ChronoBiology LETTER

DiagnosTechs™ THE LEADING LAB IN SALIVA TESTING SINCE 1989

Spring 2012 | 11th Edition

Diagnos-Techs™
Clinical & Research Laboratory
Quarterly Newsletter

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Diagnos-Techs™ Laboratory Excels in Proficiency Testing

Ning Cigielska, MS
Michael Everitt, PhD

Like most well run laboratories, Diagnos-Techs™ participates in periodic proficiency testing. This process involves analysis of test samples provided by an external source. These samples may contain nothing, or they may contain a variable amount of unknown substances to be analyzed. The Diagnos-Techs™ laboratory assessment is then compared to the known test sample contents by the external testing service.

One such area of proficiency testing is our GP-2 assay, the O&P (ova and parasites) assay performed on the stool sample submitted as part of our GI Health panel. In this assay, samples from two stool samples submitted are examined three times under the microscope by a parasitologist. Any organisms or parasite eggs discovered are then listed on the report.

We are accustomed to receiving 100% scores on these proficiency tests from the external testing source. On recent testing, however, our O&P examinations of the test sample proved even superior. Our parasitologist recognized not only the test organisms, but several additional parasites present in the test specimen – which had not been noted by the testing facility. When called to the facility's attention, the presence of these other parasites was recognized and acknowledged.

MORAL: A highly trained, knowledgeable pair of examining eyes is essential to an accurate O&P.

Diagnos-Techs™ is going **Paperless!**

In an effort to be more mindful of the environment, we will now be distributing an electronic version of our quarterly newsletter, **ChronoBiology Letter**. If you do not have an email address listed on your account, please call **Client Services at 1.800.878.3787** to add your email address.

If you prefer to receive a printed copy of our ChronoBiology Letter, please email us at diagnos@diagnostechs.com.

Obtaining Adequate Saliva for Testing

John J. White, MD, CM
Ronda Nelson, PhD

Adequate saliva for testing (to fill testing vials to required levels) may be difficult to obtain in some circumstances. Some patients have difficulty producing saliva; other patients have difficulty collecting saliva into the vials. A few suggestions may be of help in either case.

For patients who have problems salivating, saliva production may be facilitated by chewing a piece of dental grade paraffin wax (obtainable from a pharmacist); sniffing pickles, a cut lemon, vinegar, or any tart substance; or preparing a favorite savory dish (remember to collect saliva prior to enjoying!).

For cortisol testing (ASI/TAP), a cotton roll placed under the tongue is used. To ensure adequate saturation of the roll, do not remove roll and place back into the vial until the mouth has refilled with its usual amount of saliva.

In other instances, such as advanced age, mental or physical incompetence, or disinclination (an unruly or reluctant child), it may be difficult to collect saliva into the vial. In such circumstances, use of a sterile syringe (3cc is the ideal size) to siphon saliva from the cheek pouches of the patient can help. The saliva then is transferred to the proper vial. An unused (preferably sterile) syringe is necessary to ensure an uncontaminated sample. Such syringes generally can be obtained from a cooperative pharmacy.

Saliva Hormone Testing

A number of new developments in saliva testing have occurred over the past several years, coupled with serious reappraisals of serum hormone testing. In view of these, and requests from our clients, an updated review of saliva testing is presented in this issue of Chronobiology.

John J. White, MD, CM

In the past, clinical laboratory analysis of circulating body fluids has been done using serum, which necessitates a venipuncture. Since the latter part of the 20th century, saliva has been used increasingly for this purpose. Recently, a consortium of three research groups categorized 1,116 proteins secreted in saliva and stated that "[such] studies can be used to translate blood-based clinical laboratory tests into a format that utilizes saliva."[1] Saliva has been categorized as a "valuable biofluid… with the potential to extract more data than is possible currently with other diagnostic methods."[2] A non-inclusive list of current saliva testing is presented in Table 1.

