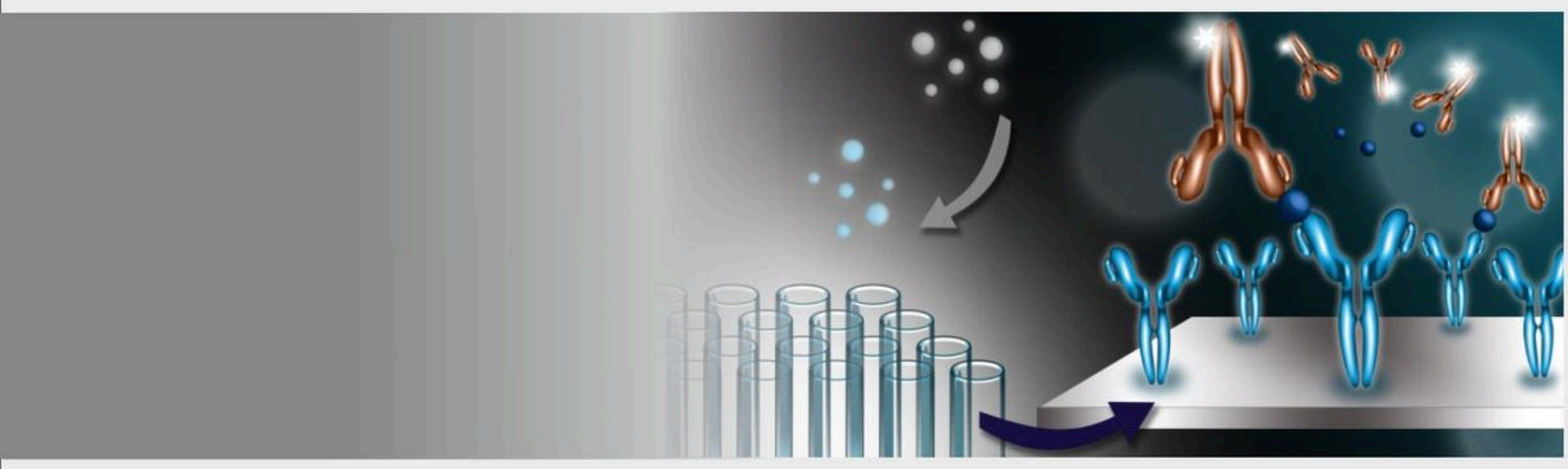
Knowledge Book

For the Laboratorians



Setting Trends...

TULIP DIAGNOSTICS (P) LTD.

a revvity company

Preface

Tulip Diagnostics (P) Ltd and its divisions, namely, Orchid Biomedical Systems, Qualpro Diagnostics, Zephyr Biomedicals, Viola Diagnostic Systems, Microxpress, Coral Clinical Systems and BioShields are ISO:13485 certified companies, involved in the manufacture and marketing of in-vitro diagnostics reagents and kits, dehydrated culture media and high technology disinfectants since 1988. The companies' commitment in building products of international standards, through indigenous R&D has accorded them virtual leadership in most product segments in the Indian marketplace. The company's state-of-art manufacturing facilities based in Goa and Uttarkhand, India, conforms to the strictest FDA (India) and GMP regulations. Most of the products are CE marked. It is this commitment to Quality, which has given the company international acclaim. Products are now exported to over 88 countries globally with an ever-increasing user base.

With three decades of experience in *in vitro* diagnostics (IVD), Tulip Diagnostics has created a strong knowledge base and believes that regular upgradation of knowledge is essential not only for better diagnosis and patient care, but also to improve the overall quality of life.

From time to time, Technical publications in form of The CRUX bimonthly magazine, The Journal of Hygiene Sciences and Technical Series on relevant topics were made available to the Laboratory professionals and clinicians as updated knowledge is vital for them to set trends in their day-to-day practice.

On completion of 30 glorious years in Diagnostic services, Tulip Diagnostics (P) Ltd., presents Knowledge book- encompassing all the publications of Technical Series in a single booklet for the benefit of professional in the health care segment.

INNOVATIVE EXCELLENCE

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BLOOD BANKING

- **Anti Human Globulin Reagent**
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KNOWLEDGE BOOK

For the Laboratorians

Anti Human Globulin Reagent

Basic Concepts and Practice

Setting Trends...

Historical Perspective

The first step towards a rational approach to blood transfusion became possible with Landsteiners discovery in 1901 of the ABO blood group system. Nearly half a century later, the next advance occurred with the introduction of serological techniques permitting the detection of incomplete antibodies, which are nonagglutinating immunoglobulin G (IgG) antibodies that sensitize red blood cells. In 1945, Coombs et al. described the use of anti human globulin test for the detection of weak and non-agglutinating antibodies in serum. Further in 1946, Coombs et al. described the use of anti human globulin test to detect in vivo sensitization of red blood cells in babies suffering from haemolytic disease of the newborn. Although the test initially was of great value in investigating Rh_o (D) haemolytic disease of the newborn, it was not long before its versatility for the detection of other IgG blood group antibodies became evident.

Although Coombs and his coworkers were instrumental in introducing the anti human globulin test to blood group serology, the principle of the test had infact been described by Moreschi in 1908. Moreschis studies involved the use of rabbit anti goat antiserum to agglutinate rabbit red blood cells that were sensitized with low non-agglutinating dose of goat anti rabbit red cell serum.

Later in 1957, Dacie et al. published data regarding the importance of anti human complement activity. Further in 1960 many reports were published indicating the need for anti human complement activity in anti human globulin serum to allow the detection of antibodies in the indirect test. Though many of the antibody specificities mentioned in these reports are now considered of little clinical significance, however one specificity that was consistently mentioned and is still considered to be clinically significant was Anti-Jk^a. Further it was also shown that the presence of anti human complement activity would enhance the reaction of some antibodies namely Anti-Fy^a and Anti-K.

Preliminary Notes-Antigen and antibody related to blood group serology

Antigens are foreign substances, which initiate an immune response in human body and react specifically with antibodies. Blood group antigens that are located on the surface of red blood cells when transferred to another individual are almost certain to carry antigens not possessed by the recipient. These foreign antigens then initiate an immune response and production of antibodies in vivo.
Antibodies are counter agents produced as a defense mechanism when foreign antigen enters human body. With respect to blood group serology it means that blood group antibodies are specific and are formed against red blood cell antigens not possessed by the recipient.

Antibody nomenclature with respect to blood group serology

Red blood cell antibodies may be naturally occurring or immune in nature. Naturally occurring antibodies are produced because of the exposure of human body to certain bacteria and plant substances whose antigenic structure resembles blood group antigens. Naturally occurring antibodies are most often IgM class of antibodies.

Immune antibodies (alloantibodies) are antibodies present in an individual due to transfusion or pregnancy. Immune antibodies most often are IgG class of antibodies, sometimes IgM or a mixture of IgM and IgG class of antibodies.

IgM class antibodies

IgM antibodies are pentamers in which five polypeptide sub units are held together by disulphide bonds and joined by J chain. IgM antibodies do not cross the placenta but are most effective in activating complement.
Sialic acid present on the red cell membranes impart to the cells, a net negative charge. As a result mutual repulsive force exists between two red blood cells known as zeta potential and the red blood cells are separated by a distance. In blood group serology IgM antibodies are termed as complete antibodies because of their ability to directly bridge the distance between two red blood cells inspite of the zeta potential and bring about visible agglutination. IgM antibodies are able to bridge this distance and bring about visible agglutination.

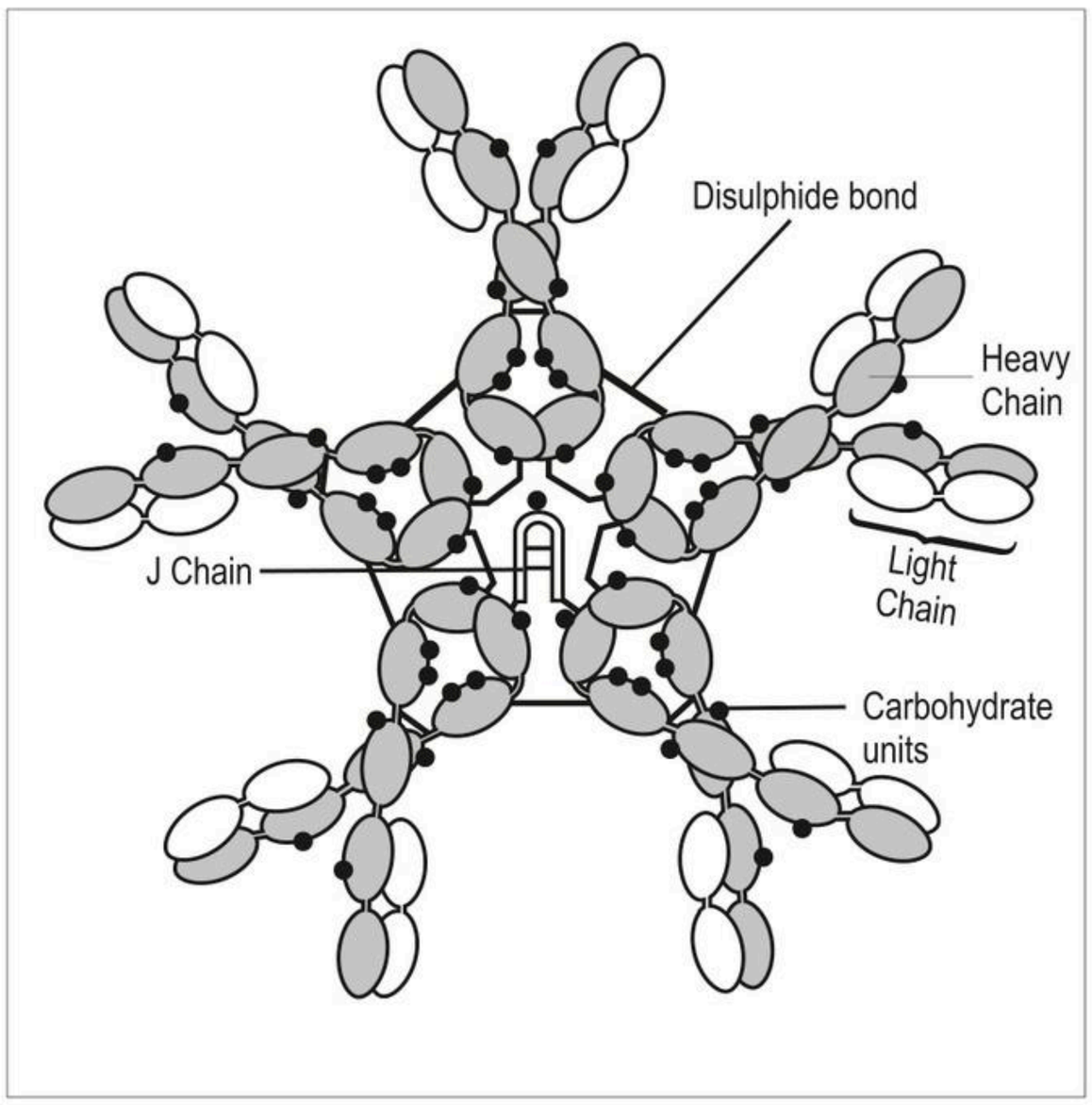

Fig1. Illustration of IgM molecule

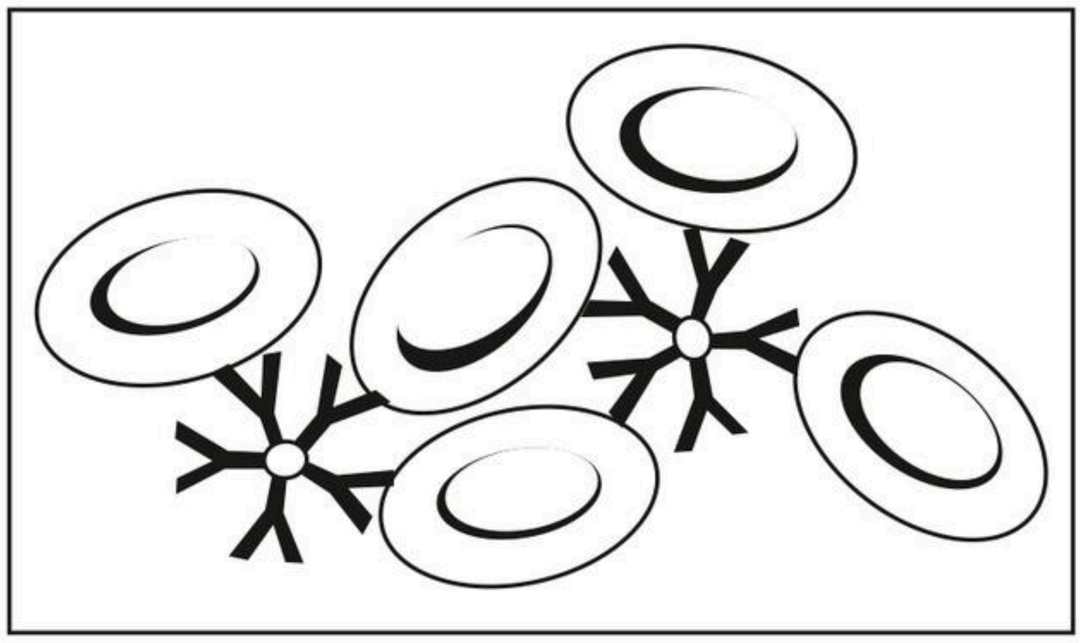

Fig.2 Illustration of complete antibodies

IgG class antibodies:

IgG antibodies are monomers with two heavy chains and two light chains. IgG is the only immunoglobulin that can cross the placental barrier, a fact which explains the role of IgG antibodies in haemolytic disease of the newborn. Four subclasses of IgG are present and they vary widely in their ability to activate complement. IgG3 molecules are highly effective in activating complement, IgG1 moderately activate complement, IgG2 slightly activate complement and IgG4 do not activate complement.

