Name number Study group

Protocol
Red blood cell count

Methods

Red blood cell count

Equipment: Bürker's counting chamber with cover glass, microscope with lamp, a cup of approx. 10–12 ml with stopper, micropipette with adjustable range of 1–5 ml and 10–100 µl, dropper with a fine tip, blood sample, Hayem's solution – beware! POISON! (Na2SO3 – 5 g, NaCl – 1 g, HgCl2 – 0.5 g, distilled water 200 ml), a vessel with disinfecting solution.

Note:
1. Hayem's solution is a highly toxic solution due to its HgCl2 content, which causes burns after ingestion and is harmful also after long-lasting skin exposure. Before handling, read the safety rules carefully!
2. Always use gloves when working with blood!

Procedure:

1. Place 4950 µl of Hayem's solution into the cup by using the micropipette.
2. Add 25 µl of blood using the other micropipette: the chosen volume (25 µl) is set on the micropipette and can be checked on its display. Fix the tip by slightly pressing the bottom of the micropipette, immerse it (approx. 1 cm) into the blood sample and gently press the button (position 1). Pull the tip from the blood – any excess of blood should be carefully removed using a pad of cotton wool. Then, the blood is expelled into the cup with Hayem's solution. The button of the micropipette is pressed completely (position 2) when the tip is immersed in the solution – the whole volume of blood is expelled. Blood is slowly expelled into the solution, and near the wall of the vessel so that it is not mixed yet. The used tip is then disposed into the vessel with disinfecting solution by pressing the ejector of the micropipette, and the device is then placed back onto the stand.
3. The cup is closed by a stopper and its content mixed by whirling movements for 1–2 min in order to get a homogenous suspension. The solution should not come into contact with the stopper.
4. Prepare the Bürker's counting chamber. On the middle prism, two grids are engraved constituting a network of smaller (1/400 mm^2) and larger (1/25 mm^2) squares for counting red and white blood cells, respectively.
5. Take a sample of the suspension from the cup into a fine dropper. The tip of the dropper is placed between the cover glass and the bottom of the chamber where the suspension is drawn by capillarity. When filling the chamber, we have to prevent the solution from overflowing into the grooves between the prisms or to the space over the other grid where the red blood cell count should be performed. If this happens, the chamber must be cleaned and refilled. Then, put the filled chamber on the microscope plate and, under steady visual control, bring the objective near to the cover glass.
6. Red blood cells are counted in a total of 40 (or 80 if a higher precision is desired) small squares. If some cells touch the margin of the square, only those lying on the upper and the right side of the square are counted, irrespective of whether they are within or outside the square, as shown in Fig. 2.

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7. Estimate the average number of RBCs in one square from the value obtained by counting 40 or 80 squares. Since the volume above the square is 1/4000 mm^3, multiply the average number by 4.109. Finally, multiply by the dilution ratio, i.e. 199 to get the number of RBCs in 1 litre of blood. The margin of error of such estimation of RBCs is ± 200,000 mm^3.

The Bürker's counting chamber and basic rules for counting RBCs

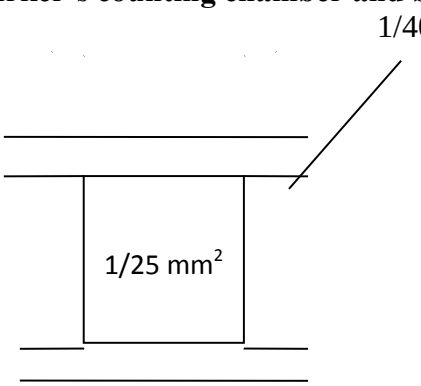

Fig. 1

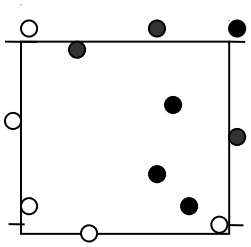

Fig. 2

Estimation of haemoglobin concentration

Equipment: Spectrophotometer Spekol, funnel and 5 ml cylinder for transforming solution, micropipette with adjustable range 10–100 µl, vessel with disinfection solution, and transforming solution (solution by van Kempen-Zijlstra: K3Fe(CN)6 – 0.2 g, KCN – 0.05 g, KH2PO4 – 0.14 g, distilled water to 1000 ml; in Drabkin's solution, KH2PO4 is replaced by NaHCO3 – 1.0 g), automatic analyser Mythic 18

Note:
1. Transforming solution is a highly toxic solution due its KCN content, which is highly toxic when inhaled, ingested or in contact with skin. Before handling, read the safety rules carefully!
2. Always use gloves when working with blood!

