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In Vivo Collection and SEM Identification of Oral Biofilm Using Indirect Composite Prototype Restorations. Clinical and Laboratory Study

Kostadin G. Georgiev[1], Ivan A. Filipov[1], Iliyan N. Dobrev[2]

[1] Department of Operative Dentistry and Endodontics, Faculty of Dental Medicine, Medical University of Plovdiv, Plovdiv, Bulgaria
[2] Department of Microbiology, University of Food Technologies, Plovdiv, Bulgaria

Correspondence:
Kostadin G. Georgiev, Department of Operative Dentistry and Endodontics, Faculty of Dental Medicine, Medical University of Plovdiv, 3 Christo Botev Blvd., 4000 Plovdiv, Bulgaria
E-mail: g.kostadin@gmail.com
Tel: +359883453823

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Background: The oral ecosystem is a dynamic environment inhabited by more than 700 microbial taxa. Recent studies report that multispecies oral biofilms develop on the surface of resin composites leading to degradation of its organic matrix and altered structural stability of the restoration.

Aim: To examine the efficacy of a novel clinical approach to investigating in vivo formed biofilms on resin composite surfaces.

Materials and methods: The clinical protocol of this study implemented indirect composite molar restorations (from resin material Filtek Z250, 3M ESPE) as intraoral biofilm carriers (test devices). We recruited for the experiment 5 consenting adult subjects with indications for indirect molar restoration. For each subject we fabricated 4 indirect restorations, 3 of which dedicated to different intraoral duration – 3, 7, and 14 days. All composite carriers were fixed temporarily for the intended time period and consecutively replaced. The detached carriers were prepared for microscope analysis at each time interval. The fourth composite carrier was used as the definitive restoration.

Results: The timeline of the biofilm formation and the microbial morphology were associated with previous studies of in vivo bacterial colonisation. A correlation between the plaque formation cycle and the DMFt indices of the subjects was established.

Conclusions: The implementation of indirect composite restorations as intraoral biofilm carrier offers valuable contribution to the real time investigation of in vivo biofilm accumulation.

BACKGROUND

More than 700 bacterial species are found in the oral environment. The hard and soft tissues in the mouth are a natural substrate used by the oral microbiota to form complex and heterogenic microcosm biofilms.[1-3] The development of oral biofilm has been studied with various methods. Two approaches are defined by Darrene and Cecile: cultivation-based and non-cultivation based methods.

The cultivation-based methods require collection of biofilm from the oral environment. Dental plaque samples can be assembled by scraping the surface of a test object with a swab[4] or with a sterile curette[5]. After collecting the samples vertexing[6] or sonification[7] can be used to disperse the cells from the biofilm. Selective media and Gram-staining are the methods of identification of microbial taxa found in oral biofilm samples.[8,9] However, the protocol of cultivation-based methods is complicated and some bacterial species can be lost during the sample collection.

Non-cultivation-based methods have been developed to give more complete evaluation of the microbiota in dental plaque samples.[9] Multispecies biofilm models can be observed by fluorescence in situ hybridization (FISH), epifluorescence microscopy, confocal laser scanning microscopy (CLSM), and scanning electron microscopy (SEM).[10-13] In recent studies, PCR has been used for identification and quantitative analysis of in vitro modulated oral biofilms.[14] Quantitative real-time PCR (qPCR) is widely used in association with optical methods (CLSM and FISH) to compare the quantitative dissemination of microbiota versus its 3D structure.[15]

AIM

The aim of this study was to examine a new clinical approach for the investigation of in vivo biofilm formation on resin composite surfaces. The composite surfaces differ significantly from tooth enamel and thus biofilm formation may show specific patterns and species diversity.

MATERIALS AND METHODS

To study the evolution of bacterial plaque with minimal disturbance of its integrity we decided to implement resin based indirect restorations which were placed on student volunteers for different time intervals. Samples were obtained from 5 adult subjects. The design of the experiment required sample collection at 3 days, 1 week, and 2 weeks. At least 4 indirect composite restorations were available for each patient, as at least 1 sample per patient was collected for the 3 different time intervals. The 4th indirect restoration from the sequence was used as a definitive restoration. The oral hygiene habits of the patients remained unchanged. All subjects reported brushing twice a day, without flossing or rinsing with oral solutions. For each time interval, the proximal surfaces of 5 indirect composite inlays were inspected. For the SEM analysis, a scanning electron microscopy (JEOL - JSM 6390, Jeol USA Inc) was used. The accelerating voltage was 20 kV. The analysis was made using fractured Au-coated samples. The observed surfaces were divided into three segments – coronal segment, medial segment and gingival segment.

