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# Stability of urine samples for samples identification: DNA-based typing Vs. steroid profile

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## Abstract

One of the crucial issues related to antidoping is the possibility of sample manipulation that can results as biochemical alteration of the sample or its substitution with a sample from another person. An athlete, as a consequence of a detected positivity, can make raise doubts about the attribution of the urine sample to his person. So, it could be necessary to introduce methods which make it possible to attribute a sample to a particular individual.
In this study, two systems of identification of urine samples were compared, one that considers the study of steroid profile and the other that it is based on forensic DNA typing. The specific problems associated with the stability of urine samples that are subjected to doping control routine were considered to highlight any efficacy limitation and difference.
It was demonstrated the utility of the DNA typing when the degradation of the urinary sample, subjected to preservation, does not allow the sample-individual linkage by steroidal profile.

## Introduction

In November 2013, the World Antidoping Agency (WADA) introduced the “steroid profile” within the athlete biological passport. The steroid profile gains information about androgen anabolic steroids (AAS) markers. Relative concentration of AAS and their ratio give origin to the urinary steroid profile that can be used for the assignment of a sample to an individual. However, steroid profile analysis can be problematic given some verified difficulties in the interpretation of profile (principally in females) and the problems related to bacterial degradation. In this work we compared the strategy of steroid profile analysis with the DNA typing of a urine sample for the identification of urine samples in doping control taking into account the stability of samples at different temperatures.

## Experimental

4 urine samples (2 males and 2 females) were aliquoted and stored at different temperatures (-20°C, 4°C, room temperature and 37°C). DNA from samples were extracted and typing of each sample were performed starting from t=0 (fresh aliquot) and up to 25 days of storage. Both the steroid profile and the DNA typing were accomplished and these two strategies of sample identification were compared
For DNA typing, DNA was extracted from urinary sediment using a PrepFiler Forensic DNA extraction Kit (Life technologies) starting from 1mL urine centrifuged at 3000g for ten minutes and after removal of the supernatant and following the protocol suggested by manufacturer. Quantification of extracted DNA was achieved by real time PCR using human DNA quantification kit (Life technologies) and following protocol from manufacturer. 1ng DNA was subsequently amplified with human DNA Identifiler kit (Life technologies) and the separation of allelic fragments were executed by an ABI Prism 310 instrument (Life technologies).
1 µL each amplified sample was mixed to 13 µL of formamide and 1 µL of internal size standard. Separation run has been made injecting each sample for 5 seconds at 15Kv Voltage and 60°C temperature run for a total of 28 minutes each run. Data analysis and typing of the STR alleles were made with a Gene Mapper software v1.0 (Life technologies).

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## Results and Discussion

Reference steroid profile analysed at t=0 consisted in Testosterone to Epitestosterone (T/E) ratio, Androsterone to Etiocholonalone (A/Etio) ratio, 5α-androstane-3α,17β-diol to 5β-androstane-3α,17β-diol (5α/5β) ratio. Also the presence of 5-α-androstan-dione (d) and of 5β-androstan-dione has been considered. We monitored the steroid profile of a sample stored at 37°C for 25 days: as expected, we found Testosterone (T) metabolized with the formation of Androsterone and Etiocholanolone (A/Etio), inversion of the ratio Androsterone/Etiocolanolone, high level of 5α-androstandione (i) and of 5β-androstandione (data not shown).
Two signs of sample degradation after storage were considered for DNA typing. First sign is the reduction and the disappearance of high MW alleles at various loci.

