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Molecular Genetic study of β–thalassemia minor syndrome

A thesis

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By

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﴿٨٣﴾ قُلْ كُلٌّ يَعْمَلُ عَلَىٰ شَاكِلَتِهِۦ فَرَبُّكُمْ أَعْلَمُ بِمَنْ هُوَ أَهْدَىٰ
سَبِيلًا ﴿٨٤﴾ وَيَسْـَٔلُونَكَ عَنِ ٱلرُّوحِ ۖ قُلِ ٱلرُّوحُ مِنْ أَمْرِ رَبِّى
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الاهداء

الى ارض عظيمة انجبتني ....تاج الشرق ومملكة العصور.....لانها الاوفى والاعز...

الى من ارتقيا مراتب الذرى.....ومرافئ الشمس....

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List of contents

List of Figures

List of Tables

List of abbreviations

No.	Abbreviation	Full name
1.	bp	Base pair
2.	DNA	Deoxyribonucleic acid
3.	HbA	Adult hemoglobin
4.	HbA_2	Adult hemoglobin subunit 2
5.	HbF	Fetal hemoglobin
6.	Kb	Kilo base pair
7.	mRNA	Messenger RNA
8.	PCR	Polymerase chain reaction
9.	RFLPs	Restriction fragment length polymorphism
10.	RNA	Ribonucleic acid
11.	α –hemoglobin	Hemoglobin type alpha
12.	β –hemoglobin	Hemoglobin type beta
13.	γ- hemoglobin	Hemoglobin type gamma
14.	δ –hemoglobin	Hemoglobin type delta
15.	MCV	Mean corpuscular volume
16.	MCH	Mean corpuscular hemoglobin
17.	PCV	Packed cell volume
18.	RBCs	Red blood cells
19.	WBCs	White blood cells

Abstract

In this study, molecular and genetic analysis of *β –thalassemia minor* in a sample of Baghdad population were made. The study included hematological screening of peripheral blood cells, pedigree analysis for carriers s' families and their relatives, hemoglobin electrophoresis, and molecular analysis of β –globin gene, all in comparison with normal people.

The first part included hematological prescreening for 100 carrier of *β –thalassemia minor* attending Central Health Public Laboratories came from Baghdad. The hematological tests were packed cell volume (PCV), mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH). Results obtained showed that PCV was 32 -38 % for carriers, MCV was 62 -65 fl for carriers, and MCH was 19 -26 pg for carriers.

Moreover, hemoglobins A, A_2, F were also determined using electrophoresis that showed, carriers had elevated levels of HbA_2 and decreased levels of HbA in comparison with normal people.

In addition, it was found that there is a morphological change of the red blood cells (RBCs), since RBCs took abnormal shapes due to the disease. The white blood cells were at normal levels in carriers. This part of the study included also, hemoglobin analysis of carrier parents and their affected children with β –thalassemia syndrome. Three electrophoresis techniques were used and results obtained showed that there is a remarkable difference in hemoglobin electrophoresis among carriers who represented blood samples taken to complete this study. Results showed that there is also a remarkable difference in hemoglobin electrophoresis in carriers and patients, in comparison with normal people.

The pedigree analysis of families with β –thalassemia syndrome was made depending on data obtained previously and family history. The analysis

showed the transfer of the trait among generations, especially in families preferring marriage of relatives.

The second part of this study included the molecular analysis at DNA level for Baghdad families with β –thalassemia, using four specific primers for the PCR amplification and specific enzyme for the restriction analysis. The results showed the presence of frameshift mutation in codon 6 of the β –globin gene in carriers with thalassemia minor (heterozygous) that led to partial depression in gene expression in β –globin gene in comparison with normal people since it occurred in one allele of the gene, which led to the presence of single DNA band after electrophoresis, while results of the normal individuals showed the presence of two DNA bands on the gel, indicating the presence of the restriction site and no changes in the gene expression occurred.

This is the first study concerning the molecular level and mutation type in Baghdad to be compared with the other parts of Iraq and with the neighboring Arab Countries.

Supervison Certificate

We certify that this thesis was prepared under our supervision, at the College of Science, Al-Nahrain University in partial fulfillment of the requirement for the Degree of Master of Science in Biotechnology.

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1.1 Introduction

Thalassemia is an inherited blood disorders combined with decrease in the production of the protein (globin), and the globin protein itself is abnormal (Losekoot *et. al.,* 1991). The red cells morphology is abnormal, compromised by smaller red cells "microcytic" and paler than normal "hypochromic".

The protein part of the hemoglobin molecule is composed of two different chains, alpha and beta, and either can be affected. There are over than 200 different mutations that can cause thalassemia (Kanavakis *et. al*., 1997). However, they can be classified into two main groups: alpha and beta thalassemia according to which of the two globin chains are affected (Winichagoon *et. al*., 1993).

The most severe form is the alpha thalassemia, results in fetal or newborn death, while β –thalassemia can be classified into three categories, the very severe symptoms of anemia which is called β *–thalassemia major,* the symptoms free and no effect on health which is called β *–thalassemia minor*, and the β *–thalassemia intermedia* which is a case between the minor and major types of this disease (Michiels and Giordano, 1996).Thalassemia minor is defined as the most common form of thalassemia, and is also called (thalassemia trait).

A person having thalassemia minor is defined as a carrier. Carriers may cause no symptoms, but changes in blood do occur. Most carriers have completely normal healthy lives (Antonio and Renzo, 2000), but children with thalassemia major develop the disease symptoms within the first year of life, and without treatment, health complications may lead to heart failure and infection, in which both considered to be the major causes of death among those untreated thalassemia major children (Yuregir *et al.,* 1995).

Thalassemia intermedia is much severe than the *thalassemia minor* but less severe than the *thalassemia major*, and children with *thalassemia intermedia* may develop some of same complications conferred by *thalassemia major*. All forms of thalassemia can not be caught from another individual who has it, and transmitted only through heredity, so the disease is passed on through parents who carry the globin gene disorder. A carrier has one normal gene and one thalassemic gene in all body cells. When two carriers become parents, there is a 25% chance of producing an affected child (thalassemia major), 50% chance of producing a carrier child, and 25% chance of producing a healthy child.

These ratios are the same for each pregnancy when both parents are carriers (Wainscoat *et al.*, 1983).

Measuring the red blood cell indices is important in the diagnosis of β –*thalassemia minor*, which reveal microcytic hypochromic anemia.The diagnosis also relies upon the hemoglobin analysis that reveals decreased amounts of hemoglobin A (HbA) with increased HbA_2 amounts of carriers in comparison with normal values. Mutation analysis of β –globin gene is useful in diagnosis of the most mutation types and also useful in testing all members of a family, who are suspected to be thalassemic carriers, and in the prenatal diagnosis (Olivieri and Nathan, 1994), on the other hand, mutations that alter the β –globin gene are detected by several PCR –based procedures, but the most commonly used methods are primer –specific amplification, with a set of probes or primers complementary to the most mutations in the population from which the individual originated. If the mutation escapes detection, a denaturating gradient gel electrophoresis followed by DNA sequencing is performed (Kanavakis *et al.*, 1997). In addition, carriers rarely cause clinical disease and they do not require any treatment unlike β –*thalassemia intermedia*

and β –*thalassemia major* patients in which they require blood transfusion. Moreover, carriers recognition is important for purpose of genetic counseling and in warning carriers about the potential risks of marriage to another carrier.

Aims of the study

1. The determination of suitable strategy for the rapid hemoglobin abnormalities analysis that cause the β –thalassemia syndrome.
2. The implementation of a useful diagnostic procedure based on family, partner analysis and carrier detection.
3. Defining the molecular and genetic basis of β –thalassemia minor, which might be useful in the future to start gene bank for this syndrome.

1.2 Literature review

1.2.1 Thalassemia

1.2.1.1 Definition and History

Thalassemia can be defined as group of inherited diseases of blood and is considered as group of disorders each result from an inherited abnormality of globin production (Giordano *et al*., 1999); these disorders can be defined as hemoglobinopathies (Gulen *et al*., 1999). Hemoglobinopathies is the description of syndromes caused due to hemoglobin synthesis disorders and can be divided into three classes:-

a. Structural variants of hemoglobin as in sickle cell anemia (HbS).

b. HPFH, is a group of disorders signed as hereditary persistence of fetal hemoglobin , which possessing the failure of normal switching phase from the fetal hemoglobin (HbF) to adult hemoglobin.

c. Failure in the synthesis of one or more of the globin chains of hemoglobin, as in thalassemias (Dacie and Lewis, 2001).

Thalassemia represents the major occurrence among the hemoglobinopathies, which means exhibiting the defect in the amount produced of one or both of globin chains resulting in erythropoiesis hemolysis with variable degree of anemia (Gulen *et al.*, 1999).

Others defined as a condition in which a reduction occur in the rate of synthesis of one or more of the globin chains leading to imbalanced globin chain synthesis, defective in the hemoglobin production, and damage to the red cells or their precursors, so that, an increase in the globin subunits occur (Bunn and Forget, 1986).

About one hundred thousand babies over the world are born with the severe forms of thalassemia each year and will survive of severe anemia

during the first year of life associated with splenomegaly and bone changes (Cooley and Lee, 1925).

Thalassemia occurs sporadically in every racial group and considered as the most public health problems in many parts of the world, it is widely distributed in the Mediterranean region, Middle East, South East Asia, China, India and North Africa (Kazazin and Boehm, 1988).

The history of thalassemia started with the discovery of Cooley and Lee, who first described the severe form of the disease combined with splenomegaly and bone changes occurring in children, in 1925. Then the disease later named thalassemia (thalassa, which is a Greek word means the sea) because the first cases observed in individuals whose ancestors traced to the lands bordering the Mediterranean. At the end of 1930s, the clinical syndrome of thalassemia had been well described, but the description of the heterozygous condition for thalassemia appeared in the Italian literature as early as 1925. The genetic character of this disorder became well known during the 1940s, the period following the late 1940s was one of rapid progress in all aspect of human hemoglobin field. In 1960 -1970 a good working model of genetics of the disorder was developed for the detection of Hb Bart's and HbH and for determination of hemoglobin amounts produced (Weatherall and Clegg, 1981; McGhee and Payne, 1995; Weatherall, 1999).

The progress in molecular techniques, using the recombinant DNA, aid in localization of genes of the human hemoglobin polypeptide chains on the related chromosomes, the γ, δ and β genes are located on chromosome 11; the α –genes are located on chromosome 16 (Deisseroth *et al*., 1975). Neel and Valentine named the mild form of Cooley's anemia "thalassemia minor", which is the heterozygous condition and is characterized of being symptoms free in most cases, while the more severe case than the thalassemia minor is

the thalassemia intermedia; which represents the conditions of low Hb and the development of the disease symptoms do occur.

Patients with thalassemia minor are called carriers and are symptoms free, while the homozygous condition called thalassemia major which represents the severe form of thalassemia (Weatherall, 1986).

1.2.2 Hemoglobin structure

Hemoglobin molecule has been found as an interesting subject to study, by biochemists, physical chemists and chemists for along time (Al-Awamy *et al*., 1986). This molecule is composed of two parts; the heme part and the protein part which is called globin. Globin has four subunits; two alpha polypeptide chains and two beta polypeptide chains, each arranged as more or less spherical subunits (Giordano *et al*., 1998, Gulen *et al.*, 1999).The complete tetrameric hemoglobin molecule has a molecular weight of 64,000 dalton. Heme part is composed of four heme groups. These are molecules composed of protoporphyrin rings, each containing an iron atom at the center (Heisman, 1992). Each of the four globin subunits contains one peptide chain together with its heme group. The most important part of the molecule is the iron atom, because the oxygen molecules attach themselves reversibly during the oxygenation and deoxygenation of hemoglobin in its normal physiological role (Dammas *et al.*, 1995).

1.2.3 Hemoglobin types

Human hemoglobin considered to be heterogenous at all stages of development, so that; different hemoglobins are synthesized in the embryo, fetus, and adult, each adapted to a particular oxygen requirements of these changing environments (Weatherall, 1996). Normal adult human hemoglobin (HbA or hemoglobin A) is composed of two alpha peptide chains and two beta peptide chains, it is written as $\alpha_2\beta_2$. The human fetus has a different type

of hemoglobin (Weatheral and Weather, 1987), which has the same molecular complexity but it is chemically distinguishable.

The human fetal hemoglobin is composed of four peptide chains: two alpha peptide chains, they are the same as in adult type, and two gamma peptide chains, it is written as $\alpha_2\gamma_2$ (Pearson, 1996).

Normal human adult has a minor hemoglobin component produced at 35 weeks of gestation, called hemoglobin A_2. It is composed of two alpha chains and two delta chains ($\alpha_2\delta_2$), which is electrophoretically distinguishable from others. The two alpha chains (HbA_2) are identical with those in the adult protein and also found in fetal hemoglobin (Gries *et al.,* 1985).

The alpha (α) chain contains 141 amino acids; delta chain (δ) contains 146 amino acids (Telen and Kaufman, 1999).The δ chain (of HbA_2) differs from the β chain (of HbA) in only 10 residues. The first eight residues and the C terminal residues (127 to 146) are the same in δ and β chains (Renney and Sharma, 1995). The symbol δ is used to indicate that these peptide chains are distinguishable in their primary structure from the beta (β) and gamma (γ) peptide chains and they are also under separate genetic control.

The α, β, γ and δ peptide chains give good indication that the genetic control is regulated by four types of structural genes (Telen and Kauman, 1999). Two abnormal hemoglobins can be identified in human; these are hemoglobin H, and hemoglobin Barts. Hemoglobin H is composed of four beta peptide chains (β_4), while hemoglobin Barts is composed of four gamma peptide chains (γ_4). These two abnormal hemoglobins are produced in certain conditions of inherited hemolytic anemia (Wickramasinnghe and Lee, 1998). Both are caused due to a relative over production of β or γ peptide chains within a cell leading to tetramer formation of the type. Table (1-1)

summarizes different types of human hemoglobins.

Table (1-1). Different types of human hemoglobins. (Telen and Kaufman, 1999).

No.	Hemoglobin occurrence	genotype
1.	Normal Adult	$\alpha_2\ \beta_2$
2.	Fetal (HbF)	$\alpha_2\ \gamma_2$
3.	(HbA_2)	$\alpha_2\ \delta_2$
4.	Hemoglobin H	β_4
5.	Hemoglobin "Barts"	γ_4
6.	Portland	$\delta_2\gamma_2$

7.	Gower	$\zeta_2 \gamma_2$
8.	Gower II	$\alpha_2 \varepsilon_2$

The Portland, Gower I, and Gower II hemoglobins are the human embryonic hemoglobins (Telen and Kaufman, 1999).

The α-peptide chain of human adult hemoglobin has 141 amino acids, and the β-chain has 146 amino acids, so the difference in number of amino acids between the two chains, α and β, is 85 pairs of amino acids, which is greater than the number of differences between the β, γ, and δ chains found in the human hemoglobins, adult (HbA), fetal (HbF), and hemoglobin A_2 (HbA_2) (Trager *et al.*, 1993).

1.2.4 Globin operon

1.2.4.1 Structure of β-globin gene cluster

The β- globin gene cluster is located on chromosome 11, which contains six genes or pseudogenes, which are spread over 60 kb. They are arranged in the order of their expression during development, as: 5'-epsilon (ε), G-gamma (γ^G), A-gamma (γ^A), pseudobeta (ψβ), delta (δ), and beta (β) -3' .(Fritsch *et al.*, 1980).

Each individual gene and its flanking regions has been sequenced

(Fritsch *et al.*, 1980).The γ^G and γ^A genes share a similar sequence and located on one chromosome, and are identical in the 5' region to the center of the large intron as shown in figure (1-1), but they show some divergence at 3' position (Slighton *et al.*, 1980).

Although there is an individual variability, the alpha genes cluster usually contains one functional ζ gene and two alpha genes designated as α_2 and α_1; it also contains four pseudogenes, duplicated genes that have lost their ability to function. These have been named ψζ, $\psi\alpha_1$, $\psi\alpha_2$, θ. Each α gene is located in a homologous region, approximately four kb long, and is interrupted by two small non –homologous regions. The β –globin gene cluster resembles the α –globin genes, in which it contains a series of single restriction fragment length polymorphisms (RFLPs). The arrangement of RFLPs or haplotypes in the beta globin cluster falls into two domains, one on the 5' side of the β –gene, occupying a region for about 32 kb from the ζ gene to the 3' end of the ψβ gene, and three common patterns of RFLPs are found. The haplotypes of the β –globin gene are similar in most populations, but they differ markedly among individuals of African region (Watson and Kendro, 1961). Figure (1-1) shows the structural organization of the globin gene cluster.

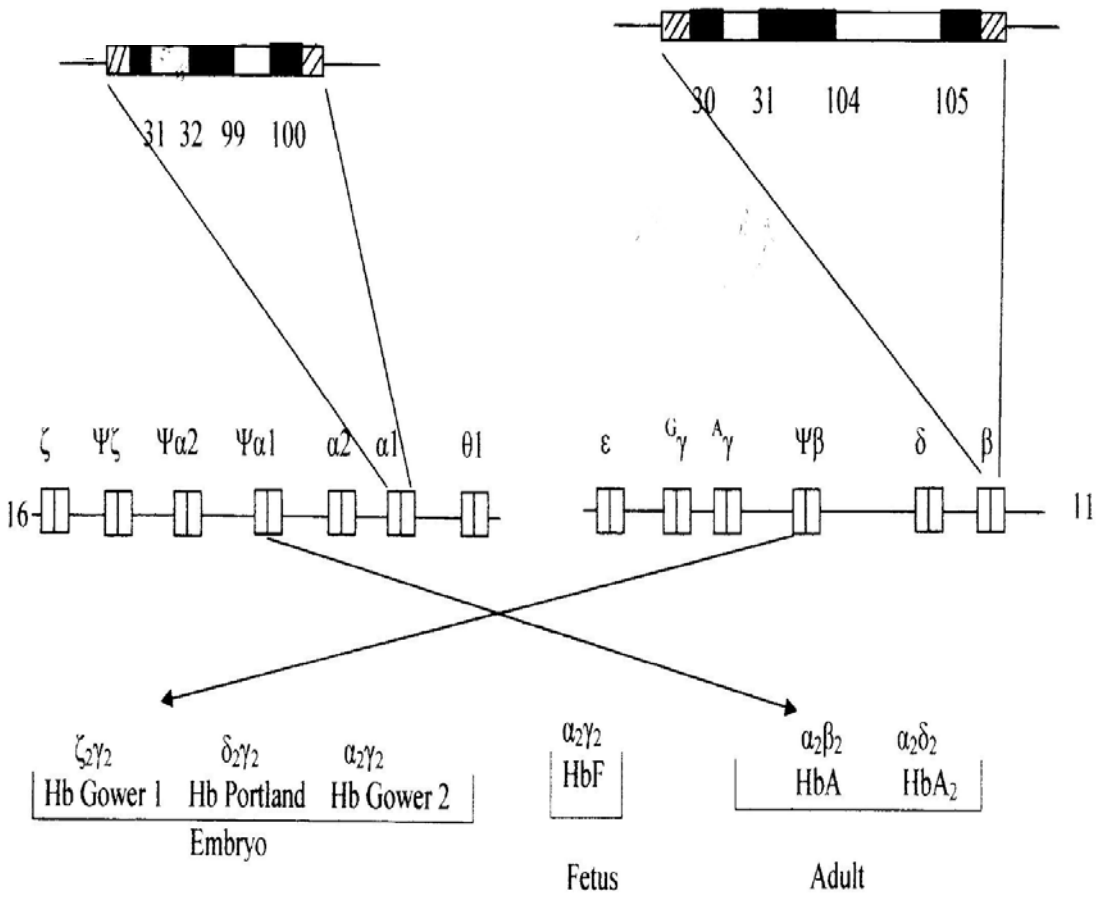


Figure (1-1). Structural organization of the globin gene cluster. On the left is the α globin cluster on chromosome 16, and on the right, the β globin cluster on 11. Each of the globin genes is composed of three exons (black boxes) and two introns (white boxes). Above, an expanded view of the α1 and β globin genes is shown. The stippled areas depict the 5' and 3' non –coding regions. The numbers refer to the α / α positions within the gene. (Dacie and Lewis, 2001).

1.2.5 Clinical syndromes of thalassemia

The clinical syndromes associated with thalassemia arise from the combined consequences of inadequate hemoglobin production and of unbalanced accumulation of one type of globin chain. The former causes anemia with hypochromia and microcytosis; the latter leads to ineffective erythropoiesis and hemolysis. Clinical manifestations range from completely asymptomatic microcytosis to profound anemia which is incompatible with life and can cause death in utero.

Table (1-2) shows the clinical syndromes of thalassemia.

Table (1-2). The clinical syndromes of thalassemia. (Dacie and Lewis, 2001).

