Haemoglobinopathies in the Diagnostic Laboratory

Barbara J Bain, Gold Coast, 2011

Image from www.dsc.discovery.com

Which variant haemoglobins are relevant?

- Detection of variant haemoglobins is important for three reasons
  - Genetic significance (relevant to offspring)
  - Clinical significance (relevant to individual)
  - Diagnostic significance
- Only alpha and beta chain variants are usually clinically or genetically relevant
- Gamma chain variants are relevant only in utero and in the neonatal period
- Delta chain variants are relevant to diagnosis

Which thalassaemias are relevant?

- β thalassaemia
- Haemoglobin H disease
- Haemoglobin Bart’s hydrops fetalis
- Carrier state for δ thalassaemia

Variant haemoglobinopathies

Variant haemoglobinopathies of genetic significance

- Haemoglobin S and haemoglobins that interact with it
  - C, D-Punjab, O-Arab, Lepore, E
- Haemoglobins that interact with thalassaemia genes
  - E, Lepore, other rare thalassaemic haemoglobinopathies
  - Constant Spring, Paksé
Variant haemoglobin S

Variant haemoglobin S is a common variant of haemoglobin that causes sickle cell disease, characterized by a missense mutation in the beta-globin gene that results in a substitution of valine for glutamic acid in the beta chain.

Variant haemoglobins of clinical significance (i)

- Haemoglobin S and haemoglobins that interact with it
  - C, D-Punjab, O-Arab, Lepore, E
- Haemoglobins that interact with thalassaemia genes
  - E, Lepore
  - Constant Spring

Variant haemoglobins of clinical significance (ii)

- Unstable haemoglobins
- High affinity haemoglobins
- Methaemoglobins
  - Low affinity haemoglobins

Variant haemoglobins of diagnostic significance

- Delta chain variants (complicate diagnosis of beta thalassaemia)
- Alpha chain variants (complicate diagnosis of beta thalassaemia)
- Alpha and beta chain variants that interfere with measurement of glycated haemoglobin
- Insignificant but confusing variants
Haemoglobin S

**Significance**
- Sickle cell disease in homozygotes and compound heterozygotes
- Lesser degree of pathology in heterozygotes
  - Complications of hypoxia
  - Renal complications

**Recognition**
- Ethnic origin and clinical history
- Blood count and film
- HPLC
- Alkaline electrophoresis on cellulose acetate
- Acid agarose electrophoresis
- Sickle solubility test
- Isoelectric focusing
- Capillary electrophoresis


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Haemoglobin S

What could look like haemoglobin S on HPLC?
- Haemoglobin Q-Thailand (α chain)
- Haemoglobin Manitoba (α chain)
- Haemoglobin Yakima
- Haemoglobin E-Saskatoon
- Haemoglobin G-Pest (α chain)
- Haemoglobin G-Waimanalo (α chain)

How is this dealt with?
- Quantity of variant
- Precise retention time
- Presence of an A2 variant
- *Obligatory alternative technique*

Haemoglobin S

Acid agarose electrophoresis

Haemoglobin S

What else can give a single band with the mobility of S (F)?

Haemoglobin C

Significance

- Interacts with S
- In homozygotes, causes a chronic haemolytic anaemia with an increased incidence of gallstones

Haemoglobin C

**Recognition**

- Ethnic origin and clinical history
- Blood count and film
- HPLC
- Alkaline electrophoresis on cellulose acetate
- Acid agarose electrophoresis
- Isoelectric focusing
- Capillary electrophoresis


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Blood count and film, C trait

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Blood count and film, C disease

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Haemoglobin C

Blood count and film, SC compound heterozygosity

Cellulose acetate electrophoresis at alkaline pH

Haemoglobins that resemble C on cellulose acetate at alkaline pH (i)

<table>
<thead>
<tr>
<th>Haemoglobin</th>
<th>Agarose gel</th>
<th>Citrate agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Mobility of C</td>
<td>Mobility of C</td>
</tr>
<tr>
<td>E</td>
<td>Mobility of A</td>
<td>Mobility of A</td>
</tr>
<tr>
<td>A₂</td>
<td>Mobility of A</td>
<td>Mobility of A</td>
</tr>
</tbody>
</table>

Haemoglobins that resemble C on cellulose acetate at alkaline pH (ii)

