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TRATAMENTO DE LESÕES DE CÁRIE EM DENTINA E ANÁLISE DO PERFIL MICROBIANO ASSOCIADO

Tese apresentada à Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas como requisito para a obtenção do título de Doutor em Odontologia, Área de Saúde Coletiva.

Orientadora: Profa. Dra. Maria da Luz Rosário de Sousa

Co-orientador: Prof. Dr. Reginaldo Bruno Gonçalves

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RESUMO

O fator mais importante para o desenvolvimento de lesões de cárie é a atividade metabólica do biofilme sobre os tecidos dentais. A partir desta definição e do desenvolvimento de novos materiais restauradores, uma abordagem mais conservadora sobre o tratamento de lesões de cárie em dentina vem sendo pesquisada.

Capítulo 1 – Objetivo: Comparar os resultados do acompanhamento clínico e radiográfico após 24 meses, de molares permanentes com lesões de cárie em dentina, com extensão igual ou maior que o terço médio, após a remoção total ou parcial de dentina cariada. Metodologia: 18 molares permanentes foram estudados de 11 adolescentes com idade entre 12 a 17 anos. Os dentes foram randomizados em dois grupos com 9 voluntários em cada um deles. No grupo experimental foi realizada a remoção parcial de dentina cariada e o forramento da cavidade com cimento de ionômero de vidro, e no grupo controle foi realizada a remoção total de dentina cariada e forramento com hidróxido de cálcio e ionômero de vidro. Ambos os tratamentos foram realizados em uma única sessão e o material restaurador utilizado foi a resina composta fotopolimerizável. Os dentes foram avaliados por meio de testes de vitalidade pulpar e exame radiográfico durante dois anos. Resultados: Nenhum dente estudado apresentou imagem radiográfica sugerindo lesão periapical em ambos os grupos, e nenhum voluntário sentiu dor sem estímulo durante o tempo de acompanhamento. Conclusão: Conclui-se que a manutenção de dentina infectada sob restaurações pode ser indicada em dentes permanentes de adolescentes e o tratamento pode ser realizado em uma única sessão sem a necessidade de reabertura do dente.

Capítulo 2 – Objetivo: comparar o perfil microbiano de lesões de cárie em dentina não detectadas clinicamente (NCD) e de lesões detectadas clinicamente (CD), por meio da técnica de Reação em Cadeia da Polimerase baseada na

Eletroforese em Gel com Gradiente Desnaturante (PCR-DGGE). Metodologia: 8 amostras de dentina cariada não detectadas clinicamente (NCD) e 8 detectadas clinicamente (CD), coletadas previamente ao procedimento restaurador (Capítulo 1), foram analisadas pela técnica de PCR–DGGE. Lesões em dentina sob esmalte íntegro foram consideradas lesões não detectadas clinicamente. Foi realizada a extração de DNA e em seguida a PCR com um conjunto de *primers* universais do 16S rRNA. Os fragmentos do 16S rDNA amplificados pela PCR foram separados pelo gel de DGGE. Os perfis microbianos foram comparados utilizando os programas Bionumerics e Primer5. Resultados: A heterogeneidade dos perfis demonstrou a nítida separação entre os grupos com baixa de similaridade entre eles (45% R>0,5). Não houve diferença estatisticamente significante entre a média do número de *amplicons* (bandas) das lesões NCD e CD (p=0,40). Conclusão: Os resultados sugerem que existem espécies bacterianas claramente diferenciadas entre lesões NCD e CD, porém com algumas sobreposições entre elas, e lesões de cárie CD possuem uma microbiota tão complexa quanto as lesões NCD.

Palavras-chave: Remoção parcial de cárie, diversidade microbiana, cárie em dentina.

ABSTRACT

The most important factor to development of caries lesion is the metabolic activity of biofilm on the dental tissues. By this definition and of the development of new restorative materials, a more conservative approach about dentin caries lesion treatment has been researched.

Chapter 1. Objective: To compare the clinical and radiographic outcomes of permanent molar teeth, with deep lesions treated by complete or partial removal of carious dentin after follow-up over a 24-month period. Methodology: 18 permanent molars of 11 adolescents, aged 12 to 17 years, were assigned to interventions by using random allocation. In the experimental group nine teeth were submitted to partial removal of carious dentin, protected with glass ionomer cement and restored with resin composite. In the control group, nine teeth were submitted to complete removal of carious dentin, protected with calcium hydroxide and glass ionomer cement and restored with resin composite. Radiographic examination and pulp vitality tests were performed 12-24 months after cavity sealing and the teeth were not re-entered. Results: No volunteer felt pain without stimulus and no teeth presented an image suggesting periapical lesion. Conclusion: The results suggest that partial removal of carious dentin can be indicated to maintain the pulp vitality and that there is no need to reopen after cavity sealing.

Chapter 2. Objective: To compare the microbial profile of non-clinically (NCD) and clinically detected dentin-related caries lesions (CD) by Polymerase Chain Reaction based-Denaturing Gradient Gel Eletrophoresis. Methodology: 8 dentin caries sample of non-clinically detected lesions (NCD) and 8 clinically detected (CD), previously collected to operative procedure (Chapter 1), were assessed by PCR-DGGE. Dentin caries lesions underlying sound enamel were considered non-clinically detected lesions. The total microbial genomic DNA of the sample was isolated. PCR was performed with a set of universal bacterial 16S rDNA primers.

The PCR-amplified 16S rDNA fragments were separated by DGGE. The groups were assessed by comparing the PCR-DGGE fingerprinting profile using Bionumerics and Primer5 programs. Results: The profile heterogeneity demonstrated the evident separation of the groups with low similarity between them (45% R>0,5). There was no statistical difference between the means of amplicons (bands) of the NCD and CD lesions (p=0,40). Conclusion: These findings suggest that there are clearly differentiated species between the groups; however, with some overlap between them, and that CD lesions had a microbiota as complex as those of NCD lesions.

Key-words: Partial caries removal; microbial diversity; dentin caries.

SUMÁRIO

1. INTRODUÇÃO

A carie dentária é definida como uma doença complexa, causada por um distúrbio no equilíbrio fisiológico entre o conteúdo mineral do dente e o fluido do biofilme dental (Ferjerskov, 2004), que resulta na destruição localizada de tecidos dentais mineralizados por ácidos, produzidos durante a fermentação de carboidratos da dieta pelas bactérias bucais (Selwitz *et al*, 2007). Os sinais iniciais no esmalte dental não são detectáveis com os exames clínicos e radiográficos tradicionais. A doença é reversível, inicialmente, podendo ser paralisada em qualquer estágio, mesmo quando da presença de cavitação de esmalte ou dentina, se na presença de condições apropriadas (Selwitz *et al*, 2007), principalmente pela exposição a diferentes formas de fluoretos e controle dos níveis de biofilme dental.

Por esta definição do processo de cárie, o fator mais importante passa a ser a atividade metabólica no biofilme, e a desmineralização do esmalte e da dentina pode ser vista como o reflexo deste processo dinâmico. A implicação desta definição é que o sinal do processo (a lesão) pode ser modificado pela alteração do biofilme, deste modo, a desorganização deste com escova e dentifrício fluoretado pode modificar a lesão (ten Cate & Featherstone, 1996).

