Review

Gunay Aliyeva*, Chingiz Asadov, Tahira Mammadova, Surmaya Gafarova
and Eldar Abdulalimov

Thalassemia in the laboratory: pearls, pitfalls, and promises

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Abstract: Thalassemia is one of the most common hereditary disorders of the developing world, and it is associated with severe anemia and transfusion dependence. The global health burden of thalassemia has increased as a result of human mobility and migration in recent years. Depending on inherited mutations, thalassemia patients exhibit distorted hemoglobin (Hb) patterns and deviated red cell indices, both of which can be used to support identification by diagnostic tools. Diagnostic approaches vary depending on the target population and the aim of the testing. Current methods, which are based on Hb patterns, are used for first-line screening, whereas molecular testing is needed for conformation of the results and for prenatal and preimplantation genetic diagnosis. In the present paper, we review the diagnostic parameters, pitfalls, interfering factors, and methods; currently available best-practice guidelines; quality assurance and standardization of the procedures; and promising laboratory technologies for the future of thalassemia diagnosis.

Keywords: HbA₂; hemoglobin; HPLC; population screening; prenatal diagnosis; thalassemia diagnosis.

Introduction

Thalassemia is among the most common hereditary disorders in the world [1–3]. Inherited through an autosomal

recessive pathway, point mutations and deletions on the genes that code for the globin chains cause decreased hemoglobin (Hb) production, leading to severe anemia [4]. Thalassemia patients depend on lifelong medical care, receiving routine blood transfusions and supplemental therapies [5]. Therefore, timely diagnosis and prevention is essential, especially in regions with high prevalence of this disorder [6–8].

The genes that encode for globin proteins are located on β - and α -globin gene clusters on chromosome 11 and 16, respectively [4] (Figure 1). Expression of each globin gene varies throughout the embryonic and fetal development, which is why the Hb patterns of newborns and adults differ from each other [9, 10]. The marked level of fetal hemoglobin (HbF) in newborns is gradually replaced by adult hemoglobin (HbA) during the first year of life. Thalassemia patients, as well as non-symptomatic heterozygous carriers of the disease, exhibit distorted Hb patterns, which have been used for diagnostic purposes since the early 1950s [11–13].

Approximately 5% of the world population are carriers of hemoglobinopathies, and 2.9% are of thalassemia [14]. Carriers are healthy individuals with only a mild anemia, and couples in which both partners are carriers are at risk of giving birth to sick children. Globally, there are 300,000–400,000 babies born each year with a Hb disorders [1–3]. Thalassemia is mainly a disease of the developing world, presenting a high incidence in the Mediterranean area, the Middle East, Transcaucasia, the Indian subcontinent and Southeast Asia [1, 2]. Nevertheless, the prevalence of thalassemia is growing substantially in non-indigenous regions, such as Northern Europe, North America and Australia, due to increased mobility and migration flows of populations in recent decades [15–18]. The global burden of hemoglobinopathies necessitates implementation of public health interventions, such as screening programs and prenatal diagnosis, even in non-indigenous countries with high rates of immigration [16].

*Corresponding author: Gunay Aliyeva, Department of Hemopoietic Pathologies, Institute of Hematology and Blood Transfusion, Baku, Azerbaijan, E-mail: galiyeva@rocketmail.com.
<https://orcid.org/0000-0002-1751-3684>

Chingiz Asadov, Tahira Mammadova, Surmaya Gafarova and Eldar Abdulalimov: Department of Hemopoietic Pathologies, Institute of Hematology and Blood Transfusion, Baku, Azerbaijan

Figure 1: Diagram of α - and β -globin gene clusters and subsequently produced hemoglobin molecules.

Genes encoding for β - (*HBB*), δ - (*HBD*) and γ -globin (*HBG1* and *HBG2*) molecules are located in chromosome 11 (β -globin gene cluster), while α -globin (*HBA1* and *HBA2*) genes are located in chromosome 16 (α -globin gene cluster).

How screening and early detection can prevent thalassemia?

