Advances in Biology & Earth Sciences
Vol.8, No.1, 2023, pp.44-51

MODERN METHODS OF LABORATORY DIAGNOSIS α AND β THALASSEMIA

K.A. Alieva, R.F. Mamedova*

Department of Genetics, Baku State University, Baku, Azerbaijan

Abstract. This article discusses abnormal globin genes in various combinations that lead to many thalassemias, including three severe thalassemias, i.e., homozygous β-thalassemia, β-thalassemia/Hb E, and fetal dropsy Hb Barth. Variations in the laboratory diagnosis of thalassemia, which require a range of tests, including erythrocyte indices and Hb and DNA analyzes, are also reviewed. It is noted that the analysis of RBCs in thalassemia using an automatic hematology analyzer is the primary screening for thalassemia, since microcytosis and a decrease in the content of Hb in erythrocytes are hallmarks of all RBCs in thalassemia, however, these two RBC indices cannot distinguish a feature of thalassemia from iron deficiency or between conditions. α- and β-thalassemia. Today, hemoglobin analysis can be performed either by automated high performance liquid chromatography (HPLC) or by a capillary electrophoresis (CE) system. These two systems provide both qualitative and quantitative analysis of hemoglobin components and assist in prenatal and postnatal diagnosis of thalassemia within a short period of time. Various molecular methods used to detect point mutations in β-thalassemia and to detect large deletions in α-thalassemia are reviewed. The article notes that all of these methods have some advantages and disadvantages. In conclusion, all service laboratories are strongly encouraged to select the method(s) that are most familiar to them and economical for their daily use.

Keywords: *thalassemia, hemoglobinopathies, diagnosis, hemoglobin analysis, DNA analysis.*

Corresponding Author: *R.F. Mammadova, Department of Genetics, Baku State University, Z. Khalilov 23, AZ 1148, Baku, Azerbaijan, e-mail: rena.mamedova2015@mail.ru*

Received*: 6 February 2023;* ***Accepted****: 9 March 2023;* ***Published****: 18 April 2023.*

1. Introduction

Hemoglobinopathies can be roughly divided into two groups: structural hemoglobin (Hb) variants (abnormal Hb) and thalassemias. These structural variants of hemoglobin are usually caused by single amino acid substitutions in the α- or β-globin chains. Most of these abnormal hemoglobins do not have clinical symptoms; however, some of these mutations can change the functional properties or stability of hemoglobin and lead to clinical disorder. Thalassemias result from a defect in the globin chain. They are classified into α-, β-, δβ- and δβγ-thalassemias according to the specific globin chains that are synthesized with the defect. From a public health standpoint, only α- and β-thalassemias are usually of concern (Calzolari et al., 1999; Weatherall and Clegg, 2001).

The main pathophysiological change in thalassemia is an unbalanced production of the globin chain. This results in the destruction of erythrocyte precursors in the bone

How to cite (APA):
Alieva, K.A., Mamedova, R.F. (2023). Modern methods of laboratory diagnosis α and β thalassemia. *Advances in Biology & Earth Sciences*, *8*(1), 44-51.

marrow or peripheral blood, resulting in chronic anemia, splenomegaly, and skeletal deformity due to bone marrow expansion (Weatherall, 1998). Homozygous or compound heterozygous states for β-thalassemia have a variable course; however, death occurs mostly in the first few years of life without blood transfusion. With adequate blood transfusion and administration of iron chelators, patients with thalassemia may develop normally and live to adulthood. Moderate β-thalassemias, such as β-thalassemia/Hb E, have a wide range of clinical manifestations from a condition compatible with normal survival and growth into adulthood without treatment, to transfusion-dependent thalassemia (TDT). The reasons for this clinical heterogeneity are not fully understood (Olivieri, 1999; Barbara, 2006).

