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Transfer, Persistence and DNA Source Attribution of Trace Biological Material in Digital Penetration Assault Cases

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I. Purpose and goals

According to current RAINN (Rape, Abuse & Incest National Network, www.rainn.org) statistics: a person in the U.S. is assaulted every 68 seconds [1], every 9 minutes that person is a child [2], and only 25 out of every 1,000 perpetrators will end up in prison (www.rainn.org/statistics/criminal-justice-system). These statistics are sobering and do not tend to improve year to year. Sexual assault (SA) is commonly thought of as penile penetration of the vagina, without consent from the victim. Only in 2011 was the Uniform Crime Report definition of rape updated from an 80-year old definition to include the following definition of rape: “penetration, no matter how slight, of the vagina or anus with any body part or object, or oral penetration by a sex organ of another person, without the consent of the victim” [3]. Penetration with any body part – specifically digital penetration (penetration with fingers) – is the subject of the proposed work.

Digital penetration as a form of sexual assault has gained much attention recently in the media with the recent highly publicized case of Dr. Larry Nassar, Michigan State University and USA Gymnastics medical doctor. According to a 2018 BBC News report, Judge Janie Cunningham stated that “we have over 265 [victims] identified.” [4]. Many victim accounts describe the use of so-called ‘medical treatments’ to disguise the assaults, mainly penetration of the vagina and/or anus (without gloves or consent). In a 2016 article in the by Gallion et.al. [5], they assessed ~1500 child patients from October 2010 to June 2013 at an outpatient clinic that provides specialized forensic medical exams to children if there are concerns or reports of sexual abuse. Out of the cases where children were able to report or describe the type of abuse, 56% (279 out of 502) indicated genital penetration, with 74% of that (207/279) involving penile penetration and 37% (104 of 279) involving digital penetration. According to personal communications with a forensic science

county laboratory in the Mountain West subregion of the country (private communication, agency asked name not to be reported), in 2020 out of 150 sexual assault cases, 47 (or 31%) of the cases had reported digital penetration (although none indicated that only digital penetration occurred, and all cases had other types of penetration reported as well the only type of assault). Valentine et. al. [6], reports that amongst 118 SA cases of groping/fondling, 47 of these cases involved digital penetration of the vagina with finger(s). Fifty-four of the cases were classified as groping without contact of bodily fluids (groping/fondling of victims' external genitalia and/or breasts). From the 118 cases, 42 sexual assault kits (SAKs) were sent to the state crime laboratory for analysis. Six of these kits resulted in full or partial STR profiles from touch contact, three of which included vaginal swabs for this analysis [6]. All of these reports, individually and collectively, highlight the frequency and significance of digital penetration thus necessitating research to be done in order to better understand and analyze this type of challenging evidence.

Digital penetration cases will be challenging due to the presence of trace amounts of male skin epithelial cells amongst an overwhelming majority of vaginal epithelial (digital penetration of a female by a male) and/or skin epithelial (digital fondling or assault to external vaginal areas). Not only is the amount of male epithelial cells a challenge in these cases, it is also the nature of the epithelial cells themselves. Surface skin that is transferred during these types of assaults is considered 'touch DNA' deposits or shed skin cells which may or may not contain DNA [7, 8]. Standard methods for the recovery of touch DNA includes swabbing the suspected area with a cotton swab and subsequently extracting the totality, or a significant portion, of the DNA from the cotton [7, 9-12]. In the cases of digital penetration, the victim's cellular material could be in vast excess with any trace cellular DNA from the assailant being masked when standard autosomal STR profiling is performed. Of course, a potential solution to this situation is the use of Y-

chromosome STR (YSTR) analysis which will essentially ignore the significant amount of female DNA and target specifically the male DNA in the sample, whether that is male epithelial cells or sperm. Only a handful of studies have been published that involve an analysis of challenging digital penetration or no-sperm sexual assault cases.

II. Project design

In this work, we sought to develop a full rapid digital penetration evidence processing workflow that would assess not only the ability to recover probative DNA profiles in digital penetration samples but to uniquely provide critical contextual information by means of mRNA body fluid identification (BFID) that will provide support for determining the nature or circumstances of the digital assault. The aims of the project were: 1) optimization of a customizable workflow for the rapid processing of digital penetration samples, 2) evaluation of a “training” set of samples consistent of non-casework voluntary digital penetration samples from 4-5 participating couples and 3) utilization the final optimized workflow for the rapid processing of digital penetrations samples to evaluate a larger number of donor sets. Work on Aim 3 is still in progress as we recruit more donor sets to continue to evaluate the workflows designed in this work and to provide the forensic community with valuable information for the routine analysis of digital penetration samples including: 1) an evaluation of the ability to obtain DNA profiles from trace biological material in digital penetration cases (from victims as well as suspects), 2) an optimization of both standard and enhanced STR typing strategies for the analysis of trace biological material, 3) a determination of the interval in which DNA profiles and BFID results can be obtained from digital penetrations samples and 4) recommendations on the time interval and optimal sampling locations for digital penetration evidence.

