Detection of potentially cariogenic strains of *Streptococcus mutans* using the polymerase chain reaction

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Streptococcus mutans is a pathogen related to the occurrence of human dental caries. The determination of total amounts of mutans streptococci, as well as the proportion related to other oral bacteria, is of interest when assessing the risk of developing caries. In this context, it is better to use a sensitive, specific and non-time consuming method such as the polymerase chain reaction (PCR), than to use culture and biochemical identification methods. In this work we identified potentially cariogenic strains of S. mutans and assessed the relationship with the dmft, DMFT or dmft/DMFT index. Using DNA isolated from dental plaque, a 192 bp sequence was identified and amplified from the spaP gene and a 722 bp sequence from the dexA gene. The results suggest that it is important to evaluate the presence of cariogenic S. mutans strains in plaque content rather than the accumulation of plaque itself. However, other factors like diet, hygiene, genetic background, the flow rate of saliva and the presence of specific antibodies, also play a key role in the development of caries.
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INTRODUCTION

Dental caries constitute an infectious disease caused by bacterial infection, in which *Streptococcus mutans* plays an important role. It is estimated that around 95% of the world's population is affected by dental caries. From 1991-1992 the World Health Organization estimated the DMFT index (number of decayed, missing and filled teeth) for Mexico to be 2.5-5.1, in 12 year old children, which is considered to be moderate to high.[1]

To prevent dental caries, plaque control is most important. This precaution limits implantation and colonization of the *Streptococcus mutans*, and subsequently prevents substantial loss of teeth.[2,3] Implantation of *S. mutans* is promoted by the presence of adhesins that interact with salivary receptors. In contrast, salivary glycoproteins of high molecular weight (agglutinins) in association with IgA, interact with the *S. mutans* adhesins blocking the attachment of *S. mutans* to the enamel, promoving the defense against infection.[4,5] It has been observed that during the initial adherence of *S. mutans* to the film covering the tooth surface, a 190 kD fibrilar adhesin, known as Ag I/II encoded by the *spaP* gene, participates. The structural gene that codes for Ag I/II is know for *S. mutans* serotype c (NG5), whose genetic product contains a marker peptide of 38 amino acids, two repeated amino acid sequences: the A region, that is found in the N-terminal region, rich in alanine and the proline P region.[6,7] Also is it has been expressed in *E. coli*, and the recombinant protein is recognized by the monoclonal antibody anti AgI/II, which reacts with a 190 kD peptide.[8,9]

It has been shown that intragastric immunization with Antigen I/II coupled to the B fraction of *Vibrio cholerae* (CTB) toxin, stimulates specific antibodies of the IgG class in sera and IgA class in the mucosa, with a type Th2 $CD4^+$ interleukin profile.[10] Similarly, when rhesus monkeys are immunized via the nasal route, antibodies are directed against two components, Ag I/II and CTB.[11] Immunization with synthetic peptides corresponding to the alanine rich region of Ag I/II, induces an antibody response that inhibits the association between this cellular surface protein and the salivary components.[6,13]

In order to assess the risk of infection and to monitor the level of colonization of an individual, different methods have been developed to detect *S. mutans*. Some of these methods estimate the number of *S. mutans* colony forming units (CFU). This is the case of

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the *Strep mutans* test, which is used to identify groups of people at risk of developing caries.[14] Characterization is also carried out using mutacins (Bacteriocins), which are used as epidemiological markers to establish the source of infection and the mechanism of transmission.[15]

Based on the fact that *S. mutans* adheres to tooth enamel through Antigen I/II and that this constitutes a genetic marker for potentially cariogenic strains, Ono *et al.* (1994) have designed a system to amplify this antigen. This was done using the polymerase chain reaction (PCR) in microbiological isolates of dental plaque (DP).[16] Using a similar procedure Igarashi *et al.,* 1996. Used the sequence of the gene that codes for dextranase (dexA) and have amplified a segment that may be used for detecting cariogenic strains of *S. mutans*.[17]

The object of this work is to set up a system that can be used to detect potentially cariogenic strains of *Streptococcus mutans*, using the polymerase chain reaction in dental plaque. The method we used is different from the one used by Ono and Igarashi, because we used dental plaque samples instead of *S. mutans* strains isolated. In this context, it will then be possible to apply this system to evaluate the risk of acquiring dental caries and subsequently for the setting up of control mechanisms in pre-school age children.