Procedure:

1. Switch the apparatus on and wait at least 15 minutes before the measurement. For estimation of haemoglobin concentration, a wavelength of 540 nm is used. The lower lever has to be turned to the left position.
2. Prepare the measured solution: put 5 ml of transforming solution into a test tube by using the funnel and 5 ml cylinder and add 20 µl of blood taken from the blood sample by means of an adjustable micropipette (for the procedure, see Exercise I.1). The absorbance is measured 10 min after mixing the solution.
3. Fill the cuvette with distilled water (blind experiment) and move cuvette inside the apparatus. Press C. Press FAKT – either 1.000 or last factor will appear on the display. Insert the correct factor for your measurement (for haemoglobin this is 22.8) using POS and INC. Press FAKT again (this inserts the value into the memory of the Spekol). Press R. Now you can read the concentration of the blind experiment.

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4. After this setting, insert the cuvette with the measured sample and read the concentration of haemoglobin (mmol/l). Multiply this value by the coefficient 16.11 to obtain the concentration of Hb in g/l.
5. Check the values obtained by the Spekol by using the Mythic 18 automatic analyser.

Note:
Cuvettes must be clean and their outer surface dry. If some solution gets onto the surface while filling the cuvette, it must be wiped off (the instrument would be damaged if the aggressive solution reaches its inside). Cuvettes may be taken only by their upper parts, optimally in lateral ground areas, never in areas where the measuring light beam passes. The meter and the fragile cuvettes are the most sensitive parts of the instrument: avoid any shock or shaking.

Calculated parameters of red blood cells

Based on the values obtained by measurements, further parameters commonly used in clinical practice can be reached by calculation:

1. average volume of red blood cell (MCV = mean corpuscular volume)
MCV = Hct / number of red blood cells (physiological values: 80–95 fl)

2. average weight of Hb in red blood cell (MCH = mean corpuscular haemoglobin)
MCH = Hb / number of red blood cells (physiological values: 27–32 pg)

3. average concentration of haemoglobin in red blood cell (MCHC = mean corpuscular haemoglobin concentration)
MCHC = Hb / Hct (physiological values: 310–360 g Hb / litre of red blood cell)

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Name number Study group

Results

Red blood cell count

Describe the method:

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Fill in the number of red blood cells (counted in 40 small squares)

…. ….. ….. ….. ….. ….. ….. ….. ….. …..

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…. ….. ….. ….. ….. ….. ….. ….. ….. …..

Average number of red blood cells per square

.....

The volume of one small square: 1/4000 μl

The number of red blood cells in one small square is multiplied by $4 * 10^9$

.....

Obtained value is multiplied by * 200

Total red blood cell /l:.....

Estimation of haemoglobin concentration

In Graph 1 note the haemoglobin concentration that you have found. The physiological range of haemoglobin concentration in men 130–175 g/l [Hb (1Fe) 8.07 – 10.9 mmol/l], and in women 120–165 g/l [Hb (1Fe) 7,45 – 10,2 mmol/l], is marked in the graph.

