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Review

Review on screening and analysis techniques for hemoglobin variants and thalassemia

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Abstract

Thalassemia involves gene mutation that causes the production of an insufficient amount of normal structure globin chains while Hb variant involves gene mutation that causes the change in type or number of amino acid of the globin chain. It has been reported that some 200 million people worldwide had hemoglobinopathies of some sort. Attempts to develop effective and economical techniques for screening and analysis of thalassemia and Hb variants have become very important. In this review, we report the different techniques available, ranging from initial screening to extensive analysis, comparing advantages and disadvantages. Some indirect studies related to thalassemia indication and treatment follow-up are also included. We hope that information on these various techniques would be useful for some scientists who are working on development of a new technique or improving the existing ones.

Keywords: Hemoglobinopathies; Hemoglobin variants; Thalassemia; Screening techniques

Contents

1. Introduction 1150
2. Initial screening techniques for thalassemia 1152
   2.1. Complete blood count (CBC) 1152
   2.2. Osmotic fragility test (OFT) 1153
3. Conventional confirmatory tests for thalassemia and Hb variants 1153
   3.1. Deoxyhemoglobin solubility test 1154
   3.2. Hemoglobin precipitation test 1154
   3.3. Brilliant cresyl blue test or new methylene blue test 1154
   3.4. Alkaline resistant hemoglobin test 1154
   3.5. Acid elution stain (modified Kleihauer–Betke test) 1155
   3.6. Ion exchange micro-column 1155

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4. Instrumental techniques for determination of thalassemia and Hb variants 1155
4.1. High performance liquid chromatography (HPLC) 1156
4.2. Electrophoresis 1156
4.3. Isoelectric focusing (IEF) 1156
4.4. Flow cytometry 1157
5. Extensive analysis techniques for thalassemia and Hb variants 1157
5.1. Polymerase chain reaction (PCR) with different formats of gel electrophoresis 1157
5.2. DNA technology: DNA probe/DNA microchip 1157
6. Indirect studies related to thalassemia indication and treatment follow-up 1158
6.1. Ferritin 1158
6.2. Skin tissue Fe concentration 1158
6.3. Magnetic behavior of erythrocytes 1158
6.4. Nuclear magnetic resonance spectroscopy 1158
7. Conclusion 1159
Acknowledgements 1159
References 1159

1. Introduction

Hemoglobin (Hb) is the molecule that carries and transports oxygen all through the body. It is composed of tetraglobin chains, two alpha and two non-alpha chains. The alpha (α) chains are encoded by the two closely related genes, alpha 1 and 2, on chromosome 16. The non-alpha chains – beta (β), gamma (γ) and delta (δ) – are encoded by a cluster of genes on chromosome 11. A fetus has a high amount of HbF ($\alpha_2\gamma_2$) as compared to other types of Hbs. A newborn has about 80% HbF and at about 1 year of age, HbF reaches a normal level. A few weeks after birth, the production of HbA ($\alpha_2\beta_2$) becomes dominant with the elevated level of HbA_2 ($\alpha_2\delta_2$) [1–3]. In normal adults, HbA is the main type of hemoglobin (96–98%) while HbA_2 and HbF are only present in 2–3% and less than 1%, respectively [4,5].

The failure in hemoglobin synthesis is a main cause of microcytosis and anemia in many population groups around the world. Hemoglobin variants (Hb variants) are characterized by the gene mutation of the globin chains that form hemoglobin (i.e., the replacement of different amino acids at a certain position). Thalassemia, which is slightly different from Hb variants, involves the gene mutation that causes production of an insufficient amount of normal structure globin chains. All types of thalassemias are considered quantitative hemoglobin disease.

Many types of hemoglobin variants have been found, depending on racial background [1–5]. Some types are not at all a problem, for example HbE heterozygous, while some types can cause severe anemia with serious clinical manifestation, for example HbS homozygous or sickel cell disease. Normally, Hb variants carriers, especially heterozygous, have no symptoms. However, combination of Hb variants and thalassemia gene on the same globin chain may result in severe symptoms. For example, combination of HbE with β-thalassemia gene becomes double heterozygote that shows symptoms similar to homozygous β-thalassemia, and combination of Hb constant spring (CS) with α-thalassemia gene will cause symptoms similar to HbH disease.

Thalassemia can be categorized into three classes – major, intermediate and minor – according to the severity of the symptoms [2,3]. The two main thalassemia syndromes (thalassemia major) are α and β thalassemias, which involve homozygous genetic defect in the α globin and β globin chain production, respectively. β-Thalassemia and sickel cell anemia have a wide distribution in tropical areas due to natural selection by malaria [6–8]. α-Thalassemia is most commonly found in Southeast Asia and Africa [1]. Related thalassemia minors or carriers are α-thal-1 (2 out of 4 globin gene deletion) and α-thal-2 (1 out of 4 globin gene deletion). Type 1 has insignificant but observable anemia while type 2 is a silent carrier without any symptoms shown [2]. Compound heterozygotes of α-thal-1 and α-thal-2 result in HbH disease. HbH is composed of β_4 chains instead of $\alpha_2\beta_2$ chains as in normal HbA. HbH is a relatively mild form of thalassemia and may go unnoticed. However, combination of HbH with HbCS, a type of hemoglobin variant of the alpha gene that has an elongated alpha chain with 31 extra amino acids, is severe and blood transfusion may be necessary. Homozygous α-thal-1 causes Hb Bart's hydrops fetalis which consists of γ_4 chains instead of $\alpha_2\gamma_2$ chains as in normal HbF. Unborn infants with Hb Bart's hydrops fetalis normally die before birth or within a short time after birth.