III. Methods

Sample collection

All body fluid samples were collected with written informed consent from volunteer participating couples using procedures approved by the University of Central Florida's Institutional Review Board. All samples were deidentified prior to testing and analysis.

Five donor couples were asked to collect various samples 1, 3, 6, 12 and 24 hours after digital penetration. Only one time interval was collected after an individual act of digital penetration. After samples were collected, donor couples were asked to abstain from sexual activity during the collection interval time frame as well as for 2-3 days after collection before starting the next time interval set. This was done to ensure that biological material detected in this study was due to the act of digital penetration and not prior or other sexual activity.

From the female donors, both external and internal vaginal swabs were collected, both pre- (just prior to digital penetration) and post-digital penetration (after specified time interval). For the external swabs, sterile cotton swabs were pre-moistened with nuclease free water prior to collection. Internal vaginal swabs were collected from lower vaginal canal regions. From the male donors, both hand surface and fingernail samples were collected, both pre- (just prior to digital penetration) and post-digital penetration (after specified time interval). Two swabs were collected at each time interval, using a swab 1/swab 2 approach where donors used swab 1 first and swab 2 second. While these swabs were individually evaluated, a composite profile was developed using profiles obtained from both swabs (i.e. if an allele was present in either swab, it was accounted for in a final overall profile).

For all samples collected from the male donors, sterile cotton swabs were pre-moistened with nuclease free water prior to collection. For the hand surface samples (dominant hand), male

donors were provided two sterile swabs with one to collect from the thumb and the second to collect from the rest of the hand. For the fingernail swabs, male donors were provided five individual small-head sterile cotton swabs with one swab used per finger of the dominant hand. While it is not typical practice to use an individual swab for each finger, we did not want to lose any biological material from repeated swab use and all swabs would be combined into a single sample during extraction.

All samples were dried overnight at room temperature. Once dried swabs were stored in sample envelopes either at room temperature or in the freezer. Upon sample receipt, all samples were stored at -20°C until needed.

Extractions

DNA/RNA co-extraction

All male donor samples (surface hand and fingernail swabs) were extracted using a DNA/RNA co-isolation protocol based on the *mirVana* miRNA isolation kit [13]. DNA was purified using the QIAGEN QIAmp DNA Investigator kit, following the manufacturer's suggested protocol. RNA was eluted into 30 µl nuclease free water. DNA was eluted into 30 µl buffer ATE. This same co-extraction protocol was used for the following sets of female donor samples (internal and external vaginal swabs): donor sets 3 and 4 (swab 2 only) and donor set 5 (swabs 1 and 2).

Organic DNA extraction

A standard non-differential organic DNA extraction [14] was used for remaining female donor samples (internal and external vaginal swabs): donor sets 1 and 2 (swabs 1 and 2), donor sets 3 and 4 (swab 1 only), with a modified elution volume of 30 µl.

DNA Analysis

Quantitation

All DNA extracts were quantified using the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems™ by ThermoFisher Scientific, Carlsbad, CA) according to the manufacturer's suggested protocol. All quantitations were performed using a QuantStudio™ 5 Real-Time PCR instrument (ThermoFisher Scientific). All RNA extracts were also quantitated following DNase treatment to ensure no residual genomic human DNA was present in the RNA extracts.

Autosomal STR amplifications

DNA was amplified using the GlobalFiler™ Express PCR Amplification Kit (ThermoFisher Scientific) according to the manufacturer's recommended protocol, with up to 1ng input and 29 amplification cycles.

Y STR amplifications

DNA was amplified using the Yfiler™ Plus PCR Amplification Kit (ThermoFisher Scientific) according to the manufacturer's recommended protocol, with up to 1ng input and 29 amplification cycles.

Y-STR pre-amplification

DNA was amplified using an in-house developed Y chromosome targeted pre-amplification method (based on [15] but modified to be suitable for use with the Yfiler™ Plus kit, manuscript in preparation). Following this pre-amplification, amplification products were purified using the

QIAGEN MinElute PCR purification kit (12 μ L buffer EB elution volume). Five microliters of the purified pre-amplification product was used in a subsequent standard Yfiler™ Plus amplification.

