CASE REPORT

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DNA Typing: An Accessory Evidence in Doping Control

ABSTRACT: A clear positive case for anabolic steroids doping was confounded by alleged urine tampering during doping control procedures. Review of the chain of custody showed no flaws, but nevertheless the athlete was adamant that the urine sample should be analyzed for DNA in order to support her contention that she was not the donor of the sample. The results obtained showed that the urine sample that scored positive for steroids contained nuclear DNA that could not be matched to the DNA obtained from the athlete's blood. On the other hand, the same urine sample contained mitochondrial DNA whose nucleotide sequences spanning the hyper variable regions HV1 and HV2 proved to be identical to those determined in mitochondrial DNA amplified from the athlete's blood. The occurrence of an extraneous genotype is compatible with exogenous nuclear DNA admixture to the athlete's urine. Alternatively, taking in consideration the mitochondrial DNA, we could not exclude that a sibling or a maternal relative of the athlete could have acted as a donor of the urine utilized for doping control and DNA analysis. Both situations point to possible tampering of the urine by the athlete. Adjudication at CAS maintained previous national and international federation decision that there was no proof of a chain of custody flaw to justify the athlete's allegation of urine substitution after collection.

KEYWORDS: forensic science, androgen, doping control, DNA typing, manipulation of results

The simultaneous detection and identification of various androgens is a commonly encountered problem in clinical androgen assays and metabolic studies (1–3) as well as in doping control of anabolic agents (4–6). A prerequisite for the identification and quantification of anabolic exogenous steroids by GC-MS is the relative retention time, the ratio between the main diagnostic ions (7). An adverse analytical finding confirmed in our lab showed the presence of metabolites of three anabolic steroids, namely methyltestosterone, stanozolol and nortestosterone. The presence of these metabolites was confirmed according to IOC guidelines, which included B-sample (another aliquot from the same sample waited for confirmation propouses) analysis in the presence of the athlete and other witnesses (7). To support a positive case in doping control some points must be considered, for example, the chain of custody for sample collection and transport to the laboratory. In the present case the athlete complained that the urine did not belong to her and so insisted that DNA analysis should be carried out.

In cases of doping control, workplace drug testing or general forensic toxicology, DNA typing has been successfully used to individualize urine samples. This is carried out by comparing DNA profiles of urine to a tissue from the individual being tested, usually blood or buccal swabs (8–10).

Although there is no free DNA in normal urine, it is common to observe a number of epithelial cells that are shed from the genito-urinary tract. Besides epithelial cells, leucocytes may also contribute as a source of DNA. In this context, it has been estimated that females afford a higher amount of quantifiable DNA than males. Typically, 14–200 ng/mL and 4–60 ng/mL of DNA could be extracted from the urine of female and male donors, respectively (11).

The relatively small quantities of DNA can be limiting for molecular probe genotyping and for this reason, PCR amplification has been elected as the method of choice (12).

However, other factors such as the storage conditions of the urine may have a significant effect on the final yield of DNA. For example, upon thawing frozen urine, cell lysis occurs and as a consequence, the released DNA becomes amenable to degradation by cellular nucleases and other insults. Thus, the already scarce nuclear DNA may be insufficient for profiling. Therefore, the necessity of investigating mitochondrial DNA haplotypes must also be considered. Mitochondrial DNA presents several advantages over nuclear DNA: it is haploid and has a monoclonal nature (matrilineal inheritance), has a high copy number, it is circular and has a smaller size. In addition, the fact that mitochondrial DNA is encased within the organelle renders it more resistant to degradation.

In a recent review, the case for mitochondrial DNA typing as applied to identity testing has been given ample support (13). A thorough discussion backed by a large body of experimental evidence leaves no doubts as to the robustness and usefulness of mitochondrial DNA as an informative forensic tool. The present

paper describes how mitochondrial DNA analysis was decisive in resolving a case of potential sample tampering.

