Age and gender related changes of salivary total protein levels for forensic application

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The authors declare that they have no conflict of interest.

KEYWORDS

forensic odontology, saliva, total protein, age, identification

J Forensic Odontostomatol 2018. May; (35): 26-33
ISSN :2219-6749

ABSTRACT

Saliva is one of the most commonly encountered biological fluids found at the crime scene. Forensic science including forensic odontology is focused on the positive identification of individuals. The salivary protein profiling can help in personalization by the changes associated with age throughout life and gender. These changes also seem to vary with the dietary habits, environmental factors and geographical areas. Thus, the aim of present study is to estimate these changes in salivary total protein concentration and profiling in individuals of Gujarat, India. The association of total protein concentration and protein content with the age, gender, tooth eruption, functions of the protein and its physiological significance are also intended for study in this population. One hundred unstimulated whole saliva samples from study subjects of Gujarat population were collected and grouped based on age and gender. Total protein concentration was determined by Bradford assay; also protein was separated and analyzed using Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE). T Test and ANOVA were used for statistical analysis. The concentration of Total Protein was found to be between 2-4 mg/ml. It showed a positive correlation with age and gender. It can be concluded more protein bands were prominently present in the adolescents group followed by children and lastly in the adults groups.More high (more than 80 kDa) and low (less than 30 kDa) molecular weight proteins are seen in children and adolescents than adults. SDS PAGE allowed identification and comparison of group variabilities in protein profiles. The total salivary protein showed an association between the parameters under this study which will aid in the individual identification in the field of forensics.

INTRODUCTION

Forensic science including forensic odontology deals with the dental evidences and its examination that is used in recognition of the crime and positive identification of an individual. Teeth, physical injuries, bitemarks, lip prints and oro-facial structures are the normal evidences used in forensic odontology investigations. Over the last decade or so there has been a growing interest in saliva and its use as a diagnostic tool as an alternative to blood or urine.[1] In recent times, the importance of saliva has been brought into focus for individualization and victim identification in mass disasters. Saliva is composed of about 99% water, 1% solid; 0.5% inorganic substances

(chloride, sodium, phosphorus, calcium, potassium, nitrogen, oxygen, bicarbonate, dissolved carbon dioxide and trace elements) and 0.5% organic substances (protein, vitamin, lipid, hormone-like substance, free amino acid, urea, microbial, shed cells and antimicrobial substances).[2] Components of saliva may serve as biomarkers because the composition of oral fluid is responsive to behavioral, mechanical, genetic or ontogenetic stimuli.[3] Saliva is often detected in scenes of crime along with bite marks or lip prints where the oral cavity may have been involved.[1] Saliva can act as an aid in cases where the direct examination of bitemarks or lip prints cannot lead to a positive conclusion due to elasticity of the skin or substrate, malocclusion, partial impressions, multiple or distorted impressions, avulsion bites leading to tear of the tissue and unavailability of records . Saliva also leads to identification of individuals associated with crime scenes like homicide, assault, child or elder abuse, poisoning, drug or alcohol abuse and other criminal cases. Saliva is a body fluid usually deposited with oral evidences when oral tissues come in contact with skin, food, clothes, cutlery, glass and cigarette. Forensic investigation and analysis of saliva is preferred due to ease of availability at a crime scene, effective collection methods and huge variation in the biological composition throughout an individual's life. The added advantage of its noninvasive method of collection even by individuals with limited training and avoidance of intrusion of private functions while collection under direct supervision, makes saliva a popular fluid for forensic analysis. Serological and cellular analysis of obtained saliva is of immense use in identification of the accused.[1]

Proteomics and genomics are gaining popularity as remarkable tools in forensics. Proteins are found in different forms based on their molecular weight, size, structure and functions. Specific array of proteins have distinctive character and function which helps to gain information of a particular individual. Molecular changes in proteins are reflected in the salivary composition and serve as genetic markers. Salivary biomarkers may be used in detecting and diagnosing systemic diseases based on the relativity between a specific disease and salivary protein changes. It also provides vital information in identifying a person involved in crime.[4] Total salivary protein concentration and protein profiling demarcates age and gender dependent differences. Thus it is essential to understand the standard baseline of the variations found in the protein profiles of a population. The major salivary proteins comprise amylases, acidic, basic, and glycosylated proline-rich proteins (PRPs), statherins, histatins, peroxidases, cystatins, and mucins constitutes the proteome. Concentration of protein in whole saliva is ~2000-4000 µg/mL.[5] The main functions of salivary proteins are protection of teeth, pH maintenance, anti-microbial and anti-fungal properties, decrease in demineralization by pellicle formation, remineralization of enamel, caries prevention, inhibition of microbial growth and defence to maintain oral health. Functions of salivary proteins may depend on the molecule's location or site of action.[6]

The identification of individuals by salivary protein profiling can aid in personalization by the changes observed associated with age throughout life and gender. The aim of present study is to estimate these changes in one hundred individuals of Gujarat population using salivary total protein. The present study quantitatively estimates the protein concentration of saliva in different age groups and gender with the help of Bradford Assay. It also focuses on the qualitative approach to determine salivary proteome of individual and pooled samples using SDS PAGE.