Entretanto, em estágios avançados da cárie, nem mesmo uma escovação cuidadosa consegue remover o biofilme de uma cavidade. Assim, a dentística restauradora tem a função de devolver a integridade do dente para que o paciente possa higienizá-lo corretamente. Porém, uma vez que a dentina se torna desmineralizada e infectada existem dúvidas com relação à quantidade de tecido cariado que deve ser removido de uma cavidade antes do dente ser restaurado.

Com o desenvolvimento de novos materiais adesivos e uma abordagem mais conservadora, uma nova fase da odontologia se iniciou (Ricketts & Pitts, 2009). Também conhecida como Odontologia de Mínima Intervenção, esta busca

prevenir e detectar doenças orais no seu estágio mais precoce com objetivo de minimizar o tratamento invasivo (Hien Ngo, 2009). Desta forma, a manutenção da vitalidade pulpar é o objetivo principal do tratamento conservador de lesões profundas de cárie em dentina (Casagrande *et al*, 2009), e remover parcialmente esta lesão pode ser uma das formas de atingir este objetivo.

Não existe evidência clara de que seja prejudicial deixar dentina cariada se ela estiver úmida e amolecida antes de selar a cavidade. Realmente, esta prudência pode ser preferível à curetagem vigorosa, porque quanto menos a polpa estiver exposta e estando a dentina selada, sem contato com o ambiente oral, maior o estímulo para paralisação da progressão da lesão (Mertz-Fairhurst *et al*, 1998). O processo reparativo de esclerose tubular e dentina terciária serão estimulados reduzindo a permeabilidade da dentina remanescente e os microrganismos estarão então em um ambiente muito diferente (Kidd, 2004).

Para Kidd (2004), a permanência ou não de dentina infectada sob uma restauração é irrelevante quando se aceita que o processo de cárie é guiado pelo biofilme e seu reflexo é a lesão nos tecidos dentais. Deste modo, a remoção parcial de dentina cariada deve ser preferível à remoção completa em lesões profundas para diminuir o risco de exposição pulpar (Ricketts *et al*, 2009; Lula *et al*, 2009; Thompson *et al* 2008; Oliveira *et al*, 2006). E, diminuir o risco de exposição pulpar significa evitar futuros tratamentos endodônticos.

Algumas técnicas foram propostas com o objetivo manter a vitalidade pulpar, entre elas: o selamento de lesões em dentina, no qual toda a dentina cariada permanece sob a restauração definitiva (Mertz-Fairhurst *et al* 1998); o capeamento pulpar indireto, no qual apenas a dentina cariada com consistência "firme e semelhante ao couro" não é removida e o cimento de hidróxido de cálcio é empregado sobre a mesma; e o procedimento de curetagem em 2 estágios ou "stepwise excavation", no qual a dentina amolecida e úmida é removida na primeira sessão, o dente é restaurado provisoriamente e reaberto após algumas

semanas para nova curetagem e restauração definitiva (Kidd, 2004). Estas duas últimas técnicas são as mais utilizadas entre os cirurgiões-dentistas, no entanto, novos estudos clínicos controlados são necessários antes que elas sejam amplamente aceitas na Odontologia e também para demonstrar a necessidade ou não da reabertura da cavidade em uma segunda sessão (Ricketts *et al*, 2009; Bjordal, 2008).

As bactérias cariogênicas isoladas da sua fonte de nutrição por uma restauração com boa integridade marginal, ou morrem ou permanecem inativas favorecendo a vitalidade pulpar (Thompson *et al*, 2008). Os microrganismos envolvidos em lesões iniciais e no desenvolvimento de novas lesões cárie já estão bem documentados (Li *et al*, 2007; Byun *et al* 2004; Munson *et al* 2004), assim como a microbiota de lesões de cárie em dentina (Loesche *et al*, 1973; Bjorndal & Larsen, 2000; Martin *et al*, 2002; Aas *et al* 2008). Esses estudos demonstraram que a dentina cariada é dominada por bactérias gram-positivas, particularmente dos gêneros *Actinomyces, Lactobacillus, Propionibacterium* e *Streptococcus*. No entanto, a diversidade bacteriana em lesões de cárie em dentina não detectadas clinicamente ainda não está bem relatada

Lesões de cárie em dentina não detectadas clinicamente são lesões sob esmalte sadio, sem cavidade aparente e que são detectadas com o auxílio do exame radiográfico (Weerheijm *et al.*, 1992). Estudos demonstraram que a microbiota dessas lesões era menos complexa quando comparada com lesões clinicamente detectáveis (Weerheijm *et al.*, 1990; de Soet *et al.*, 1995) e os autores sugeriram que os resultados demonstraram uma diferença etiológica entre as lesões. Porém, estes estudos foram realizados por meio de cultura e nenhum deles utilizou métodos de genética molecular para analisar o perfil microbiano deste tipo de lesão.

Os estudos sobre comunidades microbianas tem tradicionalmente dependido das técnicas de isolamento e cultivo. No entanto, a maior limitação destes métodos é que 50% da microbiota presente na cavidade bucal não cresce quando se utiliza

técnicas microbiológicas convencionais (Wade, 1997). Por este motivo, métodos de genética molecular baseada em genes específicos têm sido efetivamente utilizados para se obter uma identificação mais acurada (Ida *et al*, 1999).

Com o advento destes novos métodos tem sido possível reavaliar a patogênese de infecções orais, e estes têm sido usados para caracterizar a microbiota associada à abcessos alveolares, periodontites e infecções endodônticas, e em cada caso tem sido encontrado que, linhagens ainda não caracterizadas representam uma proporção substancial da microbiota presente (Dymock *et al*, 1996; Paster *et al*, 2001; Munson *et al,* 2002).

A técnica da Reação em Cadeia da Polimerase (PCR), utilizada na detecção e identificação de bactérias orais (Igarashi *et al*, 1996; Alam, *et al*, 2000) envolve a síntese *in vitro* de milhões de cópias de um segmento específico de DNA na presença da enzima *Taq* DNA polimerase. A amplificação enzimática decorre do anelamento de iniciadores que delimitam as seqüências do DNA de dupla fita, que se deseja amplificar (Igarashi *et al*, 1996). Esses iniciadores são sintetizados artificialmente, de modo que as seqüências de nucleotídeos sejam complementares aquelas que flanqueiam a região que será amplificada.

Dentre as diferentes aplicações da PCR, a PCR-DGGE (Reação em Cadeia da Polimerase associada a Eletroforese em Gel de Gradiente Desnaturante) tem a capacidade de fazer um levantamento total da comunidade bacteriana sem cultivo (Muyzer *et al*, 1995). Esta técnica tem a vantagem da ubiqüidade do lócus 16S rRNA no ambiente microbiano, o qual pode ser amplificado pela PCR e seqüenciado com um conjunto de "primer" universal. O refinamento do DGGE é que ele permite diferenciar produtos gênicos amplificados pela PCR de comprimentos similares, mas com seqüências diferentes, separados em um gel de gradiente desnaturante. A diferenciação da espécie bacteriana está baseada no seu diferencial de migração no gel como uma função do percentual de guanina mais citosina e no seu comportamento de fusão. O resultado é um gel com várias

bandas em cada "canaleta", com cada banda presumivelmente representando um diferente microrganismo dentro da comunidade microbiana de uma amostra (Muyzer *et al*, 1993).