Experimental

Reagents, Chemicals and Solutions for Steroids Analysis in Urine

The following substances: 4β-stanozolol, 3′-hydroxystanozolol, 16β-hydroxystanozol, norandrosterone, methyltestostrone metabolites (17α-methyl-5α-androstane-3α,17β-diol, M1 and 17α-methyl-5β-androstane-3α,17β-diol, M2) were a kind gift from Dr. W. Schänzer and H. Geyer from the Institute of Biochemistry, Germany Sports University, Cologne, Germany. Methyltestosterone as an internal standard was bought from Aldrich (Milwaukee, WI). All reagents were analytical grade. MSTFA was purchased from Chem Fabrik (Waldstetten, Germany). NH_4I and ethanethiol from Sigma (St. Louis, MO) tert-butylmethylether (TBME) from Tedia (Fairfield). Methanol from Tedia (Fairfield). Stock solutions were prepared in methanol at a concentration of $1000 \, ng.\mu L^{-1}$. These solutions were further diluted to yield appropriate working solutions for the preparation of the calibration standard. The solutions were sealed and frozen at $-20°C$ until use. Methyltestosterone was used as an internal standard (ISTD). Stock solution was dissolved in methanol at $1000 \, ng.\mu L^{-1}$ and diluted to $10 \, ng.\mu L^{-1}$.

Equipment and Conditions for Steroid Analysis in Urine

The experimental conditions were described elsewhere (14). Shortly, a Hewlett Packard GC (6890 series) interfaced to MSD (5973 series) equipped with a 7683HP autosampler. HP-1 Capillary column (100% methylsiloxane, $17 \, m \times 0.20 \, mm$ I.D. $\times 0.11 \mu m$ film thickness). The carrier gas was helium (0.7 mL/min, Injection pulse pressure 50 psi, 0.80 min, split 10:1). Oven: $140°C/40°C/min/180°C/3°C/min/230°C/40°C/min/300°C(2')$. Injector: $280°C$. A split/splitless deactivated glass single-taper liner from HP ($79 \, mm \times 7 \, mm$ I.D.) (cup $6 \, mm$ length $\times 1 \, mm$ hole) and an internal volume of $0.9 \, mL$ was used. Inside the liner $0.017 \, mg$ of deactivated glass wool were well compacted between 23 and 33 mm measured from its top. Interface MSD: $280°C$, quadrupole: $150°C$ to $180°C$ at $40°C/min$, then to $240°C$ at $3°C/min$ and to $300°C$ at $40°C/min$ (held 3 min). The transfer line was at $280°C$.

Mass spectra were obtained in SIM mode. The registered ions in confirmation procedure were m/z 143, 345, 270 and 450 for methyltestosterone metabolites (M1 and M2). Ions m/z 225, 315, 405 and 420 for norandrosterone (nortestosterone metabolite) and ions m/z 254, 545, 560 for 3′OH-stanozolol and 4β-hydroxystanozolol and 218, 231, 560 for 16β-hydroxystanozolol and m/z 301 and 446 for methyltestosterone-P (ISTD).

Sample Preparation for Steroid Analysis in Urine

Only the confirmation procedure will be described (for screening procedure see the method described by Geyer et al. and Andreas et al. (14,15). A urine sample obtained from the female athlete was individually processed in our routine confirmation procedure for androgen analysis. For methyltestosterone and nortestosterone metabolites, isolation of androgens is based on a clean-up using C18-column; 2 mL of urine are added to a C18 column (5). The column is washed with 2 mL of water and the adsorbed fraction is eluted with 2 mL of methanol. The methanolic eluate is evaporated to dryness and the residue is dissolved in 1 mL of 0.2 M sodium phosphate buffer pH 7.0. 5 mL of TBME are added to the aqueous phase. After shaking for 5 min and centrifugation, the organic layer is discarded and the residual organic solvent is removed under a

stream of N_2 at $40°C$. To the buffer solution $25 \, \mu L$ of ISTD and $150 \, \mu L$ of β-glucuronidase from *E. coli* are added and hydrolysis is performed for 1 h at $50°C$. The buffered solution is alkalinised with $250 \, \mu L$ of 20% potassium carbonate solution to pH 9.0 and the analytes are extracted with 5 mL of n-pentane on a mechanical shaker for 5 min. After centrifugation the ethereal layer is transferred and evaporated to dryness under vacuum.