MATERIALS AND METHODS:

Study design and saliva sampling

This study was done to examine and compare the salivary proteome in individuals during the course of dental eruption in children and throughout the adult life. Samples were analyzed individually for inter-individual variations as well as pooled to assess inter-group differences. For this study unstimulated whole saliva specimens were collected from 100 healthy individuals of Gujarati population. Written consent was taken from each participant before the collection. Whole unstimulated saliva was collected in the morning between 8 a.m. to 10 a.m. No edentulous individuals were included for the subject selection. Healthy individuals with no acute or chronic disease between the age group of 3 to 60 years were included. Any consumption of water, food or drugs one hour prior to sample collection was excluded. The subjects were equally divided into children group 1 (3 to 6 years) - children with primary dentition: 8 subjects, group 2 (7 to 12 years) – children with mixed dentition: 25

subjects, adolescents (13 – 25 years) - permanent dentition until third molar eruption: 34 subject sand adults (26-60 years) - post dental eruption: 33 subjects. Subjects were also divided on basis of gender: 53 males and 47 females. Table 1 shows age and gender distribution of the study subjects. Nearly 5 ml of saliva sample was collected in sterile eppendorf within 5 minutes time window, fellowed by addition of 0.5 M EDTA for preservation. Immediately after collection saliva samples were centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatants were gently aspirated with a pipette without disturbing the pellet. The supernatants were frozen and preserved at −20°C. Prior to analysis, aliquots were thawed and used for analysis.

Table 1. Table shows distribution of subjects for this study according to (A) age and (B) gender.

A	
Groups according to Age	**No. of subjects**
Children 3 to 6 years (Primary dentition)	8
Children 7 to 12 years (Mixed dentition)	25
Adolescents 13 to 25 years (Permanent dentition until third molar eruption)	34
Adults 26 to 60 years (Post dental eruption)	33
Total	100

B	
Groups according to Gender	**No. of subjects**
Female	47
Male	53
Total	100

Quantification of salivary proteins

The standard Bradford assay[7] suitable for measuring between 10 and 100 µg protein was used in the present study to determine the concentration of salivary proteins. The approximate protein concentration in the sample was unknown so a range of dilutions (1, 1/5, 1/10, 1/20, 1/100) were assayed randomly in 4 samples to optimize the dilution. The samples and protein standards were measured at A595 for absorbance in Vis-UV Spectrophotometer between 5 and 60 minutes. The total protein concentration was calculated using the BSA standard calibration curve. Statistical analysis ANOVA test was done to compare the estimated age and chronological age of different groups and T- test was done for gender differentiation.

Qualitative analysis by SDS-Page gel electrophoresis

Proteins from saliva were extracted by TCA - Acetone - DTT method as described earlier[8]. The pellet obtained at the end of the process was used for SDS-PAGE gel electrophoresis for separation and analysis of salivary proteins. SDS-PAGE[9] was performed in gel caster and power bank assembly (SE260 from Hoefer Inc., USA) using 15% polyacrylamide resolving gel and 5% stacking gel. 1x sample buffer was added to 5 µL of the prepared samples. Electrophoresis was carried out at constant voltage of 180 V for three hours. MAGSPIN-34 MAG Universal prestained protein ladder was used as a reference. Coomassie brilliant blue colorimetric staining protocol was used for staining the gels. Gel Documentation System Photo scanner was used for gel image analysis.

RESULTS

The proteins present in the saliva were quantified by the Bradford assay. In the present study the total protein content was calculated in all saliva samples with 20 as the optimum dilution factor. The protein concentration obtained from Bradford assay was found to be 1.75 ± 0.75 mg/ml in children having primary dentition (3-6 years). It increased with age in children having mixed dentition to 2.31 ± 0.81 mg/m. It slightly decreased to 2.25 ± 0.74 mg/ml in adolescents having permanent dentition up to the eruption of third molar (13-25 years). Protein content was found to be 2.05 ± 0.91 mg/ml in adults (26-60 years) corresponding to decrease than adolescents group as shown in Table 2.

