Immunogenic Antigens from *Streptococcus mutans* Which Stimulate Secretory IgA Response from Parotid Saliva in Children with Caries

C. Román, A. Rivera, R. Santellan, B. Teutle, A. Yañez, L. Cedillo and S. Giono

**Abstract:** The aim of this study was identify immunogenic antigens from *S. mutans* which stimulate secretory IgA response from parotid saliva with caries in children. The sample consisted of 55 children aged 6-10 years old with caries, they took a sample of unstimulated parotid saliva with Schaefer capsules. Cultured *S. mutans* ATCC 35668, the bacteria were disintegrated by sonication, electrophoresis was performed proteins obtained from the bacterium and transferred to PVDF membrane and conducted western blot analysis the collected saliva. Unstimulated parotid saliva of children with caries containing IgA antibodies reactive to antigens of 26 and 19 kDa and there IgA reactivity against 190 kDa antigen (Ag I/II) in about half of the collected sample (26/55, 47%). Unstimulated parotid saliva of children with caries containing IgA antibodies reactive to antigens of *S. mutans* of 26 and 19 kDa. IgAs reactivity against 190 kDa antigen (Ag I/II) in about half of the collected sample (26 children, n = 55). IgAs reactivity against 250 kDa antigen in the middle of the collected sample (28/55). Which may be the antigen SpA of *S. sobrinus* (210 kDa) and cross-reactive to the antigen of *S. mutans*.

**Key words:** *Streptococcus mutans* · Immunological Response · Iga · Saliva · Caries

**INTRODUCTION**

*Streptococcus mutans* has been implicated as one of the causative agents dental caries, often isolating human dental plaque, presents various cell surface antigenic substances. Among these antigens found on the cell surface antigenic protein of 190 kDa that has been variously designated as antigen I/II, B, IF, P1, SR, PAC and MSL-1. This cell surface protein antigen is involved in cell adhesion to the surface of streptococcal teeth [1]. Based on the homology of their DNA *Streptococcus mutans* is divided into seven species: *Streptococcus mutans*, *S. sobrinus*, *S. ratti*, *S. cricetti*, *S. downei*, *S. ferus* and *S. macacae*, which can be subdivided into eight serotypes a, b, c, d, e, f, g and h, of the species *S. mutans* *sobrinus* have been implicated as the primary agents in caries in humans. *S. mutans* serotype c colonies are the most frequently isolated from the human oral cavity [2].

It is believed that rich domains and proline-alanine are primarily responsible for the interaction between the antigen I/II and salivary components. Evidence for a role for antigen I/II in the adhesion is primarily based on the study of adherence of *S. mutans* coated hydroxyapatite disks salivary proteins [3].

The secretory IgA (IgAs) act as the first line of defense of the host mucosa through intervention in adherence and colonization of microorganisms. IgA antibodies inhibit the adhesion of oral streptococci to epithelial cells isolated from the oral mucosa [4].
Dental caries is an infectious disease caused by bacteria that cause mineral dissolution of tooth hard tissues by acid end products of metabolism of bacteria able to ferment carbohydrate (acidogenic theory), affecting enamel, dentin and cementum [5].

Virulence factors are those conditions or specific characteristics that make it pathogenic microbe. In the case of Streptococcus mutans, the most involved in the production of cavities are acidogenicity, aciduricidad, acidophilicidad synthesis of glucans and fructans mutans, intracellular synthesis of polysaccharides such as glycogen and producing dextranase [6].

Saliva is a fluid surrounding all hard and soft tissues of the oral cavity, the oral fluid is a single fluid mixture and components derived from various surfaces. It is produced especially by the major salivary glands: parotid 20%, 65% of the submandibular and sublingual 6-7%, however during stimulated parotid flow provides up to 50% of the total excretion depending on the circadian rhythm whereas patient and between 80% and 90% of the average daily saliva produced is capable of stimulation. Is sterile when it leaves the salivary glands, but is no longer immediately when it enters the oral cavity, mixed with crevicular fluid, food debris, desquamated cells and microorganisms of the oral mucosa [7-9]. Salivary components, flow, viscosity and buffering capacity play an important role in preventing, initiation and progression of the disease, contributing to the prevention of caries by its antibacterial effect. Some components of saliva, both immunoglobulins as not immunoglobulins contribute to this effect [10].

