

CLINICAL AND MICROBIOLOGICAL CHARACTERISTICS IN PREDICTING DENTINE CARIES PROGRESSION

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ABSTRACT

The purpose of this study was to evaluate the clinical aspect of dentine and its microbiota in predicting caries progression. The sample consisted of schoolchildren in the 7 to 14 years age group. Treatment involved cavity preparation through the clinical criterion of hardness, with the collection of carious and remnants dentine for microbiological analysis. The clinical aspect (color and consistency) of the dentine remnants was recorded and the teeth were restored using silver amalgam and glass ionomer cement as pulpal protector (baseline – BL). After 1 year the restoration was removed and after new clinical and microbiological analyses, the teeth were then restored. Microbiological samples were collected at both time-points and cultivated in sheep blood agar, in anaerobiosis for 48 hours. Bacterial growth was analyzed quantitatively. Semiquantitative

and qualitative analysis of the bacteria was performed by hybridization with genomic DNA probes and the checkerboard method.

A significant difference was observed between the aspect of dentine remnants at BL and at 1 year ($p=0.0078$). The amount of bacteria at BL and at 1 year did not differ significantly ($p=0.37$) and the microbiota of the carious dentine was predominantly composed of Gram-positive cocci. The removal of carious dentine based on the clinical criterion of hardness, followed by a well-adapted restoration, would determine the non-progression of caries. The few bacteria that still remained in the cavity would be no longer viable.

Key words: dental caries, dentine, hardness, microbiology, DNA probes.

ASPECTO CLÍNICO E MICROBIOTA DA DENTINA PÓS PREPARO CAVITÁRIO E SUA RELAÇÃO COM A PROGRESSÃO DE CÂRIE

RESUMO

Este trabalho objetivou avaliar o aspecto clínico da dentina e sua microbiota na predição da progressão de cárie. A amostra foi constituída por indivíduos na faixa etária de 7 a 14. O tratamento envolveu a remoção de tecido cariado pelo critério clínico de dureza, o qual foi coletado análise microbiológica. Após o mesmo, fez-se análise do aspecto clínico da dentina remanescente, bem como nova coleta microbiológica e restaurou-se os elementos com amálgama de prata, após forramento com ionômero de vidro. Um ano após, com a reabertura do dente, outra coleta microbiológica foi feita, bem como análise do aspecto clínico do tecido remanescente. O crescimento bacteriano foi analisado quantitativamente através de hibridização com sondas de DNA genômico e do método Checkerboard.

Observou-se diferença estatisticamente significativa entre o aspecto da dentina na linha base do estudo e um ano após ($p=0.0078$). A quantidade de bactérias na linha base, em relação a um ano depois do preparo, não diferiu estatisticamente ($p=0,37$) e a microbiota predominante da dentina cariada foi composta de cocos Gram-positivos.

A remoção de dentina cariada pelo critério clínico de dureza, favorecida pela boa adaptação da restauração determinaram a não progressão de cárie. As poucas bactérias que ainda permaneceram na cavidade após o preparo provavelmente tornaram-se inviáveis em promover o avanço do processo cariioso.

Palavras chave: cárie dentária, dentina, dureza, microbiologia, sondas DNA.

INTRODUCTION

It is known that dental caries is an infectious disease caused by tooth-adhering bacteria, which form dental biofilm. A significant amount of scientific evidence demonstrates that caries only occur if there are microorganisms present¹. Dentine caries present differences in related microbiota when compared to enamel caries, since the growth of microorganisms occurs in a much more anaerobic and protein-rich

environment. Among the microorganisms investigated in dentine caries, the gram-positive bacilli predominated in the majority of studies^{2,3,4}.

It is known that an outer layer of carious dentine is infected and not susceptible to remineralization (the collagenous fibers do not present striation, the peritubular dentine and the odontoblastic processes are absent and the tubules are infected by microorganisms) and should be removed. The inner layer,

although it is affected, should be preserved since its collagenous fibers present striation, the peritubular dentine and the odontoblastic extensions are present and the tubules are not infected by microorganisms^{5,6,7}.

