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An Overview of Beta-Thalassemia Carrier Detection in Developing Countries like Iran

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Abstract: Iran is a country with high prevalence of beta-thalassemia trait. Heterozygote screening is essential for the prevention and control of severe thalassemia diseases. In most clinical laboratories, measurment of hemoglobin A_2 and F is used as a tool for diagnosis of beta thalassemia minor by manual and automated methods. Analysis of these tests simultaneously is time-consuming and costly. Although high performance liquid chromatography (HPLC) is highly sensitive and specific, however, in developing countries where economical factors play a major role in planning for management of patients, the role of HPLC is limited. To review the evaluation of the methods determining HbA_2 and F which do not use HPLC or any automated expensive instrument and find out of any relation between these hemoglobins with red blood cells (RBC) indices this study was performed. Samples from 173 patients suspected to have β thalassemia minor were analyzed. HbA_2 and F were determined by column chromatography and alkaline denaturation Betke methods, respectively. Both hemoglobins were also measured by electrophoresis on cellulose acetate gel. RBC indices were measured by Sysmex coulter counter. The correlation coefficient between the amounts of HbA_2 obtained by column chromatography method and those fulfilled by electrophoresis method was 0.43. Correlation between HbF obtained by alkaline denaturation and electrophoresis was 0.95. While there was a reasonable correlation between HbF obtained by alkaline denaturation method and those obtained by electrophoresis, neither of them showed any correlation with RBC indices. The linear relationship between HbF obtained by manual method with that obtained using electrophoresis, is as alkaline denaturation method for HbF = 0.136+0.917(electrophoresis method for HbF). No correlation was found between amounts of HbA_2 obtained by both methods and neither of them showed any relationship with RBC indices. Either electrophoresis or chemical methods may be used for HbF. since electrophoresis technique is unsuitable for mass screening and chromatography for HbA_2 is the reference method, it is essential and must be done when performing HbA_2 electrophoresis.

Key words: Beta-thalassemia trait · Hemoglobin A_2 & F · Red blood cells (RBC) indices

INTRODUCTION

Thalassemia and hemoglobinopathies, a group of autosomal-recessive inherited human disorders, are prevalent in many parts of the world. Heterozygote screening and genetic counseling are essential for the prevention and control of severe thalassemia diseases [1].

Iran is a country with high prevalence of about 5-10% of beta-thalassemia trait [2]. It is important for reducing or eliminating the risk of mistakes in screening programs for hemoglobinopathies, including the thalassaemias. Complete screening for β-thalassemia is based on the detection of red cell indices, HbA_2, HbF and hemoglobin variant values. In particular, HbA_2 determination plays a key role in screening programs for β-thalassemia because a small increase in this fraction is one of the most important markers of β-thalassemia heterozygous carriers [3].

The 1975 International Committee for Standardization in Hematology expert panel on abnormal Hbs and thalassemias made diagnostic recommendations regarding the laboratory investigation of these conditions. Initial tests recommended include a complete blood count (CBC), electrophoresis at pH 9.2, tests for solubility and sickling and quantification of Hb A_2 and Hb F. If an abnormal Hb is identified on the preliminary tests, then

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further techniques were recommended to identify the variants. These techniques include electrophoresis at pH 6.0-6.2, globin chain separation and isoelectric focusing (IEF). Additional testing, including heat and isopropanol stability tests, was recommended for detection of unstable Hbs or Hbs with altered oxygen affinity [4].

Structural hemoglobinopathies may have an impact on the red cell indices and red cell indices are critical to the diagnosis of thalassemias. The key components of the CBC include: Hb, red blood cell (RBC) number, mean corpuscular volume (MCV), mean cell hemoglobin (MCH) and red cell distribution width (RDW) [4, 5]. The CBC (complete blood count) in the thalassemia trait is associated with high red cell count relative to hemoglobin concentration and hematocrite resulting in a marked fall in MCV and MCH. To avoid missing double heterozygotes for α and β thalassemia with normal MCV and MCH, the first set of tests includes hemoglobin A_2 quantitation [6]. However, hematological diagnosis is sometimes presumptive and in these cases, DNA analysis becomes necessary [3, 7].