The main factors are innate defense peroxidase and histatins lizozima. In vitro, these proteins are known to limit bacterial and fungal growth, or interfere with the absorption of glucose metabolism and promote aggregation and removal of the bacteria. These antimicrobial agents are synthesized and secreted by the major and minor salivary glands, but a small amount enters the oral cavity or tissue fluid polymorphonuclear leukocytes which enter via gingival crevicular fluid [11].

Specific defense factors are immunoglobulins IgG, IgM, IgA and secretory IgA (IgA-s). The most abundant immunoglobulin in the saliva, as in all human secretions,-s is the dimeric IgA is produced by plasma cells in the salivary glands located. There are two subclasses of IgA present in saliva as a major component IgA1 immunoglobulin, although the relative amount of IgA2 is higher than in other salivary secretions [12, 13].

The aim of this study was identify immunogenic antigens from S. mutans which stimulate secretory IgA response from parotid saliva with caries in children.

MATERIALS AND METHODS

We performed a descriptive cross-sectional study of a population of 55 children of 6-10 years of age who had moderate to severe caries, the degree of caries was determined by the ceo-d y CPO-D indices.

Inclusion criteria were children aged 6-10 years with clinical presence of dental caries, children who are not rehabilitated, parents who accept voluntary and signed informed consent. Exclusion criteria were the presence of chronic diseases, allergic diseases likely etiology, acute or chronic disease of the oral cavity, any other pathology at the time of the clinical history, patients with physical limitations, patients who agree to participate in the study and subjects who do not meet the age and eligibility requirements. Elimination criteria were: children who do not attend the day of the salivary sampling and not interested in making another appointment, children are not able to collect the minimum necessary to analyze saliva and children who do not understand the signs of the process of collecting [14].

Each of the patients diagnosed with dental caries study employed a scan previously sterilized material (mouth mirror and explorer) and calculations were performed dmf-t and DMF-D individually, as the sum of decayed, missing and filled either temporary or permanent, after standardization of the evaluator (Kappa 0.91). The decay rates dmf-t and DMF-D were taken according to the parameters set by the World Health Organization (WHO).

Each of the patients were sampled glandular unstimulated saliva using sterile Schaefer capsules, which are placed in the trolley immediately parotid duct outlet [15]. The samples were stored immediately in a container with ice for transport to the laboratory where they were stored at -70°C until processing.

Streptococcus mutans strain ATCC 35668 (Cat. #0969P Microbiologics. USA) was grown in Todd Hewith broth and incubated at 37°C for 24 hours, at the end massively seeded in blood agar plates and incubated again at 37°C for 24 hours. The bacteria were collected in a polypropylene tube with 6 ml of sterile distilled water and sonicated using a ultrasonic processor (Vibra Cell ™, VCX 130 USA) with pulses of 30 seconds and 5 seconds rest for 30 minutes with an intensity of 75 %. Then
Centrifuged for 10 min at 9000 G (IEC Medipsin.120. USA). The supernatant and pellet were stored separately and stored at -20°C until use.

Bacterial extracts were mixed in a 3:1 ratio with sample buffer (1.5% SDS, 3.5% of â-mercaptoethanol, 0.007% bromophenol blue) and placed in a water bath for 5 min to denature the proteins and thus to separate by their molecular weight.

Electrophoresis was performed on the soluble proteins of S. mutans in polyacrylamide gels under reducing conditions 12.5% (SDS-PAGE) for 180 min (30 min to 40 min and 150 mV to 80 mV), to determine the size of the proteins was used a molecular weight marker precasted 10-250 kDa (PageRuler™ Plus Prestained Protein Ladder, Fermentas. U.S.). Upon completion of the transfer, proteins were blocked with skim milk Sveltys 5% in PBS (blocking buffer) overnight at 4°C, then the membranes were washed 6 times with PBS-0.05% Tween 20 (PBS-T), allowed to dry and cut into 5 mm strips and stored at 4° C until use in the immunodetection.

