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The 2000–2001 GEP–ISFG Collaborative Exercise on mtDNA: assessing the cause of unsuccessful mtDNA PCR amplification of hair shaft samples

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Abstract

We report the results of Spanish and Portuguese working group (GEP) of International Society of Forensic Genetics (ISFG) Collaborative Exercise 2001–2002 on mitochondrial DNA (mtDNA) analysis. 64 laboratories from Spain, Portugal and several Latin-American countries participated in this quality control exercise. Five samples were sent to the participating laboratories, four blood stains (M1–M4) and a sample (M5) consisting of two hair shaft fragments. M4 was non-human (*Felis catus*) in origin; therefore, the capacity of the labs to identify the biological source of this sample was an integral part of the exercise. Some labs detected the non-human origin of M4 by carrying out immuno-diffussion techniques using antihuman serum, whereas others identified the specific animal origin by testing the sample against a set of animal antibodies or by means of the analysis of mtDNA regions (Cyt-b, 12S, and 16S genes). The results of the other three human blood stains (M1–M3) improved in relation to the last Collaborative Exercises but those related to hairs yielded a low rate of success which clearly contrasts with previous results. As a consequence of this, some labs performed additional analysis showing that the origin of this low efficiency was not the presence of inhibitors, but the low quantity of DNA present in these specific hair samples and the degradation.

As a general conclusion the results emphasize the need of external proficiency testing as part of the accreditation procedure for the labs performing mtDNA analysis in forensic casework.

Keywords: Mitochondrial DNA (mtDNA) polymorphism; Standardization; Collaborative Exercise; Heteroplasmy; Hair shafts; Animal identification; Cytochrome b; 12S; 16S

1. Introduction

Every year the GEP–ISFG group organizes a Collaborative Exercise with the main aim of improving standardization and serving as a Quality Control Program [1–4]. Blood stains and other biological traces (for instance, hair samples) are distributed among the participants for DNA typing and statistical evaluation of results. The results are thoroughly discussed during the GEP–ISFG annual meeting and as a consequence the quality of the trial and the quality of forensic expertise itself in the labs of the group continuously improves.

Here we report the fifth mtDNA exercise (2001–2002) carried out by the GEP–ISFG group. It is remarkable that labs performing mtDNA analysis have increased gradually: 4 in 1997, 5 in 1998, 16 in 1999, 19 in 2000 and 26 in 2001 from a total of 64 participating labs.

In this exercise together with a forensic case with bloodstains and hair shafts a non-human sample (cat) was included. The analysis of HVS-I and HVS-II regions of the mitochondrial D-Loop was carried out by 26 laboratories in blood stains and by 23 laboratories in hair shafts. Additionally, in order to find out whether sample M4 belonged to human or to non-human species, four laboratories analyzed Cytochrome b (Cyt-b), and/or 12S-RNA, and/or 16S-RNA regions of the mitochondrial genome.

2. Materials and methods

2.1. Design of the exercise and preparation of samples

For the present exercise, the Quality Assurance Unit (National Institute of Toxicology, Ministry of Justice, Madrid, Spain) submitted to all the laboratories four samples consisting of 100 μl of air dried blood with EDTA as anticoagulant embedded in FITZCO Inc. (Life Technologies) paper: M1, blood stain belonging to an M3 alleged mother; M2, blood stain belonging to an alleged M3 sibling; M3, blood stain belonging to an unidentified individual; and M4, blood stain from unidentified species origin. Furthermore, two hair fragments of 2–4 cm labeled as M5 were submitted. Notwithstanding, as many laboratories found many problems when analyzing M5 samples, a second set of 16 hair fragments was demanded. The head hairs were obtained by cutting the distal end with sterilized scissors. Studies to test the quality of the hair shafts were not previously performed.

Questions were posed as follows (Fig. 1): (a) identification of sample M3, if M1 blood stain was the biological mother and M2 a blood stain from a sibling, and (b) inquire whether the stain M4 and the hair shafts M5 may be originated by individual M3. In order to answer these questions, laboratories selected the markers (autosomal STRs, Y chromosome STRs, mitochondrial DNA (mtDNA)) according to their routine practice. M5 samples (hair shafts) were submitted for mtDNA typing.

