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TECHNICAL NOTE

PATHOLOGY/BIOLOGY

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The Use of Laser Microdissection in Forensic Sexual Assault Casework: Pros and Cons Compared to Standard Methods*

ABSTRACT: Sexual assault samples are among the most frequently analyzed in a forensic laboratory. These account for almost half of all samples processed routinely, and a large portion of these cases remain unsolved. These samples often pose problems to traditional analytic methods of identification because they consist most frequently of cell mixtures from at least two contributors: the victim (usually female) and the perpetrator (usually male). In this study, we propose the use of current preliminary testing for sperm detection in order to determine the chances of success when faced with samples which can be good candidates to undergo analysis with the laser microdissection technology. Also, we used laser microdissection technology to capture fluorescently stained cells of interest differentiated by gender. Collected materials were then used for DNA genotyping with commercially available amplification kits such as Minifiler, Identifiler Plus, NGM, and Y-Filer. Both the methodology and the quality of the results were evaluated to assess the pros and cons of laser microdissection compared with standard methods. Overall, the combination of fluorescent staining combined with the Minifiler amplification kit provided the best results for autosomal markers, whereas the Y-Filer kit returned the expected results regardless of the used method.

KEYWORDS: forensic science, laser microdissection, sexual assault, Sperm Hy-Liter Express

The samples that are more often analyzed in forensic laboratories are blood, saliva, sweat, semen, urine, hairs, teeth, bones, and tissues, or a mixture of two or more of these samples (1–3). Sexual assault cases account for almost half of all forensic analysis being performed. Most of these cases correspond to sexual assaults where the victim is a female and the perpetrator is one or more males (4).

In any forensic laboratory, casework starts with the sample submission, log, and description. A number of preliminary tests are carried out to determine the presence or absence of semen in biological stains. Two of the most common tests in Portugal are

- Phosphatesmo KM (5,6). This test is based on a colorimetric reaction. The paper strip turns from white to purple in the presence of prostatic acid phosphatase.
- Auto-fluorescence of biological stains (7). Through excitation with near-UV light—forensic light—(430 nm), the biological stains will glow green, when viewed through special goggles.

Auto-fluorescence is mainly used to pinpoint the location of biological stains over large surfaces, such as sheets. Once located, a small fraction of the stain is collected and tested with a Phosphatesmo KM strip, to assess the presence of semen.

The main goal of preliminary testing is to determine which samples are worth analyzing. We aimed to use the preliminary tests as a starting point for downstream collection of cells of interest with laser microdissection techniques and DNA analysis.

Furthermore, we compared a traditional sperm staining technique (hematoxylin and eosin—H&E) with a commercially available fluorescent kit (Sperm Hy-Liter, Independent Forensics).

Next, the samples are prepared for DNA extraction and quantification, which is followed by DNA amplification with commercial kits for genotyping. Results using the traditional approach comprise a genotyping profile that is frequently mixed depending on the amount of cells originating from the victim and one (or even more) perpetrators. Work is being done by some groups to fully automate this entire process (8). Yet, less attention has been given to methods designed to separate the genetic material from the male (perpetrator) from that of the female (victim) contained in mixed samples, can make the interpretation of results more difficult. This has been overlooked because it can be time-consuming or the nature of the sample does not allow such separation without incurring in substantial losses of samples.

At this point, we should make an important note. Whole sample analysis is the common practice in Portuguese forensic laboratories. However, it is not the most common practice worldwide. Preferential lysis, also known as differential

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extraction, is the most widely used technique of sample preparation. This technique relies on the separation of cells according to their morphology and physical properties (9).

In recent years, laser microdissection (LMD) has emerged as a valuable tool for forensic scientists (10). The main advantages of LMD are the ability to physically separate mixture components, usually sperm cells from other cells, while retaining the former and discarding the latter. By collecting only the cells of interest, the mixture separation analysis is no longer needed, streamlining the laboratory procedures. In addition, with the LMD technology, fewer cells are needed to obtain a genetic profile from any given sample.

This study was performed to evaluate the advantages and limitations of including LMD into the standard forensic procedure used for genotyping characterization of simulated sexual assault samples mixed in the laboratory. Results will be discussed in terms of the duration of the procedure, resources consumed, and quality of the results obtained.

Material and Methods

Experimental material consisted of simulated sexual assault samples containing semen and female saliva, which were prepared in the laboratory in the proportions of 1:1 (50% semen), 1:4 (20% semen), 1:9 (10% semen), and 1:19 (5% semen). Four spots of each dilution were placed in a cotton-based fabric and left at room temperature for 48 h. Preliminary tests with forensic light (λ = 430 nm) and Phosphatesmo KM for acid phosphatase detection were performed on small sample cutouts to assess the sensitivity of these tests to detect the biological spot and the presence of semen in the sample.

After 48 h, samples were viewed and photographed under forensic light. One of the stains from each dilution was then tested with the Phosphatesmo KM strips.

The other stains from each dilution were then mounted on microscopic slides, as described in the slide preparation section, stained with hematoxilyn–eosin and the commercial kit Sperm Hy-Liter Express to visually confirm the presence of sperm.

