Biological Safety
In
Hematology Laboratory

- **STANDARD PRECAUTIONS**

The clinical laboratory presents an environment with many potential risks ranging from biologic hazards to chemical or fire hazards. Safety training has become a mandatory part of responsible employee practice and training not only for employees but also for their colleagues.

One of the greatest risks associated with the hematology laboratory is the exposure to blood and body fluids.

Laboratorians must protect themselves from contamination by observing practices that prevent direct contact with body fluids or a contaminated surface, contamination, or inhalation.

Standard precautions must be adopted by the laboratory.

Standard precautions apply to blood, semen, vaginal secretions, cerebrospinal fluid, synovial fluid, pleural fluid, any body fluid with visible blood, any unidentified body fluid, unfixed slides, microhematocrit clay, and saliva from dental procedures.

Blood borne pathogens are pathogenic microorganisms that, when present in human blood, can cause disease. They include, but are not limited to, hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV).

Biological safety objectives

1. Protect workers from disease resulting For dangerous biological agents.

2. Maintain a safe working environment

3. Prevent the spread of biological contaminants outside sites the work .

4. The control of biological contaminants through management and risk assessment in the workplace.

It is important to note that the Biosafety Levels described below are not to be confused and equated with Agent Risk Groups:

Biosafety Level 1 :

Practices and safety facilities are appropriate for working with well-defined biological agents. These biological agents are not known to cause disease in healthy adult humans and pose only a minimal potential hazard for laboratory personnel and the environment.

Bio-safety Level II:

Practices and safety facilities must meet the perquisites to deal with a broad spectrum of indigenous moderate risk agents.

These agents are known to cause diseases but immunization or antibiotic treatment is available. Examples are Hepatitis B virus, the Salmonellae, Toxoplasma spp.

Bio-safety Level II+:

The term ‘Infectious Material’ applies to blood, any other body fluid, and other potentially infectious material like biopsies. All body fluids shall be considered as infectious materials. Research involving Biological Safety Levels II+ shall contact the Biological Safety Committee for appropriate authorization, guidelines and inspections. Such work can only be performed in the Biohazard laboratory in the Ground Floor

Personal Protective Equipment

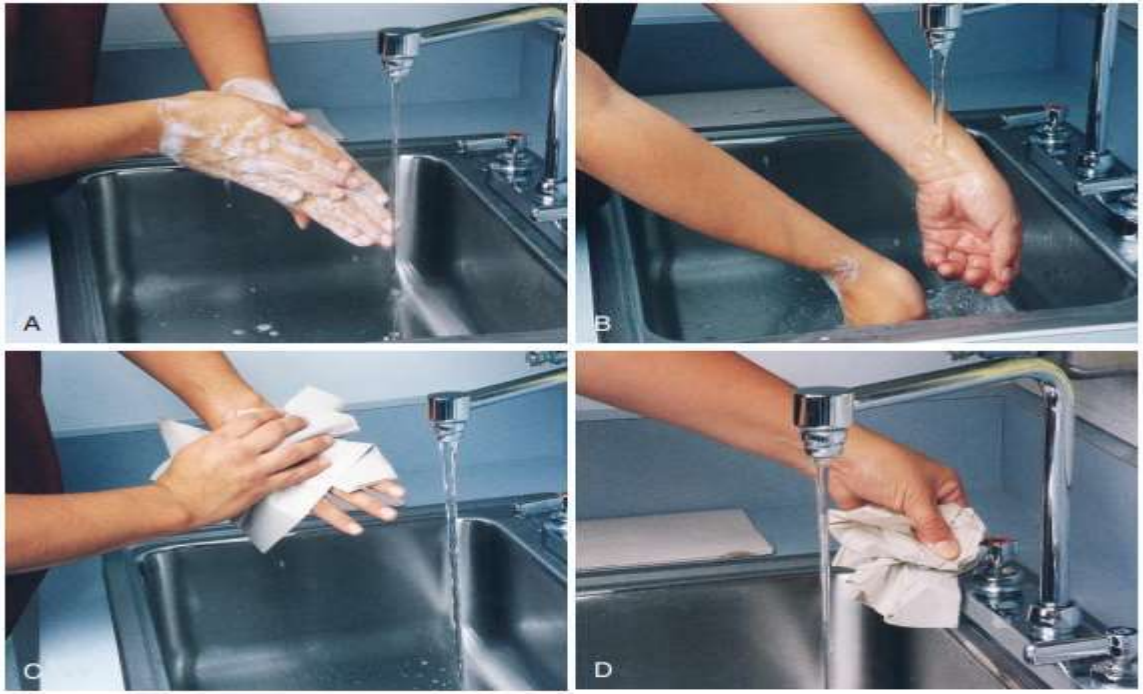
The main features of standard precautions that relate to safe hematology laboratory practice include personal protective equipment (PPE) and safety features other than PPE.

Table 1.1 Personal Protective Equipment

- Gloves
- Fluid-resistant gowns
- Laboratory coats
- Goggles
- Face shield
- Mask
- Plexiglas countertop shield

Safety Features Other Than Personal Protective Equipment:

- **Handwashing:** This is a basic yet most effective tool to prevent contamination. Soap and water must be used, and the hand washing procedure should include the wrists and at least a 10- to 15-second soap application with warm water; Hands must be washed with every patient contact, after gloves are removed, and if gloved or ungloved hands have been contaminated with a bodily fl



- **Puncture-resistant containers:** Care must be taken with contaminated sharps. Needles, blades, pipettes, syringes, and glass slides must be placed in a leak-proof, puncture-proof, properly labeled biohazard container

Laboratory Waste Management

SL No.	BIN Type	Waste Type
1. 	**RED Bin** Biohazard – Laboratory waste	Tips, tubes, Gloves, SDS PAGE gel, serological pipette, TC Flask, Plastic pertridish , tubes 15ml and 50ml etc..
2. 	**BLUE BIN** Broken Glass ware	Disinfected/Treated broken Glass wares, Any glass equipment containers, pipettes
3. 	**Halogenated Solvent Waste** White Canister, 5 Ltrs	Chloroform, Dichloromethane, Carbontetrachloride, TetraChloroethylene, Perchloroethylene …
4. 	**Non-Halogenated Solvent waste** White Canister, 5 Ltrs	Acetone, Acetonitrile, Diethylether, Ethanol, Hexane, THF, Methanol, Toluene, Methyl tert-butyl ether (MTBE), Dimethyl sulfoxide (DMSO), Dimethyl formamide(DMF) …
5. 	**White round Container**	Syringes and needles
6. 	White square Container	Sharps, slides, coverslips, blades etc…
7. 	**LARGE GREY BIN** Ethidium Bromide (Imaging Stations)	Ethidium Bromide Gel, gloves, tubes and tips which contaminated with EtBr
8. 	**Liquid Ethidium Bromide Waste** (Ground Floor Kitchen)	Buffers containing Ethidium Bromide
9. 	**Paper Recycle**	Waste papers
10. 	**GREEN BIN** (Near Lab 7 and Lab 1)	Books, papers, catalogues…
11. 	**CYAN BIN** E-waste (Ground Floor Kitchen)	Battery, CDs, pendrive, cartridge,floppy etc..
12. 	**SMALL GREY BIN** Phenol/Chloroform Solid Waste (Ground / Second Floor Fume Hoods)	Eppendorf tubes, falcon tubes, tips contaminated with phenol
13. 	New Bins For Fly Labs For Broken Bottles and Vials With Media	Broken fly vials with media in them

- **Mechanical pipetting device:** Mouth pipetting is never permitted, and other objects (e.g., pens, pencils) should be kept away from the mouth and mucous membranes.

- **Eating, drinking, and smoking:** These activities are strictly forbidden in the laboratory area. Food or drink items should not be kept in the laboratory.

- **Personal hygiene:** Regarding issues of personal hygiene, long hair must be tied back, beards must be trimmed to no more than 1 in length, fingernails must be no longer than 1/4 in. beyond the end of the finger, and there should no jewelry ornamentation of the fingers.

- **Dangling jewelry:** Earrings and necklaces and other dangling jewelry are not allowed.

- **Cosmetics:** Laboratory employees may not use cosmetics or lip balm.

Med. Lab.Tec.Department

Practical Hematology

SECOND-LINE INVESTIGATIONS IN PATIENTS WITH BLEEDING TENDENCIES

(Mixing studies)

3ed stage

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SECOND-LINE INVESTIGATIONS IN PATIENTS WITH BLEEDING TENDENCIES

The pattern of abnormalities obtained using the first-line tests often gives an indication of the underlying defect and determines the appropriate further tests required to define it.

The patterns are outlined in the following Table with suggestions for further testing.

FIRST-LINE TESTS USED IN INVESTIGATING ACUTE HAEMOSTATIC FAILURE

	PT	APTT	Fibrinogen	Platelets	Condition	Second-Line Investigation
1.	N	N	N	N	Normal haemostasis Disorder of platelet function Factor XIII deficiency Mild/masked coagulation factor deficiency Mild von Willebrand disease LMWH or direct acting inhibitor Anatomical or surgical lesion Disorder of vascular haemostasis Disorder of fibrinolysis	Specific factor assays including FXIII Platelet function (or screening such as PFA-100) VWF assay (or screening such as PFA-100) Anti-Xa assay Assays of fibrinolytic factors
2.	Long	N	N	N	Factor VII deficiency Mild liver impairment or vitamin K deficiency Early oral anticoagulation (primarily factor VII reduction) Lupus anticoagulant (with some reagents) Mild factor II, V or X deficiency	Mixing test for correction of PT Specific factor assays beginning with factor VII Test for lupus anticoagulant Liver function tests, trial of vitamin K
3.	N	Long	N	N	Factor VIII, IX, XI, XII, prekallikrein or HMWK deficiency FVIII deficiency secondary to VWD Circulating anticoagulant, e.g. lupus anticoagulant Heparin or direct acting anticoagulant Mild factor II, V or X deficiency	Mixing test for correction Specific factor assays VWF assays if factor VIII low Anti-Xa assay, anti-IIa assay Test for lupus anticoagulant
4.	Long	Long	N or Abnormal	N	Vitamin K deficiency Anticoagulants (warfarin, direct acting, heparins) Factor V, X or II deficiency Multiple factor deficiency, e.g. liver failure Combined factor V + VIII deficiency Fibrinogen deficiency/disorder Inhibition of fibrin polymerisation Hyperfibrinolysis	Mixing tests for correction Specific factor assays Thrombin time Anti-Xa assay, anti-IIa assay Liver function tests, trial of vitamin K D-dimer assay
5.	N	N	N	Low	Thrombocytopenia	Blood count and film
6.	Long	Long	N or Abnormal	Low	Massive transfusion Liver disease Disseminated intravascular coagulation	D-dimer assay Liver function tests

HMWK = high molecular weight kininogen; N = normal

Correction tests using the prothrombin time or activated partial thromboplastin time

Principle

Prolongation of the PT or APTT can be investigated with simple correction tests by mixing the patient's plasma with normal plasma.

Correction indicates a possible factor deficiency, whereas failure to correct suggests the presence of an inhibitor, but interpretation should be cautious.

Reagents

Plasmas for correction. Normal plasma contains all the coagulation factors; therefore mixing tests with normal plasma will identify the presence of an inhibitor or a factor deficiency.

PPP.From the patient and a control

Other reagents. As described for PT and/or APTT tests.

Method

- Perform

a PT and/or APTT on control, patient and a 50:50 (0.05 ml of each) mixture of the control and patient plasma.

- Perform all the tests in duplicate using a balanced order to avoid time bias.
- Note that mixing experiments to detect FVIII inhibitors may require incubation for 2 h before analysis.

Interpretation

- If the prolongation is the result of a deficiency of a clotting factor, the PT or APTT of the mixture should return to within a few seconds of normal. It is then necessary to identify the specific factor(s) that are deficient.

- If the APTT is prolonged and normal plasma fails to correct the APTT, an inhibitor should be suspected. An inhibitor screen and tests for an LAC should be performed

A case study

A 65-year-old man is admitted from the haematology clinic with bruises and gum bleeding.
All Initial investigations were normal except for prolonged APTT (80s).

Q: What is/are the next investigation(s) that you should perform?

Answer: Correction (mixing study) test using the activated partial thromboplastin time.

Q: You performed the mixing and the results were the following:

APTT normal pooled plasma: 30s
APTT patient plasma: 80 s
APTT 50/50 mix immediate : 35 s

APTT normal pooled plasma after 2hr incubation : 35s
APTT patient plasma after 2hr incubation: 82 s
APTT 50/50 mix immediate after 2hr incubation : 70 s

Discuss these results

Discussion of the results

The mixing studies indicate the presence of an inhibitor. It is time dependant inhibitors and therefore the immediate mixing study didn't detect it.

In order to allow for a time-dependant inhibitor to manifest, a 2-hour incubation is recommended prior to carrying out further investigations.

INTERPRETATION OF THE INHIBITOR SCREEN BASED ON THE ACTIVATED PARTIAL THROMBOPLASTIN TIME

Tube	Content	Clotting Time		
1	Normal plasma	Normal	Normal	Normal
2	Patient's plasma	Long	Long	Long
3	50:50 mixture, patient: normal; incubated 2 h	Normal	Long	Long
4	50:50 mixture, patient: normal; no incubation	Normal	Long	Normal
Interpretation		Deficiency	Immediately acting inhibitor	Time-dependent inhibitor

Thanks

Med. Lab.Tec.Department

Practical hematology
Lecture 12

3ed stage

Coagulation Screening Test
Prothrombin and Activated Partial Thromboplastin Time

Lecturer
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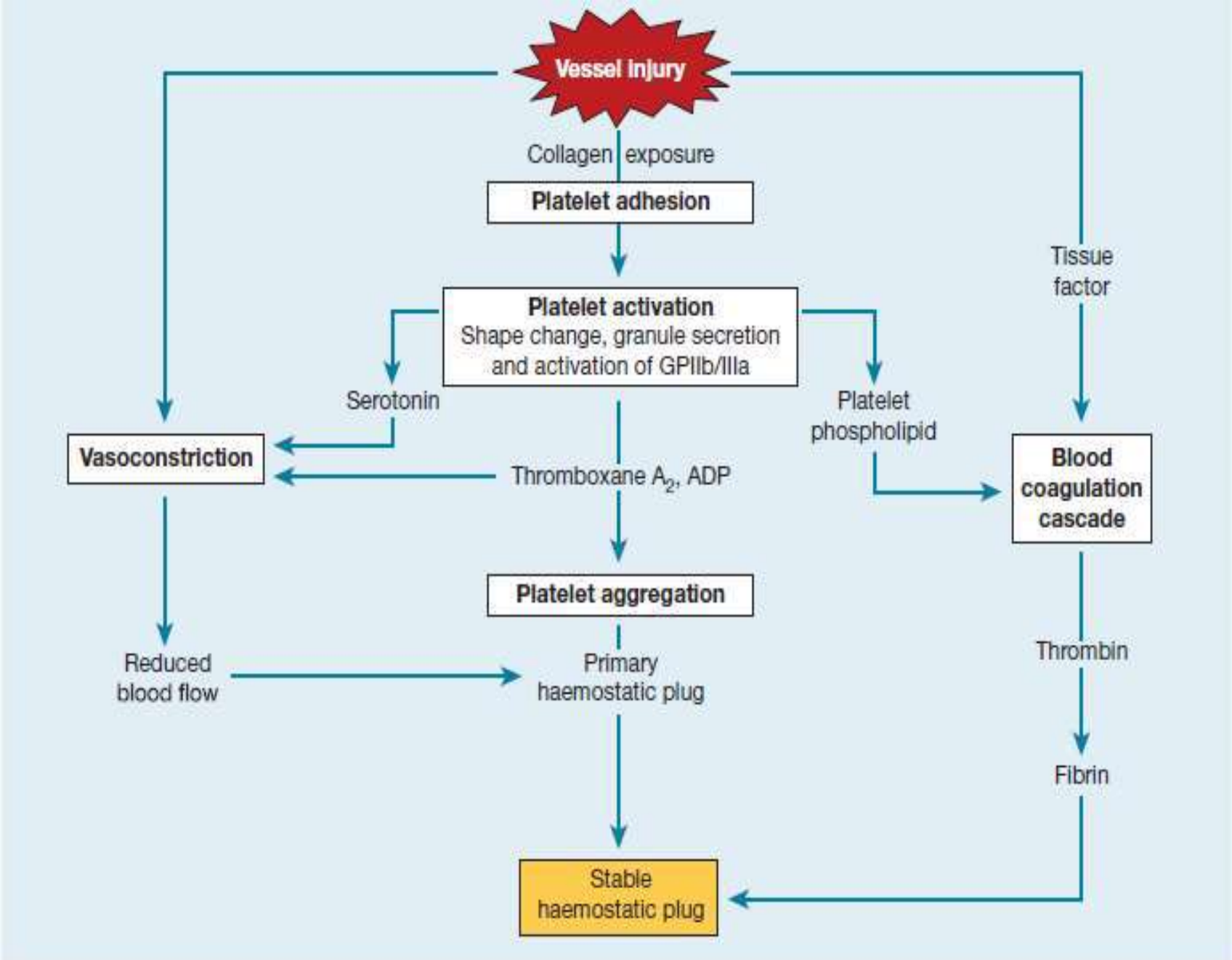

Vessel injury
Collagen exposure
Platelet adhesion
Tissue factor
Platelet activation
Shape change, granule secretion and activation of GPIIb/IIIa
Serotonin
Platelet phospholipid
Vasoconstriction
Thromboxane A_2, ADP
Blood coagulation cascade
Platelet aggregation
Reduced blood flow
Primary haemostatic plug
Thrombin
Fibrin
Stable haemostatic plug

Introduction:

Patients who experience recurrent bleeding require evaluation for the source of their bleeding disorder. Bleeding may occur because of an inherited clotting factor defect or an acquired deficiency that is secondary to some other cause**.**

Hereditary deficiencies of each of the coagulation factors have been described. Haemophilia A (factor VIII deficiency), haemophilia B (Christmas disease, factor IX deficiency) and von Willebrand disease (VWD) are the most frequent; the others are rarer.

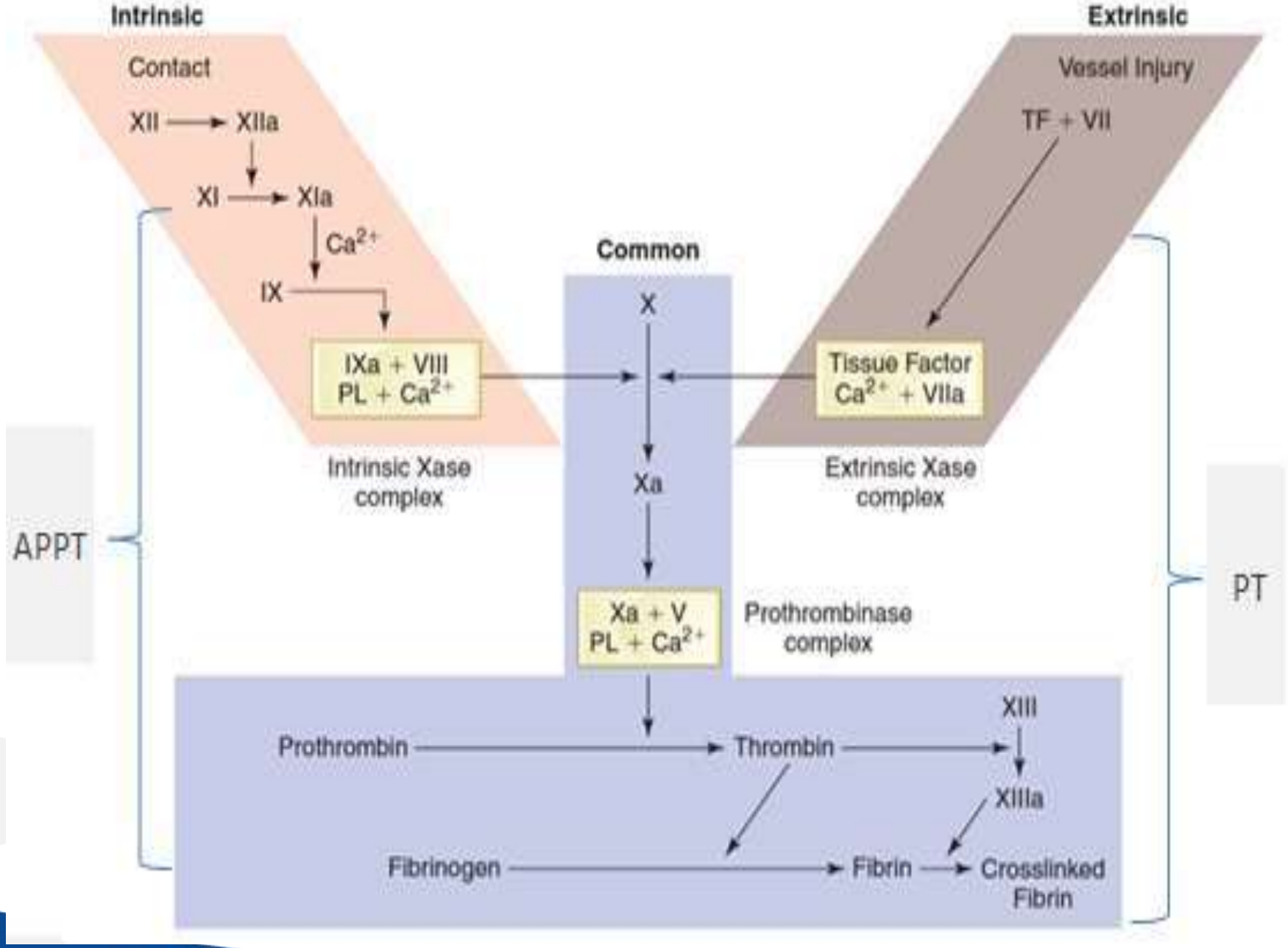
The acquired coagulation disorders are more common than the inherited disorders (e.g, Vitamin K deficiency, Vitamin K-antagonist therapy , Haemorrhagic disease of the newborn, Liver disease and Disseminated intravascular coagulation).

Laboratory Diagnosis of coagulation disorders:

A.Prothrombin Time

Principle:

The PT test measures the clotting time of recalcified plasma in the presence of an optimal concentration of tissue extract (thromboplastin) and indicates the overall efficiency of the extrinsic clotting system. Although originally thought to measure prothrombin. the test is now known to depend also on reactions with factors V, VII and X and on the fibrinogen concentration of the plasma.


Intrinsic
Contact
XII → XIIa
XI → XIa
Ca^{2+}
IX
IXa + VIII
PL + Ca^{2+}
Intrinsic Xase complex
Extrinsic
Vessel Injury
TF + VII
Tissue Factor
Ca^{2+} + VIIa
Extrinsic Xase complex
Common
X
Xa
Xa + V
PL + Ca^{2+}
Prothrombinase complex
Prothrombin
Thrombin
XIII
XIIIa
Fibrinogen
Fibrin
Crosslinked Fibrin
APPT
PT

Specimen Collection and Storage

1. Collect whole blood into vacuum tube with 3.2% sodium citrate. This requires a 9:1 dilution of blood to anticoagulant.
2. Specimens should not be obtained through a heparin lock or any other heparinized line.
3. Most routine coagulation investigations are performed on platelet-poor plasma (PPP), which is prepared by centrifugation at 2000 g for 15 min at 4C (approx. 4000 rev/min).
4. The sample should be kept at room temperature if the testing is completed within 2 h of collection and it should be kept at 4C for if tested later .

Procedure

1. Deliver 100 µl of plasma into a glass tube placed in a water bath
2. add 100 µl of thromboplastin reagent.
3. Wait1–3 min to allow the mixture to warm.
4. Then add 100 µl of warmed CaCl2 and start the stopwatch.
5. Mix the contents of the tube and When a fibrin clot forms, the timer stops, and the interval is recorded.

Note :

1. Some thromboplastins contain calcium chloride, in which case 200µl of thromboplastin is added to 100 µl plasma and timing is started immediately.
2. Carry out the test in duplicate on the patient's plasma and the control plasma. When a number of samples are to be tested as a batch, the samples and controls must be suitably staggered to eliminate the time bias.

Normal value of prothrombin time is between 11 and 16 s.

Interpretation:

The common causes of prolonged PTs are as follows:

1. Administration of oral anticoagulant drugs (vitamin K antagonists)
2. Liver disease, particularly obstructive jaundice
3. Vitamin K deficiency
4. Disseminated intravascular coagulation(DIC)
5. Rarely, a previously undiagnosed factor VII, X, V or prothrombin deficiency .Note: with prothrombin, factor X or factor V deficiency the APTT will also be prolonged.

B. Activated Partial Thromboplastin Time

The test measures the clotting time of plasma after the activation of contact factors and the addition of phospholipid and CaCl2', but without added tissue thromboplastin ,and so indicates the overall efficiency of the intrinsic pathway. To standardize the activation of contact factors ,the plasma is first preincubated for a set period with a contact activator such as kaolin, silica or ellagic acid. During this phase of the test, factor XIIa is produced, which cleaves factor XI to factor XIa, but coagulation does not proceed beyond this in the absence of calcium.

After recalcification ,factor XIa activates factor IX and coagulation follows. A standardized phospholipid is provided to allow the test to be performed on PPP. The test depends not only on the contact factors and on factors VIII and IX but also on the reactions with factors X, V, prothrombin and fibrinogen. It is also sensitive to the presence of circulating anticoagulants (inhibitors) and heparin.

Procedure:

1. To initiate contact activation, 100 µL of warmed (37° C) of the kaolin–phospholipid reagent is mixed with an equal volume of warmed PPP.
2. The mixture is allowed to incubate for the exact manufacturer-specified time, usually 3 minutes.
3. Next 100 µL of warmed 0.025 M calcium chloride is forcibly added to the mixture, and a timer is started. When a fibrin clot forms, the timer stops, and the interval is recorded.

Normal value of Activated Partial Thromboplastin Time is 26–40 s.

Interpretation

The common causes of a prolonged APTT are as follows:

1. Disseminated intravascular coagulation
2. Liver disease
3. Massive transfusion with plasma-depleted red blood cells
4. Administration of or contamination with heparin or other anticoagulants
5. A circulating anticoagulant (inhibitor)
6. Deficiency of a coagulation factor other than factor VII. The APTT is also moderately prolonged in patients taking oral anticoagulant drugs and in the presence of vitamin K deficiency. Occasionally, a patient with previously undiagnosed haemophilia or another congenital coagulation disorder presents with an isolated prolonged APTT.

Screening tests of blood coagulation

Screening tests	Abnormalities indicated by prolongation	Most common cause of coagulation disorder
Thrombin time (TT)	Deficiency or abnormality of fibrinogen or inhibition of thrombin by heparin or FDPs	DIC Heparin therapy
Prothrombin time (PT)	Deficiency or inhibition of one or more of the following coagulation factors: VII, X, V, II, fibrinogen	Liver disease Warfarin therapy DIC
Activated partial thromboplastin time (APTT or PTTK)	Deficiency or inhibition of one or more of the following coagulation factors: XII, XI, IX (Christmas disease), VIII (haemophilia), X, V, II, fibrinogen	Haemophilia, Christmas disease (+ conditions above)
Fibrinogen quantitation	Fibrinogen deficiency	DIC, liver disease

DIC, disseminated intravascular coagulation; FDPs, fibrin degradation products.