Including criteria:

1. Age: 18 – 28 years; 2. Indications for indirect tooth colored restoration of posterior non-vital tooth with more than 2 missing surfaces; 3. PI (Silness-Loe) ≤ 1; 3. Written consent form, signed by the subjects of the trial.

Excluding criteria:

1. Systematic diseases with oral manifestation; 2. xerostomia; 3. Missing teeth; 4. Periodontal diseases; 5. Unfavorable occlusion - group-function occlusal alignment, or parafunctional activity such as bruxism or clenching.

Clinical protocol (No: 06/10.11.16. approved by Ethics Committee of Medical University of Plovdiv).

First appointment. Prior to the subject's participation in the trial, a written informed consent form was signed and personally dated by the subjects and by the study dentist who conducted the informed consent discussion. At the initial examination, an individual dental status was obtained (DMFt) of each subject, followed by a plaque index assessment (Silness-Löe Index) and radiographic evaluation of the dentition (OPG). The occlusal contacts were taken under consideration before initiating the tooth preparation, using 40 microns articulating paper (Bausch Arti-Check, USA). Only non-vital teeth were prepared. No anaesthetic solutions were used during the procedure. Prior to the tooth preparation a rubber dam sheet was placed ensuring proper isolation of the operative field. The teeth were prepared for indirect composite overlays following the sequential guidelines: a minimum reduction of 1.5 mm in the proximal boxes, rounded internal line angles, no undercuts in the cavity walls, butt-joint margins, and a minimum cuspal reduction of 2 mm. Occlusal divergence of proximal and occlusal walls of at least 6° to 8° **(Fig. 5A)**. After the preparation form was achieved, a semi-arch bite impression with a PVS material was taken using two step technique with a triple tray and bite registration. The preparations were temporized and the patients were instructed to return after a week.

Laboratory fabrication. The impressions were sent to a laboratory technician and 4 composite restorations were fabricated for each patient **(Fig. 5C)**. Composite material Filtek™ Z250 (3M ESPE, USA) was used instead of indirect lab composite.

Second appointment. At the second appointment, the temporary filling was removed and after proper isolation of the operative field (rubber dam) a visual evaluation of the cavity preparation was performed. After seating the composite prototype #1, the occlusion was adjusted and the prototype cemented to the tooth with a temporary cement (RelyX Temp NE 3M ESPE, USA) **(Fig. 5B)**.

In later appointments, the following procedures were performed:

Three days of sample collection. Removal of prototype #1 after 3 days of in vivo incubation and temporary cementation of prototype #2.

One-week of sample collection. Removal of prototype #2 after 1 week of in vivo incubation and temporary cementation of prototype #3.

Two weeks of sample collection. Removal of prototype #3 after 2 weeks of in vivo incubation and definitive overlay cementation using adhesive resin cement (RelyX Ultimate 3M ESPE, USA).

The occlusal divergence of the cavity walls (6° to 8°) allowed effortless removal of the prototypes

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without disturbing the integrity of the samples. All cavities were isolated with rubber dam to avoid contamination of the plaque samples during the prototype collection and replacement. The removed samples were immediately immersed in 2.5% glutaraldehyde solution for fixation of the biofilm. After 24 hours, the samples were rinsed with 0.1M Na-acetate buffer and dehydrated with graded ethanol series.[16]

RESULTS

Using scanning electron microscopy, microbial colonisation on the proximal surfaces of the resin composite prototypes was observed. The morphology of the accumulated biofilm will be described at various time intervals of up to two weeks. Correlation between the initial stages of plaque formation and the DMFt values was observed as well.

