

2006 GEP-ISFG collaborative exercise on mtDNA: reflections about interpretation, artefacts, and DNA mixtures

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Abstract

We report the results of the seventh edition of the GEP-ISFG mitochondrial DNA (mtDNA) collaborative exercise. The samples submitted to the participant laboratories were blood stains from a maternity case and simulated forensic samples, including a case of mixture. The success rate for the blood stains was moderate (~77%); even though four inexperienced laboratories concentrated about one-third of the total errors. A similar success was obtained for the analysis of mixed samples (78.8% for a hair–saliva mixture and 69.2% for a saliva–saliva mixture). Two laboratories also dissected the haplotypes contributing to the saliva–saliva mixture. Most of the errors were due to reading problems and misinterpretation of electropherograms, demonstrating once more that the lack of a solid devised experimental approach is the main cause of error in mtDNA testing.

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1. Introduction

GEP-ISFG mtDNA collaborative exercises have been performed in the last seven years [1–5]. One of the aims of these exercises is to improve the quality and standardization of mtDNA analyses in both technical and interpretation issues. In the 2006 exercise, we analysed seven samples consisting of four blood stains (labelled M1 to M4) from a maternity case, and three simulated forensic samples, namely, a reference blood stain labelled as M5, an unknown colourless stain labelled as M6 and two unknown hair shafts labelled as M7. M6 was composed of a mixture of saliva from the donor of M5 and saliva from an unknown donor, whereas M7 consisted of two hair shafts from the above-mentioned unknown donor, but coated with saliva from the M5 donor. MtDNA haplotypes were unknown before the samples were submitted to the laboratories, so the donors were not deliberately selected.

2. Participating laboratories

The number of laboratories that analysed samples M1 to M7 is indicated in Table 1. Different DNA extraction methods, amplification and sequencing primers, purification strategies, and sequencing equipment were used among these laboratories. Only one laboratory carried out mtDNA quantification using real time-PCR [6]. As in previous exercises, there was not an apparent relation between the different technologies employed by the laboratories and the amount and type of errors.

3. Results regarding the analysis of blood stain samples

A summary of the results obtained for samples M1 to M5, including the consensus haplotypes and the number of laboratories reporting them is shown in Table 2. As a rule in this exercise, an out-of-consensus result in the report of a particular haplotype constitutes an error. For those samples that were analysed by at least five laboratories, ‘consensus’ means that at least 70% of them (rounding up decimals) report exactly the same result for a given sequence range, but the remaining 30% do not fully coincide and provide a different result to the consensus (the later only applies just in case this 30% includes at least three laboratories).

All laboratories indicated their sequence ranges; only two laboratories reported ‘extended’ haplotypes that included regions outside of classical HVS-I and HVS-II (16024–

16365 and 72–576 in one case and 16024–16569 and 1–576 in the other).

Complete results are shown as supplementary material (Tables S1–S4). Although the global success rate for samples M1–M2–M3–M4–M5 was moderate (77.5%), it is important to highlight that a substantial amount of errors were concentrated in only four inexpert laboratories (laboratories 7, 9, 10 and 12 committed errors in all blood samples; see Tables S1–S3); the success rate excluding these laboratories was 88.2%. The GEP-ISFG group has the policy of allowing any laboratory to participate in its annual quality control (QC) exercise, even those that are still in the process of implementing or have recently implemented the mtDNA analysis technique in their routine work. As a consequence, most of the out-of-consensus results are provided by these inexperienced laboratories, a recurrent situation from previous editions of the GEP-ISFG QC. In contrast, 25 laboratories did not commit any error in samples M1–M2–M3–M4–M5, while seven laboratories reported sequences with only one inconsistency with respect to the consensus haplotypes.

4. Detecting DNA saliva–saliva and hair–saliva mixtures using mtDNA

The GEP-ISFG mtDNA working group was the first consortium analysing and interpreting mtDNA sequencing profiles from sample mixtures [7]. Several parameters influence the detection of nucleotide variants in mtDNA mixtures: (i) the type of tissues contributing to the mixture (related to different mtDNA copy number per cell in different tissues), (ii) differences in mtDNA content among donors, (iii) differences in the amount of fluid from each donor present in a mixture, and (iv) technical factors that can lead to undesirable interpretation artefacts, such as the varying signal throughout the electropherograms when using dye terminator chemistries.

