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Analysis of body fluid mixtures by mtDNA sequencing: An inter-laboratory study of the GEP-ISFG working group

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Abstract

The mitochondrial DNA (mtDNA) working group of the GEP-ISFG (Spanish and Portuguese Group of the International Society for Forensic Genetics) carried out an inter-laboratory exercise consisting of the analysis of mtDNA sequencing patterns in mixed stains (saliva/semen and blood/semen). Mixtures were prepared with saliva or blood from a female donor and three different semen dilutions (pure, 1:10 and 1:20) in order to simulate forensic casework. All labs extracted the DNA by preferential lysis and amplified and sequenced the first mtDNA hypervariable region (HVS-I). Autosomal and Y-STR markers were also analysed in order to compare nuclear and mitochondrial results from the same DNA extracts. A mixed stain prepared using semen from a vasectomized individual was also analysed. The results were reasonably consistent among labs for the first fractions but not for the second ones, for which some laboratories reported contamination problems. In the first fractions, both the female and male haplotypes were generally detected in those samples prepared with undiluted semen. In contrast, most of the mixtures prepared with diluted semen only yielded the female haplotype, suggesting that the mtDNA copy number per cell is smaller in semen than in saliva or blood. Although the detection level of the male component decreased in accordance with the degree of semen dilution, it was found that the loss of signal was not consistently uniform throughout each electropherogram. Moreover, differences between mixtures prepared from different donors and different body fluids were also observed. We conclude that the particular characteristics of each mixed stain can deeply influence the interpretation of the mtDNA evidence in forensic mixtures (leading in some cases to false exclusions). In this sense, the implementation of preliminary tests with the

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aim of identifying the fluids involved in the mixture is an essential tool. In addition, in order to prevent incorrect conclusions in the interpretation of electropherograms we strongly recommend: (i) the use of additional sequencing primers to confirm the sequencing results and (ii) interpreting the results to the light of the phylogenetic perspective.

Keywords: mtDNA mixture; Preferential lysis; Sequencing; HVS-I; Haplotype; mtDNA phylogeny

1. Introduction

The analysis of mixtures of fluids is routine practise in forensic casework usually related to sexual assault cases. These analyses are generally performed using preferential lyses followed by STR genotyping. In a number of cases, however, the mtDNA analyses can be the unique strategy (e.g. when analyzing degraded or low copy number samples). In other cases, establishing the mtDNA haplotypes of the contributors can add information to the legal investigation (e.g. to exclude a maternal relationship between victim and suspect in rape cases, or when trying to obtain some information about the (geographical) origin of the mtDNA carried by the unknown offender [1]).

Theoretically, when a preferential lysis is performed on semen mixed with other fluids (e.g. saliva, blood, or vaginal fluid), DNA from the non-spermatic cells remains in the first fraction, while the nuclear DNA (nDNA) from the spermatozoa remains in the second one. This is due to the fact that in the spermatic nuclei there are rich disulfide bond proteins [2], which give relative resilience (compared to epithelial and other cells) against the enzymatic treatment employed during DNA extraction [3]. Therefore, if the preferential lysis is effective, the first fraction should contain a mixture of male (from non-spermatic cells and the mid-pieces of spermatozoa) and female mtDNAs, whereas the second fraction should not retain any mtDNA from mitochondria (note however that some mtDNA inserts from the nuclear genome [4] could be interpreted as real mtDNA).

On the other hand, the number of mtDNA copies varies depending on the cell type [5,6]. It is unknown to what extent this fact could affect the detection of minor components in unbalanced mixtures. During the 2004 GEP-ISFG mtDNA proficiency exercise [7], a mixture stain (saliva from a female and semen diluted 1:20) was studied and the mtDNA sequencing analysis yielded an unexpected consensus result: only the HVS-I/HVS-II saliva haplotype was detected, while the male autosomal STR profile was predominant. Hence, the use (exclusively) of mtDNA analysis could in this case lead to a false exclusion. Several additional experiments were performed in order to clarify these apparent contradictory results. The results of these experiments pointed to the existence of different relative amounts of nuclear and mitochondrial DNAs in saliva and semen [7].

Forensic labs have demonstrated to have a great deal of experience in analysing nDNA when performing preferential lysis, but very little in mtDNA [8]. In order to shed light on the mtDNA patterns originated when analyzing mixtures of semen with other body fluids, the mtDNA-working group of the GEP-ISFG carried out the present inter-laboratory study.

2. Materials and methods

The stains were prepared using mixtures of fluids from three healthy couples (which hereafter will be referred to as couples 1, 2 and 3), each one made-up with samples from a male and a female donor. For each couple, the males donated their semen while the female provided the saliva and blood. Samples were prepared in the Policía Científica DNA lab in Madrid (Spain) by mixing saliva or blood with the same volume of semen. In order to simulate forensic casework, in each case the samples were prepared using three semen dilutions in saline buffer: pure, 1:10 and 1:20 (see Table 1). The fresh fluids were mixed in a laminar-flow hood, shaken, and subsequently, 100 μl of the mixture were

Table 1
Samples analysed in this inter-laboratory study

Female/male number pair	Female saliva/semen mixtures	Female blood/semen mixtures
Samples analysed by participating labs		
1	50 μl of saliva + 50 μl of pure semen	50 μl of blood + 50 μl of pure semen
	50 μl of saliva + 50 μl of semen 1/10	50 μl of blood + 50 μl of semen 1/10
	50 μl of saliva + 50 μl of semen 1/20	50 μl of blood + 50 μl of semen 1/20
2	50 μl of saliva + 50 μl of pure semen	50 μl of blood + 50 μl of pure semen
	50 μl of saliva + 50 μl of semen 1/10	50 μl of blood + 50 μl of semen 1/10
	50 μl of saliva + 50 μl of semen 1/20	50 μl of blood + 50 μl of semen 1/20
3	50 μl of saliva + 50 μl of pure semen	50 μl of blood + 50 μl of pure semen
	50 μl of saliva + 50 μl of semen 1/10	50 μl of blood + 50 μl of semen 1/10
	50 μl of saliva + 50 μl of semen 1/20	50 μl of blood + 50 μl of semen 1/20
Samples analysed by coordinating lab		
4 (Female 3 + vasectomized male)	50 μl of saliva + 50 μl of pure semen	50 μl of blood + 50 μl of pure semen
	50 μl of saliva + 50 μl of semen 1/10	50 μl of blood + 50 μl of semen 1/10
	50 μl of saliva + 50 μl of semen 1/20	50 μl of blood + 50 μl of semen 1/20

Table 2
HVS-I haplotypes of donors and the number of labs that analysed each couple

Couple	Donor	HG	Haplotype	No. of labs
1	Female	T	16294T 16304C	9
	Male	H	16362C	
2	Female	K	16192T 16224C 16284G 16286G 16311C 16362C	9
	Male	H	CRS[a]	
3	Female	H	16092C 16257T 16293G 16311C	8
	Male	J	16069T 16126C 16193T 16278T 16311C	
4	Female	H	16092C 16257T 16293G 16311C	1
	Male	J	16069T 16126C 16300G	

HG, the most likely haplogroup assignation.
[a] Cambridge reference sequence [27].

applied onto bloodstain cards (Whatman) and air-dried. All samples were produced as a single lot. The HVS-I haplotypes of each donor are shown in Table 2.

Previously, one lab tested couple 1 mixtures. Saliva and blood from the female donor mixed with undiluted semen and with semen diluted 1:5, 1:10, 1:20 and 1:40 were analysed by sequencing. This test was carried out with the aim of selecting proper semen dilutions in order to avoid excessive dilutions, which would prevent the detection of the male components in the mixtures.

Fourteen labs participated in the study. Each lab analysed two couples (12 samples), while each couple was analysed by at least eight labs (see Table 2). No *a priori* information was given to the participants concerning either the mtDNA haplotypes of the original donors or the semen dilutions. The only recommendation given to the labs was to select the central region of each stain for DNA extraction in order to avoid as much as possible heterogeneity in the distribution of the fluids in the stain. The labs were requested to fill in a questionnaire with the technical details of the analysis. Each lab used their routine methods to carry out preferential lysis, cell count, nuclear and/or mtDNA quantification, PCR amplification and automated DNA sequencing (both strands) of the HVS-I region. Several labs also performed autosomal and Y-STRs typing in both fractions (see Table 3). One of the participants additionally carried out the analyses extracting the DNA using common protocols (no preferential lysis).

