

Standardization of the HbA₂ assay

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ABSTRACT

Background

A project for the standardization of HbA₂ was launched by the IFCC back in 2004.

Materials and methods

In this work we report on the state-of-the-art of the project on standardization of HbA₂. Data obtained from various EQAS studies, and from previous experimental evaluations, are presented.

Results

We have proven that biases between various commercial methods are still currently significant. We have also shown that calibration by commutable control materials may halve the inter-method variability.

Conclusions

The foundation of the reference system for HbA₂, together with a brief preliminary presentation of the proposed primary reference measurement procedure based on ID-MS are outlined.

WHY TO STANDARDIZE

The promotion of the standardization of routine laboratory methods is one of the mission statement of the International Federation of Clinical Chemistry (IFCC) general policy.

In the specific case of the methods used for the determination of hemoglobin A₂ (HbA₂), their standardization is a real need, not only an academic specific exercise. Indeed, there are several important motivations to meet this:

- most of the routine laboratory methods are poorly aligned;
- important decision limits have been proposed as markers for the diagnosis of thalassemia syndromes;
- the correct diagnosis of carriers in couples at risk is the basic instrument to reduce the burden of thalassemia syndromes;

- β -thalassemia is one of the few examples among genetic disorders in which identification of carriers is performed, mostly, by simple hematological and biochemical tests.

The evidence that the laboratory methods are not aligned may come from the information provided by the external quality assessment schemes (EQAS) or by specific investigations. We have previously reported that the overall inter-laboratory CVs evaluated from a pilot exercise in Italy with 48 Italian laboratories were in the order of 6 to 8% and that the fraction of laboratories reporting unacceptable results ranged from 17 to 32% [1]. We have also shown that data collected in Italy from a mandatory exercise performed in Tuscany and surrounding regions reported CV values between methods similar to those found in another EQAS exercise provided by a manufacturer [2].

Table 1 Performance characteristics of different methods for HbA₂ testing

Method	Between-run reproducibility	Bias versus consensus mean		r
		Slope	Intercept	
Bio-Rad D10	1.7	0.978 to 1.018	0.07 to 0.24	0.9981
Bio-Rad Variant II β -thalassemia	0.9	0.866 to 0.905	0.20 to 0.38	0.9977
Bio-Rad Variant II dual kit	1.7	1.026 to 1.047	-0.23 to -0.14	0.9995
Menarini HA8160	2.3	0.790 to 0.826	0.56 to 0.72	0.9976
Trinity Premier High Resolution	6.6	1.012 to 1.087	-0.08 to 0.24	0.9941
Trinity Premier Quick Scan	1.4	1.115 to 1.156	-0.42 to -0.24	0.9985
Sebia Capillarys 2 FP	1.9	0.898 to 0.946	-0.19 to 0.02	0.9969
Tosoh G8	1.9	1.150 to 1.180	-0.63 to -0.50	0.9992

Data were derived from reference 3. Reproducibility is expressed as CV %. For slope and intercept, the 95% confidence intervals are reported.

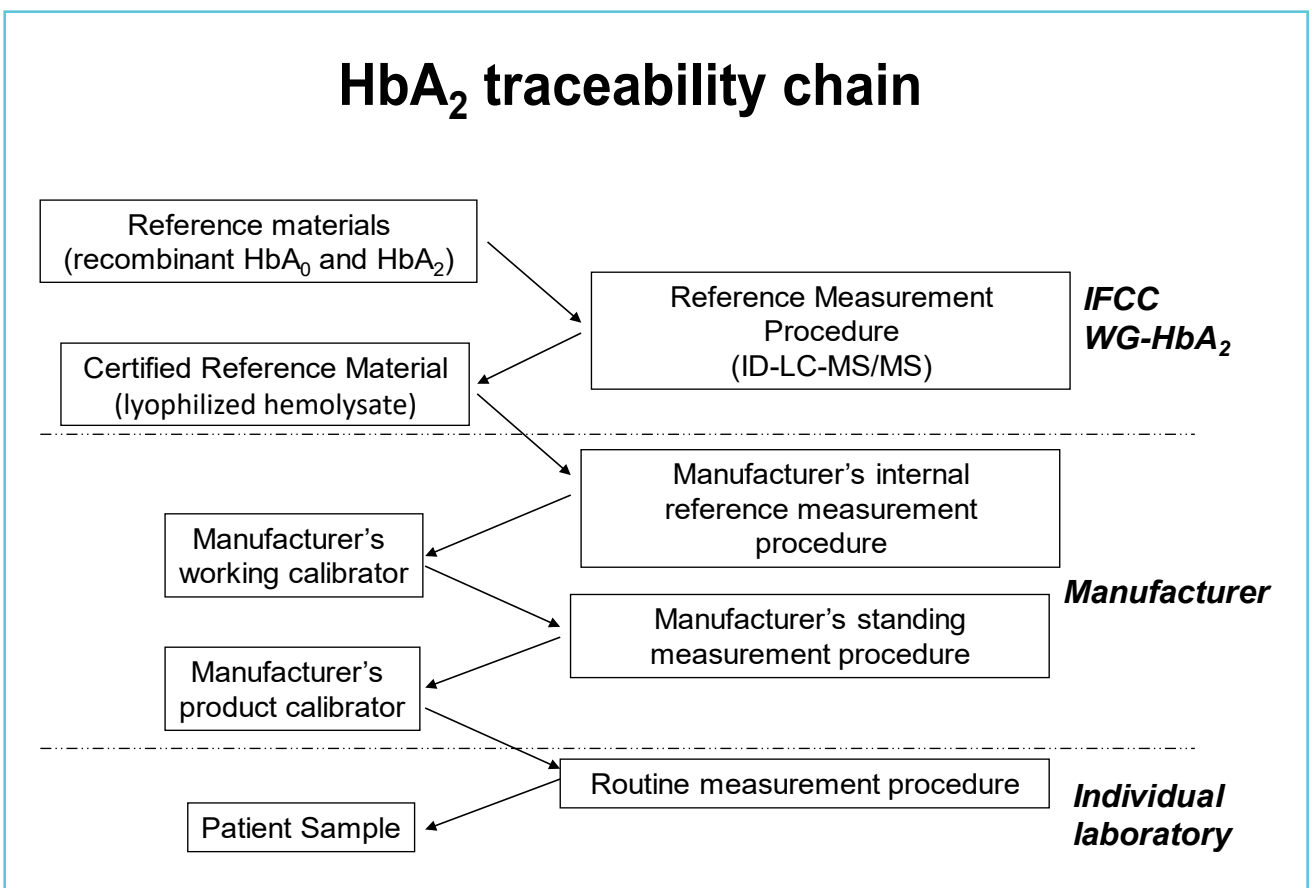
Besides that, we have recently evaluated the performance of current high-performance methods for HbA₂ by measuring 40 blood samples in double over two separate days by the HPLC and one capillary electrophoresis system [3]. We have found a mean imprecision ranging from 0.9 to 6.6 %, and a significant bias between the methods which were however quite well correlated (r between 0.9941 and 0.9995). In Table 1 the main data concerning the performance of the evaluated methods are summarized. In the same work we have tested the commutability of various control materials and we have shown that it is possible to reduce the variability between the methods by using a couple of commutable home-made control materials as calibrators. With regard to the overall variability, we have