It should be noted that α-thalassemias are also heterogeneous. The milder forms (called α-thalassemia 2 or α^{+}-thalassemia) result from the deletion of one α-globin gene and cause mild anemia in their homozygous states. Whereas α-thalassemia 1 or α^{0}-thalassemia is associated with a lack of synthesis of the α-globin chain due to the deletion of two α-globin genes on the same chromosome. In homozygous conditions, this leads to the most severe form of thalassemia, namely hydrops fetalis Hb-Bart. Compound heterozygous states for α-thalassemia 2 and α-thalassemia 1 result in Hb H disease, which varies in severity.

Thalassemias are extremely heterogeneous at the molecular level. Over 200 different β-globin gene mutations have been found in patients with β-thalassemia and are nearly as diverse in their molecular pathology of α-thalassemia. However, the world population appears to carry a few common mutations unique to a particular area, as well as varying numbers of rare mutations.

Let us turn our attention to the usual methods of diagnosing thalassemia, by which, for a definitive diagnosis, subjects with phenotypic features associated with thalassemia can first be characterized using hematological (erythrocyte counts) and biochemical tests (Hb analysis) and subsequent DNA analysis. However, this diagnostic approach will not detect individuals with normal or borderline erythrocyte indices and/or HbA2 levels, which are silent forms of thalassemia (Yilmaz *et al*., 2019). In addition, with at least 1800 mutations that cause thalassemia or abnormal hemoglobin variants have been characterized to date, identification of the mutation in samples from subjects with suspected hemoglobinopathies may require more labor-intensive methods (Singh *et al*., 2020). The application of new technologies and high throughput molecular approaches such as next generation sequencing (NGS) opens up the possibility for screening and accurate diagnosis of hemoglobinopathies (Li *et al*., 2020).

2. Laboratory diagnosis of thalassemia

Diagnosis of thalassemia and abnormal hemoglobin requires a combination of laboratory tests, including measurement of red blood cell indices with an automatic hematology analyzer, Hb analysis, and quantification of Hb A2 and Hb F. High performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) distinguish between thalassemias and carriers. They are widely used to replace manual equipment. These systems provide both qualitative and quantitative analysis of hemoglobin components with good accuracy and reproducibility. They allow us to perform prenatal and postnatal diagnosis of thalassemia in just a few minutes. The specific mutation of thalassemia can be detected using DNA analysis. Moreover, thalassemia genotyping can be performed using real-time polymerase chain reaction

(PCR) followed by melting curve analysis. Cases in which the mutation cannot be detected by the previous molecular analysis method will be sent for DNA sequencing. And in the past few years, genome sequencing using NGS has also been used to diagnose thalassemia.

Automatic hemoglobin analyzers

Due to the small amount of globin formation, thalassemic erythrocytes are microcytic and hypochromic. However, Hb, mean corpuscular volume (MCV) and mean corpuscular Hb (MCH) cannot distinguish between thalassemia and iron deficiency or between α- and β-thalassemia. Hb analysis is necessary to identify carriers of α- and β-thalassemia and the disease. Automated HPLC and CE are sensitive and accurate methods for the qualitative and quantitative analysis of hemoglobin components in erythrocytes (Stephens et al., 2015). The HPLC system is a cation exchange system that uses two double piston pumps to set up a gradient of sodium phosphate buffers with increasing ionic strength to pass through a spherical cation exchange resin column for 6.5 minutes. Hemolysate samples are determined using a spectrophotometer that measures double wavelength at 415 and 690 nm. The resulting chromatograms are separated by retention time (RT). Similarly, the CE system is based on free solution capillary electrophoresis from the cathode to the anode. The Hb components are separated in silica capillaries by their electroosmotic flow and at high voltage (9800 V) by electrophoretic mobility in an alkaline buffer. Photometry at an absorption wavelength of 415 nm is used for the direct determination of Hb fractions. The obtained electrophorograms are divided into 15 zones. Several publications on automatic hemoglobin analyzers have shown their effectiveness in the study of thalassemia and hemoglobinopathies for prenatal and postnatal diagnosis (Khongthai *et al*., 2019; Li *et al*., 2019).

