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Evaluation of Phadebas Forensic Press test paper as a source of biological material for immunochromatographic testing and DNA analysis

Davidek, Nicole Marie

BOSTON UNIVERSITY

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EVALUATION OF PHADEBAS FORENSIC PRESS TEST PAPER AS A

SOURCE OF BIOLOGICAL MATERIAL FOR

IMMUNOCHROMATOGRAPHIC TESTING AND DNA ANALYSIS

by

NICOLE MARIE DAVIDEK

B.S., University of Nevada, 2011

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Approved by

First Reader _____

Amy N. Brodeur, M.F.S.
Instructor and Assistant Director, Biomedical Forensic Sciences

Second Reader _____

Kevin Kosiorek, M.S.
Adjunct Instructor, Biomedical Forensic Sciences

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EVALUATION OF PHADEBAS FORENSIC PRESS TEST PAPER AS A

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Boston University School of Medicine, 2014

ABSTRACT

Saliva is commonly found at crime scenes and other biological fluids, such as semen, urine, blood, and fecal matter may be present alongside saliva on an article of clothing, such as underwear. Forensic testing is required to detect saliva in order to corroborate events related to a crime and to identify stains that can be used in DNA analysis. A cross-reactivity study was carried out to determine the specificity of the Phadebas® Forensic Press test for saliva stains. Whole blood, semen, urine, fecal matter, vaginal secretions, condoms, lubricants, vinegar, and PBS were all tested for reactivity with the Phadebas® paper. Only fecal matter and urine demonstrated cross-reactivity within the 60-minute test window.

As conservation of sample is a necessity in forensics, a study was carried out to determine if a cutting from Phadebas® paper performs similarly to a cutting from the original sample or stain in immunochromatographic testing with RSID™-Saliva. Testing of extracts from fabric cuttings allowed for detection of 1:100 dilutions of saliva, whereas only neat, 1:5 and 1:10 dilutions were able to be detected when using Phadebas® paper

extracts. These samples were stained and examined under a microscope to determine if enough nucleated epithelial cells were present for STR analysis. Of the 81 fabric extracts examined, 15 were shown to reach the threshold where either a partial or full STR profile would be expected. In contrast, none of the Phadebas® extracts reached the threshold, indicating that a cutting from the original stain is a more reliable source for DNA analysis.

Overall, these results support that the Phadebas® Forensic Press test is useful for detecting latent and diluted saliva stains but should be considered a screening method only due to false positive results observed with urine and fecal matter. Furthermore, using Phadebas® Forensic Press test paper as a source of biological material for immunochromatography or DNA analysis is not as successful as using the stained substrate itself, and should be avoided whenever additional sample is available for direct testing.

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ABBREVIATIONS

ALS	Alternate Light Source
DNA	Deoxyribonucleic Acid
DSM	Bio-Degradable Starch Molecule
H&E	Hematoxylin & Eosin Y
IU/μL	International Units of Activity
μL	Microliter
mRNA	Messenger Ribonucleic Acid
NECs	Nucleated Epithelial Cells
ng	Nanogram
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
pg	Picogram
PSA	Prostate Specific Antigen
qPCR	Quantitative Polymerase Chain Reaction
RCF	Relative Centrifugal Force
RSID	Rapid Stain Identification
STR	Short Tandem Repeats

1. Introduction

Saliva is routinely used as a source for DNA in forensic casework and may originate from many evidentiary sources including saliva stains, cigarette butts, drinking containers, and swabs from sexual assault kits or DNA standards (1, 2). Due to the commonality of this biological fluid, its identification is important and can play an integral part in solving a case (3).

Saliva is produced and secreted by the salivary glands located within the oral cavity. Saliva is composed mostly of water with the remainder being composed of electrolytes, digestive enzymes, bacteria, epithelial cells, red and white blood cells, and food debris (4, 5). The primary digestive enzyme present in saliva is alpha-amylase, which aids in the digestion of starch. The composition of saliva has been shown to be highly variable throughout the day and also between individuals (4, 6-9).

1.1. Forensic Techniques for Saliva Identification

To test for the presence of saliva on clothing or other items of evidence (bottles, cigarettes, etc.), current forensic methodologies target the enzyme amylase (2, 10, 11). There are two isoforms of α-amylase present in humans: salivary and pancreatic (2, 5, 9). Although the concentration and activity of α-amylase is highest in saliva, it can also be found in other body fluids including breast milk, fecal matter, urine, and semen (3, 5, 9, 10, 12). As α-amylase is present in other forensically relevant biological fluids, there have been both screening and confirmatory (or secondary screening) tests developed to aide in saliva identification.

Initial examination of an item of evidence includes a visual inspection under natural light to locate any whitish stains. If no stains are visible, an alternate light source (ALS) can be used to identify stains not visible to the naked eye (3, 5). A benefit to the use of an ALS is that latent stains can be detected without any modifications to the item of evidence, although it has been shown to be difficult to distinguish between saliva stains and those from other biological fluids or chemicals that may be present (3, 8).

Following the identification of a stain through a visual examination or with use of an ALS, the stain can be screened for the presence of saliva. Initial saliva screening methods include: the starch-iodine radial diffusion test, Phadebas® Forensic tube test and Phadebas® Forensic Press test. These tests are all based upon the level of amylase activity within a sample. The starch-iodine radial diffusion test utilizes an agar gel containing starch, in which wells are filled with sample extracts and incubated for several hours or overnight. Following incubation, the gel is dyed with an iodine solution, changing the color of the gel to a blue-purple due to the presence of the starch. If amylase is present, the blue-purple color will not appear around the sample well, indicating that amylase in the sample digested the starch in that area; the greater the diameter of the un-dyed area around the sample well, the greater the amount of amylase activity that is present within the sample (5, 13). This test can also be performed in a microcentrifuge tube with the sample and reagent in a liquid state, allowing for rapid results (14).

The Phadebas® Forensic tube and Forensic Press tests utilize a water-soluble blue dye that is attached to insoluble starch, either present in a tablet or impregnated on filter paper, respectively (3, 8, 15). The Phadebas® Forensic tube test is completed in a

microcentrifuge tube whereby the dye tablets are added directly to the supernatant resulting from the extraction of a fabric cutting or swab. The tube is then incubated and centrifuged, dissolving the dye tablet; any amylase present will react with the starch attached to the Phadebas® dye, releasing the blue dye into solution. The intensity of the resulting supernatant color change is indicative of the amount of amylase present in the sample. This method is considered semi-quantitative if the resulting supernatant is analyzed using a spectrophotometer (16).

The Phadebas® Forensic Press test uses filter paper in which the reagent dye particles are present on one side of a piece of filter paper. When moistened and applied to a saliva stain, the amylase present in the stain will digest the starch, allowing the blue dye to diffuse out onto the paper, which can be observed on the white side of the paper (3, 5, 8, 15). The advantage to the Press test is that it allows for mapping of an item of evidence, including large items of evidence, for the detection of non-visible stains.

The primary disadvantage to these screening techniques is that forensically relevant body fluids, such as semen, vaginal secretions, and blood, have been shown to generate positive results (5, 15, 17). In an effort to make the identification of saliva more robust and more specific, secondary screening techniques have been developed.

Secondary screening methods for saliva include immunochromatography, gene expression assays, and spectroscopy (5). Immunochromatographic techniques are very common in forensics as they are fairly specific, easy to use, and provide rapid results (5, 9). One such immunochromatographic assay is RSID™-Saliva, a lateral flow strip test that utilizes antibodies for the detection of human salivary α-amylase. RSID™-Saliva has

been shown to have low levels of cross-reactivity with other body fluids, such as semen, urine and breast milk, and is very sensitive (9, 12). Another immunochromatographic assay, Seratec® Amylase, which utilizes similar techniques to RSID™-Saliva, has been shown to have similar specificity and sensitivity for α-amylase (18). An additional secondary screening method, SALIgAE® has been developed that is less specific and sensitive than RSID™-Saliva (12, 14, 19). SALIgAE® is a colorimetric tube test whereby a sample is placed into a colorless solution; if saliva is present, a yellow color will be observed (12, 19). However, the mechanism for saliva identification has not been disclosed by the manufacturer (14, 19).

Two additional techniques have been recently adapted for secondary screening of body fluids, including saliva: mRNA analysis and spectroscopy. Identification of saliva using mRNA is based upon detection of the expressed genes statherin and histatin 3 in a particular sample or stain (2, 20). Spectroscopic methods are non-destructive and use light to determine spectral patterns of body fluids, including mixtures, for comparison with unknowns (21, 22). These methods, which include Raman and fluorescence spectroscopy, are relatively new to the field of forensic biology and are not yet widely used to identify body fluids, unlike the immunochromatographic techniques.

The goal of body fluid identification is not only to corroborate theories and recreate sequences of events, but also to provide a source for DNA profiling. The amount of DNA present on an item of evidence can vary from plentiful to undetectable. Due to this variation and inability to determine the amount of viable DNA present based on visual observation, preservation of stains, swabs, and other articles of evidence has

become necessary in the processing of crime scenes. It is important to determine if a single sampling from an item of interest can be used throughout the entire forensic testing process – from screening to DNA analysis – thus conserving more biological material for further analysis should more advanced methods be developed or retesting be necessary.

For analysis of saliva stains, manufacturers of various tests often recommend the direct testing of an item of evidence (e.g. fabric cuttings or portions of swabs) (15, 18, 23). In some laboratories, if primary and secondary screening tests and DNA analysis were to be performed on a single saliva stain, up to three samples might be excised from the original stain, which is not ideal with respect to evidence conservation and workflow; methods that minimize consumption of the sample or stain should be explored. To minimize sample loss, mutilation or contamination, and ensure there is an adequate amount remaining for future testing, the ability to use a filter paper transfer of a suspected stain for all tests would be optimal.