After three days

Scanning electron microscopy of 3-day-old composite specimens revealed biofilms with predomination of coccal forms. In three of the specimens (Patient 1, Patient 2, Patient 3), a highly organised microbial populations were observed with well-defined columnar microcolonies amongst intercellular matrix. Single rods and filaments were distinguished in the same specimens **(Figs 1A, B)**. In one of the specimens (Patient 5) microbial growth after 3 days was not identified **(Figs 2C, D)**. Scattered coccal forms with no specific organisation were detected on the resin surface of the last specimen reviewed – Patient 4 **(Figs 2A, B)**.

After one week

In two of the subjects (P4, P5) a slower plaque formation pattern was discovered – the subjects with minimal or no microbial growth at the previous time interval showed biofilm with prevalence of coccal forms after one week **(Fig. 3C)**. Columnar microcolonies amongst intercellular matrix were identified at 5000x magnification **(Fig. 3D)**. In the rest of the subjects (P1, P2, P3) a faster plaque maturation pattern was observed. The 7 days-old samples from these patients showed complex biofilm, containing filamentous forms penetrating the underlying coccal matrix **(Figs 3A, B)**. Columnar microcolonies were not visible anymore. More mature biofilm was accumulated near the gingival margin of the specimens.

After two weeks

At 2 weeks of surveillance, the plaque structure in the observed samples showed some notable change. Previously coccofilamentous structure, now shifted to predominantly filamentous **(Figs 4A-C)**. A more in-depth look at this change showed formation of corn cob structures at the surface of the biofilm **(Fig. 4D)**. However, subjects which at earlier point had shown low tendency of plaque formation, continued to form thin predominantly coccal biofilm.

DISCUSSION

Biofilms develop not only on the hard and soft tissues of the oral cavity, but also on the surface of different restorative materials in the operative dentistry.[16] Recent studies report that multispecies biofilms found on resin composite can cause deterioration of the surface and degradation of dentine-composite interface, eventually leading to secondary caries.[17] There is evidence that the monomers from the composite polymerisation process and some components of the adhesive systems can increase the growth of cariogenic microbiota including *S. mutans* and *Lactobacillus spp*.[18] The latter suggests that the bacterial adhesion process on composite surfaces and the factors promoting microbial adhesion should be taken under consideration. The chemical composition and surface roughness of the different restorative materials are proven to influence the microbial accumulation.[19] Based on this knowledge, materials could possibly be designed to inhibit plaque formation. To influence the microbial destruction of the biomaterials, a better understanding of the dynamics of a biofilm formation process is needed, as there is a difference between the microbial populations on restorative and natural teeth surfaces. Reproducible oral biofilm models for testing dental materials are constantly developed, using different substrates for in vivo and in vitro colonisation.[20-23] However, most of the experiments have been conducted with single-species biofilms or consortia of up to nine commensal species.[24,25] In a novel approach, oral inoculums have been used to generate complex microcosm biofilms on a composite surface in vitro. The microcosm models are incubated in a biofilm reactor, simulating the natural oral conditions.[26] The present study is a pilot study - a part of an experiment, conducted through clinical and laboratory protocols. An indirect protocol was used for the composite prototypes fabrication, thus giving better control on the proximal area of the restorations. The test samples reproduced actual composite restorations and provided a valuable insight of how the resin surface is colonised by the oral microbiota.