Interactions between α-thalassemia and β-thalassemia

To date, more than 1800 mutations have been identified that cause hemoglobinopathies (HbVar, 2019). These mutated α- and β-globin genes in various combinations cause more than 60 thalassemias (Fucharoen and Winichagoon, 1997; Fucharoen et al., 1998). The severity of the disease depends on the complexity of the interaction of genes, and even patients with identical genotypes can have completely different levels of severity. This can make it difficult to identify high-risk pregnancies and provide appropriate genetic counseling for both treatment and prenatal diagnosis.

The variability in the severity of thalassemia, especially β-thalassemia, includes many gene loci, some of which are directly associated with defects in the synthesis of α, β or γ globin, while others are associated with other genes such as KLF1, BCL11A (Sripichai & Fucharoen, 2016). A good example is seen in patients with β-thalassemia/Hb E co-inheriting α-thalassemia as they will have fewer mismatched α-globin chains resulting in fewer symptoms (Winichagoon *et al*., 1985). On the other hand, co-inheritance of triple α-globin genes (ααα) can lead to increased globin chain imbalance and severe anemia in β-thalassemia (Galanello *et al*., 1983).

These findings are important for genetic counseling, especially for couples at high risk for β-thalassemia/Hb E who are undergoing prenatal diagnosis. Here is an example of family pedigree, including hematological data of high-risk couples and their offspring: the mother is double heterozygous for Hb E and α-thalassemia 1, and the father is heterozygous for β-thalassemia. It is important to characterize the specific

mutation of paternal β-thalassemia. If the mutation is β^0-thalassemia, there is a ¼ chance that the unborn child will be a complex heterozygote for β0-thalassemia/Hb E. This will result in moderate to severe clinical symptoms with iron overload and possible TDT. A β^0-thalassemia/Hb E child who inherits α-thalassemia 1 is expected to have fewer symptoms than a β^0-thalassemia/Hb E child who does not carry α-thalassemia and may lead to TDT. Thus, the recommendation for both parents would be α-globin DNA testing with an additional β-globin DNA testing for the father. Prenatal diagnosis will allow high-risk couples to identify possible adverse pregnancy outcomes.

Hb analysis usually does not help diagnose mild forms of α-thalassemia, in which one or two (cis- or trans) α-globin genes are deleted. Excess β-globin chains (Hb H molecule) in these α-thalassemias cannot be visualized with Hb analysis. Thus, α-thalassemia is often diagnosed by exclusion, where a subject with microcytic erythrocytes, a normal Hb test, and a normal iron test is suspected to have α-thalassemia. Alpha thalassemia can also be masked in the presence of β-thalassemia, in which microcytic erythrocytes are also present. For these subjects, familial testing and DNA analysis may be the definitive diagnosis, and this is important for genetic counseling.

Molecular analysis of α and β thalassemia mutations

The advent of PCR made it possible to simplify the screening of single-basic mutations (Eisenstein, 1990). Most thalassemia mutations are point mutations. Point mutations are considered single base substitutions or minor insertions or deletions. In this article, we will briefly discuss only common DNA methods, namely allele-specific PCR, reverse dot blot (RDB), real-time PCR with melt curve analysis, and DNA sequencing.

Allele-specific PCR. This method uses two primers identical in sequence except for the 3' base, one of which is complementary to the wild type and the other to the mutant base, and must use a common primer for the opposite strand. For primer extension using Taq polymerase, which does not have 3'-5'-exonuclease (corrector) activity, a perfect match of the 3'-end of the primer with the DNA template must occur. In a normal person, the PCR result will only be visible in the reaction using the wild-type primer set. A heterozygous will generate a band using both wild-type and mutant primer sets, while a homozygous mutation individual will be negative with normal and positive mutant primer sets (Suwannakhon *et al*., 2019).

Real-time PCR with melting curve analysis. Conventional PCRs give a clear result, but require labor-intensive and lengthy post-PCR processing steps. Real-time PCR or quantitative PCR (qPCR) is widely used to detect, characterize and quantify nucleic acids. These are high productivity, automation and low risk of contamination after PCR. Currently, the use of real-time PCR with melting curve analysis for the diagnosis of thalassemia is based on two general approaches: analysis of intercalating dyes and analysis based on probes that receive a fluorescent signal from the synthesis of the product in PCR.

