RESEARCH
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IDENTIFICATION OF SALIVARY PROTEIN PROFILE FOR POTENTIAL MEDICAL APPLICATIONS

Running Title: Salivary Protein Profile for Prospective Medical Uses

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Abstract

Introduction: Saliva, a basic bodily fluid, encompasses a diverse array of proteins originating from various outlets such as salivary glands, bloodstream, and oral tissues. The protein makeup of saliva serves as valuable insight into an individual's well-being, aiding in the prompt identification, assessment, and tracking of numerous health conditions.

Aim: Leveraging the distinctive characteristics of salivary protein profiles to advance medical applications, spanning from diagnosis and monitoring to understanding the root causes of diseases.

Objective: Detecting and analyzing salivary protein patterns to uncover potential biomarkers for early detection and diagnosis.

Materials and methods: Saliva samples were collected through swabbing and drooling methods, followed by centrifugation at 16000 rpm for 15 minutes to separate any debris. The resulting clarified supernatant was preserved at -20°C until needed. Protein concentration or impurity removal prior to subsequent procedures like SDS-PAGE was frequently achieved through protein precipitation using trichloroacetic acid (TCA). Gel imaging, typically facilitated by a gel documentation device or scanner, was employed to record the positions and intensities of protein bands.

Result: Protein profiling and SDS-PAGE analysis reveal the presence of protein bands in both males and females, with higher protein concentration (mg/ml) observed in females compared to males.

Conclusion: The study successfully identified specific salivary proteins that could potentially function as biomarkers for targeted illnesses or conditions.

Keywords: Saliva, Human Salivary Proteome, Proteomics, Saliva Protein Profiling, Diagnosis, Biomarkers, Medical Applications.

Introduction:

In forensic investigations, identifying suspects is crucial, and DNA analysis is pivotal in this regard, aiding in victim and perpetrator identification. Collecting samples with sufficient intact DNA is imperative for obtaining a viable DNA profile, ensuring effective subsequent amplification and analysis (1). DNA traces found at crime scenes can undergo significant degradation due to factors like nucleases, metal presence, high temperatures, and irradiation, (2);(3) rendering the DNA profile unsuitable for forensic identification if not properly preserved. Saliva has historically been employed in forensic inquiries for DNA analysis and controlled substance detection. (4). While saliva is rich in proteins and some are more resistant to degradation compared to DNA, its additional potential applications in forensics remain largely unexplored (5);(6). Despite similar degradation rates, a significant portion of saliva remains preserved due to the abundance of salivary proteins, surpassing DNA (7);(8), thus supporting the potential use of protein profiling in forensic sciences.

Human saliva, a clear and slightly acidic (pH=6.0-7.0) substance, is produced by salivary glands and its composition is influenced by various factors such as sympathetic and parasympathetic stimulation, circadian rhythms, diet, health status, medications, among others (9);(10). The primary three pairs of salivary glands (parotid, submaxillary, and sublingual) along with minor salivary glands collectively generate 0.75 - 1.5 liters of saliva daily, peaking during the day and declining at night (11). Saliva typically contains 0.5 to 2 mg/mL of total protein content. Apart from maintaining oral cavity balance, saliva serves as an optimal medium for health surveillance (12). Saliva protein profiling finds applications in disease detection, forensic age and gender estimation, and ethnicity identification, with its composition being modulated by various conditions (13) ; (14). Compared to blood serum, amniotic fluid, cerebrospinal fluid, and bronchoalveolar lavage fluid, saliva offers noninvasiveness, cost-effectiveness, and clinical safety advantages. However, certain diseases and drug reactions may affect saliva production (15).

Exploring saliva as a diagnostic tool is warranted for several reasons. It meets the demand for an affordable, non-invasive, and user-friendly screening approach, reducing the discomfort associated with blood collection and privacy concerns linked to urine sampling (16). Saliva offers advantages in terms of collection, storage, transportation, and comprehensive sampling at a lower cost compared to serum or urine. Moreover, it is easier to manage during diagnostic procedures as it does not clot, thus streamlining the process (17). For healthcare providers, utilizing a salivary test reduces the risk of exposure to blood-borne pathogens. Patients benefit from the non-invasive collection method, which alleviates anxiety and discomfort, increasing their willingness to undergo health assessments. This heightened participation enhances the prospects of monitoring overall health and detecting illnesses at early stages (18).