Background

Laboratory testing for hormones using saliva was verified in the 1980's and 1990's, and saliva testing is becoming increasingly more utilized clinically, particularly for endocrinologic and immunologic investigations. The slow pace of its acceptance appears to have been the result of a variety of factors: most clinicians are unfamiliar with the benefits and availability of salivary testing; the majority of laboratories have made significant investments in blood analysis systems and equipment; there is an innate reluctance to waiver from serum testing, which has been the basis for most research and clinical decision making; and, until recently, there has been little criticism of existing serum testing.

Hormone testing has been carried out traditionally by assaying levels in serum. This type of testing is beleaguered by nature's mechanism of transport of hormones in the blood: over 90% of the hormones in blood are bound to proteins (binding globulins) and are inactive. The small percentage of hormones remaining are considered "free," or available to traverse the capillary membranes into the tissues and have their effect on target cells. Only the **free** form of a hormone is biologically active. There are major problems inherent in serum hormone testing: there is no assurance that the "free" hormone measured actually reaches the tissue fluid to affect its target cells; and there exists a constant interchange between "bound" and "free" hormones in the blood, a dynamic that calls into question whether any measured serum "free" values actually are meaningful.

These shortcomings have been recognized by The Endocrine Society for testosterone, the hormone with the lowest "free" fraction (<3%). In 2008, The Endocrine Society issued a position statement stating that "the manner in which most [serum] assays for TT [total testosterone] and FT [free testosterone] are currently performed is decidedly unsatisfactory."[3] Recognizing that "important discrepancies and inconsistencies in measurements are widespread," in 2010 The Endocrine Society paired with the Center for Disease Control (CDC), other clinical societies, and commercial laboratories to endorse "accuracy-based testing of testosterone and calibration of all methods traceable to a single high-level reference material."[4] These methods are being developed by the CDC using mass spectrometry. This process will measure not free testosterone, but total (free and protein bound) testosterone. At the time of this writing, only a few laboratories appear to have been so certified; those in the United States use expensive mass spectroscopy. In light of this overall problem, the editors of the Journal of Clinical Endocrinology and Metabolism have raised the question whether "all of our other routinely employed [serum] hormonal assays provide truly accurate and precise measurements."[5] Faced with major imperfections in and doubts about serum hormone testing, clinicians are seeking other forms of hormone testing that don't have protein binding and capillary transfer problems. A widespread and accurate form of hormone testing, one that is not subject to these limitations, utilizes saliva.

Some Clinical Conditions Detected Through Saliva Testing
(List not comprehensive)

- Adrenal conditions (e.g. Addison's/Cushing's, adrenal fatigue)
- Altered female hormone states (e.g. PCOS, menopause, hormonal alterations, infertility)
- Altered male hormone states (e.g. hypogonadism, hyperestrogenism)
- Metabolic disturbances (e.g. insulin resistance, diabetes)
- Neoplasms (e.g. benign, metastatic)
- Infectious conditions (e.g. HIV, hepatitis, Helicobacter pylori)
- Allergic states (e.g. asthma, mold, food)
- Drugs (e.g. marijuana, cocaine, alcohol)

Table 1: Some clinical conditions detected through salivary testing.

Saliva Testing

Salivary testing is eminently suitable for hormone testing because it does not entail the problems inherent to serum testing: it measures hormone levels in the tissue fluid, where there are no binding proteins. The 2007 edition of Greenspan's textbook, *Basic and Clinical Endocrinology*, states that "salivary cortisol reflects 'free' cortisol, and saliva testing directly measures *active* (emphasis added) steroid hormone ('free' hormone)."[6]

Salivary hormone testing was fully verified in the 1980's, at which time any problems with laboratory methods, specimen collection, and specimen transport were settled.[7,8,9,10,11] In 1994, it was reported that more than 2500 clinical and research articles dealing with the accuracy and application of salivary diagnostic tests had been published.[12] These papers emphasized that close correlation exists between salivary testing ("free" fraction) and the "free" fraction of the serum. Since then, several review articles have further verified and vindicated salivary hormone analysis.[13,14,15,16,17,18,19]

Chronobiologic Testing

Not only is saliva testing more accurate inasmuch as it selectively measures the active "free" hormone, but it also allows chronobiologic testing, which is not feasible with serum testing with its attendant venipunctures. Championed by Franz Halberg of the University of Minnesota, chronobiology is defined as the field of science that examines periodic (cyclic) phenomena in living organisms. That is, not just a single measurement, but serial measurements over time of dynamically varying vital hormones can be obtained. It is the difference between looking at a snapshot of a changing scene and watching an entire movie.

