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Salivary spectrum evaluation with Raman's Spectrometer

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Abstract

Background: Biofluids possess a lot of hidden informations. Along with serum, saliva beholds great potential which needs to be discovered. Certain physical properties of saliva need to be studied closely and thus can be put into use for various diagnostic purposes. **Aims and Objectives:** To find difference between male and female saliva through spectrometry. **Design:** 20 systemically sound subjects were selected and explained about the study. The subjects should not be consuming any medication or tobacco products. **Materials and Methodology:** Subjects were asked to collect unstimulated saliva in the disposable vials. The samples were then centrifuged with the help of speed vacuum concentrator. Then with the help of Gilson's pipette some centrifuge sample were taken and normal saline was added and was left to dry. With the help of Raman's Spectrometer 585nm the spectra was recorded and each sample were assessed 5 times. And then raw data was processed. **Result and Conclusion:** PCA (Principal Component Analysis), LDA (Linear Discriminant Analysis) and Average Spectra are derived. The LDA accuracy of our study was above 70% which is good for future research purposes as anything above 60% can be considered as important breakthrough.

Keywords: Unstimulated saliva, Raman's Spectrometer, Forensics, Gender Determination.

INTRODUCTION

The innovations like electrophoresis, chromatography, histochemistry, immunochemistry, electron microscopy, and microphysiology mark the beginning of the modern world [1, 2]. The saliva primarily as a digestive fluid composed of salts, amylase, mucin and protective proteins, serves another purpose of protecting both hard and soft tissues too. In 1960s and 1970s secretory IgA and non-immunological antibacterial systems and the proteins responsible for the regulation of calcium and phosphate levels dominated the research field [3]. Formation and role of the salivary pellicle's action with bacterial adherence and agglutination provided a clinical interest. The secretory process on a molecular level was redefined by morphologists and physiologists. The 1980s defined the structure and function of saliva, both in terms of synthesis and release of the secretory products and their specific roles in the oral cavity in health and disease. 1990s took a great turn towards genetic control of processes and products along with understanding more about its mechanisms.

1960s mark the beginning of saliva diagnostics when salivary calcium level was found to be elevated in cystic fibrosis patients [4] and since then the field has great successes with sensitive detection techniques. Saliva serves as a diagnostic fluid that can be used as an alternative to traditional blood and urine examination. Sampling methods are less invasive, simpler, safer, less stressful, repeatable and don't require special training and equipment. Collection, storing and preparations of sample of saliva could vary according to the components that needed to be analysed. Various techniques of sample collection like spitting method, spontaneous dribbling of saliva and the use of absorbent tissues as an indirect method are comfortable for the subjects as well as the clinician [5].

In dentistry, salivary biomarkers help in accurate evaluation of early onset of periodontal disease condition [6]. Biomarkers like the microbiological species, electrolytes in saliva, antibodies, inflammatory mediators, glucosyl transferase and physical characteristics of saliva help in the detection of early childhood caries [7].

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In clinical practice utilisation of these biomarkers might help the clinicians in improving the oral health. Few clinical chair side tests are in use for more precise molecular diagnostics and treatments these days. Hence, salivary diagnostics is important for disease diagnosis and clinical monitoring [8]. In case of any systemic disease saliva can help in diagnosis and monitoring as it contains more than 2000 proteins out of which 26% of these are found in blood. It has helped clinician into detection of cancer, heart and infectious diseases. Salivary analysis can be done for the diagnosis of the various conditions like:

1. Hereditary disease
2. Autoimmune disease
3. Malignancy
4. Infection
5. Monitoring of levels of hormones
6. Monitoring of levels of drugs
7. Bone turnover marker in saliva
8. Forensic Evidence
9. Oral diseases
10. Diagnosis of Oral Disease with Relevance for Systemic Diseases.

About 3000 differentially expressed proteins and peptides have been analysed in the human salivary proteome and characterised [9]. Only aim of the proteomic analysis is to distinguish between physiological and pathological conditions. It is possible to distinguish two types of proteomic platforms: top-down proteomics investigates intact naturally-occurring structure of a protein; bottom-up proteomics analyses peptide fragments after pre-digestion (typically with trypsin). Many different biomarkers may be proposed for the same pathology due to heterogeneity. The salivary proteome has been characterised in several diseases like in oral squamous cell carcinoma and oral leukoplakia, chronic graft-versus-host disease Sjögren's syndrome and other autoimmune disorders such as SAPHO, schizophrenia and bipolar disorder, and genetic diseases like Down's syndrome and Wilson disease [10, 11].