1.2. Purpose

The purpose of this experiment is threefold. First, to determine if the parameters set by the manufacturer are appropriate for discerning between saliva and various other body fluids and forensically relevant samples when using the Phadebas® Forensic Press test. Second, to evaluate the efficacy of extracting a portion of Phadebas® Forensic Press paper that exhibits a positive reaction for use as a source of α-amylase for testing with RSID™-Saliva immunoassay cards in comparison to using a cutting from the original stain. The final part of this project examines the RSID™-Saliva extracts (either from

Phadebas® paper or fabric) to determine if nucleated epithelial cells can be visualized microscopically. This could indicate whether or not the unused portion of extract would be suitable for DNA analysis, rather than collecting more of the original sample for further testing. A recommended protocol that takes into account sensitivity, specificity and sample preservation is presented.

2. Materials and Methods

Experiments were carried out in compliance with the protocol approved by the Boston University School of Medicine Institutional Review Board. A total of 7 donors - 2 males and 5 females - provided the following body fluids (as applicable): liquid saliva, urine, semen, vaginal swabs, fecal swabs, and whole blood; not all donors submitted all body fluids for analysis. Donors were asked to provide liquid saliva samples collected on three separate days. Whole blood samples were obtained from donors using a lancet, then collected in microcentrifuge tubes and refrigerated. All other samples were frozen until ready for use.

Positive controls consisted of fresh buccal swabs and negative controls were prepared using swabs moistened with deionized water. Per the manufacturer's recommendations, negative controls were monitored for 40 minutes to ensure no false positive reactions occurred. Cotton fabric (Benchmark Fabrics, Rosemont, NJ, USA) used for all experiments was pre-washed in hot tap water and air dried before use. A facemask was worn during all experiments to ensure that environmental contamination did not occur.

2.1. *Saliva Samples*

A total of 15 liquid saliva samples (5 donors, three samples per donor, 3 female and 2 male) was used. Frozen samples were thawed at room temperature and vortexed briefly before preparing the dilutions. A dilution series using each saliva sample and deionized water was generated as follows: 1:5, 1:10, 1:50, 1:100, and 1:500. All samples were vortexed to ensure homogeneity of the solution. Fifty microliters of each dilution, along with a neat saliva sample and negative control (deionized water), were applied to a specific area of a labeled fabric swatch and allowed to dry at room temperature for approximately two hours [Figure 1].
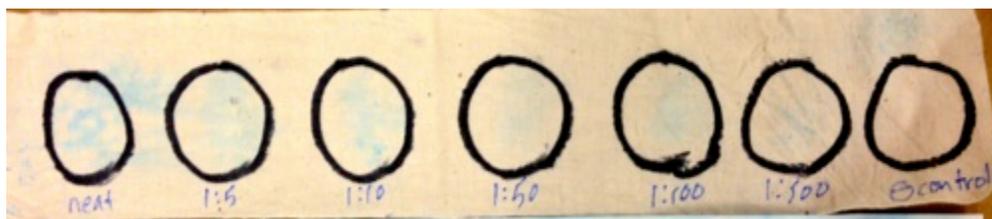
Figure 1. Preparation of Saliva Sample Fabric Swatches. Fifteen saliva samples (3 females, 2 males) were diluted with distilled water and 50μL applied of the dilutions, neat and negative control, to specific areas of labeled fabric swatches.

Once dried, the swatch was sprayed liberally with deionized water and a piece of Phadebas® paper was placed, blue reagent-side down, over all samples. The white side of the Phadebas® paper was marked to indicate placement on the swatch. The Phadebas® paper was liberally sprayed with deionized water, and a clear clipboard and weight were placed on top to apply pressure. The Phadebas® paper was checked for a reaction every five minutes, for a total of 60 minutes. The Phadebas® paper was remoistened, as necessary, during the experiment. Results were recorded at 10, 40, and 60 minutes, or when a positive result was achieved.

2.2. *RSID™-Saliva*

For saliva samples that provided a positive result with Phadebas® paper, an approximate 1cm x1cm square was cut out from the area exhibiting a positive reaction on the Phadebas® paper. Each cutting was placed in a microcentrifuge tube (Corning Inc., Corning, NY, USA) containing 200µL of RSID™ Universal Buffer and extracted for 2 hours at room temperature on an orbital shaker. After the extraction, the cuttings were removed and placed into a Costar™ Spin-X® basket (Corning Inc., Corning, NY, USA) and centrifuged at 16.1 RCF for 3 minutes. Cuttings were discarded following centrifugation. In a separate labeled microcentrifuge tube, 20µL of the supernatant was added to 80µL of RSID™ Universal Buffer and vortexed briefly. The entire 100µL mixture was added to the sample well of the RSID™-Saliva cassette (Independent Forensics, Lombard, IL, USA). Results were recorded at 10 minutes. A positive result is indicated when the RSID™-Saliva cassette shows two red lines, and a negative result is shown by the presence of a single red line at the control region.

The same procedure was repeated with cuttings from the fabric corresponding to the saliva stains that returned positive Phadebas® paper results; cuttings were taken from the center of the circle where the saliva sample was originally placed.

2.3. *Microscopy*

Following immunochromatography, approximately 130-140µL of the remaining supernatant was carefully removed from each microcentrifuge tube and discarded. Care was taken not disturb the pellet. The pellet was then resuspended in the remaining

supernatant (approximately 50μL) by mixing with a clean pipet tip. Next, 3μL of the sample/pellet was placed in a well of a labeled, 12-well microscope slide [Figure 2]. This was repeated for all Phadebas® paper and fabric samples tested with RSID™- Saliva. Slides were stained using hematoxylin and eosin Y (H&E) for easy visualization of the samples.
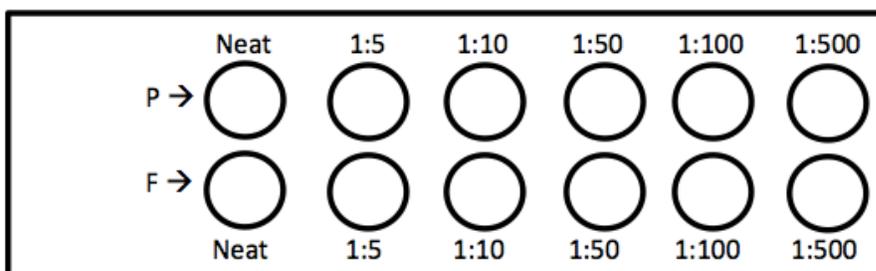
Figure 2. Microscope Slide Template. Three microliters of RSID™ Universal Buffer containing pelleted sample were applied to each well, as labeled. Only those samples that gave positive Phadebas® Forensic Press test results were included. P = Phadebas® paper; F = fabric.

Slides were heat-fixed in a 50°C oven for 30 minutes prior to staining. One drop of Hematoxylin (Thermo Fisher Scientific, Waltham, MA, USA) was applied to each slide well, and allowed to sit for three minutes. The stain was washed from the slide with methanol, and allowed to air dry before the subsequent stain was applied. One drop of Eosin Y (Acros, Waltham, MA, USA) was applied to each slide well and allowed to sit for two minutes. The stain was washed away using ethanol, and the slide was allowed to air dry before viewing.

Slides were examined on a compound light microscope for the presence of nucleated epithelial cells. The number of cells present in each sample was counted for comparison between the Phadebas® paper and fabric. Using the number of cells present

on the slides, the approximate amount of DNA was calculated (24), and a determination was made as to whether or not this amount would be expected to render a full, a partial, or no STR profile.

2.4. *Phadebas® Cross-Reactivity Study*

2.4.1. *Semen, Urine and Whole Blood*

Five semen (5 males), five urine (4 females, 1 male), and five whole blood (5 females) samples were utilized for this experiment. Frozen semen and urine samples were thawed prior to use. A neat sample and dilutions of 1:5 and 1:10 (made with deionized water) were used for the semen, urine, and whole blood samples. Deionized water was used as a negative control. All dilutions were briefly vortexed and centrifuged before use. Fifty microliters of each dilution, along with a neat sample and negative control, were applied to specific areas on labeled fabric swatches [Figure 3]. One fabric swatch per body fluid was used for each trial. The swatches were allowed to dry completely before testing.
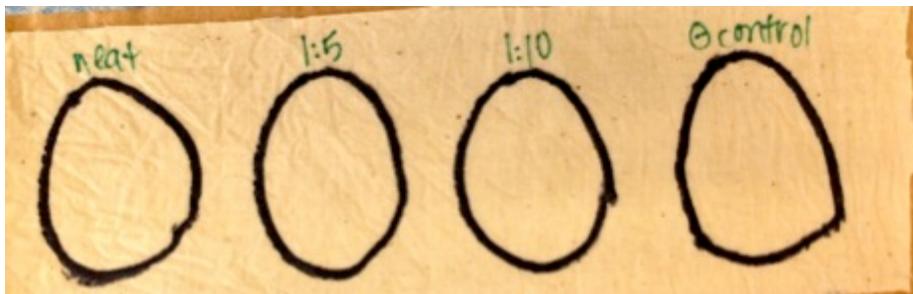

Figure 3. Preparation of Non-Saliva Sample Fabric Swatches. Samples of semen, urine, whole blood, vinegar, and PBS were diluted with distilled water and 50μL applied of the dilutions, neat and negative control, to specific areas of labeled fabric swatches.

Once dried, each swatch was sprayed liberally with deionized water and a piece of Phadebas® Forensic Press paper was placed, blue reagent-side down, over the samples. The white side of the Phadebas® paper was marked to indicate placement on the swatch. The Phadebas® paper was then liberally sprayed with deionized water, and a clear clipboard and weight were placed on top, to apply pressure. Each swatch was checked every five minutes for a reaction and remoistened, as necessary, during the experiment. Results were recorded at 10, 40, and 60 minutes, or when a positive result was achieved.