Finally, the coordinating lab performed two additional tests. In the first test, to assess how the preferential lysis works in unmixed samples, the DNA from each fluid of each donor was extracted independently (without mixing); nDNA in the extracts was then quantified, and mtDNA HVS-I was amplified. In the second test, stains composed of saliva and blood from the female donor of couple 3 mixed with semen (pure, 1:10 and 1:20) obtained from a vasectomized individual (couple 4) (see Tables 1 and 2) were prepared and analysed (preferential DNA extraction, nDNA quantification, PCR amplification and automated sequencing of the region HVS-I).

3. Results

Results concerning cell count and DNA quantification were heterogeneous between labs; nevertheless, each lab reported coherent results depending on the corresponding semen dilution, that is to say, the number of spermatozoa detected as well as the amount of nuclear or mtDNA decreased in proportion to the semen dilution. The differences in DNA quantification between labs could be attributed to stain sub-sampling (amount of sample stain taken for the analysis), or to different DNA extraction efficiency and/or to different DNA quantification methodologies.

Two labs carried out mtDNA quantification by using real time PCR. As shown in Table 4, there are some differences in the results obtained in couple 2 between the two labs. Lab no. 1 amplified a 218 bp fragment located in the HV-II segment, while lab no. 12 performed the PCR in a 287 bp fragment located in the HV-I segment. In addition, both labs used different monitoring methodologies: SYBR-Green I and Taqman probe, respectively. These two different strategies, plus the possibility of differences in the efficiency of the DNA extraction and in the amount of the final extract volume, as well as differences in the stain sub-sampling, might explain the dissimilar amounts of mtDNA copies detected.

Results concerning mtDNA haplotypes are detailed in Tables 5 and 6. In total, 312 HVS-I sequencing analyses

Table 3
Methodology

Lab	First lysis digestion time	Stain	Quantification	Detection	Edited nucleotides	Autosomal/Y-STRs
1	1 h 50 min	Erythrosine 0.5%	mtDNA RT-PCR	ABI377XL	16024–16366	No/no
2	1 h	Christmas tree	Agarose gel	ABI310	–	Yes/yes
3	2 h	Methylene blue	Quantifiler human[a]	ABI310	16033–16391	Yes/yes
4	4 h	Christmas tree	Quantifiler human[a]	ABI310	16024–16365	No/no
5	90 min	–	–	ABI3100 Avant	16050–16400	Yes/yes
6	19 h (37 °C)	Giemsa	Quantifiler human[a]	ABI310	16023–16410	Yes/yes
7	1 h (37 °C)	Korin–Stockis	Quantifiler human[a]	ABI3100 Avant	–	Yes/yes
8	90 min	Christmas tree	Quantiblot[b]	ABI310	16024–16365	No/no
9	2 h	Christmas tree	Quantiblot[b]	ABI310	16025–16365	No/no
10	2 h	Methylene blue	Quantifiler human[a]	ABI3100	–	Yes/yes
11	2 h	Haematoxylin-eosin	Quantiblot[b]	ABI310	16024–16365	No/no
12	2 h	Christmas tree	Nuclear and mtDNA RT-PCR [9]	ABI377	16024–16365	No/no
13	2 h	Papanicolau	Quantiblot[b]	ABI3100	16024–16519	Yes/no
14	2 h	Erythrosine	Quantiblot[b]/Quantifiler[a] human	ABI377	16024–16365	No/no

(−) Not reported.
[a] Quantifiler™ Human DNA quantification kit (Applied Biosystems).
[b] Quantiblot® Human DNA quantitation kit (Applied Biosystems).

Table 4
mtDNA quantification results by using two different RT-PCR strategies: (i) amplification of a 218 bp HV2 fragment and detection by SYBR-Green I and (ii) amplification of a 287 bp HV1 fragment and detection by Taqman probe

Couple	Sample	Number of mtDNA copies (μl^{-1})	
		First lysis	Second lysis
(i) Amplification of a 218 bp HV2 fragment			
2	Saliva/pure semen	4.23×10^8	Undetectable
	Saliva/semen 1:10	5.78×10^8	Undetectable
	Saliva/semen 1:20	6.41×10^8	Undetectable
	Blood/pure semen	1.32×10^8	Undetectable
	Blood/semen 1:10	4.08×10^7	Undetectable
	Blood/semen 1:20	1.8×10^7	5.32
3	Saliva/pure semen	5.15×10^{15}	100.7
	Saliva/semen 1:10	3.15×10^8	2.83
	Saliva/semen 1:20	1.12×10^8	Undetectable
	Blood/pure semen	3.6×10^8	0.693
	Blood/semen 1:10	3.6×10^7	5.67
	Blood/semen 1:20	2.06×10^7	Undetectable
(ii) Amplification of a 266 bp HV1 fragment			
1	Saliva/pure semen	8.50×10^5	4.32×10^3
	Saliva/semen 1:10	1.04×10^5	2.98×10^3
	Saliva/semen 1:20	3.38×10^4	9.46×10^2
	Blood/pure semen	1.83×10^6	1.19×10^3
	Blood/semen 1:10	2.26×10^6	3.07×10^3
	Blood/semen 1:20	1.09×10^6	4.58×10^2
2	Saliva/pure semen	1.01×10^6	4.42×10^4
	Saliva/semen 1:10	1.51×10^6	4.02×10^4
	Saliva/semen 1:20	1.32×10^6	6.38×10^4
	Blood/pure semen	1.33×10^6	2.14×10^3
	Blood/semen 1:10	8.55×10^5	1.68×10^3
	Blood/semen 1:20	1.06×10^6	1.38×10^3

were performed; 104 of them yielded a complete or partial female and male mixed profile, 142 analyses yielded only the female haplotype, 3 yielded only the male haplotype, and finally, 63 analyses were inconclusive. Therefore, the female haplotype was detected more frequently than the mixture of haplotypes.

3.1. Comparing the first and second fractions

As expected, results were more consistent in the first fraction DNA extracts than in the second ones (Fig. 1 and Table 7). As shown in Table 6, only 3 analyses gave inconclusive results in first fractions whereas a total of 60 analyses were inconclusive (no amplification, blurred sequences or contaminations) in the second ones. In addition, some participants reported to have reanalysed some samples due to DNA contaminations detected in the second fractions in the first attempt.

When comparing the three semen dilutions of the first fractions (Fig. 1(a)), most of the labs reported a mixture of male and female haplotypes for those samples containing undiluted semen (41 out of 52). In contrast, most labs detected only the female haplotype in samples prepared with diluted semen 1:10 (36 out of 52 analyses) and in samples prepared with dilution 1:20 (38 out of 52). The mixtures of male and female haplotypes detected in both 1:10 and 1:20 diluted semen samples were mainly incomplete mixtures (complete female plus incomplete male). For instance, all the mixtures detected in blood/(semen 1:20) samples were incomplete, that is to say, some of the male haplotype polymorphisms were absent (see Table 5). Therefore, the male component became less evident in proportion to the degree of the semen dilutions.

One laboratory reported only the male haplotype in a sample prepared with undiluted semen (couple 1, see Table 5). This result could be attributed to sub-sampling, probably involving a section of the mixed stain containing (by chance) low number of epithelial cells from saliva and a high number of cells from semen.

Surprisingly, one lab reported a mixture of haplotypes in a sample with semen diluted 1:20 whereas with semen diluted 1:10 only the female haplotype was detected. This only occurred in 2 out of 156 first lysis analyses; a possible explanation for this unexpected result is that samples were mistakenly mixed-up at some step in the analysis process.

In addition, the loss of male nucleotide signals was generally not uniform throughout the electropherograms (see Fig. 2), probably indicating some dependence on the fluorochrome involved and/or the nucleotide sequence of the flanking region.

One lab carried out the analyses by using both preferential and total lyses (Table 8). All DNA extracts from the second fraction yielded blurred electropherograms. The mixture of female and male haplotypes was detected in a higher number of samples when the DNA extraction was performed by total lysis than when the lysis was preferential.