MATERIALS AND METHODS

To develop a *Streptococcus mutans* detection system by PCR, a sample of dental plaque was taken from 38 children of both sexes, with an average age of 5.7 (± 1.2) years. The subjects were all volunteers and the sampling took place at the Especialidad en Odontopediatria clinic in the Universidad Autonoma de Zacatecas. The dmft (decayed missing and filled teeth) for decidus teeth, DMFT, dmft/DMFT for mixed dentition[18] and SOHI (simplified oral hygiene index) were used.[19]

DNA extraction from dental plaque by lysozyme lysis

Chromosomal DNA was obtained by the following procedure. Samples of DP were taken with sterile spoons for dentine (Medicon no. 5). The sample was then homogenized in BHI media and incubated for 18 hrs at 37ºC. The bacterial culture was span at 3000 rpm for 10 min and the resultant supernatant discarded. The cellular pellet was washed twice with 500 µl of TE (Tris-HCl 10 mM EDTA 1 Mm) at pH 8.0, and inactivated at 95ºC for 10 min. It was re-suspended in 250 µl of TE. 2 mg/ml of lysozyme was added and incubation was continued at 37ºC for 60 min and at 50ºC for 20 min. 4µg/ml of proteinase K was added and incubated at 37ºC for 60 min. Then 10% SDS was added until a concentration of 1.5 % was reached. 7.3 µl of a 4 M sodium chloride solution was added and the reaction incubated at 50ºC for 10 min. The resultant lysate was extracted once with chloroform:isoamyl alcohol (24:1) and twice with neutral phenol (phenol:chloroform: isoamyl alcohol, 25:24:1) and was precipitated with 90 % ethanol and a volume of 7.5 M ammonium acetate. The precipitated chromosomal DNA was dissolved in 50 µl TE buffer, at pH 8.0.[20]

Amplification of the 192 bp fragment from spaP gene for Ag I/II DNA fragment and the 722 bp dexA fragment by PCR

Based on the DNA sequence that codes for Antigen I/II, B, P1, spaP or Pac of *Streptococcus mutans* serotype c, obtained from the Gene Bank (Accession Number x17390), Ono *et al.,* 1994 designed a pair of oligonucleotides, to obtain a 192 bp fragment.[16] The oligonucleotides have the following sequences: upstream 5'-AAC GAC CGC TCT TCA GCA GAT ACC-3', found in the 3668 to 3698 region, and downstream 5'-AGA AAG AAC ATC TCT AAT TTC TTG-3', in the 3835 to 3859 region. A concentration of 500 nM was used for the oligonucleotides, 200 µM for the desoxynucleotides mixture (dATP, dCTP, dGTP and dTTP), 2.5 mM $MgCl_2$ and 2 U of Taq polymerase in a reaction volume of 50 µl. Amplification was carried out as follows, acoording with the procedure described by Ono *et al.* Initial denaturation was for 2 minutes at 94ºC, followed by 30 cycles with the following conditions: denaturation for 1 minute at 94ºC, annealing for 30 seconds at 57ºC and extension for 1 minute at 72ºC, with a final extension for 5 minutes at 72ºC

For the *dex A* , a 722 bp fragment was amplifying, the sequence of oligonucleotides were designed by Igarashi et al.[17] Their design was based on the gene of *S. mutans* ingbritt strain, serotype c, clone pSD2. The accession number is D49430. The sequences for the oligonucleotides are as follows: upstream 5'-TAT TAC AGC TAC TGT TGA GG-3' and downstream 5'- CAG TTT TCA TAG CTT GAG CC-3'.1.5 mM $MgCl_2$ was used and the same concentrations of oligonucleotides, desoxynucleotides and enzyme used above for the *spaP* fragment amplification. The running conditions for the reaction were performing using Igarashi *et al* protocol, as follows. Denaturation for 2 min at 94ºC, followed by 30 cycles under the following conditions: denaturation for 1 min at 94ºC, annealing for 30 sec at 52ºC and extension for 1 min at 72ºC, with a final extension for 5 min at 72ºC.