2.4.2. Fecal Matter and Vaginal Secretions

A total of 15 fecal swabs and 15 vaginal swabs were used (5 donors each, 3 swabs per donor). These samples were not diluted; the swabs were tested directly. The swabs were lightly sprayed with deionized water, and then a piece of Phadebas® paper was wrapped around the swab. The Phadebas® paper was then moistened liberally by spraying with deionized water and pressure was applied, in the same manner as the fabric swatches, to the swab for 60 minutes. Each swab was checked every five minutes for a reaction and the swabs were remoistened, as necessary, during the experiment. Results were recorded at 10, 40, and 60 minutes, or when a positive result was achieved.

2.4.3. Condoms & Personal Lubricants

Five brands of condoms were purchased from a local pharmacy for use in this experiment: LifeStyles® Skyn™ and LifeStyles® Ultra Sensitive (Ansell Healthcare, LLC, Iselin, NJ, USA), Durex® Extra Sensitive™ (Reckitt Benckiser Group plc, Slough,

Berkshire, ENG), and Trojan® Ultra Ribbed and Trojan® ENZ® Armor™ (Church & Dwight Co., Inc., Ewing, NJ, USA). The condom brands were tested in triplicate. A separate, dry swab was used to collect material from each condom's exterior and interior. Phadebas® paper was wrapped around the swab and moistened. Pressure was applied to the swab, in the same manner as previously described, for one hour and checked every five minutes, or until a positive reaction occurred. Observations were recorded at 10, 40, and 60 minutes, or when a positive result was observed.

Three brands of personal lubricants were utilized for this experiment: LifeStyles® Liquid Personal Lubricant with Aloe and Vitamin E (Ansell Healthcare, LLC, Iselin, NJ, USA), KY® Yours + Mine (McNEIL-PPC, Inc., Fort Washington, PA, USA), and NaturePlex Warm Touch (NaturePlex, LLC, Olive Branch, MS USA). Lubricant was placed in a weigh boat and swabs were rolled in the sample to evenly coat all sides of the swab tip. The swabs were allowed to air dry for 30 minutes before testing. A small piece of Phadebas® paper was wrapped around the swab and sprayed liberally with deionized water to moisten the paper. Pressure was applied to the swab, as previously described, for one hour and checked every five minutes for a reaction. Observations were recorded at 10, 40, and 60 minutes, or when a positive result was observed. Each brand was tested in triplicate.

2.4.4. Distilled White Vinegar

Two dilutions, 1:5 and 1:10, were prepared using distilled white vinegar (Whole Foods Market IP. L.P., Austin, TX, USA) and deionized water. The dilutions were

vortexed and centrifuged for three seconds before use. Fifty microliters of neat distilled white vinegar and the two dilutions were placed on specific areas of labeled fabric swatches [Figure 3] and allowed to dry completely; the negative control was 50μL of deionized water. Once dried, the samples were liberally sprayed with deionized water and a piece of Phadebas® paper was placed, blue reagent-side down, on the swatch. The Phadebas® paper was moistened thoroughly with deionized water and a clear clipboard and weight were placed on top to apply pressure. The swatches were monitored for one hour and checked every five minutes. The Phadebas® paper was remoistened as necessary during the one hour time frame. Results were recorded at 10, 40, and 60 minutes, or at the time when a positive reaction was observed. The samples were tested in triplicate.

2.4.5. Phosphate Buffered Saline (PBS)

Two dilutions (1:5 and 1:10) using PBS and deionized water were prepared, briefly vortexed and centrifuged before use. Fifty microliters of neat PBS, the two dilutions, and deionized water (negative control) were applied to specific areas of a labeled fabric swatch [Figure 3]. Samples were placed in the center of the labeled area, and allowed to air dry thoroughly before testing. Once dried, the swatch was sprayed with deionized water to moisten, and a piece of Phadebas® paper, blue-dye side down, was applied to the swatch. The Phadebas® paper was thoroughly sprayed with deionized water and a clear clipboard and weight were placed on top to apply pressure. Samples were monitored for one hour, and checked every five minutes for any reaction.

Phadebas® paper was remoistened as necessary. Results were recorded at 10, 40, and 60 minutes, or when a positive result was observed. The samples were tested in triplicate.

3. Results & Discussion

3.1. *Saliva*

Identification of saliva can be essential for corroborating events of various crimes including sexual assaults, burglaries, and homicides. Not only can saliva support one's account of the sequences of events if it is determined to present on an item of evidence, it can be used as a source for DNA analysis. In order to locate a saliva stain, there are a few methods that can be used. If the stain is visible and saliva is suspected, a direct cutting of the stain can be used in the starch-iodine radial diffusion test, SALIgAE®, Phadebas® Forensic tube test, RSID™-Saliva, or Seratec® Amylase test (12-14, 16, 23).

Furthermore, if no stains are visible, the use of an ALS or the Phadebas® Forensic Press test can be used to locate potential saliva stains (3, 5). Most of these methods for the detection of saliva, aside from using an ALS, are targeting the presence or activity of the enzyme, α-amylase.

There are a few problems associated with targeting α-amylase as a way of identifying saliva. First, amylase is a ubiquitous enzyme that is found in plants and animals, with its function across species the same: the digestion of carbohydrates (2, 14). Previous research has shown that the Phadebas® Forensic Press test does not specifically target human α-amylase and cross reacts with both dog and cat saliva, but not with plants (14, 25, 26). Second, the composition of saliva, including amylase levels, fluctuates over

time and between individuals (4, 6-9, 27). Due to this inherent variability, it can be expected that not all saliva stains will be able to be detected by the Phadebas® Forensic Press test or with the other commonly used methods mentioned above.

Finally, not only is α-amylase present in saliva, but it can also be found in other body fluids, such as semen, urine, and vaginal secretions (7, 11, 28). The type of α-amylase present in the other body fluids is primarily pancreatic α-amylase, not salivary, however the Phadebas® reagent does not distinguish between the two (9). Based upon these factors, the specificity of methods used to detect the presence of saliva have been challenged in the literature, with most methods considered screening techniques at best, including the Phadebas® Forensic Press test (5, 29, 30).

The Phadebas® Forensic Press test is both a screening test and mapping technique for evidence items where saliva is suspected (15). The Phadebas® reagent is made up of Bio-Degradable Starch microspheres (DSMs) composed of a water-insoluble blue dye cross-linked to a starch molecule, and is present on only one side (the blue reagent side) of the Phadebas® paper (15). The reagent side of the paper is placed directly on the item of evidence and moistened to start the reaction (15). In the presence of both water and amylase, the bond between the dye and starch molecule is cleaved, causing the dye to be released into solution (31). As the blue dye is released and the dye molecules accumulate, the color diffuses out and is visible on the white, non-reagent side of the Phadebas® paper (15). The speed at which this color change occurs is dependent on the amount of amylase present in the sample being tested (31). To account for variation in amylase concentrations (7, 27), the results are read after 40-minutes has elapsed (per

manufacturer's instructions) and the Phadebas® paper is removed from the item of evidence (15).

In this experiment, donors were asked to submit saliva samples from three different days, to account for intra-individual variation in α-amylase activity (7, 27). The samples tested here support this variability, as samples from the same individual were shown to have different reaction times to the Phadebas® paper [Figure 4]. This inherent variability in α-amylase levels is important to consider when determining the time frame in which the Phadebas® Forensic Press test would be expected to react with saliva stains on evidentiary items. This window is difficult to pinpoint, particularly relative to when other forensically relevant body fluids have been shown to react, as these body fluids will also show variability in their levels of α-amylase activity (7, 11, 15, 27). This variability can also have a potential effect on downstream processing, since secondary screening methods target this same enzyme (18, 23).
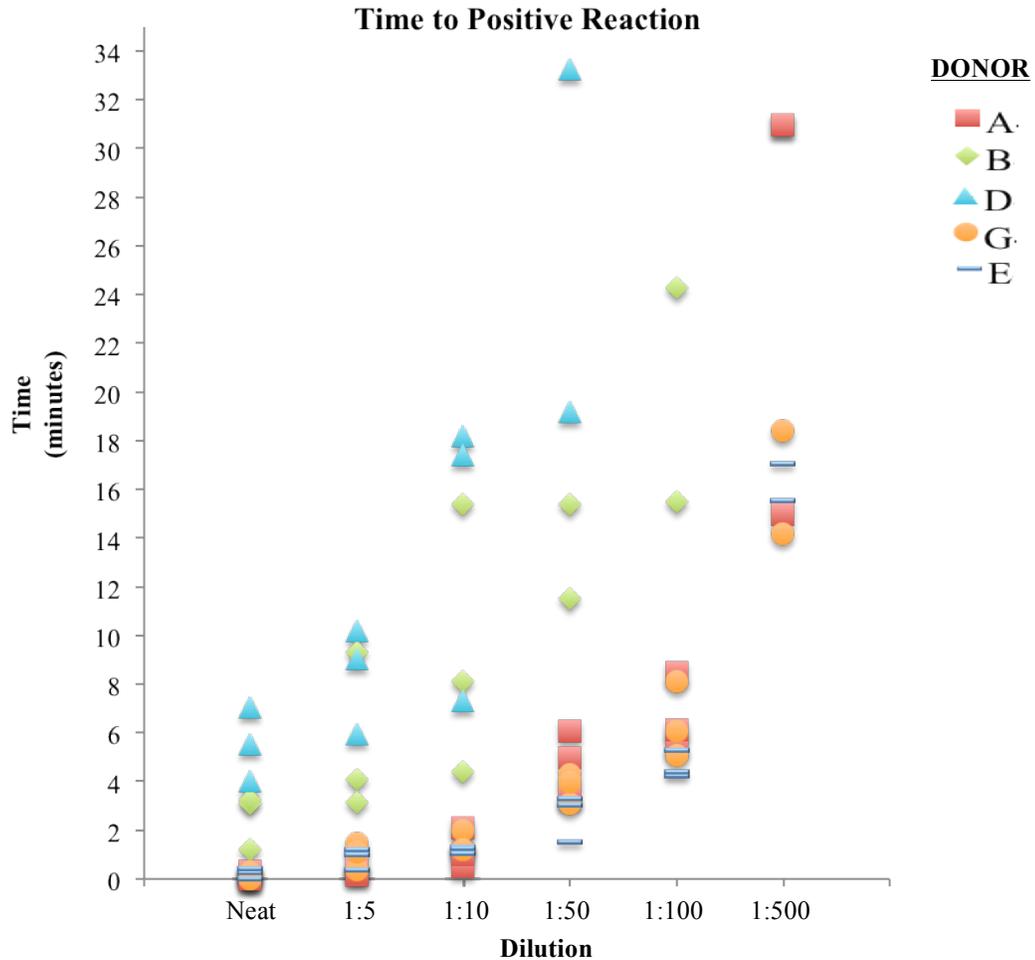
Figure 4. Variability of Reaction Time between and within Individuals. Dilutions of saliva show variability in levels of amylase activity. Time (minutes) refers to the time when a positive reaction with the Phadebas® paper was observed. Stains that reacted outside of the manufacturer's 40-minute protocol were not included.

