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Homologous Blood Transfusion Detection.

A new world in anti-doping field: Flow cytometry.

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INTRODUCTION

Blood transfusion significantly improves physical performances, due to increased red blood cell (RBC) mass and therefore augmented oxygen transport capacity [1]. A similar physiological effect can be obtained via treatment with recombinant erythropoietin (rh-EPO). With the introduction in 2001 of a method to detect directly rh-EPO in urine [2], transfusion doping practices have regained interest. Here, we describe a sensitive method for the unequivocal detection of homologous blood transfusion.

Haematological parameters of altered erythropoiesis can reveal a blood manipulation [3], but cannot confirm whether this latter is due to blood transfusion or rh-EPO abuse. In case of homologous transfusions, blood compatible for ABO- and Rh(D)- groups is transfused, but in most cases, differences exist for minor RBC antigens. Analysis of RBC antigenic expression patterns by flow cytometry is able to reveal the presence of mixed RBC populations in transfused recipients [4].

In the development of a blood transfusion doping test, several steps have been undertaken to reach quality standards which are scientifically accepted. Antibodies concentrations are optimised with standardised RBC controls and then specificity and discrimination capacities of each antibody at the optimum dilution are verified.

Validation study was made and proved that our method is absolutely specific and has a sensibility higher than 50%. The detection window is close to three months after homologous transfusion.

FLOW CYTOMETRY AND BLOOD DOPING

Device used to count small particles in flow are in use since more than 50 years [5]. Blood cells analyses are also old techniques and the most accurate ones are those using single cell

analysis [6]. Modern commercial apparatus able to analyse and separate cells appeared in the end-60's, but were slow and absolutely not user-friendly [7, 8].

The basic principles behind flow cytometer are the following: 1) A system able to aspirate, dilute and convey the liquid sample in an analysis chamber. The aim is to obtain such a dilution that all particles in the sample are physically separated, so they can be analysed individually. 2) A system able to perform the analyses using optic and/or electrical properties of the sample's particles. This system is composed of an excitation source, actually a laser beam, and devices used to detect the transmitted or emitted lights, actually CCD or photomultiplicators. 3) A system able to convert all signals in order to be usable by a data presentation software and interpretable by the operator of the instrument [9]. During the 70's flow cytometer evolved and acquired the capacities to analyse more than one parameters, leading to high-resolution apparatus [10]. Despite the apparent simplicity of these devices [11], medical use of them increased only during the 90's due to the complexity to obtain relevant and accurate controls [12, 13].

In 1972, Ekblom et al. demonstrated that blood transfusion significantly improves physical performances [1], due to increased RBC mass and therefore augmented oxygen transport capacity. The IOC declared the transfusion method as a forbidden practice in 1988. A similar physiological effect can be obtained via injections with recombinant human erythropoietin (rh-EPO). But the introduction in 2001 of a method for direct detection of rh-EPO in urine [2], reduce the abuse and this way to enhance blood oxygen transport. Unfortunately, despite the numerous risks linked to blood transfusions, these practices have regained interest.

The principle behind the homologous blood transfusions detection is to analyse minor blood groups. In most cases differences exist for minor RBC antigens, even if transfused blood is identical for A, B and Rh(D) groups. Using flow cytometry and standard reagents of immuno-haematology used in blood bank for groups' determination, we are able to analyse the RBC phenotype.

To reach quality standards mandated by the ISO 17025 norms, several steps have been undertaken to develop a direct doping test against homologous blood transfusion. Antibody concentration is optimised with phenotypes-known RBC controls furnished and certified by immuno-haematology industries. This step enables us to find conditions leading to the best separation between non-expressing and expressing RBC responses. To conclude optimisation made for each antibody bottle, we check both the specificity and discrimination. After flow cytometry analyse, the phenotype is obtained for blood samples. In order to interpret the results obtained, external controls must give clear proofs of antibodies specificity and

identification capabilities. When two or more blood groups show a mixed RBC population the sample is declared as positive. When only one blood group shows a mixed RBC population, the sample is declared as suspicious. Indeed, event if this test is not an immunoassay, it is still based on immunological recognitions, implying the demonstration of a mixed RBC population by more than one antibody.

