

ASSOCIATION OF SALIVARY PROTEINS WITH DENTAL CARIES IN A COLOMBIAN POPULATION

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ABSTRACT

Saliva has an important role in maintaining normal conditions of the oral tissues. Variability in salivary composition determines its protective characteristics against dental caries. Knowledge of the molecular content of saliva in humans is important to better understand its protective properties. The aim of the present work was to recognize protein composition in whole saliva of subjects with active caries (AC), History of caries (HC), and free of caries (H) in a Colombian population, by electrophoretic pattern, and to correlate these results with clinical diagnoses. Patients over 18 years old were selected after clinical examinations, and classified into three study groups. After patients signed the informed consent form, whole saliva samples were collected. Total protein determinations were made using the Bradford method. Individual saliva samples were

analyzed by SDS-PAGE and related to DMFT indexes. The gels were analyzed by Quantity One® 1-D software (BIO-RAD). No statistically significant difference was found between the total protein concentration and absence of prior experience of dental decay. Total protein content was higher in female subjects ($p= 0.0028$) than male, and regarding the disease, it was higher in women with HC and AC. Salivary proteins present in the majority of individuals were 101, 77, 62, 55, 44, 22 and 13 kDa in size. Association was found between 17 kDa salivary protein and AC in men. Conclusions: whole salivary proteins are very similar in the three groups, except for the 17 kDa salivary protein, which might be risk marker for dental caries.

Key words: dental caries, salivary proteins, saliva, Colombian population.

ASOCIACIÓN DE PROTEÍNAS SALIVARES CON LA CARIES DENTAL EN UNA POBLACIÓN COLOMBIANA

RESUMEN

La saliva tiene un importante papel en el mantenimiento de las condiciones normales de los tejidos orales. La variabilidad en la composición salival determina su característica protectora contra la caries dental. Conocer el contenido molecular de la saliva en humanos es importante para el entendimiento de sus propiedades biológicas. El objetivo de este trabajo fue reconocer la composición proteica de la saliva completa de sujetos con caries activa (AC), historia de caries (HC) y libres de caries (H); en una población colombiana por medio del patrón electroforético y su correlación con el diagnóstico clínico. Ciento cuarenta y cinco pacientes mayores de 18 años de edad fueron clasificados en los grupos de estudio por examinación clínica, las muestras de saliva fueron recolectadas, las determinaciones de proteína total fueron hechas por el método Bradford; la saliva individual fue separada por SDS-PAGE y relacionadas con

el índice COP. Los geles fueron analizados por el software Quantity One® 1-D (BIO-RAD). No se encontraron diferencias estadísticamente significativas entre las concentraciones de proteína total y la experiencia o no de caries dental. La cantidad de proteína total fue mayor en mujeres que en hombres ($p= 0.0028$); y con respecto a la enfermedad fue mayor en mujeres con HC y AC. Las proteínas salivares comunes entre los individuos fueron de 101, 77, 62, 55, 44, 22 y 13 kDa. Se encontró asociación entre la proteína salival de 17 kDa y la AC en hombres. Conclusiones: Las proteínas presentes en la muestra de saliva completa son muy similares en los tres grupos de estudio, excepto por la proteína de 17 kDa la cual puede ser un marcador de riesgo de caries dental.

Palabras clave: caries dental, proteínas salivares, saliva, población colombiana.

INTRODUCTION

Saliva is a clear, slightly acid mucoserous secretion, made up mainly of water (99%), with a small percentage of organic and inorganic compounds (1%)¹. Whole saliva is a mixture of fluids from the major and minor salivary glands² and gingival crevicular

fluid, which contains oral bacteria, virus, remains of food and remains of bronchial expectoration³.

Dental caries is a complex process that affects a high proportion of the world population⁴, regardless of gender, age and ethnic group, and tends to affect the population with lower socio-economic level to a

greater extent. The process depends on: 1) the interaction of protective and predisposing factors to dental caries; 2) the balance between the cariogenic and non-cariogenic microbial population, both in saliva and in plaque, and 3) the physical-chemical properties of enamel, dentine and cementum, which make the dental hydroxylapatite more or less vulnerable to acidogenic change⁵.

In developing countries, and particularly among the less privileged economic classes, there is a high proportion of dental caries experience. In Colombia the caries rate in adults is 87%⁶, and in China it has been shown that 75% of children under the age of five years already have caries experience⁷.

