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MEDICAL BIOLOGY PRACTICALS. CYTOGENETICS.
Practical 3. Solubility test and peripheral blood smear for hemoglobin S and sickled red blood cells detection

Compiled by Boris M. Sharga, Diana B. Pylypiv, Volodymir P. Feketa

Theoretical background. *Haemoglobin* (*Hb*) is a protein of *red blood cells* (*RBC*) with main duty of oxygen transport. It combines with O_2 to form unstable oxyhaemoglobin Hb.O_2 at lungs. Then, trough circulatory system it passes the O_2 to tissues and returns carbon dioxide (CO_2) from body to the lungs. *Hb* consists of 4 polypeptide chains of globins, with heme group each and 141 or 146 amino acids residues arranged in 7 or 8 helical regions joined by non-helical segments.

Haemoglobinopathies is a group of important hereditary disorders, resulting from mutations in the genes coding for the *Hb* synthesis. On the basis of genetic defect(s) involved, the haemoglobinopathies are categorized on *thalassaemias (Ts)* and *haemoglobin variants* (*Hb Vs*). Usually, the *Ts* are developed because of low production of normal globins due to mutations in regulatory genes and *Hb Vs* resulted from abnormal structure of the globins due to defects in their coding DNA sequences. Both of these conditions may be present in one patient or separately.

When *Ts* defect or deletion happened, it results in low/no production of one of the globin chains. This can cause the formation of abnormal *Hb* and anemia. There are 2 major forms of the disease, *α* - and *β* - *Ts*.

The genes for *Hb A1* and *Hb A2* are involved in *α-Ts*. This leads to low *α*-globin synthesis and a subsequent excess of *β* chains in adults and *γ* chains in newborns. The *β* chains excess form unstable tetramers *Hb H*, which have abnormal oxygen dissociation curves [8].

The mutations in the *HBB* gene on chromosome 11 cause *β-Ts*. Their severity depends on the nature of the mutations. They can prevent *β* chains formation causing most severe form, *β thalassemia major* (*βo*) or may allow low *β* chain production in case of *β thalassemia intermediate* (*β*+). In both matters there is an excess of *α* chains although, unlike in *α*-thalassaemia, the tetramers not formed. The *α* chains bind to the membranes of erythrocytes, damaging and aggregating them.

The normal haemoglobin types are:

- *Hb A* – the main form in adults (95-98% of total *Hb*) build of 2 *α* and 2 *β* protein chains (*α*2*β*2);
- *Hb A2* – typically 2-3% of *Hb* in adults, it has 2 *α* and 2 *δ* protein chains (*α*2*δ*2);
- *Hb F* – the primary *Hb* produced by the fetus, decreasing drastically soon after birth and comprising up to 2% of *Hb* of adults; it has 2 *α* and 2 *γ* protein chains (*α*2*γ*2).

The most spread of *Hb Vs* include: Hb S, Hb C, Hb E, Hb D-Punjab, Hb O-Arab, Hb G-Philadelphia. Many *Hb Vs* do not cause pathology, particularly, in heterozygotes. However, those affecting *RBC* function usually cause anaemia [8].

The *sickle cell disease* (*SCD*) is wide spread and most severe form of anemia, comprising >70% of anemias worldwide [10]. It is caused by the homozygous inheritance of the *βS*-mutation and abbreviated to as *SCD, SCD SS* or *SCA*. The *Hb* with this mutation is able to transport O_2, but once the O_2 is released, the wrong molecules stick to one another and form rod-shaped structures in the RBCs [2]. The *RBC*s become abnormal, fragile, rigid, sickle, star, holly leaf and tailed in shape (Fig.1). This decreases their flexibility and ability to move within small vessels. This leads to aggregation of the RBCs and vascular occlusions resulting in pain throughout the body, painful episodes termed for 5-6 days [6]; cerebrovascular accidents (strokes) and acute chest syndrome with inflammation of lungs, pulmonary hypertension; spleen necrosis [14]; hand and foot syndrome with possible numbness and tingling. Jaundice appears, since liver is unable to process the increased number of dead RBCs, leading to a build up of bilirubin [5]. Fatigue, pallor, irritability may also occur.