Clinically asymptomatic *Silent carriers* α^+ thalassemia trait (some cases) Rare forms of β thalassemia trait *Thalassemia minor* (low MCH and MCV, with or without mild anemia) (α^+ thalassemia trait (some cases) α^0 thalassemia trait α^+ / α^+ homozygotes β^0 thalassemia trait β^+ thalassemia trait δ / β thalassemia trait
Thalassemia intermedia (transfusion independent) Some β^+/ β^+ thalassemia homozygotes Interaction of β^0/ β^0 or β^+/ β^+ with α thalassemia Interaction of β^0/ β or β^+/ β with triple α thalassemia HbH disease α^0/ Hb Constant Spring thalassemia $\beta^0/ \delta\beta$ or $\beta^+/ \delta\beta$ thalassemia compound heterozygotes

some cases of HbE/ β thalassemia and Hb Lepore / β thalassemia Rare cases of heterozygotes for β thalassemia mutation, particularly involving exon 3.
Thalassemia major (transfusion dependent) β^0/β^0 thalassemia β^+/β^+ thalassemia β^0/β^+ thalassemia β^0/ Hb Lepore, β^+/ Hb Lepore thalassemia β^0/ HbE. β^+/ HbE thalassemia

1.2.5.1 Different forms of thalassemia

Thalassemia is extremely heterogenous at the molecular level; over 100 different mutations can cause thalassemia (Weatherall and Clegg, 1972; Weatherall, 1995).Table (1-3) shows the main groups of thalassemia, α and β thalassemia are caused by deletion and non –deletion mutations, whereas δβ – thalassemia is caused by non –deletion mutations.

Table (1-3). The main groups of thalassemia and related disorders (Al – Awamy, 2000).

No.	Type	Phenotype	Type of mutation
1.	α- Thalassemia	α^0	Deletion
			Non – Deletion
		α^+	Deletion
			Non - Deletion

2.	β - Thalassemia	β^0	Deletion
			Non – Deletion
		β^+	Normal HbA_2 type1(Silent)
			Normal HbA_2 type 2
3.	δ - Thalassemia	$(\delta)^0$ $\varepsilon_\gamma \delta\beta$ - thalassemia	Non – Deletion
4.	δβ - Thalassemia	$(\delta\beta)^0$	Non – Deletion
		$(^A\gamma\delta\beta)^0$	

1.2.5.2 Alpha –thalassemia

1.2.5.2.1 Distribution and classification

Alpha thalassemia can be found widely in the Mediterranean region, Middle East, some parts of West Africa, some regions of Indian Subcontinents and South –East Asia. In α –thalassemia, the two α –globin genes are located on chromosome 16 and due to different life stages, two abnormalities can be found, the Hb Bart's and HbH. The Hb Bart's occur in the fetus stage due to defect in hemoglobin F, which is the deficiency of α chains leading to elevated levels of γ_4 tetramers. The HbH occur in adults due to elevated β –chains levels forming β_4 tetramers. These tetramers are soluble and do not precipitate to any significant degree in the marrow, therefore do

not cause severe ineffective erythropoiesis (Schier *et al.*, 1989).There are two main groups of α –thalassemia determinants, first, there are the α^0 –thalassemia in which no α –chains are produced from either α –globin locus on an affected chromosome. Second, there are the α^+ –thalassemia, in which the out put of one of the linked pair of α –globin gene is defective (Lacerra *et al.*, 1991, Kattamis *et al*., 1996). The α^+ –thalassemias are subdivided to deletion and non –deletion types. Both α^0 –thalassemia, deletion, and non –deletion forms of α^+ –thalassemia are all heterozygous at the molecular level (Harteveld *et al.*, 2000).

1.2.5.3 δ – thalassemia

The δ –thalassemia is characterized by a reduced output of δ chains, it is characterized by reduced levels of HbA_2 in heterozygotes and an absence of HbA_2 in homozygotes. A person with δ –thalassemia has no clinical significance (Ryan *et al.*, 2000).

1.2.5.4 δβ –thalassemia

The δβ –thalassemia is heterozygous at the molecular level. In some conditions, no β or δ chains are synthesized, so the classification of these disorders is according to the structure of hemoglobin F which is produced, that is, $^{G}\gamma\,^{A}\gamma$ $(\delta\beta^0)$ and $^{G}\gamma$ $(\delta\beta)^0$ thalassemia. This method of classification is illogical and these conditions are best described by globin chains that are defectively synthesized, this simply $(\delta\beta^0)$ and $(^{A}\gamma\delta\beta)^0$ thalassemias (Weatherall *et al.*, 1989).

1.2.5.5 β –thalassemia

1.2.5.5.1 Types of β –thalassemia

Beta thalassemia can be found either as heterozygous condition (beta thalassemia minor) or as homozygous condition (beta thalassemia major) which requires frequent blood transfusions. A person of beta thalassemia minor described as a carrier of the beta thalassemia gene. Usually carriers are symptomless, while patients of the homozygous state will survive severe anemia and requires blood transfusion. The beta thalassemia intermedia is a condition between the minor and major (Ryan *et al.*, 2000).

The beta thalassemia is divided into two main varieties: in β^o thalassemia, there is a total absence of β –chain production, and in β^+ thalassemia, there is a partial deficiency of β –chain production. For descriptive aim, if the condition in which there is some β –chain production is often referred to as β^+ thalassemia, while when there is marked deficiency of the β –chain, the condition is referred to as β^{++} thalassemia, in which the deficiency is milder (Thein *et al.*, 1990).

The elevated levels of HbA_2 in heterozygotes, compromise the most common form of β –thalassemia , but there is a less common class of β –thalassemia when heterozygotes have normal levels of HbA_2 (Funcharoen *et al.*, 1988).

1.2.5.5.2 Beta thalassemia major

The (homozygous) or (compound heterozygous state) for β –thalassemia, *thalassemia major*, which produces a clinical picture described by Thamass Cooley in 1925 (Cooley and Lee, 1925). Anemia appears during the first few months of life and becomes progressively severe, so that, infants with thalassemia major fail to thrive and may have health

problems, they are also considered to be a blood transfusion –dependent (Loukopoulos *et al.,* 1990).

1.2.5.5.3 Beta thalassemia intermedia

Thalassemia intermedia is a medical term which describes those patients having phenotypes that are more sever than the thalassemia minor, but milder than the blood transfusion –dependent; the thalassemia major (Camaschella and Capellini, 1995).

The beta thalassemia intermedia syndrome involves a wide spectrum of disability and patients will survive the anemic condition later than in usual in the transfusion –dependent forms of beta thalassemia major, and they will maintain a hemoglobin level of about 6 g/dl without transfusion (Green and King, 1990).

Their growth and development is related and they become disabled with obvious skeletal abnormalities, arthritis, bone pain, and progressive splenomegaly (Dimarzo *et al.,* 1988).

At the other end of the spectrum, there are patients who remain completely asymptomatic until adult life and are transfusion –independent, with hemoglobin levels as high as 10-12 g/dl (Driscoll *et al.,* 1995). Some patients become little disabled because of the effects of hypersplenism (Perniola *et al.,* 1988).

1.2.5.5.4 Beta thalassemia minor

The heterozygous condition for β –thalassemia, *thalassemia minor* (or *trait*), represents the mild form of Cooley's anemia, named by Valentine and Neel during the 1940s. It is not usually associated with any clinical disability except in periods of stress, such as pregnancy or during severe infection when

a moderate degree of anemia may be found. The heterozygous condition characterized by reduction in the synthesis of only one β –chain, elevated levels of HbA_2 and hypochromic microcytic anemia (Kattamis, 1981). Hemoglobin values are usually in the 9-11 g/dl range, but the most consistent finding is small, poorly hemoglobinized red cells (MCV values of 50-70 fl, MCH values of 20 to 22 pg). The red cells indices are particularly useful in screening for the heterozygous carriers of thalassemia in population survey (Gurgey *et al.,* 1991).

Beta thalassemia is heterozygous at the molecular levels and due to this heterogeneity, variable hematological results of the carrier state can be estimated (Weatherall, 1995).

The bone marrow in heterozygous β –thalassemia shows slight erythroid hyperplasia with rare red cells inclusion, megaloblastic transformation due to folic acid deficiency occurs occasionally, particularly during pregnancy. Although there is a mild degree of ineffective erythropoiesis, but the red cells survival is normal or nearly so (Altay and Gurgey, 1992).

An increase in the HbA_2 level occur, reaches values of (3.5 – 7) % in carriers of β –thalassemia, the level of fetal hemoglobin (HbF) is increased in about half the patients, reaches values, usually (1 - 3) % and rarely to more than 5 %. Some carriers have the coexistence of iron deficiency with the presence of β –thalassemia minor, leading to depress the HbA_2 levels (Zhang *et al.*, 1990).

Any offspring of two beta–thalassemia gene carriers will be at risk of being homozygous for the beta –thalassemia gene (major), which is a lethal disease and blood transfusion –dependent patients (Rowley, 1976).

There are six types of β –thalassemia trait:-

1. The most common two types are β^0 and β^+ thalassemia carriers, in which both are usually symptom free with normal or slightly reduced HbA level (normal or 1 – 3 g/dl below the normal range); mild hypochromic microcytic blood picture, with low MCV and MCH values, a characteristic feature is the increase in HbA_2 level > 4 %, and in some carriers it reaches above 7.5% was associated with partial or complete deletion of β –globin gene. HbF level either normal or slightly increased with heterogenous distribution among red cells, β / α ratio is decreased.
2. A third type, severe β –thalassemia trait, is quite rare with a clinical picture similar to that of thalassemia intermedia, there is moderate anemia with splenomegaly and bony changes. MCV decreased with moderately abnormal red blood cell morphology, HbA_2 increased, with normal or slightly increased HbF level, decreased β / α ratio, and patients may need splenoctomy to reduce the severity of anemia (Khider, 1986; Ko *et al*., 1989).
3. In the fourth type "silent carrier", there is no anemia, MCH, MCV, red cell morphology, HbF, and HbA_2, all are normal, but β / α ratio is decreased (Ingram and Stretton, 1959; Schwartz, 1969).
4. A fifth type is quite carrier which had no or mild hypochromic microcytic anemia, with decreased MCV; HbF and HbA_2 are normal with decreased β / α ratio (WHO Working Group, 1982).
5. The sixth type is African, same as β^+ and β^o thalassemia carriers, but differ in that β / α ratio may be decreased or normal, as it occurs in population in which α –thalassemia is common, so α –chain may also decreased to the same extent as β – chains and β / α ratio will be normal (Chene and Schwartz, 1999).

1.2.5.6 Diagnosis of beta –thalassemia trait

Heterozygous thalassemia rarely causes clinical disease and it does not require any treatment, their recognition is important for purposes of genetic counseling (Steinberg and Dreiling, 1983), and the lack of reliable and inexpensive diagnostic technique for heterozygous β –thalassemia has been a major reason that this condition is frequently unrecognized (Rucknagel *et al.*, 1974).

The distinction between α and β –thalassemia depends on the measurement of the minor Hb (A_2, F), if these are normal, the diagnosis of α –thalassemia is most likely, although rare subjects with β –thalassemia trait also have normal level of HbA_2 and HbF (Weatherall and Clegg, 1981). Blood count, including red cell count, hemoglobin (Hb), packed cell volume (PCV) and red cell indices, considered to be valuable information and useful in the diagnosis of both alpha (α) and beta (β) –thalassemia (Harrison, 1992).On the other hand Hemoglobin electrophoresis on cellulose acetate at alkaline pH is important in the diagnosis of thalassemia to screen for HbH, Hb Bart's and presence of abnormal Hb (Brown, 1993). Whereas HbA_2 measurement could be carried out by cellulose acetate (Hamilton *et al.*, 1979) and high performance liquid chromatography (HPLC) (Wilson *et al.*, 1983).Estimation of HbF is carried out by alkaline denaturation test (Betke *et al.*, 1959), HPLC (Wilson *et al.*, 1983), immunological methods by immunodiffusion (Chudwin and Rucknagel, 1974) and ELISA (Makler and Pesce, 1980). It is worth to mention that, coexistence of iron deficiency makes the diagnosis of thalassemia trait more difficult as it makes the typical blood picture and causes reduced HbA_2 synthesis (Wasi *et al*., 1969; Kattamis *et al*., 1972; Alperin *et al.*, 1977).

Recent investigations, indicated the importance of DNA analysis and globin chain testing, to identify specific genotypes for research purposes, to differentiate an α –thalassemia carrier from β –thalassemia carrier, to identify a silent carrier gene, or to examine for family inheritance patterns with multiple genes (Dacie and Lewis, 2001).

1.2.5.7 Clinical features of β –thalassemia trait

The heterozygous state of beta thalassemia, *β –thalassemia minor*, has a clinical feature by having imbalanced globin chain synthesis. The β –globin synthesis will be slightly decreased, resulting in the production of an excess of α –globin chains (Baysal *et al.*, 1995).

The excess of α –globin chains will precipitate in the red cells precursors, this condition is much worse in the β –thalassemia major and causes many health problems, while in β –thalassemia minor; the magnitude of the excess of α –chains is much less and can be dealt with successfully by the proteolytic enzymes of the red cells precursors, in spite of that, there is a mild degree of effective erythropoiesis (Lucarelli and Galimberi, 1990).

The anemia of beta thalassemia has three major components:-

a. Hemolysis of circulating mature red cells containing α –chains inclusions.
b. Reduction in the rate of hemoglobin synthesis, resulting in hypochromic and microcytic red cells.
c. The most important component is the ineffective erythropoiesis.

The elevated levels of HbA_2, is a characteristic mark to heterozygous β –thalassemia, this elevation is caused by:-

a. A defect in β –chain synthesis leading to a relative decrease in HbA level.

b. An absolute increase in the output of δ chain both *cis* and *trans* to mutant β –globin gene (Weatherall and Clegg, 1981).

1.2.6 Molecular basis of thalassemia

The application of recombinant DNA technology to study the globin producing genes, including the study of thalassemia has revealed a great deal of different types of defects at the molecular level (Wong *et al*., 1987).

B–thalassemia is extremely heterogenous at the molecular level and for about 100 different mutations has been found in association with this phenotype, table (1 – 4). These include deletion of the β –globin gene and non –deletion mutations that may affect the transcription, processing, or translation of β –globin messenger RNA (Diaz –Chico *et al*., 1987).

Table (1 – 4). The molecular basis of β –thalassemia (Antonio and Renzo, 2000).

Population	**Type of mutation**	**Phenotype**
	Deletion	
Indian	1) 619 bp	β^0
Black	2) 135 kb	High HbA_2 β^0
Dutch	3) ~10 kb	High HbA_2 β^0
Czech	4) 4.237	High HbA_2 β^0
	Transcriptional mutation	
Black, Indian	5) –88 C→T	β^+
Mediterranean	6) –87 C→G	β^+
Japanese	7) –31 A→G	β^+
Black, Chinese	8) –29 A→G	β^{++}
Kurdish	9) –28 A→C	β^+
Chinese	10) –28 A→G	β^+

Mediterranean India Mediterranean, black Black Black Kuwait Indian	Processing mutants Splice junction 11) IVS –1 5` GT → AT 12) IVS – 1 5` GT → TT 13) IVS – 2 5` GT →AT 14) IVS – 2 3` AG →CG 15) IVS – 2 3` AG →GG 16) IVS – 2 3` -17 bp 17) IVS – 2 3` - 25bp	 β^0 β^0 β^0 β^0 β^0 β^0 β^0
Indian, Chinese Greek, N. European Greek, Algerian Mediterranean	Consensus sequence 18) IVS – 1 position 5 G→C 19) IVS – 1 position 5 G→T 20) IVS – 1 position 5 G→A 21) IVS – 1 position 6 T→C	 β^+ β^+ β^+ β^{++}
Black S.E Asian Mediterranean	Cryptic splice sites in exons 22) Codon 24 T→A 23) Codon 26 G→A 24) Codon 27 G→T	 β^+ β^{++} β^E β^+ $\beta^{Knossos}$
Mediterranean Mediterranean	Cryptic splice in introns 25) IVS – 1 position 110 G→A	 β^+ β^0
Chinese Mediterranean Mediterranean	26) IVS – 1 position 116 T→G 27) IVS – 2 position 654 C→T 28) IVS – 2 position 705 T→G 29) IVS – 2 position 745 C→G	β^0 β^0 β^+

β^0= Absence of β - globin gene product.

β^+= Some residual production of β - globin gene.

β^{++}= Reduction of β - globin gene product is very mild.

IVS = Intervening sequence

1.2.6.1 Gene deletion

Four different types of deletions affecting only the β –genes, with one exception these are rare and appear to be isolated single events; the most common type of this kind of mutation, is the 619 bp deletion at the 3' end of the β gene, but even that is restricted to the Sind and Gujarati populations of Pakistan and India, where it accounts for approximately 50% of the β –thalassemia alleles (Thein *et al.*, 1994).

The Indian 619 bp deletion removes the 3' end of the β gene but leaves the 5' end intact, while the other four deletions remove the 5' end of the β gene and leave δ –gene intact (Basak *et al.*, 1992).

Heterozygotes for the other four deletions all have usually high HbA_2 levels. It is not clear that the increased δ –gene transcription and, if so, that is only the gene in *cis* that is usually active, possibly as a result of reduced competition from the deleted 5' β gene for transcriptional factors (Cao *et al.*, 1990).

1.2.6.2 Mutations to termination codons

Base substitutions that leads to a change of an amino acid codon into a chain termination codon (non –sense mutations) prevent translation of the messenger RNA and result in β^0 –thalassemia (Giordano *et al.*, 1998). Several substitutions of this type have been described, a codon 39 mutation occurring with great frequency in the Mediterranean, and a codon17 mutation is common in Southeast Asia (Varawalla *et al.*, 1992).

1.2.6.3 RNA processing mutation

RNA processing can also be affected by different types of mutations that create new splice sites, within either the introns or exons, resulting in variable phenotypic affects, depending on the degree of which the new site is utilized in comparison with the normal splice site (Kerkhoffs *et al.*, 2000), for example the G→A substitution at position 110 of IVS -1, which is one of the commonest forms of β –thalassemia in the Mediterranean, leads to about 10% splicing at the normal site and results in a phenotype of severe β^+ -thalassemia (Varawalla *et al.*, 1992).

A mutation that produces a new acceptor site at position 116 in IVS -1 results in little or no β –globin mRNA production and the phenotype of β^0 – thalassemia (Hall *et al.*, 1991).

1.2.6.4 Transcriptional mutations

Some types of mutations which are basically, base substitutions, occur in the conserved sequences, that are located upstream from the β –globin gene, leading to the β^+ -thalassemia phenotype of each mutation, but there is a considerable variability in the clinical severity, according to the type of these different mutations which affect the transcriptional stage (Mokrydimas *et al.*, 1997).

Two mutations are in the mRNA CAP site; they are at position -88 and -87, these two mutations are close to the CCAAT box. Four mutations lie within the ATA box homology (Takihara *et al.*, 1986).

Studies showed that, a base substitution from A to C at CAP site (+1) was identified in an Indian of ancestors came from Asia, this Indian carrier have the phenotype of β –thalassemia minor but in fact he is homozygous for the mutation (Azer and Chingiz, 1995).

1.2.7 Prenatal diagnosis of β –thalassemia syndromes by PCR

The development of the PCR has had a dramatic impact on the study and analysis of nucleic acids. This development in molecular techniques of mutations analysis leads to the discovery of over than 200 mutation of the β –globin gene. Many different mutations cause β –thalassemia and its related disorders, and the most common types of mutations that cause this disease are point mutations affecting the globin gene, but some large deletions are also known. The PCR –based analytical protocols represent the basis of the prenatal diagnosis (Dacie and Lewis, 2001). Figure (1-2) summarize the PCR process.

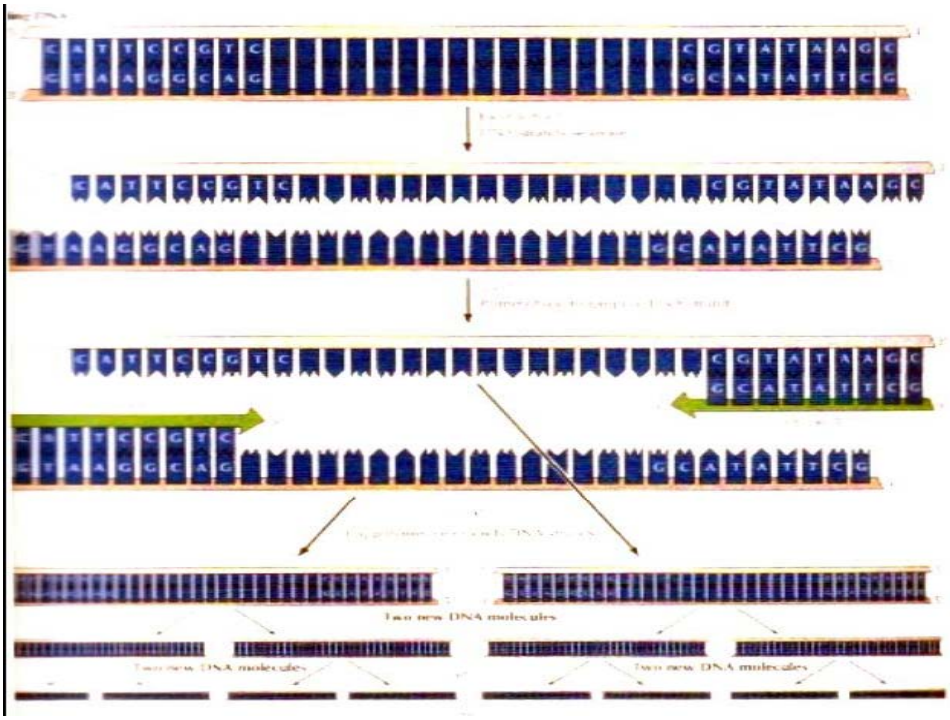


Figure (1 -2). PCR process in which target DNA is amplified. The figure shows DNA denaturation by heat, annealing with the primer, and amplification by Taq polymerase (Bartlett and Stirling, 2003).

