



Comparative analysis of cellulose acetate hemoglobin electrophoresis and high performance liquid chromatography for quantitative determination of hemoglobin A2

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Background

The present study is designed to evaluate the reliability and cost effectiveness of cellulose acetate Hb electrophoresis and high performance liquid chromatography (HPLC) in the determination of HbA2 levels.

Methods

The test population comprised 160 individuals divided into four groups: normal individuals, β -thalassemia trait (BTT) patients, iron deficiency anemia (IDA) patients, and co-morbid patients (BTT with IDA). HbA2 levels determined using cellulose acetate Hb electrophoresis and HPLC were compared.

Results

HbA2 levels were found to be diagnostic for classical BTT using either method. In co-morbid cases, both techniques failed to diagnose all cases of BTT. The sensitivity, specificity, and Youden's index for detection of the co-morbid condition was 69% and 66% for HPLC and cellulose acetate Hb electrophoresis, respectively.

Conclusion

This study revealed that semi-automated cellulose acetate Hb electrophoresis is more suitable for use in β -thalassemia prevention programs in low-income countries like Pakistan. This technique is easily available, simple and cost effective.

Key Words Cellulose acetate hemoglobin electrophoresis, Hemoglobin A2, High performance liquid chromatography

INTRODUCTION

Hemoglobin (Hb), an essential element of red blood cells, is responsible for the transport of gases throughout the body [1]. HbA is a heterotetramer of 2 α and 2 β globin subunits, with one heme embedded in each chain [2]. Normal Hb composition in the newborn is 60%–85% HbF ($\alpha_2\gamma_2$) and 15%–40% HbA ($\alpha_2\beta_2$), while HbA2 ($\alpha_2\delta_2$) is almost immeasurable [3]. During the first one and a half years of life, expression of HbA increases to more than 95% of Hb composition, accompanied by a decrease in HbF expression to less than 1% of Hb composition. At the same time, HbA2 levels undergo a slight increase to 2.5%–3.5% of Hb composition [4].

HbA2 is considered as an essential marker for the diagnosis of β -thalassemia trait (BTT). Soon after its discovery in the late 1950s [5], HbA2 was assumed to be an important parameter in the diagnosis of β -thalassemia minor state, with most researchers endorsing the view that HbA2 level $>3.5\%$ is a diagnostic feature of BTT [4]. In iron deficiency anemia (IDA), HbA2 level is mostly reduced, as IDA modulates δ chain synthesis.

According to the World Health Organization, IDA is the cause of half of all cases of anemia worldwide [6]. Anemia is also the most common disease due to nutritional deficiency in Pakistan. Thalassemias are the most common hereditary single gene disorder worldwide [7]. The two most common clinical entities, α - and β -thalassemia, are classified on the

basis of inheritance of abnormal α and β chains, respectively. On the basis of clinical severity, β -thalassemia can be classified as silent, trait, intermedia, and major. The global prevalence of BTT is estimated at around 1.5%; however, it is more common in countries falling in the “thalassemia red belt”, which includes North and South Africa, Southern Europe, the Middle East, Southeast Asia, and the Far East [8]. In the Pakistani population, the prevalence of BTT is reported to be approximately 6% [9].

Pakistan is a country where IDA and BTT are very common. Therefore, it is quite possible to find both conditions simultaneously in a single individual. In such cases of co-morbid disorder (i.e. BTT with IDA), HbA2 levels may be within the normal range. This makes the diagnosis of β -thalassemia minor somewhat challenging, and impossible by Hb electrophoresis. The presence of β -thalassemia gene (BTT) in an individual can be detected from certain parameters in the complete blood count (CBC) and confirmed by Hb electrophoresis [10, 11].

Several different Hb electrophoresis techniques are available, including manual, semi-automated, and high performance liquid chromatography (HPLC). In order to launch an extensive nationwide thalassemia prevention program, it is important to screen BTT in the Pakistani population using cost-effective, accurate, and precise methods. This study compares and evaluates the technical accuracy and efficiency of the two most widely used methods for measurement of HbA2 levels, namely, cellulose acetate Hb electrophoresis and HPLC.

MATERIALS AND METHODS

This cross-sectional multicenter analytical study was carried out at Baqai Institute of Hematology, Muhammadi Institute of Hematology, and Hussani Institute of Hematology. Samples were collected from different provinces and districts of Pakistan between January 2013 and January 2014.