We established a minimum of 50 confirmed spermatozoa in each slide to carry on with the laser microdissection (11). The minimum of 50 cells was chosen due to the capture efficiency of the LMD system, in order to effectively collect 30 cells (12). The 30 cells are the minimum recommended starting material to obtain clean and balanced genetic profiles from haploid cells. Also, we imposed a time limit for each slide: If the 50 sperm were not found within 15 min, the slide would be discarded.

For the sake of consistency and minimizing variation in the results, multiple samples were collected from the same semen and female saliva donors. Samples were collected under informed consent from these healthy volunteers. The study was approved by the Ethics Committee of the National Institute of Legal Medicine and Forensic Sciences.

Standard Procedure

DNA Extraction and Quantification—DNA from each sample was extracted using the AutoMate Express kit (Applied Biosystems, Life Technologies Corporation, 5791 Van Allen Way, PO Box 6482, Carlsbad, California 92008) with the PrepFiler TM lysis buffer (13). Quantification of DNA in the samples was performed using the Quantifiler Trio kit in an Applied Biosystems 7500 system (14). This standard procedure was selected over the preferential lysis method (9) mainly for two reasons: This is the method currently used in the Portuguese laboratories of the National Institute of legal Medicine and Forensic Sciences, thus allowing a comparison to be made. Also, sample loss with the differential lysis method is not uncommon (15). Therefore, we found it to be preferable to use the whole sample and then perform a mixture analysis rather than decrease the yield of material and as a result not being able to obtain complete profiles.

DNA Amplification and Genotyping—DNA was amplified using MiniFiler, Identifiler Plus, NGM, and Y-Filer kits (Applied Biosystems), as described in Table 1. The PCR mixture composition and thermal cycling conditions were as shown in Table 1. Thermal cycling was performed on GeneAmp 9700 Thermal Cycler (Applied Biosystems). Volumes and reaction times have been validated for use in forensic casework (Table 1).

Electrophoresis and detection methods followed the manufacturer's instructions. Samples were prepared for capillary electrophoresis using 0.5 μL Liz 500 Size Standard, 12.5 μL Hi-Di™ formamide (Life Technologies/Applied Biosystems), and 1 μL sample or allelic ladder. Injection conditions for the 3500 Genetic Analyzer (Applied Biosystems) used 1.2 kV and 12s for injection. The analysis employed a minimum threshold of 100 RFU for the 3500 Genetic Analyzer, using the GeneMapper ID-X analysis software.

TABLE 1—*Commercial kits used for DNA amplification. All reaction times and volumes have been validated for forensic routine.*

	Kit			
	MiniFiler	Identifiler Plus	NGM	Y-Filer
Volumes (μL)				
Reaction Mix	5	5	5	4.6
Primer Set	2.5	2.5	2.5	2.5
Taq Polymerase	NA	NA	NA	0.4
Sample	10	10	10	10
Final Volume	17.5	17.5	17.5	17.5
Reaction Times				
Pre-incubation	95°C–11 min	95°C–11 min	95°C–11 min	95°C–11 min
*Denaturation	94°C–20s	94°C–20s	94°C–20s	94°C–1 min
*Annealing	59°C–2 min	59°C–3 min	59°C–3 min	61°C–1 min
*Extension	72°C–1 min			72°C–1 min
Elongation	60°C–45 min	60°C–10 min	60°C–10 min	60°C–80 min
*Num. Cycles	30	28	29	30
Total Time	2 h 36 min	1 h 54m 2 0 s	1 h 57 m 40 s	3 h 01 min

Laser Microdissection Procedure

Slide Preparation—A small portion of each sample was cut out from the fabric, and cells were resuspended in 300 μL of PBS. Samples were then incubated for an hour, with agitation, at room temperature. The fabric portion was removed, and the samples centrifuged at 13,000 rpm in a Biofuge Pico (Heraeus) for 3 min. The supernatant was discarded, and the cell pellet was resuspended in 30 μL of PBS. The sample was smeared on a frame slide with PET (polyethylene terephthalate)membrane (MMI membrane slide) and fixed with alcohol 95% (v/v).

Staining—The smears were stained with hematoxylin and eosin (H&E), in a simplified procedure (6 min hematoxylin incubation and washout with distilled water plus 4-min incubation with May-Grünwald Eosin). H&E staining was chosen because it is the simplest and most widespread staining technique (12). The Sperm Hy-Liter Express (Independent Forensics) (SHL) was used in another set of samples according to the manufacturer's instructions, except for the final (optional) mounting procedure. In the latter situation, nuclei from both epithelial and sperm cells were visualized using DAPI-compatible fluorescence filters. Human sperm heads were visualized using fluorescein or Alexa 488 compatible filters.

Laser Microdissection—Laser microdissection was conducted in an Olympus Cell Cut Inverted Microscope with a fluorescence module attached. The MMI Cell Tools ® software was used to command visualization, microdissection, capturing, and reporting cells of interest. The procedure included a full slide scan for each slide with a 4× objective. Cutting was performed with the 20× objective for Sperm Hy-Liter stained samples and the 40× objective for samples stained with H&E. Microdissected sperm cells were automatically captured (glued by the mounting membrane) directly onto MMI Isolation Caps of 0.5-mL microtubes without any kind of manipulation, thus preventing sample contamination by the operator. Approximately 50 sperm cells were selected to be captured per slide. Captured sperm cells were microscopically inspected whenever necessary during the procedure to confirm integrity and relative position of the samples on the MMI Isolation Cap.