Actually, only one colour (i.e. one wavelength of detection) is used to make these analyses, but more could be used in a near future [14].

METHODOLOGY OF HOMOLOGOUS TRANSFUSION DETECTION

The aim of this paper is to present a methodology to implement the method to detect homologous blood transfusion.

To choose a flow cytometer, important points are the capacity to change settings between tubes due to proposed internal controls and the correspondence between the excitation wavelengths of the laser beam and the fluorescent probe used to stain RBC.

Antibodies used to recognise minor blood groups (primary antibodies) are commercially available in any serological laboratory furnisher, the sources of the antibodies must be checked carefully as they will completely implicate the choice of the secondary antibodies, which bear the fluorescent probe and recognise specifically the primary antibody. These secondary antibodies are easy to access when contacting furnishers of antibodies or flow cytometry products. Isotonic phosphate buffer is recognised as being the best buffer available for staining, washing and suspension steps.

Finally, external RBC controls are commercially available. Great care must be taken when choosing them, as the pattern of blood groups must be known in order to prepare mixes of RBC and predict the results. In the first steps of implementation, the prediction of the results are very important as they are the only way to check that an antibody gives a clear separation of the RBC population. A very important point is the knowledge of the expression type of each RBC control. Expressions can be homozygous or heterozygous. Homozygous expression of a blood group means that the corresponding antithetic group is not expressed. In heterozygous expression, both antithetic groups are expressed, but with a low number of sites on the RBC surfaces, leading to weak signals. For the antigens we use, the pairs are C and c, E and e, Fy^a and Fy^b, Jk^a and Jk^b, S and s.

During the optimisation of antibodies concentrations and staining conditions we recommend to also use mixes of RBC controls having heterozygous expression in order to familiarise with the limits of separation that can be obtained depending on the antibodies.