<table>
<thead>
<tr>
<th>Haemoglobin</th>
<th>Cellulose acetate, alkaline</th>
<th>Acid agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>AC</td>
<td>AC</td>
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<tr>
<td>AE</td>
<td>AE</td>
<td>AE</td>
</tr>
<tr>
<td>AA₂</td>
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<td>AFSC</td>
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<tbody>
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<td>C</td>
<td>Mobility of C</td>
<td>Mobility of C</td>
</tr>
<tr>
<td>CHarlem</td>
<td>Mobility of S</td>
<td>Mobility of S</td>
</tr>
<tr>
<td>OArab</td>
<td>Mobility very slightly on the C side of S</td>
<td>Mobility very slightly on the S side of A</td>
</tr>
</tbody>
</table>

### Haemoglobins that resemble C on cellulose acetate at alkaline pH (iv)

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<tr>
<td>AFSC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Haemoglobin C

**Acid agarose electrophoresis**


Haemoglobin C

**HPLC**

BioRad

Variant II

Haemoglobin C

Isoelectric focusing


Haemoglobin E

Significance

• Interaction with $\beta$ thalassaemia can lead to $\beta$ thalassaemia intermedia or major
• Homozygosity gives mild microcytic anaemia
• Interaction with haemoglobin S gives mild sickle cell disease

From Weatherall and Clegg, the Thalassaemia Syndromes
Haemoglobin E

Recognition

- Ethnic origin and clinical history
- Blood count and film
- HPLC
- Alkaline electrophoresis on cellulose acetate
- Acid agarose electrophoresis
- Isoelectric focusing
- Capillary electrophoresis


Haemoglobin E

Blood count and film E trait

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Haemoglobin E

Blood count and film E homozygosity

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**Haemoglobin E**

**Blood count and film E/β thalassaemia**


**Cellulose acetate electrophoresis at alkaline pH**


**Cellulose acetate electrophoresis at alkaline pH**

A + C


**Acid agarose electrophoresis**

A + C

Haemoglobin E trait

Cellulose acetate, alkaline pH

<table>
<thead>
<tr>
<th>Trait</th>
<th>Acid agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>C trait</td>
<td>C trait</td>
</tr>
<tr>
<td>C disease</td>
<td>C disease</td>
</tr>
<tr>
<td>E trait</td>
<td>E trait</td>
</tr>
<tr>
<td>AFSC</td>
<td>FASC</td>
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</table>

Haemoglobin E disease

Cellulose acetate, alkaline pH

<table>
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<tr>
<th>Trait</th>
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<tbody>
<tr>
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<tr>
<td>E trait</td>
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Haemoglobin E

HPLC
BioRad
Variant II


Haemoglobin E

Isoelectric focusing

Haemoglobin D

Which haemoglobin D?

• An extract from an email
• I am a lucky parent of 6 children, the youngest of whom has been diagnosed with having haemoglobin D trait through the recent NHS screening programme
• My background (so I have assumed till last week) is ‘white’ English/ Irish/ Scottish and I can trace my “roots” to the early 1800s locally in Lancashire where I live and earlier down part of my fathers side to South West Scotland to at least the mid 1600s.

Haemoglobin D

Which haemoglobin D?

• I need to know what variant of haemoglobin D I carry and so do my children for different reasons.
• have a burning desire to find out what strain of haemoglobin D but I hope that it is not Punjab / Los Angeles for my children's sake.

Haemoglobin D

Which haemoglobin D?

• Only haemoglobin D-Punjab/D-Los Angeles matters
• It is not very fair to tell people that they have ‘haemoglobin D’ that might or might not matter.
Haemoglobin D-Punjab

**Significance**
- Interaction with haemoglobin S leads to sickle cell disease

**Recognition**
- Ethnic origin
- Blood count and film
- HPLC
- Alkaline electrophoresis on cellulose acetate
- Acid agarose electrophoresis
- Isoelectric focusing
- Capillary electrophoresis

**Afro-Americans** 0.01%

**Afro-Caribbeans** 0.4%

**Pakistan** 1%

Also known as D Los Angeles

Map from http://www.vr3.co.uk/vr3/images/world_map.jpg

**Also China, Thailand, Indonesia**

**Also Norway, Denmark, Sweden, Holland, Germany, France, Italy, Portugal, Greece, Turkey, ex-Yugoslavia, Lebanon, Iran, United Arab Emirates**

**Also Cuba, Venezuela**

Also known as D Los Angeles

Map from http://www.vr3.co.uk/vr3/images/world_map.jpg
Haemoglobin D-Punjab

Homozygous D

From: Lichtman’s Atlas of Hematology, McGraw-Hill

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Cellulose acetate electrophoresis, alkaline pH