Regardless of the type of microorganism, some studies state that bacteria must be totally removed during cavity preparation in order to impede caries lesion progression^{8,9}. Others, however, report that even when all the soft carious tissue is not removed, the residual bacteria will be rendered harmless, if there is good sealing^{4,10}.

The clinical criterion of hardness must be considered at least as a criterion to remove carious dentine during cavity preparation¹¹. This method has the advantage of removing disorganized or infected dentine and confers resistance to the preparation since it prevents softening or shifting of the dentine remnants after restoration¹².

Conversely, histological studies have shown that hardness is not an adequate parameter to determine the removal of carious dentine and that contamination persists, with bacterial percentages varying from 36 to 94% in deep caries^{11,13}.

It is also known that due to the tubular structure of dentine, there is bacterial penetration inside the tubules, precluding total removal of the pathogenic microorganisms present, and allowing a number of them to remain viable¹². Nevertheless, the bacterial remnants of non-softened dentine (clinical criterion of hardness) are not capable of causing lesion progression if the cavity is well sealed^{1,10,14}.

In this sense, bacteria would persist after the removal of carious dentine. However, the metabolic and growth capacity of these microorganisms may be impaired after isolation in the oral environment through sealing of the cavity.

Some other questions remain unanswered: Are the microorganisms that remain in the dentine remnants viable? Does this fact allow caries progression? Is there a relation between the dentine aspect and the type of microorganism?

Thus, the purpose of this study was to identify the microbiota present in the carious dentine and compare it to that found in dentine remnants (BL and 1 year), in order to verify its relation with caries progression. Furthermore, a relation was sought between the dentine aspect (both carious and remnant) and the microbiota encountered.

MATERIALS AND METHODS

Ethical considerations

This research was approved by the Research Ethics Committee of the Federal University of Rio Grande do Norte (protocol number 100/02). All relevant information about the study was provided to the parents and/or guardians of the schoolchildren, who then authorized their participation by signing an informed consent form, as per National Health Council Norms (resolution 196/96).

Sample studied

This study sample was composed of 34 schoolchildren from the public system in Natal-RN-Brazil, in the 7-to 14-years age group, who presented caries lesions with cavitation located only on the occlusal surfaces of permanent molars and with radiolucency in the middle third of the dentine, confirmed by bite-wing radiographic examination.

The study was developed in the Department of Dentistry of Federal University of Rio Grande do Norte (UFRN) and consisted of a clinical trial to evaluate the degree of progression of caries during the one-year follow-up by visual and the radiographic examination (bite-wing radiograph).

Microbiological analysis

Prophylaxis was performed with pumice stone and water on the selected teeth, which were then anesthetized, photographed and isolated. The rubber dam and tooth surface were disinfected with 1% NaOCl. The first layer of carious dentine was removed with a small spoon excavator and discarded, since contact with dental biofilm could also lead to the presence of bacteria unrelated to carious dentine. The remaining layers were collected with another excavator spoon, being scored according to the dentine aspect: 1-very soft; 2-soft; 3-medium hard; 4-hard. Their contents were dispensed into individual sterile eppendorf tubes containing a cryoprotector [Brain Heart Infusion + Dimethyl Sulfoxide] to preserve the dentine collected. Next, these samples were submitted to microbiological examination to determine the presence of bacteria through hybridization with genomic DNA probes and the checkerboard method¹⁵.

Cavity preparation was finalized with carbide drills 245 (SS White, Rio de Janeiro-RJ, Brazil). After concluding the preparation (medium hard dentine), the clinical aspect of the dentine remnants was

scored (1- light yellow; 2- yellow; 3- light brown; 4- dark brown; 5- black). Material was collected for microbiological analysis of the dentine remnants immediately after cavity preparation. The cavity was filled with 0.05 ml saline solution for one minute, and then its contents were withdrawn from the cavity with a sterile insulin syringe. This solution was placed into a sterile eppendorf to evaluate microbiota in the dentine through seeding in sheep blood agar plates with 48-hour incubation, in anaerobiosis, at 37°C. After bacterial growth in the blood agar plates, colony-forming units were counted (quantitative analysis). They were removed with a sterile metal instrument. The colonies were placed into a sterile eppendorf with a cryoprotector (BHI + DMSO) to determine existence and predominance of bacterial types (semi-quantitative and qualitative analysis). Probes for twenty three bacteria related to dentine caries and environmental contamination were employed (Table 1).