For each sample, 5 mL of whole blood was collected and divided into 3 mL of anticoagulated blood for CBC (in commercially available vacuum container tubes containing anti-

coagulant ethylenediaminetetraacetate [EDTA]) and 2 mL in vacuum container gel tubes for serum. Complete blood counts, serum ferritin, HPLC, and cellulose acetate Hb electrophoresis tests were performed on all blood samples.

The total test population consisted of 160 individuals divided into four groups, each containing 40 individuals: Group A comprised normal individuals, Group B comprised individuals with β -thalassemia trait, Group C individuals, with IDA, and Group D individuals, with the co-morbid condition, i.e. β -thalassemia minor with IDA.

CBC was performed on an automated analyzer (Sysmex KX-21, Tokyo, Japan). Serum ferritin was measured using an enzyme-linked immunoassay (ELISA) kit manufactured and supplied by DiaMetra. Cellulose acetate Hb electrophoresis was performed using a semi-automated technique (Interlab Roma Microtech Series 648iso, Rome, Italy). HPLC was performed using a Bio-Rad variant HPLC system with the variant β -thalassemia short program reorder pack (Bio-Rad laboratories, Hercules, CA, USA), according to the manufacturer's instructions.

Results were analyzed using the SPSS statistical software version 17 (Chicago, IL, USA). One-way ANOVA was used to compare the groups. Results were calculated and reported as mean \pm SD. *P* value < 0.05 was considered statistically significant.

The sensitivity, specificity and Youden's index value [12] were calculated as follows:

$$\text{Sensitivity} = [\text{True positive} / (\text{true positive} + \text{false negative})] \times 100$$

$$\text{Specificity} = [\text{True negative} / (\text{true negative} + \text{false positive})] \times 100$$

$$\text{Youden's index} = (\text{sensitivity} + \text{specificity}) - 100$$

RESULTS

CBC results demonstrated mild anemia in patients with BTT, and moderate to severe anemia in patients with IDA (Table 1). RBC counts and red cell distribution width were found to differ significantly between BTT and IDA cases. However, no CBC parameters could reliably discriminate for the presence of the co-morbid condition (BTT+IDA).

Compared with normal individuals, HbA2 levels in both

Table 1. Comparison of CBC parameters and serum ferritin levels from normal individuals, and from cases of β -thalassemia trait (BTT), iron deficiency anemia (IDA) and the co-morbid condition (BTT+IDA). All values are mean \pm SD.

	Normal	BTT	IDA	BTT+IDA	<i>P</i>		
					Normal vs. BTT	Normal vs. IDA	Normal vs. BTT+IDA
Hb (g/dL)	13.1 \pm 1.2	11.5 \pm 1.2	7.5 \pm 1.2	9.2 \pm 1.5	0.011	0.001	0.001
RBC ($\times 10^6 / \mu\text{L}$)	4.7 \pm 0.5	5.8 \pm 0.5	3.3 \pm 0.8	4.2 \pm 0.6	0.001	0.001	0.001
MCV (fL)	87.8 \pm 6.9	71.4 \pm 5.5	68.9 \pm 9.1	72.7 \pm 10.3	0.001	0.001	0.001
MCH (pg)	27.9 \pm 3.1	20.2 \pm 2.2	20.2 \pm 1.9	22.2 \pm 3.1	0.001	0.001	0.001
MCHC (%)	31.8 \pm 1.8	27.9 \pm 2.4	26.1 \pm 2.6	28.4 \pm 2.1	0.001	0.001	0.001
RDW (%)	14.3 \pm 2.7	16.0 \pm 1.7	20.4 \pm 4.2	16.9 \pm 2.1	0.403	0.001	0.076
Ferritin (ng/mL)	95.1 \pm 15.0	113.0 \pm 12.0	2.7 \pm 1.8	7.1 \pm 1.7	0.081	<0.001	<0.001

Abbreviations: BTT, β -thalassemia trait; IDA, iron deficiency anemia.

Table 2. Comparison of cellulose acetate Hb electrophoresis and high performance liquid chromatography.