Atualmente, a técnica da PCR-DGGE tornou-se uma importante ferramenta para estudos de comunidades bacterianas complexas em uma variedade de habitats, incluindo bolsas periodontais, saliva e canais radiculares (Zijnge *et al*, 2003; Li *et al*, 2005; Rôças *et al*, 2004). No entanto, ainda não foi utilizada para o levantamento do perfil microbiano de lesões de cárie em dentina detectadas e não detectadas clinicamente.

Além das considerações anteriores relacionadas à manutenção da vitalidade pulpar e a diversidade bacteriana de lesões de cárie em dentina, deve haver preocupação também com o bem-estar do paciente e a realidade dos serviços públicos de saúde no Brasil. Estudos demonstram que tratamentos realizados em lesões de cárie profunda, com a remoção parcial da dentina infectada, resultaram em quantidade menor de exposições pulpares (Bjorndal, 2001, 2002). Resultados estes que beneficiam tanto o paciente, pela não necessidade de se submeter a novas intervenções, quanto à população como um todo, pois muitos casos que seriam encaminhados para tratamento endodôntico deixam de ser, o que diminui a espera pelo atendimento nos serviços públicos e torna menos oneroso o atendimento odontológico no ambiente do Sistema Único de Saúde, permitindo que seus recursos sejam direcionados para outras prioridades em saúde bucal.

Assim, os objetivos deste estudo foram comparar os resultados do acompanhamento clínico e radiográfico após 24 meses, de molares permanentes com lesões de cárie em dentina, com extensão igual ou maior que o terço médio, após a remoção total ou parcial de dentina cariada; e comparar o perfil microbiano de lesões de cárie em dentina não detectadas clinicamente (NCD) e de lesões detectadas clinicamente (CD), por meio da técnica de Reação em Cadeia da

Polimerase associada a Eletroforese em Gel de Gradiente Desnaturante (PCR-DGGE).

Os objetivos específicos deste estudo serão apresentados na forma de capítulos:

I. Twenty-four months of follow-up after partial removal of carious dentin: a randomized clinical trial.

II. Microbial Diversity Analysis of Non-Clinically and Clinically Detected Dentin-Related Caries Lesions.

CAPÍTULO I

Twenty-four months of follow-up after partial removal of carious dentin: a randomized clinical trial.

Partial removal of carious dentin: a longitudinal study

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Abstract

Objective: The aim of this study was to compare the clinical and radiographic outcomes of permanent molar teeth with deep lesions treated by complete or partial removal of carious dentin after follow-up over a 24-month period.

Methods: A total of 20 adolescents from Piracicaba, São Paulo, Brazil were screened; 11 had at least one deep carious lesion in permanent molars. 18 permanent molars were assigned to interventions by using random allocation. In the control group, nine teeth were submitted to complete removal of carious dentin, protected with calcium hydroxide and glass ionomer cement and restored with resin composite. In the experimental group nine teeth were submitted to partial removal of carious dentin, protected with glass ionomer cement and restored with resin composite. Radiographic examination and pulp vitality tests were performed 12-24 months after cavity sealing and the teeth were not re-entered.

Results: A total of 16 teeth of the 9 volunteers were included in the final statistical analysis. One volunteer of the experimental group felt pain during the pulp vitality test after 12 months, however, the symptoms stopped after the stimulus ended and the tooth did not present an image suggesting periapical lesion. No volunteer felt pain without stimulus and no teeth presented an image suggesting periapical lesion.

Conclusions: The results suggest that partial removal of carious dentin can be indicated to maintain the pulp vitality and that there is no need to reopen after cavity sealing.

Key words: Partial caries removal, deep caries lesion, permanent teeth.

Introduction

With the development of new adhesive materials and a more conservative approach, a new era of minimally invasive dentistry has dawned (Ricketts & Pitts, 2009). Also known as Minimal Intervention Dentistry (MID), in practice, it seeks to prevent and detect oral diseases at the earliest stage in order to minimize invasive treatment; and where surgical intervention is indicated, the least invasive restorative technique is used (Hien Ngo, 2009). Thus, maintenance of pulpal vitality is the primary objective of conservative treatment of deep carious lesion (Casagrande *et al*, 2009), with partial caries removal being one of the possibilities of achieving this goal.

Dental caries is a infection process caused by acids from bacterial metabolism diffusing into enamel and dentin and dissolving the mineral. There are many possibilities for intervening in this continuing process to arrest or reverse progression of the lesion (Featherstone, 2008) and one of these could be to modify the biofilm (ten Cate and Featherstone, 1996). At an advanced stage of caries a cavity can retain the biofilm and careful brushing cannot remove it. Thus, the role of operative dentistry is to restore the integrity of the tooth so that the patient can clean effectively. (Kidd, 2004).

The treatment of deep carious lesions approaching healthy pulp presents the practitioner with a significant challenge, and recent concepts in caries management have not yet been adopted in everyday practice (Thompson *et al*, 2008; Doméjean-Orliaguet *et al*, 2009). Although partial caries removal would appear to be preferable to complete caries removal in deep lesion, in order to decrease the risk of pulp exposure (Ricketts *et al*, 2009; Thompson *et al*, 2008; Oliveira *et al*, 2006; Kidd, 2004), in a recent survey, only approximately 20% of network dentists favored partial caries removal techniques in deep lesions (Oen *et*

al, 2007). Thompson *et al.* (2009) suggested that before this concept can be accepted by the dental profession, additional clinical trials will be necessary.

In addition to decreasing the risk of pulp exposure, a more conservative approach could be a patient-friendly treatment to the repair reversible damage of the disease (Mickenautsch, 2005). Furthermore, avoiding endodontic treatment, which could be the next step after pulp exposure, would reduce time and cost. Therefore, in community health dentistry, more patients would be treated with the same financial resources.

In contries in development, where the financial resources for health are limited and the major part of the population depends on the Public Health Services, the difficulty of the access to specialized treatment is considered a risk factor for tooth loss. Patients without alternative prefer tooth extraction to enduring the pain.

However, whilst there is evidence that partial caries removal can be the elective treatment, there is insufficient evidence to indicate whether it is necessary to re-enter and excavate further in the stepwise excavation (Ricketts *et al* 2009). Considering that after sealing, the level of colonization in partial caries removal is similar to that in complete caries removal for all microorganisms (Lula *et al* 2009; Orhan *et al*, 2008; Ricketts, 2008) and carious lesions can be arrested if the restoration margins remain sealed (Mertz-Fairhurst *et al* 1998; Falster *et al*, 2002), the aim of this study was to compare the clinical and radiographic outcomes of permanent molar teeth with deep lesions after partial caries removal in a single session or complete caries removal and definitive restoration, during follow-up over a 24- month period.

Materials and Methods

This twenty-four month randomized controlled clinical study was conducted in groups of adolescents residing in Piracicaba, Sao Paulo, Brazil. The approval for

the study was obtained from the Human Research Ethics Committee of Piracicaba Dental School, State University of Campinas (Report n. 102/2006). All adolescents were instructed regarding oral hygiene procedures and received dental care at the Dentistry Clinic of Piracicaba Dental School where this study was carried out.