- F + S
- A + C + G
- A + C
- A + D-Punjab
- A
- γ variant, A + F
- A, F, S, C


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Haemoglobins that resemble S on cellulose acetate at alkaline pH

<table>
<thead>
<tr>
<th>Cellulose acetate, alkaline pH</th>
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</tr>
</thead>
<tbody>
<tr>
<td>A, S</td>
<td>A, S</td>
</tr>
<tr>
<td>A, D</td>
<td>A, D</td>
</tr>
<tr>
<td>S, D</td>
<td>S, D</td>
</tr>
<tr>
<td>AFSC</td>
<td>FASC</td>
</tr>
</tbody>
</table>


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Acid agarose electrophoresis

Haemoglobin D-Punjab

HPLC BioRad
Variant and variant II


Haemoglobin D-Punjab

Isoelectric focusing

Haemoglobin Lepore

Significance

- Interaction with haemoglobin S leads to relatively mild sickle cell disease
- Interaction with β thalassaemia leads to thalassaemia intermedia or major
- Homozygosity leads to thalassaemia intermedia or major
**Haemoglobin Lepore**

**Significance**
- Haemoglobin Lepore homozygote

From: Weatherall and Clegg, the Thalassaemia Syndromes

**Recognition**
- Ethnic origin
- Blood count and film
- HPLC
- Alkaline electrophoresis on cellulose acetate
- Acid agarose electrophoresis
- Capillary electrophoresis

**Haemoglobins that resemble S on cellulose acetate at alkaline pH**

<table>
<thead>
<tr>
<th>Cellulose acetate, alkaline pH</th>
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</thead>
<tbody>
<tr>
<td>A, S</td>
<td>A, S</td>
</tr>
<tr>
<td>A, Lepore</td>
<td>A, Lepore</td>
</tr>
<tr>
<td>S, Lepore</td>
<td>S, Lepore</td>
</tr>
<tr>
<td>AFSC</td>
<td>FASC</td>
</tr>
</tbody>
</table>

Haemoglobin Lepore

HPLC BioRad
variant and  Variant II


Haemoglobin O-Arab

Significance
- Interaction with haemoglobin S leads to sickle cell disease
- Interaction with βthalassaemia, which is rare, can cause haemolytic anaemia
- Homozygosity, which is rare, can cause compensated haemolysis or haemolytic anaemia

Recognition
- Ethnic origin (Arabs, Greeks, eastern Europeans, Kenyans, Sudanese, North Africans)
- HPLC
- Alkaline electrophoresis on cellulose acetate
- Acid agarose electrophoresis
Haemoglobin O-Arab

Cellulose acetate electrophoresis, alkaline pH


Haemoglobin O-Arab

Acid agarose electrophoresis


Haemoglobin O-Arab

HPLC BioRad
Variant and Variant II


Haemoglobin Constant Spring
Haemoglobin Constant Spring

**Significance**
- An α chain variant resulting from mutation of the termination codon that is synthesized at a greatly reduced rate
- Similar significance to α+ thalassaemia
- However homozygotes (α<sup>CS</sup>α/ α<sup>CS</sup>α) can have anaemia and splenomegaly
- Compound heterozygotes with α<sup>0</sup> thalassaemia have haemoglobin H disease

**Recognition**
- Ethnic origin
  - Most frequent in South East Asia (Thailand, China, Vietnam, Laos, Cambodia, India, Malaysia, Indonesia)—1-8% in Thailand
  - Also Arabs, Greeks, Sicilians, Indians
- Blood count
  - more anaemia but less microcytosis than in α+ thalassaemia; MCV can be low or clearly normal but MCH is reliably reduced (cells are overhydrated)

- Blood film (basophilic stippling)
- HPLC, doublet or triplet (unstable, heterozygotes~2%, homozygotes~5%)
- Alkaline electrophoresis on cellulose acetate (slower than A<sub>2</sub>)
- Acid agarose electrophoresis
- Can be confused with haemoglobin Paksé

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**High affinity haemoglobins**

**Significance**
- High Hb is necessary for normal oxygen delivery
- Venesection is probably not a good idea (although sometimes patients appear to feel a benefit)
- The thrombotic risk does not appear to be high
- Misdiagnosis can be dangerous
High affinity haemoglobins