Population control programs of β -thalassemia were first applied in Mediterranean at-risk populations in the late 1970s [8, 19]. Since then, most of the countries with a high prevalence of thalassemia set up national or partial prevention programs for either β - or α -thalassemia screening [8]. Although most of these programs are performed on a voluntary basis, in some countries premarital screenings are mandatory [20–24]. Population screenings can target newborns, adolescents, couples in premarital or preconception stages and pregnant women. While newborn screening aims to prevent morbidity and mortality via early detection and timely provision of necessary medical care, premarital and preconceptional screenings are able to prevent the conception or birth of affected children [7, 25]. Identified carrier couples are referred to genetic

counselling for the application of further procedures of prenatal or preimplantation genetic diagnosis [26–29].

Heterozygous carriers of thalassemia are typically known to have microcytic hypochromic anemia and, ideally, screening procedures would include an evaluation of the red blood cell (RBC) indices followed by Hb pattern analysis. Recommended cut-off values for mean corpuscular volume (MCV) and mean corpuscular Hb concentration (MCH) are 78–79 fl and 27 pg, respectively [7, 25, 30]. Samples below these values are further investigated by HbA₂ quantification for the identification of β -thalassemia carriers [31] (Table 1). Individuals with HbA₂ values above 3.4–3.6% are identified as carriers of β -thalassemia, which is followed by a definitive diagnosis via DNA analysis [25, 30]. On the other hand, individuals with decreased RBC indices and normal HbA₂ values are suspected of heterozygous α -thalassemia if iron deficiency is excluded. Normocytic indices, together with respective abnormal Hb fractions, indicate the presence of variants.

Table 1: Diagnostic parameters of the commonest hemoglobinopathies.

Hemoglobinopathy	Red blood cell indices	Hb patterns
β -Thalassemia carrier	↓ MCV, ↓ MCH, ↑ RBC	↑ HbA ₂
β -Thalassemia major/intermedia	↓ Hb	↑ HbF
α -Thalassemia carrier	Normal	Normal
α -Thalassemia carrier	↓ MCV, ↓ MCH	Normal or low HbA ₂
HbH disease	↓ MCV, ↓ MCH	3%–30% HbH
$\delta\beta$ -Thalassemia carrier	↓ MCV, ↓ MCH	Normal or low HbA ₂ , ↑ HbF
Hb Lepore	↓ MCV, ↓ MCH	Low HbA ₂ , mildly increased HbF, and 5%–15% abnormal Hb fraction
HbS carrier	Normal	35%–40% HbS
HbS homozygote	↓ Hb, normal MCV & MCH	5%–10% HbF, 75%–95% HbS
HbS/ β -thalassemia	↓ Hb, ↓ MCV, ↓ MCH	5%–10% HbF, 65%–95% HbS
HbS/ $\delta\beta$ -thalassemia	↓ MCV, ↓ MCH	25%–35% HbF, 65%–75% HbS
HbE carrier	Normal	25%–30% HbE
HbE homozygote	↓ MCV, ↓ MCH	5%–15% HbF, 85%–95% HbE
HbE/ β -thalassemia	↓ Hb	40%–60% HbF, 40%–60% HbE

Because there is only a small numerical difference in HbA₂ levels of normal individuals and β-thalassemia carriers, precision of the quantification is important. Despite the existing precise cut-off values, several interfering factors are known to complicate the interpretation of the results (Table 2). RBC indices should be evaluated within 6 h of sample collection due to the hemolysis and increased cell permeabilization over time. Interpretation of the RBC count in pregnant women and in iron-deficient patients responding to iron supplementation need to be made with caution. Various genetic and acquired factors are known to be associated with borderline HbA₂, and values above 3.2 require DNA analysis for further investigation. The presence of sickle Hb (HbS) can lead to falsely elevated HbA₂ levels in most high performance liquid chromatography (HPLC) systems [32]. Severe iron deficiency, on the other hand, can reduce HbA₂ levels by up to 0.5% [31]. Elevated HbF levels, commonly associated with hereditary persistence of fetal hemoglobin (HPFH) and δβ-thalassemia, can also be observed in bone marrow disorders and pregnancy [33].