The determining role of saliva in the caries process is related to its composition, because colonization of the tooth surface by cariogenic microorganisms is initiated by their interaction with the proteins in the acquired film⁸, and to the different functions of these components in facilitating bacterial coaggregation⁹.

In vitro studies have shown that salivary proteins can interact with oral bacteria in different ways to promote bacterial adherence to the saliva film, cell aggregation, cell inhibition or death, and to protect the hard and soft tissues in the oral cavity. Several saliva components serve in one or more of these processes, generating redundancy regarding their functions. However, this does not imply that proteins with equal functional roles contribute to the same degree¹⁰.

The salivary flow rate, the ability to maintain the hydrogen ion balance, antimicrobial activity, the aggregation of microorganisms and the cleanliness within the oral cavity, the immune response, and calcium phosphate binding proteins interact to inhibit or revert the demineralization of exposed dental surfaces.

Due to these component-mediated functions that have been attributed to saliva, when some researchers evaluated the effect of saliva on dental caries, they considered that in addition to dental caries being a multifactorial disease induced mainly by bacteria, it may be equally influenced by hereditary saliva factors. Similarly, it has been reported that although the general composition of saliva is similar in different individuals, there are significant genetic differences among them¹¹.

As the complex interaction among proteins may contribute to salivary functions that either protect from or increase the risk of dental caries, the question as to which are the factors that protect some people from the disease or the factors that make others susceptible

to it, are still unanswered¹². Further studies are needed on the components of saliva that might be related to caries experience in different individuals. Therefore, the aim of this study was to recognize differences in the protein composition of whole saliva in a sample of individuals from the Colombian population with active caries, a history of caries and without caries, by determining electrophoresis patterns and correlating the results with the clinical diagnoses.

MATERIALS AND METHODS

Population and sample

One hundred and forty five individuals over 18 years of age, without any kind of systemic compromise, were selected after clinical analysis. According to the state of oral health of the participants determined using the DMFT index (decayed, missing and filled), the individuals were classified into three groups: healthy (H) (49 individuals completely free of caries), history of caries (HC) (49 subjects with amalgam or resin fillings and currently free of caries) and active caries (AC) (47 patients with multiple cavities, including enamel and dentine).

Saliva collection

Samples of saliva were taken by salivation without stimulation, after patients had signed informed consent forms. Patients should not have eaten or ingested anything and should not have smoked for 2 hours prior to sample collection. For each individual, 3 to 5 ml of saliva were collected in glass jars. Each sample was clarified by centrifugation at 10000xg for 10 minutes. The precipitate made up of epithelial cells and detritus was discarded. To each ml of the supernatant containing the fraction of interest, was added 0.1 ml of a protease inhibiting solution, with pH 7.5: Tris (0.1M), Na₂EDTA (2%), n-propanol (10%), phenylmethylsulfonyl fluoride (2 mM)¹³. Finally the samples were stored at -20°C until they were used (maximum 1 week, to avoid protein degradation).

Total Protein Concentration

Total protein in the sample was calculated according to the method of Bradford¹⁴, where its concentration was determined by interpolation of the absorbances of each sample on the calibration curve.

Electrophoresis of saliva samples

Salivary proteins were separated by electrophoresis under denaturing conditions (sodium dodecyl sulfate

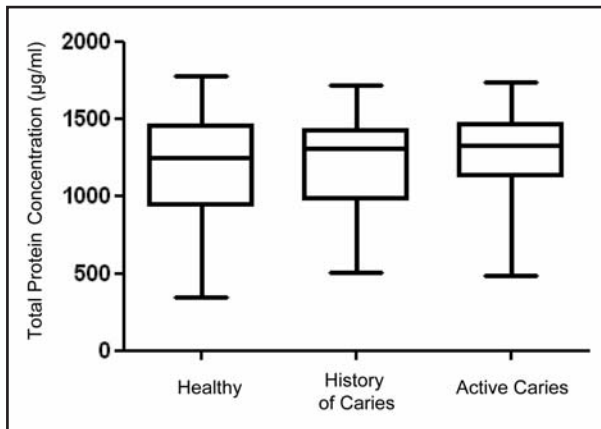


Fig. 1. Total Protein Concentration. Interquartile distribution of total protein concentration in subjects for each study group.