Sample size

The volunteers of this study took part in a previous study in which the prevalence of clinically undetected caries lesions was verified in Piracicaba, Brazil (Rando-Meirelles & Sousa, 2010). All adolescents that had at least one occlusal deep lesion in this previous study were invited to participate in the present study.

In order to take part of it, male and female adolescents had to be among 12 to 17 years old and at least one occlusal deep carious lesion in permanent molar with a radiographic image equal or greater in extension than the middle third of the dentin. They were not admitted to the study if any of the following criteria were present: proximal, buccolingual or palatolingual lesion and radiographic image suggesting periapical lesion in the tooth in the study; spontaneous pain or sensitivity to vitality tests; insufficient address or unwillingness to return for follow-up. 20 volunteers were screened and 11 were found to meet the eligible criteria (Figure 1).

Randomization

The adolescents had an equal probability of assignment to the groups. The sample was randomized using a random-number table, for a total of 9 teeth in the control group and 9 teeth in the experimental group (Figure 1). The adolescent that had more than one lesion had probability to be allocated in the 2 groups, as volunteers 1, 7 and 8 (Table 1).

Figure 1. Flow diagram of the progress through the study phases according to the CONSORT statement (Moher *et al.*, 2001).

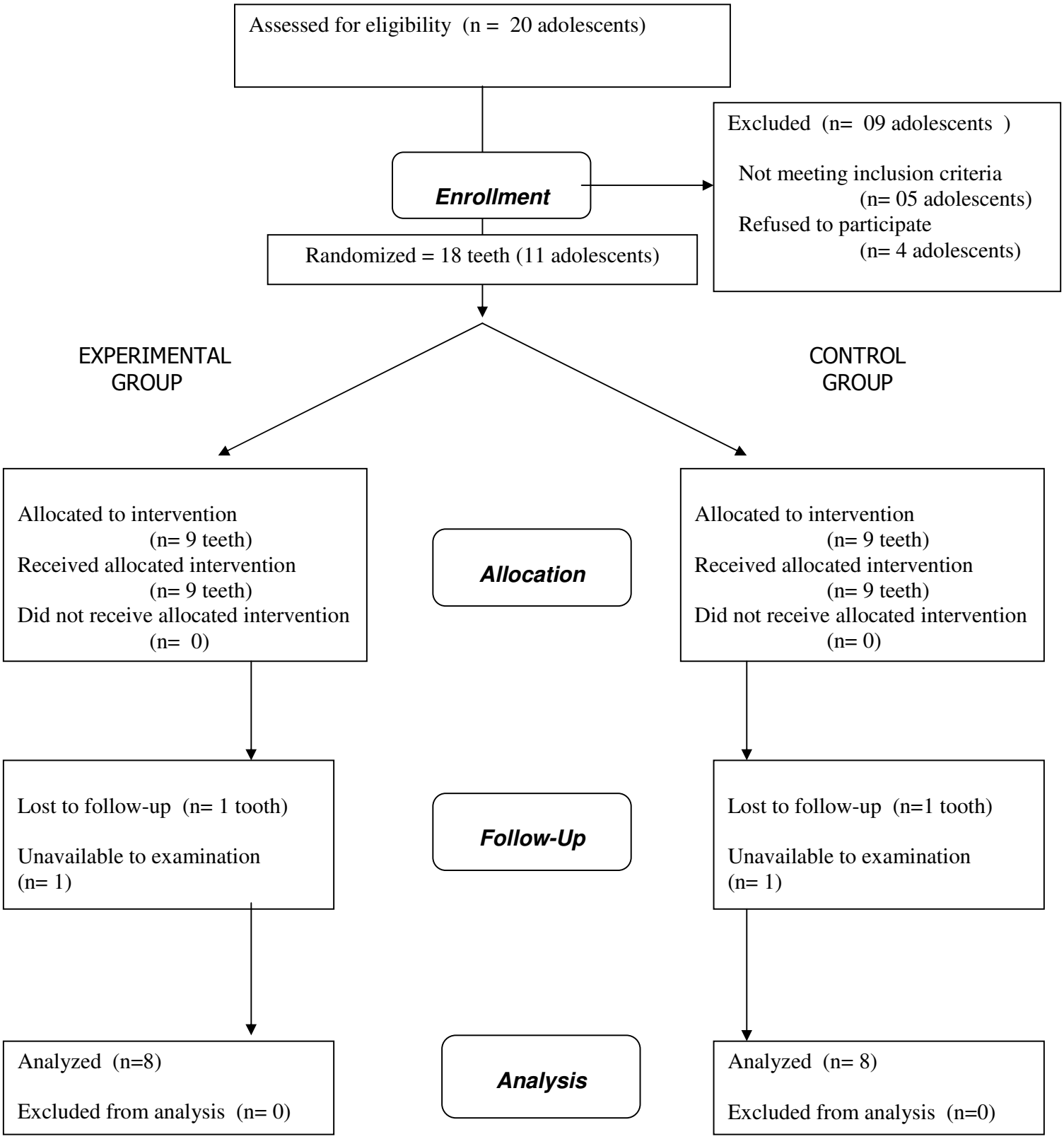

Table 1. Characteristics of the sample. Piracicaba, Brazil. 2007.

VOLUNTEER	AGE	GENDER	TOOTH	FOLLOW-UP (MONTH)
1	17	M	17	24
1	17	M	27	24
1	17	M	37	24
2	15	F	47	18
3	12	M	16	18
4	15	M	46	X
5	15	M	36	X
6	12	M	17	18
6	12	M	26	18
7	13	F	46	24
7	13	F	47	24
7	13	F	37	18
8	12	F	27	24
8	12	F	16	24
9	12	F	36	18
9	12	F	46	18
10	12	M	26	12
11	12	F	16	12

Restorative Procedure

The 18 permanent molars were randomized into two groups of 09 teeth, with regard to the type of treatment to be performed (control and experimental groups).

The area to be treated were anesthetized and isolated with rubber dam. Some of the teeth had no cavity; that is to say, the enamel was integral and in these cases, access to the lesion was performed with burs at high revolutions per minute.

In the control group, conventional treatment was performed, with complete removal of carious dentin (TR) using burs at low revolutions per minute and excavator. The complete removal of carious dentin meant removal all of soft tissue until hard dentin. For lining, calcium hydroxide cement (Dycal, Caulk/Dentsply, Rio de Janeiro, Brazil); as base, conventional glass ionomer cement (Vidrion F, S.S.White Brazil, Rio de Janeiro, Brazil); and for the definitive restoration, an

adhesive system and resin composite (Single Bond and Filtek Z 350, 3M, St Paul, MN, USA) were used.

In the experimental group, the carious dentin was partially removed (PR) with an excavator for this purpose. The peripheral carious dentin; that is, the adjacent walls were completely removed; however, the carious dentin under the pulp wall was not removed. Conventional glass ionomer cement (Vidrion F, S.S.White Brazil, Rio de Janeiro, Brazil) was used for lining, and the definite restoration was performed with an adhesive system and resin composite (Single Bond and Filtek Z 350, 3M, St Paul, MN, USA).

