
Detection of Haemoglobin S Among Libyan Children in Sebha Medical Centre

Abdulgader Saleh Ali,* Alghanay Abobaker Alghanay,** Saad Mohammed Othman,**
Mohammed Mahmud Ahmed,**

Abstract:

Background: Sickle cell anaemia is caused by a single-base mutation leading to substitution of amino acid valine for glutamic acid at the sixth position of the β -globin chain. In the homozygous (Hb SS) both genes are abnormal, whereas in the heterozygous (Hb AS) only one chromosome carries the gene. Clinical presentation of sickle cell disease occurs frequently in children admitted in the Department of Paediatrics in Sebha Medical Centre.

Objective: The main aim of this study, was to apply the alkaline cellulose acetate electrophoresis technique to investigate the presence of haemoglobin S in blood collected from these children.

Materials and Methods: Blood samples were collected in EDTA anticoagulant tubes from 19 child (males = 13 and females = 6), suspected with sickle cell anaemia, and used for haematology and haemoglobin alkaline electrophoresis (pH 8.4) analysis.

Results: The haematological indices results were: total haemoglobin ($X = 7.27 \pm 1.35$ g/dl), RBC ($X = 2.56 \pm 0.53 \times 10^6/\text{mm}^3$), MCV ($X = 88.74 \pm 8.05$ fl), MCH ($X = 29.87 \pm 2.40$ pg) and Total leukocytes count (TLC) ($X = 13.69 \pm 6.31 \times 10^6/\text{mm}^3$). The results of haemoglobin fractions as separated by alkaline electrophoresis were: 7 (36.8%) children had a haemoglobin fraction migrated in the position of Hb A, and 8 (42.1%) children had a single abnormal fraction migrated in the position of Hb S. Whereas, 4 (21.0%) children had two fractions migrated in the position of Hb A and S.

Conclusion: We conclude that a large prospective study would lead to better understanding of the distribution of these genetic disorder among Libyan populations, at least in this region of the country. Consequently, better genetic counselling and more efficient way of utilising the health care resources as well as improving family planning programmes could be achieved.

Key words: Haemoglobin S, sickle cell anaemia, Libyan children.

Introduction:

The inherited disorders of haemoglobin "haemoglobinopathies", are genetic disorders of structure and synthesis of one or more of the globin polypeptide chains,^{1,2,3} without inherited disorders of haem moiety.² Haemoglobinopathies can be divided into a number of overlapping groups: the structural haemoglobin variants involves; substitution, addition, or deletion of one or more amino acids of the globin chain; the thalassemia, a disorder result of quantitative defect in production of globin chain; combination of both disorders, resulting in a complex haemoglobinopathies; and hereditary persistence of fetal haemoglobin, an asymptomatic disorder.² Haemoglobin S is the most important structural abnormality of haemoglobin chain that leads to sickle cell disease. It is a major health problem which causes high morbidity and mortality in many

countries. In fact, this disease is caused by a single-base mutation of adenine to thymine that results in substitution of valine for glutamic acid at the sixth position of the β -globin chain.^{2,3,4}

Sickle cell anaemia (Hb SS), is chronic, moderate to severe haemolytic anaemia in homozygous person, both genes are abnormal and inherited from both parents.^{2,3} The pathophysiological outcome of the homozygous (Hb SS) can be described by haemolytic anaemia, and sickle cell crisis, as a result of polymerisation of Hb S within the red blood cells.² The primary cause of the clinical manifestations are complication from a moderate to severe anaemia associated with chronic haemolysis and the consequences of vaso-occlusion of the microcirculation plugged

*) Department of Biochemistry, Faculty of Medicine, Sebha University, Sebha, Libya.

