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Identification of Sperm and Non-Sperm Male Cells in Cervicovaginal Smears Using Fluorescence In Situ Hybridization: Applications in Alleged Sexual Assault Cases

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ABSTRACT: The identification of spermatozoa or constituents of seminal fluid is critical in the evaluation of alleged sexual assault victims. However, failure to identify sperm and/or elevated levels of acid phosphatase can occur for a variety of reasons. Molecular techniques, such as molecular cytogenetic analysis offers new approaches to improve on the identification of male cells in alleged sexual assault cases.

Fluorescence in situ hybridization (FISH) with a Y chromosome specific DNA probe was applied to archival cervicovaginal smears from 41 alleged sexual assault cases to identify Y-bearing (male) cells. FISH identified Y-bearing sperm and non-sperm cells in 78% of the cases previously confirmed to have sperm. FISH also identified Y-bearing non-sperm male cells in 39% of the cases in which cytology did not detect spermatozoa; in one of these instances, it also detected sperm. Cervicovaginal acid phosphatase levels, determined at the time of the cervicovaginal smears, were also compared with the presence or absence of Y-positive cells. Application of this technique can detect non-spermatozoic male cells in routine cervicovaginal smears of sexual assault victims.

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Sexual assault is one of the most under-reported crimes in the United States, and relatively few assailants (4%) are prosecuted [*1*, National Institute of Justice, personal communication]. It is usually an unwitnessed crime and statements by victims may be the only direct evidence that assault has occurred [*2,3*]. Almost all victims suffer emotional trauma as a result of the assault, but only 20% to 45% of victims show evidence of bodily injuries [*4,5*]. While the victims may feel fortunate not to have physical injuries this, ironically, may later be used against them if the case goes to court [*1*].

After a sexual assault is reported, in addition to the physical examination the unequivocal presence of spermatozoa and an elevated acid phosphatase in the cervicovaginal specimen are the crucial physical evidence most often sought to corroborate the testimony of the assault victim [*4,6–8*]. Negative results have been used to suggest that no sexual contact or ejaculation occurred [*1*]. Therefore, obtaining specimens containing spermatozoa or an elevated acid phosphatase that confirm recent sexual contact may make the difference between the alleged assailant being charged and convicted or going free.

For medicolegal reasons, it is crucial to enhance the sensitivity of detecting evidence of an assault. Advances in molecular genetics using DNA probes have had a significant impact on clinical diagnosis and patient management and its role in pathology and forensic medicine is emerging. One of these areas of molecular genetic technology is fluorescence in situ hybridization (FISH). This technique involves using a DNA probe(s) specific for a chromosome or chromosomal region labeled with non-radioactive fluorescent molecules and hybridizing it to their homologous regions in cells. This permits the rapid identification of a chromosome(s) in interphase nuclei by the observation of a fluorescent dot(s) under a

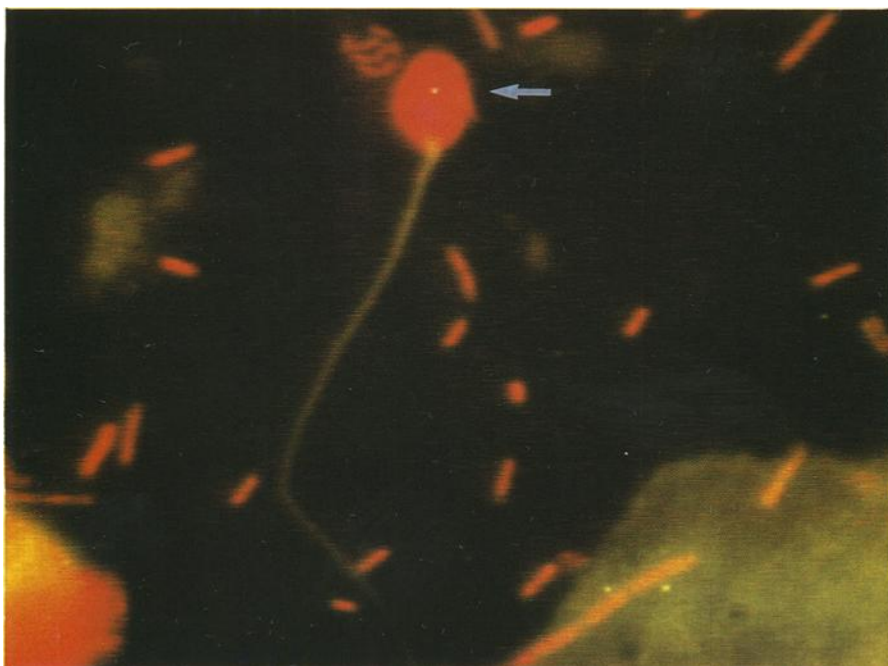

FIG. 1—*Propidium iodide stained sperm with a Y chromosome fluorescent signal* (arrow).