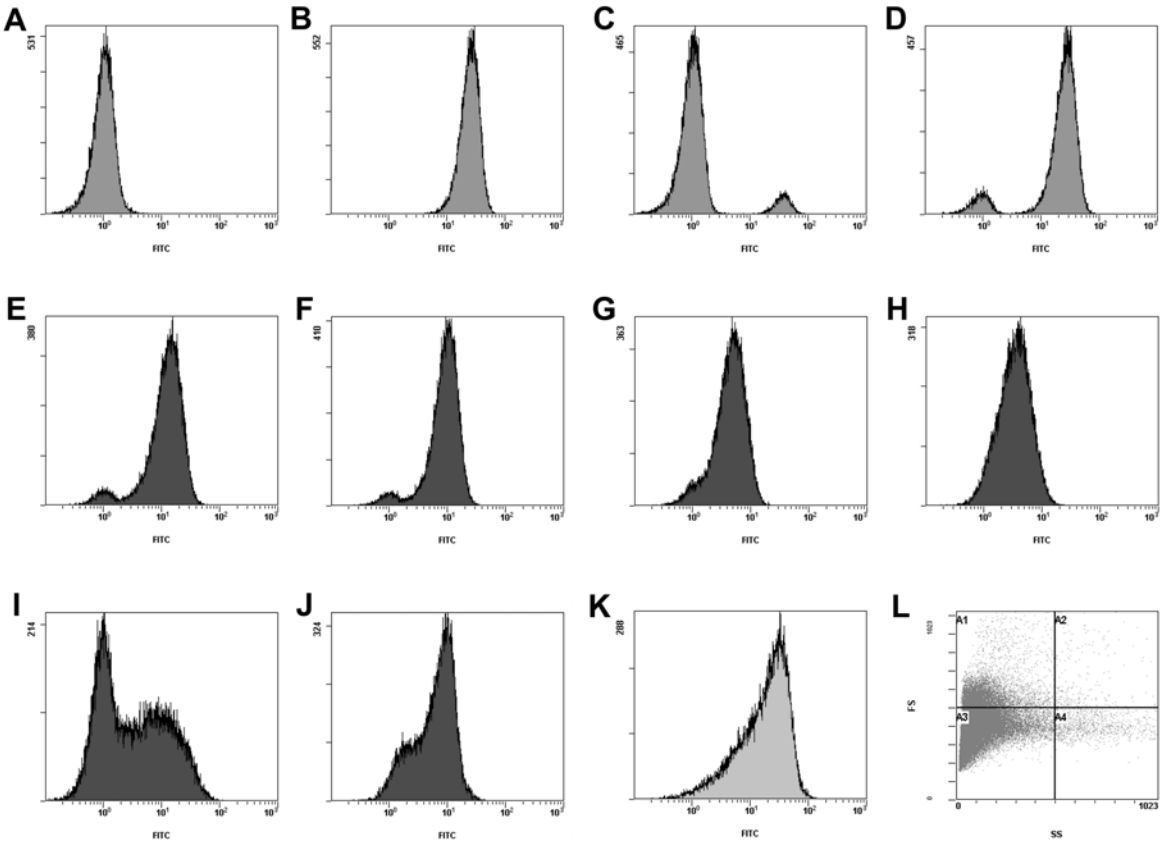

Figure 1: Results obtained with our system (Flow cytometer: FC500 from Beckman-Coulter, primary antibodies from DiaMed). A) Histogram of a single RBC population without expression. B) Single RBC with a strong expression. C) Double RBC population with a minor population having a strong expression. D) Double RBC population with a minor population lacking expression. E) Same situation as D, but the main population has a normal expression. F) Same situation as D, but the main population as a weaker expression than in E. G) Same situation as D, but with a heterozygous or weak expression. H) Same as D, but the expression is too weak to distinctly see the peaks. I) Single expressing RBC population, but there was not enough primary antibody. J) Same as I, but with too much primary antibody. K) Single RBC population, but the RBC expression is very wide due to heterozygozity and/or to a bad conservation of the blood. L) Forward – Sideward scatergram, this graphic show the size versus internal composition of all analysed events. This result is obtained from a freshly withdrawn blood stored 48 hours at 4°C and diluted in an isotonic buffer without staining and shows a figure used to control the state of conservation of the blood.

Figure 1 show different histogram obtained with our system (FC-500 from Beckman-Coulter working under CXP 2.0 as flow cytometer and all primary antibodies from DiaMed company). On this figure, the importance of optimal dilution is perfectly clear. Situations with two peaks are clearly distinguishable in at least two histograms and will lead to a positive final result.

Internal controls are necessary to make any interpretation. The two first controls are RBC stained with anti-glycophorin-A and isotypic control. Their aim is to prove that the gating only contains RBC lineage. The first tube contains RBC stained with the isotypic control of anti-glycophorin-A and is used to set the gating around the RBC population. As samples are often not transported or withdrawn in the same time and conditions, the FS-SS scatergram (Size versus internal composition, figure 1 L) can change between individuals and the gate or the voltage settings must be up-tuned to contain only RBC, with the less aggregates as possible. This gating strategy must be developed in each laboratory depending on the apparatus and user habits and experience. The second internal control is RBC stained with anti-glycophorin-A antibody. The gate is kept at the same position than the first internal control and the interesting result is in the histogram. Only one clear peak is on the right, and the left part of this peak is not superimposed with the right part of the previous histogram. This shows that only RBC are present and that an expression induce a rightward shift of the peak.

The third and last internal control is RBC stained only with the secondary antibodies used to recognise each primary antibody and bearing the fluorescent tag. Only one peak on the left side of the histogram must be seen. Optimally, the whole peak is visible, and voltage settings of the fluorescent signals can be up-tuned to see completely the left part of the peak. The gating graph (figure 1 L) is also used to have an idea of the conservation state of the blood, but this implies good experience.

