Research Article

Trends in General Medicine

Hb A2 Reference Limits in Moroccan Healthy Volunteers

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Received: 14 Jun 2023; **Accepted:** 20 Jul 2023; **Published:** 26 Jul 2023

Citation: Marzouki N, Quiddi W, Rabi A, et al. Hb A2 Reference Limits in Moroccan Healthy Volunteers. Trends Gen Med. 2023; 1(1): 1-5.

ABSTRACT

HbA2 determination plays a key role in screening programs for hemoglobinopathy and reducing diagnosis mistakes. The normality area for Hb A2 is often wide. It is advisable for each laboratory to validate the State of Hb A2 for local population because low values with microcytosis can indicate deficiency anemia or alpha-thalassemia. However, high level is for beta-thalassemia or other hemoglobin variant. We have performed a prospective study in search of local reference values for hemoglobin A2 and F. We have used the most powerful methods for hemoglobin fractions analysis: Capillary electrophoresis and HPLC. Five hundred healthy transfusion center's donor interviewed, selected and collected for current study. Genetic analysis show no mutation in alpha and beta-globin gene. Exclusion and inclusion criteria well established and respected. Significant correlation detected ($p<0.05$) between capillary electrophoresis and HPLC dual program A2/F (D10) after outlier's exclusion. Local reference limit found here correlate well with interval confidence of sebia and biorad.

Keywords

Reference limit, D 10 – HPLC, Capillarys 2 flex piercing, Capillary electrophoresis.

Introduction

The inherited hemoglobinopathies are a large group of disorders that include thalassemia and hemoglobin variants. Accurate determination of the carrier phenotype is essential for detecting couples at risk for producing offspring with hemoglobinopathy. Definitive diagnosis of hemoglobin abnormalities relies heavily on the clinical laboratory and there are multiple indications for screening and testing [1].

A Scandinavian group conceived the concept of reference values in the 1970s. Then by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC-LM) and the National Committee for Clinical Laboratory Standards (NCCLS) (now Clinical and Laboratory Standards Institute (CLSI) in the United States during the 1980s. The Guide for correct performance of the tests (GBEA) [2], the ISO 15189 [3] and the directive 98/79 / CE [4] prescribe in various titles the mention of reference limits on analytical reports and laboratory reagent leaflets. Exhaustive lists of various variability factors (pre analytical, exclusion and partition) described in IFCC and CLSI [5,6]. There is a great need for reference values that are representative of healthy humans and presented in a manner that they can be utilized by all laboratories [7]. The need to reference both the level of standardization and measurement uncertainty in medical standards is only beginning to enter into the concerns of the professionals involved [8]. Recently Ichihara proposed a quick and simple method for determining the reference interval and the outliers [9].

Before confidence intervals determination, subjects first selected after exclusion of individuals with increased body mass index, history of chronic or metabolic disease, acute illness, or use of prescribed medication a month before sample collection. The objective was to control pre analytical factors and minimize their effects. Next there was statistical removal of outlier values followed by statistical analysis to calculate adult (18–60 years) reference intervals. Generally, data from more than 120 sample collected for most of the partitioned age groups, resulting in very robust data with narrow 95% CI. Partition criteria designed to classify reference individuals into different subclasses. The two most common are age and sex [10].

In our study, reference limits of Hb A2 and F calculated by capillarys 2 flex piercing (sebia). Simultaneously, high performance liquid chromatography dual program A2/F on (D10, biorad) used. There are the most powerful methods in hemoglobin fractions identification and thalassemia diagnosis. Robust statistical analysis by IBM-SPSS used to remove outlier values and detected narrow confidence limits around the obtained reference limits. Correlative tests permit results comparison between technics and populations' groups.