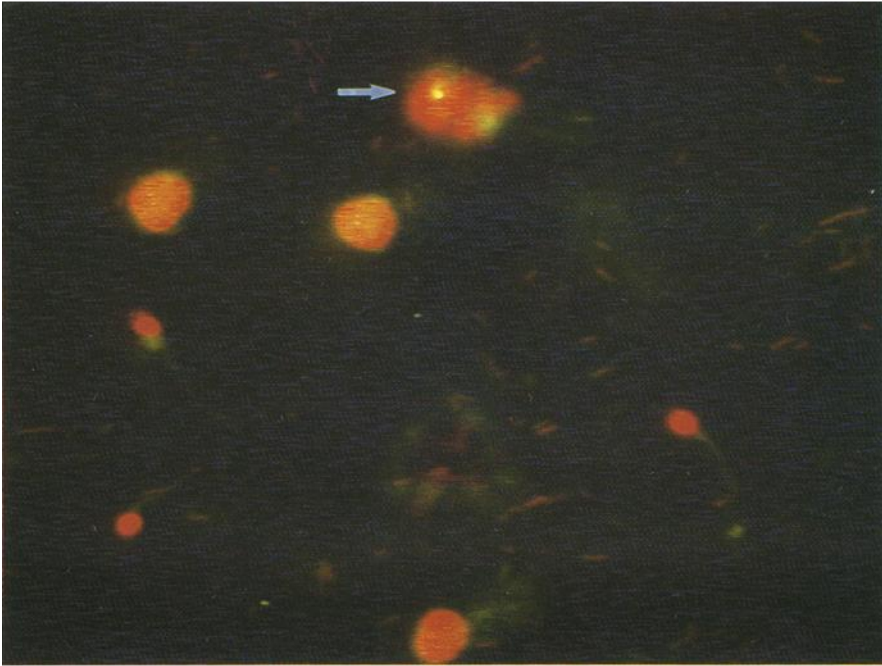

FIG. 2—*Low power view (40X) showing propidium iodide stained sperm, bacteria and a Y-positive non-sperm cell* (arrow).

UV microscope. An advantage of FISH is that it is a relatively simple and rapid laboratory procedure with high specificity and sensitivity in detecting human chromosomes and/or chromosomal regions in dividing and non-dividing cells.

A retrospective study utilizing FISH with Y-chromosome specific DNA probes was performed to identify non-sperm male cells and sperm in cervicovaginal smears of alleged rape cases. The results of this preliminary investigation are presented and the applicability of FISH as a technique for acquiring evidence in alleged rape cases is discussed.

Material and Methods

Cytology/Chemistry

We retrospectively reviewed 41 archival cervicovaginal smears and accompanying cervicovaginal acid phosphatase levels from alleged sexual assault cases of women cared for at North Carolina Baptist Hospital from 1988 to 1993. The acid phosphatase levels were determined enzymatically by the Dupont Automated Chemistry Analyzer and determined to be elevated if the level was over 50U. Semen free cervicovaginal smears taken from two women with known sexual histories of abstinence were used as negative controls. All cases were rescreened by a cytotechnologist for the presence or absence of spermatozoa. The coverslips were gently removed by soaking the slides in xylene (two to ten days) and destained in 5% HCl for 20 minutes. The slides were coded and blinded prior to FISH analysis.