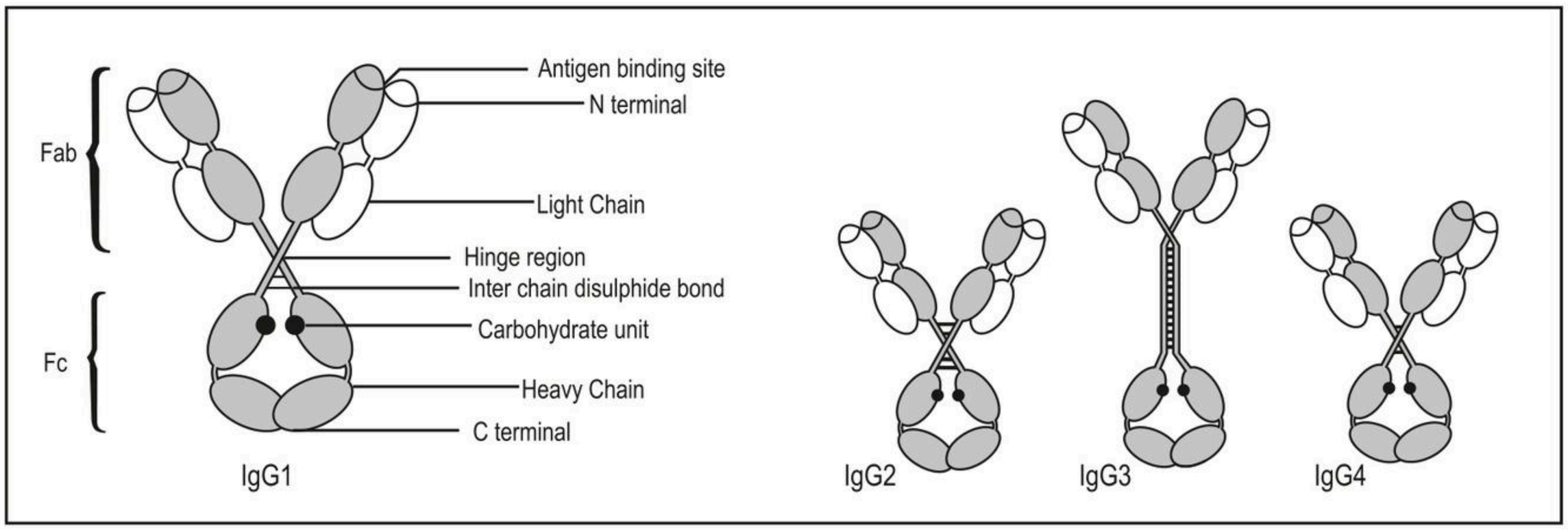

Fig. 3 Structure of four subclasses of IgG

IgG antibodies are also known as incomplete antibodies because they cannot bridge the distance between two red blood cells and bring about direct agglutination like the IgM immunoglobulins. Antibodies that do not agglutinate red blood cells directly are termed as incomplete antibodies or sensitizing antibodies and the red blood cells that have such antibodies on their surface are known as sensitized cells. Incomplete antibodies sensitized onto red blood cells can be agglutinated by anti human globulin reagent through the usage of enhancement medium to visualize agglutination.

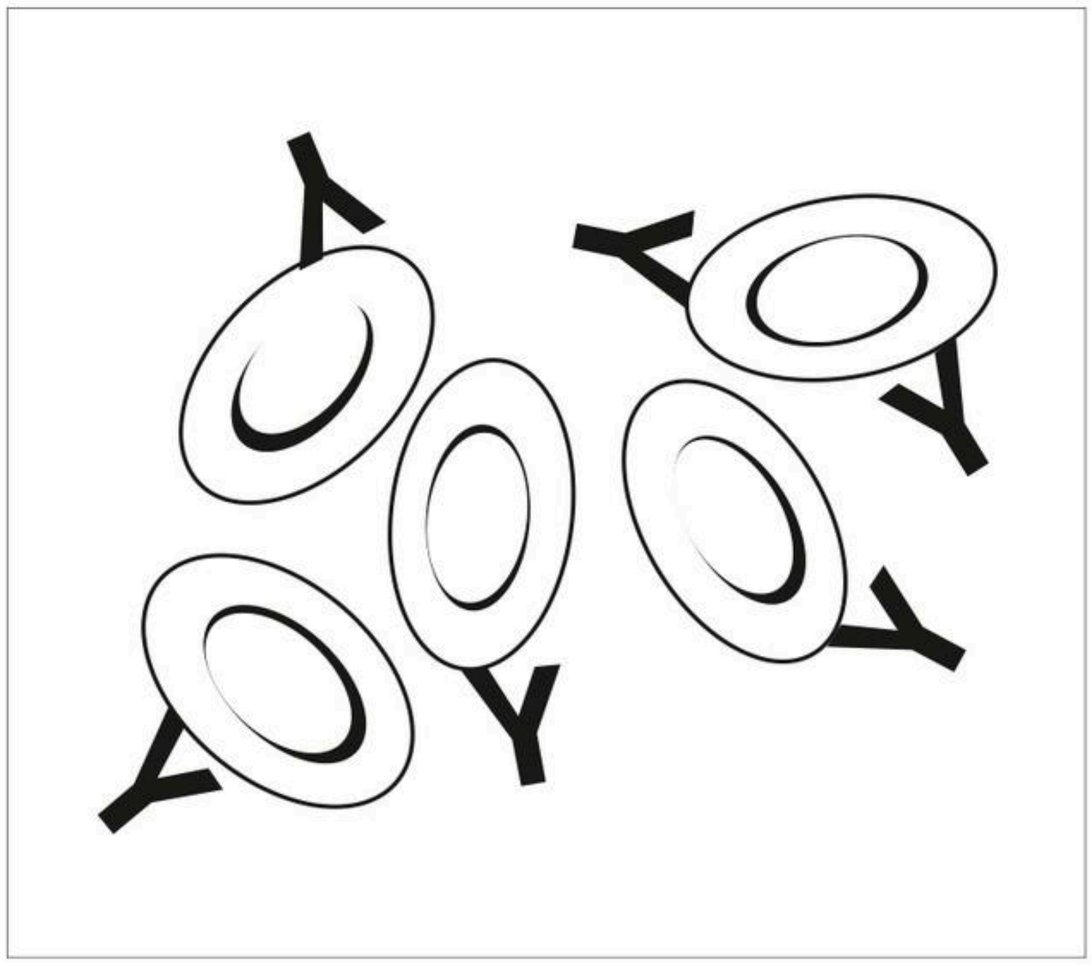

Fig . 4 Illustration of red blood cells sensitized with incomplete antibodies

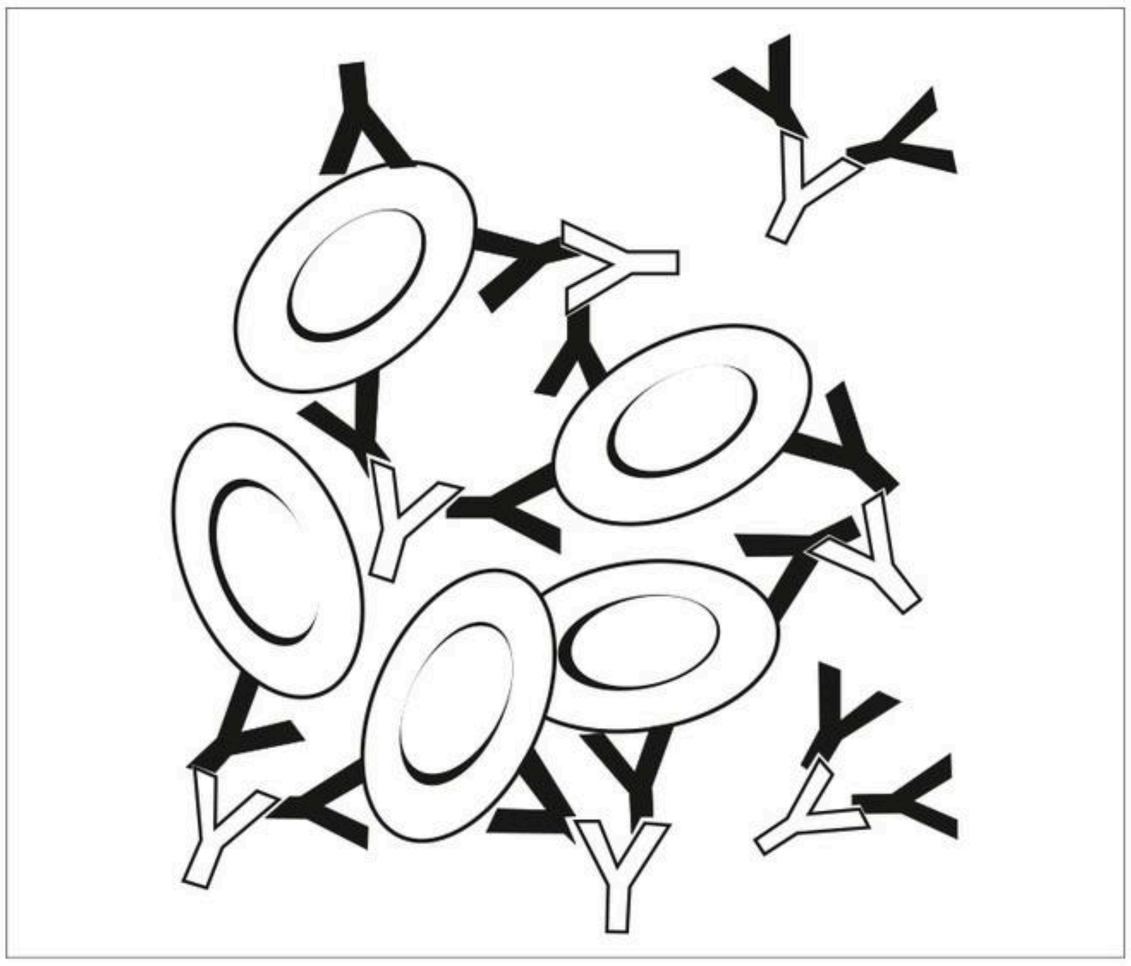

Fig. 5 Illustration of IgG sensitized red blood cells agglutinated by AHG reagent

Role of enhancement medium

An enhancement medium can either be a protein (e.g. Bovine serum albumin) or enzymes (e.g. papain, bromelin, ficin) or low ionic salt solution (LISS) or charged molecules (polybrene) that reduce zeta potential, enhance antibody uptake, enhancement in agglutination reactions.

Inorder to clearly understand antibody enhancement methods, it is important to consider the basic aspect of haemagglutination reactions. Agglutination is a reversible chemical reaction and occurs probably in two stages:

- Sensitization i.e. attachment of antibody to red blood cell membrane antigen.
- Formation of bridges between the sensitized red blood cells to form lattice (crosslinking) to visualize agglutination.

Thus various factors that affect these two stages can be manipulated to enhance agglutination.

Enhancement techniques

- **Albumin additives**
  Bovine serum albumin, a dipolar protein has been used routinely for many years as an enhancement medium. The enhancement effect of albumin is attributed to its influence on the second stage of agglutination by reducing the net negative charge on the red blood cell membranes thereby potentiating agglutination. Bovine serum albumin is available as solutions of 22% or 30% concentration.

- **Enzymes**
  Treating red blood cells with certain enzymes enhances agglutination of antibodies with red blood cell antigens. Most commonly used enzymes in immunohaematological procedures are papain, bromelin and ficin belong to thiol protease class of enzymes.

  Proteolytic enzymes reduce the red blood cell surface charge by cleaving sialic acid molecules on red blood cell membrane, lowering the zeta potential and enhance agglutination. Enzyme treatment also causes spicule formation on the red blood cell membrane. This greatly increases the potential number of contact points for antibodies to react with corresponding antigens on red blood cell membrane.

  The reaction of Anti-P, Lewis and Kidd blood group antibodies are stronger with enzyme treated red blood cells. Another important observation is that Lewis and Kidd blood group antibodies may lyse enzyme treated red blood cells.

- **Positively charged molecules**
  In the presence of positively charged polymers such as hexadimethrine bromide (Polybrene®), protamine sulphate and poly-L-lysine, normal red blood cells exhibit spontaneous aggregation. Thus any red blood cells sensitized with antibody will exhibit enhanced agglutination.

  The action of these charged molecules may be due to neutralization of negative charge contributed by sialic acid and release of water molecules that cover red blood cell membrane thereby enhancing agglutination.

  Polybrene® generally is added to red blood cells that have been incubated with antibody at low ionic strength and low pH.

