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Short communication

Investigation on the application of DNA forensic human identification techniques to detect homologous blood transfusions in doping control

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ABSTRACT

Homologous blood transfusion is an illicit practice used by athletes to improve the delivery of oxygen to tissues and, as such, it is banned in sports. The current method of detection is based on the flow cytofluorimetric phenotypic identification of red blood cells mismatch of minor blood group antigens between the donor and the recipient. The selectivity of this method to clearly identify transfused samples is related to the number of blood group antigens tested. Despite the fact that several different antigens are investigated, two individuals sharing the expression of the same minor blood group antigens pattern cannot be distinguished. We tested the possibility to use a different approach based on DNA forensic human identification techniques. Analysis of the DNA short tandem repeats (STRs) demonstrated its suitability in detecting mixed whole blood samples simulating homologous blood transfusion in 100% of the samples tested, ensuring the capability of clearly detecting mixed blood cell populations also on samples where the fraction of the minoritary population is as low as 2.5%.

1. Introduction

Homologous blood transfusion is a practice, banned by the World Antidoping Agency (WADA) [1], that some athletes may illicitly use to improve the delivery of oxygen to the tissues. At present, the method of detection in doping control is based on the identification, by cytofluorimetry, of mixed red blood cells (RBCs) populations based on differences of expressions of minor blood group antigens on erythrocytes surface [2–4]. The current screening test is performed by testing eight antigens belonging to four different human blood groups (big C, small c and big E of the Rh group, Jka and Jkb of the Kidd group, Fya and Fyb of the duffy group and big S of the MNS group). An individual can either express or not express the blood group antigens on the surface of his/her RBCs. In all cases in which the donor and the recipient have a different antigenic expression pattern for minor blood groups antigens, a double population of cells (expressing and non-expressing) can be detected at one or more antigens.

While the sensitivity of the method clearly depends on the donor/recipient ratio at the time of the blood sample collection, the selectivity to clearly identify one or more double populations depends on the number of blood group antigens analysed: the broader the range of antigens tested, the higher the probability is to detect “donor cells” in the recipient's blood [5]. Nonetheless, it is self-evident that such an approach generates a “false-negative” result in case the donor and the recipient share the same haplotype of the target antigens. Indeed, based on the anonymous blood samples analysed by our laboratory to detect doping by blood transfusions, we built up a database and found that many blood samples, received from different athletes, shared precisely the same eight antigens pattern. Even expanding the number of antigens analysed, a practice that has already been implemented also by other several WADA-accredited antidoping laboratories, we found that, as expected, the frequency of individuals sharing the same haplotypes decreased but, after all, a certain number of individuals with the same exact haplotypes still persisted.

The above observation promoted us to explore the validity of alternative/complementary approaches for the detection of homologous blood transfusions, focusing in detail on the methods using DNA-based forensic human identification techniques, and specifically on the analysis of mixed DNA profiles [6].

Forensic human identification is based on the analysis of portions of DNA called microsatellites (short tandem repeats, STRs) [7]. Each STR locus is constituted by a wide number of alleles on the basis of the number of repeated sequences. Each STR

Abbreviations: STRs, short tandem repeats; WADA, World Antidoping Agency; RBCs, red blood cells; PCR, polymerase chain reaction; RFU, relative fluorescence unit.

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locus of an individual is composed of two alleles that can be different (heterozygous) or same (homozygous) on the basis of the number of repeated elements. As 16 loci are normally tested in human identification, and also considering the wide number of alleles present at each locus, the probability that two different individuals share the same haplotype pattern is virtually zero.

Practically, DNA may be extracted from whole blood and amplified by polymerase chain reaction (PCR) into a master mix containing the primer set of all the 16 loci of the identification panel (D8, D21, D7, CSF, D3, TH01, D13, D16, D2S, D19, vWA, TPOX, D18, D5, FGA and Amelogenin used for sex determination). Each primer is specific for a certain locus. PCR amplification is extremely specific and generates a very high number of fragments containing the alleles of interest. Amplified samples can then be analysed by a capillary electrophoresis system to separate the fragments locus by locus [8]. Alleles detection is made by a five-colour detector system. A size standard constituted by fragments of known molecular weight is added to the mixture to align the runs. An allelic ladder sample is also used to get the correct typing in comparison with the unknown sample.