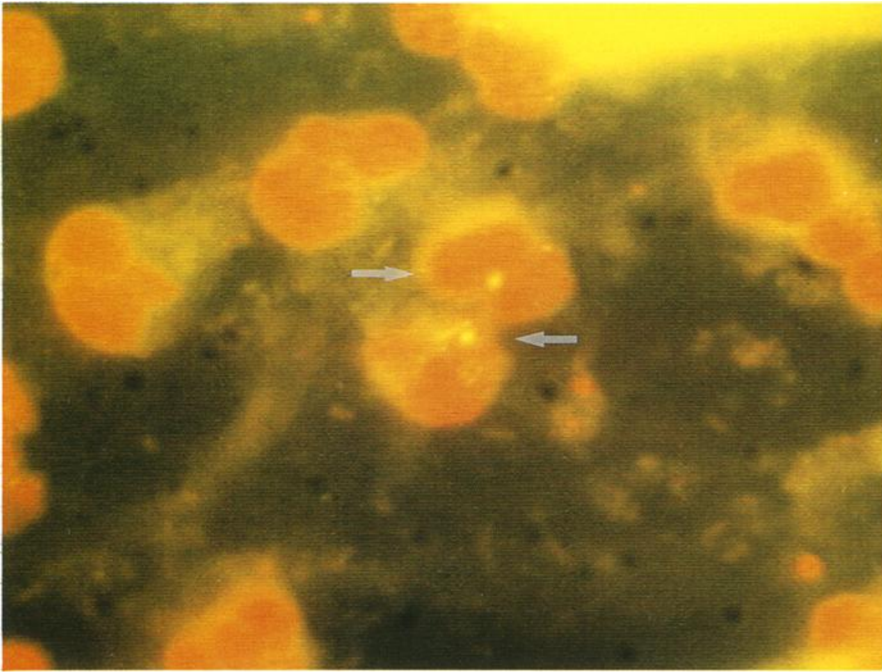

FIG. 3—*Two segmented neutrophils each with a Y chromosome fluorescent signal* (arrow).

Fluorescence In Situ Hybridization

Slides were dehydrated through an ethanol series, treated with pepsin (0.4%/4XSSC) at 37°C for 20 minutes followed by an ethanol dehydration. Denaturation was in 50% formamide/2XSSC at 70°C for 2 minutes followed by dehydration through an ethanol series and the slides were air dried. The biotinylated DNA probe (1.5 μL/30 μL) [Y chromosome specific DNA cocktail probe (DYZ3/DYZ1) (Oncor)] was denatured at 70°C for 5 minutes and chilled quickly. The hybridization mixture was added to each slide, covered with a glass coverslip and sealed with rubber cement. Slides were incubated at 37°C overnight in a humidified chamber. Post-washing was done in 65% formamide/2XSSC at 43°C for 20 minutes, followed by two washings in 2XSSC at 37°C and a final wash in 1XPBD at room temperature. A blocking reagent was added to each slide followed by the addition of fluorescein-labeled avidin and incubation in a humidified chamber at 37°C for 20 minutes. Slides were washed 3 times in 1XPBD at room temperature. Cells were stained with propidium iodide and covered with a glass coverslip prior to microscopic analysis.

The slides were then examined under a fluorescent microscope. Cells were easily visualized. Non-sperm (epithelial and inflammatory) cells were scored as positive and of male origin if a fluorescent signal indicating the presence of the Y chromosome was identified. Sperm were identified both morphologically and/or with a fluorescent signal.

The chromosome Y cocktail probe is a mixture of alpha and classical satellite DNA [DYZ3/DYZ1]. The DYZ1 is highly specific for the repeated alphoid DNA located at the centromere of human chromosome Y while DXZ3 hybridizes to the short repeats related to the AATGG in the pericentric heterochromatin of chromosome Y [9]. The hybridization efficiency of the Y probe using FISH has been examined in our laboratory on cytogenetically normal males (46,XY) and females (46,XX) uncultured tissue samples (n = 180). In each