Results concerning autosomal STRs were as expected (Fig. 3). In the first lysis, all labs detected a mixed STR profile in those stains of blood or saliva mixed with undiluted semen. Many labs detected the male component in the second lysis. In cases where the semen was diluted 1:10 or 1:20, the preferential extraction was less useful, with the female autosomal STR profiles frequently obscuring that of the male in second fractions. Therefore, patterns of nDNA from semen were clearly different from those obtained for the mtDNA: DNA extracts yielded a mixture of profiles when analysed by autosomal STR typing, but showed only the female mitochondrial haplotype. Firstly, the capabilities of mixture detection may be different in the two analytical systems (STR fragment analysis *versus* sequencing). Secondly, detecting the minor component of a mixture also depends on the overall concentration of the mixture. And thirdly, differences in relative amounts of nuclear and mtDNAs in semen and in saliva or blood are also possible.

A number of participants did not obtain sufficient signal for one or more Y-STR loci in diluted semen mixtures. As expected, Y-STR alleles were obtained in both first (from non-spermatic male cells) and second (from spermatozoa) fractions. In first fraction, DNA extracts from samples prepared with diluted semen (in which only the female autosomal STR and mtDNA profiles were detected) a partial or complete Y-STR profile was identified. These results demonstrate once again the usefulness of performing Y-STR analyses in forensic

Table 5
First fraction results

Couple	Sample	mtDNA profile	Conclusion	No. of labs
1	Saliva/pure semen	16294T/C 16304C/T 16362C/T	Female + male	7
		16294T/C 16304C/T 16362C	Incomplete female + male	1
		16362C	Only male	1
	Saliva/semen 1:10	16294T/C 16304C/T 16362C/T	Female + Male	4
		16294T/C 16304C/T, 16304C/T 16362C/T	Female + incomplete male	4
		16294T 16304C	Only female	1
	Saliva/semen 1:20	16294T/C 16304C/T 16362C/T	Female + Male	3
		16294T 16304C 16362C/T, 16294T/C 16304C/T	Female + Incomplete male	2
		16294T 16304C	Only female	3
		Blurred	Inconclusive	1
2	Saliva/pure semen	16192T/C 16224C/T 16284G/A 16286G/C 16311C/T 16362C/T	Female + male	1
		16192T 16224C/T 16284G 16286G, 16311C 16362C, 16192T/C 16224C/T 16284G 16286G 16311C 16362C, 16192T 16224C/T 16284G/A 16286G/C 16311C/T 16362C/T	Female + incomplete male	3
		16192T 16224C 16284G 16286G 16311C 16362C	Only female	5
	Saliva/semen 1:10	16192T 16224C 16284G 16286G 16311C 16362C	Only female	9
	Saliva/semen 1:20	16192T 16224C 16284G 16286G 16311C 16362C	Only female	9
3	Saliva/pure semen	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male	7
		16069T/C 16092C/T 16257T/C 16293G/A 16311C	Female + incomplete male	1
	Saliva/semen 1:10	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male	2
		16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G 16311C, 16069T/C 16092C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C, 16092C/T 16257T/C 16293G 16311C	Female + incomplete male	3
		16092C 16257T 16293G 16311C	Only female	1
		Blurred	Inconclusive	1
	Saliva/semen 1:20	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311 C	Female + male	1
		16069T/C 16092C/T 16257T 16293G 16311C, 16069T/C 16092C/T 16126C/T 16193T/C, 16257T/C 16278T/C 16293G 16311C	Female + incomplete male	2
		16092C 16257T 16293G 16311C	Only female	5
1	Blood/pure semen	16294T/C 16304C/T 16362C/T	Female + male	5
		16294T 16304C 16362C/T, 16294 T/C 16304 T/C, 16294T 16304 C/T	Female + incomplete male	3
		16294T 16304C	Only female	1
	Blood/semen 1:10	16294T 16304C	Only female	9
	Blood/semen 1:20	16294T 16304C	Only female	9
2	Blood/pure semen	16192T/C 16224C/T 16284G/A 16286G/C 16311C/T	Female + male	3
		16192T/C 16224C/T 16284G 16286G 16311C 16362 C/T, 16192T/C 16224C/T 16284G/A 16311C/T 16362C/T, 16192T 16224C/T 16284G/A 16286G/C 16311C (edited up to 16350)	Female + incomplete male	3
		16192T 16224C 16284G 16286G 16311C 16362C	Only female	3
	Blood/semen 1:10	16192T 16224C 16284G 16286G 16311C 16362C	Only female	9
	Blood/semen 1:20	16192T 16224C 16284G 16286G 16311C 16362C	Only female	8
		Blurred	Inconclusive	1
3	Blood/pure semen	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male	6
		16069T/C 16126C/T 16193T/C 15257C/T 16278T/C 16293G/A 16311C	Incomplete female + male	1
		16092C 16257T 16293G 16311C	Only female	1
	Blood/semen 1:10	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male	2
		16069T/C 16092C/T 16126C/T 16193T/C, 16257T 16278T/C 16293G 16311C	Female + incomplete male	1
		16092C 16257T 16293G 16311C	Only female	5
	Blood/semen 1:20	16092C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C, 16069T/C 16092C/T 16193T/C 16257T/C 16278T/C 16293G 16311C, 16092C/T 16257T 16278T/C 16293G 16311C	Female + incomplete male	3
		16092C 16257T 16293G 16311C	Only female	5
Total				156

Table 6
Second fraction results

Couple	Sample	Haplotype	Conclusion	Lab
1	Saliva/pure semen	16294T/C 16304C/T 16362C/T	Female + male	4
		16294T 16304C	Only female	1
		16362C	Only male	1
		No amplification	Inconclusive	1
		Blurred	Inconclusive	1
		Contamination	Inconclusive	1
	Saliva/semen 1:10	16294T/C 16304C/T 16362C/T	Female + male	2
		16294T/C 16304C/T	Female + incomplete male	2
		16294T 16304C	Only female	3
		No amplification	Inconclusive	1
		Blurred	Inconclusive	1
	Saliva/semen 1:20	16294 T/C 16304 C/T 16362 T/C	Female + male	1
		16294T 16304C	Only female	5
		No amplification	Inconclusive	1
		Blurred	Inconclusive	1
		Contamination	Inconclusive	1
2	Saliva/pure semen	16192T 16224C/T 16284G 16286G 16311C/T 16362C/T, 16192T 16224C 16284G 16284G/C 16311C 16362C/T	Female + incomplete male	3
		16192T 16224C 16284G 16286G 16311C 16362C	Only female	2
		No amplification	Inconclusive	1
		Blurred	Inconclusive	2
		Contamination	Inconclusive	1
	Saliva/semen 1:10	16192T 16224C 16284G 16286G 16311C 16362C	Only female	5
		No amplification	Inconclusive	1
		Blurred	Inconclusive	2
		Contamination	Inconclusive	1
	Saliva/semen 1:20	16192T/C 16224C/T 16284G/A 16286G/C 16311C/T 16362C/T	Female + male	1
		16192T 16224C 16284G 16286G 16311C 16362C	Only female	5
		No amplification	Inconclusive	1
		Blurred	Inconclusive	1
		Contamination	Inconclusive	1
3	Saliva/pure semen	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male	1
		16069T/C 16092C/T 16126C/T 16193T/C 16257T 16293G 16311C, 16092C/T 16257T 16293G 16311C	Female + incomplete male	3
		16092C 16257T 16293G 16311C	Only female	1
		Blurred	Inconclusive	2
		Contamination	Inconclusive	1
	Saliva/semen 1:10	16069T/C 16092C/T 16193T/C 16257T 16293G 16311C, 16069T/C 16092C/T 16126C/T 16193T/C 16257T 16278T/C 16293G 16311C	Female + incomplete male	2
		16092C 16257T 16293G 16311C	Only female	4
		Blurred	Inconclusive	1
		Contamination	Inconclusive	1
	Saliva/semen 1:20	16069T/C 16092C/T 16257T 16293G 16311C, 16069T/C 16092C/T 16193T/C 16257T 16278T/C 16293G 16311C	Female + incomplete male	2
		16092C 16257T 16293G 16311C	Only female	4
		Blurred	Inconclusive	1
		Contamination	Inconclusive	1
1	Blood/pure semen	16294T/C 16304C/T 16362C/T	Female + male	2
		16294T/C 16304C/T	Female + incomplete male	1
		16362C/T	Incomplete female + male	1
		16294T 16304C	Only female	1
		No amplification	Inconclusive	1
		Blurred	Inconclusive	2
		Contamination	Inconclusive	1
	Blood/semen 1:10	16294T 16304C	Only female	7
		No amplification	Inconclusive	1
		Blurred	Inconclusive	1
	Blood/semen 1:20	16294 T 16304 C/T 16362C/T	Female + incomplete male	1
		16294T 16304C	Only female	6
		No amplification	Inconclusive	1
		Blurred	Inconclusive	1