For stanozolol metabolites, the same procedure described above was used except for the washing step with TBME and the final extract was obtained using TBME instead of n-pentane.

Derivatization Prior to GC-MS Analysis for Steroid Analysis in Urine

Previously to GC-MS analysis, N, O-TMS and O-TMS derivatives were formed. The dried residues were kept inside desiccators containing P_2O_5/KOH during 20 min, dissolved in $100 \, \mu L$ of MSTFA-NH_4I-2-mercaptoethanol (1000:2:6, v/w/v) and heated at $60°C$. Three μL of each sample were injected into the GC-MS system (14–15).

DNA Typing

DNA Extraction—Total (nuclear and mitochondrial) DNA was extracted from urine using the organic method, essentially as described by Vu et al. (11). Total blood nuclear and mitochondrial DNA was extracted with the organic method following standard procedures described elsewhere (16).

STR Typing—Total nuclear DNA was subjected to PCR amplification using primers for the following STR single locus loci: F13A01 (6p34.3-p25.1), F13B (1q31-q32.1), D5S818 (5q23.3-32), LPL (8p22), CSF1P0 (5q33.3-q34), TPOX (2p25.1-pter), TH01 (11p15.5), vWA (12p12-pter), D16S539 (16q24-qter), D13S317 (13q22-q31), FGA (4q28), D3S1358 (3p), D18S51 (18q-21.3), D8S1179 and D21S11. For sex investigation of the urine sample amelogenin primers (Xp22-22.3 and Y) and Y chromosome loci DYS19, DYS434, DYS437, DY390 and DY393 were also used for amplification. The sequences of the primers and the conditions for amplification are all described in the Short Tandem Repeat DNA Internet Database (17). These loci had all been validated by several international accreditation agencies and are routinely used in most laboratories conducting forensic genotyping.

Mitochondrial DNA Typing—The sequences of the primers used for PCR were: HV1 forward 16144, 5′TGACCACCTGTAGTA-CATAA 3′, HV1 reverse 16410, 5′ GAGGATGGTGGTCAAGGG 3′, HV2 forward 155, 5′ TATTTATCGCACCTACGTTC 3′, HV2 reverse 381, 5′ CTGGTTAGGCTGGTGTTAGG 3′. Primer sequences were based on the numbering system of Anderson (18). These sequences and the PCR programs were obtained from Steighner & Holland (19). The reactions were carried out in a Perkin-Elmer GeneAmp PCR 9700 thermal cycler.

Electrophoresis of the PCR products under non-denaturing conditions silver staining for the conformational determinations (SSCP) was carried out essentially as described by Menezes et al. (20).