**) Department of Paediatric, Faculty of Medicine, Sebha University, Sebha, Libya.

by sickle cells.³ Whereas, The sickle cell trait (Hb AS) is a heterozygous state, as the abnormal gene from one parent and the normal from the other parent,^{2,4,5} when both parents have sickle cell trait, one in four their children will develop sickle cell anaemia.⁴

Persons with sickle cell trait are usually asymptomatic and have normal haemoglobin and red cell survival. There are rare complications in Hb AS patients, but they need genetic counselling.² However, individuals with inherited two β -globin genes homozygous (homozygous) have sickle cell anaemia, and more than 90% of their total haemoglobin is Hb S.³ The sickle cell disease occurs mainly in Africans,^{4,5} where 25% of the population carry the gene, but it has also been found in India,⁶ the Middle East,^{4,7} the Caribbean and southern of the USA.⁴ Haemoglobin S (Hb S) is becoming the most common genetic abnormality in France.⁸ The incidence of sickle cell anaemia among Libyan population is unknown, at least in Sebha region. Nonetheless, one electrophoretic study of abnormal haemoglobin conducted in Benghazi area, indicated that the incidence of the sickle cell gene is low, and the thalassaemic gene is high.⁹ The haematological and clinical presentation of sickle cell disease is occurring frequently in children in the southern region of Libya, and the diagnosis mainly based on these presentations. Hence, we aimed to use the cellulose acetate electrophoresis technique for the identification of haemoglobin S variant in these children.

Materials and Methods:

Patients and Sample collection

Blood samples were collected from Libyan children (Female = 13, Male = 6), suspected with sickle cell anaemia, admitted in the paediatric Department of Paediatrics at Sebha Medical Centre, Sebha, Libya, and also from their parents after explaining to all the objective of this work. The blood was collected in tubes with EDTA anticoagulant. It was confirmed that none of these children and parents had recent blood transfusion.

Haematological analysis

The haematological analysis of total haemoglobin (Hb), red blood cells (RBC's), mean corpuscular volume (MCV), and the mean corpuscular haemoglobin (MCH) were done on the same day using automated cell

counter (Sysmex, KX-21N, Sysmex cooperation, Kobe, Japan).

Preparation of haemolysates for electrophoresis

For the haemoglobin electrophoresis all the samples were treated as previously described,¹ 1ml from each sample was removed to clean tube filled with normal saline and mixed by inversion, then centrifuged at 2500 rpm for 10 minutes. After that, 0.2-0.3 ml of red blood cells were withdrawn and washed 3 times with normal saline. Then 6-10 volumes of distilled water with one-half volume of toluene were added to the packed cells. After gentle mixing, cells were allowed to haemolyse for 5-10 minutes. The material (debris) was precipitated by centrifugation for 5-10 minutes. Hereafter, the haemoglobin concentration of the clean supernatant was standardised to 7g/l, and transferred to clean tubes to be stored frozen until the electrophoresis analysis.

Quality Control

As with any analytical procedure a control sample was run with each batch. The control samples were from three different known haemoglobin (Hb A,F,S) had been prepared as the following: (1) Haemoglobin A control was prepared from adult individuals, (2) Haemoglobin F control was prepared from a cord blood and (3) Haemoglobin S control was prepared using blood collected from a child had been diagnosed with sickle cells disease according to the results obtained from the Biosciencia (GmbH, Postfach 1628, Mainz, Germany).

Analytical Procedure

Cellulose acetate electrophoresis: Helena electrophoresis system (Helena Laboratories, Beaumont, Texas, USA) was used to separate the haemoglobin in small samples of haemolysates prepared from whole blood using Titan cellulose acetate plates, specially designed for this purpose. Electrophoresis was at 350 V for 30 minutes in Super-Heme buffer (tris-EDTA-boric acid), pH 8.4 and ionic strength 0.025.¹⁰ The three control samples of different haemoglobins variants (A,F,S) were applied with each plate as markers for the identification of the haemoglobin bands. The electrophoretic procedure was carried out as previously described and recommended by the manufacturer without significant modifications, and at the end of the procedure plates were

stained in Ponceau S, de-stained then dried in hot oven.¹⁰

Qualitative evaluation of haemoglobin bands

The haemoglobin bands appeared on oven dried transparent plates were inspected visually for the presence of abnormal haemoglobin (Hb S). The haemoglobin control samples were used as markers to help in the identification of the haemoglobin bands.