Complications of *SCD* include: neurological (seizures, cerebrovascular accident, meningitis), pulmonary (acute pulmonary infarction, pneumonia, atelectasis, acute chest syndrome), musculoskeletal (avascular necrosis, osteomyelitis, hand and foot syndrome), visual (blindness, retinopathy), genitourinary (eclampsia, nocturia, hematuria), dermatological (stasis ulcers of hands, ankles, feet), other organs (splenomegaly, acute hepatomegaly, gall stones) [5].

In heterozygotes, with one sickle gene and one normal adult *Hb* gene (*Hb AS* or 'sickle cell trait') partial erythrocytes sickling may appear at low oxygen pressure.

Hb S is an abnormal *Hb* produced from a point mutation in the *HBB* gene that results in replacement of glutamic acid by valine at the position 6 on the *β*-globin chain of subunit (*βS*) of the haemoglobin molecule [11]. *SCD* is inherited as autosomal recessive trait with 25% chance of offspring with disease in family, where both parents are carriers of one sickle cell allele:

A- allele for normal *HbA,* healthy condition;

S – allele for *HbS*, disease in homozygote ***SS***

Couple genotypes, P:	mother ♀***AS*** × ♂***AS*** father
Gametes, G:	***A, S*** and ***A, S***
Children, F:	***AA*** 25% (healthy) ***AS*** 50% (carriers) and ***SS*** 25% (diseased)

However, *SCD* can also result from the inheritance of *βS* in combination with other *HBB* mutations, the two most common being a second structural *β*-globin variant *βC* (*SCD SC*) [12] and one of the many *β-Ts* mutations that lead to the insufficient production of normal *β*-globin (*SCD S/β-thalassaemia*) [13]. The *Hb D-Punjab* and *Hb O-Arab* can result in *SCA* when combined with *Hb S* in offspring also [15, 16].

Both: *SCD* and *Ts* genotypes evolved in people living in humid Tropical and Mediterranean climates with endemic malaria. These provide a degree of resistance to *Plasmodium* parasites, the causal agents of the disease.