Declined values: anaemia; raised values: dehydration, polycythaemia

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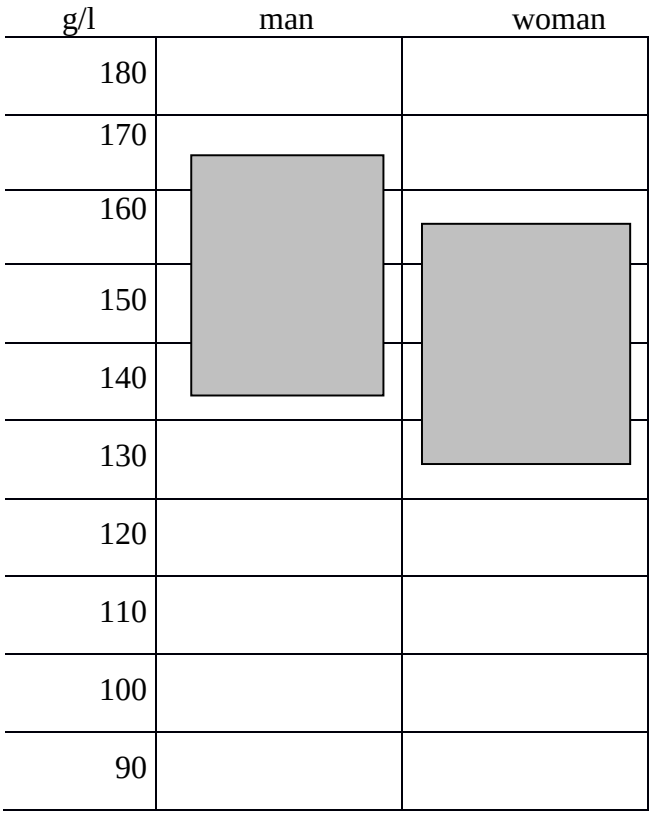
Name number Study group

Graph 1. Haemoglobin concentration (g/l)



Calculated parameters of red blood cells

1) **Average volume of red blood cell (MCV = mean corpuscular volume)**

MCV = Haematocrit / number of red blood cells (physiological values: 80–95 fl)
Calculation: MCV =

2) **Average weight of Hb in red blood cell (MCH = mean corpuscular haemoglobin)**

MCH = haemoglobin / number of red blood cells (physiological values: 27–33 pg)
Calculation: MCH =

3) **Average concentration of haemoglobin in red blood cell (MCHC = mean corpuscular haemoglobin concentration)**

MCHC = haemoglobin / haematocrit (physiological values: 310–360 g/l)
Calculation: MCHC =

Conclusion

According to your results, draw a conclusion about your specimen. Suggest the gender of your unknown patient and attempt to diagnose him/her.

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Name number Study group

Protocol
Determination of blood groups by slide method

Methods

Equipment:

Standard sera of group A (anti-B, i.e. beta), group B (anti-A, i.e. alpha) and group 0 (anti-A and anti-B, i.e. alpha and beta), slides, dropper

Procedure:

1. Drop each serum on the slide. Be careful not to mix them!
2. Using a dropper with a fine tip, take a drop of blood from the blood sample.
3. The corner of another slide is put into the blood drop and a small amount of blood is transferred to one of the sera and mixed by the same corner. In the same way, but using always another corner of the slide, transfer blood to the remaining test sera and mix the samples carefully.
4. Agglutination may be speeded up (2–10 min) by carefully rocking the slide to all four sides. The agglutination appears as visible flakes floating in the transparent serum. The result may be tested by microscopic observation: in the case of a positive reaction, the rod cells will form larger clusters.

Note:

Do not confuse the terms agglutination and coagulation, as these designate totally different physical and/or chemical events. Unfortunately, sometimes coagulation does appear in the test drop, usually when too much blood is added and mixed only slightly or not at all. To prevent this, the ratio of blood to test serum should be around 1:10.

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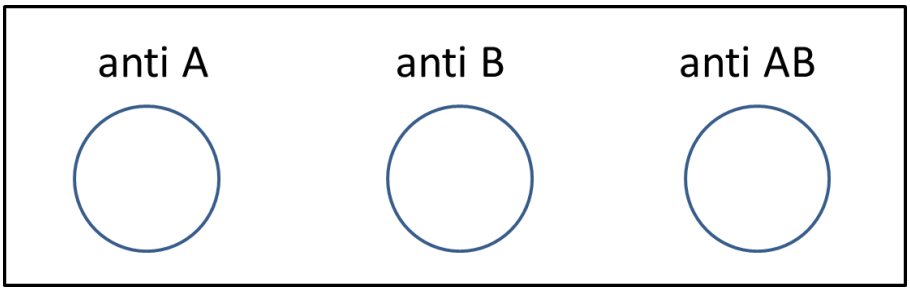
Name number Study group

Results

Into the scheme below draw the reaction observed in your conducted experiment.