Results:

The haematological indices were investigated in a group of male and female Libyan children suspected with sickle cell anaemia, and compared to the reference values in children at the same age group.¹¹ Table 1. Illustrates; the mean value of the haematological indices results as the following: The mean value of total haemoglobin was 7.27 ± 1.35 g/dl, it was lower than the reference value in children ($11.5 - 15.5$ g/dl) of the same age group. The age group of our children was between 6 and 12 years. The mean value of the red blood cells in this group of patients were $2.56 \pm 0.53 \times 10^6/\text{mm}^3$, it was also lower than the reference value ($4.0 - 5.2 \times 10^6/\text{mm}^3$) of the same age group. However, the haematological indices results represented by the mean corpuscular volume (MCV) was 88.74 ± 8.05 fl and the mean corpuscular haemoglobin (MCH) was 29.87 ± 2.40 pg, both were within the reference value (77 - 95 fl and 25 - 33 pg), respectively, of the same age group.

All of these children were suspected with sickle cell anaemia according to previous positive solubility test results. However, the percentage analysis of all the separated haemoglobin fractions shown that: 7 (36.8%) children (boys & girls) had haemoglobin fraction (single band) migrated in the position of Hb A; and 8 (42.1%) children (boys & girls) had a single abnormal fraction migrated in the position of Hb S; and 4 (21.0%) children (boys & girls) had two fractions migrated in the position of Hb A and S. All migrated corresponding to the haemoglobin positions (Hb A,F,S) of the control samples.

Table 2. Presents the possibilities of the inheritance of haemoglobin A and S from parents to children. They are unrelated families, for three families; fathers and mothers had only single bands of Hb A variant, and none of their tested children appeared to carry the abnormal haemoglobin gene since no band in the position of Hb S appeared. In another four families, two fathers were Hb S trait and two normal (Hb A) mothers, the children were Hb S trait or Hb SS disease. In five families, mother's blood only tested, the Hb AS variants were detected in all. Their tested children had Hb SS disease gene. Unfortunately, we were unable to collect blood from their fathers, beside their mothers. However, Figure 1. illustrates, only a representative example of general mobility of haemoglobin fractions of both the control samples and samples of two families as separated by electrophoresis procedure: In the control sample position 1, there was a single band of adult haemoglobin (Hb A), and in the control position 2, there was again a single band of fetal haemoglobin (Hb F), and in the control position 3, there was a single band of haemoglobin S (Hb S). However, in the position 4, for a mother of suspected child, two bands were detected corresponding to Hb A and S positions, and in the position 5, her child, a single band corresponding to Hb S was detected. Unfortunately, for this family, we were unable to collect blood from the father. In positions 6, 7 and 8, of a father, a mother and their suspected son, respectively, single bands of adult haemoglobin (Hb AA) were detected.

Table 1: The mean values of the haematological indices results in the suspected children with sickle cell anaemia.

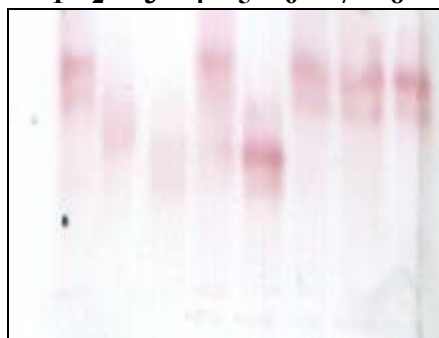
Age (years)	$X = 8.18 \pm 3.22$
Hb (g/dl)	$X = 7.27 \pm 1.35$
RBC ($10^6/\text{mm}^3$)	$X = 2.56 \pm 0.53$
MCV (fl)	$X = 88.74 \pm 8.05$
MCH (pg)	$X = 29.87 \pm 2.40$
TLC ($10^6/\text{mm}^3$)	$X = 13.69 \pm 6.31$

Table 2: the possibilities of the inheritance of haemoglobin A and S from parents to their children as detected by the cellulose acetate electrophoresis. B = boys and G = Girls.