Detection – Capillary Electrophoresis

Capillary electrophoresis was performed on amplified DNA products. An 11 μ L reaction mix containing 9.6 μ L Hi-Di formamide, 0.4 μ L GeneScan™ 600 LIZ™ dye Size Standard v2.0 and 1 μ L of amplified DNA or Allelic Ladder was added to the wells a 96-well plate and covered with a plate septa (Applied Biosystems™ by ThermoFisher). Samples were injected onto a 3500 Genetic Analyzer with POP-4™ polymer and Module J6 (15s injection, 1.2 kV, 60°C), and analyzed using the GeneMapper™ ID-X v1.6 and v1.7 software (Applied Biosystems™ by ThermoFisher).

RNA Analysis

DNase Treatment

All RNA extracts were treated with Zymo DNase I (Zymo Research, Irvine, CA). First, 2 μ L of Zymo DNase I and 2 μ L of Zymo DNA digestion buffer were added to each sample tube (Zymo Research). Samples were vortexed and centrifuged, and then incubated at room temperature for 15 minutes. After incubation, the RNA extracts were stored at -20°C until needed.

Reverse Transcription

DNase treated RNA extracts were reverse transcribed into cDNA using the SuperScript™ IV Vilo™ Master Mix kit (Invitrogen™ by ThermoFisher). A 20 μ L reaction mix containing 4 μ L of SuperScript™ IV Vilo™ Master Mix, 6 μ L of nuclease-free water and 10 μ L of sample was added to 0.2 μ L MicroAmp™ tubes (Applied Biosystems™ by ThermoFisher). Samples were reverse

transcribed using the following program: 25°C 10 min; 50°C 10 min; and 85°C 5 min. A negative control (nuclease free water) was included in each reverse transcription PCR reaction. All cDNA reverse transcription products were stored at -20°C until needed.

mRNA Body Fluid Identification Assay Amplification and Detection

All cDNA products were amplified using a 12-plex mRNA body fluid identification multiplex system. The 12-plex system contained mRNA biomarkers for the identification of blood, semen, saliva, vaginal secretions, menstrual blood, nasal secretions and skin. The 12.5 µL reaction mix contained 6.25 µL of QIAGEN® Multiplex Master Mix, 1.75 µL of nuclease free water, 1.25 µL of QIAGEN® Q Solution, 1.25 µL of an in-house CE primer mix and 2 µL of reverse transcribed cDNA product. Amplifications were performed in 0.2 µL MicroAmp™ tubes (Applied Biosystems™ by ThermoFisher) using the following cycling program: 95°C 15 min; 33 cycles of 94°C 30 sec; 55°C 90 sec (+0.2°C/cycle); 72°C 45 sec; and 72°C 30 min. A positive control (previously tested cDNA product) and negative control (nuclease free water) were included with each amplification. All amplified cDNA products were stored at 4°C until needed. Capillary electrophoresis was performed on amplified cDNA products. An 11 µL reaction mix containing 9.7 µL Hi-Di™ formamide, 0.3 µL GeneScan™ 500 LIZ™ dye Size Standard and 1 µL of amplified cDNA was added to a 96-well plate and covered with a plate septa (Applied Biosystems™ by ThermoFisher). Samples were injected onto a 3500 Genetic Analyzer with POP-4™ polymer and Module G5 (8s injection, 1.2 kV, 60°C), and analyzed using the GeneMapper™ ID-X v1.6 and v1.7 software (Applied Biosystems™ by ThermoFisher).

High Resolution Melt (HRM) Analysis

High Resolution Melt analysis was performed on reverse transcribed cDNA products using the QIAGEN Type-IT HRM PCR kit. A 20 μ L reaction mix containing 10 μ L of QIAGEN HRM PCR Master Mix, 6 μ L nuclease-free water, 2 μ L of an in-house primer mix (Table 4), and 2 μ L of reverse transcribed cDNA product was added to a QIAGEN Strip Tube. All samples were amplified using the QIAGEN RotorGene Q with the following cycling program: 95°C 5 min; 40 cycles of 95°C 5 seconds, 58°C 35 seconds, 72°C 20 seconds; HRM from 74°C to 91°C using 0.2°C temperature increments. A positive (cDNA reverse transcription product from RNA, previously run with positive detection of body fluids) and negative (nuclease free water) control were included in each HRM run. Sample data was analyzed using the Rotor-Gene Q Series Software v2.2.3.