Agarose gel electrophoresis

The PCR amplification products of *spaP* and dextranase were analyzed by electrophoresis in neutral 2% agarose gels, buffered with TBE (0.04 M Tris, 0.04 M boric acid and 1mM $Na_2EDTA(2H_2O)$. The gel was run at 80 Volts. (Photograph 1)

RESULTS

Thirty-eight (38) subjects, from 3 to 7 years of age were studied at random. 79% had dental caries and 21% of the subjects were healthy. They were clinically examined and the *dmft/DMFT* and SOHI indices deter-

Table 1. Patient distribution according to the dmft/DMFT and SOHI.

dmft/DMFT	Number of Patients	Percentage	SOHI	Number of Patients	Percentage
0	8	23.68%	0	1	2.63%
1 - 2	7	18.42%	< 1	12	31.57%
3 - 5	12	28.94%	1 - 2	19	50.00%
6 - 9	7	21.05%	> 2	6	15.78%
> 10	4	7.89%			

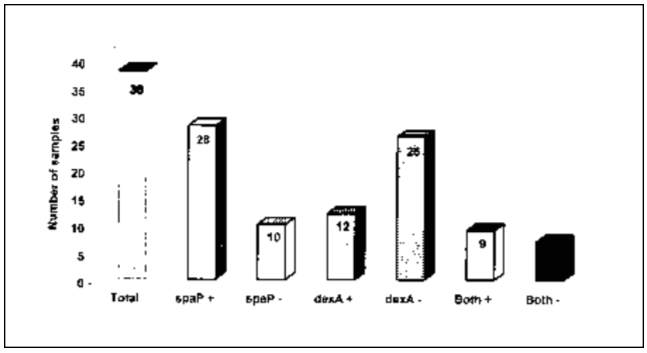


Figure 1. Relative caries experience according with Grainger and Nikiforuk[21] in our population of study (1960).

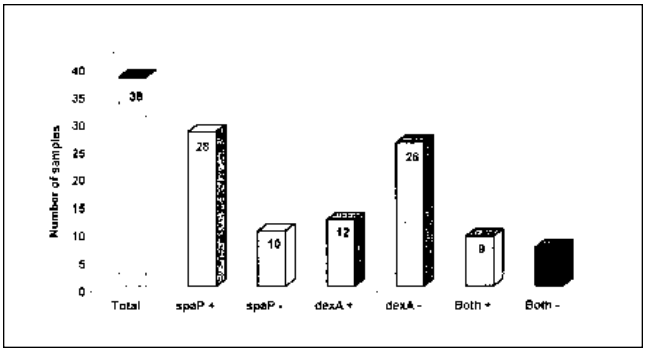


Figure 2. Identification of spaP and dexA in dental plaque samples in 38 childrens using PCR technique.

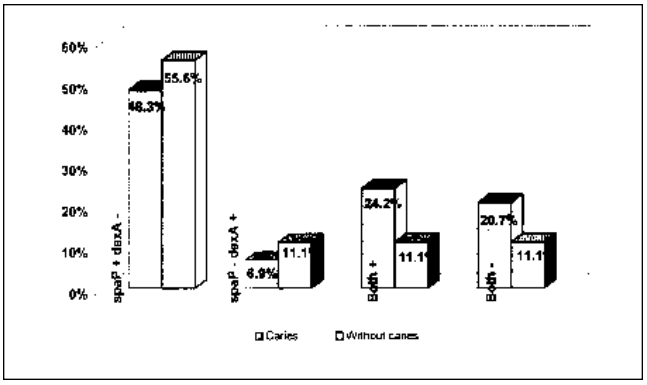
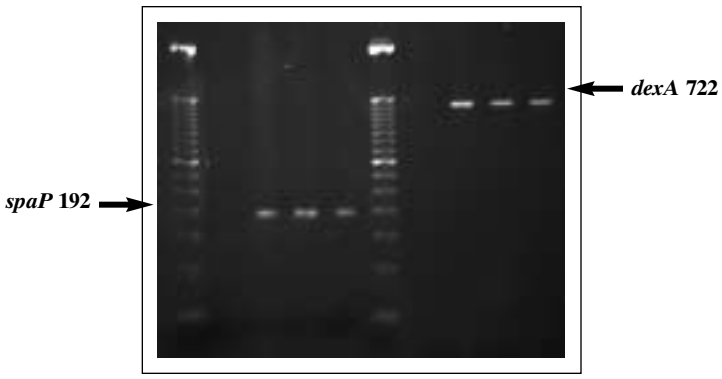