The M6 sample consisted of a 50:50 mixture (80 µL) of saliva from the M5 donor plus saliva from an unknown donor. The presence of a DNA mixture in M6 sample was questioned to the participating laboratories. A total of 18 out of 26 laboratories reported the correct mixture haplotype, and only two laboratories dissected the mixture haplotype into the two potential contributing haplotypes, namely, one sequence belonging to haplogroup U5b (16051G 16189C 16270T 73G 146C 150T 263G 309.1C 315.1C) and another mtDNA probably belonging to haplogroup H (263G 315.1C). Dis-

Table 1
Number of participating laboratories in the mtDNA GEP-ISFG collaborative exercise of 2006–2007

| Maternity case | | | | Forensic case | | |
|------------------|------------------|------------------|------------------|------------------|---------------------|--|
| M1 (blood stain) | M2 (blood stain) | M3 (blood stain) | M4 (blood stain) | M5 (blood stain) | M6 (saliva mixture) | M7 (hair shaft contaminated with saliva) |
| 36 | 36 | 40 | 36 | 36 | 26 | 33 |

Table 2
Consensus haplotypes and number of laboratories reporting them

| Samples | Consensus haplotypes (16024–16365 and 73–340) | No. of laboratories/total laboratories (%) |
|----------|---|--|
| M1–M2–M4 | 16188T 16311C 152C 263G 309.1C 315.1C | 30/36 (83.3) |
| M3 | 93G 151T 263G 315.1C | 33/40 (82.5) |
| M5 | 16051G 16189C 16270T 73G 146C 150T 263G 309.1C 315.1C | 24/36 (66.7) |
| M6 | 16051R 16189Y 16270Y 73R 146Y 150Y 263G 309.1C 315.1C | 18/26 (69.2) |
| M7 | 263G 315.1C | 26/33 (78.8) |

crepancies were mainly due to poor quality electropherograms, nomenclature problems, clerical errors or misinterpretation/misreading of the electropherograms (see Table 3).

Hair shafts are frequently covered with other fluids (like blood or vaginal fluid in rape cases) coming from a different donor, a fact that is often unperceived by the forensic analyst if a previous morphological study is not carried out. Therefore, it is recommended to wash the hair shaft before carrying out the DNA extraction in order to remove possible contaminant agents and to analyse both the liquid and hair samples separately. This procedure prevents the haplotype from the contaminating fluid to predominate or even mask the signal coming from the hair shaft, thus leading to a false exclusion. The hair shaft can be washed using a cotton swab or carrying out a preferential lysis [8].

The M7 sample consisted of two hair shafts from an unknown donor that were deliberately wet with saliva from the M5 donor, thus emulating a quite typical forensic sample. The aim of the analysis of the M7 sample was to know if the hair shafts could belong to the M5 donor. Twenty-six out of 33 laboratories reported the consensus haplotype (the one from the hair donor) for the M7 sample. One laboratory reported the haplotypes from the saliva and hairs separately; the saliva traces were firstly collected by rubbing the surface of the hair shaft using a cotton swab, and secondly the hair shafts were washed several times before DNA extraction.

Regarding the five laboratories that reported non-consensus sequences, two of them detected either the mixed haplotype (matching both haplotypes coming from the saliva donor and from the hair shaft) or only the saliva haplotype, which would

Table 3
M5, unknown donor and M6 consensus haplotypes and out of consensus haplotypes reported for the M6 mixture

| Donor | Haplotype |
|---------------|---|
| M5 donor | 16051G 16189C 16270T 73G 146C 150T 263G 309.1C 315.1C |
| Unknown donor | 263G 315.1C |
| Consensus M6 | 16051R 16189Y 16270Y 73R 146Y 150Y 263G 309.1C 315.1C |

| Lab. ID | Reported haplotype | Error | Comments concerning electropherogram |
|---------|---|--|---|
| 1 | 16051R 16189Y 73R 146Y 150Y 263G 309.1C | 16270Y and 315.1C omitted | Only F electropherogram available |
| 2 | 73R 146Y 150Y 263G C8TC6 and C7TC6 | Nomenclature and HVS-I omitted | 16051R, 16189Y, and 16270Y in electropherograms |
| 3 | 263G 315.1C | M5 not detected | Presence of 146Y and other unclear positions |
| 4 | 16189Y 16270Y 73R 146Y 150Y 263G 309.1Y 310Y 315.1C | 16051R omitted | 16051R in electropherograms |
| 5 | 263G | 315.1C omitted, M5 not detected | Unavailable |
| 6 | 16189Y 16270Y 73R 146C 150T 263G 309.1C 315.1C | 16051R omitted, 146Y 150Y not detected | Low quality electropherograms |
| 7 | 16051G 16189Y 16270T 73G 92A 146C 150T 263G | HVS-II-PolyC omitted, 146Y 150Y not detected, 92A detected | Low quality electropherograms (deficient purification?) Phantom mutation |
| 8 | 51R 16189Y 16270Y 73R 146Y 150Y 263G 309.1C/-315.1C | Clerical error | 16051R in electropherograms |