1.2.8 Prevention

The different forms of thalassemia can be prevented by two ways; the first way is the genetic counseling, which is screening the whole population when they are still in school and warning carriers about the risk of marriage to another carrier (Basorga and Benz, 1988). Many efforts, around the word in which thalassemia occurs widely, are directed toward developing prenatal diagnosis programs (Chui and Waye, 1998), this involving screening of mothers at their first prenatal visit, screening the fathers in cases in which the mother is a thalassemia carrier, and offering the couple possibility of prenatal diagnosis and therapeutic abortion if they are both carriers of a gene for severe form of thalassemia (Lam *et al.*, 1997).

A prenatal diagnosis can be carried out at the 18^{th} week of pregnancy (Brambati *et al.*, 1991) by utilizing fetal blood sampling and globin chain synthesis analysis. These methods have been applied successfully in many countries resulting in a reduction in the birth rate of homozygous β – thalassemic in many parts of the Mediterranean (Alter, 1985).

Fetal DNA analysis is helpful to determine the hemoglobin disorder in utero, this can be done by using DNA derived from amniotic fluid, but this analysis can be done relatively late in pregnancy and the amniotic fluid cells have to grown in culture to obtain enough DNA (Wichramasinghe and Lee, 1998).

Chorionic villus sampling is another way utilizing the use of DNA and can be done in the ninth week of pregnancy. It can be considered to be the major method for the prenatal diagnosis of thalassemia (Rodeck, 1993).

The progress in DNA technology, give a great deal to facilitate the development of prenatal diagnosis programs (Chehab *et al*., 1987), this

includes the polymerase chain reaction (PCR), which allows small amounts of DNA to be rapidly amplified.

The PCR technique together with oligonucleotide probes and non – radioactive labeling techniques, help in reducing the technology required for prenatal diagnosis and otherwise it develop the "dot plot" analysis to determine whether a fetus has inherited a severe form of thalassemia (Nico *et al.*, 1999).

2.1 Materials

2.1.1 Apparatus

Various apparatus have been used as follow:

No.	Apparatus	Company / Country
1.	Agarose gel electrophoresis system	SEBIA / France
2.	Autoclave	Memmert / Germany
3.	Cellulose acetate electrophoresis system	Shandon / England
4.	Light microscope	Olympus / Philippines
5.	Laminar flow hood	Telstar / Spain
6.	Microcentrifuge	Beckman / Germany
7.	MS9 system	CERGY –PONTOISE/ FRANCE
8.	PCR system	Primus 69 / Germany
9.	pH meter	WTW / Germany
10.	Sensitive balance	Sartorius / England
11.	Shaker –incubator	Thermo / Germany
12.	Spectrophotometer	Cecil / England
13.	Universal centrifuge	Universal 16A/ Germany
14.	Variant system	BIO –RAD / U.S.A
15.	Vortex mixer	Stuart / England
16.	Water bath	Memmert / Germany

2.1.2 Chemicals

- **Shandon / Germany**

Sodium diethylbarbiturate, diethylbarbituric acid, tris (hydroxymethyl) aminomethan, ethylendiamine tetra acetic acid, boric acid, sodium chloride, chloroform.

- **SEBIA / France**

Alkaline buffer, tris-Barbital buffer, amidoblack stain (stock solution), ethylene glycol, citric acid, ethanol, acetic acid, hemolysing solution.

- **BIO-RAD/ England**

Elution buffer 1, elution buffer 2, wash solution.

- **AB ANALITICA / USA**

Solution 1 (lysis Reagent), Solution 2 (washing buffer 2), solution 3 (washing buffer 3).

2.1.3 Blood samples collection

Blood samples were collected from 100 patients and their family members and relatives attending the Central Health Public Laboratories who came from Baghdad.

2.1.4 Buffers and solutions

2.1.4.1 Estimation of HbA_2 (Betke, *et al*, 1959)

The following buffers used for HbA_2 estimation:

2.1.4.1.1 Barbitone buffer (pH 8.9)

Sodium-diethylbarbiturate	5.15 gm
Diethylbarbituric acid	0.92 gm
Distilled water	1000ml

Sodium-diethylbarbiturate and Diethylbarbituric acid were dissolved in 500 ml distilled water, pH was adjusted to pH 8.9 and the volume was completed to 1000 ml with distilled water.

2.1.4.1.2 Tris (pH 8.9)

Tris(hydroxymethyl)amino methane	14.5gm
Ethylene diamine tetra acetic acid	1.5 gm
Boric acid	0.9 gm
Distilled water	1000ml

Tris(hydroxymethyl)amino methane**,** ethylene diamine tetra acetic acid and boric acid were dissolved in 500 ml distilled water, pH was adjusted to pH 8.9 and the volume was completed to 1000 ml with distilled water.

2.1.4.1.3 Lysate solution

Blood samples were collected into anticoagulant covered tubes, using Na_2EDTA as anticoagulant.Red cells were washed three times with 0.85% saline and were lysed by addition of two volumes of distilled water.

2.1.4.1.4 Normal saline

Sodium chloride	8.5 g
Distilled water	1000ml(pH7)as final volume

2.1.4.2 Hb electrophoresis by cellulose acetate paper (Dacie and Lewis, 2001)

The following buffers and solutions were used for Hb electrophoresis by cellulose acetate paper.

2.1.4.2.1 Barbitone buffer pH 8.6

Sodium diethylbarbiturate	5.15gm
Barbitone (diethylbarbituric acid)	0.92gm
Distilled water	1000ml

Sodium-diethylbarbiturate and barbitone were dissolved in 500 ml distilled water, pH was adjusted to pH 8.9 and the volume was completed to 1000 ml with distilled water.

2.1.5 Hb electrophoresis by agarose gel (HYDRAGEL HEMOGLOBIN (E) K20 KIT)

2.1.5.1 Lysate solution

Blood samples were collected in anticoagulated tubes using Na_2EDTA as anticoagulant, washed two times with ten volumes of saline. Hemolyze ten µl packed red cells with one hundred and thirty µl Hemolyzing Solution and vortexed for ten seconds and incubated for five minutes at room temperature.

2.1.5.2 Agarose gel

agarose	0.8 g/100ml
Alkaline buffer	pH 8.5±0.1

Gel plates ready for use, used for the electrophoresis.

2.1.5.3 Tris –barbital buffer

Barbital	2.45%
Sodium barbital	13.73%
Sodium azide	0.13%

Each fifty ml was diluted up to one liter with distilled or deionized water then used as electrophoresis buffer.

2.1.5.4 Working solution for electrophoresis

The working solution for electrophoresis contains the electrophoresis buffer and sodium azide.

2.1.5.5 Amidoblack stain

Amidoblack	0.4g/ 100ml
Ethylene -glycol	6.7%

Used to stain gels. The working staining solution is stable for one month.

2.1.5.6 Staining solution diluent

This was used for the preparation of the amidoblack staining solution.

2.1.5.7 Destaining solution

Each vial of stock destaining solution to be diluted up to hundred liters with distilled or deionized water. It is convenient to dilute only one ml of the stock solution to one liter .After dilution, the working destaining solution contains:

Citric acid 0.05 g/100ml

The stock of destaining solution was stored at room temperature or refrigerated.It is stable until the expiration date indicated on the kit package or destaining solution vial label.Working destaining solution is stable for one week at room temperature in closed bottle.

2.1.5.8 Hemolyzing solution

Hemolyzing solution is a buffer with additives, nonhazardous at the concentration used, necessary for optimum performance, usually stored at room temperature or refrigerated, it is stable until the expiration date indicated on the kit package or Hemolyzing solution vial label.

2.1.5.9 Fixative solution

This solution contains (vol. /vol.):

60% ethanol
10% acetic acid
30% distilled or deionized water

Mixed well, stored at room temperature, tightly capped to prevent evaporation and it was discarded after three months.

2.1.6 MS9 devise (automatic full digital cell counter) (BIO – RAD)

This technique was used for obtaining complete blood picture (Hb, PCV, MCV,MCHC, MCH, RBC count,WBC count, platelets count, and differential count).The technique depends on electrical impedance technology as a principle of its work.

2.1.7 Hb analysis by variant beta thalassemia short program

This technique is used for automatic Hb separation to obtain different Hb structure with their percentage in a blood sample. Different Hb molecules can be separated by this devise such as: (HbA, HbA_2, HbF, HbS, HbC, and HbD), utilizing the principle of cation –exchange of High Performance Liquid Chromatography (HPLC).

2.1.8 Leishman stain (Frei *et al*., 1995)

Leishman powder	1.5 gm
Methanol (absolute)	1000 ml

The mixture was heated to 50˚C and allowed to cool at room temperature and shaked several times during the day after standing for 24 hour, it was filtered to obtain clear stain.

2.1.9 Isolation of DNA from whole blood

The AB ANALITICA kit was used to isolate DNA from whole blood.The kit contains:

Solution 1 (lysis reagent)
Solution 2 (washing buffer 2)
Solution 3 (washing buffer 3)
Filter columns
Tubes and caps

Solution 1 should be shaken before use to resuspend the binding resin.

2.1.10 PCR Amplification

The following reagents were used for PCR amplification.

2.1.10.1 Primers

Primers were provided by Alphadna Company, ready to use and the sequences of these primers are listed in table (2-1)

Table (2-1). Primers sequences used in PCR amplification.

P5	CCAACTCCTAAGCCAGTGCC
P7	CTTTCCCTAATCTCTTTCTTTCAGGGC
P10	CACTGACCTCCCACATTCCC
P12	CTGAGACTTCCACACTGATGC

2.1.10.2 (10x) PCR buffer

This buffer was provided by aj ROBOSCREEN Company and is made of 10mM Tris – HCl (pH 8.3), 50 mM KCl, and 0.0001% gelatin.

2.1.10.3 dNTPs mixture

Provided by aj ROBOSCREEN Company with concentration of 2.5µmol / sample.

2.1.10.4 Taq polymerase

Provided by aj ROBOSCREEN Company in a concentration of 5u /µl of the enzyme.

2.1.10.5 *Bsu* 36I

Restriction enzyme, provided by Promega Company, in a concentration of 10u / 5µl.

2.2 Methods

2.2.1 Sterilization methods

- Autoclaving : buffers and solutions were sterilized by pressure vessel (autoclave) at 121˚ C and 15 Ib/ In^2 for 15 minutes.
- Dry heat: a laboratory oven was used for glassware sterilization. Glassware were placed in the oven at 180˚ C for 2 hours.

2.2.2 Preparation of Hb electrophoresis by cellulose acetate paper (Dacie and Lewis, 2001)

1. Blood lysate prepared according to (2.1.4.1.3).
2. Distilled water added to the precipitate at twice the volume of the precipitate, shaked by hands for two minutes , then left for an hour.
3. Five or six drops of chloroform is added then shaked for four minutes then centrifuged at 3500 rpm for 15 minutes.
4. The lysate was removed by Pasteur pipette.
5. With the power supply is still disconnected, the compartments of electrophoresis tank were filled with barbitone buffer, the wicks were soaked and

positioned in place.

6. The cellulose paper was soaked in barbitone buffer for at least 5 minutes. The paper was immersed slowly to avoid trapping air bubbles and ensure even saturation of the membrane.
7. The membrane was placed between two pieces of absorbent papers.
8. A small volume (10 µl) of each sample was placed into the sample well.
9. The applicator was dipped into the sample wells, and the samples were applied to cellulose-acetate membrane approximately 2 cm from one end of the membrane, the tips of the applicator were allowed to remain in contact with the membrane for 3 seconds.
10. The cellulose-acetate membrane was then placed across the bridge of the tank so that the membrane surface is in contact with the buffer, with the line of the cathode end.
11. The power supply was connected and run at 280-300 volt for 20 minutes or until a visible separation was obtained.
12. The power supply was disconnected and the cellulose -acetate membrane was removed .Followed by HbA_2 estimation steps.

2.2.3 Estimation of HbA2 (Marengo and Rowe, 1965)

1. Following the electrophoresis of blood samples carried according to (2.2.2), the HbA_2 estimation was done by cutting the strip containing the HbA band and placed in a universal bottle containing 20 ml of distilled water. The HbA_2 band was also eluted from strip in a universal bottle containing 4 ml of distilled water.
2. The universal bottles were placed on rotating mixer for 15 minutes.
3. The absorbance was read at 413 nm for HbA2 and HbA.
4. The percentage of HbA_2 was calculated as following:-

$$\%HbA_2 = \{A^{413}\ HbA_2 / [A^{413}HbA_2 + (A^{413}HbA \times 5)] \times 100$$

2.2.4 Preparation of blood films on slides (Ingram and Minter, 1969)

1. A drop of patients' blood was placed on in the center of a glass slide and spread on the glass in a monolayer.
2. The blood film was stained with Leishman stain for 5-7 minutes.
3. The film was then washed with distilled water for 10 minutes, dried, and examined.

2.2.5 Variant beta thalassemia short program

2.2.5.1 Specimens collection

At least 5µl of venous blood are required for this test. Whole blood specimens should be collected in a vacuum blood collection tube containing EDTA as an anticoagulant. Patient specimens are stable for 7 days when stored at 2-8 ºC.

2.2.5.2 Sample preparation and analysis

1. Five µl of whole blood from each patient sample was pipetted into separate 1.5 ml sample vial, then 1.0 ml of Hemolysis Reagent was added to each sample vial, covered parafilm and mixed by inversion.
2. The sample vial was placed into the VARIANT devise. The hemolysate are stable for 24 hours when stored at 2-8 ºC.
3. Then the analysis was carried out by using the β- thalassemia short program in which samples were placed in the devise and the analysis done automatically on them.

2.2.6 Preparation of Hb electrophoresis by agarose gel

2.2.6.1 Sample preparation

1. Anticoagulated blood was centrifuged at 5000 rpm for five minutes, the plasma was discarded.
2. The red blood cells pellets washed two times with ten volumes of saline.
3. Ten µl of washed packed red cells were hemolyzed with one hundred and thirty µl of Hemolyzing Solution, then vortexed for ten seconds and incubated for five minutes at room temperature.

2.2.6.2 Procedure

I. Migration step:

1. HYDRAGEL K20 applicator was placed on a flat surface and raised the part of the applicator carrier with the numbered notches.
2. One hundred and twenty µl distilled water was pooled on the lower third of the frame printed on the HYDRAGEL K20 applicator carrier.
3. The HYDRAGEL agarose gel plate was unpacked.
4. One thin filter paper was rolled onto the gel surface to absorb the excess of liquid. Remove the paper immediately .Do not leave the filter paper for a too long contact with the gel to avoid its dehydration.
5. The plate was placed (the gel side up) with its edge against the stop at the bottom of the printed frame.
6. The gel was bended and lowered down onto the water pool; with no air bubbles are trapped. Water is spread underneath the entire gel plate and the gel is lined up with the printed frame.
7. The applicator carrier was lowered with the numbered notches down to the intermediate position with the switch in high position.

8. The applicator was placed on a flat surface with the well numbers in the right –side –up position.
9. Ten µl of hemolyzed sample was added into the applicator wells; loaded within two minutes. Then it was placed on the applicator carrier and lowered, so that the applicator contacts the gel surface for one minute, then the switch was turned to rise up the applicator, after that it was removed and then discarded.
10. The gel was placed into an appropriate electrophoresis chamber according to the polarity indicated on the gel, the lower side of the gel on the cathodic side.
11. The chamber was plugged to the power supply.

Migration condition	K20
Volume of buffer per compartment	150 ml
Total buffer volume	300 ml
Migration time	15 minutes
Constant voltage	165 v
Initial current (per gel)	7±2 mA

12. After migration, the chamber was unplugged and the gel plate was removed.

II. Fixation:

The gel plate was dried completely in the incubator –dryer at 80º C (for ten minutes minimum).

III. Staining –Destaining:

1. The dried and cooled gel was immersed in the staining solution for five

minutes, and then it was placed into three baths containing destaining solution until the background was completely colorless and clear.

2. The gel was dried completely with hot 80ºC airs

2.2.7 MS9 (automatic full digital cell counter)

2.2.7.1 Principle

The MS9 principle is impendancemetrie:-

- Mechanical structure:-

The measuring chamber is made of 2 parts: one part (1) is to receipt the dilute in which are the blood cells to analyze and count. The other part filled with detergent, is the aspiration part, (2) in which a vacuum is applied (6) the blood cell dilution of the first part cross by aspiration a calibrated aperture (4) which separates both of them.

- Electrical structure:-

Two electrodes (3) are in both of these two parts of measuring chamber. A constant current (5) is established between these two electrodes across the aperture.

- Phenomena :-

When a cell, coming from the whole blood dilution part, passes by a simple mechanical aspiration, to the aspiration part (1) because of its different resistivity in front the diluent one, the cell disturbs the constant current established between the two electrodes and generates a pulse (3). Figure (2-1) illustrate the MS9 principle.

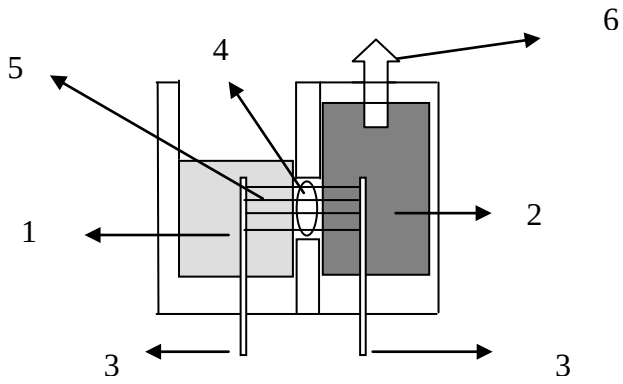


Figure (2-1). The MS9 principle

2.2.8 DNA isolation from whole blood

1. Two hundred µl of whole blood (EDTA –treated) was added in a 2 ml tube.
2. Five hundred µl of solution 1 was transferred to blood sample.
3. The sample was mixed gently by inverting the tube several times, until a complete homogenous mixture was obtained, and incubated at 60ºC for four minutes. At the end of the incubation, the tube was inverted several times (at least ten times) to ensure the complete binding of DNA to the resin.
4. The filter columns were placed into the two ml centrifuge tubes with the lysate was transferred to the filter. The cap of the tube was closed and centrifuged at 14,000 rpm for 1 minute. The filter retained the matrix and the DNA bound to it, while the filtrate contained proteins and other contaminants.
5. The filter was taken out and the filtrate was discarded, then the filter was replaced in the test tube and five hundred µl of solution 2 was added. The cap of the tube was closed, vortexed for few seconds and centrifuged at 14,000 rpm for 1 minute.
6. The filter was removed to new tube, and the filtrate was discarded, then five hundred µl of solution 3 was added to the filter. The cap closed and

incubated for two to four minutes at room temperature and centrifuged at 14,000 rpm for 1 minute.

7. Five hundred µl of ethanol 80% was added, so that any salt traces were removed from the sample.
8. The filter was removed and placed into a new 1.5 ml Eppendorf and 100 µl of 65-70ºC preheated water was added, vortexed for few seconds to resuspend the resin and then the tube was allowed to stand for 2-5 minutes at 65-70ºC to obtain the complete DNA releasing. The tube was vortexed again and centrifuged at 14,000 rpm for 1 minute, to avoid DNA breaking, the resin was resuspended with a pipette's tip instead of vortexing.
9. The filter was removed; the DNA contained in the filtrate is stable for some weeks if stored at 4ºC and for at least one year if it is stored at -20ºC.

2.2.9 PCR amplification

The following reagents were mixed in a sterile 0.2 ml eppendorf tube as given in table (2 -2).

Table (2 -2). Reagents and their addition order used in β –globin PCR amplification.

Addition order	component	Volume	concentration
1.	D.W	12.7 µl	—
2.	10 x PCR buffer	5 µl	1x
3.	$MgCl_2$	1.5 µl	25mM
4.	dNTPs	0.5 µl	10mM

5.	primer	1.5 µl from each primer	10 pmol for each primer
6.	*Taq polymerase*	0.3 µl	1.5 u /R
7.	DNA	2 µl	—
total		25µl	—

Amplification was performed in Primus 96 PCR device and programmed for 40 cycles (94 ºC for 5 minutes, 92 ºC for 1 minute, 62 ºC for1.5 minute and 72 ºC for 2.5 minutes).

2.2.10 Restriction

The following reagents were mixed in sterile 0.5 ml eppendorf tube as given in table (2-3).

Table (2-3).Reagents and their addition order used in restriction technique.

