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CASE REPORT

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Successful DNA Typing of a Urine Sample in a Doping Control Case Using Human Mitochondrial DNA Analysis

ABSTRACT: In a doping control case, a urine sample was tested positive for nandrolon. We were asked by the athlete to perform DNA investigations on the questioned urine sample and compare these to a fresh blood sample taken from the athlete in order to detect or rule out manipulation and/or switching of the samples. The urine sample had been collected nine months prior to the investigation and had been stored at 4°C. In a first approach, nuclear DNA systems were investigated that failed with the exception of the Amelogenin system. Due to the high copy number of mitochondrial DNA molecules and the robustness of the mitochondrial genome, we investigated the HVR I and HVR II regions of mitochondrial DNA and obtained reproducible and clear sequencing results for both the blood and the urine samples. Due to the identical sequences, it could not be excluded that the blood sample and the urine sample were from the same individual or an individual having the same maternal lineage.

KEYWORDS: forensic science, doping control case, manipulation, stored urine sample, human mitochondrial DNA, individualization

Due to cases of positive tested urine samples in doping control cases and alleged manipulation or switching of samples, several studies on the individualization of fresh and stored human urine samples were carried out ten years ago using nuclear DNA markers (1,2). At this time in early 1990, investigations were carried out with 200 mL of urine using multi-locus probes (3). Brinkmann et al. (1992) (4) reported the possibility of typing urine samples using single locus probes, PCR-VNTR (variable number of tandem repeat) polymorphisms, and short tandem repeat (STR) systems. The results of these investigations showed that the success rate of DNA typing is dependent on several factors, e.g., the sex of the urine sample, the storage conditions, quantity, quality and age of the urine sample, and the DNA polymorphisms used. In general, the success rate is greater in females than in males and is greater using fresh rather than stored urine. The establishment of the new generation of STR markers has increased the success rate of individualization of urine samples. The volume for successful amplification could be decreased to 5 to 20 mL of urine even after a storage period of several weeks. Recently analysis of the human mitochondrial DNA has been validated for forensic purposes and seems be a powerful tool where conventional DNA markers (5–7) have failed. Here we describe a case of a doping control sample that was tested positive for nandrolon. The application of human mitochondrial DNA analysis on the stored urine sample and comparison of the results with the blood sample were carried out in order to investigate whether a switching or manipulation had occurred.

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Materials and Methods

Materials

Comparative DNA investigations were carried out on a fresh blood sample taken from the athlete and the questionable urine sample (25 mL), which had been collected nine months prior to the investigation and stored at 4°C. On arrival at our institute, the urine sample was at room temperature and had a cloudy appearance.

Extraction

DNA extraction of the blood and urine samples was performed on different days to avoid cross-contamination. DNA was extracted from 200 μL of fresh blood using the Chelex method (8). The liquid fraction of the urine sample was centrifuged for 30 min at 5000 rpm to pellet the cellular material, and the supernatant was discarded. DNA extraction of the urine sample (15 mL) was carried out applying the All-Tissue DNA Kit (GEN-IAL, Troisdorf, Germany) according to the manufacturerís protocol. A second extraction of the urine sample (8.5 mL) was performed separately to confirm the amplification results of the first extraction.

Amplification

Short Tandem Repeat (STR) Systems—The investigations on the human genomic DNA were carried out using the well-established STR systems TH01, VWA, D21S11, D8S1179, D12S391, D18S51, and the sex-specific Amelogenin system. The amplification was performed in singleplex reactions according to the published protocols.

TABLE 1—*Sequencing results of the hypervariable region I + II of the human mitochondrial DNA.*

HVR I + II	Position	Position	Position	Position	Position	Position	Position
	73	150	263	303–315 C-Stretch	16343	16362	16390
Reference sequence	A	C	A	—	A	T	G
Blood sample	**G**	**T**	**G**	Length heteroplasmy*	**G**	**C**	**A**
Urine sample	**G**	**T**	**G**	Length heteroplasmy*	**G**	**C**	**A**

NOTE: bold type = deviation from the reference sequence; — = no length heteroplasmy; * = length heteroplasmy: describes the occurrence of different length variants within the "C-Stretch." An exact assignment of the number of cytosine is not possible, and the sequencing reaction breaks off after the "C-stretch."