To avoid misinterpretations due to microbial contamination [15, 16], bloods are first diluted in a buffer containing antibiotics and staining procedure is made in a buffer containing sodium azide. Each histograms obtained with the staining using anti-blood groups antibodies are classified as uncertain situation, single or double population. Single population histogram shows only one peak on the left or right side of the histogram (figure 1 A or B). Double population histogram shows two clearly separated peaks (figure 1 C or D). Uncertain situations show histograms with two overlapping peaks (figure 1 E or F) or with a shoulder (figure 1 G). Histograms with a very large peak (figure 1 K) must be treated carefully as they

can be due to a single heterozygous expression or to an overlap of two wide peaks depending on the blood groups tested and on the analytical conditions.

Three results for each blood sample are admitted: Negative, Suspicious or Positive. Negative samples have no histograms showing double population. Positive samples have two or more histograms showing double populations. Other situations are considered as suspicious samples.

For confirmation purposes, we repeat the analysis for one sample only on double population or suspicious antigens. In order to improve the interpretation and to prove that a double peak or a suspicious peak is not due to non-optimal antibody concentration as shown in figure 1, parts I or J, we make also analyses with half and double concentration. The aim is to obtain a potential better separation than the one observed. Normally and based on our experience, signals obtained with a bad antibody concentration can only give suspicious histograms. But as one suspicious histogram is enough to render the entire sample as suspicious, all laboratories must be confident with their results. One other reason to use multiple antibody concentration is to avoid the possible situation that a bad antibody concentration gives rise to a double peak histogram, even if this possibility has a minimal probability to arise.

To make a validation study, anonymous bloods samples from the local blood bank volunteers were used. They were stabilised and RBC were counted. Phenotypes of each blood samples were determined using classical tools – ID cards from DiaMed SA, based on agglutination reaction - and not flow cytometry. All mixes were randomly chosen based on A-, B- and D-expression identity. Four users interpreted separately 140 blood samples containing different percentages of minor RBC population. We obtained 19 samples with 5% of minor RBC population, 25 samples with 3%, 30 samples with 1.5%, 32 samples with 0.5% and 34 samples with 0% (pure bloods, negative sample). The database data were extracted to classify each mix as positive, suspicious or negative, leading to a prediction for the observed results.

On the 34 negative samples, none of the users gave back a positive or suspicious result. 100% specificity was obtained with this methodology and our method.

Table 1 gives mean values, standard deviation and extreme ranges obtained after interpretation of all 106 positive samples.

Positive sample returned as positive (Real positive)

Percent of minor population	**0.5%**	**1.5%**	**3%**	**5%**	**all samples**
Predicted value	*81.3%*	*80.0%*	*80.0%*	*78.9%*	*80.2%*
Observed mean value	28.9%	62.5%	68.0%	61.8%	53.5%
Standard deviation	21.3%	8.3%	0.0%	2.6%	9.1%
Minimum	0.0%	53.3%	68.0%	57.9%	41.5%
Maximum	50.0%	73.3%	68.0%	63.2%	63.2%

Positive sample returned as suspicious (real suspect)

Percent of minor population	**0.5%**	**1.5%**	**3%**	**5%**	**all samples**
Predicted value	*18.8%*	*16.7%*	*12.0%*	*10.5%*	*15.1%*
Observed mean value	20.3%	15.0%	19.0%	27.6%	19.8%
Standard deviation	10.7%	5.8%	3.8%	2.6%	4.0%
Minimum	9.4%	10.0%	16.0%	26.3%	15.1%
Maximum	34.4%	23.3%	24.0%	31.6%	23.6%

Positive sample returned as negative (false negative)