Radiographic Follow-up and Pulp Vitality Test

For diagnosing the lesion and including the tooth in the study, periapical and interproximal (bitewing) radiographs were taken. Teeth that presented a radiolucent image equal to or greater in extension than the middle third of the dentin, however, without attaining the pulp chamber, and which did not have an image suggesting periapical lesion, were included in the study.

Radiographic follow-up was performed every six months over a period of 24 months, and periapical and interproximal radiographs were taken.

Pulp vitality tests were performed with refrigerated gas (Aerojet, Rio de Janeiro, Brazil). These tests were performed in the first step before the restorative procedure, and at all the stages of radiographic follow-up.

Criteria for clinical and radiographic evaluation

The two types of study treatment were evaluated clinically according to marginal integrity (MI) and vitality tests (VT); radiographically according to eventual changes in periapical tissue (PT) and caries lesion (CL). The criteria ranged from the best rating to the worst one. The result was considering good, satisfactory or unsatisfactory, if at least one of the criterion described in table 2 was present.

Table 2. Criteria for clinical and radiographic evaluation.

	Criteria			
	Clinical		**Radiographic**	
	Marginal Integrity	**Vitalily Tests**	**Periapical Tissue**	**Caries Lesion**
Good	Without failure	No sensitivity	No image suggesting periapical lesion	Apparent arrest of the carious process
Satisfactory	Partial failure	Sensitivity during test with total remission of the simptons	No image suggesting periapical lesion	Apparent arrest of the carious process
Unsatisfactory	Total failure	Spontaneous sensitivity	Image suggesting periapical lesion	Increase of the lesion

Statistical analyses

The Mann Whitney test was used to compare the mean follow-up time between the two groups.

Results

The characteristics of the sample are shown in detail in Table 1. The final sample consisted of 16 teeth of 9 patients, as two volunteers withdrew from the research, one being the control and the other the experimental group.

Table 3 demonstrates the number of teeth followed-up in each group, the success rate after radiographic and clinical examinations. Only one patient reported sensitivity during the thermal test, however, with total remission of symptoms immediately after removal of the stimulus. This patient, a 12-year old boy, was part of the experimental group, and the treated tooth was the maxillary left permanent first molar (26).

Table 3. Rate of success after radiographic examination (RE) and pulp vitality test (VT) in the experimental group (PR) and control group (TR).

	Treament group and test							
	PR				**TR**			
	RE		**VT**		**RE**		**VT**	
	N	%	n	%	n	%	N	%
Good	8	100	7	87.5	8	100	8	100
Satisfactory	0	0	1	12.5	0	0	0	0
Unsatisfactory	0	0	0	0	0	0	0	0
TOTAL	8	100	8	100	8	100	8	100

In the control group (TR) the mean follow-up time was 21 (sd=3.21) months and in the experimental group (PR) it was 18.7 (sd=5.01) months. This difference was not statistically significant (p=0.36).

Discussion

The partial removal of carious dentin has been indicated as one step of the stepwise excavation and has already been documented (Leksell *et al*, 1996; Bjørndal *et al*, 1997, 1998, 2000; Maltz *et al*, 2002; Kidd, 2004). However, the need to re-enter the cavity has been questioned (Ricketts *et al*, 2009) and the results of this study suggest that there is no need for re-entering the cavity in the teeth of young patients. These results are in agreement with those of three other studies, Mertz-Fairhurst *et al* (1998) in permanent teeth, Ribeiro *et al* (1999) and Lula *et al* (2009) in deciduous teeth.

The cavity sealing after partial removal of carious tissue may modify bacterial growth and drastically reduce the presence of cariogenic bacteria, and their persistence does not seem to be a reason for reopening of cavities in deciduous and permanent teeth after partial caries removal (Lula *et al*, 2009;

Oliveira *et al*, 2006). In addition, it does not depend on the type of filling material used (Mertz-Fairhurst *et al*, 1998; Ribeiro *et al*, 1999; Franzon *et al*, 2007) because the aim is to effectively seal the lesion from the oral environment.

The possible indication of partial removal of deep carious lesions in a single session, as part of the public service protocol, would bring advantages to the patient and the service. The benefit to the patient would be a more conservative and simultaneously a less invasive approach to caries treatment, reducing widespread patient dental anxieties (Mickenautsch, 2005). For the service, decreasing the treatment from two sessions to one session would be reduce both time and cost, allowing a larger number of patients to be treated, as well as avoiding pulp exposure and probably future need for endodontic treatment. The endodontic treatment should be indicating when all possibilities of less invasive treatment had been used. Hommez *et al* demonstrated that when the coronal restoration was good in teeth with a good endodontic treatment, 22.5% of these teeth had apical periodontitis. It meant in that study that of the 182 endodontically treated teeth, 41 had no success.

During the vitality tests, one tooth presented painful symptoms after 12 months of the treatment, but the painful sensation ceased immediately after the stimulus was removed. In this case, the first permanent molar was the affected tooth and the patient was twelve years old. The lesion was clinically undetected and could be diagnosed after the radiographic examination. The lesion was soft and wet, as were the majority of the lesions included in this study. This symptom could be associated with reversible pulpitis. This diagnosis implies that the pulp is vital, but has some areas of inflamed tissue that will heal after conservative vital pulp therapy (Sigurdson A, 2003). Mild trauma with subsequent inflammation can cause small regions of neurogenic inflammation and sufficient mechanical damage to stimulate a nerve sprouting reaction (Byers *et al*, 1988) and thereby possibly

cause exaggerated response to vitality tests, indicating more severe inflammation than is actually present.

Fifty percent of the teeth treated were non-clinically detected lesions; it means that the lesions, in which the enamel was apparently sound, were detected by radiographic examination and not during visual examination. It shows that partial caries removal can be used in both clinically undetected and clinically detected lesions.

Recents concepts as partial removal of carious dentin have not yet been adopted in everyday practice (Thompson *et al*, 2008; Doméjean-Orliaguet *et al*, 2009). Thompson suggests that before this concept can be accepted by the dental professionals, additional clinical trials will be necessary. Therefore, our results add evidences to previous studies (Mertz-Fairhurst *et al*, 1998; Ribeiro *et al*, 1999; Lula *et al* 2009) and provide to dental profession subsidy to make decisions in caries management.

Maintenance of pulpal vitality is the primary objective of the conservative treatment of deep carious lesions (Casagrande *et al*, 2009) and the results of this study showed that the partial removal of dentin caries in a single session in permanent teeth can be the elective treatment to attain this objective.

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CAPÍTULO II

Microbial Diversity Analysis of Non-Clinically and Clinically Detected Dentin-Related Caries Lesions.

