**Evaluation of the co-existence of the red fluorescent plaque bacteria *P.gingivalis* with *S. gordonii*****and *S. mutans* in white spot lesion formation during orthodontic treatment**

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**Abstract**

**Background:** Early detection of white spot lesions (WSLs) around brackets during orthodontic treatment is important for treatment and prevention. But it is unclear whether red fluorescent plaque (RFP) bacteria *P.gingivalis* and its co-existence with *S mutans* and *S.gordonii* has any significant influence on this.Therefore the role of this bacterial co existence and WSLs formation during one year of fixed orthodontic therapy was evaluated.

**Methods:** Fourteen 12 to 22 year old (mean 15±3 years) consecutive patients attending the University of Liverpool dental hospital were recruited for this study. Quantitative Light-induced Fluorescence (QLF) was used to identify RFP and enamel demineralisation, respectively, on anterior labial surfaces before and after placement of fixed orthodontic appliances. Bacterial composition was determined by denaturing gradient gel electrophoresis (DGGE) following nested PCR amplification of the 16S rRNA V2-V3 hypervariable region.

**Results:** WSLs were recorded on 4.2% of tooth surfaces and WSL development was not associated with RFP bacteria *P.gingivalis* presence. Differences in RFP bacteria *P. gingivalis* presence with *S. mutans* and *S. gordonii*,were observed before and after appliance placement. Intra subject changes in plaque flora between visits were not significantly associated with WSL development (p>0.05). However, DGGE profiles indicated that apart from *S.mutans*, *S.gordonii* might also have a role in human enamel demineralisation.

**Conclusions:** Fixed orthodontic brackets in adolescents may play a role in altering bacterial composition around brackets during orthodontic treatment and it is plausible that *S.gordonii* also have a role in human enamel demineralisation. Combinatorial approach of QLF technology and DGGE may be useful in determining bacterial composition during orthodontic therapy which could inform clinical interventions.

Keywords: quantitative light-induced fluorescence, denaturing gradient gel electrophoresis, dental plaque, dental caries

**Introduction**

Development of enamel demineralisation known as white spot lesions (WSLs) adjacent to orthodontic brackets remains a significant clinical problem [1]. WSLs can be seen as early as four weeks following placement of fixed orthodontic appliances and the overall prevalence been reported to be between 2 and 96 % [1]. WSLs, which are early signs of demineralisation, may or may not lead to the development of caries which is a common chronic infectious disease [2]. The aetiology of dental caries is described by the specific plaque hypothesis, nonspecific plaque hypothesis, and ecological plaque hypothesis [3] and demineralisation occurs at early stages of caries development when plaque is present for sufficient time [4]. Fixed orthodontic appliances creating plaque retentive areas which present an oral hygiene challenge leading to increasing volume and numbers of bacteria [5] that may cause WSLs formation and caries. Therefore early detection of WSLs around brackets during orthodontic treatment is important for treatment and prevention. Quantitative Light-induced Fluorescence (QLF™, Inspektor Research Systems, BV, The Netherlands) is a non-invasive diagnostic tool, used for longitudinal quantification of incipient lesions on smooth surfaces with quantitative assessment of early demineralisation, as well as dental plaque [6,7]. Heavy plaque deposits fluoresce deep red [6, 7], probably due to porphyrins present, particularly in Gram-negative anaerobes known as red fluorescent plaque (RFP) [6]. It was reported that combination of intrinsic characteristics of different bacteria, such as *Porphyromonas gingivalis,* in the mature biofilm are responsible for the red auto fluorescence than the characteristics of a single species [8] It was also suggested that the red fluorescent plaque could be more cariogenic [9, 10], But it is unclear that the RFP bacteria *P.gingivalis* and its co-existence with *Streptococcus gordonii* or *Streptococcus mutans* have any significant role in white spot lesion formation during orthodontic treatment.It was reported that *P. gingivalis* requires a preformed streptococcal substratum for its incorporation into a biofilm [11] and displays species specificity, forming biofilms with *S. gordonii* [12] but not with *S. mutans* [11] . Certain species; such as *Streptococcus sanguinis*, were associated with health, while others, such as *S. mutans*, are associated with caries [13]. Orthodontic appliances have also been implicated as a risk factors for inflammation of the gingiva and their withdrawal improved gingival health and reduced subgingival *P. gingivalis* [14]. Therefore the aims of this study were: (i) to identify presence or absence of *P.gingivalis* along with *S. gordonii* and *S. mutans* in adolescents undergoing fixed orthodontic treatment; (ii) to determine the incidence of WSLs and evaluate its relationship with the identified bacterial composition. Cariogenic bacteria have traditionally been identified using culture-based methods, but have now been largely superseded by molecular methods which includes those that are non-cultivable [15] and have been used in this study.

