DETECTION OF HEMOGLOBINOPATHIES IN HYPOCHROMIC, MICROCYTIC AND SICKELED CELL BLOOD FILMS BY HEMOGLOBIN ELECTROPHORESIS

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ABSTRACT

background: The hemoglobin molecule (Hb) is protein carries a negative charge that render it attracts to anode pole causing separate it and moving toward the positive pole of electric current, affected by the movement of this type of charge (negative or positive) and strength as well as the molecular weight of hemoglobin type. Until these days, there are more than 300 known genetic mutation may occur in hemoglobin molecule. Some of them with clear clinical implications that threat health, especially for heterozygous states.

Objective: To detect some of hemoglobinopathies such as β- thalassemia and sickle cell anemia cases by hemoglobin electrophoresis technique in population sample identified from Nassiriyah governorate in Iraq.

Methods: Eighty samples of blood were drawn from subjects who suspected to have hemoglobinopathies and examined by Hb electrophoresis technique in College of medicine – Thiqar University. The diagnosis of hemoglobinopathies was made on the basis of hemoglobin electrophoresis, sickling tests, and family studies. Persons, who have low level of hemoglobin concentrations, accompanied with elevation of abnormal hemoglobin percentage, were involved in this study. Fifty eight cases of hemoglobinopathies were diagnosed, 30 of β0 (73.5%) as β- thalassemia, and 28 of ^0(35%) as sickle cell disease carriers.

Results: Iraqi β-thalassemia and sickle cell anemia carriers were identified in hematology sections in Nassiriyah governorate hospitals. Recent hemoglobin protein study revealed the presence of different common defected hemoglobin types associated with these disorders, distributed as the following: 15 subjects (18.7 %) were (HbAS) and 8 (10%) were (HbFS) sickle cell minor carriers, 7(8.8%) were (HbS) sickle cell disease, while thalassemic major were: 9 (11.3%) with (HbF) , and 12 (15%) with (HbAF) type. Thalasemia minor hemoglobin (HbA2) type represents 7 (8.8%). However, there are also 22 subjects were have normal Hb electrophoresis.

Conclusion: The investigations described below manifested a rapid and simple method which allows quantitative analysis of the proportions of the various hemoglobins forms present. Hemoglobin gel electrophoresis is a simple and convenient technique for the study of the hereditary hemoglobinopathies in alkaline pH (4.8 to 6.8). We suggest extending its usage to the detection of other hemoglobin disorders.

Introduction

β- thalassemia and sickle cell disease represent the most frequent hemoglobinopathies. Thalassemia syndrome is a series of genetic disorders in the hemoglobin (Hb) synthesis characterized by reduced rate of production of one or more of the globin chains of hemoglobin. β- thalassemias

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are autosomal recessive inherited defects that results mainly from mutations that decreasing (β+) or eliminating (β0) the reflects the extreme insolubility of α-globin, that present in relative excess because of decreased β-globin synthesis. Insoluble α-globin precipitates in developing erythroblasts, leading to marked ineffective erythropoiesis [1]. β-thalassemias patient characterized by hypochromic, hemolytic anemia,(see Fig.2) and dependence on blood transfusions to sustain life [2] β-thalassemia is widely distributed throughout the world, with considerable frequencies in the eastern mediterranean countries, including Iraq [14–17] and Arabian gulf region [18]. It has been estimated that 3% of the world’s population, or 200 million people, in addition to almost 150 thousand affected individuals born annually. Carrying of β-thalassemia gene, reflects a wide spectrum of clinical manifestations ranging from β-thalassemia intermedia to severe, transfusion-dependent β-thalassemia major. The clinically important feature of β-thalassemia is its interaction with other hemoglobinopathies like sickle cell disease in co-inheritance feature improves the hematologic parameters of heterozygous β-thalassemia [19].

Sickle cell disease (SCD) is a protean disorder caused by elevations of intraerythrocyte and total blood viscosity. Hypoxia induced gelation of hemoglobin S (HbS) deforms the erythrocyte and its membrane and causes massive cation loss as well as increased erythrocyte surface expression of adhesion molecule receptors [20]. The concomitant lack of deformability and enhanced stickiness, lead to arise hemolytic anemia and acute vaso-occlusion; organ damage develops from recurrent erythrocyte sickling, chronic the hemolysis, and progressive endothelial vasculopathy [21]. Like other parts of the country Hemoglobinopathies, are an important problem in Nassiriyah province (In south of Iraq with a population of around 1.5 million and with more than 400 registered patients) (Fig. 1). The emergence of these disorders partially due to consanguineous marriages that are common in this area. The aim of this article is to use hemoglobin gel electrophoresis for detecting of some hemoglobinopathies forms. This approach may contribute treatment and possibly prevent the transmission of the hemoglobin mutation, by identifying the carriers since their offspring are at risk of inheriting the mutation.