Human Mitochondrial DNA—For the human mtDNA investigations, the hypervariable regions HVR I (15997 to 16401 nt) and HVR II (029 to 408 nt) were analyzed according to the recommendations of the International Society for Forensic Genetics (ISFG) (9). Amplification conditions were as follows: 5 μL extract, 10 pmol of each primer (HVR I: M13-15997 to M13-16401/HVR II: M13-029 to M13-408), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 2.5 μL 10xPCR buffer (SERAC, Bad Homburg, Germany), and 1 U Taq DNA polymerase (SERAC, Bad Homburg, Germany) in a reaction volume of 25 μL and overlaid with oil. Temperature profile: 95°C–1 min, 60°C–1 min, 72°C–3 min using 30 cycles for the blood sample and 32 cycles for the urine sample. Purification of the amplification products was done using Microcon 100 columns.

Sequencing of the Human Mitochondrial DNA—Sequencing reactions were performed in both directions using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt) according to the manufacturer's protocol. Sequencing products were analyzed on an ABI 310 Genetic Analyzer using the sequencing module Seq POP6 Rapid (1 mL), filterset A and the software Data Collection (Version 1.0.2), Sequence Analysis (Version 3.3), and Sequence Navigator (Version 1.0.1). Sequencing results were evaluated both manually and automatically with the Sequence Navigator. Sequences of the blood sample and the urine sample were compared against each other as well as with the reference sequence (10).

Results and Discussion

Quality Assurance

In general, internal controls are necessary to guarantee the correctness of the results, and various controls were also used in this case for the extraction, amplification, and interpretation of the results.

First, the blood and urine samples were extracted by different persons on different days. In addition, DNA extraction of the urine sample was performed twice with 15 and 8.5 mL to check and confirm the correctness of the results. Both urine samples showed clear results for the Amelogenin system and the hypervariable regions HVR I and HVR II, indicating that a volume below 10 mL is sufficient for successful mitochondrial DNA analysis. To exclude contamination of the extraction reagents, an extraction blank was used. The amplification was controlled by including positive and negative controls, which gave the expected results. The interpretation of the sequencing results was carried out both manually and computer-aided and cross checked to confirm the results. The sequencing data obtained were checked against all employees to exclude contamination caused by the laboratory personnel.

STR Systems

DNA typing of the blood sample showed reproducible and clear results in all systems investigated, whereas DNA typing of the urine samples failed in all STR systems; only the Amelogenin system gave a clear XY pattern showing that the sample must have come from a male.

Human Mitochondrial DNA

Due to the high copy number of mitochondrial DNA molecules in each cell and the robustness of the mitochondrial genome, we investigated the non-coding regions of the human mitochondrial DNA (mtDNA) hypervariable region I (HVR I) and II (HVR II) as described by the DNA Commission of the International Society for Forensic Genetics (ISFG) (9). Both regions could be successfully amplified and sequenced in the blood sample as well as in the urine samples. The sequencing results of the urine samples were compared with those of the blood sample and with those of the reference sequence (10). The sequences of the urine and the blood samples were identical in the sequenced regions and showed differences in HVR I at three positions (*nt* = 16343, 16362, 16390) and in HRV II at four positions (nt = 73, 150, 263, and 303 to 315/length heteroplasmy) compared to the reference sequence (Table 1).

Conclusions

Due to the identical sequences, the urine and blood samples could not be excluded as potentially originating from the same individual or an individual having the same maternal lineage. The determination of the specific haplotype frequency in a database of 1964 unrelated individuals (Germany, Switzerland, Austria, France) revealed that this haplotype did not occur in this database and could therefore be considered as uncommon. The results of the mtDNA analysis confirmed that the sample could have come from the suspected athlete concerned and under the specific circumstances involved manipulation and/or switching of the urine sample could be excluded. As a consequence, it can be stated that—although mtDNA analysis is less discriminative than STR typing—the application of this method can be a useful tool for investigating urine samples in order to detect possible manipulation and verification of mixed urine samples in cases where STR analysis failed. To our knowledge, this is the first case of successful mtDNA typing of a stored urine sample in an official doping control case and the first case in Germany where an official genetic analysis was carried out to find out if manipulation and/or switching of the samples had occurred.

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