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# Polymerase Chain Reaction for detection of *Streptococcus mutans* and *Streptococcus sobrinus* in dental plaque of children from Cartagena, Colombia*

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## SUMMARY

***Objectives:*** To detect the presence of *Streptococcus mutans* and *Streptococcus sobrinus* in dental plaque of children from Cartagena and correlate it to dental caries precavity stages, applying a standardized PCR-based technique for epidemiological purposes.

***Methods:*** Descriptive study using a non-probabilistic sample of 50 children between 3 and 5 years of age, preschoolers from a Caribbean population in Colombia. Criteria for selection were that children should exhibit plaque accumulations on the surface of the cervical margins of the rearmost molars, and placed in one of two study groups: carious lesions and sound surfaces. Dental plaque samples from both groups were subjected to molecular analysis and statistical analysis was applied to determine the difference between the two groups using the frequencies of presence of *S. mutans*, *S. sobrinus* or both in the two groups applying Fisher's exact test for association between the presence of microorganisms and the state of the tooth surface from where the dental plaque was taken.

***Results:*** The frequency of *S. mutans* in carious lesions was 76% and 24% in healthy surfaces. The frequency of *S. sobrinus* in carious lesions was 81.9% and 18.1% in caries-free surfaces. There was statistical significance between the presence of *S. mutans* and the presence of caries (p=0.001) and between the presence of *S. sobrinus* (p=0.02) and the presence of caries. There was no statistical significance between the presence of caries and the simultaneous presence of both microorganisms (p=0.08).

***Conclusions:*** The presence of *S. mutans* and *S. sobrinus* in dental plaque samples is highly prevalent and associated to non cavitated carious lesions, being the molecular identification of these microorganisms by PCR a sensitive, fast, and easy to use detection method for the mutans group of oral bacteria.

***Keywords:*** *Polymerase Chain Reaction; Dental plaque; Streptococcus mutans; Streptococcus sobrinus; Preschool.*

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***Reacción en cadena de la polimerasa para la detección de Streptococcus mutans y Streptococcus sobrinus en placa dental de preescolares de Cartagena, Colombia***

## RESUMEN

***Objetivos:*** Detectar la presencia de *Streptococcus mutans* y *Streptococcus sobrinus* en placa dental de niños de Cartagena y relacionarlo con la caries dental en estadíos precavitacionales, estandarizándose la técnica basada en PCR para detectar a nivel epidemiológico la presencia de estos microorganismos.

***Métodos:*** Estudio de tipo descriptivo, a partir de una muestra no probabilística de 50 niños entre 3 y 5 años de los hogares infantiles de una población del Caribe colombiano. Como criterios de selección se tuvo en cuenta que los niños presentaran acumulaciones de placa dental sobre la superficie de los márgenes cervicales de los últimos molares. El estudio se dividió en dos grupos: lesiones cariosas y superficies sanas. Las mediciones se realizaron a partir de muestras de placa dental,

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sometidas a análisis molecular. El análisis estadístico se realizó a partir de las frecuencias de presencia de *S. mutans*, *S. sobrinus* o ambos en las lesiones cariosas y superficies sanas, utilizando la prueba exacta de Fisher para la asociación entre la presencia de los microorganismos y el estado de la superficie dental donde se encontró la placa.

***Resultados:*** La frecuencia de *S. mutans* para las lesiones cariosas fue 76% y 24% en superficies sanas. Con respecto a *S. sobrinus,* la frecuencia en lesiones cariosas fue 81.9% y 18.1% en superficies sin caries. La presencia de caries estuvo asociada significativamente en forma individual con *S. mutans* (p=0.001) o con *S. sobrinus* (p=0.02), pero no fue significante con la presencia simultánea de los dos microorganismos (p=0.08).

***Conclusiones:*** La presencia de *S. mutans* y *S. sobrinus* en muestras de placa bacteriana se considera un factor de asociación con la presencia de lesiones de caries precavitacionales, siendo la identificación molecular por PCR un método sensible, rápido y de fácil uso para la detección de bacterias del grupo *mutans*.