![Figure 1: Iraq map illustrates Nasseryiah province south east of Iraq](image)
Material and methods

Subjects
A total of eighty low hemoglobin level (under 10 mg/dl) subjects suspect to have hemoglobinopathies and voluntaries with sign and symptoms of anemia, whom attended to Nassiriyah hospitals, recruited in this study. All were ethnic Arabs, with ages ranging between (1.2- 34) years (median age of 7.8 years) (see table 1). They included 38 males and 42 females. Informed consent was obtained from all subjects, and the study was approved by College of Medicine, Thiqar University. 3 ml whole blood samples were collected in EDTA coated tubes from patients during their attendance to hospitals, then uses immediately.

Hb
The level of hemoglobin was checked by using of Reflotron Plus (Roche, Germany) Roche diagnostic GmbH machine.

Blood film
Complete blood count was determined by using coulter Micro diff II machine according to manufacturer instructions in addition to routine examination of peripheral blood films.

Sickling test
50 μl of well-mixed whole blood was added to 4 ml of the phosphate buffer / sodium hydrosulfite solution (one tube for each test and each control). The tube was covered with a cap or parafilm, and mixed three or four times. Then incubated in the reading rack for 10 to 20 minutes at room temperature. Positive: If hemoglobin S is present (or any other sickling hemoglobin), the solution will be turbid and the lines on the reading rack are not visible.

Electrophoresis:
Electrophoresis of the hemoglobin solutions was carried out in an apparatus (Hellabio), utilizing alkali denaturation technique as the following: Venous blood was drawn from fasting individual into EDTA treated vacuntair tube. About 100-200 μl of whole blood was added to a tube with 10 ml saline, centrifuge. 30 μl of the sediment was mixed with 130 μl hemolyzing solution. Then 5 μl of each hemolysate was applied across the slits and left 20 to 30 seconds to give time to be absorbed. The gel was place into the tank with samples on the cathodic side, and run 200 volt for 20 minutes. Dry it completely with hot air (less than 60ºC) and stained it for 5 minutes with proteins staining solution. The film was distained for 5 minutes in 3 distaining solution baths. The film is dried again with hot air. The results were (fig 3,4, and 5) the standards were evaluated and analyzed by Hellabio software. The standards were done by using HbAFSA2 and HbAFS protein HalloBio.

RESULT & DISCUSSION

Low hemoglobin concentration is the common manifestation of anemia caused by many environmental factors such as malnutrition and hemorrhagic conditions or by hereditary factors such as hereditary persistence of fetal hemoglobin (HPFH) and hemoglobin-pathies (HbP). Approximately more than 5,000 patients affected by major forms of (HbP) disorders; mainly β-thalassemia major and sickle cell disease, clearly manifested among inbred ethnic groups live in Iraq. Such a large number of severely affected patients represent an enormous human suffering for many families and they need of intensive supportive therapy with little or no chances of being cured. This fact make the using of new techniques for detection, prevention and treatment of such disorders consequences acquired a highly priority. In this study, we found that the age of HbA2, HbAF, and HbAS carriers was older than HbF and HbS hemoglobin type carriers. The 70 unrelated blood samples derived from Iraqi β-thalassemia and sickle cell carriers were analyzed to elicited several forms of sickling and β-thalassemia hemoglobin protein pattern as the following: Thalassemia Trait: The presence of mild microcytosis, in the absence of iron
deficiency, suggests the presence of β-Thalassemia [25]. These carriers or individuals with β-thalassemia trait are essentially normal, although they can usually be detected by screening red cell indices that demonstrate a reduced mean corpuscular volume (MCV) and reduced mean corpuscular hemoglobin value (MCHV), gel electrophoresis precisely determine the elevation of hemoglobin A2 level thalassemia minor (figure 4C and E, table 1). Our result here came to be in consistent with [26]. This Thalassemia intermedia pattern may results from elevation of fetal hemoglobin (HbF) to around 30-50% as illustrated in (figure 4C)

**Thalassemia disease:** Thalassemia gene carriers characterized by the absence of S form of hemoglobin (HbS) having a high concentration (70- 90%) of fetal form of hemoglobin (HbF) accompanied with HbA2 hemoglobin pattern as lower as than normal (less than 1%)(see fig 4D), and a highest percentage of hypochromia and reticulocytes obtained by blood film monitoring.