As neonatal Hb composition differs from adults, testing of newborns requires a different approach. In healthy newborns, samples are typically composed of approximately 75% HbF, and 0%–1% Hb Bart’s, and the remaining fraction is HbA. While elevated HbF levels can possibly be associated with β-thalassemia major, Hb Bart’s levels above 25% indicate the presence of α-thalassemia (HbH disease) [34–37].

Although current screening algorithms are able to efficiently reveal carriers or affected babies, laboratories in resource-lacking regions may not always be equipped with the necessary sophisticated assays. Evaluation of the red cell indices and iron status – supported by cheaper assays such as an osmotic fragility test, detection of HbH inclusions, evaluation of blood smears for microcytic hypochromic anemia, and a sickle solubility test – can be reliable alternatives in such circumstances.

Molecular diagnosis

Molecular diagnosis of thalassemia is mainly applied for confirmation of screening results, for clarification of complicated cases, and for prenatal diagnosis. It is recommended that, all positive screening results are confirmed through DNA analysis, and results should be interpreted altogether, including the evaluation of hematology and family history, if necessary [25, 30]. Currently, there are more than 650 thalassemia mutations included in the IthaGenes database, of which around 390 account for β-thalassemia and the rest for α-thalassemia [38]. The main types of the mutations are point mutations, large deletions and duplications [4]. Despite the broad range and general heterogeneity, thalassemia mutations are commonly population-specific, with each population having a specific spectrum of mutations [2, 7, 39–41]. Therefore, information about the ethnic origin of the patient facilitates the selection of the best diagnostic

Table 2: Factors interfering with laboratory diagnosis of thalassemia.

Factors associated with increased HbA ₂	Factors associated with borderline HbA ₂ in β-thalassemia carriers	Factors associated with increased HbF
KLF1 mutations	Mutations: -101 [C > T]	HPFH
α-gene triplication	-92 [C > T]	δβ-thalassemia
Variants eluting with HbA ₂	CAP + 1 [A > C]	BCL11A mutations
Antiretroviral therapy in patients with HIV	+33 [C > G]	HBS1L-MYB mutations
Hyperthyroidism	+1480 [C > G]	Bone marrow malignancies
Megaloblastic anemia	IVS-I-6 [T > C]	Aplastic anemia
Hypertrophic osteoarthropathy	IVS-II-844 [C > G]	Fanconi anemia
<i>Pseudoxanthoma elasticum</i>	PolyA mutations	Erythropoietic stress
	Presence of: α-thalassemia	Pregnancy
	δ-Thalassemia	HbF inducing therapies
	α-Chain variants	
	δ-Chain variants	
	Severe iron-deficiency	
	Sideroblastic anemia	
	Erythroleukemia	
	Aplastic anemia	
	Lead poisoning	

strategy. Phenotypic expression and clinical manifestation of the mutations vary depending on the type of mutation and its location on the gene. β -thalassemia mutations are categorized as β° , β^+ , and β^{++} (silent) mutations, according to their clinical severity. On the other hand, point mutations occurring on the α -globin genes are associated with more severe α -thalassemia phenotypes compared to deletional mutations. Thus, determination of the genotype is essential for prediction of the clinical severity of thalassemia [42–44].

Prenatal diagnosis of carrier couples became available in the early 1980s, as soon as the molecular basis of thalassemia was characterized [45, 46]. Amniotic fluid and chorionic villi are the main choices as a source of fetal DNA, although various alternatives have been developed to date [47–49]. Prenatal diagnosis enables prevention of thalassemia through termination of affected pregnancies, whereas preimplantation genetic diagnosis is a useful alternative for couples who have medical contraindications to abortion or who are against the termination of pregnancy due to the ethical or religious beliefs [28, 29, 50].

Current methods in the laboratory

Various methods are available for Hb pattern analysis and molecular diagnosis of thalassemias (Table 3). The early methods of Hb pattern analysis, such as cellulose acetate electrophoresis (CAE) and isoelectric focusing (IEF), have been gradually replaced by automated

systems, such as HPLC and capillary electrophoresis (CE). CAE and IEF are relatively cheaper, and can be used as provisional methods, and provide satisfactory results in variant Hb detection; however, they are not recommended for HbA₂ quantification due to insufficient precision [25, 31]. Diagnosis of β -thalassemia carriers is solely based on the quantification of HbA₂, with a very small numerical difference between carriers and healthy individuals. Laboratories located in regions with a higher prevalence of β -thalassemia should therefore consider systems that provide higher precision, such as HPLC and CE. In addition to their precision, these systems are automated, high-throughput and require very small sample volumes.