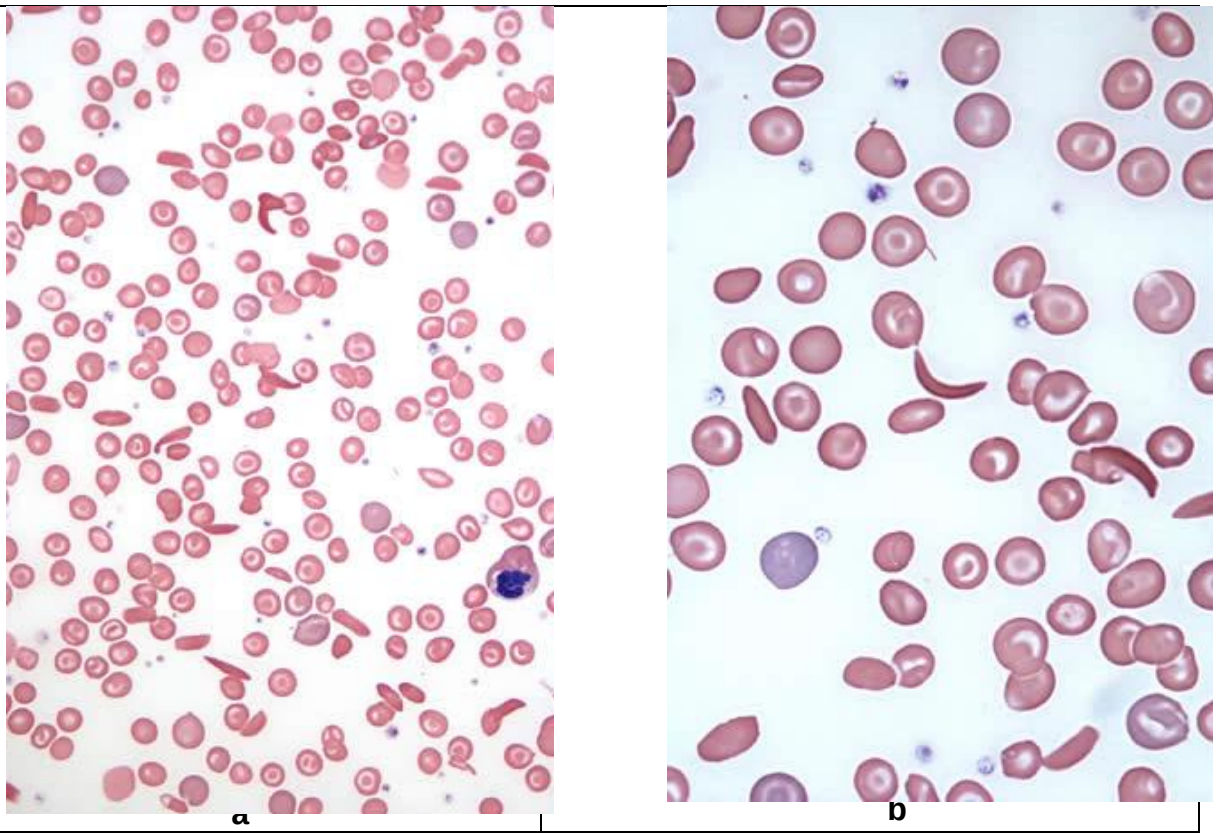

Fig. 1. Blood smears from sickle cell disease patients: a, b - adults, c, - neonate.

Modified from Keith Chambers, Leicester Royal Infirmary. Creative Commons BY-SA [7].

There are several methods in use to diagnose *SCA*. They are based on estimation of the presence of *Hb S* and/or abnormally-shaped *RBC*. These tests include: *Hb solubility test*, *Hb electrophoresis*, *isoelectric focusing*, *high-performance liquid chromatography (HPLC)*, *density-based separation by centrifugation in multiphase systems* [2], and/or a *peripheral blood smear*. It is possible to diagnose *SCA* in *molecular genetic tests* searching for 2 copies of the *Hb S* gene. However, this technique is expensive and is used mainly, in prenatal diagnosis to identify *Hb Vs* and determine the probability of *SCD* child birth.

The *Hb* solubility test is based on the relative insolubility of *HbS* when it interacts with strong reducing agent in a patient's blood sample mixed with a reagent containing saponin, 2.3M potassium phosphate buffer and sodium hydrosulfite.

Saponin acts as detergent, destroing the membranes of *RBC* to release *Hb*. Sodium hydrosulfite is reducing agent and detaches the O_2 from *Hb*. The *Hb S*, if present, will form liquid crystals providing turbidity to the solution. Deoxygenated *Hb S* is insoluble in concentrated phosphate buffer and precipitates. Other *Hbs* are more soluble in the reducing agent. Particularly, deoxygenated *Hb A* is soluble at this conditions and tube remains transparent. We can see through the tube the black lines on a card placed behind the sample (*negative result* of the test). In a *positive* screen the lines are not visible because of turbidity. This indicates the *Hb S* or another sickling *Hb* presence.

At least 20% amount of wrong *Hb* is necessary for this result. Either homozygotic for this mutation individual ("sickle cell anemia") or heterozygotic one ("sickle cell trait", in which *Hb S* is usually 30-45%) may have *positive results*. It can also be observed for *Hb C Harlem* and *Hb C Georgetown*.

The test is prone of error in cases of blood with hyperlipidemia; extreme leukocytosis; erythrocytosis; multiple myeloma; high plasma protein levels at hyperglobulinemia. These may mislead to *false positive results*.

False negative results are observed in anemic individuals with *Hb* < 7.0 g/dL. To overcome this problem, packed erythrocytes (0.01 mL) may be used. *False negative* screen may occur, if *Hb S* concentration is less, than 20%; at high fetal *Hb* or after recent blood transfusion also. The blood samples from infants younger than 6 months (they have high level of *Hb F* and low levels of other *Hbs*, particularly, *Hb S*) and normal *Hb* specimens from people with recent blood transfusion have *false negative* result in this test also.