After the completion of the analysis, in the report edited by the Quality Assurance Unit, the following items were specified: (a) M2 and M3 belong to identical twins, (b) M1 belongs to their biological mother, (c) M4 is a non-human blood (cat; *Felis catus*), and (d) M5 consists of hair shafts coming from a sister of M2 and M3.

The analysis of HVS-I and HVS-II regions of the mitochondrial D-Loop was carried out by 26 laboratories in blood stains to establish whether the individuals M1–M3 were maternally related and by 23 laboratories to establish whether the hair shafts and M3 had the same haplotype (see Table 1).

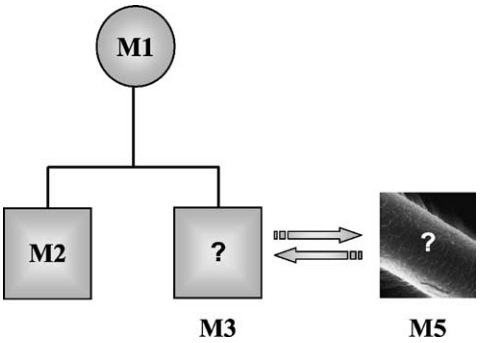

Fig. 1. The diagram explain the parental relationship between samples analyzed in 2001–2002 GEP–ISFG Collaborative Exercise.

Additionally, in order to find out whether sample M4 belonged to human or to non-human species, four laboratories analyzed Cytochrome b, and/or 12S-RNA, and/or 16S-RNA regions of the mitochondrial genome.

2.2. DNA extraction and purification

The use of the extraction protocol usually employed by each laboratory was recommended by the Quality Assurance Unit.

Among the various protocols and techniques used to extract DNA from the blood stains, the most frequent was proteolitic digestion and phenol/chloroform purification (eight laboratories) followed by inorganic extraction with the chelating resin Chelex-100 (seven laboratories). Nine laboratories performed further purification by using centrifugal filter devices (Centricon, Microcon).

Most laboratories used phenol/chloroform or Chelex resin for DNA extraction from hair samples (see Table 2).

2.3. Cyt-b, 12S and 16S amplification

Primers used by laboratories for the amplification of these loci are either published by Kocher et al. [7] (Cyt-b and 12S) or designed by the labs themselves.

2.4. mtDNA D-loop amplification

For the blood samples, a high percentage of laboratories used independent direct amplification of HVS-I and HVS-II with the primers previously described by Wilson [5] and, in less proportion, with those described by Vigilant et al. [6]. Regarding the number of cycles, 51.8% of laboratories used 35–36 cycles, 33.4% used 30–32 cycles and 14.8% used 38–40 cycles. Primers and PCR strategies for analyzing hair samples are summarized in Table 2.

2.5. Sequencing methodology

Automated DNA sequencing was used by all of the laboratories except one (manual sequencing), either with dye terminators (22 laboratories) or dye primers (three laboratories). About half of the laboratories used capillary electrophoresis (ABI 310, and one lab ABI 3100) and the rest used vertical electrophoresis in polyacrylamide gels (ABI 377).

3. Results

3.1. Cyt-b, 12S, and 16S sequencing results for species identification

Based on classical Ag–Ab immuno-diffusion techniques, some labs reported the following statements: ‘‘non-human blood’’ or ‘‘blood of animal origin’’ or ‘‘blood of cat’’.

Individual labs decided to implement additional techniques in order to find out the origin of sample M4. Four laboratories specified that the sample came from *F. catus*, by performing mtDNA analysis (one laboratory Cyt-b, 12S, 16S; two only 16S and one only Cyt-b). These labs carried out the species identification by using BLAST search (http://www.ncbi.nlm.nih.gov/BLAST). The results obtained clearly demonstrate the effectiveness and robustness of the method for the species identification.

3.2. D-loop analysis

3.2.1. Blood samples

The consensus sequence was established when at least three labs reached the same results. The sequences were reported as differences to the Cambridge Reference Sequence (CRS) and each laboratory had to include in the report some details about procedures and equipment. This included: extraction method, PCR methodology (conditions, number of cycles, primers, Taq polymerase, thermal-cycler), purification procedure, sequencing methodology (manual or automated; with dye primer or dye terminators), type of Sequencer, method of sequence analysis and edition.

