Y-STR AS PROOF OF RAPE WHEN SPERM CELLS CANNOT BE FOUND

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Summary

Identification of spermatozoa is the biological evidence most often sought in the examination of rape victims. Absence of spermatozoa usually terminates biological investigations, and the victim's testimony can therefore be contested. We assessed the utility and reliability of PCR amplification using Y-chromosomal STR polymorphisms in specimens taken from female victims of sexual assault with negative cytology.
Overall, when sperm cells could not be found, Y-chromosome STRs were detected and demonstrated sexual penetration in 28.8% of 104 swabs obtained from 79 victims. In the population of victims examined more than 48 hours after the sexual assault, Y-STR were still observable in 30% of cases. These results show that swabs can be taken from victims for Y-chromosome DNA typing even after a long lapse of time between the sexual assault and medical examination.

Introduction

Identification of spermatozoa is the biological evidence most often sought in examination of alleged sexual assault victims. The identification relies on Papanicolaou staining of smears. The sensitivity of the detection can be improved by limited proteinase K digestion prior to examining slides. The basis for this limited digestion is the increased sensitivity to proteases of epithelial cells compared to sperm cells. Under mild digestion, the flagella is destroyed, but the very compacted sperm cell nuclei are still undigested. This difference of sensitivity is also used to prepare DNA from sperm cells. After mild digestion and sperm nuclei washing steps, more stringent lysis conditions allow extraction of the male DNA component, which is characterized by means of autosomal STR analysis. In the best cases a pure male DNA profile can be obtained. In less favorable examinations, depending on the efficiency of the epithelial cells digestion and the number of sperm cells, a mixed profile can be obtained and, providing the female DNA profile is available, a partial male profile can be deduced.
However, in many cases, the male component of the DNA mix cannot be interpreted, or is even absent. In practice, when no sperm cell is visible on cytological examination, male autosomal DNA analysis fails, and failure to demonstrate the presence of spermatozoa terminates the biological investigation. In such cases, the testimony of the victim is open to contentation.

Absence of spermatozoa in specimens taken from victims of sexual assault can be explained by a number of factors including penetration without ejaculation, an oligospermic or azoospermic assailant, a non penile penetration, or a prolonged post-coital interval. In this regard, vaginal inflammation, salivary enzymes and anal bacteria accelarate the sperm cell lysis. The longer the interval between intercourse and the sampling, the fewer the sperm cells detected. This well known fact often leads the doctors of the forensic unit to not take swabs beyond three days.

However, failure to demonstrate the presence of spermatozoa does not preclude the presence of Y-chromosome from male DNA, resulting either from the presence of lyzed sperm cells or male epithelial cells. It should be pointed out that in both these cases, the male DNA will be obtained in the so called "epithelial fraction", which is the one obtained after mild protease digestion.
The objective of this study was to evaluate the feasability of Y-STR analysis in negative specimens from cytologic examination. Molecular techniques using Y-chromosome-specific DNA probes are new tools

capable of identifying Y-bearing sperm and non-sperm cells. As shown in this work, Y-STRs can provide evidence, particularly in cases of negative cytology [1, 2] and in instances involving old samples leading to difficult DNA extraction [3].
The postulate would be to use a single step digestion, under conditions designed to lyse either the male epithelial and sperm cells. In the second step, Y-STR analysis was perfomed. The rationale for this protocol was that theoretically, the primers designed to hybridize to Y-STR sequences would not hybridize to female DNA. Thus, Y-STR analysis should still be possible when the male to female DNA ratio is well below the 1/50 threshold for detection of male autosomal STRs.

Most of the human Y-chromosome does not recombine during meiosis and remains unchanged from generation to generation. Paternal lineages [4] and Y-chromosome polymorphism in different populations [5] can be established with Y-primers. In fact, these primers hybridize to polymorphic sequences of Y-chromosome organized into large interspersed tandemly repeated arrays. A set of seven primer pairs - DYS 19, DYS 389, DYS 390, DYS 391 DYS 392, DYS 393 and DYS385 - is currently used to research Y-chromosome in forensic studies and to determine paternity [6]. With such a set the haplotype diversity is in the 0.997 range in European populations. Many more are available and useful determination of individuals can be achieved with up to 19 markers. The experimental procedures for multiplex amplification of various sets of Y-STR markers are described in the literature.
For this study, which was designed to evaluate the possibility of Y-STR analysis, we focused on three markers : DYS389, DYS393 and AMG.

2. Materials and Methods

2.1. Selection of Specimens

One hundred and four swabs without spermatozoa detected by cytology were collected from 79 alleged female victims of sexual assault. The sites where the specimens were taken were cervicovaginal, anal or oral. For 20 women, multiple swabs were available, corresponding to various sites. All swabs were blind-collected among the multiple samples taken from each victim. All specimens used for cytology were excluded. Cytology used conventional Papanicolaou to stain the smears. The swabs for Papanicolaou staining were not the ones used for DNA extraction.

