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CASE REPORT

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Substitution of Human for Horse Urine Disproves an Accusation of Doping*

ABSTRACT: In order to detect switching and/or manipulation of samples, the owner of a stallion asked our lab to perform a DNA test on a positive doping urine sample. The objective was to compare the urine DNA profile versus blood and hair DNA profiles from the same stallion. At first, 10 microsatellite markers were investigated to determine the horse identity. No results were obtained when horse specific markers were typed in the urine sample. In order to confirm the species origin of this sample we analyzed the mitochondrial cytochrome *b* gene. This analysis from blood and hair samples produced reproducible and clear PCR-RFLP patterns and DNA sequence match with those expected for horse, while the urine sample results were coincident with human. These results allowed us to exclude the urine sample from the questioned stallion and determine its human species origin, confirming the manipulation of urine sample.

KEYWORDS: forensic science, positive doping, urine, DNA typing, microsatellites, horse, species-specific identification, mitochondrial DNA, cytochrome *b*

Urine is the sample of choice for drug screening in sports. Doping in performance horses is defined as the "illegal application of any substance, except normal diet, that might modify the natural and present capacities of the horse at the time of the race" (1), and is normally performed in order to increase the horse's performance. In sports horses, the inoculation of caffeine is considered illegal. Caffeine is not a regular part of a horse's diet, and is among the drugs that may be suministrated to increase the performance. Caffeine is an alkaloid which can be obtained from the wastes of tea, or coffee or it may be prepared synthetically. Both caffeine and theobromine are naturally occurring members of the methylxanthine family and, whilst caffeine has an effect on predominantly the central nervous system, theobromine is associated with diuretic responses.

Manipulation of urine sampling in sports drug testing is a violation of anti-doping rules and is consequently sanctioned by regulatory authorities. In some instances, the origin of the submitted samples may be challenged because of the medical, legal, and economic consequences of a positive drug test (2,3). Numerous analytical strategies have been used, including gas chromatography-mass spectrometry for steroids and metabolites, gas chromatography-nitrogen/phosphorus detector analysis, high-performance liquid chromatography-UV fingerprinting, and DNA-STR (short tandem repeat) analysis.

Urine DNA originates from the presence of intact desquamative epithelial cells of the urinary tract. However, only 20–30% of samples yield a sufficient DNA to interpret amplification results. Separating the cells from micro-organisms and other urine components, prior to DNA extraction, increases the number of successfully typed samples (4–7).

DNA profiling is useful in the certification of a specific animal identity (8–11). In addition, DNA sequence analysis allows for identifying the species source of unknown casework urine specimens. Mitochondrial DNA typing (mtDNA) can be used to differentiate among animal species (10,12–15). Such discriminatory tests have important applications in agriculture, food technology, and an important and growing role in forensic sciences (e.g., in the detection of illegal import, export, and hunting of endangered species, and stealing of livestock animals) (10,12,15–17).

Here we report a case of species determination in blood, hair, and urine samples from a positive caffeine doping case in an American Trotter breed horse. In order to detect switching and/or manipulation of the samples, the owner of the stallion asked our lab to perform a DNA test on the positive doping urine sample. The objective was to compare the DNA profile of the urine to fresh blood and hair samples taken from the questioned stallion.

Material and Methods

Animal Samples

The casework involved blood, hair, and urine samples that were received by our laboratory to determine if they belonged to the same individual. Peripheral blood was obtained by sterile jugular puncture from the questioned horse, stored with anticoagulant at 20°C until the analysis. Hairs were taken from the tail and mane and were sent to us in a plastic envelope. The urine sample positive

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for doping had been collected three months prior to study and had been stored frozen at −20°C in the Centre of Research and Control of Doping (University of La Plata, Argentina).

DNA Isolation

Whole DNA from blood lymphocytes was isolated by the DNAzol® method (Invitrogen, Carlsbad, CA) (18).

To extract the DNA from hair, the bulbs were cut and placed into a 0.5 mL tube, and incubated at 95°C with NaOH (200 mM) for 10 min. An equal volume of neutralizing solution (Tris–HCl 100 mM, 200 mM, pH = 8.5) was added and hair remnants were separated from the suspension. This DNA was used as template in PCR reactions without additional purification.

For DNA isolation, the total volume of urine (10–15 mL) was centrifuged at 2000×*g* for 10 min. The pellet obtained was washed twice with water and subjected to DNAzol® extraction procedure (Invitrogen), following suggested modifications (19).