β-Thalassemia major is not as widely spread throughout Southeast Asia as α-thalassemia, but the β chain hemoglobinopathy HbE and HbTak are quite common. In HbE, the 26th amino acid of a normal β chain, glutamine, is replaced by lysine [1,2,4]. HbTak is another common Hb variant found in Asian population which is due to an insertion of the dinucleotide CA into codon 146 [CAC → CA(CA)C] resulting in elongation of the β chain by 11 amino acids. HbS and HbC are the main Hb variants in Africa but the

Table 1
Causes of different types of thalassemia and examples of hemoglobin variants and the areas commonly found

Thalassemia/Hb variants	Type	Cause/severity	Prevalent regions
Alpha-thalassemia	α-thal 2 trait or heterozygous α-thal 2 ($-\alpha/\alpha\alpha$ or α^+)	Only one out of four genes is deleted/No symptoms (silent carrier)	Southeast Asia, Africa
	α-thal 1 trait or heterozygous α-thal 1 ($-/\alpha\alpha$ or α^0)	Two deleted genes are on the same chromosome/observable anemia	
	homozygous α-thal 2 ($-\alpha/-\alpha$)	Two deleted genes are on different chromosomes/observable anemia	
	α-thal 1 /α-thal 2 or HbH disease ($-/-\alpha$ or α^+)	Three out of four genes are deleted and excess β-chains form β_4/significantly big liver and spleen	
	α-thal 1 /α-thal 1 or homozygous α-thal 1 or Hb Bart's hydrops fetalis ($-/-$)	There is no alpha chain production and infants have γ_4 instead of normal HbF ($\alpha_2\gamma_2$)/die before or at birth	
Hb variants related to mutation in alpha globin chain	HbCS	Alpha chain has extra 31 amino acids	Southeast Asia
	HbMahidol (HbQ)	$\alpha^{74Asp\rightarrow His}$	Southeast Asia
	HbThailand	$\alpha^{56Lys\rightarrow Thr}$	Thailand
Beta-thalassemia	β^+ thal heterozygote (β^+/β)	Less production of beta chain/insignificant symptoms	Mediterranean, Middle East, India, Southeast Asia, and also found in West and North Africa, West Asia, Italy, Greece, East and Central Europe and USA
	β^+ thal homozygote (β^+/β^+)	Less production of beta chain/need some blood transfusion	
	β^0 thal heterozygote (β^0/β)	Complete failure on beta chain production/need some blood transfusion	
	β^0 thal homozygote (β^0/β^0)	Complete failure on beta chain production/severe symptoms and need blood transfusion often	
Hb variants related to mutation in beta globin chain	HbE	$\beta^{26Glu\rightarrow Lys}$	Southeast Asia
	HbC	$\beta^{6Glu\rightarrow Lys}$	Africa, USA, Mediterranean and Caribbean
	HbS	$\beta^{6Glu\rightarrow Val}$	

most common major hemoglobinopathy is HbS homozygosity. Both HbS and HbC affect the solubility of the hemoglobin by polymerization. HbC forms crystals and makes the red blood cell rigid which causes hemolytic anemia, while deoxygenated HbS polymerizes and forms fiber structure. Similar to HbE, the glutamine at the sixth position on the β chain in HbC and HbS is substituted by lysine and valine, respectively [4,9]. Table 1 summarizes the causes of different types of commonly found Hb variants and shows the areas of their prevalent existence [8].

In 1995, it was reported that nearly 200 million people worldwide had thalassemia or Hb variants of some type [1]. Intermarriage causes various combinations of globin chain defects. Even though carriers of some types of hemoglobin defection do not have any health effect, if in combination with thalassemia trait, then there would be a 25% chance that their children could have thalassemia symptoms [10].

Thalassemia patients in severe cases normally have iron overloading of tissues and malfunctioning liver and spleen due to increased iron collection from blood transfusion treatment [11,12]. The symptoms (i.e., abnormal facial features, big tummy and pale) and the treatment processes (i.e., regular blood transfusion) usually have an affect mentally, physically and economically on the patients and their society [13,14]. The lack of education and knowledge about the disease, along with the neglect of couples to have family planning, causes this genetic disease to spread widely. Therefore, in many countries, couples are now encouraged to have their health checked before or at the very beginning of pregnancy [15,16]. Attempts to develop effective and economic techniques for thalassemia screening and analysis have become very important, especially in the countries that have populations with high percentage of thalassemia trait, high birth rate and low funding [17,18].

There are many techniques that have been used to screen and diagnose for hemoglobin variants and thalassemia, mostly done in combinations. In this article, the authors attempt to report the different techniques available, ranging from initial screening to extensive analysis, including a few indirect studies. Screening techniques are the group of techniques that can initially indicate a defect in hemoglobin synthesis. Positive results from these tests need confirmation by a more extensive analysis technique. Negative results normally help in cutting down the number of subjects that need to be further diagnosed by a more advanced and complicated testing. Extensive analysis techniques can give more precise information about types of Hb variants or types of thalassemia. They normally involve higher technologies and instrumentation, and therefore are more expensive than screening techniques. In some cases, it is necessary to perform a more extensive test instead of a combination of many cheaper techniques, such as in prenatal diagnosis where sampling procedures are difficult and the amount of sample is limited. The flow chart shown in Fig. 1 summarizes the techniques for diagnosis of hemoglobinopathies that are commonly used in most laboratories. Different laboratories may have differ-