Table 6 (*Continued*)

Couple	Sample	Haplotype	Conclusion	Lab
2	Blood/pure semen	16192T/C 16284G/A 16286G/C 16362C/T	Female + male	1
		16192T 16224C/T 16284G/A 16286G 16311C/T 16362C	Female + incomplete male	1
		16192T 16224C 16284G 16286G 16311C	Only female	1
		No amplification	Inconclusive	1
		Blurred	Inconclusive	3
		Contamination	Inconclusive	2
	Blood/semen 1:10	16192T/C 16224C 16284G 16286G 16311C 16362C	Female + incomplete male	1
		16192T 16224C 16284G 16286G 16311C 16362C	Only female	3
		No amplification	Inconclusive	2
		Blurred	Inconclusive	1
		Contamination	Inconclusive	2
	Blood/semen 1:20	16192T/C 16224C/T 16284G 16286G 16311C 16362C, 16192T 16224C/T 16284G/A 16286G/C 16311C/T 16362C/T	Female + incomplete male	2
		16192T 16224C 16284G 16286G 16311C 16362C	Only female	3
		No amplification	Inconclusive	2
		Blurred	Inconclusive	1
		Contamination	Inconclusive	1
3	Blood/pure semen	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male	3
		16069T 16126C 16193T 16278T 16311C	Only male	1
		No amplification	Inconclusive	1
		Blurred	Inconclusive	2
		Contamination	Inconclusive	1
	Blood/semen 1:10	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male	1
		16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G 16311C	Female + incomplete male	1
		16092C 16257T 16293G 16311C	Only female	3
		No amplification	Inconclusive	1
		Blurred	Inconclusive	1
		Contamination	Inconclusive	1
	Blood/semen 1:20	16092T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + incomplete male	1
		16092C 16257T 16293G 16311C	Only female	2
		No amplification	Inconclusive	1
		Blurred	Inconclusive	3
		Contamination	Inconclusive	1
Total				156

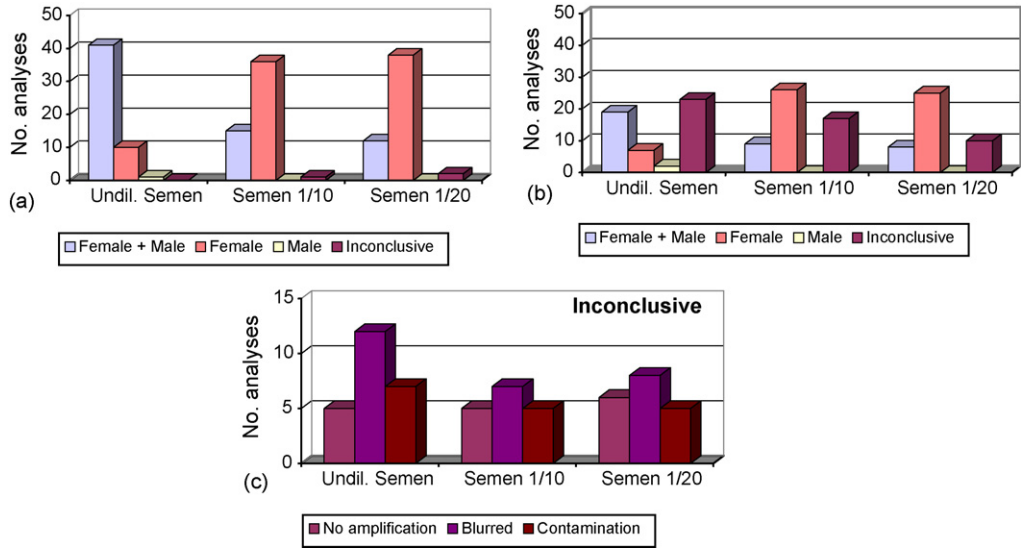

Fig. 1. Haplotype results in the first (a) and second fractions (b and c).

Table 7
Comparison between results obtained in first and second DNA extracts

Result	First fraction	Second fraction
Female + male haplotypes	68	36
Female haplotype	84	58
Male haplotype	1	2
No amplification	0	16
Blurred sequence	3	27
Contamination	0	17

male/female mixtures where the female cell contribution is predominant.

3.2. Comparison of tissues

Concerning the first fraction, no difference between saliva/undiluted semen and blood/undiluted semen mixtures was observed; most of the labs reported the female/male mixture. However, when comparing the samples prepared with 1:10 diluted semen, the female haplotype was mostly detected in blood/semen stains whereas a mixture of haplotypes was detected in half of the saliva/semen stain analyses (Fig. 4(1)). This difference between saliva and blood was still evident in the 1:20 semen samples but not so apparent as in the 1:10 dilutions.

Therefore, it seems that the number of mtDNA copy number per cell may be higher in blood than in saliva.

3.3. Comparison of donors

Results obtained in couples 1 and 3 were similar but very different from those obtained for couple 2 (Fig. 5). In first fractions, most labs detected a mixture of male and female haplotypes from couples 1 and 3 when the samples were prepared with undiluted semen. In contrast, only half of the analyses performed in samples from couple 2 yielded a mixture (Fig. 5(1a)). The differences between both couples 1 and 3 and couple 2 are more pronounced in samples with semen diluted 1:10 since all labs detected only the female haplotype in couple 2, while approximately half of the analyses yielded a mixture of haplotypes in couples 1 and 3 (Fig. 5(1b)). Finally, in samples containing semen diluted 1:20, only the female haplotype was detected in most of the analyses in the three couples, thus minimizing the differences between donors (Fig. 5(1c)). Therefore, our results suggest that different donors contribute different amounts of mtDNA.

Consistent with other results, the high amount of inconclusive data for the second fractions did not allow any clear conclusion to be reached (Fig. 5(2)).

3.4. Semen from a vasectomized individual

The blood and saliva from the female donor of couple 3 were also mixed with semen from a vasectomized individual (couple 4). As indicated in Table 9, in first and second fractions, a mixture of male and female haplotypes was detected in all samples prepared with undiluted semen. In samples with diluted semen only the female haplotype was detected.

In the first fractions, when comparing the results of couple 4 with the ones obtained in couple 3 (both couples with the same female donor), no differences were observed (see Fig. 5(1)). These results point to the fact that there may be no differences between mixtures coming from unvasectomized and vasectomized donors, at least, as far as mtDNA is concerned. We are aware, however, that more mixtures prepared using samples from other vasectomized individuals would be needed in order to derive definitive conclusions.