Thanks For Listening

Med. Lab.Tec.Department

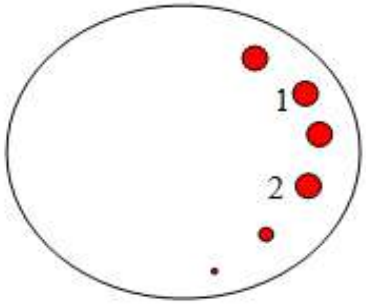
Practical hematology

Lecture 15

Coagulation Studies

3rd stage

(Bleeding Time)



Lecturer
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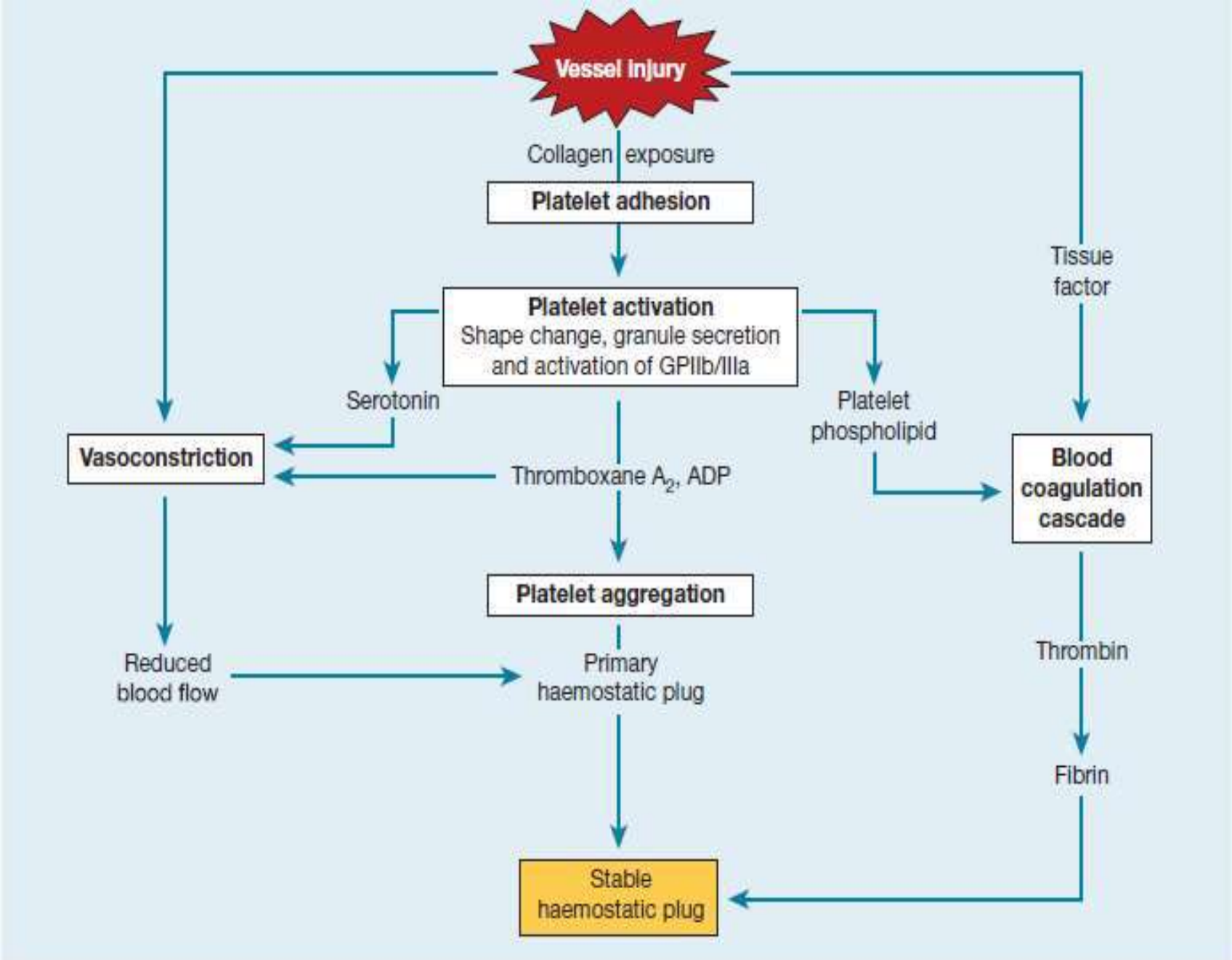
Introduction:

Coagulation is a complex network of interactions involving vessels, platelets, and factors. The ability to form and remove a clot depends on many synergistic forces.

Similar to a balance scale, hemostasis relies on a system of checks and balances between thrombosis and hemorrhage that includes procoagulants and anticoagulants (Hemostasis arrests bleeding from a vessel wall defect and simultaneously maintains fluidity within the circulation).

Hemostasis involves the following 4 interrelated steps:

1. Vasoconstriction (contraction of injured blood vessels).
2. Platelet plug formation.
3. Formation of a blood clot.
4. Fibrinolysis (dissolution of the clot).


Vessel injury
Collagen exposure
Platelet adhesion
Tissue factor
Platelet activation
Shape change, granule secretion and activation of GPIIb/IIIa
Serotonin
Platelet phospholipid
Vasoconstriction
Thromboxane A_2, ADP
Blood coagulation cascade
Platelet aggregation
Reduced blood flow
Primary haemostatic plug
Thrombin
Fibrin
Stable haemostatic plug

Bleeding Disorders.

Bleeding disorders may be inherited or acquired—the acquired defects being more common. Disorders due to platelet and vessel wall defects are more common than coagulation disorders that are due to deficiencies of clotting factors.

Tests For Hemostasis:

1. **Bleeding Time (BT)** is the time interval between the skin puncture and spontaneous, unassisted (i.e. without pressure) stoppage of bleeding. The BT test is an *in vitro* test of platelet function.

The BT is a simple test used as a routine before every minor and major surgery (e.g. tooth extraction), biopsy procedures, and before and during anticoagulant therapy, whether or not there is a history of bleeding.

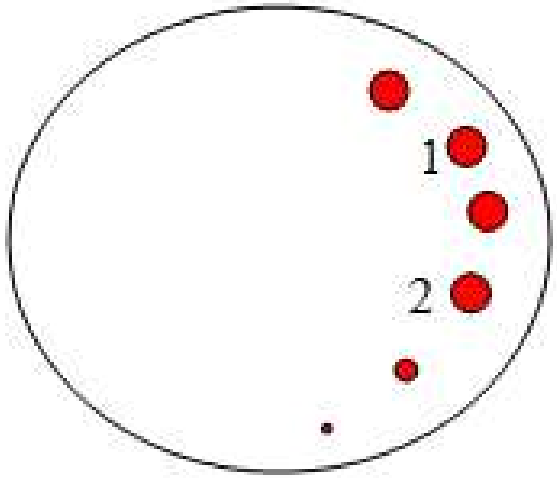
A. DUKE'S method

Materials and Instruments:

1. Sterile disposable lancets.
2. Filter paper or tissue paper.
3. Stopwatch.
4. Alcohol prep pads.

Procedure:

1. Clean the ear lobe with alcohol prep and allow to dry.
2. A standardized puncture (3 mm. deep) of the ear lobe is then made with the use of a sterile disposable lancet.
3. The stopwatch is started at the moment of the puncture.
4. Absorb the emerging blood with a filter paper or tissue every 30 seconds. Don't apply pressure on the ear lobe.
5. When bleeding ceases the stopwatch in halted and the bleeding time is estimated as in (fig.1).



The normal bleeding time (2-5) minutes.

A.Standardized template method(Ivy "method)

Materials:-

1. Sphygmomanometer
2. 70% Alcohol, or alcohol swabs
3. Cotton
4. Template bleeding time device
5. Sterile lancet
6. Filter paper
7. Stop watch

Procedure

1. Clean the skin over the front of the forearm with 70% alcohol.
2. Apply a sphygmomanometer cuff around the patient's arm above the elbow, raise the pressure to 40 mm Hg and maintain it there till the end of the experiment.
3. Clean the skin area once again. Grasp the underside of the forearm tightly, make a 1–3 mm deep skin puncture, about 5–6 cm below the cubital fossa. Note the time.
4. Remove the blood every 30 seconds by absorbing it along the edges of a clean filter paper by gently touching the wound with it, till the bleeding stops. This is the end-point.

Normal bleeding time with this method 2- 9 minutes.

Factors affecting Bleeding time:

1. Age : Neonates and children longer than adults.
2. Sex : Females longer than males.
3. Lateral aspect longer than Medial aspect.
4. Transverse longer than Longitudinal incision.
5. Cold prolongs BT (important if temp < 25 C)
6. Platelets < 80 000/ul prolongs BT.
7. Venostasis, if a cuff not inflated then shorter, if overinflated longer.

Prolonged bleeding time is associated with:

1. Vascular diseases (e.g. scurvy).
2. Quantitative Platelets disorders (thrombocytopenia).
3. Qualitative platelets defects (e.g. thrombasthenia)
4. Ingestion of Aspirin and other anti-platelet drugs.
5. Von-Willebrand disease (vWD).
6. Severe deficiencies of factor:- V, XI, VIII, and fibrinogen.

Thanks For Listening

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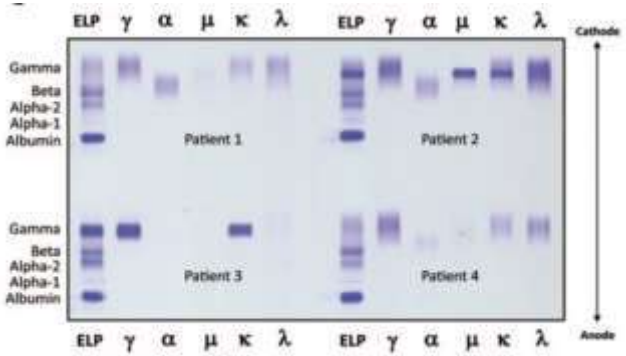
Practical hematology

Lecture 14

Diagnosis of plasma cell disorder

3rd stage



Lecturer

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Introduction:

Paraproteinaemia: This is the presence of a monoclonal immunoglobulin band in the serum. Normally, serum immunoglobulin's are polyclonal and represent the combined output from millions of different plasma cells. A monoclonal band (M-protein), or paraprotein, reflects the synthesis of immunoglobulin from a single clone of plasma cells. This may occur as a primary neoplastic disease or secondary to an underlying benign or neoplastic disease affecting the immune system.

Multiple myeloma

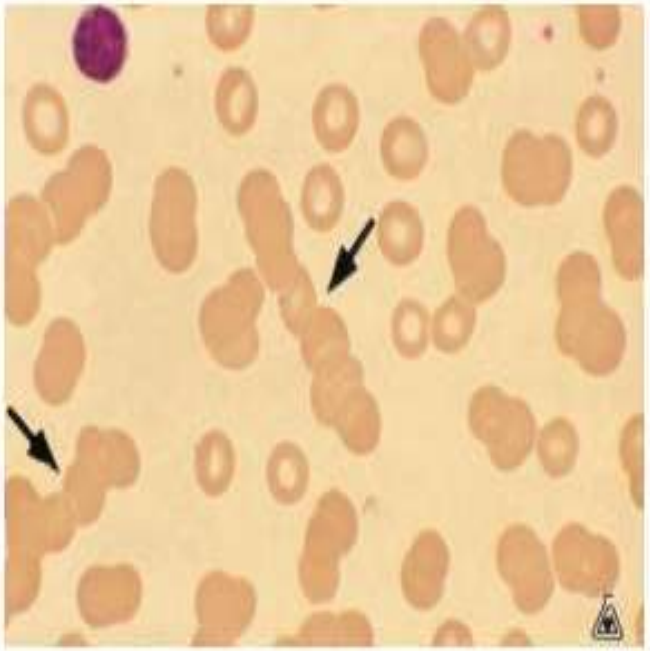
Is a neoplastic disease characterized by plasma cell accumulation in the bone marrow the presence of monoclonal protein in the serum and/or urine and, in symptomatic patients, related tissue damage.

Diagnosis of plasma cell disorder (multiple myeloma):

1. Clinical features

2. Hematologic Studies

Table (1): Overview of Major Disorders	
	Multiple myeloma
Predominant cell type	Plasma cells in marrow
Main symptoms	Bone pain, Thirst, Fatigue
Significant laboratory findings	Calcium↑↑ Hyperviscosity (↑ESR) Monoclonal spike IgM Rouleaux formation
Organ involvement	Kidneys, Bone marrow



3. Laboratory Studies

Any patient suspected of having a plasma cell disorder should have a serum protein electrophoresis (SPEP) and immunofixation electrophoresis (IFE) for quantifying and typing the M-component, respectively, as well as quantitative measurement of albumin and polyclonal serum immunoglobulins.

Clinical Protein and Immunofixation Electrophoresis

Electrophoresis is a laboratory method fundamental to the diagnosis and management of plasma cell disorders. Serum protein electrophoresis (SPEP) is commonly used to detect the presence of circulating monoclonal proteins.

Immunofixation electrophoresis (IFE), a related technique, is used to confirm that the restricted bands observed by protein electrophoresis are monoclonal, as well as to characterize the types of monoclonal antibody present.

A. Principle of Electrophoresis

Electrophoresis is a method of separating proteins based on their physical properties.

Serum is placed on a specific medium, and a charge is applied. The net charge (positive or negative) and the size and shape of the protein commonly are used in differentiating various serum proteins.

The proteins are stained, and their densities are calculated electronically to provide graphical data on the absolute and relative amounts of the various proteins.

Further separation of protein subtypes is achieved by staining with an immunologically active agent, which results in immunofixation.

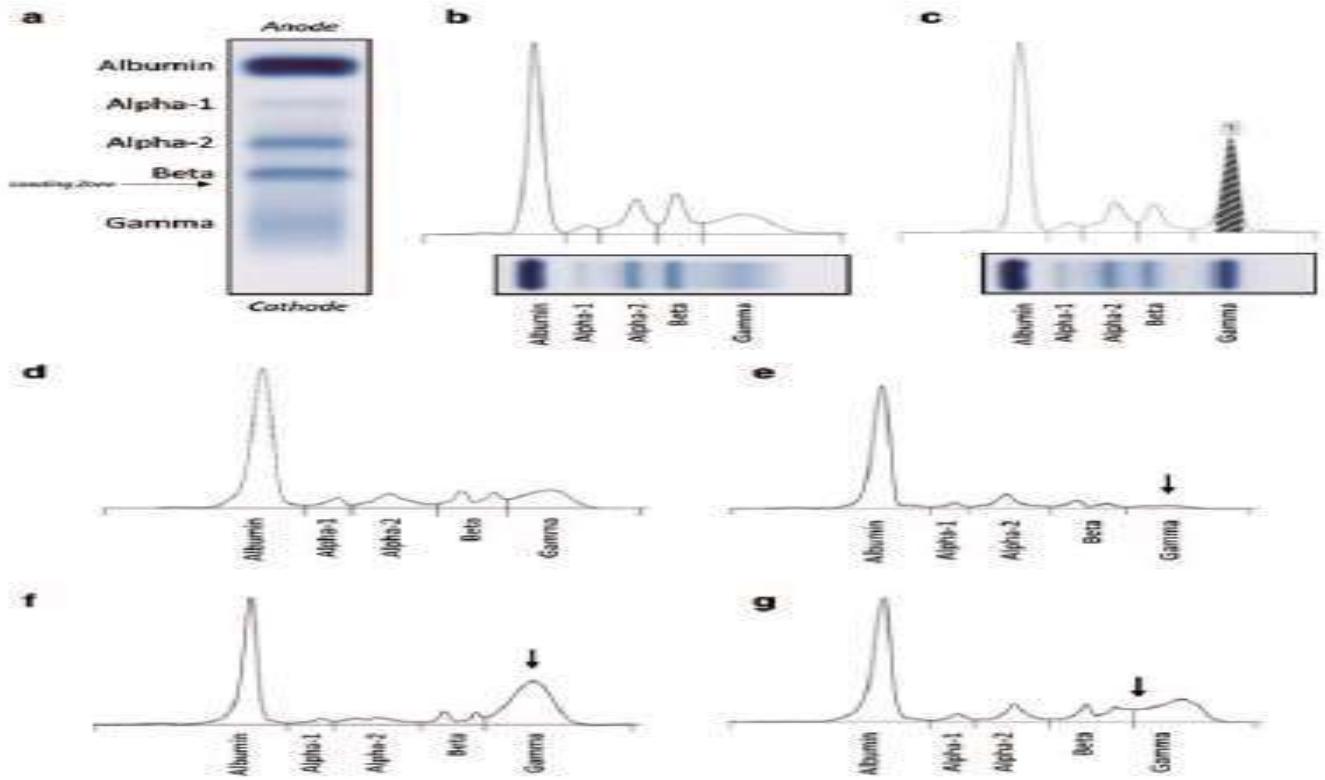
Components of Serum Protein Electrophoresis

The pattern of serum protein electrophoresis results depends on the fractions of two major types of protein: albumin and globulins. Albumin, the largest peak, lies closest to the positive electrode. The next five components (globulins) are labeled alpha1, alpha2, beta1, beta2, and gamma. The peaks for these components lie toward the negative electrode, with the gamma peak being closest to that electrode.

After the protein migration protocol is completed, gels are dried, stained, washed, and dried again. An image of the gel is then made using either a scanner or an integrated digital imaging system. An *electropherogram* is then generated. This is a visual representation of the specimen migration pattern, in which the area under the curve (AUC) of specific fractions and/or abnormal bands is proportional to the band intensity on the gel.

To calculate the quantity of fractions in mass concentration (e.g., g/dL), the relative percent of individual AUCs is multiplied by the serum total protein concentration performed separately using chemical methods or refractometry. This method is used to quantify the M-spike at baseline and to monitor a patient's disease course over time.

Monoclonal immunoglobulins are also referred to as *monoclonal antibodies*, *monoclonal proteins*, *M-proteins*, and/ or *M-spikes* usually lie on **GAMMA FRACTION. However, and IgA, IgM, and sometimes IgG also can be identified in the beta fraction.**



Figure(2) Serum protein electrophoresis**. a** Protein electrophoresis of a normal serum specimen.Normal albumin, alpha-1, alpha-2, beta, and gamma fractions are observed. **b** An electropherogram derived from the same specimen as in **a** (oriented with albumin to the *left* and gamma fraction to the *right*). **c** An electropherogram derived from the serum of a patient with a distinct monoclonal protein in the gamma region. Note the gate (*shading*) applied over the M-spike, used in quantification. Electropherograms from capillary-based platforms demonstrating: **d** normal pattern; **e** hypogammaglobulinemia;

f polyclonal hypergammaglobulinemia; and **g** beta–gamma bridging (due to increased polyclonal IgA in liver disease).

A.Immunofixation—Gel Electrophoresis

While protein electrophoresis permits the identification of discrete abnormal bands in serum and urine, IFE can be used to confirm whether such bands represent monoclonal immunoglobulins, and if so to provide clonal characterization. Clinical laboratory IFE methods share initial steps with protein electrophoresis, except each patient specimen is initially migrated in multiple consecutive lanes (often six) on the gel. After protein migration, a template grid of channels is placed on top of the gel, so that each channel is aligned directly above a migration lane.

In the first channel, an acid solution is applied to precipitate all serum proteins and create what is essentially the patient's SPEP pattern used for comparison purposes.

In subsequent channels, antibodies directed against heavy chains (γ, α, μ) and/or light chains (κ, λ) are applied separately. Antisera to δ and ε heavy chains can also be used when an IgD or IgE

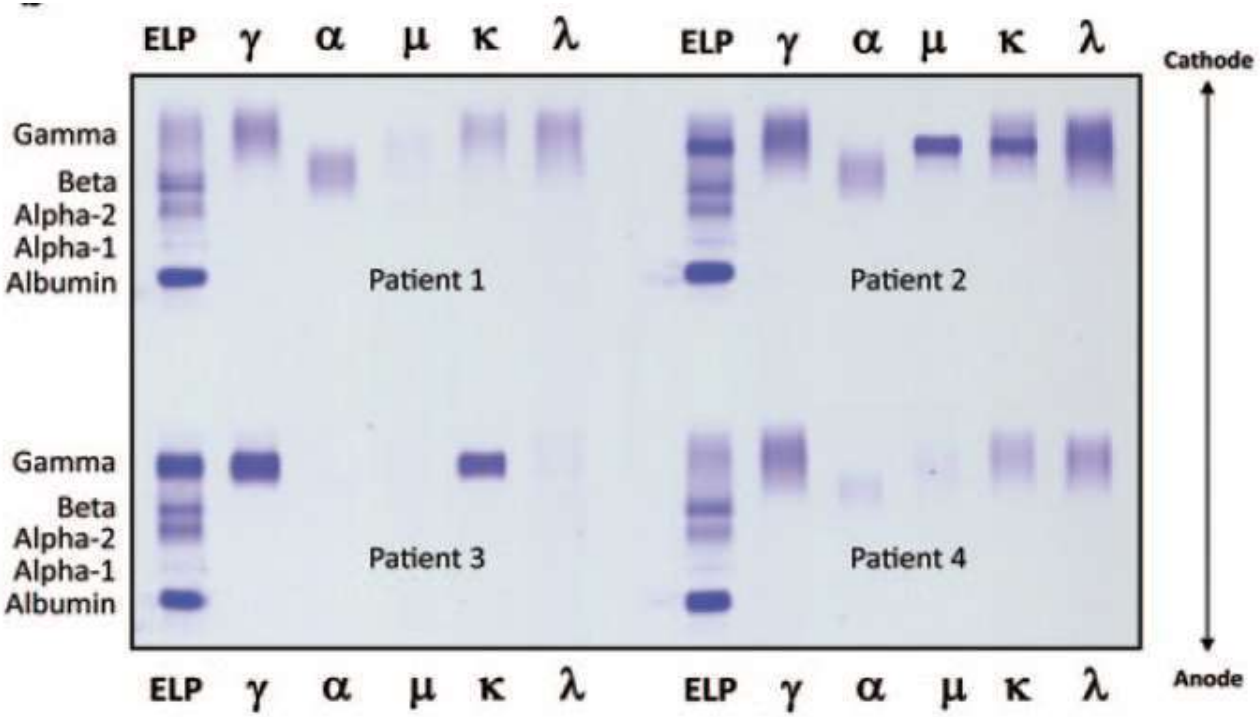


Figure (3): Serum Immunofixation Electrophoresis (*IFE*)from four patients).

Note that on these gels, albumin is oriented toward the bottom whereas the gamma region is oriented toward the *top*. *Patients 1* and *4* show normal patterns of polyclonal immunoglobulin expression. *Patient 2* has a monoclonal IgM-kappa. *Patient 3* has a monoclonal IgG-kappa, with suppression of other polyclonal immunoglobulins

Thanks For Listening

Med. Lab.Tec.Department

Practical Hematology

Lecture 13

Iron stain

3rd stage

Lecturer
Dr. Muqdad M Najem
Asst.Lec. Iman.H.Jirjees

EVALUATION OF IRON STORES IN BONE MARROW

- Marrow examination often should include evaluation of the iron stores, especially if the patient is anaemic. The examination is accomplished by staining a marrow film or section by the Prussian blue technique.

- Bone marrow aspirate should be stained when evaluating iron stores in the differential diagnosis of anaemia.

EVALUATION OF IRON STORES IN BONE MARROW

- Marrow macrophages (seen best in the aspirate particle preparation) are evaluated for storage iron, and
- erythroblasts (best evaluated in the direct film or concentrate) are examined for the presence of iron granules in the cytoplasm (sideroblasts).
- The proportion of normal late erythroblasts that contain one to four small Prussian blue granules is extremely variable (3–69 percent) in normal subjects.
- Pathologic ring sideroblasts are characterized by an increased number of iron granules (> 4) arranged in a ring encircling at least 1/3 of the nucleus, reflecting accumulation of iron in mitochondria.

Summary of Siderocytes and sideroblasts

Siderocyte	Mature red cell containing one or more siderotic granules (Pappenheimer bodies)
Normal sideroblast	Nucleated red cell containing one or more siderotic granules, granules few, difficult to see, randomly distributed in the cytoplasm, reduced proportion of sideroblasts in iron deficiency and anaemia of chronic disorders

Summary of Siderocytes and sideroblasts

Abnormal sideroblasts	Cytoplasmic iron deposits (ferritin aggregates): increased granulation, granules larger and more numerous than normal, easily visible and randomly distributed, proportion of sideroblasts usually parallels the percentage saturation of transferrin (e.g. haemolytic anaemia, megaloblastic anaemia, iron overload, thalassaemia disorders)
	Mitochondrial iron deposits (non-ferritin iron): ring sideroblasts in inherited and acquired sideroblastic anaemias

Differential Diagnosis of Bone Marrow Ring Sideroblasts

Clonal	Non-Clonal
Myelodysplastic Syndromes with ringed sideroblasts	X-linked sideroblastic anemia
MDS/MPN overlap syndromes	Alcoholism
	Drug induced sideroblastic anemia
	Copper, deficiency Lead poisoning Zinc toxicity

Method for staining siderotic granules (Prussian blue stain or perl's reaction)

Requirements :

1. **Methanol, absolute.**
2. **Staining jar.**
3. **Potassium ferrocyanide.**
4. **Concentrated Hydrochloric acid.**
5. **Eosin powder.**
6. **A source of running tap water.**

Method for staining siderotic granules (Prussian blue stain or perl's reaction)

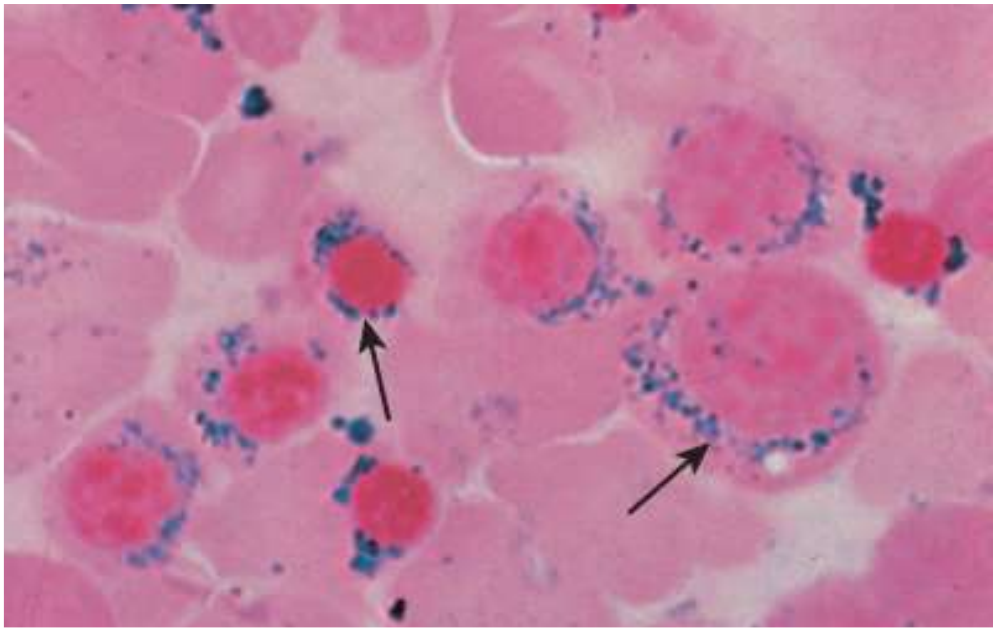
Preparation of reagents :

1. **Solution A: 47 mmol/L Potassium Ferrocyanide. (2 g in 100 mls of dH2O).**
2. **Solution B: 0.2 M HCL: done by adding 2 mls of Conc. HCL to 98 ml of d H2O.**
3. **Working solution prepared just before use: by mixing equal volumes of solutions A and B.**
4. **1% Eosin: 1 g of Eosin powder in 100 ml dH2O.**

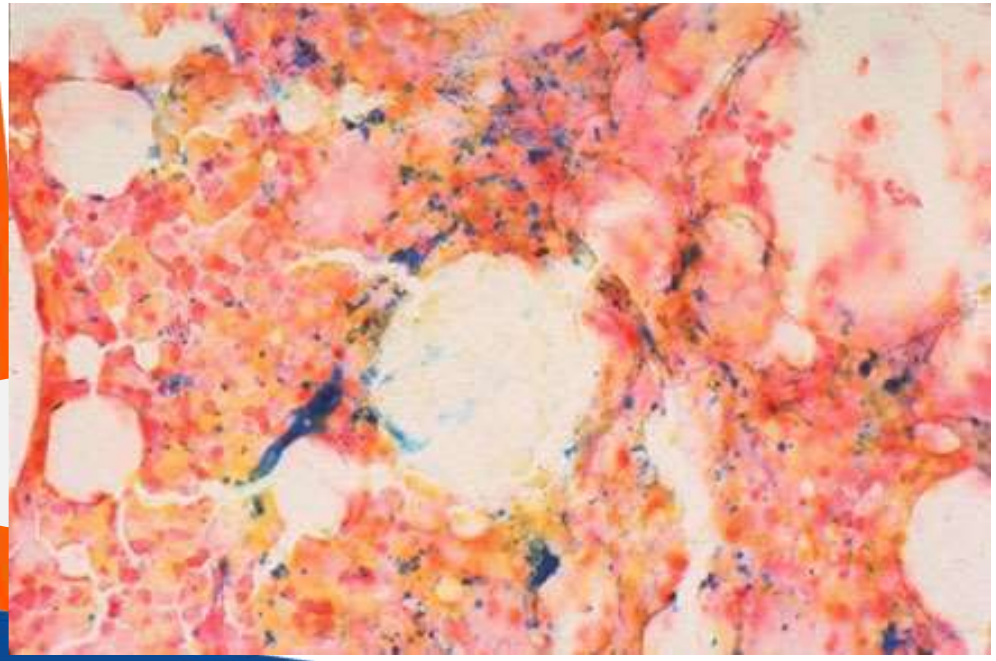
Method for staining siderotic granules (Prussian blue stain or perl's reaction)

Procedure :

1. Fix Air dried slides in Absolute methanol for 10-20 minutes.
2. When dry, place the slides in the working solution prepared in3 above.
3. Leave for 10 minutes at RT (~20C).
4. Wash in running tap water for ~ 20 minutes.
5. Counterstain with 1% eosin for 10-15 sec.
6. Wash with Tap water.
7. Allow to dry.



