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Collecting Saliva and Measuring Salivary Cortisol and Alpha-amylase in Frail Community Residing Older Adults via Family Caregivers

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Abstract

Salivary measures have emerged in bio-behavioral research that are easy-to-collect, minimally invasive, and relatively inexpensive biologic markers of stress. This article we present the steps for collection and analysis of two salivary assays in research with frail, community residing older adults-salivary cortisol and salivary alpha amylase. The field of salivary bioscience is rapidly advancing and the purpose of this presentation is to provide an update on the developments for investigators interested in integrating these measures into research on aging. Strategies are presented for instructing family caregivers in collecting saliva in the home, and for conducting laboratory analyses of salivary analytes that have demonstrated feasibility, high compliance, and yield quality specimens. The protocol for sample collection includes: (1) consistent use of collection materials; (2) standardized methods that promote adherence and minimize subject burden; and (3) procedures for controlling certain confounding agents. We also provide strategies for laboratory analyses include: (1) saliva handling and processing; (2) salivary cortisol and salivary alpha amylase assay procedures; and (3) analytic considerations.

Video Link

The video component of this article can be found at http://www.jove.com/video/50815/

Introduction

The theoretical perspective guiding lifespan developmental science emphasizes the need to integrate biological, behavioral, social, and contextual factors into the study of health and behavior[1]. Contemporary theoretical models champion the notion that individual differences in biological sensitivity and susceptibility to contextual factors may moderate risk versus resilience, and can forecast morbidity and mortality in vulnerable populations. Considerable research attention has focused on these issues early in life development[1], but only recently have investigators been able to test the core ideas in studies with medically and cognitively frail older adults[2-4]. One reason for this trend is the development of procedures to noninvasively measure differences in neurobiological activity including the hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system (ANS) within and across individuals via oral fluids (saliva)[5,6].

Salivary measures can allow for the collection of normative longitudinal data, to compare changes between different treatment groups, to compare effectiveness of interventions, or can be extended to include measurement of coregulation or activity between multiple individuals. The data obtained from these measures can shed light on stress-linked biological processes in health conditions common in older adults including cancer, heart disease or neurocognitive disorders[7]. Saliva has received growing attention as a biospecimen due to the perceptions of sample collection as an easy to learn, inexpensive, minimally invasive, and acceptable[8]. Nonetheless, the conventional methods of collection requiring participants to drool through a straw into a small tube have been shown to be too complicated or undesirable for older, frail participants. New methods are required that can standardize the preanalytical phase of collection and processing to deliver the most accurate and meaningful results. The field of salivary bioscience is advancing rapidly and the purpose of this presentation is to provide an update of developments and a practical roadmap for investigators interested in integrating these measurement approaches into research on aging. In this protocol we demonstrate (1) procedures for collection of salivary samples in cognitive impaired older adults by family caregivers in the home setting, (2) the procedures for measuring HPA axis and ANS activity via salivary cortisol and salivary alpha amylase (sAA), and (3) representative individual differences in salivary cortisol and sAA data.

Protocol

1. Recruitment

1. Obtain appropriate Institutional Review Board approval of collection protocol with special attention to consenting subjects with impaired capacity and the collection of biological specimens[9-10].
2. Exclude participants with potential for blood leakage into saliva by assessing oral health (*i.e.* "Did you observe bleeding gums in the past two days?")[11].

2. Preparation of Saliva Collection Supplies

1. Prior to scheduling home visit to consented participants, gather and assemble necessary supplies: 4 Salimetrics Children's Swabs (SCS) , 4 swab storage tubes, 4 color-coded tube caps, 5 white labels, 1 plastic zip-lock bag, saliva collection instruction sheet, 5 nitrile gloves, and storage box.
2. Apply nitrile glove. Using gloved hand, place 4 SCS into 4 storage tubes and cap with yellow, orange, purple, and blue color caps, respectively.
3. Place label on storage tube to indicate time of collection (on awakening- yellow cap, 30 min post awakening-orange cap, mid day before lunch-blue cap, evening before dinner-purple cap).
4. Place the 4 capped storage tubes plus saliva collection instruction sheet and 4 nitrile gloves in zip-lock bag and label bag with subject ID and date of collection.
5. Schedule short home visit to subjects previously consented and on day prior to saliva sampling. On arrival to home provide plastic bag of supplies and instruction sheet.