seen that after calibration the CVs were reduced from 6.8 to 3.4 %, from 6.6 to 4.6 %, and from 6.7 to 3.0 % for HbA₂ to concentrations lower than 3.0 %, between 3.1 and 4.5 % and above 4.6 %, respectively.

HOW TO BUILD A ROBUST STANDARDIZATION SYSTEM

In 2004, the IFCC approved a project for the development of a complete reference system for HbA₂, as shown in Fig. 1. At the top of the traceability chain, pure recombinant hemoglobins (HbA and HbA₂) are used to calibrate the reference measurement procedure. This reference measurement procedure is based on peptide mapping and isotopic dilution mass spectrometry

Figure 1 IFCC reference system and traceability chain for HbA₂ measurement



(ID-MS), as described previously [2]. Specific tryptic fragments of the δ and α chains (i.e. δ T2 and α T5) are selected as signature peptides representing either HbA₂ or total hemoglobin, and quantified. The principle of the method together with the main performance characteristics are under publication. According to the traceability chain reported in Fig. 1, the reference measurement procedure will then be used to assign the HbA₂ value to certified reference materials which will be produced in collaboration with the Joint Research Centre (JRC).

We have already prepared and characterized one pilot batch of this material consisting of a stabilized hemolysate in the lyophilized form [4]. The material was found to be quite stable with respect to HbA₂ content for at least seven years when stored at -20°C, commutable with the majority of routine methods and to have a total hemoglobin concentration and methemoglobin (MetHb) content similar to that of fresh blood. Manufacturers will then use these certified reference materials to calibrate their methods and to assign values traceable to the primary reference measurement procedure to their calibrators. Finally, the laboratorians using a routine method traceable to a reference measurement procedure will hopefully provide a more robust and accurate result.

TIME-SCALE FOR IMPLEMENTING THE REFERENCE SYSTEM

The first major step is to publish and validate the reference measurement procedure. The principle of this novel method is under publication at the moment of writing this report. The validation of the method according to the various ISO standards will require more work and the involvement of another laboratory because the minimum number of three laboratories is required in order to assign the values to any certified reference material. We estimate at least

one more year to accomplish this second step. Almost in parallel we hope to be able to prepare the certified material to be used as calibrator in collaboration with the JRC. So, it is not unlikely that a couple of years will be necessary in order to have the manufacturers align their methods.

CONCLUSION

Some are of the opinion that the novel molecular techniques such as next generation sequencing (NGS) may substitute the use of HbA₂ in the field of thalassemia screening and diagnosis. We have performed a survey among various opinion leaders and apparently this will not be the case, at least in the next coming years. Indeed, the following points were drawn:

1. Measuring a protein is not the same as sequencing the DNA. Measuring HbA₂ is a functional analysis at protein level, and it will never be replaced by DNA technology.
2. NGS requires a lot of work for the interpretation and, in many cases, the significance of novel SNPs or variants of unknown significance is not known. In addition, it is really critical to know who is going to perform this interpretation.
3. There are still unresolved ethical problems in handling "incidental findings" with DNA technologies.
4. NGS is very expensive and it is unlikely that it could be implemented in poor countries where the burden of thalassemia syndromes is greater than in some richer countries.
5. On the contrary, measuring HbA₂ is simple and fast and it has to be considered that in prenatal screening, as for couples at risk, it is desired to have the result in 1 or 2 days.

As such it can be concluded that HbA₂ assay still remains a gold standard for thalassemia screening and consequently its standardization is a major and topical issue.

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