clearly lead to a false exclusion in real casework in the case the hair shaft would belong to a suspect.

5. Statistical interpretation of the results: match–mismatch criteria and database searching

The GEP-ISFG mtDNA exercise also collects information regarding interpretation of the mtDNA matching evidence in the simulated forensic cases emulating the work carried out by the laboratories in real forensic situations. A plethora of different interpretations were used in this part of the exercise. This is to some extent expected and reflects the lack of standards and consensus among forensic geneticists. On the other hand, some laboratories only reported the match or mismatch status between reference and unknown samples avoiding any kind of statistical interpretation. On the contrary, other laboratories reported the number of matches of one specific haplotype in a specific database (mainly SWGDAM; www.fbi.gov/hq/lab/fsc/april2002/mtDNA.htm; [9]) or the frequency of the haplotype in that database, and finally, a few laboratories calculated a likelihood ratio value by applying the Balding and Nichols correction method [10] and using either their own or the SWGDAM database. A different and independent exercise carried out by the GEP-ISFG group on interpretation (data not shown) also highlights the lack of consensus among laboratories and the key role of chosen databases (specially the SWGDAM) for haplotype frequency and likelihood ratio estimation.

The interpretation of the number of nucleotide differences among haplotypes also differed substantially between laboratories. Some of the laboratories adopt a simplistic rule that considers two haplotypes as being different if there are two or more different nucleotide positions between them. Others are reluctant to adopt this convention mainly because the mutation rate in the mitochondrial genome dramatically varies among nucleotides; therefore, evaluating an exclusion/inclusion solely by the number of nucleotide differences can be problematic (see [11] for a review). The type of tissues or fluids involved in the haplotype comparison also plays a role since some of them are more prone to mutation than others. In problematic cases where enough information to establish an exclusion or an inclusion is not available, it might be necessary to enlarge the mtDNA fragment under study, in some cases analysing the complete control region sequence (from 16024 to 16569 and from 1 to 576) or looking for particular polymorphic positions in the coding region [12–15].

Concerning databases, some laboratories used their own (generally containing small amounts of profiles) while others used published data or the SWGDAM database. At the time of this collaborative exercise, the EMPop database (<http://www.empop.org/>) was not operative; during the last GEP-ISFG meeting however this database was recommended (especially for those laboratories lacking their own databases) instead of the SWGDAM since the former contains a higher number of haplotypes and the data were carefully checked before their inclusion in the database [11,16–18]. However, laboratories need to be aware of potential problems related to

the representativeness of external databases in their casework routine (see [11] for some caveat).

6. Conclusions and recommendations in relation to methodology

We have detected several aspects of the methodology that could help improve the results in routine casework and future editions of the GEP-ISFG exercise.

Only one laboratory carried out a specific quantification of the amount of mtDNA contained in the samples considered in this exercise. MtDNA quantification allows us to control the analysis process more accurately. The main advantage of quantifying mtDNA is that the results of this assay can assist in deciding the best strategy for posterior analysis (e.g. PCRs producing short amplicons in critical samples *versus* long amplicon PCRs in good quality samples). It also provides adjustments in the amount of target mtDNA for the PCR (avoiding the unnecessary loss of mtDNA in critical samples) and provides information regarding the convenience of handling low and high mtDNA content samples separately in order to prevent cross-contamination. Additionally, knowing the mtDNA content in forensic samples may help to detect contamination, especially in those samples with low amount of DNA: if an unexpected high amount of mtDNA is observed in a casework sample (a hair shaft, for instance), we may suspect contamination occurred when the sample was originated (e.g. a hair shaft mixed with vaginal fluid) or during collection and/or the analytical process. There are several examples of mtDNA quantification protocols [6,19], some of which can even give information about the presence of PCR inhibitors as well as the degree of mtDNA degradation.