The protocol provided by the manufacturer recommends a 40-minute window for analysis of a suspected saliva stain, with the first 10 minutes serving as a window in which to distinguish cross-reactivity that may occur later with other relevant body fluids (15). Of the 81 neat saliva stains and dilutions tested, a majority of reactions (90%) occurred within 20 minutes. Only 5% of the remaining samples generated positive results with the 40-minute experimental window set by the manufacturer [Figure 5].

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Distribution of Saliva Reaction Times
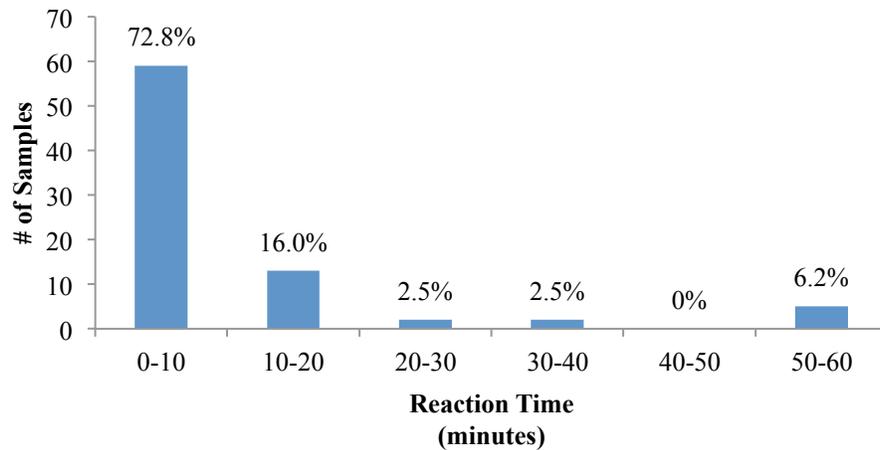
Figure 5. Distribution of Positive Reaction Times for Saliva Samples. Of the 81 saliva samples and dilutions tested, positive results were observed most often within 20 minutes (90%).

Previous research has shown the sensitivity of Phadebas® paper to be equal to approximately a 1:100 dilution, or 0.5µL of saliva in a 50µL stain, and this research supports that conclusion. Although positive results were obtained in more dilute samples (1:500), three of these samples yielded positive results after the manufacturer's recommended 40-minute experimental window.

Based upon these results, as well as others (8, 17, 29), a 40-minute time frame is adequate for saliva screening of most stains that may be encountered during casework. Due to individual variation in amylase activity (7, 27), some saliva stains may not react within the 40-minute time frame of the protocol, indicating that false negative results could occur (15). If evidence is strongly suspected to have saliva present in a particular area, but no visible stains are present and the results of the Phadebas® Forensic Press test are negative, it may be beneficial to conduct further testing with secondary screening

methods such as RSID™-Saliva or Seratec® Amylase, as sensitivity has been shown to be much higher with these immunochromatographic assays (9, 12, 18, 23, 29).

All positive and negative controls provided expected results within the manufacturer's 40-minute time frame.

3.2. *RSID™-Saliva*

Previous studies have shown that the Phadebas® reagent can cross-react with other body fluids, such as semen, urine, and breast milk (5, 15, 17). Due to this lack of specificity, additional analysis is required to further characterize a stain as saliva, if deemed probative. A common assay used as a secondary screening method for human saliva is RSID™-Saliva.

RSID™-Saliva is an immunochromatographic assay that is highly specific in the identification of human saliva by targeting human salivary α-amylase (9, 23, 29, 32). The RSID™ cassette has three regions: sample well, test region and control region. The assay utilizes mobile, dye-labeled, monoclonal antibodies to detect and bind to salivary α-amylase antigens present in the sample. Once bound, the antigen-antibody complexes migrate up the cassette and bind to stationary polyclonal antibodies present in the test region. As more of these antibody-antigen-antibody complexes accumulate, a red line appears indicating a positive result. Unbound antibodies from the sample well serve as a control to indicate whether or not the test is running properly by migrating past the test region and binding to stationary antibodies in the control region (9, 12, 32).

In order minimize the amount of original sample used the RSID™-Saliva kit utilizes a Universal Buffer that is used as both an extraction and running buffer. Additionally, the extract obtained from an item of evidence using the Universal Buffer can also be used with all three RSID™ kits (saliva, semen, and blood) (33).

In comparing the use of fabric versus Phadebas® paper as a source for RSID™-Saliva testing, this experiment showed that more positive results were observed when fabric was used [Tables 1 and 2]. Out of the 81 saliva samples tested, 59 fabric samples returned positive results, in comparison to the 17 Phadebas® paper samples. Furthermore, RSID™-Saliva was able to detect saliva stain down to a 1:100 dilution on a fabric cutting, whereas only a 1:10 saliva dilution could be detected on the Phadebas® paper cuttings. These results indicate that although some detectable levels of amylase are transferred to the Phadebas® paper, use of the original fabric sample provides a more reliable source for detection of saliva.

Table 1. Results of RSID™-Saliva Testing of Phadebas® Paper. Cuttings of Phadebas® paper were taken from regions where a saliva stain generated a positive reaction. The cuttings were extracted and tested for the presence of amylase using RSID™-Saliva. Results were recorded after 10 minutes had elapsed. Positive results are indicated by (+) and negative results are shown as (-).														
Donor	**A**			**B**			**D**			**G**			**E**		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Neat	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
1:5	+	+	-	-	-	-	-	-	-	+	-	+	-	-	+
1:10	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:500	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. Results of RSID™-Saliva Testing of Fabric Swatches. Fabric cuttings were taken from the approximate center of saliva stains that generated positive results. The cuttings were extracted and tested for the presence of amylase using RSID™-Saliva. Results were recorded after 10 minutes had elapsed. Positive results are indicated by (+) and negative results are shown as (-).															
Donor	A			B			D			G			E		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Neat	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:5	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+
1:10	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+
1:50	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+
1:100	+	+	-	-	-	-	-	-	-	+	-	+	+	+	-
1:500	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The manufacturer states that the RSID™-Saliva card can detect down to one microliter of saliva (23), and the results shown here indicate a greater level of sensitivity (0.5µL in a 50µL stain). Other studies have also reported that the RSID™-Saliva card can detect significantly lower levels of saliva, with a limit of detection of 1:500 to 1:1000 dilutions (9, 29).

Whether a Phadebas® paper sample will provide a positive result when tested with RSID™-Saliva could depend on the location from where the Phadebas® paper cutting was taken. The Phadebas® dye diffuses out from the point of contact with a stain containing amylase, and although the blue color on the white side of the paper increases in intensity as the test progresses, the speckled appearance of the reagent side of the Phadebas® paper disappears the longer the paper is in contact with the sample, until the paper becomes white [Figure 6].
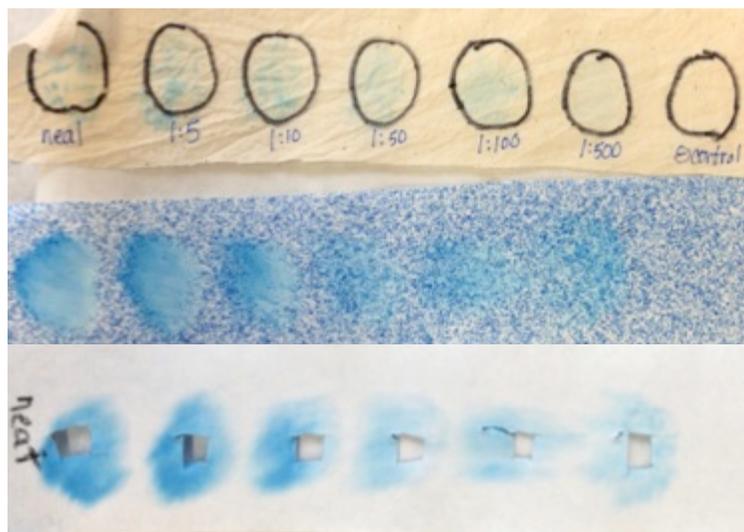
Figure 6. Comparison of Coloration of Positive Phadebas® Results.
Fabric swatch with dried saliva stains following testing with Phadebas®
paper (Top). Darker blue regions indicate positive results from the reagent
side (middle) and white (bottom) side of the Phadebas® paper. Holes
correspond to the areas where the samples were taken from.

Cuttings tested with RSID™-Saliva in this experiment were taken from the
approximate center of areas showing a positive Phadebas® result in an attempt to
correspond with the original deposition of the stain on the fabric. It is possible that
although samples were taken from areas that were in contact with the original stain, the
target analytes may have migrated to other areas of the filter paper due to capillary action
and were no longer present at the point of contact, leading to a negative RSID™-Saliva
result.