2.2 DNA extraction

DNA extraction was performed in one single step. Swabs were rehydrated for 2 hours in H2O at room temperature, centrifuged in Forensic tubes (Polylabo, Strasbourg, France) for 10 minutes at 10 000 g. The cell pellet was washed once in H2O, centrifuged as above and digested for 2 hours at 56°C in the presence of proteinase K (20 µg/0.15 ml final), DTT (4mM final) to allow digestion of sperm nuclear proteins and Chelex resin (4% final) to chelate divalent ions inhibiting the PCR. The tubes were then incubated at 100°C for 8 minutes, centrifuged for 3 minutes and the supernatants were used for amplification.

Additional controls were performed with sterile swabs (2 in each extraction series) and known positive swabs in cytological examination (4 to 8 in each series), known negative swabs from the female staff (4 in each series). A total of 12 different extractions were carried out in separate series.
Amplifications were performed in a 2400 or 9700 thermocycler (Perkin Elmer, Foster City, CA) after an initial denaturation step of 3 minutes at 94°C, for 40 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 60 seconds at 72°C, followed by a final elongation step of 7 minutes at 72°C. The PCR mix was as follows: 0.25 µM of amelogenin primers, or 0.1 µM of DYS389 primers, 1IU Taq polymerase (Boerhinger, Mannheim), 0.2 µM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl pH 8.3 for 25 µl reactions. The PCR products were detected on an ABI 310 instrument (Perkin-Elmer). The primer sequences are as in Kayser and al.[6].
To minimize the risks of contamination, all the DNA extraction and amplification steps were performed by a female technician, even if this does not ensure against secondary DNA transfer. We also performed Y-

STR typing of all the males in the laboratory of Molecular Biology before establishing the procedure routine.

3. Results

3.1. Characteristics of the Subjects

The characteristics of the alleged victims are summarized in table I. Briefly, the age range ran from 3 to 75 years old and the median was 22. The number of assailants was more than one in 14% (11/79) of the cases. The assailant was a stranger 41.8% (33/79) of the time. Vaginal, oral and anal penetration was alleged in respectively 72.2% (57/79), 29.1% (23/79) and 13.9% (11/79) of the cases. Perineal trauma occurred in 30.4% (24/79) of the cases and other physical injuries were seen in 17.7% (14/79) of the cases. The range of the lapse of time between alleged sexual assaults and the medical examinations was from 2 to 192 hours. The median was 20 hours. In two cases the lapse was not known. None of the 79 victims had any sexual intercourse during the interval between the sexual assault and the medical examination.

Victims age	median: 22 (range: 3 to 75 years old)
Assailant: single	68/79 (86%)
Assailant not known by the victim	42/79 (62%)
Vaginal penetration	57/79 (72%)
Oral penetration	23/79 (21%)
Anal penetration	11/79 (14%)
Perineal trauma	24/79 (30%)
Other physical injuries	14/79 (18%)
Lapse of time between the assault and medical examination	median: 20 hours (range: 2 to 192 hours)

Table I.

3.2 Y chromosomal DNA analysis

Y-chromosome STRs were amplified from the DNA samples prepared as described in the Material and Methods section. The most sensitive marker was DYS393. In 24/104 (23.1%) swabs, amplification made it possible to detect the Y-chromosome STR DYS393, whereas DYS389 and AMGY were detected in 11/104 (10.6%) and 6/104 (5.8%) of DNA samples, respectively. Seventy four swabs (71.2%) were not suitable for amplification of Y chromosome material. Discrepancies were noted in 5 swabs in which DYS393 was negative and DYS389 was positive. Y DNA was detected in 25 cases of vaginal penetration, in 3 cases of anal penetration and in 2 cases of oral penetration. Figure 1 shows the lapses in time between the alleged sexual assault and the medical examinations according to the Y DNA findings.

Overall sensitivity	DYS393	24/104	23%
	DYS 389I / DYS 389II	11/104	11%
	AMG	6/104	6%
	None	74/104	71%
Discrepancies	DYS389 + / DYS 393 -	5/80	6%

Table II.

3.2 Y chromosomal DNA analysis

Y-chromosome STRs were next amplified from the same DNA samples with the Yplex 6 kit under the recommended procedure (30 cycles). Overall, the results were superimposable to the results obtained with the in house procedure (40 cycles). The difference of sensitivity is probably due to the higher Taq amount required in the Yplex procedure.