The increase in hemoglobin F level as another parameter in screening beta thalassemia is variably associated to the presence of beta thalassemia trait and is more typical in presence of delta-beta thalassemia and of hereditary persistence of fetal hemoglobin. The determination of HbF is relevant for the final diagnosis of various physiopathological conditions. In some investigators opinion its importance will increase in the following years, because of the proliferation of novel approaches for the induction of HbF synthesis as a cure for thalassemia syndromes [8]. Quantitation of HbA_2 remains the most reliable for the diagnosis of β thalassemia trait. This can be measured by microcolumn chromatogray, electrophoresis at alkaline pH followed by elution and high performance liquid chromatography (HPLC). Till recently electrophoresis was considered the gold standard for measurement of HbA_2 [6, 9].

The technique is however, laborious, time-consuming and unsuitable for mass screening. Microcolumn chromatography and HPLC are becoming popular [6]. HPLC is emerging as the method of choice for the initial screening of thalassemias and haemoglobinopathies and quantification of Hemoglobins (Hbs) like HbA, HbA_2 and HbF. Since it is expensive, in the developing countries where economical factors play a major role in planning for management of patients, the role of HPLC is limited [10]. HPLC is a sensitive and precise method for detecting thalassemia and abnormal Hbs. It has become the preferred method for thalassemia screening because of its speed and reliability [11, 12]. However, information is quite limited about using such a system to study the complicated α-thalassemia and β-thalassemia syndromes in Southeast Asia [13].

Each country considering its economic conditions and health service policies should find the best including automated instrumental methods or not, to performance of screening program for beta thalassemia minor. The main aim of this study arises here. Authors report an investigation carried out on HbA_2 levels meanwhile assessment and finding correlation between alkaline denaturation Betke et al and the cellulose acetate electrophoresis for HbF.

To see the necessity of the testing simultaneously all of them and find out any relation between these parameters and red blood cells (RBC) indices, samples from patients suspected to have β thalassemia minor were analyzed. The results were also discussed.

MATERIALS AND METHODS

Human Study Subjects: 173 adult blood samples collected for routine thalassemia screening were examined. None of the patients had malignant tumor, or acute infectious disease. The patients signed written informed consent.

Sampling and Assays: Based our laboratory condition, HbA_2 and F were determined by column chromatography and alkaline denaturation methods, respectively. Both hemoglobins were also measured by electrophoresis on cellulose acetate gel. The columns contain resin DE-52 were used for estimation of HbA_2. Determining of HbF was included alkaline stability test. The Helena electrophoresis system (Helena Laboratories, Beaumont, TX, USA) was used to electrophorese hemolysates on Titan III cellulose acetate plates at 350 V for 25 min in an alkaline buffer. The results were analyzed according to manufacturer's guidelines. The densitometer was Helena Jonior 24. Erythrocyte indices and Hb concentrations were determined using an automatic cell counter, the Sysmex coulter counter Kx21.

Thalassemic individuals have a reduced MCV and one study has suggested that an MCV of 72 fl is maximally sensitive and specific for presumptive diagnosis of thalassemia syndromes [14] but the sensitivity of mean corpuscular hemoglobin (MCH) <27 pg and mean corpuscular volume (MCV) <80 fl has been

shown as a screening test in first step of screening of beta-thalassemia trait in Iran [2]. According to that survey and some other surveys, testing for beta-thalassaemia trait should be carried out when the mean cellular haemoglobin (MCH) is <27 pg [2, 15, 16]. For all of the patients that they had certain signs of hypochromia and microcytosis (MCH <27 and MCV <80), hemoglobins A2 and F were determined by microcolumn chromatography and alkaline denaturation respectively. In elevated cases in these parameters, electrophoresis on the acetate gel was performed.

Statistical Analysis: Comparison of Quantitative values for Hb A_2 and HbF in related mentioned methods was obtained by the linear regression equation by using Minitab analysis. Correlation coefficient, determination of "r" and drawing of graphs were obtained by Microsoft Office Excel 2003.