The choice of method is generally made based on need and conditions of each laboratory, including the volume of workload, the spectrum of common hemoglobinopathies in a particular location, the sample source (adult or newborn), ease of handling, local availability of the diagnostic systems and their cost. In addition to first-line screening methods, laboratories are recommended to be equipped with a second method to confirm the results of variant Hb identification [25, 30]. Although identification of variant Hb requires confirmation by a second method of choice, increased HbA₂ levels accompanied by red cell indices typical of a β -thalassemia carrier do not need further confirmation. Quantification of HbF levels, which are essential in the diagnosis of thalassemia major or intermedia, HPFH, and $\delta\beta$ -thalassemia, can be done by a conventional alkali denaturation test alongside with HPLC and CE [33, 51].

Table 3: Current methods used in laboratory diagnosis of thalassemia.

Hemoglobin pattern analysis	Molecular diagnosis	Supplementary methods
Cellulose acetate electrophoresis (CAE)	Point mutations	Sickle solubility
Citrate agar electrophoresis	Restriction enzyme PCR (RE)	Detection of HbH inclusions
Isoelectric focusing (IEF)	Amplification refractory mutation system (ARMS)	Heinz body inclusions
High performance liquid chromatography (HPLC)	Reverse dot blot hybridization	Alkali denaturation
Capillary electrophoresis (CE)	Real-time PCR	HbF intracellular distribution
	Denaturing gradient gel electrophoresis (DGGE)	Osmotic fragility test
	High resolution melting analysis (HRMA)	Globin chain synthesis
	Pyrosequencing	Globin chain separation
	Sanger sequencing	DCIP for HbE screening
	Next generation sequencing (NGS)	Oxygen dissociation curve
	Deletions	Hb stability test
	Gap PCR	Quantification of HbA ₂ by microcolumn chromatography
	Multiplex ligation probe amplification (MLPA)	Mass spectrometry
	Microarray	
	Southern blotting	
	Array comparative genome hybridization (aCGH)	

Methods for molecular diagnosis of thalassemias are highly variable, which is a reflection of the heterogeneous mutational basis of these disorders. Advantages and limitations of currently available methods for point mutations, as well as deletions, have been previously reviewed [30]. Choice of the molecular method should be made based on the mutation spectrum of the particular population. Relatively less expensive multiplex methods are generally chosen as the first line of screening for mutations (e.g. amplification refractory mutation systems [ARMS], reverse dot blot hybridization, gap PCR), followed by more comprehensive investigations, including Sanger sequencing for point mutations and multiplex ligation probe amplification (MLPA) for deletions. These two latter methods are able to reveal unknown mutations. Sanger sequencing is recommended to be performed by at least two sets of primers in both forward and reverse directions [30].

Molecular testing can be used as a definitive diagnosis and for the clarification of complicated cases, such as in newly transfused patients or in coinheritance of different hemoglobinopathies. Nevertheless, identification of the genotype is essential in couples prior to prenatal or preimplantation genetic diagnosis [28, 30].

Supplementary tests of thalassemia

Additional tests used in the diagnosis and monitoring of thalassemia patients can be divided into iron-related and non-iron-related tests. Evaluation of iron metabolism is essential in the monitoring of thalassemia patients. These patients commonly have iron overload as a result of ineffective erythropoiesis or serial blood transfusions, and they exhibit increased serum iron and ferritin levels [52]. Excess iron is accumulated in organs, causing heart failure; portal and hepatocyte iron loading; and endocrine dysfunctions, such as diabetes, hypogonadism, thyroid dysfunction and growth retardation [53–60]. Therefore, evaluation of iron status, as well as the functionality of organs susceptible to iron accumulation, is part of the routine medical care of thalassemia patients [61, 62]. Alongside common tests for the evaluation of iron status, such as serum iron, serum ferritin, transferrin, transferrin saturation, and total iron binding capacity (TIBC), the relatively cheaper, faster, and easy-to-perform zinc-protoporphyrin (ZnPP) test has been a key tool to screen for iron deficiency in thalassemia-specialized laboratories [63].