4. Discussion

Absence of spermatozoa in specimens from victims of sexual assault can be explained by a number of factors including a prolonged post-coital interval, an oligospermic or azoospermic assailant, a vasectomized or orchidectomized assailant, penetration without ejaculation, digital penetration, use of a condom, douching after intercourse, use of spermicidal agents, menstruation, vaginal inflammation [1]. Spermatozoa are rapidly destroyed in the mouth by salivary enzymes and in the anus by bacterial enzymes [7]. False negative findings due to excessive inflammation or haemorrhage can now be avoided by using proteinase K treatment before cytological examination [8].
A cytological examination is usually performed in sexual assault investigations. Failure to demonstrate the presence of spermatozoa is frequent [9,10] but does not exclude the presence of male DNA (Y-chromosome). Exfoliated male epithelial cells resulting from penetration were identified using Fluorescence In Situ Hybridization, which does not make the identification of an assailant possible [1,2], and there is one rape case reported in the literature where Y-STR analysis was successfully made [11]. However, no systematic investigation demonstrating the interest of the method had been carried out until now. Nonetheless, encouraging data have been published: based on positive PSA test results on traces from rape cases, in which 35% of male autosomal DNA typing failed. Y STR typing was possible in half of these failed tests [12]. These data possibly reflect the sensitivity of Y-STR typing in a mix with amplification of male/female cell ratios of up to 1:2000, whereas the limit of male DNA detection is 1:50 for autosomal STR typing [13].

Cases reported here, having negative cytology but with male DNA detected by Y-chromosomal STR polymorphisms, can be explained by either a very low amount of sperm cells [13] or identification of Y-bearing non-sperm cells. Non-sperm male cells could be epithelial or inflammatory cells that are indistinguishable from the victims' cells with conventional cytology.
Our data suggest that sensitivity to detection using Y STR amplification could be higher than cytology. Regarding all the positive cases in this study we cannot exclude the eventuality that spermatozoa were absent in specimens used for cytology and present in specimens used for molecular biology. This study has now to be extended to Y-STR analysis on the same exact swabs as the ones used for cytological examination. As for now, our data should by no means be regarded as a rigorous comparison of the sensitivity of cytology vs PCR.

Under our experimental conditions, sensitivity of amplification was DYS393 > DYS389 > AMG. The increased sensitivity of the DYS393 marker as compared to DYS389 cannot be due to amplification of a X-chromosome homologue of DYS393 since it has never been observed on control female DNA under our experimental conditions (see figure 2 bottom panel). Thus, the increased sensitivity is probably due to a lower yield of amplification for DYS389. Among the factors responsible for this yield, the hybridization of primers is the most important. We did not specifically focus on this relative decrease, because with a commercially available primer mixes we obtained a sensitivity identical to the one with the in house protocol. Amelogenin appeared to be the least sensitive marker (see figure 2, top panel). However, the absence of detection of Y material is probably due to the competition between X and Y hybridization sites. The amelogenin primers hybridize to both the X and Y sequences, and Y-chromosomes are likely not to be detected when the amount of X material exceeds, by far, the amount of Y-chromosome material [13]. Differential lysis should be prefered to Y-STR typing each time cytologic analysis shows a reasonable amount of sperm cells because of the accuracy of identification. Probabilities of random matches are usually in the 10^{-6}-10^{-10} range with STR, whereas they are in the 10^{-4}-10^{-6} range with Y markers [15]. Although differential lysis allows typing of rapists by autosomal STR analysis and comparison with offenders databases [15], differential lysis was expected to fail due to the absence of detected sperm

cells on Papanicolaou stained smears. With the method used here, Y chromosome detection is not hampered by the loss of sperm cells during the washing steps. Moreover, any male non-sperm cell DNA extracted with this technique, is lost with the differential lysis. Thus, this method provides evidence of penetration, independently of ejaculation, sperm cell count, yield of differential lysis and proportion of female cells in the sampling.

Concluding remarks:

Our data show that Y chromosome analysis provides evidence of the presence of male cells in up to 28,8% of alleged female victims of sexual assault with negative Papanicolaou staining. This is useful for non-penile penetration as well. Presence of Y DNA in cases with negative cytology can provide proof of sexual contact and could be used to corroborate the testimony of female sexual assault victims. Additional evidence (Y-typing of consensual partners) is needed because recent consensual intercourse before sexual assault or before examination cannot be excluded. This method showed the feasibility of haplotype determination on swabs initially characterized as ‘negative’. Although not tested here, the method is likely to be simplified by multiplex amplifications as described in [16] or commercial kits as described here. Careful evaluation of the PCR conditions is still to be achieved for detection of a low copy number of Y-DNA material. Y-STR profiles are not recorded in the French database for convicted sexual offenders. Consequently, autosomal STR analysis should be considered when the cytology is positive. An important point of our study is that the Y-chromosome was detected in 33% of victims examined more than 96 hours after the sexual assault (Fig. 1). Spermatozoa are rarely detected in such intervals, especially in oral or anal swabs [17]. Our results show that swabs ought to be taken from victims for Y-chromosome DNA typing even after long lapses of time between sexual assaults and medical examination.