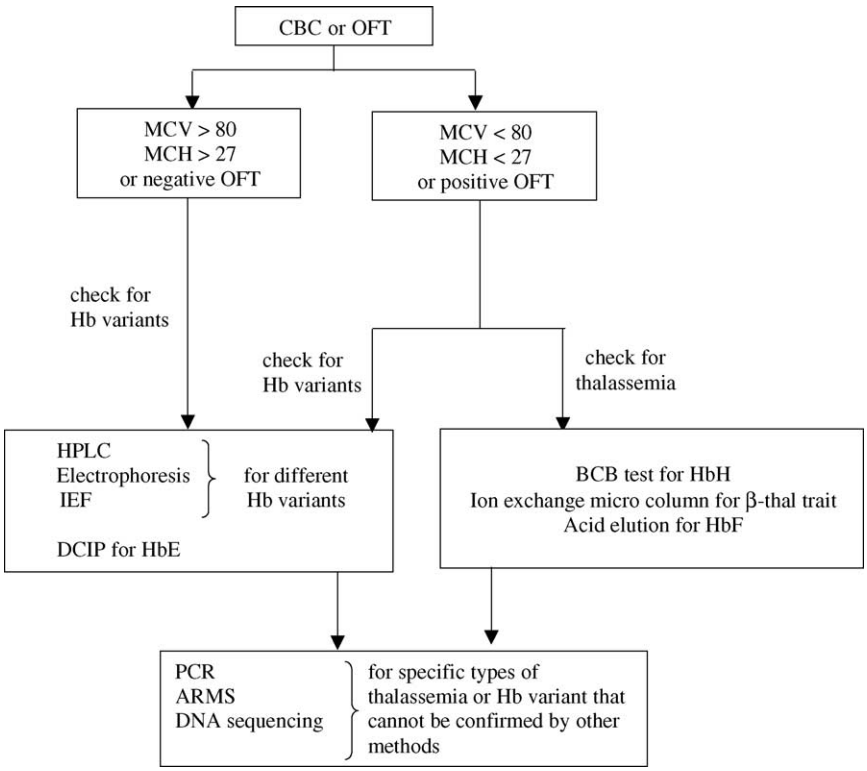

Fig. 1. Flow chart summarizes the normal process of thalassemia and Hb variants diagnosis (see text).

ent choices of analysis techniques, depending on availability of instrumentation and funding. In the developing countries where economic restrictions do not allow for the use of the relatively more expensive technologies, the development of low cost analysis techniques will always be needed. It is hoped that this will be useful information for those who are interested in learning about thalassemia and Hb variants diagnosis techniques for general knowledge, for the development of a new technique or for the improvement of the existing ones.

2. Initial screening techniques for thalassemia

Initial screening techniques are defined as techniques that are simple and relatively low cost which can indicate the possibility of having thalassemia. These techniques should involve the least sample pretreatment and be rapid, and may not need special instrumentation. This would lead to low cost and high sample throughput analysis. They provide a "yes/no" type answer. Positive samples need further confirmatory test while negative samples can be eliminated from further complicated and expensive testing. The complete blood count (CBC) or the alternative osmotic fragility test (OFT) can be used to screen for thalassemia. The negative result eliminates the possibility of having thalassemia, but does not completely exclude the possibility of having Hb variants. Therefore, if necessary, Hb variants testing is needed. Positive results reveal the possibility of having either thalassemia or Hb variants. These screening techniques cannot provide the information on the exact type of hemoglobinopathies, but can help in cutting down the number of samples from unnecessary complicated and expensive testing.

2.1. Complete blood count (CBC)

Complete blood count, a screening test involving the measurement of important characteristics of the blood, has been used as part of the diagnosis process for many diseases, including blood disorders, heart disease, kidney problems and nutritional status [1,19]. The main features of the blood normally tested in the CBC are the total white blood cell count (WBC), red blood cell count (RBC), hematocrit (Hct), hemoglobin (Hb), red cell distribution width (RDW), peripheral blood smear and other important erythrocyte indices (EI), namely mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) [19–22]. Among these parameters, MCV and MCH are the most important ones that can indicate the existence of thalassemia trait, i.e., when MCV < 80 and MCH < 27.

Table 2 summarizes the tests performed in the CBC, the calculation needed for each and the approximate normal cut-off level. However, due to the similar low red blood cell count between the patients with thalassemia (iron overloading) and the ones with iron deficiency, it has been suggested that in the geographic regions where iron deficiency rate is high, the cutoffs for thalassemia interpretation should be adjusted to more suitable values by using a receiver operator char-

Table 2
Summary of tests done for CBC and the interpretation of results for thalassemia and hemoglobinopathies indication

Test	Activity or calculation	Normal level
WBC[a]	Number of white blood cells in cubic millimeter (microliter) of blood	4500–10000 cells μl^{-1}
RBC	Number of red blood cells in cubic millimeter (microliter) of blood	Male 4.7–6.1, female 4.2–5.4 million cells μl^{-1}
Hct	MCV × RBC	Male 40.7–50.3%, female 36.1–44.3% (varies with altitude)
Hb	Spectrophotometric measurement at 540 nm of the cyamethemoglobin (Hb bound with cyanide)	Male 13.8–17.2 g dl^{-1}, female 12.1–15.1 g dl^{-1}; using 64,500 g/mole Hb
MCV	10 (Hct/RBC)	80–95 fl
Mentzer index	MCV/RBC	Less than 13 favors thalassemia over iron deficiency
MCH	(10 Hb)/RBC	27–31 (>30) pg per cell
MCHC	(100 Hb)/Hct	28–33%
Peripheral smear	Visual inspection for shape and number of red blood cells by staining with colored chemicals	
Erythrocyte sedimentation rate[a]	The rate that RBCs settled in the tube within 1 h (mm h^{-1})	Varies with age, gender and pregnancy; male age/2, (female age + 10)/2
Platelet count[a]	–	100000–300000 cells μl^{-1}
Fibrinogen[a]	–	200–400 mg dl^{-1}

[a] Parameters that are normally tested in CBC, but are not directly related to determination of thalassemia and Hb variants.

acteristic (ROC) curve [23]. The ROC curve is the plot of the true-positive results (*Y*) against the false-positive results (*X*) for the various sets of results used for constructing the decision threshold. It is used to statistically determine an optimal cutoff point for the medical tests [24–27], which in this case should better differentiate thalassemic microcytosis from non-thalassemic ones (i.e., iron deficiency patients). Other tests such as erythrocyte sedimentation rate, platelet count, and fibrinogen may also be done along with the CBC [28,29].