For immunodetection the strips were incubated overnight with the saliva samples to a 1/50 dilution in blocking solution, the strips were washed immediately with PBS-T 6 times, one minute each wash, the term was incubated with anti- IgA (human IgA ) conjugated to biotin (4701 Tago, USA) diluted 1:500 in blocking solution for 2 hours at 37°C, subsequently washed with PBS-T and incubated in streptavidin-peroxidase solution (streptavidin peroxidase SIGMA 52438, USA) for 2 hours at 37°C, after washed with PBS-T and developed with the solution of peroxidase (40 mg diaminobenzidine, 20 ml of phosphoric acid pH 7.4 and 10 ul of hydrogen peroxide 30%) to observe bands, the reaction was stopped with distilled water, the strips were allowed to dry for analysis.

Once all the data collected, will be captured in SPSS v. 20, which was determined fashion and standard deviation of the variables dimensional scales.

RESULTS

The results reported for the age variable were: X = 8.6 ± 1.2 years with a range of 4, where the minimum age was 6 years and the maximum 10 years. Of the 55 patients who formed the sample, 29 were male (52.7%) and 26 were female (47.3%).

Regarding the epidemiological indices to record caries average CEO obtained a 5.6 with a standard deviation of ± 3.1 indicating that the children were with modified CEO according to WHO criteria, on the other hand was obtained CPOD of 2.6 with a standard deviation of ± 1.6.

Electrophoretic separation of protein S. mutans ATCC 35668 obtained by sonication at 75% intensity on polyacrylamide gels showed 12.5% protein bands ranging from 250 to 13 kDa, a total of 29 bands were found proteins of which 19 bands were well defined with the following molecular weights: 87, 69, 64, 55, 54, 50, 46, 44, 41, 38, 35, 29, 28, 27, 26, 25, 14 and 13 kDa. Also 10 faint bands with weights molecular of 250, 190, 130, 126, 82, 72, 34, 31, 23, 19 kDa (Figure 1).

Fig. 1: Polyacrylamide gels under reducing conditions (SDS-PAGE 12.5%) with the separation of protein S. mutans ATCC 35668 and stained with Coomassie blue. MWM: molecular weight marker, Line 1 and 2 and their corresponding protein profile molecular weight S. mutans ATCC 35668 obtained by sonication.
Identification of IgA antibodies by the technique of Western blotting was performed for 55 parotid saliva samples, 100% of them showed IgA reactivity against some of the antigens of *S. mutans* ranging from 250 to 19 kDa.

Immunodetection in some proteins of *S. mutans* were observed quite clear showing well defined bands and other questionable activity tenuous, both were included in the analysis. The antigenic proteins of *S. mutans* that gave positive reaction IgA antibodies had higher immunogenicity of 26 kDa present in 100% of the samples (55 children), 19 kDa on 98% (54 children), 250 kDa in 51% (28 children) and 190 kDa present in 47% (26 children) of the samples (Figure 2).

The recognition bands 250, 190 and 130 kDa appeared in 31 children, either together or in different combinations. Antigenic proteins that are less recognition obtained 72, 50, 29 kDa present in 6 children, 82 and 41 kDa on 5 children and finally 87, 34, 31, 27, 23 kDa identified in a child (Table 1).

The number of reactive bands IgA against *S. mutans* ATCC 35668 varied from 2 to 12 with an average of 5.3 and standard deviation of ± 2.6.

**DISCUSSION**

*Streptococcus mutans* is considered the main etiological agent of dental caries, this due to its ability to adhere to the tooth surface, sucrose accumulation in dental plaque as well as producing and tolerate high concentrations of acids, which cause demineralization of the enamel. Children who become infected with *S. mutans* prematurely mutans are more likely to develop tooth decay and the initial establishment of *S. mutans* appears to be associated with the eruption of the first teeth, however the eruption of the first molars, which usually takes place between 19 and 30 months of age, provided start retentive surfaces that favor biofilm formation by various microorganisms, including including *S. mutans* and Lactobacillus sp. This has been called the window
period of infection and it was found that after about 30 months of age without colonization of *S. mutans* risk for acquiring this organism is decreased [15-19].

*S. mutans* has several cell surface antigens including a cell surface antigenic protein of approximately 190 kDa named in different ways, among them are antigen I / II, B, IF, P1, SR, PAc, MSL-18, the Glucosyltransferases (gtf) of 170 kDa which is an enzyme that synthesizes glucans from sucrose, the glucan binding proteins (GbpB) 60 kDa antigenic protein of 13 kDa called D antigen, a protein of 39 kDa (Ag III), a protein 29 kDa antigen (antigen) and an antigenic protein of 70 kDa (antigen C). The influence of antibodies for glucosiltransferaza (GTF), glucan binding proteins (GbpB) and the antigen I / II in colonization and accumulation of *S. mutans* on erupting tooth surfaces has not been fully documented [20-22].