- **Polyethylene Glycol (PEG)**
  PEG is a water-soluble polymer used as an additive to increase antibody uptake. Its action is to remove water molecule that takes up more space around the red blood cell membrane, thereby enhancing antibody uptake and reaction strength.

- **LISS (Low Ionic Salt Solution) and LISS additives**
  LISS (approximately 0.03 M) greatly increases stage 1 uptake of antibody to red blood cells, as compared to normal saline (approximately 0.17M). To prevent lysis of red blood cells at such a low ionic strength, a non-ionic substance such as glycine is incorporated in LISS.

  LISS additives may sometimes contain macromolecules (e.g. BSA) in addition to ionic salts and buffers. Addition of macromolecules to LISS reduces ionic strength with shortened incubation times.

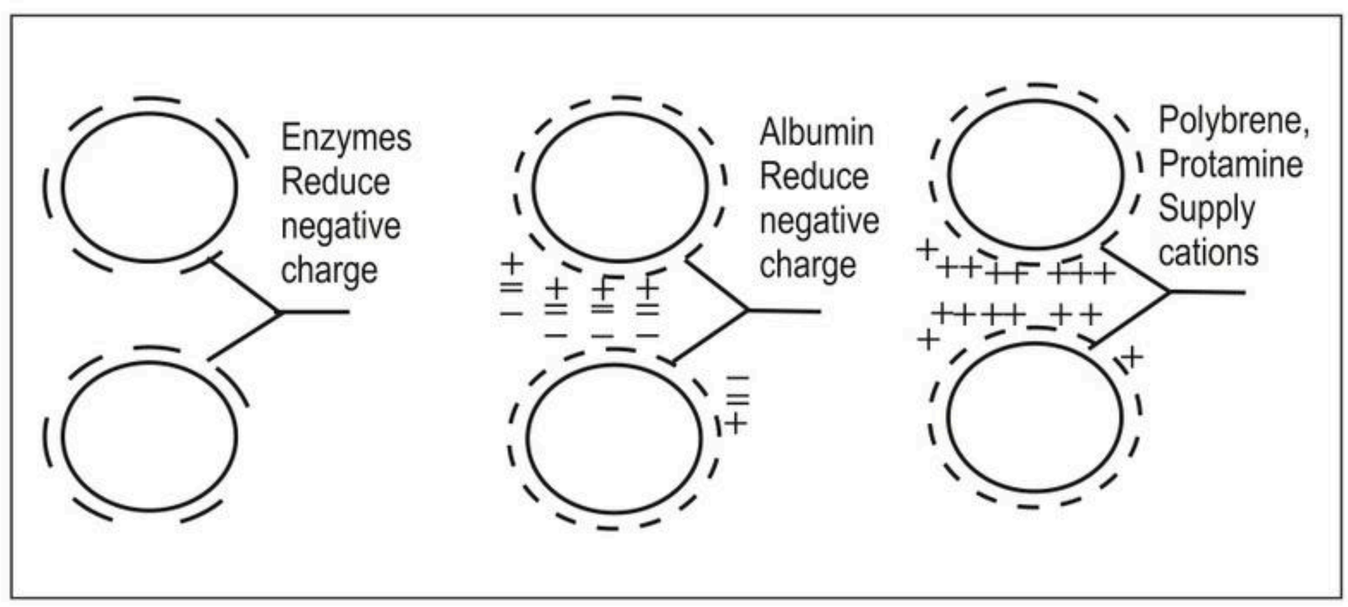

Fig. 6 Illustration of Antibody Enhancement

Complement

An observable phenomenon that may result from red blood cell antigen-antibody interaction is haemolysis. This requires co-operation of another defense mechanism of body known as the complement system. Complement is the name given to a set of approximately twenty serum proteins, which in response to antigenic stimulus react with one another in a sequential cascade.

The most important complement component is C3b, which can be, activated either by the classical or the alternate pathway. The antigen-antibody interactions activate the classical pathway that is of great significance in blood group serology.

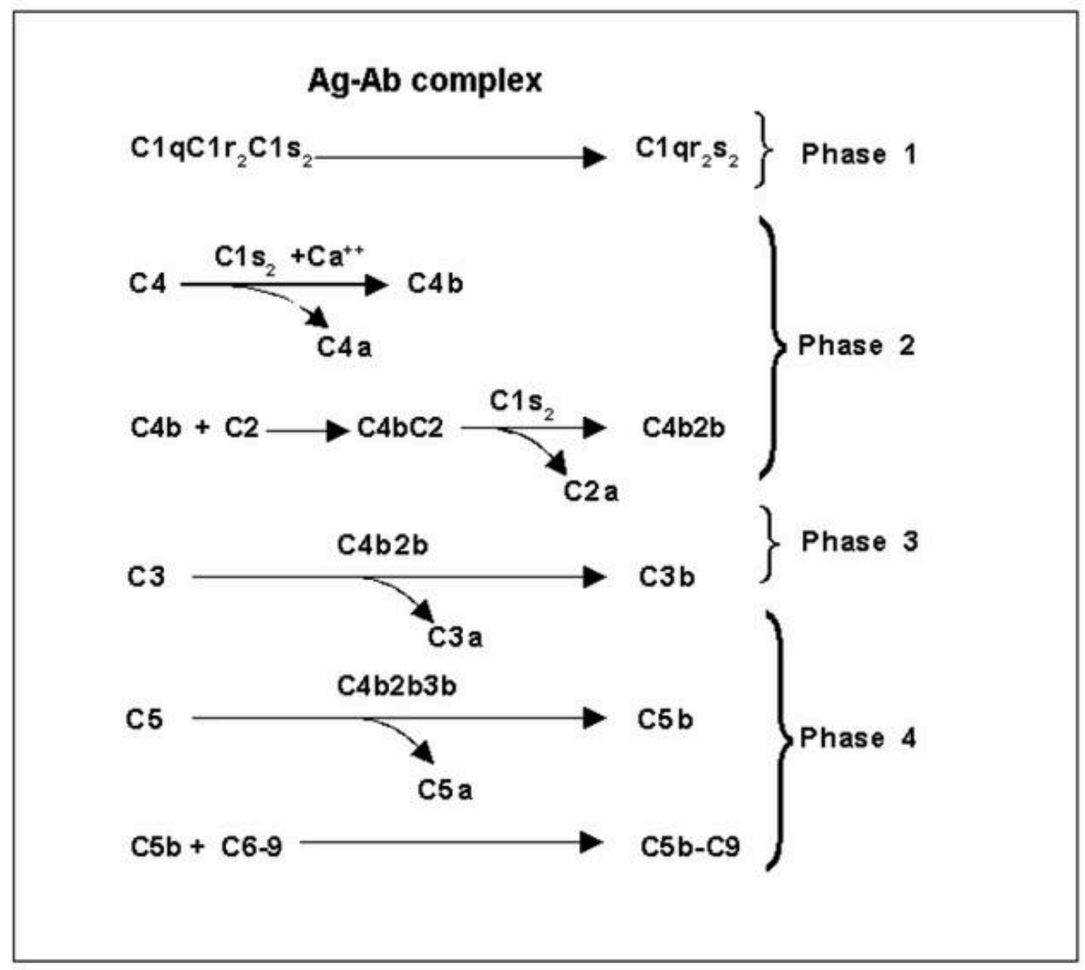

Fig. 7 Activation of Classical complement pathway

Immunoglobulin requirements for activation of classical pathway

The mode of activation of classical pathway is through binding of C1q to CH2 domain of IgM or to the CH2 domain of IgG1 or IgG3 antibodies. The C1q molecule has six Fc binding sites and inorder to make a firm bond with antibody molecule, atleast two of these sites must bind to antibody.

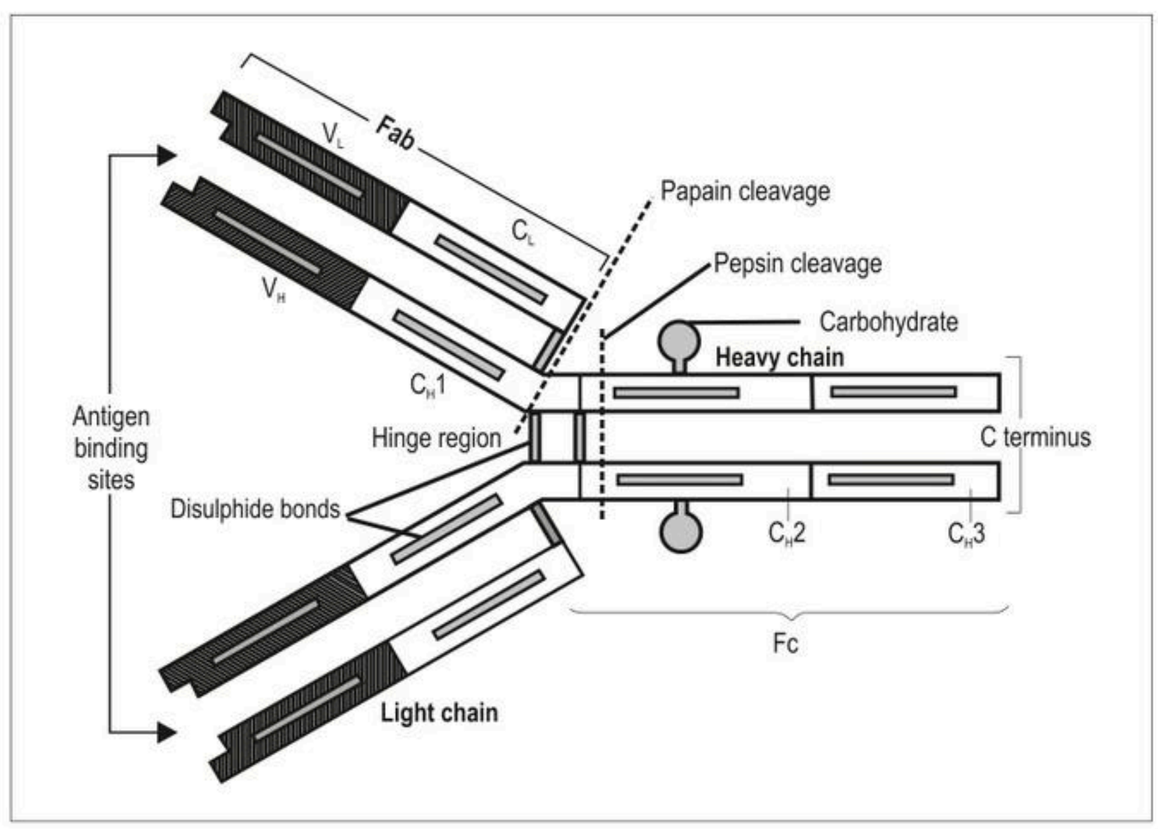

Fig.8 Illustration of immunoglobulin molecule

In case of IgM antibodies, when the molecule is in planar or star form, there is only a single binding site for C1q, as a result it cannot bind firmly and activate C1q. However when combined with antigen, IgM molecule usually assumes staple form with the F(ab) at right angle with Fc. The distortion produced by this movement exposes an additional C1q binding site. As a result IgM antibody combined with its corresponding antigen bind to two C1q sites on single IgM molecule.

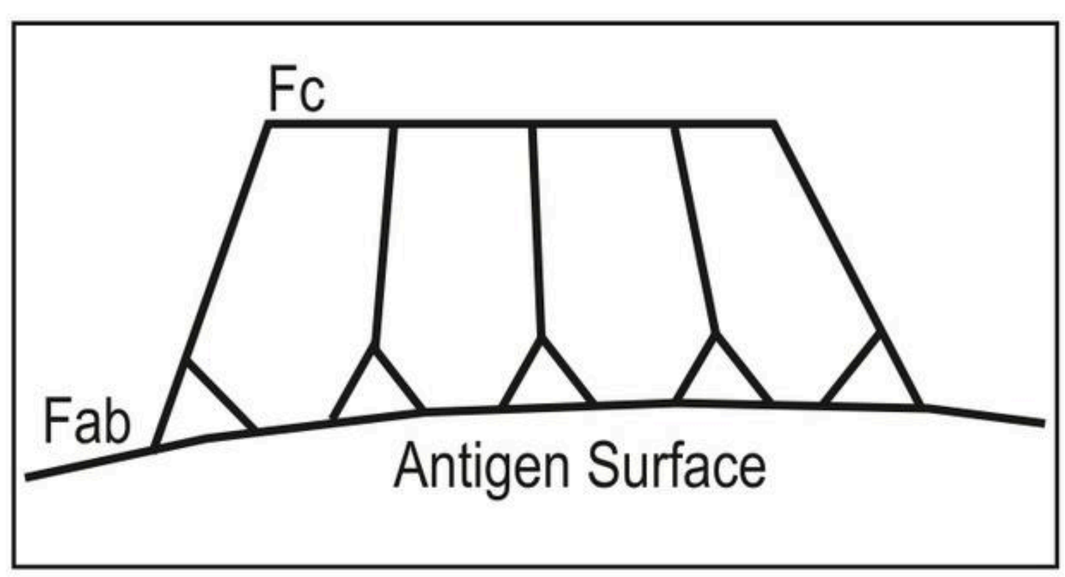

Fig 9. Illustration of staple form-IgM molecule

In case of IgG antibodies, two IgG antibodies must be present on antigen surface at a distance of 20-30 nm apart from each other. This is the maximum span of the C1q molecule. It is for this reason IgM is considered more effective in activating C1q than IgG molecule.