Clinical Monitoring and Therapeutic Procedures

After the collection process, pulpal protection was performed with glass ionomer cement (Vidrion F, SS White, Rio de Janeiro-RJ, Brazil) and cavity varnish (Cavitine, SS White, Rio de Janeiro-RJ, Brazil) and the tooth was restored with silver amalgam (Velvalloy, SS White, Rio de Janeiro-RJ, Brazil) and radiographed.

The filling was removed 1 year after restoration and re-assessed using dentine remnant scores for comparison with baseline (BL) data. Sample collection for microbiological analysis was performed employing the same technique as for BL. The aim of this procedure was to compare the microbiota present at different times. A new restoration was performed in the same session.

Statistical Analysis

Differences in the clinical appearance of the dentine between the two time-points (BL and 1 year) were evaluated with Bowker’s test at a significance level of 5%. Wilcoxon’s test was used to evaluate the amount of bacteria as determined by the microbiological analysis. Friedman’s non-parametric test and the sign test were used to determine if there was a difference in the type of bacteria present in the carious dentine at BL and after 1 year.

The correlation between the clinical aspect of carious dentine, as well as that of dentine remnants with

Table 1: List of bacterial species and a reference of oral organisms for obtaining genomic DNA probes.

<i>Bacteria Investigated</i>	<i>Reference</i>
GRAM-POSITIVE	
<i>Streptococcus anginosus</i>	ATCC 33397
<i>Streptococcus sobrinus</i>	ATCC 33478
<i>Streptococcus sanguis</i>	ATCC 10556
<i>Streptococcus oralis</i>	ATCC 35037
<i>Staphylococcus epidermidis</i>	ATCC 14990
<i>Staphylococcus aureus</i>	ATCC 12598
<i>Streptococcus gordonii</i>	ATCC 10558
<i>Streptococcus mutans</i>	ATCC 25175
<i>Streptococcus mitis</i>	ATCC 49456
<i>Peptostreptococcus anaerobius</i>	ATCC 27337
<i>Peptostreptococcus micros</i>	ATCC 33270
GRAM-NEGATIVE	
<i>Veillonella parvula</i>	ATCC 10790
GRAM-POSITIVE BACILLI	
<i>Lactobacillus casei</i>	ATCC 7469
<i>Propionibacterium acnes</i>	ATCC 11827
<i>Actinomyces naeslundii</i>	ATCC 12104
<i>Actinomyces israelii</i>	ATCC 12102
<i>Actinomyces viscosus</i>	ATCC 43146
<i>Actinomyces odontolyticus</i>	ATCC 17929
<i>Bifidobacterium dentium</i>	ATCC 15423
GRAM –NEGATIVE BACILLI	
<i>Prevotella intermedia</i>	ATCC 25611
<i>Prevotella melaninogenica</i>	ATCC 25845
<i>Porphyromonas gingivalis</i>	ATCC 33277
<i>Prevotella nigrescens</i>	ATCC 33563

ATCC – (American Type Culture Collection, Rockville, MD, USA)

the presence of bacteria, was determined with Spearman’s correlation coefficient for a significance level of 95%.

RESULTS

Caries progression after 1 year of observation was the main end-point analyzed in this study. None of the 34 teeth presented caries progression, as revealed both by radiography and visual examination (followed the opening of the cavity).

Concerning the clinical aspect of carious dentine, of the 34 teeth 6% (n=2) were very soft, 67% (n=23) were soft and 27% (n=9) were medium hard. Regarding the appearance of dentinal remnants at BL, 38% (n=13) were yellow, 54% (n=18) were light brown and 8% (n=3) were dark brown. After

Table 2: Number of teeth analyzed, median (log), confidence interval and its level of significance in relation to the amount of bacteria at baseline (BL) and after 1 year.

Time-point	n	Median	CI 95%	p
BL	32	0.52	-0.1974 to 1.385	0.37
1 Year	32	0.56	-2.696 to 14.040	

Table 3: Number of teeth analyzed, median (log), confidence interval and its level of significance in relation to the semiquantitative and qualitative analysis of the types of microorganisms at baseline (BL) and after 1 year.