IV. Findings

Digital Penetration Samples

The goal of this work was to develop a full rapid digital penetration evidence processing workflow that would assess not only the ability to recover probative DNA profiles in digital penetration samples but to uniquely provide critical contextual information by means of mRNA body fluid identification (BFID) that will provide support for determining the nature or circumstances of the digital assault. Initially, five donor couples were used as a “training” sample set in order to evaluate collection time intervals in which successful DNA and RNA profiling results could be obtained from digital penetration samples. The sample collected from these donor couples included samples from both the male and female participation, pre- and post-digital penetration at 1, 3, 6, 12 and 24 hours. For samples collection from the female, the objective was to identify male DNA (YSTR analysis) from skin (mRNA BFID). For the samples collected from

the male donor, the objective was to identify female DNA (aSTR analysis) from vaginal secretions (mRNA BFID).

Female Internal and External Vaginal Swabs

For the female internal and external vaginal swab samples, the goal was to identify the presence of male ‘perpetrator’ skin cells transferred to the female ‘victim’ during digital penetration. Given the likely trace amounts of touch/skin cells amongst an overwhelming amount of female DNA, DNA analysis of these samples was limited to Y-STR analysis. Figure 1 (Appendix C) provides Y-STR profile recovery for the female internal and external vaginal swab samples using both the standard and enhanced (Y chromosome target pre-amplification, YTPA) analysis.

For the internal samples using standard Y-STR analysis (Yfiler™ Plus), alleles were observed for donor sets at the 1 and 3 hour time intervals. At 6 hours, full profiles were obtained for the male donor in two of the five donor couples. At 12 and 24 hours, alleles were observed for three of the five donor couples including a full profile from one donor couple out to 24 hours. The male cells here are shed skin cells not sperm cells and therefore will be more susceptible to degradation by the harsh female vaginal environment. The ability to recovery profiles out to 24 hours post digital penetration was both surprising and encouraging. Our laboratory has extensive experience in enhanced YSTR analysis methods including the use of Y chromosome targeted pre-amplification for use with challenging and late reported sexual assault evidence. We wanted to evaluate the possible improvement in allele recovery with the used of this YTPA enhanced method compared to standard analysis. Samples where ~15 alleles or less were recovered using standard analysis were then processed using enhanced YSTR analysis and the improvement in allele

recovery is shown (Appendix C, Figure 1, top panel) as evidence by the increase in allele recovery shown in the peach-colored bars. An improvement in allele recovery was observed in ten of the fifteen samples in which enhanced YSTR analysis was performed. In some cases this resulted in the allele recovery when initially no profile was obtained from the same sample using standard analysis.

For the external vaginal samples, similar results were observed (Appendix C, Figure 1, lower panel) with full YSTR profiles of the male ‘perpetrator’ detectable even out to 24 hours post-digital penetration. For the 1 and 3 hour time intervals, full or nearly full profiles were obtained from a larger number of donor couple sets. Again, the improvement in allele recovery using enhanced YSTR analysis can be seen.

For these internal and external vaginal swabs, the recovery of a YSTR profile from the male donor represents transfer of skin cells from the male ‘perpetrator’ during digital penetration. However, without the identification of the body fluid source of origin as skin for these samples, then it is also possible that the DNA could have originated from other sexual activity (e.g. saliva and/or semen). The probative value of the identification of skin using the CE or HRM BFID assays is still somewhat limited due to the presence of skin from the female donor in all of these samples as well. The identification of skin using these assays does not confirm the presence of male skin. However, one of the advantages of the use of mRNA profiling for BFID for these samples is the availability of cSNP assays which permit an association of donor to body fluid. Using these advanced assays, a mixture of skin could be detected and then linked to body the male and female donors, confirming the presence of skin from both donors. Future work is aimed at using these assays for the evaluation of the female internal and external samples. For the current work, CE and HRM assay results show an identification of skin and an absence of saliva or semen. For the

purposes of this controlled study in which donors were asked to abstain from other sexual activity, the absence of the presence of saliva or skin for all of the samples evaluated supported the source of the male YSTR profiles as being from male skin cells.

Despite the limitations of the CE and HRM assays, the BFID results from the internal and external vaginal samples are provided in Figure 2 (Appendix C). For this work, only three of the five donor couples were available for analysis as a DNA-only organic extraction was used for the internal and external vaginal samples for the other two couples and therefore mRNA fractions from these donor couples was not available for testing. Skin was detected in ~73% of the internal vaginal samples and ~93% of the external vaginal samples. As was observed for the DNA analysis of these samples, more successful results were obtained from the external vaginal samples with detection of skin in all but one sample.