Adrenal Cortisol Testing

One principal application of chronobiology is circadian cortisol output assessment. Assessment of the adrenal gland's diurnal/circadian rhythm using multiple cortisol samplings throughout the day can only be accomplished, with absolute accuracy, via non-stressful saliva testing.

In 2008, The Endocrine Society published guidelines for the diagnosis of Cushing's Syndrome.[20] One of the four recommended initial screening tests is late night *salivary* cortisol measurement (on two consecutive days). Ample references are provided to show that "free cortisol in the blood is in equilibrium with cortisol in the saliva"; that "the concentration of salivary cortisol does not appear to be affected by a change in the rate of salivary production"; and that "an increase in blood cortisol is reflected by a change in the salivary cortisol concentration within a few minutes." The accuracy of a single salivary cortisol assay has been attested to and validated. It logically follows that cortisol samples collected at other times of the day must be equally valid, using proper technology. Such technology can be by either (costly) mass spectrometry or (economical) ELISA techniques; both "yield a 92%-100% sensitivity and a 93%-100% specificity."[20]

Diagnos-Techs™ salivary hormone testing laboratory was the first in the United States to provide, commercially, a temporal adrenal cortisol profile for clinical use (offered since 1987). Hormone assays are carried out using the ELISA technology, and results and standards are checked regularly using mass spectrometry. Diagnos-Techs™ has earned the Joint Commission's Gold Seal of Approval and is CLIA (Clinical Laboratory Improvement Act) and COLA (major laboratory accreditation organization) approved. The salivary test panel offered by Diagnos-Techs™ truly constitutes a chronobiologic investigation. It includes four cortisol evaluations throughout the day to detect possible stressful disturbances in the circadian cortisol rhythm. Chronobiologic salivary cortisol testing has been used clinically most prominently by the United States government. Panels that measure cortisol and other hormones have been relied upon clinically to assess objectively the circadian adaptation of aircrew to transmeridian flight;[21] to create physiologic profiles of soldiers experiencing military survival training;[22] and to evaluate circadian shifts in astronauts before flight (including the proposed MARS project astronauts).[23,24]

Cycling Woman's Hormone Assessment

Another prime example of chronobiologic evaluation is a cycling female hormone evaluation. Because of the wide variations of the two principal cycling hormones, estradiol and progesterone, throughout the follicular and luteal phases of a woman's monthly cycle, no single day's hormonal sample can in any way be representative of the cycle. The Diagnos-Techs™ Cycling Female Homone Panel (FHP™) entails a single saliva collection every 2-4 days depending on the length of the woman's cycle. A representative graph illustrating the vagaries of a woman's hormones throughout her cycle is depicted in Figure 1. Ordering the expanded version of the FHP™ adds 7 measurements of pituitary hormones (follicular stimulating hormone [FSH] and luteinizing hormone [LH]) which are measured around the

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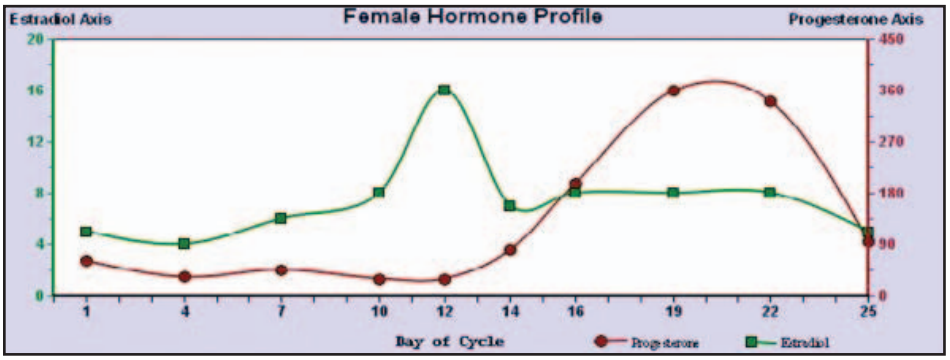