Materials & Methods

Design Study

This was a prospective study using data from 500 whole blood normal volunteers for reference values screening. The patient's selection due to their healthy state. The samples collected over a period of 8 weeks, and analyzed within 24 hours of each other. Some samples excluded after blood cell counts. They were microcytic, hypochromic and / or anemic. HPLC analysis detected fortuitous diabetic. Hb A1C calculated simultaneously by D10 A2/F program. Hb A1C>6% increase Hb F level (Hb F>1%). Moreover, heterozygosity for Hb S, for Hb C or HPHF observed in some healthy individuals. Whole blood of 320 samples (199 male and 121 female) collected, after biased cases exclusion and normal genetic test. They have year age span 18-60 y. They used to establish a reference interval for Hb A2 and Hb F with Capillarys 2 Flex piercing and HPLC dual program (A2/F) by D10. These samples had hemoglobin, MCH, and MCV levels within the normal range (Sysmex XE-5000, America, Mundelein, it) and they have no hemoglobin variant and no mutation in alpha and beta-globin gene. This study considered quantity and quality assessment project for south Moroccan population.

HPLC

HPLC analysis was performed using dual program (A2/F) by D10 (BioRad, marne la coquette, France), which separates hemoglobin fractions by cation exchange chromatography using his alkaline gradient. HbA2 and Hb F single point calibrators performed daily to adjust and ensure proper retention times and to establish calibration parameters for accurate quantification. Low and high controls evaluated at the front and the end of each run.

Capillary zone electrophoresis (CE)

CE was performed following the manufacturer's guidelines for the Sebia Capillarys 2 Flex piercing system using reagents provided in the Capillarys Hemoglobin (E) kit (Sebia,Norcross,GA).The instrument analyze EDTA whole blood for hemoglobin variants. The lysed red cells electrophoresed in alkaline buffer (pH 9.4) allowing separation directed by pH and endosmosis. Detection of hemoglobin fractions using absorbance at 415 nm. CE not require daily calibration, but normal Hb A2 control analyzed daily through each capillary before additional quality control if required (as AFSC, AF or abnormal Hb A2) and in the end of runs. By this way, we ensure proper charge and function of the capillaries.

Statistical analysis

A statistical study carried out on SPSS to calculate means, standard deviations and reference limits. Analysis applied the T-test, Chi-2, spearman, Anova test and test of independent samples (levene test). Correlations among quantities assessed by a Spearman correlation matrix. The algorithm looked for the occurrence of any statistically significant correlation between quantities (Hb A2; Hb F), using a Spearman correlation matrix. Two quantities were considered correlated if the Spearman correlation coefficient was statistically significant (P <0.001). Qualities correlations examined by Anova and T- test. The test result under scrutiny considered eligible as a reference value, if results fell within the current reference intervals. Hb A2 and Hb F means values calculated and their difference between M and F groups and age class groups studied by IBM SPSS Statistics. Variance analysis of two Hbs (A2, F) also examined for three age class groups. Intra and intergroup correlation verified by levene test. We quantify intergroup difference by $\mathbf{R = t^2 / t^2 + (N1+N2-2).}$ R-value indicate population size effect and explain why group's difference presents in the same population [11].

Results

Five--Hundred-blood donor from transfusion center accepted analysis. 62% are men and 38% of female. 46.2% donors have an age less than 30 years (44.7% mal / 49% female). In this age class, we have more female than mal. 41,1% have an age between 30 and 45 years (44.2% men/ 35% female; (table 1). However, over 45 years old, we have only 12.7% donor (11.1% men/16,6% women). The largest percentage (71.5%) corresponds to mal donor with age between 30 and 45 y. 42.1% women (largest group of female) are age < 30 years was. Sex = f (age class) Correlation studied by Chi-2 test. Chi-2 value is 2,29.

Table 1: Frequencies of age class and sex groups (M, F).

		Percentage	Percentage valid	Cumulative percentage
Valid	M	65.9	65.9	65.9
	F	34.1	34.1	34.1
	< 30	47.2	47.2	47.2
	between 30 and 45	33.4	33.4	80.6
	> 45	19.4	19.4	19.4
	Total	100.0	100.0	100.0

Hb A2 reference interval established using 320 samples after bias exclusion. Hb A2 has normal distribution (mean 2.66%; ecartype: 0.245) (figure 1). The values from both groups combined to establish a single Hb A2 reference range. The reference interval showed a modest shift: analysis by HPLC demonstrated a normal range of <3.35%. However, analysis by CE produced a normal range of <3.4%.

Capillary electrophoresis shows that reference limits of hemoglobins are HbA (96.3 - 98%), HbA2 (2, 00 - 3, 40) and HbF (0, 00-0, 5) (table 2). HPLC reference limits: HbA (95, 65 - 97, 64), HbA2 (2, 36 - 3, 35) and HbF (0-1) (Table 2).