Percent of minor population	**0.5%**	**1.5%**	**3%**	**5%**	**all samples**
Predicted value	*0.0%*	*3.3%*	*8.0%*	*10.5%*	*4.7%*
Observed mean value	50.8%	22.5%	13.0%	10.5%	26.7%
Standard deviation	29.5%	9.2%	3.8%	0.0%	12.1%
Minimum	15.6%	13.3%	8.0%	10.5%	13.2%
Maximum	84.4%	33.3%	16.0%	10.5%	40.6%

Table 1: results of validation study separating each type of positive sample. Four types of positive sample are analysed and interpreted. 0.5%, for a mix of two bloods containing a minor population representing 0.5% of RBC number. 1,5% for a mix with 1.5% of minor RBC population. 3% and 5% for mixes containing respectively 3% and 5% of the minor RBC population.

A global mean sensibility of 53% was obtained. Suspicious sample were found at a level of 20% and the false negatives, represent 27%. More interest is found with the results for each type of mixes. We observe that the sensibility is higher than 60% for each type of mixes, except for the ones with 0.5% of minor RBC population.

With the results given in table 1, we can simulate the detection window of our anti-doping method based on scenarios. Knowing that the RBC life is 120 days and that the disappearance rate of a RBC population is perfectly linear [17], we can calculate the percentage of a minor population depending on the initial amount transfused. Figure 2 present plots of the sensibility versus the days post transfusion for two transfusions, showing that we have more than 60% of chances to detect a homologous blood transfusion during a period of nearly 3 months after the doping practise.

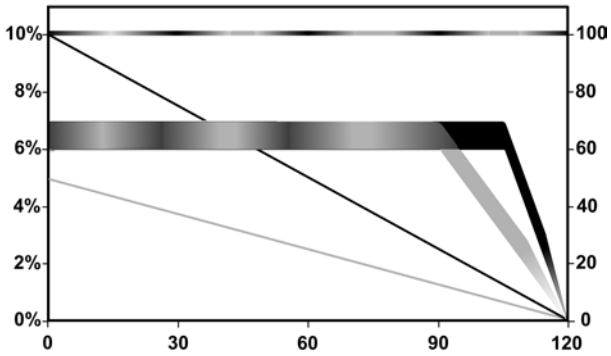

Figure 2: Simulation of the sensibility and specificity of the presented methodology on two different theoretical homologous transfusions using our system as a function of elapsed time after the blood doping. In black is the scenario of a 10% injection of foreign RBC, in grey is the scenario for a 5% transfusion. Continuous lines show the percentage of minor RBC population. Upper line shows the specificity of our method and grey and black ribbons show the approximate sensibility.

Minor population representing less than 1.5% can be detected, but as the sensibility decrease rapidly, difficulties arise due to this loss of specificity and are not related to the appearance of a detection limit. The real limit of detection is depending on the situation as a minor peak can be partially hidden in the left tail of the main peak and on the laboratory experience to be able to distinguish between a flat tail on the left and a second minor peak overlapping. But in any case, as soon as the peaks are not clearly separated, any histogram will be classified at most as suspicious.

CONCLUSIONS

The presented methodology to detect homologous blood transfusion doping using flow cytometry enables any WADA-accredited laboratory to implement a method, which must be then, as usual, fully accredited by official organisation. But as positive or negative controls are not accessible *stricto senso*, which is not usual in anti-doping settings, we use external controls to check the specificity and identification capacities and internal controls to check the gating strategy and interpretability of the results.

Our method, not presented in details in this paper, is 100% specific. No false positive or false suspect rose with our settings and our systems. This optimal specificity is accessible because of strict interpretation criterion and these criterion lead to a sensibility higher than 50% for all samples used in validation study. These samples included some difficult sample in which only

0.5% of the whole RBC were from another source and the sensibility for these sample was around 30%. On all other sample, the sensibility is higher than 60%. These results lead to a detection window around three months after homologous blood transfusion, depending on the amount of blood injected.

This methodology will not eradicate this form of blood doping, but it will become so hazardous for cheaters that its utilisation will decrease greatly in a very short delay.

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