***Palabras clave:*** *Reacción en Cadena de la Polimerasa; Placa dental; Streptococcus mutans; Streptococcus sobrinus; Preescolar.*

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Dental caries in Colombia is still considered a public health problem, given that 65.8% of Colombians present at least one carious lesion, according to the National Study on Oral Health, Colombia 1998[1]. For children five years of age, the national prevalence of caries was at 54.8%, reporting similar results in this age group in the Caribbean region (55.6%). Although this study did not evaluate dental caries in children younger than five years of age, regional studies[2] evidence similar epidemiological behavior. Dental caries in children with temporary dentition is considered a preventable disease because of the short amount of time these teeth will be present in the mouth and because of the many prevention campaigns carried out in schools where children spend most of their time.

Dental caries is classified as a multifactorial pathology, where a dynamic process is developed on the microbial deposits upon the dental surface on which there is the intervention of biological factors like microorganisms, cariogenic diet, and a susceptible host, which interact over time to produce the mineral loss caused by the imbalance between the fluids from the dental plaque and the minerals from the tooth[3].

There is consensus among researchers worldwide on the usefulness of a caries diagnostic system that permits detecting initial stages of the disease, capable of being halted merely through the use of preventive measures. However, to successfully develop these prevention actions, it is necessary to know within each population the role played by the microorganisms associated to dental plaque deposited on a carious lesion and how these contribute to revert the process in the presence of plaque sweeping through dental brushing or to accentuate the demineralization.

In this regard, the *Streptococcus mutans* and *Streptococcus sobrinus* are the microorganisms associated most frequently to development of carious lesions[4] due to their capacity of adhering to the enamel surface and forming a bio-film facilitated by extracellular polysaccharides they produce by using sugar in the diet as a substrate, which favors demineralization measured by the acid products from bacterial metabolism[5]. These two microorganisms are the most frequently isolated from dental plaque in humans, as shown by the prevalence reported in epidemiological studies[6]. For example, Hirose[7] in 1993, established that the proportion of *S. sobrinus* in subjects with caries is greater than in healthy individuals. Likewise, Okada[8] reported that the index of Decayed, Missing, or Filled Teeth (DMFT) of children positive for *S. mutans* and *S. sobrinus* was significantly higher than in children positive only for *S. mutans*. Additionally, PM Corby[9] in 2005 points to *S. mutans* as the main indicator agent of dental caries, present in 90% of the subjects with active caries, but with a much lower frequency in subjects free of caries. From another perspective, the presence of *S. mutans* has also been identified as a predictive factor for cariogenic risk[10].

Although there have been diverse methods published to detect and identify cariogenic microorganisms, these are mostly time consuming and costly to carry out [11]. Among these, there are direct microscopy, microbiological cultures, enzymatic tests, and DNA-based tests. Of these, tests based on Polymerase Chain Reaction (PCR) surge as an alternative to conduct epidemiological and clinical studies, due to its high sensitivity and specificity, besides its rapidity when obtaining results. For bacteria from the *mutans* group, different genes have been successfully used to identify the different bacterial species in this group.

This study sought to detect the presence of *S. mutans* and *S. sobrinus* in dental plaque in children from Cartagena and determine its association with the presence of dental caries in precavitation stages; thereby, standardizing a PCR-based technique to detect the presence of these microorganisms.

## MATERIALS AND METHODS

This was descriptive, cross-sectional study from a non-probabilistic sample of 50 children between three and five years of age, cared for at foster homes of the Colombian Institute for Family Welfare (ICBF for its name in Spanish) in Cartagena, La Boquilla regional office, whose population is Afro-descendant from the Colombian Caribbean. The mean age of the participants was 4.2 years (SD=0.87), with a frequency of 58% for the group of four-year-olds, followed by the five-year olds with a frequency of 32%, and lastly, by the group of three-year-olds with a frequency of 10%. A total of 44% of the participants were females and 56% were males.

Sample size was calculated based on the historical tendency[12,13] from the studies with most similarity with the procedures used in this study. The participants were selected according to the following criteria: children aged between three and five years, residing in the geographical area of the target population of study, officially registered in foster homes of the region, and that parents accepted to participate in the study by signing an informed consent. The study excluded children who presented some type of physical or motor disability that would hinder their following instructions to obtain the samples of dental plaque, as well as children receiving antibiotic treatment within the three months prior to the study[14].

***Instruments.*** For each individual included in this study a selection criteria format was initially filled out including indicators for diagnosis of caries with ICDAS II criteria, selecting only codes 0, 1, and 2 to determine their inclusion in the study. This procedure was carried out by an internationally calibrated examiner (Kappa 0.78) in the visual criteria of caries. Additionally, to store the data obtained from the molecular tests, another format was designed containing the following variables: molecular detection of microorganisms (*S. mutans, S. sobrinus* or both), state of dental surface (with caries, without caries), sex, and age.

