TECHNICAL NOTE

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Use of a Y Chromosome Probe as an Aid in the Forensic Proof of Sexual Assault

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ABSTRACT: Currently, the most common procedures for the forensic identification of semen that may be present due to a sexual assault include the microscopic identification of spermatozoa, acid phosphatase activity, or the detection of PSA. However, not all cases of sexual assault result in the deposit of semen. Fluorescent In Situ Hybridization (FISH) has been found to be a very sensitive and specific method for detection of the Y chromosome from male cells. This study was undertaken to demonstrate the presence of epithelial cells of male origin in the postcoital vaginal tract using a commercially available probe. Results identified Y chromosome in intact epithelial cells on postcoital Days 1 through 4, and on Day 7. Additionally, Y chromosome positive epithelial cells were identified in vaginal swabs obtained following intercourse with no ejaculation. The method developed in this study demonstrates that FISH is a sensitive method for the identification of the presence of male epithelial cells in the postcoital vagina.

KEYWORDS: forensic science, fluorescent in situ hybridization, FISH, semen, sexual assault, Y chromosome

The forensic laboratory has numerous ways of detecting biological evidence left by a male individual following a sexual assault case. The most common method is the microscopic detection of spermatozoa by the Nuclear Fast Red-Picroindigocarmine (Christmas Tree) Stain. Semen identification using a presumptive acid phosphatase test, and semi-quantitative PSA test are also employed (1–3). Although these methods can be very useful in semen identification, there are problems that are associated with each of them. The Christmas Tree Stain is dependent upon the presence of sperm, but in many cases there is no ejaculation by an assailant. Additionally, the number of sperm present regularly decreases after the event, even in cases where there is ejaculation. The presumptive acid phosphatase test, and semi-quantitative PSA test levels also are dependent upon ejaculation and decrease in sensitivity rapidly after the event.

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The purpose of the research described herein is to use various Y chromosome probes to aid in the forensic proof of sexual assault. Fluorescent In Situ Hybridization (FISH) is a very sensitive and specific method for the detection of the Y chromosome from blood lymphocytes, bone marrow specimens, and epithelial swab specimens from the skin, buccal cavity, and vagina (4–6). This study examines the FISH detection of the Y chromosome in epithelial cells for extended amounts of time after sexual assault as reported in earlier studies by Rao, and Pettenati (4,6). During sexual assault there are various ways in which male epithelial cells can be deposited. This can occur during penetration, ejaculation or from the saliva of the assailant. In this study DNA X and Y chromosome probes were used to detect specific regions of gene sequences of the X and Y-chromosomes in interphase nuclei of intact epithelial cells. Two commercially available kits were used to obtain the initial data and to serve as a guideline for further studies that utilized a kit from a third vendor. The Y chromosome probe assay results were compared to results from nuclear fast red/picroindigocarmine stain, acid phosphatase, and the Seratec™ PSA Semiquant Test.

Materials and Methods

Postcoital Swab Collection

Vaginal swabs were collected in accordance with approved guidelines for the protection of human subjects. Samples were obtained at 24, 48, 72, 96, and 168 hours post coitus. Only one sample was obtained for each coital event, as this best mimics the one forensic sample that would be taken after a sexual assault. "Abstinence samples" were obtained three weeks or more after the previous sexual contact. Coital events for swabbing were at least three days apart. For sampling, the cotton swab was inserted approximately 1 in. into the vaginal canal by the volunteer, rotated for 5–10 s and then carefully removed. Swabs were air dried for one to two hours at room temperature, transported to the research holding area and frozen until use.

Buccal Swab Collection

Sterile swabs were also used to collect samples of the buccal cavity in accordance with approved guidelines for the protection of human subjects. These were taken from both male and female volunteers for use as positive and negative controls. These swabs were also used to develop FISH protocols, before coital sample trials.