2. Materials and methods

For the purpose of this work we created two databases. The first database is made of 58 samples of normal and non-transfused patients. The second database has been created simulating homologous transfused samples by mixing randomly and in different proportions the donor samples of the first database two by two (also taking into account the matching for the ABO-Rh system). 24 samples of this second database were mixed at 5% of the donor. Blood samples were first analysed by the currently approved citofluorimetric procedure to detect blood transfusions, using a FC500 Beckman Coulter cytofluorimeter. The classical eight minor blood group antigen panel was also expanded analysing five more antigens (small e, small s, big Kell, Lewis a and Lewis b) thus improving the selectivity of the method. Samples sharing identical blood group haplotypes were mixed at 40%, 25%, 10% and 5% of the donor and re-analysed for homologous blood transfusion detection.

Also for forensic human identification analysis, DNA was extracted and purified from whole blood of normal and mixed samples at 40%, 25%, 10% and 5% using a Prepfiler Forensic DNA Extraction Kit (Applied Biosystems). DNA was subsequently amplified by polymerase chain reaction (PCR) using AMPFlSTR Identifiler PCR amplification Kit (Applied Biosystems). PCR was performed with 1 ng estimated DNA. A total of 28 cycles were used for amplification. Separation of the amplified STR fragments was made with a capillary electrophoresis system ABI Prism 310 (Applied Biosystems). Runs were made at 15 kV, 60 °C, and at injection time of 5 sec. Alleles identification for each locus was made by comparison to an allelic ladder using Gene Mapper ID v.3.2 software. Transfused samples were detected by the presence of three or four different alleles for a single genetic locus (Fig. 1).

3. Results

As for the basic database (normal and non-transfused samples), the flow cytofluorimetric analysis of minor red blood cell group antigens had shown that out of a total of 58 samples, 24 individuals shared an identical blood groups haplotypes with at least one individual of the database (41.4% of the total). Identical haplotypes belonged to eight different groups of haplotypes (data not shown). As expected, for each of the samples sharing the same haplotype and mixed at 5% of the donor, no double population of