Male Hand Surface and Fingernail Swabs

For the male hand surface and fingernail swab samples, the goal was to identify the presence of female ‘victim’ vaginal secretions transferred to the male ‘perpetrator’ during digital penetration. DNA analysis for these samples involved the use of autosomal STR (aSTR) analysis using the Globalfiler™ Express kit. Male donors were not asked to exclude any normal daily functions such as hand washing and/or showering. Therefore it is likely that biological material would be quickly lost from these samples as a result of these activities. However, underneath the fingernail samples were included as the fingernail may provide some protection from sample loss for extended intervals compared to hand surface samples.

The results from the aSTR analysis of the hand surface and fingernail samples collected 1 – 24 after digital penetration are represented in Figure 3 (Appendix C). To evaluate the presence

of the female donor in these samples, the number of unique female alleles (i.e. not shared alleles with the male donor, as determined by evaluation of reference STR profiles) was evaluated and expressed as the percent of unique female allele recovery. For a majority of samples, high levels (75-100%) of unique female alleles were observed for both the hand surface and fingernail samples. The presence of female DNA was detected in 42 of the 50 samples (84% of the samples tested), with more than 50% unique female alleles recovered in 93% of the 42 female-DNA positive samples.

These allele counts however do not easily show the relative amounts of male and female DNA in each sample. Figure 3 (bottom panel), includes the M:F (male major:female minor) or F:M (female major:male minor) ratios for each of the samples. These ratios were calculated based on quantitation data. In several samples, the amount of total human and male DNA were approximately equal and therefore the DNA was designated as from the male donor only. For the fingernail samples at 1 hour, the female donor was the major contributor for four of the five donor couples. For one donor couples (couple 5), the female donor continued to be the major donor for the fingernail 3 and 6 hour samples as well as the hand surface 1, 3 and 6 hour samples.

The detection of vaginal secretions on the hand surface or in the fingernail samples would represent more intimate contact with the victim as opposed to casual contact. Therefore the transfer and persistence of vaginal secretions was evaluated in the male surface hand and fingernail samples. Vaginal secretions was not detected in any of the male surface hand samples using the CE or HRM assays. Vaginal secretions was detected in numerous fingernail samples (Figure 4, Appendix C) from 1 to 24 hours post-digital penetration. Vaginal secretions was detected in all fingernail samples collected 1 hour after digital penetration. Variable results were observed in the 3 – 24 hour samples, however vaginal secretions was detected in 24 hour samples for three of the

five donor couples. These samples again will largely be impacted by the activities of the male donor after digital penetration.

Conclusions

The results from this initial data set demonstrate that male DNA can be detected in female internal and external vaginal samples out to 24 hours post-digital penetration in some cases. The results also demonstrate that female DNA can be detected from underneath a male perpetrator's fingernails also out to 24 hours post-digital penetration, although successful detection of vaginal secretions in these samples is more variable past the 1 hour time frame. This "training" set of samples will be used to refine the collection time intervals for future donor couples, including collection intervals beyond 24 hours. Additionally, based on the lack of results from the hand surface from the male donor samples, this sample type will no longer be evaluated for the additional donor sets.

Rapid Co-extraction

A main goal of the current work was to develop and optimize of a customizable workflow for the rapid processing of digital penetration samples. The methods and findings described above used a standard DNA/RNA co-extraction workflow for the processing of the initial five donor couple digital penetration samples. This was done in order to ensure the best results were obtained for this initial set of samples as it would be used to determine refined collection time intervals for the additional sample work. However, while these samples were being evaluated, the workflow for the rapid processing of digital penetration samples was developed and the performance of this

workflow was evaluated with single source and admixed body fluid samples as well as non-casework voluntary digital penetration samples.

