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Use of forensic investigations in anti-doping☆

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ABSTRACT

The fight against doping is mainly focused on direct detection, using analytical methods for the detection of doping agents in biological samples. However, the World Anti-Doping Code also defines doping as possession, administration or attempted administration of prohibited substances or methods, trafficking or attempted trafficking in any prohibited substance or methods. As these issues correspond to criminal investigation, a forensic approach can help assessing potential violation of these rules.

In the context of a rowing competition, genetic analyses were conducted on biological samples collected in infusion apparatus, bags and tubing in order to obtain DNA profiles. As no database of athletes' DNA profiles was available, the use of information from the location detection as well as contextual information were key to determine a population of suspected athletes and to obtain reference DNA profiles for comparison.

Analysis of samples from infusion systems provided 8 different DNA profiles. The comparison between these profiles and 8 reference profiles from suspected athletes could not be distinguished.

This case-study is one of the first where a forensic approach was applied for anti-doping purposes. Based on this investigation, the International Rowing Federation authorities decided to ban not only the incriminated athletes, but also the coaches and officials for 2 years.

1. Introduction

The World Anti-Doping Code establishes a harmonisation of the anti-doping rules across all sports and countries in the world [1]. It provides a framework for rules, regulations and practice of sport.

Doping is defined in this document through a number of articles, in Sections 2.1–2.8. Despite the broad scope of this code, the fight against doping is mainly focused on the first definition, which is the detection of a prohibited substance in an athlete's biological sample. For this purpose, blood and urine specimens are collected in and out of competition and submitted to a variety of analytical tests designed to highlight the presence of a banned substance (e.g. stimulants, anabolic steroids, exogenous erythropoietin (EPO), corticosteroids, and monitoring of blood transfusion) [2].

Noteworthy, other doping offences include the use or attempted use by an athlete of a prohibited substance or method, possession of prohibited substances or methods, trafficking or attempted trafficking in any prohibited substance or methods, or administration or attempted administration of any prohibited method or substance. However, these violations of the Anti-Doping Code are barely investigated as traditional analytical methods cannot provide relevant information on these offences.

Since such issues correspond closely to criminal cases under forensic investigation, relying on a similar approach may benefit the fight against doping to help assessing potential violation of these rules. Indeed, collecting items, examining evidences and interpreting results other than dope testing biological samples would allow assessing the use, administration and possession of prohibited substances or methods by drawing links between seized prohibited substances and/or medical equipments and an athlete or his entourage [1]. Also, a drug intelligence approach used for tackling drug-trafficking networks could also be applied to identify and dismantle doping products-trafficking networks to which an athlete or his entourage may be linked [3].

Nevertheless, while these techniques have a great potential in the fight against doping, their use remains marginal. Therefore, this paper describes a recent doping case where a forensic approach proved successful in providing evidence that led to suspension for

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anti-doping violation rules of several athletes and related technical staff. As such, investigation methods novel to the field of anti-doping will be presented in this case study.

2. Case report

In July 2007, during the Rowing World Cup on the Rotsee Lake (Lucerne, Switzerland), a plastic bag containing different types of medical equipment was found by a local resident in a waste container. As a witness had seen a team official throwing away the incriminated plastic bag into the compost bin, he decided to report this to the International Rowing Federation (FISA). Considering this fact and as the probability that elite rowers were involved in the use of these equipments was high, the Federation decided to transmit the material to the Swiss Laboratory for Doping Analyses (LAD) for investigations (Fig. 1). FISA asked the LAD to undertake extensive analyses to investigate on a potential anti-doping rule violation and to evaluate involvement of suspected athletes in this case.

The first aim was to establish if there was a violation of anti-doping rules. The drug present in the plastic bag consisted of 12 bottles of Neoton®, 10 bottles of Esafosfina®, a bottle of aminocaproic acid, 4 vials of Panagin®, 2 vials of inosine and 2 boxes of Biotad® tablets. Analysis on the composition of these products revealed that there were compounds used for faster recovery only, and none of them could be considered as doping agents. However, 4 syringes, 4 needles and 13 used intravenous infusion items were found alongside these products. According to the World Anti-Doping Code, the use of an intravenous system constitutes a violation of the anti-doping rules [2].