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Fig. 1. Delays between alleged sexual assault and examination according to the Y DNA findings. The numbers in the boxes represent the number of negative (empty boxes) or positive (filled boxes) victims for the presence of Y DNA material.

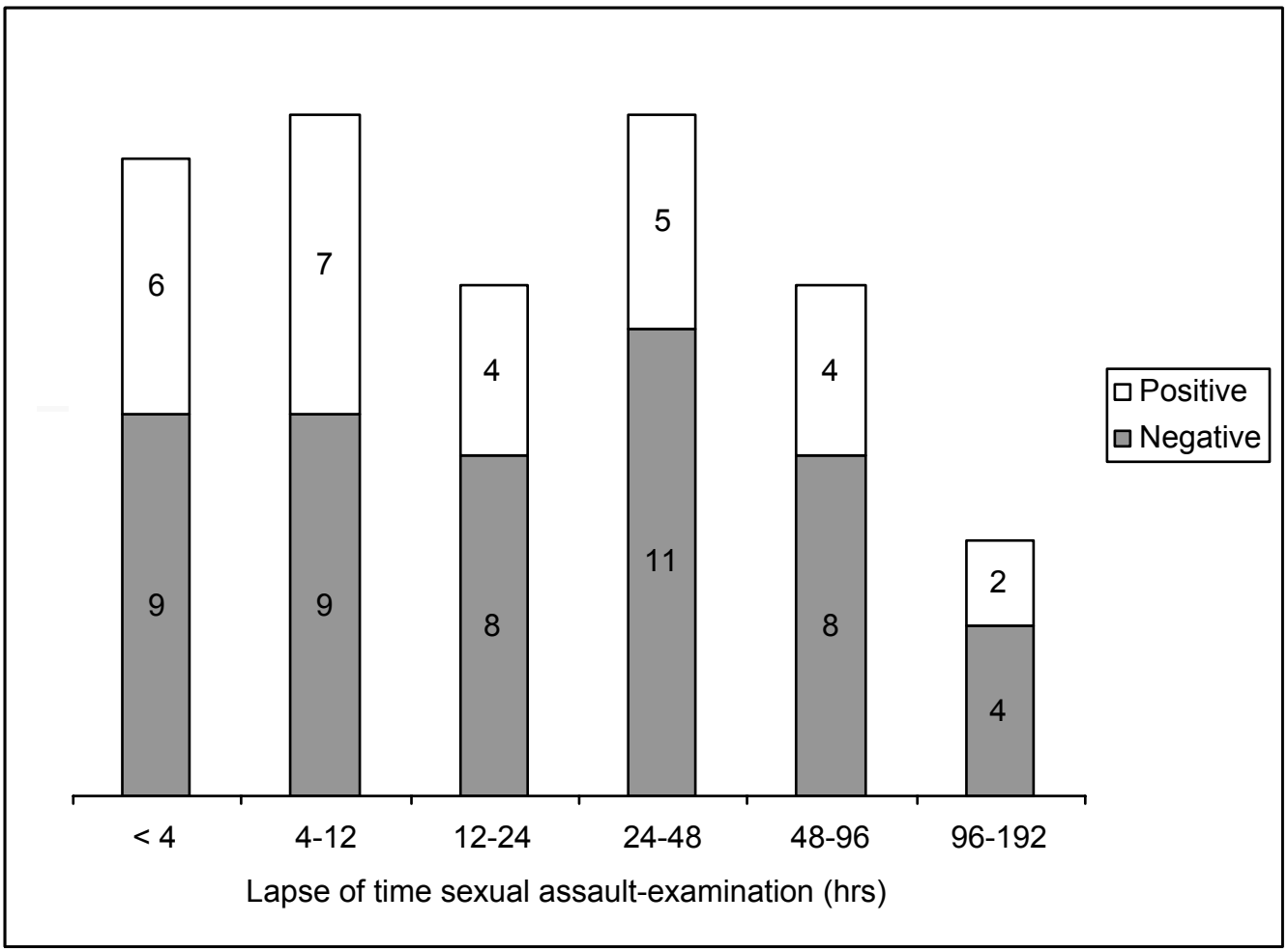

Figure 2
Y STR amplification of casuistic material (three top panels). Top panel : Amelogenin amplification, arrow : Y-specific AMG-allele. Bottom panel, control female DNA amplified with DYS393 primers. Top scale : size calculated from the Rox 500 marker. Vertical scales : arbitrary RFU.