surface microhardness change (%SHC) according to $\%SHC = 100(VHN_{baseline} - VHN_{after})/VHN_{baseline}$. This procedure was carried out by a single blind and trained examiner.

2.8. *Statistical analysis*

The variations of the cariogenicity variables and red fluorescence values of biofilms according to the sucrose concentration were assessed using one-way ANOVA, and this was followed by Tukey's test to identify statistically significant differences between the groups. Pearson's correlation coefficient was also calculated to evaluate associations between the *R/G* values and the cariogenic properties of the biofilms.[19] All statistical procedures were performed using the PASW Statistics 18.0, (SPSS, IBM Corporation, Somers, NY 10589, USA), and the level of significance was set at $\alpha = 0.05$.

3. Results

The QLF-D results are presented as the *R/G* values of the dental biofilms in Table 1. These values increased consistently as the sucrose concentration increased from 0.05% (=0.91) to 0.5% (=2.56), being differed significantly with the sucrose concentration except for the between 0.05% and 0.1%. Examples of the fluorescence images of the 10-day-matured dental microcosm biofilms that formed on the enamel surface in artificial saliva supplemented with sucrose at different concentrations are given in Fig. 2.

The cariogenicity of the biofilms increased with the sucrose concentration. All of the analysed variables except for the total bacteria CFUs varied approximately linearly (either increasing or decreasing) with the sucrose concentration (Table 1). The aciduric bacteria CFUs in the microcosm biofilms increased significantly with the sucrose concentration ($p < 0.0001$). However, the total bacteria CFUs did not follow this tendency, not consistently increasing with the sucrose concentrations. The mean values of the supernatant pH significantly

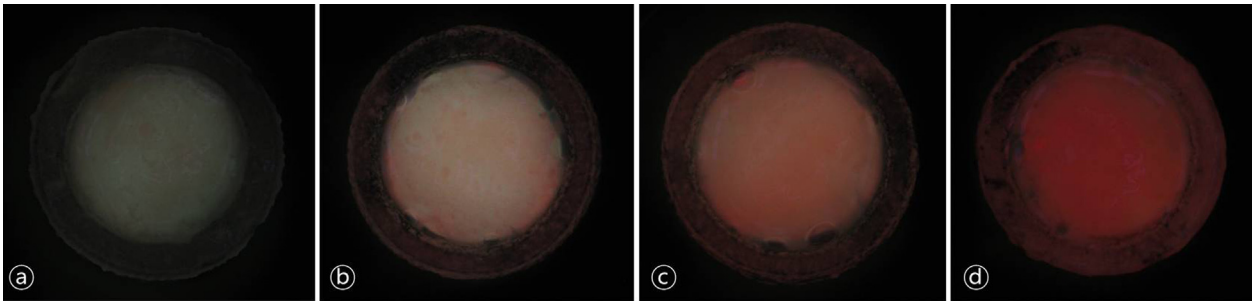

Fig. 2 – QLF-D fluorescence images of dental microcosm biofilms formed on the enamel discs after 10 days in artificial saliva supplemented with sucrose at different concentrations: 0.05% (a), 0.1% (b), 0.2% (c), and 0.5% (d).

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Table 2 – Correlations (*r* values) of the red fluorescence intensity (quantified as the R/G value) with cariogenicity variables of dental microcosm biofilms.

R/G	Cariogenicity variable			
	Total bacteria CFUs	Aciduric bacteria CFUs	Supernatant pH	%SHC
r	0.30	0.83*	−0.95*	0.90*
p	0.073	<0.0001	<0.0001	<0.0001

r is the Pearson correlation coefficient.
* $p < 0.01$.

decreased with increasing sucrose concentration, indicating that the biofilm acidogenicity increased with the sucrose concentration ($p < 0.0001$). The %SHC also increased with the sucrose concentration, indicating greater mineral loss in the enamel.

Table 2 lists the correlation coefficients between the R/G values measured by the QLF-D and all other outcome variables associated with cariogenicity. The R/G values showed significant correlations with cariogenic properties except for the total number of bacteria ($p < 0.05$). Strong correlations were identified between the R/G values and the number of aciduric bacteria ($r = 0.83$, $p < 0.0001$), the supernatant pH ($r = -0.95$, $p < 0.0001$), and the %SHC ($r = 0.90$, $p < 0.0001$).

4. Discussion

The present study assessed the direct association between the red fluorescence observed by the QLF-D and the cariogenicity of dental microcosm biofilms by forming pathogenic biofilms with various levels of cariogenicity on enamel surface and measuring the severity of biofilm-induced demineralisation.

This study evaluated whether differences in the cariogenicity of the biofilms influence the red fluorescence intensity using a well-controlled biofilm model. Dental microcosm biofilms with various levels of cariogenicity were produced by adjusting sucrose concentration. This reflects the clinical situations where biofilms with different levels of cariogenicity can be formed depending on the frequency of diet, sucrose content of food, and their sites within oral cavity. Also, the biofilms were matured for the same period of time so as to exclude possible influences of maturation differences in their properties due to the previous study's finding that changes to physical properties of a biofilm such as its thickness, density, and volume that resulted from maturation can affect the fluorescence intensity.[6] By culturing the biofilms for the same period of time, but changing the sucrose concentrations exposed to the biofilms, it was possible to assess the red fluorescence intensity according to the biofilm cariogenicity.