As red residue was visible inside the infusion tubing, potentially corresponding to blood traces, it was decided to conduct genetic analyses on these biological samples after collection, in order to obtain DNA profiles that could later be compared to DNA profiles from suspected athletes (Fig. 2). However, since no database of athletes' DNA profiles was available, and to avoid profiling all rowers who took part in this competition, an evaluation of the contextual information available was crucial. Indeed, this kind of information was taken into account when determining which athletes should be targeted. As a first indication, the medical material was found in a rubbish bin located behind the hotel where two federations' teams were staying, namely Nation A and B. Moreover, the drug packaging and the plastic bag containing it provided additional and relevant

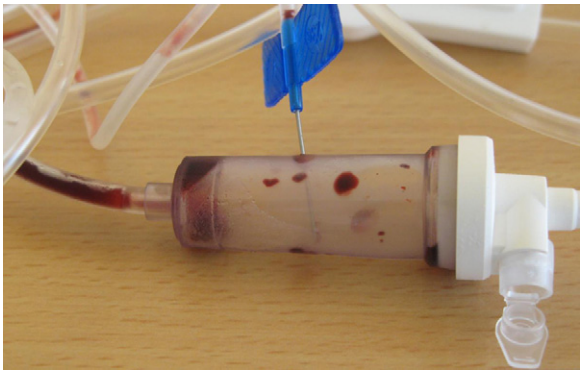

Fig. 2. Red residues inside the tubing systems.

information. Indeed, the inscriptions were written in Cyrillic alphabet and only Nation A was from a country using this alphabet (Fig. 3). According to these observations, the FISA decided to target only athletes from this nation.

Thereafter, the LAD submitted all parts of the perfusion systems (bottles, syringes, plastic tubes and needles) containing biological samples to the Forensic Genetics Unit (UGF). This material was analysed for DNA profiling in order to identify the source donors of the blood collected on the perfusion systems. Afterwards, FISA decided to collect anti-doping tests blood samples on rowers of the Nation A team to obtain reference DNA profiles for comparison.

3. Materials and methods

DNA extraction was performed on 10–100 μL of liquid blood collected on the infusion tubing. If dry, the blood was directly rinsed with the buffer used for DNA extraction. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen AG, Hombrechtikon, Switzerland) following the manufacturer's instructions. DNA extracts were concentrated to about 25 μL using Microcon 30 spin columns (Millipore AG, Zug, Switzerland). They were quantified with a real-time PCR in order to set-up the DNA amplification protocols. This was performed with the Quantifiler Human DNA Quantification kits using a qPCR ABI 7300 according to the manufacturer's instructions (Applied Biosystems, Zug, Switzerland) in half reaction volume. The mean DNA concentration of the blood stains was 17.59 ng/μL, ranging from 0.04 (dried blood in a plastic tube) to 66.84 ng/μL (liquid blood in a needle). Reference samples were analysed with the same protocol but in a dedicated room.

DNA amplifications were carried out with the AmpFℓSTR® SGM Plus® PCR Amplification Kit from Applied Biosystems, following the manufacturer's instructions, in half reaction volume. This kit amplifies 10 Short Tandem Repeat loci (D3S1358, VWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01

Fig. 1. Overview of the material present in the dustbin.

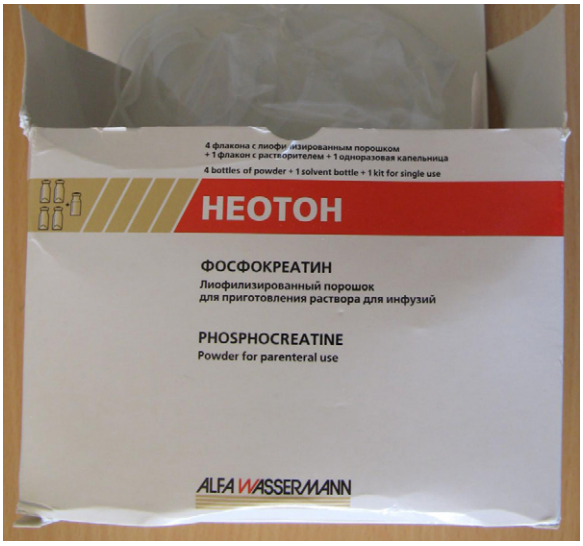

Fig. 3. Cyrillic alphabet present on several drugs packaging.