Many laboratories use an automated CBC machine which can provide many blood parameters in one run. However, the high cost limits its use in many hospitals around the world. Osmotic fragility test, an alternative screening test for thalassemia, is therefore performed instead.

2.2. Osmotic fragility test (OFT)

The main purpose of this technique is to diagnose the hereditary spherocytosis and it is also useful for screening of thalassemia. This simple test utilizes osmosis, the movement of water from lower to higher salt concentration region, to test for the osmotic resistance of the red blood cell [30]. A single hypotonic saline solution can be prepared from dilution of a Tyrode's solution, which is composed of NaCl, KCl, $CaCl_2{\cdot}6H_2O$, $MgCl_2{\cdot}6H_2O$, $NaHCO_3$, NaH_2PO_4, glucose and distilled water [31]. Whole blood is thoroughly mixed with this solution. In a hypotonic condition, the concentration of salt on the outside of a cell is lower than that on the inside, resulting in net water movement into cells. Normal red blood cells are broken within 1–2 min and the mixture then turns clear and reddish. Abnormal red blood cells have deviated osmotic resistances as compared to normal red cells. Spherocytes and erythrocytes with various membrane defects may show decreased osmotic resistance. However, red blood cells of thalassemia have higher osmotic resistance and thus have slower rupture rate, therefore the mixture remains turbid even after 1–2 h [32]. This technique can be carried out in one test tube and it is also called one tube method. Different laboratories may be using slightly different recipes for preparation of hypotonic salt solution, but all are normally based on the same concept of kinetic osmotic fragility. The OFT is a quick preliminary and very economic test before performing further studies of the blood cells.

3. Conventional confirmatory tests for thalassemia and Hb variants

These are useful tests to confirm the existence of certain Hb variants or abnormal level of some Hb types. Confirmatory tests for Hb variants include deoxyhemoglobin solubility test (DST) for detection of HbS and dichlorophenol indophenol precipitation test (DCIP) for detection of HbE. HbH disease which relates to α-thalassemia can be detected by DCIP and brilliant cresyl blue test (BCB). Alkaline resistant hemoglobin test (ART) and acid elution stain (AES) are used for detection of abnormal levels of HbF, which can help identify some types of thalassemia. The ion exchange microcolumn technique is used to quantify the amount of HbA_2 and HbF to identify β-thal trait, E-trait and EE homozygotes. These conventional techniques are relatively low cost and do not require complicated instrumentation. However, some of these techniques may need a highly experienced operator to translate the results. Therefore, availability of more modern instrumentation that can provide more precise information with less requirement of an experienced operator and less usage of toxic chemicals diminishes the use of some of these conventional techniques such as DST and ART.

3.1. Deoxyhemoglobin solubility test

This is a simple test for HbS based on its insolubility in a potassium phosphate saponin buffer solution (composed of K_2HPO_4, KH_2PO_4, saponin and distilled water). Turbidity would be observed within 5 min if the whole blood containing HbS were mixed with sodium hyposulfite and saponin buffer. This test can discriminate samples with HbS from samples with almost all other hemoglobins except Hb Bart's and some rare sickle Hb such as C-Georgetown and S-Travis. Therefore, if a positive test result is shown (i.e., high enough turbidity that newsprint cannot be seen through the test mixture when placed behind the tube), then a follow-up test by electrophoresis is recommended. A false-negative result may be from a high anemic condition [33–35].

3.2. Hemoglobin precipitation test

Some hemoglobin variants such as HbH (β_4 with α-thalassemia) and Hb Köln ($\beta^{98Val\rightarrow Met}$) are classified as unstable hemoglobins which can be precipitated by heating or adding a chemical such as isopropanol or dichlorophenol indophenol [2,36]. The heat stability test can be carried out at either medium temperature (50 °C) for 1–2 h or at high temperature (68 °C) with chemical reaction aids for 1 min. Although taking longer time, the medium temperature stability test is very simple. The clear supernatant of erythrocyte hemolysate in Tris buffer medium, obtained after removing plasma, hemolyzing with distilled water and removing stroma, is placed in the 50 °C water bath for 1 h. Normal hemolysates remain completely clear, while unstable hemoglobins cause flocculation of various turbidities. The test can be done much faster by using chemicals, i.e., KCN and $K_3Fe(CN)_6$, to form hemolysate cyanmethemoglobin. In a phosphate buffer medium, this hemolysate cyanmethemoglobin is agitated rapidly in the 68 °C hot water bath. After 1 min, normal hemolysate may show slight cloudiness and therefore this high temperature method, even though very fast, may need high experience in interpretation in order to avoid a false-positive reading [19,37].

Another way to demonstrate the instability of Hb is with isopropanol precipitation. Packed erythrocytes, cold deionized water and CCl_4 (1:1:1.5 ratio) are placed in a closed tube and vortexed for a few minutes, followed by centrifugation. The clear supernatant is then mixed with isopropanol–Tris buffer at a control temperature of about 37 °C. Unstable hemoglobins cause more turbidity over time, while normal hemoglobins remain clear for at least 30 min. The isopropanol test is reported to have some limitations on the subjects that contain ≥5% HbF, and those that are inappropriately preserved (i.e., unrefrigerated or too old samples) may give false-positive results. Adding anticoagulating reagent can help reduce the false reading but it is suggested that the samples with high HbF should be tested by heat stability, as it is not interfered by HbF [19,38,39].