Figure 1: Female Hormone Panel (FHP™) result for a cycling 32-year-old woman. The pre-ovulatory estradiol surge is seen, followed by elevated progesterone during the luteal phase of the cycle.

time of ovulation. These FSH and LH assessments are valuable particularly for women with fertility problems, pre-menopausal symptoms, and menopausal distress.

Andropause Assessment

It is becoming more evident that investigation of hypogonadism in men, sometimes termed "andropause," cannot be limited to a single serum testosterone assessment. Such a measurement is unreliable and fraught with error as previously noted. As well, overall problems with deficiencies of predecessor hormone supply and reduced activity of the underlying testosterone pathway co-reactions, the amount present of the potent male hormone dihydrotestosterone, and the dynamics of the controlling pituitary hormones (FSH and LH), should be taken into account prior to any possible recommendations for testosterone therapy. Aromatization of testosterone to estradiol and conversion of the androstenedione pathway to estriol and estradiol (common in older men) are not accounted for without measuring these other hormones. All these values should be known and addressed prior to pronouncing the testes unresponsive and prescribing testosterone therapy.

A representative and thorough investigation that can provide a sound basis for therapy recommendations is the Expanded Male Hormone Panel™ (eMHP™) from Diagnos-Techs™.

Inasmuch as andropause symptomatology can result not only from testosterone deficiency, but from vascular, psychologic, and neurological problems as well, a broad and thorough investigation should include an adrenal stress assessment (ASI™).

Summary

- Serum hormone testing, for years the staple and standard for hormone testing, has been called into serious question by endocrine experts due to uncertainty in distinguishing protein-bound from "free" forms of a hormone, and uncertainty in how much "free" or available hormone actually crosses the capillary barrier into the tissue fluid;
- Saliva hormone testing has attracted considerable interest because it measures hormones in the tissue fluid itself (i.e., the "free" form of the hormone), thereby providing more accurate results than serum testing;
- The accuracy of saliva testing was established during the 1980's, and since then has been verified continually by, literally, thousands of scientific papers;
- The most accurate laboratory methodologies for saliva analysis are ELISA techniques and mass spectrometry;
- Salivary hormone testing for clinical use has been available commercially since 1987 (from Diagnos-Techs™). It is not merely a research tool. It has been used by the United States government, and it is recommended by The Endocrine Society for the diagnosis of Cushing's Syndrome. There has been a steady increase in saliva testing laboratories and saliva hormone testing since the technique was introduced over two decades ago by Diagnos-Techs™;
- Salivary testing is an accurate and accessible method for antibody assessment which is an important element in the investigation of food allergies and intestinal parasitic diseases;
- Salivary hormone testing is non-invasive, economic, private, accurate, and non-stressful. It is the only feasible method to carryout chronobiologic testing, which is very valuable in assessing stress and the vagaries in hormone cycling. Such testing is nearly impossible in clinical practice using serum testing and its requisite serial venipuncture;
- In view of the demonstrable benefits of saliva hormone testing, and the doubts regarding the "Gold Standard" serum testing, saliva hormone testing deserves to be considered the "Platinum Standard" for hormone testing.

For references please see back page.

The Therapy Corner

In an effort to further broaden the perspective and service provided by Diagnos-Techs™, we are adding a new section to the Chronobiology Newsletter called The Therapy Corner. In this section, we will provide background material, interpretive guidelines, and therapeutic strategies for a variety of conditions, with particular focus on conditions identified by our tests. In this issue, we discuss Clostridium difficile infection.

