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ORIGINAL ARTICLE

DIAGNOdent measurements of cultures of selected oral bacteria and demineralized enamel

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Abstract

Objective. Carious tissue fluoresces with a wavelength different from sound tissue when stimulated by light with a wavelength of 655 nm. This difference is thought to have a bacterial origin rather than indicating demineralization. This study aimed to measure fluorescence emitted by normal cultivable caries-associated bacterial flora and typical porphyrin-producing bacteria with DIAGNOdent, and to verify earlier findings that demineralization of the dental hard tissue does not affect DIAGNOdent readings. ***Material and methods.*** Bacterial samples were collected from five occlusal caries lesions in three subjects. From these, mixed anaerobic flora, *Lactobacilli* and mutans *Streptococci* were cultured in up to three different kinds of culture medium. Colonies of *Lactobacilli* and mutans *Streptococci* were also measured after transferring them to glass slides. Laboratory teaching strains of *Prevotella spp., Porphyromonas gingivalis* and *Actinomyces odontolyticus* were cultured anaerobically and fluorescence measured directly after an appropriate incubation period. Sound enamel surfaces of 15 extracted premolars were demineralized and changes in fluorescence measured. ***Results.*** DIAGNOdent readings > 20 were only obtained from young colonies of *Prevotella* and from colonies of mutans *Streptococci* cultured on mitis–salivarius–bacitracin agar. Higher measurements were obtained as the bacterial colonies aged. Lower measurements were obtained after transferring colonies to glass slides. Demineralization of enamel did not affect the DIAGNOdent measurements. ***Conclusions.*** The change in fluorescence measured with DIAGNOdent has a bacterial origin rather than occurring as a result of demineralization. The measurements are presumably dependent on bacterial metabolites rather than bacteria themselves, and probably record synergistic effects during the carious process rather than the quantity or species of bacteria involved.

Key Words: *Demineralization, dental caries, fluorescence, oral bacteria*

Introduction

Auto-fluorescence of dental hard tissue was first described in the 1920s [1]. Since then, attempts have been made to use this property to differentiate between sound and carious dental hard tissue. In 1998, Hibst & Gall [2] described the successful use of red light (655 nm) to differentiate between sound and carious tissues and this was the basis for the development of the DIAGNOdent instrument (KaVo, Biberach, Germany). When using light with an excitation wavelength of 655 nm, a more intense fluorescence in the 700–800 nm wavelength region was observed from a carious lesion compared with that at a sound spot of enamel [3]. The origin of this difference in fluorescence was speculated to be caused by the bacterial content of the lesion and not due to demineralization. In a study carried out by König *et al.* [4] the similarity between the fluorescence spectrum from bacterial porphyrins and from carious lesions was described. These findings have been further supported by later studies [3,5]. Porphyrins, which are bacterial breakdown products, are therefore thought to be responsible for the increase in DIAGNOdent readings accompanying carious lesion formation.

Because of the possible bacterial origin of the fluorescence measured with DIAGNOdent, new areas of application for the instrument have been discussed.

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The possibility of assessing caries activity has been suggested [6], as well as using the instrument to verify the removal of infected tooth substance prior to placement of restorations [7]. The in vitro and in vivo performance of the instrument has been quite extensively investigated [8]. Some studies have compared the bacterial content of lesions with DIAGNOdent measurements [9,10], but the origin of the fluorescence signal has not been fully described. The ability of the oral microflora to emit red fluorescence when different excitation wavelengths were used has been described [5,11]. In a study on cultures of single species of caries-associated bacteria, they were found to emit red fluorescence when an excitation wavelength of 405 nm (± 20 nm) was used [12]. Other studies have, however, concluded that some interaction between bacterial species and their surroundings is needed so that red fluorescence can be measured [13,14]. Very little has been published on how cultures of different bacterial species respond to the laser light of the DIAGNOdent instrument. It is, therefore, important to investigate the origin of the fluorescence measured with the DIAGNOdent instrument.