Abstract

Objective: The aim of this study was to compare the microbial profile of non-clinically and clinically detectable dentin-related caries lesions using PCR-based denaturing gradient gel electrophoresis (DGGE). Methodology: 11 volunteers, from 12 to 17 years old, participated in this study. Carious dentin samples with extension equal or greater than the middle third of the dentin were collected from sixteen permanent molars, 8 being of non-clinically detectable caries lesions (NCD) and 8 of clinically detectable caries lesions (CD). The samples taken from the diseased dentin tissue were collected at points apical to the enamel-dentin junction. The DNA from each sample was purified and the 16S rDNA PCR was performed. After that, a Nested-PCR with primers containing a 40GC-clamp was processed. The *amplicon* products were defined in a gradient gel after electrophoresis and a band profile was defined for each sample. The microbial profile was compared by *Bionumerics* and *Primer5* software, and the ANOSIM and SIMPER statistical tests were used. Results: The profile heterogeneity demonstrated the evident separation of the groups with low similarity between them (45% R>0,5). There was no statistical difference between the means of *amplicons* (bands) of the NCD and CD lesions (p=0,40). Conclusion: These findings suggest that there are clearly differentiated species between the groups; however, with some overlap between them, and that CD lesions had a microbiota as complex as those of NCD lesions.
Key words: Dentin caries microbiology, molecular analysis, PCR-DGGE, non-clinically caries lesions.

Introduction

Dental caries is one of the most prevalent chronic diseases in the world (Anusavice, 2002; World Health Organization - WHO, 2002) and the microbial population involved in the lesions is known to be highly complex and variable (Martin *et al*, 2002; Munson *et al*, 2004). The microorganisms related to initial lesions and early caries development have been extensively documented in the literature (Napimoga *et al*, 2004; Li *et al*, 2007; Aas *et al* 2008), as well as those in dentin caries lesions (Bjorndal & Larsen, 2000; Martin *et al*, 2002; Munson *et al* 2004; Chhour *et al* 2005). These studies demonstrated that the carious dentin is commonly dominated by gram-positives bacteria, particularly those of the *Veillonella*, *Actinomyces, Lactobacillus, Propionibacterium* and *Streptococcus* genera.

Carious dentin may be described and classified as two closely related habitats. The former may be composed of soft tissue, a heavily infected, necrotic, and irreversibly demineralized superficial zone; the latter is found in deeper, less infected and reversibly damage tissue (Fusayama, 1979). Although they have divergent properties, Munson *et al* (2004), using culture and molecular analyses, demonstrated that there were no significant differences between these two types of lesions as regards the microbial profile.

When comparing the microbiota between non-clinically detectable (lesion underlying sound enamel) and clinically detectable dentin caries, one study demonstrated that the former had a less complex microbiota (de Soet *et al*, 1995), nevertheless, this study used only culture methods to obtain its results. Non-clinically detectable dentin caries are considered lesions in dentin underlying intact enamel and is diagnosed by radiographic examination.

The major limitation of culture studies is that around 50% of the oral microbiota do not grow in conventional culture media in a laboratory (Wilson *et al*, 1997), and due to this impediment, molecular methods have been used. The

advent of new molecular methods has made it possible to reevaluate the pathogenesis of oral infections and a substantial proportion of the microbiota are from species have not yet been characterized (Dymock D *et al.*, 1996; Wade *et al.*, 1997; Paster *et al.*, 2001; Munson *et al.*, 2002). The Polymerase Chain Reaction (PCR) is the basis for many other techniques in diagnostic microbiology, such as PCR-DGGE (Denaturing Gradient Gel Electrophoresis).

The PCR-DGGE is capable of surveying entire bacterial communities without cultivation. The DGGE approach is based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of DNA denaturants. These 16S rDNA fragments with similar lengths but different base-pair sequences can be separated based on their differential migration in the gel as a function of percent of guanine plus cytosine (G+C content) and melt behavior (Muyzer *et al*, 1993). This technique has become an important tool for studying complex bacterial communities in a variety of habitats, including environmental biofilms, periodontal pockets, endodontic infections (Zoentendal *et al* 1998; Zingle *et al* 2003; Roças *et al* 2004).

Therefore, although there are some reports in the literature, up to now, little evidence has been described as regards the microbiota of non-clinically detectable dentin caries. In view of this, the aim of the study was to compare the microbial profiles of non-clinically detectable and clinically detectable carious dentin based on the PCR-DGGE technique.

Methodology

Sample – Sixteen samples of carious dentin from permanent molars were analyzed in 11 adolescents ranging from 12 to 17 years of age. Theses adolescents were selected in a previous study, in which an epidemiological survey of this age group was conducted in the Municipality of Piracicaba, São Paulo, Brazil (Rando-Meirelles & Sousa, 2010). Of these sixteen samples, 8 were from teeth with

clinically detected dentin caries (CD) and 8 from teeth with non-clinically detected dentin caries (NCD), that is to say, detected by radiographic examination. The volunteers included in this study presented teeth with occlusal caries lesions in dentin with a radiographic image equal or greater in extent than the middle third of dentin, and in the cases of non-clinically detected lesions, teeth that presented sound enamel on visual examination. An informed consent was obtained from all volunteers, and the study was approved by the Ethics Committee of the Piracicaba Dental School-UNICAMP (Protocol No. 102/2006).

Sample collection – All the volunteers were submitted to local anesthesia and rubber dam isolation of the area to be treated. In the teeth with clinically sound enamel, surface cleaning was performed with sterile water and a Robinson brush at low speed, and minimum opening required for sample removal was performed at high speed. In the teeth in which enamel was not intact and the lesions were apparent, the most superficial lay of carious dentin was removed and discarded, and then, the samples were collected.

The samples were harvested with sterile curettes just below the enamel-dentin junction and immediately stored in RTF solution at -20°C.

DNA purification and manipulation – the samples were kept in water-bath at 37°C for 10 minutes, and vortex-mixed for 30 seconds. The bacterial cells were precipitated at 20.000g for 10 minutes, the supernatant was discarded. The DNA from each sample was purificated using a chloroform/isoamyl alcohol, CTAB and Proteinase K protocol (Saito *et al*, 2006; Kuipers *et al*, 1999; Smith *et al*, 1989).

Each sample was analysed for the genomic-DNA concentration (50 nm) by spectrophotometry (GeneQuant^{TM} 1300 – General Eletric Healthcare). The DNA samples were kept at -20°C until evaluation. The 16S rDNA of each sample was amplified with the primers D88 and E94 described by Paster *et al* (2001).

Some genomic DNA from different bacteria species (*S. mutans*, *P. endodontalis*, *L. acidophylus*, *P. vulgatus*, *E. faecalis*, *S. gordonii*, *S. sanguinis*) were used to prepare the reference standard product for the band normalization.

PCR DGGE – A pair of primers bac1 and bac 2 (Rupf *et al*, 1999) with a 40 GC clamp added to the 5' end of the bac1 (Li *et al*, 2005) were used for this study.

The nested-PCR was prepared with a 50 µL final solution with H_2O milli-Q – 38.25 µL; PCR Buffer 10X - 5 µL; dNTP 10 mM – 0.5 µL; $MgCl_2$ 50 mM – 2.5 µL; Forward Primer 10 µM– 1.0 µL; Reverse Primer 10 µM – 1.0 µL; TAQ DNA polimerase 5U/µL (0.25 µL – Taq DNA Platinum); DNA template from the 16S rRNA PCR product - 1 µL (~100 ng). The *nested*-PCR conditions were: 95°C initial denaturation for 5 minutes and 30 cicles of 95°C for 1 minute, 56°C for 1 minute, 72°C for 2 minutes and a final extension of 72°C for 30 minutes.