**Materials and Methods**

Study design

A prospective, non-interventional, 12-month longitudinal cohort investigation of WSLs incidence and presence of supragingival RFP *P. gingivalis* with *S. gordonii* and *S. mutans* among adolescents undergoing fixed orthodontic treatment.

Participants

Fourteen [8 females and 6 males] 12-22 years old (mean 15 ± 3 years) consecutive patients attending the University of Liverpool dental hospital were recruited and identified by the abbreviations P1-P14. ToothcareTM, QLF images and plaque samples were taken during the routine appointments for 12 months from placement of orthodontic appliances.

Exclusion criteria

Participants, receiving antibiotic therapy less than eight weeks prior to or at during the study, were excluded.

Ethical approval

Ethical approval was granted by the North West Ethics Committee, Liverpool; U.K. (reference 09/H1005/63). Participants and/or parents/guardians were given verbal and written information. Those willing to participate signed consent/assent forms.

Detection of WSLs and RFP

WSLs were assessed using QLF images obtained at base line (before appliance placement) and every second orthodontic appointment (≈12 weeks). Images of individual anterior segment teeth (canine to canine) were captured, stored and analysed with customised software. Demineralisation was quantified by fluorescence loss from the enamel surface. The difference between original pixel values and reconstructed sound pixel values of fluorescence (below 95% threshold) estimated gave resulting fluorescence loss in demineralised enamel. This difference was expressed as a change in fluorescence radiance (ΔF, %). The area of demineralised enamel (mm2) was also calculated. Multiplication of these variables gave ΔQ,%

RFP was identified with ToothcareTM, a hand held device based on QLF principles. The labial/buccal surfaces of the maxillary and mandibular six anterior teeth (central incisors, lateral incisors and canines) were scanned for RFP, with lateral incisors examined first, followed by central incisors and canines. Once RFP was identified on a single tooth, plaque was removed aseptically. The plaque was transferred into sterile 1.5 ml Eppendorf® tubes and stored at -20oC prior to molecular analyses. RFP was identified and samples collected before placement of orthodontic brackets and at every follow up appointment (Table 1).

Molecular analyses

*P. gingivalis* ATCC 33277 was maintained on fastidious anaerobic agar (FAA), (Bioconnections, UK) supplemented with 5% horse blood (TCS Biosciences, UK), while *S. gordonii* DL1 and *S. mutans* NCTC 10449on brain heart infusion agar (BHI). Bacteria were grown anaerobically (N2:CO2:H2, 80:10:10) at 37°C.

Isolation of genomic DNA from RFP and standard reference cultures were carried out using MasterPureTM complete DNA and RNA purification kits (Epicentre Biotechnologies, UK) according to slight modification of manufacturer’s protocol; pre-treatment with 5μl Proteinase K solution (0.18 mg/ml, [50 mM Tris-Hcl (pH 7.5)], 100 mM NaCl, 0.1 mM EDTA, 10 mM CaCl2, 0.1% Triton® X-100, 1 mM dithiothreotol in 50% glycerol solution), and addition of 30μl of 10% SDS. Samples were mixed by inversion, incubated at 550 C for 60 minutes, centrifuged (2 minutes at 13,000 x g), supernatant discarded and the cell pellet used for extraction.

Nested polymerase chain reaction **(**PCR) was used to amplify the V2-V3 region of the 16S rRNA gene. 16S rRNA gene conserved primers 27F and 1492R were used to amplify a larger region [16] followed by nested PCR reaction [using GC-clamp primers, 357F-GC and 518R [17] and the product of first PCR reaction as template] to amplify the hyper variable region. All primers were obtained from Sigma-Aldrich®. The expected product length (~235bp) was confirmed on 2.5% agarose gels stained with ethidium bromide.

Denaturing gradient gel electrophoresis (DGGE) (Bio-RAD, Germany) was used to identify profiles of bacteriain RFP samples, with *P. gingivalis* ATCC 33277, *S. gordonii* DL1 and *S.mutans* NCTC 10449 as references. Parallel gradient gels containing 7.5% (w/v) acrylamide (acrylamide/bisacrylamide:37.5:1) were cast containing linear gradients of the denaturants urea and formamide, increasing from 30% (top of gel) to 60% (bottom) (100% denaturants corresponded to 7M urea and 40% [v/v] deionised formamide). Nested PCR products of the hyper variable region (15µl) were loaded on gels, run at 60V for up to 16 h at constant temperature (600C) in TAE buffer (1x Tris-acetate-EDTA), then stained in ethidium bromide (0.1µg ml–1) to allow visualization of DNA under UV light.