Transfusion dependence of most of these patients make them susceptible to transfusion-transmitted infections. Therefore, together with liver function tests,

thalassemia patients need to occasionally be tested for viral hepatitis [64–66]. These disorders are also associated with increased indirect bilirubin levels due to red cell hemolysis [67, 68].

Thalassemia patients are prone to hypercoagulability and thrombotic events [69]. This is related to several factors, including procoagulant activity of hemolyzed circulating RBCs, increased platelet activation, coagulation factor defects, depletion of antithrombotic factors, and endothelial inflammation [70, 71]. In addition to minor variations in screening tests (e.g. PT, aPTT) and marked thrombocytosis, these patients commonly exhibit decreased levels of protein C and coagulation factors, and elevated D-dimer [72–76].

Considering all of these, thalassemia is a good example of how a single nucleotide substitution in a gene can cause serial pathophysiological abnormalities, leading to the deviations in normal values of dozens of metabolites.

Guidelines, protocols and standardization

Although each country has its specific need-based prevention and screening programs, there are a lot of similarities between the regulation of the laboratory processes across the world. Guidelines produced by the British Committee for Standards in Haematology (BCSH) covers almost all aspects of the screening and diagnosis, although it was written to be a source for the NHS Sickle Cell and Thalassemia Screening Programme [25]. In addition to the BCSH guidelines, more recent handbooks for laboratories that handle antenatal and newborn samples can be accessed from the program resources [37] (Table 4). Documentation from the Association of Public Health Laboratories (APHL) provides guidance for screening, confirmation and follow-up of hemoglobinopathies, with the aim of improving the capabilities of the United States Newborn Screening and Genetics in Public Health Program [36]. The guidance document is mainly focused on laboratory diagnosis of newborn samples, and it provides local algorithms for the newborn screening program. A resourceful set of best-practice guidelines for carrier identification and prenatal diagnosis has recently been published by the European Molecular Genetics and Quality Network (EMQN) [30]. The document provides necessary information on molecular methods and the overall process for prenatal diagnosis. Recommendations for the measurement of HbA₂ and HbF, provided by the International Council

Table 4: Publications on regulation of diagnostic laboratory procedures of thalassemia.

Publication	Organization	Type	Year	Location	Ref.
NHS Sickle Cell and Thalassaemia Screening Programme: Handbook for antenatal laboratories	Public Health England	Handbook	2017	UK	[37]
NHS Sickle Cell and Thalassaemia Screening Programme: Handbook for newborn laboratories	Public Health England	Handbook	2017	UK	[37]
Developing a reference system for the IFCC standardization of HbA ₂	International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Working Group on Standardization of Hemoglobin A2	Report on standardization	2017	International	[79]
ICSH recommendations for assessing automated high-performance liquid chromatography and capillary electrophoresis equipment for the quantitation of HbA ₂	International Council for the Standardization of Haematology (ICSH)	Recommendations	2015	International	[78]
Hemoglobinopathies: Current Practices for Screening, Confirmation and Follow-up	Association of Public Health Laboratories (APHL) & Centers for Disease Control and Prevention (CDC)	Guidance document	2015	USA	[36]
EMQN Best Practice Guidelines for molecular and haematology methods for carrier identification and prenatal diagnosis of the haemoglobinopathies	European Molecular Genetics Quality Network (EMQN)	Guidelines	2014	Europe	[30]
ICSH recommendations for the measurement of Haemoglobin A ₂	International Council for the Standardization of Haematology (ICSH)	Recommendations	2012	International	[31]
ICSH recommendations for the measurement of Haemoglobin F	International Council for the Standardization of Haematology (ICSH)	Recommendations	2012	International	[33]
Prevention of Thalassaemias and Other Hemoglobin Disorders. Volume 2: Laboratory Protocols	Thalassaemia International Federation (TIF)	Protocols	2012	International	[77]
Significant haemoglobinopathies: guidelines for screening and diagnosis	British Committee for Standards in Haematology (BCSH)	Guidelines	2010	UK	[25]

for the Standardization of Haematology (ICSH), and laboratory protocols published by Thalassemia International Federation (TIF) are targeted to an international audience who have varying needs [31, 33, 77, 78]. The latter is a good resource for detailed protocols of Hb pattern analysis and molecular techniques.