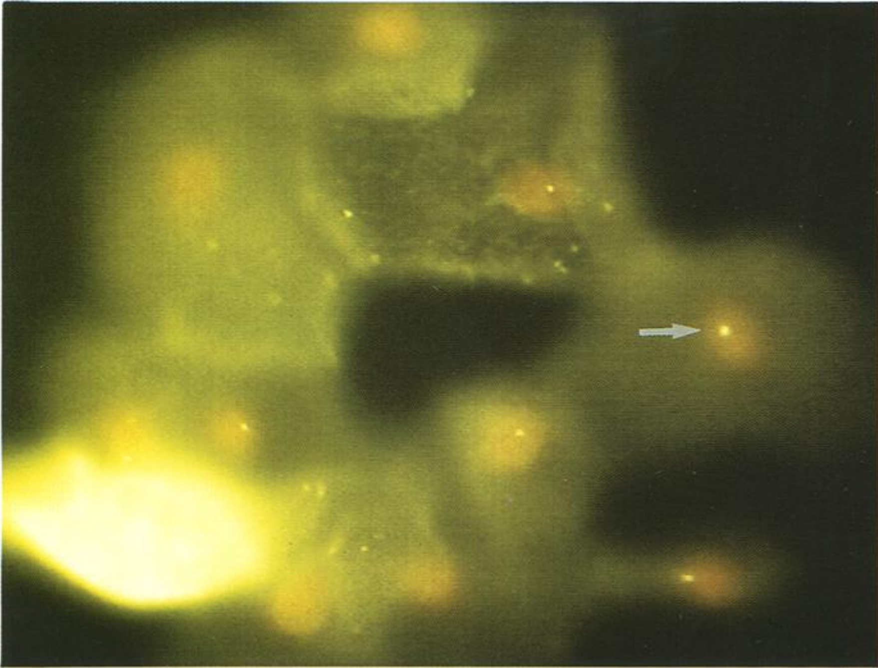

FIG. 4—*A cluster of Y chromosome positive* (arrow) *squamous epithelial cells identified in a cervicovaginal smear previously negative for sperm on cytology and with non-elevated acid phosphatase.*

instance, 100 interphase cells per case were counted for the presence or absence of a Y signal. These studies demonstrated the Y probe to be present in 99.97% in of cell in males and 0.001% in females.

Results

Cytology/Chemistry

On routine cytologic examination of the Papanicolaou stained smears, 23/41 cases were positive for spermatozoa, 19 of which had elevated acid phosphatase levels (Table 1). In 18/41 cases, spermatozoa were not identified; however, there was one instance of a slightly elevated acid phosphatase level (61.8U).

Fluorescence In Situ Hybridization

Spermatozoa were identified in 17/23 (74%) of the smears previously positive for sperm on routine cytologic examination (Table 1, Fig. 1). It was noted that the propidium iodide used to counterstain the cells highlighted the spermatozoa, enabling easier visualization of the spermatozoa when compared to routine screening using the Papanicolaou stain.

Non-sperm cells (epithelial and segmented neutrophils) exhibiting one distinct fluorescent signal for the Y probe were observed in 16/23 (70%) cases previously reported positive for spermatozoa by the Papanicolaou stain (Table 1, Figs. 2, 3). Non-sperm cells were not evident in the remaining seven cytologically sperm positive cases, however FISH identified sperm in three of these instances. In situ hybridization was unsuccessful in two cases.

TABLE 1—*Comparison of cytological findings and acid phosphatase levels with FISH.*

Cytology/Chemistry		FISH			
	Total Cases	Sperm (+) Non-sperm cell (+)	Sperm (+) Non-sperm cell (−)	Sperm (−) Non-sperm cell (+)	Sperm (−) Non-sperm cell (−)
Sperm (+)/AP > 50	19	12	2	2	3[a]
Sperm (+)/AP < 50	4	2	1	0	1
Sperm (−)/AP > 50	1	0	0	0	1
Sperm (−)/AP < 50	17	1	0	6	10
Total	41	15	3	8	15

[a]Hybridization was unsuccessful in 2 of the cases.
AP — acid phosphatase level (U); non-sperm cell — epithelial and/or inflammatory cells.

Of the 18 cases negative for spermatozoa on the Papanicolaou stained smears and with non-elevated acid phosphatase levels, FISH identified either sperm and/or non-sperm cells with a Y-specific fluorescent signal in 7 (39%) cases (Table 1, Fig. 4). In the single instance in which the acid phosphatase level was slightly elevated with no sperm identified cytologically, FISH did not detect any Y-bearing cells. Y-bearing cells were not identified in the negative controls with FISH.

FISH was difficult to perform on these archival slides because of the age of the slide, the presence of residual mounting solution, the presence of dried mucus layers and loss of cells after coverslip removal and during the hybridization procedure. These factors obscured and/or interfered in the overall analysis.

Discussion

In alleged rape cases, the absence of laboratory evidence does not disprove the sexual assault but makes the victim's claim more difficult to prove and the case more difficult to prosecute [*4*]. The identification of spermatozoa, motile or nonmotile, and the elevation of cervicovaginal acid phosphatase are the two forms of evidence most often used to corroborate the testimony of the assault victim [*8*]. In addition, seminal material has also been shown by analyzing for the presence of the p30 glycoprotein of the semen. However, obtaining positive evidence that confirm a recent sexual assault is often difficult with current methods.