Addition order	components	volume
1.	Sterile D.W	11.8 µl
2.	10X buffer	2µl
3.	Bovin Serum Albumin (BSA)	0.2µl
4.	DNA	10µl
5.	Restriction enzyme	1µl
Total		25µl

All mixed together then put in water bath at 37º C for three hours, and then the tube were placed in deep freeze to stop any more reactions.

3. Results and Discussion

In this study 100 β –thalassemia carriers and their families attending Central Public Health laboratory have been subjected to following analysis to ascertain their status:

1. Pedigree analysis of families with β –thalassemia syndrome.
2. Red blood cells indices.
3. Hemoglobin analysis (Al –Awamy, 2000).

3.1 Pedigree analysis of families showing β –thalassemia syndrome

Pedigree analysis might show how presumably healthy looking parents will give rise to children with β –*thalassemia major,* thus in the following six samples, it was possible to demonstrate the risk behind marriage of β –*thalassemia minor* individuals. The squares with light and dark sides represent a male with *thalassemia minor*, and the circles with light and dark sides represent female with *thalassemia minor*, dark square and circle represent an affected male and female with *β –thalassemia major*, respectively. The six examples as listed below, summarized all forms of the disease inheritance that found in families under this study, and some cases showed the coexistence of another inherited disease beside the β –thalassemia syndrome.

3.1.1 First case: is represented by a family composed of eight individuals as in figure (3-1)

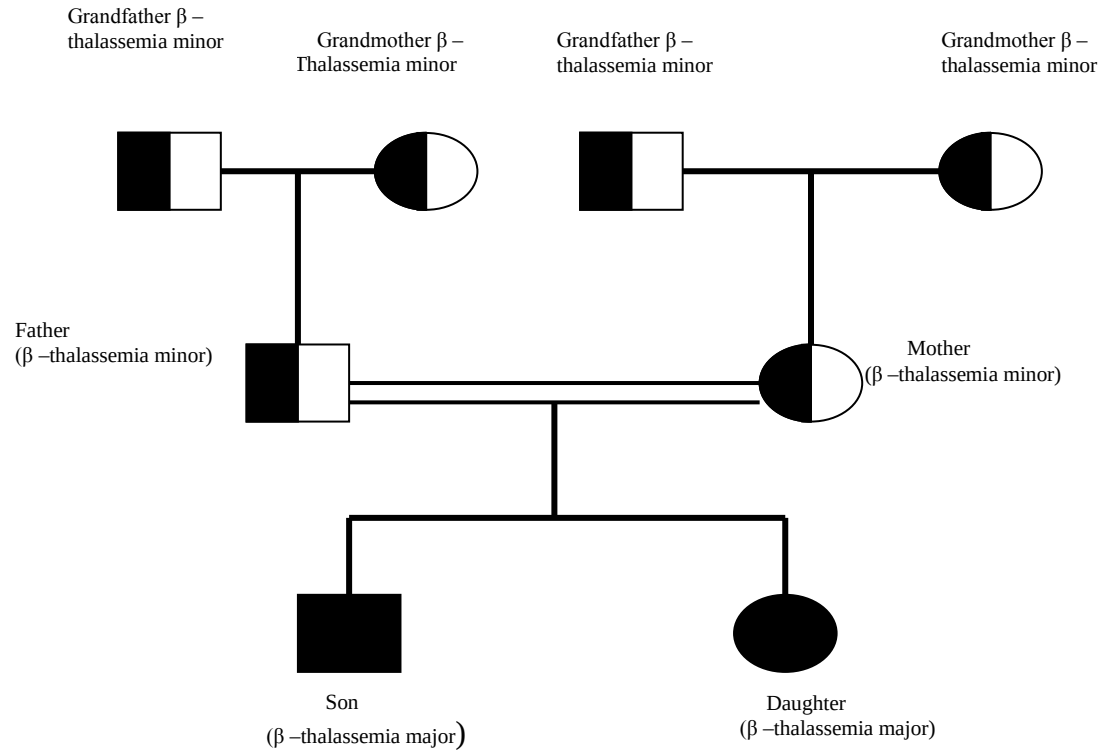


Figure (3-1) Pedigree analysis of the first case.

Figure (3-1) indicates that the father who is a carrier (*β-thalassemia minor*) married to his cousin who was a carrier of *(β-thalassemia minor*), their offsprings showed β-*thalassemia major* symptoms (daughter and son). According to the history of the family, both grandfathers were carriers of *β-thalassemia minor.* They were married to grandmothers who were normal; so both of the progenies (father and mother) were β –thalassemia carriers and because of the society traditions, the father was married his cousin and the frequency of the *β –thalassemia major* increased and their offsprings showed *β –thalassemia major* symptoms.

3.1.2 Second case: is represented by a family composed of eleven individuals as in figure (3-2)

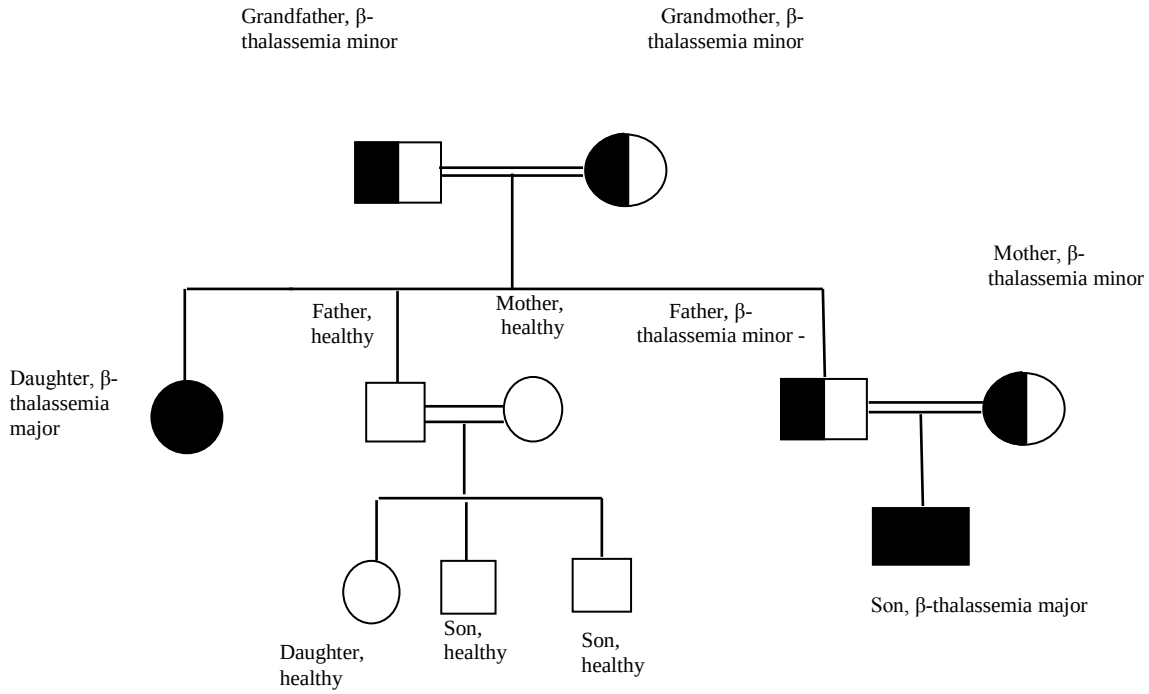


Figure (3-2) Pedigree analysis of the second case.

Figure (3-2) indicates that, the grandfather who is *β –thalassemia minor*, married to his cousin (grandmother) who is *β –thalassemia minor* too, their offsprings showed variable results. They produced a daughter with *β –thalassemia major* symptoms, a son who is normal person, married to his cousin who is normal too and produced normal offsprings, while the third son (the father) who is a *β –thalassemia minor* married to his cousin (the mother) who is a *β –thalassemia minor*, so the frequency of the syndrome to major type of β –thalassemia increased, and their offspring showed *thalassemia major* symptoms.

3.1.3 Third case: is represented by a family composed of ten individuals as in figure (3-3)

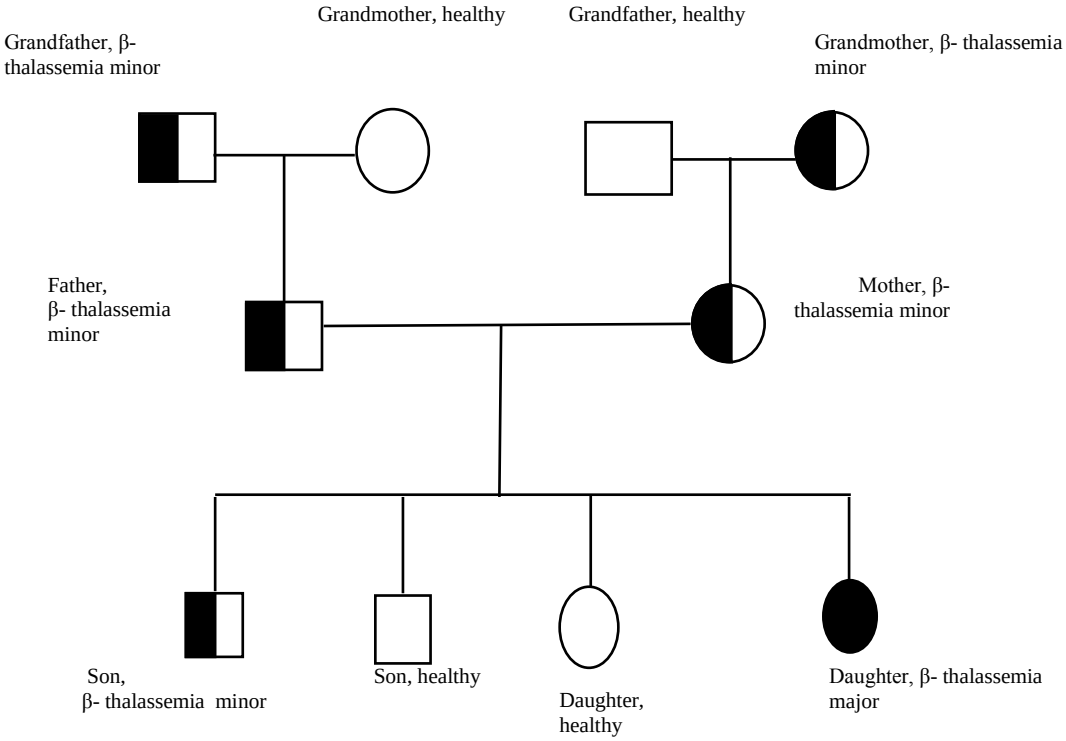


Figure (3-3). Pedigree analysis of the third case

Figure (3-3) indicates that grandparents from the father (*β –thalassemia minor*) side were healthy (grandmother), and β – thalassemia carrier (grandfather), while grandparents from the mother (*β –thalassemia minor*) side were healthy (grandfather), and β –thalassemia carrier (grandmother). When both parents in which both are carriers, were married, their offsprings were as the following, a son who is *β –thalassemia minor*, a daughter showed the *β –thalassemia major* symptoms, and two healthy children (son and daughter).

3.1.4 Fourth case: is represented by a family composed of eight individuals as in figure (3-4)

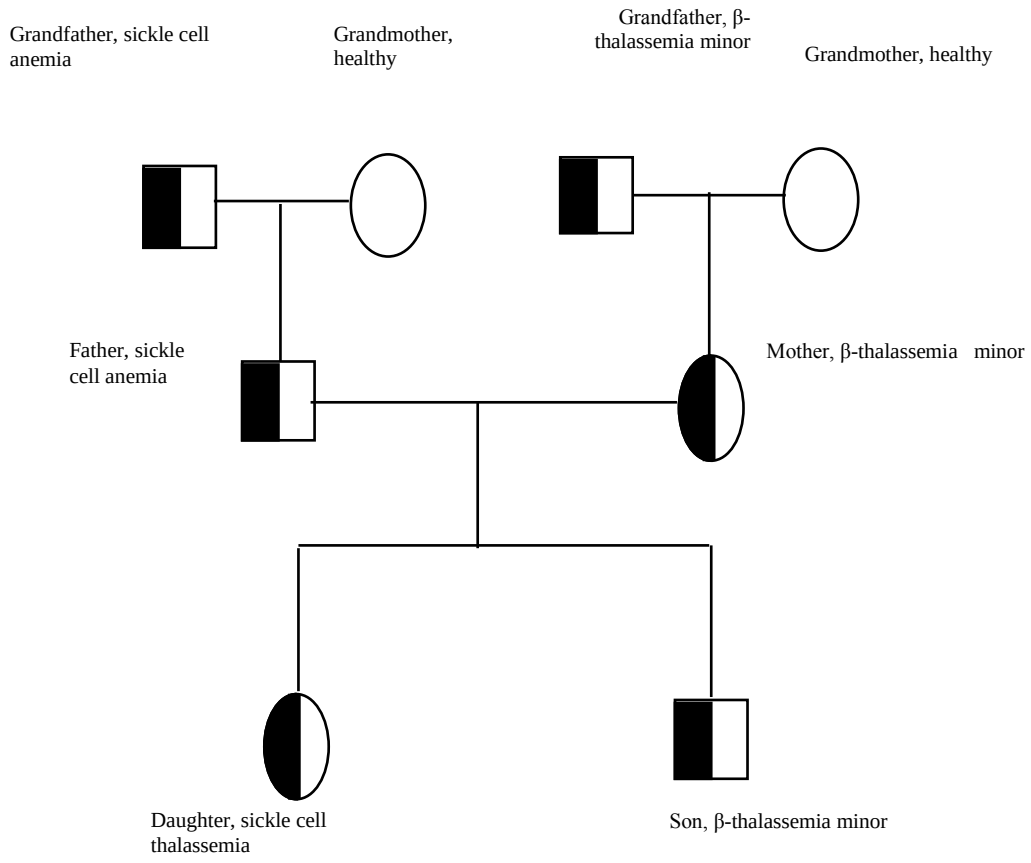


Figure (3-4). Pedigree analysis of the fourth case

Figure (3-4) indicates that, the grandparents from the father (sickle cell anemia) side were, healthy (grandmother), and a sickle cell anemic (grandfather), while the grandparents from the mother (*β –thalassemia minor*) side were, healthy (grandmother), and a *β –thalassemia minor* (grandfather). When both parents were married, their offspring were a daughter with sickle cell thalassemia and a son with *β –thalassemia minor*.

3.1.5 Fifth case: is represented by a family composed of seven individuals as in figure (3-5)

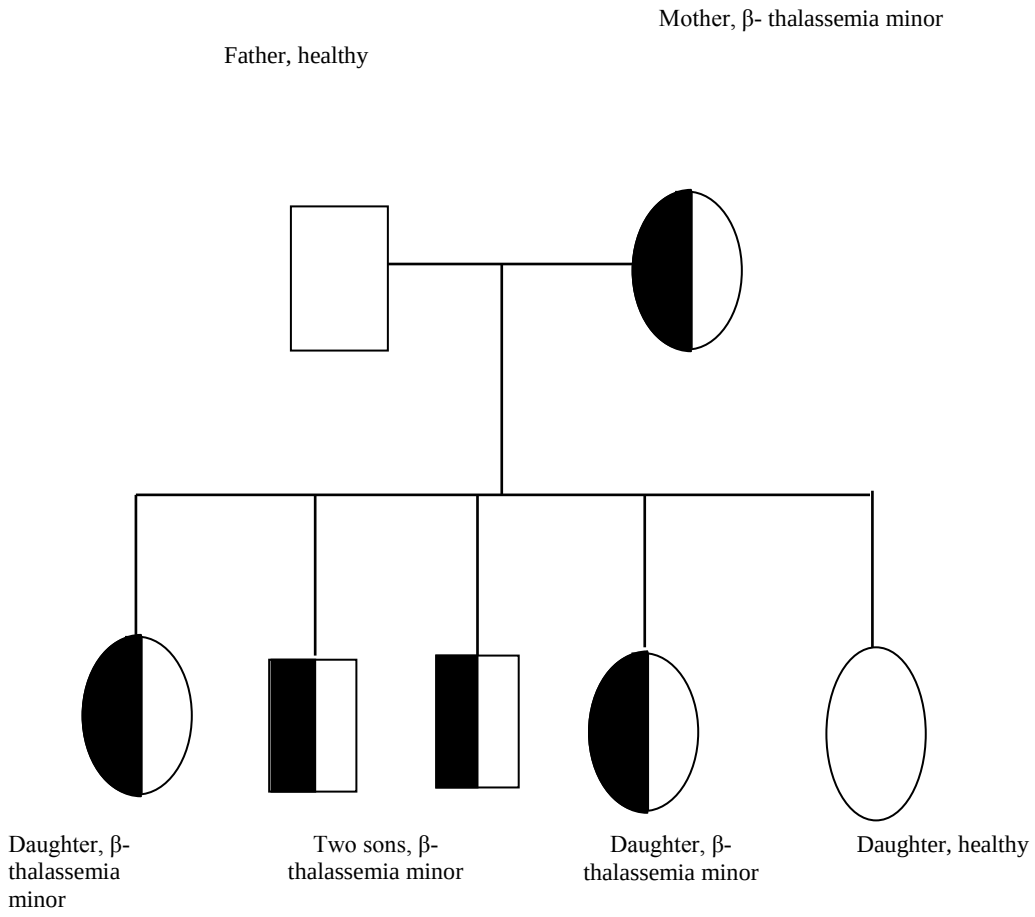


Figure (3-5). Pedigree analysis of the fifth case.

This figure indicates that, the father (normal) was married to β – *thalassemia minor* female (mother), their offspring were, only one normal daughter while the other children are all carriers of the β –thalassemia gene.

3.1.6 Sixth case: is represented by a family composed of ten individuals as in figure (3-6)

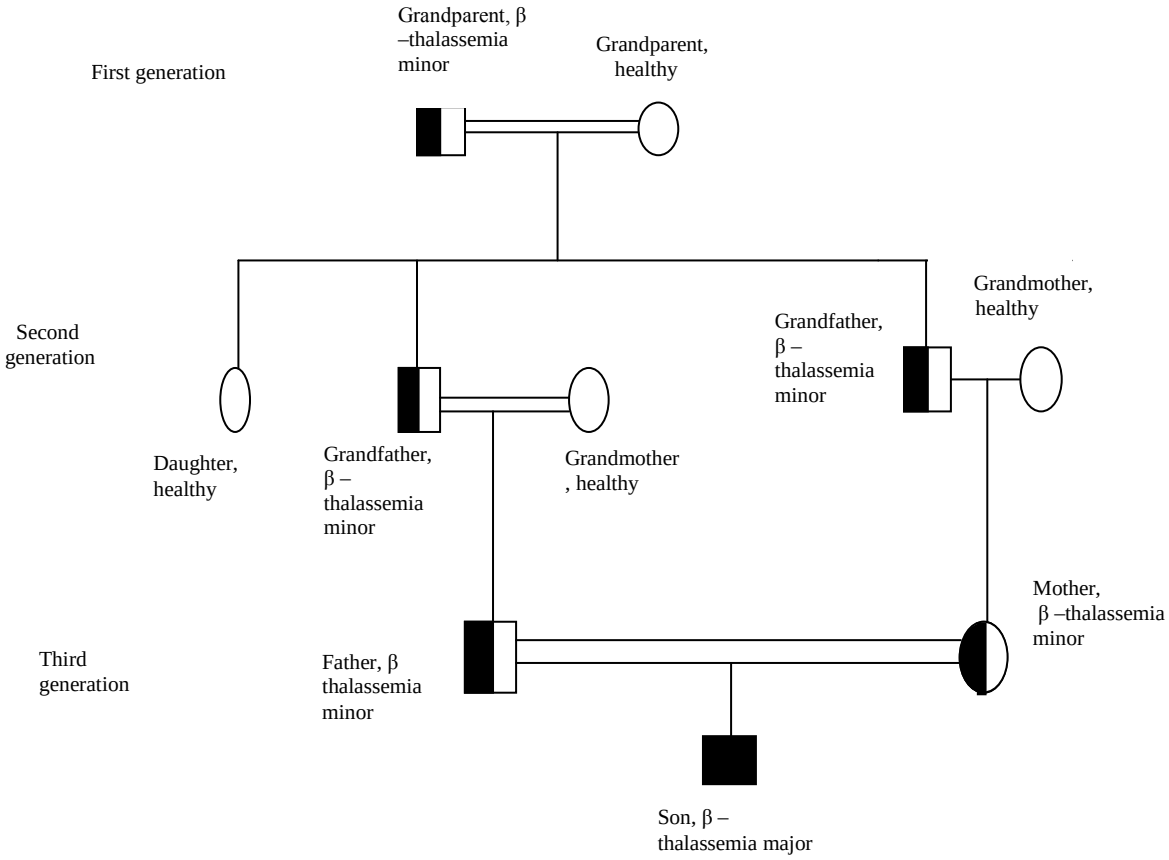


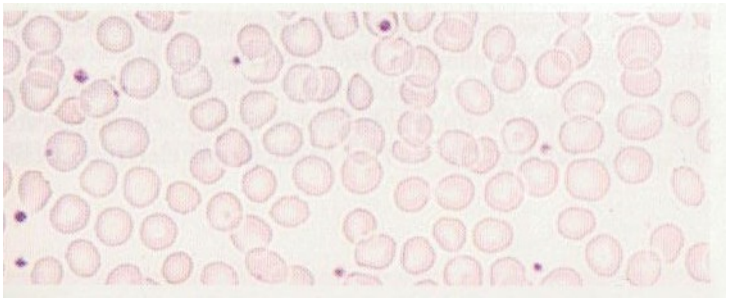
Figure (3-6).Pedigree analysis of the sixth case

Figure (3-6) indicates that, the parents of the grandfathers were, healthy (mother) and *β –thalassemia minor* (father), when they got married due to society traditions, the offsprings of the second generation were, a healthy daughter , and two sons who are *β –thalassemia minor*. When those two sons got married, the offspring of each one, representing the third generation were, *β –thalassemia minor*, and when they married due to society tradition, the frequency of the major type increased and they produced a son, showed *β –thalassemia major* symptoms.