A previous study indicated that saliva stains that returned positive Phadebas®
results within 25 minutes gave positive results when analyzed further with RSID™-
Saliva (29). In this experiment, a more limited positive correlation of Phadebas®
Forensic Press test results and RSID™-Saliva results was observed. If a Phadebas® result
was observed within the 40-minute time frame, testing of a fabric cutting from the

original stain using RSID™-Saliva almost always provided a positive result. Additionally, if a saliva stain provided positive results prior to one minute during the Phadebas® Forensic Press test, further analysis of a cutting from the positive area on the Phadebas® paper yielded a positive result with RSID™-Saliva every time. This correlation indicates that if Phadebas® paper is to be used as the source for RSID™-Saliva testing, stains exhibiting positive results prior to one minute should perform similarly to a fabric cutting.

Previous research has also shown that the RSID™-Saliva assay can be performed without the use of the RSID™ buffer, but instead with the supernatant generated during DNA extraction (34). Since sample conservation is key, use of the DNA extraction supernatant can eliminate an additional extraction step and minimize sample loss. Optimization of the extraction procedure is necessary in order to determine if the Phadebas® paper can provide enough sample for down-stream processing.

3.3. *Microscopy*

One purpose of identifying saliva and other biological fluids in a stain is to identify a viable source for DNA, and it is important to determine if a single sampling from an item of evidence is sufficient to obtain a full STR profile. The most efficient processing of saliva evidence for the purposes of generating an STR profile would allow for the same sample to be used throughout the screening, confirmatory, and DNA analyses without ever needing to resample the original stain or swab.

Overall, the fabric extracts contained far more nucleated epithelial cells (NECs) than the Phadebas® paper extracts. Of the 81 extracts examined, 21 of the Phadebas® samples contained NECs, in comparison to the fabric extracts where 65 of the samples contained NECs [Tables 3 and 4].

Table 3. Number of Nucleated Epithelial Cells per Phadebas® Sample. A 3µL aliquot of extract from each Phadebas® paper sample analyzed using RSID™-Saliva was examined for the presence of NECs. Samples were stained using H&E and viewed under a microscope at 400x magnification. The numbers of NECs present in each sample were counted. n/a indicates that the sample was not extracted.

Donor	A			B			D			G			E		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Neat	0	0	4	1	3	11	0	1	0	0	0	0	4	0	32
1:5	1	0	1	0	2	0	0	0	2	0	0	0	0	0	0
1:10	0	0	3	1	3	8	2	1	0	0	0	0	0	0	0
1:50	0	0	0	0	0	0	1	n/a	0	0	0	0	0	0	0
1:100	0	1	0	0	1	n/a	n/a	n/a	n/a	0	0	0	0	0	0
1:500	0	0	0	0	1	n/a	n/a	n/a	n/a	0	0	0	0	0	0

Table 4. Number of Nucleated Epithelial Cells per Fabric Sample. A 3µL aliquot of extract from the RSID™-Saliva assay of the fabric cuttings was examined microscopically for the presence for NECs. Samples were stained with H&E and viewed under a microscope at 400x magnification. The numbers of NECs present in each sample were counted. n/a indicates that the sample was not extracted.

Donor	A			B			D			G			E		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Neat	0	92	3	6	5	143	78	139	1000+	32	20	64	624	3	54
1:5	2	46	0	11	7	91	14	16	139	1	8	21	94	125	1000+
1:10	4	23	3	3	7	43	4	0	12	0	9	11	204	3	216
1:50	1	1	3	2	3	2	19	n/a	23	2	4	0	3	0	45
1:100	0	1	1	3	0	n/a	n/a	n/a	n/a	3	1	1	13	0	4
1:500	0	0	0	0	2	n/a	n/a	n/a	n/a	3	2	0	4	0	10

Due to the abundance of NECs present in neat saliva, it would be expected that an extract from positive Phadebas® paper corresponding to a neat saliva stain would contain

NECs; however, in this experiment, out of the 15 neat samples tested, only 7 of the 3μL pellet extracts of Phadebas® cuttings from neat saliva contained NECs. In contrast, the 3μL extracts from all but one fabric cutting of neat saliva stains contained nucleated epithelial cells. Although about half of the extracts from Phadebas® paper of neat saliva samples contained epithelial cells, the numbers of cells present were typically far lower than the number of cells present in the fabric extracts of the same sample [Figure 7].
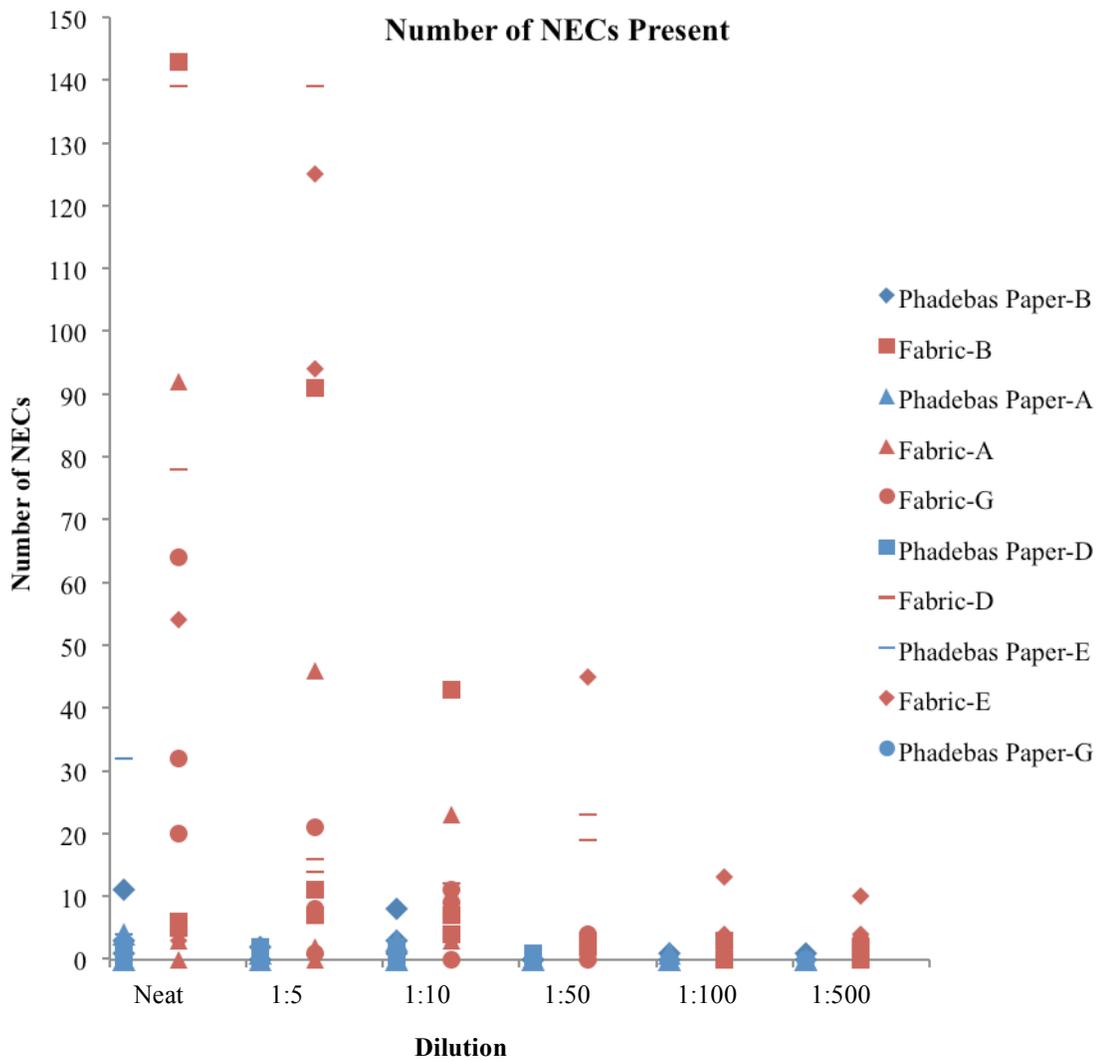

Figure 7. Comparison of NECs Present in Phadebas® Paper and Fabric Extracts. The numbers of NECs present in the 3μL fabric extracts were greater than those present in the Phadebas® extracts. The five fabric samples in which >200 NECs were present were not included.

Since only 3μL of extract was utilized, there is a possibility that the aliquot examined was not representative of the extract as a whole. After centrifugation, the pellets from the fabric cuttings were highly visible, but those for the Phadebas® cuttings were typically unable to be observed by the naked eye. Due to the difficulty in determining where the pellet was located in the extract, it is possible that the aliquot removed for the generation of the slide may not have contained any NECs, even if such cells were present in the extract. Microscopic examination of the whole extract pellet would give a better indication of whether or not a Phadebas® paper cutting is a sufficient alternative to a direct cutting of a saliva stain.

The source of DNA from saliva for STR analysis is shed epithelial cells from the oral cavity (1, 8) and if sufficient nucleated epithelial cells (NECs) are present in an extract of the Phadebas® paper, it would suggest that the Phadebas® paper itself could provide enough DNA for an STR profile. Similar to amylase levels, the amount of epithelial cells present in a saliva sample varies by individual, as some will shed far more epithelial cells than others (1, 35), causing the amount of DNA to be highly variable in saliva samples. Research has previously shown that Phadebas® paper has the potential to be used as an alternative to a fabric cutting for PCR-based DNA analysis (8). Some dyes, such as those found in denim, are known to inhibit the PCR process (24). Although the Phadebas® reagent utilizes a dye, previous research has concluded that the dye used does not inhibit DNA PCR analysis (8), supporting the use of Phadebas® paper as an alternative to fabric cuttings containing known inhibitors during forensic casework processing.