Horse DNA Typing

All DNA samples were used as template for PCR amplification of the STR panel recommended by the International Society for Animal Genetics (ISAG) for equine identification. These markers have been tested in several Horse Comparison Tests with a high degree of concordance among labs (ISAG; http://www.isag.org.uk/). PCR amplification of 12 microsatellites was performed on a thermal cycler (MJ Research, Boston, MA and Bio-Rad Laboratories Inc., Hercules, CA) in three multiplexes: (i) AHT4-AHT5-HMS6-HMS7-HTG4-HTG6-VHL20; (ii) ASB2-HMS3-HTG10; (iii) TKY333-TKY394, following the recommendations of the ISAG. The positive controls consisted of horse samples previously typed by our lab and standardized during the International Horse Comparison Test of the ISAG (2005–2006). Each casework sample was genotyped in a MegaBACE1000 automated sequencer (GE Healthcare) using ET550-R as Molecular Size Standard (GE Healthcare). The analysis and genotype assignments were made by using the MEGABACE Genetic Profiler Software Suit version 2.2 (GE Healthcare). Binsets were standardized using control DNAs from ISAG 2005–2006 International Horse Comparison Test.

Species-Specific Determination by RFLP Analysis of PCR Products

For cytochrome *b* (Cyt *b*) amplification a universal pair of primer oligonucleotides was used: L14816 5′-CCATCCAACAT CTCAGCATGATGAAA-3′ and H15173 5′-CCCCTCAGAATGA-TATTTGTCCTC A-3′ (20), which allow amplification of a *c.* 358 bp fragment in almost all mammalian species. The amplification reactions were carried out under known conditions (15). Reference DNA samples from horse (NZ6) and human (NZ1) species were provided by the CIGEBA DNA bank (15), and used as DNA species-specific controls.

For restriction fragment length polymorphism (RFLP) tests, 10 microliters of PCR products were separately digested with 2.5 units of *Hae*III and *Hin*fI restriction enzymes (Invitrogen) in a final volume of 20 μL for at least 2 h at the temperature recommended by the suppliers. Restriction fragments were resolved by electrophoresis in 6% (w/v) acrylamide–bis acrylamide (19:1) nondenaturing gels, 1× tris–borate EDTA (TBE), 0.1% (w/v) ammonium persulfate, and 0.01% (v/v) TEMED, at 120 V and ethidium bromide stained. The fragment pattern sizes were determined by comparison to the 100 bp DNA Ladder (Invitrogen).

DNA Sequencing and Sequence Analysis

In order to confirm the results obtained by PCR-RFLP analysis, all PCR products were sequenced using DYEnamic ET Dye Terminator Kit (GE Healthcare) and the same universal Cyt *b* primers with a MegaBACE 1000 automated sequencer (GE Healthcare). The nucleotide sequences obtained were analyzed by applying BLASTN 2.2.16 (21) program against the database (GenBank, EMBL, DDBJ, PDB).

Results and Discussion

Horse DNA Identification

Only blood and hair samples produced results in the 12 microsatellites used for horses (ISAG). Identical genetic profiles were evident in blood and hair. By contrast, urine sample did not produce any results, even though positive controls were seen. Several explanations for negative results were possible, including low DNA quality in the urine sample, or the presence of inhibitors of the PCR reaction in the template solution. We also considered that the species origin of the urine was other than horse. For this last possibility, we proceed to amplify mitochondrial Cyt *b* to determine the species origin of the urine sample.

Species-Specific Determination

To determine the species origin of the urine, we applied the mtDNA Cyt *b* typing using a validated PCR-RFLP method (15). All reactions resulted in a single fragment of the expected size (*c.* 358 bp). The findings allowed rejection of several hypotheses: the presence of PCR inhibitors, low quality or quantity of DNA from urine sample. As a human source of urine is easily obtained we included human DNA control.

Restriction fragment length polymorphism analysis of blood and hair showed a perfect match to those from the equine DNA control.