To standardize the collection of samples of dental plaque, a pilot test was performed in which a periodontal probe (Williams) was used with which the weight in grams was calibrated for each substrate, which permitted establishing the amount of plaque necessary to obtain efficient DNA, which was validated by using reference strains of *S. mutans* and *S. sobrinus*; thus, verifying the correct molecular identification for each microorganism.

***Clinical examination.*** For each participant, the samples of dental plaque were obtained from carious lesions and from healthy dental surfaces. After obtaining the sample of dental plaque, each participant was subjected to a clinical exam to detect dental caries, which permitted classifying the participants into two groups: with caries, without caries. This procedure was conducted by a sole examiner considered the international gold standard for the diagnosis of dental caries with ICDAS II visual criteria. The exam was performed with the aid of a portable dental unit with artificial light and air, as recommended by Ekstrand in 2001[15] and ratified in the ICDAS II working document in 2005[16].

***Procedure for sample processing and analysis.*** The samples were collected in sterilized Eppendorf tubes containing 500 µl of Tris HCl 0.5M, pH: 8.0, and transported at room temperature to the microbiology laboratory at Universidad de Cartagena. The Eppendorf tubes were coded in a way that the technician in charge of processing and analyzing the samples was unaware of their origin in relation to the state of the dental surface from where these were obtained. To obtain the genomic DNA, for the samples of dental plaque and the pure cultures from the control strains, we used a procedure previously standardized in our laboratory.

Briefly, the samples of dental plaque were probed via five 0.9-second pulses and then two washes with Tris HCl 0.5 M pH 8.0 and centrifuged at 13,000 rpm for five minutes (Spectrafuge 16M, Labnet); thereafter, the resulting button was resuspended in 250 µl of TE buffer, and subjected to a heating cycle followed by freezing and thawing and finally centrifuged at 13,000 rpm for 15 minutes.

The supernatant was discarded and the precipitate was washed twice with isopropanol, centrifuged, discarding the supernatant on every occasion. The final button containing bacterial DNA, was left to dry at

**Table 1**
**Oligonucleotide sequences used in specific amplification of genetic fragments of *S. mutans* and *S. sobrinus****

| Gene | Microorganism | Sequence | T° Alignment | Size pb | Localization |
|---|---|---|---|---|---|
| *gtfb*- F | *S. mutans* | 5'ACTACACTTTCGGGTGGCTTGG3' | 59.5 | 517 | 793-814 |
| *gtfb*-R | *S. mutans* | 5'CAGTATAAGCGCCAGTTTCATC3´ | 59.5 | 517 | 1288-1309 |
| *gtfI* –F | *S. sobrinus* | 5'GATAACTACCTGACAGCTGACT3´ | 59.5 | 712 | 871-892 |
| *gtfI*–R | *S. sobrinus* | 5'AAGCTGCCTTAAGGTAATCACT3' | 59.5 | 712 | 1561-1582 |

*Oligonucleotides obtained from literature[11]

room temperature until complete evaporation of the alcohol, and finally resuspended in 50 µl of TE buffer and stored at -20ºC until its PCR analysis.

To extract DNA from control strains, nearly five colonies from a pure culture in nutrient agar were resuspended in an Eppendorf tube with 100 µl of Tris HCl 0.5 M, pH 8.0. The resuspended samples were subjected to the same procedure described previously to obtain DNA from samples of dental plaque.

For molecular analysis, we amplified a fragment of Chromosomal DNA via PCR technique using a thermocycler (Gene-amp PCR system 2.400) and an amplification kit (GoTaq Green Master Mix, Promega). The oligonucleotides used as primers to amplify *glucosyltransferase-B* (*gtfb*) and *glucosyltransferase-I* (*gtfI*) genes are shown in Table 1. Sequences of genes used were obtained from literature[11], from the GenBank, access numbers M17361 for *gtfb* and D90213 for *gtfI*. The specificity of the oligonucleotides used was verified with the *primer 3* program. The ATCC 25175 (*S. mutans*) and ATCC 33478 (*S. sobrinus*) control strains were used as positive amplification controls. The PCR products were subjected to electrophoresis in agarose gel at 1.5%.