Family no.	Offspring	Hb	Father	Mother
			Hb	Hb
1	B1	SS	AS	AS
	G1	AS		
2	B1	SS	AS	AS
	B2	AA		
3	G1	AS	AS	AS
4	B1	SS		AS
5	G1	AA	AA	AA
6	G1	SS		AS
7	B1	AA	AA	AA
8	B1	SS		AS
9	G1	SS	AS	AS
	G2	AA		
	B1	SS		
	B2	AS		
	B3	AA		
10	B2	AS		AS
11	B1	SS		AS
12	B1	AA	AA	AA
	B2	AA		

Positive (+)

1 2 3 4 5 6 7 8



Negative (-)

Figure 1: Photomicrograph representative of cellulose acetate membrane at pH 8.4, showing relative positions of various haemoglobins. The common haemoglobin found were of (1) Control (Hb A), (2) Control (Hb F), (3) Control (Hb S), a family of (4) a mother, and (5) a son, another family of (6) a father, (7) a mother, and (8) a son.

Discussion:

Blood diseases could be monitored by complete blood count (CBC) test. The red blood cell (RBC) count is a common one; it reflects the actual number of red blood cells per volume of blood, its increase and decrease a sign of abnormality.¹² In these children, our result shows lower values than the reference range of the same age group, as expected in condition of suspected anaemia. The estimation of total haemoglobin is another common test for the evaluation of blood

diseases; again, our results were lower than the reference range of the same age group, an indication of suspected anaemia. On the other hand, the haematological indices (PCV, MCHC, MCH, and MCV) results were within the reference range. Unaltered values of MCHC²¹ and higher value of PCV,²¹ and lower values of MCV and MCH^{21,22} were previously reported. Our findings may exclude the possibility of haematological diseases caused by megaloblastic anaemia, iron deficiency

anaemia, thalassemia or hereditary spherocytosis, a relatively rare congenital disorders.^{4,12}

The cellulose acetate electrophoresis is playing an important role in routine clinical diagnosis and in life science research.¹⁰ It separates proteins based on their net charges, the haemoglobin analysis is a typical one,^{1,10} and indicate to which group the haemoglobin variant belongs (e.g. confirm haemoglobin S variant).¹³ In this Medical Centre, diagnosis of haemoglobin disorders had been increasingly difficult and mainly based on the haematological and clinical presentations, because of the lack of the suitable technique (e.g. electrophoresis). Hence, it is for the first time the alkaline cellulose acetate electrophoresis was used to confirm the diagnosis of nineteen children from twelve unrelated families by the detection of haemoglobin S. In alkaline conditions, haemoglobin S can be separated from other haemoglobin variants.¹⁰ However, in some cases, other techniques as citrate agar electrophoresis or HPLC chromatography are recommended to differentiate Hb S from Hb D, co-migrate at the same speed.¹ But, unlike Hb S and Hb C; Hb D is not found in every ethnic population.¹ In fact, Hb D has been connected with Indians,¹⁶ Asians¹⁷ and Berber-speaking populations.²⁰ In spite of this, we think further analysis of beta-globin Haplotype gene mutation associated with the haemoglobinopathies in this country is required. A previous study of five hundred and forty-five subjects from Benghazi, indicated that Hb AD trait were only found in two subjects, and that the incidence is low (19). In this study, we relayed on alkaline cellulose acetate electrophoresis and detected single bands of haemoglobin S in 42.1% of tested children, previously tested positive with the solubility test. It is well known that in alkaline conditions, haemoglobin S can be separated from other haemoglobin variants,¹⁰ confirming the diagnosis of sickle cell anaemia.¹³ Sickle cell anaemia, a sever form of anaemia, as a result of red blood cells disorder, which characterised by an abnormal cells shape, leading to premature destruction of RBC.² Moreover, it is a hereditary disease which can be passed along to children through their parent's genes.⁴ However, in about 21.0% of these suspected children, we detected fractions of both Hb A and S, indicating that they are haemoglobin S gene carriers (sickle cell trait), again they had positive solubility test, a simple