DNA Extraction, Quantification, Amplification, and Genotyping

Extraction was performed using the Arcturus PicoPure DNA Extraction kit (Applied Biosystems). This extraction kit was selected because it was specifically designed for LMD samples and provides a DNA yield of up to 100% in such samples. The portion of the cap where cells were present was removed with a sterile scalpel blade and placed into a 0.2-mL microtube containing 20 μL of the extraction solution. The samples were incubated for 3 h at 65°C, and the proteinase K was inactivated at 95°C for 10 min (16). DNA amplification and genotyping follow the same procedure as for the standard method, except for the sample volume which was altered from 5 to 10 μL in order to maximize the starting DNA quantity (Table 1).

TABLE 2—*Results of sperm investigation of slide smears under hematoxylin and eosin (H&E) and fluorescent Sperm Hy-Liter staining. Each symbol represents a slide. (+: 50 or more sperm cells were found within the allocated time; −: <50 sperm cells or none was found within the allocated time). Tissue means that the sample was obtained from a fabric cutout; Direct means that a stain was directly placed onto the microscope slide.*

	Sperm concentration			
Sample	50%	20%	10%	5%
H&E				
Tissue	++–	++–	—	—
Direct	+++	+++	++–	+–
Sperm Hy-Liter				
Tissue	+++	+++	+–	—
Direct	+++	+++	+++	+++

Results

Regardless of the outcome of the forensic light and Phosphatesmo KM preliminary tests, visualization of the sperm cells is important to determine whether enough cells are present to generate a DNA profile. The Sperm Hy-Liter kit has proven to be far more effective and sensitive at detecting sperm cells, as it is based on fluorescence. Sperms cells were discovered and singled out far more easily and quickly than with H&E staining (Table 2).

Standard Protocol

Using the standard procedure, we were able to determine the genotyping profile in all mixed samples independently of the semen-to-epithelial cells proportion. Several artifacts, including stutters, adenylation, *locus* imbalance, off-scale peaks, spikes/pull-ups, among others, were however observed particularly in samples containing high DNA amounts. All these artifacts render the genotyping analysis virtually impossible, which required a second amplification procedure to be performed using less DNA template. These results are in line with the ones commonly obtained in real forensic samples and provided us a comparison with the LMD protocol.

In this context, previous studies suggest that optimal range for a mixture analysis can be anywhere between 50%, with equal amounts of both contributors (type A mixture), and 20%, with a

TABLE 3—*Quantification with the Quantifiler Trio kit. Samples were prepared in v/v (saliva/semen). Results are reported in ng/μL.*

	Quantity (ng/μL)											
	1:1 (50%)			1:4 (20%)			1:9 (10%)			1:19 (5%)		
	T. Large Autosomal	T. Small Autosomal	Y	T. Large Autosomal	T. Small Autosomal	Y	T. Large Autosomal	T. Small Autosomal	Y	T. Large Autosomal	T. Small Autosomal	Y
Sample 1	16.07	9.93	4.18	24.62	16.07	2.69	42.15	30.36	2.71	37.65	25.82	1.22
Sample 2	23.5	14.44	4.65	21.08	14.54	2.14	30.28	20.64	1.89	31.36	19.8	0.8
Sample 3	19.14	11.2	3.34	33.21	22.99	3.85	26.25	17.78	1.51	25.36	19.37	1.06
Mean	19.57	11.86	4.06	26.30	17.87	2.89	32.89	22.93	2.04	31.46	21.66	1.03
Ratio	7.75		1	15.27		1	27.41		1	51.74		1