The first approach relies on DNA intercalating fluorescent dyes, such as SYBR Green I, to bind to double-stranded DNA (dsDNA) and undergo conformational changes that increase their fluorescence. In the presence of single-stranded DNA (ssDNA) or dyes in free solution, they will not fluoresce. After the completion of the amplification reaction, the thermal cycler program generated a melt curve by increasing the temperature in small increments and monitoring the fluorescent signal at each step.

When 50% of double-stranded DNA is split into single-stranded DNA, the so-called melting point (Tm) occurs. Different size or GC content in PCR products showed different Tm peak. Therefore, for the genotyping of α-thalassemia, multiplex GAP-PCR with melting curve analysis was developed. Primers were designed to specifically amplify two deletion fragments, the -SEA and -THAI deletions, and two normal fragments, the ψζ- and α2-globin gene. Melting curve analysis distinguishes between α-thalassemia 1 heterozygotes, α-thalassemia 2 homozygotes, Hb H disease, and α-thalassemia 1 homozygotes.

A second, probe-based assay is currently widely used to detect point mutations. The TaqMan assay is a fluorescently labeled oligonucleotide probe. The TaqMan assay utilized the 5' exonuclease activity of thermostable Taq polymerases. The probe consisted of a fluorescent reporter at the 5' end and a quencher at the 3' end. The reporter's fluorescence is quenched due to its proximity to the quencher. However, during the anneal/extension step in the PCR reaction, the probe hybridizes to the target region. Taq exonuclease activity from 5' to 3' will cleave the reporter resulting in a fluorescent signal that is proportional to the amount of PCR product in the sample. This method can be applied to diagnose β-thalassemia, multiplex probe-based fluorescence melting curve analysis (FMCA), which is a powerful tool for detecting Tm-based point mutations generated by thermal denaturation of probe-target hybrid (Huang et al., 2011, Xiong et al., 2011).

Direct DNA sequencing. Mutations can be identified by sequencing the PCR product using the Sanger dideoxy termination method. This requires the production of a single strand of DNA as a template. There are a number of methods for this. An aliquot of amplified DNA can be subjected to another round of PCR in the presence of one primer strand by the following methods:

A) the original PCR product can be denatured and rapidly cooled so that the two strands remain separated;

B) one of the primer chains is phosphorylated at the 5'-end, and the PCR product is treated with lambda exonuclease, which cleaves the 5'-phosphorylated strand of double-stranded DNA;

C) biotin can be included at the 5' end of one of the primer strands, allowing the PCR product to be adsorbed onto the streptavidin-coated magnetic beads, which can then be processed to denature the duplexes and allow the non-biotinylated strands to be removed from the beads.

Alternatively, PCR products can be subcloned into a vector for sequencing, but this method allows the detection of PCR artifacts. This allows the identification of new or rare mutations present in the population (Korf & Rehm, 2013).

Multiplex ligation dependent probe amplification

Multiple ligation dependent probe amplification (MLPA) is a multiplex PCR technique that detects any deletions or duplications in screened areas. This method has been proven to find known and unknown deletions in undisclosed cases after performing conventional methods. The MLPA method is easy to use and only requires a thermal cycler and CE equipment (Schouten *et al*., 2002; Lei *et al*., 2019).

MLPA begins with the DNA denaturation/hybridization step. The DNA is denatured and subjected to incubation with a mixture of MLPA probes. MLPA probes consist of two separate oligonucleotides (LPO and RPO). Two probe oligonucleotides are hybridized to adjacent target sequences. The probes can then be ligated during the

ligation reaction. Only ligated probes will be amplified during the PCR reaction, the amount of probe ligation products is a measure of the amount of target sequences in the sample. The amplification products are separated by CE.