Similarly, the dichlorophenol indophenol (DCIP) precipitation test is also used widely to screen for HbE and HbH. DCIP can oxidize HbE and HbH faster than any other type of hemoglobin, and therefore it can be used to screen for HbE and HbH. Interpretation of results can be difficult since it involves observing the cloudiness in a deep blue color of DPIC solution. However, a reducing agent may be added to overcome this problem. For example, in the AOAC standard titration method for ascorbic acid, the color of an oxidant DCIP is changed from dark blue to light blue on the way to the end point pink [40]. Therefore, if a small amount of ascorbic acid were added to the DCIP thalassemia test, then the observation could be made more accurately under the light blue condition.

Hemoglobin precipitation tests can be used to screen for some hemoglobin variants but they may not be able to speciate the types of hemoglobins (i.e., HbE and HbH show similar results). Further tests are needed to pinpoint the exact type.

3.3. Brilliant cresyl blue test or new methylene blue test

Both tests are based on the same procedures but with different reagents. They are specifically performed for HbH diagnosis, which cannot be indicated using other techniques such as affinity column and electrophoresis. Polymerase chain reaction can be used to diagnose for HbH, but the cost is higher than these simple color tests. HbH is unstable and it precipitates in the red cells, giving the appearance of many small golf balls inside the cells that can be observed when staining the blood film with brilliant cresyl blue ($C_{17}H_2OClN_3O$) or new methylene blue ($C_{18}H_{22}ClN_3S{:}SClZnCl_2$) [41,42]. The incubation time of blood and the reagents (brilliant cresyl blue in sodium citrate media) takes about 1 h in a controlled temperature setting of about 37 °C [43]. This test is very useful to confirm for α-thalassemia involving HbH inclusion body. However, the technique yields low sensitivity for α-thal trait and therefore it should only be used as a confirmatory test, but not for screening of α-thalassemia.

3.4. Alkaline resistant hemoglobin test

This is a test for abnormal level of fetal hemoglobin (HbF). Normally hemoglobins are denatured at alkaline pH such as in NaOH solution and they can be precipitated readily with saturated ammonium sulfate ($(NH_4)_2SO_4$) solution. However, HbF is not denatured as easily and remains soluble. Differences in alkaline resistance of the normal Hb and fetal Hb allow for rapid testing for the amount of HbF in blood. The procedure consists of a few experimental and calculation steps as reported before [19,44,45]. A suspended mixture of Hb-cyanide–ferricyanide (or cyanmethemoglobin) is prepared by adding packed red cells, obtained from centrifugation of whole blood in isotonic saline solution, into a cyanide–ferricyanide solution (KCN and $K_3Fe(CN)_6$ in distilled water). Then NaOH is added and the solution is mixed

for a few minutes before adding the saturated $(NH_4)_2SO_4$ solution. Coagulated protein can be removed by filtering the mixture until a clear filtrate is obtained. The percent of alkaline resistant hemoglobin is calculated based on the absorbance of the filtrate (Df) and the absorbance of the 1:10 dilution of the original cyanmethemoglobin without NaOH and $(NH_4)_2SO_4$ added (Db) at 540 nm, using the following equation: (100 Df)/(10 Db). In a normal person more than 1 year old, the percentage of HbF should be expressed as being less than 1–2% by using this method. Higher levels of HbF will be suspected of having a hemoglobin disorder of some kind. Although the method was found to mistakenly yield lower results for a subject with HbF higher than 30% of total hemoglobin, such as in umbilical cord blood of newborns, this method was sufficiently sensitive and reproducible for measuring 1–10% HbF, providing that final cyanmethemoglobin concentration is higher than 480 mg/100 ml [46]. In the cases where high amount of HbF is present, an alternative method such as immunological determination of HbF, e.g., by the gel precipitation or immuno-diffusion, involving the use of monoclonal antibody against HbF, may be used to avoid incorrect results obtained from the alkaline resistant hemoglobin test [8,47,48].

3.5. Acid elution stain (modified Kleihauer–Betke test)

This is a simple test for HbF and Hb Bart's. After smearing a blood sample on the slide and letting it dry, the slide is immersed in an 80% alcohol solution (ethyl, methyl or propyl alcohol) for 2–3 min. After that, the slide is immersed in a staining solution of Amido Black 10B ($C_{22}H_{14}N_6O_9S_2Na_2$) prepared in alcohol with pH adjusted to 2.0. After 3 min, the slide is washed under running water for 1 min. In the acidic condition, HbA, HbA_2, HbE, and HbH will be eluted out of the blood cells, leaving the cells empty (ghost cells) and showing no color. HbF and Hb Bart's can tolerate acid and are stained by the Amido Black 10B, showing dark blue color of the cells which can be observed under the microscope.

There are a few precautions that need to be taken when working with this technique. If the slide is left dry for too long, HbA will not be eluted out. The concentration of alcohol is also important because alcohol higher than 85% will cause HbA to stay in the cell, while alcohol lower than 65% will cause vacuolization of HbF. In addition, if the pH of the solution is higher than 2.5, HbA will not be eluted. All these cases will show false results [49,50].

The drawbacks of this technique are time consuming and subjected to human error. Another possible way of detection of HbF is flow cytometry which is more precise as described later.

3.6. Ion exchange micro-column

In the regions where economic restriction does not allow for the use of a relatively higher cost instrument such as HPLC, a cheaper method such as this ion exchange micro-column along with other inexpensive tests can be used in combination to diagnose the type of thalassemia. This technique is based on ion exchange chromatography as a simplified version of high performance liquid chromatography. The use of diethylaminoethyl DEAE anion exchanger, packed in a relatively cheap and small syringe, and Tris–HCl mobile phase can be adapted to separate HbA_2 and HbF effectively. The relative amounts of these Hbs can be estimated by calculating the peak areas of the absorbance, measured at 415 nm, of fractions eluted from the column. It has been shown that the results obtained from the batchwise micro-column are in agreement with those from HPLC, though the method lacks automation and yields lower precision [51–55]. However, the result from ion exchange micro-column technique is acceptably accurate and precise and can be used to confirm some types of thalassemias such as β-thalassemia trait. In addition, with its simplicity and low cost, some laboratories perform this technique together with the OF tests as regular screening techniques, especially where thalassemia cases related to abnormal ratio of HbA_2 and HbF is commonly found such as in Thailand.