The exposure concentration of sucrose was related to biofilm cariogenicity. The biofilms formed in BMM supplemented with sucrose at a higher concentration had a microbial composition with an increased number of acid-resistant bacteria and a decreased enamel surface microhardness due to the higher acidogenicity of the biofilms. The *in situ* study of Aires et al.,[20] also found that increasing the sucrose concentration from 1% to 40% increased the biofilm acidogenicity and decreased the surface microhardness. Therefore, it can be assumed that increasing the sucrose concentration increases the cariogenic potential of the biofilm due to enhanced substrate diffusibility, thereby promoting the development of dental caries.[21]

The red fluorescence detected by the QLF-D (quantified as the R/G value) also showed to increase in proportion to the cariogenicity of biofilms. As emission of red fluorescence is caused by bacterial porphyrin, the metabolites of bacteria, it can be suggested that the cariogenicity of biofilm is correlated with the production of porphyrin compounds within the biofilm. The production of metabolites can be affected by differences in the metabolic ability resulted from signalling interactions between bacteria.[22] However, exact mechanisms of porphyrin compounds related to biofilm cariogenicity could not be identified from the results of this study; thus, further studies need to be investigated.

The results of this study with regard to the bacteria count show that the R/G values were not significantly correlated with the total bacteria count ($p > 0.05$), while there was a strong correlation with the number of aciduric bacteria ($r = 0.83$, $p < 0.0001$). The aciduric bacteria within the biofilms contribute to the qualitative characteristics, and hence an increase in the composition of the aciduric bacteria could mean that the cariogenic potential of the biofilms is increased.[23] Therefore, from the strong correlation between the red fluorescence intensity and the number of aciduric bacteria, it can be assumed that the red fluorescence intensity may be more associated with qualitative rather than quantitative characteristics of the biofilms, and this is also corroborated by a previous finding that the auto fluorescence of the biofilm could be induced by its intrinsic characteristics.[13,24]

This study confirmed the potential that the QLF-D is a meaningful device for plaque assessment due to its ability to focus on pathogenic biofilms as a whole rather than on a single caries-associated species and to evaluate the cariogenicity of the biofilms. According to the previous study that isolated pure cultures of plaque *in vivo*, there was no significant correlation between the red fluorescence of plaque and the single species within the plaque.[13] Based on this result, it can be assured that examining single species of the plaque might be of little relevance to the fluorescence of a real dental microcosm biofilm consisting of a very large number of unknown types of oral bacteria. Also, the cariogenicity of dental biofilms is determined by interactions between the various bacterial species constituting the dental biofilm rather than by the properties of a single species. Thus, evaluation of cariogenicity should not be limited to simply identifying a specific causative microorganisms and counting their numbers in biofilms, but rather investigate the metabolic activity of biofilms.[22]

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Furthermore, high-cariogenic plaque with its pH values lower than the critical pH could be identified by the red fluorescence intensity detected from the plaque using the QLF-D. When the sucrose concentration was 0.5% in the present study, the pH was below the critical pH 5.5. The *R/G* value was also the highest in this group, and differed significantly from the *R/G* values for all of the other groups (*i.e.*, with sucrose concentrations below 0.5%), for which the pH values were higher than the critical pH.

In addition, the red fluorescence intensity showed positive correlations with %SHC, the degree of damage on the biofilm-induced lesion ($r = 0.90$, $p < 0.0001$); therefore, it is possible to determine the cariogenicity of biofilms by assessing red fluorescence intensity of plaque using the QLF-D system. A previous study concluded that noncavitated enamel lesions reveal characteristic fluorescence emission bands in the red spectral region, which may derive from porphyrin compounds.[25,26] This can be related to our finding that red fluorescence intensity increases with the severity of caries lesions although the evaluation subjects were different. The assessed subjects were biofilms in this study whereas enamel was the evaluation subject in the previous study. However, this study can still be meaningful as a pre-clinical research from preventive perspectives for assessing plaque that is a causal factor for development of dental caries rather than evaluating the developed caries on enamel.

It should be borne in mind that the amount and type of porphyrin synthesis can differ with the bacteria growth conditions; thus, the findings need to be interpreted and applied properly to different situations.[22,24,27] It should be considered that there are limits to the ability to directly apply a particular *R/G* value obtained under the *in vitro* culture condition to actual clinical situations. Further clinical studies are required to assess the observed range of fluorescence colours of *in vivo* plaque and evaluate the associations to use red fluorescence as a predictor of plaque cariogenicity.

It has been found that the fluorescence of underlying material can also confound the fluorescence emitted by dental biofilm, and hence the apparent fluorescence of biofilm could depend on the type of underlying material.[28] Our protocol involving the use of bovine enamel – which is considered an acceptable alternative to human enamel – minimized the potential for the underlying material to influence the detected plaque fluorescence. Using this bovine enamel alternative to human enamel and the microcosm biofilm model used in this study allows for narrowing the gap between the *in vitro* and *in vivo* study.

The QLF-D results obtained in this study indicated that the intensity of red fluorescence differed with the cariogenicity of the biofilms. This suggests that if this device can be used as a plaque assessment device, both quantitative assessment (*e.g.*, analyzing the plaque distribution) and also qualitative assessment can be performed, which would detect the cariogenicity of the plaque more accurately than by simply checking the current oral hygiene status. Also, clinical use of the QLF-D may make it possible for patients to check their own oral hygiene status, and providing a quantitative numerical value for their risk of caries development could increase their motivation. In addition, dentists could use the device to distinguish high-risk patients with poor oral hygiene, detect areas with especially high cariogenic potential in the oral cavity, and perform intensive preventive treatments.

5. Conclusion

It can be concluded that the significant association between cariogenic properties and the red fluorescence intensity detected by the QLF-D, as confirmed in this study, means that this device can be used to detect different levels of cariogenicity by assessing the red fluorescence intensity emitted by dental biofilms.

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