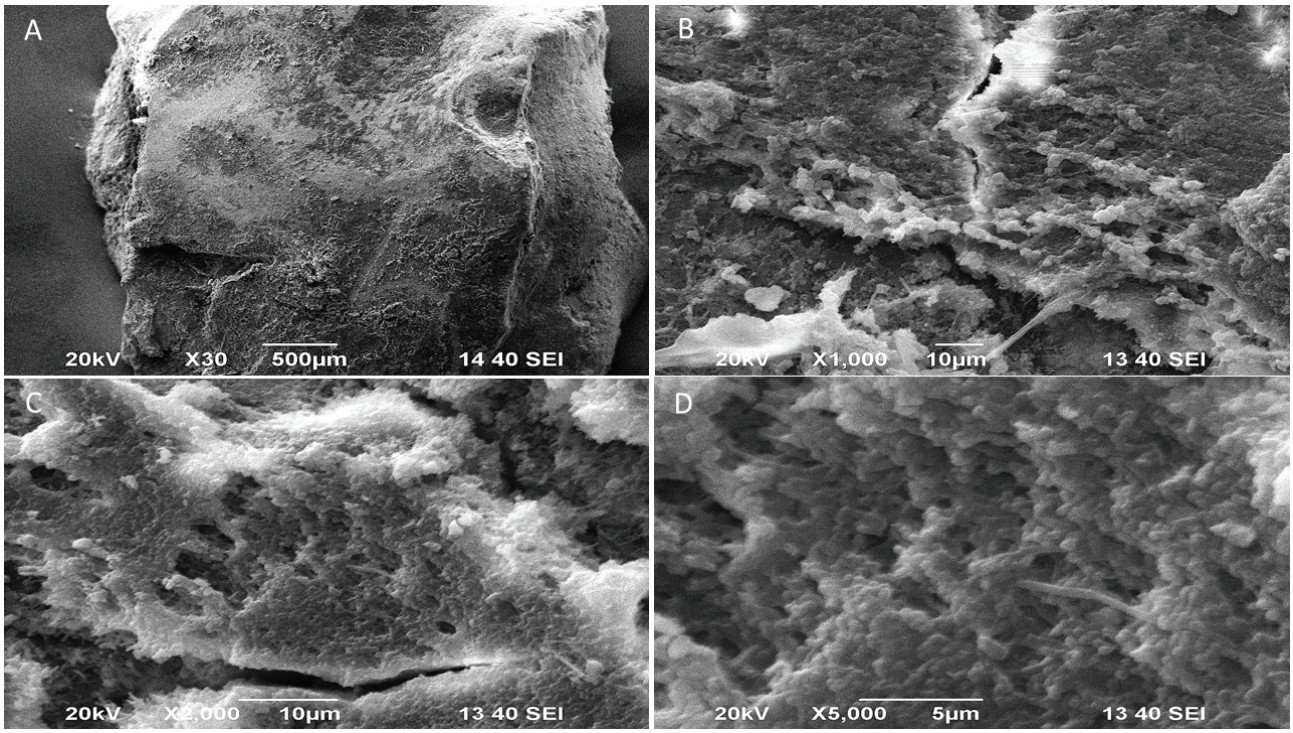

Figure 1. Scanning electron micrographs of resin composite surfaces after having been 3 days in vivo. Highly organised microbial populations with well-defined columnar microcolonies were distinguished **(A-D)**. Single rods and filaments were observed under higher magnification values **(C, D)**.