Ringed Sideroblasts



Iron stain in
macrophages

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Practical hematology

Lecture 12

Diagnosis of the Acute Leukemia's

3ed stage

Lecturer
Muqdad M Najem
Iman.H.Jirjees

- Acute leukemia is a heterogeneous group of hematopoietic neoplasms resulting from clonal proliferation of immature precursor cells (blasts).
- The genetic aberrations resulting in the blockage of differentiation and uncontrolled proliferation of the blasts may occur at different developmental stages, including the pluripotential stem cells, and the progenitors committed to the lymphoid or myeloid lineages.
- A diagnosis of acute leukemia requires 20% blasts in the PB or BM. The exceptions are acute myeloid leukemia with recurrent genetic abnormalities where the presence of the genetic abnormality defines acute leukemia.

The diagnosis of acute leukemia requires the following:

1. Morphological assessment
2. Determination of cytochemical and immunophenotypic features
3. Evaluation of genetic abnormalities.

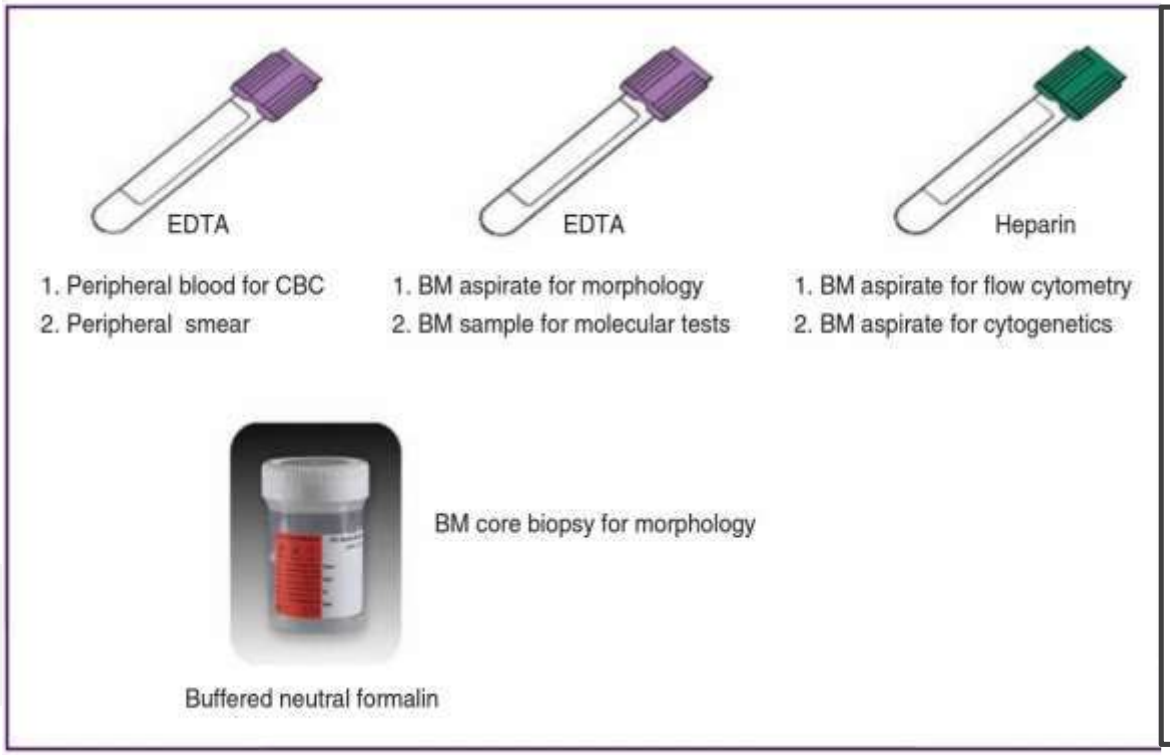


FIGURE 1: Sample requirement for diagnostic work-up of acute leukemia. BM, bone marrow; CBC, complete blood count; EDTA, ethylenediaminetetraacetic acid.

A. PB examination

should be correlated with the results of complete blood count (CBC).

- PB of acute leukemia patients almost always shows features of bone marrow failure, including anemia, thrombocytopenia, and neutropenia.
- Blasts are usually present, but the number varies widely.
- A manual 200-leukocyte differential is recommended unless the total count is too low to permit this.
- Enumeration of PB blasts, their morphology, presence of lineage-defining morphological features such as Auer rods in myeloblasts.

B. BM examination: Aspirate and biopsy

- A BM differential count is performed on a Wright–Giemsa stained aspirate smear by counting 500 nucleated cells. Enumeration of the blasts is essential for diagnosis.
- Trephine biopsy is valuable for assessment of marrow cellularity, megakaryocyte distribution and morphology, presence of fibrosis, and other abnormal cells. In the event of a dry tap due to a packed marrow or associated marrow fibrosis, the biopsy is crucial for diagnosis of acute leukemia.

C. Cytochemical stains are a useful adjunct to identify blast lineages.

- Myeloperoxidase (MPO) and Sudan Black B stains are specific for myeloblasts; it is usually negative or weakly positive in monoblasts and promonocytes. Lymphoid blasts, erythroid blasts, and megakaryoblasts are MPO and SBB negative.
- Nonspecific esterases (NSE) show diffuse reactivity in monoblasts and monocytes.
- Naphthol AS-D chloroacetate esterase (CAE) is a reliable specific esterase for the granulocytic lineage from the promyelocyte stage onward.
- A positive cytochemical stain is helpful, but a negative reaction does not exclude a myeloid lineage.

D. Immunophenotyping:

- Leukaemic and normal cells of different types express characteristic nuclear, cytoplasmic and cell surface antigens. This is referred to as the immunophenotype of the cell. Characterization of the immunophenotype is referred to as immunophenotyping and is achieved by means of labelled antibodies that recognize specific epitopes of cellular antigens.
- Immunophenotyping is an integral part of work-up for acute leukemia. It not only defines the lineage of the leukemia cells but also identifies unusual phenotypic features that provide clues for specific types of acute leukemia

D. Immunophenotyping:

- **The technique employed for immunophenotyping is usually flow cytometry, although Immunohistochemistry is an essential tool when tissues require assessment, whether these be lymph nodes, trephine biopsy specimens or other tissues.**

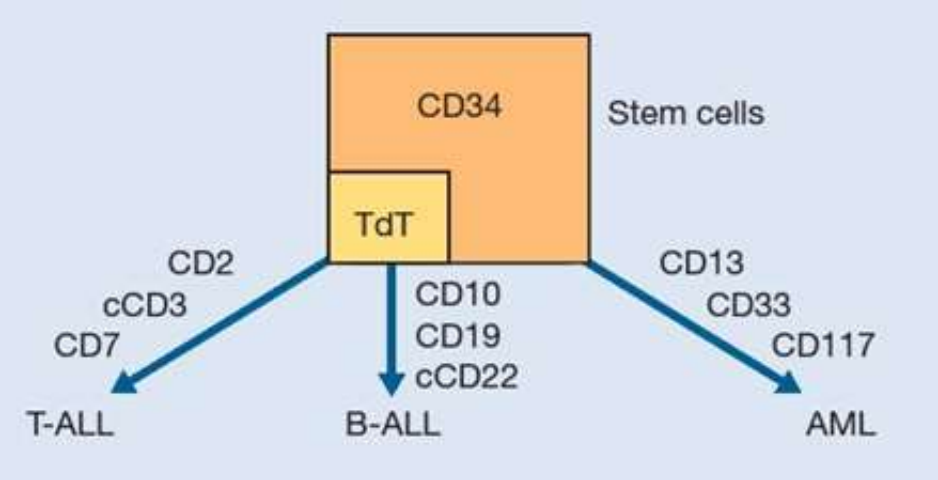


Figure 2 Development of three cell lineages from pluripotential stem cells giving rise to the three main immunological subclasses of acute leukaemia. CD34 is expressed on most stem cells whereas TdT expression defines a lymphoid lineage. Three surface markers which are seen characteristically in T-ALL, B-ALL and AML are shown. AML, acute myeloid leukaemia; B-ALL, B-cell acute lymphoblastic leukaemia; c, cytoplasmic; HLA, human leucocyte antigen; T-ALL, T-cell acute lymphoblastic leukaemia; TdT, terminal deoxynucleotidyl transferase.

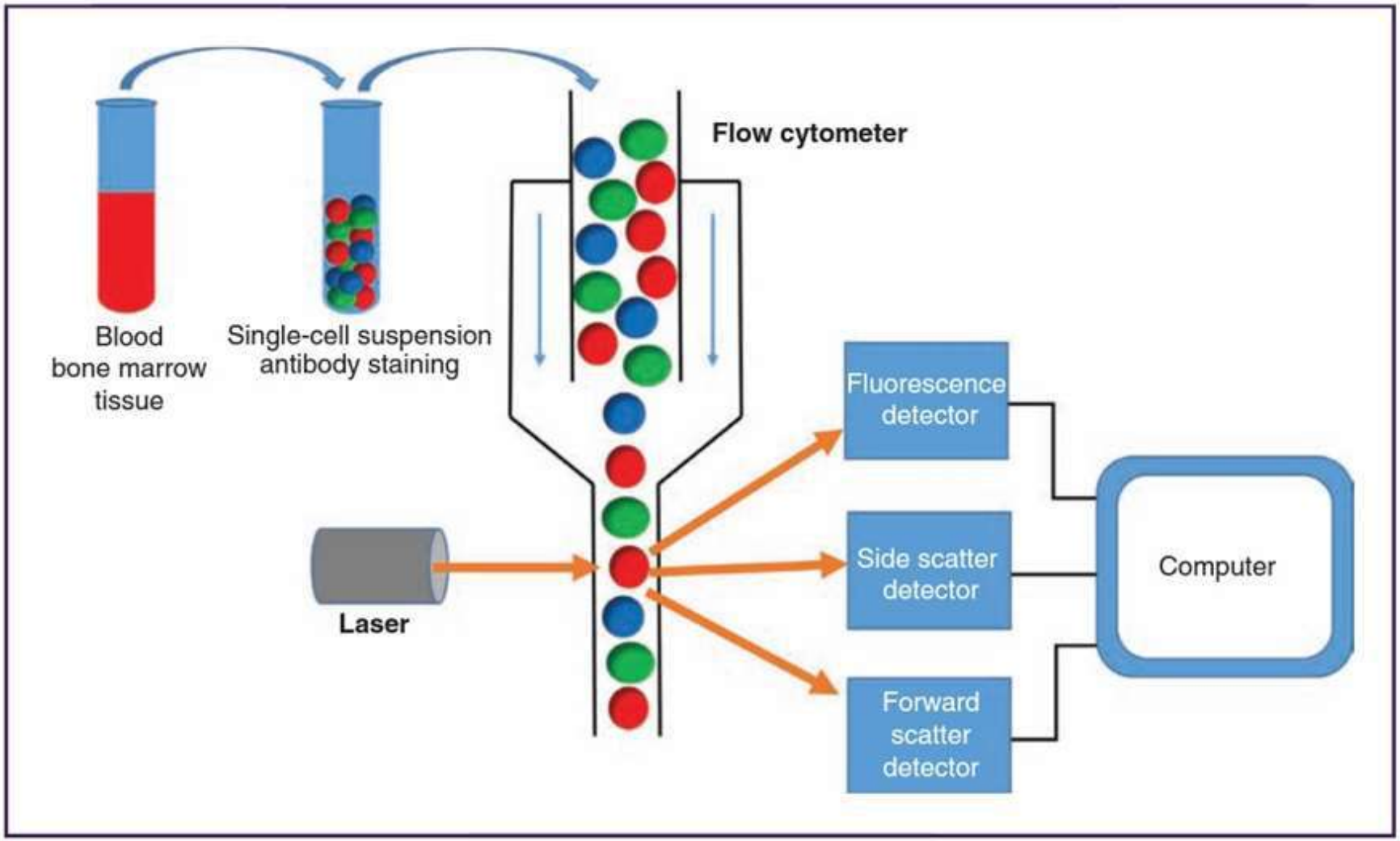


FIGURE 3: A schematic illustration of flow cytometry immunophenotyping. A single-cell suspension is prepared from blood, bone marrow aspirate, or solid tissue, and stained with fluorochrome-conjugated antibodies. The cells are then loaded on to the flow cytometer and run through a very narrow nozzle that allows the cells to go past the laser light one cell at a time.

E. Genetic analysis

- Genetic analysis is of increasing importance in haematological neoplasms, being relevant to:

(i) identification of aetiological factors;
(ii) diagnosis and classification
(iii) determination of prognosis; and
(iv) identification of MRD.

- Techniques include conventional cytogenetic analysis, fluorescence in situ hybridization (FISH), molecular genetic analysis of DNA, molecular genetic analysis of ribonucleic acid (RNA), and immunological techniques that depend on recognition of a protein encoded by a specific gene.

Sudan Black Stain

Requirements:

1. Staining Jar with a lid for fixation.
2. Formalin (10%).
3. Ethanol-absolute.
4. Ethanol (70%).
5. Hydrated Disodium Hydrogen phosphate ($Na_2HPO_4.12H_2O$).
6. Crystaline phenol.
7. Sudan black powder.
8. Leishman stain.

Preparation of reagents:

A. Phenol phosphate buffer:

- First prepare a solution of $Na_2HPO_4.12H_2O$, by dissolving 0.3 gm of its powder in 100 ml of dH_2O. and put this in a glass bottle.
- Then let crystalline phenol liquefy by heating to 68 C in a water bath, then take 16 gm of this liquefied phenol and add it on top of the solution above.
- Mix vigorously.

B. Sudan Black solution:

Dissolve 0.2 gm of Sudan black powder in 67 ml of absolute ethanol.

C. Working solution of Sudan black stain:

Add 40 mls of buffer (in A) to 60 mls of the Sudan black solution (in B). This working solution could be used for 2-3 months.

Procedure:

1. Fix air-dried slides in Formalin vapour for 10-20 minutes. This could be done easily by soaking a piece of Gauze in formalin and pitting it at the bottom of staining jar , and then put the slides in the Jar and put the lid on, so that the slides will be fixed in formalin vapour.
2. Wash in running tap water for 1 minute.
3. Allow to dry.
4. Immerse in the SB working solution prepared in C above for 1 hour.
5. Rinse in 70% ethanol for 2 minutes. This done by removing the slides from the working solution jar and immersing them in 70% ethanol solution.

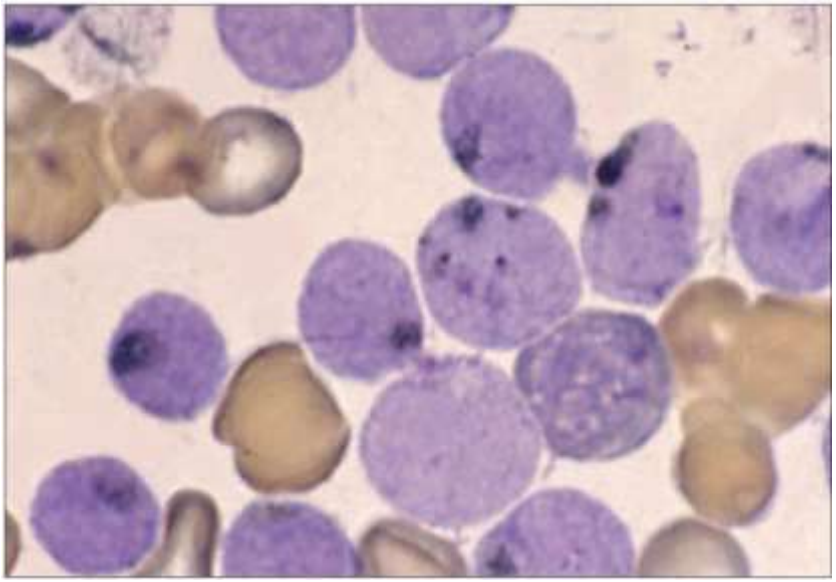
Procedure:

6. Wash in Distilled water for 2 minutes.
7. Allow to dry.
8. Counterstain with Leishman stain (2 minutes with undiluted and another 2 minutes in diluted stain).
9. Always run a normal control bone marrow with the tested one.

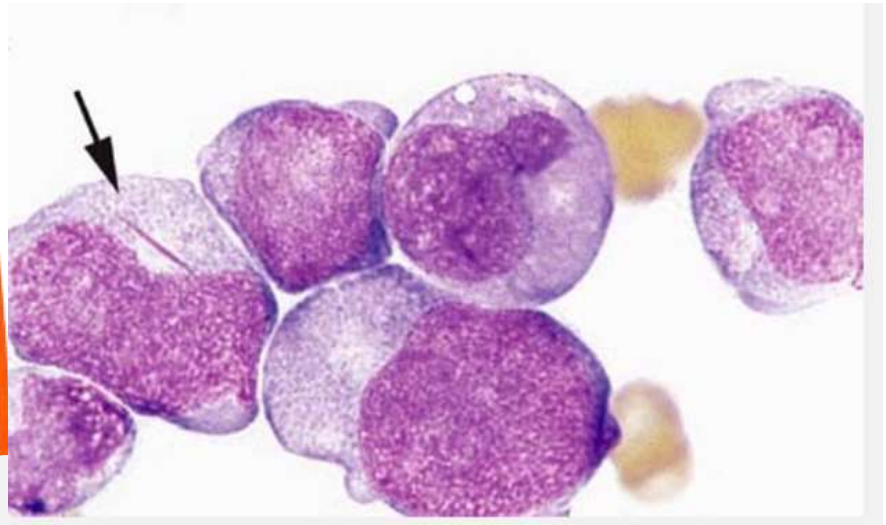
SBB staining is another useful technique for the differentiation of AML from ALL. SBB stains cellular lipids. The staining pattern is quite similar to that of MPO; SBB staining is possibly a little more sensitive for the early myeloid cells.

Interpretation

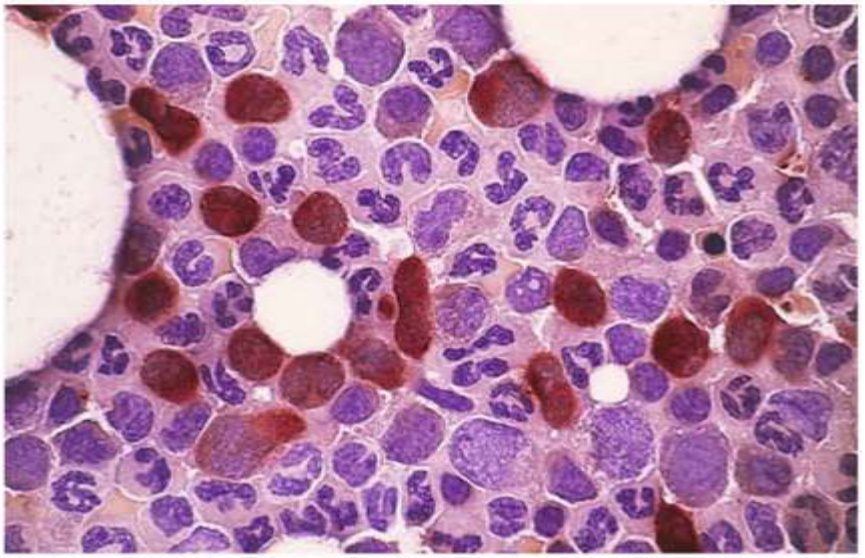
- SBB staining is another useful technique for the differentiation of AML from ALL. SBB stains cellular lipids. The staining pattern is quite similar to that of MPO; SBB staining is possibly a little more sensitive for the early myeloid cells.
- Granulocytes (neutrophils) show a positive reaction to SBB from the myeloblast through the maturation series. The staining becomes more intense as the cell matures as a result of the increase in the numbers of primary and secondary granules. Monocytic cells can demonstrate negative to weakly positive staining due to various changes that occur during differentiation. Lymphoid cells generally do not stain. In ALL, fewer than 3% of the blast cells show a positive reaction.



Bone marrow aspirate film from a patient with FAB M1 AML showing Sudan black B activity that identifies the blast cells as myeloid



Auer rod (cytoplasmic needlelike red crystal) (arrow) in AML



Nonspecific esterase reaction clearly differentiates the monocytes

Med. Lab.Tec.Department

Practical hematology
Lecture 11

3ed stage

Acidified Serum Lysis Test
(HAM Test)

Lecturer

Muqdad M Najem

FIBMS -Haematopathology

and

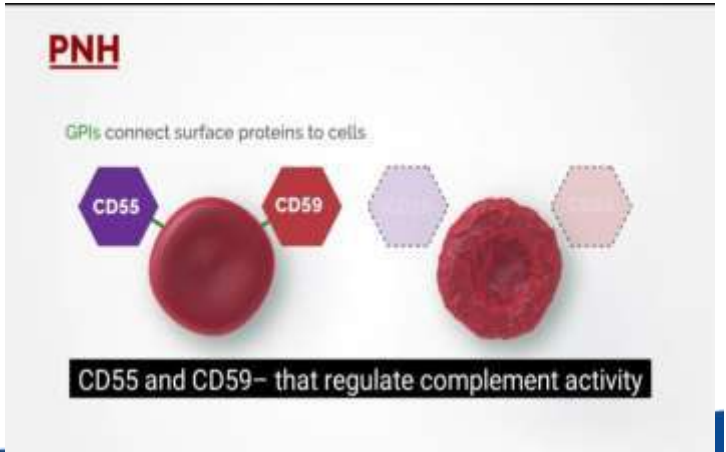
Asst.Lec. Iman.H. Jirjees

Introduction:

The Ham's test is used to confirm a diagnosis of paroxysmal nocturnal hemoglobinuria (PNH) :

PNH is an acquired clonal disorder resulting from a somatic mutation occurring in a haemopoietic stem cell.

The characteristic feature of cells belonging to the PNH clone is that they are deficient in several cell-membrane–bound proteins including red cell : CD55, CD59, that have roles in the protection of the cell against complement mediated attack.



PRINCIPLES:

The patients red cells are exposed at 37c to the action of normal or the patient own serum suitably acidified to the optimum PH for lysis.

The patient's serum is acidified using 0.2N HCl. A 50% solution of the patient's cells is added to tubes containing the patient's acidified serum, unacidified serum, and normal ABO-compatible serum. A normal red cell control is run. Normal red cells will not hemolyze, but cells from patients with PNH will hemolyze with acidified serum from the patient and from normal ABO compatible serum.