The main objectives of this study were: (i) to measure the fluorescence of the normal cultivable caries-associated bacterial flora and typical porphyrin-producing bacteria with DIAGNOdent; and (ii) to verify earlier findings suggesting that demineralization of the dental hard tissue does not affect DIAGNOdent readings [4].

Material and methods

The study was approved by The Ethics Committee, Huddinge Hospital (402/02), Stockholm, Sweden and by the National Bioethics Committee in Iceland (02-151-S2).

Bacterial samples

Bacterial samples were obtained from five occlusal lesions in three subjects aged between 18 and 30 years. All lesions were scheduled for restorative treatment at the Dental School Clinic, University of Iceland. Dentine involvement of all lesions was verified by visual and radiographic examination and by DIAGNOdent measurements >20. Samples from carious dentine were collected using a sterile dental bur. The bur was then inserted into a sample tube containing reduced transport fluid and sent to the laboratory for bacterial analysis.

Bacterial analysis

Specimens were mixed on a vortex mixer for 15 s and 100-µl aliquots of each specimen inoculated on to both selective and non-selective media. Rogosa agar (Difco, Detroit, MI) was used to culture *Lactobacilli* following aerobic incubation. Mitis–salivarius–bacitracin agar (MSB; Difco) [15] was used for culture of mutans *Streptococci* following incubation in candle jars at 37°C for 48 h. Samples on blood agar were incubated in an anaerobic cabinet (Don Whitley Scientific, Shipley, UK) at 37°C for 1 week in order to obtain a mixed flora that would include anaerobes in the original sample. For further analysis, cultures of *Lactobacilli* were also inoculated onto blood agar plates and cultures of mutans *Streptococci* were inoculated on to blood agar plates and in broth and incubated in a candle jar for 48 h at 37°C. Furthermore, cultures of mutans *Streptococci* from all five samples were inoculated on to one MSB agar plate and incubated in a candle jar for 72 h. These samples were used to test the relationship between maturation of the mutans *Streptococci* and fluorescence. In addition, laboratory teaching strains of *Prevotella spp., Porphyromonas gingivalis* and *Actinomyces odontolyticus* were cultured anaerobically on Fastidious Anaerobic blood agar (FAA; LabM, Bury, UK) for 1 week. To identify young colonies of *Prevotella spp.*, the anaerobic blood agar plates were examined in a dark room using long-wave ultraviolet light (366 nm) as used routinely in the laboratory for this purpose, and as described by Myers *et al.* [16] and by Shah *et al.* [17]. This was done to examine the relationship between the reduction in fluorescence under UV light shown by *Prevotella spp.* with maturation of the colonies and fluorescence measured by DIAGNOdent on the same cultures. As expected, the black-pigmented colonies of *P. gingivalis* did not show fluorescence under UV light [17].

DIAGNOdent measurements

All DIAGNOdent measurements were performed directly after an appropriate incubation time for each type of bacterial culture as described above. The same DIAGNOdent instrument and a B-tip were used for all measurements and the instrument was calibrated against a ceramic standard before measurements were carried out. The DIAGNOdent instrument was then used in the same way as in the clinical situation. The standard value for the underlying material was determined by measuring colony-free parts of the agar and glass slides separately in order to allow intrinsic variation in fluorescence to be measured. Then the tip was placed as closely to the colonies as possible, without direct contact, and the peak value of the DIAGNOdent readings was registered. All colonies were measured twice and the average determined.

Cultures of *Lactobacilli* and mutans *Streptococci* on both selective media and blood agar and the anaerobic

culture on FAA were measured both directly on the agar plates and after transferring colonies with a sterile loop to glass slides. The broth cultures of mutans *Streptococci* were only measured on glass slides. Colonies on the MSB agar plates were measured with DIAGNOdent after incubation for 24, 48 and 72 h. Cultures of the laboratory strains of *Prevotella spp.*, *P. gingivalis* and *A. odontolyticus* were only measured directly on the agar plates.

Teeth

The material comprised 15 premolar teeth extracted from young adolescents on orthodontic indication. Only visually sound teeth were used. The buccal surfaces of each tooth were used for measurements.

