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Oral Metabolic Biomarker Analysis for Improved Dental Caries Risk Assessment

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Abstract

Dental caries is the most prevalent chronic disease in the world. The presence or absence, the level of expression, as well as the post-translational modification of multiple peptide biomarkers in saliva, are theoretically altered by diseases or interventions. **Objective:** This study aimed to define and discover caries-associated saliva native biomarkers using new mass spectrometric techniques. **Methods:** Saliva samples were collected from caries-active or caries-free subjects. Saliva peptidome was extracted with ethanol and then was desalted with Oasis HLB sample extraction SPE cartridges (Waters). Dental caries-associated native peptide biomarkers in saliva were identified and characterized by Waters Acquity UPLC M-Class System with Xevo G2-XS Q-Tof mass spectrometry with alternative scanning LC-MS (LC-MSe), allowing for normalized label-free quantitation. **Results:** Six peptides were identified to be different in saliva when comparing caries-active and caries-free groups. Among them, five peptides were higher in expression level, while another one was lower in the caries-free group compared with the caries-active one. Five native peptides of higher expression in the caries-free group were identified as novel proline-rich antimicrobial peptides (PrAMPs). These novel and native PrAMPs have variant and complementary properties (multi-charged, highly basic or acidic, and hydrophobic or hydrophilic) of a perfect AMP and they could be potential therapeutic agents against multidrug-resistant pathogenic bacteria as well as dental caries biomarkers. **Conclusions:** This study confirmed that UPLC Q-TOF MS-based

peptidomics is an effective method for screening distinctive peptides from the saliva of adult patients with dental caries. Clinical Significance: These novel PrAMPs may be important biomarker candidates, which has key implications in preventive, diagnostic, and therapeutic modalities for dental caries.

Keywords: saliva, mass spectrometry, peptidomics, caries risk assessment, metabolic biomarker, dental caries.

Introduction

Dental caries continues to be a major threat to warfighter dental health and readiness. Dental caries can lead to severe oral pain and dysfunction and interfere with a service member's ability to eat, communicate, sleep, and perform assigned duties, both in deployed settings and in garrison. The current dental care efforts include clinical and radiographic exam. They are effective in detecting evidence of past or current disease or verifying periodontal health, but provide only limited information about patients and sites at risk for future breakdown. CAMBRA (Caries Management by Risk Assessment) is a commonly used screening method for assessing future caries risk, but it is mainly subjective and speculative without definitive proof. The disease remains highly prevalent among military recruits and remains difficult to prevent. (Leiendecker, Martin, & Moss, 2011).

Moreover, dental caries is a primary contributor to anatomic space infections of endodontic origin, which ranked as the second leading cause of high-severity dental emergencies (behind fractured teeth) during Operation Enduring Freedom in Afghanistan from 2011 to 2012 (Wojcik et al., 2015). Importantly, this sequela carries the high potential for medical evacuation from an air, land, or sea operational environment, necessitating undue safety risk, cost, logistical planning, and impacts to the mission.

A recent study found that 91 percent of the U.S. adults aged 20-64 had a history of dental caries in permanent teeth (Dye, Thornton-Evans, Li, & Iafolla, 2015). The cause of the disease is multifactorial. However, the primary etiology for dental caries is often linked with dental plaque, wherein certain acid-producing, oral bacteria flourish and can readily dissolve enamel, dentin, or cementum, leading to the chronic and progressive

destruction of dental hard tissue (caries) in a susceptible host. It is also known that the whole saliva represents a complex balance of factors derived from local and systemic sources (Sugimoto, Wong, Hirayama, Soga, & Tomita, 2010). Therefore, we can predict that plaque and saliva collection can potentially aid in early diagnosis, caries risk assessment, and evaluation of preventive treatment outcomes.

To understand the cariogenic potential of the microbial community at different stages of the dental caries process, it is important to learn about the activities of peptides, proteins, and metabolites relevant to the bacteria environment (Lorenzo-Pouso et al., 2018; Nyvad, Crielaard, Mira, Takahashi, & Beighton, 2013). Differential changes and thus biomarkers discovery at peptidome, proteome and metabolome levels would imply the defining biochemical and ecological processes in the dental host and microbial community for dental caries etiology.