This workflow (visual overview provided in Appendix C, Figure 5) included a modified RNeasy® Plus Micro kit (QIAGEN) extraction, following the manufacturer's suggested protocol, with additional modifications for optimization and recovery of DNA through the use of the included gDNA eliminator columns. Swab tips were cut from the end of a dried body fluid swab and placed into 1.5 mL microcentrifuge tubes. For bloodstains, a 1/4th cutting of a whole stain was used. To each sample, 350 µL of Buffer RLT Plus and 3.5 µL of 1 M DTT were added followed by a brief vortex and incubation at room temperature for five minutes. The stain or swab piece was then placed into a spin basket and back into its original tube, then centrifuged at 13,000 rpm for three minutes. The spin baskets and accompanying swab or stain piece were discarded, and the sample lysate was transferred to a gDNA Eliminator Mini Spin Column inside of a 2 mL collection tube. Sample tubes were centrifuged at 10,000 rpm for thirty seconds. The gDNA Eliminator Mini Spin Column was then transferred to a clean collection tube and set aside at room temperature for later processing. To the flow-through that contained the RNA, 350 µL of 70% ethanol was added, using the micropipette to gently mix the sample. The full sample volume in this collection tube was then transferred to an RNeasy MinElute® Spin Column and centrifuged at 10,000 rpm for 15 seconds. The flow-through in the collection tube was discarded and the RNeasy MinElute® Spin Column placed into a new collection tube. The RNA was then washed twice, first with 700 µL of Buffer RW1, then with 500 µL of Buffer RPE, centrifuging at 10,000 rpm for 15 seconds and replacing the collection tube after each buffer addition. Next, 500 µL of 80% ethanol was added to each sample, and samples were centrifuged at 10,000 rpm for two minutes. The flow-through in the collection tube was discarded and the RNeasy MinElute® Spin

Column placed into a new collection tube. The samples were then centrifuged again at 10,000 rpm for five minutes to remove any residual ethanol. Following centrifugation, the RNeasy MinElute® Spin Columns were placed in a clean 1.5 mL microcentrifuge tube and 14 µL of RNase-free water was added to the spin column membrane. The tubes were centrifuged at 13,000 rpm for one minute to elute the RNA from the spin column membrane. The RNeasy MinElute® Spin Column was then discarded, and the RNA extract was stored at -20°C until needed. The previously stored gDNA Eliminator Mini Spin Columns were then processed for DNA recovery. To each column, 500 µL of Buffer AW2 was added and the tubes were centrifuged at 10,000 rpm for 30 seconds. The flow-through in the collection tube was discarded and the gDNA Eliminator Mini Spin Column placed into a new collection tube. Next, 500 µL of 80% ethanol was added to each sample tube, and the tubes were centrifuged at 10,000 rpm for 30 seconds. The flow-through in the collection tube was discarded and the gDNA eliminator spin column placed into a clean 1.5 mL microcentrifuge tube. To elute the DNA, 25 µL of Buffer ATE was added to the gDNA Eliminator Mini Spin Column membrane and allowed to incubate for one minute. After incubation, the tubes were centrifuged at 13,000 rpm for one minute. The gDNA Eliminator Mini Spin Column was then discarded, and the DNA extracts stored at 4°C until needed.

We are currently in the process of testing the full workflow using a small sub-set of 1 hour samples samples for couple 1 were evaluated using this full rapid workflow as we collect samples from the additional donor sets. The results of the developed rapid workflow will be compared to the standard methods used for the “training” set in order to determine the most suitable protocols for use with the digital penetration samples.

Future Work

While the results of the first five donor couples was highly successful, the work will continue to expand the number of donors used and further evaluate and refine the time intervals in which successful results can be obtained from digital penetration evidence. Additionally, the newly developed rapid co-extraction method will be evaluated for use with digital penetration samples.

Currently, analysis of the internal and external vaginal samples collected from the female are evaluated for the presence of ‘skin’. The BFID assays used eliminate the source of male DNA as being from saliva or semen. However, there is no direct confirmation of the presence of male ‘skin’ since female skin cells will also be present. The use of an mRNA cSNP assay permits not only detection of body fluids present but an association between the donor and body fluid source as well. cSNP analysis will detect this mixture and allow a confirmation of the donor genotypes within skin. This work is currently underway with all sample sets and will be the subject of a future publication.

V. Implications for criminal justice policy and practice in the United States

Sexual assault evidence involving vaginal intercourse is routinely processed for DNA analysis and often results in the identification of the semen donor. The transfer and persistence of semen components in the vaginal tract is well characterized and standardized biological evidence processing workflows have been established. However, in cases of digital penetration of the vagina without semen ejaculation, it is more challenging to find physical evidence identifying the perpetrator and supporting the act itself. This is due to the fact that in many instances only trace quantities of biological material are transferred and that it is often difficult to distinguish any DNA found as having originated from sexual activity versus that arising from innocent social interaction. This work begins to fill this gap by establishing how long DNA evidence linking a perpetrator to

an act of digital penetration can be recovered and whether, and for how long, direct physical evidence of the act itself (via body fluid specific markers) can be obtained to support the occurrence of the offense. This work will continue to further establish optimal time intervals for the collection and analysis of digital penetration evidence.