Thorough exploration of the human saliva proteome offers a pathway to deepen our comprehension of pathophysiology and lay the groundwork for identifying potential disease biomarkers. With the subsequent identification of various clinically relevant biomarkers influenced by diseases, proteomics provides prospective benefits not typically offered by conventional diagnostic methods (19). Saliva proteome analysis shows significant promise as a diagnostic tool and aids in disease progression tracking and therapeutic monitoring (20). In recent years, numerous global research institutions have focused on studying proteins, particularly those sourced from saliva. Despite substantial interest in this domain, a notable number of healthcare professionals remain unaware of its methodologies and applications. Thus, the aim of this investigation is to identify and analyze patterns of salivary proteins to unveil potential biomarkers for early disease detection, diagnosis, or monitoring.

Materials and methods:

Sample collection:

Eight participants aged 21 to 30 were recruited from Chennai's Saveetha Dental College and Hospitals. Saliva samples were collected between 9 am and 12 pm using an enhanced unstimulated spitting method, surpassing standard protocols. Before collection, participants abstained from eating, drinking, smoking, or oral hygiene for at least 15 minutes. They rinsed their mouths with water, tilted their heads back for 60 seconds, and expelled 0.1-1 ml of saliva into sterile containers. Subsequently, samples were promptly chilled on ice and stored at -80°C for further examination.

Saliva sample processing for protein isolation and mass spectrometry:

All chemicals utilized in this study were of analytical grade or higher. Saliva samples underwent centrifugation at 16000 rpm for 15 minutes to precipitate any debris present in the saliva. The resulting clarified supernatant was then stored at -20°C until further analysis.

Trichloroacetic acid (TCA) precipitation of proteins:

TCA precipitation serves to concentrate protein samples and remove impurities like salts and detergents before proceeding to subsequent applications such as SDS-PAGE or 2D-gels. However, it's important to note that TCA precipitation denatures the protein and should not be used if the protein needs to remain in a folded state.

SDS-PAGE technique:

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) was utilized to evaluate saliva's protein composition. Each sample was loaded with 5-10µg of protein onto a 10% acrylamide gel, serving as a sieve for protein migration under an electric field. The gel, acting as a porous matrix, facilitated protein migration during electrophoresis. Subsequently, the gel was typically photographed using gel documentation equipment or a scanner to capture the protein bands' locations and intensities.

SDS-PAGE, a widely utilized technique, provides insights into a protein's molecular weight and quantity:

1. Bands refer to the dark horizontal "bars" representing stained proteins within the gel. As proteins migrate through the gel, they are separated based on molecular weight, with each band indicating proteins of specific molecular weights.
2. A "protein molecular weight marker" or "ladder" is typically included on one side of the gel for reference.
3. A dense, dark band indicates a protein of high abundance in the sample, while a faint, narrow band suggests the presence of only a trace amount of that protein.
4. Lanes featuring a single band may indicate the presence of a single protein in the sample, whereas lanes with multiple bands suggest the presence of multiple proteins.

The duration of this study was **3 months.**

Results:

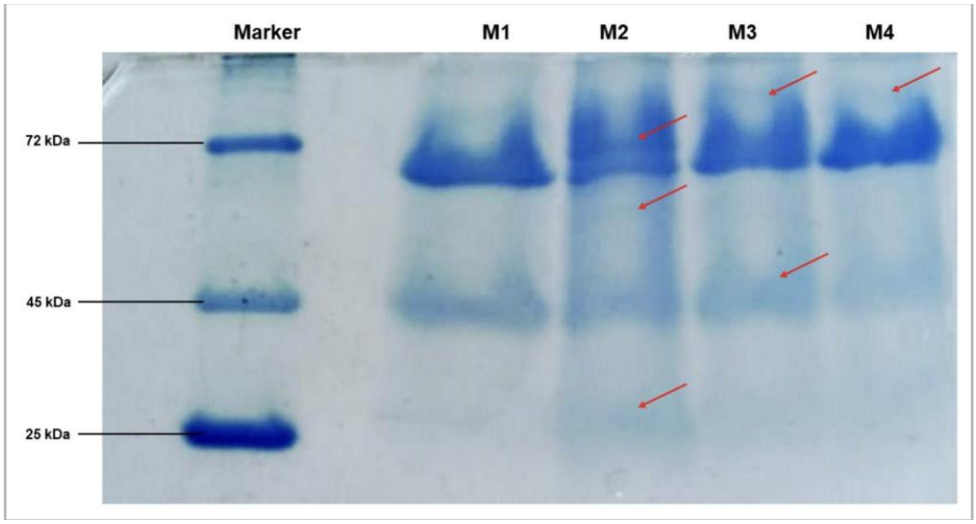

Fig 1. Protein profile pattern of Male saliva samples in One dimensional SDS-Polyacrylamide Gel Electrophoresis.