3.5. Preferential lysis from unmixed fluids

In order to better understand the patterns of preferential lyses in unmixed fluids, one lab carried out differential DNA extractions of blood, saliva and semen from each donor,

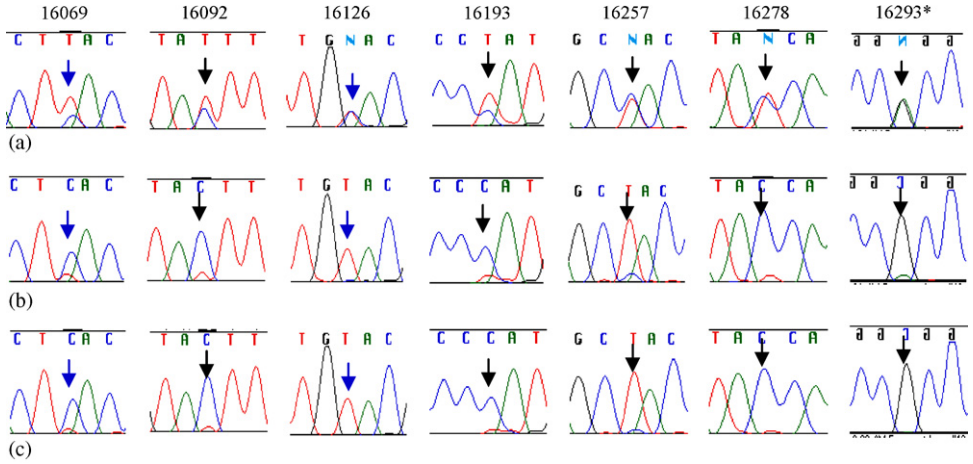

Fig. 2. Electropherograms of blood/semen samples (first fraction) from couple 3: (a) blood/undiluted semen; (b) blood/semen 1:10; (c) blood/semen 1:20. In samples prepared with semen diluted 1:10, the nucleotide position 16,126 does not show any mixture, whereas in the other positions, the male/female mixture is detected. Asterisk (*) indicates sequence with reverse primer.

Table 8
Haplotype results from the lab that performed the analyses with and without preferential lysis

Couple	Sample	Haplotypes	Conclusion
Total lysis			
2	Saliva + undiluted semen	16192T/C 16224C/T 16284G 16284G/C 16311C/T 16362C/T	Female + incomplete male
	Saliva + semen 1/10	16192T/C 16224C 16284G 16286G 16311C 16362C	Female + one male polymorphism
	Saliva + semen 1/20	16192T 16224C 16284G 16286G 16311C 16362C	Only female
	Blood + undiluted semen	16192T/C 16224C/T, 16284G 16284G/C 16311C/T 16362C/T	Female + incomplete male
	Blood + semen 1/10	16192T 16224C 16284G 16286G 16311C 16362C	Only female
	Blood + semen 1/20	16192T 16224C 16284G 16286G 16311C 16362C	Only female
3	Saliva + undiluted semen	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male
	Saliva + semen 1/10	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male
	Saliva + semen 1/20	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male
	Blood + undiluted semen	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male
	Blood + semen 1/10	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male
	Blood + semen 1/20	16069T/C 16092C 16257T 16293G 16311C	Female + one male polymorphism
First fraction			
2	Saliva + undiluted semen	16192T 16224C 16284G 16286G 16311C 16362C	Only female
	Saliva + semen 1/10	16192T 16224C 16284G 16286G 16311C 16362C	Only female
	Saliva + semen 1/20	16192T 16224C 16284G 16286G 16311C 16362C	Only female
	Blood + undiluted semen	16192T 16224C 16284G 16286G 16311C 16362C	Only female
	Blood + semen 1/10	16192T 16224C 16284G 16286G 16311C 16362C	Only female
	Blood + semen 1/20	16192T 16224C 16284G 16286G 16311C 16362C	Only female
3	Saliva + undiluted semen	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male
	Saliva + semen 1/10	Blurred	Inconclusive
	Saliva + semen 1/20	16092C 16257T 16293G 16311C	Only female
	Blood + undiluted semen	16069T/C 16092C/T 16126C/T 16193T/C 16257T/C 16278T/C 16293G/A 16311C	Female + male
	Blood + semen 1/10	16092C 16257T 16293G 16311C	Only female
	Blood + semen 1/20	16092C 16257T 16293G 16311C	Only female

Results of second lyses have not been included because all samples yielded blurred sequences.

quantified the nuclear DNA, and then amplified the HVS-I mtDNA segment, the results of which were checked by agarose gel electrophoresis stained with ethidium bromide.

Results from nuclear DNA quantification are shown in Table 10. Results of HVS-I amplification from blood and saliva are shown in Fig. 6 and those from semen in Fig. 7. nDNA was detected in both first and second fractions (Table 9) and HVS-I amplicons were obtained in both factions (Fig. 6). Neither blood nor saliva are fluids containing cells resistant to the enzymatic action of the DNA extraction chemicals. These results demonstrate that, as expected, preferential lyses performed in saliva and blood were not efficient since second fractions yielded DNA (not all cells were lysed or DNA recovered by the first fraction).

With respect to the analysis of semen samples, it would be expected to detect mtDNA only in the first fraction and nDNA in both fractions (from non-spermatic cells in the first one and from spermatozoa in the second one). The results confirmed this hypothesis. We detected nuclear DNA in both fractions and, as expected, the nDNA concentrations were higher in the second fractions than in the first ones (see Table 10). We carried out HVS-I mtDNA PCRs by using 0.4 ng of DNA as template. MtDNA amplicons were only obtained in first fractions and not in the second ones (see Fig. 7(a) and (b)). These amplicons were sequenced in order to verify that the resulting haplotypes matched the haplotypes of the donors.

In addition, we also performed HVS-I amplifications from second fractions by using increasing amounts (4 and 20 ng) of nDNA template. We only detected a slightly higher signal when the DNA template amount was increased 50 times (see Fig. 7(c) and (d)). The sequencing analysis (in order to determine whether the resulting haplotypes were consistent with the ones from the donors) was not possible due to the low yield of the amplicons. It could be inferred that these amplicons come from nuclear mitochondrial insertions (NUMTs); however, this hypothesis is unlikely to happen (see e.g. Ref. [10]).

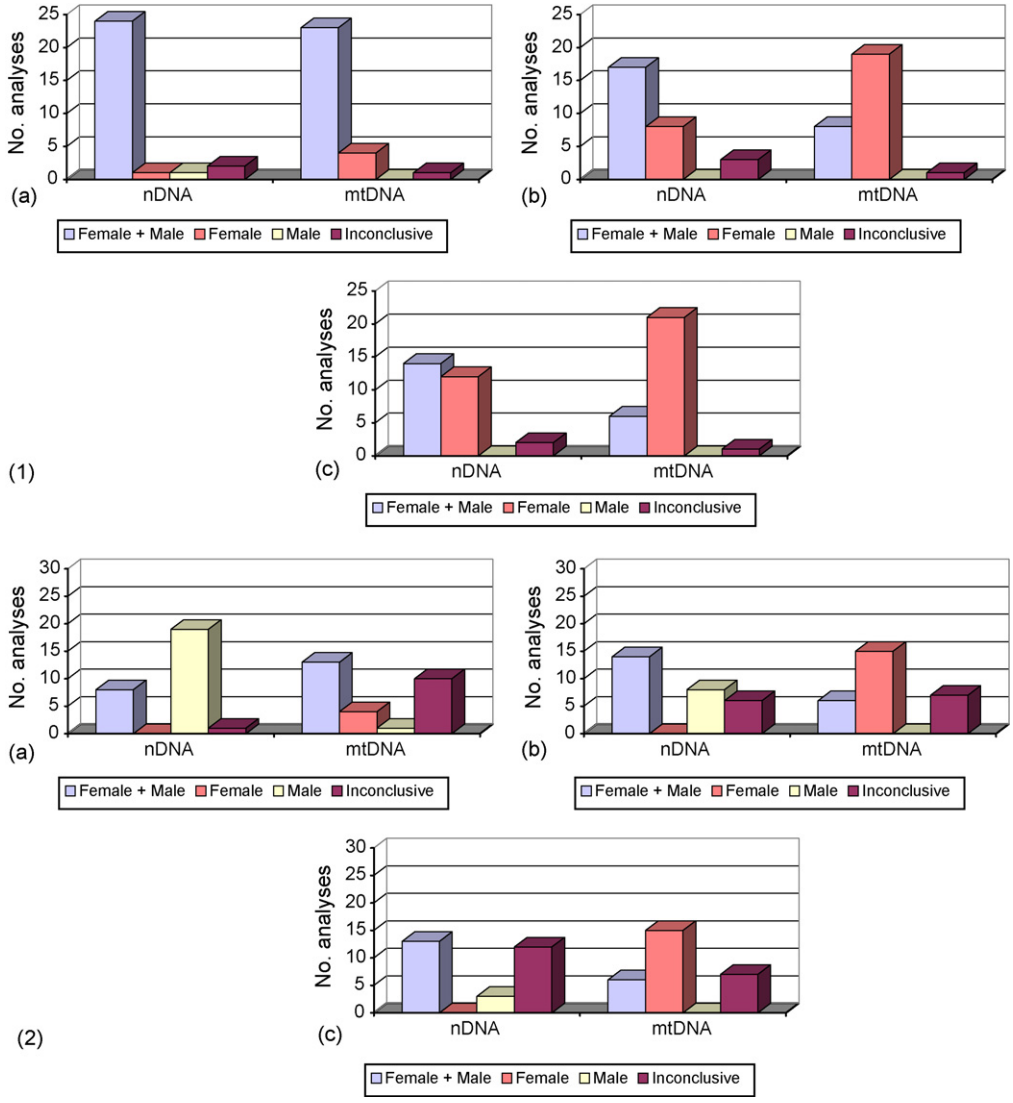

Fig. 3. Comparison between nuclear and mitochondrial results:(1) first fraction; (2) second fraction. (a) Samples prepared with pure semen; (b) samples prepared with semen diluted 1/10; (c) samples prepared with semen diluted 1/20.