Table 1
Number of laboratories participating in mtDNA analysis in the GEP–ISFG Collaborative Exercise 2001

Type of analysis	No. of participant labs	No results	Results	
			No consensus	Consensus
mtDNA in blood	26	0	5	21
mtDNA in hair	23	10	10	3

Table 2
Extraction and amplification methods used by Laboratories for the analysis of M5 hair shaft sample analysis

Lab	Extraction method	Primers	No. of cycles	Results
A	P/C/I-Microcon	L15997-H16401 and L0048-H0408; L15997-H16236 and L16159-H16395; L0048-H0285 and L0172-H0408	36	Low efficiency PCR
B	P/C/I-Centricon	L15997-H16401 and L0048-H0408	36	Unsuccessful amplification
C	P/C/I-Centricon	L15997-H16236 and L16159-H16395; L0048-H0285 and L0172-H0408	36	Consensus[a]
D	P/C/I-Microcon	L15996-H16401 and L0029-H0408	36	16224C/T; 16252C/A; 16311C/T; 263G; 315.1C
E	Other	L15996-H16401 and L0048-H0408	38	16069T/C; 16126C; 16278T/C; no results in HVS-II
F	P/C/I-Centricon	L15997-H16071 and L16055-H16139 and L16131-H16218 and L16209-H16303 and L16287-H16379 and L16347-H16401	36	16311C; unsuccessful HVS-II amplification with usual primers
G	Chelex	L15997-H16236 and L16159-H16395; L0048-H0285 and L0172-H0408	36	Consensus[a]
H	Chelex-Wizard Minipreps	Seminested: 1st L15997-H16401 and 2nd L15997-H16236; nested: 1st L15997-H16401 and 2nd L16159-H16235; L0048-H0408	36 + 36; 36	[b]
I	P/C	L15997-H16236 and L16159-H16395; L0048-H0285 and L0172-H0408	32	Consensus[a]
J	Chelex	L15997-H16401; L0048-H0408	35	HVS-I: not determined; HVS-II: 73G; 146C; 152C; 195C; 263G; 315.1C
K	Chelex	L15997-H16401; L0048-H0408	36	No results
L	Chelex/Puregen	L15996-H16401; L0029-H0408	30	Unsuccessful amplification
M	P/C-EtOH precipitation	Seminested: 1st L15997-H16401 and 2nd L15997-H16236; seminested: 1st L15997-H16401 and 2nd L16159-H16395; seminested: 1st L0048-H0408 and 2nd L0048-H0285; seminested: 1st L0048-H0408 and 2nd L0172-H0408	30 + 30	16311C; 263G; 309.1C; 309.2C; 315.1C[c]
N	P/C-Microcon	L15926-H16401; L0048-H0580	40	16223T; 16311C; 16325C/T; 16362C/T; HVS-II: insufficient material for secuencing
O	Chelex	L16263-H16401; L0034-H0186	40	HVS-I: unsuccessful amplification; HVS-II: without results
P	P/C/I-Centricon	L15996-H16401; L0029-H0408	35	Unsuccessful amplification
Q	P/C/I-Ultrafree MC	L15990-H16391; L0034-H0370; four sub-regions re-amplified	32; 32 + 32	16311C; 263G; 309.1C; 315.1C[d]
R	Chelex	L15997-H16401 and L15997-H16255 and L16209-H16401; L0140-H0366	38	HVS-I: CRS; HVS-II: 263G; 315.1C
S	P/C/I-Microcon	L15997-H16395; L0048-H0408	30	Low efficiency PCR
T	P/C/I-Ultrafree	Nested: 1st L15926-H16498 and 2nd L15996-H16401	35 + 25	HVS-I:[e]; HVS-II: no results
U	Chelex	L15996-H16401; L0029-H0408	30	Unsuccessful amplification
V	P/C/I-Centricon	Non-specified	36	Unsuccessful analysis
W	P/C/I-Centricon	Non-specified	36	Unsuccessful analysis

Last column shows the results or comments reported by laboratories.