Currently, standard forensic protocols call for between 0.75-2.5ng of DNA to be used for the generation of an STR profile (36, 37), with a general recommendation of 1ng of DNA. These amounts of DNA have been shown to minimize artifacts that may increase the difficulty of interpretation of an STR profile (36-39), or result in improper inclusions or exclusions of individuals. For this experiment, the number of cells present in each sample was multiplied by 6.7 picograms, which was previously determined to be the approximate amount of DNA present in a single cell (24), and then converted to nanograms (ng). As the optimal range for generation of an STR profile is between 0.75-2.5ng, 0.75ng (approximately 112 epithelial cells) was used as the threshold value to determine if a particular sample would be expected to generate a full STR profile.

Only nine of the fabric samples contained enough NECs to meet the 0.75ng threshold within the 3μL sample examined. The greatest dilution of saliva that met this threshold was a 1:10 dilution; none of the Phadebas® paper samples met this criteria.

A second threshold, 0.25-0.75ng, was set to indicate whether or not a partial profile could likely be obtained with the sample. Six fabric samples met these criteria, with the lowest concentration being a 1:10 dilution; again, all of the Phadebas® paper samples were below this threshold. All remaining samples, 66 in total, were judged to contain insufficient amounts of DNA for generation of either a full or partial STR profile.

The fabric utilized in this experiment could have had an impact on the amount of epithelial cells transferred to the Phadebas® paper. Previous research has shown that less absorbent materials, such as leather or painted wood, provided full STR profiles for dilutions down to 1:500 from Phadebas® paper samples corresponding to saliva stains.

Phadebas® paper samples corresponding to saliva stains on more porous materials tested (denim, cotton and wood) resulted in levels of DNA that were insufficient to produce profiles (8), in concordance with the results observed during this experiment, which utilized a porous fabric as well.

Previous research has indicated that in order to obtain an adequate quantity of epithelial cells from saliva for generation of an STR profile, a combination of two swabs (one moistened and one dried) was required (40). This 'double swab technique' rehydrates and loosens cells with the first swab and the subsequent application of the second, dry swab against the stain provides an optimal location for these cells to adhere in sufficient quantities for generation of a DNA profile (40). Since the Phadebas® paper is gently laid upon the fabric, there is no movement of the paper against the stain or epithelial cells present, which could explain why epithelial cell transfer from the fabric to the Phadebas® paper was minimal.

Despite the low numbers of epithelial cells present within these samples, it is possible that STR profiles could be obtained with lower than the optimal value of 0.75ng of DNA (36-39). Research has shown that DNA profiles can be obtained with as little as 125pg of DNA (38, 39, 40), indicating that with further testing, some Phadebas® samples could meet this threshold, allowing for generation of a partial or full STR profile when another sample is not available.

3.4. *Phadebas® Cross-Reactivity Study*

3.4.1. Semen

Within the 60-minute time frame allotted for the experiment, there were no semen samples that gave a positive result, indicating the Phadebas® paper has no cross-reactivity with semen. The literature varies in concluding whether or not semen stains will cross-react with Phadebas® paper. Several studies, in agreement with these results, report no reactivity with semen and the Phadebas® reagent (3, 17, 42, 43). A few other studies provided instances where a single semen sample had a higher level of α-amylase than a positive control buccal swab (9, 11, 17), but all other semen samples tested returned negative results.

It has been suggested that higher than normal levels of amylase found in semen samples could be an artifact of the collection process (44). Since saliva can be used as a lubricant during masturbation (9, 45), its use can potentially contribute to the amount of amylase detected in donated semen samples. In case work, samples could not only have saliva present due to masturbation, but also as a result of prior consensual sex (46-48), increasing the likelihood of saliva contamination on swabs from sexual assault kits or with other items of evidence.

If Phadebas® paper is used to locate stains on an item of evidence, it must be sensitive enough to detect the saliva present, and still allow for semen testing to be subsequently performed (7, 10). In cases where both semen and saliva are expected to be present, the manufacturer indicates that Phadebas® paper can be used in conjunction with semen screening, and recommends that the AP Spot test be conducted on the Phadebas®

paper after a stain has been analyzed for saliva (15). The use of a similar product for the detection of amylase, Red Starch paper, has shown that screening for both saliva and semen can be performed on the paper (10). Since semen has previously been shown not to generate positive results with the Phadebas® paper, (3, 17, 42, 43), in concordance with the current study, any positive results observed before AP Spot testing are indicative of saliva.

The levels of amylase typically found in semen have been previously reported to be between 3-200 IU/L, which is far below the average level found in saliva of 263,000 to 376,000 IU/L (7, 15, 42). Although no positive results were observed during the 60 minutes, some semen stains showed diffuse blue coloring on the Phadebas® paper after it dried, mimicking a positive reaction [Figure 8].

Figure 8. Positive Result with Neat Semen Sample. Diffuse coloring visible after reaction with 50µL of neat semen and drying of Phadebas® paper.

As long as a stain is in contact with the moistened Phadebas® paper, any active amylase present in the stain will continue reacting with the Phadebas® reagent. Since the reaction is water-dependent, it is only stopped once the paper has dried. To eliminate the possibility of positive results with semen, the 40-minute time frame established by the

manufacturer should be strictly followed, and samples suspected to contain both saliva and semen should undergo further analysis.

3.4.2. *Urine*

The results from the cross-reactivity study with urine show that neat urine stains can have similar levels of amylase activity as those in dilute saliva stains [Table 5]. Of the 15 urine stains tested, nine neat and one 1:5 dilution returned positive results within the 60-minute experimental period. Of the 10 samples where positives were recorded, only four were within the recommended experimental window of 40 minutes, with the remaining six positive results occurring between 40 and 60 minutes. These results support other studies that show no neat urine samples generated a positive Phadebas® results within 10 minutes (17, 29). Two studies obtained positive results with one or more samples with the Phadebas® or Red Starch paper (10, 17), but these occurred after the 10-minute cut off established to eliminate positive reactions due to non-saliva body fluids.

Table 5. Results of Cross-Reactivity Study with Urine. Five urine samples (4 females, 1 male) were diluted (1:5 and 1:10) with distilled water. The dilutions and neat sample were tested for reactivity with Phadebas® paper. Positive results are indicated by (+) followed by the time that this reaction occurred (minutes:seconds). Negative results are shown as (-), and indicate that no reaction was observed at the end of 60-minute test period.

	Neat			1:5			1:10		
Donor	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
A	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-
C	(+) 53:00	(+) 53:00	(+) 45:00	-	-	-	-	-	-
D	(+) 46:00	(+) 55:00	(+) 35:35	-	-	-	-	-	-
E	(+) 31:35	(+) 31:35	(+) 37:55	(+) 56:26	-	-	-	-	-

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The color change observed on the Phadebas® paper from reactions with urine stains was less intense than those present with neat saliva stains [Figure 9A and 9C], and for all but one stain (D-3) occurred outside the recommended 40 minute experimental time frame. However, one donor's neat urine stains did generate a color change similar to that of a neat saliva stain [Figure 9B], and also returned positive results within the 40-minute time frame.
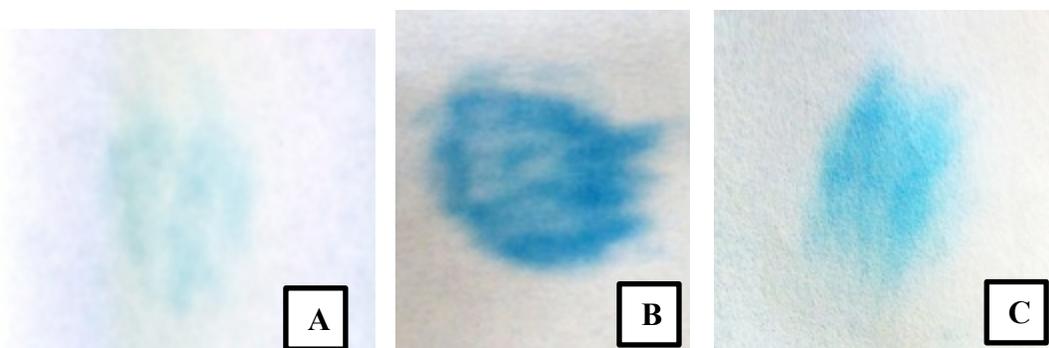

Figure 9. Comparison of Neat Urine Stain Reactions. Results at the end of 60-minute experiment. (A) Diffuse positive reaction with 50μL neat urine stain. (B) Positive reaction observed with a 50μL neat saliva stain. (C) Intense positive reaction from 50μL neat urine stain.

The inconsistent results when testing urine for cross-reactivity is likely due to the varying levels of α-amylase between individuals – certain individuals have high enough α-amylase levels for detection, and others have levels that are too low. The level of amylase activity in urine has been previously reported to be between 263-940 IU/L, which is lower than the average level found in saliva of 263,000 to 376,000 IU/L (7, 15), indicating that screening techniques should be able to discriminate between saliva and urine stains.

The Phadebas® chemistry is based on the enzymatic activity of amylase; thus, reactions can occur with samples that contain pancreatic amylase (17, 25, 29), such as

urine (49, 50), in addition to samples containing salivary amylase. In cases of sexual assault, where the underwear of an individual may need to be analyzed, there is a possibility of a mixture of body fluids to be present. The stains present could be due to semen, vaginal secretions, urine, fecal matter, or saliva. To address the possibility of positive results observed with Phadebas® paper, secondary screening techniques are necessary to eliminate any non-probative stains.

Based upon the results of this experiment, it is possible for a neat urine stain to obtain a positive result within the manufacturer's 40-minute time frame; however, the 10-minute window for cross-reactivity is shown to be a sufficient threshold in discriminating between urine and saliva. In cases where urine and saliva are suspected to be present, and a positive reaction is observed, especially if the reaction occurs after 10 minutes, the stain should be further characterized with a secondary screening technique.