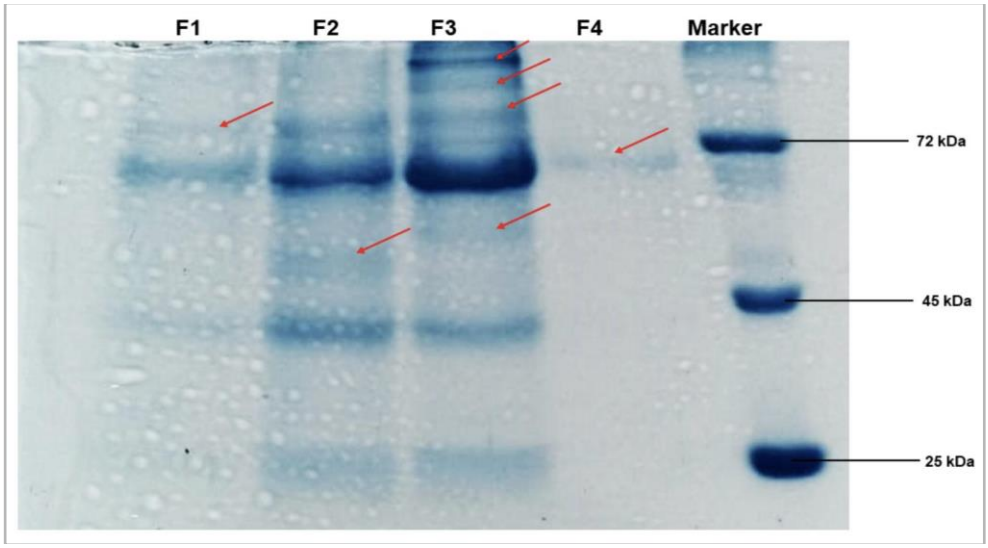

Fig 2. Protein profile pattern of Female saliva samples in One dimensional SDS-Polyacrylamide Gel Electrophoresis.

In the male samples, M4 presents a predominantly pure solution of the 75 kDa protein, while M3 displays two protein bands, one at 75 kDa and the other at 50 kDa. Sample M2 showcases three protein bands. Conversely, the female sample F3 exhibits the highest protein concentration across four bands at 60 kDa, 75 kDa, 80 kDa, and 85 kDa.

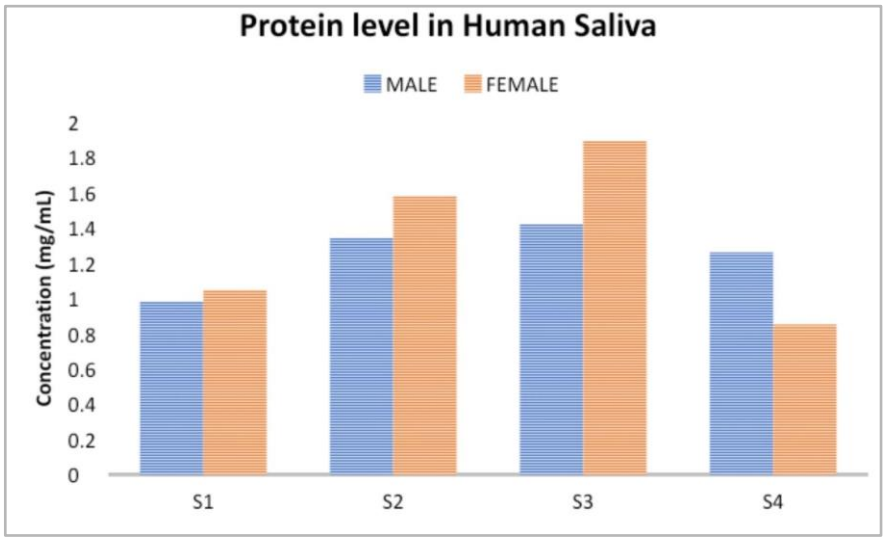

Fig 3. Protein content in human saliva samples.

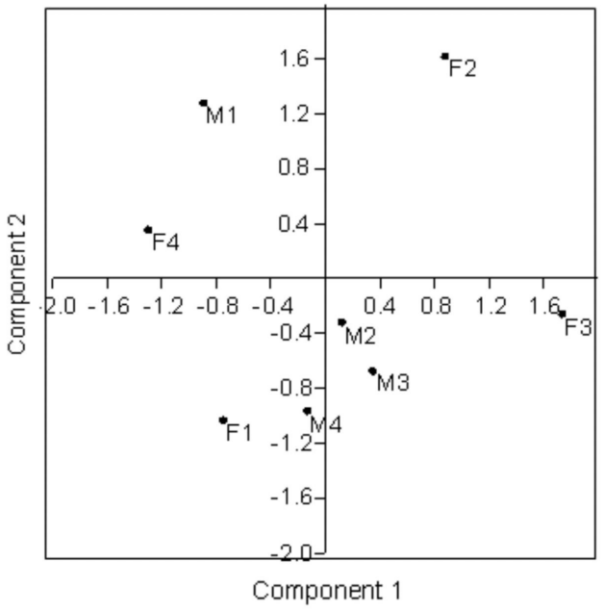

Fig 4. Principal Coordinate analysis of protein content among male and female saliva samples.