VI. Participants and other collaborating organizations

Erin Hanson

- Project Role: Principal Investigator (PI)
- Contribution to the project: experimental design, performed laboratory experiments, data analysis, preparation and submission of reports and abstracts
- Funding support: UCF employee
- Collaborated internationally: No
- Travelled to foreign country: No

Jack Ballantyne

- Project Role: Co-Principal Investigator (Co-PI)
- Contribution to the project: experimental design, perform laboratory experiments, data analysis, preparation and submission of reports and abstracts, presentation of data to the forensic community
- Funding support: UCF employee
- Collaborated internationally: No
- Travelled to foreign country: No

Lauren Crawford

- Project Role: Graduate Research Assistant
- Contribution to the project: experimental design, perform laboratory experiments, data analysis, preparation and submission of reports and abstracts, presentation of data to the forensic community
- Funding support: UCF employee
- Collaborated internationally: No
- Travelled to foreign country: No

VII. Changes in approach from original design and reason for change, if applicable

No changes were made from the original design.

Appendix A. References

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Appendix B. Products

- Transfer, Persistence & DNA Source Attribution of Trace Biological Material in Digital Penetration Assault Cases. Crawford L and Hanson E. 2025 NIJ Forensic Science R&D Symposium (at 2025 AAFS conference). Baltimore, MD. February 2025.
- Optimization of Capillary Electrophoresis and High Resolution Melt mRNA Profiling Assays for Body Fluid Identification. L. Crawford and E. Hanson. University of Central Florida Student Scholar Symposium (Poster) Mar. 2025.
- Two manuscripts currently in preparation: 1) development and optimization of optimized rapid co-extraction screening workflow, 2) summary of transfer, persistence & DNA source attribution from initial digital penetration donor sets.

Appendix C. Figures and tables

Figure 1. YSTR analysis of internal and external vaginal swabs collected 1 – 24 hours after digital penetration. Allele recovery (Yfiler™ Plus) for standard analysis (blue bars) versus enhanced YTPA analysis (peach bars) is shown. Maximum allele recovery is 27 alleles. Values provided represent a composite profile created from allele recovery data from swabs 1 and 2 for each time intervals. C = couple or donor set number.