3. Saliva Collection Instruction

1. Review instruction sheet (see **Figure** 1 for example). Emphasize the need to record start and stop time of saliva collection.
2. Record the name, dosage, and schedule of all prescription and OTC medications taken by the participant within the last 48 hr[12].
3. Instruct participant to tilt head forward to allow saliva to pool on the floor of mouth.
4. Instruct saliva collector (SC) to apply nitrile glove to dominant hand (only required for hand holding swab)[9].
5. Instruct SC to uncap the swab storage tube to retrieve oral swab. Do not remove the basket insert from the tube.
6. Instruct SC to place the oral swab under the front of the tongue of the older adult to absorb about 3 drops (75 µl) of pooled saliva, and to HOLD the oral swab in place for 2 min to ensure that it is saturated. Swab will become opaque when saturated.
7. Have the SC return the saturated oral swab back into the basket of the swab storage tube and replace the cap.
8. Have the SC immediately place tube in storage box and place in freezer. Discard glove in trash bin.
9. Have SC record start and stop times on saliva instruction sheet.

4. Provide Additional Instructions and Answer Questions

1. Instruct SC that they will be collecting saliva sample on four occasions the following day. The times of collection are on awakening, 30 min post awakening, mid morning, and evening[13,14].
2. Instruct SC to avoid sample collection within 1 hr after a major meal.
3. Have SC instruct patient to rinse mouth with water 10 min prior to saliva collection.
4. Discard samples visibly contaminated with blood.
5. Instruct SC that saliva samples should remain in freezer until retrieved by study team. Confirm that study team will schedule a time and day to retrieve the frozen samples and bring new saliva collection supplies for any future collection date.

5. Saliva Handling and Processing

1. Once samples are removed from subject's freezer, keep frozen during transport to lab using insulated freezer bag.
2. On arrival to lab, replace white label on tube with sequential barcode.
3. Confirm sequential barcode matches subject ID, date of collection, and time of collection.
4. Return saliva sample to freezer (-20 °C) to precipitate the mucins.

6. Optional: Salivary Cortisol Assay Protocol

1. Ensure all salivary assay kit reagents are at room temperature.
2. After thawing saliva samples, vortex and centrifuge at 1,500 x g for 15 min.
3. Prepare wash buffer following kit instructions.
4. Pipette 25 µl of standards and controls into appropriate wells of plate layout. Standards and samples should be assayed in duplicate.
5. Pipette 200 µl enzyme conjugate into wells.
6. Mix plate by placing on rotator for 5 min at 500 rpm (then incubate at room temperature for 55 min).
7. Wash and blot plate with wash buffer 4x.
8. Add 200 µl tetramethylbenzidine (TMB) solution .
9. Again mix the plate for 5 min at 500 rpm then incubate in the dark for an additional 25 min.

10. Add 50 µl stop solution and mix for 3 min at 500 rpm.
11. Within 10 min of adding stop solution wipe off plate bottom. Read with a plate reader at 450 nm.
12. Calculate cortisol concentration using the average optical density. Subtract the average optical density in nonspecific binding wells from the average optical density in zero, standard, control, and sample wells.
13. Estimate the concentrations of the controls and unknowns using software capable of logistics.

7. Optional: Salivary Alpha Amylase Assay Protocol

1. Set plate reader to incubate at 37 °C, and read in center measurement kinetic mode at 1 min and 2 min.
2. Use 405 nm filter and no reference filter.
3. Dilute saliva samples with the alpha-amylase diluent provided.
4. Prepare 1:200 dilution by first preparing a 1:10 dilution of the saliva by pipetting 10 µl of saliva into 90 µl alpha-amylase diluent. Then, further dilute by pipetting 10 µl of the 1:10 dilution into 190 µl alpha-amylase diluent (1:20).
5. Heat alpha-amylase substrate solution to 37 °C using a preheated microtiter plate incubator.
6. Add 8 µl of diluted saliva samples to individual wells.
7. Simultaneously add 320 µl of preheated (37 °C) alpha-amylase substrate solution.
8. For reading kinetically in 37 °C plate reader, immediately place plate in reader and start reader.
9. Subtract 1 min readings from the 3 min reading and multiply by the conversion factor.
10. Calculate amylase activity as ΔAbs./min x TV x DF /MMA x SV x LP = U/ml. Where ΔAbs./min = Absorbance difference per minute ; TV = Total assay volume (0.328 ml) ; DF = Dilution factor ;MMA = Millimolar absorptivity of 2-chloro-p-nitrophenol (12.9) ;SV = Sample volume (0.008 ml) ; LP = Light path = 0.97(specific to plate received with kit).