Discussion:

The results of salivary protein profiling reveal that females exhibit higher protein concentrations (mg/ml) compared to males. Using the SDS-PAGE method to assess protein size and quantity, it was observed that M4 from the male sample constitutes a relatively pure solution of the 75 kDa protein, while M3 contains both a 75 kDa and a 50 kDa protein. In contrast, F3 from the female sample exhibited the highest protein content across bands at 60 kDa, 75 kDa, 80 kDa, and 85 kDa, represented by four distinct bands.

Saliva serves as a valuable diagnostic tool due to its simplicity, safety, cost-effectiveness, and non-invasiveness, offering significant translational and clinical application potential (21). Zhang A's study aimed to streamline proteomic methods for comprehensive saliva protein analysis and understand saliva proteomics' role in biomarker development (22). Human saliva harbors clinically significant proteins, with approximately 30% of blood proteins also detectable in saliva (23). A multitude of medically relevant analytes in saliva are progressively emerging, including biomarkers for diverse ailments such as cancer, autoimmune disorders, viral and bacterial infections, cardiovascular conditions, and HIV (24) ; (25). While whole saliva aids in diagnosing diverse illnesses, gland-specific saliva is beneficial for evaluating major salivary gland pathology. Salivary protein profiling in disease individuals yields vital clinical insights for diagnosis and prognosis (26).

Tumors often remain undetected until they advance, attributed to vague symptoms and complex diagnostics, necessitating invasive measures as they progress. Early molecular diagnosis is imperative for improving survival rates. (27). T. Jarai et al. aimed to devise a rapid, sensitive mass spectrometric method for identifying differentially expressed proteins as tumor-specific biomarkers in Head and Neck Squamous Cell Carcinoma (HNSCC) patients' saliva. (27,28). Whole saliva samples from HNSCC patients and healthy individuals were analyzed using SDS-PAGE, MALDI TOF/TOF mass spectrometry, and the Mascot database. Annexin A1, beta- and gamma-actins, cytokeratin 4 and 13, zinc finger proteins, and P53 pathway proteins were uncovered as potential indicators, crucial in tumor formation and novel to saliva detection. Salivary proteomics presents a non-invasive approach for cancer detection and therapy monitoring, aiding in early-stage tumor identification with reproducibility.

Discovering biomarkers for noninvasive prediabetes/diabetes detection aids in preventing or delaying progression to diabetes and its consequences. This study aimed to characterize the human salivary proteome in type 2 diabetes to identify potential biomarkers (29). Whole saliva from control and type 2 diabetes individuals was analyzed using 2D-LC-MS/MS, revealing 487 novel proteins. Sixty-five proteins exhibited over a 2-fold difference in abundance between control and type 2 diabetes samples, primarily associated with metabolic and immunological response pathways. This comprehensive investigation provides insight into disrupted pathways in diabetic saliva, laying the groundwork for novel noninvasive diabetes screening and monitoring techniques.

Sjögren's syndrome (SS), a chronic autoimmune disorder characterized by dry mouth and dry eyes, lacks early diagnostic indicators, often leading to delayed diagnosis (30). Salivary proteomics has emerged as a promising avenue for identifying disease biomarkers to enhance SS diagnosis. Through 2DE and MALDI-TOF-MS analysis, 28 significantly different protein sites were identified in SS samples. Notable alterations in α-amylases precursor, carbonic anhydrase VI, β-2 microglobulin, glyceraldehydes-3-phosphate dehydrogenase, epidermal fatty acid binding protein, and immunoglobulin k light chain were observed (31). These unique saliva protein biomarkers hold potential for a simple, precise, and non-invasive SS diagnosis tool.

Clinical proteomics aims to elucidate connections between crucial molecular physiological agents, proteins, and distinct physiopathological conditions. Integrating proteomic findings into saliva diagnostics will link molecular analytes to treatment monitoring, therapeutic efficacy, and disease progression tracking. Future progress in salivary diagnostics could revolutionize screening, risk evaluation, and therapy oversight for various health ailments, enabling more personalized interventions prior to disease advancement.

Conclusion:

The objective of the study was to uncover salivary protein patterns with potential medical applications. Through comprehensive procedures including sample collection, processing, protein separation, identification, and data analysis, the research successfully pinpointed specific salivary proteins that could potentially act as biomarkers for targeted diseases or conditions of interest.

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Ethical clearance:

Conflict of interest: All the authors declare that there was no conflict of interest in the present study

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