Metabolomic or metabolic profiling is an emerging discipline that investigates all the metabolites and their metabolic pathways in a given biological system. Many proteins in the 120-159 kDa molecular weight range in saliva (Castro, Tovar, & Jaramillo, 2006) function during the adhering and aggregating of cariogenic bacteria into a plaque biofilm. However, other proteins in saliva, for examples defensins and the cathelicidins, may also contribute to the defense against microorganisms (Dale, & Fredericks, 2004).

Transcriptional responses to dental caries results in functional changes to the proteome implicated in response to the stimulus. Describing and quantifying proteome/peptidome-wide changes in abundance is crucial towards understanding biological phenomenon more holistically, at the level of the entire system. To date, there is no comprehensive evidence showing that salivary proteins/peptides could serve as potential indicators for the early diagnosis of the risk factors causing dental caries. Therefore, proteomics/peptidomics indicate the promising direction of future investigations of such factors, including diagnosis and thus prevention in dental therapy. Recent advances in mass spectrometric technology have led to a new era in metabolic and peptide biomarker discovery at all levels, from small to large biomolecules, that will potentially have a huge impact on future disease diagnosis, monitoring, and risk assessment.

The presence or absence, the level of expression, as well as the posttranslational modification of multiple biomarkers in saliva and dental plaque, are theoretically altered by diseases or interventions that can be detected with modern mass spectrometry (MS) (Al-Tarawneh, Border, Dibble, & Bencharit, 2011). The aim of this study was to define and discover caries-associated biomarkers by using the technology of mass spectrometry. The null hypothesis was that saliva samples among caries-active and caries-free population do not have significant biomarkers to differentiate them.

Materials and Methods

The Institutional Review Board at Wilford Hall Ambulatory Surgical Center, Joint Base San Antonio, Lackland, TX, USA approved this protocol (#FWH20200034H).

Sample collection

The participants in the biomarker portion of the study were selected from the following criteria: patients meeting the following inclusion criteria included 18-60 year old subjects in good physical health, who have been diagnosed by their primary dental provider as 1) having active dental caries (30 subjects) or 2) being caries-free with low risk for dental caries (30 subjects, control group). Excluded subjects were patients who were pregnant, patients who have periodontitis, and patients with a history of chemotherapy, radiotherapy, immunotherapy, or surgery. Participants were recruited through the United States Air Force (USAF) annual dental examination, which is a critical component of preventive health care and is considered as part of the standard of care in the Department of Defense health care system.

Once the examining dentist, Assistant Investigator (AI), categorized the eligible participants into the appropriate caries-risk level, the subject was informed about the research effort and given an option to participate. Upon the subject's consensus, the AI captured the written Informed Consent and HIPAA forms from the subject at this time. The subject had the opportunity to ask questions about the study, then the subject was scheduled for the sample collection with Primary Investigator (PI). The subjects were also screened based on their dental record and on their recent annual dental exam. Once screened, subjects were contacted via phone using a pre-generated script. The subject

was given an opportunity to reconsider the participation until the sample collection appointment. At the subsequent appointment, the PI confirmed the study criteria once again and began sample collection. A participant could voluntarily withdraw from the study at any time.

Unstimulated saliva and dental plaque were collected. At the sample collection site, subjects were instructed to spit 2 mL of unstimulated saliva into 10 mL Falcon tubes, which were placed in a Styrofoam cooler. After the saliva collection, dental plaque was collected, pooling plaque from the facial surfaces of the maxillary right and left dentition (#2, #3, #4, #5, #6, #11, #12, #13, #14, and #15 according to the universal nomenclature system) using sterile toothpicks. The pooling allowed enough material (250 µg proteins) for mass spectrometry and to get a representative sample from all chosen teeth, given that microbial composition may change depending on sampling site. One toothpick of plaque samples (proteomics samples) was placed in a 1.5 ml cryovial on ice, containing 500 µL of PBS (phosphate buffer saline, pH 7.4), and the other toothpick (metabolomics sample) in an empty cryovial on ice. All samples were stored at -80 °C until testing. A total of 60 subjects were recruited, and they were categorized into two groups (30 subjects per group): one, the experimental group was those with active dental caries, and two, the control group was those who are caries-free and at low risk for dental caries.