[a] 16311C 263G 315.1C.

[b] HVS-I: incomplete edition from two DNA extracts—from 15,998 to 16,236 = CRS; from 16,160 to 16,235 = CRS. HVS-II: sequences from three DNA extracts: 152C 182T 263G 309.1C 315.1C; 309.1C 315.1C; 263G 309.1C.

[c] Length heteroplasmy in 309.1C 309.2C.

[d] Length hetroplasmy in 303–315.

[e] HVS-I: incomplete edition (from 16,112 to 16,370 position = CRS).

Twenty-one out of the 26 laboratories (~81%) which studied these samples obtained coincident results. The consensus sequence was 16311C 263G 315.1C in samples M1–M3. The maternal relationship between these samples was confirmed by nuclear DNA analysis (data not shown). One laboratory reported the consensus haplotype without the insertion 315.1C probably due to miswriting. One laboratory just differed in a single polymorphism [M1: 263G 315.1C; M3: 263G 315.1C] while another one reported incorrect typing results for one sample [M2: 16095C/G 16127N 16311C 263G]. Finally, two laboratories reported erroneous haplotypes.

3.2.2. Hair sample

The results reported by laboratories are summarized in Table 2.

Ten out of the 23 laboratories (~43%; Table 1) which studied hair samples (M5) did not obtain or report any result even after the analysis of the second set of hair shafts. A great number of these laboratories stated the difficulties in the analysis of these samples (poor yield in PCR, difficulties in achieving good sequencing results). As it can be seen in Table 2, there is no correlation between the different DNA extraction methodologies and the success rate in mtDNA amplification. The consensus sequence, obtained only by three laboratories (labs C, G and I in Table 2) was: 16311C 263G 315.1C. It was known after the completion of the exercise that M5 came from a sister of M2 and daughter of M1 and, therefore, M5 should carry the same mitochondrial DNA.

Two laboratories, that used nested PCR strategies, reported the consensus haplotype but included insertions in HVS-II poly-C stretch (labs M and Q). One lab (F) obtained only HVS-I consensus sequences. And finally, seven labs (D, E, H, J, N, R and T) reported non-consensus sequences that will be discussed later.

With the aim of clarifying the poor success in the analysis of sample M5, further studies (including increasing on the DNA input on the PCR reaction, evaluation of Taq polymerase inhibitors and the use of shorter amplicons) were performed by different labs as described below.

3.3. Additional remarks on hair sample analysis: inhibition studies and amplification efficiency

In a first approach, it was considered by some labs that the low efficiency in the amplification of M5 samples might be explained as a mispairing due to a point mutation in the annealing site of some of the primers used. Due to the fact that low yield was observed after the amplification of the four mtDNA sub-regions analyzed, this hypothesis was ruled out. Two laboratories decided to carry out additional analysis in order to explain the possible causes of this low efficiency. Due to its particular relevance, the results are explained in the next sections.

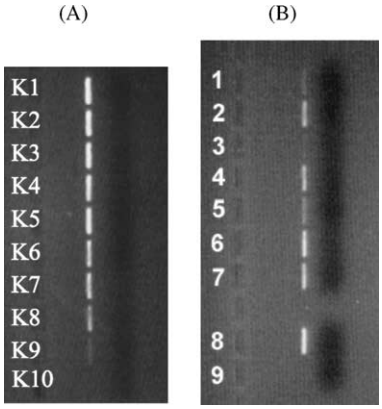

Fig. 2. Inhibition studies. (A) HVS-IA amplification results with different DNA dilutions from K562 cell line. (K1) 1 ng DNA template; (K2) 0.1 ng; (K3) 0.05 ng; (K4) 0.01 ng; (K5) 5 pg; (K6) 2.5 pg; (K7) 1.2 pg; (K8) 0.6 pg; (K9) 0.15 pg; (K10) negative control. (B) Results of HVS-IA amplification of control DNA with DNA hair extract (extract 1: from 3 cm hair shaft and extract 2: from 7 cm hair shaft). (1) 10 μl DNA hair extract 1; (2) 9 μl DNA hair extract 1 + 1 μl K562 (1.2 pg); (3) 10 μl DNA hair extract 1; (4) 9 μl DNA hair extract 1 + 1 μl K562 (0.6 pg); (5) 5 μl DNA hair extract 2 + 5 μl H_2O; (6) 5 μl DNA hair extract 2 + 4 μl H_2O + 1 μl K562 (1.2 pg); (7) 5 μl DNA hair extract 2 + 4 μl H_2O + 1 μl K562 (0.6 pg); (8) positive control; (9) negative control.