Cellular Extraction Procedure

The cotton swab head was cut off and placed in a tube with 1ml of PBS pH 7.4, and vortexed for 10 s. The tubes were incubated at room temperature for 45 min. Subsequently, the tube was vortexed again for 10 s, sonicated for 10 s, and vortexed again to maximize cell retrieval. The cotton remnants were removed from the tube and the tube was centrifuged at 14 000 rpm for 3 min. For the buccal cells, the supernatant was taken down to 100 (μL, the tube was vortexed for 8 s, and 60 (μL of the cell suspension was removed and spread on a 22 by 22 mm area of an X-tra™ charged microscope slide. For the postcoital cells, the supernatant was removed to 100 (μL and then placed in a separate tube for subsequent PSA and acid phosphatase studies, and stored at −20°C. The remaining 40 (μL of the cell pellet was used for spermatozoa identification.

Both the postcoital and buccal FISH slides were allowed to dry at room temperature. The dry slides were placed in a coplin jar containing 200 mL of ice cold 3:1 methanol/glacial acetic acid in the −20°C freezer and incubated for one hour. After this time the slides were then retrieved, air-dried overnight, and placed in a covered slide box in the −20°C freezer.

Vysis™ CEP® X SpectrumOrange™/Y SpectrumGreen™ DNA Probe Kit For Fluorescence In-Situ Hybridization

The assay was performed according to the manufacturer's recommendations. The basic procedure consisted of denaturation, hybridization, and post-hybridization washing. This was followed by a counterstaining using DAPI II; 10 (μL of DAPI II counterstain was applied to the target area of the slide in low light conditions. A glass 22 by 22 mm cover slip was applied carefully to the slide and the slide was subsequently stored at −20°C prior to signal detection.

Detection

A fluorescent microscope (NIKON) with 60X and 100X objectives was used to detect the signals. One hundred twenty-five cells were enumerated on each slide and the probe signal intensity was noted. Signal was recorded as bright, dim, compact, or diffuse (any background staining was noted). On the microscope, DAPI/Green/Orange filters were used to detect the CEP® X/Y probes. This filter configuration allowed the simultaneous excitation and emission of the X SpectrumOrange™, Y SpectrumGreen™, and DAPI counterstain.

Roche™ Digoxigenin labeled Human Chromosome Y Painting Probe with Anti-digoxigenin-fluorescein, Fab fragments detection

The Human chromosome Y painting probe, DIG-labeled Roche™ protocol was modified to use FISH on interphase cells. The cellular extraction and fixation procedure of the Roche™ protocol was modified removing the metaphase preparation of the cells.

The following controls were used: 1) a male buccal slide preparation with Y chromosome painting probe mixture and subsequent addition of FITC Anti-Digoxigenin labeled antibody; 2) a female buccal slide preparation with Y chromosome painting probe mixture and subsequent addition of FITC Anti-Digoxigenin labeled antibody; 3) a male buccal slide preparation with hybridization buffer but no probe, with subsequent addition of FITC Anti-Digoxigenin labeled antibody. The specimen DNA was denatured as in the previous protocol. The manufacturer's recommended protocol consisting of hybridization, washing, and detection with labeled antibody was then followed. Microscopy was performed as previously stated.

Locus Specific Identifier (LSI®) SRY Spectrum Orange™/ CEP® X Spectrum Green™ Probe Assay

The manufacturer's recommended protocol was followed, which consisted of denaturation, hybridization, wash, and detection as previously stated.

Nuclear Fast Red-Picroindigocarmine Stain

Semen identification was confirmed by evidence of spermatozoa, from a swab smear on a glass slide, and stained by the nuclear fast red/picroindigocarmine method (7). The slides were examined microscopically at 400X.

Acid Phosphatase Test

The extracted samples were tested for the presence of acid phosphatase using a modification of a previously published protocol (8) with α naphthyl phosphate as the substrate.