SAMPLES:

1. **Red cells of the patient: obtained from defibrinated blood, heparinized, oxalated, citrated, or EDTA blood** ***(test can be carried out satisfactorily on cells which have been stored at 4c for up to 2-3 weeks in ACD or Alsever's solution if kept sterile*****).**

2. **Serum of patient: best obtained by defibrination. (*Because in PNH patient if it is obtained from blood allowed to clot in ordinary way at 37 c, it will almost certainly be markedly lysed*).**

3. **Normal serum: should be obtained by defibrination. Serum derived from blood allowed to clot spontaneously at room temperature or at 37c can be used.**

N.T:

- **Normal serum known to be strongly lytic to PNH cells is to be preferred to patient serum, the lytic potentiality of which is unknown.**

- **HAM test is positive in patient with HEMPAS (Hereditary Erythroblastic multinuclearity) associated with a positive acidified-serum test only if normal plasma is used and no if patient plasma is used.**

- **Serum should be used within few hours of collection; their lytic potency is retained for several months at -70c, but at 4c and even at-20c deteriorate within days.**

METHOD:

1. **Deliver 0.5ml of fresh normal serum group AB or ABO compatible with the patient blood into 6 glasses test tubes.**
2. **Place 2 tubes (tubes no.3) at 56c for 10-30 minutes to inactivate complements and keep other tubes at room temperature.**
3. **Add to 2 tubes (tubes no.2) 0.05ml of 0.2mol/l of HCL ,and 0.05ml of HCL to tubes no.3**
4. **At the same time wash samples of patient RBC and normal control RBC twice and prepare 50% suspensions in normal saline of each sample.**
5. **Add 0.05 ml of patient RBC suspension to tubesT1,T2,T3 and of normal control RBC suspensions to tubes C1,C2,C3**

METHOD:

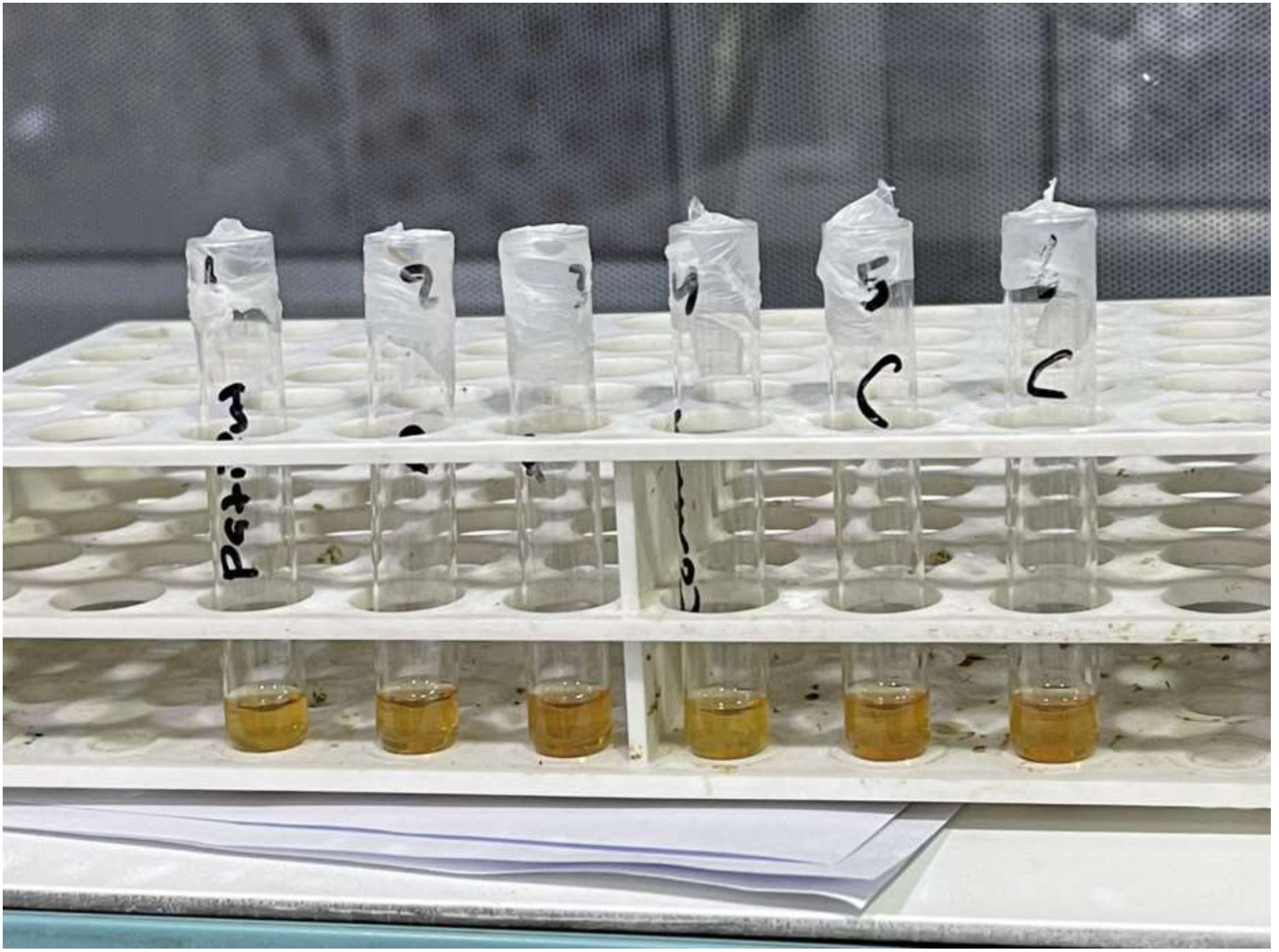
6. Mix the tubes well and put them at 37c in the waterbath for 1 hr.
7. After 1 hr centrifuge the tubes at 2000-3000rpm for 5 minutes.
8. Add 0.05 ml of each cell suspensions to 0.55ml D.W. to prepare standard for subsequent quantitative measures of serum.
9. Retain 0.5 ml of serum to be used as a blank.
10. Deliver 0.3 ml of supernatants of each cell lines (T1, T2, T3, C1, C2 and C3) and of the blank and 100% lysed cell suspensions into tubes containing each 5ml of 0.4ml/l Ammonia or Drabkin's reagent.
11. Measure absorbance in each tube by spectrometer at wave length 540nm and then estimate % of lysis of each sample.

Reagent	Test(ml)			Control(ml)		
	T1	T2	T3	C1	C2	C3
Fresh normal serum	0.5	0.5	0	0.5	0.5	0
Heat inactivated normal serum	0	0	0.5	0	0	0.5
0.2 mol/l HCL	0	0.05	0.05	0	0.05	0.05
50% patient RBC	0.05	0.05	0.05	0	0	0
50% normal RBC	0	0	0	0.05	0.05	0.05
MgCl (250 mmol/l:23.7g/l)	0.01	0.01	0.01	0.01	0.01	0.01
Lysis(in a positive modified test)	Trace (2%)	+++ (30%)	-	-	-	-

RESULTS:

1. **RBC from patient with PNH will undergo definite although incomplete lysis in acidified serum (tube-2).**
2. **No lysis or match less lysis will be visible in the inacidified serum (tube-1).**
3. **No lysis will be brought about by acidified inactivated serum (tube-3).**
4. **Normal control samples of cells should not undergo lysis in any of 3 tubes.**
5. **In PNH 10-15% lysis is usually obtained when lysis is measured as librated Hb. Exceptionally there may be as match as 80% lysis or as little as 5%.**


Parameter values
Scales



N.T.:

- In PNH it is characteristic that young cells (reticulocyte-rich) population such as in upper RBC layer obtained by centrifugation undergoes more lysis than the RBC derived from mixed whole blood.
- The sensitivity of HAM test can be improved by the addition of magnesium to the test to enhance the activation of complement.
- Addition of 10uL of 250mmol MgCl (final concentration=4mmol) to each tube prior to incubation.

Thanks
For
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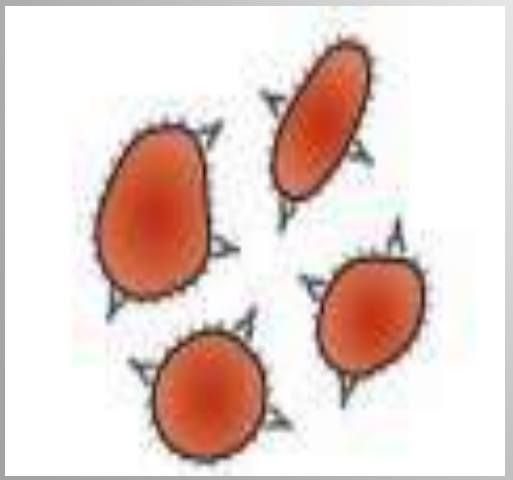


Med. Lab.Tec.Department

PRACTICAL HEMATOLOGY

LECTURE TEN

DIRECT ANTIGLOBULIN TEST

Asst.Lec.Iman. H. Jirjees

Introduction and principles

- The antihuman globulin test, which is also referred to as the Coombs test, is based on the principle that antihuman globulins (AHGs) obtained from immunized nonhuman species bind to human globulins such as IgG or complement, either free in serum or attached to antigens on red blood cells (RBCs).
- The DAT should be performed on every patient to detect the presence of hemolysis has been established to distinguish immune from non-immune hemolytic anemia.
-

The DAT is used primarily for the investigation of

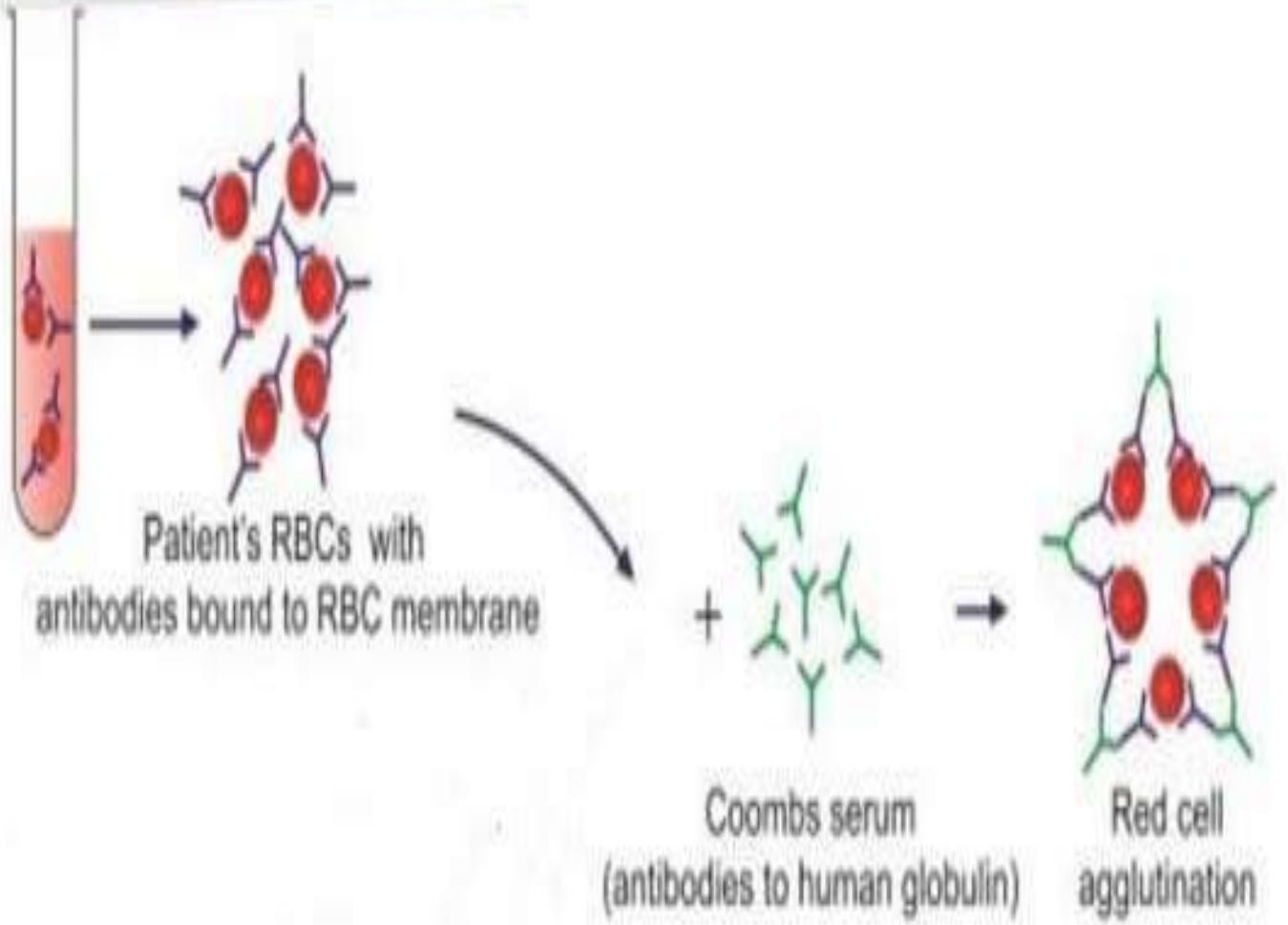
1. hemolytic transfusion reactions.
2. hemolytic disease of the fetus and newborn (HDFN).
3. autoimmune hemolytic anemia (AIHA).
4. drug-induced immune hemolysis.

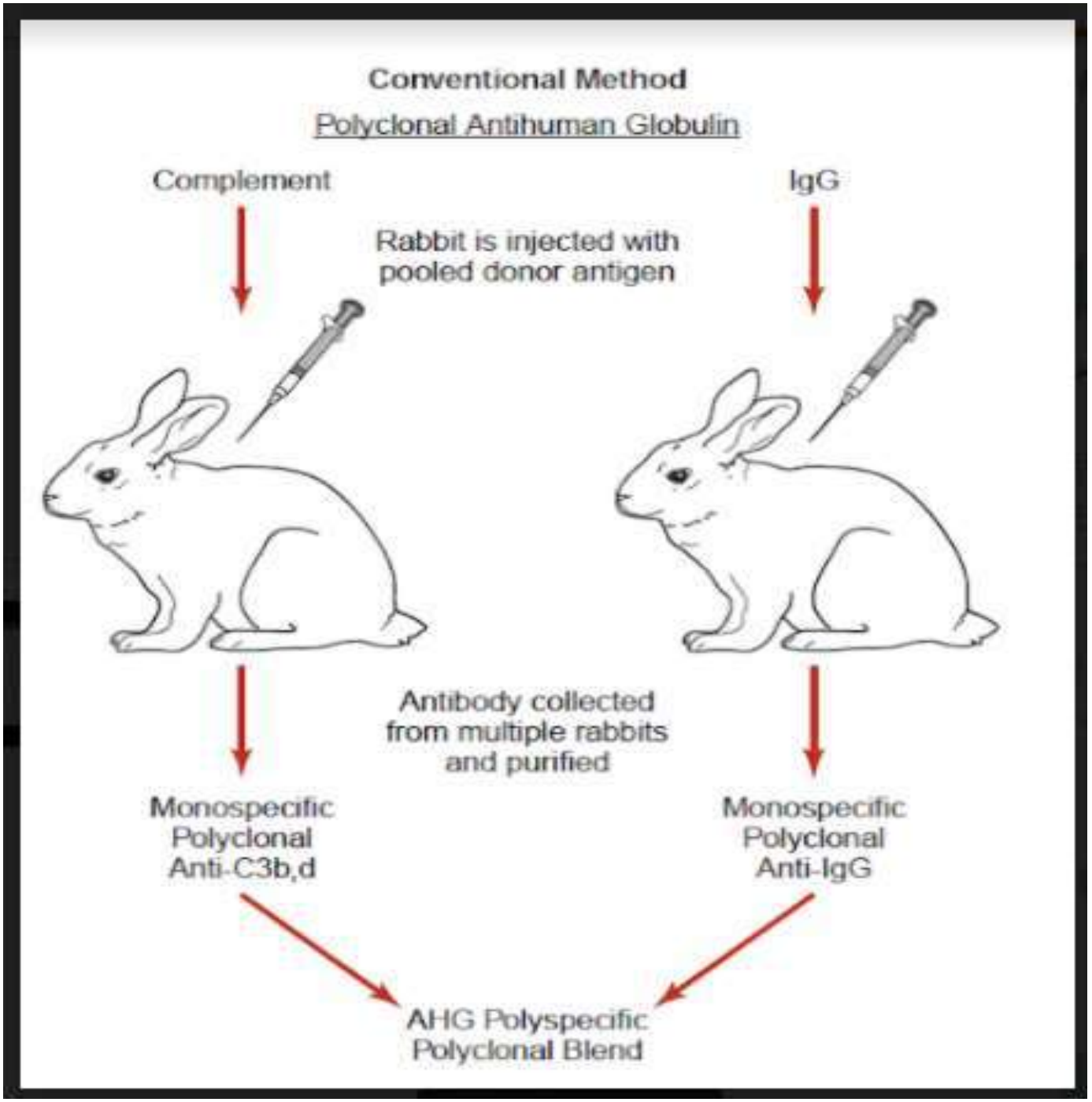
THE PRINCIPLES OF THE DAT

- The DAT is based on the detection of antibodies attached to red cells that do not produce direct agglutination. Most of the antiglobulin reactivity is directed at the heavy chains or the complement component, thus bridging the gap between adjacent red cells to produce visible agglutination. The strength of the observed agglutination is usually proportional to the amount of bound protein.

- The DAT is performed by testing freshly washed red cells directly with antiglobulin reagents containing anti-IgG and anti-C3d. Polyspecific anti-IgG,-C3d and monospecific anti-IgG or anti-C3d can be used.

Direct antiglobulin test (DAT) (Coombs test)

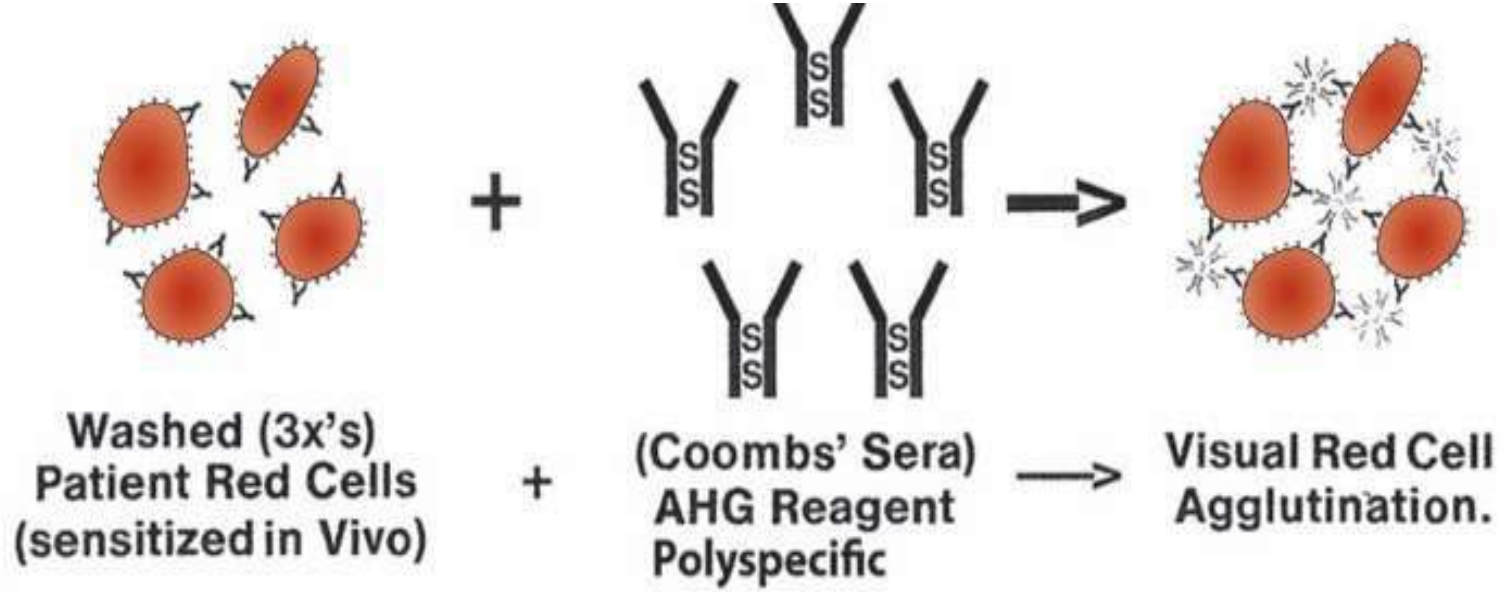



Conventional Method
Polyclonal Antihuman Globulin
Complement
IgG
Rabbit is injected with pooled donor antigen
Antibody collected from multiple rabbits and purified
Monospecific Polyclonal Anti-C3b,d
Monospecific Polyclonal Anti-IgG
AHG Polyspecific Polyclonal Blend

- The red cells need to be washed to remove free plasma globulins and complement; otherwise, the antiglobulin reagent can be neutralized, leading to a false-negative result.
- The saline used for washing the red cells should be at room temperature; washing red cells with warm (e.g, 37° C) saline can result in the loss of red-cell-bound, low-affinity IgG.

- The red cells should be tested immediately after washing to prevent false-negative results due to the elution of IgG. Although any red cells may be tested, EDTA-anticoagulated blood samples are preferred. The EDTA prevents in vitro fixation of complement by chelating the calcium that is needed for C1 activation.

- The DAT can be initially performed with a polyspecific antihuman globulin (AHG) reagent that is capable of detecting both IgG and C3d. If the results are positive, tests with monospecific reagents (anti-IgG and anticomplement) need to be performed to appropriately characterize the immune process involved and determine the diagnosis.



METHODS AND MATERIALS

- Whole blood sample.
- Test tubes.
- Pipettes .
- Saline.
- Centrifuge.
- Antiglobulin reagent (polyspecific).

PROCEDURE

1. Dispense 1 drop of a 2% to 5% suspension of red cells into tube.
2. Wash tube three or four times with saline. Completely decant the final wash.
3. Immediately add (1-2) drops of polyspecific AHG serum and mix. Centrifuge for 10–60 s.

RCF (*g*)	100	200–220	500	1000
Time (s)	60	25–30	15	8–10

- Examine for agglutination after gently resuspending the button of cells. A concave mirror and good light help in macroscopic readings. If the result appears to be negative, confirm this microscopically.

Interpretation

- 1.The DAT is positive when agglutination is observed after centrifugation. Monospecific AHG reagents are needed to confirm which globulins are present.

- 2. The DAT is negative when no agglutination is observed.


Thanks
for
listening

Med. Lab. Tec.Department

3ed stage

Practical hematology

Lecture Nine

Erythrocyte Inclusions (Red Cell Inclusions)

Asst. Lec. Iman. H. Jirjees

Introduction and Principles:

- The cytoplasm of all red cells is free of debris, granules ,or other structures. Inclusions that find their way into the cytoplasm are the result of distinctive conditions .This section summarizes four of the most common red cell inclusions Howell-Jolly bodies, siderotic granules/Pappenheimer bodies, basophilic stippling ,and Heinz bodies.

Objectives:

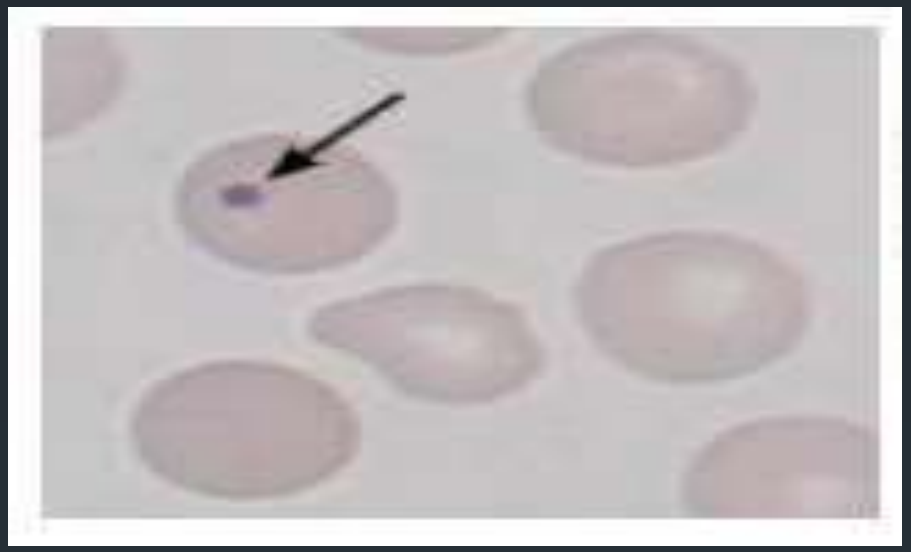
- At the end of this lecture the students will be able to

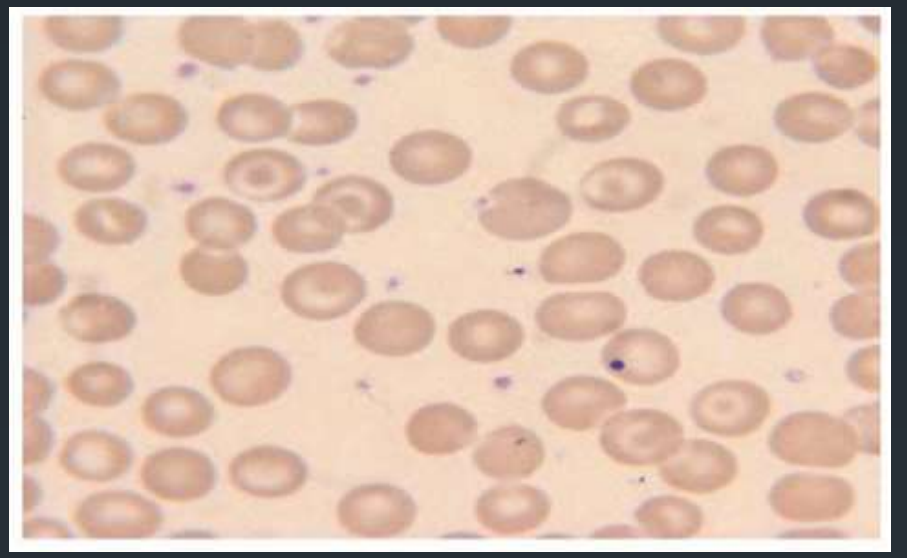
1. Identify to the types of red cell inclusions.
2. Differentiate between them.
3. The significant condition of each type.

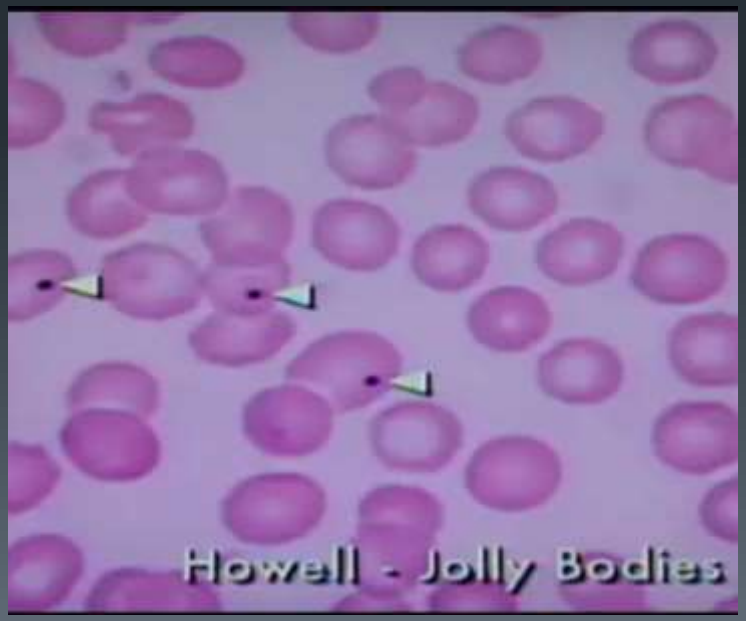
Types of Inclusions:

- **Howell-Jolly bodies:**
- are remnants of DNA that appear in the red cell as round, deep purple, non deformable structures 1 to 2 μm in size. They are eccentrically located in the cytoplasm and are seen when erythropoiesis is rushed. The spleen usually pits these inclusions from the cytoplasm, yet when the bone marrow is responding to anemic conditions, the spleen cannot keep pace with Howell-Jolly body formation. They appear in the blood following splenectomy. They can be a normal finding in neonates (in whom the spleen is functionally immature).