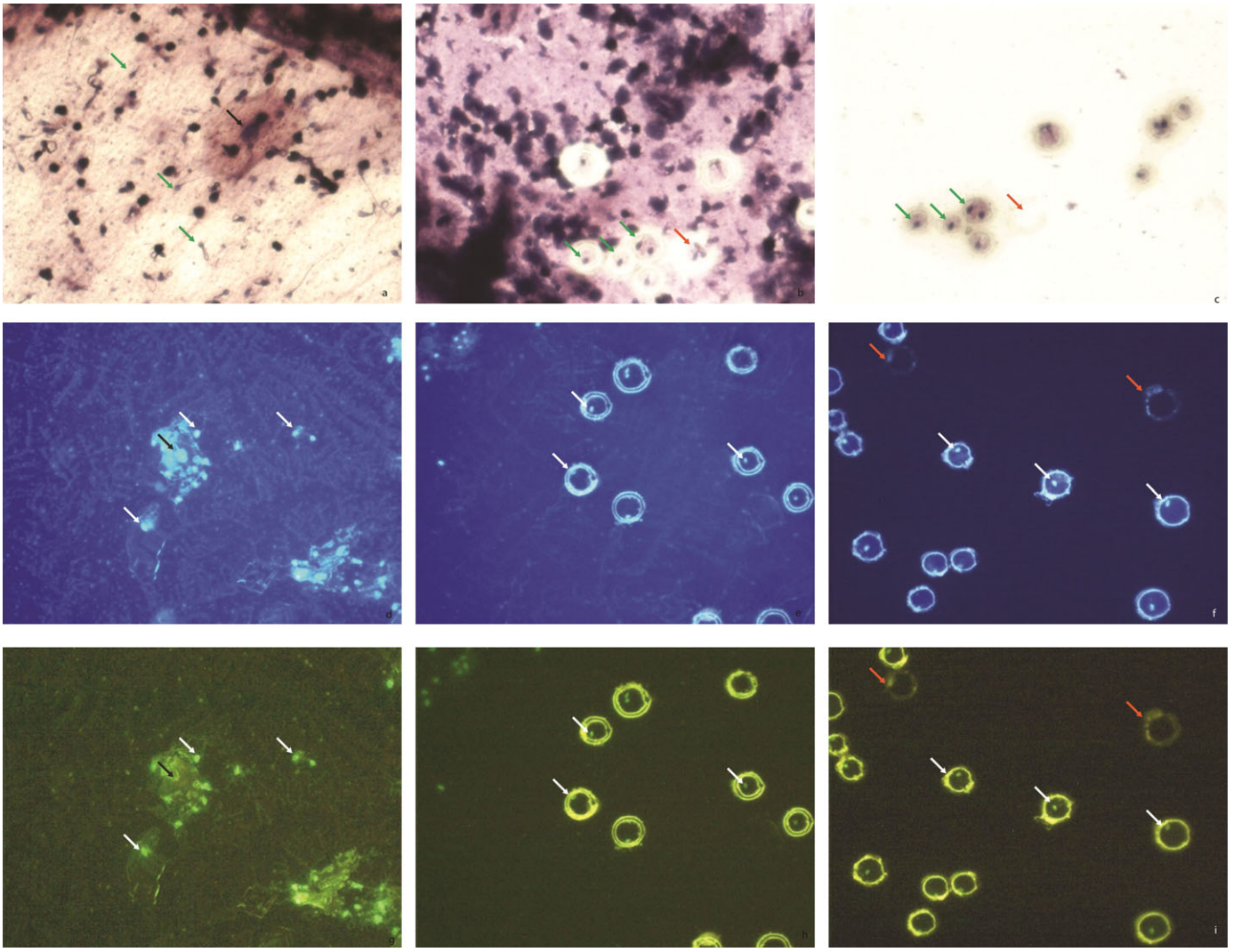

FIG. 1—*Visualization of cells for laser microdissection. a, b, and c—Hematoxylin and eosin staining. d to i—Sperm Hy-Liter Express staining. d, e, and f—DAPI staining for cell nucleus. g, h, and i—Sperm-specific staining with sperm head antibodies. First column—Slide view. Second column—view after cutting. Third column—inspection view (after cell collection). Black arrow: Epithelial cell nuclei. Green and white arrows: Sperm. Red Arrow: Imperfect cut, cells not collected. [Color figure can be viewed at wileyonlinelibrary.com]*

major and a minor contributor (type B mixture) (Table 3) (17). With less than 20% for the minor contributor, allele dropout may occur or a true peak may be considered a stutter (18).

Laser Microdissection

The laser capture microdissection is a "contamination free" process. Once the staining is performed, there is no more physical manipulation of the cells on the mounting membrane besides placing and removing the slide from the stage of the dissection microscope. Laser microdissection and sample collection was made with an automated system into contaminant-free microtubes. The capture procedure was not always an easy task to be performed. It relies very much on the operator skills, on the quality of mounting membranes and sticky microtube caps, and on fine calibration of the equipment prior to each session. In our hands, some of the cuts were not completed, leading to failures in capturing targeted cells from the slide. The capture efficiency (i.e., number of cells captured vs. targeted) was roughly between 0.8 and 1 per session. Nonetheless, the minimum requirements of 30 sperm cells to perform genotyping analysis were always met (12,19) (Fig. 1).

Although the procedure is less time-consuming and more cost-effective, the H&E staining of the samples is not as effective as when the samples were fluorescently stained with the SHL kit. H&E staining does not provide the same level of discrimination: Sperm cells could only be found when the proportion of semen-to-epithelial cells was above 20%, whereas the SHL kit allowed detection of sperm cells in concentrations down to 10% of semen. The identification of the sperm cells was readily achieved with the SHL kit with less than 10 min to find and collect 50 sperm cells per slide, as opposed to the 25 min required with the H&E staining (Table 4).

Overall, the standard procedure was found to be the less time-consuming, but it always yielded mixture genotyping profiles. In the lower semen proportions (10% and 5%), the genotyping mixture was not always apparent: the big peak imbalance and stochastic effects produced several artifacts, such as pull-ups and allele dropouts. In those samples, it was not possible to recover the genetic profile of the minor contributor. Nevertheless, the