Seratec™, PSA Semiquant®

The assay was performed according to manufacturer's recommendations (9) with the following modifications. The post-coital swab samples were prepared as indicated previously and the supernatant from the cellular PBS extract was saved and frozen at −20°C for the PSA identification. Two hundred microliters of each postcoital supernatant was placed into the test well of an appropriately labeled Seratec™ PSA Semiquant® slide. Supernatant from a known semen sample obtained using the cell extraction method cited was used as the positive control and PBS was used as a negative control. The slides were allowed to incubate at room temperature for 10 min and read.

Results and Discussion

Vysis™ CEP® X SpectrumOrange™/Y SpectrumGreen™ DNA Probe Kit For Fluorescence In-Situ Hybridization on Buccal Epithelial Smears and Vysis™ LSI® Y Chromosome for Postcoital Cell Identification

The Vysis™ CEP® X SpectrumOrange™/Y SpectrumGreen™ DNA Probes and the Vysis™ LSI® Y Chromosome kit were the first sets of Y chromosome FISH probes used in this study to obtain positive XY signals in male buccal smears, and positive XX signals in the female buccal smears. Preliminary identification of both male and female cells in our hands was confirmed by performing FISH on a female buccal slide and a male buccal slide. Slide screening of male cells yielded only the expected Orange X and Green Y signals. In the same respect, slide screening of female cells yielded only two orange X labels in all of the scanned cells. These findings were repeated using buccal swabs as controls during processing of the post-coital samples.

Roche™ Digoxigenin labeled Human Chromosome Y Painting Probe with Anti-digoxigenin-fluorescein, Fab fragments detection

Control Slide Identification—In this study the Roche™ Y chromosome paint probe was used to develop most of the postcoital cell identification. Three control slides using buccal cells were run with the experiment, which included identification and enumeration of the male and female buccal slides for Y signal as performed in the

Vysis™ experiments. Five different postcoital slides from each of the time points of Day 1, 2, 3, 4, 7, and AA (> 21 days or abstinence) were assayed by the Roche™ Y chromosome paint probe. During each experiment control, slides of male and female buccal cells were run for further analysis of specificity and sensitivity. The control performed in each experiment for the Anti-Digoxigenin fluorescein Fab fragment non-specific binding was a slide that had the antibody but no probe for each experiment, and this was negative for any signal fluorescence in all controls.

The Roche™ Y DNA probe and data from the control slides indicated no false positive identification in any experiment. The FISH assay adapted to Y chromosome paint signal epithelial cell identification yielded specific results on the control slides.

Five swabs were analyzed for each postcoital time point. One swab only was obtained from a single coital event. In addition, two swabs procured immediately after a non-ejaculate penetration were obtained. Table 1 shows a summary of samples from each coded source in the study.

TABLE 1—*Roche™ cellular Y signals for each postcoital sample, each day. Identification of how many Y signals in epithelial cells were found from each slide, for each day or instance, counting a total of 125 cells per slide. Each number represents a separate postcoital episode.*

Postcoital Cell Identification	
Days after Coital Event	Y Signal Cell Count/125 Cells
Day 0 non-ejaculate	1, 3
Day 1	1, 6, 1, 6, 4
Day 2	3, 4, 4, 3 also 0/29
Day 3	3, 1, 3, 4, 1
Day 4	2, 3, 3, 4, 2
Day 7	2, 4, 2, 1, 3
> = 21 Days	0, 0, 0, 0, 0