and FGA) plus the gender marker Amelogenin. Amplified DNA was analysed with an ABI 3100 Genetic Analyzer from Applied Biosystems following standard procedures. For each stain and reference sample, DNA profiles were validated, with a second result obtained either with an independent DNA extraction or with two independent amplifications of the same DNA extract. The least concentrated sample was amplified with an increased number of PCR cycles (34) to enhance the sensitivity of the amplification. For this sample, a consensus DNA profile was built from 5 amplifications using the guidelines from Castella et al. [4].

4. Results and discussion

4.1. DNA analyses

Forensic methods can provide useful tools in investigating the possible violation of anti-doping rules by using techniques based not only on the analysis of a sample provided by the athlete, but by the presence of other evidence. Only a few cells are necessary to establish a DNA [5,6]. Indeed, sufficient biological material might be collected on the neck of a drug flask, inside an infusion tube, syringe, blood bag or urine sample to provide a DNA profile. As a matter of fact, the two main matrices for doping analyses, which are urine and blood, are compatible with DNA profiling. Therefore, after comparison with a reference sample of an athlete, the source of a biological sample may be assessed.

Presently, forensic genetics has already been used in anti-doping to identify individuals by their DNA profiles obtained from urinary extracts. In most of the cases, the aim of DNA profiling was to demonstrate that a urine sample really belonged to an athlete or conversely to investigate whether an athlete really gave genuine urine and not a negative urine hidden in a pocket [7,8].

Although DNA profiling is a very powerful tool for identification purposes, its potential for the fight against doping remains widely unexploited. Indeed, it may provide relevant evidence on violation of anti-doping rules where traditional techniques would prove totally inefficient.

4.2. Application to the case

In the particular context of the case, the motivation for FISA to use DNA analysis was to determine if one or more anti-doping rule violations occurred. According to the examination of the seized material and the preliminary analytical results, further investigations had to focus on the possible use of a prohibited method rather than a prohibited substance. Also, FISA had to evaluate whether the use of the intravenous infusion equipment was medically justified or not. Indeed, infusion is only allowed for legitimate medical treatment but prohibited for enhancing recovery [2]. However, as the seized substances were not determined as doping agents, direct detection and quantification methods in doping samples would not prove relevant. In consequence, obtaining the identity of the persons who used these equipments for doping purpose was mandatory.

As a first step, the location of the medical equipment allowed focusing on a limited number of athletes. Accordingly, examination of the Cyrillic alphabet appearing on several items, including two bags containing drugs, syringes, ampoules, perfusion bottles, packing tape and infusion systems, provided useful information for reducing the population of athletes potentially incriminated. Indeed, this alphabet is found in Eastern European countries such as Bulgaria, Russia or Ukraine. As Nation A was using the Cyrillic alphabet, it was decided to conduct tests on athletes of their team. These two pieces of information were very important for limiting the number of analyses required and to shorten the time necessary to find the perpetrators. If the perfusion systems and the drug packaging had been transmitted to the LAD alone, reducing the number of suspects to such a low number would have been difficult and investigations would need to have been extended to many more athletes (Fig. 4).