Howell-Jolly bodies





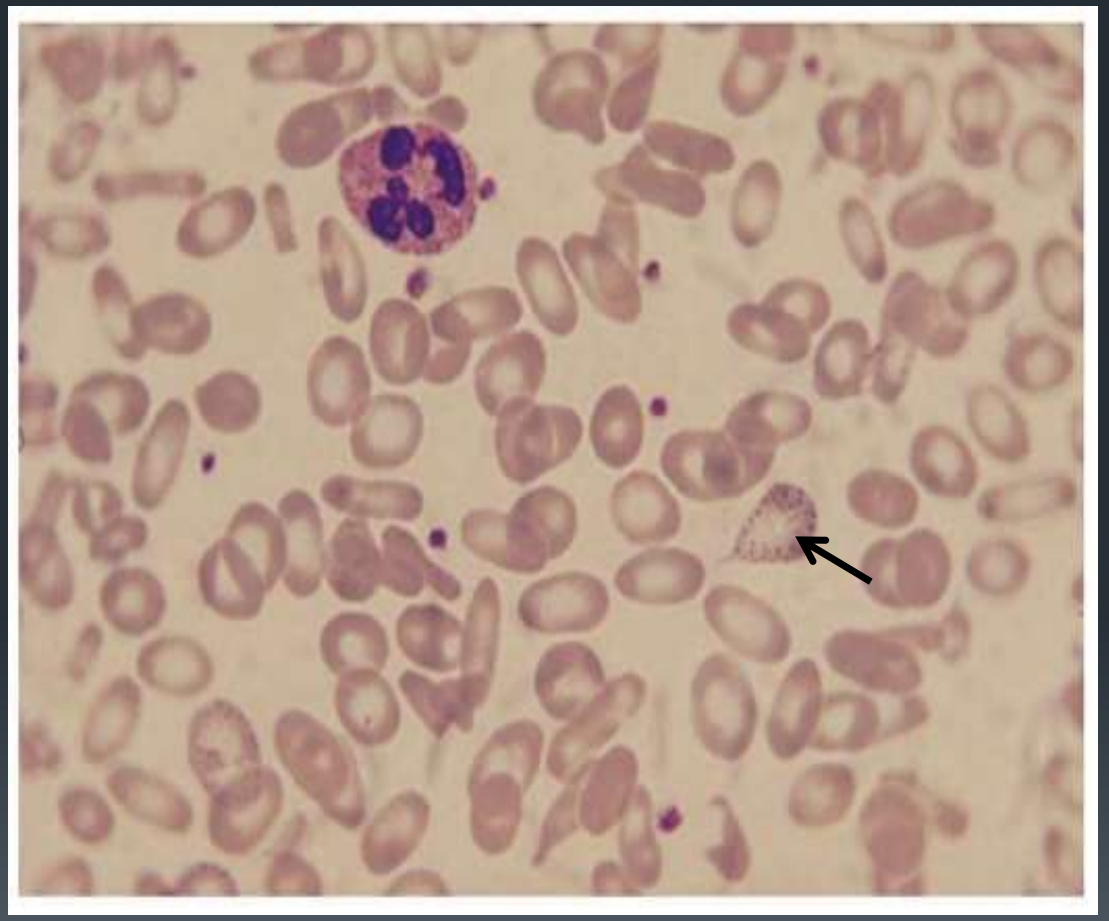


Basophilic stippling

- is a result of RNA and mitochondrial remnants. These remnants appear as diffusely basophilic granules located throughout the cytoplasm and are either dustlike or coarse in appearance.
- Increased numbers are seen in:

- Thalassemia trait and major
- Hemolytic anemia
- Megaloblastic anemia
- Heavy metal poisoning (coarse basophilic stippling) :Lead, zinc, arsenic, silver, mercury.

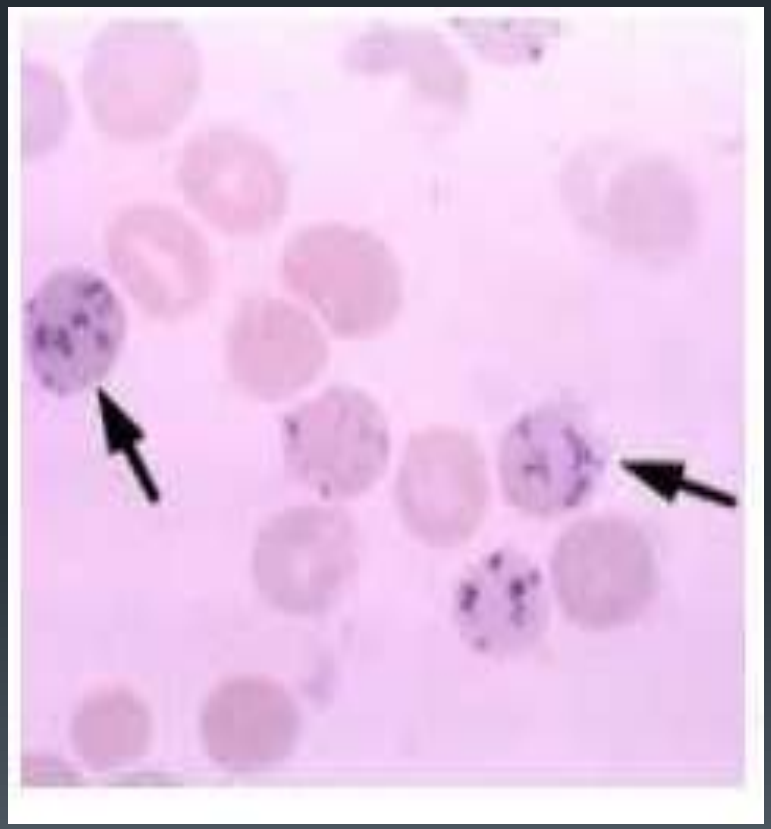
Basophilic stippling

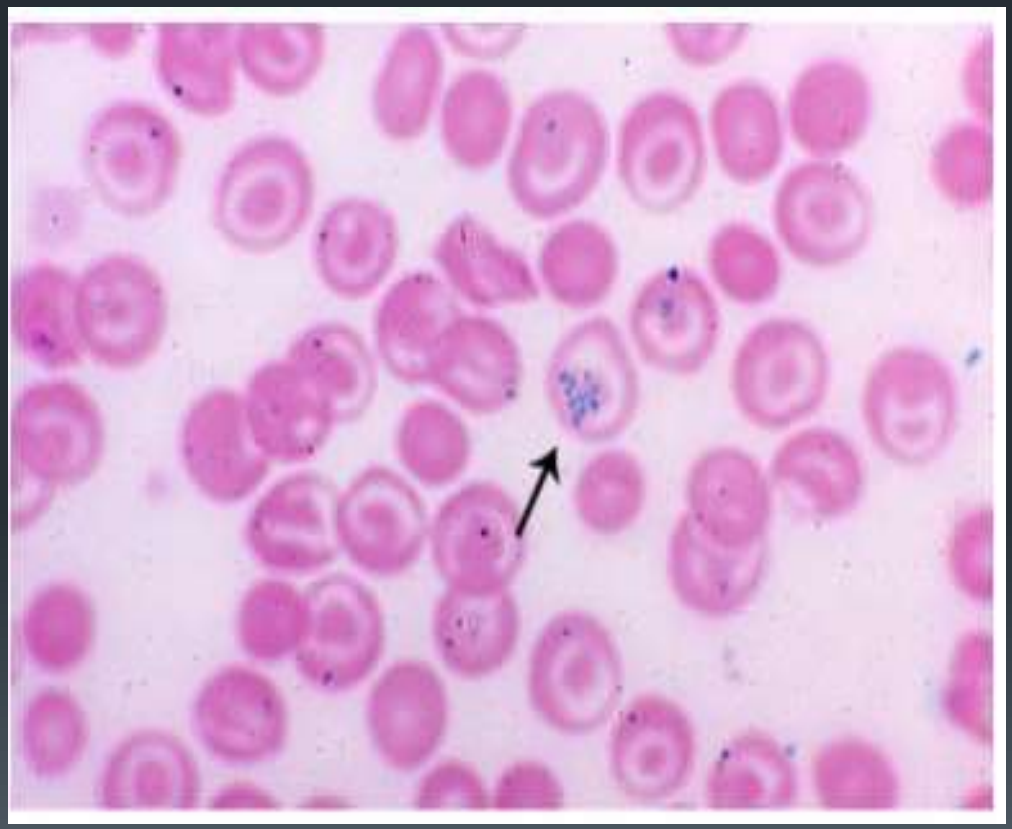


Siderotic granules/Pappenheimer bodies:

- Are seen in the iron loading processes such as hereditary hemochromatosis and iron loading anemias subsequent to transfusion therapy. They appear as small beaded inclusions, light purple and located along the periphery of the red cells. Siderotic granules may also be viewed in thalassemic conditions and in patients post splenectomy.

Siderotic granules/Pappenheimer bodies:



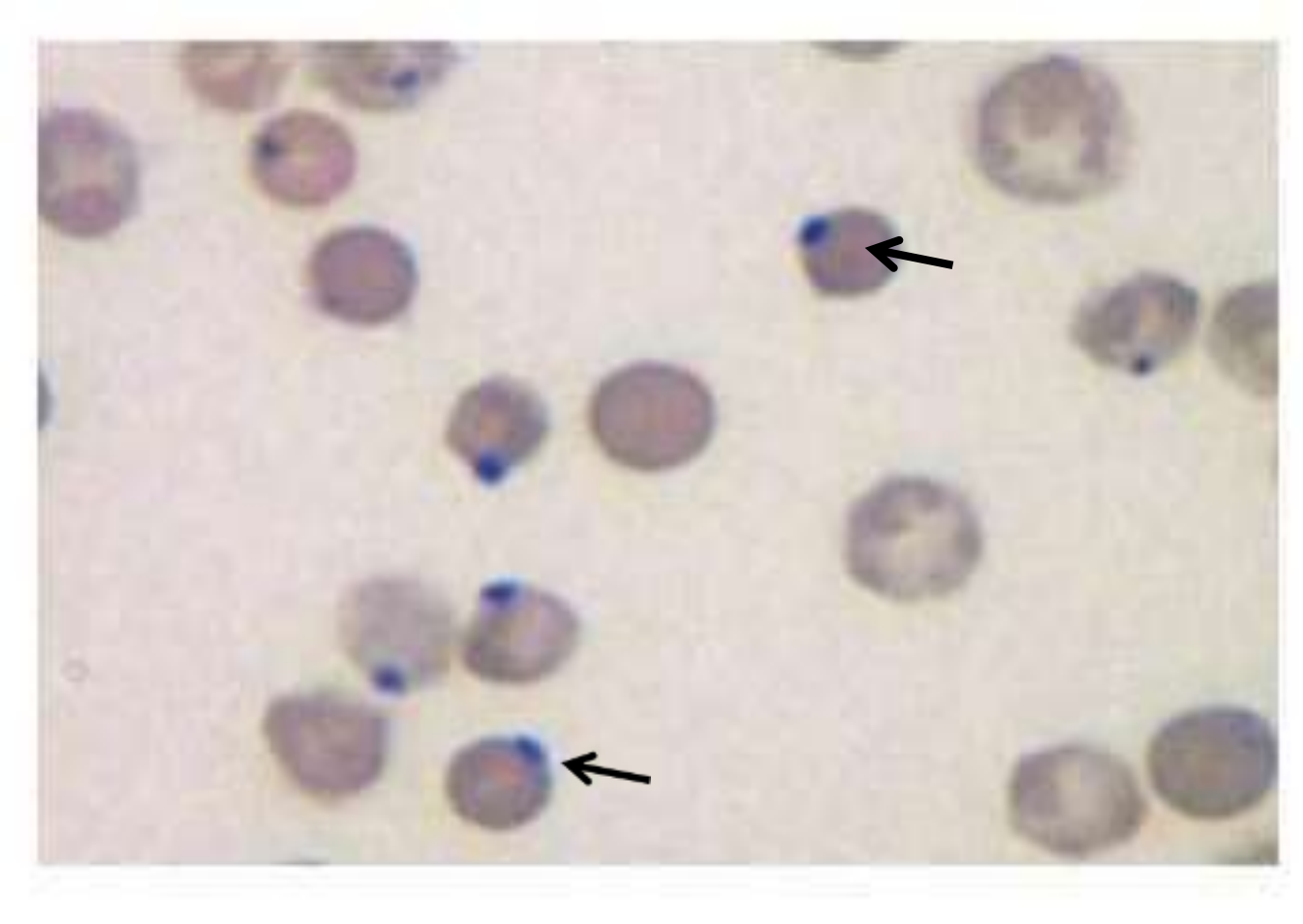


Heinz bodies:

- result from denatured hemoglobin and are defined as large structures approximately 1 to 3μm in diameter located toward the periphery of the red cell membrane. To visualize the actual Heinz body inclusion, staining with a supravital stain such as new methylene blue. Heinz bodies are seen in:

- Oxidative stress like glucose-6-phosphate dehydrogenase deficiency, glutathione synthetase deficiency.
- Unstable hemoglobins.

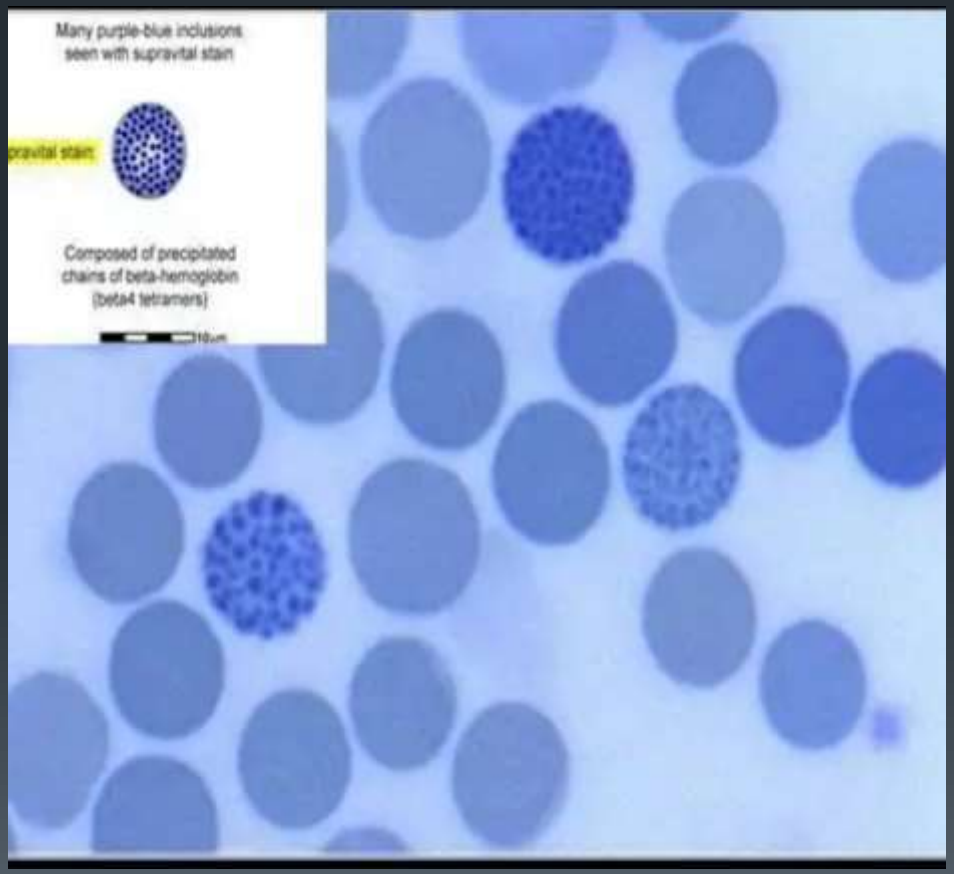
Heinz bodies

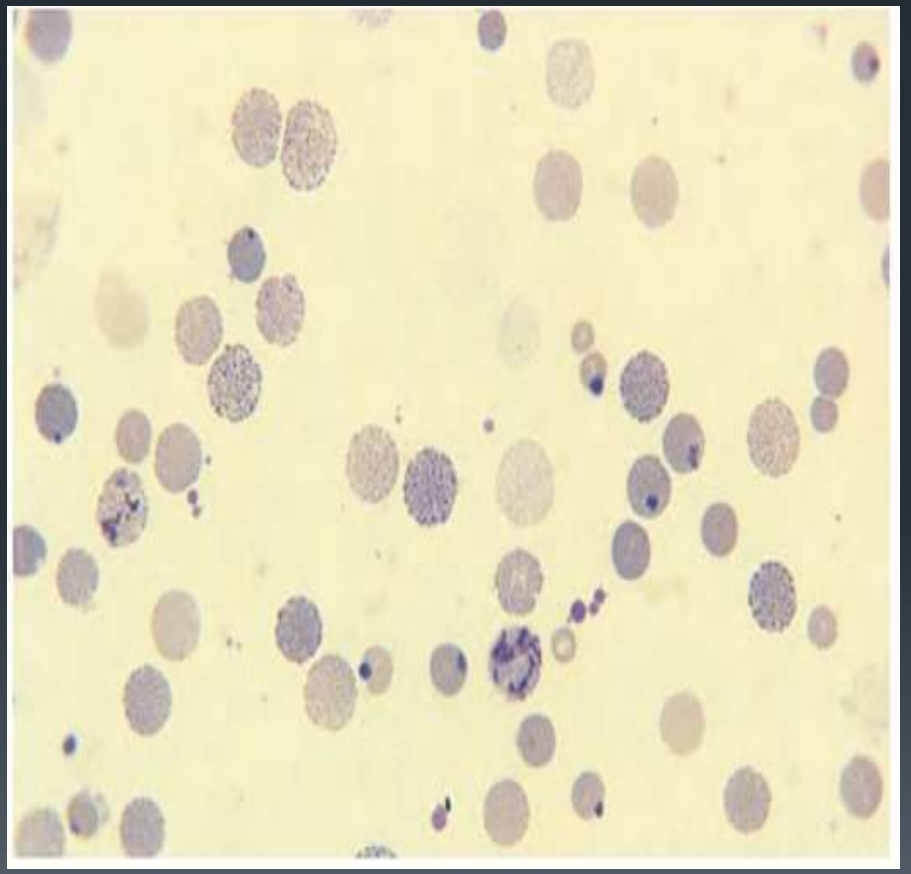


Hemoglobin H (golf ball):

- is composed of 4 beta tetramers, indicating that chains are present in excess as a result of impaired alpha -chain production. Exposure to methylene blue, or new methylene blue, results in denaturation and precipitation of abnormal hemoglobin. Brilliant cresyl blue causes the formation of a large number of small membrane-bound inclusions, giving the cell a characteristic "golf ball–like" appearance when viewed by light microscopy. These changes are seen most frequently in alpha -thalassemia but also can be found in:
- Hb H disease.
- Acquired Hb H disease (myeloproliferative, myelodysplastic).

Hemoglobin H (golf ball):



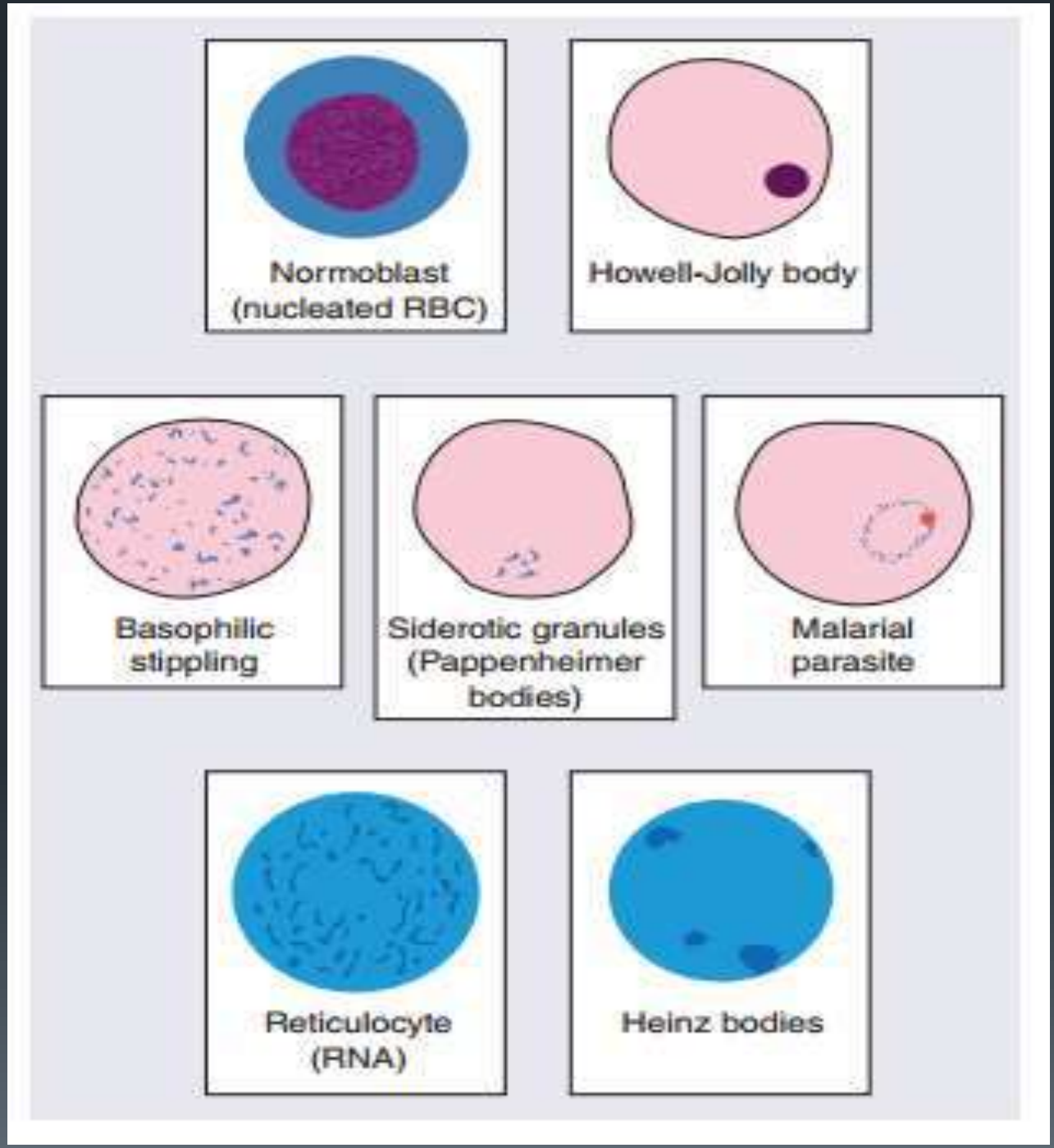


Summary of Inclusions

Summary of Inclusions	
Inclusion	**Composition**
Howell-Jolly body	DNA in origin
Basophilic stippling	RNA remnants
Siderotic granules/ Pappenheimer bodies	Iron
Heinz bodies	Denatured hemoglobin
Hb H body (supravital only)	β-Globin tetramers (β4)

Common RBC Inclusions	Cartoon Image	Inclusion	May be associated with
Howell Jolly Bodies		DNA	Hyposplenism Asplenism Severe hemolytic anemia
Heinz Bodies	 *Supravital stain*	Hemoglobin	G6PD deficiency Oxidant drugs Unstable hemoglobin
Pappenheimer Bodies		Iron deposits	Thalassemia Sideroblastic anemia Hemolytic anemia Post-splenectomy
Hemoglobin H Inclusion	 *Supravital stain*	Hemoglobin	Hemoglobin H disease
Basophilic Stippling		Ribosomes	Lead poisoning Thalassemia Sickle cell anemia MDS

Rashidi H MD, Yee N, Nguyen J MD et al. HematologyOutlines.com


Normoblast
(nucleated RBC)
Howell-Jolly body
Basophilic
stippling
Siderotic granules
(Pappenheimer
bodies)
Malarial
parasite
Reticulocyte
(RNA)
Heinz bodies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Med. Lab. Tec.Department

Practical hematology
Lecture 8

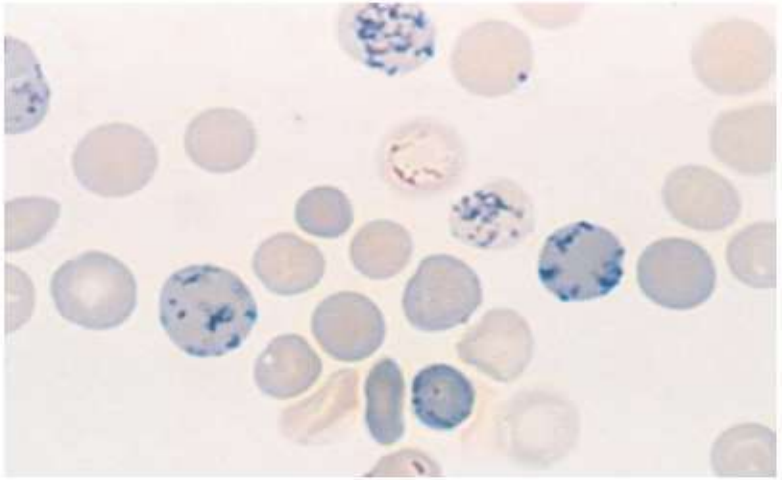
3ED STAGE

RETICULOCYTE COUNT

Lecturer
Asst.Lec. Iman.H. Jirjees

RETICULOCYTE COUNT

- The reticulocyte is the last immature red blood cell stage . Normally, a reticulocyte spends 2 days in the bone marrow and 1 day in the peripheral blood before developing into a mature red blood cell. The reticulocyte contains remnant cytoplasmic ribonucleic acid (RNA) and organelles such as the mitochondria and ribosomes of blue stained granulofilamentous material after new methylene blue, (fig 3). The reticulocyte count is used to assess the erythropoietic activity of the bone marrow.



Reagents and Equipment

1. New Methylene Blue (Supravital Stain).
2. Test tubes
3. Microscope slides.
4. Microscope with(100 X oil immersion objective).
5. Transfer pipettes.

PROCEDURE

1. Mix equal amounts of blood and new methylene blue stain 2 to 3 drops, or approximately 50 µL each), and allow to incubate at room temperature for 3 to 10 minutes.
2. Remix the preparation.
3. Prepare two wedge films.
4. In an area in which cells are close together but not touching ,count 1000 RBCs under the oil immersion objective lens1000X total magnification). Reticulocytes are included in the total RBC count (i.e., a reticulocyte counts as both an RBC and a reticulocyte).

5. To improve accuracy, have another laboratorian count the other film; counts should agree within 20%.
6. Calculate the % reticulocyte count:

$$\text{Reticulocytes (\%)} = \frac{\text{number of reticulocytes} \times 100}{1000 \text{ (RBCs counted)}}$$

For example, if 15 reticulocytes are counted,

$$\text{Reticulocytes (\%)} = \frac{15 \times 100}{1000} = 1.5\%$$

Sources of Error and Comments

1. If a patient is very anemic or polycythemic, the proportion of dye to blood should be adjusted accordingly.
2. An error may occur if the blood and stain are not mixed before the films are made. The specific gravity of the reticulocytes is lower than that of mature red blood cells, and reticulocytes settle at the top of the mixture during incubation.
3. Moisture in the air, poor drying of the slide, or both may cause areas of the slide to appear refractile, and these areas could be confused with reticulocytes. The RNA remnants in a reticulocyte are not refractile.

4. Other red blood cell inclusions that stain supravitally include Heinz, Howell-Jolly, and Pappenheimer bodies. Heinz bodies are precipitated hemoglobin ,usually appear round or oval, and tend to adhere to the cell membrane . Howell-Jolly bodies are round nuclear fragments and are usually singular. Pappenheimer bodies are iron in the mitochondria whose presence can be confirmed with an iron stain, such as Prussian blue (fig 4).