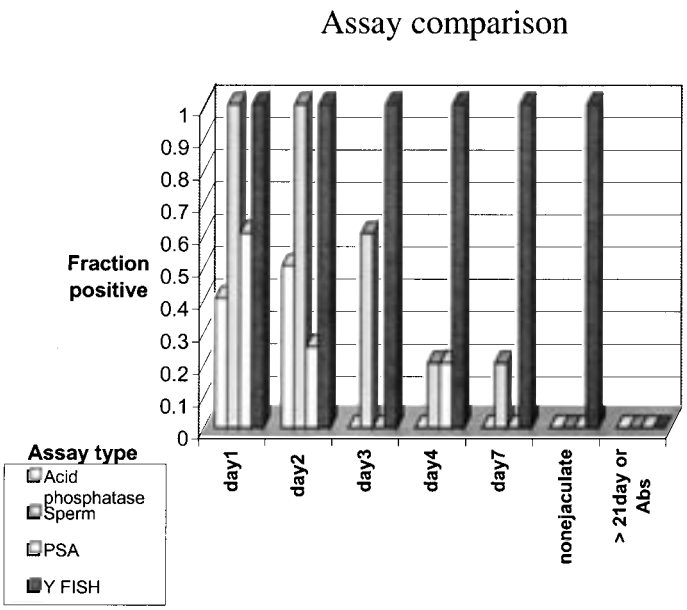

FIG. 1—*Comparison of acid phosphatase, sperm, PSA and Y chromosome FISH. Graphic representation of the comparison of the Roche Y chromosome method, acid phosphatase, prostate specific antigen and spermatozoa detection.*

TABLE 2—*Comparison of Roche™ Y chromosome FISH method to current forensic methods of identifying sexual assault evidence. Each sample was obtained from a separate coital event on the day indicated. Comparison of the Roche Y chromosome method to acid phosphatase assay, prostate specific antigen assay and spermatozoa detection.*

Sample ID	Acid Phos.	PSA	Sperm	Y FISH Signal
Day 1				
AK22	+	+	+	+
AK22	Negative	Negative	+	+
AK22	Negative	Negative	+	+
AJ16	+	+	+	+
XR37	Negative	+	+	+
Day 2				
PF31	Negative	Negative	+	+
XR37	+	Negative	+	+
AK22	+	Negative	+	+
AJ16	Negative	+	+	+
PF37*	Negative	Negative	Negative	Negative
Day 3				
PF31	Negative	Negative	+	+
AK22	Negative	Negative	Negative	+
AK22	Negative	Negative	+	+
AJ16	Negative	Negative	Negative	+
XR37	Negative	Negative	+	+
Day 4				
VD18	Negative	Negative	+	+
XR37	Negative	Negative	Negative	+
AK22	Negative	+	Negative	+
PF31	Negative	Negative	Negative	+
VL81	Negative	Negative	Negative	+
Day 7				
PF37	Negative	Negative	+	+
XYZ	Negative	Negative	Negative	+
XYZ	Negative	Negative	Negative	+
AJ16	Negative	Negative	Negative	+
XR37	Negative	Negative	Negative	+
Non-Ejaculate				
PLAB	Negative	Negative	Negative	+
PLAB	Negative	Negative	Negative	+
> 21 Days or Abs.				
ZD92	Negative	Negative	Negative	Negative
ZD92	Negative	Negative	Negative	Negative
XYZ	Negative	Negative	Negative	Negative
KC23	Negative	Negative	Negative	Negative
VD18	Negative	Negative	Negative	Negative

* Pf37 Day 2 slide had negligible cells on slide for FISH and sperm ID.

After performing FISH with the Y chromosome DNA probe on the postcoital samples, the pellets of each were analyzed for spermatozoa, and the supernatants were analyzed for PSA and acid phosphatase. Results of this comparison can be found in Table 2 and in Fig. 1.The Roche™ Y chromosome identification of postcoital cells yielded positive Y signals for each day. Days one, three, four, and seven were all positively identified, with all five swabs for each day, yielding Y signal positive cells. Day 2 had four sample positive Y signals and the fifth swab had negligible quantities of any cells on the slide, so results were not valid, as all other determinations were based on the 125-cell count per slide. A very important finding was from the postcoital non-ejaculate immediate swabbings of the vagina. Two samples were taken and both samples were positive for the Y signal. This indicates there is identification of epithelial cells or cells from penis penetration only, and no ejaculate fluid. As expected, these two samples were negative for PSA, acid phosphatase, and spermatozoa. This is an important finding because many rapists have sexual dysfunction including delayed ejaculation, and may be oligospermic or aspermic. Also,

the act may not be committed to the extent of male ejaculation. The greater than or equal to 21-day specimens yielded no findings of the Y signal. This group was mostly comprised of actual abstainers. Further analysis must be undertaken for the time between Day 7 and Day 21 to ascertain the last day of detection of the Y signal positive epithelial cells. The average number of cells identified from the postcoital swabs for each day was calculated, with no significant decrease in Y signal from Day 1 to Day 7.