RESULTS AND DISCUSSION

Quantitative values for HbF by electrophoresis/ elution were in agreement with values obtained using the alkaline denaturation. The linear regression equation of HbF measured by alkaline denaturation and those with the other mentioned method was y = 0.136 + 0.917x, with a correlation coefficient of 0.95. Based on the results, while there was a reasonable correlation between hemoglobin F obtained by alkaline denaturation method and those obtained by electrophoresis, neither of them showed any correlation with the mean cell volume (MCV) and mean cell hemoglobin (MCH) of patients. Figure 1 shows the good relation between two mentioned method for HbF.

We also compared HbA_2 measured by micro column chromatography with the method of electrophoresis. Column used in our study was containing resin DE-52. No correlation was found between amounts of HbA_2 fulfilled by both methods and neither of them showed any relationship with RBC indices. Correlation coefficient of this relation was 0.43. This week relation is shown in Figure 2.

Correlation between each of these hemoglobins with RBC indices is shown in Table 1.

This study also showed that although linear regression analysis of HbF measured by Electrophoresis had good correlation with values obtained by the alkali denaturation method, the mean values of HbF detected by both methods in men were substantially higher than women. When doing both methods for mean values of HbF regardless the sex, these parameters were same (Table 2).

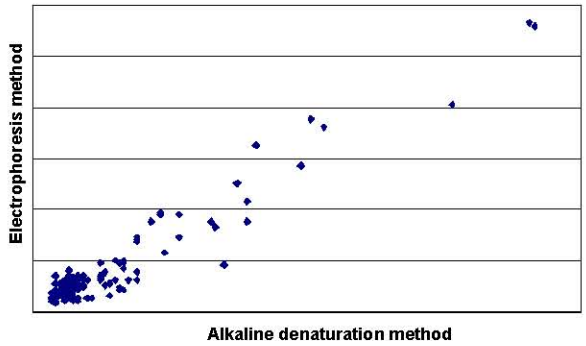

Fig. 1: The linear relationship between HbF by two difference methods

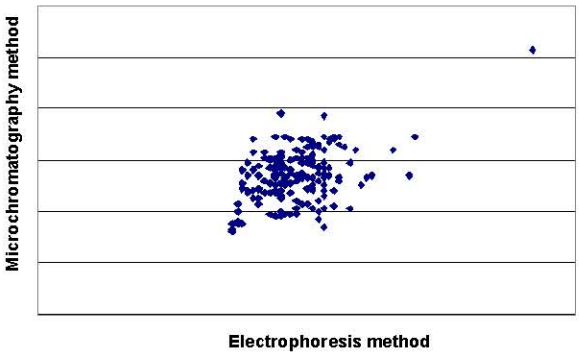

Fig. 2: Relation between two different methods for HbA_2

On the other hand, the mean values of HbA_2 detected by micro column technique were substantially higher than those determined by the electrophoresis method (Table 3).

The linear regression equation found in our study for HbF measured by alkaline denaturation Betke et al and those with electrophoresis method was y = 0.136 + 0.917x, with a correlation coefficient of 0.95. We study the effect of the sex on mean values of Hb F by two mentioned methods. The outcomes for men were higher than those for women. Regardless this effect, mean values of HbF by both methods, was same.

In one study quantitative values for Hb A_2, Hb E, Hb F and Hb Bart's by HPLC were in agreement with values obtained using the other conventional methods (Table 4). The linear regression equation of HbA_2 and HbE measured by HPLC that they found (VARIANT BTS program) with those measured by electrophoresis/elution was y = 0.274 + 1.019x, with a correlation coefficient of 0.99. HbF measured by HPLC (BTS program) was also compared with the method of Betke et al; the linear regression equation was y = 0.218 + 1.380x, with a correlation coefficient of 0.99. Although linear regression analysis of HbF measured by HPLC in their study showed excellent correlation with values obtained by the alkali

Table 1: Comparison of HbA_2 and HbF levels measured by micro column chromatography, alkaline denaturation and electrophoresis separately with the RBC indices