***Statistical analysis.*** The information was initially stored in an Excel matrix (Microsoft Office 2007) and the subsequent statistical analysis was done with the STATA® program for Windows® version 10.0. For the univariate analysis, we used frequencies and percentages with confidence intervals of 95%. For the bivariate analysis, we used Fisher's exact test, assuming a decision limit of 0.05.

***Ethical considerations.*** This study gathered samples of dental plaque by using a calibrated probe with a group of preschool children. This action did not involve direct contact with support tissue of the tooth, which diminished risks to the physical integrity of the participants. Nevertheless, because this research was conducted with under-age subjects, we adhered to the normative ethical aspects from the Helsinski Declaration, Edimburg 2000 modification and to the technical, scientific, and administrative norms for research on human beings, Resolution N° 8430 of 1993 from the Ministry of Health of the Republic of Colombia. In this regard, the inclusion of each participant required a signed informed consent form from each parent or legal guardian. The consent form contained information related with the objectives of the current study and the procedures that would be performed on the child, appertaining to gathering the samples and the information required. The Project was approved by the Institutional Ethics Committee at Universidad de Cartagena, with the prior concept from the research committee which considered this a minimum-risk research.

## RESULTS

Figure 1 shows the PCR amplification products for *S. mutans* and *S. sobrinus*, with sizes of 517 bp (*gtfb*) and 712 bp (*gtfI*), respectively. These results show that the amplification of the fragment corresponding to *S. mutans gtfb* (517 bp) and *S. sobrinus gtf1* (712 bp) genes took place in the ATCC strains, which could be distinguished by PCR methods because of the difference of sizes of the respective bands.

In the study, it was found that the frequency of *S. mutans* in subjects with caries was at 76% (CI 95%: 59-

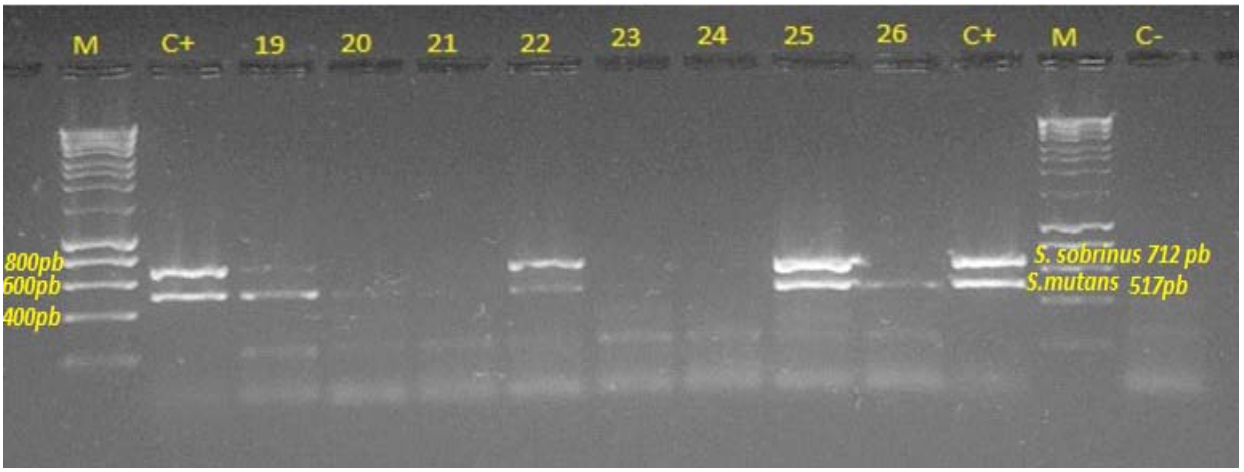


**Figure 1. Multiple PCR to detect *S. mutans* and *S. sobrinus* in dental plaque samples**

Specific fragments of *S. mutans* (*gtfb* gene) and of *S. sobrinus* (*gtfI* gene) were amplified via PCR from samples of dental plaque. Lanes 1 and 12: Molecular weight marker. Lanes 2 and 11: Positive controls for *S. mutans* (ATCC 25175) and *S. sobrinus* (ATCC 33478). Lanes 3-10: Product of amplification of dental plaque samples (children from #19 to #26). Lane 13: Negative control.