An important point to note is that all examples of immune Anti-D fail to activate complement. The reason being that the D antigenic sites are too far apart from each other on the red blood cell surface. As discussed two IgG molecules must be at a distance of 20-30 nm apart from each other so as to activate C1q. Since D antigenic sites are too far apart from each other, the bound IgG molecules cannot activate C1q.

Complement binding antibodies

Complement components may attach to red blood cells in vivo or in vitro via one of the probable mechanisms

- Complement binding alloantibody (blood group antibodies present in individual due to transfusion or pregnancy) specific for red blood cell antigen may cause sensitization of complement component on red blood cell surface due to transfusion.

Complement binding alloantibodies when present in low concentrations bring about the attachment of complement component upto the C3 stage, but produce little or no haemolysis. Further activation of C3 occurs only when complement is powerfully activated due to high concentration of antibodies, leading to the generation of large amount of C3b. Certain antibodies such as Anti-Jk^a are seldom lytic but invariably activate complement thereby leading to the deposition of complement components on the red blood cell surface.

Complement binding by human red cell Antibodies

System	Antibody	Readily detectable haemolysis*	Positive antiglobulin test (anti-complement)
ABO	anti-A anti-B ant-A,B	many	many
	anti-A_1	none	very few
	anti-HI	none	few
	anti-H^t	all	all
Lewis	anti-Le^a	some	all
	anti-Le^b	few	many
P	anti-P_1	few	few
	ant-PP_1 P^k	all	all
	anti-P (auto-)	all	all
	anti-P (allo-)	many	all
Ii	anti-I anti-i } auto-		
Sd	anti-Sd^a	none	some
Rh	anti-D	none	none
	anti-c, E, etc.	none	none
K	anti-K	none	many
Fy	anti-Fy^a	none	many
Jk	ant-Jk^a	few	all
MNSs	anti-M, -N	none	none
	anti-S, -s	none	some
Lu	anti-Lu^a, -Lu^b	none	none
Di	anti-Di^a	few	few-
Xg	anti-Xg^a	none	many

- About 10-20% of patients with warm AIHA (Autoimmune haemolytic anaemia) demonstrate in vivo sensitization with complement component C3.
- In cold haemagglutinin disease, the cold reactive autoantibody can react with red blood cell antigens at temperature upto 32^{0}C range (thermal amplitude 10-32^{0}C). When the patient is exposed to cold temperature, red blood cell is sensitized with autoantibody, which activates complement. If the red blood cells escape haemolysis, they return to the central circulation where the temperature is 37^{0}C and the autoantibody dissociates from the cells, leaving complement component C3d firmly bound to the red blood cell membrane.

Basic concepts of Anti human Globulin testing

Two major class of antibodies react with red blood cell antigens namely the complete antibodies and the incomplete antibodies.

Complete antibodies, or saline agglutinins will agglutinate red blood cells suspended in saline, which are usually IgM class of antibodies.

Incomplete antibodies do not react in saline but require special techniques to agglutinate red blood cells; which are IgG class of antibodies.

After combining with their corresponding antigen, blood group antibodies have the ability to activate complement. Thus sensitization of incomplete antibodies or complement component can occur either in vivo or in vitro. Anti human globulin reagent is used to detect IgG or complement sensitized red blood cells.

For detecting in vivo antibody or complement sensitization DAT (Direct Antiglobulin Test) is used whereas for detecting in vitro antibody or complement sensitization IAT (Indirect Antiglobulin Test) is used.

Anti Human Globulin Reagents

Polyspecific Anti human globulin (Coombs) Reagent:

The term 'polyspecific' reagent refers to reagent system containing blend of antibodies for more than one antigen i.e. blend of Anti human-IgG, Anti human C3b, Anti human C3d. Polyspecific reagent may be a blend of:

- Polyclonal Anti human-IgG, polyclonal Anti human C3b, polyclonal Anti human C3d.
- Polyclonal Anti human-IgG, monoclonal Anti human C3b, monoclonal Anti human C3d.
- Polyclonal Anti human IgG, monoclonal Anti human C3d.

ICSH Recommendations for Polyspecific Anti Human Globulin reagents

ICSH has recommended certain specifications, which a commercially available Anti human globulin reagent should comply:

Specificity:

The reagent should only agglutinate red blood cells sensitized with antibodies and /or coated with significant levels of complement components.

- Potency of Anti-human IgG antibodies:
  The potency of Anti-human IgG component should be evaluated by serological titration (chequer board titration).
- Specificity and potency of Anti human complement antibodies:
  A polyspecific reagent should contain Anti-C3c and Anti-C3d at controlled levels to avoid false positive reactions or a suitable potent monoclonal Anti-C3d. It should contain little or no Anti-C4 activity. The assessment of these qualities requires red cells specifically coated with complement components for evaluating the serological potency.
- All the evaluations should be done in parallel with international reference reagents.

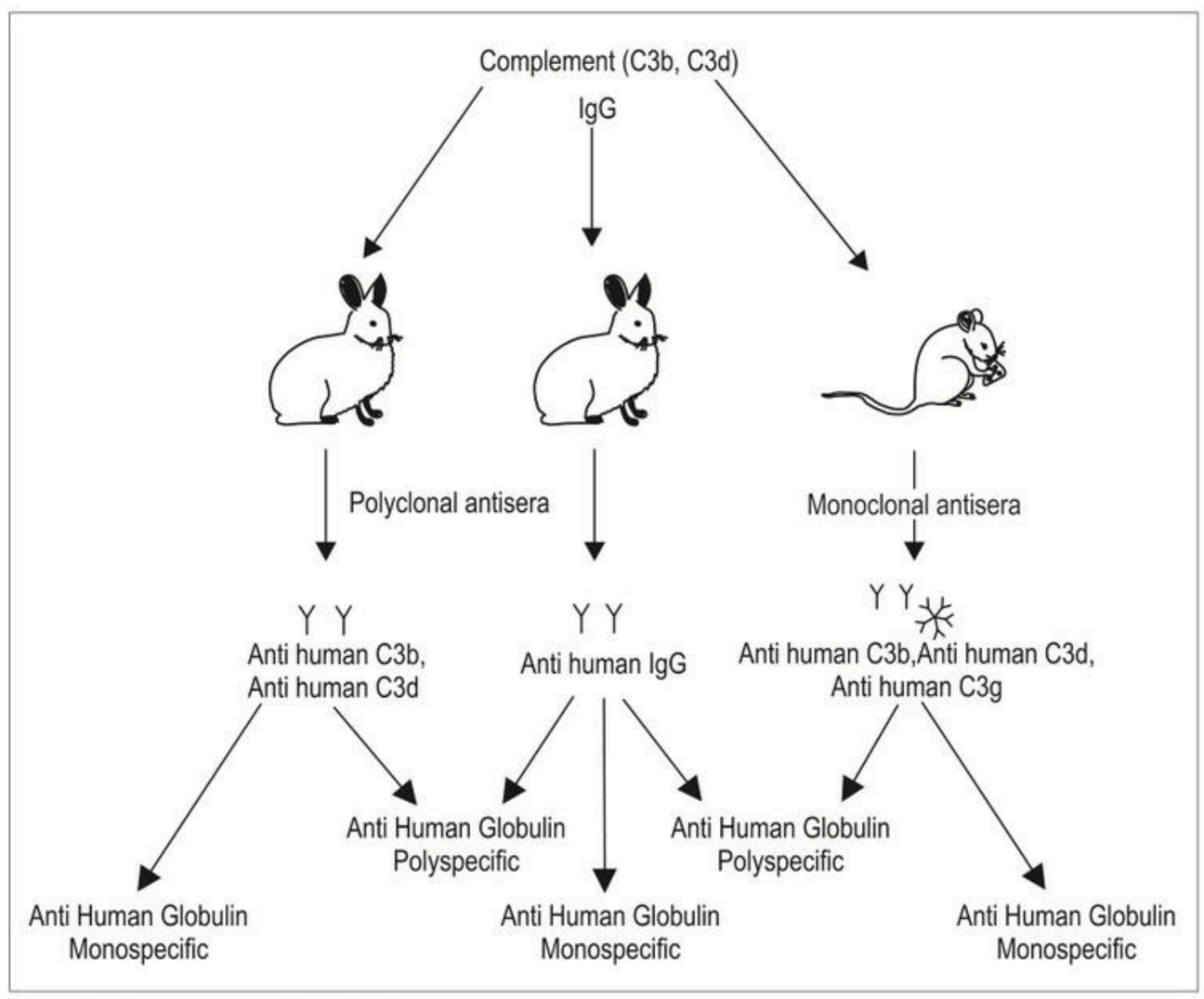

Fig. 10 Anti Human Globulin Manufacture

Polyclonal Anti human-IgG is usually prepared in rabbits, though when large volumes of antibody are required sheep or goats may be used. In production of polyclonal Anti human-IgG, animals are hyperimmunised to produce high titer, high avidity Anti human IgG class antibodies. After achieving the desired specificity and titer, the animals are bled for the production batch of reagent.

With monoclonal antibodies, the hybrid clones are screened for antibodies with the required specificity and affinity. The antibody secreting clones may then be propagated in culture or by innoculation into mice, in which case the antibody is collected in ascites. Monoclonal anti human complement may be either IgM or IgG class of antibodies.

Thus separate blends of Anti human-IgG and Anti human complement antibodies are made, and each pool is then adsorbed with A_1, B and O red blood cells to remove heterospecific antibodies. The total antibody content of each pool is determined and then pools are analysed by titration method to calculate the optimum antibody dilution for Anti human IgG component and Anti human complement component.

Optimum concentration of Anti human IgG component is ascertained by chequer board titration method. In chequer board titration dilutions of Anti human IgG are reacted with various dilutions of Anti-D IgG sensitized cells. This is a critical step because excess Anti human IgG may lead to prozoning during test returning false negative results, correspondingly if Anti human IgG concentration is less than optimum it may lead to false negative results with weakly sensitized cells. The evaluation procedure should also involve parallel testing with international reference reagent (e.g. W.H.O. RIIIM – Polyspecific Anti human globulin serum, available from Central Laboratory of Netherlands Red Cross Transfusion Service, Amsterdam).

Similarly optimum concentration of Anti human Complement is ascertained by sensitizing complement to O Rh_o (D) positive red blood cells by the sucrose method, preparing various dilutions of complement sensitized cells. The optimum concentration of Anti human complement component is evaluated by reacting dilutions of Anti human complement with dilutions of cells sensitized with different levels of serially diluted complement in parallel with international reference reagent.

Once the required optimum performance characteristic for each component is ascertained, reagent blend of Anti human IgG and Anti human Complement antibodies is prepared and run as a system in parallel with the international reference reagent. Thus the final product is one that contains optimum concentration of Anti human IgG and Anti human Complement antibodies for broad spectrum reactivity.

Dilutions of IgG Sensitized Cells

Dilutions of AHG	N		16		32		64		128		256		512		1024		2048		4096	
N	3.5	3.5	3.5	3.5	3.5	3.5	2.5	3.5	2.5	2.5	1.0	1.0	1.5	1.5	1.0	1.5	1.0	1.5	G^r	2.0
1:2	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	2.0	2.0	2.0	2.0^s	1.5	1.0^s	1.5	1.0^s	2.0	2.0	1.0	1.0
1:4	3.5	3.5	3.5	3.5	3.5	3.5	2.5	3.0	1.5	2.0	1.5	1.5	1.5	1.0^s	1.0^s	1.0	1.0^s	1.0	1.0^w	1.0^w
1:8	3.5	3.5	3.5	3.5	3.0^s	3.0^s	2.0^w	3.0	1.5	1.5	1.0^s	1.5	1.0^s	1.5	1.0^s	1.0	1.0^w	1.0^s	1.0^w	1.0
1:16	3.5	3.5	3.5	3.0^s	3.0^s	2.5	2.0	2.0	1.5	1.5	1.5	1.5	1.0^s	1.0^s	1.0^s	1.0^s	1.0^w	1.0	1.0^w	1.0
1:32	3.5	3.5	2.0	3.0^s	3.0^s	2.5	2.0	2.0^w	2.0	1.0^s	1.5	1.0^s	1.0^s	1.0	1.0	1.0	1.0^w	0.5	1.0^w	Gr
1:64	3.5	3.5	2.0^s	2.0	2.0	2.0	2.0	1.5	1.0^s	1.0^s	1.0^s	1.0^s	1.0^s	1.0^w	Gr	0.5	0.5	0.5	Gr	---

W.H.O. R III M Commercially available Polyspecific AHG

Fig. 11 Chequer board titration results

Monospecific Coombs Reagent:

Monospecific reagents for use in anti human globulin test are prepared by similar procedure, however the reagent contains antibody of only one specificity.

- Monospecific Anti human IgG reagent contains only polyclonal Anti human IgG
- Monospecific Anti human C3b reagent contains only polyclonal Anti human C3b or monoclonal Anti human C3b.
- Monospecific Anti human C3d reagent contains only polyclonal Anti human C3d or monoclonal Anti human C3d.