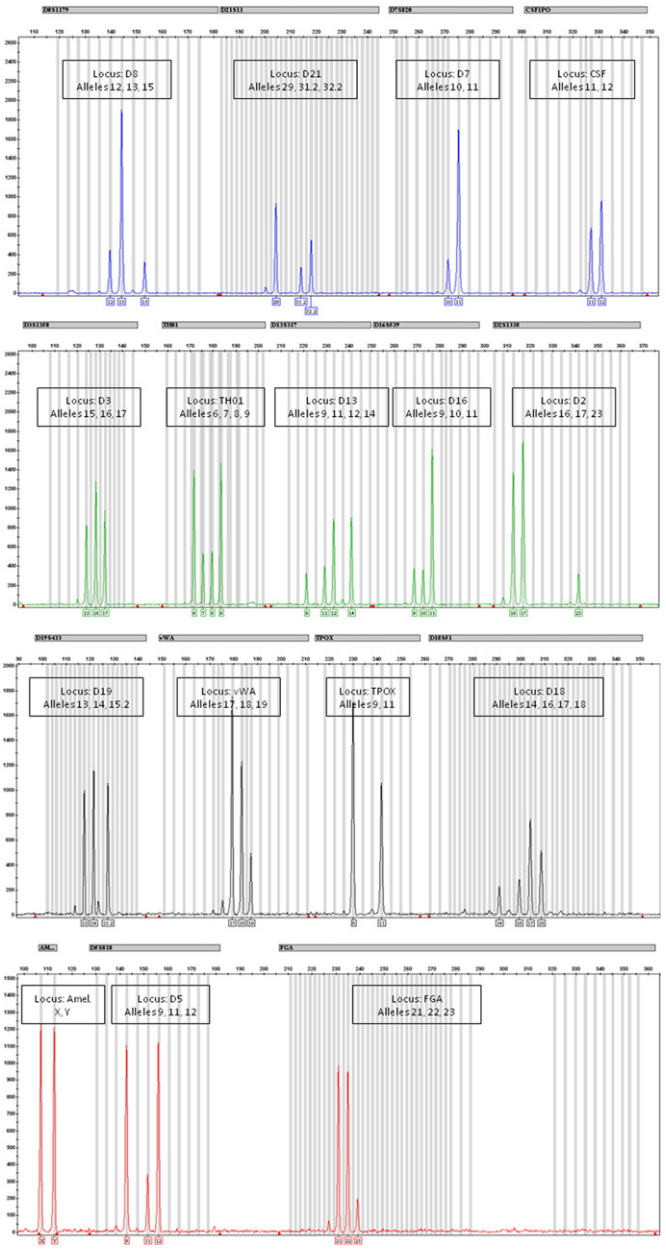

Fig. 1. A mixed sample (5% of the donor) analysed using a DNA-based forensic identification technique as described in the present paper. Triple and quadruple alleles emerge at various loci as a result of a mixture between two different blood sources.

erythrocytes were detected with the flow cytofluorimetric method for all 13 antigens analysed thereby mimicking “false negatives” results. On the contrary, the analysis of the same 24 samples, based on forensic identification, has shown that it is possible to detect a mixed sample in all cases considered. We identified triple and quadruple alleles at various loci to assess that a sample was the result of a mixture (Table 1).

When the donor is at 5%, an average of 11 different loci showing triple and quadruple alleles (and minimum number of 7) are always detected. Alleles belonging to the donors were considered only when their threshold levels were higher than 100 relative fluorescence units (RFU). In most cases the signals were very abundant to allow a typing of minor alleles with a threshold level higher than 150 RFU. Limit of detection (LOD) of the donor contribution was also calculated by analysing mixed samples and progressively reducing the percentage of the

Table 1
Frequency of hetero- and homozygotes in 58 normal (non-transfused samples) and the frequency of multiple alleles detected at different loci in 24 transfused samples at 5% of the donor.

	Normal (non-transfused) samples database $n=58$			Transfused samples database $n=24$		
Locus	**Detectable alleles**	**Frequency of heterozygotes**	**Frequency of homozygotes**	**Fr. 4 alleles detected**	**Fr. 3 alleles detected**	**Fr. 3+4 alleles detected**
FGA	28	0.976	0.024	0.33	0.63	0.96
CSF1PO	10	0.833	0.167	0.13	0.75	0.88
D2S1338	14	0.810	0.190	0.25	0.63	0.88
D18S51	23	0.857	0.143	0.42	0.46	0.88
TH01	10	0.857	0.143	0.25	0.58	0.83
D19S433	15	0.833	0.167	0.38	0.46	0.83
D16S539	9	0.833	0.167	0.33	0.46	0.79
vWA	14	0.881	0.119	0.29	0.50	0.79
D21S11	24	0.857	0.143	0.29	0.42	0.71
D3S1358	8	0.738	0.262	0.25	0.46	0.71
D5S818	10	0.762	0.238	0.17	0.54	0.71
D7S820	10	0.786	0.214	0.25	0.38	0.63
D13S317	8	0.786	0.214	0.17	0.46	0.63
TPOX	8	0.619	0.381	0.13	0.33	0.46
D8S1179	12	0.786	0.214	0.17	0.29	0.46

contribution of the donor. We found that this procedure has good sensitivity as donor alleles are clearly detectable even when their contribution is as low as 2.5%.