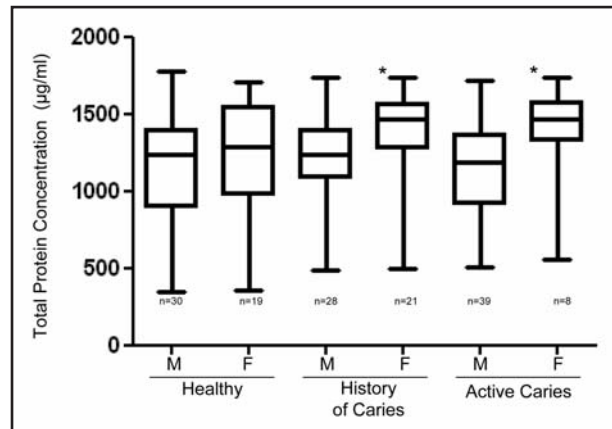


Fig. 2. Total protein concentration per sex. Interquartile distribution of total protein concentration in subjects for each study group distributed according to sex. n = number of individuals. * $p < 0.05$ Significant differences by the Mann-Whitney test.

– polyacrylamide gel electrophoresis, SDS-PAGE), using a wide range molecular weight marker, in gels with 10% polyacrylamide concentration. The gels were dyed with a Coomassie blue solution (0.25%, w/v). After staining the gel for five minutes, it was destained with a solution of methanol, acetic acid and distilled water. Then it was processed in a gel dryer for analysis by means of software, in which the approximate molecular weight of the bands in the samples was calculated by interpolation within the calibration curve on which the relative mobility of each marker protein (Rf) was plotted against the logarithm of molecular weight.

Analysis of the information

The gels were analyzed with the software *Quantity One® 1-D Analysis* (BIO-RAD), which was used for determining the molecular weight of the protein bands in each sample.

Statistical Analysis

Based on an inferential statistical analysis, data distribution did not show normality in all groups with the *Shapiro Wilk* test, therefore different non-parametric statistical tests were applied by means of the *Stata 6 software*. The differences between groups were evaluated by means of *Kruskal-Wallis* ($p < 0.05$) and *U- Mann-Whitney* ($p < 0.05$) tests to obtain the associations among the different study variables. The *chi-square* test was used to observe the presence of individual salivary proteins according to gender and study groups.

TABLE 1. Interquartile distribution of total protein concentration ($\mu\text{g/ml}$) in each of the groups studied.

Statistics	Healthy ($\mu\text{g/ml}$)	Caries History ($\mu\text{g/ml}$)	Active Caries ($\mu\text{g/ml}$)
Median	1244	1309	1327
Range	(342-1779)	(507-1712)	(484-1737)
Percentile 25	939.5	979	1122
Percentile 75	1461	1422	1462
Average	1196	1198	1262
SD	362.4	299.8	311

RESULTS

Total Protein Concentration

Average total protein concentration in the saliva of the individuals studied ($\mu\text{g/ml} \pm \text{SD}$) was 1219 ± 323.9 . The medians among groups were very similar. The *Kruskal-Wallis* test showed that there is no statistically significant difference between the amount of protein and dental caries experience ($p = 0.4204$) (Fig. 1). Table 1 shows the interquartile distribution.

Regarding gender, average total protein concentration was higher ($\mu\text{g/ml} \pm \text{SD}$) in the female population (1328 ± 320.52) than in the male population (1173 ± 316.51), (*Mann-Whitney* $p = 0.0028$). When it was observed between groups by *Kruskal-Wallis*, it showed differences ($p = 0.0015$) between total protein quantity in men and women in groups HC ($p = 0.0039$) and AC (0.0004), correlated to the highest medians in the female population (Fig. 2).

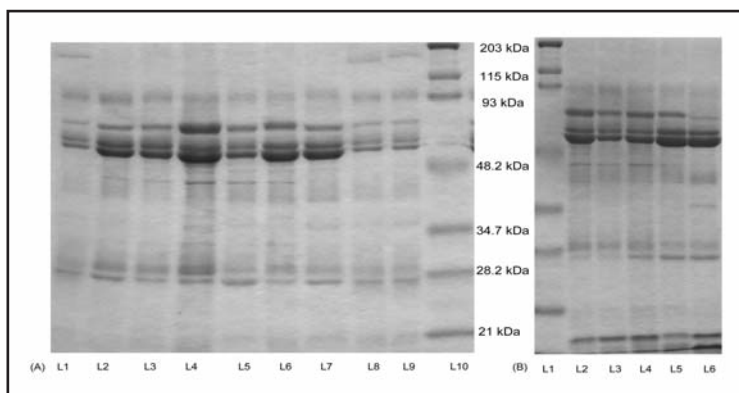


Fig. 3. Polyacrylamide gel electrophoresis in denaturing conditions. Whole saliva samples from each individual in the study were separated in denaturing conditions in 10% polyacrylamide gels. Healthy individuals: A. Lines (L): L1 and L7. History of Caries: L3, L4, L8 and L9. Active Caries: L2, L5, L6. L10: mol weight marker. B. L1: mol. weight marker. Active Caries: L2 and L3. History of Caries: L4, L5 and L6.