Table 2. Table shows the total protein concentration in saliva samples obtained by Bradford Assay of various age groups. The average protein concentration was found to be increasing form adolescent group to children up to adults. Whereas in pooled samples concentration decreased from children to adolescent to adult group. The association of protein concentration with the eruption of teeth can also be assessed.

Group	Average Total Protein Concentration (mg/ml)	Total Protein Concentration in pooled samples (mg/ml)
Children 3 to 6 years (Primary dentition)	1.75 ± 0.75	2.60
Children 7 to 12 years (Mixed dentition)	2.31 ± 0.81	3.68
Adolescents 13 to 25 years (Permanent dentition until third molar eruption)	2.25 ± 0.74	3.25
Adults 26 to 60 years (Post dental eruption)	2.05 ± 0.91	2.71

ANOVA statistical analysis was done for the age groups. The P value was found to be 0.60(P > 0.05) showing no significant difference between the age groups. Total Protein content in pooled saliva samples was done for each group. It showed saliva protein concentration obtained from children (3- 6 years) was 2.60 mg/ml, increased in children (7-12 years) at 3.68 mg/ml and decreased to 3.35 mg/ml in adolescents (13-25 years). Further protein concentration was found to decrease at 2.71 mg/ml in adults (26-60 years).

Our results showed higher salivary total protein in female than males as shown in Table 3.

Table 3. Table shows the total protein concentration in saliva samples obtained by Bradford Assay of gender groups. The average protein concentration and total protein concentration in pooled samples was found to be increased in female group than male group.

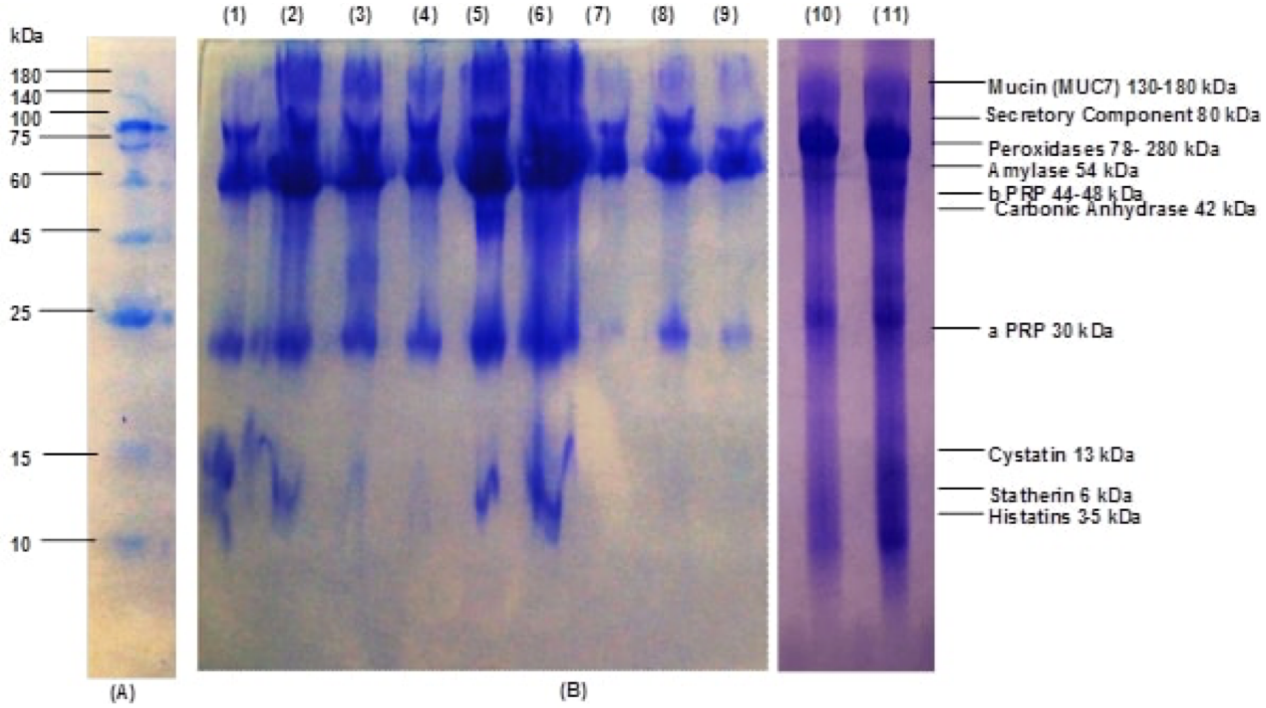
Group	No. of subjects	Average Total Protein Concentration (mg/ml)	Total Protein Concentration in pooled samples (mg/ml)
Female	47	2.25 ± 0.85	3.06
Male	53	2.06 ± 0.80	2.60