Bacteria Investigated	Time-point	n	Median	CI 95%	p
<i>S. anginosus</i>	BL	34	0.41	0.08 to 0.73	0.35
	1 year	34	0.64	0.2 to 1.0	
<i>S. oralis</i>	BL	34	0.32	0.05 to 0.59	0.38
	1 year	34	0.17	0.04 to 0.31	
<i>S. mitis</i>	BL	34	0.29	-0.022 to 0.61	0.78
	1 year	34	0.26	0.11 to 0.42	
<i>V. parvula</i>	BL	34	0.03	-0.03 to 0.09	0.37
	1 year	34	0.088	-0.01 to 0.18	
<i>S. aureus</i>	BL	34	0.11	-0.07 to 0.3	0.14
	1 year	34	0.47	0.09 to 0.84	
<i>S. gordonii</i>	BL	34	0.03	-0.03 to 0.09	0.75
	1 year	34	0.06	-0.02 to 0.14	
<i>S. sobrinus</i>	BL	34	0.23	-0.05 to 0.52	0.91
	1 year	34	0.29	0.07 to 0.51	
<i>S. epidermidis</i>	BL	34	0.03	-0.03 to 0.09	0.31
	1 year	34	0.12	0.003 to 0.232	

the 1-year experimental period, 27% (n=9) presented yellow, 46% (n=16) presented light brown and 27% (n=9) presented dark brown (p=0.0078). Dentin exhibited darkening but no softening after the follow-up period.

In the checkerboard analysis of the microbiota of the carious dentine, 18 (78.26%) of the 23 species tested were detected. Of these, the predominant were Gram-positive cocci (*S. gordonii*, *P. anaerobius*, *S. aureus*, *S. mitis*, *S. anginosus*, *S. oralis*, *S. sanguis*, *S. sobrinus*, *P. micros*, *S. mutans* and *S. epidermidis*) followed by the Gram-positive bacilli group (*L. casei*, *P. acnes*, *A. naeslundii* and *A. israelii*). A few species of Gram-negative cocci (*V. parvula*) and Gram-negative bacilli (*P. gingivalis*, *P. intermedia*) were identified at low levels. In the Gram-positive cocci group, there was a predominance of *S. sobrinus* followed by *S. anginosus* and

in the Gram-positive bacilli group *A. naeslundii* predominated.

After cavity preparation, only 11 (47.82%) of the 23 bacteria evaluated were found (*S. anginosus*, *P. anaerobius*, *S. oralis*, *S. mitis*, *V. parvula*, *S. aureus*, *S. sanguis*, *P. gingivalis*, *S. gordonii*, *S. sobrinus*, *S. epidermidis*). Of these, 2 (8.69%) were found only in the 1 year samples (*P. gingivalis* and *P. anaerobius*), 1 (0.43%) was found only at BL (*S. sanguis*) and the remaining 8 (34.78%) were detected both at BL and at 1 year. The predominant group in this bacterial analysis were the Gram-positive cocci with lower scores than those of the carious dentine group. *S. anginosus* predominated, followed by *S. oralis* and *S. sobrinus*. No Gram-positive bacilli were observed, as opposed to carious dentine.

Patients who still presented bacteria after cavity preparation (BL and 1 year), did not present caries recurrence (as observed by radiography and visual examination after the opening of the cavity).

The quantitative comparison of the presence of bacteria in the carious dentine and after cavity preparation (BL and after 1 year), showed a significant difference both at BL (p < 0.001) and after 1 year (p = 0.012).

However, when the mean number of bacteria found after cavity preparation at BL and 1 year were compared, there was no significant difference between these two periods (p > 0.05) (Table 2). Of the 32 cases, (since two were not analyzed because of sample loss) 5 (16%) presented bacteria after cavity preparation (BL) and 27 (84%) did not. After 1 year, all 5 cases cited above exhibited a reduction in the number of bacteria. Of the 27 cases that did not present bacteria after cavity preparation (BL), 19 (70%) remained bacteria-free after 1 year and 8 cases (30%) presented bacteria after 1 year, even when they did not present any at BL. The semiquantitative and qualitative analysis of the presence of bacteria in dentine caries at BL and after 1 year showed no significant difference between bacteria types at these two time-points (Table 3).