Direct Anti Human Globulin Test (DAT)

DAT is used to detect in vivo sensitization of red blood cells with immunoglobulin, complement or both. A positive DAT, with or without shortened red blood cell survival, may result from:

- Autoantibodies to intrinsic red blood cell antigens.
- Alloantibodies in recipients circulation reacting with antigens on recently transfused donor red blood cells.
- Alloantibodies in donor plasma, plasma derivatives or blood fractions, which react with antigens on red blood cells of transfusion recipients.
- Alloantibodies in maternal circulation, which cross placenta and sensitize foetal red blood cells (HDN).
- Antibodies directed against certain drugs, which bind to red blood cell membranes (e.g. Penicillin)
- Adsorbed proteins, including immunoglobulins, which attach to abnormal membranes or red blood cells modified by therapy with certain drugs, notably those of cephalosporin group.
- Complement components or rarely IgG bound to red blood cells after administration of drugs such as quinidine and phenacetin may induce drug/anti drug interaction.
- Non red blood cell immunoglobulins associated with red blood cells in patients with hypergammaglobulinaemia or recipients with high dose of intravenous gammaglobulin.
- In-patients with organ transplantation, passenger lymphocytes of donor origin produce antibodies directed against ABO or other antigens on the recipient's cells, causing a positive DAT.
- Patients receiving ALG (antilymphocyte globulin) or ATG (antithymocyte globulin) of animal origin may develop a positive DAT within a few days, apparently related to high titer heterophile antibodies in these products and the presence of corresponding antibodies in animal derived AHG sera.

Major applications of DAT in blood group serology

Haemolytic disease of the newborn (HDN)

In HDN foetal red blood cells in vivo are sensitized with IgG alloantibody of maternal origin thereby demonstrating a positive DAT with cord red blood cells. The most commonly observed HDN is due to Rh_o (D) incompatibility between mother and foetus.

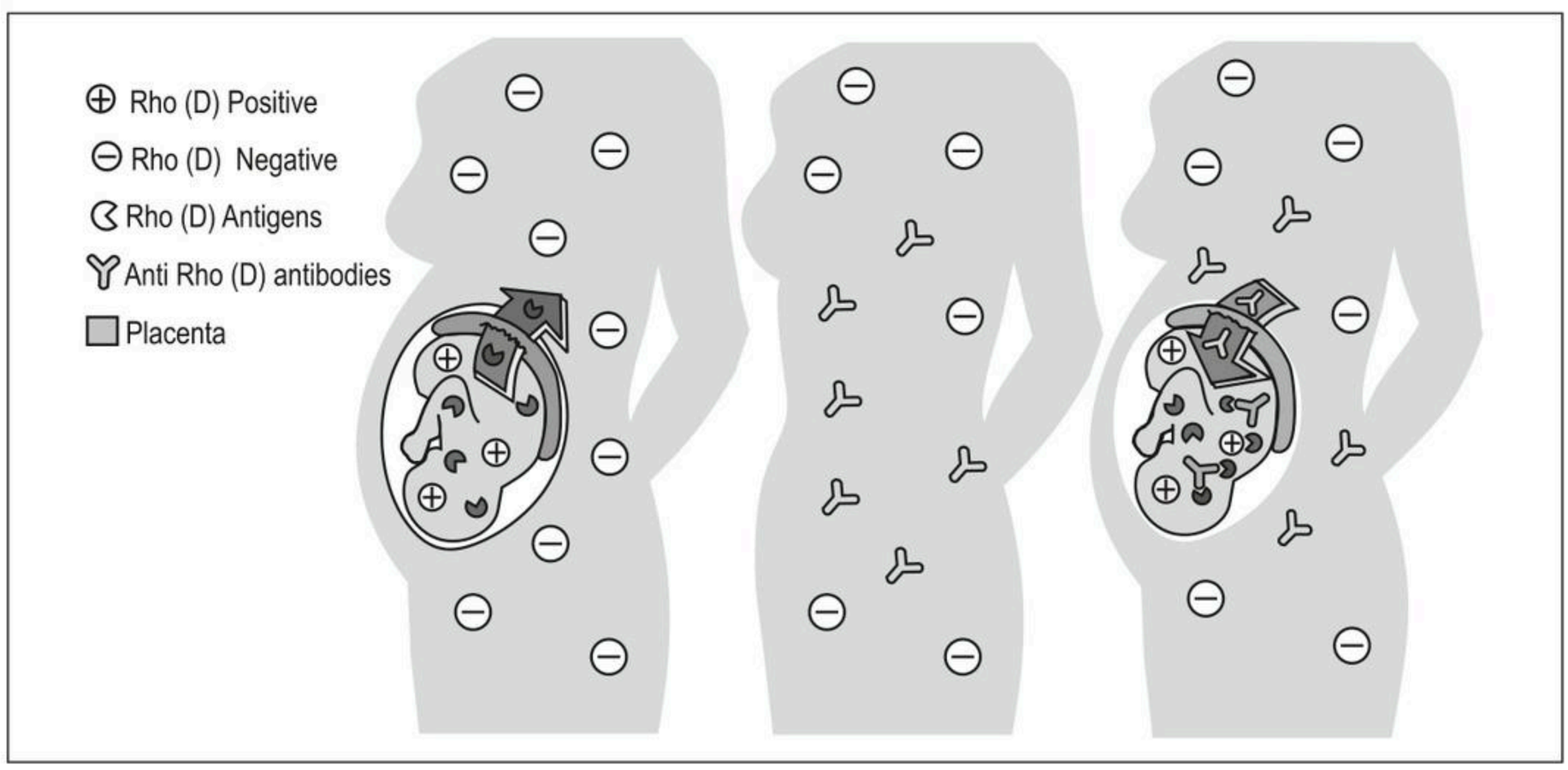

Fig. 12 Illustration of HDN

If the father is Rh_o (D) positive and the mother is Rh_o (D) negative and during first pregnancy their progeny inherits Rh_o (D) positive red blood cell antigens. During parturition, the foetal red blood cells can enter mother's circulation providing antigenic stimulus for the production of Anti-D antibodies. These Anti-D antibodies normally will not have any effect during the first Rh-incompatible pregnancy unless the mother has Anti-D antibodies by previous incompatible blood transfusions.

During subsequent pregnancy, for the same couple, if the foetus is Rh_o (D) positive again, the Anti-D antibodies will be activated along with the presence of Anti-D antibodies from the first pregnancy already in the circulation. Since the IgG antibodies cross the placental barrier, these circulating Anti-D will sensitize and destroy foetal Rh_o (D) positive cells. This process is demonstrated by a positive DAT on cord red blood cells.

Transfusion Reactions

A patient will demonstrate positive DAT, if serum contains antibodies against red blood cell antigens of donor red blood cells. Likewise antibody present in donor plasma may also react with recipient red blood cells thereby demonstrating positive DAT.

Other immune haemolytic diseases

A positive DAT may be observed due to acquired haemolytic anaemia probably because of autoantibodies directed against individuals own intrinsic red blood cell antigens.

Classification of Autoimmune Haemolytic Anaemia:

- Warm Autoimmune Haemolytic Anaemia (WAIHA)
  - Primary (idiopathic)
  - Secondary (to conditions such as lymphoma, SLE, carcinoma, drug therapy

- Cold Agglutinin Syndrome (CAS)
  - Primary (idiopathic)
  - Secondary (to conditions such as lymphoma, mycoplasma pneumonia, infectious mononucleosis)

- Mixed type AIHA
  - Primary (idiopathic)
  - Secondary (to conditions such as SLE, lymphoma)

- Paroxysmal Cold Haemoglobinuria (PCH)
  - Primary (idiopathic)
  - Secondary (to conditions such as syphilis, viral infections)

- DAT Negative AIHA
  - Primary (idiopathic)
  - Secondary (to conditions such as lymphoma, SLE)

Drug induced haemolytic anaemia:

Also certain drugs namely penicillin, procainamide, cephalosporins may also be associated with immune red blood cell destruction thereby demonstrating a positive DAT.

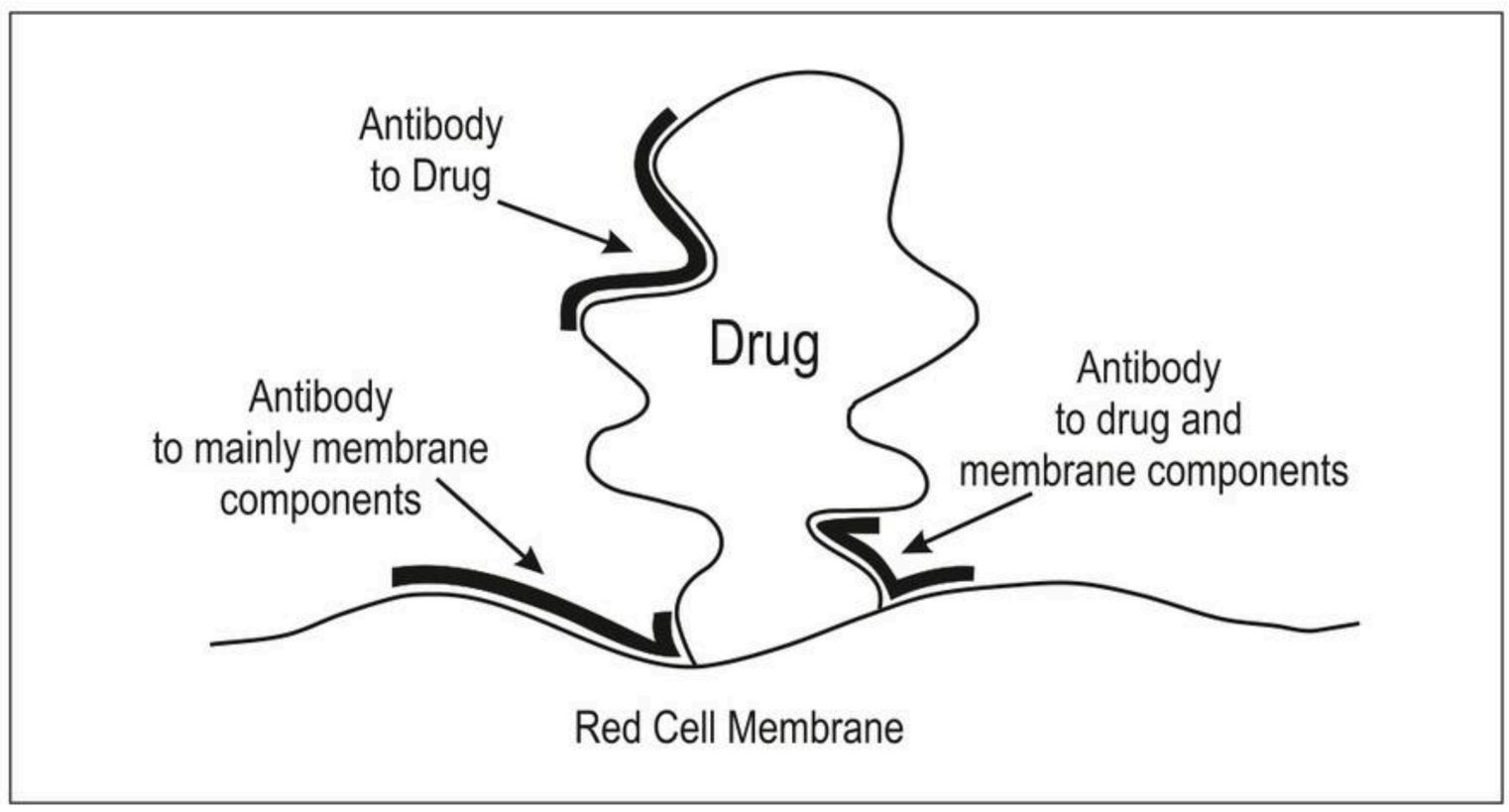

Fig. 13 Illustration of Drug induced antibody reactions

Importance of serological studies in DAT positive results

As per AABB technical manual, three investigation approaches are helpful in evaluation of positive DAT:

- Test the DAT positive red blood cells with Monospecific Anti human-IgG and Monospecific Anti-C3d reagents to characterize type of proteins sensitized with red blood cell membrane.
- Test serum/plasma to detect and identify clinically significant antibodies to red blood cell antigens.
- Test eluate prepared from sensitized red blood cells with a panel of reagent red blood cells to define whether the sensitized protein is immunoglobulin or complement component. Elution frees antibody from sensitized red blood cells and recovers antibody in usable form. When only complement is sensitized, eluates are frequently non-reactive.

Probable serological findings with DAT positive- AIHA/Drug induced HA:

Parameter	WAIHA	CAS	Mixed type AIHA	PCH	Drug induced AIHA
DAT positive result	IgG/ IgG + C3/ C3	Mostly C3	IgG +C3	Mostly C3	IgG/IgG + C3
Immunoglobulin Type	IgG (sometimes IgA or IgM rarely alone	IgM	IgG, IgM	IgG	IgG
Eluate	IgG	Non reactive	IgG	Non reactive	IgG
Serum	• May React by IAT • May haemloyze enzyme treated red cells at 37°C • Mostly agglutinate enzyme treated red cells at 37 °C • May agglutinate untreated red cells at 20°C • Rarely agglutinate untreated Cells at 37 °C	• IgM haem-agglutinating antibody reactive at 4 °C usually react at 30°C in albumin	• IgG IAT reactive antibody • IgM haem agglutinating antibody usually react at 30 - 37°C in saline, also may react at 4°C saline	• IgG biphasic haemolysin (Donath Landsteiner antibody)	• IgG antibody similar to WAIHA
Specificity	Usually Rh Specificity	Usually Anti-I but can be Anti-i, rarely Anti-Pr	Usually specificity unclear, can be Anti-I, Anti-i or other cold agglutinin specificities	Anti-P (non-reactive with p and P^x red cells	Specificity often Rh related

Indirect Anti human Globulin Test (IAT)

In IAT procedures, serum or plasma is incubated with red blood cells, washed to remove unbound globulins. Agglutination that occurs after addition of Anti human Globulin reagent indicates reaction between antibody in the serum and antigen present on the red blood cell membrane.