The polyacrilamide gels were prepared according the DCode™ Universal Mutation Detection System's manual recommendations. A 30-70% linear denaturing (urea and formamide) gradient was prepared in an 8% (w/vol) polyacrilamide gel. The PCR product of each sample and from the control was loaded in each lane. The electrophoresis was set to 60V at a 60°C constant temperature for 16 hours.

PCR-DGGE microbial profile analyses – after electrophoresis, the gels were rinsed and stained in a fresh solution containing 0.2% silver nitrate, fixed and scanned in an *ImageScanner* PowerLook 1120 USG (Amersham Biosciences). The microbial profile from each sample was compared with Bionumerics software 5.1 (Applied Maths NV. Belgium). The tolerance in relation to the band position was 0.8%. The analyses were made upon the algorithm UPGMA (Unweighted Pair-Group Mothods using Arithmetic Averages) and Jaccard index similarity index. Cophenetic correlation coefficient was used to evaluate the consistency among the clusters. Multivariated analyse (PCoA) and the similarity analysis (ANOSIM) were made with *Primer5* for Windows version 5.2.6 (Plymouth Marine Laboratory,

Primer-E, United Kingdom) and the banding pattern richness were analyzed with EstimateS for Windows 8.2.

The t test was used to compare the mean of the number of *amplicons* between the groups (CD and NCD) and among the samples.

Results

Table 1 shows the general characteristics of the studied sample, such as age, gender, number of the tooth from which the sample was collected, and whether the lesion was clinically detected (CD) or non-clinically detected (NCD).

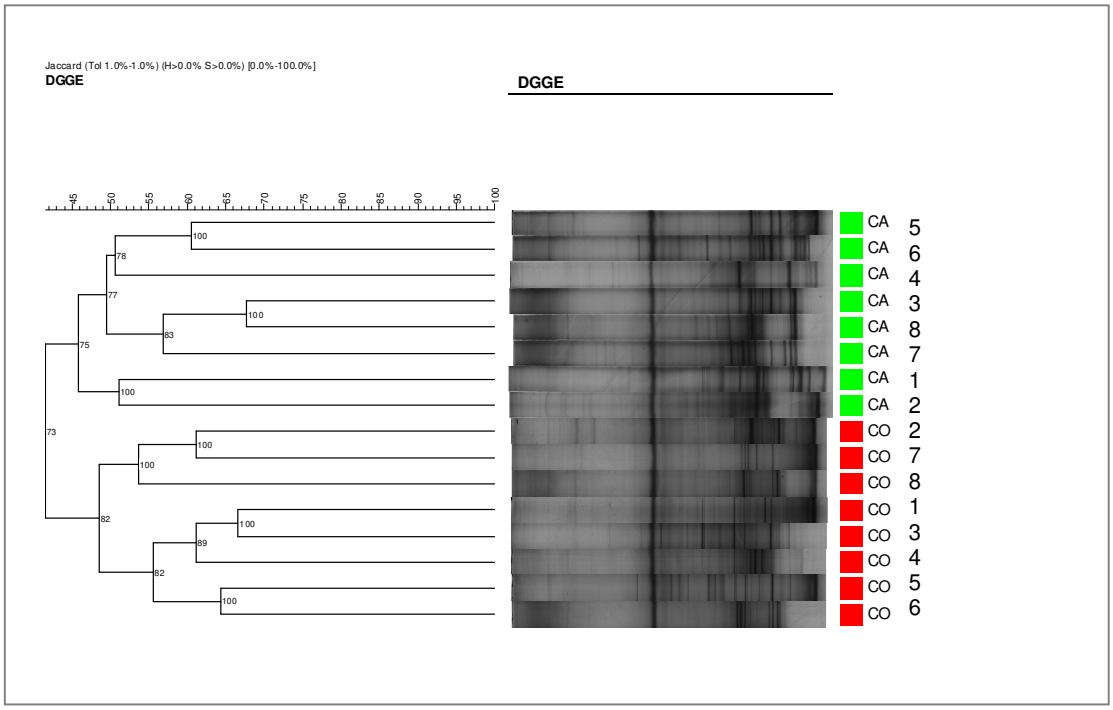
Table 1. Characteristics of the sample.

SAMPLE	VOLUNTEER	AGE	GENDER	TOOTH	LESION TYPE*
CO1	1	17	M	27	NCD
CO3	1	17	M	37	NCD
CO2	2	15	F	47	NCD
CA1	3	12	M	16	CD
CA2	5	15	M	36	CD
CA3	6	12	M	17	CD
CA4	6	12	M	26	CD
CA5	7	13	F	46	CD
CA6	7	13	F	47	CD
CO4	7	13	F	37	NCD
CO5	8	12	F	27	NCD
CA7	8	12	F	16	CD
CO6	9	12	F	36	NCD
CO7	9	12	F	46	NCD
CO8	10	12	M	26	NCD
CA8	11	12	F	16	CD

* NCD – non-clinically detected lesion
CD – clinically detected lesion

Discriminations of the PCR products were made on DGGE gels combining the microbial profiles of different groups present in CD and NCD lesions.

Classification done by the hierarchical method allowed an elucidative dendogram to be constructed, containing the sequences in which divisions or unions occurred of the different profiles of the microbial communities revealed by DGGE. The heterogeneity of these profiles is represented by the position of their knots in the similarity baseline. Figure 1 demonstrates the clear separation between the groups, and which of the samples show similarity between them. This figure also illustrates the similarity between samples from the same volunteer. The samples of CD lesions CA3 and CA4 are from volunteer 6 and presented a similarity of 49%; CA5 and CA6 are from volunteer 7 and presented a similarity of 60%; and NCD lesions CO1 and CO3 are from volunteer 1 and presented a similarity of 67%; CO6 and CO7 are from volunteer 9 and presented a similarity of 48%. Distortion of the original matrix was quantified by the cophenetic correlation coefficient, which expressed values that guarantee the reliability of interpretation of the clustering generated by the UPGMA method [r>0,8] (Rohlf, 2000).



Non-clinically detected lesion;
Clinically detected lesion.

Figure 1. Clusters analysis by *fingerprints* DGGE of microbial communities in dentin caries lesions

The richness of rDNA *amplicons* signifies the number of band separated by the DGGE gel in each sample. This number was compared between the studied groups (NCD and CD lesions), and also among the samples from a same volunteer. When the *amplicons* richness in each type of lesion was analyzed, the mean in the group of NCD lesions was 32.5 (sd=2.9) and in the CD lesions was 30.6 (sd=4.9), without statistically significant difference between them (p=0.40). When the samples from the same volunteer, as from volunteer 7 (samples CA5, CA6 and CO4), and from volunteer 8 (samples CA7 and CO5), which presented both NCD and CD lesions were analyzed, no statistical significance was found either (Table 1).

Table 1. Richness of *amplicons* in samples of volunteers with different lesions types.

Volunteer	CD lesions	NCD lesions	
	n of *amplicons*	N of *amplicons*	
7	33	36	P=0,29
8	32	35	P=0,31

Principal Components Analysis was performed with the same set of data used in the hierarchical classification (Figure 2). This indirect gradient analysis method consists of a multivariate statistical technique that allowed the distribution of the microbial community profiles along the rank axes based on the presence and absence of bands detected by DGGE. The percentage values associated with each rank axis (X, Y) inform the explainability of these axes throughout the changes perceived in the community structures within the rank space.