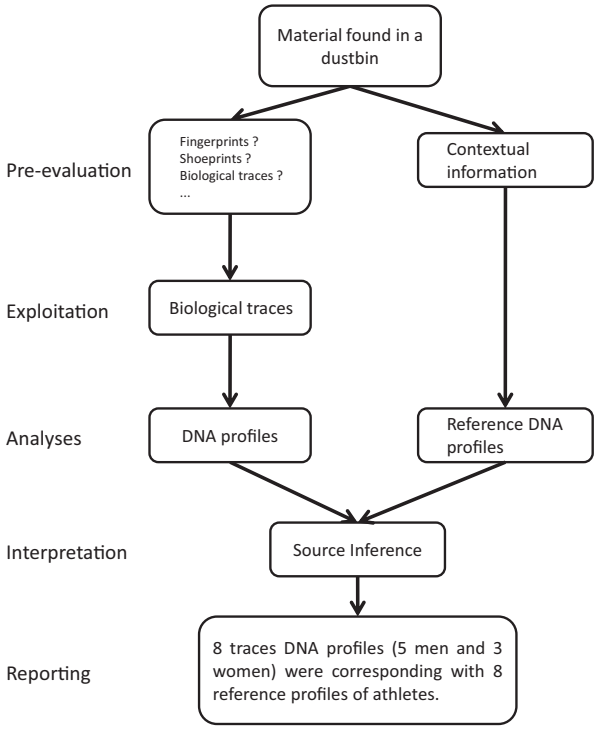

Fig. 4. Forensic investigation approach.

Subsequently, DNA profiling was conducted by the UGF on 10 biological samples collected on the parts of perfusion systems (bottles, syringes, plastic tubes and needles) and 30 reference blood samples from athletes of Nation A present at the competition. It was possible to determine 8 different DNA profiles coming from the different samples and due to the presence of a gender marker, 5 of these profiles were determined as male and 3 as female (Fig. 5).

In order to compare these with references profiles, collection of blood samples from athletes for DNA profiling was divided in three rounds. On the first round of testing, 9 athletes from Nation A were controlled at a training camp. After comparison, DNA profiles of 2 athletes among the 9 could not be distinguished from the trace DNA profiles, considering the 10 corresponding loci. On the second round, 3 athletes from Nation A were tested and one of the DNA profiles obtained was undistinguishable from a third trace DNA profile. Finally, the third round concerned 18 athletes of Nation A and 5 DNA profiles of these athletes could not been distinguished from the last 5 traces DNA profiles related to this doping case.

Considering these results, a Bayesian approach was used to evaluate the statistical probability of the evidence. The DNA evidence is assessed with a likelihood ratio (LR) [9]. This metric estimates the probability of a DNA match under two alternative hypotheses that are:

H_1: the DNA profile originated from the suspect.
H_2: the DNA profile originated from an unknown person unrelated to the suspect.

The value of the likelihood ratio is defined by an equation representing the probability (Pr) of the DNA evidence (E) given Hypothesis 1 (H_1) or Hypothesis 2 (H_2):