**Recognition**
- Clinical features
- Family history
- Blood count
- Electrophoresis, HPLC
- Oxygen dissociation curve

**Cellulose acetate electrophoresis, alkaline pH**
- Adult Hb 200 g/l

**Acid agarose electrophoresis**

**Isoelectric focusing**
- Variant with similar mobility to F

High affinity haemoglobins


**Significance of these findings**
- High affinity haemoglobin (haemoglobin Yakima)
- Asymmetric hybrids
- Not all high affinity haemoglobins are detectable by these techniques
- Oxygen affinity studies are needed

**Published case history of the first patient in whom this variant haemoglobin was recognised**
- 1954, aged 31, Swedish ethnic origin
- Routine blood test
  - RBC ≤ 7.3 x 10^{12}/l, Hb ≤ 220 g/l, Hct ≤ 0.55 l/l, WBC and platelets normal
High affinity haemoglobins

- Blood gases normal
- Red cell mass 71 ml/kg (NR 26-32)
- 1955-1965 – treated repeatedly with $^{32}$P (a total of 72 mCi)
- 1964, aged 41, two daughters polycythaemic
- Investigations led to discovery of Hb Yakima (38%)
- Urinary erythropoietin high
- $^{32}$P stopped

High affinity haemoglobins

- 1975, aged 52, Hb 99 g/l, hypocellular marrow
- Transfusion dependent
- 4 months later, myelodysplastic features
- Hb Yakima fell to 33%, A to 43%
- Hb F rose to 34%
- 1976 AML
  
  Jones et al. (1967) J Clin Invest, 11, 1840
  Bagby et al. (1978) Blood, 52, 350

Haemoglobin M

Oxygen dissociation curve
Novy et al. (1967) J Clin Invest, 46, 1848

18/08/2011
**Haemoglobin M**

**Significance**
- Causes blue tinge to skin
- Possible misdiagnosis as cyanosis due to hypoxia
- Does not deliver O₂ so Hb may be high
- Can be α, β or γ chain variant

**Recognition**
- Clinical features
- Brown blood
- Spectrometry after oxidation

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**Methaemoglobins**

**Blue patient**
- ? Cyanosis (could be low oxygen affinity)
- ? Methaemoglobinaemia (is blood brown?)
  - Exogenous
  - Enzyme deficiency
  - Variant haemoglobin (? blue from birth—? getting better or not—? blue from ~ 6 months)

**Drug-induced methaemoglobinaemia**

*From Interactive Haematology Imagebank, courtesy of Wiley-Blackwell and Professor L. Hirst*
Methaemoglobins

Unstable haemoglobins

Significance
- Chronic haemolytic anaemia
- May be exacerbated by oxidant stress
- Most common is haemoglobin Köln
Unstable haemoglobins

Recognition
• Clinical features
• Blood count and film
• Heinz body preparation
• Electrophoresis and HPLC
• Test for unstable haemoglobin

Unstable haemoglobins

FBC of two patients with haemoglobin Köln:

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>127</td>
</tr>
<tr>
<td>MCV</td>
<td>106</td>
</tr>
<tr>
<td>MCH</td>
<td>31.1</td>
</tr>
<tr>
<td>MCHC</td>
<td>293</td>
</tr>
<tr>
<td>Platelet</td>
<td>55</td>
</tr>
<tr>
<td>count</td>
<td>128 x 10⁹/l</td>
</tr>
</tbody>
</table>

Unstable haemoglobins


Unstable haemoglobins

HPLC BioRad
Variant and Variant II


The β thalassaemias

Why does β thalassaemia matter?
- Diagnostic confusion in heterozygotes if not distinguished from iron deficiency
- Occasional diagnostic confusion in thalassaemia intermedia
- Clinically and economically significant disease in homozygotes and compound heterozygotes (including with haemoglobin E)
- Therefore genetic significance

From: Bain, Haemoglobinopathy Diagnosis, Wiley-Blackwell

Unstable haemoglobins

Denatured Köln
Köln + A

E only A + C F + S A + C

Isopropanol test

From: Bain, Haemoglobinopathy Diagnosis, Wiley-Blackwell
Why does β thalassaemia matter?

- Beta thalassaemia major – a medical problem

Why does β thalassaemia matter?

- Beta thalassaemia major – a social and economic problem

Why does β thalassaemia matter?

- Beta thalassaemia major – a social and economic problem

Why does β thalassaemia matter?