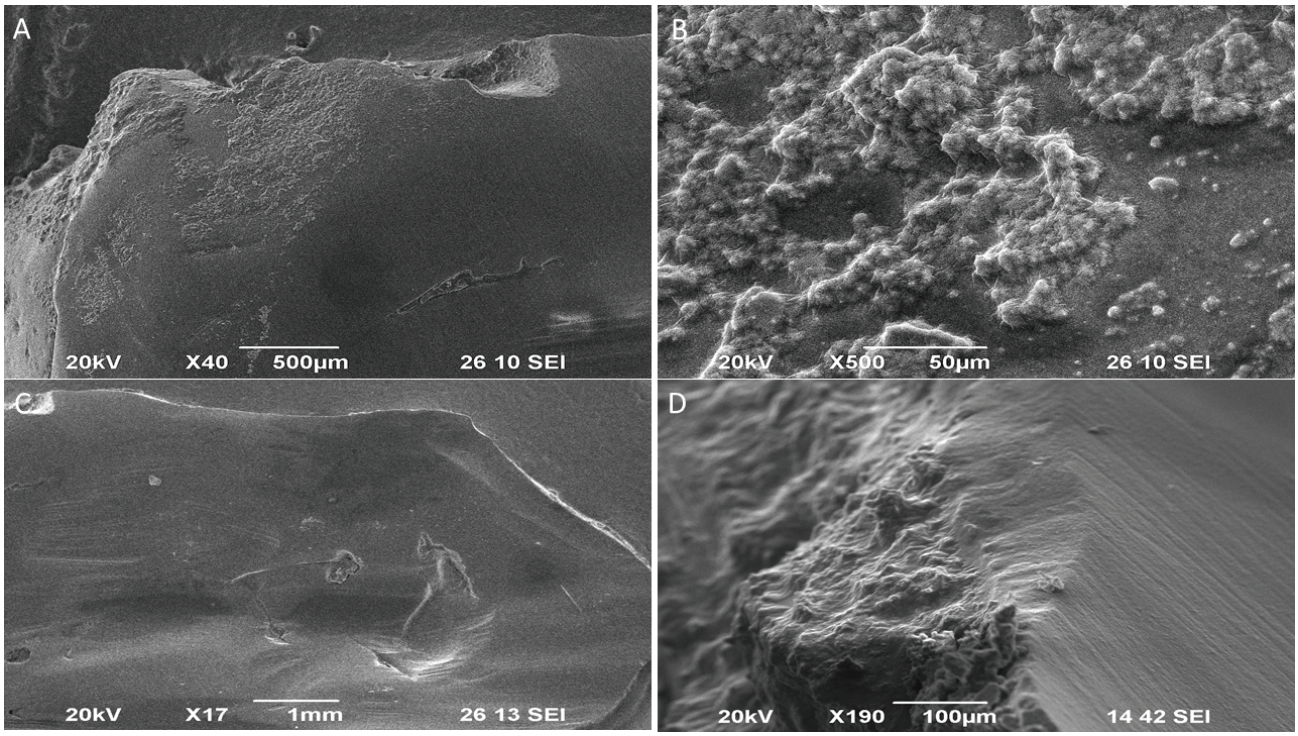

Figure 2. Scanning electron micrographs of resin composite surfaces after having been 3 days in vivo. In one of the subjects we observed poor plaque accumulation: single coccal colonies were found near the gingival margin of the composite specimen **(A, B)**. A sample from another subject could not provide any microbial growth at all after 3 days of incubation **(C, D)**.

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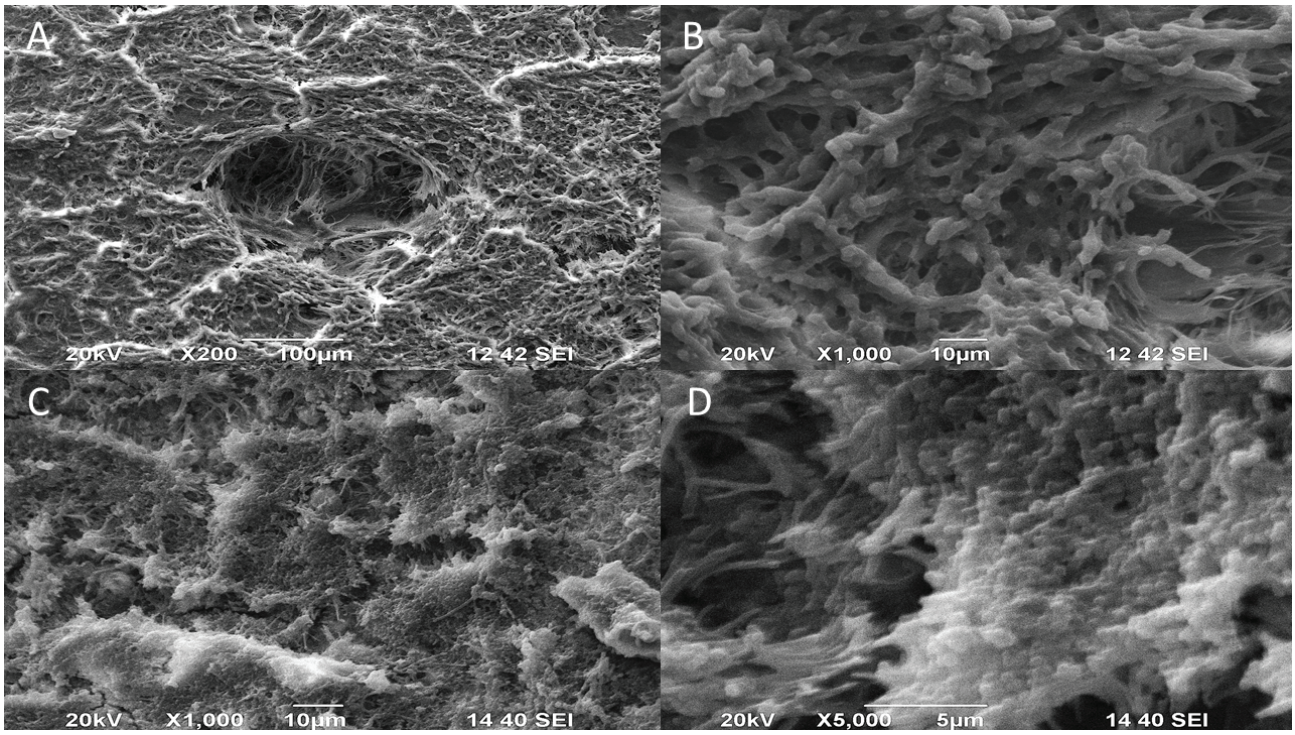