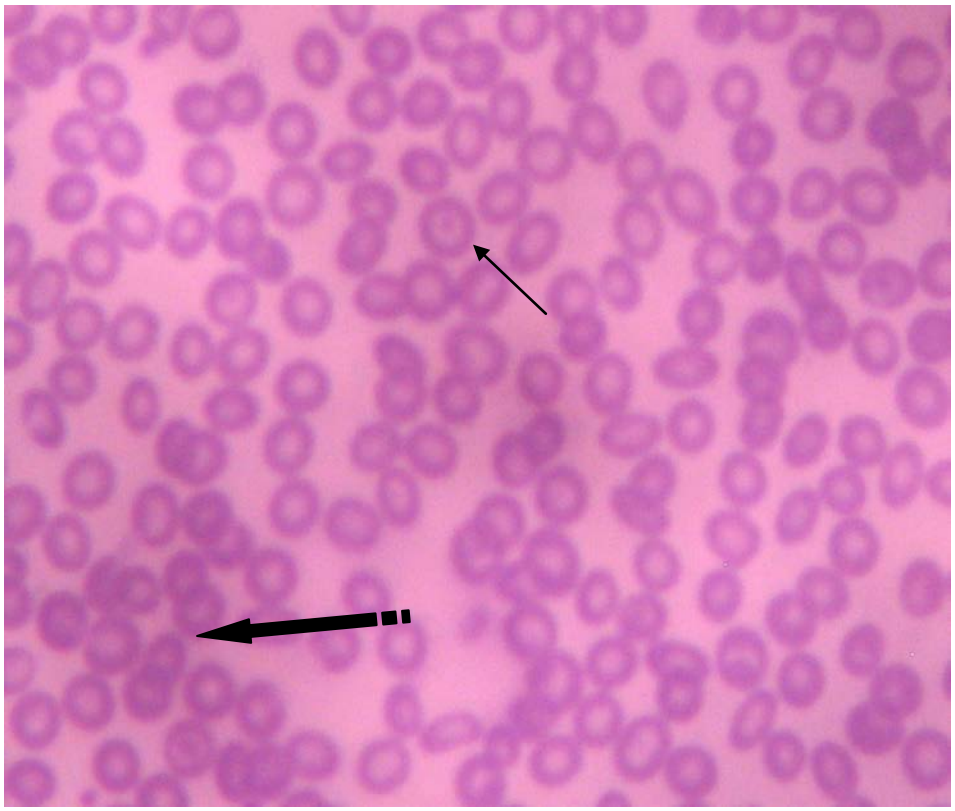
3.2 Hematological analysis of β –thalassemia

3.2.1 Complete blood picture (blood count and film)

The blood count and film provide valuable information useful in the diagnosis of both α and β thalassemia, it is also useful to investigate the presence of other structural variants combined with thalassemia, by observing the morphological characteristics of the red blood cells and the presence of other abnormal cells in the blood smear, for this all the families were investigated for blood count and film. Generally, in most carriers the results showed the same values, in which, carriers have blood count of low MCH (19 -26) pg, MCV (62-65) fl, Hb (11- 12) g/dl, PCV (32-38) %, these values are low as compared with normal values, while blood film showed microcytic hypochromic red blood cells, and few target and oval cells in comparison with normal blood film.

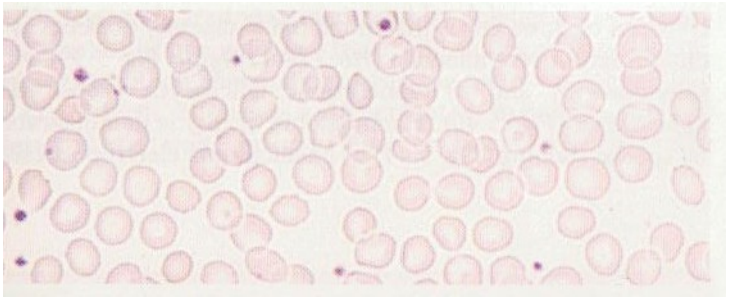


(A)

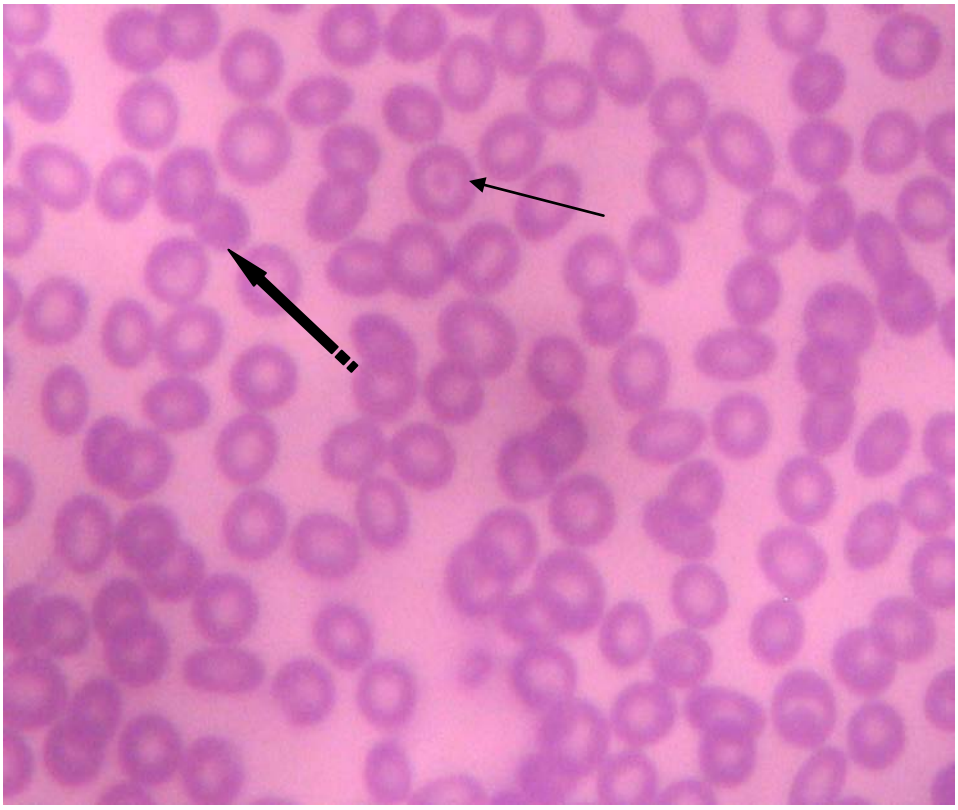


(B)

Figure (3-7). (A) Atlas photograph (Allan and John, 2003). (B) Microscopic photograph (x400) of blood film taken from the mother who is β –*thalassemia minor* showing target cell pointed by solid arrow ⟶ and anisocytosis pointed by discreet –dotted arrow ⇢



(A)



(B)

Figure (3-8). (A) Atlas photograph (Allan and John, 2003). (B) Microscopic photograph (x 400) of blood film taken from the father who is β –*thalassemia minor* showing target cell indicated by solid arrow ⟶, and anisocytosis pointed by discreet –dotted arrow ⇢ .

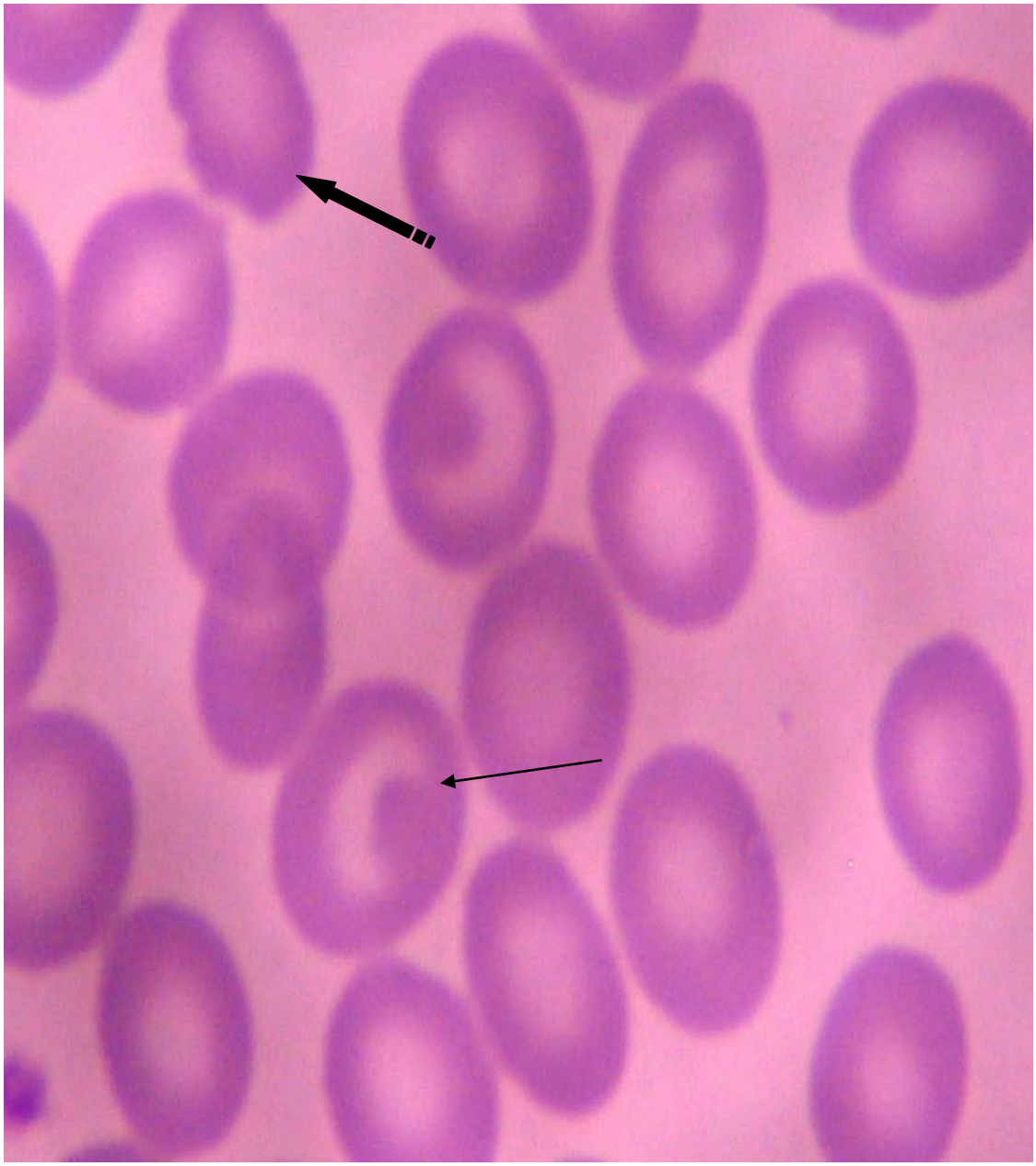
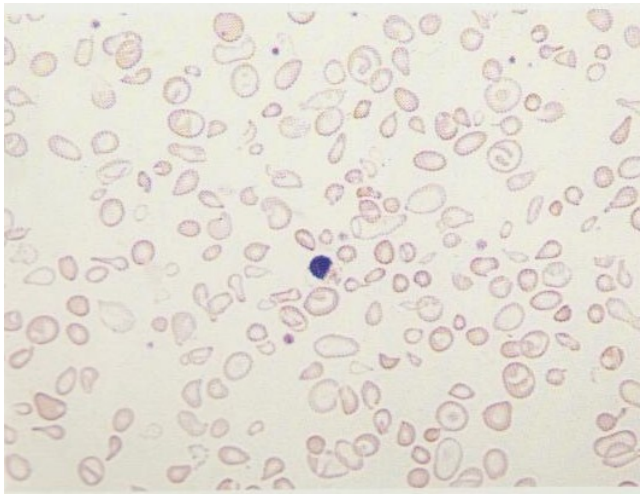
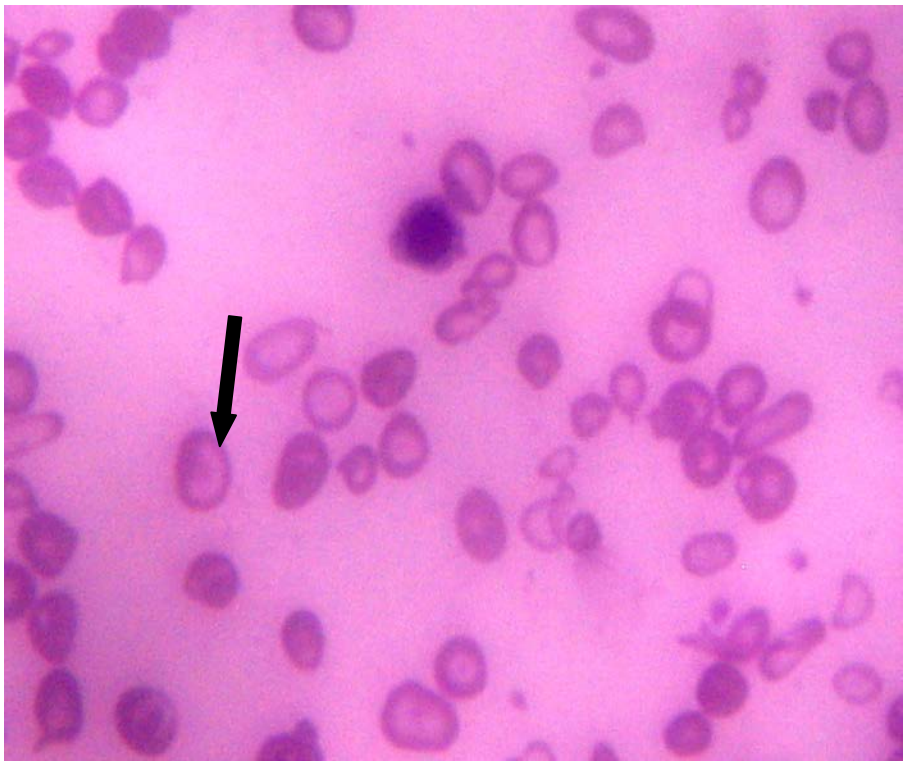


Figure (3-9). Microscopic photograph (x1000) of blood film taken from the mother who is β –*thalassemia minor* showing target cell pointed by solid arrow ⟶ , and anisocytosis pointed by discreet –dotted arrow ⇢ .



(A)



(B)

Figure (3-10).Microscopic photograph (x400) from Sandoz atlas. (B) Microscopic photograph (x400) of blood film taken from the patient who is β –*thalassemia major* showing target cell pointed by solid arrow ➡.

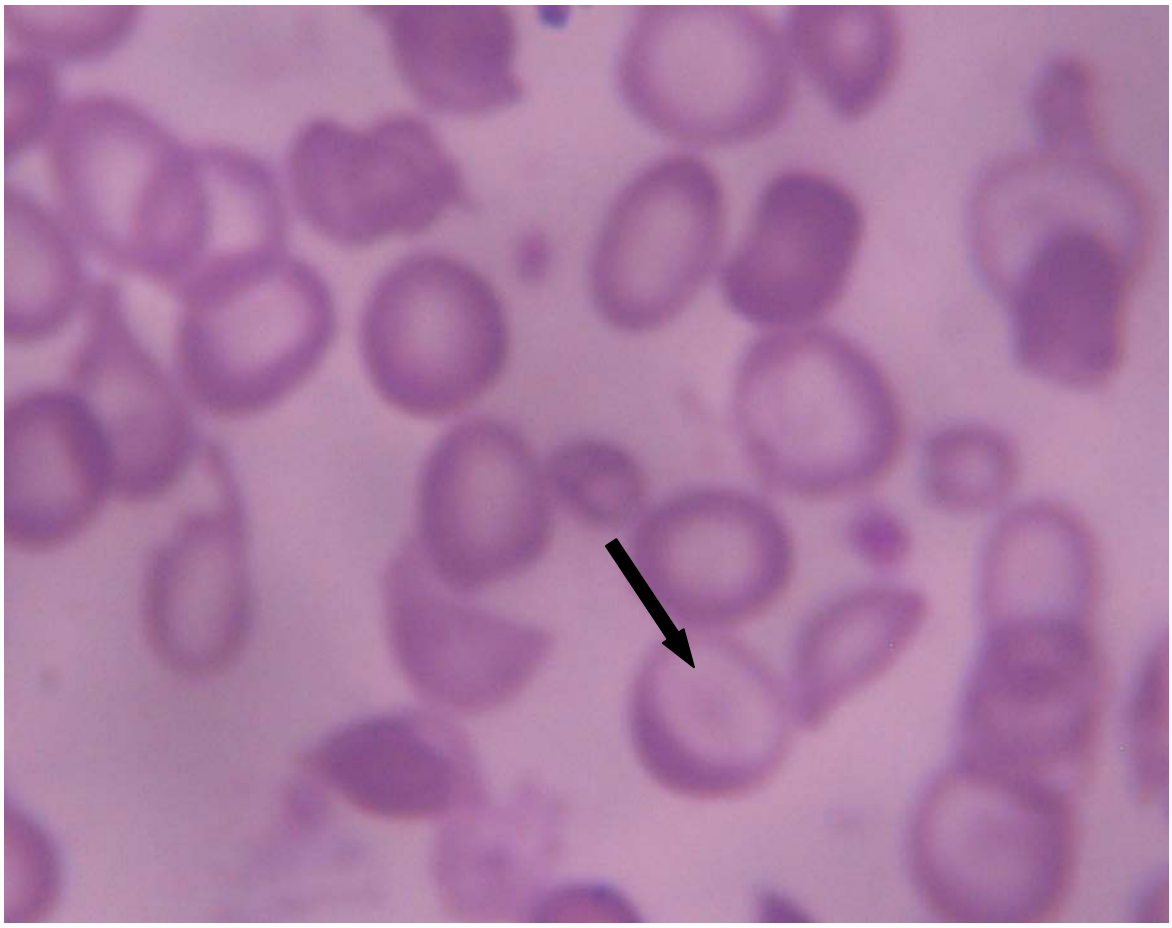


Figure (3-11).Microscopic photograph (x1000) of blood film taken from the patient who is β –*thalassemia major* showing target cell pointed with solid arrow ⟶ .

It was concluded that a carrier of the β –*thalassemia minor* is suspected when microcytic hypochromic RBCs, and target cells were also found in blood sample smear in which they are the common feature in this type of disease, but β -*thalassemia major* patient is suspected when severe

microcytic anemia is found, poikilocytes, cells fragments, and anisocytosis were found in blood sample smear.

The main reason for the presence of such type of cells in blood is the great need for red blood cells to perform the normal function in oxygen and nutrient supplementation, since there is a mild level of blood hemolysis with mild reduction in HbA level. The only difference between the minor and major type of this disease is the percentage of occurrence of these abnormal cells in blood which is affected by the type of the disease as a result of its severity. Their percentage is high in patients with *thalassemia major* and less in patients with *thalassemia intermedia* and less in *thalassemia minor*.

3.2.2 Red blood cells indices in β –thalassemia

Red blood cells indices in β –thalassemia carriers were obtained by measuring MCH, MCV, PCV and Hb. Table (3-1) showed red blood cells indices in parents who are both β –*thalassemia minor* and their affected child who is β –*thalassemia major*.

Table (3-1).Red blood cells indices in parents who are both *β –thalassemia minor* and their affected child who is *β –thalassemia major* (Allan and John, 2003).

Red blood cells index	Female (normal range)	Male (normal range)	β –thalassemia minor (carrier)		β –thalassemia major (affected)
			Mother	Father	Son
Mean Corpuscular Volume (MCV) fl	80-95	80-95	64.34	77.69	70.16

Mean Corpuscular Hemoglobin (MCH) pg	27.5-33.2	27.5-33.3	19.6	25.66	21
Packed Cell Volume (PCV) %	36-48	42-52	36.1	43.9	33.4
Hemoglobin (Hb) g/dl	12-16	14-18	11.03	14.5	10
Red blood cells count (RBCs)	3.8 -5.8 x 10^{12}	4.5-6.5 x10^{12}	5.61 x10^{12}	5.65 x10^{12}	4.76 x10^{12}

fl =10^{-15}

pg =10^{-12}

dl =10^{-2} (Dacie and Lewis, 2001).

The mean values for MCV were obtained for carrier (mother and father) and their affected child (*β –thalassemia major*) were almost similar but lower than mean values of the control. The mean values for MCH, PCV, and Hb were obtained for carrier (mother and father) and their affected child (β –*thalassemia major*) were lower than mean values of normal, while the red blood cells counts were in normal ranges.