4. Discussion

Based on the above approach, the study of mixed samples in forensic human identification appeared to be very helpful potentially in detecting samples suspected for blood transfusion. An individual's DNA profile comprises a collection of genotypes, one for each marker. Each genotype consists of an unordered pair of alleles, one inherited from the father and one from the mother. When both alleles are identical the individual is homozygous for that marker, and only a single allele value is observed, while, for a heterozygote for that marker a couple of alleles are observed. In a diploid genome like the human one, only these two conditions are possible. The presence of three or four alleles at a certain locus can be due to contamination or can be the consequence of a transfusion practice. Also a transfusion practice can generate mixed DNA profiles showing less than three alleles and this can occur when the donor is homozygous for one allele and the recipient is also homozygous for the same or when the donor and the recipient are homozygous for different alleles on the same locus. That is the reason why only the presence of at least three alleles at a certain locus is informative to generate a suspect for transfusion. We show here that this test has the highest sensitivity as possible since 100% of the simulated transfused samples with the donor at 5% were clearly without the occurrence of false negatives. Also, no interference due to the background noise was detected. Moreover, while calculating the percentage frequency of the appearance of triple and quadruple alleles in all loci analysed (excluding Amelogenin locus that only attends for sex determination), we also found that loci such as FGA, CSF1PO, D2S1338, D18S51, TH01, and D19S433 are endowed with a higher diagnostic value for our purpose as they present a high frequency of triple or a quadruple allele (more than 80%) in all the cases considered. On the contrary, loci such as TPOX and D8S1179 seem to be less as they presented low frequencies of triple and quadruple alleles (see Table 1). The fact that a locus can have more or less diagnostic value for the purpose of this study is a consequence of the variability of the locus expressed as the number of different alleles and also the percentage of heterozigosity at that locus. In general terms, the higher the number of different alleles in the locus, the higher the probability to detect three or four alleles at that locus when mixing two samples. Moreover the higher the heterozygosity the more the probability to detect multiple alleles. As a result of this study, FGA locus was found to be the most informative one because of its high number of different alleles (28) and also for the high heterozigosity shown. Loci such as CSF1PO and TH01, despite presenting a low number of alleles (10), were highly informative anyway because of their high heterozygosity, meanwhile loci such as D21S11 or vWA did not show much high power discrimination despite the high number of different detectable alleles.

To find loci with high informative power allowed to develop a reduced panel to be applied in the screening of the transfused sample without losing the specificity and sensitivity of the test.

A limitation of this test is the impossibility to detect mixed blood samples from two identical twins, since the shared genetic information is identical. Another possible limitation is represented by transfusion practices using erythrocytes concentrates that have been leukoreduced first. Leukoreduction is the removal of the majority (but not all) of the white blood cells to create concentrated packed red blood cells. Since the DNA in a blood sample is extracted from white blood cells, this can represent a challenge for the application of this test. However, forensic DNA techniques are becoming more and more sensitive with time and further improvements might allow overcoming this problem soon. As a matter of fact we are now testing a more sensitive approach in this direction (to quote a couple, the use of very high sensitive PCR amplification kits and the use of quantitative real time PCR).

In summary, although DNA forensic human identification is normally applied to the study of mixed samples as a consequence of contamination. This study demonstrates that the methods based on such techniques can represent a novel, powerful tool that can also be applied in doping control for the detection of homologous whole blood transfusions with a very high power of discrimination among the transfused samples. This is achieved thanks to the high number of loci analysed and most of all thanks to their high polymorphism. This work also reveals the potential utility of molecular biology-based techniques in doping control analysis.

References

[1] The World Anti-Doping Code International Standard, Prohibited List 2012, World Anti-Doping Agency, Montreal, Canada, 2011, Available from: ⟨http://www.wadaama.org/en/Science-Medicine/Prohibited-List⟩.
[2] M. Nelson, H. Popp, K. Sharpe, M. Ashenden, Haematologica 88 (2003) 1284–1295.

[3] M. Nelson, M. Ashenden, M. Langshaw, H. Popp, Haematologica 87 (2002) 881–882.
[4] S. Giraud, N. Robinson, P. Mangin, M. Saugy, Forensic Sci. Int. 179 (2008) 23–33.
[5] P.A. Arndt, B.M. Kumpel, Am. J. Hematol. 83 (2008) 657–667.
[6] T.M. Clayton, J.P. Whitaker, R. Sparkes, P. Gill, Forensic Sci. Int. 91 (2008) 55–70.
[7] J.M. Butler, (Ed.), Short tandem repeat analysis for human identity testing, Current Protocols in Human Genetics, 2004, (Chapter 14: Unit 14.8).
[8] J.M. Butler, E. Buel, F. Crivellente, B.R. McCord, Electrophoresis 25 (2004) 1397–1412.