and rapid test for the detection of haemoglobin S, but it doesn't differentiate between sickle cell trait from sickle cell disease (14). In a heterozygous subject presenting with pathological manifestations, more sophisticated biochemical tests or molecular biology investigations may be necessary to determine the cause of the disease.⁸ In general, when both parents or one of them is a carrier of defected gene, that gene could be passed to their children, as a result, sickle cell trait or sickle cell anaemia manifestation.^{2,4} Figure 2. Shows the proposed possibilities of passing haemoglobin S gene from parents with sickle cells trait to their children.

On the other hand, approximately 36% of these children had single band of haemoglobin A; such findings excluded the sickle cell anaemia, as previously been diagnosed with the solubility test. The common reported causes of false positive solubility test are; abnormal plasma proteins in whole blood samples, RBC's contain Heinz bodies, too high or too low PCV, old reagents¹⁵ and polycythemia.¹⁸ Despite, that the number of cases wasn't large, we presume that the method was capable of detecting the haemoglobin S, but this result may not reflect the incidence of sickle cell anaemia or the prevalence of haemoglobin S in this region. Also, our screening of the literature for the prevalence of haemoglobin S in this region came out with nothing. Thus, further studies based on better conditions should be conducted to set up its prevalence in the area.

Conclusion:

We may conclude that the analytical work for detecting haemoglobin S variant can be undertaken by a simple manual technique, and that a large prospective study would lead to better understanding of the distribution of this genetic disorder among the Libyan population, at least in this region of the country. Consequently, leading to better genetic counselling and more efficient way of utilising the health care resources, besides improving family planning programmes; since, we have high rate of consanguinity and extensive families.

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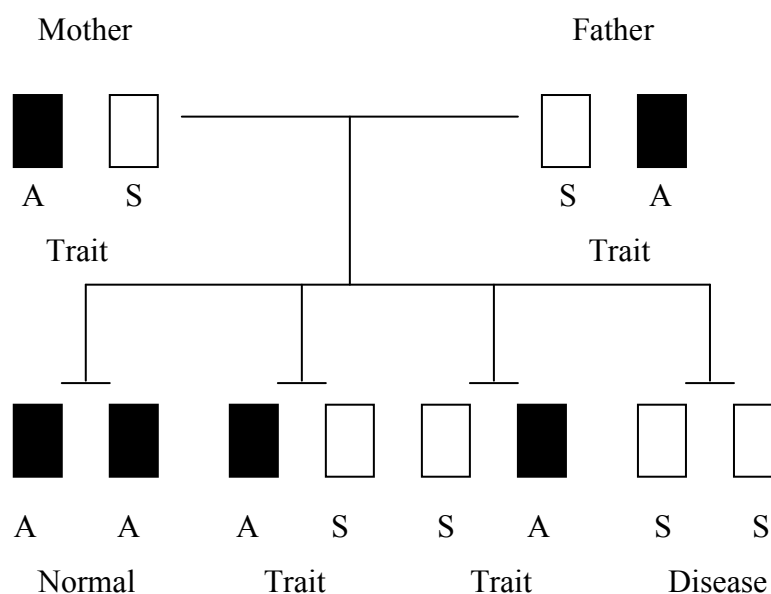


Figure 2. The possibilities of the inheritance of haemoglobin S from parents by their children.

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