Laboratory Diagnosis of Hemoglobinopathies and Thalassemia

Archana M Agarwal, MD
Medical Director, Hematopathology and RBC Laboratory
ARUP Laboratories
Assistant Professor of Pathology
University of Utah Department of Pathology

Learning Objectives

• Understand the pathophysiology of hemoglobinopathies

• Recognize the most important expected test results in hemoglobinopathies and thalassemias

• Understand different testing methodologies

• To be able to direct ordering physician to appropriate tests for these disorders

Hemoglobin (Heme+Globin)

• Hemoglobin is a tetramer composed of 4 globin molecules; 2 alpha globins and 2 beta globins or beta like globins

• The alpha globin chain is composed of 141 amino acids and the beta globin chain is composed of 146 amino acids

• Each globin chain also contains one heme molecule
Normal Adult Human Hemoglobin Composition

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Structure</th>
<th>% of Normal Adult Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>$\alpha_2\beta_2$</td>
<td>&gt;96%</td>
</tr>
<tr>
<td>Hb A2</td>
<td>$\alpha_2\delta_2$</td>
<td>~2.5%</td>
</tr>
<tr>
<td>Hb F</td>
<td>$\alpha_2\gamma_2$</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

Hemoglobinopathy (structural)

- Due to mutations in either alpha or beta globin
- **Structural** – substitution, addition or deletion of one or more AAs in the globin chain
  - i.e. HbS, HbC, HbE, HbD, HbO, etc...
- Over 1000 identified
  - Majority are benign & discovered incidentally
  - Pathogenic mutations can cause
    - Change in physical properties (sickling, crystalizes)
    - Globin instability (Heinz body formation, lower expression)
    - Altered oxygen affinity

Thalassemia (quantitative)

- A quantitative decrease in the production of alpha or beta globin chain
  - Large deletions, point mutations, small insertion/deletion that leads to decreased transcription or an unstable transcript
- Beta thalassemia results from mutations in beta gene(s)
  - Pathogenesis a result of the **free alpha subunits**
  - Two classes: $\beta^0$ and $\beta^+$
- Alpha thalassemia results from large deletions in the alpha gene(s)
  - Pathogenesis a result of the **free beta subunits**
Demographics: Thalassemias

- Found most frequently in the Mediterranean, Africa, Western and Southeast Asia, India and Burma
- Distribution parallels that of Plasmodium falciparum

Classification & Terminology: Alpha Thalassemia

- Normal \( \alpha\alpha/\alpha\alpha \)
- Silent carrier - \( \alpha/\alpha\alpha \)
- Minor /trait \( -\alpha/-\alpha \)
- Hb H disease \( --/-\alpha \)
- Barts hydrops fetalis \( --/-- \)

Clinical Presentations of Alpha Thalassemia

- A single deletion (\( \alpha\)-thalassemia minor)
  - silent carrier state
  - RBC morphology and hemoglobin concentrations are usually normal
- Two gene deletion (\( \alpha\)-thalassemia minor)
  - Mild microcytic anemia
- Three gene deletion (hemoglobin H disease)
  - Precipitated \( \beta \) chains—Hb H
  - Patients have moderate anemia, marked microcytosis, splenomegaly, and bone marrow erythroid hyperplasia
- Four gene deletion (Hydrops fetalis)
  - Not compatible with life (barring very early intervention)
  - Hemoglobin is primarily comprised of \( \gamma \) (Bart’s), which has a very high affinity for O2 and is a poor oxygen transporter
Classification & Terminology: Beta Thalassemia

• Normal \( \beta/\beta \)
• Minor / trait \( \beta/\beta^0 \)
  \( \beta/\beta^+ \)
• Intermedia \( \beta^0/\beta^+ \)
• Major \( \beta^0/\beta^0 \)
  \( \beta^+/\beta^+ \)

Clinical Significance of \( \beta \) Thalassemia

• Heterozygous asymptomatic
• Homozygous \( \beta^0 \) is a severe disorder associated with transfusion dependent hemolytic anemia
• Homozygous \( \beta^+ \) is a heterogenous disorder
  – severity depending on mutation and % of HbA
  – Increased HbA = decreased severity

Sickle Cell Anemia

• Single nucleotide base change codes for valine instead of glutamic acid at the 6th position from the N-terminus of the \( \beta \)-globin chain
• Affects the shape and deformability of the red blood cell
• Leads to veno-occlusive disease and hemolysis
Peripheral Smear: Sickle Cell Anemia

Hb E
- 2nd most prevalent hemoglobin variant
  - 30,000,000 worldwide
  - 80% in Southeast Asia
- Hb E trait: microcytosis (mean MCV=65fl). No anemia
- Hb E disease: MCV =55-65fl with minimal anemia
- *On HPLC has similar migration pattern as Hb A2