TABLE 4—*Time per step for each procedure. Values are reported in minutes.*

	Laser Microdissection					
	Sperm Hy-Liter Express		H&E		Standard method	
Procedure	Task	Time	Task	Time	Task	Time
Resuspension	PBS	60	PBS	60		
Staining	Solution 1	12	Ethanol 95%	45		
	Solution 2	17	Dry	5		
	Solution 3	17	4× Eosin	5		
	Solution 4	17	Wash and Dry	10		
			6× Hematoxylin	5		
LMD	Scan (Whole slide)	10	Scan (Whole slide)	5		
	Collection	10	Collection	25		
Extraction	Incubation—PicoPure	180	Incubation—PicoPure	180	Incubation	40
	Proteinase K inactivation	10	Proteinase K inactivation	10	PrepFiler Extraction	30
Quantification					7500 Real-Time PCR Syst.	60
DNA amplification	Minifiler	156	Minifiler	156	Minifiler	156
	Identifiler Plus	114.3	Identifiler Plus	114.3	Identifiler Plus	114.3
	NGM	117.4	NGM	117.4	NGM	117.4
	Y Filer	181	Y Filer	181	Y Filer	181
Electrophoresis	3500 Genetic Analyzer	40	3500 Genetic Analyzer	40	3500 Genetic Analyzer	40
Results Analysis	Genemapper ID-X 1.4	5	Genemapper ID-X 1.4	15	Genemapper ID-X 1.4	25
Total time (Minifiler)		534		561		351

TABLE 5—*Cost per step for each procedure. Values are in euros and calculated per sample, according to the methods described in this article. The cost was calculated using the ordering price for each reagent, using the manufactures' websites.*

	Laser Microdissection					
	Sperm Hy-Liter Express		H&E		Standard method	
Procedure	Task	Costs (per sample)	Task	Costs (per sample)	Task	Costs (per sample)
Resuspension	PBS	0.19	PBS	0.19		
Staining	Solution 1	13.3	Ethanol 95%	0.03		
	Solution 2		Dry			
	Solution 3		4× Eosin	0.33		
	Solution 4		Wash and Dry			
			6× Hematoxylin	0.82		
LMD	Scan (Whole slide)	4.68		4.68		
	Collection	5.5		5.5		
Extraction	Incubation—PicoPure	1.54	Incubation—PicoPure	1.54	Incubation	3.67
	Proteinase K inactivation		Proteinase K inactivation		PrepFiler Extraction	6.69
Quantification					Quantifiler Trio	4.41
DNA amplification	Minifiler	21.98	Minifiler	21.98	Minifiler	21.98
	Identifiler Plus	9.45	Identifiler Plus	9.45	Identifiler Plus	9.45
	NGM	11	NGM	11	NGM	11
	Y Filer	17.73	Y Filer	17.73	Y Filer	17.73
Electrophoresis	3500 Genetic Analyzer	3.16	3500 Genetic Analyzer	3.16	3500 Genetic Analyzer	3.16
Results Analysis	Genemapper ID-X 1.4		Genemapper ID-X 1.4		Genemapper ID-X 1.4	
Total cost (Minifiler)		50.35		38.23		39.91
Total cost (Identifiler Plus)		37.82		25.7		27.38
Total cost (NGM)		39.37		27.25		28.93
Total cost (Y Filer)		46.1		33.98		35.66

standard procedure is by far the fastest protocol, in which we are able to obtain results less than 6 h after sample collection (59.09% of the SHL) (Table 4).

Laser capture microdissection with the H&E staining proved to be effective in order to isolate male genetic profiles from recovered sperm cells. However, the separation was still not perfect: some *loci* displayed tri-allelic and tetra-allelic patterns and there was significant dropout, due to stochastic effects. Additionally, the H&E staining may inhibit PCR amplification (12). It was, nonetheless, the most cost-effective procedure per sample (Table 5), but also the slowest; a result can only be obtained 9h21 min after starting sample processing using the Minifiler kit (Table 4).

Our data show that staining samples with the SHL kit prepared for laser microdissection are highly reliable and provided the most consistent results, without the need for performing subsequent confirmatory analysis. It is, nevertheless, the more expensive method per sample analyzed (Table 5). The time required to obtain a final result using the SHL fluorescent staining is less, yet within the same range (~9 h), to that needed to perform the same task using the H&E staining (Table 4). Taking all this into consideration, one may conclude that considerable delay in obtaining genotyping results using laser capture microdissection may be attributed to steps prior (staining) and after (extraction) microdissection itself, which account for 23% and 36% of the total estimated time to complete the procedure,