3.3.1. Inhibition studies

Firstly, the highest dilution of good quality genomic DNA producing a visible signal in mtDNA PCR was established (Fig. 2A). This was performed by amplifying HVS-IA region from serial dilutions of K562 cell line DNA (from 1 ng to 0.15 pg), being the result in the range of 1/800 to 1/1600 (corresponding to 1.2–0.6 pg genomic DNA).

Secondly, the presence or absence of inhibitors was determined in DNA extracts obtained from hair shafts by amplifying a mixture of these extracts with good quality DNA (1.2 and 0.6 pg).

All the inhibition series were performed with the same PCR protocol (36 cycles). Agarose gel electrophoresis was performed to estimate the fragment size and quality of the PCR products. As it can be seen in Fig. 2B in none of the cases PCR inhibition was observed.

3.3.2. Amplification efficiency

Four strategies were adopted in order to evaluate amplification efficiency. Firstly, a light microscopy study of the set of hair fragments was carried out. Some of them showed either no medulla or non-continuous medulla. In order to establish a possible correlation between the amplification success and hair morphology determined by microscopic examination, independent amplifications of extracts coming from non-medullated hairs and extracts of medullated hairs were performed. A slightly higher yield in the DNA extracts coming from non-medullated hairs was observed (data not

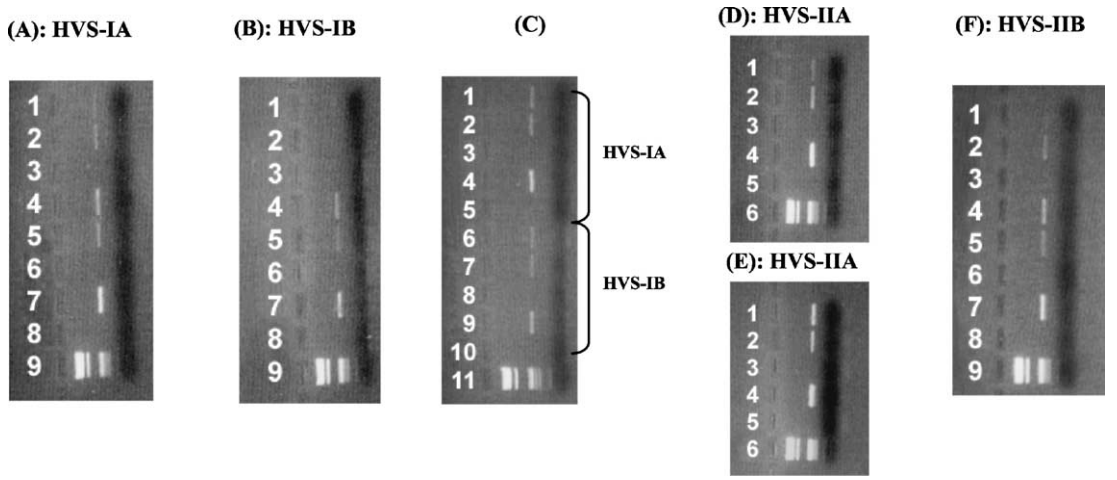

Fig. 3. Amplification efficiency. (A) HVS-IA and (B) HVS-IB amplification results with extracts from 3 cm hair shaft (1 and 2) and 7 cm (4 and 5). Extraction blanks (3 and 6). Positive and negative controls (7 and 8). MWM (9). (C) HVS-IA (1–5) and HVS-IB (6–10) amplification results with extracts from 15 cm hair shaft. Extraction blanks (3 and 8). Positive controls (4 and 9). Negative controls (5 and 10). (D) HVS-IIA amplification results with extracts from 3 cm hair shaft and (E) from 7 cm. DNA hair extracts (1 and 2). Extraction blanks (3). Positive controls (4). Negative controls (5). MWM (6). (F) HVS-IIB amplification results with extracts from 3 cm hair shaft (1 and 2) and from 7 cm (4 and 5). Extraction blanks (3–6). Positive controls (7). Negative controls (8). MWM (9).

shown), which is according to the results reported by Lynch et al. [8].