The total protein concentration in females was found to be 2.25 ± 0.85 mg/ml and in males was found to be 2.06 ± 0.80 mg/ml. T test statistical analysis was done for the gender groups. P value was found to be 0.28 (P > 0.05) showing no significant difference between females and males. Total protein content in pooled saliva samples was found to be 3.06 mg/ml and 2.6 mg/ml in females and males respectively.

The total protein concentration in pooled samples was greater than the average total protein concentration in all the age as well as in gender groups.

The SDS PAGE of each age group and pooled samples from each group shows the protein bands that are identified according to their molecular weight[10] as shown in Figure 1.

Figure 1: SDS-PAGE gel of salivary protein (A) shows the Prestained Protein Ladder gel (B) shows saliva samples of different groups (1) Age 3 (2) Age 7 (3) Age 12 (4) Age 13 (5) Age 18 (6) Age 25 (7) Age 27 (8) Age 46 (9) Age 60 (10) Pooled female sample (11) Pooled male sample



Our results showed that more protein bands were prominently present in the adolescents group followed by children with mixed dentition of age group 7-12 years followed by children with primary dentition of age group 3-6 years and lastly in the adults groups. High molecular weight protein band like Mucin (MUC7- 130 to 180 kDa) was present in both groups of children and adolescents but absent in adults. Secretory component of IgA (80 kDa), peroxidases (78 kDa), amylase (54 kDa) protein bands were seen in all the age groups. aPRP- proline rich protein band (30 kDa) and bPRP- proline rich protein band (44-48 kDa) were also seen in all the age groups. We observed the presence of carbonic anhydrase (42 kDa) only in the adolescent group and children with mixed dentition of age group 7-12 years saliva samples but its absence in adults. Low molecular weight protein bands like cystin (13 kDa), statherin (6 kDa) and histatin (3-5 kDa) bands are not very clearly seen in the adults but seen in both groups of children and adolescents. The SDS-PAGE gels of the pooled samples of all the age groups showed similar protein bands as compared to the individual sample. All protein bands in female pooled samples as well as in male pooled samples which are similar to the bands seen in individual samples.

DISCUSSION

The concentration of total protein in whole saliva obtained in the study using Bradford Assay was found to be in the normal range between 2.0-4.0 mg/ml.[5] The dilution factor 20 was found to be most appropriate in our study for the concentration of salivary proteins according to the absorbance range as it showed absorbance in the optimum range (<1). Each individual sample from all age groups contained a specific and different protein concentration which may help in differentiating each person. The age and total protein concentration showed a positive correlation. Our study shows difference in protein concentration of young, adolescents and adults. This is in accordance with results shown by Katie P. Wu et al.[11] Our results show linear increase in protein concentration with age until development of permanent teeth. This is in accordance with the studies by earlier reports by Nagler and Hershkovich,[12] Deshpande et al.[13,14] and Vibhakar et al.[6] Our results are also in

accordance with results by Kalipatnapu et al.[15] showing that protein content increase until middle age and remains constant in adults, further it decreases with advancing age. Our study also shows that children aged 7-12 years with mixed dentition show more protein concentration than children aged 3-6 years with primary dentition because of differences in salivary gland development. Katie P. Wu et al. also concluded that age 12-14 years show more protein concentration than 3-11 years.[11] Our study also shows the same results until the age of 14 years. Statistical analysis shows no significant difference between the age groups. These results were in accordance with study results by Shiv kumar et al.[3] Higher protein concentration was found in females than in males. This was also shown in studies by Dodds et al.[16] Statistical analysis showed no significant difference between males and females similar to results by Shivkumar et al.[3] and Vibhakar et al.[6] but in contrast with results by Dodds et al. which stated that significant sex differences in salivary protein concentrations exist.[16]

SDS PAGE showed high individual variability of the saliva protein band patterns in our study. The present study showed that the protein profiles differ with the age groups, which can be correlated to the teeth eruption in children (Primary and mixed dentition), adolescents (Permanent dentition until third molar eruption) and adults (Post dental eruption). This is in accordance with the study by M. Morzel et al.[17] More high and low molecular weight proteins are seen in both groups of children and adolescents than adults. Adolescent and children saliva were seen to contain both higher (more than 80 kDa) and lower molecular weight protein (less than 30 kDa). However, lower molecular weight protein lesser than 25 kDa was not observed in adults and was found mostly in children and adolescent saliva samples. These results were also reported by Shivkumar et al.[3] as they stated that children contained high molecular weight proteins and low molecular weight proteins of >90 kDa and <30 kDa respectively. Deshpande et al.[13] also showed the number of peaks of high molecular weight proteins (>70 kDa) observed in primary, mixed and permanent dentition did not show any statistically significant difference, though the average number of peaks in permanent dentition was higher than primary and mixed dentition age groups.