It has been estimated that the cost for chemicals and materials per test of the micro-column technique is approximately five times less than that of HPLC. Even though the total analysis time per run is longer than automated HPLC (4 h versus 20 min), many ion exchange micro-columns can be set up and run at the same time. Therefore, the total analysis time of, e.g., 50 tests using multiple micro-columns at one time is less than performing 50 continuous HPLC runs (16 h using HPLC and 4 h using ion exchange micro-columns). In addition, an attempt to reduce the analysis time per run and to make the micro-column technique more automated has been carried out. A flow injection analysis system was joined together with a much smaller ion exchange column to improve the analysis time for hemoglobin typing as compared to the batch process [56]. More work needs to be done, but the preliminary results have suggested that the flow based and reduced volume ion exchange column system has the potential to improve the analysis time per run and to greatly reduce the amount of blood sample needed for the analysis.

4. Instrumental techniques for determination of thalassemia and Hb variants

These techniques involve modern technologies of complicated instrumentation. They can be automated and are usually faster and more reliable but more expensive than the conventional techniques. Even though these techniques can provide detailed information and can help in diagnosis of many types of Hb variants, there are a few exceptional Hb variant cases that cannot be identified with these techniques, and more extensive confirmatory tests are needed. Most instrumental techniques can perform qualitative and quantitative analysis, but with limited ways to accurately quantitate the signals, such as in gel electrophoresis, these techniques have been

used mainly for diagnosis of Hb variants rather than for detection of abnormal level of Hbs in thalassemia diagnosis, as shown in the flow chart in Fig. 1.

4.1. High performance liquid chromatography (HPLC)

In high performance liquid chromatography, particle size of the stationary phase packed in the column is quite small (about 2–5 μm). High pressure is required to force the mobile phase to continuously flow through the column. As the sample solution flows with the liquid mobile phase through the stationary phase, the components of the sample will migrate according to the non-covalent interactions of the compounds with the stationary phase. The degree of interactions determines the degree of migration and separation of the components (i.e., the component with a stronger interaction with the mobile phase than with the stationary phase will have a shorter retention time and thus will be eluted from the column first and vice versa) [57,58].

HPLC has become a very important tool for thalassemia and Hb variants diagnosis because of its ability to accurately and rapidly qualitate and quantitate different types of Hbs. However, in most laboratories, HPLC has been used for diagnosis of Hb variants rather than for quantification of normal Hb or thalassemia diagnosis, except for the case of prenatal analysis. The HPLC technique requires a very small amount of blood samples (μl), therefore, it is very suitable for prenatal diagnosis of thalassemia [59–63] where sample may be limited and difficult to obtain. There are many reports showing the agreement of results obtained from HPLC and those obtained from other techniques such as the globin synthesis technique, isoelectrofocusing, carboxymethylcellulose chromatography and DNA sequencing [59–64].

Anion exchange resin DEAE and gradient Tris–HCl buffer solution, pH 8.5–6.0, is a widely used stationary–mobile phase system for HbA_2 and HbF quantification to effectively diagnose β-thalassemia and Hb Bart's hydrop fatalis that occur frequently in Southeast Asia [62,65]. The system can also separate other Hb variants such as HbS, HbC and HbJ [66,67]. Cation exchangers, such as CM-cellulose (CMC) and silica supported with carboxylic acid residues with bis-Tris–KCN developer, can also be used for the same purpose [60,68,69]. The system based on carboxymethylated poly(vinyl alcohol) resin and sodium phosphate buffer solution as a stationary–mobile phase has been developed for separation and quantification of St-HbA1c, which is a marker of blood glucose regulation in diabetic patients [70].

The ratio of different globin chains (e.g., β:γ for β-thalassemia diagnosis) can also be determined with HPLC using a reverse phase C18 column and shows similar results to those obtained from CMC which is normally employed for this purpose [59]. Determination of Hb types using HPLC has gained high popularity over globin chains determination using CMC because of HPLC's relatively easier and faster analysis which is a result of its having fewer sample preparation steps and an automated data analysis system [61]. HPLC has an overall performance better than electrophoresis.

4.2. Electrophoresis

Electrophoresis is one of the widely used techniques for analyzing hemoglobin variants based on the movement of different Hb or different globin chains, containing different charges, in the electric field. At an alkaline pH, Hb is negatively charged and will move toward the anode (positively charged) terminal. Electrophoresis of total Hb is different from electrophoresis of separated globin chains. To perform electrophoresis of globin chains, a few steps need to be done in order to obtain free globin chains. First, heme is removed from hemoglobin by treating with mercaptoethanol. Then the four globin chains are split apart without denaturing them using 8 M urea. A cellulose acetate membrane is mainly used in alkaline pH electrophoresis. Normal operating voltage is about 250 mV and the approximate run time is about 90 min. After that, the membrane needs to be stained, de-stained and air dried before separation of globin chains can be observed. The main limitation of electrophoresis at alkaline pH is the inability to differentiate HbA_2, HbC, HbO and HbE from one another, nor can HbD, HbG and Hb Lepore be differentiated from HbS [33,71,72]. Therefore, it is normally used to screen for some types of Hb variants. The confirmatory test can be done using electrophoresis in acidic media.