Figure 3. Scanning electron micrographs of resin composite surfaces after having been 1 week in vivo. The one-week samples from three of the subjects showed complex biofilm containing filamentous forms penetrating the underlying coccal forms **(A, B)**. The subjects with minimal or no microbial growth at the previous time interval showed biofilm with prevalence of cocci after one week **(C)**. Columnar microcolonies amongst intercellular matrix were identified at 5000x magnification **(D)**.

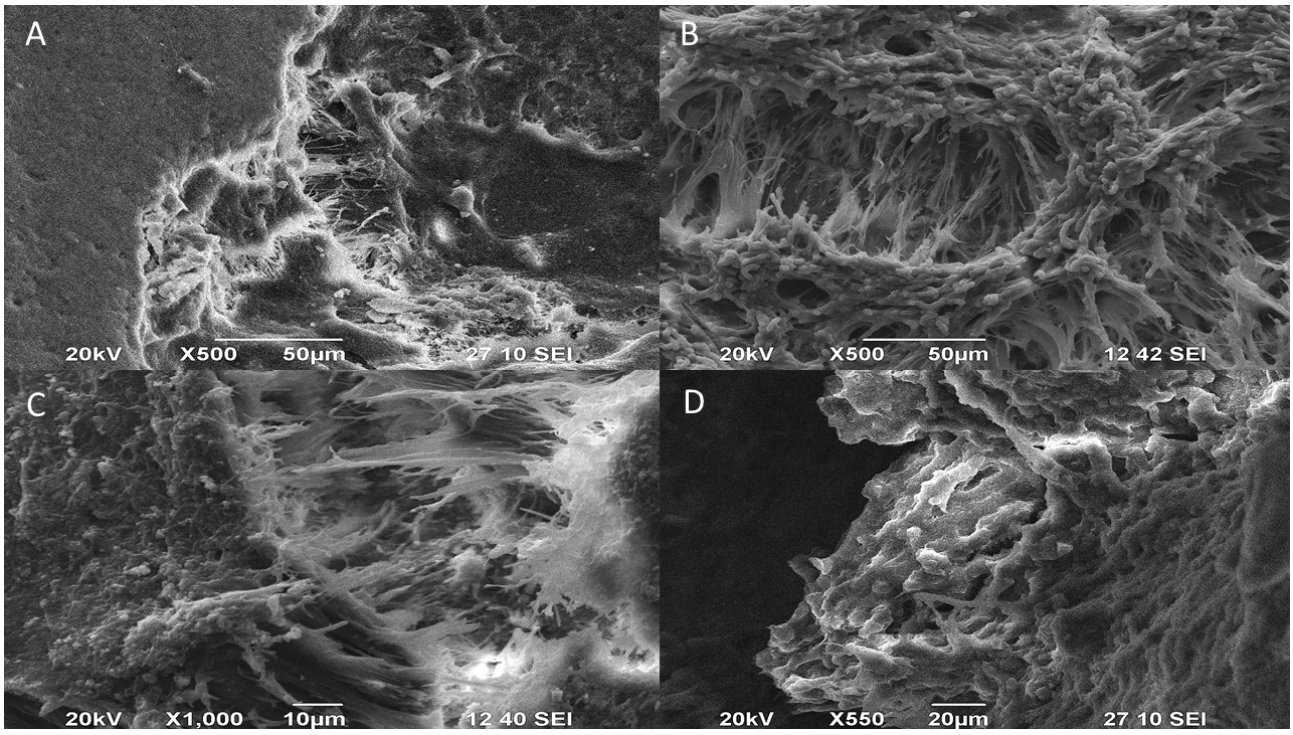

Figure 4. Scanning electron micrographs of resin composite surfaces after having been 2 weeks in vivo. Previously coccofilamentous structure shifted to predominantly filamentous **(A, B)**. Well organised extracellular matrix was observed between the biofilm layers **(B, C)**. We were able to identify corn cob structures at the surface of the biofilm **(D)**.