Table (3 -2). Red blood cells indices of parents who are, healthy (father), and β –*thalassemia minor* (mother) and their offspring who is β –*thalassemia minor* (daughter). (Allan and John, 2003).

Red blood cells index	Female (normal range)	Male (normal range)	β – *thalassemia minor*	Healthy	β –thalassemia minor
			Mother	Father	daughter
Mean Corpuscular Volume (MCV) fl	80-95	80-95	63.44	90.04	66.72
Mean Corpuscular Hemoglobin (MCH) pg	27.5-33.2	27.5-33.3	21.20	29.86	22.30
Packed Cell Volume (PCV) %	36-48	42-52	36.8	39.8	36.5
Hemoglobin (Hb) g/dl	12-16	14-18	12. 3	13.2	12.2
Red blood cells count (RBCs)	3.8 -5.8 x 10^{12}	4.5-6.5 x10^{12}	5.80 x10^{12}	4.42 x10^{12}	5.47 x10^{12}

The mean values for MCV, MCH, PCV, and Hb obtained for carriers (mother and daughter) were almost similar but lower than mean values of

normal, while these values for the healthy (father) were within the normal range. The red blood cells count of both parents and their daughter were within the normal range. It was concluded that carriers demonstrate low MCV, MCH values in relation to severity of anemia; they represent the ratio between red blood cells with PCV and red blood cells with Hb respectively that gives good indication for the red blood cells hemolysis as a result of the disease. Carriers also demonstrate morphological changes in red blood cells that are less severe than in affected individuals.

Moreover, erythroblasts are normally not seen and carriers are symptoms free (Camaschella and Cappellini, 1995). So, red blood cells appearance and their indices are important in the diagnosis of β –thalassemia (Antonio and Renzo, 2000). These cells are found to perform the normal function in oxygen supplementation to the body and since there is a mild level of blood hemolysis with reduced rate of HbA level in *β –thalassemia minor* syndrome, in spite of the mild Hemolysis, the bone marrow of a carrier is normal and the life age of the red blood cell is normal or nearly so.

β –thalassemia carrier is suspected when there are hypochromic microcytic red blood cells, anisocytsis, few target and oval cells. The rate of these cells differs among β –thalassemia population, they are found in high levels in patients of *β –thalassemia major*, and less in patients of *β –thalassemia intermedia,* and *β –thalassemia minor*.

3.2.3 Hemoglobin abnormality

All the families who were represented by samples within Baghdad population and grouped according to their family relationship (father, mother, and children) were investigated for hemoglobin abnormality, the results showed various hemoglobin abnormalities, e.g. elevated levels of HbA_2 in β –

thalassemia carriers combined with elevation in HbF levels in some carriers, whereas, decreased amount of HbA was observed as shown in table (3 - 3). These results illustrated the abnormal hemoglobins percentages in parents with β –*thalassemia minor* (trait), and in their son who is affected with β –*thalassemia major*.

Table(3-3). The abnormal hemoglobins percentages of individuals of a family.

Hemoglobin type	Normal ranges %	β–*thalassemia minor* (carriers)		β–*thalassemia major*
		father	mother	patient
HbF	0.5 -1.5	0.6	1.6	95.1
HbA_2	1.8 -3.5	5.5	6.5	3.8

The increase in HbA_2 levels is due to over – production of α and δ subunits and the elevation of HbF is due to over production of its main components (α_2 γ_2 subunits). The increased requirements of the body for oxygen and food demands, which are not fully satisfied by the main hemoglobin (HbA); force the body to increase the HbA_2 and HbF levels in blood, all this, is due to the reduction in β –chain formation or deformation in this protein that result in HbA malfunction (Antonio and Renzo, 2000). A suspected person could be identified as having the β –thalassemia or as a healthy one, by using hematological analysis standards of a healthy person then compare it with those of the suspected one, so a person with β-thalassemia can be identified as homozygous or heterozygous by measuring

HbA, HbA_2, HbF levels and observing the presence or absence of the β –chain in blood samples in regard with standard values of healthy people. All individuals of families under this study were investigated for the hemoglobin abnormality, and their mean percentages were obtained as in table (3-4).

Table (3- 4) shows the mean percentage of hemoglobin in families that were investigated for hemoglobin abnormality (Antonio and Renzo, 2000)

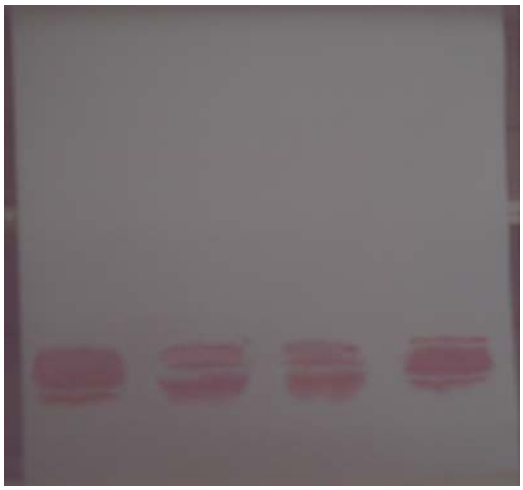
Hemoglobin type	Normal ranges %	β –*thalassemia minor* (carriers)		β –*thalassemia major* (patients)
		Father	Mother	Patient
HbF	0.5 -1.5	11.7	11.8	17.6
HbA_2	1.8 -3.5	5.9	5.1	7.2
HbA	96 -98	96	97	90.9

It was concluded that the increase in HbA_2 levels as compared with the normal levels, is due to the excess in the production of α_2 δ_2 (the main subunits) in order to satisfy the body requirements of oxygen that could not be fully satisfied by HbA, during different life stages.

3.2.3.1 Hemoglobin analysis by cellulose acetate electrophoresis

The hemoglobin analysis by cellulose acetate is simple and mostly used in laboratories for the detection of hemoglobin disorders. Blood samples from carriers and their relatives were taken and hemoglobins were separated

and subjected to electrophoresis by cellulose –acetate paper to identify abnormal hemoglobins. All 100carriersand their families who were represented by samples within Baghdad population and grouped according to their family relationship (father, mother, and children) were investigated for hemoglobin analysis by cellulose –acetate paper. The results obtained were shown in figure (3- 12) which illustrated hemoglobin analysis of parents who are *β –thalassemia minor* and their child (patient) who is *β –thalassemia major* in comparison with normal person (control).



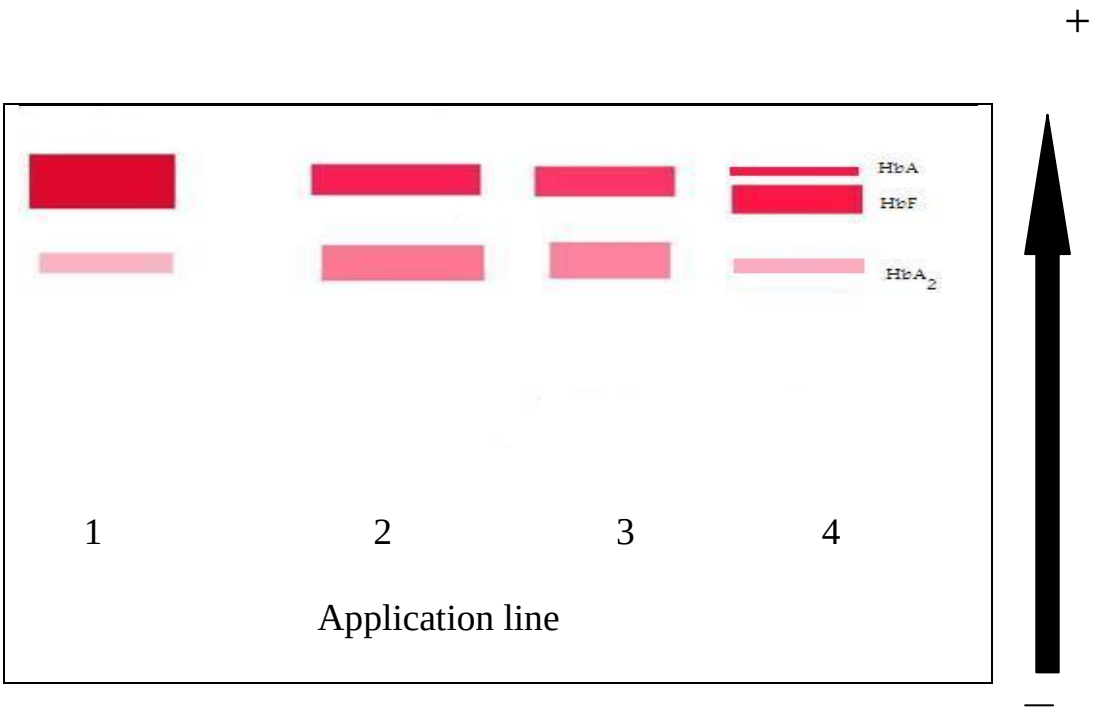
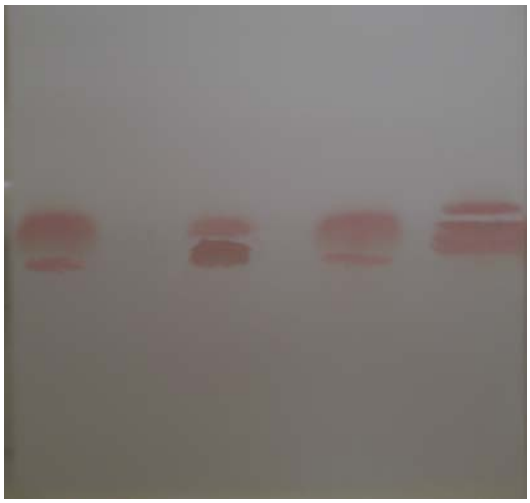


Figure (3 -12). Hemoglobin electrophoresis by cellulose – acetate paper. Line (1) control, line (2) and line (3) β –*thalassemia minor* (father and mother respectively), and line (4) β –*thalassemia major* (affected child).

It is concluded that there is a defect in the β –globin chain production leading to an excess in HbA_2 production in both parents who are both carriers, while there is HbA band in blood sample of their child who is *β –thalassemia major*, this is normal due to blood transfusion.

Results shown in figure (3- 13) illustrate hemoglobin analysis of parents, normal (mother) and a *β –thalassemia minor* (father), their offspring are, normal son and a β –*thalassemia minor* daughter.



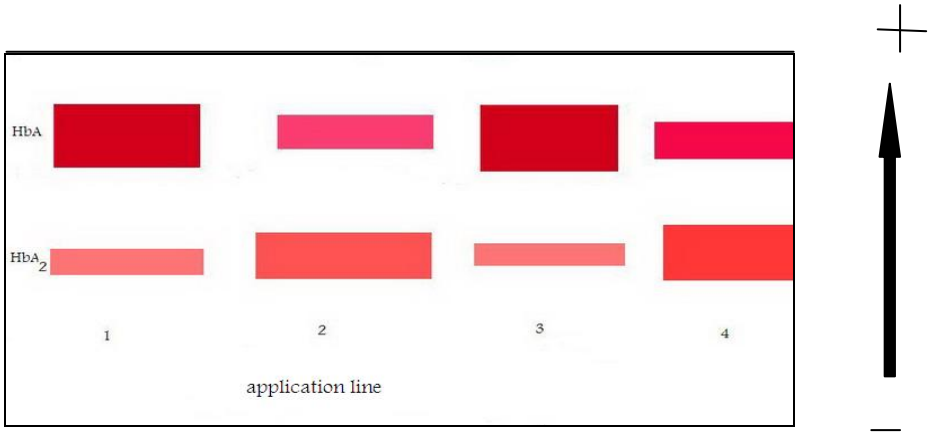


Figure (3 -13).Hemoglobin electrophoresis by cellulose –acetate paper. Lines (1) and (3) normal (mother and son respectively), lines (2) and (4) β –*thalassemia minor* (father and daughter respectively).

It is clear from this figure, that there is high level of HbA_2 in both of the father and his daughter blood samples in comparison with the normal (control), indicating the hyperproduction of this type of hemoglobin as a reflection for β –thalassemia syndrome.

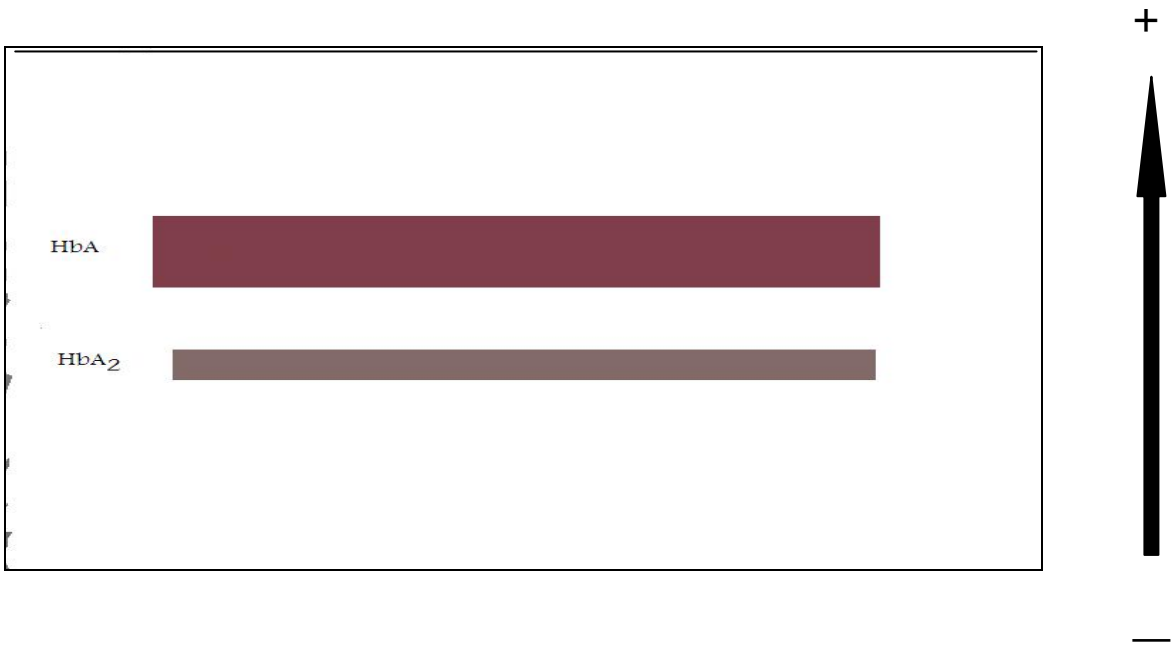


Figure (3-14). Hemoglobin electrophoresis by cellulose –acetate paper, shows the HbA_2 hemoglobin elution from HbA hemoglobin.

3.2.3.2 Hemoglobin analysis by agarose gel electrophoresis

The HYDRAGEL HEMOGLOBIN (E) K20 kit is used for this purpose. This method is used for the separation of the normal hemoglobin (HbA) and hemoglobin abnormalities (S or D and C or E) by electrophoresis on alkaline agarose gels as illustrated in the appendix I.

This technique is useful for separating human hemoglobin molecules according to their electrical charge and / or shape. The agarose gel is more accurate technique in detecting the hemoglobin abnormalities than cellulose – acetate electrophoresis; hence it was used during this study to analyze hemoglobins in individuals under study. Figure (3 -15) shows hemoglobin analysis for β –thalassemia carriers (parents) that indicate an increase in HbA_2 level due to defect in β –globin gene, and the absence of β –globin band for β –thalassemia patient (children).

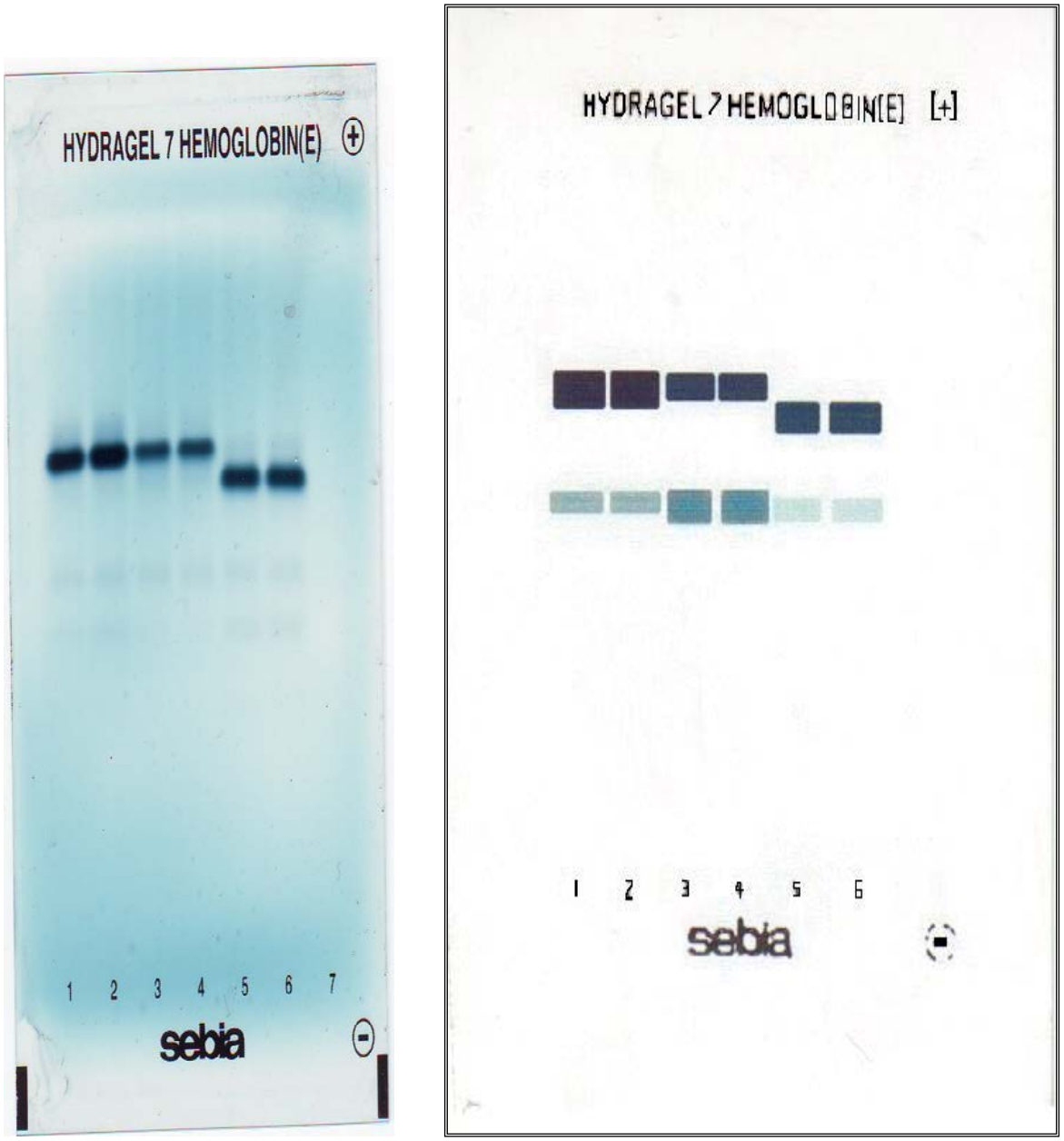


Figure (3 -15). Hemoglobin electrophoresis using agarose gel, line (1) normal (control), line (2) normal son, lines (3) and (4) β –*thalassemia minor* (father and mother respectively), lines (5) and (6) β –*thalassemia major* (daughter and son respectively).

3.2.3.3 Hemoglobin analysis by High Performance Liquid Chromatography (HPLC)

The VARIANT β –thalassemia Short Program represents the automated High Performance Liquid Chromatography (HPLC). The HPLC is being used increasingly as the initial diagnostic method of hemoglobinopa - thies (Dacie and Lewis, 2001).

It is very accurate technique and provides a provisional identification of a large proportion of variant hemoglobins using a very small amount from each blood sample.

This program provides an area percent determination of hemoglobins A_2 and F, as well as qualitative separation of normal and commonly occurring abnormal hemoglobins. Other less frequently variants may also elute within the established analyte identification windows. Figures (3-16), (3-17) and (3-18) show the hemoglobin separation of a family in which parents are carriers of β –*thalassemia minor*, and their son who was β –*thalassemia major*. The obtained percentages for father were 5.6% for HbA_2 and 0.0% for HbF, while the percentages for mother were 6.2% for HbA_2 and 1.8% for HbF. On the other hand, the child gave percentages of 3.7% for HbA_2 and 91.8% for HbF.