At a lower pH of about 6.0, a better separation of different hemoglobins is obtained. Those Hbs that co-migrate in alkaline pH electrophoresis can be separated in acidic media. Nevertheless, the main technique for Hb quantification by densitometric scanning of the gel is still somewhat difficult and unreliable [33] and therefore electrophoresis technique has been used mainly for detection of Hb variants rather than measuring level of Hb in thalassemia diagnosis. It is highly specific in the detection of certain Hb disorders such as sickle cell disease. Even though the electrophoresis in acidic media is quite a powerful technique in separation of many types of Hbs, please keep in mind that not all Hb variants can be separated by electrophoresis in acidic media. For example, Hb Okayama cannot be separated using electrophoresis, but can be done so in HPLC [73].

Capillary electrophoresis is the new format of electrophoresis where separation takes place in a small fused silica capillary. It is rapid, easily automated and consumes low amounts of reagents, as compared to conventional gel electrophoresis. It also offers much higher throughput as compared to HPLC [74]. However, some researchers found that CE has higher instrumentation cost and is less accurate as compared to automated HPLC [75].

4.3. Isoelectric focusing (IEF)

This technique is based on the electrophoresis technique but with a higher degree of separation. Different Hbs migrate in a pH gradient to the point where their net charges are zero.

The order of migration is the same as in alkaline electrophoresis but the narrower bands obtained from this method (IEF) allow for the resolution of HbC, HbE, HbO, HbS, HbD and HbG [76,77].

Two different formats of IEF, thin layer gel and capillary, have been reported [78–81]. Cossu et al. [82] applied the immobilized pH gradient method (IPG) with a thin layer gel that has a pH range of 6.7–7.6 to differentiate heterozygous from homozygous β-thalassemia in newborns. The group suggested the use of umbilical cord blood because it contains only HbF, HbA and acetylated HbF (HbFac) and the ratio of HbA:HbFac or HbF:HbA is used instead of the conventional β:α ratio in the IEF of globin chains.

Capillary IEF showed very promising performance both in qualitative and quantitative aspects. A single IEF run can replace the main tests that normally have to be carried out in combination for qualitative and quantitative analysis of Hbs, for instance, alkaline and acid electrophoresis for major Hb variants, ion exchange chromatography for HbA_2 quantification and alkaline resistant test for HbF [78,81]. It has been proven to have a comparable performance to chromatography or radioactive globin chain methods [79] and can be used for analysis of hemoglobin variants in adult and newborn [83].

4.4. Flow cytometry

Even though acid elution stain test seems to be simple, it is rather time consuming and subject to human error. The more precise and sensitive quantification of HbF can be done using the instrumental based flow cytometric technique [84,85]. The interested component of the cell is bound to a fluorescence label. Light scattering can identify the cell population of interest. Fluorescence intensity is measured to quantify the component of interest. The discovery of monoclonal antibody production has extended the use of flow cytometry. Antibody against HbF tagged with fluorescent dye can be used to specifically determine the amount of HbF. It has been demonstrated that detection of both a fetal cell surface antigen and HbF using two different monoclonal antibodies and two colored dyes is a precise way to identify the fetal cells [86]. The technique called gradient centrifugation has been proved to enrich the fetal cells from the adult blood and can extend the sensitivity of the flow cytometric analysis of HbF [87].

5. Extensive analysis techniques for thalassemia and Hb variants

These are advanced techniques used to detect thalassemia and Hb variants. They are complicated and expensive and therefore are used in the cases for which there are no other ways to accurately confirm or identify the types of thalassemia or Hb variants. They involve DNA technology that can provide in-depth detailed information of gene mutation.

5.1. Polymerase chain reaction (PCR) with different formats of gel electrophoresis

Polymerase chain reaction is a technique that allows a small amount of DNA to be amplified in vitro. The process is composed of cycles of the three following steps: perform heat denaturing to separate the DNA sequence target into two strands, anneal each strand to the specific primers and then extend the polymerase chain from the primer termini [10]. Once there are enough of the DNA target sequences produced, further analysis can be performed. Gel electrophoresis is commonly done following the PCR to separate different DNA fragments. Many additional methods can be coupled with gel electrophoresis and PCR to obtain better information such as those described briefly here.

Direct DNA sequencing of PCR products is quite a straightforward method to indicate the mutation site [88,89].

The restriction fragment length polymorphism (RFLP) technique can differentiate between different DNA sequences based on the length of fragments yielded by a particular enzyme restriction and can indicate the mutation point of a gene in thalassemia patients [90,91].

The amplification refractory mutation system (ARMS-PCR), also known as allele specific PCR, is another technique that has been introduced to be used for thalassemia diagnosis. This technique utilizes two PCR reactions: one contains a primer specific for the normal allele and the other contains one for the mutant allele. Gel electrophoresis is then employed to separate specific DNA bands. Diagnosis of genotyping is based on whether there is amplification in one or both reactions (i.e., the band in normal reaction only indicates normal allele, the band in mutant reaction only indicates mutant allele, and bands in both reactions indicate a heterozygote) [92–94]. ARMS-PCR is more accurate as compared to RFLP.

Single stranded conformation polymorphism (SSCP) is the technique that was developed based on the fact that the mobility in gel electrophoresis of single strands of DNA drastically depends on nucleotide sequence. Single stranded DNA is produced by adding one primer at a concentration higher than another primer in the PCR step. After the primer with lower amount is used up, the reaction will continue producing only the product of the excess primer. The mobilities of single strands are then compared [95]. Single stranded DNA may also be produced by denaturing double stranded DNA, as in the technique called denaturing gradient gel electrophoresis (DGGE). The DGGE technique utilizes the gradient of low to high pH to denature different gene fragments and retard their mobilities in gel. A mobility shift can be detected even with a slight difference in the base pair sequence [92,96,97].