Figure 3. Detection of DBP samples positive and negative for spaP and dexA by PCR, in patients with or without dental caries.

mined. The *dmft/DMFT* values in this study group were from 0 to 13 with a mean of 4.1, and for the SOHI index, values were from 0 to 2.5 with a mean of 2. In the case of the *dmft/DMFT* index a high percentage, 28.94 %, of the total number of patients, had values from 3 to 5, whereas only 7.89 % had more than 10. For the SOHI, 50% of the study group had values from 1 to 2 and only 2.63% were zero (Table 1).The relative caries experience was determined according with Grainger and Nikiforuk[21] and are summarized in the Figure 1.

With the DP samples, a 192 bp segment of the *spaP* gene was amplified that codes for *S. mutans* AgI/II. Another segment of 722 bp was amplified from the *dexA* gene of the dextranase enzyme Figure 1. From the DP samples obtained, 28 were positive for the *spaP* gene that codes for Ag I/II, and 10 were negative. In the case of the gene that codes for dexA, 12 were positive and 26 negative, and from the total samples 9 were positive for both genes and 7 were double negatives (Figure 2). With respect to the presence of caries, from 30 patients with caries experience (representing 78.9% of the sample), 48.27% were *spaP* + *dexA* -, 6.89% were *dexA* + *spaP* -, 24.16% were positive for both and 20.68% were double negative. For the 8 subjects without caries (representing 23.68% of the sample), 55.5% were *spaP* + *dexA*-, 11.11% were *dexA* + *spaP* -, 11.11% were positive for both and 11.11% were double negative (Figure 3).

In accordance with the *dmft/DMFT* index, 6 subjects were positive for *spaP,* with an index of zero and the rest had a homogenous distribution, except for 3



Photograph 1. PCR products identified from dental plaque: lanes 1 and 6 Molecular Weight Markers DNA 50pbm lanes 2 and 7 negative control, lanes 3-5 *spaP,* lanes 8-10 *dexA.*

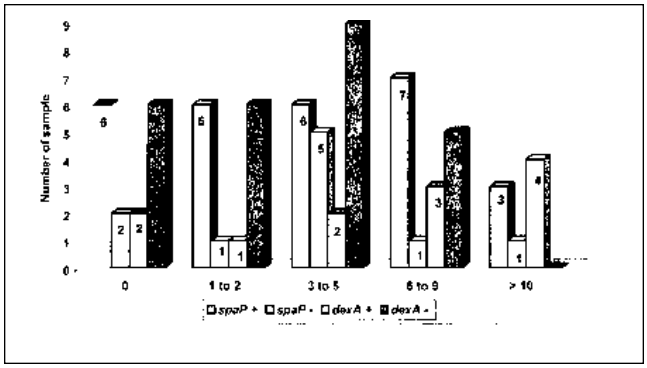


Figure 4. Distribution of DBP samples positive and negative for spaP and dexA by PCR technique, in relation to the dmft/DMFT index.

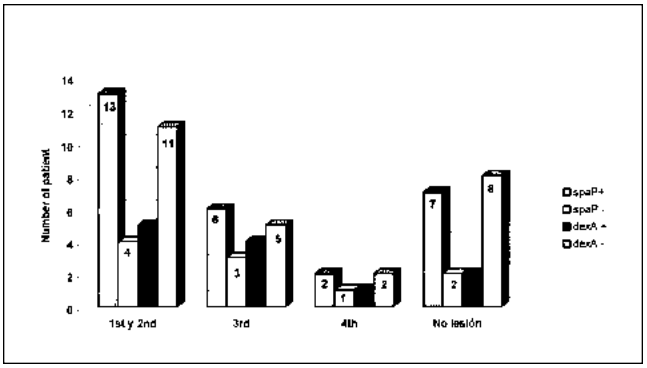


Figure 5. Detection of spaP and dexA in DBP by PCR technique, showing the degree of lesions found in patients with dental caries and clinically healthy patients.