The *Hb D-Punjab* and *Hb O-Arab* are not detecteble by this test.

Reference tube show *negative result*. Often 2 tubes are used: one for *positive* and another for *negative result* standard.

Experiment 1. Solubility Test for Hemoglobin S

Principle: The *Hb S* is not soluble when combined with sodium dithionite, a reducing agent. The saponin in whole blood sample lyses the *RBC* and *Hb* is released. *Hb S*, if present, will form liquid crystals and give a turbidity to the solution. The transparent solution is seen, when *Hb S* is absent or present at the amount less than 20%. Turbidity can be observed for *Hb C Harlem*, *Hb C Georgetown*, *Hb C Ziguinchor*, and *Hb S Travis* in this test also. Other *Hbs* are more soluble at these conditions.

Reagents and equipment

Cautions! Avoid contact of blood and reagents with eyes, mouth and skin. **Do use gloves!**

Calibrated test tubes, 12 × 75 mm in diameter; micropipet, 20 -100 μL; disposable micropipet tips; pipets, 2.0 mL; paper-board and test tube holder (tubes should be held on distance 2.5 cm from the reading card; reading card should have 16-18-font straight black lines printed 0.5 cm apart on white carton); day light lamp; gloves.

Stock solution: Saponin 5 g; KH_2PO_4, crystals 84,5 g; K_2HPO_4, anhydrous 108 g; Add distilled water to 500 mL. (The reagent should be stored in fridge at 4°C not more than 1 month).

Working solution: Add 5 mg sodium dithionite ($Na_2S_2O_4$) to 1 mL of stock solution.

Specimen: The whole blood treated by EDTA, heparin or sodium citrate to prevent coagulation in tube is suitable for this experiment. It is acceptable to store the specimen at 4ºC for up to 3 weeks before use in the test.

Reference tubes: The specimen from a patient should be compared with "sickle cell trait" (30-45% *Hb S*) and a negative "healthy" sample from adults with known *Hb AA* as controls.

Procedure

1. Take the specimen and reagents from the fridge and allow them to attain room temperature before the test.
2. Pipet 2 mL of working solution into the test tube.
3. Add 20 μL of whole blood specimen to the test tube.
4. Mix well and place the test tube with reference tubes for 6 minutes in the tube holder at room temperature.
5. Observe the turbidity or its absence by looking onto reading card through the test tube.

Results

1. *Negative result*: suspension is transparent, the lines on reading card is clearly visible
2. *Positive result*: suspension is turbid, the lines on reading card is not visible through it.
3. The test does not allow differentiate between the genotypes *Hb S* ("sickle cell disease", *S/S*) and *Hb S* "sickle cell trait", *A/S*). To distinguish between these 2 genotypes, a *Hb* electrophoresis at alkaline pH should be performed. The electrophoresis at alkaline pH (and, occasionally, at acidic pH) allows to separate the *Hb S* from *Hb C Ziguinchor*, *Hb C Harlem*, *Hb C Georgetown* and *Hb S Travis*, which are resulting in turbidity in this test also.

Experiment 2. Differentiation between the genotypes *Hb S* "sickle cell disease"(*S/S*) and *Hb S* "sickle cell trait"(*A/S*)

Principle : The method is based on a modification of the sodium dithionite hemoglobin solubility test by Louderback et al. (1974) [9], allowing to differentiate the *Hbs* "sickle cell disease" (genotype *SS*), "sickle cell trait" (genotype *AS*) and

normal *Hb* individuals (genotype *Hb AA*) by results of *Hb* dissolving followed by centrifugation.

Reagents and equipment

Cautions! Avoid contact of blood and reagents with eyes, mouth and skin. **Do use gloves!**

Calibrated test tubes, 12 × 75 mm in diameter; micropipet, 20 -100 μL; disposable micropipet tips; pipets, 2.0 mL; paper-board and test tube holder (tubes should be held on distance 2.5 cm from the reading card; reading card should have 16-18-font straight black lines printed 0.5 cm apart on white carton); day light lamp; clinical or serological centrifuge, gloves.