**Sickle cell – Thalassemia disease:** The transmission of the sickling gene from one parent and that of thalassemia from the other parent results another variant of sickle cell-thalassemia disease. The severity of this condition varies according to the amount of normal beta globin produced by the beta globin gene so the condition appears as less severe than sickle cell anemia. Hemoglobin gel electrophoresis may yield a pattern of 65 % sickle hemoglobin and 34% F hemoglobin as in (fig 5E). The presence of normal (A) hemoglobin in combination with more than 90% S hemoglobin so far has only been encountered in sickle cell-thalassemia disease.

**sickling trait:** The electrophoretic hemoglobin pattern was that of the sickling trait illustrated in (fig. 5). Although most data indicate that HbAS has no significant effect on clinical morbidity or mortality other than a mild anemia. Sickle cell-thalassemia disease was suspected but ruled out by the absence of reticulocytosis and by an electrophoretic pattern of hemoglobin which is typical of the sickling trait as in (fig 5C). Certain studies suggest that the fractional HbS content of sickle trait erythrocytes may influence the severity of certain clinical complications [24]. The amount of S hemoglobin in sickle cell trait carriers has been found to vary from 34 to 39 % (fig 5C), without any apparent correlations to the severity of their clinical manifestations. We encountered several instances of low hemoglobin values in patients with a positive sickling test, caused by a variety of anemias superimposed on the sickling trait. Electrophoretic analysis of the hemoglobin of some individuals revealed that even in the absence of a positive sickle cell test by blood film test, erythrocytes may still contains small amounts of S hemoglobin in consent with [22]. The reduction of the HbS level in the sickle cell trait associated with α thalassemia can be explained by a greater affinity of B^A than B^S chains for α chais in limited supply [23].

**Sickle cell anemia (SCA):** Electrophoresis of hemolysates of patients with sickle cell anemia produced a characteristic pattern which consisted of a major component of hemoglobin S shows variations from (70 to 91 %) (Fig 5D) and usually with reciprocal values for F hemoglobin (Data not shown). Fetal hemoglobin (HbF) is a major contributor to the remarkable phenotypic heterogeneity of sickle cell anemia and influences the levels of disease severity [24]. Since in some patients with the disease, the minor component was less than 5 % on even absent, quantitation of the fetal fraction from gel electrophoresis. Our results indicated that SCA patients’ blood film is characterized by low rate of hypochromic and reticulocytes cells by microscopic diagnoses.

By indirect investigations, we revealed that some anemia patient may suffered from transient hemolysis due to a pyogenic infection, occurrence of G6PD (glucose - 6 - phosphate dehydrogenase deficiency), but
not due to heritable hemoglobin disorder. In those individuals hemolysis was ceased after the appropriate treatment. It may be argued that the high consanguinity rate among homozygous individuals may have affected the actual frequencies of hemoglobinopathies emergence. The significant stimulus to carry out such studies was the demonstration that hemoglobin in hemoglobinopathies patients is electrophoretically different from normal adult hemoglobin and their assessment does not require a complex apparatus and special skills necessary for common detection methods. Another advantage is that hemoglobin electrophoresis can be used for mass screening purposes since multiple samples can be separated simultaneously.

The results presented here, may provide a good reliable foundation for introducing early screening measures as well as marriage/genetic counseling and planning for a regional preventive program for hemoglobinopathies in Nasseriah, that help the affected families in improving their medical services via helping their clinicians and genetic counselors in evaluating their variants and designing their treatment regimens.