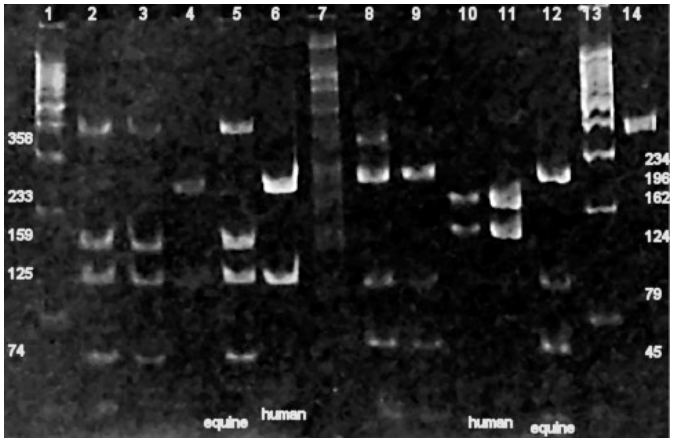

FIG. 1—*PCR-RFLP gel showing the band patterns obtained with the digestion of the Cyt* b *PCR products from all casework samples. Lines 2–6 (*Hae*III); lines 8–12 (*Hinf*I) restriction patterns; lines 1, 7, and 13: MW; lines 2 and 8: blood; lines 3 and 9: hair; lines 4 and 10: urine; lines 5 and 12: equine control; lines 6 and 11: human control; line 14: PCR without digestion. As shown in the photograph, lines 4 and 10 showed the same band patterns than the human control DNA (lines 6 and 11). Numbers on the left and right side of the figure indicates the band sizes of the digested PCR products in comparison to the molecular size marker (100 bp DNA ladder).*

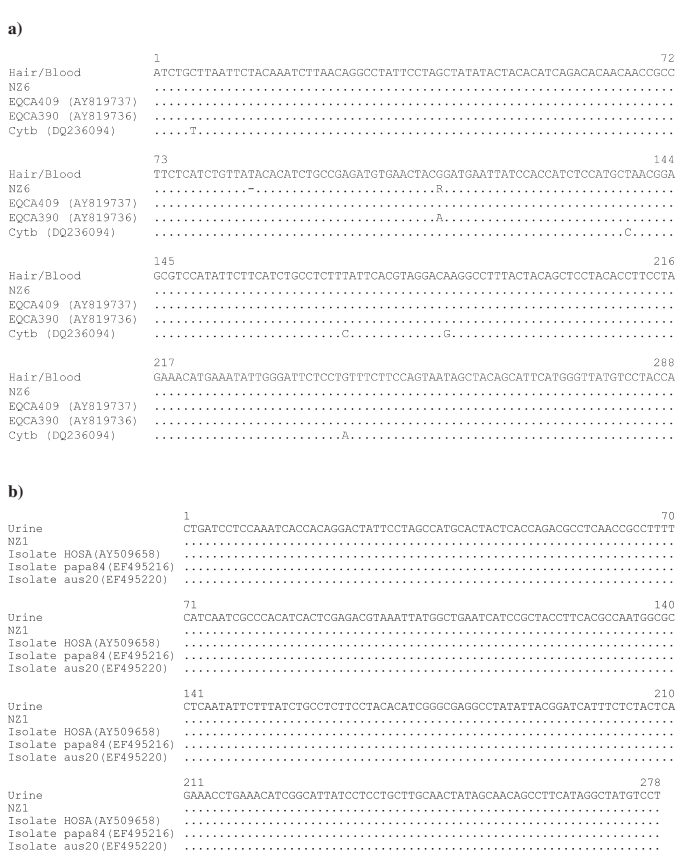

FIG. 2—*DNA sequence alignments of the Cyt* b *sequences obtained from the casework samples in comparison to the Cyt* b *sequences retrieved from database. Dots indicate identity to the first sequence in a given nucleotide position. Accession numbers are shown between brackets. Sequence results from species controls DNA used in the PCR-RFLP analysis are included. (a) Hair and blood consensus Cyt* b *sequence in comparison to reported* Equus caballus *Cyt* b *sequences: NZ6 (equine species-specific control), AY819737, AY819736, and DQ236094. (b) Urine Cyt* b *sequence in comparison to human Cyt* b *sequences: NZ1 (human species-specific control), AY509658, EF495216, and EF495220.*

On the other hand, the band patterns obtained from urine resembled those from the human DNA control (NZ1; Fig. 1). The applicability of this method to the forensic field was demonstrated by simulated casework conditions where different types of samples, including problematic specimens such as hair, bone samples, bristles, and feathers were investigated to identify the species (15,20).

In order to confirm the PCR-RFLP results, Cyt *b* amplicons from each casework sample were sequenced. The sequences derived were used to identify their biological species origin by aligning to the Cyt *b* gene sequence entries reported in public nucleotide databases (Figs. 2*a* and 2*b*). Blood and hair sequences were 99–97% identical to reported equine Cyt *b* (22; accession no. AY819737, AY819736, and DQ236094), while those derived from urine sample were 100% identical to human database sequences (22; accession no. AY509658, EF495216, and EF495220) as is shown in Figs. 2*a* and 2*b*.