Sample preparation

A saliva sample (1.2 mL) was aliquoted and centrifuged at 18,000×*g* for 20 min at 4°C. A saliva supernatant (1.0 mL) was pipetted into new 5mL tube. Freeze-cold ethanol (2.0 mL) with 1% formic acid was added to the saliva supernatant, immediately vortexed, incubated on ice for 20 min, and vortexed again. Finally, the sample was centrifuged at 18,000×*g* for 20 min at 4°C. Supernatant (2.7 mL) for each sample was aliquoted and dried by a Speed Vac concentrator. Oasis HLB extraction cartridges were activated with 1 mL acetonitrile and then 1 mL 50% acetonitrile / 0.1% formic acid in water. After equilibration with 2 x 1 mL 0.1% formic acid in water, Oasis HLB extraction cartridges were ready for use. Speed Vac dried samples were reconstituted with 1 mL 3% acetonitrile / 0.1% formic acid and centrifuged at 18,000×*g* for 5 min at 4°C. Supernatants were then loaded onto the ready-to-use HLB cartridges for desalting and purification.

Sample-loaded cartridges were washed with 3 x 1 mL 3% acetonitrile / 0.1% formic acid. Peptides were eluted from the cartridges with 3x 220 µL 50% acetonitrile / 0.1% formic acid. Peptides eluate was dried using a Speed Vac concentrator and then reconstituted in 50 µL 3% acetonitrile / 1% formic acid for LC-MS analysis.

LC-MS characterization

The samples from the experimental and control groups were evaluated and compared with one another. LC-MS analysis for peptidome samples were performed in Ultra Performance Liquid Chromatography (UPLC) Quadrupole Time-of-Flight (Q-Tof) MS system. The differential peptidome among the two groups were analyzed by UPLC Q-Tof MS from which the sequence of these peptidomes were confirmed by LC-MS^E. For every LC-MS^E experimental run, all ions were streamed through a collisional cell, inside which a dual scan mode was activated that alternated between low and high energies. For the low energy scan, minimal collision energy (< 5 eV) was applied, and ions were minimally fragmented. The data for the low-energy, collision-induced dissociation provided mass quantification of the non-fragmented "precursor" ion. For the high energy scan, ions underwent fragmentation, after which the fragments were analyzed in the Q-ToF MS. The high energy data provided information about the ion fragmentations and their masses for identification. A search of the peptide spectra in the SwissProt database using a Progenesis QIP search engine was conducted to identify known proteins. Comparison of the correlation variance and fold change in abundance were performed between the two groups.

Results

Six peptides were identified to be different in saliva when comparing caries-active and caries-free groups. Among them, five peptides were higher in expression level in caries-free group, while another one was lower in caries-free group compared with caries-active. Five native peptides of higher expression in in the caries-free group were identified as experimental m/z values: 912.14, 1014.50, 978.50, 658.36, and 1009.01 respectively. MSe spectra of these dental caries biomarkers were shown in Figures 1-5. Most, if not all, b ions, y ions and associated derivative ions were determined manually in the MSe

spectra. These data combined with target MS/MS spectra and data-dependent acquisition data were used to decipher their full sequences by de novo peptide sequencing technology. That is, peptide sequencing were performed based on their MS/MS spectra without prior knowledge of the amino acid sequence. Their full sequences were successfully identified and results of their sequences (redacted due to potential intellectual property and pending patent submissions) demonstrated that they are novel PrAMPs. These novel and native PrAMPs have all the properties (multi-charged, highly basic or acidic, and hydrophobic or hydrophilic) of a perfect AMP and they could be potential therapeutic agents against multidrug-resistant bacteria as well as dental caries biomarkers.