Next generation sequencing

The development of sequencing technology makes a huge contribution to the study of the human genome. NGS technologies have been able to sequence the entire human genome with ultra-high performance, scalability and speed at a level that is not possible with Sanger sequencing technology. Most NGS platforms consist of three main steps:

a) preparation of the first library using random DNA fragmentation followed by ligation using special linkers;

b) library amplification using clonal amplification and PCR methods;

c) sequencing using incorporation of fluorescently labeled nucleotides by DNA polymerases or ligation processes.

NGS has enabled researchers to diagnose and understand complex diseases through whole genome sequencing, exome sequencing, or targeted gene panels (Yang *et al.*, 2013; Stark *et al.*, 2016). Recently, the NGS method has been used to screen for thalassemia. The targeted approach of NGS was designed to cover entire regions encoding globin genes, their key regulatory regions and modifier genes such as KLF1, BCL11A, HBS1L and MYB. Preliminary evidence suggests that NGS can be much more accurate than traditional thalassemia diagnosis through complete blood count (CBC), Hb testing, Hb typing, and selection for genotyping. A preliminary study using PCR-NGS among 57229 cases was conducted in Guangxi, China, and identified 458 unusual or new mutations that could not be detected by conventional methods.

3. Conclusion

Laboratory diagnosis of thalassemia requires a number of tests, including RBC indices and Hb and DNA tests. Although low MCV and MCH are characteristic of RBCs in thalassemia, these two RBC indices do not distinguish between thalassemia and iron deficiency, or between α- and β-thalassemia. Today, Hb analysis can be performed using an HPLC or CE system. Using these automated systems, both qualitative and quantitative analysis of hemoglobin components can be obtained, which will help to carry out both prenatal and postnatal diagnosis of thalassemia within a few minutes. DNA analysis has been used to detect point mutations in β-thalassemia and to detect large deletions in α-thalassemia. Limitations of traditional methods for diagnosing thalassemia are missed diagnoses due to normal or borderline erythrocyte indices and/or Hb A2 levels, various labor-intensive methods may be required to detect mutations in thalassemia, which has over 1800 varieties. As noted, the NGS method has recently been introduced to screen for thalassemia. NGS screening should cover more loci, including genetic modifiers that have a significant impact on clinical manifestations, which is important for accurate diagnosis and treatment of thalassemia. However, the NGS method still has some limitations, including high cost. All service labs were strongly encouraged to choose the methods they are most familiar with and economical for routine use.

References

Barbara, J.B. (2006). The α, β, (and (thalassemias and related conditions. In *Haemoglobinopathy Diagnosis*, ed. Barbara J. B. (London: Blackwell), 63–138.

Calzolari, R., McMorrow, T., Yannoutsos, N., Langeveld, A., & Grosveld, F. (1999). Deletion of a region that is a candidate for the difference between the deletion forms of hereditary persistence of fetal hemoglobin and δβ-thalassemia affects β-but not γ-globin gene expression. *The EMBO Journal*, *18*(4), 949-958.

Fucharoen, S., Winichagoon, P. (1997). Hemoglobinopathies in Southeast Asia: molecular biology and clinical medicine. *Hemoglobin*, *21*(4), 299-319.

Fucharoen, S., Winichagoon, P., & Thonglairum, V. (1988). β-thalassemia associated with α-thalassemia in Thailand. *Hemoglobin*, *12*(5-6), 581-592.

Galanello, R., Ruggeri, R., Paglietti, E., Addis, M., Melis, M. A., Cao, A. (1983). A family with segregating triplicated a globin loci and β-thalassemia. *Blood*, 62, 1035–1040.

HbVar, (2019). A Database of Human Hemoglobin Variants and Thalassemias. Available at: http://globin.bx.psu.edu/cgi-bin/hbvar/counter (accessed August 13, 2019).

Huang, Q., Liu, Z., Liao, Y., Chen, X., Zhang, Y., & Li, Q. (2011). Multiplex fluorescence melting curve analysis for mutation detection with dual-labeled, self-quenched probes. *PloS one*, *6*(4), e19206.