Currently, there are two external quality assessment programs available for proficiency testing of hemoglobinopathy laboratories. The Sickle Cell and Other Hemoglobinopathies Proficiency Testing Program (HbPT), provided by the United States Centers for Disease Control and Prevention as a part of Newborn Screening Quality Assurance Program (NSQAP), assesses laboratory proficiency by evaluating the results of five blind-coded newborn samples sent by HbPT 3 times per year. The Abnormal Hemoglobins Program of United Kingdom National External Quality Assessment Service (UK NEQAS) is flexible, and participants can register for individual parts of the program, based on their needs. The specimens – which are designated as sickle screening (SS), adult (AH), and

liquid newborn (LN) – are available for choice and are distributed 6 times per year. Both programs are aimed to assess the performance of non-molecular techniques.

Because high precision is needed in HbA₂ quantification, standardization of HbA₂ measurements are crucial for accurate diagnoses. Established in 2004, the International Federation of Clinical Chemistry and Laboratory Medicine Working Group on Standardization of HbA₂ (IFCC WG-HbA₂) has aimed to develop an international reference system, including a reference measurement procedure and certified reference material [79]. Since it was established, the working group have evaluated several mass spectrometry-based approaches as reference measurement procedures [79–81]. In addition to the reference measurement procedure, certified reference material is also needed as an integral element of a reference measurement system. At present, the only internationally recognized reference material for HbA₂ is the World Health Organization International Reference Reagent, provided by the United Kingdom National Institute for Biological Standards and Control

(89/666, NIBSC, UK). It was introduced more than 25 years ago, and it is expected to be depleted in the near future. Thus, IFCC WG-HbA₂, aims to develop a new replacement reference material, which is supposed to be produced at a minimum of two levels of HbA₂ fraction and in large enough batches to serve for at least 5–10 years [79, 82].

Promises

Currently available methods have been through a lot of developments, and they almost cover all aspects of thalassemia and variant Hb diagnoses. Despite this, the application of the recent advances promises increased efficiency and throughput, which would improve the efficacy of laboratory procedures. Recent studies that evaluate next-generation sequencing (NGS) as a tool for the screening of thalassemia carriers, demonstrated higher sensitivity and specificity compared to conventional methods [83]. However, the cost of it is not comparable to traditional screening methods. NGS promises shorter turnaround time and comparable precision, but the inability to investigate gene rearrangements, large deletions, and duplications is currently its major drawback in the genotyping of Hb disorders. Nevertheless, single-cell whole-genome analysis is being successfully applied to preimplantation genetic diagnoses [84, 85]. NGS has also been evaluated for use in non-invasive prenatal diagnosis (NIPD). Although it is not as informative as in fetal aneuploidies and in genetic traits absent from the mother and paternally inherited by the fetus (e.g. RhD gene mutations), the application of NIPD in thalassemias can identify pregnancies where the fetus has not inherited paternal mutations, removing the need for an invasive test in 50% of at-risk pregnancies [86, 87].

In addition to developments in molecular techniques, mass spectrometry – a recent advance in protein chemistry – is a promising approach in the quantification of Hb fractions. Various studies have evaluated its performance in the diagnosis of Hb variants, as well as thalassemias. Despite the promising results achieved in variant Hb identification, newborn dried blood spot analysis, and HbA₂ quantification, further evaluation and validation is still needed for the application in clinical practice [80, 88–90].

Conclusions

Thalassemia is a complex disease, necessitating a comprehensive approach for its laboratory testing and diagnosis. Population screening in high-prevalence areas require

high-throughput testing algorithms and methods, which adds extra burden to its diagnosis. Despite this, current best-practice guidelines and protocols are capable of the successful regulation of complex laboratory procedures, together with necessary EQA programs. There are several innovative approaches currently being introduced that would be capable of increasing the efficacy and ameliorating the throughput and precision of laboratory diagnoses of thalassemia and other hemoglobinopathies.

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