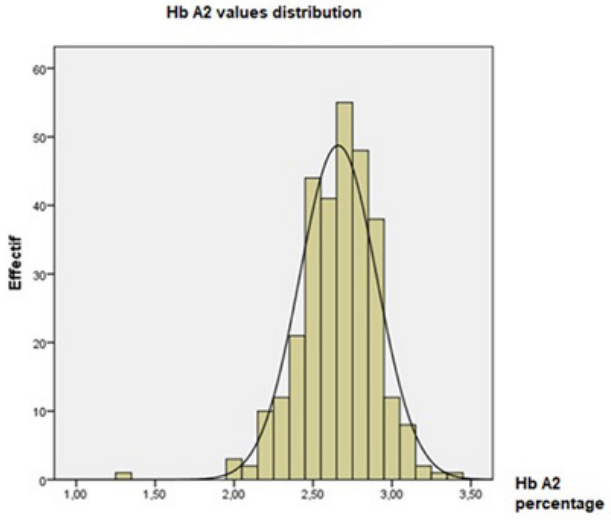

Figure 1: Hb A2 distribution of samples analyzed with CE.

Table 2: Local Hemoglobin CE values compared to sebia and HPLC values compared to Biorad results.

Hb	Sebia CE result	Our CE result	Biorad HPLC result	Our HPLC result
Hb A	96.8 - 97.80%	96.3 - 98 %	95.6 - 97,5 %	95,65 - 97,64%
Hb A2	2.20 - 3.20%	2, 00 - 3, 40%	2,5 - 3, 40%	2, 36 - 3.35%
Hb F	0.00 - 0.50%	0.00– 0.50%	0 – <0.8%	0.00 - 1%

The significance (Sig.) value calculated by spearman test looking for possible HbA2 and HbF reference values correlation. Significance is equivalent to '.000'for two tests (table 3).

Table 3: Correlation between Hb A2 on Ec and Hb F on D10.

		Hb A2 D10 Value	Hb F D10 Value	Approximate meaning
Interval	**R.pearson**	522	290	000[c]
Ordinal	**Correlation of Spearman**	484	356	000[c]
Number of valid observations		320	320	

CE HbA2 means values (M and F groups) studied (table 4). We used, in addition, D10 HbF Values means to compare between M and F groups (table 4). Then, variance analyzed for (HbA2, HbF) values of three-age class groups. The equal variance for CE and HPLC Hb A2 values of three age class groups detected (p>0.05) by Levene Test (table 5). Correlation intra and inter age class group tested for Hb A2 and Hb F values (CE/ HPLC) by ANOVA test (Table 6). The variances are not equal for CE and HPLC Hb F values of three age class groups (Table 7).

Table 4: Means difference of Hb A2 values by CE and HPLC in men and females.

	SEX	N	Means	Ecart type	standard error means
CE Hb A2	M	199	2,6683	,26429	,01874
	F	121	2,6410	,20005	,02000
D10 Hb A2	M	199	3,0131	,37299	,02644
	F	121	3,0290	,32013	,03201

Table 5: ANOVA test: Correlation intra and inter age class group for CE and HPLC Hb A2 and Hb F values.

		SQUARES SUM	ddl	MEAN SQUARE	F	Sig.
CE Hb A2	Intergroups	,146	2	,073	1,218	,297
	Intragroups	17,697	296	,060		
	Total	17,842	298			
D10 HbA 2	Intergroups	,052	2	,026	,204	,815
	Intragroups	37,657	296	,127		
	Total	37,709	298			
CE Hb F	Intergroups	,104	2	,052	,636	,530
	Intragroups	24,307	296	,082		
	Total	24,412	298			
D10 Hb F	Intergroups	1,346	2	,673	1,061	,347
	Intragroups	187,756	296	,634		
	Total	189,102	298			

Discussion

The selection of a reference population is not easy. The groups of reference individuals are rarely homogeneous and no way allow removing all bias. The current reference intervals in use at our laboratory derived mainly from manufacturer suggestions, in some cases modified according to literature data or practical experience. We started analysis according to guide protocol [2,3 and 4] by excluding samples having Hb < 12 g/dl (CI: 14, 53 - 14, 96), MCH < 27 pg (28, 94 - 29, 39) and MCV < 80 fl (85,71 - 86,76). Normal distribution found in Hemoglobin (figure 1), MCV and MCH values. In first selection, after cell blood count, microcytic, hypochromic and / or anemic patients excluded from analysis.