3.4.3. Whole Blood

All blood samples gave negative results within the 60-minute time frame allotted for this experiment. In a scenario where expired blood is suspected, it may be important to identify the presence of saliva in order to determine the likely sequence of events. Blood-saliva mixtures could also be present in cases of sexual assault or physical assault, if trauma were to occur during the act. Thus, either body fluid should be able to be detected without affecting the subsequent detection of the other.

Whole blood has been shown to decrease α-amylase activity in saliva, and the interference is speculated to be due to its high protein content (28), indicating that if a

stain containing both blood and saliva were found, a false negative could occur with Phadebas® paper. Previous Phadebas® research performed using mixtures of both saliva and blood yielded positive results (3, 29, 43), indicating that the presence of blood does not interfere with the Phadebas® chemistry. In the present experiment, the color of the bloodstain rendered it difficult at times to determine if a false positive result had occurred [Figure 10].
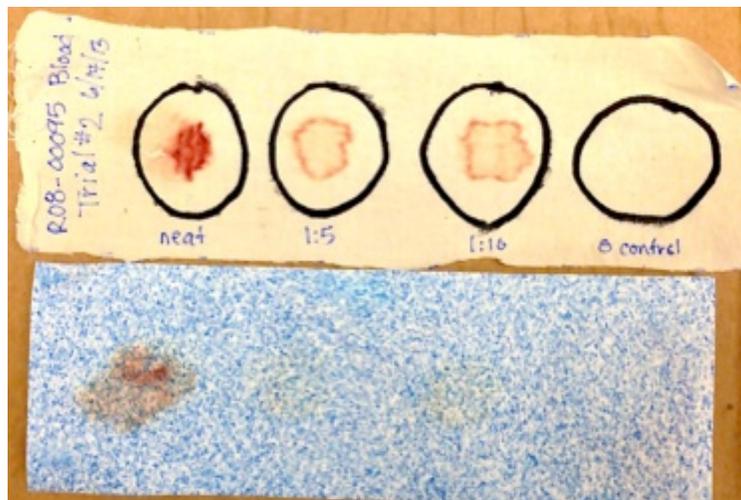
Figure 10. Transfer of Whole Blood onto Phadebas® paper. Samples of whole blood were diluted with distilled water and 50μL applied of the dilutions, neat and negative control, to specific areas of labeled fabric swatches. Transfer of sample as seen at the end of the 60-minute cross-reactivity study.

The transfer of blood onto the Phadebas® paper was also reported in a previous study (3). Although the blood did not generate a positive result, the transfer could interfere with the interpretation of a positive result. If the red color from the blood was strong enough to be visualized on the white side of the Phadebas® paper, this could mask any blue color that would normally appear with a reaction with saliva.

3.4.4. *Fecal Swabs*

Positive reactions were achieved with 14 out of the 15 fecal swabs tested during the course of this experiment [Table 2]. Eight of the 15 samples generated a positive result within the first 10 minutes, making it the one sample type other than saliva that reacted within this window. All positive reaction times were within the 40-minute time frame for the testing recommended by the manufacturer, thus, the Phadebas® paper is shown here to be unable to distinguish between these two types of body fluids. These results are in concordance with other studies (10, 17, 29), and potential cross-reactivity is acknowledged by the manufacturer (15).

Table 6. Results of Cross-Reactivity Study with Fecal Swabs. Three swabs from 1 male and 4 females were tested for reactivity with Phadebas® paper. Positive results are indicated by (+) followed by the average time (minutes:seconds) required for the reaction to be observed. Negative results are shown as (-).		
Donor	**1**	**2**	**3**
A	(+) 3:00	(+) 2:50	(+) 2:20
B	(+) 1:45	(+) 1:30	(+) 1:45
D	(+) 20:00	(+) 19:30	(+) 18:30
E	-	(+) 16:00	(+) 18:00
F	(+) 8:00	(+) 7:00	(+) 10:00

Despite high cross-reactivity, distinct differences between fecal swabs and saliva stains can aid the analyst in determining if a stain is contaminated with fecal matter and thus, is appropriate for saliva testing (9). The fecal stains on swabs were highly visible, in comparison to positive control buccal swabs, where no stains were visible. Distinct colors and odors were also present, which have also been used in characterizing a stain as fecal material versus saliva (9, 10, 26). However, it cannot be assumed that all fecal matter

stains would be apparent on an article of clothing (17), particularly on dark fabrics or items heavily stained with blood, soil or other contaminants.

Fecal material can contain concentrations of amylase at similar levels to those found in saliva (26). The amount of amylase present in fecal samples is due to the combination of both salivary and pancreatic α-amylase, although the level of pancreatic amylase is significantly higher than that of salivary α-amylase (51). Salivary and pancreatic α-amylase cannot be distinguished by the Phadebas® paper as the chemistry of the Phadebas® reagent is based upon the enzymatic action of amylase, which does not vary between these two isoforms (29). Since salivary and pancreatic α-amylase have the same function, it would be expected for the two samples to react similarly to the Phadebas® paper. Consequently, if both fecal matter and saliva are expected to be present on a sample, the use of the Phadebas® Forensic Press test will only be able to identify that amylase is present, and further characterization of the stain will be necessary (9, 15).

3.4.5. Vaginal Swabs

Of the 15 vaginal swabs tested, none gave a positive result within the 60-minute time frame for these experiments, indicating that the Phadebas® paper can discriminate between vaginal secretions and saliva. Vaginal secretions have relatively low levels of α-amylase (less than 100 IU/L) (26, 28). Based upon these values, typical vaginal samples would not be expected to generate false positive results. Other studies have also reported that vaginal secretions did not react with the Phadebas® paper, which further supports the

10-minute cut-off for discriminating saliva from other forensically relevant stains (25, 29). In contrast, several studies reported that vaginal samples may contain levels of α-amylase that could cause false positive reactions with Phadebas® paper (26, 28), but false positive results were obtained with only a single sample (17).

In cases of sexual assault, saliva and vaginal secretions may be present on swabs or on collected items of evidence, such as underwear. If oral assault is suspected to have occurred on a female victim, testing for the presence of saliva is often conducted in order to support the allegations.

Previously, Lugol's iodine has been used to discriminate between vaginal secretions and saliva. Lugol's iodine stains glycogenated epithelial cells a chocolate brown color; samples where greater than ten percent of the nucleated cells present contain glycogen are presumptively considered to be vaginal in origin (52, 53). This method does not confirm the presence of vaginal secretions, as oral samples and penile swabs can contain low amounts of glycogenated cells (53-55). RSID™-Saliva has been shown to not cross-react with vaginal secretions (9, 12). Thus, the combination of positive Phadebas® and RSID™-Saliva results, is highly indicative of saliva (52, 54).

3.4.6. *Condoms & Personal Lubricants*

None of the brands tested generated positive results, indicating that neither the lubricants, nor the condoms themselves, interact with the Phadebas® chemistry in the absence of biological material. These results are similar to those reported previously (10)

and support the use of Phadebas® paper on swabs from condoms or sexual assault kits where lubricants may be present and saliva is suspected.

It is becoming increasingly necessary to be aware if condoms and/or lubricants were used during a sexual assault case, as perpetrators are now often utilizing condoms when committing these acts (10, 56-58). The use of a condom by an assailant can limit the amount of assailant DNA recovered, unless the condom itself is retrieved (56, 58). If it is suspected that a condom was used during a sexual assault, or if a condom is recovered from a sexual assault and saliva is suspected to be present, either from oral contact or through other means, any method used in the testing for the presence of saliva should, ideally, not interact with condoms or lubricants.

Condoms are composed of latex or non-latex materials, including polyurethane and lamb caecum, which are then given a particulate coating, similar to that found in latex exam gloves, to aid in application of lubricant and to prevent self-adhesion (57). The majority of condoms are pre-treated with water or silicone-based lubricant (56, 57, 59).

Silicone-based lubricants are composed of polydimethysiloxane (PDMS), and are water insoluble. This property allows for these types of lubricants to be detected for a significant period of time following use (56, 57). Alternatively, water-based lubricants are composed of polyethylene glycol (PEG) (56). These lubricants are water-soluble, making their persistence in sexual assault cases rare, as the body readily absorbs these types of substances (57). Both types of lubricants are often combined with a spermicide (57), most commonly nonoxynol-9 (56, 59).

Previous research with lubricants has shown that lubricants can result in false negative semen results or completely inhibit testing with immunochromatographic techniques, such as ABAcard® p30 or Seratec® Semi-quant PSA card (59, 60). This was reported to be due to the viscosity of the lubricant, which did not allow for the sample to migrate up the cassette membrane (59, 60). Research has also shown that certain condom lubricants can result in false positives when tested using p30 immunoassay cards (59, 60), particularly lubricants that contained nonoxynol-9 (59). In the present study, only one of the condom brands contained a spermicidal lubricant, Trojan® Enz® Armor™, and did not generate different results from the other condoms and lubricants tested; no cross-reactivity was observed with any of the condoms and lubricants brand tested.

3.4.7. *Distilled White Vinegar*

None of the distilled white vinegar stains analyzed during this experiment generated a positive result with Phadebas® paper, indicating that there is no cross-reactivity with common household vinegar.

The findings of physical trauma during a sexual assault examination can be important, but genital trauma is not always readily visible (45, 61, 62). The use of toluidine blue dye has been incorporated into sexual assault examinations in some hospitals to aid in the detection of physical trauma in the genital region (45, 62, 63). Since the outermost layer of the skin does not contain nuclei, damage to the skin can expose deeper tissue containing nuclei allowing for nuclear stains, such as toluidine blue, to be applied for visualization of lacerations or trauma present (45). After the application

of toluidine blue, a 1% acetic acid solution or medical lubricant may be used to remove excess dye (45, 62, 63).

Furthermore, vaginal douches commonly are a mixture of vinegar and water (64, 65). It is possible that following a sexual assault a victim may use a douche before seeking medical attention (66). Due to the likelihood of sexual assault kit swabs having exposure to vinegar, either through the use of douches or in medical procedures, it is important to assess whether or not vinegar reacts with the Phadebas® paper (45).