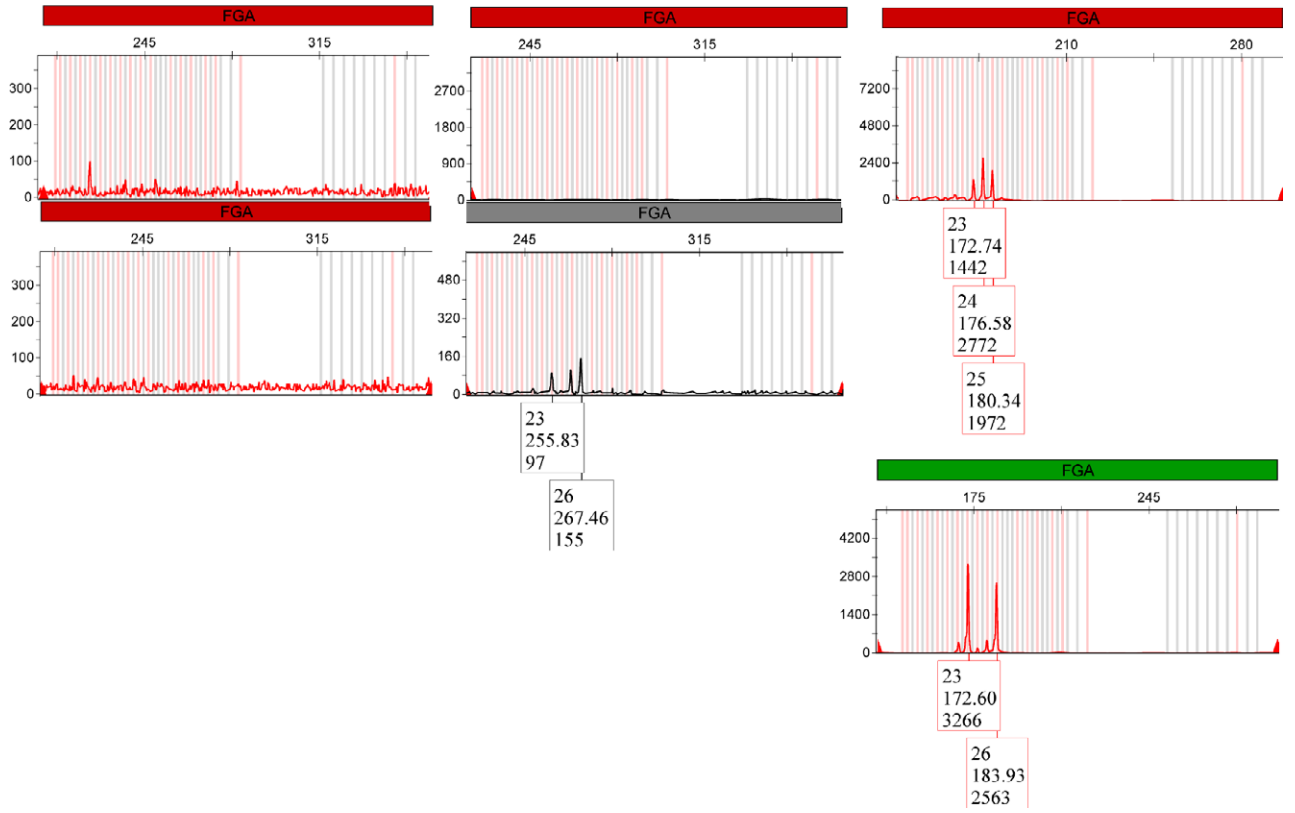

FIG. 2—*Electropherograms of the FGA marker in samples analyzed with LMD. Left column, Identifiler Plus kit; middle column, NGM kit; right column, Minifiler kit. top row, H&E staining; bottom row, SHL staining. Correct genotype for this marker is 23 and 26. [Color figure can be viewed at wileyonlinelibrary.com]*

respectively. It is also worth mentioning that SHL staining coupled to laser microdissection is only possible if the dissection system incorporates an epifluorescence microscope, which significantly increases the initial budget for the investment.

The quality of the results is of utmost importance: Low quality or bad genotyping results may render the evaluation of evidence difficult or even impossible. Whenever this is the case, more costs must be added for repeated sample processing, which can easily reach and exceed the amount required to process mixed samples using laser capture microdissection with the SHL fluorescence staining. Of course, from this equation we are excluding the initial investment spent in the acquisition of the laser microdissection system coupled to a fully motorized epifluorescence microscope which can easily reach more than $169,500.

Results obtained using several kits of autosomal *loci* and a semen concentration of 50% show that all kits amplified a mixture (M) in all *loci*. Identifiler Plus and NGM have in common 10 loci: D8S1179, D21S11, D3S1358, TH01, D16S539, D2S1338, D19S433, vWA, D18S51, and FGA. In these loci, the kits performed almost identically for all the tested semen concentrations, when using the standard method. When the semen proportion was lowered to 20%, in some *loci,* only the major contributor (m) was amplified. This trend was followed by other *loci*, when the tested concentrations dropped to 10% and even further to 5%. However, the Minifiler kit led to mixture profiles in the 20% concentration, with the first dropouts occurring at 10%.

The LMD with the SHL staining produced the best results (Fig. 2), and successful amplification of all *loci* was achieved using the Minifiler kit. With Identifiler Plus and NGM amplification kits, results were only slightly better with SHL than using the H&E staining.

Several artifacts were observed in most amplifications, regardless of staining technique or amplification kit utilized, with the notable exception of the conjunction of SHL staining and Minifiler amplification (Fig. 2). Allele dropout and drop-in were the most common artifacts. The FGA marker of Fig. 2 was chosen to be representative of the results obtained in this study because it is one of the few markers present in all autosomal amplification kits.

Finally, Y Filer produced results with all methods. However, when using the standard method in the lowest semen concentration DYS19, DYS439, DYS392, Y GATA H4, and DYS438 started to dropout (*, Table 6).

Discussion

Here, we aimed to determine the sensitivity and the existence of any correlation between the sperm concentration, preliminary testing results, and microscopic viewing. The tested sperm dilutions are within or below the expected range usually found in casework stains.