<table>
<thead>
<tr>
<th>Hemoglobinopathies</th>
<th>Hb(A5)</th>
<th>Hb(S)</th>
<th>Hb(FS)</th>
<th>Hb(F)</th>
<th>Hb(AF)</th>
<th>Hb(A2)</th>
</tr>
</thead>
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<tr>
<td>n</td>
<td>15</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Age range year</td>
<td>28.3-34</td>
<td>2-28</td>
<td>1.8-5.2</td>
<td>1-2.8</td>
<td>2-6</td>
<td>2.5-7</td>
</tr>
<tr>
<td>Hb g/dl</td>
<td>10.04 ± 1.35</td>
<td>9.3 ± 1.5</td>
<td>8.3 ± 1.8</td>
<td>5.3 ± 1.4</td>
<td>7.4 ± 1.6</td>
<td>9 ± 1.3</td>
</tr>
<tr>
<td>(8-12)</td>
<td>(7.8-12.2)</td>
<td>(6.2-12)</td>
<td>(3-8)</td>
<td>(5.5-10.8)</td>
<td>(6.8-10.8)</td>
<td></td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>2.67 ± 1.06</td>
<td>1.83 ± 0.53</td>
<td>3.74 ± 1.19</td>
<td>6.1 ± 1.9</td>
<td>3.3 ± 0.79</td>
<td>2.90 ± 0.87</td>
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<td>(1.2-4.06)</td>
<td>(1.96-3.2)</td>
<td>(2.5-4)</td>
<td>(4-10)</td>
<td>(2-4.8)</td>
<td>(2-3.88)</td>
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<td>Hb F (%)</td>
<td>48.2 ± 8.19</td>
<td>33.6-60</td>
<td>86.62 ± 5.8</td>
<td>40.15 ± 6.77</td>
<td>28.8-48</td>
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<td>Hb A2</td>
<td>1.27 ± 0.45</td>
<td>0.92 ± 0.51</td>
<td>0.83 ± 0.66</td>
<td>1.58 ± 0.71</td>
<td>9.33 ± 2.49</td>
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<td>(0.53-2.02)</td>
<td>(0.22-2.0)</td>
<td>(0.1-2.02)</td>
<td>(0.8-3.2)</td>
<td>(6.2-12.3)</td>
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<td>Hb S</td>
<td>42.41 ± 6.70</td>
<td>30.8-55</td>
<td>78.4-92.06</td>
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<tr>
<td></td>
<td>(55-63)</td>
<td>78.4-92.06</td>
<td>30.8-50.2</td>
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</table>

Table 1: Representative values, the number, age range, reticularity presence, abnormal Hb range, and concentration of AF,S and A2 hemoglobinopathies patient involved in this study.
Detection Of Hemoglobinopathies In Hypochromic, Microcytic And Sickeled Cell Blood Films By Hemoglobin Electrophoresis

**Figure 2: Representative characteristics of Beta thalassemia and sickle cell disorders**: left view is a normal blood film picture, normal red cells (erythrocytes) showing little variation in size and shape, an approximately round outline and a small area of central pallor in some of the cells. While the medium view represents Beta thalassemia blood film showing hypochromia and marked microcytosis, anisocytosis and poikilocytosis, and right picture summarized clearly sickle cell anemia marked features.

**Figure-3: The characterization of hemoglobinopathies by gel electrophoresis**, the different molecular weight of hemoglobin molecules types make travel of electrically charged proteins seems at different locations. (A) panel 2 (AS) sickle cell disease hemoglobin pattern panel 3 HbAF hemoglobin type pattern Panel 4 represents HbFS , and Panel 5 a normal pattern. (B) panel 2 HbAS pattern, panels 3,4 and, 5 depicted (AF) thalassemic hemoglobin pattern. All results were compared with panel 1 (control band).
Figure 4: Different indices of beta thalassemia, patient samples were analyzed by hemoglobin electrophoresis in comparison with control (A) normal individuals (B, C, D, and E) representative examples of different patterns reflected the facts of hemoglobin protein defects in different thalassemia.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control</th>
<th>Hb-normal</th>
<th>Hb/AF</th>
<th>Hb/F</th>
<th>Hb/A2</th>
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</thead>
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<tr>
<td>Hb/A</td>
<td>97.5-98.5</td>
<td>97.25</td>
<td>96-98</td>
<td>33.13</td>
<td>48.00</td>
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<tr>
<td>Hb/S</td>
<td></td>
<td></td>
<td>0.1%</td>
<td>35.84</td>
<td>51.94</td>
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<tr>
<td>Hb/F</td>
<td></td>
<td></td>
<td>0.1%</td>
<td>77.83</td>
<td>77.29</td>
</tr>
<tr>
<td>Hb/A2</td>
<td>1.5-3.5</td>
<td>2.75</td>
<td>1.5-3.5</td>
<td>0.04</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Figure 5: Different indices of sickle cell anemia, patient samples were analyzed by hemoglobin electrophoresis in comparison with control (A) normal individuals (B, C, D, and E), representative examples of different patterns reflected the facts of hemoglobin protein defects in sickle cell anemia patients.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control</th>
<th>Hb-normal</th>
<th>Hb/AS</th>
<th>Hb/S</th>
<th>Hb/FS</th>
</tr>
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<tbody>
<tr>
<td>Hb/A</td>
<td>97.5-98.5</td>
<td>97.25</td>
<td>96-98</td>
<td>69.35</td>
<td>65.61</td>
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<td>Hb/S</td>
<td></td>
<td></td>
<td>0-1%</td>
<td>34.39</td>
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<tr>
<td>Hb/F</td>
<td></td>
<td></td>
<td>0-1%</td>
<td>91.80</td>
<td>91.80</td>
</tr>
<tr>
<td>Hb/A2</td>
<td>1.5-3.5</td>
<td>2.75</td>
<td>1.5-3.5</td>
<td>1.12</td>
<td>1.07</td>
</tr>
</tbody>
</table>
REFERENCES