- Beta thalassaemia major – a medical problem

From Weatherall and Clegg, The Thalassaemia Syndromes
The economic argument for screening – Cyprus

- Total population of country - 600,000
- 600 transfusion dependant persons with β thalassaemia major, i.e. 1 in 1000
- 600 patients require 20,000 units blood per year
- Without premarital screening 50 new cases per year would result
- This would require 46,000 units of blood per year by 2021 (13% of population would donate just for treatment of β thalassaemia major)

What can be achieved– Cyprus

- Prevention programme commenced in 1978
- A collaboration between the medical profession and the church
- Couples who apply for a marriage licence must have a haemoglobinopathy screen prior to the licence being granted
- Proof that the test has been undertaken and the results of the test explained is given to the couple by the screening centre
- Prenatal diagnosis made available and acceptable

What can be achieved– reduction of disease burden

- Cost effectiveness
  Calculations in the Cypriot model predicted the cost of 8 weeks of screening was equivalent to one week of treatment for all thalassaemics
- Burden for families
  Before programme:
  Many families suffered fatalities of their children/siblings
  Many women terminated any/all pregnancies for fear of a thalassaemia major child - but 75% of these were likely to be healthy infants

The outcome of screening

Prevention of thalassaemias - TIF, 2003 (With thanks to Dr Barbara Wild)
The outcome of screening

Bozkurt (2007)
Hemoglobin, 31, 257

A broader view

When can screening be done?

• Adolescence
• Pre-marriage
• Preconceptually
• Antenatally

How can screening be done?

• Universal testing based on red cell indices and quantification of haemoglobin A2
• Universal screening and selective testing based on red cell indices (MCH or MCV?)
• Selective screening based on assessment of ethnic origin
Which methods are satisfactory for A₂ quantification?

- HPLC, microcolumn chromatography, capillary electrophoresis, even cellulose acetate electrophoresis and elution
- **NOT** scanning densitometry or isoelectric focussing

Why might one prefer the MCH?

Why are red cell indices important?

- Because they can be used for screening, reducing the number of more definitive tests that need to be done
- Because beta thalassaemia trait is not the only cause of an increased haemoglobin A₂ percentage

Might we be misled?

- Can indices be misleading?
  - Silent β thalassaemia
  - Liver disease and drugs that cause macrocytosis
- Can haemoglobin A₂ be misleading?
  - Reduced by iron deficiency
  - Increased by HIV infection and its treatment
HIV infection and its treatment


Why does α thalassaemia matter?

- Alpha^0 thalassaemia homozygosity
  - a fetal problem
  - a maternal problem

α globin genes

The four alpha genes on chromosome 16

One alpha gene can be deleted

This is α^+ thalassaemia heterozygosity
The commonest form is written as −α^2/αα
One alpha gene can also be effectively lost through gene fusion
\[ \alpha_2\alpha_1 \]
This is also \( \alpha^+ \) thalassaemia heterozygosity. It is written as \( -\alpha^{3.7}/\alpha\alpha \).

Two alpha genes can be deleted, one on each chromosome 16
\[ \alpha_1 \]
This is \( \alpha^+ \) thalassaemia homozygosity. The commonest form is \( -\alpha^{4.2}/-\alpha^{4.2} \).

One alpha gene can be effectively lost from each chromosome 16 through gene fusion
\[ \alpha_2\alpha_1 \]
This is also \( \alpha^+ \) thalassaemia homozygosity. It is written as \( -\alpha^{3.7}/-\alpha^{3.7} \).

Two alpha genes can be deleted on one chromosome
\[ \alpha_2 \quad \alpha_1 \]
This is \( \alpha^0 \) thalassaemia heterozygosity; common variants include \( -^{\text{SEA}}/\alpha\alpha \) and \( -^{\text{MED}}/\alpha\alpha \).
One alpha gene can be lost from one chromosome 16 and two from the other

\(\alpha^1\)

This is compound heterozygosity for \(\alpha^+\) and \(\alpha^0\) thalassaemia

All four alpha genes can be lost

This is \(\alpha^0\) homozygosity, e.g. \(-SEA/-SEA\)

Diagnosis needed

- Haemoglobin Bart’s hydrops fetalis
- Haemoglobin H disease
- Alpha\(^0\) thalassaemia trait

Diagnosis not needed

- Alpha\(^+\) thalassaemia

Diagnosis of \(\alpha^0\) thalassaemia heterozygosity

- Ethnic origin
- Red cell indices (MCH < 25 pg)
- DNA analysis
- DNA analysis of at risk fetus
- Offer of termination of pregnancy
Diagnosis of α0 thalassaemia – which ethnic origins matter?


Conclusions

• A variety of techniques is needed
• Well informed laboratory staff are essential
• Interpretation must be in the context of clinical features, ethnic origin and blood count and film
• Always keep in mind the purpose of the test in that patient

The End

Image from www.dsc.discovery.com