Salivary biomarkers have potential to facilitate breakthroughs in epidemiologic studies, management of emergency situations and detection and surveillance of diseases by health workers. Recently an increasing number of studies on salivary biomarkers have been published as a consequence of the impressive development of advanced technologies. There are several salivary biomarkers of cancer and other Ionizing Radiation-associated diseases have been identified, few salivary biomarkers of exposure and no biomarker of susceptibility or effects specific to IR have been reported so far [12]. Further studies are needed to fully assess the potential of saliva as a source of biomarkers in the radiation research field. Table 1 shows other relevant studies on salivary biomarkers.

Table 1: Studies on salivary biomarkers

S. no.	Year	Researcher	Study title	Conclusion
1	2015	Nedeljka Ivković *et al*. [13]	Biomarkers of Stress in Saliva	Chromogranin A (CgA) and α-amylase enzyme can be used as alternative indices of adrenergic activity during stress reactions, due to their stability in saliva and reliability of the obtained values.
2	2017	Rajesh Babu *et al*. [14]	Forensic application of saliva: Evaluation of association of gender with the refractive index of saliva among the young adults of Western India	There is a significant difference found between male and female salivary refractive index value.
3	2018	Y.L. XU *et al*. [15]	Discovery and identification of fatigue-related biomarkers in human saliva.	30 fatigue-related protein markers are identified from saliva. Fatigue assessment model for emergency physicians using salivary biomarkers are also established.
4	2018	Hajer Jasim *et al*. [16]	Saliva as a medium to detect and measure biomarkers related to pain	They were first to demonstrate a correlation between the Glutamate concentration in stimulated whole saliva and blood.
5	2019	Maxime François *et al*. [17]	Current State of Saliva Biomarkers for Aging and Alzheimer's Disease	Main findings of salivary biomarkers of aging and AD includes various proteins, metabolites, and alterations to DNA and miRNA.

Aim of the Study: To evaluate few physical properties, we decided to undertake a study with an aim to study the Raman Effect using spectrometer in saliva of systemically sound individuals and then ascertain the trends by close observation of the spectrum.

The objectives were:

1. To study the spectrum using Raman's Spectrometer **(Figure 3)** in the salivary matrix of 20 systemically sound individuals in the range of 20-30 years
2. To assess the spectrum pattern in females
3. To assess the spectrum pattern in males
4. To evaluate whether there exists any difference of spectra between the two genders.

MATERIALS AND METHOD

Around 20 individuals within the age range of 20-30 years, systemically sound and no history medication were a part of this study. Informed consent was taken after approval from the Institutional Ethical Committee clearance. Individual with infection of upper respiratory tract and tobacco users were not a part of the study. Care was taken that there was no lesion active or passive present in the oral cavity. About 5ml saliva was collected in a specialised disposable vial by spitting method only after rinsing the mouth clean of debris.

Figure 1: Speed Vacuum Concentrator for obtaining a clear supernatant of the saliva.

The obtained salivary vials were then centrifuged with the help of speed vacuum concentrator (figure 1) for 3 hours. The obtained supernatant was taken out with the help of Gilson's pipette in a separate vial. Normal saline was added to eliminate the stringiness of the matrix.

Using Raman's Spectrometer (585nm) the spectra was recorded by keeping the following parameters constant: Spectral centre- 1300nm, Integration- 5 seconds, Accumulation – 10 seconds Laser power- 30, Objective lens- 50x. (Figure 2)

And in order to assess the reliability of the readings each sample was assessed five times. Raw data was processed by keeping baseline and then filtered for noise reduction. It was normalized and interpreted with calibrated wavelength range of 400nm – 1800nm. This study was conducted in November, 2018.