Phosphatesmo KM strips proved to be highly sensitive to the phosphatous acid present in the seminal fluid. Also, the reaction intensity, as measured by the obtained color in the strip, appears to be concentration dependent, something that was expected in order to provide some insight regarding the semen quantity in the samples

Visualization of the sperm cells and their collection is important for downstream analysis. A minimum number of cells were

TABLE 6—*Quality of results using the LMD/SHL, LMD/H&E, and Standard protocol with several commercial kits (Identifiler Plus, NGM, Minifiler, Y Filer). Results shown by locus and respective average peak height (results averaged from a minimum of three separate electropherograms, in RFUs). +: Successful amplification; −: no amplification; M: mixture; m: major contributor only; *: successful amplification but beginning to dropout. The standard procedure has results shown by semen concentration (%): 50/20/10/5.*

	Identifiler Plus			NGM			Minifiler			Y Filer		
Kit and Method	LMD/SHL	LMD/H&E	Standard	LMD/SHL	LMD/H&E	Standard	LMD/SHL	LMD/H&E	Standard	LMD/SHL	LMD/H&E	Standard
Locus												
D8S1179	306	172	M/M/M/m	230	242	M/M/M/m						
D21S11	100	<100	M/M/M/m	333	220	M/M/M/m	3105	3076	M/M/M/M			
D7S820	<100	<100	M/m/m/m				5742	2568	M/M/m/m			
CSF1PO	<100	<100	M/m/m/m				5241	8963	M/M/m/m			
D3S1358	862	280	M/M/M/M	293	201	M/M/M/M						
TH01	331	426	M/M/m/m	<100	244	M/M/m/m						
D13S317	<100	<100	M/M/m/m				2395	2204	M/M/M/M			
D16S539	143	124	M/M/m/m	298	349	M/M/m/m	4652	2128	M/M/M/m			
D2S1338	<100	<100	M/M/m/m	<100	<100	M/M/m/m	3006	3423	M/M/M/m			
D19S433	180	233	M/M/m/m	423	380	M/M/m/m						
vWA	200	238	M/M/M/M	411	407	M/M/M/M						
TPOX	<100	<100	M/M/M/M									
D18S51	<100	<100	M/m/m/m	274	<100	M/m/m/m	3865	8597	M/M/M/m			
D5S818	<100	<100	M/M/M/m									
FGA	<100	<100	M/M/m/m	<100	210	M/M/m/m	2914	2062	M/M/m/m			
D1S1656				352	246	M/m/m/m						
D12S391				<100	232	M/m/m/m						
D10S1248				535	633	M/M/M/m						
D22S1045				532	472	M/M/M/m						
D2S441				442	250	M/M/m/m						
DYS456										928	315	+/+/+/+
DYS389I										364	420	+/+/+/+
DYS390										508	189	+/+/+/+
DYS389II										336	244	+/+/+/+
DYS458										493	608	+/+/+/+
DYS19										278	134	+/+/+/*
DYS385 a/b										800	471	+/+/+/+
DYS393										701	181	+/+/+/+
DYS391										936	600	+/+/+/+
DYS439										422	532	+/+/+/*
DYS635										<100	300	+/+/+/+
DYS392										222	143	+/+/+/*
Y GATA H4										315	469	+/+/+/*
DYS437										645	589	+/+/+/+
DYS438										183	325	+/+/+/*
DYS448										176	129	+/+/+/+

established to ensure that a balanced profile was obtained. Furthermore, we imposed a time limit to find the minimum number of cells, so that microdissection would not become a time-consuming technique. We found that 20% was the lowest dilution in which sperm could be found with the H&E staining, whereas that limit was 10% with the Sperm Hy-Liter kit (stains found in tissue / cloth). However, if further refinements are made to the staining, mainly the fixation (20), more cells can be found. Other similar studies have tested spermatozoa detection under several conditions, including mixture with different body fluids, substrate cutouts, and semen concentration. Their results pave the way for sperm detection on samples with even lower sperm concentrations (21).

The standard procedure is well established and produced the expected results. It is the fastest procedure, despite not being the most cost-effective by a narrow margin. It confirmed mixture genetic profiles when the semen-to-saliva proportion was 20–50%, which can be interpreted with a certain degree of confidence. Below the 20% proportion, dropouts increased significantly. The quantification of total DNA amounts in complex forensic samples is of utmost importance to assess if dropouts or other artifactual phenomena may be occurring (22). Given that the DNA amount is a limiting factor for genotype profiling of mixed sexual assault samples, we and others considered that the quality of the forensic experimental results would increase using laser capture microdissection to differentially collect only sperm cells.

When performing laser capture microdissection of forensic samples, the minimum acceptable number of sperm cells required is 30. Even within this range, allele dropouts occurred when we used H&E staining. This occurred probably because H&E staining has inhibitory effects on the amplification process, which are particularly relevant with low template DNA levels; DNA degradation might also have happened under these testing conditions (12).

In our hands, the Sperm Hy-Liter Express fluorescence staining followed by laser capture microdissection proved to be by far the most effective method. It generated good quality genetic profiles from sperm cells using the Minifiler amplification kit. Capture guidance of sperm cells fluorescently stained with Sperm Hy-Liter Express was better than that obtained with H&E staining when DNA amplification was performed with the less effective Identifiler Plus kit. The best results of this series were obtained combining laser microdissection of fluorescently stained sperm cells with the Sperm Hy-Liter Express kit and DNA amplification with the Minifiler kit. Of note is the good

performance of the Y Filer amplification system in all protocols used in this study, including DNA obtained by the standard method and by laser capture microdissection. Under these conditions, all alleles were successfully amplified although we observed dropouts in some *loci* when the 5% semen proportion was tested using the standard method.