When a correlation was sought between the clinical aspect presented by the carious dentine as well as

the dentine remnants after cavity preparation and the type of bacteria encountered, no significant correlation was observed ($p > 0.05$, $r = -0.01258$ to 0.3774 ; $p > 0.05$, $r = -0.03541$ to 0.2546 , respectively).

DISCUSSION

Glass Ionomer Cement is the only restorative material that has the capacity to bond chemically to the teeth surfaces¹⁶, providing a suitable cavity seal when used for pulp protection. Moreover, some authors have shown there is mineralization of the dentin under glass ionomer cements^{17,18}, favoring the color change and the persistence of dentine hardness found in this study.

Based on the scientific proof that caries is an infectious disease, it was determined that its treatment should consist of the complete removal of bacteria to achieve dentine sterility and avoid its recurrence^{8,9,19}. Thus, diverse studies were performed to identify suitable criteria for removing carious dentine that would guarantee the absence of microorganisms^{20,21}, since clinicians have great difficulty in determining which dentine must be removed during cavity preparation.

The clinical criteria of coloration, hardness and caries-indicating dye are used to achieve a bacteria-free cavity preparation and impede caries progression. These criteria, however, are empirical and subject to individual variations.

Coloration is not a reliable parameter, since dentine may be pigmented in cases of dormant caries, or as a result of physiologic defense reactions of the organism and must be preserved, not removed. Clinicians did not find an association between color of dentin and presence of cariogenic microorganisms^{4,12,22}.

In relation to the clinical criterion of hardness, although it is not scientifically precise, it must be considered at least as a limit to determine the interruption of carious dentine removal until a more accurate method is developed^{11,23}.

It is also known that due to the tubular structure of dentine, there is bacterial penetration inside the dental tubes, making the total removal of pathogenic microorganisms impossible. Thus, some viable microorganisms inevitably remain¹². For this reason, this study opted for the removal of carious dentine based on the clinical criterion of hardness and evaluated microbiota and the progression of caries through a 1-year follow-up, to evaluate whether the routinely-used clinical criterion is adequate to avoid caries recurrence.

The permanence of bacteria occurs after carious dentine removal using any of the 3 clinical criteria (coloration, hardness, caries-indicating dye). However, in keeping with the literature^{10,14}, this study reveals that bacterial remnants of non-softened dentine (hardness criterion) are not capable of causing lesion progression if the cavity is well sealed, since even if bacteria are present, there are no nutrients available for them to grow and multiply and destroy dental tissue.

Therefore, the clinical characteristic of dentine hardness seems to be a good indicator of dentine caries progression, since softened dentine was removed from all 34 teeth, leaving them with a rigid aspect and not a single case of caries recurrence was detected during the 1-year follow-up.

The clinical characteristic of coloration does not seem to be a good indicator of caries progression, since none of the teeth that presented a rigid aspect, regardless of dentine coloration (light, light with dark spots or dark), presented caries recurrence.

The coloration presented by dentine remnants has no significant correlation with the type of microbiota, further confirming the fact that the clinical criterion of coloration is not effective in determining the removal of carious dentine. These results are in agreement with study by Banerjee et al.²².

Scientific evidence demonstrates that caries only occur in the presence of microorganisms²⁴. Among the microorganisms found in dentine caries, a great majority of authors report a predominance of Gram-positive bacilli^{2,4,19}. Bjorndal and Larsen¹⁹ found a predominance of Gram-positive bacilli in carious dentine, followed by streptococci. Before dentine removal, after 4-6 months, when it was harder and more pigmented, the total number of colony-forming units was reduced, as was the frequency and proportion of Lactobacilli, with the microbiota dominated by *A. naeslundii* and various streptococci. This species, which predominated after cavity preparation, did not represent the typical microbiota of deep lesions, confirming the possibility of no progression of the caries lesion after cavity preparation. Dental lesions do not necessarily present the same bacteriologic aspect, due to the different availability of nutrients, which vary according to the size of cavity opening. In this sense, the microbiota of the carious dentine was investigated to compare this microbiota with that found after cavity preparation. In this study Gram-positive cocci were predomi-

nant, followed by Gram-positive bacilli, probably because mid-depth cavities were investigated, as opposed to most studies, which studied deep cavities. After cavity preparation, no Gram-positive bacilli were recovered and the predominance was of Gram-positive cocci at lower scores. These microorganisms would not be typical of carious dentine lesions, as affirmed by Bjorndal and Larsen¹⁹ and for this reason, no caries recurrence occurred in any of the samples.