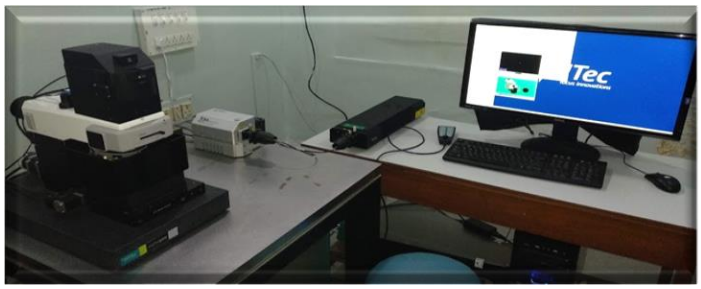


Figure 2: Raman's Spectrometer analysing the spectrum of salivary matrix

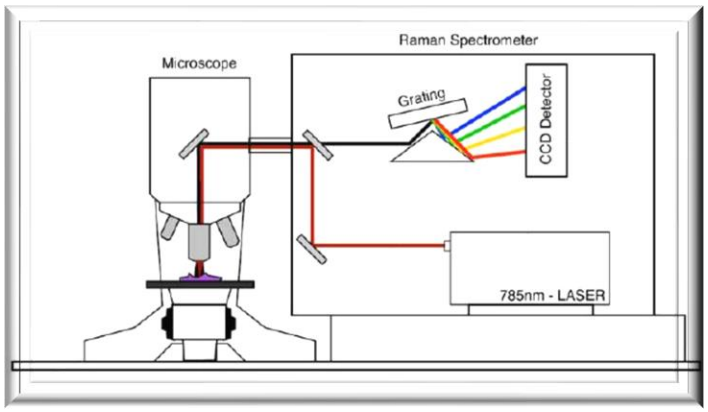
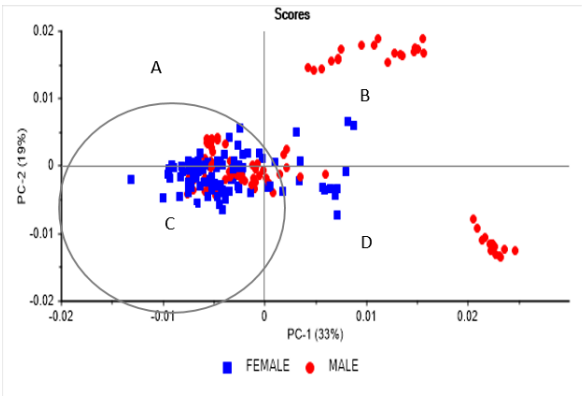
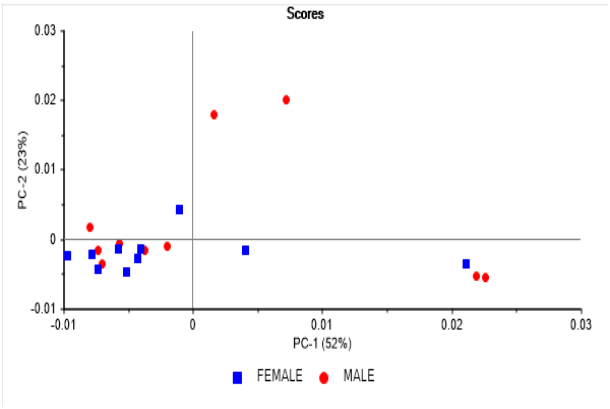


Figure 3: Schematic representation of Raman's Spectrometer. Image courtesy- researchgate.net

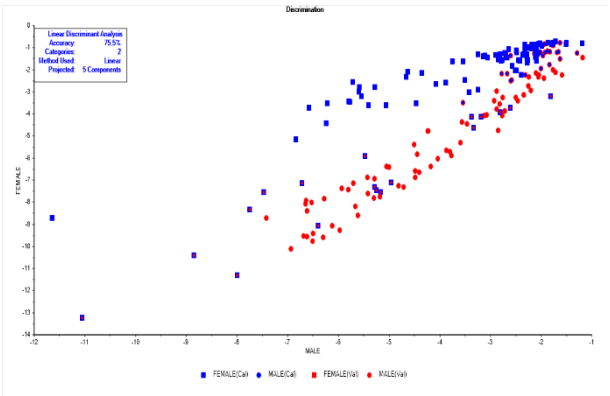
Analysis: PCA (Principal Component Analysis), LDA (Linear Discriminant Analysis) and Average Spectra were derived.



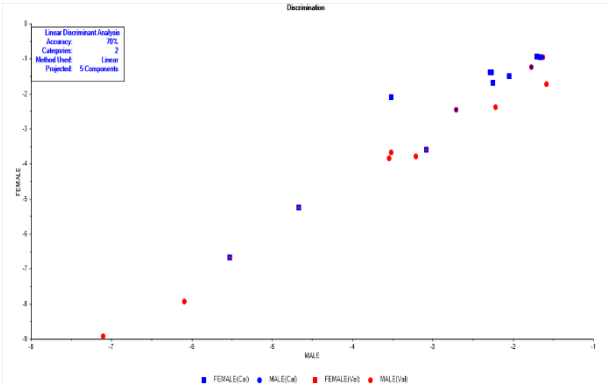
Graph 1: Showing PCA 1: Depiction of each sample assessed five times. i.e. 20 individual x 5times= 100 plots. The distribution of these samples appears within a fixed range. And those not within the range indicate presence of either impurity or burnt [laser] out samples.



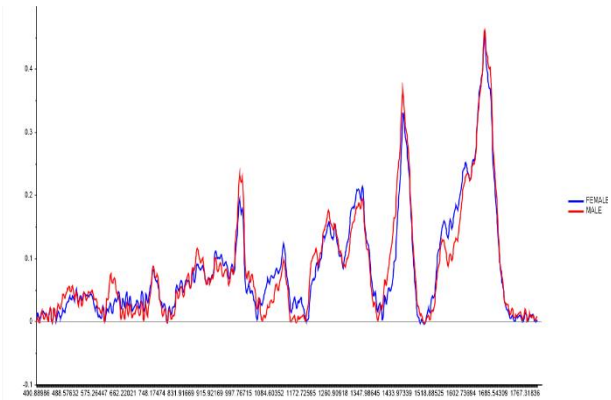
Graph 2: Showing PCA 2: The mean distribution of all the samples i.e. 20 plots.