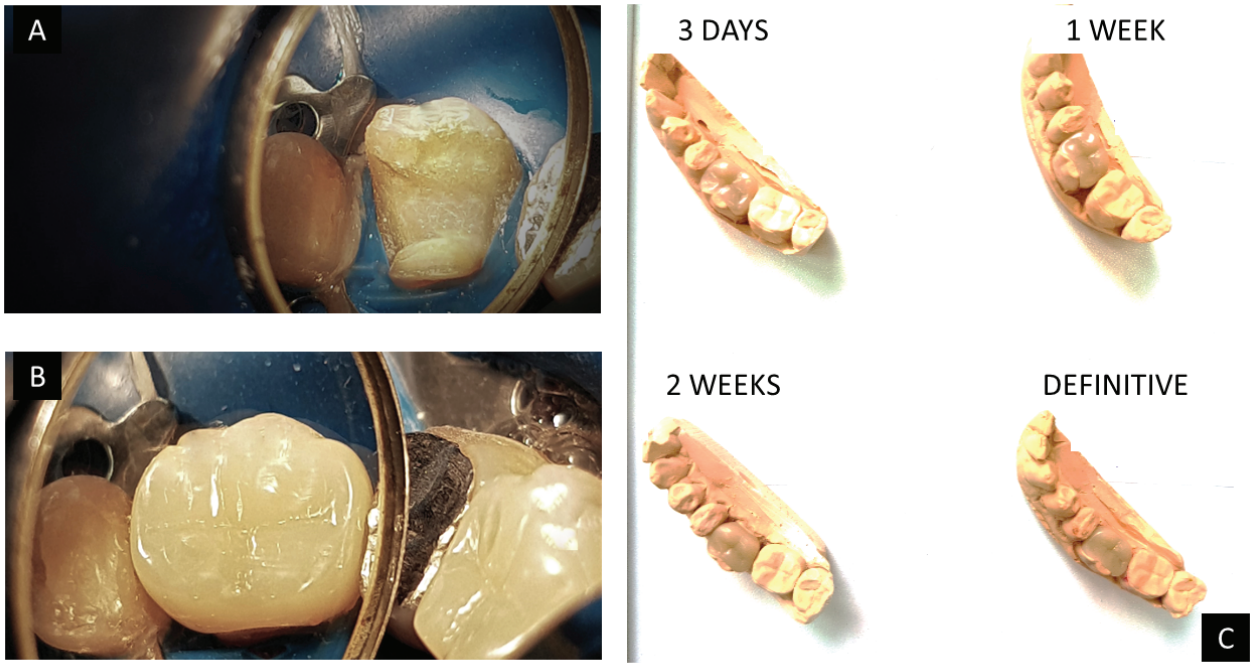

Figure 5. The clinical protocol included: preparation of the cavity for indirect resin composite overlay **(A)**; Four composite prototypes were fabricated for each subject **(C)**; The test samples for 3 days, 1 and 2 weeks were seated in the cavity with temporary cement **(B)**.

The timeline of the biofilm formation and microbial morphology were associated with previous studies of in vivo bacterial colonisation.[27-30] In the 3 days samples of the study, predomination of coccal forms was observed (an example of early colonial forms). The one-week samples showed complex biofilm containing filamentous forms (an example of late colonial forms) penetrating the underlying coccal matrix. Columnar microcolonies were not visible anymore. After two weeks, the coccofilamentous structure shifted to predominantly filamentous, thus indicating the end of the plaque maturation cycle. A correlation between the plaque formation cycle and the obtained DMFt indices was established. Slower plaque formation pattern was detected in subjects with a lower grade of DMFt index (DMFt/P4=13, DMFt/P5=10). Subjects with accelerated plaque maturation (P1, P2, P3) showed higher values of the DMFt index (DMFt/P1=22, DMFt/P2=20, DMFt/P3=18). The higher prevalence of dental caries may have an impact on the inherent microbiota of the individual, thus affecting the biofilm accumulation cycle on the oral surfaces. Implementing SEM observation allows defining only the microbial distribution within the plaque during the stages of maturation.[31-33] According to our findings of the microbial colonization on resin composite surfaces - after a period of 2 weeks, a mature multilayered biofilm will cover the proximal surface of the resin restoration. Coronally - near the contact point - the attached colonies were found to be smaller and less mature. Closer to the gingival margin of the restorations, conglomerating colonies with many filamentous forms were observed, which indicated the final stage of the biofilm maturation cycle.