Prior to DIAGNOdent measurements, the teeth were rinsed with water and then air-dried in a gentle air-stream for 5 s [18]. The same DIAGNOdent instrument and a B-tip were used for all measurements and used according to the manufacturer's instructions. A visually sound spot was chosen on each tooth and baseline measurements performed. All teeth were then embedded in wax (Tenax wax; S.S. White materials, Prima Dental Group, Gloucester, UK), leaving only a small window of accessible enamel surface for demineralization. In order to produce artificial subsurface lesions, the teeth were immersed in a demineralization solution according to Buskes *et al*. [19] for 2 weeks. The solution contained 3 mM $CaCl_2 \cdot 2H_2O$, 3 mM KH_2PO_4, 50 mM CH_3COOH, 10 M KOH and 2–50 μM Methylhydroxydiphosphonat (MHDP), at pH 5. The teeth were then washed thoroughly with water, the wax removed and a DIAGNOdent measurement performed at the demineralized spot.

As a verification of real demineralization (i.e. that artificial lesions and not erosive defects were created), QLF images were captured at baseline and after demineralization using the Inspektor™ Pro intraoral fluorescence system (Inspektor Dental Care Research and Development, Amsterdam, The Netherlands). The purpose of performing analysis of the sound areas at baseline was to prove that the whole area used for the DIAGNOdent measurements was sound, without mineral defects of any kind. All images were captured under standardized conditions. The analysis of the images was done by one analyst using Inspektor Pro 2.0.0.38. In this way, the average loss of fluorescence (ΔF) in the artificial lesions was obtained.

Statistical analysis

The data were analysed using Statistica version 7 (StatSoft Scandinavia AB, Uppsala, Sweden). The Wilcoxon Matched Pairs Test was used for analysing the effect of demineralization on DIAGNOdent and QLF readings. The effect of maturation of mutans *Streptococci* on their auto-fluorescence was analysed by using Friedman's ANOVA and Kendall's coefficient of concordance. DIAGNOdent measurements of different bacterial species on different agar types are presented as medians, with minimum and maximum values.

Results

Table I presents an overview of the DIAGNOdent measurements of the test bacteria: mutans *Streptococci*, *Lactobacilli* and the mixed anaerobic cultures. The standard values for all agar types and glass

Table I. DIAGNOdent measurements for different bacterial species cultured on selective and non-selective media.

Bacteria	Agar type	DIAGNOdent readings on agar plates Median/min/max	DIAGNOdent readings on glass slides Median/min/max
Lactobacilli	Rogosa agar	0/0/0	0/0/0
Lactobacilli	Blood agar	0/0/1	0/0/0
Mutans *Streptococci*	MSB	79/49/79	49/30/54
Mutans *Streptococci*	Blood agar	0/0/0	0/0/0
Mutans *Streptococci*	Broth		1/0/1
Anaerobic culture	FAA blood	0/0/1	0/0/0
Prevotella spp. (young culture; UV-fluorescent)	FAA blood	18/15/22	ND
Prevotella spp. (older culture; non-UV-fluorescent)	FAA blood	0/0/0	ND
P. gingivalis (non-UV-fluorescent)	FAA blood	2/0/4	ND
A. odontolyticus	Blood agar	0/0/0	ND

ND = test not performed.

slides were tested. MSB agar plates showed a standard value of 20, which was subtracted from the value for the bacteria before inclusion in the Table. The standard values for other culture media and for glass slides were 0. Figure 1 shows how the DIAGNOdent measurements of colonies of mutans *Streptococci* grown on MSB agar increased between 24 and 72 h of incubation, although this increase was not statistically significant ($p = 0.052$; Friedman's ANOVA). Young *Prevotella spp.* colonies grown on FAA, which showed pink fluorescence under UV light, also fluoresced when measured with DIAGNOdent (Table I). *Prevotella* colonies that did not fluoresce under UV light, which were usually older cultures, did not fluoresce either when measured with DIAGNOdent. *P. gingivalis* did not show any fluorescence under UV light and gave DIAGNOdent readings of 0–4. The instrument did not measure any fluorescence from colonies of *A. odontolyticus.*

The creation of artificial subsurface lesions on sound enamel was verified by QLF measurements. Analysis of QLF images showed significant loss of fluorescence over the test period ($p < 0.001$; Wilcoxon Matched Pairs Test), while DIAGNOdent readings did not change significantly ($p = 0.722$; Wilcoxon Matched Pairs Test). The results are presented in Figure 2.