Blood 1



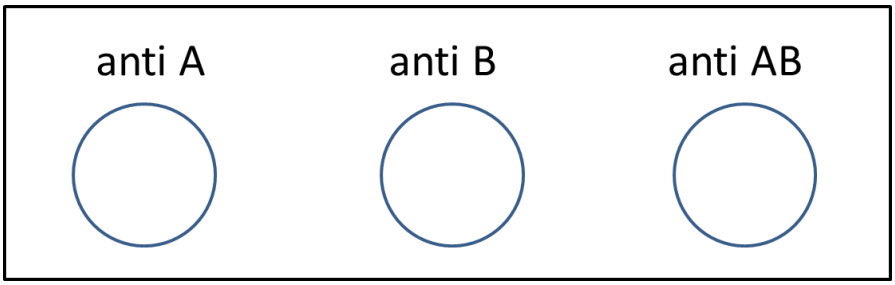
Blood group determined in your experiment:
This particular blood group is found in ……% of population.
Based on Mendel's laws suggest all the possible combinations of the parent's blood groups.

…….. …… ……..…… ……..

Suggest combinations of parent's blood groups unable to produce your patient's blood group.

…….. …… ……..…… ……..

Blood 2



Blood group determined in your experiment:
This particular blood group is found in ……% of population.
Based on Mendel's laws suggest all the possible combinations of the parent's blood groups.

…….. …… ……..…… ……..

Suggest combinations of parent's blood groups unable to produce your patient's blood group.

…….. …… ……..…… ……..

Conclusion

Propose possible blood groups of acceptors compatible for receiving your patient's blood products – blood plasma and packed RBCs.

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Name number Study group

Protocol

Erythrocyte sedimentation rate

Methods

Equipment: Stand with a set of sedimentation pipettes, rubber cups, sucking rubber cylinders, six samples of blood:

- anti-coagulated human blood with full plasma
- anti-coagulated bovine blood with full plasma
- anti-coagulated horse blood with full plasma
- anti-coagulated human blood, the plasma of which is totally replaced by physiological solution
- anti-coagulated horse blood, the plasma of which is totally replaced by physiological solution
- anti-coagulated human blood with low R.B.C. (Ht = 0.29)

Procedure:

1. Mix well the anti-coagulated undiluted blood (**use gloves!**) and pour 2–3 ml into the first rubber cup which is placed in the stand in such way that the tip of the sedimentation pipette is exactly in the centre.
2. The rubber cylinder is put on the upper end of the pipette and used to suck up the blood. By slightly pressing the cylinder, a small amount of air is expelled from the pipette, the lower end of which is then immersed into the cup filled with blood. Release gradually the cylinder to suck blood up to the mark 0 (height 200 mm). In that moment, press the pipette down towards the bottom of the rubber cup. Then, with the hand which was used to suck up the blood, fix the pipette in this position by tightening its screw. In the same way, the remaining two pipettes are filled with the other samples.
3. Read the sedimentation values every 15–20 minutes so that you obtain at least 3 values (in clinical practice, values after 1 and 2 hours are recorded).

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Name number Study group

Results

Graph of erythrocyte sedimentation rate (choose different colours for each specimen):

Time (min)	15	30	45	60	75	90	105	120
human blood with full plasma								
Human erythrocytes with physiol. solution								
bovine blood with full plasma								
horse blood with full plasma								
human blood with low R.B.C.								
horse erythrocytes with physiol. solution								

List all the factors influencing the erythrocyte sedimentation rate:

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Conclusion

Explain the differences between the blood samples you worked with.

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Name number Study group

Protocol
Osmotic fragility test

Methods

Equipment: Stand with 13 test tubes, solution of 1% NaCl, distilled water, physiological solution (0.9% NaCl), 2 graduated pipettes (10 ml), dropper, anti-coagulated human blood, gloves.

Procedure:

1. Twelve test tubes are filled, by using two pipettes, with decreasing amounts of 1% solution of NaCl and increasing amounts of distilled water according to the table below. The last test tube is filled with the physiological solution (0.9% NaCl). Always use a clean pipette for each test tube.