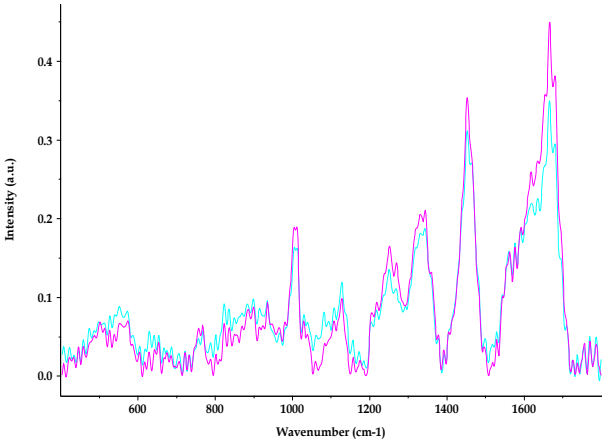
Graph 3: Showing Quadrant C of Graph 1 in Linear Discriminant Analysis (LDA), Refined and manageable distribution of data of all the samples assessed five times each.



Graph 4: Showing LDA of Quadrant C- Refined Mean distribution of all the samples.



Graph 5: Showing Spectrum of male and female [20 individuals x 5reading=100 plots] salivary matrix. The blue line indicates 10 male individuals in the study whereas the red on shows female readings



Graph 6: Showing Average Spectrum of male and female taken in the study.

RESULTS

PCA shows the distribution of samples within a fixed range and those which are not within the clusters were maybe due to impurity in the sample or any other physical defects. LDA showed more refined and manageable distribution of data. It was seen that a pattern formation in male and female group, can be distinguished clearly. The LDA accuracy of our study was above 70% which is good for future research purposes as anything above 60% can be considered as important breakthrough.

The Graphs of both the male and female were obtained separately, so a random spectrum of any gender can be ascertained and try to match it with both. Whichever graph comes near accurate to the random one will be our apparent match for that gender.

DISCUSSION

The secretion of saliva are influenced by various factors such as the nutritional intake, seasonal variation, circadian rhythm, BMI and others [18]. Prodan et. al [19] study shows the difference between components of salivary secretion of males and females. In that study it was mentioned about the total protein concentration, amylase, chitinase and secretory IgA quantity variation between male and female. It was suspected that these changes in composition of male and female saliva reflected in the value obtained. There are certain drawbacks regarding the use of saliva samples, an extensive number of samples is required to reach statistical significance.

Lednev [20] and his team analysed sixty samples of samples in their laboratory, thirty male and thirty female using a standard bench-top Raman spectrometer. They were looking for trends and characteristics that differentiated between multidimensional data between the male and the female samples. They reported details in the American Chemical Society journal, Analytical Chemistry. Team members and graduate students Claire Muro and Luciana de Souza Fernandes also used the same system to analyse other body fluids including peripheral blood and sweat as well as vaginal fluid and semen.

Takeda *et al*. [21] in 2009 used Nuclear Magnetic Resonance (NMR) Spectroscopy to determine differences between the urine and saliva samples of different donors based on the detection and comparison of different metabolites. Certain compounds like acetate, formate, glycine and pyruvate, were found in higher concentrations in male samples, showing difference between male and female bodily fluids.

In 2015, scientists at the University of Albany (Huynh *et al*.) developed a biocatalytic assay approach to analyse amino acids in fingerprints to determine the gender of the donor [22]. The study had an accuracy of 99%, with the gender differences believed to be due to the higher concentration of amino acids in fingerprints deposited by females.

In 2016, researchers at the University of Albany (Muro *et al*.) have highlighted the possibility of using portable Raman Spectroscopy to determine the gender of an individual based only on their saliva in real-time [23]. The study utilised a total of 48 saliva samples from both male and female donors of multiple ethnicities, depositing the samples onto aluminium foil and drying overnight. Samples were then subjected to Raman analysis and the chemical signatures scrutinised to determine whether or not the saliva of male donors differed from that of female donors.

CONCLUSION

There is a significant difference found between male and female saliva using Raman's Spectrometer. This will be attributed due to the presence of different protein concentrations, hormone levels, as well as IgA of males and females. Hand-held devices can be designed to distinguish the two genders. Thus can prove to be helpful in forensics and consequently help detection at the scene of crime. Since this study is purely done on the basis of assessment of Raman spectrometry, further studies are required to verify the particular components that are responsible for the change in Spectrum between male and female saliva.

Conflicts of Interest

The authors declare no conflict of interest.

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