$$LR = \frac{Pr(E|H_1)}{Pr(E|H_2)} \quad (1)$$

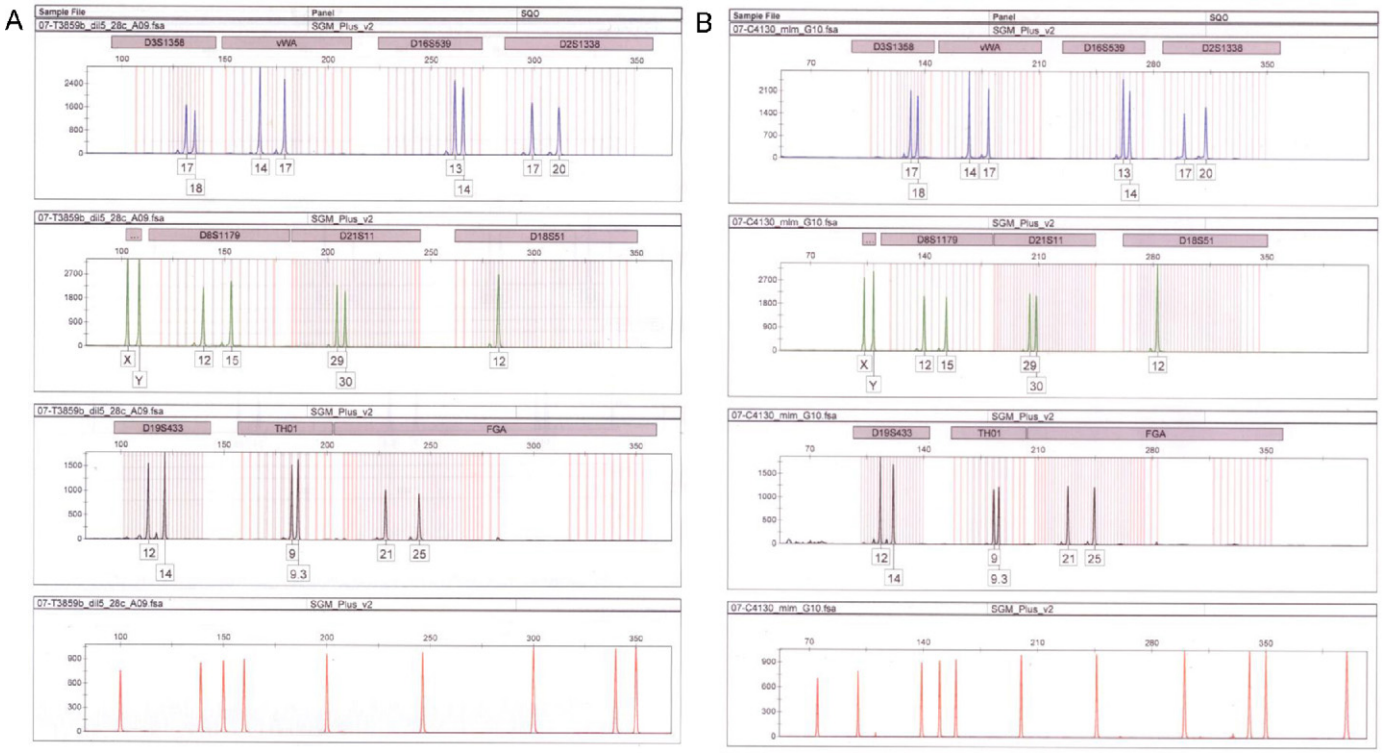

Fig. 5. DNA profile from residues found in a transfusion system (A) and a reference blood sample (B).

So to translate this ratio for a better understanding in court, a verbal scale was developed by Evett to express the weight of the evidence (Table 1) [10].

In this case study, LR values larger than 1 billion were reported for the 8 DNA matches observed. These values represent an adequate and conservative way of expressing the draw of the evidence when 10 loci SGM Plus DNA profiles are concerned [11]. On a verbal scale, DNA analyses provided an extremely strong support to the hypothesis, according to which eight suspected athletes were at the origin of the 8 matching blood stains.

Noteworthy, DNA analyses could be differentiated from anti-doping analyses since a DNA profile by itself is useless. Indeed, a reference is always needed. In other words, while the presence of a doping substance in the athlete biological fluids can prove doping, a DNA profile is only useful when it can be compared to reference material. In a forensic case, the profile would have been compared to a database. However, in the anti-doping field, DNA analyses are still not widespread and no databases have been built. An idea would be to introduce the DNA profile in the biological passport to provide a nearly exhaustive database useful to solve such cases. As it is still only a proposition, it was necessary to use other sources of information as in the forensic field, namely the contextual information and physical evidence left behind by the users. In this case, such information was really crucial to reduce the circle of suspected athletes and target the cheaters.

Table 1
Verbal scale representing the support to H_1.

LR	Verbal scale
>1 to 10	Limited evidence to support
10 to 100	Moderate evidence to support
100 to 1000	Moderately strong evidence to support
1000 to 10,000	Strong evidence to support
>10,000	Very strong evidence to support

Also, the question of eligibility of DNA analyses in the anti-doping field has never been discussed before. According to Swiss law, agreement of the athletes themselves or an order by a magistrate to conduct this type of analyses would be required [12]. In the context of the case, no magistrate could order the expertise and the athletes would certainly not have given their approval. However, in the world of professional sport, all athletes have to sign a contract, namely an Athlete's Commitment form, including several obligatory points. Actually, the FISA's form includes that: "*the athlete is willing to submit to ANY tests* (*blood, urine, gas, etc.*) *carried out by FISA*" [13]. Therefore, the Federation, on the advice of FISA head doctor, decided that it was possible to conduct tests without seeking specific consent.