mtDNA Sequencing—DNA was sequenced using the DYEnamic ET Terminator Cycle Sequence kit (Amersham Biosciences in an ABI automatic DNA sequencer. Homology searches were carried out using NCBI's (National Centre for Biotechnology Information) BLAST network service and the GenBank databases.
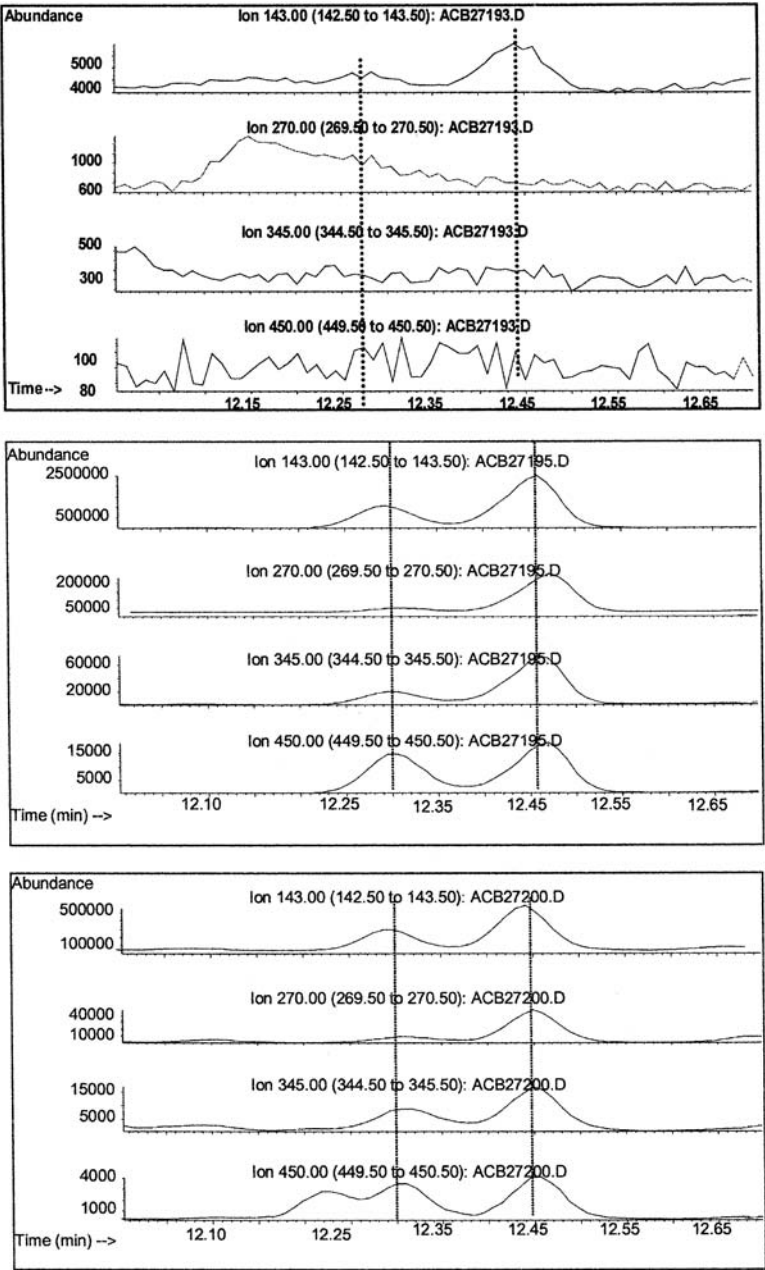
FIG. 1—*GC-MSD SIM results (a) urine blank (BU), (b) suspect sample and (c) positive control (STD1). Chromatograms m/z 143, 270, 345 and 450 of the bis-O-TMS of methyltestosterone metabolites.*

Results

Chromatography and Specificity

As an example of those steroids found in the suspicious sample a representative chromatograms of SIM analysis of the sample spiked with methyltestosterone main metabolites and internal standard and of the urine blank are shown in Fig. 1. There are no chromatographic peaks interfering with the analytes or internal standard. With the sample processing and chromatographic conditions described, analytes and internal standard were well resolved from each other.

From Fig. 1 (methyltestosterone metabolites), it can be seen that satisfactory resolution and symetrical peaks were obtained. The presence of the exogenous steroids was suspected after the presence of their main metabolites in the urine sample. They

TABLE 1—*Relative retention time of the diagnostic ions of the analytes observed in the suspicious sample and positive control.*

| | Relative Retention Time | |
Substance	Sample	Positive Control
Norandrosterone	0.61 ± 0.00	0.61 ± 0.00
Methyltestosterone-M1	0.82 ± 0.00	0.82 ± 0.00
Methyltestosterone-M2	0.83 ± 0.00	0.83 ± 0.00
3′OH-Stanozolol	1.44 ± 0.00	1.43 ± 0.00
4β-OH-Stanozolol	1.46 ± 0.00	1.45 ± 0.00

were characterized by comparison of the relative retention times (Table 1) and the ratio of the relative abundances of their main diagnostic ions (GC/MS SIM acquisition, Table 2) after analysis by

