Clinical Chemistry 65:12
1481–1483 (2019)

Editorials

Dried Blood Spots May Improve Detection of Blood Doping

Holly D. Cox[1*]

In 2019, investigations have found at least 40 blood bags, 5 cross-country skiers, 5 cyclists, and 1 physician involved in a highly sophisticated blood doping scandal *(1)*. The findings highlight the need for improved detection of blood doping practices. Athletes use blood doping methods to increase hemoglobin mass and, thus, oxygen delivery to muscle cells to obtain an unfair advantage. Hemoglobin mass can be increased by a variety of methods, including blood transfusions (autologous or allogeneic), recombinant human erythropoietin preparations (EPO)[2], or by a growing number of erythropoiesis-stimulating agents *(2)*. Often the methods are combined or used in very small doses, called microdoses, to evade detection. Because of the wide variety of blood doping methods, an indirect method capable of detecting all forms of blood doping was developed. The method measures 2 blood parameters—hemoglobin concentration [Hb] and reticulocyte percentage (Ret%)—and the OFF score, which is defined as [Hb] $-60 \times \sqrt{}$Ret% *(3)*. Any method that stimulates erythropoiesis will cause a significant increase in immature red blood cells, called reticulocytes, as well as an increase in [Hb]. Blood withdrawal, before autologous transfusion, also stimulates erythropoiesis and will increase Ret%. Additionally, negative feedback mechanisms cause a prolonged suppression of Ret% after blood transfusion or upon cessation of EPO injections, which can last for up to 3 weeks *(4–6)*. In 2009, the World Anti-Doping Agency (WADA) implemented longitudinal measurement of the 2 blood parameters as part of the hematological module of the athlete biological passport (ABP) program. In the ABP program, an athlete's blood parameters are measured by any antidoping laboratory and entered into a database that contains the previous blood data for that athlete. The ABP program uses individual baseline variation with a Bayesian statistical model to predict normal limits for each parameter with a 99.9% probability threshold *(7)*. After implementation, the method was successful at detecting gross blood manipulations that were used at the time *(8, 9)*. Over the past decade, however, blood doping methods have become increasingly complex to avoid large perturbations in athlete blood profiles.

Previous work demonstrated that transfusion of 1 to 2 U of blood could be detected by a blinded investigator using the ABP method when 10 blood collections were obtained over a 42-week period *(10)*. Unfortunately, athletes often transfuse smaller volumes of blood, and the number of blood samples collected from athletes is substantially less owing to several limitations. Additionally, blood doping routines are now designed to schedule large EPO doses and blood withdrawal during out-of-competition months when testing is less frequent *(11)*. Thus, one major deficiency in the current ABP testing program is the small number of samples collected, especially during the out-of-competition months. The low frequency of blood sample collection is because of the unique preanalytical challenges of blood collection in the field of antidoping. Blood is not collected in hospitals or clinics; it is collected in the field before or after sporting events and often in remote locations that are a long distance from the nearest antidoping laboratory. Because of legal restraints regarding strict chain of custody regulations, the blood cannot be manipulated outside of the laboratory. To maintain the accuracy of reticulocyte counts and other blood parameters, the labile samples must be shipped to the antidoping laboratory in an expedited and temperature-controlled manner *(12)*. Thus, the cost of shipment is a major barrier. Depending on the location, it is often not possible for blood to arrive within the required time and temperature limits. Additionally, a phlebotomist must be scheduled to collect the venous blood sample. This greatly hinders unannounced and out-of-competition testing, which are more likely to detect a blood manipulation event. Finally, increasingly frequent venous blood collections may be considered invasive for healthy individuals.