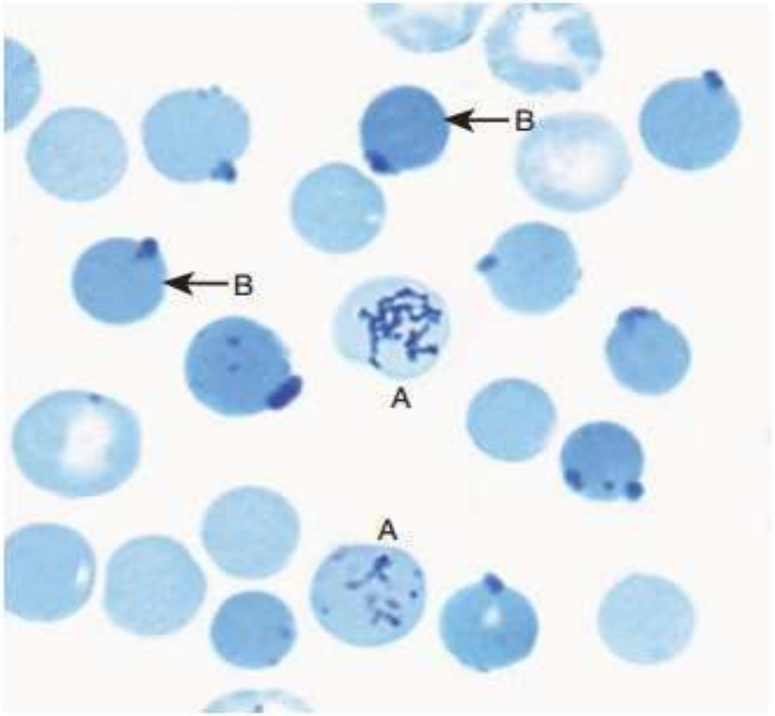


Figure (4) Reticulocytes (A)and Heinz bodies (B)stained with supravital stain (peripheral blood X1000).

Conditions Associated With..

Decreased reticulocyte count	Increased reticulocyte count
Aplastic anemia	hemorrhagic
Iron deficiency anemia	hematonic
Sideroblastic anemia	Hemolytic

The normal value:

0.5-2.5%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Med. Lab.Tec.Department

PRACTICAL PHYSIOLOGY

LECTURE SEVEN

3ed stage

Erythrocyte Sedimentation Rate

Prepared by Asst. Lec. Iman. H. Jirjees

Modified Westergren Erythrocyte Sedimentation Rate

- The ESR is the rate at which erythrocytes settle out of anticoagulated blood in 1 hour. This test is based on the fact that inflammatory and necrotic processes cause an alteration in blood proteins, resulting in aggregation of RBCs, which makes them heavier and more likely to fall rapidly when placed in a special vertical test tube.

The settling or sedimentation of red cells in a sample of anticoagulated blood occurs in 3 stages:

i. In the first stage, the RBCs pile up (like a stack of coins), and form rouleaux that become heavier during the first 10-15 minutes.
ii. During the second stage, the rouleau (pleural of rouleaux) being heavier (see below) sink to the bottom. This stage lasts for 40–45 minutes.
iii. In the third stage, there is packing of massed bunches of red cells at the bottom of the blood column. This stage lasts for about 10–12 minutes.

Objectives:

- Upon completion of this session the students will be able to:

1. Estimate the ESR of a given blood sample.
2. Count the Reticulocyte.
3. Indicate the significance condition of each test.

Materials and Instruments:

1. 3.8% sodium citrate as a diluting solution.
2. 3 ml of whole anticoagulated blood. (EDTA as anticoagulant).
3. Westergren's pipette (A straight glass pipette 30 cm long and 2.55 mm diameter, graduated from 0-200 and open at both ends).
4. Westergren's pipette rack (Fig.1).
5. Glass pipettes 2 ml and 0.5 ml.
6. Plain test tubes.
7. Disposable syringe, alcohol prep.

Procedure

1. Use well-mixed blood collected in EDTA and dilute at four parts blood to one part 3.8% sodium citrate (e.g., 2 mL blood and 0.5 mL diluent). Alternatively, blood can be collected directly into special sedimentation test tubes containing sodium citrate. Standard coagulation test tubes are not acceptable ,because the dilution is nine parts blood to one part sodium citrate.
2. Place the diluted sample in a 200-mm column with an internal diameter of 2.55 mm or more.

1. Place the column into the rack and allow to stand undisturbed for 60minutes at room temperature(18 to 25° C) .Ensure that the rack is level.
2. Record the number of millimeters the red blood cells have fallen in 1 hour. The buffy coat should not be included in the reading. Read the tube from the bottom of the plasma layer to the top of the sedimented red blood cells (Fig.2). Report the result as the ESR, 1 hour x mm.

Figure (2) Erythrocyte sedimentation rate (ESR), 1 hour =93 mm which is elevated above the reference intervals.

Sources of Error and Comments

1. If the concentration of anticoagulant is increased, the ESR will be falsely low as a result of sphering of the RBCs, which inhibits rouleaux formation.
2. The anticoagulants sodium or potassium oxalate and heparin cause the red blood cells to shrink and falsely elevate the ESR.
3. A significant change in the temperature of the room alters the ESR.
4. Even a slight tilt of the pipette causes the ESR to increase.

Sources of Error and Comments

5. Blood specimens must be analyzed within 4 hours of collection if kept at room temperature (18 to 25° C). If the specimen is allowed to sit at room temperature for more than 4 hours, the red blood cells start to become spherical, which may inhibit the formation of rouleaux. Blood specimens may be stored at 4° C up to 24 hours prior to testing, but must be rewarmed by holding the specimen at ambient room temperature for at least 15 minutes prior to testing.
6. Bubbles in the column of blood invalidate the test results.
7. The blood must be filled properly to the zero mark at the beginning of the test.

TABLE (1) FACTORS AFFECTING THE ERYTHROCYTE SEDIMENTATION RATE (ESR)

Category	Increased ESR	Decreased ESR
Blood proteins and lipids	• Hypercholesterolemia • Hyperfibrinogenemia	• Increased bile salts • Increased phospholipids • Hyperglycemia
Red blood cells	• Anemia • Macrocytosis	• Polycythemia • Sickle cells • Spherocytosis • Thalassemia
White blood cells	• Leukemia	• Leukocytosis (marked)
Drugs	• Heparin • Penicillamine • Theophylline • Vitamin A	• Adrenocorticotropic hormone (corticotropin) • Cortisone • Salicylates
Clinical conditions	• Acute heavy metal poisoning • Acute bacterial infections • Malignancy • Multiple myeloma • Pregnancy • Rheumatoid arthritis	• Cachexia
Specimen handling	• Refrigerated sample not returned to room temperature	• Clotted blood sample Delay in testing
Technique	• High room temperature • Tilted ESR tube • Vibration	• Bubbles in ESR column • Low room temperature • Narrow ESR column diameter

The normal value

Age	17-50 years		51-60 years		61-70 years		>70 years	
Sex	**Men**	**Women**	**Men**	**Women**	**Men**	**Women**	**Men**	**Women**
ESRvalue	≤ 10	≤ 12	≤ 12	≤ 19	≤ 14	≤ 20	≤ 30	≤ 35


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Med. Lab. Tec.Department

Practical hematology

Lecture 6

3ed stage

Platelet Count

Asst.Lec. Iman. H. Jirjees

Introduction and Principle:

- Platelets are non-nucleated blood cells, minute round or oval discs 2-4μm in diameter. They are formed in the bone marrow from megakaryocytes, which are extremely large cells of the hemopoietic series in the bone marrow that fragment into platelets either in the bone marrow or soon after entering the circulating blood.

Introduction and Principle:

- A platelet count is the number of platelets in 1 liter (L) or 1 microliter (µL) of whole blood. Platelets adhere to foreign objects and to each other, which makes them difficult to count .They also are small and can be confused easily with dirt or debris.

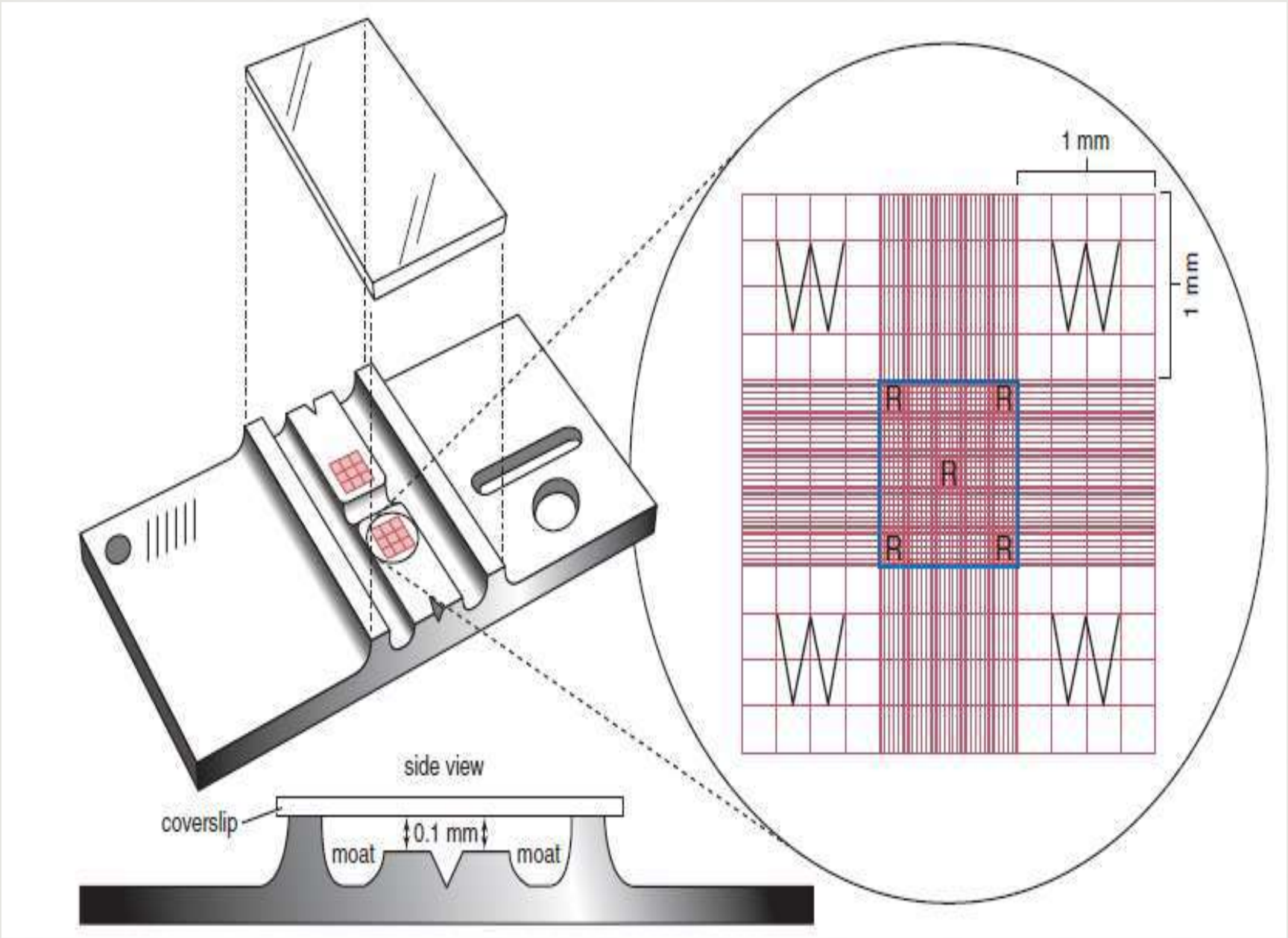
Objectives:

At the end of this session the students must be able:

- 1. To determine the platelet count of a provided blood sample.
- 2. Indicate the significance of any abnormal result.

Materials and Instruments:

1. Whole fresh blood with EDTA as anticoagulant.
2. Hemocytometer .
3. Cover slip.
4. Micropipette.
5. Dilution fluid (Ammonium oxalate 1%).
6. Petri dish.
7. Filter paper.
8. Microscope.


1 mm
1 mm
R
R
R
R
R
side view
coverslip
0.1 mm
moat
moat

Procedure:

1. Make a 1:100 dilution by placing 20 µL of well-mixed blood into 1980 µL of 1% ammonium oxalate in a small test tube.
2. Mix the dilution thoroughly and charge the chamber.
3. Place the charged hemacytometer in a moist petri dish containing moistened filter paper with normal saline for 15 minutes to allow the platelets to settle.

4. Platelets are counted using the 40X objective lens (400X total magnification). The platelets have a diameter of 2 to 4 µm and appear round or oval, displaying a light purple sheen when phase-contrast microscopy is used. The shape and color help distinguish the platelets from highly refractile dirt and debris. “Ghost” RBCs often are seen in the background.

5. Count the number of platelets in the 25 small squares in the center square of the grid. The area of this center square is 1 mm

6. Platelets should be counted on each side of the hemacytometer, and the difference between the totals should be less than 10%.

7. The accuracy of the manual platelet count should be verified by performing a platelet estimate on a Wright-stained peripheral blood film made from the same specimen General reference intervals for males and females according to age groups can be found on the inside front cover of this text.

Calculations:

- Calculate the platelet count by using one of the equations given earlier. Using the first equation as an example, if 200 platelets were counted in the entire center square:

- Cells counted X Dilution factor

- Area (mm2) X Depth (0.1)

$$\frac{200 \times 100}{1 \times 0.1}$$

$$= 200{,}000/\text{mm}^3 \text{ or } 200{,}000/\mu\text{L}$$

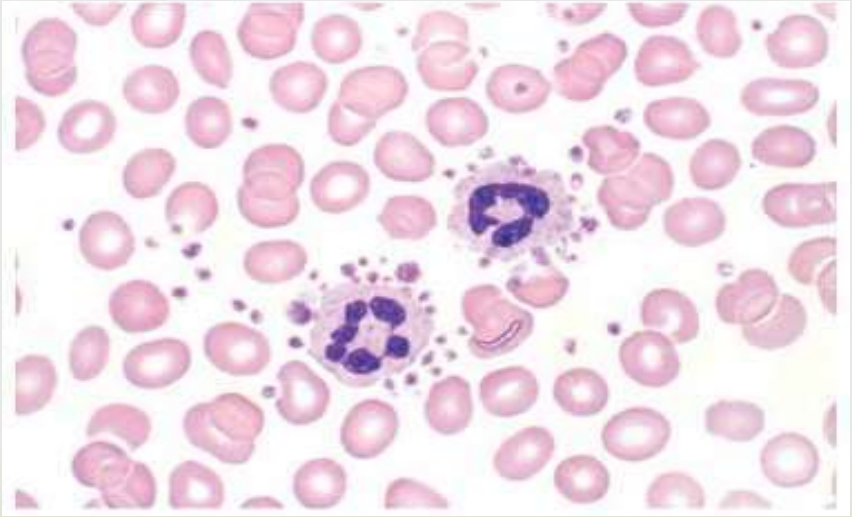
$$\text{or } 200 \times 10^3/\mu\text{L or } 200 \times 10^9/\text{L}$$

Sources of Error and Comments:

1. Inadequate mixing and poor collection of the specimen can cause the platelets to clump on the hemacytometer. If the problem persists after redilution, a new specimen is needed. A skin puncture specimen is less desirable because of the tendency of the platelets to aggregate or form clumps.
2. Dirt in the pipette, hemacytometer, or diluting fluid may cause the counts to be inaccurate.
3. If fewer than 50 platelets are counted on each side, the procedure should be repeated by diluting the blood to 1:20. If more than 500 platelets are counted on each side, a 1:200 dilution should be made. The appropriate dilution factor should be used in calculating the results.

4. If the patient has a normal platelet count, the 5 small, red blood cell squares may be counted. Then, the area is 0.2 mm on each side.

5. The phenomenon of “platelet satellitosis” may occur when EDTA anticoagulant is used. This refers to the adherence of platelets around neutrophils, producing a ring or satellite effect (Figure 1). Using sodium citrate as the anticoagulant should correct this problem. Because of the dilution in the citrate evacuated tubes, it is necessary to multiply the obtained platelet count by 1.1 for accuracy



The Result:

Age	Adult Man	Adult Women	6-12years	2-6 years	1year	3-6 Months
Platelet count $\times 10^9$ /L	280±130 × 10^9 /L		170-450	200-490	200-550	200-550

Age	2Months	1Months	14 day	7 day	3day	Birth
Plateletcount $\times 10^9$ /L	210-650	200-500	170-500	160-500	210-500	100-450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Practical hematology

Lecture 5

3ed stage

Blood Cell Count

Red and White Blood Cell Count

Assist.Lec. Iman. H. Jirjees

Introduction and principles:

- Manual cell counts are performed using a hemacytometer ,or counting chamber.

- The principle for the performance of cell counts is essentially the same for white blood cells (WBCs), red blood cells (RBCs), and platelets; only the dilution, diluting fluid, and area counted vary. Any particle (e.g., sperm, CSF, etc…) can be counted using this system.

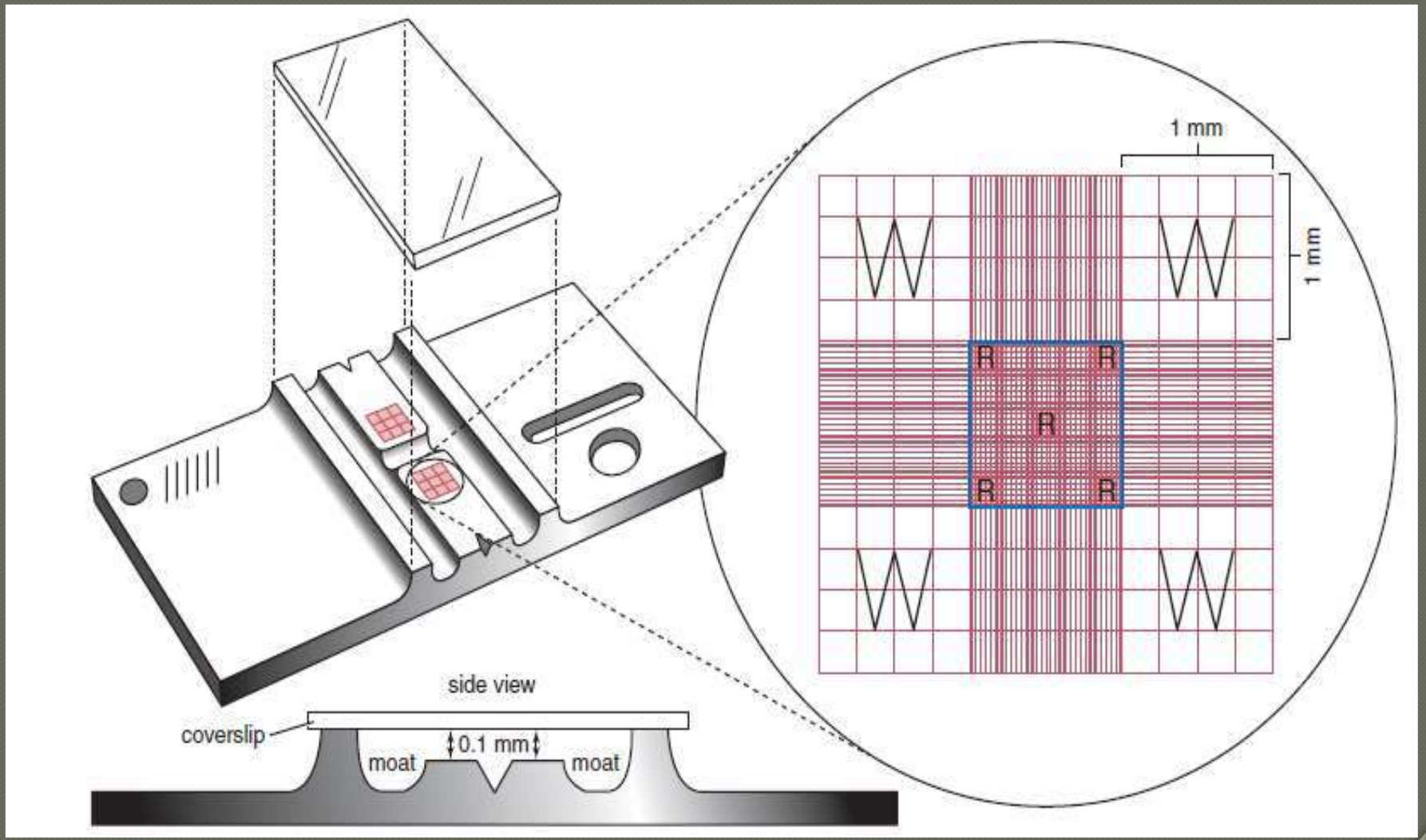
Objective:

- At the end of this session the students will be able to do WBC, RBC count and to indicate the significance of the test in assessing patient's

Materials and Methods:

1. Hemacytometer (counting chamber)
2. Micropipette.
3. Cover slip.
4. Dilution fluid (hayme's solution or isotonic salin for RBC and Turk's solution or 1% ammonium oxalate or 3% acetic acid or 1% hydrochloric acid for WBC).
5. Blood sample.
6. Microscope.

Hemacytometer (counting chamber)



Procedure:

- **1.** Clean the hemacytometer and coverslip with alcohol and dry thoroughly with a lint-free tissue. Place the coverslip on the hemacytometer.
- **2.** Make a 1:20 dilution by placing 25 µL of well-mixed blood into 475 µL of WBC diluting fluid or 1:100 dilution by placing 20 µL of well-mixed blood into 1980 µL of RBC diluting fluid in a small test tube .
- **3.** Cover the tube and mix by inversion.

Procedure:

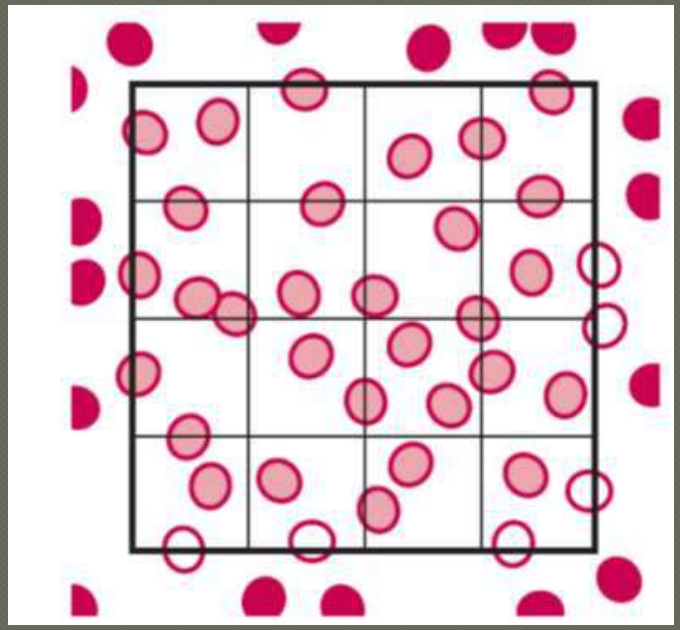
- **4.** Allow the dilution to sit for 10 minutes to ensure that the uncounted blood cells have lysed. The solution will be clear once lysis has occurred. WBC counts should be performed within 3 hours of dilution.
- **5.** Mix again by inversion and fill a plain microhematocrit tube.
- **6.** Charge both sides of the hemacytometer by holding the microhematocrit tube at a 45-degree angle and to the coverslip edge where it meets the chamber floor.

Procedure:

- **7.** Leave the chamber undisturbed on a bench for at least 2 min for the cells to settle, but not much longer, because drying at the edges of the preparation initiates currents that cause movement of the cells after they have settled.
- **8.** While keeping the hemacytometer in a horizontal position, place it on the microscope stage.
- **9.** The microscope focus by using the low-power (10X) objective lens (100X total magnification) for WBCc and high-power (40X) objective lens (400X total magnification) for RBCc, The cells should be distributed evenly in all of the squares.

Procedure:

- **10.** For a 1:20 dilution, count all of the cells in the four corner squares and 1:100 dilution count all cells in the 80 central small squares , starting with the square in the upper left-hand corner. Cells that touch the top and left lines should be counted; cells that touch the bottom and right lines should be ignored as shown in figure (2).



Procedure:

- **11.** Repeat the count on the other side of the counting chamber. The difference between the total cells counted on each side should be less than 10%. A greater variation could indicate an uneven distribution, which requires that the procedure be repeated.
- 12. Average the number of counted cells on the two sides .Using the average, calculate the cells count using one of the equations given earlier.

Calculations:

- The general formula for manual cell counts is as follows and can be used to calculate any type of cell count:

$$\text{Total count} = \frac{\text{Cells counted X Dilution factor}}{\text{Area (mm2) X Depth (0.1)}}$$

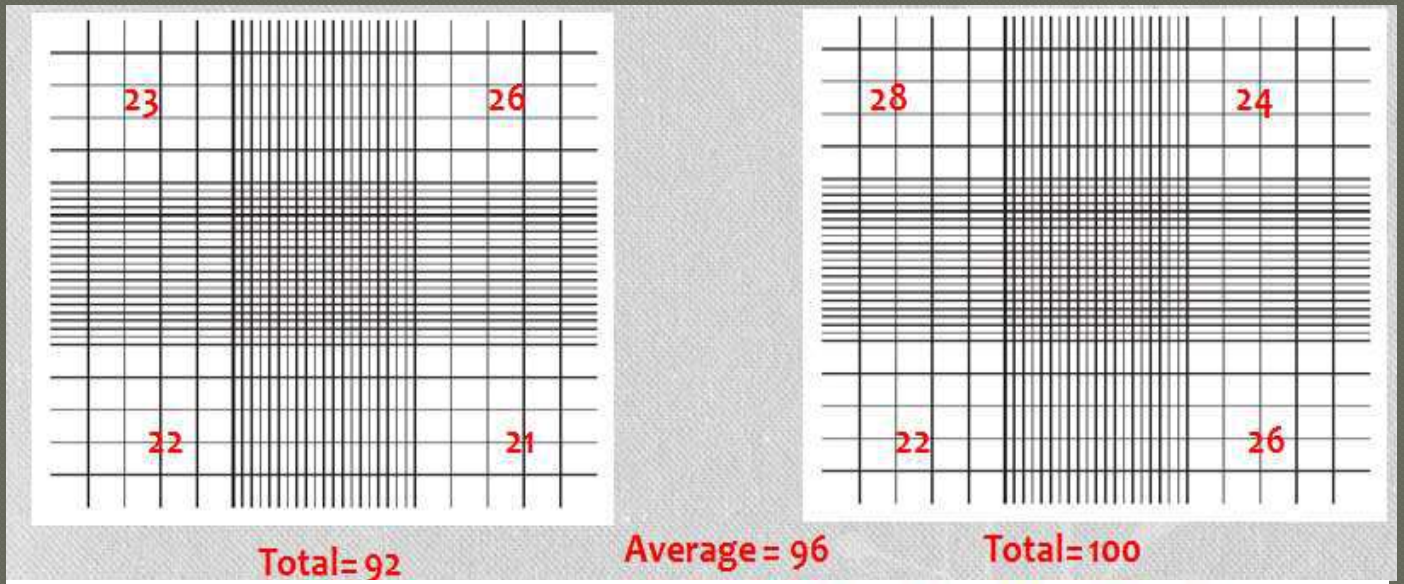
- OR

$$\text{Total count} = \frac{\text{cells counted X dilution factor X 10}}{\text{Area (mm2)}}$$

- The calculation yields the number of cells per mm^3. One mm^3 is equivalent to one microliter (µL). The count per mL is converted to the count per liter (L) by multiplying by a factor of 10^6.