samples which had a value equal 0 greater than 10. In the case of those samples that were negative for Ag I/II, one had a value greater than 10 and the rest were distributed within the different ranges established. For *dexA*, two positive samples corresponded to patients with *dmft/DMFT* indices equal to zero. The distribution of positive and negative samples with respect to the *dmft/DMFT* is shown in Figure 4. Here it is important to point out that some of the samples belonging to the same individuals were positive for both *dexA* and s*paP*, or were negative for both. In agreement with the occurrence of large caries, we found that of the 28 patients with samples positive for *spaP*, 13 had primary and second degree lesions, 6 had third degree lesions, 2 had fourth degree lesions and 7 patients did not have lesions. Likewise, 5 samples were positive for *dexA* and the corresponding patients had primary and second degree lesions, 4 had third degree lesions, 1 fourth degree lesions and 2 patients did not have lesions (Figure 5).

Discussion:

In this study we set up a system to detect potentially cariogenic strains of *Streptococcus mutans* using the polymerase chain reaction and DP cultures. The system has high specificity and sensitivity, and by using specific genetic markers it was possible to detect *S. mutans*. The markers we used were the *spaP* gene, which codes for AgI/II and *dexA*, which codes for the dextranase enzyme. Using this technology it is also possible to evaluate the presence of *S. mutans* in clinical samples in a very short time, without using selective media or isolating the microorganism. Furthermore, we have set up an immediate and qualitative system for controlling and assessing the risk of acquiring dental caries. By using this technique it is believed that the prognosis will help those patients with potentially cariogenic strains. In addition, it is important to point out that Hajishengallis *et al.,* 1996, 1998, have demonstrated that Ag I/II plays an important role in the initial implantation of *S. mutans* onto the tooth surface. These authors found that AgI/II, when coupled to the A2/B subunit of cholera toxin and administered orally, produces a protective immune response.[12,22] These findings coincide with our data, since the presence of a considerable quantity of samples positive for *spaP* correspond to patients with first and second degree lesions or with the absence of lesions. Similarly, there is further correlation between the characteristics of bacterial colonization and tooth decay. Such that, the lesion involves deep tissue and there is a change in the microbial flora which allows the implantation of bacteria with greater resistance to environmental acid and a lower requirement for oxygen. In contrast, the presence of bacterial strains in DP, that present *spaP* and *dexA* genes, promote the presence of caries by the adherence of S. *mutans* to teeth via the interaction of AgI/II with tooth film proteins. Hence, the bacteria become united with the tooth, and the dextranase enzyme allows greater bioavailability of carbohydrates for bacterial metabolism.

When the patients used in this study were analyzed individually, no relationship between the SOHI and *dmft/DMFT* indices was found. We found that some of the subjects with a large quantity of DP did not have high *dmft/DMFT* values, and vice versa. Hence, the results of this work appear to contradict the findings of other investigators that have shown that the larger the quantity of plaque, the larger the *dmft/DMFT* value. From the clinical findings, it may be established that there is no direct relationship between the SOHI and *dmft/DMFT* indices. It is therefore necessary to evaluate the quality of DP, in other words when dental organs are examined bacterial content rather than the quantity of plaque present, should be assessed. By means of this technology (PCR) it is possible to detect populations or individuals at risk. However, to protect this risk population efficient means of control must be established which will modify the buccal environment, and an efficient impact program should include the use

of oral antiseptics to control bacterial flora residing in the plaque, as well as a DP control technique.

Finally, when considering the design of alternative measures of prevention and control, it is important to understand that there are multiple interrelated factors involved in the development and evolution of dental carries. For instance, the microenvironment in the oral cavity, which has a direct influence on strategies used by infectious microorganisms to evade the host's defense barriers. The mechanism used by *S. mutans* to colonize the surface of teeth involves synergistic or antagonistic relationships between different microorganisms found in the DP. It is anticipated that, based on the results presented in this work, it will be possible to identify individuals and populations that are at risk of developing dental caries. In this way it will be possible to increase preventive measures in populations that, due to their dental development, immunological characteristics, eating habits and genetic predisposition, are most susceptible.

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