Representative Results

The protocol is meant to provide one example of how salivary biomeasures can be collected in the community setting. The procedures are designed to measure the diurnal profile of salivary alpha amylase and salivary cortisol concentrations (**Figure 2**).

Analytic Strategy

Cortisol results have been shown to be positively skewed. Therefore it is suggested to use log transformation to the data to yield a normal distribution. Several strategies are available to model differences in diurnal rhythms of cortisol and alpha amylase. The first strategy is to plot the awakening response (*i.e.* the diurnal change of alpha amylase or cortisol between waking and 30 min post-waking)[15]. The second strategy is to plot total cortisol or alpha amylase concentration over the day(area-under-the-curve or AUC) . The resulting summary parameters can be grouped into two general categories: 1) the measure of the magnitude of response or 2) the measures of the pattern of response over time. Growth curve modeling may be useful for studies in which multiple samples are collected in individuals across the day[16,17]. A growth curve can fitted to allow for individual differences in the diurnal cycle by adding predictors at the individual or measurement level[18].

Saliva Collection **Subject ID_____**

Cap Color	When to collect sample	Collection Start/ Stop Time		Comments
Yellow	 MORNING Upon awakening	____:____ ☐ a.m.	____:____ ☐ a.m.	
Orange	30 Minutes after awakening	____:____ ☐ a.m.	____:____ ☐ a.m.	
Blue	Mid Day	____:____ ☐ p.m.	____:____ ☐ p.m.	
Purple	 NIGHT Just before bed	____:____ ☐ p.m.	____:____ ☐ p.m.	

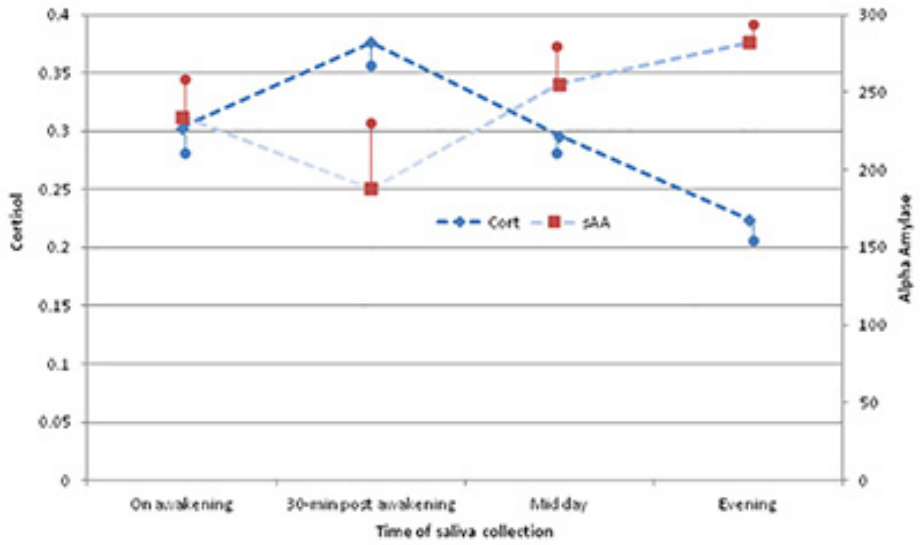
1) Remove color coded cap and retrieve swab.

2) Place end of swab under tongue for 2 minutes.

3) Place wet swab back into tube and reseal

4) Place in storage box in freezer.

Figure 1. Instructions for home sample salivary data collection. Illustration describing the schedule for collection of saliva by the family caregiver. Click here to view larger image.



*Note: Cortisol concentration in uL/dL. Alpha Amylase concentrations U/ml.
*Representative values reflect mean plus standard error of mean from 8 subjects.
*These data are intended to approximately represent the results that might be found
*using the protocol presented in this article.

Figure 2. Representative salivary cortisol and salivary alpha amylase data. Graphical representation of salivary cortisol and alpha amylase values and standard errors from 8 reflect subjects. These data are intended to represent the results that might be found using the protocol presented. Note: Cortisol concentration in µl/dl. Alpha Amylase concentrations U/ml. Click here to view larger image.