Discussion

Discovery of the six peptides between caries-active and caries-free population rejects the null hypothesis. Abundance-changed biomarkers discovery at the peptidome level would imply the biochemical and ecological processes in the dental host and microbial community. Here five native proline-rich peptides in saliva were identified as novel peptides biomarkers associated with dental caries. These discovered novel proline-rich (more than 50% proline composition) antimicrobial peptides (PrAMPs) were found higher in expression level in the caries-free group compared with caries-active. PrAMPs have attracted particular attention due to unique mechanism of killing bacteria without cell membrane disruption. PrAMPs represent an avenue in antibiotic development to counter the rapid increase in multidrug-resistant pathogenic bacteria. Some PrAMPs interact with ribosomes to inhibit translation. The binding of PrAMPs blocks and destabilizes the initiation complex of bacterial protein synthesis, thus preventing entry into the elongation phase (Seefeldt *et al*., 2015). Our findings provide a basis for the future development of this class of potent antimicrobial agents.

PrAMPs show a variety of modes of actions, including a bacteria-killing mechanism shift at high concentration, non-lytic mechanisms, as well as possessing different intracellular targets and lipopolysaccharide binding activity. PrAMPs display the ability to not only modulate the immune system via cytokine activity or angiogenesis but also

possess properties of penetrating cell membranes and crossing the blood brain barrier, suggesting a role as potential novel carriers.

The analysis of saliva samples at the proteomics and metabolomics level has the potential to discover additional biomarkers with further studies. Also, LC-MS characterization of the collected plaque samples in this study will be used to verify the findings of saliva samples. Based on previously published findings (Fine, 2015; Martínez-Gomis *et al*., 1999; Sinha *et al*., 2013; Sharma *et al*., 2017; Lorenzo-Pouso *et al*., 2018), the potential use of lactoferrin and lactoferrin-derived AMPs in vitro candidates can be investigated to verify the feasibility of using these AMPs as in vivo indicators of an individual patient's caries risk.

Dental caries disease poses threats to combat readiness of our fighting forces during military operations. As a diagnostic application of these discoveries, construction of a proof-of-concept, fluorescence-based lateral flow assay (LFA) platform will be performed for the detection and quantification of oral disease biomarkers (such as those associated with dental caries and periodontitis) in saliva. Custom antibodies against the biomarkers discovered in this study will be procured from a commercial source. The resulting identities of the novel peptides will be further evaluated using custom antibodies which can be used to produce an LFA to test for the presence of the novel peptides in saliva samples. This LFA platform can be used as a point-of-care diagnostic tool to quantify oral disease biomarkers. The platform may enable dentists and clinicians to better assess oral health, predict the progression of oral diseases, and guide treatment decisions.

Conclusion

This study demonstrated that mass spectrometry-based peptidomics is an effective method for screening distinctive peptide biomarkers as well as discovering novel natitve AMPs in dental caries. These discovered novel PrAMPs could have the therapeutic potential in a number of complex processes such as inflammation, wound repair, ischemia-reperfusion injury, and angiogenesis. Moreover, they may be important biomarker candidates, which may have key implications in preventive, diagnostic, and therapeutic modalities for dental caries. Results of this study may improve caries-risk

assessment, preventive management and effective and sustainable treatment for dental caries, and therefore upgrade the oral health and readiness of our Active Duty members.

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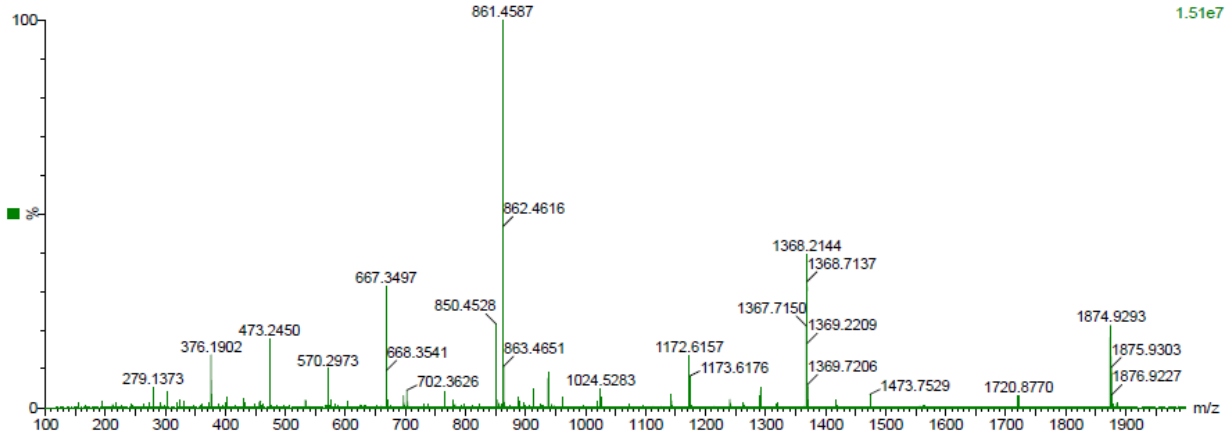

Figure 1. MSe spectra of experimental m/z value 912.14 peptide found higher in expression level in caries-free group compared with caries-active one.