Buffer solution is a 280 g/L $(NH_4)_2SO_4$ solution, the pH of which is adjusted to 7.1 ± 0.1 with K_2HPO_4 (1 mol/L).

Working solution Add 1 g of Na dithionite, the reducing agent ($Na_2S_2O_4$), plus 1 g of detergent saponin to 100 ml of buffer solution. Dissolve the reagents by vigorous shaking. The working solution can be stored at refrigerator temperature (2 to 8ºC) and is stable for as long as 1 month in tightly stoppered bottle.

Specimen: 0.1 ml of whole blood sample containing anticoagulant (heparin, EDTA, ACD, etc.)

Reference tubes: Blood sample obtained from an individual with known normal adult *Hb* (genotype *AA*) should be used as control throughout both steps of the experiment.

Procedure

1. Brought the working solution to room temperature.
2. Add the working solution to the 2-ml line of a calibrated 12 × 75 mm test tube.
3. Add to this tube 100 μL of the wholeblood testspecimen. Blood sample containing anticoagulant (heparin, EDTA, ACD, etc.) may be used.
4. Mix the test sample thoroughly with the working solution by inverting the tube several times.
5. Allow to stand at room temperature for 5 min.
6. The tube is then examined visually in the tube holder near to a light source and on a distance 2.5 cm from the reading card.

Results

1. If the solution became turbid, the lines cannot be seen through the tube, then the specimen is either heterozygous or homozygous for *Hb S*.
2. Differentiation of heterozygosity is determined by centrifuging the tube in a clinical or serological centrifuge for 3 mm at 3400 to 5000 rpm.
3. Examine the specimen with the same light source to indentify the following possibilities in centrifuged tubes:
   a) the supernatant is *clear, pink*. There is a small amount of *red* precipitate, then the sample is *heterozygous* for *Hb S*.

b) the supernatant solution is *clear, yellow*. There is a large amount of *red* precipitate, then the sample is *homozygous* for *Hb S*.
c) the uniform *red* supernatant with only a small amount of *white* precipitate then the sample is *homozygous* for *Hb A*.

Experiment 3. Presence of sickling phenomenon in blood sample

Principle: The *RBC* containing sickle hemoglobin (*Hb S*) become sickle shaped, when deoxygenated by strong reducing agent, as sodium metabisulfite. RBC with normal *Hb A* will not sickle [4].

Reagents and equipment

Cautions! Avoid contact of blood and reagents with eyes, mouth and skin. **Do use gloves!**

Blood collected in K2 EDTA tube; 2% Sodium metabisulfite solution in distilled water; DPX Mountant; clean glass slide; cover slip; glass rods; light microscope.

Procedure

1. Place a drop of whole EDTA anticoagulated blood on the glass slide. **Cautions**! Handle all samples as infectious.
2. Add 2 drops of 2% sodium metabisulfite.
3. Mix well with a glass rod.
4. Place a cover slip on a mix and press it gently to remove any of air bubbles from the blood.
5. Using the glass rod seal the preparation on perimeter of cover slip with DPX Mountant.
6. Check the preparation for the presence of sickle cells - "holly oak leaf" form of RBCs after 30 min under magnification 100-400 ×. Count sickled cells in 10 microscope fields and calculate the sickle cell index (SCI) [1], the percent of sickle cells from total red cell population. Percent of sickle cells is increased with age and correlated with disease severity, especially at hemolytic complications.
7. Re-examine the same preparation for sickling phenomenon after 24 hours.
8. Make the preparation variant according to steps 1-5, but without adding of 2% sodium metabisulfite and use this to compare with blood preparation, treated by this salt solution.

Results

1. Interpret results as “positive” if sickle cells present or as “negative”, if they are absent.
2. Can you observe the difference in results between the 30 min and 24 hrs exposition experiments?

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