Discussion

To advance our understanding of the meaning of individual differences in biological sensitivity and susceptibility in frail older adults investigators will need to engage caregivers in the collection of biological specimens. These collection protocols need to be minimally invasive, easy to accomplish, and yield quality specimens. Since one major challenge is ensuring that caregivers collect the samples at the correct time family caregivers designated as saliva collectors should be screened for ability to understand written and oral instructions of the 8-step saliva collection procedure.

The knowledge gained from this endeavor is likely to be most informative when biological sensitivity and susceptibility are studied in the context of the daily lives. The protocol described herein is appropriately matched to vulnerable and under-represented community samples. The findings reveal that with minimal training caregivers of frail older adults were very capable of collecting repeated saliva samples of high quality. Few sample collections were missed due to caregiver mistakes, the volumes of the majority of sample gathered was sufficient for assay, and assays revealed the expected diurnal patterns of variation for salivary cortisol and alpha-amylase.

Nevertheless, our experience reveals that several steps are necessary when collecting saliva in these high-risk community samples to promote adherence, minimize participant and caregiver burden, and reduce attrition and missing data. We offer the following specific recommendations and suggestions for the next generation of studies. Saliva collectors must be educated on the value of the data collection effort and their contribution to science and knowledge building. In this protocol the caregivers serving as saliva collectors were prompted by a telephone call each day they agreed to obtain measures, and follow-up calls confirmed data collection or made back-up plans to reschedule another day during the same week. In addition saliva collectors were given the opportunity to practice with the project staff and were given a card with a phone number to call if they had any questions. To ensure compliance with evening collection, saliva collectors were reminded to place collection materials by the night stand.

This protocol may be appropriate for use in other samples and can contribute to an understanding about the role of various biological pathways involved in stress-related disorders in other under-represented populations. The measurement time points in this protocol were designed to capture the diurnal profile across the day[6,18]. The nonlinear and diurnal nature of salivary cortisol[6] and alpha amylase[13] pattern requires multiple sampling points to create adequate statistical models. The recommended sampling design for salivary cortisol and alpha amylase involves sampling immediately upon waking, 30 min post waking, midday, and immediately prior to bed[14,19].

Other experiments interested solely on the awakening challenge response may only require on-awakening and 30 min post awakening. For studies measuring diurnal patterns, the minimum sampling is 3 samples each day for 2 days in a row. Additional physical measures of stress

activation, activity of the sympathetic and parasympathetic nervous system, or psychological or behavioral manifestations of stress can also be built into the protocol.

Consistency in collection methods is important in order to avoid introducing unsystematic error into study data[20]. Since saliva collected by placing a swab underneath the tongue on yields results similar to those from whole saliva collected by passive drool, and because we were interested in assessing both cortisol and alpha-amylase from a population from which passive drool would be difficult to collect, we only used samples of saliva collected via a swab placed under the tongue for our protocol. Given the higher rates of xerostomia[3] in the older adult population and the effect of saliva flow rate on levels of salivary assays it is also important to note the time needed to collect saliva, in order to estimate the flow rate (ml/min)[21]. Multiply assay results (U/ml) by the flow rate in order to express the results as output per unit of time (U/min), to allow for comparison in data analysis.

In order to minimize the risk of food residue that can change salivary pH or composition[11,12] , participants who have eaten within 1 hr should rinse their mouth with a small amount of water prior to providing a specimen. Consideration of access to food and drink should be carefully planned in the study design.

Finally it is important to consider important potential confounders of the stress response including age, gender, personality traits, use of nicotine/alcohol/caffeine, or use of medications (*e.g.* psychotropics) on individual responses to stress. For a more thorough overview of the use of salivary hormone assessment in health sciences see Granger and colleagues[22], and for a discussion of individual differences in assessment of salivary hormones, see Schultheiss and Stanton, 2009[23].

Disclosures

In the interest of full disclosure, DAG is founder and serves as the Chief Scientific and Strategy Advisor at Salimetrics LLC (State College, PA) and SalivaBio LLC (Baltimore, MD). These relationships are managed by the policies of the committee on conflict of interest at the Johns Hopkins University School of Medicine, and Arizona State University's Office of Research Adherence and Integrity.

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