	HbA_2 chromatography	HbA_2 electrophoresis	HbFalkaline denaturation	HbF electrophoresis
MCV	r=-0.07	r=0.05	r=0.03	r=0.07
MCH	r=0.05	r=0.19	r=0.02	r=0.07

Table 2: Comparison of mean values of HbF regarding and regardless the sex by two methods (Electrophoresis and alkaline denaturation)

		HbF regarding the sex	
	HbF regardless the sex	Men	Women
Electrophoresis	1.4	1.8	1.1
Alkaline denaturation	1.4	1.8	1.1

Table 3: Comparison of mean values of HbA_2 regarding and regardless the sex by two methods (Electrophoresis and micro column chromatography)

		HbA_2 regarding the sex	
	HbA_2 regardless the sex	Men	Women
Electrophoresis	4.8	4.9	4.7
Micro column chromatography	5.4	5.4	5.3

Table 4: Comparison of HbA_2, HbE and HbF level measured by HPLC (BTS program of VARIANT) and the conventional methods

		HbF, %			HbA_2/E, %		
Phenotype	n	VARIANT	Betke *et al.*,	P	VARIANT	Elect/elut[a]	P
Normal	192(HbF) 116(HbA_2)	0.7±0.82	0.5±0.54	<0.0001	2.6±0.38	2.3±0.65	<0.0001
β-thalassemia trait	39	1.8±1.05	1.3±0.82	<0.0001	5.9±1.35	5.9±1.36	0.8583
Hb E trait	60	1.5±1.73	0.9±1.25	<0.0001	27.2±3.93	26.7±3.17	0.2238
β-thalassemia/HbE	30	35.2±12.41	25.4±8.92	<0.0001	59.4±12.92	56.3±13.63	0.0277

[a]Electrophoresis/Elution

denaturation method, the mean values of HbF detected by HPLC were substantially higher than those determined by the alkali denaturation method (Table 4).

These observations note that either electrophoresis or method of Betke et al may be used for HbF. Based on our study neither of two assessed hemoglobins showed any relationship with RBC indices. One previous study in patients with homozygous sickle cell(SS) disease On simple correlation coefficient analysis had negatively correlated HbA_2 with both HbF and MCV, red cell count (RBC) correlated negatively with MCV. Less consistent were positive correlations between HbA_2 and RBC, between HbF and both MCV and haemoglobin, Partial correlation coefficient analysis clarified some of these relationships [17].

In one another survey for identification of Hb E variant which is prevalent in north India laboratory investigations were based on RBC indices and high performance liquid chromatography (HPLC). A negative correlation was found to exist between levels of HbA(2) and RBC indices including the MCV and MCH. A similar correlation was seen between levels of HbF with Hb, RBC count and MCV [18]. These relationships are in agreement with our findings even if differences in methods and aims to be exist.

Various indices utilizing these CBC components have been developed with a view to providing a mathematical derivation to efficiently screen patients with microcytosis for further hematologic studies to confirm β-thalassemia. According a new formula the % microcytic - % hypochromic - RDW is the most reliable index evaluated, with 100% sensitivity and 92.6% specificity [5].

The no linear regression analysis and weak relation was found in our study between amounts of HbA_2 fulfilled by micro column chromatography and electrophoresis. This notes that the both methods estimation of HbA_2 must be done when beta thalassemia screening program.

Different methods of micro column chromatography have been compared with other methods including cellulose acetate electrophoresis, column chromatography on DEAE-Sephadex and starch block. Although all of them found to be reliable and reproducible, the

microcolumn chromatographic method using DE-52 cellulose was the most rapid, reproducible, economical and well suited for large scale surveys [19].

The results of one another study for comparison between microchromatography on a DEAE cellulose column and conventional cellulose acetate electrophoresis showed a lowest misclassification rate with a greater reliability. Therefore, the use of the microchromatographic procedure for beta-thalassemia screening was recommended [15]. Among three different methods to assess hemoglobin A2 concentration (microchromatography and cellulose acetate electrophoresis with further elution of the bands or measured by densitometry) a statistical significant difference (P less than 0.05) had been found between densitometry and the other two methods by the other investigators. Microchromatography and the elution method showed no statistical difference [20].