Appendix

Figure 1: HelloBio software screen: the software presents the following: A- the hemoglobin types distribution B- gel band pattern and C- hemoglobin percentage table
كشف اعتلالات الهيموغلوبين في الأشخاص حاملي كريات الدم الحمراء المنجملة والصغيرة الشاحبة بواسطة الترحل الكهربائي للهيموغلوبين

د. أحمد عبد الكاظم ناصر* د. طالب حسن علي** د. مويض ناجي مجيد***

الملخص
الخلفية: جزء من الهيموغلوبين (خضاب الدم) بروتين يحمل شحنة كهربائية سالبة وذلك يجعلها تتفاعل مع التيار الكهربائي وتحرك نحو القطب الموجب عند مروحة الترحل الكهربائي. تتأثر حركتها هذه بنوع الشحنة (سالبة أو موجبة) وقوتها وكذلك بالوزن الجزيئي لنوع الهيموغلوبين. هناك أكثر من 300 فئة وراثية معروفة حتى اليوم قد تحدث لجزء الهيموغلوبين بعضها ذات تداعيات سرية واضحة تهدد الصحة خاصة في حالات تامن المزج.

الاهداف: تكشف بعض من اعراض هيموغلوبين الدم مثل الانتقادات تأثيره على الدورة الدموية وتقلص الدم المنجل عن طريق استخدام تقنية الترحل الكهربائي للهيموغلوبين لعينة من مجتمع مدينة الناصرية في العراق.

الطريق: ثمانية عينة من الدم سُجِّيت من أفراد يشتبه بتعانين من قصر الدم من مدينة الناصرية فحصوا بالتقنية المتقطعة في كلية الطب - جامعة ذي قار، من العينات شُخصت 8 من المرضى. كانت لحالات الهيموغلوبين الدم كان من بين 30 عينة 37% على أنها نادس من هيموغلوبين HbAS و 28% على أنها تأثيرها، HbFS و 3% على أنها قصر الدم المنجل، HbSS و 12% كلما كانت معروفة بقيل الأشخاص ممن لهم مستويات منخفضة فقط في البحث.

النتائج: هام قصر الدم المنجل والبيتا تأثيرها كانا من مراجعات وحدات فحوص الدم في مستشفيات مدينة الناصرية. دراسة بروتين هيموغلوبين الحالية للأفراد ذوي مستويات الهيموغلوبين الوراثية كشفت عن وجود إدخال خطأ في تعالي مناطق هيموغلوبين معينة مختلفة وشائعة ينتج عنها ظهور اعتلالات هيموغلوبين مزوجة كالآتي: 15% من المرضى يحملون قطر الدم المنجل ويحملون نعطات هيموغلوبين HbAS (18.7٪) و 20٪ منهم قصر الدم المنجل الطيفي بينما 7 مراعي HbS 12٪ و 7 مراعي هب هب HbFS كانوا على جميعهم HbA2 في مرضى HbS و 7 مراعي HbA2 كانوا كالآتي: 9٪ HbA2 و 12٪ من حجم العينة يحملون هب HbA2 في مرضى HbS و 7 مراعي HbA2 كانوا كالآتي: 9٪ هب HbA2.

المستنتاجات: الطريقة التي أُستخدمت في البحث تتيح لمواجهة اعتلالات الدم في هذا البحث بطريقة سريعة تسمح بتقديم الكمي والنسب الدقيقة لأشكال بروتين هيموغلوبين الموجودة. فالترحل الكهربائي للهيموغلوبين الشائع في البلاد هو طريقة سهلة ونوعية لدراسة اعراض هيموغلوبين المروثة في الوسط القاعدي في البلاد (8-6.4). نقترح تعميم استخدام التقنية المتقطعة لتشمل تحديد اعتلالات الدم الأخرى.