A qualitative analysis of the bacteria found in carious dentine and in dentine after cavity preparation (BL and 1 year) revealed that bacteria found in carious dentine is much more abundant and diversified than that found in dentine remnants after cavity preparation. A predominance of Gram-positive cocci was observed in carious dentine, followed by Gram-positive bacilli. Cavity preparation eliminated Gram-positive bacilli, leaving only a reduced amount of the same Gram-positive cocci found in carious dentine. Despite the persistence of bacteria after preparation, no caries recurrence was observed at the end of the experimental period. Given that a satisfactory clinical seal was observed in all samples after 1 year of follow-up, bacteria would be rendered non-viable by adequate sealing. In addition, Gram-positive cocci had lower scores and are not related to caries progression¹⁹. These findings would confirm that cavity preparation, based on the clinical criterion of hardness, eliminates Gram-positive bacilli, the microorganisms that predominate in dentine caries and are probably responsible for caries progression. Only the Gram-positive cocci that may be part of the affected dentine would remain. Since Gram-positive cocci are susceptible to remineralization they can persist.

A proper cavity seal reduces the microbiota even in situations of incomplete removal of the carious tissue^{4,9,25}, potentially contributing to the non-progression of the caries.

S. anginosus was the most prevalent and most significant bacteria both in the carious dentine and the dentine remnants after preparation, followed by *S. sobrinus*, suggesting that these organisms have a role in the pathogenesis of medium-size dentine caries.

S. sanguis was the only bacteria found exclusively at BL. It is considered an initial colonizer of dental biofilm. The fact that it no longer appeared after 1 year, when the microbial deposits and cavity seal were removed, further supports this notion.

P. gingivalis and *P. anaerobius* were the two species found only after 1 year, conceivably due to the fact that they perform proteolysis efficiently and thus manage to survive in the nutrient-poor environment of the bottom of a sealed cavity, that is, in dentine. They were not detected at BL, conceivably because the collection method was not adequate for low levels. Negative bacteriological examinations may signify the presence of microorganisms at an undetectable level or at lower numbers in the specimens studied.

The patients who still presented bacteria after cavity preparation (BL and 1 year), did not present problems of caries recurrence (as observed by radiography and through opening of the cavity), suggesting that the bacteria that remain in the cavity after preparation are not viable to continue the carious process, provided that the cavities are properly sealed, as affirmed by Knight et al.²⁵ and Silva et al.¹⁰.

The clinical parameter (hardness) seems to be sufficient for the non-progression of caries, provided that the cavity is correctly sealed. This result is in accordance with Ricketts et al.²⁶ who found a relation between the level of dentine infection and the visual scores used in occlusal caries diagnosis.

The current clinical criteria for carious dentine removal lead to the persistence of bacterial remnants in the cavity. For this reason special attention must be given to sealing the cavity to impede access to nutrients essential for bacterial survival and proliferation.

The microbiota found in the carious dentine of mid-depth cavities is composed mainly of Gram-positive cocci, followed by Gram-positive bacilli. After cavity preparation the gram-positive bacilli were not recovered and the Gram-positive cocci became predominant in lower quantities than those of carious dentine. Gram-positive cocci in small quantities have no relation to caries progression and are likely a part of the microbiota of the affected dentine.

No correlation was observed between bacteria type and the aspect of carious dentine or with dentine remnants after cavity preparation (BL and 1 year). There was no correlation between the clinical criterion of coloration and caries progression, however, the clinical aspect of hardness proved to be effective in determining the non-progression of caries.

CORRESPONDENCE

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