Has reported a possible correlation between different alleles of DRB1 * 4 (human leukocyte antigen) and significant activity to cell surface antigens of *S. mutans* in healthy adults. Finding increased reactivity to five or six bands with a molecular weight of 170-190 kDa. These bands represent cell surface antigens, such as the SA II (with a molecular weight of 190 kDa) and glucositransferasa. Both antigens were involved in initial adhesion of *S. mutans* as prime candidates for the development of vaccine against caries [23].

In this study, 26 samples showed IgA reactivity against antigens of 190 kDa and no reaction to antigens of 170 kDa, which makes us differ in results, because the study population were children aged 6-10 years of age and sample unstimulated saliva of the parotid gland. Also described electrophoretic protein patterns within certain genotypes and serotypes of *S. mutans* to reveal the IgA antibody reactivity against different antigenic proteins of *S. mutans* in adults without cavities and see if there is difference between the collected saliva. As a result of the electrophoretic separation of proteins in *S. mutans*, *S. sobrinus*, *S. salivarius* and *S. mitis* were found protein bands from 21.000 to 210.000 Da. In the immunoblot reactions were observed against 210 kDa protein in all subjects except for a 7 year old girl. La saliva parotídea mostró mayor número de bandas (6 a 20) contra *S. mutans* KPSK2 (serotipo C) con un promedio de 17 bandas [24]. En este trabajo se encontraron reactividad de IgAs contra antígenos de *S. mutans* en cantidades que van de 2 a 12 bandas por individuo, lo cual no coincide con otros reportes, que mencionan que esto puede deberse a la diferencia de edades, debido a que otros trabajos analizaron muestras de adultos sin caries y aquí se trabajaron muestras de niños con caries, siendo importante considerar que en los niños su sistema inmunológico se encuentra en desarrollo. The study evaluated the relationship between the secretory immune system and dental caries in children aged 3-5 years with varying degrees of decay, reported IgA reactivity to antigenic proteins of 39, 59, 97 and 150 kDa in almost all saliva samples. They mention that the lack of reactivity of antibodies against 185 kDa band (Ag I / II) suggests an immature immune system and that is one of the most important antigens, highly immunogenic *S. mutans* [20]. In the present work we report molecular-weight proteins that do not match those reported by other authors, but IgA were detected against 39 of 190 kDa antigen in 27 samples, perhaps because our population is older and are caries moderate.

Analyzing the antigens *S. mutans* UA 159 against IgA of children with moderate and severe decay was observed IgA reactivity against antigens of 77.17, 51, 47.4, 43.9, 35.81, 35.24, 28.98, 18.25, 71, 54.41, 52.02, 35.02, 33.57, 33.58, 20.65 kDa (in children with moderate decay). 77.17, 65.27, 48.33, 47.47, 36.70, 35.16, 35.81 kDa (in children with severe tooth decay). Being reported that low molecular weight antigens will correspond to enzymes involved in amino acid synthesis and protein translation glycolytic expressed as important factors in the multiplication of the bacteria and the development of caries, it can interact with proteins of 26 and 19 kDa which may correspond to the proteins of 28.98 and 18.25 kDa previously reported, which are in all individuals of our population, possibly because they are going through a process of active caries [25].

The low presence of IgA antibodies against antigens of *S. mutans* high molecular weight (190 kDa) is due to the young age of our population and the lack of maturity of the immune system or decrease in the expression of 190 kDa antigens from the microorganism to go unnoticed by the host immune attack. A study suggesting future with healthy patients of the same age to compare the production of antibodies and collected data that will help in monitoring on the adaptive immune response against *S. mutans*.

**CONCLUSION**

Unstimulated parotid saliva of children with cavities containing IgA antibodies reactive to antigens of *S. mutans* of 26 and 19 kDa. IgAs no reactivity against 190 kDa antigen (Ag I / II) in about half of the collected
sample (26 children, n = 55). IgAs no reactivity against 250 kDa antigen in the collected sample (28 children, n = 55). Which may be the antigen SpA of *S. sobrinus* (210 kDa) and cross-reactive to the antigen of *S. mutans*.

**REFERENCES**