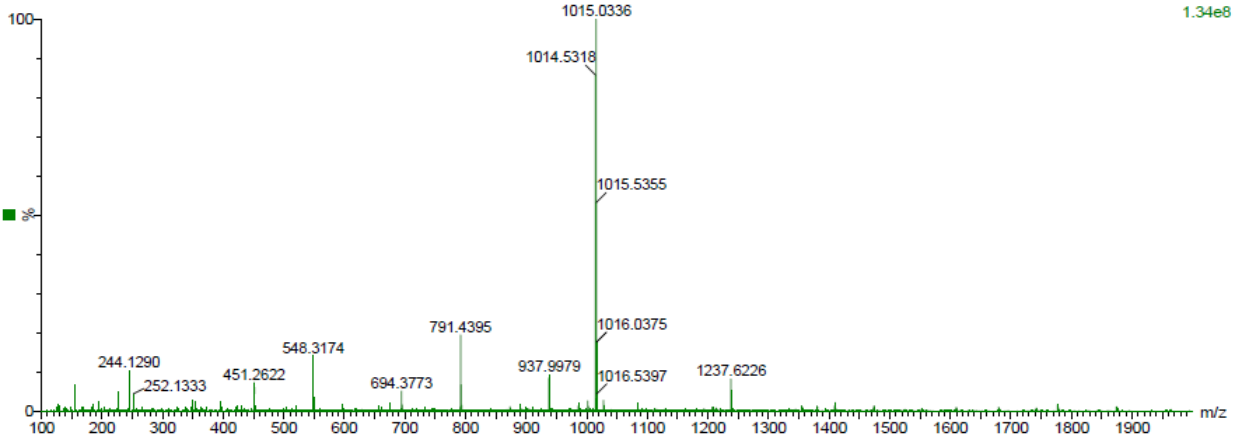

Figure 2. MSe spectra of experimental m/z value 1014.50 peptide found higher in expression level in caries-free group compared with caries-active one.

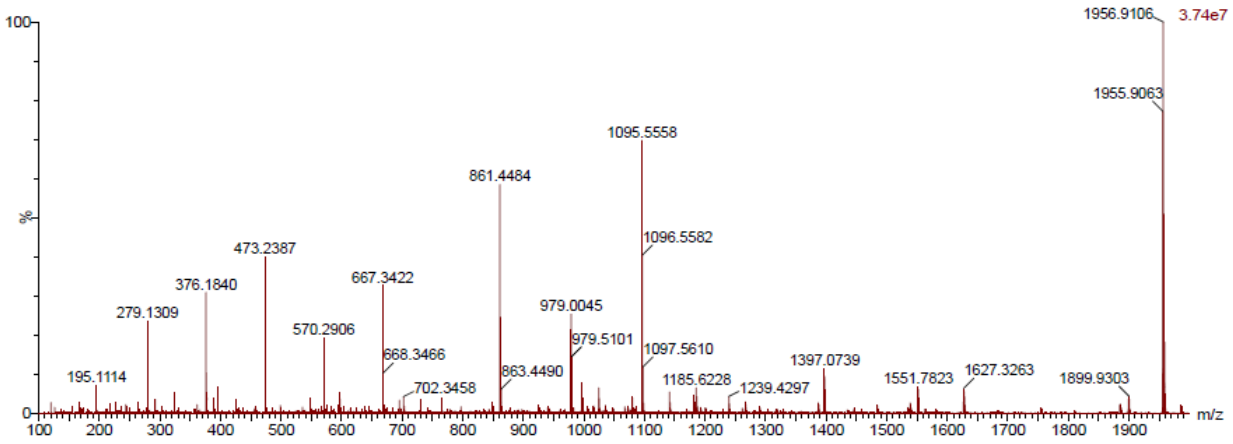

Figure 3. MSe spectra of experimental m/z value 978.50 peptide found higher in expression level in caries-free group compared with caries-active one.