Secondly, amplifications of DNA extracts from hair shafts of increasing length were performed. As shown in Fig. 3A–F, efficiency of amplification improved as more centimeters of hair were used for DNA extraction. These results also allow to reject the presence of inhibitors as the possible cause of the low PCR efficiency [9,10].

Thirdly, when the amount of Taq polymerase is doubled from 1 to 2 U, amplification efficiency improves accordingly (Fig. 4). Finally, a laboratory (F) developed a multiplex PCR system of short fragments using six primers pairs to obtain overlapping HVS-I fragments of approximately 100 bp [11]. The efficiency of amplification improved as the length of the amplicon diminished (Fig. 5) concluding that degradation is the cause of the low efficiency in the PCR.

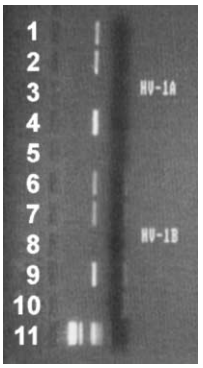

Fig. 4. Amplification efficiency with DNA extract from 15 cm hair shaft and 2× Taq. HVS-IA (1–5) and HVS-IB (6–10). Extraction blanks (3 and 8). Positive controls (4 and 9). Negative controls (5 and 10). MWM (11). Compare with 3C.

4. Discussion

The number of GEP laboratories analyzing mitochondrial DNA in blood stains increases every year. In 1998, 4 blood samples were studied and all the participating laboratories (five) obtained the same consensus sequence, except one

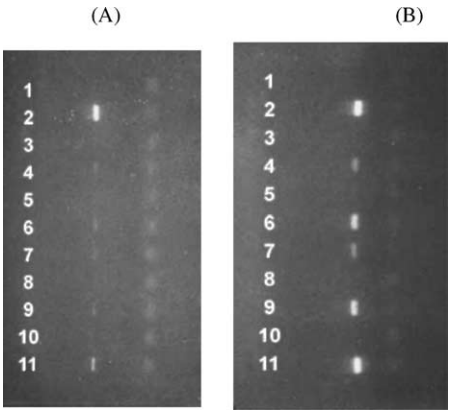

Fig. 5. Amplification efficiency: (A) HVS-IA amplifications (278 bp amplicon) and (B) L15997-H16071 amplifications (113 bp amplicon) with DNA extract from hair shaft of this Collaborative Exercise (11) and with other hair shafts extracts (4, 6, 7 and 9). Extraction blanks (3, 5, 8 and 10). Negative and positive controls (1 and 2).

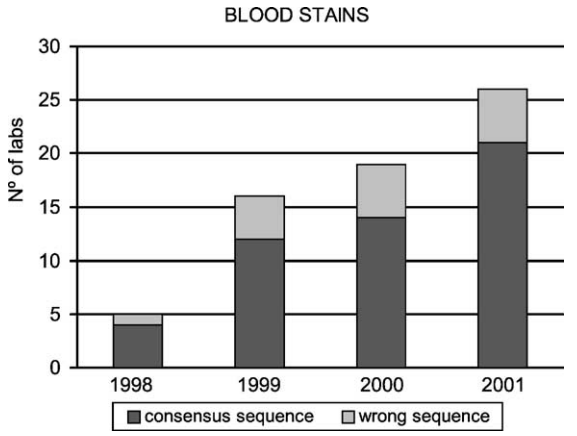

Fig. 6. Number of laboratories analyzing mtDNA in blood stains and success ratio. It is important to mention that wrong sequence results can be generally attributed to those labs which recently adopted the Collaborative Exercise.

which failed in one sample. In 1999, five blood samples were analyzed and 12 out of the 16 participating labs agreed with the results obtained. In the 2000 GEP Collaborative Exercise, 19 labs studied four blood samples, 15 of them also analyzed a hair shaft specimen. Fourteen succeeded in blood stains and 9 in hair shaft mtDNA analysis. In the present exercise, 21 out of the 26 laboratories which studied blood samples obtained a consensus sequence (Fig. 6). Only one out of the five laboratories reporting incorrect results had participated previously in the GEP mtDNA Collaborative Exercise.