Tube number	1	2	3	4	5	6	7	8	9	10	11	12
NaCl 1% (ml)	6.3	6.0	5.7	5.4	5.1	4.8	4.5	4.2	3.9	3.6	3.3	3.0
H_2O (ml)	3.7	4.0	4.3	4.6	4.9	5.2	5.5	5.8	6.1	6.4	6.7	7.0
% NaCl	0.63	0.60	0.57	0.54	0.51	0.48	0.45	0.42	0.39	0.36	0.33	0.30

Table for preparation on NaCl solutions of decreasing osmotic pressure

2. Each test tube is carefully mixed.
3. Two drops of blood are placed into each tube and the content is again carefully mixed. After this, do not move the stand or the test tubes so as not to interrupt the sedimentation of non-haemolysed red cells.
4. After approx. 2 hours (min. after 30 minutes) of standing at room temperature, estimate the range of haemolysis by examining the colour of the supernatant solution above the sediment cells and the intensity of turbidity of the cell suspension. The tube with the normal physiological solution serves as the control since blood taken some time ago may already be partially haemolysed.

In the lowest concentrations of NaCl a complete haemolysis occurs, i.e. all red cells disintegrate. The content of the tube is quite pellucid (a text can read through the tube). At a certain concentration, however, the haemolysis is not complete, as a portion of the blood cells (i.e. those with the highest resistance) did not haemolyse, which appears as a slight turbidity in the lower part of the tube – incomplete haemolysis. The lowest concentration where a small fraction of cells remained non-haemolysed designates the s.-c. *maximal resistance*. On the other end of the concentration scale one finds a tube where the haemolysis is hardly distinguishable: the solution above the sediment is only slightly coloured by haemoglobin

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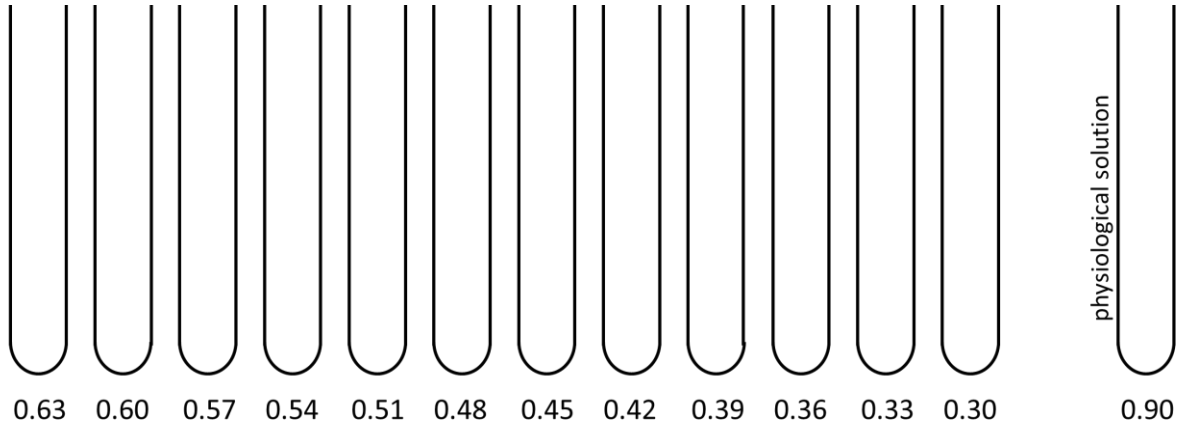
released from the least resistant cells. The concentration where osmotic haemolysis just begins to appear is designated as the *minimal resistance*. When non-haemolysed erythrocytes form a non-transparent sediment on the bottom of the test tube with a yellowish fully transparent NaCl solution above it, such a test is designated as no haemolysis.

The *osmotic resistance range* is the difference between the maximal and minimal resistance value.

Results



- Minimal osmotic resistance was observed in ……%NaCl (physiological: 0.4 - 0.44 %)
- Maximal osmotic resistance was observed in……%NaCl (physiological: 0.3 - 0.34 %)
- Mark osmotic resistance range.

Conclusion

Note down all the types of haemolysis known to you (there are 6 types) and explain their mechanism.

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