In conclusion, we demonstrated that the alleged horse urine was of a human origin, indicating the manipulation of samples. This evidence supports the innocence of the breeder suspected of horse doping.

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References

1. Ungemach FR. Doping control in race horses. Tierarztl Prax 1985;13(1):35–53.
2. Vu NT, Chaturvedi AK, Canfield DV. Genotyping for DQA1 and PM loci in urine using PCR-based amplification: effects of sample volume, storage temperature, preservatives, and aging on DNA extraction and typing. Forensic Sci Int 1999;102(1):23–34.
3. Thevis M, Geyer H, Mareck U, Sigmund G, Henke J, Henke L, et al. Detection of manipulation in doping control urine sample collection: a multidisciplinary approach to determine identical urine samples. Anal Bioanal Chem 2007;388(7):1539–43.
4. Brinkmann B, Rand S, Bajanowski T. Forensic identification of urine samples. Int J Legal Med 1992;105(1):59–61.
5. Prinz M, Grellner W, Schmitt C. DNA typing of urine samples following several years of storage. Int J Legal Med 1993;106(2):75–9.
6. Junge A, Steevens M, Madea B. Successful DNA typing of a urine sample in a doping control case using human mitochondrial DNA analysis. J Forensic Sci 2002;47(5):1022–4.
7. Nakazono T, Kashimura S, Hayashiba Y, Hara K, Miyoshi A. Successful DNA typing of urine stains using a DNA purification kit following dialfiltration. J Forensic Sci 2005;50(4):860–4.
8. Marklund S, Ellegren H, Eriksson S, Sandberg K, Andersson L. Parentage testing and linkage analysis in the horse using a set of highly polymorphic microsatellites. Anim Genet 1994;25(1):19–23.
9. Tozaki T, Kakoi H, Mashima S, Hirota K, Hasegawa T, Ishida N, et al. Population study and validation of paternity testing for thoroughbred horses by 15 microsatellite loci. J Vet Med Sci 2001;63(11):1191–7.
10. Hsieh HM, Huang LH, Tsai LC, Kuo YC, Meng HH, Linacre A, et al. Species identification of rhinoceros horns using the cytochrome *b* gene. Forensic Sci Int 2003;3:1–11.
11. Moodley Y, Baumgarten I, Harley EH. Horse microsatellites and their amenability to comparative equid genetics. Anim Genet 2006;37(3):258–61.
12. Bataille M, Crainic K, Leterreux M, Durigon M, de Mazancourt P. Multiplex amplification of mitochondrial DNA for human and species identification in forensic evaluation. Forensic Sci Int 1999;99(3):165–70.
13. Branicki W, Kupiec T, Pawlowski R. Validation of cytochrome *b* sequence analysis as a method of species identification. J Forensic Sci 2003;48(1):83–7.
14. Zhou B, Zhang L, Wu MY. DNA-based technique and species identification. Fa Yi Xue Za Zhi 2003;19(4):245–8.
15. Bravi CM, Liron JP, Mirol PM, Ripoli MV, Peral-Garcıa P, Giovambattista G. A simple method for domestic animal identification in Argentina using PCR-RFLP analysis of cytochrome *b* gene. Leg Med 2004;6:246–51.
16. Palumbi SR, Cipriano F. Species identification using genetic tools: the value of nuclear and mitochondrial gene sequences in whale conservation. J Hered 1998;89(5):459–64.
17. Ross HA, Lento GM, Dalebout ML, Goode M, Ewing G, Mclaren P, et al. DNA surveillance: web-based molecular identification of whales, dolphins, and porpoises. J Hered 2003;94(2):111–4.
18. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Protein sequence similarity searches using patterns as seeds. Nucleic Acids Res 1997;25(17):3389–402.
19. Villegas Castagnasso EE, It V, Díaz S, Liron JP, Kienast ME, Ripoli MV, et al. Estudio de diversidad gentica en animales domsticos. Analecta Veterinaria 2004;24:11–3.
20. Parson W, Pegoraro K, Niederstatter H, Foger M, Steinlechner M. Species identification by means of the cytochrome *b* gene. Int J Legal Med 2000;2:23–8.
21. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
22. Meece JK, Reynolds CE, Stockwell PJ, Christensen JE, Reed KD. Identification of mosquito blood meal source by terminal restriction fragment length polymorphism (T-RFLP) profile analysis of the cytochrome *b* gene. J Med Entomol 2005;42(4):657–67.

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