Concerning the time it takes to execute the presented protocols, the standard procedure is the most manageable. Results may be achieved in less than 6 h. However, considering the artifacts that often occur, the need for confirmation analysis is recurrent which may undermine this advantage. The standard method may still keep the time advantage if the sample has to be re-injected in the genetic analyzer, yet if the confirmation procedure requires new DNA amplification it becomes the slowest method.

On the other hand, data from this study indicate that the LMD technique is straightforward. Results of low quality most frequently indicate that the sample is degraded, and thus, undertaking a new analysis will not give better results. Moreover, after slide preparation, sample degradation is greatly reduced and the slides can be stored for postponed analysis as desired. To achieve good results, microdissection slide preparation is the most sensitive procedure. DNA extraction using the Arcturus PicoPure extraction kit is the longest step of this procedure, which takes more than 3-h incubation. Long waiting periods often allow the performance of other useful tasks, such as preparing new slide batches or data analysis.

Most laboratories consider that the greatest hurdle in using a Laser Microdissection system in forensic casework is the initial investment on the acquisition of the equipment which can easily reach more than $169,000, besides the relatively high price of the consumables. Taking into consideration only the running costs per sample submitted to LMD, it increases by about 21% when compared to the standard protocol. The Olympus Cell Cut Microdissection System used in this study required special slides with a PET membrane and special microtubes with sticky caps for automatic sample collection to avoid losing the microdissected sample and any kind of sample manipulation by the operator. Other microdissection systems, like the Laser Microdissection and Pressure Catapulting system by Palm (Carl Zeiss, Germany), can use standard glass microscopic slides and the collection method uses normal Eppendorf tubes. Savings in these two consumables are enough to place the running costs of LMD at the same level or even below the standard method.

Conclusions

Existing preliminary testing can be a good indicator to determine whether forensic samples are eligible candidates to undergo microdissection procedures. We encourage laboratories to perform similar analysis with their preliminary tests, even if they do not plan to use microdissection in the foreseeable future. The knowledge gained can be of use to decide the best course of action for each sample, even with more traditional methods (differential lysis, chelex extraction)(etc.).

The whole sample analysis and mixture interpretation method used in this study are generally accepted for forensic analysis of sexual assault samples in Portugal because it can generate results relatively fast, which can be easily replicated by many labs, and is of low cost. Nonetheless, a comparison of LMD with the most commonly used method of differential extraction is of significant importance to help validate the conclusions of this work and provide laboratories with a more accurate comparison with their own methods. However, forensic samples are hardly ever in pristine conditions and performing analysis with low quantity or degraded samples is challenging. Working near the threshold of recommended cells collected and allowing some sample degradation to occur proved to be detrimental to achieve results of undisputed quality. However, it was possible to recover complete autosomal profiles using the combination of LMD, staining with SHL and posterior analysis with the MiniFiler amplification kit. Despite analyzing less markers, the ability to obtain solid results from all loci (an order of magnitude greater compared to the other used autosomal kits) allows a profile to be singled out, validated and statistically valuated.

The LMD technique using mixed samples fluorescently stained with the Sperm Hy-Liter Express kit provided clean, individual genotyping profiling, when used in conjunction with the Minifiler amplification kit, thus preventing the necessity for more sophisticated mixture analysis. With this method, good results were achieved using minimal cell numbers, yet we recommend collecting more than the 50 cells per sample of this study to increase the analysis robustness. Increasing the number of cells of interest collected is crucial to improving the quality of genotyping results obtained with faster and cheapest autosomal DNA amplification kits.

The Olympus Cell Cut Microdissection System used in this comparative study has relatively higher running cost than other microdissection systems, because it requires MMI special slides and collection microtubes. Yet, it has the advantage of being fully automatized and "hands-free," which contributes to dramatically decrease contamination probability of the samples by the operator. Other systems allow the use of common slides and microtubes, which significantly decrease microdissection running costs to the level of the standard procedure. Nevertheless, these techniques designed to minimize costs often have the problem of contamination, losing or destroying nucleic acids in the dissected sample as major drawbacks, as all procedures are performed in slices with coverslips removed and they use either out-of-focus UV laser power to catapult the sample (Palm system from Carl Zeiss) or a high-power IR laser to melt the transfer film in order to collect glued samples (Arcturus XT from Molecular Devices). It is worth noting that the versatility offered by laser microdissection systems facilitates the analysis of various sample types (bone, hair) (etc.) (23).

LMD methods are more time-consuming than standard procedures, but provide end results of better quality. Recent studies encourage, whenever possible, the use of high-sensitive high-reproducible methods to avoid the need for performing mixture genotyping analysis (24). Most of the rape cases are perpetrated by single individuals, which genetic profile can be easily resolved with LMD (25). Furthermore, recent studies have been successful in amplifying Y-STR haplotypes from a single sperm cell, which can be useful in rape cases with multiple perpetrators (26–28).

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