Hb C
- Mutation in β-globin gene (ΔGlu→Lys)
- Seen predominantly in blacks: Gene prevalence in US black population is 2 to 3%
- May confer malaria resistance
- Often asymptomatic, mild anemia, splenomegaly
- Blood smear shows many target cells, rare intracellular crystals
- Hb S/C disease causes moderate to severe anemia and hemolysis
Diagnosis

• Indications for Testing
  – Hemolytic anemia; family history of hemoglobinopathy

• Laboratory Testing
  – Initial testing – CBC with peripheral smear
  – Polychromasia, spherocytes, schistocytes, sickle cells, Heinz bodies, basophilic stippling; however, the lack of any of these cells does not rule out hemolytic anemia
  – Many hemoglobinopathies can be diagnosed using electrophoretic or high performance liquid chromatography (HPLC) techniques, but some may be missed
  – Genetic testing

Importance of CBC

• Thalassemias
  – Red cell indices are critical to diagnosis
  – Hypochromic microcytic anemia
    • MCV (mean corpuscular volume or size of the cell) is key
    • RDW (red cell distribution width) changes are variable
    • Increased RBC count → one distinguishing factor between thalassemias and other microcytic anemias

• The RBC count in thalassemia is either normal or on higher side of normal
• MCV usually less than 70 in
• The RDW is usually in the normal range

Distinguishing Features Between Iron Deficiency and Thalassemia

• The RBC count in thalassemia is either normal or on higher side of normal
• Low RBC count
• MCV usually more than 70
• RDW is usually more than 17
Diagnosis of Thalassemias

High-Pressure Liquid Chromatography

- Cation Exchange
- Analytical cartridge contains negatively charged silica
- Buffers contain Na+ and K+ ions
- Hemolysates contain positively charged hemoglobin
- Hemoglobin binds to negatively charged silica at injection
- Na+ and K+ concentration increased and separates hemoglobin fragments from silica

Normal Patient Chromatograms
Summary of HPLC

**Advantages**
- Fast
- Small amounts of sample
- Accurate quantitation of A2

**Disadvantages**
- Hemoglobin E cannot be separated from A2
- Hemoglobin H and Barts elute too quickly from column

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Capillary Electrophoresis

[Image of Capillary Electrophoresis equipment and diagram]

http://www.sebia-usa.com

Phoresis Reports

[Images of Phoresis reports]

http://www.sebia-usa.com
Alkaline and Acid Gel Electrophoresis

- Electrophoresis (pH 8.4 (alkaline) and pH 6.2 (acid) on agarose gels)
- Slow, labor-intensive, and inaccurate in the quantification of low-concentration Hb variants (e.g., Hb A2) or in the detection of fast Hb variants (Hb H, Hb Bart's)
- The precision and accuracy of Hb A2 measurements using densitometric scanning of electrophoretic gels is poor, especially when compared with HPLC techniques

Isoelectric Focusing

- IEF is an electrophoretic technique with excellent resolution
- IEF is an equilibrium process in which Hb migrates in a pH gradient to a position of 0 net charge
- The Hb migration order of IEF is the same as that of alkaline electrophoresis with better resolution

Molecular Analysis

- Alpha thalassemia
  - Multiplex ligation dependent probe amplification (MLPA) and multiplex PCR
  - Alpha globin sequencing
- Beta thalassemia
  - Beta globin sequencing
  - The test examines the complete beta globin coding sequence, the splice sites and other intronic regions known to harbor mutations, the proximal promoter region, and the 5' and 3'UTR regions
  - Clinical sensitivity is up to 97% based on the ethnicity
  - Beta globin deletion testing by MLPA
**α–Thalassemia Diagnosis**

- Hb gel/HPLC migration patterns
  - Not helpful for α–Thalassemia, unless β4 (Hb H) and γ4 (Hb Barts) are present
- Genetic analysis
  - MLPA: will identify all deletions and duplications
  - Multiplex PCR for 7 common deletions-only 7 common deletion
  - Alpha globin sequencing
    - PCR amplification followed by bidirectional sequencing of the complete protein coding sequence with exonic/intron boundaries, proximal promoter region, 5' and 3' untranslated regions, and polyadenylation signal
    - Only useful in 5-10% of cases where alpha thal is due to point mutation

**β–Thalassemia Diagnosis**

- HPLC: Elevated HB A2 diagnostic
- Molecular analysis: Complete beta globin coding sequence, the splice sites and other intronic regions known to harbor mutations, the proximal promoter region, and the 5' and 3'UTR regions
- Clinical sensitivity is up to 97% based on the ethnicity
- Beta globin del/dup in some cases (about 5%) where beta thalassemia is due to large deletions

**Sickle Cell Disease Diagnosis**

- Sickledex test (Screening test)
  - Deoxygenated Hb-S is insoluble in a concentrated phosphate buffer solution and forms a turbid suspension
  - Normal Hemoglobin A and other hemoglobins remain in solution
  - It does not differentiate between Sickle Cell Disease (S/S) and Sickle Cell Trait (A/S)
Sickle Cell Disease Diagnosis

Simplified Algorithm

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