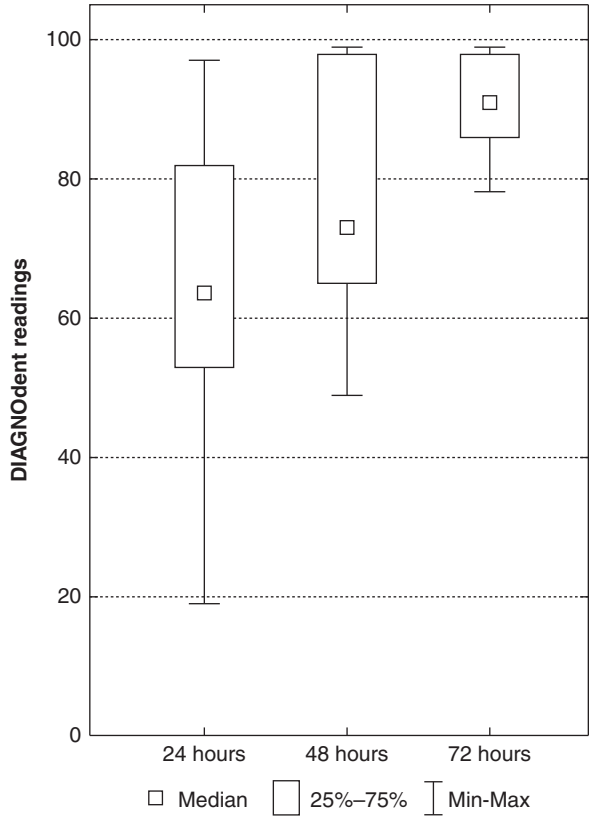

Figure 1. Box plot showing DIAGNOdent measurements of mutans *Streptococci* colonies on MSB agar after 24, 48 and 72 h of incubation. No significant difference: $p = 0.052$ (Friedman's ANOVA).

Discussion

Cultivable bacterial species suspected to play a major role in occlusal caries are principally *Lactobacilli* and mutans *Streptococci* [20]. *Actinomyces* species have also been implicated in the initial caries process, although they are most often linked to root surface caries [21]. As an initial investigation of how bacterial cultures respond to light with a wavelength of 655 nm, mutans *Streptococci*, *Lactobacilli* and *Actinomyces* species and mixed anaerobic cultures were chosen. However, the microbiota of occlusal dentin caries is more complex than was previously thought and contains organisms that are difficult to isolate and identify, as well as non-cultivable bacterial species [22,23] that may play an important role in the fluorescence emitted from the carious lesion and recorded with the DIAGNOdent instrument. The present study, therefore, has certain limitations as it only focused on cultivable bacterial species.

As mentioned earlier, the fluorescence from carious dental hard tissue measured with DIAGNOdent is thought to be caused by porphyrins, a bacterial breakdown product. It was, therefore, thought important to investigate how cultures of typical porphyrin-producing bacteria, such as *Prevotella spp.* and