A validation study in which vinegar cross-reactivity was tested with Phadebas® paper yielded negative results for all samples tested (25), but no other studies regarding cross-reactivity with vinegar could be identified. Previous studies have tested the cross-reactivity of vinegar with other common forensic assays, such as catalytic color tests for blood and semen, and showed that vinegar does not generate false positives during testing (67, 68). However, dilutions of 3% of vinegar have been shown to generate false positive semen results with ABAcard® p30 (69).

Common household vinegar is typically between 4-7% acetic acid (70), which is stronger than the 1% dilutions typically used in sexual assault examinations (45, 62, 63). Both commercial and homemade douching products can vary in the amount of vinegar in the solution (65, 71, 72). The dilutions in this study ranged from 5% to less than 1%, indicating that the amount of vinegar that may be encountered during a sexual assault examination should not react with Phadebas® paper. However, if the victim utilized a douche following the sexual assault, it is possible that the concentration of vinegar could be significantly higher on swabs collected.

3.4.8. PBS

All PBS stains tested with Phadebas® paper gave negative results within the 60-minute time frame for this experiment. These results indicate that positive results achieved with dilutions generated with PBS are not due to the addition of PBS.

As it is common for PBS to be used to generate dilutions or negative control samples (8, 9), it is important to ensure that the Phadebas® paper does not intrinsically react with this solution. It has previously been reported that Red-Starch paper does not cross-react with PBS (10), and although the Phadebas® Forensic Press test is based upon the same chemical interaction, the direct testing of the Phadebas® paper with PBS is necessary. The results presented here, in concordance with previous studies (8, 10), demonstrate that both PBS and water are appropriate for use with Phadebas® paper.

4. Conclusions

In sexual assault cases, items of evidence may need to be screened for both saliva and semen (10). As it is possible that saliva could have been used as a lubricant for intercourse (46, 48) or its presence could be due to oral assault (29), determining the composition of biological stains is important to substantiate a victim's story or a case theory.

In the cross-reactivity study, the Phadebas® Forensic Press test showed cross-reactivity with urine and fecal matter within 60 minutes, and after the 60-minute experimental time frame with semen. Although all of these body fluids were shown to cross-react to some degree, positive reactions within the manufacturer recommended 40-

minute test window were observed only with urine and fecal matter. Fecal matter was the only biological sample that cross-reacted within the 10-minute window outlined in the protocol for discriminating between saliva and other amylase-containing body fluids. Therefore, any stain that may contain both saliva and fecal matter requires additional analysis to determine the source of the positive result. No positive results were obtained with whole blood, vaginal swabs, condoms/lubricants, vinegar and PBS.

Due to the potential for cross-reactivity with some body fluids, the evidence/case information is crucial in order to properly evaluate results of the Phadebas® Forensic Press Test. For instance, if underwear were being examined, it would be expected that urine, fecal matter, semen, or vaginal secretions might be present. Visual examination would be key, as fecal matter and urine both have distinct features that could distinguish them from other body fluids present – although these features may not always be present due to washing of the item or other environmental factors (9, 10, 17). Saliva, unless suspected based on case information, may not believed to be present on an item of evidence; however, it has been detected on waistbands, collars, cuffs, and around button holes as a result of normal everyday wear and activities (73). The Phadebas® Forensic Press test is shown here to be a sufficient method for screening an article of evidence for biological fluids, and any positive result observed should be analyzed further using secondary screening or confirmatory methods.

It is important to conserve sample when processing an item of evidence to ensure enough remains for subsequent testing, DNA analysis or independent testing by another party. Therefore, the most efficient protocol would use a single sample from an item of

evidence to perform both primary and secondary biological screening for characterization of the stain, as well as DNA analysis, to eliminate unnecessary sample loss.

To test the efficacy of a single sample protocol, saliva stains on fabric and Phadebas® paper exposed to saliva stains were tested using RSID™-Saliva and microscopically examined for the presence of nucleated epithelial cells. The results of the RSID™-Saliva experiment indicate that the use of a fabric cutting, versus a cutting from a positive area of Phadebas® paper corresponding to a stain, provides a more consistent and reliable source of amylase for use with this assay. The microscopic examination of 3μL of extract pellets indicated that only the fabric samples contained enough nucleated epithelial cells to generate a full STR profile using current DNA analytical techniques.

In conclusion, using Phadebas® Press test paper as a source of biological material for immunochromatography or DNA analysis is less than optimal and should be avoided when additional sample is available for direct testing.

4.1. *Future Directions*

Although only fecal matter and urine provided positive results in this study, further testing is required to determine if detection of saliva by the Phadebas® Forensic Press test is inhibited by the presence other biological fluids, chemicals or products in a stain or on a swab. It was shown here that blood was visibly transferred to the Phadebas® paper. Since this could interfere with interpretation of results, the extent of the effect blood may have on interpreting a Phadebas® result if a stain consisting of both saliva and blood were to be present should be determined.

Since saliva can be found in the presence of other body fluids, the single-source protocol should be expanded to determine if Phadebas® paper could be used in both primary (catalytic color tests) and secondary (immunoassays) screening tests for other body fluids, such as blood and semen.

The only interaction observed from either a condom or lubricant with the Phadebas® paper was that the lubricant created a spot, similar to an oil-spot on paper. Due to the water insolubility of silicone-based lubricants, the presence of this type of lubricant could result in false negatives if saliva present is unable to interact with the Phadebas® paper. Additional testing is required in order to determine if the presence of a silicone-based lubricant will inhibit the Phadebas®-saliva interaction.

Although the use of Phadebas® paper as a source of biological material did not prove to be highly successful, optimization of the single source protocol may improve sample transfer and recovery. First, the optimum location from where cuttings from the positive region of the Phadebas® paper should be taken should be studied. It is possible that cuttings in this experiment were taken from areas that no longer contained the components from the original saliva stain due to sample migration, which would diminish the amount of both amylase and epithelial cells present for additional testing. In addition, the type of fabric may have also played a role in the amount of amylase and epithelial cells transferred to the Phadebas® paper and in the extraction of the samples. In order to address the variation in substrates that may be encountered in case work, additional types of fabrics and non-porous surfaces should be tested to determine the scope of this interference with testing.

The use of Phadebas® paper in secondary saliva screening with immunochromatography should also be tested using the Seratec® Amylase assay. The Seratec® Amylase test is an immunoassay card that targets salivary α-amylase, is run similarly to the RSID™-Saliva card and also provides results in 10 minutes (18). In-house validation of the Seratec® Amylase card shows its sensitivity to be 1:1000, which is similar to those reported with RSID™-Saliva (9, 29), but no research has been published to corroborate these results. Research is also needed to determine if the RSID™-Saliva buffer is appropriate for DNA extraction and quantification.

The microscopic analysis of the aliquots from both the fabric and Phadebas® paper provided only an estimate of the amount of DNA present in the entirety of the sample extracts. Further quantitative testing of the Phadebas® paper extracts using qPCR is required to evaluate the use of Phadebas® as a source for DNA analysis in place of a swab or cutting from a sample or stain. Additionally, the presence of a DNA stabilizer in RSID™ Universal Buffer indicates that the DNA present in the sample should be preserved and able to be processed using common DNA extraction techniques and quantified using qPCR, with little degradation. In-house validation of the performance of an RSID™ extract (sample + RSID™ Universal Buffer) followed by Chelex® DNA extraction reported that STR profiles were obtained from samples that gave positive RSID™-Saliva results (32).

LIST OF JOURNAL ABBREVIATIONS

Am J Emerg Med   American Journal of Emergency Medicine

Am J Hum Genet   American Journal of Human Genetics

Am J Public Health   American Journal of Public Health

Anal Biochem   Analytical Biochemistry

Ann Emerg Med   Annals of Emergency Medicine

ANZFSS   The Australian and New Zealand Forensic Science Society

Biochem Med Metab B   Biochemical Medicine and Metabolic Biology

BMB Rep   Biochemistry and Molecular Biology Reports

Clin Chem   Clinical Chemistry

Digest Dis Sci   Digestive Diseases and Sciences

Fertil Steril   Fertility & Sterility

Forensic Sci   Forensic Science

Forensic Sci Int   Forensic Science International

Forensic Sci Int Gen   Forensic Science International: Genetics

IDOG   Infectious Diseases in Obstetrics and Gynecology

JAIDS	Journal of Acquired Immune Deficiency Syndromes
J Forensic Sci	Journal of Forensic Science
J Forensic Sci Soc	Journal of the Forensic Science Society
JOGNN	Journal of Obstetric, Gynecologic & Neonatal Nursing
Med Gen Med	Medscape General Medicine
Sci Justice	Science & Justice
Women Health	Women & Health

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VITA

NICOLE MARIE DAVIDEK

1988

317 Allston St. #16
Brighton, MA 02135
(775) 762-2781
nicole.davidek@gmail.com

EDUCATION

M.S. Biomedical Forensic Sciences   expected January 2014

   Boston University School of Medicine   Boston, MA

   3.67 current GPA

B.S. Biology with a minor in Nutrition   May 2011

   University of Nevada, Reno   Reno, NV

   3.86 GPA, graduated with Honors, *Cum laude* and with Distinction

RESEARCH

Master's Thesis
   Evaluation of Phadebas® Forensic Press Test as a Source of Biological Material for Immunochromatographic Testing and DNA Analysis

Undergraduate Thesis
   Comparison of the ROCK2/RhoA Ca^{2+}-sensitization pathway in both cytosolic and membrane fraction of murine colon smooth muscle
   Presented at Biology Honor's Day 2011

AWARDS/PROFESSIONAL MEMBERSHIPS

Phi Kappa Phi Honors Society, *Member*   2011-Present

Golden Key Honors Society, *Member*   2011-Present

UNR, College of Science Dean's List   2007-2011