The bacterial composition of the RFP was determined by the number of bands detected and the distances travelled. Raw TIFF files were imported and analysed with TotalLab™ TL120 DM 1-D gel analysis software (Nonlinear Dynamics). The profiles from known strains were used as standards.

Statistical analysis

The categorical data were subjected to statistical analyses using chi-square test (GraphPad Software, San Diego, CA) to assess associations between bacteria and WSL development. A *p* value of < 0.05 was considered statistically significant.The sample size has been selected based on the probability of identifying plaque constituents which are common in the target population. If any given plaque constituent is present in at least 10% of the population, then using the binomial distribution, it can be calculated that the chosen sample size would give greater than 95% probability (statistical power) that the constituent would be present in at least one participant in the sample.

**Results**

A total of 573 QLF images were obtained from 14 participants at baseline and every other visit (four of which had only upper fixed appliances). One participant (P5) relocated from Liverpool and had only one set of QLF images taken following the placement of the fixed appliance. Four participants (28.6%) did not show any signs of demineralisation (WSLs) throughout the study. Of the ten remaining participants, six (60%) showed signs of demineralisation at the first appointment (3-6 months) and four at the last appointment (12 months after placement). Participants with early signs of demineralisation developed larger lesions at multiple sites by the end of the study, whereas the participants that demonstrated demineralisation later on during their treatment ended the study with smaller lesions on a single site. The overall incidence of WSLs was 4.2%, with mean number 1.7 ± 1.5 per participant, ranging from 0.08mm2 to 5.62mm2, with a median of 0.76mm2. No lesions were visible bye eye using standard clinical procedures.

Eighty RFP samples in total were collected with a mean of 5.71±2.76 samples per participant (Table 1). Variations in total numbers (mean per participant was 7.43 ± 1.40) of visits were due to non attendance at appointments. Labial surfaces of upper right lateral incisors (35% of total) followed by upper right canines (17.5% of total) were most common sites for RFP. Less than 10% of total RFP samples were collected at any one of the other sites with upper left central incisors showing no RFP at any visit. 16.4% of the sites where RFP was detected (n=80) showed signs of demineralisation identified with QLF with the remainder showing no signs of demineralisation using QLF. The overall incidence of WSLs was not related to presence of RFP (p >0.05) (Figure 1).

PCR products of the expected size (~1,500bp and ~235bp respectively) were obtained from the RFP samples from all participants after the first and second stages of nested PCR., Overall, 71/80 (89%) samples demonstrated bands corresponding to both *S. mutans* NCTC 10449 and *S. gordonii* DL1 and all 14 participants produced at least one sample with both. In 4/80 (5%) samples, bands corresponding to *S. mutans* NCTC 10449 were present whilst bands corresponding to *S.gordonii* DL1 were absent. Similarly, in 5/80 (6%) samples, bands corresponding to *S.gordonii* DL1 were detected whilst *S. mutans* NCTC 10449 was absent. However, the two Streptococci strains were never absent at the same time (Table 2). *P.gingivalis* was absent from all 80 RFP samples. Three additional bands from RFP samples that didn’t correspond to reference bacteria were also observed (data not shown).One of these unidentified bands was relatively common (Observed in samples from 86% of participants), with the other two were relatively rare (<28% of participants).

Eighteen of 24 WSLs in 9 participants were associated with RFP samples (Table 2). Sixteen of which (89%) produced bands corresponding to both *S. mutans* NCTC 10449 and *S. gordonii* DL1. Interestingly, 2/3 RFP samples from P13 associated with WSL (samples 4 and 6) produced bands corresponding to *S.gordonii* DL1 but not *S. mutans* NCTC 10449. However, no statistically significant changes (p >0.05) in the composition of dental plaque, with respect to *S.mutans* NCTC 10449, and *S.gordonii* DL1, were associated with the development of WSLs.