	Normal	BTT	IDA	BTT+IDA	P		
					Normal vs. BTT	Normal vs. IDA	Normal vs. BTT+IDA
HPLC							
HbA2	2.9±0.2	4.4±0.5	1.9±0.5	2.7±0.4	0.001	0.001	0.874
HbF	0.5±0.3	0.5±0.3	0.6±0.2	0.5±0.2	0.001	0.078	0.641
HbA	96.7±0.8	95.1±0.7	97.5±0.4	96.7±0.5	0.001	0.031	0.990
Cellulose acetate Hb electrophoresis							
HbA2	2.9±0.1	5.6±0.7	1.6±0.6	3.0±0.7	0.001	0.001	0.932
HbF	0.3±0.2	0.6±0.3	0.6±0.2	0.5±0.2	0.013	0.014	0.153
HbA	96.8±0.3	93.8±1.0	97.8±0.6	96.5±0.8	0.001	0.014	0.786

Abbreviations: BTT, β -thalassemia trait; IDA, iron deficiency anemia; HPLC, high performance liquid chromatography.

Table 3. Comparative analysis of HbA2 levels measured using cellulose acetate Hb electrophoresis and high performance liquid chromatography.

	HPLC	Hb-EP	P
Normal	2.87±0.17	2.89±0.13	0.793
BTT	4.42±0.53	5.58±0.75	0.001
IDA	1.93±0.47	1.63±0.57	0.36
BTT+IDA	2.74±0.37	3.04±0.66	0.061

Abbreviations: BTT, β -thalassemia trait; IDA, iron deficiency anemia; Hb-EP, Hb electrophoresis; HPLC, high performance liquid chromatography.

the classical BTT and IDA groups were found to differ by a highly significant level when measured by either HPLC or cellulose acetate Hb electrophoresis. However, results for the group with the co-morbid condition (BTT+IDA) were unsatisfactory using either technique (Table 2).

The sensitivity, specificity, and Youden's index of HPLC and cellulose acetate Hb electrophoresis to detect normal individuals and BTT cases were all 100%. In contrast, in the co-morbid (BTT+IDA) group, Youden's index was 69% for HPLC and 66% for cellulose acetate Hb electrophoresis (Table 4). Therefore, the results of this study showed that both cellulose acetate Hb electrophoresis and HPLC can reliably diagnose normal and classical BTT individuals, but neither technique can diagnose BTT in co-morbid cases (Table 3).

DISCUSSION

β -thalassemia and IDA are the two most common microcytic hypochromic anemias among the Pakistani population [13, 14]. Pakistan has a population of 160 million and an annual population growth rate of 3% [15]. The Pakistani population is divided into many ethnic groups, the major five of these being Punjabi, Pathan, Sindhi, Balochi, and Urdu-speaking; these are further subdivided into castes or 'Biradris' [14, 15]. These ethnic groups have a very strong tradition of marriages within Biradris. Marriages amid close

Table 4. Sensitivity, specificity, and Youden's Index of HbA2 levels in different patient groups for cellulose acetate Hb electrophoresis and high performance liquid chromatography.

	Sensitivity	Specificity	Youden's Index
HPLC			
Normal	100%	100%	100%
BTT	100%	100%	100%
BTT+IDA	69%	100%	69%
Cellulose acetate Hb electrophoresis			
Normal	100%	100%	100%
BTT	100%	100%	100%
BTT+IDA	66%	100%	66%

Abbreviations: BTT, β -thalassemia trait; IDA, iron deficiency anemia; HPLC, high performance liquid chromatography.

relatives, especially among first cousins, are a very common practice in Pakistani culture [14, 15]. Inter-family marriages are a very common cause of genetic diseases such as β -thalassemia major. Because of social and religious factors, it is inevitable to develop awareness about such practices to avoid these types of devastating illnesses [16].

β -thalassemia major is a fatal disease that develops only when both parents are heterozygous for the β -thalassemia gene (β -thalassemia minor) [9]. Around 5,000 new β -thalassemia patients are added every year to the existing population of Pakistan [13]. To halt the propagation of the β -thalassemia gene in a homozygous state (β -thalassemia major), an extensive prevention program was initiated in Pakistan in 1994, based on increased awareness of thalassemia and improved detection rates for β -thalassemia minor, particularly with respect to prenatal diagnosis. However, despite further technical advancements, the incidence of children born with β -thalassemia major has not reduced [9].