Applications of IAT

IAT determines in vitro sensitization of red blood cells and is used in following situations:

- Detection of incomplete antibodies to potential donor red blood cells, pregnant women, blood donors.
- Identification of antibody specificity using a panel of red blood cells with known antigenic profile.
- Determination of red blood cell phenotype using known antisera (e.g. D^u testing)
- Titration of incomplete antibodies.

Probable sources of Error in Antihuman Globulin Testing

False Negative Results

- Neutralization of Antihuman Globulin Reagent
- Failure to wash cells adequately to remove all serum/plasma. Fill tube atleast three fourth full of saline for each wash.
  - If increased serum volumes are used, routine wash may be inadequate. Wash additional times more than three or four wash phases.
  - Contamination of Anti human globulin reagent by extraneous protein. Do not use finger or hand to cover tube. Contaminated droppers or wrong reagent dropper can neutralize entire vial of Anti human globulin reagent.
  - High concentration of IgG paraproteins in test serum (cryoglobulin). Wash additional times more than three or four wash phases.

- Interruption in testing
  - Bound IgG may dissociate from red blood cells or leave too little IgG to detect or may neutralize Anti human globulin reagent. Perform the test immediately.
  - Agglutination of IgG coated cells will weaken. Centrifuge and read immediately.

- Improper reagent storage
  - Anti human globulin reagent may loose reactivity if frozen. Reagent may become bacterially contaminated. Store at the recommended storage condition.
  - Excess heat or repeated freeze/thaw cycles may cause loss of reactivity of Anti Human globulin reagent. Replace the reagent back to the recommended storage condition.

- Improper procedure
  - Overcentrifugation may pack cells so tightly that agitation required to resuspend cells breaks up agglutinates. Undercentrifugation may not be optimal for agglutination. The optimum centrifugation speed should be ascertained for each centrifuge.
  - Failure to add test serum, enhancement medium or Anti human globulin reagent may lead to negative test result. Follow the manufacturers instructions meticulously.
  - Too heavy red cell concentration may mask weak agglutination. Too light suspension may be difficult to read.
  - Improper/insufficient serum:cell ratio

- Complement
  - Rare antibodies, notably Anti-Jk^a and Anti-Jk^b may only be detected when polyspecific Anti human globulin reagent is used and active complement is present.

- Saline
  - Low pH of saline solution can decrease sensitivity of Anti human globulin test. Optimal wash solution for most antibodies is pH 7.0-7.2. It has been observed that commercially available infusion saline/ saline stored in plastic containers can seriously compromise the sensitivity of anti human globulin test. Saline stored in plastic containers and further autoclaved leads to leaching of certain chemicals which shifts the pH to the acidic side and impacts the sensitivity of anti human globulin test. Preferably use phosphate buffered saline as wash solution or suspending medium.
  - Some antibodies may require saline to be at specific temperature to retain antibody on red blood cell. Use 37^0C or 4^0C saline.

False Positive Results

- Particles or Contaminants
  - Dust or dirt in glassware may cause clumping of cells. Fibrin or precipitates in test serum may similarly produce cell clumps that mimic agglutination

- Improper procedure
  - Overcentrifugation may pack cells so tightly that they do not easily disperse and appear positive
  - Centrifugation of test with polyethylene glycol or positively charged polymers prior to washing may create clumps that do not disperse.

- Cells with positive DAT result
  - Cells that are positive by DAT will also be positive in any indirect antiglobulin test. In such case antibodies should be eluted from the sensitized cells.

- Complement
  - Complement components, primarily C4, may bind to cells from clots or from CPDA-1 donor segments during storage at 4^0C and occasionally at higher temperature. For DATs, use red blood cells anticoagulated with EDTA, ACD or CPD
  - Samples collected in scratched glass tubes can lead to spurious activation of complement.
  - Complement may attach to cells in specimens collected from infusion lines used to administer dextrose-containing solutions. Strongest reactions are seen when large bore needles are used or when sample volume is less than 0.5 ml.

Coombs Control Cells/Complement Coated Cells

Coombs control cells should be used routinely in direct and indirect Anti Human Globulin test. Coombs control reagent is Anti-D IgG sensitized, washed and made upto a 5% suspension. Coombs control cells are used for:

- Procedural validation of tests employing Coombs reagent. Coombs control cells are added after performing Anti Human Globulin test. To a negative result after addition of Coombs control cells, agglutination indicates that AHG was indeed added and that it has not been neutralized.

- Functional validation of Coombs reagent. The performance of Coombs reagent can be validated as a quality control measure on routine basis.

Similarly complement coated cells can also be prepared in vitro. Thus complement coated cells can also be used for functional validation of Coombs reagent.

Now with commercially available red blood cell stabilizing solution, the Coombs control cells and complement coated cells can be prepared in situ and stored in cell stabilizing solution for long term use and storage.

Appendix

Indirect Anti human Globulin Test for the detection of Red blood cell antibodies

● Saline Phase Indirect Anti Human Globulin Test

Specimen:

Serum or plasma may be used. Preferably freshly collected serum should be used.

Reagents:

1. Normal saline
2. Polyspecific AHG or Anti-IgG reagent.
3. Coombs control cells.
4. Donor cells/ reagent red blood cells

Procedure:

1. Add two drops of serum to properly labeled test tubes.
2. Add one drop of reagent red blood cells or donor red blood cells as a 2-5% saline suspension to each tube and mix.
3. Centrifuge and observe for haemolysis and agglutination. Grade and record the results.
4. Incubate at 37^{0}C for 30-60 minutes.
5. Centrifuge for 15-20 seconds at approximately 900-1000 g and observe for haemolysis and agglutination. Grade and record the results.
6. Wash the red blood cells three or four times with saline and completely decant after the final wash.
7. Add AHG reagent to the dry cell button according to the manufacturers instructions. Mix well.
8. Centrifuge for 15-20 seconds at approximately 900-1000 g and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coombs control cells.

● Albumin Phase Indirect Anti Human Globulin Test

Specimen:

Serum or plasma may be used. Preferably freshly collected serum should be used.

Reagents:

1. Normal saline
2. Bovine albumin (22% or 30%).
3. Polyspecific AHG or Anti-IgG reagent.
4. Coombs control cells.
5. Donor cells/ reagent red blood cells

Procedure:

1. Add two drops of serum to properly labeled tubes.
2. Add an equivalent volume of 22% or 30% bovine albumin (unless manufacturers directions state otherwise).
3. Add one drop of 2-5% saline suspended reagent or donor red blood cells to each tube and mix.
4. Incubate at 37^{0}C for 15-30 minutes.
5. Centrifuge and observe for haemolysis and agglutination. Grade and record the results.
6. Wash the cells three or four times with saline and completely decant after final wash.
7. Add AHG to dry cell button according to the manufacturers instruction. Mix well.
8. Centrifuge and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coombs control cells.

● LISS Phase Indirect Anti Human Globulin Test

Specimen:

Serum or plasma may be used. Preferably freshly collected serum should be used.

Reagents:

1. Normal saline
2. LISS
3. Polyspecific AHG or Anti-IgG reagent.
4. Coombs control cells.
5. Donor cells/ reagent red blood cells

Procedure:

1. Wash reagent or donor red blood cells three times in normal saline and completely decant saline after last wash.
2. Resuspend the cells to a 2-5% suspension in LISS.
3. Add two drops of serum to properly labeled tube.
4. Add two drops of LISS suspended red blood cell suspension and incubate according to manufacturers direction. Typically this is 10-15 minutes at 37^{0}C.
5. Centrifuge according to manufacturers direction.Typically this is 15-30 seconds at 900-1000g and observe for haemolysis and agglutination by gently resuspending the cell button. Grade and record results.

6. Wash the cells three or four times with saline and completely decant after final wash.
7. Add AHG to dry cell button according to the manufacturers instruction. Mix well.
8. Centrifuge and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coombs control cells.

● PEG Enhanced Indirect Anti Human Globulin Test

Specimen:

Serum or plasma may be used. Preferably freshly collected serum should be used.

Reagents:

1. Normal saline
2. PEG (20% in PBS)
3. Polyspecific AHG or Anti-IgG reagent.
4. Coombs control cells.
5. Donor cells/ reagent red blood cells

Procedure:

1. For each sample to tested, mix 2 drops of test serum, 4 drops of 20% PEG in PBS, and 1 drop of 2-5 % red blood cell suspension.
2. Incubate according to manufacturers directions. Typically this is 37^0C for 15 minutes.
3. Do not centrifuge.
4. Wash the cells four times with saline and completely decant after the final wash.
5. Add AHG to dry cell button according to the manufacturers instruction. Mix well.
6. Centrifuge and observe for reaction. Grade and record the results.
7. Confirm the validity of negative tests by adding Coombs control cells.

● LIM (Low Ionic Medium-Polybrene) Indirect Anti Human Globulin Test

Specimen:

Serum or plasma may be used. Preferably freshly collected serum should be used.

Reagents:

1. Normal saline
2. Low ionic Medium (LIM): To a 500 ml volumetric flask add 25 g of dextrose and 1 g of $Na_2EDTA\,2H_2O$. Fill flask to 500 ml mark with distilled water.
3. Polybrene (Commercially available)
4. Resuspending medium: 0.2 M trisodium Citrate, 5% dextrose; Working solution made by mixing 60 ml of 0.2 M trisodium citrate with 40 ml of 5% dextrose.
5. Washing solution (for Antihuman Globulin Testing): 0.01M trisodium citrate
6. Polyspecific AHG or Anti-IgG reagent.
7. Coombs control cells.
8. Donor cells/ reagent red blood cells

Procedure:

1. Prepare 1% suspension of donor or reagent red blood cells in the serum used for testing.
2. Add 1.0 ml of LIM solution. Mix and incubate for 1 minute at room temperature.
3. Add 0.1 ml of 0.05% Polybrene to each tube and mix.
4. Centrifuge according to manufacturers directions. Typically this is 10 seconds at 900-1000 g and decant the supernatant fluid. Do not resuspend cell button.
5. Add 0.1 ml of resuspending solution. Shake tube gently and observe for persistent agglutination. If strength of agglutination is weak, examine the test and a known negative control macroscopically. Do not recentrifuge.
6. If desired, the Anti Human Globulin test may be performed as follows:
   a) Add 0.05 ml of resuspending solution to each tube and mix well.
   b) Wash the cells three times with 0.01 M trisodium citrate solution.
   c) Add two drops of Anti-IgG to the dry button and mix.
   d) Centrifuge for 15 seconds at 900-1000 g. Read and record the results.
   e) Add one drop of Coombs control cells to each negative tube.

Interpretation of results for Anti Human Globulin Tests:

1. The presence of agglutination/haemolysis after incubation at 37^0C constitutes a positive test.
2. The presence of agglutination after addition of AHG reagent constitutes a positive test.
3. Anti Human Globulin tests are considered negative when no agglutination is observed after initial centrifugation and positive result with Coombs control cells. If after addition of Coombs control cells a negative result is observed then the test is invalid and must be repeated.
4. For the LIP (Low Ionic Polybrene) procedure, agglutination that persists after addition of resuspending solution indicates a positive result.

Controls:

1. The procedure used for the detection of unexpected antibodies in pretransfusion testing should be checked daily with weak antibodies.
2. When LIP technique is used test an unknown serum against reagent red blood cells, an inert serum should be tested against a random cell sample for comparative purposes.

Notes:

1. The incubation time and volume and concentration of red cells incubated are those given in literature. In all cases, the manufacturers package insert should be strictly adhered to.
2. For the PEG procedure:
   a) Omit centrifugation after 37^{0}C incubation, as red blood cells will not resuspend readily.
   b) Use monospecific Anti-IgG rather than polyspecific AHG to avoid unwanted positive reactions due to C3-binding antibodies.
3. LISS additive and PEG solutions are available from various commercial sources. Manufacturers instruction should be followed when using these reagents.

● Papain -One stage Enzyme technique/ Two stage Enzyme technique

Specimen:

Serum to be tested. Preferably freshly collected serum should be used.

Reagent:

1. Reagent red blood cells
2. Polyspecific AHG or Anti-IgG reagent.
3. Coombs control cells.
4. Donor cells/ reagent red blood cells

Procedure for One stage enzyme technique:

1. Add two drops of serum to an appropriately labeled tube.
2. Add two drops of 2-5% saline suspension of reagent red blood cells.
3. Add two drops of papain solution and mix well.
4. Incubate at 37^{0}C for 30 minutes.
5. Centrifuge and gently resuspend the cells, observe for agglutination. Grade and record the results.
6. Wash the cells four times with saline and completely decant after the final wash.
7. Add AHG to dry cell button according to the manufacturers instruction. Mix well.
8. Centrifuge and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coombs control cells.