In cases of sexual assault, recovery of spermatozoa using cervicovaginal smears has been reported to be from 46% to 71% [*10,11*]. The time interval between intercourse and the collection of the cervicovaginal smear adversely affects the detection of spermatozoa. Motile spermatozoa have been recovered from the vagina after 3 to 24 hours [*12–14*], and from the cervix, 110 hours to 7 days [*15,16*]. After 3 hours, only 50% of the smears will have motile sperm [*7,17*]. After 72 hours, only 50% of the cervicovaginal smears will have any spermatozoa present [*7*]. Nonmotile spermatozoa have been found in the cervicovaginal secretions 14 to 17 days [*16,18,19*] and in the cervix up to 12 days after intercourse [*18*]. Other factors which adversely affect the recovery of spermatozoa from cervicovaginal specimens include douching after intercourse, urination, and oral contraceptive pills which alter the cervicovaginal environment.

Additional complications can be incurred if the assailant is oligospermic or even azospermic, resulting in a possible elevated acid phosphatase but no spermatozoa. Many convicted perpetrators report sexual dysfunction and lack of ejaculation during the sexual assault resulting in neither an elevated acid phosphatase level nor spermatozoa [*11*]. Similarly, if the act of assault is interrupted with penetration but no ejaculation within the victim, spermatozoa will not be recovered and the acid phosphatase will not be elevated [*20*]. However, penile epithelial cells will still exfoliate into the vagina. If the assailant has had a previous vasectomy, no spermatozoa will be present. Cellular debris or menstruation may obscure the spermatozoa causing erroneous false negative interpretation of the cervicovaginal smear [*16,21*].

An elevated acid phosphatase is the other important indicator of the presence of semen and recent intercourse. As mentioned, acid phosphatase may be elevated with the absence of spermatozoa. Significant levels of acid phosphatase cannot be found 12 hours after intercourse in 50% of cervicovaginal swabs [*22*]. In one study, no positive results were noted after 36 hours [*7*].

In the present study, FISH with Y-chromosome specific DNA probes was used to identify Y-bearing (male) sperm and non-sperm cells in cervicovaginal smears on previously confirmed and non-confirmed sexual assault cases as determined by conventional cytopathology. Overall in the 41 cases, both sperm and/or non sperm cells were identified by FISH in 63% of the cases whereas cytology identified sperm in 56%. FISH identified both sperm

and/or non-sperm cells in 83% of the cases where cytology demonstrated the presence of spermatozoa.

In general, this technique compared well with conventional cytologic analysis. Certainly, the two techniques are diagnostically complementary. In some aspects, the use of FISH may be superior to conventional cytology since non-sperm male cells are identifiable. FISH identified Y-bearing non-sperm male cells in 39% of the cases in which cytology did not detect spermatozoa and the acid phosphatase levels were not elevated. In one instance, FISH detected a Y-bearing sperm in a cytologically sperm negative smear. The non-sperm male cells morphologically appeared to be epithelial or inflammatory in nature, most likely originating from the penis.

To better examine the accuracy of FISH in comparison to conventional staining, a control study on cervicovaginal smears from women with known sexual history is currently in progress in our laboratory. This prospective controlled study has established the ability of FISH to serve as a potential new technique for identifying male cells in cervicovaginal smears (Rao et al., in preparation). This study includes the use of multicolor FISH to demonstrate clearly the presence of X and Y probes in non-sperm male cells to rule out possible non-specific hybridization.

Using FISH on routine cervicovaginal smears with no elaborate preparation, non-sperm Y-bearing cells can be identified and additionally enhance the screening for spermatozoa. FISH may also be useful in cases of child abuse when the young victim's testimony is unclear, denial of assault is questioned or if the assault is of a digital or oral nature. The identification of the Y chromosome may also complete the investigation of a homicide in which sexual assault is questioned.

Sexual assault is prevalent in our society. Sensitive and specific laboratory evidence is crucial in corroborating a victim's testimony, convincing the jury of recent sexual contact and sexual assault, and prosecution of the perpetrator. The technique using FISH is a simple laboratory procedure that can utilize commercially available kits, a fluorescent microscope and, in the case of sexual assault, air-dried or destained Papanicolaou smears. The technique is highly specific and sensitive.

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