Electrophoresis of saliva samples

The gels obtained were used for analyzing each sample (Fig. 3). Salivary proteins present in individuals (100%) were of molecular weights 101, 77, 62, 55, 44, 22 and 13 kDa. The molecular weights of the less common proteins were 168 to 32 kDa (Table 2).

On correlating the results of the clinical diagnosis to the electrophoretic patterns of salivary proteins in the population studied using *Kruskal-Wallis*, no statistically significant difference was found, as these proteins were common among the study groups ($p=0.3893$).

Regarding gender, on doing the analysis of proportions, the *chi-square* test showed that the saliva of men more frequently contained proteins of molecular weights 37kDa ($p=0.047$), 29kDa ($p=0.009$) and 26kDa ($p=0.011$). The rest of the proteins are common to the two sexes. On comparing individual proteins by gender and by study group, statistically significant differences were only found for the 17 kDa protein, which appeared more frequently in men with AC ($p=0.037$) (Table 2).

DISCUSSION

This study evaluated the protein constitution of saliva in a sample of individuals from the Colombian population with active caries, a history of caries and without caries, by means of determining electrophoretic patterns and their correlation with clinical diagnosis, for which whole

saliva from each individual that participated in the study was used.

Most studies conducted to describe protein components of saliva have used saliva fractions, by taking samples directly at the salivary glands, e.g. from the parotid glands¹⁵⁻¹⁹, the minor salivary glands²⁰ or comparing saliva from the parotid, whole saliva and crevicular fluid²¹; thus, most of these studies have characterized saliva according to the glands it proceeds from. Studies of whole saliva are important firstly because it is what naturally covers teeth, and secondly because it contains a uniform mixture of secretions from the major and minor salivary glands, enabling the

observation of a wide range of its biological and physiological characteristics, as well as variability among population groups²². Due to the abovementioned reasons, studies of whole saliva in recent years have been preferred^{22,24,25-28}, and they have attempted to elucidate the global effect of saliva on oral health, with the aim of using it as a means to diagnose other diseases (by determining the presence, absence, reduction or increase of any of its components).

In contrast to this study, other similar studies divided the population studied into two groups-healthy subjects and subjects with caries. This study was designed with three study groups, by adding the group with a history of caries, based on the possibility of salivary protein content being different from that of caries-free subjects because they had had the disease at some time in their lives, i.e. salivary protein similar to the active caries group. The aim was to observe whether the disease could be explained by the presence or absence of salivary proteins in these individuals, who were used for a first attempt at characterization of the salivary composition of our population.

Total protein concentration results do not show statistically significant differences when they are associated to the state of oral health in the individuals studied, in contrast to Yue et al. 2002, who reported that the quantity of protein was lower in subjects who were susceptible to caries than in those who were resistant. This might be due to the high variability in our population, since salivary

TABLE 2. Proteins by molecular weight found in the three study groups.

Protein kDa	168	101	77	62	55	44	39
n (%)	9(6.20)	145(100)	145(100)	145(100)	145(100)	145(100)	92(63.4)
H:CH:AC	2:4:3	49:49:47	49:49:47	49:49:47	49:49:47	49:49:47	28:35:29
M:F	6:3	97:48	97:48	97:48	97:48	97:48	59:33
H= M:F	2:0	30:19	30:19	30:19	30:19	30:19	16:12
CH= M:F	2:2	28:21	28:21	28:21	28:21	28:21	18:17
AC= M:F	2:1	39:8	39:8	39:8	39:8	39:8	25:4
Protein kDa	37	32	29	26	22	17	13
n (%)	59(41)	19(13)	82(56.5)	134(92.4)	145(100)	62(42.75)	145(100)
H:CH:AC	17:20:22	5:6:8	28:26:28	41:49:44	49:49:47	19:20:24	49:49:47
M:F	*35:24	11:8	*49:33	*93:41	97:48	40:22	97:48
H= M:F	8:9	2:3	15:13	28:13	30:19	8:11	30:19
CH= M:F	11:9	3:3	13:13	28:21	28:21	12:8	28:21
AC= M:F	16:6	6:2	21:7	37:7	39:8	*20:3	39:8

H: healthy, CH: caries history, AC: active caries. M: male. F: female
 The percentages in bold indicate the proteins common to all individuals.
 * $p < 0.05$ by Chi-square.

secretion is regulated by mechanisms that include physiological, psychological and pathological factors^{21,29}, which reflect differences in quantity and composition of salivary flow.