Secondly, Automatic Hb A1C result, obtained simultaneously by HPLC, excluded Samples with HbA1 C> 6 percentage (exclusion of fortuitous diabetic). High level of HbA1c increase Hb F level and decrease by consequent Hb A2 percentage. Then, we analyze each technic result individually. Three samples are heterozygous for Hb S. five others others for Hb C. Some normal individuals have hereditary persistence of hemoglobin F (HPHF/ hb F >1%; Hb A1C<6%), and one has Hb A2 equal to 1.30% which is low than current reference intervals and indicate a delta-thalassemia (condition in normal count cell patient). We delete these cases from studying reference limit.

In this step, 320 samples respect procedure [2, 3] with normal genetic test for alpha and beta-globin, and ready for statistical analysis. Significant correlation observed between CE and HPLC hemoglobin results (Table 3).

Maximum percentage of blood donor in transfusion center of Marrakech (in this period) is 71, 5% with age class < 30 years old and 2/3 are men. I think that is concordant with availability of this sex group with low age in our population. However, this result can also coincide with recruiting period and sampling bias. Sex and age class correlation studied by Chi-2 test.The minimum theoretical size is 12, 71. Chi-2 = 2, 92 (< 3.84), no reject of H0 hypothesis. So, No significant correlation detected between age classes and sex of participant.

Table 6: Levene Test on equal variances: The equal variance for CE and HPLC Hb A2 values of three age class groups (p>0.05).

		F	Sig.	t	ddl	Sig. (bilateral)	Difference of means	Difference of standard error	Confidence interval (95 %)	
									Inferior	Superior
Hb A2 on CE	Hypothesis of equal variances	3,598	,059	,911	297	,363	,02734	,03000	-,03170	,08638
	Hypothesis of inequal variances			,998	251,925	,319	,02734	,02741	-,02664	,08132
Hb A2 on D10	Hypothesis of equal variances	,490	,484	-,365	297	,715	-,01593	,04367	-,10187	,07000
	Hypothesis of inequal variances			-,384	227,260	,702	-,01593	,04152	-,09775	,06588

Table 7: The variances are not equal for CE and HPLC Hb F values of three age class groups.

		F	Sig.	t	ddl	Sig. (bilateral)	Difference of means	Difference of standard error	Confidence interval (95%)	
									Inferior	Superior
HbF (D10)	Hypothesis of equal variances	8,779	,003	-1,668	297	,096	-,16240	,09735	-,35399	,02919
	Hypothesis of inequal variances			-1,518	156,379	,131	-,16240	,10695	-,37364	,04885
HbF (EC)	Hypothesis of equal variances	15,385	,000	-2,012	297	,045	-,07025	,03491	-,13894	-,00155
	Hypothesis of inequal variances			-1,584	116,392	,116	-,07025	,04435	-,15808	,01759

HbA interval is slightly wider in our population (96.3-98) comparing to sebia [1] result (96.8-97.8) (table 2). The patients with 96.3 <Hb A< 96.8% are normal. The same for patients with 97.8% <Hb A<98%. Hemoglobin A2 is also wider. Patients with 2% <Hb A2<2.2% and 3.2% <HbA2<3.4% are also normal. Hb F < 0, 5% in the two study.

Values of Hb A are (95, 65 - 97, 64) by HPLC (table 2). Hb A2 is between 2.36% (≅2.5%) and 3.35% (≅3.40%) in our population. HPLC Hb A and Hb F results varied slightly from CE values of our patients. Nevertheless, difference is important for inferior limit of Hb A2. CE inf limit is 2% but it is 2.36% in HPLC. Moreover, sup limit is 3.35% (hplc) near to CE value (3.40%). HbA2 Means values studied by CE and HPLC in men and female (table 4). We show no difference of HbA2 values calculated by CE in M and F groups (= 2.6%). The same result obtained by HPLC. Comparing CE and HPLC result, HPLC values are slightly important than CE result in the two groups (M and Female).