Example:

When a 1:20 dilution is used;



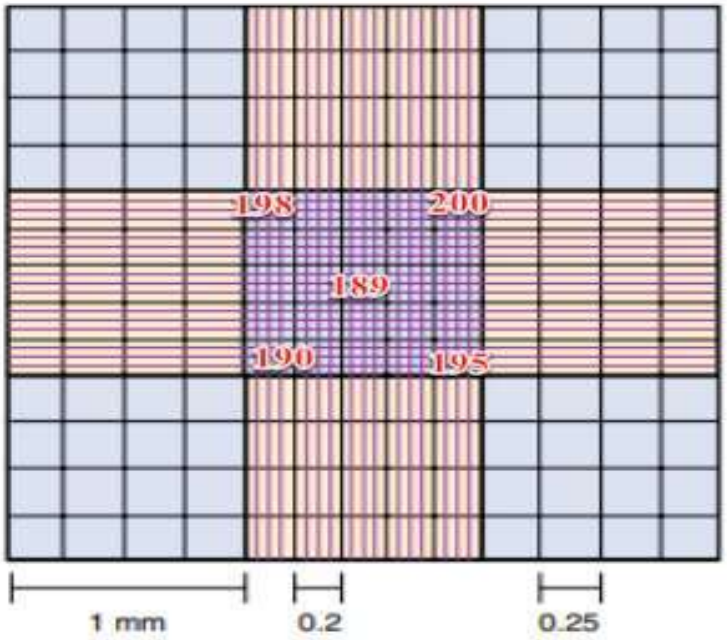
$$\text{WBC count} = \frac{\text{cells counted} \times \text{dilution factor}}{\text{area counted } (\text{mm}^2) \times \text{depth}}$$

$$= \frac{96 \times 20}{4 \times 0.1}$$

$$= 4800/\text{mm}^3 \text{ or } 4800/\mu\text{L or}$$

$$4.8 \times 10^3/\mu\text{L or } 4.8 \times 10^9/\text{L}$$

When a 1:100 dilution is used



$$\text{RBC COUNT} = \frac{\text{Cells counted} \times \text{Dilution factor}}{\text{Area (mm2)} \times \text{Depth (0.1)}}$$

$$= \frac{972 \times 100}{0.2 \times 0.1}$$

$$= 4.86 \times 10^{12}/\text{L Or } 4860000/\mu\text{L}$$

$$\text{Or } 4860000/\text{mm}^3$$

Sources of Error and Comments

- 1. The hemacytometer and coverslip should be cleaned properly before they are used. Dust and fingerprints may cause difficulty in distinguishing the cells.
- 2. The diluting fluid should be free of contaminants.
- 3. If the count is low, a greater area may be counted (e.g., 9 mm^2) to improve accuracy.
- 4. The chamber must be charged properly to ensure an accurate count. Uneven flow of the diluted blood into the chamber results in an irregular distribution of cells. If the chamber is overfilled or underfilled, the chamber must be cleaned and recharged.

Sources of Error and Comments

- 5. After the chamber is filled, allow the cells to settle for 2 minutes before counting. Any nucleated red blood cells (NRBCs) present in the sample are not lysed by the diluting fluid.
- 6. If any of the following filling defects occur, the preparation must be discarded and the filling procedure must be repeated using another clean dry chamber:
- • Overflow into moat
- • Chamber area incompletely filled
- • Air bubbles anywhere in chamber area
- • Any debris in chamber area.

Sources of Error and Comments

- 7.To obtain a coefficient of variation of 5%, it is necessary to count about 400 cells; in practice, it is reasonable to count 100 white cells. The 100 cell count can be achieved by counting the cells in the four larger corner squares

The Result:

Age	Adult Man	Adult Women	6-12years	2-6 years	1year	3-6 Months
RBC×10^{12} /l	5±0.5 ×10^{12} /l	4.3±0.5	4.6±0.6	4.6±0.6	4.5±0.6	4.7±0.6
WBC× 10^{9} /l	4.0-10.0×10^{9} /l		9±4	10±5	11±5	12±6

Age	2Months	1Months	14 day	7 day	3day	Birth
RBC×10^{12} /l	3.7±0.6	4.2±1.2	4.9±1.3	5.1±1.2	5.3±1.3	6.0±1.0
WBC× 10^{9} /l	10±5	12±7	14±8	14±8	15±8	18±8

Thanks for listening

Med. Lab. Tec.Department

PRACTICAL HEMATOLOGY

Lecture Four

3ed stage

Determination of Hematocrit (Hct)

And

Blood Indices

Prepared by Asst.Lec. Iman. H. Jirjees

Introduction and principle:

- **Microhematocrit**
- The hematocrit is the volume of packed red blood cells that occupies a given volume of whole blood. This is often referred to as the packed cell volume(PCV). It is reported either as a percentage (e.g., 36%) or in liters per liter (0.36 L/L).

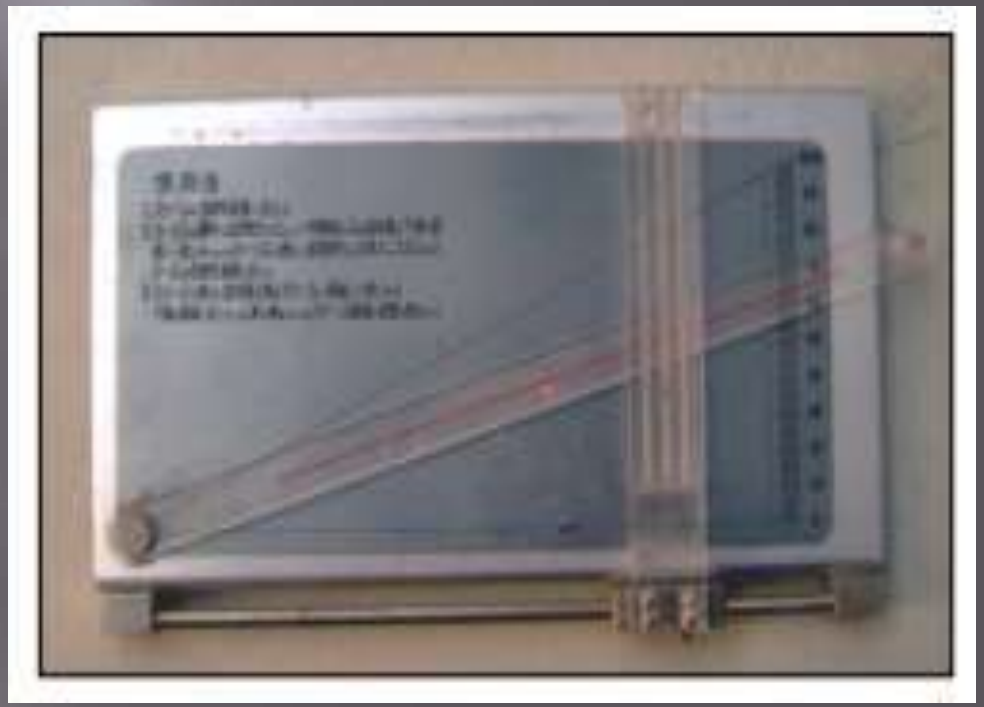
Objectives:

- At the end of this session the attendees will:

1. Gain the knowledge and skills how to estimate packed cell volume.
2. Indicate the significance abnormal PCV.
3. Differentiate between the different types of anemia.

Materials and Equipment's:

1. Microhematocrit centrifuge.
2. Microhematocrit reader



3. Microhematocrit Capillary tubes.

a. Plain-blue tip for Ethylene Diamine Tetra Acetic acid (EDTA)tubes.

b. Heparinized-red tip for capillary collection specimens.

4. Plastic seal or clay.

Procedure:

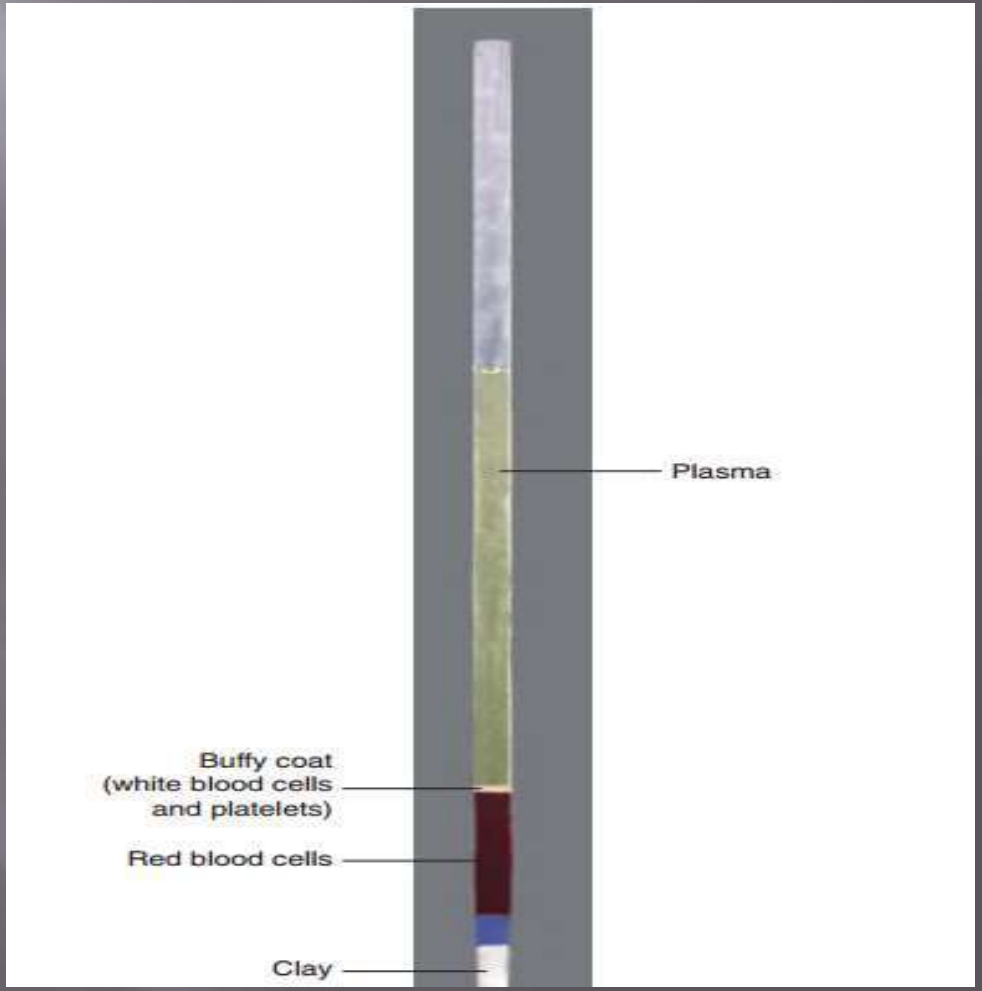
1. Fill two plain capillary tubes approximately three quarters full with blood anticoagulated with EDTA or heparin. Alternatively, blood may be collected into heparinized capillary tubes by skin puncture. Wipe any excess blood from the outside of the tube.
2. Seal the end of the tube with the colored ring using nonabsorbent clay. Hold the filled tube horizontally and seal by placing the dry end into the tray with sealing compound at a 90-degree angle. Rotate the tube slightly and remove it from the tray. The plug should be at least 4 mm long.

Procedure:

3. Balance the tubes in a microhaematocrit centrifuge with the clay ends facing the outside away from the center, touching the rubber gasket.Tighten the head cover on the centrifuge and close the top.
4. The centrifuge used for the capillary tubes provides a centrifugal force of c12 000 g and 5 min centrifugation results in a constant PCV. When the PCV is >0.5, it may be necessary to centrifuge for a further 5 min.
5. Determine the haematocrit by using a microhaematocrit reading device. Read the level of red blood cell packing; do not include the buffy coat (WBCs and platelets) when taking the reading (fig. 3).

Procedure:

6. The values of the duplicate haematocrits should agree within 1% (0.01 L/L).



Sources of Error and Comments:

1. Improper sealing of the capillary tube causes a decreased haematocrit reading as a result of leakage of blood during centrifugation. A higher number of red blood cells are lost compared with plasma due to the packing of the cells in the lower part of the tube during centrifugation.
2. An increased concentration of anticoagulant (short draw in an evacuated tube) decreases the haematocrit reading as a result of red blood cell shrinkage.

3. A decreased or increased result may occur if the specimen was not mixed properly.
4. Because the PCV gradually increases with storage, the test should be performed within 6 h of collecting the blood sample, but a delay of up to 24 h is acceptable if the blood is kept at 4°C.
5. The time and speed of the centrifugation and the time when the results are read are important. Insufficient centrifugation or a delay in reading results after centrifugation causes haematocrit readings to increase. Time for complete packing should be determined for each centrifuge and rechecked at regular intervals.

6. The buffy coat of the sample should not be included in the haematocrit reading because this falsely elevates the result.
7. A decrease or increase in the readings may be seen if the microhaematocrit reader is not used properly.
8. Many disorders, such as sickle cell anemia, macrocytic anemias, hypochromic anemias, spherocytosis, and thalassemia, may cause plasma to be trapped in the red blood cell layer even if the procedure is performed properly. The trapping of the plasma causes the microhaematocrit to be 1% to 3% (0.01 to 0.03 L/L) higher than the value obtained using automated instruments that calculate or directly measure the haematocrit and are unaffected by the trapped plasma.

9. A temporarily low haematocrit reading may result immediately after a blood loss because plasma is replaced faster than are the red blood cells.
10. The fluid loss associated with dehydration causes a decrease in plasma volume and falsely increases the haematocrit reading.
11. Proper specimen collection is an important consideration. The introduction of interstitial fluid from a skin puncture or the improper flushing of an intravenous catheter causes decreased haematocrit readings.

Rule of three:

- When samples are analyzed by automated or manual methods, a quick visual check of the results of the haemoglobin and haematocrit can be done by applying the "rule of three." This rule applies only to samples that have normocytic normochromic red blood cells. The value of the haematocrit should be three times the value of the haemoglobin plus or minus 3: a value discrepant with this rule may indicate abnormal red blood cells, or it may be the first indication of error.

For example, the following results are obtained from patients:

Case 1

- HGB = 12 g/dL
- HCT = 36% (0.36 L/L)
- According to the rule of three,
- HGB (12) X 3 = HCT (36)
- An acceptable range for the haematocrit would be 33% to 39%. These values conform to the rule of three.

Case 2

- HGB = 9 g/dL
- HCT = 32%
- According to the rule of three,
- HGB (9.0) X 3 = HCT (27 versus actual value of 32)
- An acceptable range for haematocrit would be 24% to 30%, so these values do not conform to the rule of three.

Case 3

- HGB = 15 g/dL
- HCT = 36%
- According to the rule of three,
- HGB (15) X 3 = HCT (45 versus obtained value of 36)
- An acceptable range for haematocrit would be 42% to 48%, so these values do not conform to the rule of three.

Interpretation

- If values do not agree, the blood film should be examined for abnormal red blood cells; causes of false increases and decreases in the haemoglobin and/or haematocrit values should also be investigated.
- In the second example, the blood film reveals red blood cells that are low in haemoglobin concentration (hypochromic) and are smaller in volume (microcytic), so the rule of three cannot be applied. If red blood cells do appear normal, possible causes of a falsely low haemoglobin concentration or a falsely elevated haematocrit should be investigated.
- In the third example, the specimen is determined to have lipemic plasma causing a falsely elevated haemoglobin concentration, and a correction must be made to obtain an accurate haemoglobin value.

- When an unexplained discrepancy is found, the sample processed before and after the sample in question should be checked to determine whether they conform to the rule. If they do not conform, further investigation should be done to find the problem.
- A control sample should be run when such a discrepancy is found. If the instrument produces appropriate results for the control, random error may have occurred.

Red Blood Cell Indices:

- The mean cell volume (MCV), mean cell hemoglobin (MCH) ,and mean cell hemoglobin concentration (MCHC) are the RBC indices. These are calculated to determine the average volume and hemoglobin content and concentration of the red blood cells in the sample. In addition to serving as a quality control check, the indices may be used for initial classification of anemia's.

Mean Cell Volume

- The MCV is the average volume of the red blood cell, expressed in femtoliters (fL), or 10^{-15}L

$$\text{MCV} = \frac{\text{HCT (\%)} \times 10}{\text{RBC count } (\times 10^{12}/\text{L})}$$

- For example, if the HCT =45% and the RBC count =5 $\times 10^{12}$/L ,the MCV =90 fL
- The reference interval for MCV is 80 to 100 fL. RBCs with an MCV of less than 80 fL are microcytic; those with an MCV of more than 100 fL are macrocytic.

Mean Cell Hemoglobin

- The MCH is the average weight of hemoglobin in a red blood cell, expressed in picograms (pg), or 10^{-12}g:

$$\text{MCH} = \frac{\text{HGB (g/dL)} \times 10}{\text{RBC count} (\times 10^{12}/\text{L})}$$

- For example, if the hemoglobin = 16 g/dL and the RBC count =5 $\times 10^{12}$/L, the MCH =32 pg.
- The reference interval for adults is 26 to 32 pg. The MCH generally is not considered in the classification of anemia's.

Mean Cell Hemoglobin Concentration

- The MCHC is the average concentration of hemoglobin in each individual red blood cell. The units used are grams per deciliter (formerly given as a percentage):

$$\text{MCHC} = \frac{\text{HGB (g/dL)} \times 100}{\text{HCT (\%)}}$$

- For example, if the HGB =16 g/dL and the HCT =48%, the MCHC =33.3 g/dL.
- Values of normochromic red blood cells range from 32 to 36 g/dL; values of hypochromic cells are less than 32 g/dL, and values of "hyperchromic" cells are greater than 36 g/dL. Hypochromic red blood cells occur in thalassemias, iron deficiency ,and other conditions.
- The term hyperchromic is a misnomer: a cell does not really contain more than 36 g/dL of hemoglobin, but its shape may have become spherocytic, which makes the cell appear full.

Red Cell Size Distribution Width (RDW)

- This automated method of measurement is helpful in the investigation of some hematologic disorders and in monitoring response to therapy. The RDW is essentially an indication of the degree of anisocytosis (abnormal variation in size of RBCs). Normal RBCs have a slight degree of variation.
- Normal range of RDW
- As coefficient of variation= 12.8±1.2%
- As standard deviation= 42.5±3.5 fl

$$\text{RDW (CV\%)} = \frac{\text{Standard deviation of RBC size} \times 100}{\text{MCV}}$$

Reference value:

Age / Parameter	Adult Man	Adult Women	6-12years	2-6 years	1year	3-6 Months
PCV (l/l)	0.45±0.05 (l/l)	0.41±0.05	0.40±0.05	0.37±0.03	0.34±0.04	0.35±0.05
MCV (fl)	92±9 fl		86±9	81±6	78±6	76±8
MCH (pg)	29.5±2.5 pg		29±4	27±3	27±2	27±3
MCHC (g/l)	330±15 g/l		340±30	340±30	340±20	330±30

Age / parameter	2Months	1Months	14 day	7 day	3day	Birth
PCV (l/l)	0.35±0.07	0.43±0.10	0.51±0.2	0.54±0.12	0.56±0.11	0.60±0.15
MCV(fl)	95±8	104±12	105±19	107±19	105±13	110±10
MCH(pg)	30±3	33±3	34±3	34±3	34±3	34±3
MCHC(g/l)	320±35	330±40	330±50	330±50	330±40	330±30

Thanks for listening

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PRACTICAL HEMATOLOGY

Lecture Two

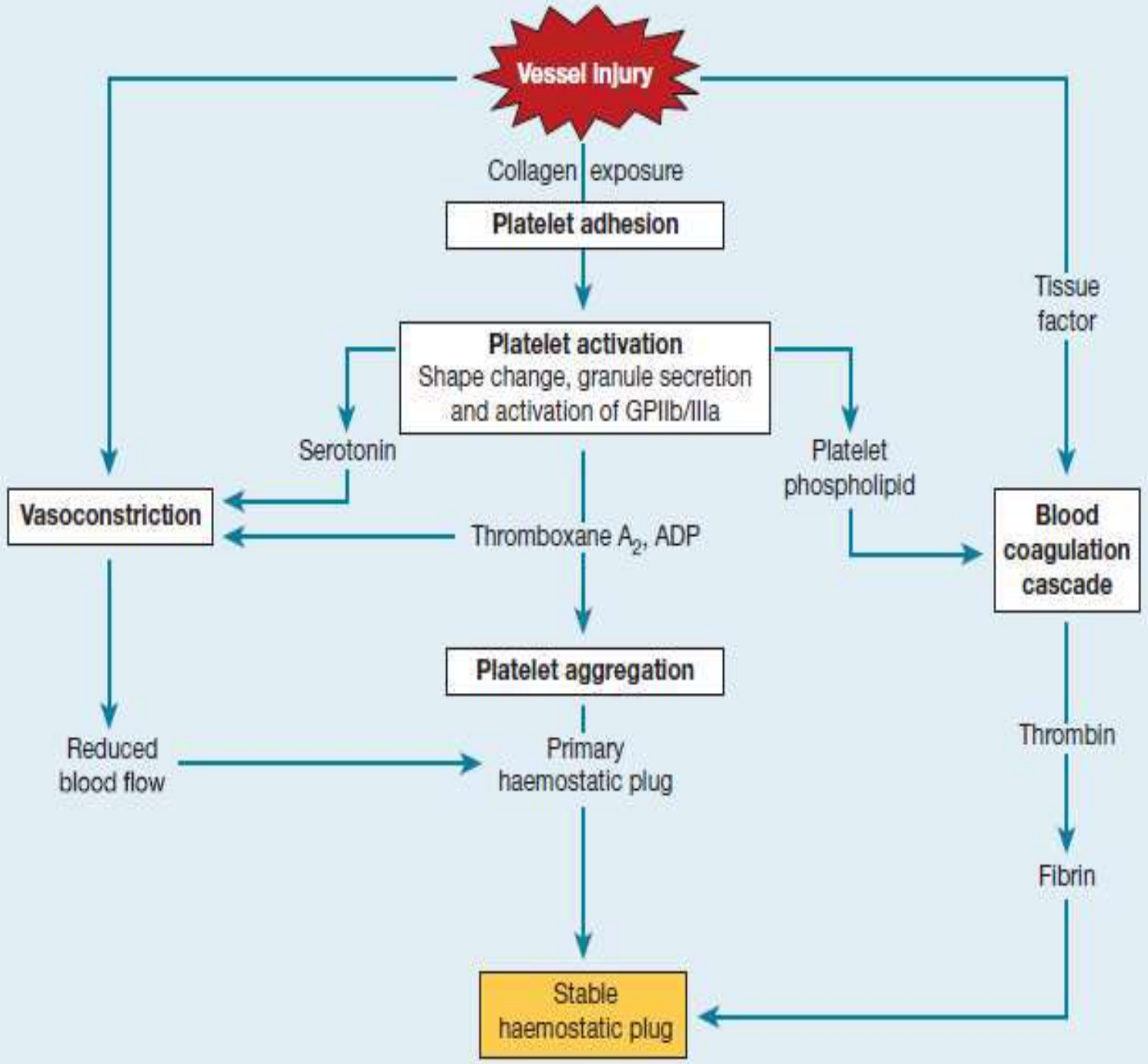
Anticoagulants

Assist. Lec. Iman. H. Jirjees

- Anticoagulants is a substances which prevent blood clotting /coagulation, and allow separation of the blood into cells and liquid (plasma) components.

- The anticoagulants commonly used in hematology laboratory are:

1. **Ethylene Diamine Tetra-Acetic acid (EDTA).**
2. **Trisodium Citrate.**
3. **Heparin.**
4. **Sodium fluoride.**
5. **Oxalate.**


Vessel injury
Collagen exposure
Platelet adhesion
Tissue factor
Platelet activation
Shape change, granule secretion and activation of GPIIb/IIIa
Serotonin
Platelet phospholipid
Vasoconstriction
Thromboxane A_2, ADP
Blood coagulation cascade
Platelet aggregation
Reduced blood flow
Primary haemostatic plug
Thrombin
Fibrin
Stable haemostatic plug

1. Ethylene Diamine Tetra-Acetic acid (EDTA)

- The sodium and potassium salts of EDTA are powerful anticoagulants.
- EDTA acts by its chelating effect on the calcium molecules in blood.
- To achieve this requires a concentration of **1.2** mg of the anhydrous salt per ml of blood .
- Accordingly, the International Council for Standardization in Haematology recommends the dipotassium salt at a concentration of 1.50–2.2 mg/ml of blood; the tripotassium salt may be accepted as an alternative.

- they are especially suitable for routine haematological work.
- Excess of EDTA, irrespective of which salt, affects both Red cells and leucocytes, causing shrinkage and degenerative changes.
- Blood films made from EDTA blood may fail to demonstrate basophilic stippling of the red cells in lead poisoning.

2. Trisodium Citrate

- changing in free calcium ion concentration affect coagulation test results.

- For coagulation studies, 9 volumes of blood are added to1 volume of 109 mmol/l sodium citrate solution (32 g/l of Na3C6H5O7.2H2O*). 9:1
- For the erythrocyte sedimentation rate (ESR), 4 volumes of blood are added to 1 volume of the sodium citrate solution (109 mmol/l) and immediately well mixed with it. 4:1

3. Heparin

- Lithium or sodium salt
- heparin at a concentration of 10–20 iu/ml of blood is a commonly used anticoagulant for chemistry, gas analysis and emergency tests.
- The best anticoagulant for osmotic fragility tests and is suitable for immunophenotyping.
- heparin is not suitable for blood counts as It often induces platelet and leucocyte clumping.

- Nor should it be used for making blood films as it gives a faint blue coloration to the background when the films are stained by Romanowsky dyes, especially in the presence of abnormal proteins.
  - It inhibits enzyme activity and it should not be used in the study of polymerase chain reaction with restriction enzymes.

Order of Draw:

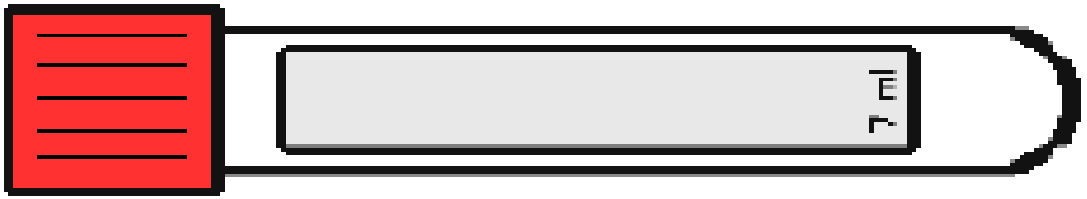
The order of draw is the same for specimens collected by syringe or tube holder; the purpose is to minimize crosses contamination when multisample specimens are collected that avoid possible test result error due to additive carryover.