Visualization of PCR products before the extension reactions is also a useful practice; the selection of good amplicons and the adjustment of the PCR product volume for the sequencing reaction help to guaranty a better sequence performance and to reduce the presence of artefacts in the electropherograms.

Concerning the length of mtDNA fragments studied, the analysis, when restricted to the classical HVS-I and HVS-II, may yield limited information in some forensic cases, such as those where the haplotype of unknown and reference samples match one of the most frequent in a reference population (i.e. the M7 haplotype is 263G 315.1C, the most common in Europe). Analysing the complete control region is the common strategy to increase the discrimination power of mtDNA. For example, it can be easily inferred from the data contained in the Human Mitochondrial Genome Database (<http://www.genpat.uu.se/mtDB/>; [20]) that there are at least 60 polymorphic sites between nucleotides 16365–16569 and 1–72, and 77 additional variants between nucleotides 341 and 574. Some polymorphisms are very informative for haplogroup assignment (e.g. 72C) while others have high mutation rate, and therefore are highly variable among populations (appearing in different haplogroup backgrounds), substantially increasing the discrimination power (e.g. 16519) [11]. Analysing the complete control region does not necessarily involve additional efforts. The

whole control region can be initially amplified in a single PCR reaction by using a single primer pair, following by sequencing shorter fragments using internal primers (i.e. [21]). These strategies do not require new technology, equipment or special training.

Analysis of SNPs located in the coding region also allows increasing the discrimination power of the mtDNA test in a forensic casework. There are several kinds of multiplexes designed to differentiate between H sub-haplogroups [13,14], East-Asian and Native-American haplogroups [15] and West European haplogroups [13,22]. Although no new equipment is usually necessary for these analyses, further technical training might be necessary to read and interpret the results properly.

Concerning the analyses of fluid mixtures, it is worth mentioning that additional non-mitochondrial genetic markers should be studied whenever possible for two main reasons: (a) autosomal markers are generally more informative than mtDNA and (b) the results of the present exercise indicate a level of success lower than desirable (69.2% in M6 sample). MiniSTRs analyses are a good choice in the study of forensic mixtures, and could also be a valuable tool for the genotyping of telogen hairs [23]. It is also recommended to carry out preliminary microscopic examinations of the hair strands aimed to detect possible contaminants and therefore evaluate appropriate protocols for decontamination.

7. Recommendations to avoid errors

The types of errors and their most probable causes are summarized in Table 4. Omission and misdocumentation of nucleotide variants are the most typical discrepancies. Omission of variants is mainly due to clerical errors (HVS-II-polyC stretch forgotten in the report but present in the electropherograms) and poor quality electropherograms (most of them due to the lack of double strand sequencing strategies and the use of additional primers in samples with length heteroplasmy at homopolymeric tracks). Deficient electro-

pherograms were also the cause of a clear example of a phantom mutation: laboratory 9 reported 16469G in all samples.

In order to improve our results, in the present edition of the GEP-ISFG exercise we emphasized several simple recommendations that would have prevented most of the errors [24,25].

7.1. L and H strand sequencing in presence of length heteroplasmy

In order to read both the L and the H strands throughout all the hypervariable segments in presence of length heteroplasmy around position 16189 in HVS-I and around position 310 in HVS-II, it is necessary to use internal sequencing primers (e.g. L16209, H 16164) [26]. The results of the present exercise demonstrate that single strand analysis/reading is a common source of error (Table 2 and Supplementary material): the percentage of consensus results obtained for samples M1, M2, M3 and M4 was clearly superior to that of the M5 sample, despite the fact that all the samples had similar characteristics and contained a sufficient amount of mtDNA. This fact can be attributed to the presence of T16189C in sample M5 which produced an unstable poly-C stretch; thus, for instance, two laboratories reported the profile 16051G 16189C 73G 146C 150T 263G 309.1C 315.1C, but omitted the 16270T variant.