The precision and accuracy of Hb A_2 measurements using densitometric scanning of electrophoretic gels is poor, especially when compared with HPLC techniques [21]. A recent College of American Pathologists [22] hemoglobinopathy survey showed a CV of 33.6% for densitometric scanning of electrophoretic gels at a Hb A_2 concentration of 2.41%. For column chromatography, the CV was 14.6% at a mean Hb A_2 concentration of 3.21% and for HPLC, the CV was 4.3% at a mean Hb A_2 of 3.47% [22].

Despite the imprecision, 296 of 387 laboratories participating in the College of American Pathologists Hemoglobinopathy Survey program reported results for Hb F, Hb A_2 and Hb identification using electrophoretic methods [22]. Some laboratories, including ours, use a combination of electrophoresis and micro column chromatography to identify and quantify Hb.

Hemoglobin electrophoresis at alkaline pH (8.6) reveals the following bands-HbA,F,S/D/G, C/E/O^{Arab} and fast -moving hemoglobins (like HbN,J,H and Barts) which migrate anodal to HbA. The migration of hemoglobin A_2 is the same as that of HbC/E/ O^{Arab}. Hemoglobin A_2 being less than 7 percent cannot be accurately quantified by densitometry and requires elution of the band and spectrophotometric measurement. Moreover electrophoresis technique is slow, labor-intensive and time-consuming therefore unsuitable for mass screening [4, 6, 21].

In specific clinical circumstances to find the laboratory tests which are most useful in the screening of β thalassemia trait, the most important technical aspects of each method as well as some possible error factors should be considered [15].

Even if different methods be employed in HbA_2 level detection, the mean values of those methods depend on the different laboratories and techniques. Genetic factors both related and unrelated to the beta- and alpha-globin gene clusters, iron metabolism, endocrinological disorders and some types of anemia, together with intra- and inter-laboratory variations in HbA_2 determination, may cause difficulties in evaluating this measurement in screening programs for β thalassemia trait [3]. All techniques determining hemoglobins A_2 and F have limitations, but in general, there is a good agreement between the three techniques including microcolumn chromatography, electrophoresis at alkaline pH and high performance liquid chromatography (HPLC) when properly standardized [6].

Some investigators have developed a rapid, simple and inexpensive enzyme linked immunosorbent assay (ELISA) for the quantitation of HbA_2, which can be used in carrier screening programs in developing countries like India. In a limited trial for beta-thalassaemia carrier screening, the results obtained with ELISAs were compared with those obtained with the microcolumn chromatography method (r = 0.89) which can be used in carrier screening programs [23].

The others compared the fast protein liquid chromatography (FPLC) technique with the conventional cellulose acetate electrophoresis (CAE) used routinely in most laboratories. The cut-off value for the diagnosis of beta-thalassaemia trait by both the techniques was 3.8 per cent and 27 heterozygotes were identified. As both techniques gave comparable results, CAE could be the cost effective method of choice for routine screening for beta-thalassaemia trait [24].

In other survey the FPLC results were highly correlated (r = 0.985, $P<0.001$) with those of HPLC for quantification of HbA_2 as well as cellulose acetate electrophoresis (r =0.977) and microcolumn chromatography (r = 0.980). In addition, the FPLC method was simple, rapid, low cost and reproducible [25].

CONCLUSION

Relationship between HbF obtained by manual method with that obtained using electrophoresis, is linear so either electrophoresis or chemical methods may be used for HbF.

Validation of both methods (microcolumn chromatography and acetate gel electrophoresis) for quantitation of hemoglobin A_2 in our country based on our findings, do not allow omitting one of them and both methods must be done. The author suggests here regarding find out of newer RBC index(RDW), as the most

reliable index in carrier screening programs [5], evaluate correlation between this highly sensitive and specific index with amount of HbA_2 and HbF for further investigation in detection of subjects with beta thalassemia trait [26, 27, 28].

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