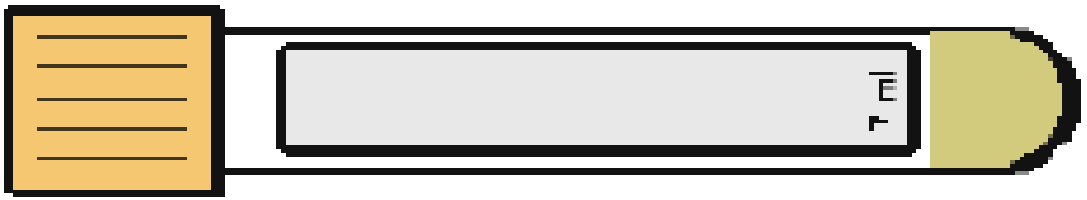
Recommended order of draw for plastic vacuum tubes

Order of use[a]	Type of tube/usual colour[b]	Additive[c]	Mode of action	Uses
1	Blood culture bottle (yellow-black striped tubes)	Broth mixture	Preserves viability of microorganisms	Microbiology – aerobes, anaerobes, fungi
2	Non-additive tube			
3	Coagulation tube[d] (light blue top)	Sodium citrate	Forms calcium salts to remove calcium	Coagulation tests (protime and prothrombin time), requires full draw
4	Clot activator (red top)	Clot activator	Blood clots, and the serum is separated by centrifugation	Chemistries, immunology and serology, blood bank (cross-match)
5	Serum separator tube (red-grey tiger top or gold)	None	Contains a gel at the bottom to separate blood from serum on centrifugation	Chemistries, immunology and serology

Recommended order of draw for plastic vacuum tubes

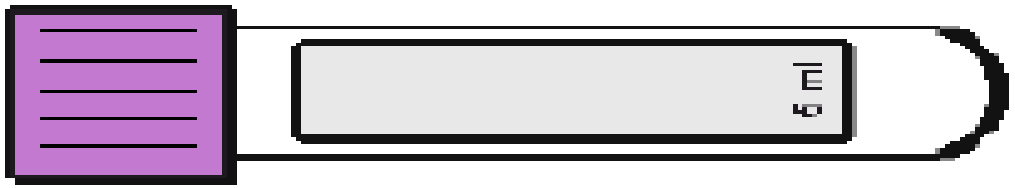
6	Sodium heparin (dark green top)	Sodium heparin or lithium heparin	Inactivates thrombin and thromboplastin	For lithium level use sodium heparin, for ammonia level use either
7	PST (light green top)	Lithium heparin anticoagulant and a gel separator	Anticoagulants with lithium, separates plasma with PST gel at bottom of tube	Chemistries
8	EDTA (purple top)	EDTA	Forms calcium salts to remove calcium	Haematology, Blood Bank (cross-match) requires full draw
9	Blood tube (pale yellow top)	Acid-citrate-dextrose (ACD, ACDA or ACDB)	Complement inactivation	HLA tissue typing, paternity testing, DNA studies
10	Oxalate/fluoride (light grey top)	Sodium fluoride and potassium oxalate	Antiglycolytic agent preserves glucose up to five days	Glucoses, requires full draw (may cause haemolysis if short draw)

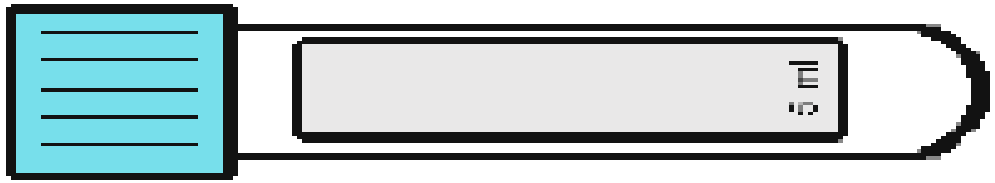
Red Top	
ADDITIVE	None
MODE OF ACTION	Blood clots, and the serum is separated by centrifugation
USES	Chemistries, Immunology and Serology, Blood Bank (Crossmatch)

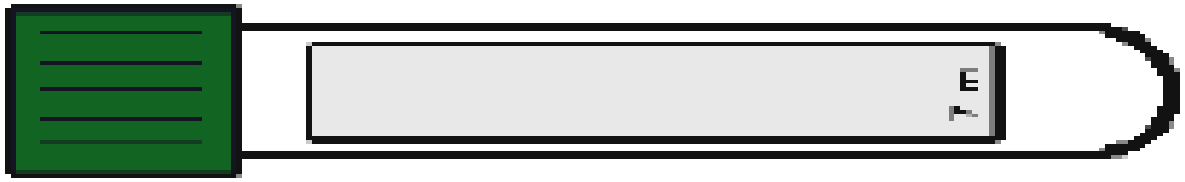
Gold Top	
ADDITIVE	None
MODE OF ACTION	Serum separator tube (SST) contains a gel at the bottom to separate blood from serum on centrifugation
USES	Chemistries, Immunology and Serology

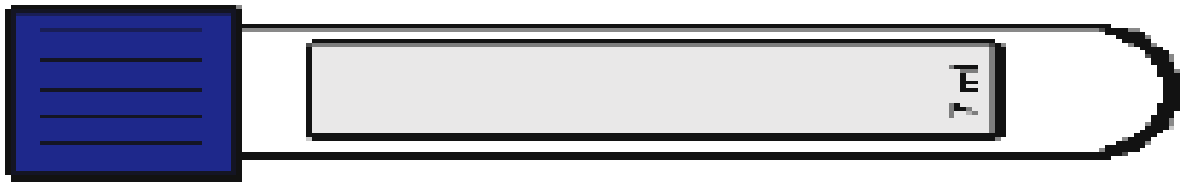
Light Green Top	
ADDITIVE	Plasma Separating Tube (PST) with Lithium heparin
MODE OF ACTION	Anticoagulates with lithium heparin; Plasma is separated with PST gel at the bottom of the tube
USES	Chemistries

Red-Gray Top	
ADDITIVE	Serum Separating Tube (SST) with clot activator
MODE OF ACTION	Forms clot quickly and separates the serum with SST gel at the bottom of the tube
USES	Chemistries

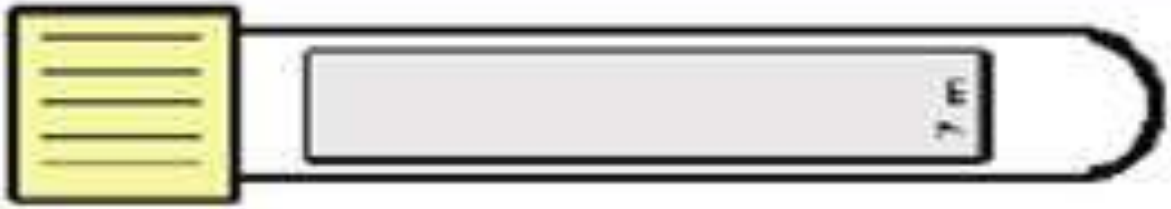
Purple Top	
ADDITIVE	EDTA liquid
MODE OF ACTION	Forms calcium salts to remove calcium
USES	Hematology (CBC) and Blood Bank (Crossmatch); requires **full draw** - invert 8 times to prevent clotting and platelet clumping

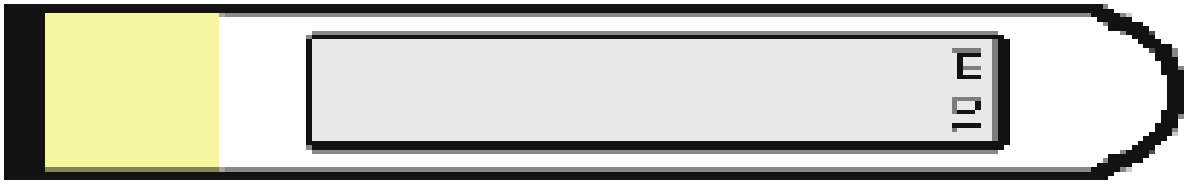
Light Blue Top	
ADDITIVE	Sodium citrate
MODE OF ACTION	Forms calcium salts to remove calcium
USES	Coagulation tests (protime and prothrombin time), **full draw** required

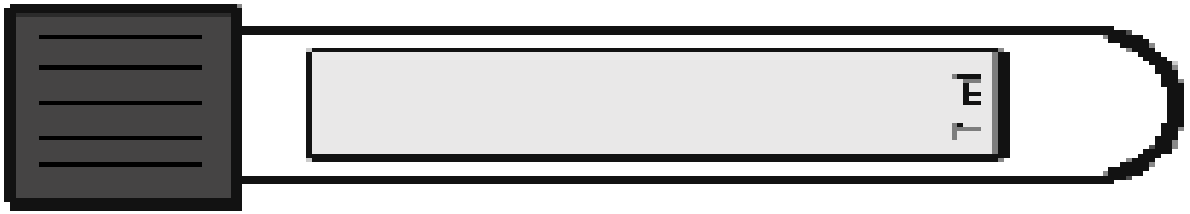
Dark Green Top	
ADDITIVE	Sodium heparin or lithium heparin
MODE OF ACTION	Inactivates thrombin and thromboplastin
USES	For lithium level, use sodium heparin For ammonia level, use sodium or lithium heparin

Dark Blue Top	
ADDITIVE	Sodium EDTA
MODE OF ACTION	Forms calcium salts Tube is designed to contain no contaminating metals
USES	For lithium level, use sodium heparin Trace element testing (zinc, copper, lead, mercury) and toxicology

Light Gray Top	
ADDITIVE	Sodium fluoride and potassium oxalate
MODE OF ACTION	Antiglycolytic agent preserves glucose up to 5 days
USES	For lithium level, use sodium heparin Glucoses, requires **full draw** (may cause hemolysis if short draw)

Yellow Top	
ADDITIVE	ACD (acid-citrate-dextrose)
MODE OF ACTION	Complement inactivation
USES	HLA tissue typing, paternity testing, DNA studies

Yellow - Black Top	
ADDITIVE	Broth mixture
MODE OF ACTION	Preserves viability of microorganisms
USES	Microbiology - aerobes, anaerobes, fungi

Black Top	
ADDITIVE	Sodium citrate (buffered)
MODE OF ACTION	Forms calcium salts to remove calcium
USES	Westergren Sedimentation Rate; requires **full draw**

COLOUR CODE	TUBE TYPE	DETERMINATIONS	INVERSIONS
Blue Purple 	Blood Culture	Aerobic followed by anaerobic	8-10 Times
Light Blue 	Sodium Citrate	For coagulation determinations on plasma specimens	3-4 Times
Red 	Serum	For serum determinations in chemistry	5-6 Times
Gold 	SST™ II Advance	For serum determinations in chemistry - with gel separator	5-6 Times
Orange 	RST	For serum determinations in chemistry - with Thrombin based clotting agent and gel separator	5-6 Times
Green 	Heparin	For plasma determinations in chemistry	8-10 Times
Light Green 	PST™ II	For plasma determinations in chemistry with gel separator	8-10 Times
Lavender 	EDTA	For whole blood hematology determinations	8-10 Times
Pink 	Cross Match	Crossmatch tubes for blood transfusion patients	8-10 Times
Grey 	NaF/NaEDTA	For glucose determinations	8-10 Times
Royal Blue 	Trace Element	For trace element, toxicology and nutrient determinations	8-10 Times


Thanks
For
Listening

Med. Lab. Tec.Department

3ed stage

Practical hematology

Lecture Three

Hemoglobin Determination

Assist. Lec

Iman. H. Jirjees

Introduction and Principle:

- Hemoglobin is the pigment inside the red blood corpuscles which bind with oxygen and carries the oxygen from the lungs to the tissues.

- The amount of hemoglobin can be estimated by different methods, The cyanmethaemoglobin (haemoglobincyanide) method for haemoglobin determination is the reference method.

Objectives:

- 1- At the end of this session the students will be able to estimate hemoglobin concentration in a provided blood sample.
- 2- Identify to the normal value of hemoglobin according to age.

Materials and Methods:

- Colorimetric method.
  - Spectrophoto-meter.

- **Drabkin's solution.**
- **Principle:**

cyanmethemoglobin reagent (drabkin's solution) consist of :

- potassium cyanide
- potassium ferricyanide

(drabkin's solution + blood)

- potassium ferricyanide + hemoglobin iron (ferrous Fe^{2+})

potassium cyanide + methemoglobin (ferric Fe^{3+})

cyanmethemoglobin

Diluent (Drabkin solution)

- The diluent should be clear and pale yellow in colour.
- When measured against water as a blank in a spectrometer at a wavelength of 540 nm, absorbance must be zero.
- If stored at room temperature in a brown borosilicate glass bottle, the solution keeps for several months.
- If the ambient temperature is higher than 30°C, the solution should be stored in the refrigerator but brought to room temperature before use.
- It must not be allowed to freeze.
- The reagent must be discarded if it becomes turbid, if the pH is found to be outside the 7.0–7.4 range or if it has an absorbance other than zero at 540 nm against water blank.

Method(procedure):

1. Make a 1 in 201 dilution of blood by adding 20 µl of blood to 4 ml of diluent.
2. Stopper the tube containing the solution and invert it several times.
3. Let the test sample stand at room temperature for at least 5 min (to ensure the complete conversion of haemoglobin to haemiglobincyanide).
4. Then pour it into a cuvette and read the absorbance in a spectrometer at 540 nm or in a photoelectric colorimeter with a suitable filter against a reagent blank.

Method(procedure):

5. The absorbance of the test sample must be measured within 6 h of its initial dilution. The absorbance of a commercially available HiCN standard (brought to room temperature if previously stored in a refrigerator) should also be compared to a reagent blank in the same spectrometer or photoelectric colorimeter as the patient sample.

6. The standard should be kept in the dark and, to ensure that contamination is avoided, any unused solution should be discarded at the end of the day on which the ampoule is opened.

Calculation of Haemoglobin Concentration

$$\text{Hb (g/l)} = \frac{{}^{*}A^{540}\text{ of test sample}}{A^{540}\text{ of standard}} \times \text{Conc. of standard} \times \frac{\text{Dilution factor (201)}^{\dagger}}{1000}$$

Sources of Error and Comments:

1. Cyanmethaemoglobin reagent is sensitive to light. It should be stored in a brown bottle or in a dark place.
2. A high WBC count (greater than 20 X 10^9/L) or a high platelet count (greater than 700 X 10^9/L) can cause turbidity and a falsely high result. In this case, the reagent-sample solution can be centrifuged and the supernatant measured.
3. Lipaemia also can cause turbidity and a falsely high result. It can be corrected by adding 0.01 mL of the patient's plasma to 5 mL of the cyanmethaemoglobin reagent and using this solution as the reagent blank

1. Cells containing Hb S and Hb C may be resistant to haemolysis, causing turbidity; this can be corrected by making a 1:2 dilution with distilled water (1 part diluted sample plus 1 part water) and multiplying the results from the standard curve by 2.
2. Carboxyhaemoglobin takes 1 hour to convert to cyanmethaemoglobin and theoretically could cause erroneous results in samples from heavy smokers. The degree of error is probably not clinically significant, however.
3. Because the haemoglobin reagent contains cyanide, it is highly toxic and must be used cautiously. Consult the safety data sheet supplied by the manufacturer. Acidification of cyanide in the reagent releases highly toxic hydrogen cyanide gas. A licensed waste disposal service should be contracted to discard the reagent; reagent-sample solutions should not be discarded into sinks.

Result:

Age	Adult Man	Adult Women	6-12years	2-6 years	1year	3-6 Months
Hemoglobin Concentration g/l	150±20	135±15	135±20	125±15	126±15	126±15

Age	2Months	1Months	14 day	7 day	3day	Birth
Hemoglobin Concentration g/l	112±18	140±25	165±4	175±4	180±30	180±40

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Practical Haematology

Lecture one

3ed stage

Collection of Blood (phlebotomy)

Lecturer

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- **Phlebotomy** – the collection of blood .

- Is one of the most common invasive procedures in health care.

- Each step in the process of phlebotomy affects the quality of the specimen and is thus important for preventing laboratory error, patient injury and even death.

Physiologic Factors Affecting Test Results

Certain physiologic variables under the control of the patient or the phlebotomist may introduce preanalytical variation in laboratory test results, these factors include:

- Stress
- Diet
- Smoking
- Posture
- Diurnal Rhythm
- Exercise

Materials and Methods:

- **Methods:**

There are three methods for collection of blood samples:

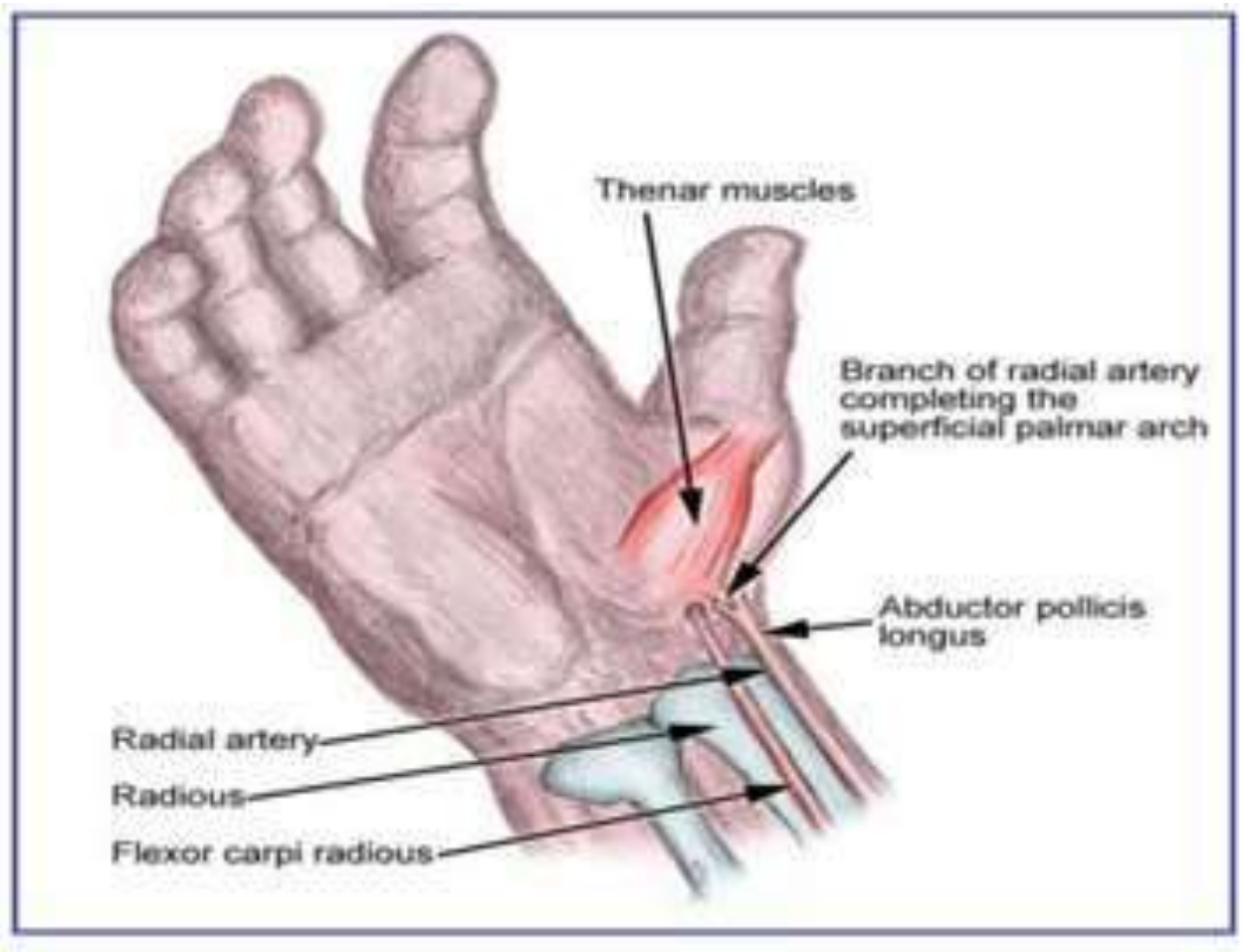
i. **Arterial blood sampling:**

ii. **Skin puncture (Capillary sampling):**

iii. **Venepuncture:**

Arterial blood sampling:

- Arterial blood is needed for special tests such as blood pH, gas levels.
- radial artery
- Femoral artery
- Brachial artery

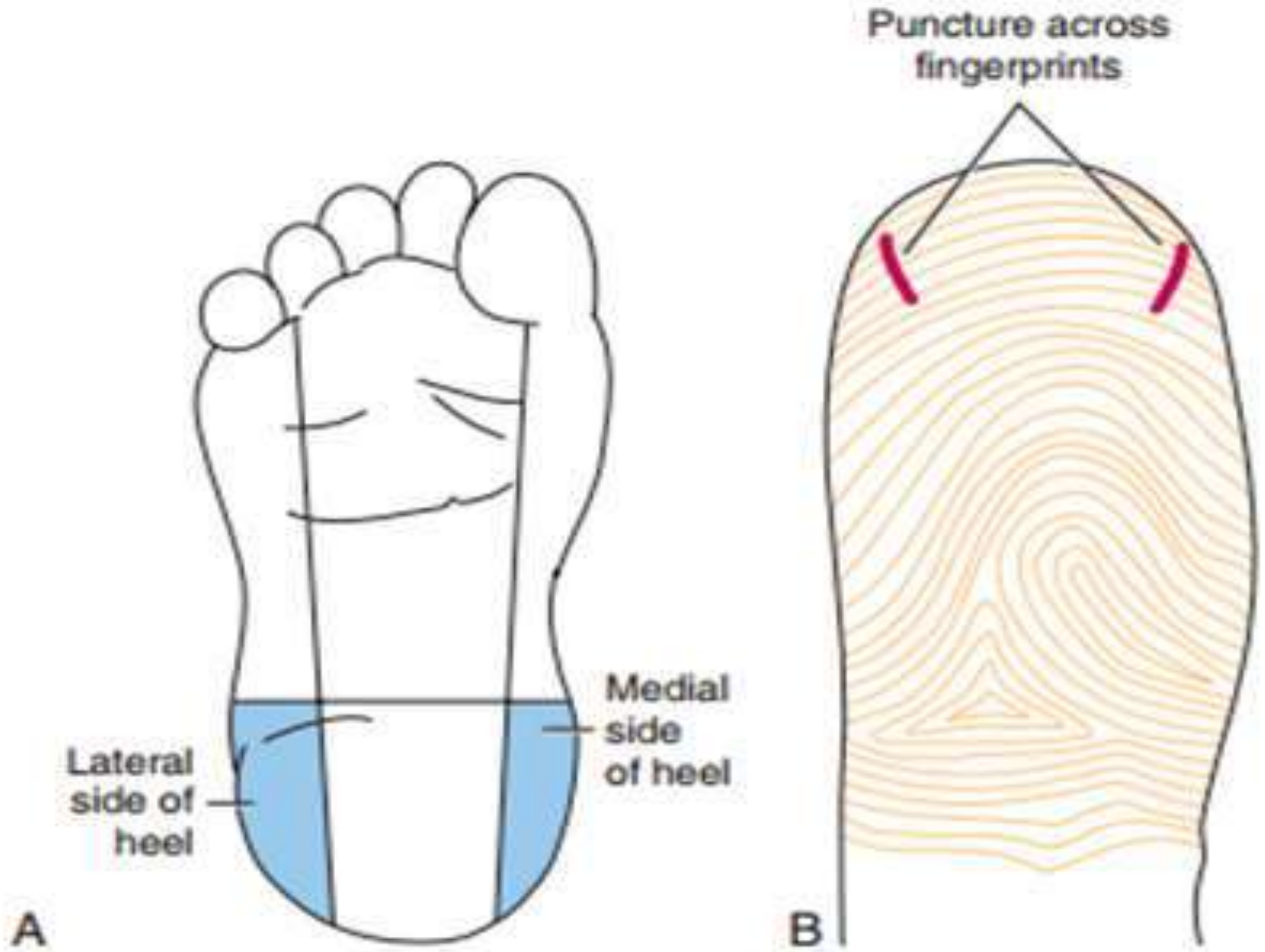


Skin puncture (Capillary sampling):

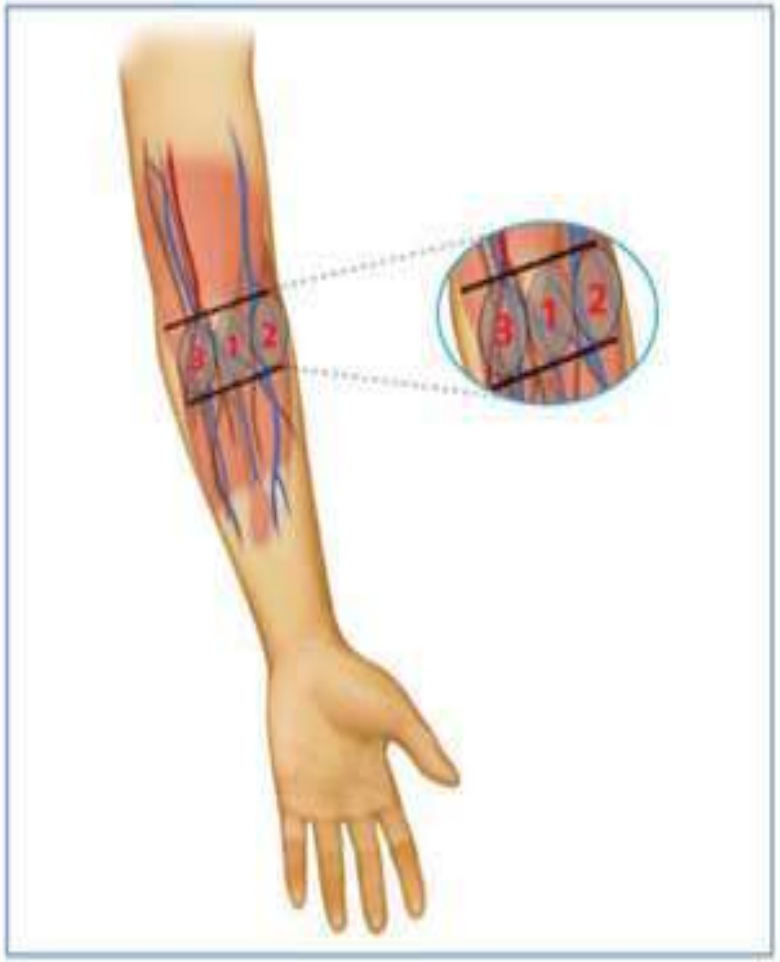
Capillary sampling from a
finger
heel
ear lobe
may be performed on patients of any age, for specific tests that require small quantities of blood.

- The site of choice for skin puncture:

Condition	Heel-prick	Finger-prick
Age	Birth to about 6 months	Over 6 months
Weight	From 3–10 kg, approximately	Greater than 10 kg
Placement of lancet	On the medial or lateral plantar surface	On the side of the ball of the finger perpendicular to the lines of the fingerprint
Recommended finger	Not applicable	Second and third finger (i.e. middle and ring finger); avoid the thumb and index finger because of calluses, and avoid the little finger because the tissue is thin


Puncture across fingerprints
Medial side of heel
Lateral side of heel
A
B

Venepuncture:



First; Veins in the medical aspect (Centre) , i.e., **Median** and lateral aspect of the medical cubital veins

Second; Veins in the lateral aspect (Outer) , i.e., **Cephalic** vein and accessory cephalic vein.

Third; Veins in the medial aspect (Inner), i.e. , **Basilic** vein and medial aspect of the medial cubital vein

Steps of phlebotomy (Blood drawing)Step

1 – Assemble equipment
Collect all the equipment needed for the procedure and place it within safe and easy reach on a tray or trolley, ensuring that all the items are clearly visible.

2 – Identify and prepare the patient

3 – Select the site

4 – Perform hand hygiene and put on gloves

5 – Disinfect the entry site

Steps of phlebotomy (Blood drawing)Step

Unless drawing blood cultures, or prepping for a blood collection, clean the site with a 70% alcohol swab for 30 seconds and allow to dry completely (30 seconds).

- Note: alcohol is preferable to povidone iodine, because blood contaminated with povidone iodine may falsely increase levels of potassium, phosphorus or uric acid in laboratory test results

Steps of phlebotomy (Blood drawing)Step

6 – Take blood "Venepuncture"

7 – Fill the laboratory sample tubes

8 – Draw samples in the correct order

9 – Clean contaminated surfaces and complete patient procedure

10 – Prepare samples for transportation

11 – Clean up spills of blood or body fluids

Complications Encountered in Venipuncture

1. Petechiae
2. Allergies
3. Nerve Damage
4. Seizures
5. Vomiting
6. Ecchymosis (Bruise)
7. Hematoma
8. Fainting (Syncope)
9. Hemoconcentration
10. Hemolysis


Thanks
For
Listening