TABLE 2—*Relative abundance of the diagnostic ions of the main nortestosterone metabolite (norandrosterone) observed in the suspicious sample and positive control.*

Sample	Parameters	Ions (m/z)			
		405	420	225	169
Suspicious	Mean	100	77	35	72
	STD	—	0.82	0.50	0.26
	CV (%)	—	0.01	1.42	0.36
Positive control	Mean	100	66	30	70
(STD1–STD3)	STD	—	0.96	0.82	2.52
	CV (%)	—	0.02	0.03	0.04

SIM acquisition with those obtained by analysis of the positive control (STD1-STD3). The relative retention time of the analytes observed in the suspect sample does not differ by more than 1% from that of the same substance in the positive control standard analyzed (Table 1). The relative abundance of diagnostic ions do not differ by more than 20% from those observed in the positive control standard (Table 2). According to the IOC these evidences constitute an adverse analytical finding (7).

Several studies have shown that the steroid profile parameters, especially the steroid ratios could be used for confirmation of steroid misuse. Therefore as described before, administration of some exogenous steroids can change these endogenous steroid ratios hindering their use as markers of the individual providing the sample. Recently Geyer et al. (6) described some of these effects.

The athlete challenged the doping control system to prove through DNA analysis that the urine sample was hers. The Federation (Confederação Brasileira de Desportos Aquáticos, CBDA) accepted the request.

Genotyping

Nuclear DNA—The results of the amplification of nuclear DNA from the urine sample at several loci and the comparison of the allelic patterns between urine and blood are shown in Table 3.

Table 3 shows that of the 14 loci, only 5 were amplified in urine sample B. Four of these loci produced a pattern that could not be matched to that obtained from the blood nuclear DNA and, therefore, indicated a clear exclusion. A conservative analysis might have considered that locus TPOX could have been an inclusion, assuming that allele 12 in the athlete's blood did not amplify (allele dropout). However, in view of the 4 the remaining loci in the urine that generated alleles distinct from those amplified from the blood nuclear DNA, the only possible interpretation is that of a mismatch.

In sample A, only loci LPL and D5S818 could be amplified. Of these, D5S818 was a match, but LPL was not, indicating again that the urine nuclear genotype was different from that of the athlete's blood. Unfortunately there were no comparable loci that could be simultaneously amplified in the two urine samples.

Because of the relatively poor amplification with nuclear DNA, mitochondrial DNA analysis was carried out. The PCR products were either investigated by conformational studies, or by direct sequencing. The results of the SSCP analysis are shown in Fig. 2.

The results in Fig. 2 show that the PCR products obtained from the urine and blood generated essentially identical profiles (lanes 1–4). In contrast, the unrelated individual added as a control (lane 5) produced a distinct pattern. The same results were observed for the hypervariable region II (lanes 6–10). The SSCP analysis carried out with urine sample A produced the same comparative results,

TABLE 3—*Results of the amplification of nuclear DNA from the urine sample at several loci and the comparison of the allelic patterns between urine and blood.*

| Locus		Athlete	Sample B	Sample A	Freq.
F13A01	al.1	6	NA	NA	
	al.2	4			
F13B	al.1	10	NA	NA	
	al.2				
LPL	al.1	14	NA	12	
	al.2			10	
D5S818	al.1	12	NA	12	0.337
	al.2	11		11	0.357
CSF1PO	al.1	11			
	al.2		NA	NA	
TPOX	al.1	8	12	NA	
	al.2		8		
TH01	al.1	9.3	7	NA	
	al.2	7	6		
vWA	al.1	16	NA	NA	
	al.2				
D16S539	al.1	9	NA	NA	
	al.2				
D13S317	al.1	12	NA	NA	
	al.2	10			
FGA	al.1	27	24	NA	
	al.2	18	20		
D3S1358	al.1	18	18	NA	
	al.2	14	17		
D18S51	al.1	13	NA	NA	
	al.2	12			
D21S11	al.1	34	29	NA	
	al.2	31	28		
Amelogenin		XX			
DYS19		NA		NA	
DYS434		NA		NA	
DYS437		NA		NA	
DYS390		NA		NA	
DYS393		NA		NA	