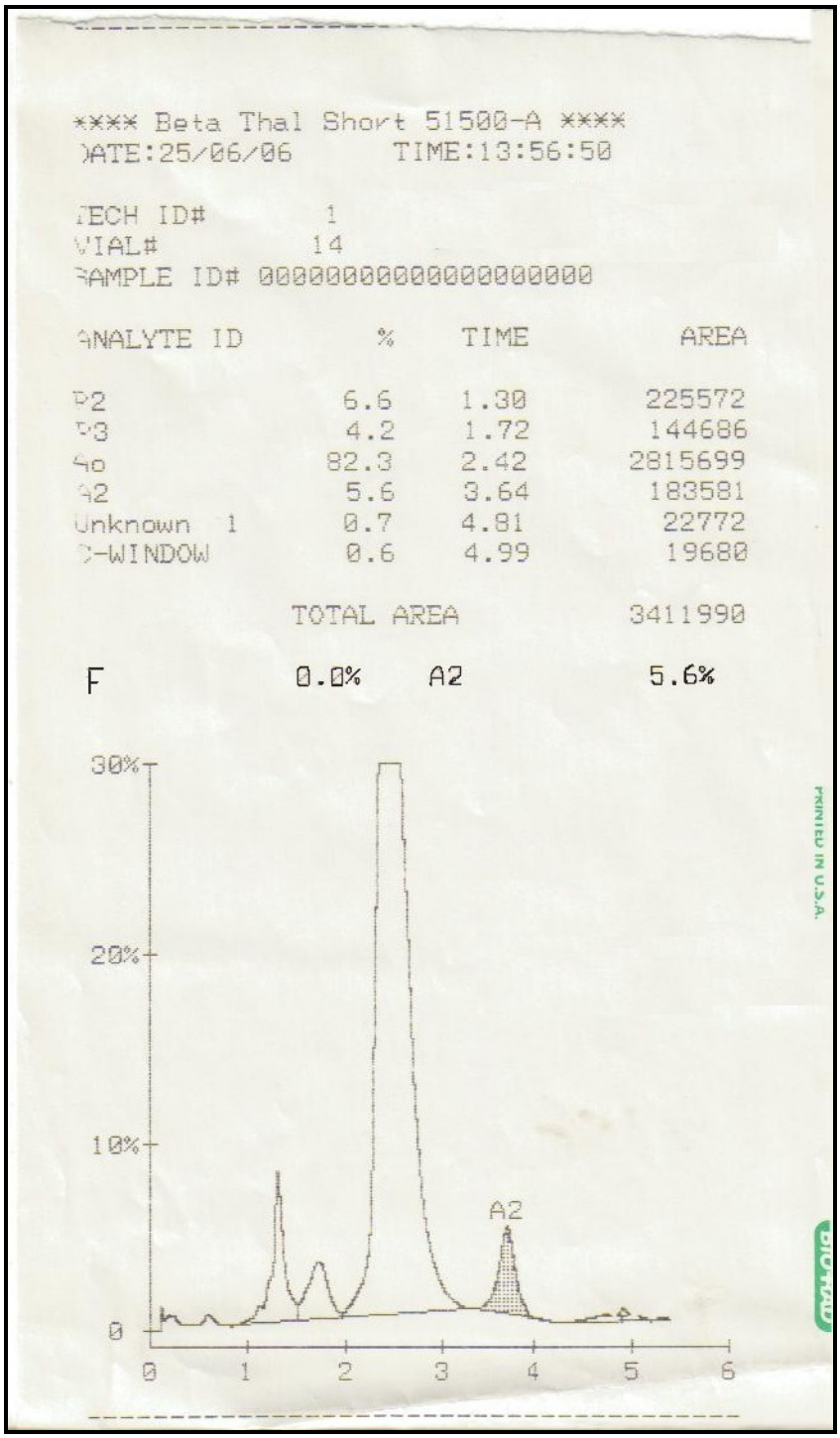


Figure (3-16).Hb separation by HPLC of β –*thalassemia minor* (father).

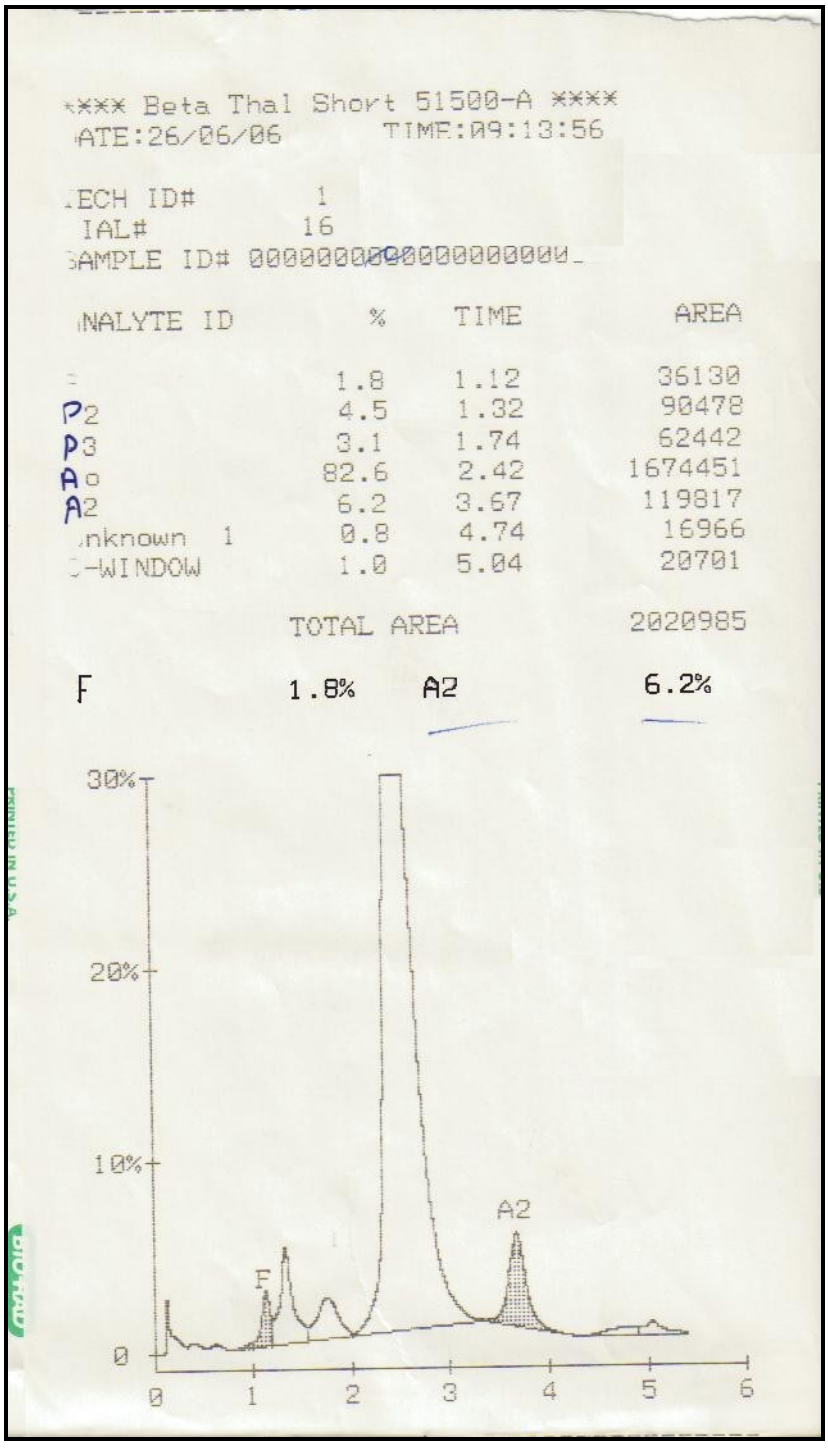


Figure (3-17).Hb separation by HPLC of β –*thalassemia minor* (mother).

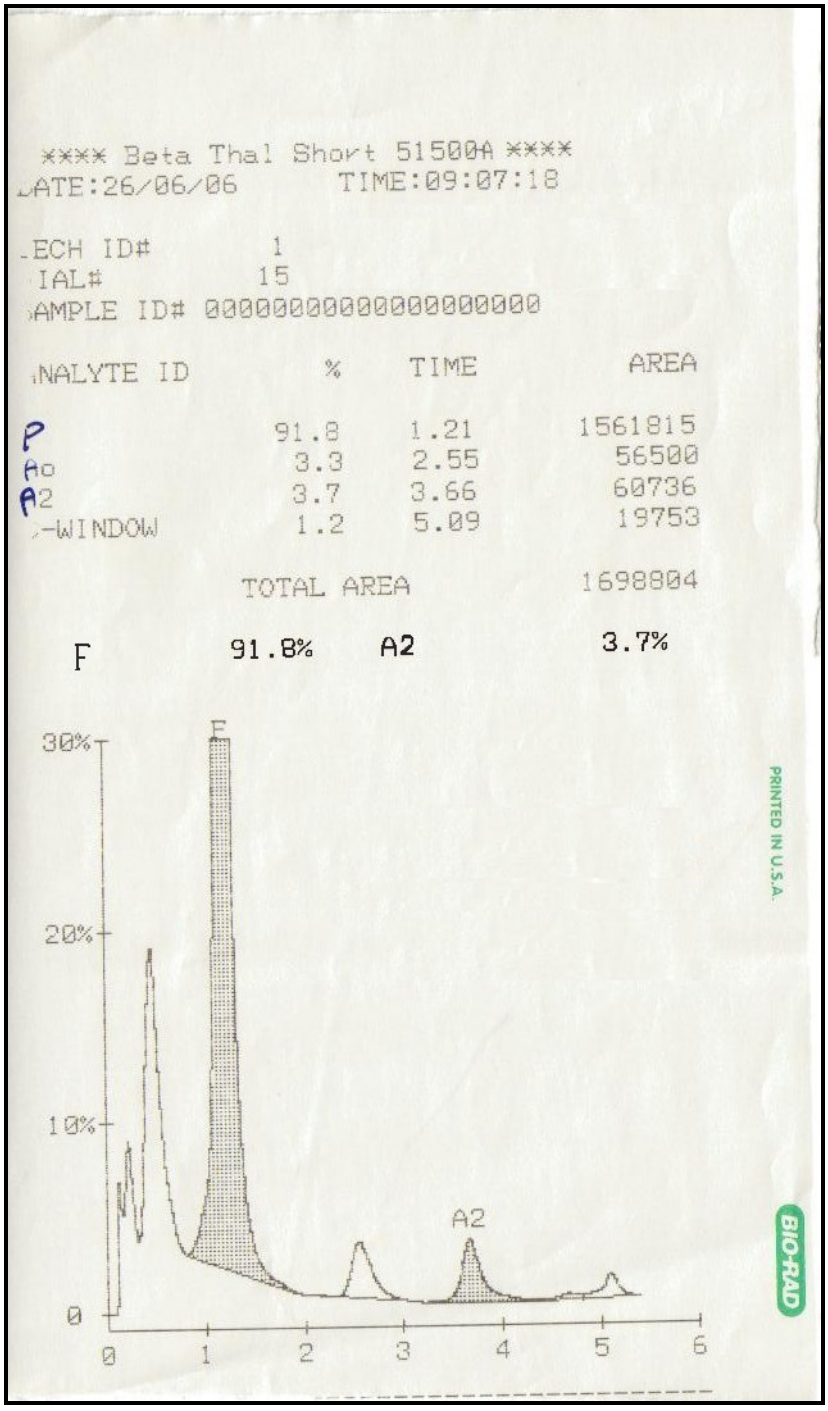


Figure (3-18).Hb separation by HPLC of β –*thalassemia major* (child).

The parents were carriers of the β –*thalassemia minor* showed elevated levels of HbA_2, while their child who was β –thalassemia major showed elevated level of HbF.

3.3 β –globin chain analysis using Polymerase Chain Reaction (PCR)

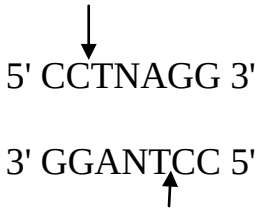
The diagnosis of β –thalassemia has progressed widely during 1980s from the phenotype diagnosis to genotype diagnosis. The PCR reaction involves using a very small amount of DNA, in which it can be amplified millions of times *in vitro*. This technique is very useful in the detection of thalassemia mutation among population (Beris, *et al.*, 1991).

In this study four specific primers were used to detect β –globin gene in members of two families as shown in figures (3-19) and (3-20). Amplification was performed in Primas for 40 cycles (94 ºC for 5 minutes, 92 ºC for 1 minute, 62 ºC for1.5 minute and 72 ºC for 2.5 minutes).

At the beginning, this study was initiated by screening genomic DNA sample of normal healthy and clinically diagnosed individual, the isolated DNA was subjected to *in vitro* gene amplification using the four specific primers. These primers are designated (P5, P7, P10, P12) are complementary to a defined region in β –globin gene.

Each two primers will amplify a specific fragment of the β –globin gene, in which P7 and P10 will amplify the 616bp fragment of the β –globin gene, P5 and P12 will amplify the 799bp fragment of the β –globin gene, while P5 and P10 will amplify the whole β –globin gene. Results obtained from the normal blood sample amplification, as in figure (3-20) showed that only the 616bp fragment was amplified while the 799bp fragment showed no response for amplification. Then the PCR product was subjected to restriction

analysis using specific restriction enzyme (*Bsu*36 I), it cut at the site:



N represents any base, and according to the supplier of the restriction enzyme, normal DNA sample contain the restriction site and it will be digested (as shown on the agarose gel) into two fragments (or more depending on the number of the restriction site in the same sample), while β – thalassemic individual do not have the restriction site due to a mutation that altered the restriction site and caused the disease, so it will not be digested on electrophoresis, and this disappearance of the restriction site mean the presence of a frameshift mutation in codon 6 of the β –globin gene (Haig and Kazazian, 2002), but this will need DNA sequencing of the carrier and patient DNA to know the main cause of the frameshift in codon 6.

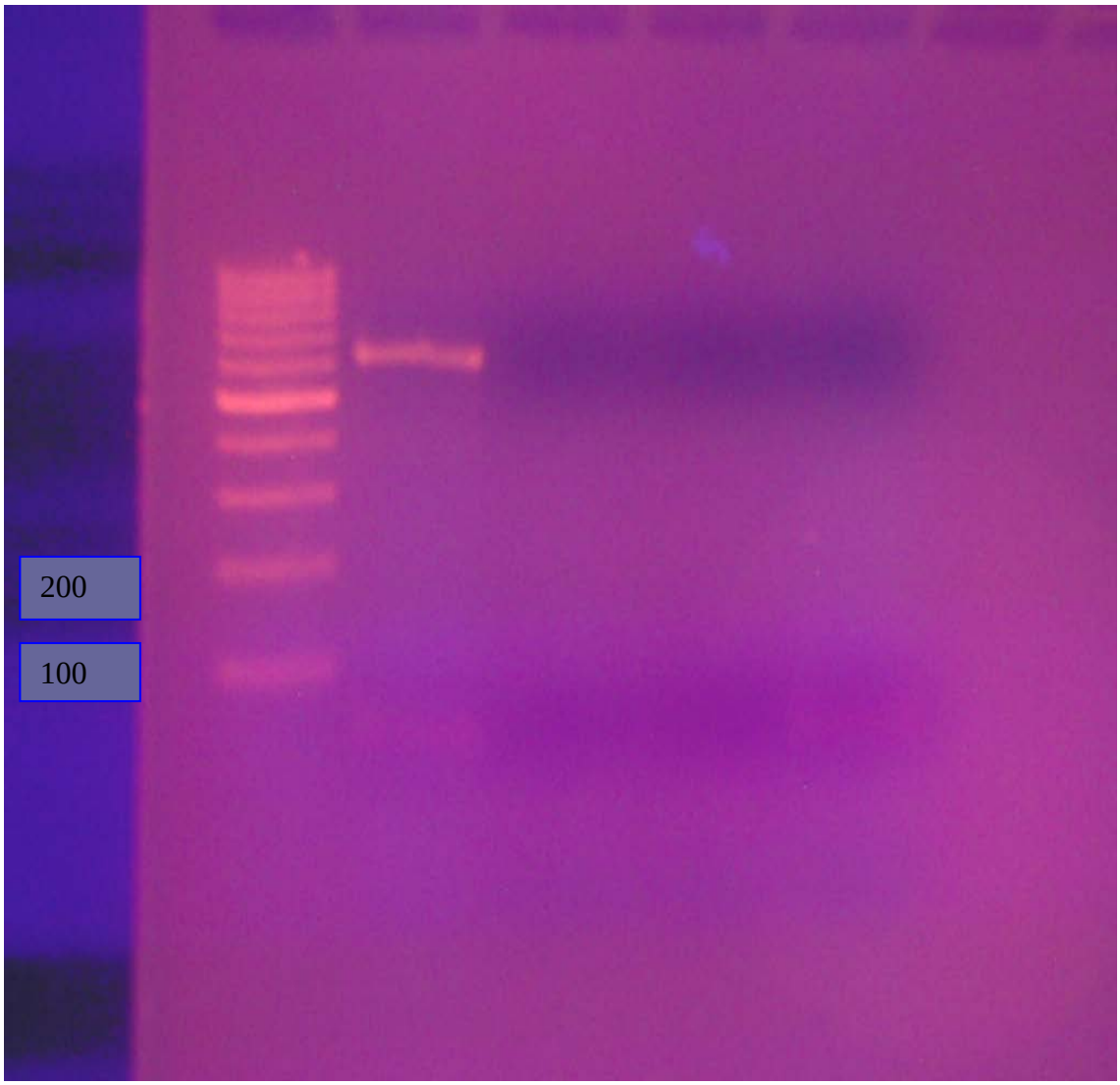


Figure (3-19). PCR amplification of beta –globin DNA of normal healthy individual. Ethidium bromide stained agarose gel (1%) of the PCR product, 400mA,100V,45 min. Line (1) marker DNA, line (2) 616bp fragment, lines (3) and (4) 799bp fragment, line (5) negative control. Numbers on the left indicate bp.

First family, represented by three individuals subjected to PCR amplification and results obtained as in figure (3-20). This figure showed the amplification of the 616bp fragment of the β –globin gene.

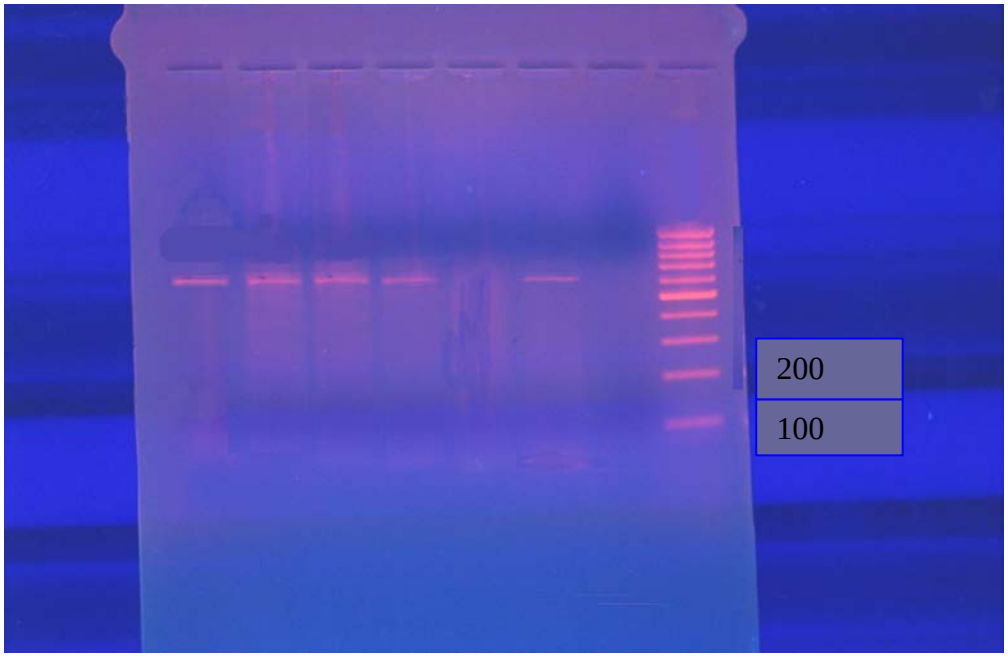


Figure (3-20). PCR amplification of the first family showing β –thalassemia syndrome. Ethidium bromide stained agarose gel (1%) of the PCR product, 400mA, 100V, 45 min. Line (1) β –*thalassemia minor* (positive control), lines (2) and (3) β –*thalassemia minor* (father and mother respectively), line (4) normal (616 bp fragment), line (5) normal (799bp fragment), line (6) β –*thalassemia major* (child), line (7) negative control, line (8) marker DNA. Numbers on the right indicate bp.

Second family, represented by five individuals, subjected to PCR amplification and results obtained as in figure (3-21).