4. Discussion

The analysis of forensic mixtures from rape cases using mtDNA is not routine practice in forensic casework but can be necessary when nuclear DNA is absent due to the poor or sub-optimum quality of samples, or when a maternal relationship between the evidence and reference samples is under investigation. In rape cases, labs usually perform preferential lysis in order to try to separate the victim and suspect STR profile. Nevertheless, differential lysis is not a useful tool for separating mtDNA from mixtures since theoretically all mitochondria should remain in first fractions. However, this type of DNA extraction is carried out very often in sexual assault cases. Several labs of the GEP-ISFG have studied blood/semen and saliva/semen mixtures by using the routine methodologies for analysing mixtures (preferential DNA extraction and autosomal and Y-STRs genotyping) and mtDNA (HVS-I automated sequencing).

First, the analyses of autosomal STRs allowed us to verify that, as expected, preferential lyses are less efficient than desirable (specially when semen is diluted) [11], since some labs detected the female nuclear DNA in second fractions. Regarding mtDNA, we found that, although it is impossible to separate the female component from the male one, better and more reliable results were obtained in first fractions than in the second lyses (3 inconclusive results in first fractions and 60 in the second ones). We observed three different types of inconclusive results: no amplification, blurred sequences and/or contamination. The three are expected findings if the lysis is effective. In this case, contamination could be more evident than in samples with high mtDNA content. Consequently, we recommend focusing more on the results obtained in first fractions than on the ones obtained in second fractions when interpreting mixtures from real forensic casework. Note that a good quality electropherogram does not rule out the presence of contamination; therefore, the prevention of

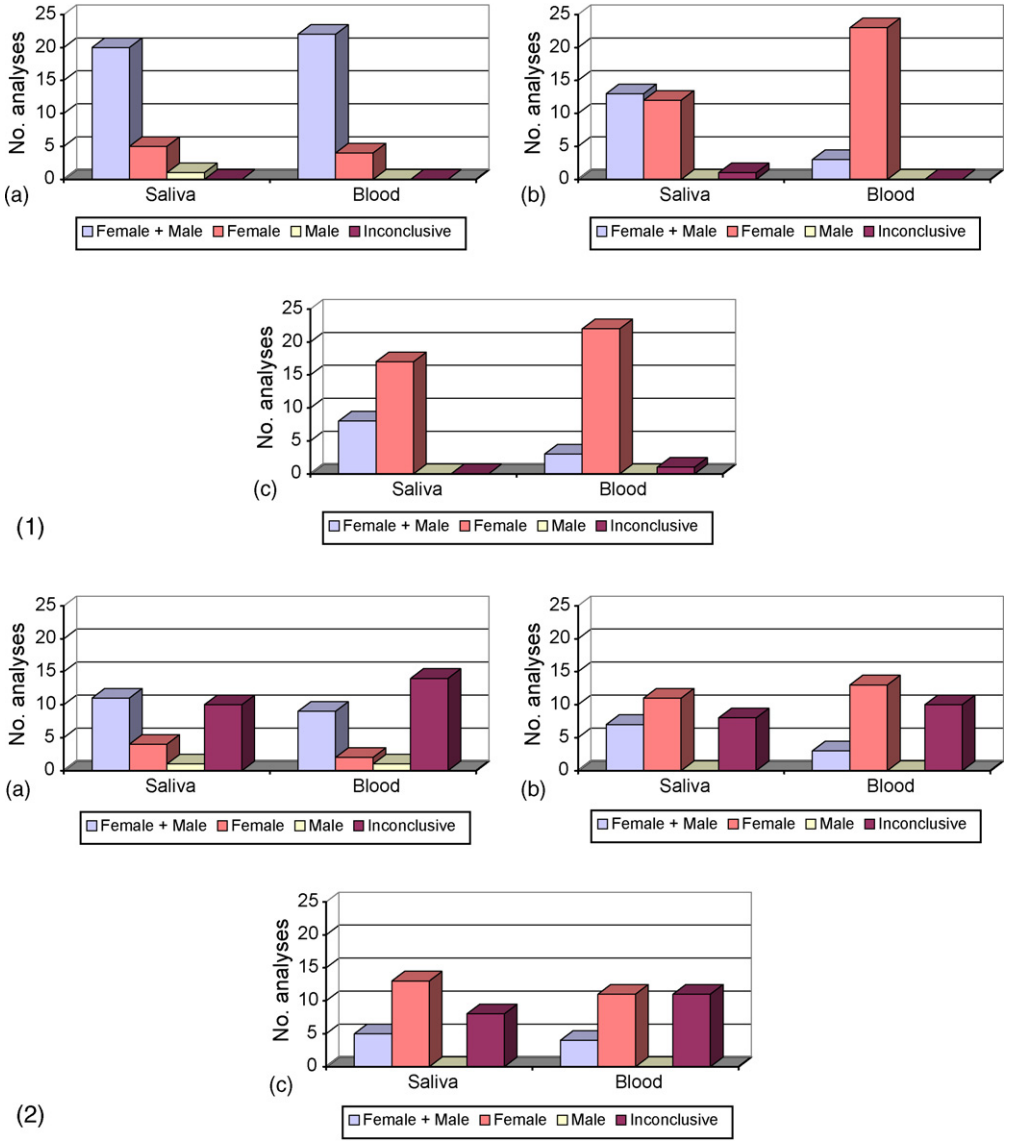

Fig. 4. Haplotype results in the first (1) and second (2) fractions. Comparison between saliva/semen and blood/semen samples. (a) Samples prepared with undiluted semen; (b) samples prepared with semen diluted 1:10; (c) samples prepared with semen diluted 1:20.

contamination should be carefully monitored throughout all the different steps of the process.

Several techniques can be used prior to sequencing in order to separate different mtDNA amplicons, such as cloning, electrophoretic separation of single strand conformational polymorphisms (SSCPs) [12], heteroduplex formation followed by electrophoresis or denaturing high-performance liquid chromatography [13]. Cloning is a technique that is not usually available in forensic labs, and requires expanded sequencing, mainly when individuals are not equally represented in the mixture [14]. SSCP or heteroduplex analyses do not allow you to distinguish all mtDNA variants. However, the separation of female and male nuclear DNAs will improve when using e.g. laser-capture microdissection (LCM) technology [15]. This new tool would enable us to obtain at least the male mtDNA component, and the combination of labelling and LCM the recovery of female mtDNA [16]. However, it is also worth mentioning that this technique may be prone to incidences of undesirable contamination [17].