The initiative of some labs to carry out the analysis of coding mtDNA regions to inquiry into the specific origin of sample M4 (*F. catus*) was also welcome in the GEP group. Although it is a well-known practice for scientists working on molecular systematic, only a few forensic labs use mtDNA typing for this aim, being probably one of the best options to determine of specific biological origin of a sample.

Conventional systems are inexpensive, fast and useful whenever particular animal species have to be identified from forensic samples such as blood. However, in some occasions, the unavailability of antisera against certain species, the absence of data which can give a hint about its origin and the nature of the evidence itself (hair, bone) preclude the application of these traditional techniques.

Hair samples belonging to the 2001–2002 Collaborative Exercise revealed problems concerning the lack of PCR amplification results. Non-consensus haplotypes reported by some laboratories showing either a high number of sequence heteroplasmies (labs D, E, N in Table 2) or wrong sequences very common in Caucasian populations (labs H, J, R, T in Table 2) indicate the possibility of a contamination originated from the analyst or its environment. This is understandable given that many laboratories included in the GEP group do not perform analysis of forensic samples, as they are mainly devoted to paternity tests, where samples are usually not so troublesome. For this reason, in the 2001–2002 GEP meeting, the importance of stretching the application of measures against contamination was emphasized. Furthermore, labs D, H and N participated for the first time in this hair mtDNA Collaborative Exercise.

It is well known that human hair shafts are unsuitable for nuclear DNA analysis because the nucleus of the cells has degenerated. In contrast, the presence of numerous mitochondria provides a rich source of mtDNA. Nevertheless, hair samples belonging to the present Collaborative Exercise showed an unusual low success rate for mtDNA PCR. In fact, some laboratories which succeeded in previous exercises with this kind of samples, failed to obtain results. For this reason, further studies were performed in order to show the possible causes. In a first approach—because inhibition was mentioned by some laboratories as the possible cause of the low efficiency—a series of inhibition analysis were carried out with these sample. As can be seen in Fig. 2, the presence of inhibitors was rejected because the control DNA (K562) mixed with DNA extract from hair sample yielded PCR product. On the other hand, it was shown that the amplification efficiency improved when PCR was performed with DNA obtained from increasing amount of hair (from 3 to 15 cm hair shaft). Moreover, this result supported the absence of inhibitory activity.

In a second approach, PCR reactions with primer pairs designed to amplify HVS-I overlapping fragments of approximately 100 pb were performed by one lab. Both, increasing of starting material (Fig. 3) and decreasing the length of the amplicons (Fig. 5) improved the PCR efficiency. These results lead us to conclude that low copy mtDNA number and high degradation were the causes of this poor PCR efficiency. Environmental effects (weathering), drugs and cosmetics are capable of affecting the state of hair and the regulation of its life cycle. However, the correlation between these factors and the mtDNA amplification success rate has not been demonstrated [5,11]. In this case, the donor did not take any type of drug and was not under cosmetic treatment, but presented alopecia and hair weakness related problems. Perhaps, a thorough microscopic study would have helped to correlate morphological hair features with the amount of mtDNA available for analysis. We also suggest the incorporation of mtDNA quantification systems (quantitative PCR), as it would allow a previous evaluation of the number of initial copies of DNA template.

Analysis of mtDNA in criminal casework specially when it involves low copy number of degraded material requires to be perfomed in highly specialized labs with experience. The exercise emphasizes the importance of independent proficiency testing as an essential part of any accreditation procedure. There are very few official proficiency testing schemes where mtDNA is included and in our opinion external proficiency testing should be required to any laboratory performing mtDNA sequencing in casework.

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