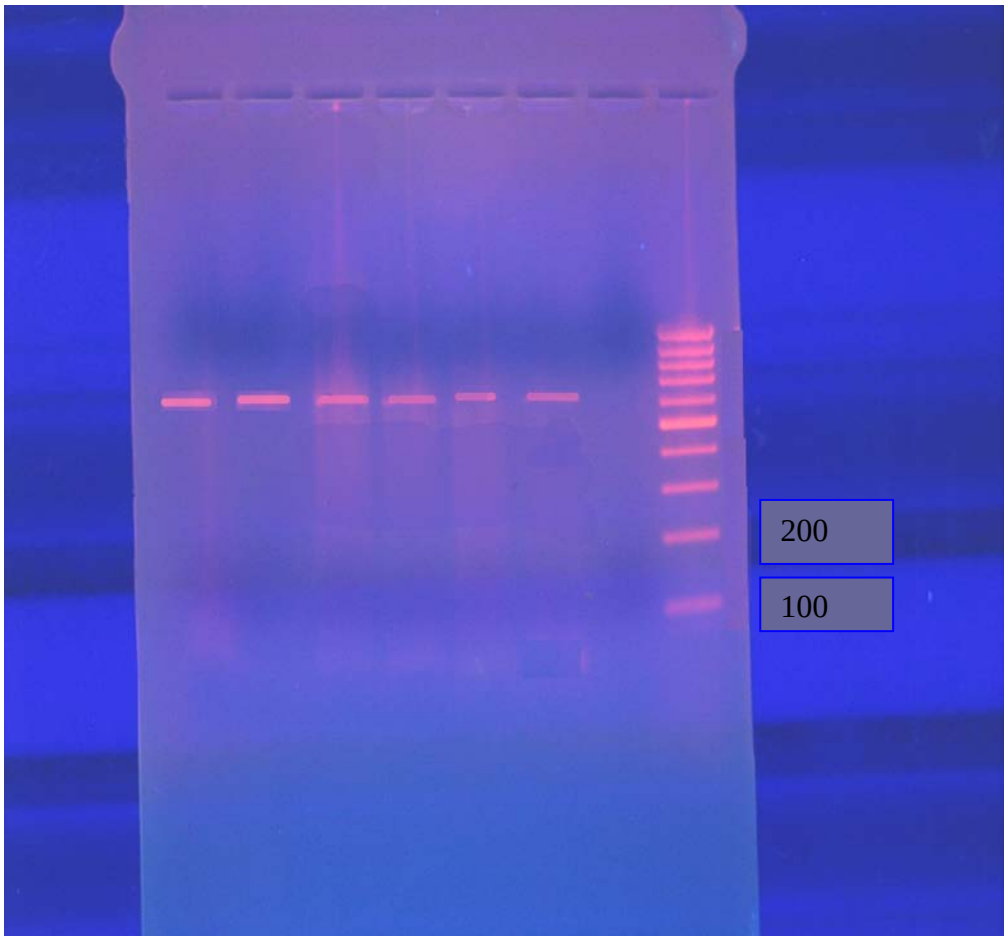


Figure (3-21). PCR amplification of the second family showing β – thalassemia syndrome. Ethidium bromide stained agarose gel (1%) of the PCR product,400 mA,100V, 45 min. Line (1) normal (control), line (2) normal (father), lines (3), (4), (5), and (6) β –*thalassemia minor* (mother, son, daughter, son respectively), line (7) negative control, line (8) marker DNA. Numbers on the right indicate bp.

Members of the two families were subjected to restriction technique with the *Bsu*36 I restriction enzyme.

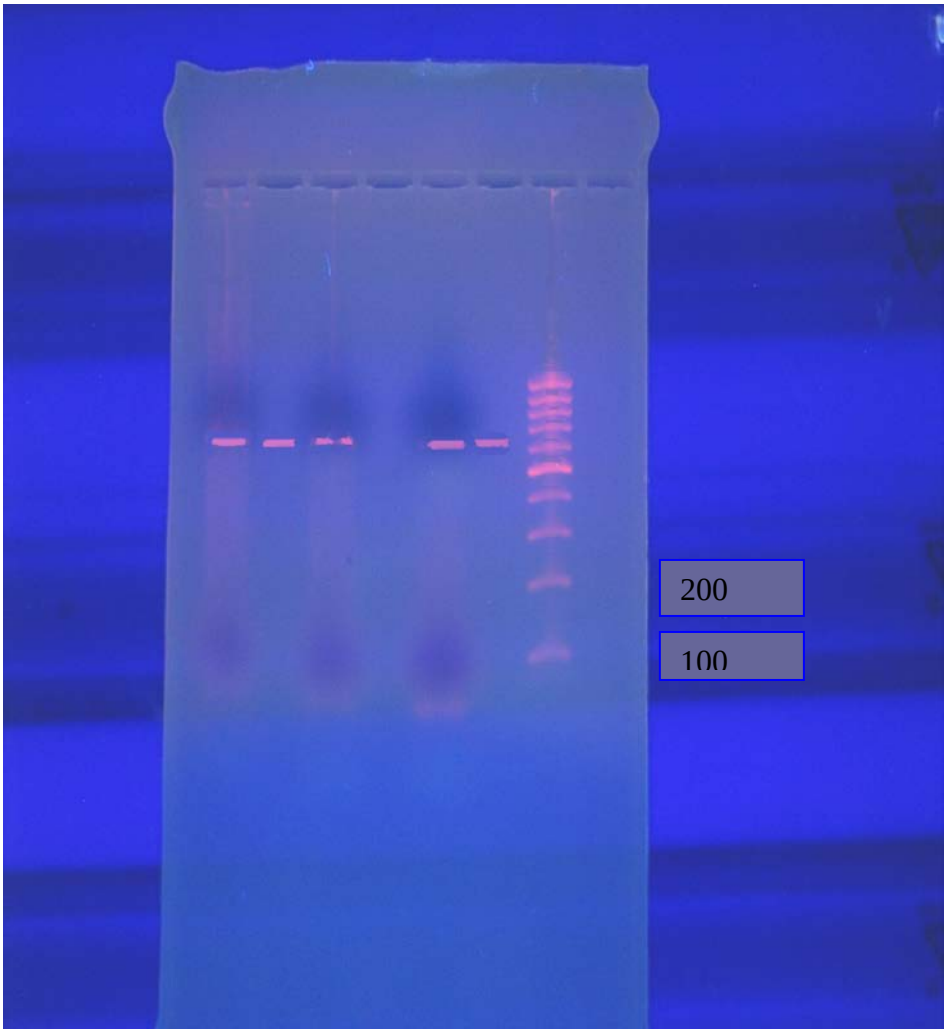


Figure (3-22). Restriction analysis of the first family. Ethidium bromide stained agarose gel (1%), 400mA, 100V, 45 min. Lines (1) and (2) β –*thalassemia minor* (father and mother respectively), line (3) normal (control), line (4) negative control, line (5) β –*thalassemia major* (child), line (6) β –*thalassemia minor* (control), line (7) marker DNA. Numbers on the right indicate bp.

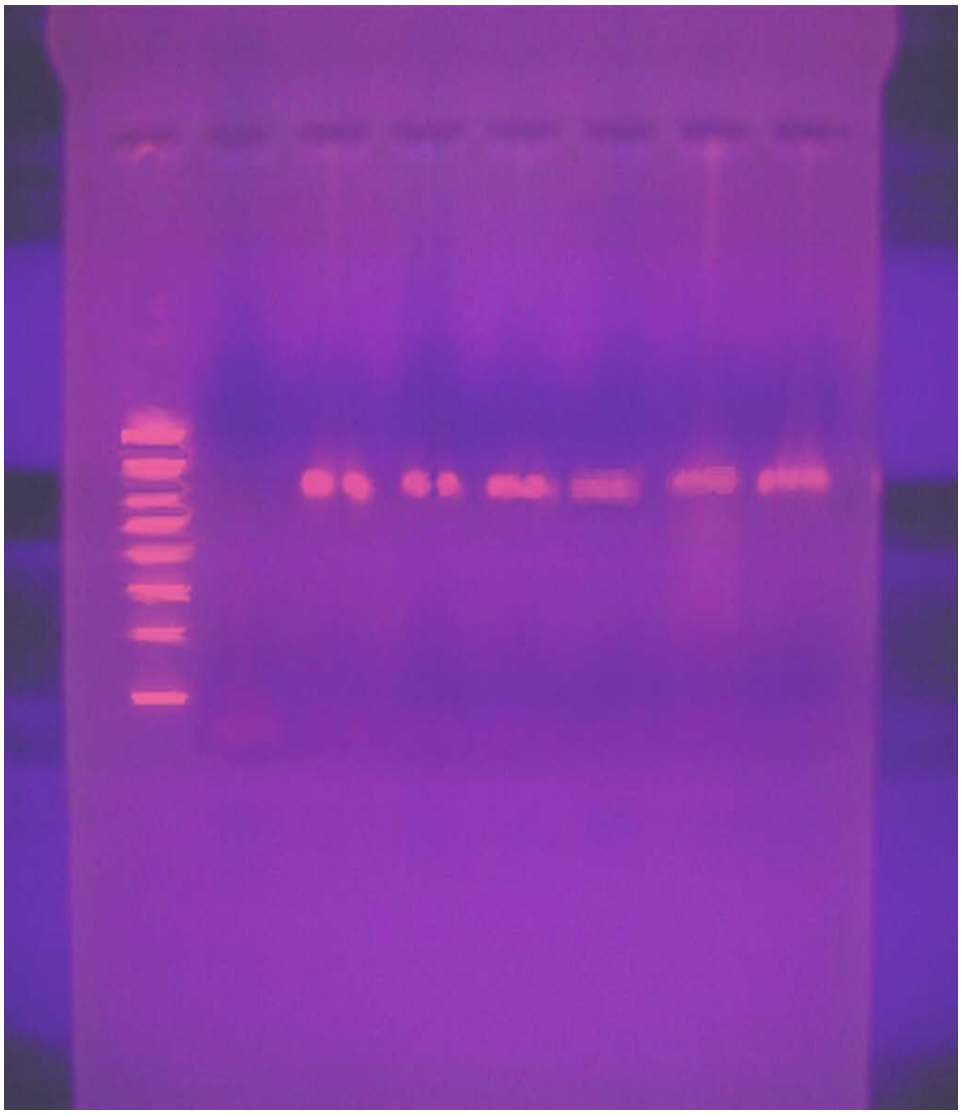


Figure (3-23). Restriction analysis of the second family. Ethidium bromide stained agarose gel (1%), 400mA, 100V, 45 min. Line (1) marker DNA, line (2) negative control, line (3) normal (control), line (4) normal (father), line (5) β –*thalassemia minor* (mother), lines (6), (7) and (8) β –*thalassemia minor* (son, daughter, and son respectively). Numbers on the left indicate bp.

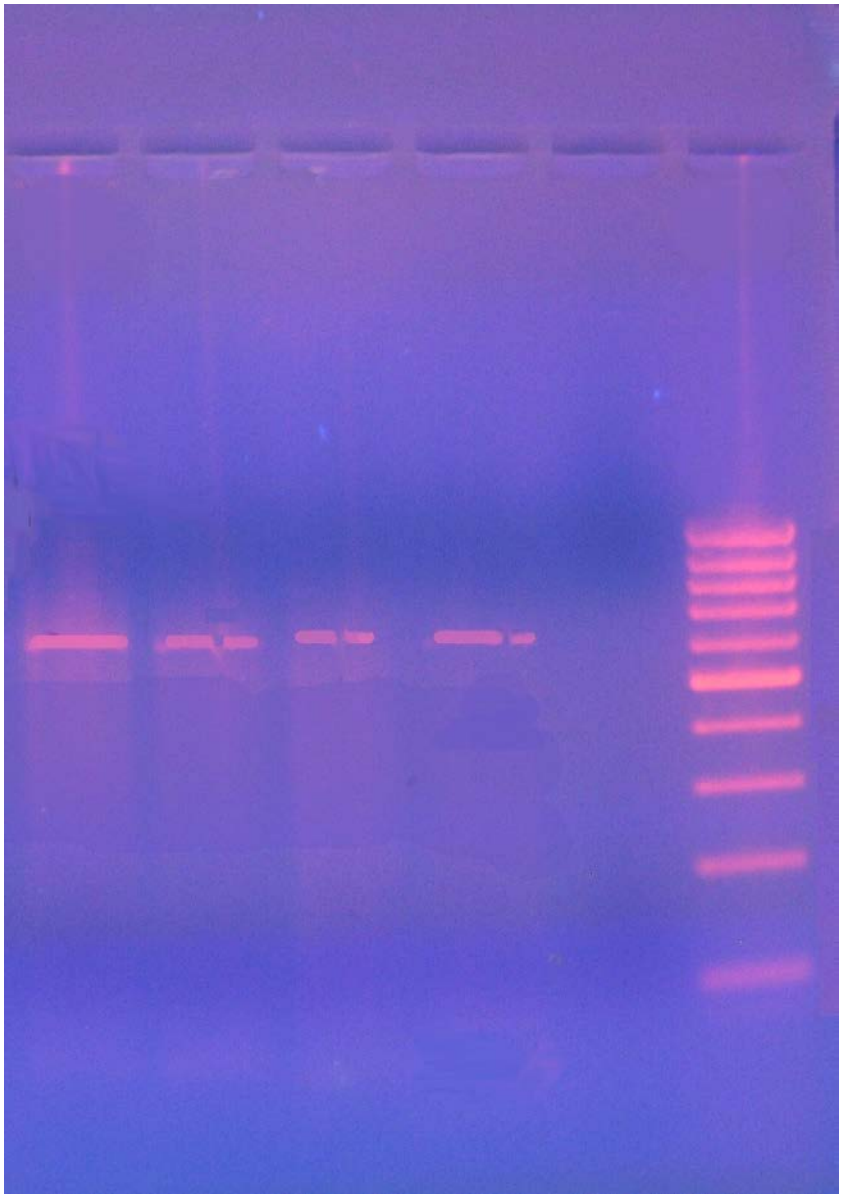


Figure (3-24). Restriction analysis of the third family. Ethidium bromide stained agarose gel (1%),400mA, 100V, 45 min. Lines(1) and(3) β – thalassemia minor (daughter and father, respectively), line(2) normal (mother), line(4) normal (control), line(5) negative control, line(6) marker DNA. Numbers on the right indicate bp.

Conclusions

1. Blood films of carriers showed hypochromic microcytic red blood cells with the presence of target cells, while blood count showed that red blood cells count were within normal ranges, but the red blood indices were lower than the normal levels.
2. The VARIANT β –thalassemia Short Program had proved to be more sensitive and accurate than cellulose acetate paper electrophoresis and agarose gel electrophoresis in detection of β –thalassemia, so it is recommended for the detection and analysis of β –thalassemia.
3. Hemoglobin electrophoresis of HbA_2 in carriers with β –thalassemia minor showed elevated levels of HbA_2 than normal ratio due to reduction in β –chain formation, which resulted in malfunction of the normal hemoglobin HbA.
4. Amplification of the 616bp and 799bp fragments of the β –globin gene of normal healthy clinically recognized individual by using specific PCR primers, in which results showed that amplification of the 616bp fragment only while the 799bp fragment did not response to amplification.
5. The 616bp fragment was subjected to specific restriction enzyme and result obtained subjected to electrophoresis, showed two DNA bands, indicating that the normal sample have the restriction site, while most carriers showed only single DNA band after restriction indicating the presence of frameshift mutation in codon 6 that altered the restriction site of the specific restriction enzyme (*Bsu*36 I) used in this study, while some carriers showed two DNA bands indicating the presence of the restriction site, so the disease caused by another type of mutation.

Recommendations

1. Amplification of the whole gene using specific primers to define other types of mutation that cause the disease followed by DNA sequencing of the carriers gene.
2. Including the other parts of Iraq in such types of study, and define the most popular types of mutations that cause this disease.

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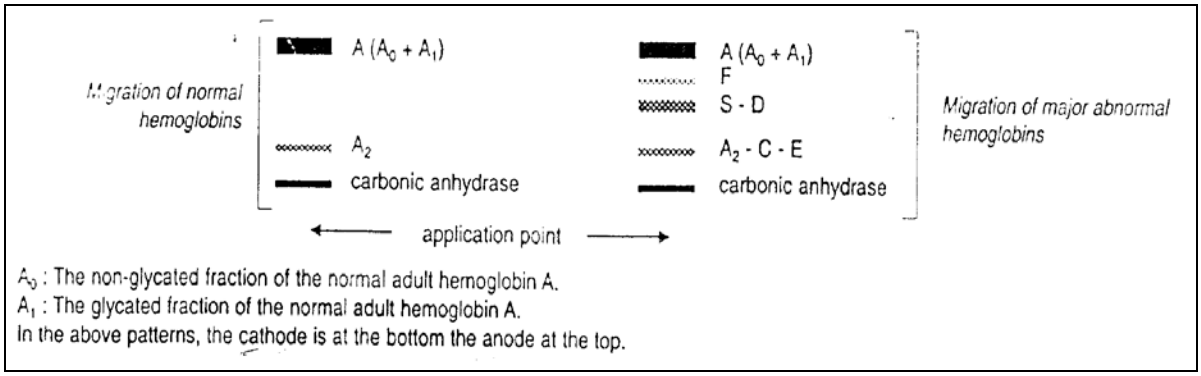
Appendix I

The HYDRAGEL HEMOGLOBIN (E) K20 kit.

Agarose gel electrophoresis. The migration of normal and major abnormal hemoglobins.



A_0 : The non-glycated fraction of the normal adult hemoglobin A.
A_1 : The glycated fraction of the normal adult hemoglobin A.
In the above patterns, the cathode is at the bottom the anode at the top.

الملخص

في هذه الدراسة تم اجراء تحليل جزيئي ووراثي لفقر دم البحر الابيض المتوسط في مئة عينة من حاملي المرض من سكان مدينة بغداد. الدراسة تضمنت، التحليل الاولي للدم و دراسة شجرة العائلة لحاملي المرض واقاربهم(pedigree analysis) والتحليل الكهربائي لنوع الهيموغلوبين، وتحليل جين البيتا غلوبين على المستوى الجزيئي، كل ذلك تم بالمقارنة مع الاشخاص الطبيعيين.

الجزء الاول من الدراسة تضمن اجراء الفحص المختبري لمئة حامل للثلاسيميا نوع β- minor، والذين قدموا الى مختبر الصحة العام المركزي في بغداد. التحليل الاولي للدم تضمن PCV، MCV، و MCH. النتائج اظهرت ان PCV كان ٣٢-٣٨% للناقلين بصورة عامة بينما النسب للاشخاص الطبيعيين كانت ٣٦-٤٨% للاناث و٤٢-٥٢% للذكور ، وMCV كان ٦٢-٦٥fl للناقلين و٨٠-٩٥fl للاشخاص الطبيعيين، و MCH كان ١٩-٢٦pg للناقلين و٢٧,٥-٣٣,٢ pg للاشخاص الطبيعيين.

علاوة على ذلك تم تحليل Aو A_2 وF ، اظهرت النتائج ارتفاع نسب هيموغلوبين A_2 ونقص بنسب A في الناقلين هيموغلوبين بالمقارنة مع الاشخاص الطبيعيين.

هيموغلوبين

بالاضافة لذلك، وجد بانه هناك تغيرات مظهرية لكريات الدم الحمراء (RBCs) حيث انه هذه الخلايا تكون اصغر من الحجم الطبيعي وشاحبة اللون كنتيجة لهذا المرض، بينما كريات الدم البيض كانت في المستويات الطبيعية للناقلين.

تضمن هذا الجزء من الدراسة ايضا"، تحليل الهيموغلوبين للاباء الناقلين واطفالهم المصابين بتناذر البيتا ثلاسيميا، باستخدام ثلاثة تقنيات للتحليل الكهربائي واظهرت النتائج بانه هناك اختلاف بسيط بنسب الهيموغلوبين بين الناقلين الذين مثلو عينات الدم التي اخذت لاكمال هذه الدراسة.بالاضافة لذلك كان هناك اختلاف واضح بنسب الهيموغلوبين بين الناقلين والمصابين، بالمقارنة مع الاشخاص الطبيعيين.

كما تمت دراسة العوائل المصابة بتناذر البيتا ثلاسيميا بالاعتماد على بيانات تم الحصول عليها مسبقا" وعلى تاريخ العائلة حيث اظهرت الدراسة كيفية انتقال هذه الصفة عبر الاجيال، خصوصا" في العوائل التي تفضل زواج الاقارب.

الجزء الثاني من الدراسة تضمن اجراء التحليل الجزيئي على مستوى الدنا وذلك باستخدام تقنية التفاعل المتسلسل لانزيم البلمرة (PCR) واربعة بوادئ خاصة لهذا التفاعل، بلاضافة لاستخدام انزيم خاص لغرض القطع ا (*Bsu*36 I) .

اظهرت نتائج القطع وجود طفرة في الكودون السادس لجين البيتا غلوبين في الناقلين والتي ادت الى تثبيط جزئي في عملية التعبير الجيني لجين البيتا غلوبين بالمقارنة مع الاشخاص الطبيعيين حيث ان

هذه الطفرة حدثت في اليل واحد لهذا الجين مما ادى الى ظهور حزمة واحدة للدنا عند ترحيل العينة على الهلام، بينما اظهرت نتائج ترحيل دنا الشخص الطبيعي وجود حزمتين على الهلام مؤكدة وجود موقع القطـــــــع وعـــــــدم حـــــــدوث اي تغييـــــــر فـــــــي التعبيـــــــر الجينـــــــي.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

دراسة وراثية جزيئية لتنادر ثلاسيميا β –minor

رسالة

مقدمة الى كلية العلوم في جامعة النهرين

وهي جزء من متطلبات نيل درجة الماجستير
في علوم التقانة الاحيائية

من قبل

رقية حازم عبد اللطيف العزي

بكالوريوس تقانة إحيائية - كلية العلوم – 2004

جامعة النهرين

بإشراف

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