To assign the ultrastructural morphology to certain bacterial taxa, DNA extracts of the plaque colonies are subjected to Human Oral Microbe Identification Microarray (HOMIM) analysis, based on 16S rRNA.[26] HOMIM analysis will be used in a following experiment to compare the microbial species populating in vivo and in vitro generated oral biofilms.

CONCLUSION

In this study, a novel clinical approach to investigating plaque accumulation on resin surfaces in vivo was presented. Temporary composite overlays were placed on 5 adult patients with indications for aesthetic indirect restorations in the distal area. Three different time periods were established to confirm earlier findings regarding the biofilm maturation cycle. It was estimated that after a period of 2 weeks, a mature multilayered biofilm will cover

the proximal surface of a resin composite restoration. Coronally, near the contact point, the attached colonies were found to be smaller and less mature. Closer to the gingival margin of the restorations, conglomerating colonies with many filamentous forms were observed, which marked the final stage of the biofilm maturation cycle.

ACKNOWLEDGMENTS

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Взятие пробы ин виво и СЭМ - идентификация оральной биоплёнки с использованием косвенных композитных прототипных восстановлений. Клиническое и лабораторное исследование

Костадин Г. Георгиев[1], Иван А. Филипов[1], Илиян Н. Добрев[2]

[1] Кафедра оперативной стоматологии и эндодонтии, Факультет дентальной медицины, Медицинский университет - Пловдив, Пловдив, Болгария
[2] Кафедра микробиологии, Университет пищевых технологий, Пловдив, Болгария

Адрес для корреспонденции: Костадин Г. Георгиев, Кафедра оперативной стоматологии и эндодонтии, Факультет дентальной медицины, Медицинский университет – Пловдив, бул „Христо Ботев" № 3, 4000, Пловдив, Болгария
E-mail: g.kostadin@gmail.com
Tel: +359883453823

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Введение: Экосистема полости рта представляет собой враждебную среду, в которой обитает более 700 микробных таксонов. Чтобы выжить, пероральная микробиота ассоциируется в сложных биоплёнках с метаболическими преимуществами. Твёрдые и мягкие ткани рта являются естественным субстратом для накопления оральной биоплёнки. Недавние исследования показывают, что биоплёнка, составленная из многих видов микроорганизмов, развивается на поверхности полимерных композитов, что вызывает ухудшение органического матрикса и компрометирует долговечность эстетических реставраций. Последнее предполагает, что на процесс бактериальной адгезии на композитных поверхностях и на факторы, способствующие микробной адгезии, следует обратить особое внимание.

Цель: Целью данного исследования является изучение нового клинического подхода в исследовании образования биоплёнки ин виво на полимерных композитных поверхностях. Композитные поверхности значительно отличаются от зубной эмали, и, таким образом, в формировании биоплёнки могут проявиться различия в зависимости от специфики типов и разновидностей.

Материалы и методы: С целью исследования развития бактериальной бляшки с минимальным нарушением её целостности мы решили применить косвенные восстановительные конструкции на основе смолы, которые были поставлены группе добровольцев из числа студентов на различные сроки. Испытательные образцы были копиями подлинных композитных восстановительных конструкций и обеспечили ценные сведения о том, как поверхность смолы колонизируется пероральной микробиотой.

Результаты и заключения: Временная шкала формирования биоплёнки и морфология микроорганизмов были связаны с предыдущими исследованиями бактериальной колонизации ин виво. Установлена корреляция между циклом формирования бляшек и DMFt – индексами объектов.