Correlation intra and inter age class group for CE and HPLC (table 5) HbF values indicate that R (r) is 0.06 (CE) and 0.08 (D10). Statistical R-value shows that group's difference is under little size effect. P-value < 0.05 indicated a significant correlation between capillary electrophoresis and HPLC results table (5). This is a significant correlation between reference values of HbA2 and HbF found by CE and HPLC. Similar findings obtained in a more recent study performed with different HPLC (Bio-Rad Variant) and CE (Sebia Capillarys 2) methods. Therefore, these two methods are complemental and well correlated.

We show no significant difference of CE HbA2 means values (M and F groups). The same result for HPLC. Comparing CE and HPLC result the difference is around 0.4% and HPLC values are slightly more important than CE result in the two groups (M and Female).

No significant differences in D10 HbF Values means (0.1%) between M and F groups. The same for CE values (0.05% of difference). Group of female values are slightly more important for two test. The equal variance noted for CE and HPLC HbA2 values of three age class groups (("Sig." >0.05) (table 6). Nevertheless, the variance are not equal for CE and HPLC HbF values ("Sig." < 0.05) (table 7). No correlation intra and intergroup (age class) for CE and HPLC HbA2 values. This difference between groups from the same population can be explicated by little size effect {R is 0.09 (CE) and is 0.03 (D10) for Hb A2}.

Conclusion

Heterozygous hemoglobin variants and diabetic individuals discovered fortuitously in transfusion center donors. We can avoid this event by programming HPLC test for healthy voluntaries before blood donation. It will be more efficacious, especially for hemoglobinopathy patients' transfusion. Local HbA2 reference limit Precision allows greater resolution between patients with and without thalassemia. A future study using clinical data and large well-conducted direct study would be very helpful to indicate a more exact HbA2 value to confident thalassemia diagnosis. In our routine analysis of Hb fractions by EC and HPLC results, the two methods are compatible and never contradictor whether in normal or pathologic patients. Neither the original population nor the statistical treatment of data known in detail before the current study. Up to now, the most studies proved that HPLC and CE methods were highly correlated and complementarity of their result can help clinicians and biologists interpret these reports.

Limitations

Future studies containing more individuals and clinical features, are desirable for more HbA2 values precision.

What is Already Known on This Topic

Hemoglobin A2 (HbA2) is a normal variant of hemoglobin A that consists of two alpha and two delta chains (α2δ2) and is found at low levels in normal human blood. Hemoglobin A2 may be increased in beta thalassemia or in people who are heterozygous for the beta thalassemia gene.

HbA2 exists in small amounts in all adult humans (1.5-3.1% of all hemoglobin molecules) and is approximately normal in people with sickle-cell disease. Its biological importance is not yet known. The quantification of HbA2 is challenging. Its level may be low or high if one or more globin genes are mutated. βthalassemia carriers are identified by an HbA2 value ≥3.5%.However, some carriers have a slight increase in HbA2. Therefore, a careful determination of the HbA2 level is necessary to avoid diagnostic pitfalls.

Genetic factors both related and unrelated to the β- and α-globin gene clusters, iron metabolism, endocrinological disorders, and some types of anemia, together with intra and inter-laboratory variations in HbA2 determination, may cause difficulties in evaluating this measurement in screening programs for hemoglobinopathies. Therefore, knowledge of all these issues is important for reducing or eliminating the risk of mistakes in screening programs for hemoglobinopathies.

What This Study Adds:

knowledge of normal Hb A2 values in our population not studied before. It is important for reducing or eliminating the risk of mistakes in screening programs for hemoglobinopathies. In normal Moroccan volunteers Hb A2 varied between 2% and 3,40% by cappilary electrophoresis and between 2,36% and 3,35% by HPLC.

In our patients analysis of Hb fractions by CE (Capillary electrophoresis) and HPLC results, the two methods are compatibles and never contradictor whether in normal or pathologic patients.

Acknowledgements

We would like to thank all the individuals who participated in this study, especially the blood and information donors. We also thank all the staff of the transfusion center who helped to carry out this work and especially the head nurse and head of the Marrakech transfusion center Pr Sif Essalam Mohamed. Finally, my thanks to my colleague Dr Imane harkati for his help in the statistical treatment of this work.

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