Clostridium difficile

January 2012

John J. White, MD, CM
Brandy Webb, ND

In our Gastrointestinal Health Panel (regular or expanded), Diagnos-Techs™ provides analysis of submitted desiccated stool specimens for the Clostridium difficile antigenic toxins (A and B). This assay has a sensitivity of 93.1% and a specificity of 99.2%.[1] It is an accurate and valuable test when C. difficile proliferation is present.

Clostridium difficile is a spore-forming bacterium with broad distribution. It comprises a usually manageable portion of the normal bowel flora. Occasionally, an increase in C. difficile population may occur, usually accompanied by diarrhea. An estimated three million cases of C. difficile infection occur in the U.S. each year, with rising mortality due to increasingly virulent strains and rising host vulnerability.[2]

Clostridium difficile infection has four clinical forms: 1) short term over-colonization (frequently occurs after a stay in a healthcare facility or following an intense antibiotic course); 2) acute diarrhea; 3) fulminant diarrhea (may be associated with pseudo-membranous colitis and leukocytosis, and may lead to death); and 4) recurrent or persistent infection (defined as C. difficile infection occurring within sixty days of initial diagnosis and treatment; recurrence/persistence rates for C. difficile are estimated to be 20-30%).[2]

Recent evidence underscores the importance of screening for Clostridium difficile infection (CDI) in non-hospitalized patients. Community-acquired CDI cases comprise up to 41% of total CDI cases, and patients are more likely to be younger, female, and have no recent hospitalization or exposure to antibiotics.[3] Moreover, they are more likely to experience milder symptoms and thus go undiagnosed.[3]

The most current definition of Clostridium difficile infection is the presence of symptoms (usually diarrhea) and a positive stool test for C. difficile toxins.[4] The enzyme immunoassay stool test for C. difficile toxins A and B offered by Diagnos-Techs™ (lab code "GP3Cd") is a convenient option for C. difficile testing, and it is available as part of the more comprehensive GI panels offered by Diagnos-Techs™ ("GI-1" and "GI-2") for cases where screening for other infectious causes is prudent.

Upon detection of Clostridium difficile toxins, treatment must be selected appropriate to the severity of presentation. In all cases, any offending antimicrobial agent should be discontinued as soon as possible (unless very strongly indicated otherwise). In the mildest form (clinical signs and symptoms are few and mild), non-pharmacologic therapies designed to rebalance the intestinal flora usually are sufficient. The first step is to initiate substantive therapy with high quality probiotics. Overall, probiotic therapy is the mainstay in CDI except in those cases involving immunocompromised or critically ill patients (administration in these cases may result in infection of the bloodstream).[4] Benefit has been demonstrated with supplementation of Saccharomyces boulardii, bifidobacteria, and Lactobacillus species in cases of C. difficile.[4,5] One key to successful probiotic therapy is proper dosing; aim for 100 billion CFUs per day for at least four weeks to achieve adequate colonization in the GI tract (lower doses of probiotics may be prescribed for several additional weeks). Some forms of fiber, especially fructooligosaccharides (FOS) and inulin, frequently are included in probiotic formulas to act as a food source for beneficial gut flora and promote their proliferation; in this context, these forms of fiber are often termed "prebiotics".[5]

Antimicrobial herbs are another mainstay of comprehensive Clostridium difficile treatment protocols, since they can combat infection without further predisposing to C. difficile. Antimicrobial herbs possess antiseptic activity, but their primary action is to augment the body's own defense mechanisms to assist in fighting the infection. Helpful herbs include echinacea, calendula, myrrh, garlic, artemisia, berberis, and hydrastis; these can be taken as tinctures or encapsulated preparations. Many of these herbs have the added benefit of anti-inflammatory or tonifying (strengthening) effects. Any drugs and herbs designed to halt peristalsis (e.g., some antidiarrheals) should be avoided, because these can mask symptoms and possibly result in toxic megacolon.[4] In view of the 20-30% incidence of persistent or recurrent infection, routine clinical and laboratory follow-up is

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Therapy Corner Continued

recommended two weeks following completion of probiotic and antimicrobial therapy.