An important aspect of our FISH analysis is that it was done concurrently with acid phosphatase, PSA, and spermatozoa detection on the same samples (Table 2). This table shows all results for this study. FISH results identified Y chromosome postcoital cells up to Day 7 and were also positive in the non-ejaculate postcoital samples. While the spermatozoa identification was successful primarily at Day 1 and 2, with one sperm detected at Day 4 and Day 7, FISH identified all of the valid postcoital samples (excluding day 2 PF37) as being positive for evidence of sexual contact. Acid phosphatase identification was only positive in two out of five samples for both Days 1 and 2. PSA was positive in three out of five samples in day one, and in one of five in days two, and four, as indicated by Table 2. These method limitations are consistent with prior literature (2,4,10). One of the Day 2 samples did not contain many cells and was negative by all assays. This reflects a sampling error that is one of the variables of casework. Since this sample represents a sampling error rather than a technique error, it was not used in the analyses of each individual technique.

The application of FISH for use in detecting the Y chromosome has been successful in many cases, and could be considered a valuable method for proving sexual contact with a male (4,6). Various cells have been used for detection of the Y chromosome including epithelial cells (5,6). In this study, developing FISH with various types of Y chromosome probes for epithelial cell identification, and comparing the outcome to current methods from the same samples, gives excellent information regarding the use of this DNA based method for forensic purposes.

Preliminary analysis of the buccal and postcoital cellular extractions from the dried swabs revealed correct signal identification with all Y chromosome DNA probes used, and was consistent with past literature (5,6). No false positives in the male or female slides were observed. This is very important and is an issue in any method used for forensic identification. Although there are checks and balances such as PSA, spermatozoa, and acid phosphatase determination, a method with no false positives instills confidence in the method. False negative cell rates fall between 3 and 5% for male and female cells in the buccal identification. In each of the four male and four female slides, there was correct identification of the X and Y signal.

An important aspect of the Roche™ experiments as in the Vysis™, was to use control slides to validate and confirm Y signals and eliminate any reason to believe cross-reactivity, false positives, or nonspecific binding of the probe or antibody has taken place. In each Roche™ run the control slides, as stated in the results, were run for DNA probe hybridization specificity and sensitivity.

Control slides included four male and four female buccal controls. The Roche™ Y average signal positive for the male cells was 72.8%. This was somewhat lower than the Vysis™ LSI® enumeration, but correctly determined the Y signal in each of the four male slides, and no Y signal in the female slides. No false positive signals were seen. The Roche™ probe FISH method described herein is a cost effective method with high specificity and sensitivity for the analysis of Y chromosome positive cells after sexual contact with a male.

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ERRATUM

Erratum/Correction of Dziegelewski M, Simich JP, Rittenhouse-Olson K. Use of a Y Chromosome Probe as an Aid in the Forensic Proof of Sexual Assault. J Forensic Sci 2002;47(3): 601–604.

On page 602, in the first column, first and third paragraphs:

. . . 100 (μL, 60 (μL, 100 (μL, 40 (μL and 10 (μL . . .

should read

. . . 100 μL, 60 μL, 100 μL, 40 μL and 10 μL, respectively.

The Journal regrets this error. Note: Any and all future citation of the above-referenced paper should read: Dziegelewski, M, Simich JP, Rittenhouse-Olson K. Use of a Y Chromosome Probe as an Aid in the Forensic Proof of Sexual Assault. [published erratum appears in J Forensic Sci 2002 Sept.;47(5)] J Forensic Sci 2002 May;47(3):601–604.