Certain parameters in the CBC can indicate classical BTT (Table 1); however, to confirm the diagnosis of BTT, Hb electrophoresis should be also performed to determine HbA2 levels. In this study, HbA2 levels <3.5% were considered normal whereas levels >3.5% were considered to indicate BTT, as shown in Table 2 [17]. ICSH recommends that β -tha-

lassemia minor should be diagnosed by determining HbA2 levels using techniques such as cellulose acetate Hb electrophoresis or HPLC [11]. Cellulose acetate Hb electrophoresis can be performed by manual or semi-automated methods. Although conventional cellulose acetate Hb electrophoresis (manual) is a satisfactory method, it is laborious, time consuming, and requires considerable technical expertise [11], whereas semi-automated cellulose acetate Hb electrophoresis is a comparatively easier technique for HbA2 analysis. Furthermore, the chances of error are small for the semi-automated technique compared to conventional cellulose acetate Hb electrophoresis method. On the other hand, HPLC is a sensitive and recommended method for the diagnosis of hemoglobinopathies because of its simplicity, shortened assay time, and accurate quantification of Hb [11].

In our study, we found that both HPLC and cellulose acetate Hb electrophoresis detected elevated HbA2 levels in classical BTT cases. No statistically significant difference was observed between the two techniques (Table 3). The sensitivity, specificity, and Youden's index was 100% for detection of normal individuals and BTT using both techniques (Table 4). Based on these findings, we conclude that both techniques are equally effective for the detection of BTT [18].

In the IDA group, patients were diagnosed on the basis of microcytic hypochromic anemia on CBC, peripheral blood morphology, and low levels of serum ferritin (Table 1). Patients with IDA also exhibited low HbA2 levels (mean 1.93 ± 0.473 on HPLC and 1.63 ± 0.570 on cellulose acetate Hb electrophoresis). These results suggest that neither method is superior for the detection of IDA (Table 3).

In the Pakistani population, the prevalence of the β -thalassemia gene and of IDA is estimated to be 6% and 43%, respectively [14, 18]. Individuals with the β -thalassemia gene, especially females of child-bearing age, may develop IDA. This co-morbid condition can result in individuals with BTT displaying normal HbA2 levels, resulting in missed diagnoses. The importance of diagnosis of the co-morbid condition can be understood by the fact that if an individual with the co-morbid condition marries an individual with BTT, it may result in the birth of a child with β -thalassemia major. Indeed, such marriages are one of the most significant causes of the propagation of the β -thalassemia gene in the Pakistani population, and have hindered the thalassemia prevention program in Pakistan. To prevent the propagation of the β -thalassemia gene through this mechanism, it is critical to identify all cases of the co-morbid condition [14].

In this study, mean HbA2 levels for the co-morbid individuals (IDA+BTT) group were 2.74 ± 0.367 and 3.04 ± 0.658 for HPLC and cellulose acetate Hb electrophoresis, respectively. Both techniques failed to diagnose BTT, as they have low levels of serum ferritin (Table 3). Comparing the two techniques, we found no significant difference ($P=0.06$) in the diagnosis of BTT (Table 3). This might mislead the pathologists and consultants, as both of these techniques will label the co-morbid patient as normal individual. In a country like Pakistan, where the rate of β -thalassemia minor is high and the etiology of IDA is common, any case of mild to

moderate anemia for which Hb electrophoresis results are normal should not be reported as normal until the probability of iron deficiency is ruled out [14, 19].

This study was designed to evaluate cellulose acetate Hb electrophoresis and HPLC as specific and cost-effective diagnostic tools for BTT and the co-morbid condition. The sensitivity of HPLC to detect co-morbid cases was 69%; the corresponding figure for cellulose acetate Hb electrophoresis was 66%. Both techniques showed a specificity of 100% (Table 4). Therefore, we conclude that both techniques are suitable for the determination of HbA2 levels in cases of BTT and the co-morbid condition.

HPLC is an expensive procedure for a low-income country such as Pakistan, where healthcare infrastructure does not provide amnesty to the poor and non-affording people. Our study revealed that cellulose acetate Hb electrophoresis and HPLC are equally effective in the diagnosis of BTT. Thus, cellulose acetate Hb electrophoresis is suitable for use in the nationwide β -thalassemia prevention program. This method is easily available, technically easy to perform, and cost effective. It is recommended that simple, easily available, and cost-effective methods such as the single tube osmotic fragility test, CBC, and cellulose acetate Hb electrophoresis be used for the screening and diagnosis of BTT.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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