NA = not amplified.
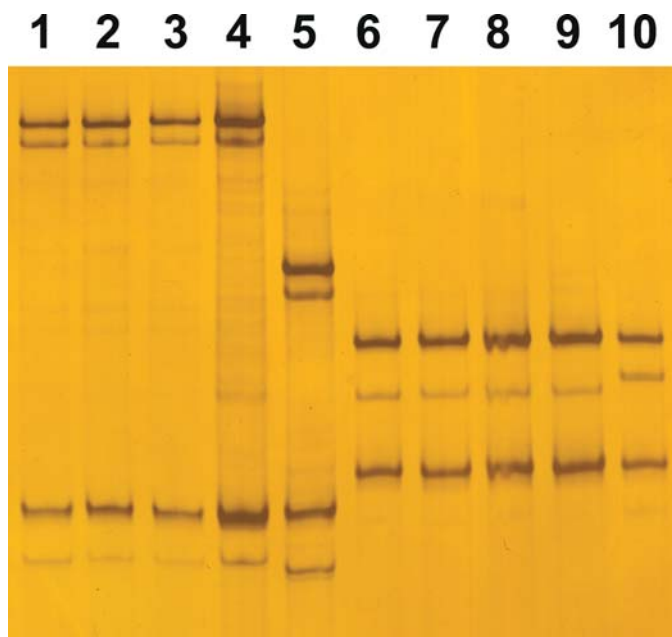
FIG. 2—*SSCP of mitochondrial DNA PCR products amplified from urine and blood. Lanes 1–5 correspond to hypervariable region I (HV1); lanes 1–3, urine sample B; lane 4 athlete's blood; lane 5, mtDNA from the blood of an unrelated individual (control); lanes 6–10 hypervariable region II; lanes 6–8, urine sample B; lane 9, athlete's blood; lane 10, unrelated individual.*
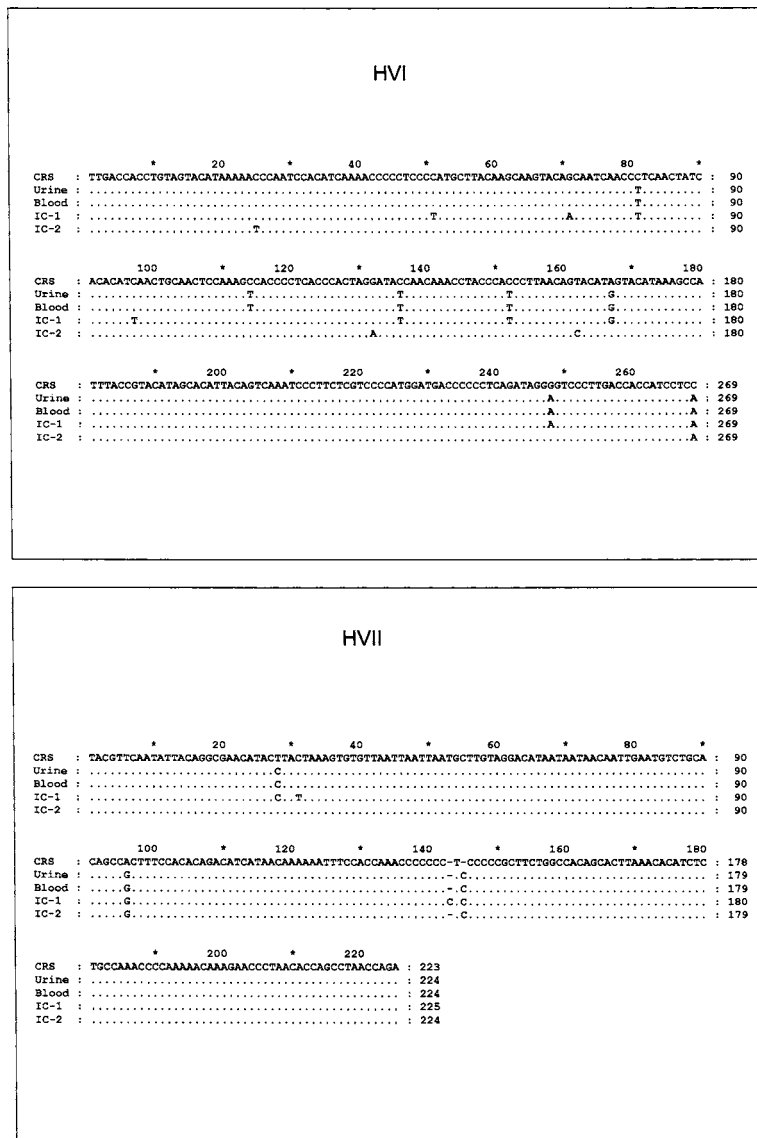
FIG. 3—*Nucleotide sequence of the hypervariable region of mitochondrial DNA amplified from urine sample B and the athlete's blood. a: Hypervariable sequence I (HVI). The dots represent identity between nucleotides in the several samples. The annotated nucleotides represent differences with regards to CRS. CRS stands for Cambridge Reference Sequence (Anderson sequence). IC-1 and IC-2 are sequences from unrelated donors which were included as controls. b: Hypervariable sequence II.*