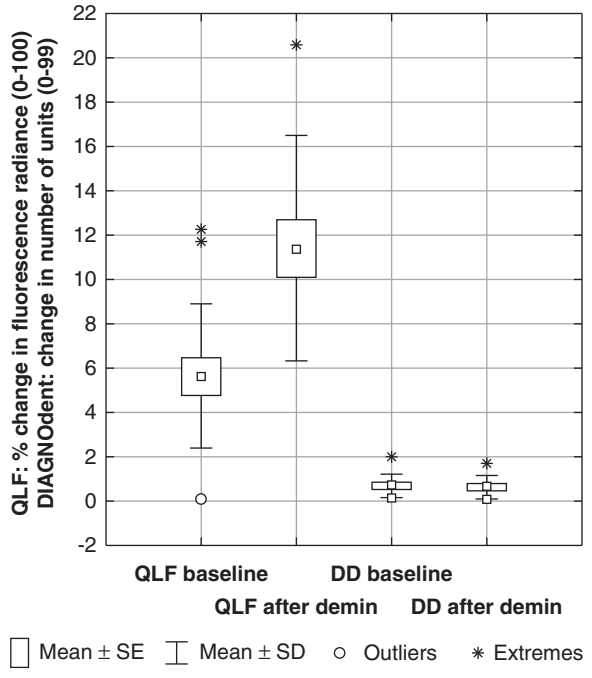

Figure 2. Box plot showing QLF and DIAGNOdent (DD) readings before and after demineralization. Significant difference seen with analysis of QLF images: $p < 0.001$ (Wilcoxon Matched Pairs Test). No significant difference seen with analysis of DIAGNOdent readings: $p = 0.722$ (Wilcoxon Matched Pairs Test).

P. gingivalis, responded to the laser light. Both these bacterial species are proteolytic, porphyrin-producing anaerobes which have been detected in coronal carious lesions [24].

To investigate the influence of different culture media on bacterial ability to emit fluorescence, the bacteria obtained from the carious samples were inoculated onto two kinds of media. Mutans *Streptococci* were also cultured in broth. The DIAGNOdent instrument was not able to measure any fluorescence from *Lactobacilli* and the anaerobically incubated mixed cultures from dentine caries, while colonies of mutans *Streptococci*, when cultivated on MSB agar, gave high readings. When the same bacteria were cultivated on blood agar or in broth, however, no change in fluorescence was measured. To some extent these findings may contradict the results of a study by Lennon *et al.* [12], in which red fluorescence was only observed from colonies of *Lactobacilli* and not from mutans *Streptococci* when examined under violet light (407 nm). The DIAGNOdent readings obtained from colonies of mutans *Streptococci* from MSB, observed in this study, increased with the age of the culture. The MSB agar contains Trypan blue, which is a moderately fluorescent substance in itself. The bacteria seem to be able to accumulate this substance in the colony, resulting in increased DIAGNOdent readings. It is, therefore, clear that the ability of bacteria to emit fluorescence, when excited with light with a wavelength of 655 nm, is highly dependent on the substrate. This was further illustrated by the decline in DIAGNOdent readings when colonies were removed from the agar culture medium and placed on glass slides.

Young colonies of *Prevotella spp.* are known to emit visible fluorescence when viewed under UV light, while *P. gingivalis* colonies do not [16,17]. Fluorescence emitted by these bacterial species measured with DIAGNOdent was somewhat similar, despite the difference in excitation wavelength. Young colonies of *Prevotella spp.* gave moderate DIAGNOdent readings, which declined rapidly as the colonies became black. Colonies of *P. gingivalis* gave very low DIAGNOdent readings even when cultured on blood agar. The porphyrin iron complex, haem, which can be metabolized by black-pigmented bacteria, is found in blood agar and it was hoped that this would induce porphyrin production by the bacteria. The low DIAGNOdent readings when black-pigmented pathogens were measured were, therefore, somewhat unexpected. This, however, somewhat supports the conclusion made by Shigetani *et al.* [14] in a study on *S. mutans* that some interaction of the bacteria with the tooth substrate is needed so that the difference in fluorescence accompanying carious lesion formation can be measured.

Demineralization of sound enamel and formation of artificial subsurface lesions had no significant effects on the DIAGNOdent readings. This failure to demonstrate that demineralization *per se* is the cause of the increase in DIAGNOdent measurements confirms results from earlier studies [4,25,26].

From this in vitro study, bearing in mind its limitations, it can be concluded that demineralization itself does not affect the fluorescence of dental hard tissue when excited with light with a wavelength of 655 nm. Thus, the change in fluorescence measured with DIAGNOdent is likely to have a bacterial origin rather than resulting from demineralization. The measurements are presumably dependent on bacterial metabolites rather than bacteria themselves and probably record synergistic effects during the carious process rather than the quantity or species of bacteria involved.

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Declaration of interest: The authors report no conflicts of interest.

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