We have also tried to correlate the results of our study with the functions of the protein[18,19] and the physiological significance of the same. Mucin provides protection against bacterial protease activity, provides viscoelasticity and lubrication. The mucin protein band absent in adults may suggest that the adults have decreased viscoelasticity and are more prone to bacterial colonization. The secretory component of IgA is known to play a role in immune functions. Peroxidases are bactericidal and fungicidal agent and help prevent decalcification of enamel. Amylase is known to have function of hydrolysation (digestion) of starch and protection by selective binding to microorganisms. Proline Rich Proteins help in remineralization and caries protection. All these protein families are found in all the groups in our study and so these functions can be correlated to physiological functions in all ages but the concentration may differ with age. The protein band of carbonic anhydrase was found in adolescents and children with mixed dentition of age group 7-12 years. The function of carbonic anhydrase is known to be protective in nature involved in salivary pH regulation. The low carbonic anhydrase in adults can be associated with an increase in prevalence of caries. Statherins inhibit crystal growth of calcium phosphate salts and inhibit its precipitation. It also binds to bacteria and hydroxyapatite. Histatins also bind hydroxyapatite, complex with metal ions, inhibit crystal growth of calcium phosphate salts and stimulate wound-closure. Similarly, cystatins also weakly bind to hydroxyapatite. These protein bands are seen only in both children groups and adolescents but absent in adults. This can be correlated to the eruption and maturation of teeth in children and adolescents up to the eruption of the third molar at the age of around 25 years. Histatin bands are also seen in children and adolescents but absent in adults. Histatins are anti-fungal (potent C. albicans growth inhibitors) and anti-bacterial (inhibits activity of *P. gingivalis* associated with forms of periodontal disease). Histatin 1 protects tooth enamel and pellicle formation whereas Histain 5 is the most potent candidacidal. The absence of histatin band in the adults may be associated with the increased susceptibility of

adults to periodontal and fungal (candidial) diseases. This aspect has to be further studied for more accurate differentiation and individualization. Presence or absence of certain protein family bands in children, adolescents or adult saliva samples needs to be further investigated for its putative physiological significance.

CONCLUSION

The age and total protein concentration showed a positive correlation with age and gender. Protein content increases from children with primary dentition to children with permanent dentition until adolescence, further remains constant in adults and decreases with advancing age. It is concluded that there was no significant difference between the age groups as well as between females and males. Inter- and intra- group variability of the protein profiles by SDS-PAGE allowed the quantification and comparison of protein profiles from all individual and group samples successfully. It can be concluded more protein bands were prominently present in the adolescents group followed by children with mixed dentition of age group 7-12 years followed by children with primary dentition of age group 3-6 years and lastly in the adults groups. More high (more than 80 kDa) and low (less than 30 kDa) molecular weight proteins are seen in both groups of children and adolescents than adults. It was thus concluded in our study that there is correlation of the total protein concentration and protein content with the age, gender, tooth eruption, salivary gland development, functions of the protein and its physiological significance.

Saliva has a great potential in this field which has to be further explored. The importance of the protein component in saliva was highlighted in the present study. It also shows that it may prove to be of great importance in investigations where DNA may not be obtained. Since whole saliva can be collected quickly and non-invasively from many crime scenes, they may prove to be useful protein profiling media for age estimation or gender determination and criminal investigation by the forensic expert. This study establishes age related changes in human salivary total protein levels and also constructs a catalogue for age estimation gender determination using saliva which will aid in individual identification in forensic cases.

ACKNOWLEDGEMENTS

The authors thank Dr. Mohinder Singh Dahiya for the scientific guidance and acknowledge support from Gujarat Forensic Sciences University.

SOURCE OF FUNDING

Authors sincerely acknowledge the financial and infrastructural support from Gujarat Forensic Sciences University, Gandhinagar, Gujarat.

DISCLAIMERS

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the University.

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