Khongthai, K., Ruengdit, C., Panyasai, S., & Pornprasert, S. (2019). Analysis of deletional Hb H diseases in samples with Hb A_2-Hb H and Hb A_2-Hb bart's on capillary electrophoresis. *Hemoglobin*, *43*(4-5), 245-248.

Korf, B.R., Rehm, H.L. (2013). New approaches to molecular diagnosis. *Jama*, *309*(14), 1511-1521.

Li, Y., Liang, L., Tian, M., Qing, T., & Wu, X. (2020). Electrophoresis features and genotypes of Hb bart's hydrops fetalis. *Scandinavian Journal of Clinical and Laboratory Investigation*, *80*(2), 129-132.

Li, Z., Shang, X., Luo, S., Zhu, F., Wei, X., Zhou, W., ... & Xu, X. (2020). Characterization of two novel Alu element-mediated α-globin gene cluster deletions causing α 0-thalassemia by targeted next-generation sequencing. *Molecular Genetics and Genomics*, *295*, 505-514.

Olivieri, N.F. (1999). The β-thalassemias. *New England Journal of Medicine*, *341*(2), 99-109.

Singh, N., Hira, J.K., Chhabra, S., Khadwal, A.R., Das, R., & Sharma, P. (2019). Misdiagnosis of double heterozygous εGγ (Aγδβ) 0-thalassemia/β++ thalassemia as homozygous β-thalassemia: a pitfall for molecular diagnostic laboratories. *Blood Cells, Molecules & Diseases*, *81*, 102394-102394.

Sripichai, O., Fucharoen, S. (2016). Fetal hemoglobin regulation in β-thalassemia: heterogeneity, modifiers and therapeutic approaches. *Expert Review of Hematology*, *9*(12), 1129-1137.

Stark, Z., Tan, T.Y., Chong, B., Brett, G.R., Yap, P., Walsh, M., ... & Cowie, S. (2016). Melbourne Genomics Health Alliance A prospective evaluation of whole-exome sequencing as a first-tier molecular test in infants with suspected monogenic disorders. *Genet. Med.*, *18*, 1090-1096.

Stephens, A.D., Colah, R., Fucharoen, S., Hoyer, J., Keren, D., McFarlane, A., ... & International Council for Standardization in Haematology (ICSH). (2015). ICSH recommendations for assessing automated high- performance liquid chromatography and capillary electrophoresis equipment for the quantitation of HbA_2. *International Journal of Laboratory Hematology*, *37*(5), 577-582.

Suwannakhon, N., Pangeson, T., Seeratanachot, T., Mahingsa, K., Pingyod, A., Bumrungpakdee, W., & Sanguansermsri, T. (2019). Noninvasive prenatal screening test for compound heterozygous beta thalassemia using an amplification refractory mutation system real-time polymerase chain reaction technique. *Hematology Reports*, *11*(3), 8124.

Weatherall, D.J. (1998). 4 Pathophysiology of thalassaemia. *Baillière's Clinical Haematology*, *11*(1), 127-146.

Winichagoon, P., Fucharoen, S., Weatherall, D., & Wasi, P. (1985). Concomitant inheritance of α- thalassemia in β°- thalassemia/hb e disease. *American Journal of Hematology*, *20*(3), 217-222.

Xiong, F., Huang, Q., Chen, X., Zhou, Y., Zhang, X., Cai, R., ... & Xu, X. (2011). A melting curve analysis–based PCR assay for one-step genotyping of β-thalassemia mutations: a multicenter validation. *The Journal of Molecular Diagnostics*, *13*(4), 427-435.

Yang, Y., Muzny, D.M., Reid, J.G., Bainbridge, M.N., Willis, A., Ward, P.A., et al. (2013). Clinical whole-exome sequencing for the diagnosis of Mendelian disorders. *N. Engl. J. Med.*, 369, 1502–1511.

Keskin, E. Y., Acar, Ö., & Özbas, H. (2021). Missed diagnosis of β-thalassemia trait in premarital screening due to accompanying HbA2-Yialousa (HBD: c. 82G> T). *Journal of Pediatric Hematology/Oncology*, *43*(1), e103-e104.