Procedure for Two stage enzyme technique:

1. Add one drop of washed packed cells and one drop of papain reagent to appropriately labeled tube
2. Incubate at 37^{0}C for 30 minutes.
3. Wash the papain treated three times with isotonic saline and prepare 2-5% cell suspension.
4. To an appropriately labeled tube add one drop of papain treated red blood cell suspension and two drops of serum under test.
5. Mix well and incubate at 37^{0}C for 30 minutes.
6. Centrifuge and gently resuspend the cells, observe for agglutination. Grade and record the results.
7. Wash the cells four times with saline and completely decant after the final wash.
8. Add AHG to dry cell button according to the manufacturers instruction. Mix well.
9. Centrifuge and observe for reaction. Grade and record the results.
10. Confirm the validity of negative tests by adding Coombs control cells.

Antibody Titration studies

● Antibody Titration for characterizing type of antibody in serum

Specimen:

Serum (antibody) to be titrated.

Reagents:

1. Red blood cells that express the antigen(s) corresponding to the antibody specificity(ies), in a 2-5% saline suspension. Uniformity of cell suspensions is very important to ensure comparability of results.
2. Normal saline. (Dilutions may be made with albumin if desired).

Procedure:

The master dilution technique for titration studies is as follows:

1. Label ten test tubes according to the serum dilution (e.g. 1 in 1, 1 in 2, etc.).
2. Deliver one volume of saline to all test tubes except the first.
3. Add an equal volume of serum to each of the first two tubes (undiluted and 1 in 2).
4. Using a clean pipette, mix the contents of the 1 in 2 dilution several times, and transfer one volume into the next tube (1 in 4 dilution).
5. Continue the same process for all dilutions, using a clean pipette to mix and transfer each dilution. Remove one volume of diluted serum from the final tube and save it for use if further dilutions are required.
6. Label ten 10 x 75 mm or 12 x 75 mm tubes for the appropriate dilutions.
7. Using separate pipettes for each dilution, transfer two drops of each diluted serum into the appropriately labeled tubes, and add one drop of red blood cell suspension.
8. Mix well and test by serologic technique appropriate to the antibody.
9. Examine test results macroscopically, grade and record the reactions. The prozone phenomenon may cause reactions to be weaker in the more concentrated serum preparations than in higher dilutions; to avoid misinterpretation of results, it may be preferable to examine first the tube containing the most dilute serum and proceed through the more concentrated samples to the undiluted specimen.

Interpretation:

1. Observe the highest dilution that produces 1+ macroscopic agglutination. The titer is the reciprocal of the dilution. If there is agglutination in the tube containing the most dilute serum, the endpoint has not been reached, and additional dilutions should be prepared and tested.
2. In comparative studies, a significant difference in titer is three or more dilutions. Variations in technique and inherent biologic variability can cause duplicate tests to give results that differ by one dilution in either direction.
3. Titer values alone can be misleading, without additional evaluation of strength of agglutination.
   The observed strength of agglutination can be assigned a number and the sum of these numbers for all tubes in a titration study represents the score. The arbitrarily assigned threshold for significance in comparing scores is a difference of 10 or more between different test samples.

Examples of Antibodies Titers, Endpoint and Scores

		Reciprocal of Serum Dilution											
		1	2	4	8	16	32	64	128	256	512	Titer	Score
Sample 1	Strength:	3+	3+	3+	2+	2+	2+	1+	±	±	0	64(256)	
	Score:	10	10	10	8	8	8	5	3	2	0		64
Sample 2	Strength:	4+	4+	4+	3+	3+	2+	2+	1+	±	0	128(256)	
	Score:	12	12	12	10	10	8	8	5	3	0		80
Sample 3	Strength:	1+	1+	1+	1+	±	±	±	±	±	0	8(256)	
	Score:	5	5	5	5	3	3	3	2	2	0		33

Notes:

Titration is a semiquantitative technique and technical variables greatly affect the results. Hence care should be taken to achieve the most uniform possible practices.

1. Careful pipetting is essential. Pipettes with disposable tips that can be changed after each dilution is recommended.
2. Optimal time and temperature of incubation, time and force of centrifugation must be used consistently.
3. The age, phenotype and concentration of test red blood cells influence the results. When the titers of several antibody containing sera are to be compared, all should be tested against red blood cells (preferably freshly collected) from the same donor. If this is not possible, the tests should use a pool of reagent red blood cells from donors of the same phenotype. When a single serum is to be tested against different red blood cell samples, all samples should be collected and preserved in the same manner, and diluted to the same concentration before use.
4. Completely reproducible results are virtually impossible to achieve. Comparisons are valid only when specimens are tested concurrently. In prenatal testing of sequential serum samples to detect changing antibody activity, samples should be frozen for comparison with subsequent samples. Each new sample should be tested in parallel with the immediately preceding sample. In tests with a single serum against different red blood cell samples, material from the master dilution must be used for all tests.
5. Measurements are more accurate with large volumes than with small volumes, a master dilution technique gives more reliable results than individual dilutions for a single test. The volume needed for all planned tests should be calculated and an adequate quantity of each dilution prepared.

● Antibody Titration studies for early detection of Haemolytic disease of the newborn

Specimen:

Serum for titration (containing potentially significant unexpected antibodies to red blood cell antigens, 1 ml). If possible, test the current sample in parallel with the most recent previously submitted (preceding) sample from the current pregnancy.

Materials:

1. Anti Human IgG reagent
2. Dilute bovine albumin (approximately 6% w/v), optional: 22% (w/v) bovine albumin, 1 ml; isotonic saline, 3 ml.
3. Micropipettes or equivalent: 0.1-0.5 ml delivery, with disposable tips.
4. Red blood cells: Group O reagent RBCs with double dose expression of antigen to which the serum contains antibody (use R_2R_2 RBCs when titrating Anti-D); wash three times and dilute to a 2% suspension with isotonic saline.

Quality Control:

1. Test the preceding sample in parallel with the current sample.
2. Prepare dilutions using separate pipette for each tube. Failure to do so will result in falsely high titers due to carry-over.
3. Confirm all negative reactions with Coombs control cells.

Procedure:

1. Using 0.5 ml volumes, prepare serial dilutions of serum in saline or 6% albumin. The initial tube should contain undiluted serum and the doubling dilution range should be from 1 in 2 to 1 in 2048 (total of 12 tubes).
2. Place 0.1 ml of each dilution into appropriately labeled 10 or 12 x 75 mm test tubes.
3. Add 0.1 ml of RBC cell suspension to each dilution.
4. Gently agitate the contents of each tube; incubate at 37^0C for 1 hour.

5. Wash the tubes four times with saline; completely decant the final wash supernatant.
6. To the dry RBC buttons thus obtained, add Anti-IgG according to the manufacturers direction.
7. Centrifuge as for haemagglutination tests.
8. Examine the results macroscopically; grade and record the reactions.
9. Add one drop of Coombs control cells to all negative tests; recentrifuge and examine the tests macroscopically for mixed field agglutination; repeat antibody detection tests when tests with Coombs control cells are nonreactive.

Results:

The titer is reported as the reciprocal of the highest dilution of serum at which 1+ agglutination is observed. A titer greater than or equal to 16 is considered significant and may warrant monitoring for HDN by cordocentesis, high resolution ultrasound, or examination of the amniotic fluid for bilirubin pigmentation.

Notes:

1. Titration studies should be performed upon initial detection of the antibody; save an aliquot of the serum (frozen at –20^{0}C or colder) for comparative studies with the next submitted sample.
2. When the titer is less than16 and the antibody specificity has been associated with HDN, it is recommended that repeat titration studies be performed every 2-4 weeks, beginning at 18 weeks gestation; save an aliquot of the serum (frozen at –20^{0}C or colder) for comparative studies with the next submitted sample.
3. When the decision has been made to monitor the pregnancy by an invasive procedure such as amniocentesis, no further titrations are warranted.
4. Each institution should develop a policy to ensure some degree of uniformity in reporting and interpreting antibody titers.
5. For antibodies to low incidence antigens consider using paternal RBCs.
6. Do not use enhancement techniques (albumin, PEG, LISS) or enzyme treated RBCs, because elevated titers may be obtained.
7. LISS should not be used as diluent in titration studies; nonspecific uptake of globulins may occur in serum-LISS dilutions.
8. Failure to obtain the correct results may be caused by incorrect technique, notably; failure to use separate pipette tips for each dilution or failure to mix thawed frozen serum.

Use of Sulfhydryl Reagents to distinguish between IgM and IgG antibodies

Specimen:

2 ml of serum to be tested

Reagents:

1. Phosphate buffered saline at pH 7.3
2. 0.01 M dithiothreitol (DTT) prepared by dissolving 0.154 g of DTT in 100 ml of pH 7.3 PBS. Store at 2-8^{0}C.

Procedure:

1. Dispense 1 ml of serum into each of two test tubes.
2. To one tube, labeled as control, add 1 ml of pH 7.3 PBS.
3. To the other tube, labeled as test, add 1 ml of 0.01 M DTT.
4. Mix and incubate at 37^{0}C for 30-60 minutes.
5. Test the antibody activity in each sample by titration against red blood cells of appropriate phenotype.

Effect of Dithiothreitol on Blood Group Antibodies

Test Sample	Dilution					Interpretation
	1/2	1/4	1/8	1/16	1/32	
Serum + DTT	3+	2+	2+	1+	0	IgG
Serum + PBS	3+	2+	2+	1+	0	
Serum + DTT	0	0	0	0	0	IgM
Serum + PBS	3+	2+	2+	1+	0	
Serum + DTT	2+	1+	0	0	0	IgG+IgM*
Serum + PBS	3+	2+	2+	1+	0	

* May also indicate only partial inactivation of IgM

Notes:

1. Sulfhydryl reagents used at low concentration may weaken antigens of Kell system. For investigation of antibodies in Kell system, it may be necessary to use alkylation with iodoacetic acid, followed by dialysis.
2. Gelling of serum or plasma sample may be observed during treatment with DTT. This can occur if the DTT has been prepared incorrectly, and has a concentration above 0.01 M. Gelling may also occur if serum and DTT are incubated too long. An aliquot of the sample undergoing treatment can be tested after 30 minutes of incubation, if the activity thought to be due to IgM has disappeared, there is no need to incubate further. Gelled samples cannot be tested for antibody activity because overtreatment with DTT causes denaturation of all serum proteins.

Elution Techniques

● Citric Acid Elution Method

Specimen:

Packed DAT positive red blood cells washed six times with saline

Reagents:

1. Elution solution: citric acid (monohydrate), 1.3 g, KH_2PO_4 0.65 g saline to 100ml, store at 4^0C.
2. Neutralizing solution: Na_3PO_4, 13.0 g; distilled water to 100 ml; store at 4^0C.
3. Supernatant saline from final wash of the red blood cells to be tested.

Procedure:

1. Chill all reagents to 4^0C before use.
2. Place 1 ml of packed red blood cells in a 13 x 100 mm test tube.
3. Add 1 ml of eluting solution and note the time.
4. Stopper the tube and mix by inversion for 90 seconds.
5. Remove the stopper and promptly centrifuge the tube at 900-1000g for 45 seconds.
6. Transfer supernatant fluid to a clean test tube and add 5-6 drops of neutralizing solution; save red blood cells for use in adsorption studies if needed.
7. Check pH; adjust it, if necessary, to pH 7.0 by adding more neutralizing solution.
8. Centrifuge at 900-1000 g for 2-3 minutes to remove precipitate that forms after neutralization. Harvest the supernatant eluate and test it in parallel with supernatant saline from final wash.

Notes:

1. Once the red blood cells have been rendered DAT-negative, they may be tested for the presence of blood group antigens, except those of the Kell blood group system. Expression of antigens in the Kell system is markedly weakened after citric acid treatment.
2. Citric acid modified red blood cells may also be treated with protease and used in autologous adsorption studies.

● Cold Acid Elution

Specimen:

Packed DAT positive red blood cells washed six times with saline

Reagents:

1. Glycine-HCl (0.1M, pH 3.0), prepared by dissolving 3.75 g of glycine and 2.922 g of sodium chloride in 500 ml of distilled water. Adjust pH to 3.0 with 12N HCl. Store at 4^0C.
2. Phosphate buffer (0.8 M, pH 8.2), prepared by dissolving 109.6 g of Na_2HPO_4 and 3.8 g of KH_2PO_4 in approximately 600 ml of distilled water. Adjust pH, if necessary, with either 1N NaOH or 1N HCl. Dilute to a final volume of 1 litre with distilled water. Store at 4^0C.
3. Normal saline, at 4^0C.
4. Supernatant saline from final wash of red blood cells to be tested.

Procedure:

1. Place the red blood cells in 13 x 100 mm test tube and chill them in an ice bath for 5 minutes before adding glycine-HCl.
2. Add 1 ml of chilled saline and 2 ml of chilled glycine-HCl to 1 ml of washed red blood cells.
3. Mix and incubate the tube in an ice bath for 1 minute.
4. Quickly centrifuge the tube at 900-1000 g for 2-3 minutes.
5. Transfer the supernatant eluate into a clean test tube, and add 0.1 ml of pH 8.2 phosphate buffer for each 1 ml of eluate.
6. Mix and centrifuge at 900-1000 g for 2-3 minutes.
7. Transfer the supernatant eluate into a clean test tube, and add test in parallel with the supernatant saline from the final wash.