When comparing the results obtained in first lyses from the three dilutions of semen (pure, 1:10 and 1:20), most labs detected the mixture of haplotypes in samples prepared with undiluted semen but not in those prepared with diluted semen (where only the female haplotype was mainly detected). The results also suggest that 1:10 dilution can lead to the loss of the male signal in the sequence electropherograms. mtDNA analysis of semen is complex because this fluid contains several cell-types (spermatozoa and round cells) at different (unpredictable) concentrations. Several approaches have been undertaken to determine the mtDNA content in spermatozoa [18–21] but so far, there is still no complete agreement on these results, which is probably due to the different methodologies applied. Our results indicate that the amount of mtDNA in semen relative to the amount of mtDNA in saliva or blood could

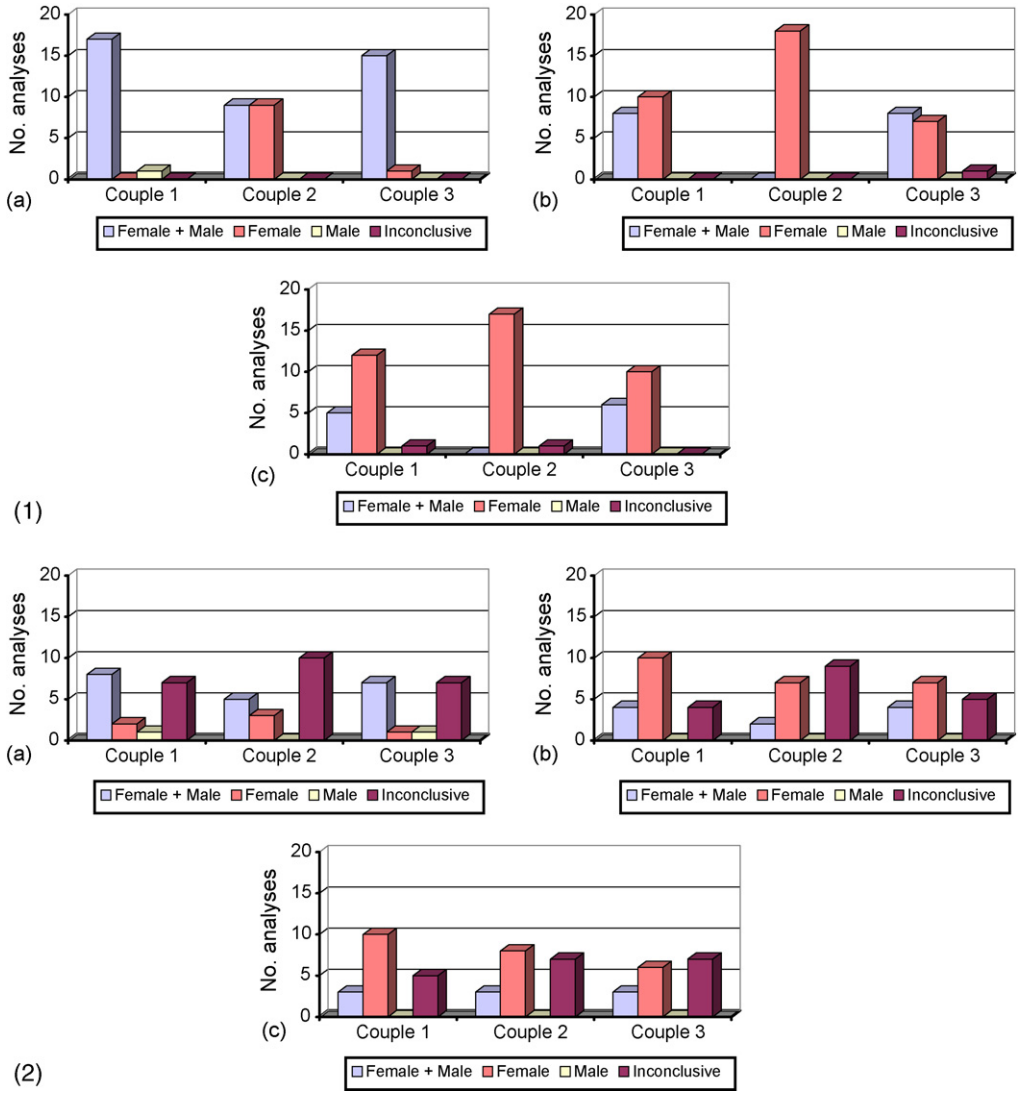

Fig. 5. Haplotype results in the first (1) and second (2) fractions. Comparison between couples. (a) Samples prepared with undiluted semen; (b) samples prepared with semen diluted 1:10; (c) samples prepared with semen diluted 1:20.

Table 9
Haplotype results from couple 4

Sample	Haplotypes	Conclusion
First fraction		
Saliva + undiluted semen	16069T/C 16092C/T 16126C/T 16257T/C 16293G/A 16300A/G 16311C/T	Female + male
Saliva + semen 1/10	16092C 16257T 16293G 16311C	Only female
Saliva + semen 1/20	16092C 16257T 16293G 16311C	Only female
Blood + undiluted semen	16069T/C 16092C/T 16126C/T 16257T/C 16293G/A 16300A/G 16311C/T	Female + male
Blood + semen 1/10	16092C 16257T 16293G 16311C	Only female
Blood + semen 1/20	16092C 16257T 16293G 16311C	Only female
Second fraction		
Saliva + undiluted semen	16069T/C 16092C/T 16126C/T 16257T/C 16293G/A 16300A/G 16311C/T	Female + male
Saliva + semen 1/10	16092C 16257T 16293G 16311C	Only female
Saliva + semen 1/20	16092C 16257T 16293G 16311C	Only female
Blood + undiluted semen	16069T/C 16092C/T 16126C/T 16257T/C 16293G/A 16300A/G 16311C/T	Female + male
Blood + semen 1/10	16092C 16257T 16293G 16311C	Only female
Blood + semen 1/20	16092C 16257T 16293G 16311C	Only female

Table 10
Quantification of nuclear DNA after preferential lysis from unmixed blood, saliva and semen from each donor, in DNA extracts from 0.7 cm × 0.7 cm of stain

Sample	First lysis (ng/μl)	Second lysis (ng/μl)
Blood, female couple 1	1.8	1
Blood, female couple 2	1.4	1.3
Blood, female couple 3	2	2.6
Saliva, female couple 1	0.15	Undetectable
Saliva, female couple 2	0.03	0.03
Saliva, female couple 3	1	0.02
Semen, male couple 1	5.4	81.5
Semen, male couple 2	1.6	55.6
Semen, male couple 3	8.2	106.3

be below the threshold of detection when unbalanced forensic mixtures, that are analysed by standard sequencing procedures, are being studied.

In addition, the lab that coordinated the present study analysed the same types of mixtures using a vasectomized man as the semen donor (couple 4: the same female donor as in couple 3 and a vasectomized male). Results obtained from couple 4 (see Table 9) are not significantly different from those obtained in couple 3 (see Table 5 and Fig. 5(1)). In both couples, the mixture of male and female haplotypes was detected in samples prepared with undiluted semen. As described in Ref. [22], during the last phases of spermiogenesis, the number of mtDNA copies declines in such a way that each mature sperm mitochondrion could contain, on average, only one copy of mtDNA. The main contribution of mtDNA molecules in semen may come from non-spermatic cells which would explain our results. This fact would be consistent with the findings reported elsewhere [23] where, after measurements of mtDNA/β-globin gene ratio by quantitative PCR (qPCR), the authors concluded that the majority of sperm mitochondria are almost totally devoid of mtDNA, and that many spermatozoa probably do not contain any mtDNA at all.

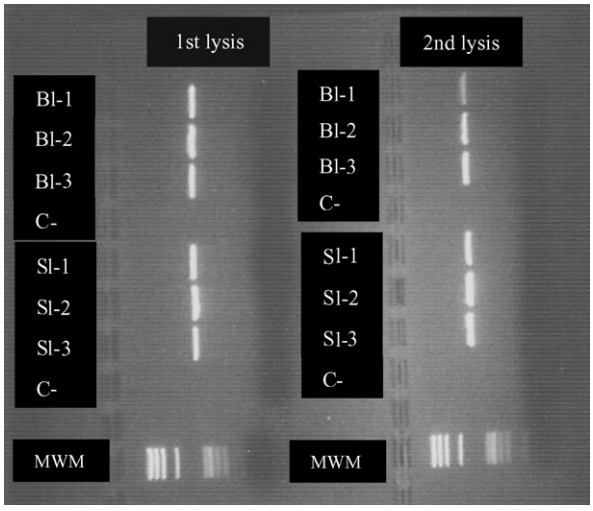

Fig. 6. HVS-I amplification results of blood (Bl) and saliva (Sl) from each female donor (1–3) after preferential lysis. "C−" indicates the negative controls of each DNA extraction. "MWM" indicates molecular weight marker.