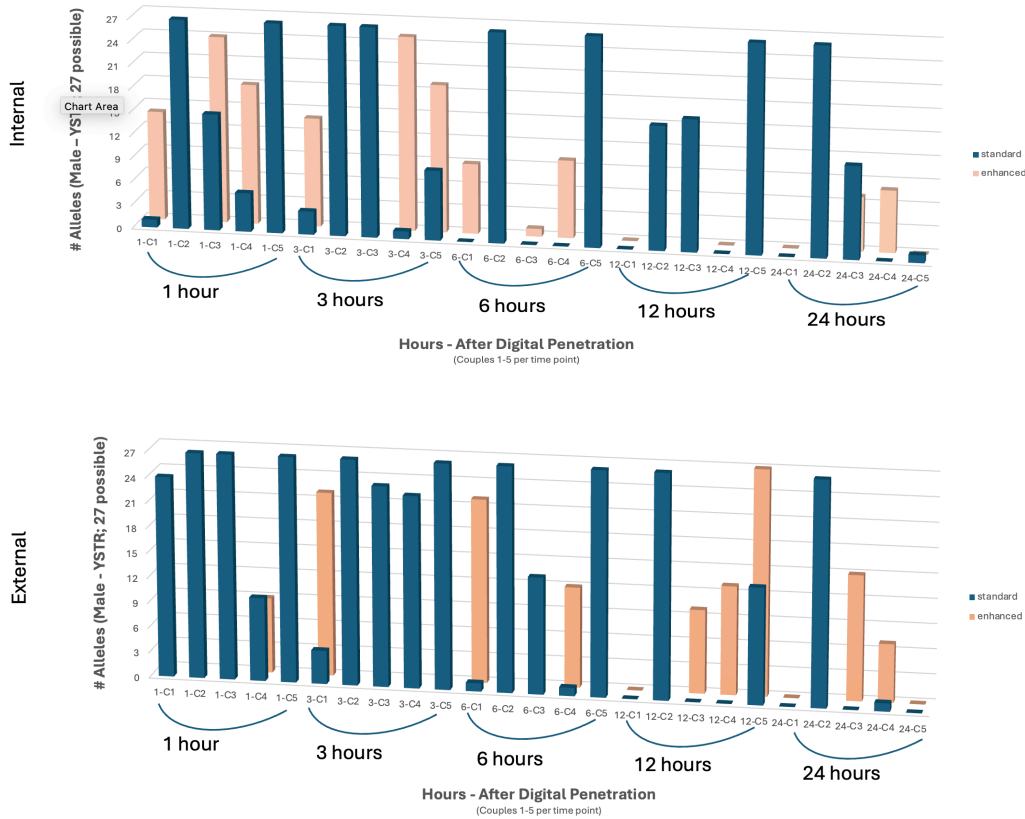


Figure 2. Skin detection in internal and external vaginal swabs collected 1 – 24 hours after digital penetration using CE and HRM BFID assays. Successful skin detection is presented by peach color cells (dark peach (++) – detected using both methods; light peach (+- or -+) – detected using one of the two methods; grey (--) – not detected using either method. Only three of five donor couples are shown as the other two couples were extracted using a DNA-only non-differential extraction and therefore no mRNA fraction was available for BFID testing.

Time	Skin detection (mRNA) - internal			Time	Skin detection (mRNA) - external		
	Couple 2	Couple 3	Couple 5		Couple 2	Couple 3	Couple 5
1 hr	+-	++	-+	1 hr	++	++	++
3 hr	--	++	+-	3 hr	--	++	++
6 hr	--	++	-+	6 hr	++	++	+-
12 hr	++	++	++	12 hr	++	++	++
24 hr	--	++	--	24 hr	++	++	++

Figure 3. aSTR analysis of male hand surface and fingernail swabs collected 1 – 24 hours after digital penetration. Recovery of unique female alleles (within M/F admixtures) (Globalfiler™ Express) is shown. Values are expressed as percents which are number of unique alleles observed out of the total possible (listed in the % of ## row in the top table). The bottom table shows the M:F or F:M ratios for each of the samples. The presence of a grey ‘male’ cells indicate the quantitates for total human and Y targets were approximately equal and therefore no ratio was calculated.

Sample	Time interval	% of Unique Female Alleles Observed				
		Couple 1	Couple 2	Couple 3	Couple 4	Couple 5
		% of 29	% of 24	% of 23	% of 23	% of 28
Hand	1	100	100	100	100	100
	3	7	100	78	4	100
	6	83	100	0	4	100
	12	100	92	57	100	64
	24	0	8	96	100	100
Fingernail	1	100	100	100	100	100
	3	0	100	96	70	100
	6	100	100	0	0	100
	12	79	100	0	100	86
	24	0	25	0	78	100

Sample	Time interval	M/F or F/M Ratio (Total Yield)				
		Couple 1	Couple 2	Couple 3	Couple 4	Couple 5
Hand	1	5:1 (F:M)	5:1 (M:F)	2:1 (M:F)	3:1 (M:F)	90:1 (F:M)
	3	male	2:1 (M:F)	male	20:1 (M:F)	100:1 (F:M)
	6	9:1 (M:F)	5:1 (M:F)	18:1 (M:F)	8:1 (M:F)	1.1:1 (F:M)
	12	2:1 (M:F)	31:1 (M:F)	95:1 (M:F)	4:1 (M:F)	male
	24	male	male	male	167:1 (M:F)	male
Fingernail	1	4:1 (F:M)	4:1 (M:F)	2:1 (F:M)	5:1 (F:M)	60:1 (F:M)
	3	male	3:1 (M:F)	24:1 (M:F)	1:1 (M:F)	5:1 (F:M)
	6	male	2:1 (M:F)	6:1 (M:F)	male	9:1 (F:M)
	12	male	10:1 (M:F)	67:1 (M:F)	4:1 (M:F)	male
	24	6:1 (M:F)	male	9:1 (M:F)	48:1 (M:F)	male

75-100%
50-74%
25-49%
1-24%
0%

F:M ratio
M: F ratio

Figure 4. Vaginal secretions detection in male hand surface and fingernail swabs collected 1 – 24 hours after digital penetration using CE and HRM BFID assays. Successful vaginal secretions detection is presented by green colored cells (dark green (++) – detected using both methods; light green (+- or -+) – detected using one of the two methods; grey (--) – not detected using either method.

Time	Vaginal Secretions (mRNA)				
	Couple 1	Couple 2	Couple 3	Couple 4	Couple 5
1 hr	++	+-	+-	++	-+
3 hr	--	++	++	--	--
6 hr	++	--	--	--	--
12 hr	--	++	--	--	--
24 hr	+-	+-	--	--	+-

Figure 5. Optimized RNeasy® Plus Micro Kit Co-Extraction Workflow for DNA and RNA