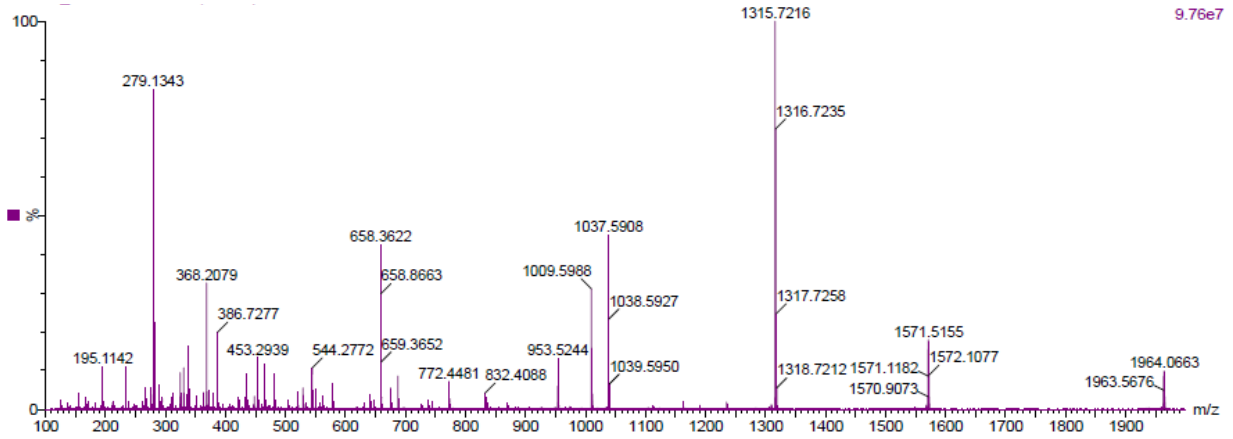

Figure 4. MSe spectra of experimental m/z value 658.36 peptide found higher in expression level in caries-free group compared with caries-active one.

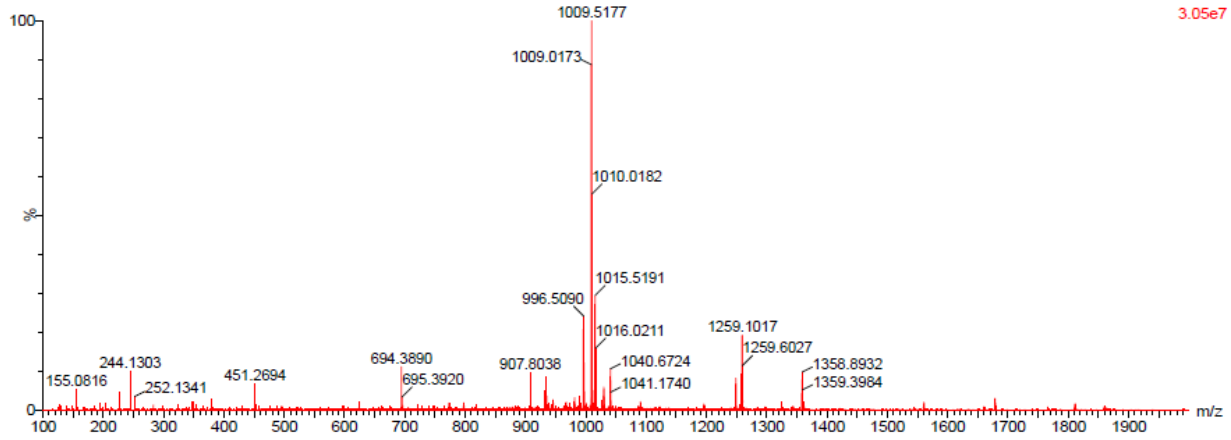

Figure 5. MSe spectra of experimental m/z value 1009.01 peptide found higher in expression level in caries-free group compared with caries-active one.