Forensic post-coital samples are usually in worse conditions of preservation than fresh semen samples, they are generally diluted (e.g. samples collected through vaginal washings) and mixed with a high quantity of female cells. In addition, these samples are also of sub-optimum quality because most of the time their collection is not carried out immediately after the sexual assault. It is then not surprising to detect only the female mtDNA from forensic mixtures (as occurs in diluted semen samples analysed in the present study). Therefore, as inferred from our results, when mtDNA analysis is undertaken in a rape case, the observation of exclusively the victim's profile is not a full evidence for the absence of a second potential contributor of DNA.

One lab carried out the mixture analysis (couples 2 and 3) using both total and preferential DNA extraction. In this case, the lab carried out both DNA extractions by Chelex-100 [24], a method that usually yields less DNA than the organic one. More haplotype mixtures were observed when the DNA extractions were performed by total lysis rather than by preferential lysis (see Table 8). Therefore, it is possible that the preferential lysis renders less DNA than the none-preferential one. Since it is not possible to separate male and female mtDNA by preferential lysis, in forensic casework it may be more appropriate to perform both types of lyses: the preferential lysis to analyze nDNA and total lysis to analyze mtDNA.

In addition to the mtDNA content in semen, we have seen that the type of fluid in the mixture is also important. We detected both female and male haplotypes in more tests when the semen was mixed with saliva than when it was mixed with blood (Fig. 4). Therefore, it seems that the number of mtDNA copy number (per volume) in blood could be higher than in saliva. This is an important parameter to take into account when evaluating results in actual forensic casework with e.g. samples containing blood from the victim (in addition to vaginal fluid). The male mtDNA may be masked by the high amount of female mtDNA, resulting in false exclusions. The knowledge of the specific type of fluids involved and hence their cellular content and the number of mtDNA molecules per cell is of great interest. The implementation of preliminary tests with the aim of identifying each fluid is an essential tool when a mixture of different fluids is supposed.

We also found that the loss of male mtDNA signal is not uniform in all nucleotide positions (see Fig. 2). The analysis of the mtDNA in mixtures can benefit from a phylogenetic interpretation of the electropherogram profiles [25]. Thus, the phylogenetic perspective could be useful to detect particular diagnostic variants (which may remain undetected) in the mtDNA profile of the contributors, or even to infer the haplotype of the donors (this was in fact the result obtained by one lab in Ref. [7] and have resulted to be useful in real cases; author's unpublished data). Therefore, the presence of e.g. two bases (apparent heteroplasmy) in a stable diagnostic nucleotide position (or in several positions) could support the hypothesis of the presence of two (or more) different haplotypes in the same sample (indicating for instance the minimum amount of contributors to the stain), and even in some cases it could be possible to assign the haplogroup of each contributor (although

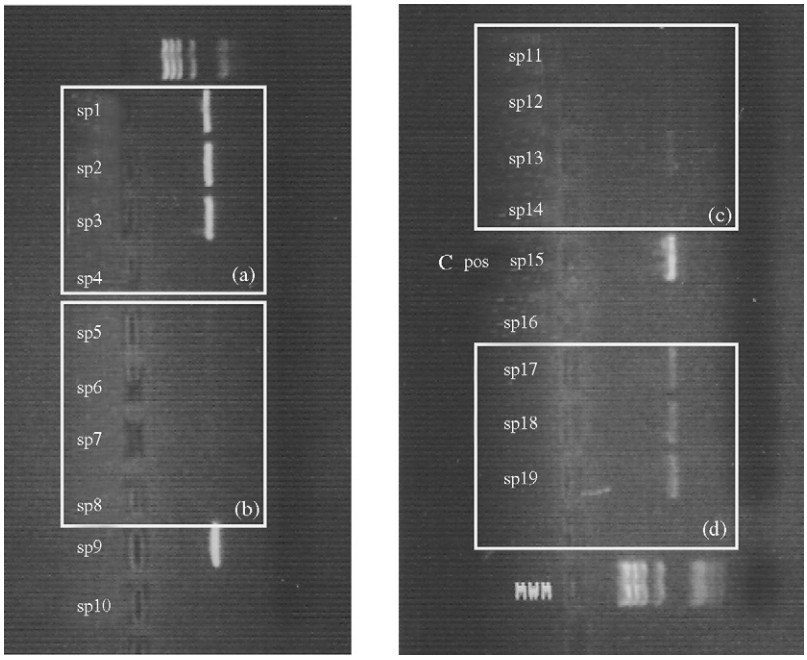

Fig. 7. HVS-I amplification results of semen from each male donor (1–3) after preferential lysis. (a) First fraction amplifications results using 0.4 ng of nuclear DNA (sp1, sp2 and sp3 correspond to the male donors from couples 1 to 3 respectively; sp4 is the DNA extraction negative control). (b) Second fraction amplifications results using 0.4 ng of nuclear DNA (sp5, sp6 and sp7 correspond to the male donors from couples 1 to 3, respectively; sp8 is the DNA extraction negative control; sp9 is an amplification positive control with 0.2 ng; sp10 is a negative control of the amplification). (c) Second fraction amplifications results using 4 ng (10×) of nuclear DNA (sp11–13 correspond to the male donors from couples 1 to 3, respectively; sp14 is a DNA extraction negative control; sp15 is a positive control of the amplification with 0.2 ng; sp16 is a negative control of the amplification). (d) Second fraction amplifications results using 20 ng (50×) of nuclear DNA (sp17–19 correspond to the male donors from couples 1 to 3, respectively).

it could be difficult e.g. to assign private polymorphisms to the particular haplotypes of each contributor) [17].

The technical quality of electropherograms is also an important issue when analyzing mixtures. We strongly recommend using the same guidelines as those for haplotypes showing point heteroplasmies [26]; note that heteroplasmy is in fact a 'natural' mixture. In addition to obtaining electropherograms without background noise in the two strands, it would also be desirable to carry out the sequencing by using additional primers in order to confirm the mixed nucleotide positions. The same approach can be used when one of the haplotypes in the mixture shows a length heteroplasmy and, in fact, there are several primers already designed which allow us to read the electropherograms behind the length heteroplasmy zone. However, the presence of an insertion or deletion polymorphism in one component of the mixture can obscure the sequencing analyses greatly. In these cases, it would be necessary to design new primers in order to prevent overlapping peaks beyond the insertion or deletion.

It is also important to quantify the male and female nDNA in order to facilitate the interpretation of the mixed profile [9]. The present study has allowed us to prove that female fluids mixed with semen dilutions over 1:10 can be very difficult to interpret and that, although better results are obtained when fluid mixtures are balanced, there are variations depending on the type of fluid and the specific donors who contributed the mixture.

5. Conclusions

The analysis of mtDNA from semen mixed with other fluids is not an easy task when using standard sequencing procedures. On the one hand, the technique has some limitations (e.g. different ability of sequencing chemistries to detect point mixtures, low sensitivity in unequal mixtures), and the experience and expertise of individual analysts can be essential for the interpretation of the results. On the other hand, we have seen that these mixtures have special natural characteristics such as the different number of mtDNA copies in different fluids and contributors to the mixture. In addition, the presence of point heteroplasmy, insertions or deletions in one of the haplotypes can complicate the interpretation of the results.

We have demonstrated that the diverse content of mtDNA copies in different body fluids can cause masking of one of the mtDNAs to occur. Furthermore, we may also obtain a set of partial and non-conclusive results, which warns us about the need to be careful when interpreting these results. In order to minimize the number of problems associated with the analysis of mixtures by means of mtDNA standard sequencing, we strongly recommend the identification of the type of fluid in order to know the cell types and the specific content of mtDNA copies. In addition, there are several factors that we must bear in mind: the high risk of contamination, mainly in second fractions; the loss of base signal (maybe stochastically) in some nucleotide positions and not in others; the type of fluids

involved in the mixture; and the possibility of differences in mtDNA content among donors.

Finally, we strongly recommend the use of additional sequencing primers to corroborate the typing data. It is also important to view the results from a phylogenetic perspective [17,28–34] that could help to interpret the mixed sequence patterns and could also prevent undesirable mistakes.

Acknowledgements

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