**Table 2**
**Relationship between the presence of *S. mutans*, *S. sobrinus,* and *S. mutans/ S. sobrinus* in children with and without caries**

| Microorganism | Groupwith caries n=26 | (%) | CI 95% | Groupwithout caries n=24 | (%) | CI 95% | Total |
|---|---|---|---|---|---|---|---|
| ***S. mutans*** | | | | | | | |
| Yes | 19 | 76.0[a] | 59-93 | 6 | 24 | 7-41 | 25 |
| No | 7 | 28 | 10-46 | 18 | 72 | 54-90 | 25 |
| ***S. sobrinus*** | | | | | | | |
| Yes | 9 | 81.9[b] | 58-100 | 2 | 18.2 | -5- 41 | 11 |
| No | 17 | 43.6 | 27-59 | 22 | 56.4 | 40-72 | 39 |
| ***S. mutans/S. sobrinus***[c] | | | | | | | |
| Yes | 7 | 77.8 | 50-100 | 2 | 22.2 | -5-49 | 9 |
| No | 19 | 46.3 | 31-61 | 22 | 53.7 | 38-68 | 41 |

a. p=0.001; b. Sp=0.02; c. Simultaneous presence of both species in the same sample

93) and 24% (CI 95%: 7-41) in individuals without caries (Table 2). With respect to *S. sobrinus,* the frequency in carious lesions was at 81.9% and 18.2% on healthy surfaces. When relating the presence of *S. mutans* and the presence of dental caries, statistical significance (p=0.001) was obtained, likewise for *S. sobrinus* (p= 0.02), while for the simultaneous presence of the two microorganisms there was no significant association with dental caries (p=0.08) (Table 2).

With respect to the relationship between the presence of *S. mutans* and *S. sobrinus* with age, the results show that there was no statistical significance for the presence of *S. mutans* (p=0.23); however, the presence of *S. sobrinus* was indeed significantly associated with age

**Table 3**
**Relationship between the presence of *S. mutans* and *S. sobrinus* with age**

| Age (years) | *S. mutans* | | | | *S. sobrinus* | | | |
|---|---|---|---|---|---|---|---|---|
| | Yes | % | No | % | Yes | % | No | % |
| 3 (5) | 1 | 20.0 | 4 | 80.0 | 0 | 0.0 | 5 | 100.0 |
| 4 (29) | 17 | 58.6 | 12 | 41.4 | 11 | 37.9* | 18 | 62.1 |
| 5 (16) | 7 | 43.8 | 9 | 56.3 | 0 | 0.0 | 16 | 100.0 |
| Total | 25 | 50.0 | 25 | 50.0 | 11 | 22.0 | 39 | 78.0 |

*p=0.004

(p=0.004), in such a manner that all the cases in which the presence of this bacteria was detected corresponded to the group of 4-year olds, with a frequency of 37.9%; p=0.004 (Table 3).

## DISCUSSION

This study found that the presence of dental caries in the children analyzed was significantly associated to the presence of any of the two species studied, although there was no association with the simultaneous presence of both species (Table 2). Even though these results were obtained from a cross-sectional design, which did not sufficiently guarantee the validity of the causal association between the presence of cariogenic microorganisms and the state of the dental surface, they may be a useful epidemiological approximation to design preventive measures tending to cause an imbalance of the dental plaque architecture that can impede the action of these microorganisms through the dental structure. This would contribute to diminishing the occurrence of dental caries in vulnerable populations as that evaluated in this study.

In this sense, it is recognized that in the etiology of dental caries the streptococcal bacteria of the *mutans* group play an important role, as the major cariogenic agents, although caries is not manifested in all surfaces colonized by these. It is important to recognize the distribution of *S. mutans* and *S. sobrinus,* which are the most commonly found species in humans, and to achieve their correct identification and differentiation from other species, considered an important step in understanding the early phases of bacterial colonization of dental plaque[17].

The difference observed in this study between frequencies of *S. mutans* and *S. sobrinus* colonization in dental plaque of surfaces with and without caries coincide with a study reported by Kanasi[18] in 2010, where *S. mutans* and *S. sobrinus* were positively associated with caries, although said study only evaluated caries in cavitational state. Other studies conducted in Brazil[11] in 2007 and in Japan[12] in 2002 with cavitational caries also found differences between the prevalence of caries and infection with *S. mutans*. When relating the presence of *S. mutans* and *S. sobrinus* with caries during early childhood, statistical significance (p<0.001) was also found[4].