7.2. Reading the electropherograms

A substantial amount of errors was caused by artefacts produced by automatic reading of the electropherograms but without carrying out further visual inspection. The 5'-ends of the sequencing electropherograms are common hotspots for errors and therefore these segments should be systematically reviewed by visual inspection. If for some reason some part of the electropherogram cannot be unambiguously read (and there is no more sample available for further sequencing), it is

Table 4
Types of discrepancies in the present study

| Type of error | Cause | Number of times |
|-----------------------------|---|-----------------|
| Position omitted | Clerical error | 10 |
| | Poor quality electropherograms/only one electropherogram per region | 8 |
| | Undetermined (no electros available) | 14 |
| Incorrect position reported | Phantom mutation | 6 |
| | Poor quality electropherograms | 6 |
| Typing error | Confusion | 6 |
| Nomenclature | – | 2 |
| Unresolved bases (Ns) | Poor purification | 2 |
| | Poor quality electros | 2 |
| | Length heteroplasmy and only one strand sequenced | 3 |
| Different haplotype | Unwashed hair (not decontaminated) | 2 |
| M6 mixture not detected | Poor quality electropherograms | 1 |
| | Undetermined (no electros available) | 1 |
| Mixed bases not reported | Poor quality electros | 4 |

mandatory to clearly report the real reading range in that specific sample.

7.3. Data entry or editing

This exercise also recorded several examples of documentation errors: (i) reporting the 16189 nucleotide as different from rCRS instead of the 16188T polymorphism, (ii) 051G instead of 16051G, (iii) nomenclature errors such as C8TC6 instead of 309.1C 315.1C, (iv) missing nucleotides (16270 instead of 16270T), (v) reporting the rCRS base instead of the one in the sample (16189T instead of 16189C), etc. Correcting such errors is feasible; for instance, haplotypes can be electronically transferred to the final report and can be double checked by two independent analysts.

7.4. A posteriori QC

It has been demonstrated several times that *a posteriori* inspection of the mtDNA profiles to the light of the phylogeny or simple database searches contributes significantly to prevent a high proportion of the errors in mtDNA reports. Here we just indicate three different related steps that should be followed in this regard:

- Verify if the polymorphisms observed in our haplotypes have been already described in the literature or in databases. It is possible to carry out a quick search in several web sources (e.g. mtDB (www.genpat.uu.se/mtDB), EMPOP (<http://www.empop.org/>), and SWGDAM (www.fbi.gov/hq/lab/fsc/april2002/mtDNA.htm)). This verification process takes only few minutes and can help to prevent a great amount of errors and misleading interpretations [27]. Nevertheless, the above databases are not fully exhaustive so it is possible that common variants reported in the population or anthropological literature are still not recorded [27,28].
- Checking the haplotype from a phylogenetic perspective helps to detect common errors such as sample mix-up or contamination (see, for instance [18,24,25]). The mtDNA phylogeny is continuously improving and therefore, nomenclature and branching patterns (especially at the tips) are continuously changing. Therefore, it is fairly difficult to be knowledgeable about phylogeny; some publications should be used as references but always bearing in mind the last update; thus for instance, we have good sources for the European [29,30], Asian or Native-American [31,32] and African [33,34] phylogenies.

In the case of samples showing sequence heteroplasmy, it is instructive to check the rate of mutation of the nucleotide position where the heteroplasmy is located, as this helps the interpretation of results. The mutation rate is not uniform throughout the mtDNA molecule and there are some positions that are prone to accumulate changes, i.e. hotspots such as 16189 in HVS-I or 152 in HVS-II. We are aware that a specific mutation rate for each nucleotide position has not been

established yet. Some useful information can be gathered in [11,35–37].

The use of phylogenetics as a tool for *a posteriori* checking can help to detect a great number of errors. Unfortunately, a substantial proportion of the laboratories show obvious difficulties in using this approach. In this regard, it is worth mentioning that the EMPOP web resource has a variety of different tools that could assist forensic geneticists in different tasks related to error detection.

8. Final remarks

In general, the outcome of the GEP-ISFG consortium exercise reflects only a modest improvement in its global outcome; the knowledge acquired in previous editions regarding the most common causes of errors helped to prevent the incidence of common mistakes in those laboratories with more experience. Since the electropherograms were also submitted by the majority of the laboratories, most of the errors could be catalogued. The causes of errors were similar to those in previous exercises, mainly comprising edition mistakes, lack of electropherogram quality at the 5'- and 3'-ends of the sequence, as well as nomenclature deficiencies. The exception this time is the apparent lack of contamination problems. We can conclude that in general the laboratories still lack solid devised experimental approaches and protocols, the keystone for preventing errors in mtDNA casework. The results of a QC are not anecdotic since these generally mirror the quality of the casework forensic practice. A great effort is still needed among forensic practitioners regarding the methodological and the theoretical framework in order to improve the health of the (somehow 'damaged') mtDNA test.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2007.10.010.

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