**Discussion**

Demineralisation/development of WSLs is a major risk associated with orthodontic treatment. Early identification allows intervention to begin sooner and possible reversal of the demineralisation process [18]. In this study, the prevalence of end-point WSLs was 4.2% of anterior labial surfaces, a value at the lower end of the reported prevalence range of WSLs amongst orthodontic patients ( 2-96% ; [1]). However, 60% of participants developed some signs of demineralisation on one or more surface(s) of any study tooth during 12 months of fixed treatment. The medial value of WSLs was < 0.76mm2, which is small and subclinical. The results reinforce the sensitivity of QLF to detect enamel demineralisation at early stages when it is relatively easy to promote remineralisation using oral hygiene instruction and/or daily sodium fluoride mouthwashes.

In the present study, ToothcareTM, was used to identify supragingival RFP. The data shows that the upper right lateral incisors were the most common site for RFP (35% of the total samples collected) followed by the upper right canines and the lower left laterals are in agreement with reports that the lateral incisors and canines were most common sites to show demineralisation following orthodontic treatment [19]. The site of RFP was not associated with development of WSLs. However, the participants brushed their teeth before their appointments, so presence of RFP may have been under-reported. However, the results from this study correlates well with a recent study where RFP was not reported to be associated with cariogenicity [20].

Nested PCR amplification of the hypervariable 16S rRNA gene region using GC-rich primers [17] and DGGE analyses has allowed the separation of unique 16S rRNA gene sequences, producing fingerprints for each species [21]. DGGE band profiles of the collected RFP were compared with band profiles of three known bacteria and none had bands that corresponded to *P. gingivalis* ATCC 33277. This was as expected, because *P.gingivalis* is a predominantly subgingival organism found in the gingival crevice [22]. *S. mutans* NCTC 10449 and *S. gordonii* DL1 bands were identified in the majority of samples from all 14 participants, while a minority of samples, *S. mutans* NCTC 10449 specific bands were present while those corresponding to *S.gordonii* DL1 were absent and *vice versa*. They were however, not both absent at the same time. The association of WSLs to specific bacterial flora was not statistically significant, but, interestingly, two samples showing WSLs from P13 had *S.gordonii* DL1 present but not *S. mutans* NCTC 10449. This result indicate the need for further analyses with large sample sizes to establish a potential role for *S.gordonii* DL1 in human enamel demineralisation. This is important as *S.gordonii* is early plaque colonisers that facilitates the attachment of other bacteria to tooth surfaces [23] and has previously been implicated in caries in animals [24].

*S. mutans* can co-aggregate with *S.gordonii* and play a role in the development of older plaque [25], suggesting that some of the sampled RFP is newly formed. Moreover, *S. mutans* has been studied extensively in relation to cariogenesis. It was also reported that *S.gordonii* may inhibit the colonisation of certain *S.mutans* species [26]. The finding that *S.gordonii* were almost always detected alongside *S.mutans* may indicate that any inhibitory effect on *S.mutans* might not affect dental caries development or demineralisation in some participants and the presence of both *S. mutans* and *S. gordonii*, suggests that both species may have a pathogenic role in development of dental caries in adolescent orthodontic patients. It was suggested that the concentration and presence of certain strains of *S.mutans* that are resistant to proteases are the main causative factors for dental caries [27]. Further work is needed to establish if the participants who developed WSLs harboured different strains and/or concentrations of *S. mutans* in their plaque. Interestingly, three bands that did not match the migration distances of bands representing *P.gingivalis, S.mutans* or *S.gordonii* were detected and may represent other bacterial species present in RFP from these participants. However, there was no statistically significant (p>0.05) association detected between the presence of these bands and the development of WSLs. But, further analyses to establish the identity of such species among larger sample size studies will be required to ascertain role of these bacteria.

**Conclusions**

The present study provides evidence that the presence or absence of RFP associated bacteria *P.gingivalis* with *S.mutans* and *S.gordonii* was not correlated significantly to the occurrence of WSLs during one year of fixed orthodontic therapy. However, the results suggest that the role of  *S.gordonii* in human enamel demineralisation should be pursued in future studies. Further work utilising larger sample sizes and investigation of non-RFP bacterial compositions is also warranted. The detection of RFP using ToothcareTM was found to be a quick and easy method of detecting RFP but techniques allowing image capture (e.g.QLFD) may be more useful.

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**Conflict of interest**

The authors declare that there is no conflict of interest pertaining to this manuscript.

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**Table legends**

**Table. 1.** Chart detailing numbers of; visits, RFP sample collection, QLF image collection and WSLs identification for all the participants.

**Table. 2.** The distribution of DGGE profile bands and their corresponding bacterial composition, based on the controls, and the incidence of WSLs.

**Figure legends**

**Figure. 1.** The incidence of WSLs and its relationship with the presence of RFP