On the other hand, previous studies conducted by our research team (data not shown), found that AC individuals had a higher *Streptococcus mutans* count in saliva samples, increasing the risk of dental caries and possibly indicating differences compared to the protein content of caries-free patients, which could be correlated to prior references that indicate the presence of different salivary levels of oral streptococci may be influenced by individual salivary proteins³⁰.

The average total protein content in saliva in the individuals studied (1219 ± 323.9) ($\mu\text{g/ml} \pm \text{SD}$) was very close to that reported by Banderas et al. 2002, (1374 ± 0.45), although they did not correlate total protein concentration between patients with or without caries²². What has been observed in this study is very high standard deviation, this can be explained by biological variability, since the concentration of salivary protein may vary due to source of saliva (the higher the flow, the lower the concentration, and the opposite), the state of stimulation, salivary flow or simply because the sample was not taken at a precise time of day (circadian variation)²³.

Banderas et al. 2002, observed that total protein concentration tended to be higher in women than in men, though without statistically significant results. This study demonstrated statistically significant differences regarding women having higher protein concentration in saliva than men. As explained above, this may be due to the large number of factors that interact to regulate the processes of salivary secretion, which affect both the quantity and the concentration of the salivary components. One of these factors might cause coincidences in the women who took part in the study, with their total protein concentration being higher than the concentration found for men.

Our research team has conducted several studies on whole saliva, which, together with this one, have established the electrophoretic profiles of the population. There are 14 bands of molecular weight in the samples separated using electrophoresis under denaturing conditions, and so far no association has been found between these bands and dental caries experience, as has been reported in other studies³¹.

The electrophoretic profiles found per individual show 8-14 protein bands, in the range of 13 to 168 kDa, with individual variability matching what has been found in most studies: high inter and intra

individual²⁶ variability, without this variability being associated to the state of oral health of the individuals, or with their sex.

Although this study did not evaluate the intensity of the bands, when the electrophoretic runs of individual samples were observed, small differences in staining were noticed on comparing each protein band between individuals, which may correspond to differences in protein concentration, in contrast to the findings of Ruhl et al. 2005, who did not observe any differences regarding this variable²⁷.

On observing the electrophoretic runs per individual, small qualitative variations were noticed, which may correspond to polymorphism due to possible alterations in the peptide composition of each protein. Salivary proteins are the product of multiple genes and multiple alleles, which produce a wide range of phenotypes, explaining the biological variability between individuals. Indirect evidence suggests that protein polymorphism may affect the interactions with oral microorganisms, explaining why different salivary proteins are observed in groups with or without caries.

Examination of the polymorphisms of salivary proteins is interesting not only in determining the role of salivary composition in the state of oral health, but also because they may define the contribution of the environment and genetic factors to the etiology of dental caries¹⁷. In this study, these polymorphisms were evident particularly in the proteins with larger molecular weight 168 and 101 kDa, but they were not associated with dental caries experience, as has been described in other studies¹⁷.

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5. Banderas et al. 2002, found a significant correlation between total protein content and number of bands present corresponding to salivary proteins per sex²². In this study, this correlation was observed only for certain proteins 37, 29, 26 and 17kDa, which were more often present in men, and the latter in active caries. There is a greater number of these proteins in males with active caries, and the value is only significant for the 17kDa protein. This suggests that these proteins might be a caries risk marker.
6. All the differences among the different studies might be explained by the genetic diversity among individuals from different geographic regions, since in some of the subjects the salivary proteins contribute in a different way to the risk of oral pathology in different populations and at different times, while in other places they do not. Moreover, fluorine supplements may intervene in this variability. They are not the same all over the world, and their distribution and accessibility depend on each country^{23,17}.
7. The scope of the study did not enable the proteins to be identified, as this requires the use of specific antibodies and other essential chemicals. Identifying them is the next step to complete this research. Further work of this kind will enable a more complete characterization of the salivary components and their interactions, which will make a real contribution towards establishing what the effect of salivary components is on a complex, dynamic process such as dental caries, contributing to the search for therapeutic tools that will help control the disease.

CORRESPONDENCE

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