For more severe cases of Clostridium difficile infection—those that present with acute diarrhea and the distinct possibility of fulminant infection and death—pharmacologic therapy must be considered. The current initial recommendation is the use of:

Metronidazole (Flagyl) 250mg PO QID (or 500mg PO TID) for 10-14 days[4,6]

OR

Vancomycin (Vancocin) 125mg PO QID for 10-14 days[4,6]

Metronidazole typically is used for mild-to-moderate CDI, while vancomycin generally is reserved for severe CDI.[4] A newer drug, fidaxomicin (Dificid), dosed at 200mg PO BID for 10 days, can be prescribed in severe cases as an alternative to metronidazole or vancomycin.[6,7] Fidaxomicin was approved by the FDA in May 2011 and has been demonstrated to be two- to eight-fold more potent than metronidazole and vancomycin.[8] Administration of antibiotics should be accompanied or followed by four weeks of potent probiotics as described previously. Again, clinical and laboratory follow-up should be obtained about two weeks following completion of therapy.

As mentioned, Clostridium difficile has a significant rate of persistence or recurrence; moreover, patients who have one recurrence are far more likely to have additional recurrent episodes.[4] Recommended treatment for the first recurrence of CDI is usually the same regimen used for the initial episode, but it may need to be modified according to the severity of the clinical presentation.[4,7,9] Nitazoxanide (Alinia) has been shown to have activity against C. difficile and may be considered in some cases (typically dosed at 500-1000mg PO BID for 10-14 days).[9,10] Recent studies have shown that proton pump inhibitor (PPI) use is a significant risk factor for recurrence of C. difficile-associated diarrhea.[11] Discontinuing PPI use in favor of alternative treatments (dietary and lifestyle modification, herbs, etc.) should be advised in recalcitrant patients.

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"Saliva Testing" In Wikipedia

John J. White, MD, CM and Brandy Webb, ND

Until recently, Wikipedia, the online encyclopedia, contained little about saliva testing, and that little was erroneous and incomplete. It was an evident lapse that needed to be addressed, updated, and corrected. We are very pleased to report that a new and more complete overview of saliva testing has been up and running since October 2011 on Wikipedia.

It reviews saliva testing from many points of view. It highlights some of the more than 1200 different substances in saliva for which testing has been done, and it discusses the various conditions that can be evaluated using saliva biomarkers. These include metabolic disturbances, neoplasms, infectious conditions, drugs and allergic states, as well as aberrations in male and female hormones, stress hormones, and antibodies familiar to our clients at Diagnos-Techs™. Several links to other appropriate topics have been added, and there are over sixty peer-reviewed, scientific articles referenced within the article.

We recommend this addition to Wikipedia to you as a source of additional knowledge about saliva testing, and as a resource for you and your patients. It contains a wealth of well-referenced information to help you with your practices.

DiagnosTechs™ THE LEADING LAB IN SALIVA TESTING SINCE 1989

Issue #11 Chronobiology Letter is published quarterly by Diagnos-Techs™ Laboratory, Inc. in Kent, WA, USA as an educational resource for our health care clients. The content in this newsletter is for informational purposes only and is not to be construed as medical advice.

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- Have your **account number** or **accession number** ready before you call.
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- **Review patient medication list.** This will help your patients to understand what to avoid before taking our tests. We do not discuss medications directly with patients.
- **Tips for saliva production.** In order to produce enough saliva, suggest to your patients to chew dental grade paraffin wax.

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- ☐ If not possible, refrigerate samples and ship within 3 days.
  No ice bags are required during shipping.
- ☐ Write the patient's name and address on the outside of the box.
- ☐ Include all samples, test form and, if applicable, a check or a copy of the front and back of insurance card together inside the supplied box. Please be sure to seal the box with clear tape OR the UPS shipping label (US only).

- **US Domestic:** Deliver completed test kit box to any **UPS** location. **www.UPS.com/dropoff** Return shipping to Diagnos-Techs™ is **PRE-PAID**. Kits will arrive within three business days of shipment.
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Saliva Hormone Testing Continued

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