i.e., the patterns of urine and the athlete's blood were undistinguishable. These results indicated that the PCR products amplified from blood and urine had the same primary structure.

In order to confirm the homology between the PCR products amplified from the mtDNA from urine and blood, direct nucleotide sequencing of the amplicons was carried out. These results are shown in Fig. 3a and b.

The results shown in Fig. 3a–b show that the mtDNA sequences obtained from the urine sample B and the athlete's blood were identical. Variations in the sequence could only be observed in IC-1 and IC-2. IC-1 and IC-2 were samples of mitochondrial DNA obtained from unrelated individuals which were added as internal controls.

Conclusions

Taken together, the results of nuclear DNA genotyping and mtDNA SSCP and sequence analysis suggested that the complete genotype of the urine sample is not the same as the genotype determined in the athlete's blood sample. Although few nuclear DNA loci were amplified in the urine (five in sample B and two in sample A), emphasis was placed on the genotypic differences between urine and blood, so that the conclusion of a mismatch was plausible. Contamination of the urine is always a concern. Nevertheless, internal controls revealed that the genotype found in the urine sample could not be matched to any of the laboratory's staff (results not shown).

Due to the fact that the nuclear DNA profile found in the urine did not match the athlete's allelic pattern it might have originated from admixture to the urine sample. The source of this haman DNA was not identified. On the other hand, the mtDNA analyzed in the urine and blood indicated that the urine could have originated from the athlete herself, or from any member of the athlete's maternal lineage, ascendant or descendant. The possibility that an individual who is not maternally related might have been the donor of the urine sample cannot be discarded. However, this hypothesis is unlikely

since the mitochondrial profile which was found for the present analysis is fairly uncommon, as based on the SWGDAM mtDNA database.

In conclusion, two interpretations are compatible with the DNA analysis:

- The urine originated from the athlete; in this case foreign DNA was added to the urine sample by means unknown. Such conclusion, however, would imply that the nuclear loci amplified in the urine sample originated from different donors, i.e., there was no single locus in the urine that could indicate a mixed genotype.
- The urine did not originate from the athlete; in this case someone belonging to the maternal lineage was the donor of the urine sample.

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