As there was just a presumption against the athletes of the Nation A team, it was decided to perform a doping control only on several athletes in order to ascertain whether or not the evidence was pointing at the right athletes' group. As results showed similarities between some samples of controls and profiles obtained on the residues found in the infusions material, further investigations were carried out to draw links between the traces' DNA profiles and references coming from the suspected athletes. Based on these results and the positive DNA matches, FISA concluded that 8 rowers had violated anti-doping rules.

A last aspect of the investigation was to ascertain if the team doctor was aware of and involved in administration of the intravenous infusions. Indeed, Article 2.8 of the World Anti-Doping Code also prohibits assisting, encouraging, aiding, abetting, covering up or any other type of complicity [1]. Since the rowers claimed that they obtained and used the equipment themselves, the team doctor seemed not to be implicated. However, in a subsequent testimony, he admitted his implication after the National Federation officials recognised the doping offence. If not, the presence of fingerprints of the doctor on the intravenous equipment may have been investigated. Nevertheless, it would require a lot of work to implement fingerprints techniques and education of the Doping Control Officer on sampling and

preservation of evidence with such materials. In this case, the persons who were in contact with the materials did not take particular care during the collection. It would be interesting to evaluate the possibility of using this approach in the context of anti-doping, while keeping in mind that, as with DNA, no database is available for fingerprints. Alternatively, it would have been possible to take samples from the outside of the perfusion system and drug packages in order to look for the DNA of the epithelial left by the person who touched this material. Once again, special care is necessary to avoid contamination and the success of these DNA analyses are not guaranteed, due to environmental conditions.

Following this case, several other investigations were conducted using DNA analyses in an anti-doping context. A huge number of urine samples were compared with other samples coming from the same athletes to highlight the practice of giving negative urine hidden in pockets as reported in some countries. Another famous case which required DNA analysis was the "Puerto case" where several professional cyclists admitted blood withdrawal in order to inject it later through transfusion. These cyclists were all sanctioned. However, an unidentified blood pocket remained and DNA analysis established a positive correlation between plasma DNA present in this pocket and DNA of Alejandro Valverde. Also, it was demonstrated that this blood pocket contained EPO. The Court of Arbitration (CAS) decided to ban him for two years from all sports competitions [14].

Since that case, FISA and two other International Federations (the International Cycling Union (UCI) and the International Gymnastics Federation (FIG)) have collaborated to settle the, so called, "No Needle Policy". The use of needle must be medically justified, appropriate, administered by a certified medical professional, declared to the competition doctor and the disposal of used needles shall be conform to recognized safety. The purpose of this policy is to prevent the culture of the injection. Athletes become accustomed to this method and it may be the beginning of a gradual shift toward doping habits.

5. Conclusion and perspective

Through forensic investigation, in particular DNA analysis, FISA authorities were able to establish that 8 rowers were involved in this doping case. The analysis of the equipment provided evidence on the use of a prohibited method. Given the number of athletes implicated and the conflicting explanation from the Nation A Federation, the FISA hearing panel decided to ban not only the eight rowers for all competitions during two years but also the coaches and officials of the National Federation [15].

This case showed that the forensic approach might bring a new perspective to the anti-doping field, especially with the support of DNA analyses. Other forensic areas such as fingerprints might also provide some crucial information which, combined with traditional detection methods, would enforce evidence by linking a person with an object like a prohibited drug bottle or packaging. The use of criminal analysis could also allow identification of networks of organised doping and highlight athletes who might be connected with this activity. This will necessarily go through awareness and education of the Doping Control Officer. The International Federations should also pay special attention to the possibility of using these techniques to provide additional evidence in cases where there are still doubts about a doping offence.

The legitimacy of DNA tests in anti-doping control should also be discussed, for example with the publication of a DNA testing policy which could include guidelines for DNA analyses, authorising the use of DNA profiling in order to prove a form of doping. As a further perspective, this document might be included in the World Anti-Doping Code.

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