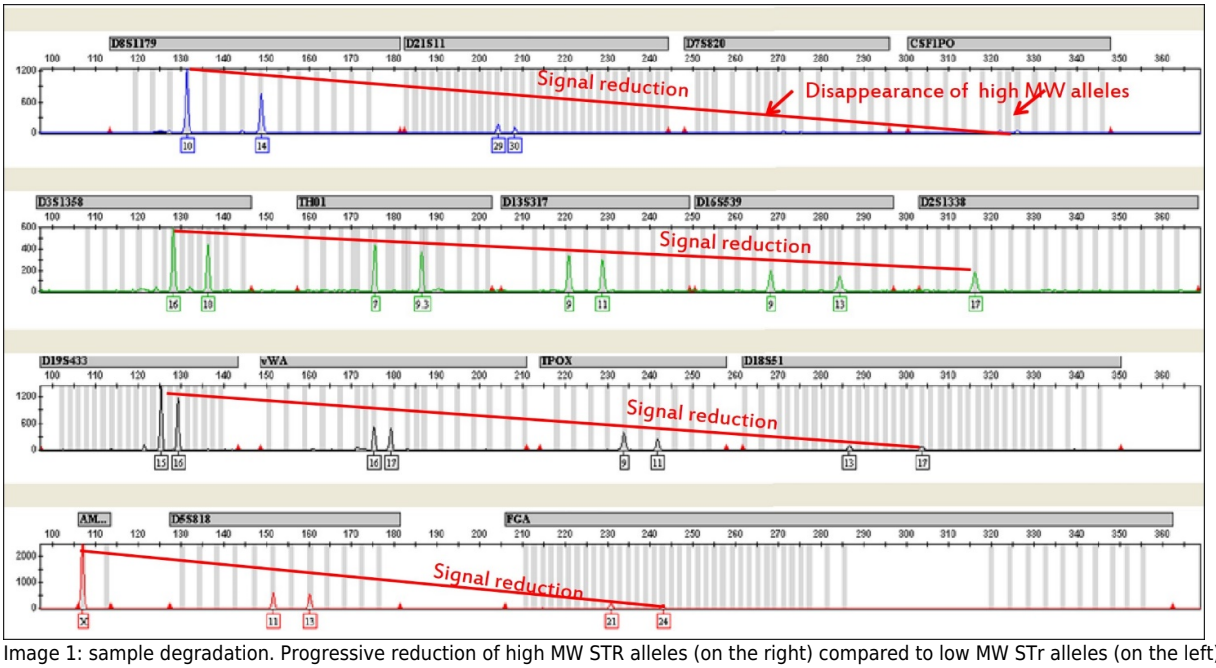

Image 1: sample degradation. Progressive reduction of high MW STR alleles (on the right) compared to low MW STr alleles (on the left).

The second sign is the altered expression of stutters.

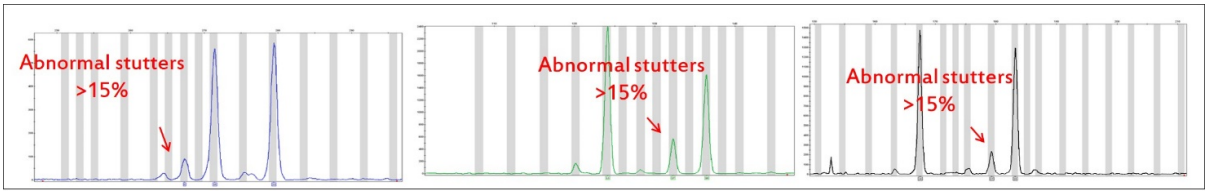

Image 2: Signal of degradation: the appearance of abnormal stutters with height >15% of the main peak

Extreme storage conditions lead to formation of strong signals of degradation; however they don't affect the final DNA profile that can be still determined for most samples even at long storage times. The typing of a certain number of loci is always obtained so to allow the identification of a sample anyway. Compared analysis steroid profile Vs. DNA typing is illustrated in Table 1.


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| | τ=0 | | | | τ= 1 (4 days) | | | T=2 (5 days) | | | τ=3 (6 days) | | | τ=4 (7 days) | | | τ=5 (8 days) | | | τ=6 (11 days) | | | τ=7 (18 days) | | | 8 (25days) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PH | PS | DNA | | PH | PS | DNA | PH | PS | DNA | PH | PS | DNA | PH | PS | DNA | PH | PS | DNA | PH | PS | DNA | PH | PS | DNA | PH | PS | DNA |
| F 1 | 6-6,5 | | | 4°C F 1 | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | |
| F 2 | 6-6,5 | | | F 2 | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | |
| M 1 | 6-6,5 | | | M 1 | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | |
| M 2 | 5-5,5 | | | M 2 | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | |
| | | | | -20°C F 1 | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | |
| | | | | F 2 | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | |
| | | | | M 1 | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | |
| | | | | M 2 | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | |
| | | | | 37°C F 1 | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 8.75 | | | 9.25 | | | 9.25 | | |
| | | | | F 2 | 6.25 | | | 6.25 | | | 6.25 | | | 8.5 | | | 8.75 | | | 9 | | | 9.75 | | | 9.25 | | |
| | | | | M 1 | 6.25 | | | 6.25 | | | 6.25 | | | 8.25 | | | 8.25 | | | 8.25 | | | 9 | | | 8.75 | | |
| | | | | M 2 | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.5 | | | 5.75 | | |
| | | | | T.A. F 1 | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | |
| | | | | F 2 | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.75 | | | 7.25 | | |
| | | | | M 1 | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 6.25 | | | 7.25 | | |
| | | | | M 2 | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.25 | | | 5.5 | | | 8.75 | | |

Table1: Summary table of steroid profile (SP) and DNA profile (DNA) at different times and different storage conditions. Here pH of urine samples are reported (pH). For steroid profile green boxes mean a complete profile while red boxes mean degradation. For DNA typing green boxes mean a complete typization (15 loci STR+Amelogenin), yellow boxes mean a partial typization (9-15 loci) and red boxes mean an invalid typization (0-8 loci).

Worst storage conditions seems affect only steroid profiles as DNA profiles are more stable at these conditions and identification of the sample can be achieved also in case of incomplete typing. Also bacterial degradation, has less influence on the DNA results.
Furthermore, it was demonstrated (data not shown) that also in condition of strong sample degradation, it was possible to achieve full DNA profiles by increasing the amount of sample urine for the extraction of DNA. However, full DNA profiles were obtained starting from 10 mL from females urine and 20 mL from males urine. Fully reliable DNA profiles were so obtained also for urine sample stored in the common procedures for doping control (refrigerated and frozen).

## Conclusions

Our data show that DNA typing is a more reliable and stable method for the identification of urine sample in doping control and it allows a more secure system for the correlation of a urine sample to an individual or the correlation of two urine sample, every time it is requested. However, the analysis of steroid profiles is still preferable for an initial screening investigation as the profile is routinely monitored in all urine samples and it can be obtained in less time than a DNA profile.

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