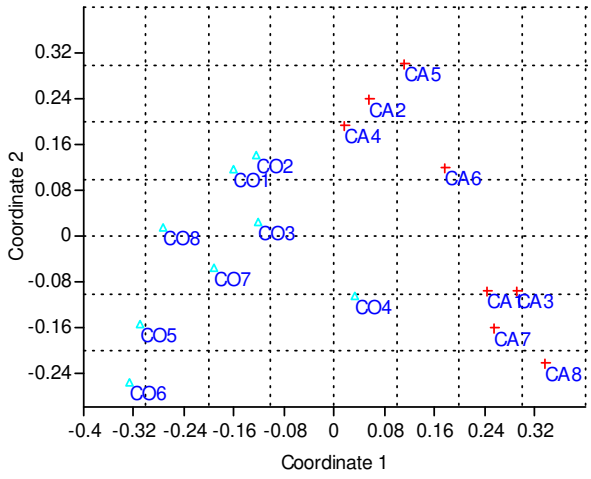


Figure 2. Principal Coordinates Analysis (PCoA) with Bray-Curtis similarity index.

The analyses of similarities (ANOSIM) were performed with the sets of data generated by DGGE in order to compare the community structures of the different microbial groups studied. The distance matrix was produced without data transformation and using *Bray-Curtis* (Legendre and Legendre, 1998) as distance measure. The R values obtained using 1000 permutations expressed the separation level of the microbial community structures ranging in a scale of 0 (indistinguishable) to 1. Values of $R>0.75$ were interpreted as indicative of well separated groups; $R>0.5$ as groups with overlapping, but clearly differentiated and $R<0.25$ as groups with hardly any separation, in accordance with the *Primer5* program manual (Clarke and Gorley, 2001). In this study, the descriptive analysis demonstrated the value $R>0.5$ ($p<0.001$), which means that the clinically and non-clinically detected caries lesions presented clearly differentiated species, but with species present in the two groups.

Discussion and Conclusions

The microbiota associated with caries lesions have traditionally been identified by culture methods; however, molecular analysis methods are increasingly being used for this purpose (Munson *et al,* 2002; Li *et al,* 2006; Aas *et al*, 2008). The intention of this work was to study non-clinically detected dentin caries, because although there are studies that have documented the microbiota of carious dentin by molecular techniques, none of them have researched these lesions underlying sound enamel and without direct contact with the oral environment, as occurs in this type of lesion.

The advantages of the use of molecular techniques, especially DGGE, to survey the microbial diversity related to caries lesions include their ability to amplify regions of 16S rDNA directly from environmental samples, without cultivation of these samples (Muyzer, 1993; Santegoeds *et al*, 1996; Meroth *et al,*

2003), and furthermore, the detection of more bacterial species, particularly those that are anaerobic and non-cultivable.

A limitation of the DGGE technique was considered to be the analysis of diversity patterns being restricted to visual comparisons and interpretations (Li *et al*, 2005). However, in this study, the Bionumerics program, which has the ability to acquire DGGE gel images and transfer them to an analytical software and record the banding patterns, was used and enabled a more precise comparison to be made.

In this study the DGGE profiles richness analysis demonstrated that there was no statistically significant difference between the NCD and CD lesions ($p=0.40$) with regard to the mean numbers of detected *amplicons*, demonstrating that both types of lesion presented complex microbiota. There was no difference when of the microbial profiles of lesions between the different volunteers were compared, and also when the comparison was made between different lesions from the same volunteer (volunteers 6 and 7). These results differed from those of previous studies (Weijkheim *et al*, 1990; de Soet *et al*, 1995) which, by culture methods, demonstrated a less complex microbiota in non-clinically detected dentin caries when compared with clinically detected lesions. The results of the present study demonstrated that carious dentin in non-clinically detected lesions were not isolated from the oral environment even with the enamel apparently sound, since their microbiota was as complex as that of lesions that were in direct contact with this environment. And the data also confirm the importance of biofilm, that when it is in disequilibrium results in the loss of minerals, leading to the dissolution of dental tissues (Kidd, 2004), even when it is far from the lesion.

Although a maximum of 41 *amplicons* were detected on the DGGE gel in all the samples in this study, one cannot conclude that this is the total number of species found in caries lesions, because unrelated bacterial species may have similar or identical migration distances in gel (Muyzer, 1999) or two

phylogenetically related bacterial species may have close band positions on DGGE gel (Hayes *et al*, 1999).

Classification done by the hierarchical method allowed the construction of a dendogram containing the sequences in which divisions or unions occurred of the different profiles of the microbial communities revealed by DGGE. The heterogeneity of these profiles demonstrated clear separation between the groups, with a similarity of 45% between them. Within the CD group, the samples with greater similarity were samples 5 and 6 from volunteer 7, which demonstrated a similarity of 60% between them, and samples 3 and 4 from volunteer 6 which demonstrated 68% similarity, suggesting that the microbiota of CD lesions from the same volunteer have greater similarity. In both cases the reliability of the clustering interpretation generated by the UPGMA method (unweighted pair-group) was 100%. In the NCD group, samples 1 and 3 of volunteer 1 demonstrated 66% similarity with 100% reliability, values close to those of the lesions in group CD, nevertheless, samples 6 and 7 of volunteer 9 demonstrated 49% similarity between them with 86% reliability, demonstrating a lower percentage of similarity.

In view of the results, it could be concluded that there are clearly differentiated species between two groups, however, with some overlapping between them, and that CD caries lesions have a microbiota as complex as that of NCD lesions.

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CONCLUSÕES

Em síntese, os resultados evidenciam que a manutenção da dentina infectada sob restaurações que permitem o isolamento da mesma do substrato oral é viável e o tratamento pode ser realizado em uma única sessão, não sendo necessária a reabertura da cavidade. Este procedimento economiza tempo e recursos financeiros, o que em serviços públicos se traduz em mais pacientes atendidos e recursos destinados a outras prioridades.

As técnicas moleculares permitem uma visão globalizada da composição microbiana que dificilmente seria obtida por técnicas tradicionais e, portanto, possuem alta aplicabilidade na caracterização das comunidades bacterianas associadas às lesões de cárie em dentina tanto naquelas não detectadas clinicamente quanto nas detectadas clinicamente.

Considerando o delineamento do estudo e as técnicas empregadas, conclui-se que:

1. A remoção parcial de dentina cariada e o subseqüente tratamento restaurador em sessão única, em dentes com lesões de cárie com extensão igual ou maior que o terço médio da dentina, pode ser empregada em adolescentes, não sendo necessário que o procedimento seja realizado em duas etapas como descrito em protocolos anteriores;
2. A remoção parcial de dentina cariada, como descrito neste estudo, pode ser realizada tanto em lesões de cárie não detectadas clinicamente quanto em lesões de cárie detectadas clinicamente;
3. Lesões de cárie em dentina não detectadas clinicamente possuem microbiota tão complexa quanto as lesões detectadas clinicamente.
4. Apesar de complexas, estas lesões apresentam espécies claramente diferenciadas entre si, porém com algumas sobreposições entre eles.

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