Although *S. mutans* is not considered the primary colonizer in the formation of dental plaque, its characteristics of acidogenicity, aciduricity, formation of extracellular polysaccharides, turn it, along with *S. sobrinus*, into the microorganisms with greatest presence in mature plaque, which is capable of producing acids that cause the imbalance between the microorganisms and the tooth; thus, causing mineral loss. Hence, the presence of these two bacterial species in dental plaque is the main cause of most carious lesions[19].

In this study, the presence of *S. mutans* and *S. sobrinus* was observed only as of four years of age, and in most of the children in whom its presence was detected, there were associated to the presence of caries. Recent studies indicate that *S. mutans* can colonize the mouths of pre-dentate infants and that transmission can occur both vertically and horizontally [20]. In this regard, Okada[19] reported the age of the first finding of *S. mutans* is between 49 and 75 months, while for *S. sobrinus* it is between 49 and 81 months, which supports -in part- the results of the current study, given

that it permits us to confirm that *S. mutans* colonization is lower in younger children, revealing that in the group of three-year olds the colonization was almost null, while in the group of five-year olds most of the carious lesions were colonized. With relationship to *S. sobrinus* in the group of three-year olds no colonization was found, coinciding with Okada in the sense that the colonization of *S. sobrinus* is more latent than that of *S. mutans*. Because of this, it is important to determine the age in which colonization of cariogenic bacteria occurs and to compare the information with that reported by some clinical studies, which show that cariogenic risk is correlated with the age when colonization occurs[21].

In the results obtained via the PCR technique standardized in this study in samples of dental plaque, the pattern obtained was consistent with other results[22] in dental plaque, indicating that this method was relevant, easy to use, and quick in detecting *Streptococcus* from the *mutans* group. Previous studies aimed at detecting cariogenic bacteria initially used saliva as a source; however, the tendency in recent studies is to use dental plaque. For example, Sánchez[23] sought to determine if the correlation between caries and the presence of microorganisms was more (or less) significant when these were analyzed in plaque or in saliva, which is relevant when trying to estimate the risk. This is how it was determined that the correlation between the presence of *S. mutans* detected from the culture of dental plaque and the presence of caries was high ($p<0.009$), which does not occur when using saliva as culture source[23].

Studies using saliva as a source of cariogenic bacteria show that the presence of the microorganisms studied from the *mutans* group was at 100%[13]. Given that the intention of the studies is to relate the presence of cariogenic bacteria and dental caries to confirm risk in subsequent analytical studies, using saliva as a source of the bacteria studied does not permit establishing an effective association. Thereby, although the presence of *S. mutans* is high in saliva, it is lower on the surface of the dental enamel, where this bacteria actually manifests its capacity to produce acids and the subsequent demineralization process is different[24]. Bearing the aforementioned in mind, this study used bacteria from dental plaque as source rather than bacteria from saliva.

Studies published recently and similar to ours, used real-time PCR to determine that high levels of *S. mutans* correlate with the presence of caries during early childhood, but only in cavitation caries, showing that with high levels de *S. mutans* in dental plaque, there is a high incidence of caries[4,25]. However, these studies suggest the use of precavitation caries as a variable to conduct an analysis coherent with prevention, given that upon finding a relationship with the early stages of the disease, it is possible to conduct more efficient specific prevention.

The intention of standardizing a method to detect cariogenic microorganisms for *S. mutans* and *S. sobrinus* in dental plaque of preschool children precisely seeks to understand the risk of dental caries and its implications to keep these children healthy during the different stages of life. In this regard, many methods have evaluated the risk of caries including the host, cariogenic bacteria, diet, age, antecedents of caries, and social factors as variables for analysis, which mostly serve to identify individuals with low risk of developing caries, but which are not as effective in predicting those with greater risk of developing caries in the future. From this perspective, the evolution of molecular identification techniques has served to obtain more reliable results; thus, recent studies coincide in demonstrating that the presence of *S. mutans* is the main etiological factor for dental caries[26].

From the results obtained in this study, it is observed that the presence of *S. mutans* and *S. sobrinus* in dental plaque is associated to development of precavitation carious lesions, with molecular identification through PCR being a sensitive, rapid, and easily used method to detect cariogenic bacteria from the *mutans* group.

***Conflicts of interests.*** The authors declare having no conflict of interest with the institution where the sample was obtained for the study (ICBF in Cartagena), given that there was no labor or financial relationship**.**

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