Owing to these limitations, dried blood spot (DBS) sample collections could significantly reduce costs, increase sample collection frequency, and improve blood stability. Collection of capillary blood from finger-prick, arm, or ear does not require a phlebotomist, but can be performed by a doping control officer. Blood samples spotted onto DBS cards are easily collected in remote

[1] Sports Medicine Research and Testing Laboratory, Salt Lake City, UT.
* Address correspondence to the author at: Sports Medicine Research and Testing Laboratory, 560 Arapeen Dr., Suite 150A, Salt Lake City, UT 84108. Fax 801-994-9455; e-mail hcox@smrtl.org.
Received September 27, 2019; accepted September 30, 2019.
Previously published online at DOI: 10.1373/clinchem.2019.311902

[2] Nonstandard abbreviations: EPO, recombinant human erythropoietin; [Hb], hemoglobin concentration; Ret%, reticulocyte percentage; WADA, World Anti-Doping Agency; ABP, athlete biological passport; DBS, dried blood spot; ALAS2L, 5′-aminolevulinate synthase 2 linear form; ALAS2L+C, 5′-aminolevulinate synthase 2 linear plus circular form.

locations and shipped at ambient temperature to the laboratory. Increased collection of blood samples also benefits the athlete because it provides a better estimation of natural baseline variability under multiple locations and conditions, such as altitude training. Just as increasing pixel density improves the resolution of an image, visualization of normal and cheating athlete blood profiles will improve with increasing sample number.

Thus, there is an important need for methods to detect blood doping in the DBS matrix. The first method, published in 2017, measured cell-specific protein markers by mass spectrometry to count cells, including immature reticulocytes, to monitor changes in erythropoiesis *(13)*. This method was later demonstrated to improve the detection of blood transfusion over current methods of reticulocyte measurement *(6)*. In this issue of *Clinical Chemistry*, Salamin and colleagues describe a new RNA-based method to monitor erythropoiesis in DBS *(14)*. The method measures the linear and circular forms of the RNA transcript for *ALAS2*[3], which is a reticulocyte-specific enzyme involved in the heme biosynthesis pathway. The method is a revision of an earlier method by the same group to measure linear transcripts of *ALAS2*, *CA1*, and *SLC4A1* (or Band 3) in RNA stabilized blood tubes *(15)*. The previous method demonstrated statistically significant changes after blood transfusion, from 9 to 15 days and 6 to 15 days posttransfusion for *ALAS2* and *SLC4A1*, respectively. The new method was adapted for extraction of 20-μL DBS spots and includes the measurement of *ALAS2* linear (ALAS2L) and *ALAS2* linear plus circular forms (ALAS2L+C). Circular RNAs have the advantage of being more resistant to degradation, are abundantly expressed, and are often cell type and tissue specific *(16)*.

The authors demonstrate acceptable repeatability (<20%) over 5 days when measured in 3 volunteers. In stability experiments, combining the linear and circular forms of *ALAS2* significantly improved the stability over the linear form alone when measured for 3 weeks at room temperature and 24 h at 37 °C. Additional method validation experiments will be needed before use of the assay, including linearity and response to increasing hematocrit %.

Methods for detection of blood doping require stable longitudinal measurements over time and between laboratories. The reported method does not use calibration standards or reference material, but rather relies on normalization of the *ALAS2* C_T value to the mean C_T value of 5 housekeeping genes. Using finger-prick blood, the authors demonstrate longitudinal stability of the method in 6 volunteers for 7 weeks. The intra-individual variability was between 13% and 42.4% for ALAS2L+C. Although this variability is larger than the current measurements for Ret%, higher variability is acceptable, provided the response to blood manipulation is significantly larger as well. Measurement of the ratio of *ALAS2* to endogenous housekeeping genes does allow for correction to changes in plasma volume, which is a frequent problem with current hemoglobin measurements. Athletes frequently experience extremes in hyperhydration and hypohydration, which can affect plasma volume.

Additionally, Salamin and colleagues demonstrate the response of *ALAS2* to 2 forms of erythropoietic stimuli: blood withdrawal and EPO administration. In both experiments, measurement of the combined linear and circular forms of *ALAS2* resulted in a greater response. After blood withdrawal, the increase in ALAS2L+C was statistically significant on days 6, 9, and 15. The response in DBS correlated well with that observed in the RNA blood stability tubes and was larger than the Ret% response. After EPO administration, ALAS2L+C increased 9-fold at day 7 postadministration, which was larger than the Ret% increase of 2-fold. In future work, the method may be improved by the measurement of 1 or 2 more gene transcripts and testing the *ALAS2* response to changes in altitude, which is always a challenge for blood doping detection methods.