5.2. DNA technology: DNA probe/DNA microchip

Analysis of nucleic acids has led to the understanding of the gene expression that controls Hbs formation. This information is more detailed as compared to information obtained

from protein analysis that normally only suggests type and amount of different Hbs production. The advance of DNA studies and fabrication technology together has led to the development of methods for diagnosis using a DNA microchip. Normally the segment of a gene of interest first has to be amplified by PCR to obtain a sufficient amount prior to hybridization with allele specific oligonucleotide probes that are immobilized on the solid phase or chip [98–100]. The bound target gene can be detected using either labels such as fluorescent substances [101,102] or electronic transducers such as piezoelectronic and ion sensitive field effect transistors (ISFETs) [103]. The attempt to pinpoint the DNA sequences that become over-expressed in a patient has become important because it can lead to the cure or prevention of the disease. In this case the precision and accuracy of detection requires highly sophisticated devices. Therefore, development of a highly sensitive and accurate device or method of detection is currently an important research trend.

One example of devices that has been used commonly is a cytometer. Cytometry is a laser based technique that allows for analysis of physical properties and fluorescence intensity of an individual cell in a heterogeneous environment. The image can differentiate different types of cells or DNA sequences that are labeled with different colors by comparing the ratios of fluorescence of different targets. With the aid of a computer, detection and visualization of many different probes can be done simultaneously [104,105].

6. Indirect studies related to thalassemia indication and treatment follow-up

These studies are not intended to be used for thalassemia diagnosis. However, the relevance of the variable of interest and the existence of thalassemia may help open up a new way to economically test for thalassemia or treatment follow-up.

6.1. Ferritin

Ferritin is the iron storage protein and its level in serum directly relates to the amount of iron stored in the body, which is important for red blood cell production. Normal ranges of ferritin are 12–300 and 12–150 ng ml^{-1} for male and female, respectively [106]. The technique commonly used to quantify ferritin is immunoassay [107,108]. A significantly high level of ferritin is found in patients with iron overload and this may help differentiate thalassemia patients from those with iron deficiency, both of which will have a low red blood cell count [109]. However, please note that iron deficiency does not exclude thalassemia disorder. In addition, any inflammatory disorder can cause a high level of ferritin. Therefore, long term monitoring of ferritin would be necessary, if its level is chosen to be observed, to gain any additional information for thalassemia diagnosis or treatment follow-up [110].

6.2. Skin tissue Fe concentration

Thalassemia patients have an increased amount of iron storage that has been reported to cause an increased risk of cardiovascular disease due to arterial stiffness [111–113]. Evaluating the level of iron in the body over time is one way to obtain additional information for thalassemia diagnosis and to evaluate the efficiency of the treatment. The X-ray spectrometric techniques (diagnostic X-ray and X-ray fluorescence spectrometry) have been employed to rapidly and non-invasively quantify the amount of iron overload in the skin of thalassemia patients [114,115]. Since the amount of iron found in the outer body skin correlates to the amount of iron overloaded in the liver, heart and spleen, the results from the skin can be used as markers for iron-overload organs. However, as mentioned previously, it is necessary to keep in mind that iron deficiency does not exclude thalassemia.

6.3. Magnetic behavior of erythrocytes

A study in physics based on observation of the change in magnetic behavior of Hb at different states (i.e., normal, oxidized and reduced states) has been conducted [116]. The reduced form of Hb induces paramagnetism while the oxidized form of Hb shows diamagnetic behavior. It was found that normal state Hb from β-thalassemia minors has lower diamagnetic response as compared to that of Hb from iron deficiency patients. This may indicate a low oxygen intake of β-thalassemia minor blood.

6.4. Nuclear magnetic resonance spectroscopy

The nuclear magnetic resonance technique is based on the magnetic properties of some nuclei that when placed in the magnetic field, would take up radio frequency energy that matches the magnetic field strength and later re-emit that energy [117]. The phenomenon is known as nuclear magnetic resonance (NMR) because it involves the nucleus in a magnetic field that has its strength in resonance with the applied radio frequency. Originally NMR spectroscopy was used for the study of composition of chemical compounds. Later, the technique was developed into the imaging technology, magnetic resonance imaging (MRI), that became a major breakthrough in medical fields because it can reveal the image of the parts of the body and seems to be the most sensitive means at present. Contrast medium such as gadoteric acid may be introduced to obtain better results [118].

NMR has been widely applied to study body iron overload [119–128]. NMR spectroscopy has been employed mainly for study of iron level in the fraction of tissue in vitro, while NMR imaging has been used mainly for determination of iron in vivo. So far, there has been no report on health hazards directly related to NMR and therefore the NMR technique is considered a safe and non-invasive way to study body iron content.

7. Conclusion

There are many different techniques available for thalassemia diagnosis, but used alone they may not be able to satisfactorily answer every question. Therefore, it is quite common to utilize more than one technique to ensure the diagnostic result. Fig. 1 summarizes techniques commonly used for diagnosis of Hb variants and thalassemia in most laboratories. If MCV, MCH or OFT screening test reveals a normal result, the possibility of having thalassemia can be eliminated, but analysis of Hb variants should be done. If an abnormal result is obtained from the screening test, there is a possibility of having either Hb variants or thalassemia case. If Hb variants tests do not show any abnormal results, thalassemia tests should still be performed. Choices of techniques depend mainly on budget and equipment available. For example, some countries with limited budget such as Thailand, Indonesia and The Philippines utilize OFT rather than MCV and MCH (from automated CBC machine) for thalassemia screening. It should be pointed out that even in laboratories equipped with high technology and many years of experience, quite a few false diagnoses were reported, which resulted in the births of thalassemia children or the abortions of unaffected fetuses [93]. It is very important to take precaution in every step of the diagnostic procedures to ensure the most accurate diagnosis. For example, be sure to use a fresh blood sample, always perform sufficient sample cleaning, and always run duplicate tests with suitable controls. Developments in chemical analysis methodologies are still very useful to this field.

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