Quantitative RNA methods are not yet performed in antidoping laboratories, although implementation of the methods poses no obvious obstacles. For protein-based measurements, WADA currently requires 2 orthogonal methods of measurement for screening and confirmation of adverse findings. With the detection of blood doping potentially moving from cellular measurements on an automated hematology analyzer to RNA amplification and protein mass spectrometry methods, it may be helpful to have at least 2 complementary methods of measurement. Thus, the publication of a second independent method for the detection of blood doping in DBS samples will further support the potential to test this application in the field.

[3] Human genes: *ALAS2*, 5′-aminolevulinate synthase 2; *CA1*, carbonic anhydrase 1; *SLC4A1*, solute carrier family 4 member 1 (Diego blood group).

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

Authors' Disclosures or Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

References

1. Cycling News. Austrian doping: a complete history of Operation Aderlass. https://www.cyclingnews.com/features/austrian-doping-a-complete-history-of-operation-aderlass/ (Accessed September 2019).
2. Jelkmann W, Lundby C. Blood doping and its detection. Blood 2011;118:2395–404.
3. Gore CJ, Parisotto R, Ashenden MJ, Stray-Gundersen J, Sharpe K, Hopkins W, et al. Second-generation blood tests to detect erythropoietin abuse by athletes. Haematologica 2003;88:333–44.
4. Damsgaard R, Munch T, Morkeberg J, Mortensen SP, Gonzalez-Alonso J. Effects of blood withdrawal and reinfusion on biomarkers of erythropoiesis in humans: implications for anti-doping strategies. Haematologica 2006;91:1006–8.
5. Morkeberg J, Belhage B, Ashenden M, Borno A, Sharpe K, Dziegiel MH, Damsgaard R. Screening for autologous blood transfusions. Int J Sports Med 2009;30:285–92.
6. Cox HD, Miller GD, Lai A, Cushman D, Eichner D. Detection of autologous blood transfusions using a novel dried blood spot method. Drug Test Anal 2017;9:1713–20.
7. Sottas PE, Robinson N, Saugy M. The athlete's biological passport and indirect markers of blood doping. Handb Exp Pharmacol 2010:305–26.
8. Zorzoli M, Rossi F. Implementation of the biological passport: the experience of the international cycling union. Drug Test Anal 2010;2:542–7.
9. Zorzoli M, Rossi F. Case studies on ESA doping as revealed by the Biological Passport. Drug Test Anal 2012;4:854–8.
10. Pottgiesser T, Sottas PE, Echteler T, Robinson N, Umhau M, Schumacher YO. Detection of autologous blood doping with adaptively evaluated biomarkers of doping: a longitudinal blinded study. Transfusion 2011;51:1707–15.
11. Lundby C, Robach P, Saltin B. The evolving science of detection of 'blood doping'. Br J Pharmacol 2012;165:1306–15.
12. Robinson N, Kirchbichler A, Banuls O, Mader M, Aikin R, Sottas PE, D'Onofrio G. Validation of a blood stability score as an easy-to-use blood sample quality index. Int J Lab Hematol 2016;38:685–93.
13. Cox HD, Eichner D. Mass spectrometry method to measure membrane proteins in dried blood spots for the detection of blood doping practices in sport. Anal Chem 2017;89:10029–36.
14. Salamin O, Gottardo E, Schobinger C, Reverter-Branchat G, Segura J, Saugy M, et al. Detection of stimulated erythropoiesis by the RNA-based 5′-aminolevulinate synthase 2 biomarker in dried blood spot samples. Clin Chem 2019;65:1563–71.
15. Salamin O, Mignot J, Kuuranne T, Saugy M, Leuenberger N. Transcriptomic biomarkers of altered erythropoiesis to detect autologous blood transfusion. Drug Test Anal 2018;10:604–8.
16. Nicolet BP, Engels S, Aglialoro F, van den Akker E, von Lindern M, Wolkers MC. Circular RNA expression in human hematopoietic cells is widespread and cell-type specific. Nucleic Acids Res 2018;46:8168–80.