Notes:

1. Keep glycine in ice bath during use, to maintain correct pH.
2. Phosphate buffer will crystallize during storage at 4^0C. Redissolve it at 37^0C before use.
3. Addition of phosphate buffer restores neutrality to the acidic eluate. Unneutralized acidity may cause haemolysis of the reagent red blood cells used in testing the eluate. The addition of 22% bovine albumin (one part to four parts of eluate) may reduce such haemolysis.

● Glycine-HCl/EDTA Elution

Specimen:

Packed DAT positive red blood cells washed six times with saline

Reagents:

1. Disodium EDTA (10% w/v): Na_2EDTA, 2 H_2O, 10 g distilled water to 100 ml.
2. Glycine-HCl (0.1 M at pH 1.5): Glycine 3.754 g; NaCl 2.922 g; distilled water to 500 ml; adjust to pH 1.5 with 12 N HCl; store at 4^0C.
3. TRIS base: Hydroxymethyl aminomethane, 12.1g; distilled water to 100 ml.
4. Supernatant saline from final wash of the red blood cells to be tested.

Procedure:

1. Mix 4 ml of glycine-HCl and 1 ml of EDTA in 16 x 100 mm test tube.
2. Immediately add 1 ml of washed red blood cells and mix well.
3. Incubate at room temperature for 1-2 minutes.
4. Centrifuge the tube at 900-1000 g for 2-3 minutes.
5. Transfer the supernatant eluate into a clean test tube and adjust to pH 7.5 with 1 M TRIS base.
6. Mix and centrifuge at 900-1000 g for 2-3 minutes.

7. Transfer the supernatant eluate into a clean test tube, and test it in parallel with the supernatant saline from the final wash.

Notes:

1. Once the red blood cells have been rendered DAT-negative, they may be tested for the presence of blood group antigens, except those in the Kell system. Treatment with glycine-HCl/EDTA denatures Kell system antigens.
2. Red blood cells modified with glycine-HCl/EDTA may be treated with protease and used in autologous adsorption studies.

● Heat Elution

Specimen:

Packed DAT positive red blood cells washed six times with saline

Reagents:

1. 6% bovine albumin, prepared by diluting 22% or 30% bovine albumin with saline.
2. Supernatant saline from final wash of the red blood cells to be tested.

Procedure:

1. Mix equal volumes of washed packed cells and 6% bovine albumin in 13 x 100 mm test tube.
2. Place the tube at 56^{0}C for 10 minutes. Agitate the tube periodically during this time.
3. Centrifuge the tube at 900-1000 g for 2-3 minutes, preferably in a heated centrifuge.
4. Immediately transfer the supernatant eluate into a clean test tube, and test in parallel with supernatant saline from final wash.

● The Donath-Landsteiner Test

Specimen:

Serum separated from freshly collected blood sample maintained at 37^{0}C.

Reagents:

1. Freshly collected normal serum, to use as a source of complement
2. 50% suspension of washed group O red blood cells that express the P antigen.

Procedure:

1. Label three sets of three 10 x 75 mm test tubes as follows: A1-A2-A3; B1-B2-B3; C1-C2-C3.
2. To tubes 1 and 2 of each set, add 10 volumes of the patient's serum.
3. To tubes 2 and 3 of each set, add 10 volumes of fresh normal serum.
4. To all tubes, add one volume of 50% suspension of washed P-positive red blood cells and mix well.
5. Place the three 'A' tubes in a bath of melting ice for 30 minutes, and then at 37^{0}C for 1 hour.
6. Place the three 'B' tubes in a bath of melting ice, and keep them in melting ice for 9 minutes.
7. Place the three 'C' tubes at 37^{0}C, and keep them at 37^{0}C for 90 minutes.
8. Centrifuge all tubes, and examine the supernatant fluid for haemolysis.

Interpretation:

The Donath Landsteiner test is considered positive when the patients serum, with or without added complement, causes haemolysis in the tubes that were incubated first in melting ice and then at 37^{0}C (i.e. tubes A1 and A2), and there is no haemolysis in any of the tubes maintained throughout at 37^{0}C or in melting ice. The A3, B3 and C3 tubes serve as a control for complement activity and should not manifest haemolysis.

Notes:

1. The biphasic nature of the haemolysin associated with PCH requires that serum be incubated with cells at cold temperature and then at 37^{0}C.
2. Active complement is essential for demonstration of the antibody. Because patients with PCH may have low levels of serum complement, fresh normal serum should be included in the reaction medium as a source of complement.
3. To avoid loss of antibody by cold autoadsorption before testing, the patient's blood should be allowed to clot at 37^{0}C, and the serum separated from the clot at this temperature.

Chequer Board titration for quality control of Anti-IgG potency in polyspecific AHG reagent and evaluation of complement potency with complement coated cells

Reagents and materials required for chequer board titration:

1. Anti-D IgG reagent with albumin titer 256-512.
2. Polyspecific AHG reagent
3. Freshly collected O Rh_o D positive cells
4. Normal saline
5. 12 x 100 mm and 12 x 75 mm test tubes
6. Pipettes 1 ml and 5 ml
7. Table centrifuge
8. Timer
9. Water bath or laboratory incubator

Reagent Preparation Procedure:

Preparation of 2% cell suspension

1. Collect 2 ml of freshly drawn venous blood in a clean 12 x 100 mm test tube with suitable anticoagulant.
2. Centrifuge at 3000 rpm for 2-3 minutes to form a cell button.

3. Discard the supernatant.
4. Resuspend the cell button in 5 ml of normal saline.
5. Centrifuge at 3000 rpm for 2-3 minutes.
6. Repeat the washing of cells (Step 4 and 5) twice more so that the cells are washed three times.
7. After the final centrifugation, remove the supernatant without disturbing the cell button.
8. Take 0.5 ml of packed cells and resuspend them in 25 ml of normal saline to get a 2% cell suspension.

Dilutions of Anti-D (IgG) reagent

1. Take a set of ten, 12 x 100 mm test tubes and number them from 1 to 10.
2. Add 2 ml of normal saline to each of the tubes from tube number 2 to 10.
3. Add 2 ml each of Anti-D (IgG) reagent to tube number 1 and 2.
4. Mix the content of tube number 2 and transfer 2 ml of diluted reagent to tube number 3 with the help of pipette.
5. Continue this serial dilution till tube number 10.
6. Discard 2 ml of diluted reagent from tube number 10.

Cell Sensitization:

1. To each of the above dilutions of Anti-D (IgG) add 2 ml of well mixed freshly prepared 2% cell suspension.
2. Mix well all the tubes and cover them with aluminium foil.
3. Incubate the tubes at 37^{0}C for 30 minutes, with periodic mixing.
4. Centrifuge the tubes at 3000 rpm for 2-3 minutes.
5. Remove the supernatant and resuspend the cell button in 5 ml of normal saline.
6. Centrifuge at 3000 rpm for 2-3 minutes.
7. Repeat the washing (Step 4 and 5) atleast four times.
8. Resuspend the cell button from each tube in 2 ml of normal saline to get a 2% suspension of sensitized cells.

Note:

Thorough washing of sensitized cells (after incubation) is very important as even slight traces of free Anti-D IgG can lead to false negative results.

Dilutions of Anti Human Globulin Reagent:

1. Take a set of six, 12 x 100 mm test tubes and number them from 1 to 6.
2. Add 2.5 ml of normal saline to each of the tubes from tube number 2 to 6.
3. Add 2.5 ml of Polyspecific AHG reagent to tube number 1 and 2.
4. Mix the content of tube number 2 and transfer 2.5 ml of the diluted reagent to tube number 3.
5. Continue this serial dilution till tube number 6.
6. Discard 2.5 ml of diluted reagent from tube number 6.

Preparation of Complement coated cells:

Reagents and material required:

1. LISS solution
2. Buffered saline
3. O group red blood cells 50% suspension
4. Inert O group serum
5. Test tubes 12 x 100 mm
6. Table centrifuge
7. Water bath or laboratory incubator

Preparation of 50% cell suspension of O group red blood cells

1. Collect 1 ml of freshly drawn venous blood in a clean 12 x 100 mm test tube containing suitable anticoagulant.
2. Centrifuge the tube at 3000 rpm for 2-3 minutes to form cell button.
3. Discard the supernatant.
4. Resuspend the cell button in 5 ml of buffered saline.
5. Centrifuge at 3000 rpm for 2-3 minutes.
6. Repeat the washing of cells (Step 4 and 5) twice more so that the cells are washed three times.
7. After the final centrifugation, remove the supernatant without disturbing the cell button.
8. Add 1 ml of buffered saline to the packed red blood cells to get a 50% O group red cell suspension.

Collection of inert O group serum

1. Collect 2 ml of freshly drawn venous blood in a clean 12 x 100 mm test tube.
2. Immediately centrifuge at 3000 rpm for 2-3 minutes.
3. Collect 0.5 ml of serum in a clean test tube.

Sensitization of O group red blood cells

1. Place 8.5 ml of LISS into a 20-25 ml container.
2. Add 0.5 ml of fresh inert O group serum to it.
3. Mix well and add 1 ml of 50% O group red cell suspension.
4. Mix thoroughly and incubate at 37^{0}C for 30 minutes with occasional further mixing.
5. Centrifuge at 3000 rpm for 2-3 minutes to form a cell button.

6. Discard the supernatant and resuspend the cell button in 20 ml buffered saline.
7. Centrifuge at 3000 rpm for 2-3 minutes.
8. Repeat the washing of cells (Step 6 and 7) thrice more so that cells are washed four times.
9. After the final centrifugation, remove the supernatant without disturbing the cell button.
10. Add 14.5 ml of buffered saline to packed red blood cells to obtain 2-3% suspension of complement coated cells.

Chequer board titration:

1. Take a set of sixty, 12 x 75 mm test tubes, number and arrange them as shown below in the table:

Dilution of AHG	3% Rho (D) + ve sensitized cells								
N	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
N									
1:2									
1:4									
1:8									
1:16									
1:32									

2. Add 0.2 ml each of N (neat) AHG in the respective tubes.
3. Similarly add dilutions of AHG in their respective tubes (horizontal rows).
4. Referring to the above mentioned table add 0.2 ml each of 2% suspension of sensitized cells with Anti-D (IgG) dilutions in their respective tubes (vertical rows).
5. Mix well all the tubes.
6. Centrifuge the tubes at 3000 rpm for 20 seconds.
7. Gently dislodge the cell button and observe for agglutination.
8. Chart the results in the above given table.

Complement potency titration:

DILUTION OF AHG	2% CELL SUSPENSION OF COMPLIMENT COATED CELLS
N	
1:2	
1:4	
1:8	
1:16	
1:32	

1. Add 0.2 ml neat AHG reagent.
2. Similarly add dilutions of AHG in respective tubes.
3. Referring to the above mentioned table add 0.2 ml each of 2% suspension of complement coated cells to all the tubes containing AHG reagent with dilutions.
4. Mix well all the tubes.
5. Centrifuge the tubes at 3000 rpm for 20 seconds.
6. Gently dislodge the cell button and observe for agglutination.

Preparation of Coombs Control cells

Reagents and Materials required:

1. Anti-D (IgG)
2. Freshly collected O Rh_o (D) positive cells
3. Normal saline
4. 12 x 100 mm test tubes
5. Pipettes 1ml and 5 ml
6. Table Centrifuge, timer.
7. Water bath or laboratory incubator.

Procedure:

Since commercially available Anti-D (IgG) reagents have albumin titer of 256-512, diluting Anti-D (IgG) reagent 1:40 to 1:50 in normal saline is enough to achieve sensitization with O Rh_o(D) positive cells.

1. Take equal volume of 1:40 to 1:50 diluted Anti-D (IgG) in a 12 x 75 mm test tube and 2% cell suspension of O Rh_o (D) positive cells.
2. Mix well and incubate at 37^0C for 15 minutes. Periodic mixing during 15 minutes interval ensures thorough sensitization.
3. Remove the supernatant and resuspend the cell button in 5 ml of normal saline.
4. Centrifuge at 3000 rpm for 2-3 minutes.
5. Repeat the washing(Step 3 and 4) atleast four times.
6. Resuspend the cell button in normal saline to obtain a 2% suspension of Coombs control cells.

REACTIONS OF COMMON ERYTHROCYTE ANTIBODIES*

	Antibody For	Saline Medium	Albumin Medium	AHG Test	Enzyme Test	In Vitro Hemolysis	Optimal °C 4	24	37
Usually IgM	H, I	M	S	F	S	F	M	S	F
	i	M	S	F	S	S	M	S	F
	A, B, A, B	M	F	F	M	S	M	S	F
	Lu^a	M	S	F	F	N	M	S	M
	Lu^b	S	S	M	F	N	R	S	M
	M, N	M	S	S	F	N	M	M	F
	P_1	M	S	S	S	F	M	S	F
	PP_1P^k	M	M	M	M	M	S	M	M
	Le^a, Le^b	M	S	S	M	S	M	S	F
Usually IgG	S, s	S	S	M	S	N	F	S	M
	K, k, Js^a, Js^b	F	S	M	F	N	F	S	M
	C, D, E, c, e,	S	S	M	M	N	F	S	M
	Fy^a, Fy^b	F	F	M	F	N	N	F	M
	Jk^a, Jk^b	F	S	M	M	F	N	S	M

*M = Most (>20%), S = Some (5-20%), F = Few (1-5%), R = Rare (<1%), N = Not reported.

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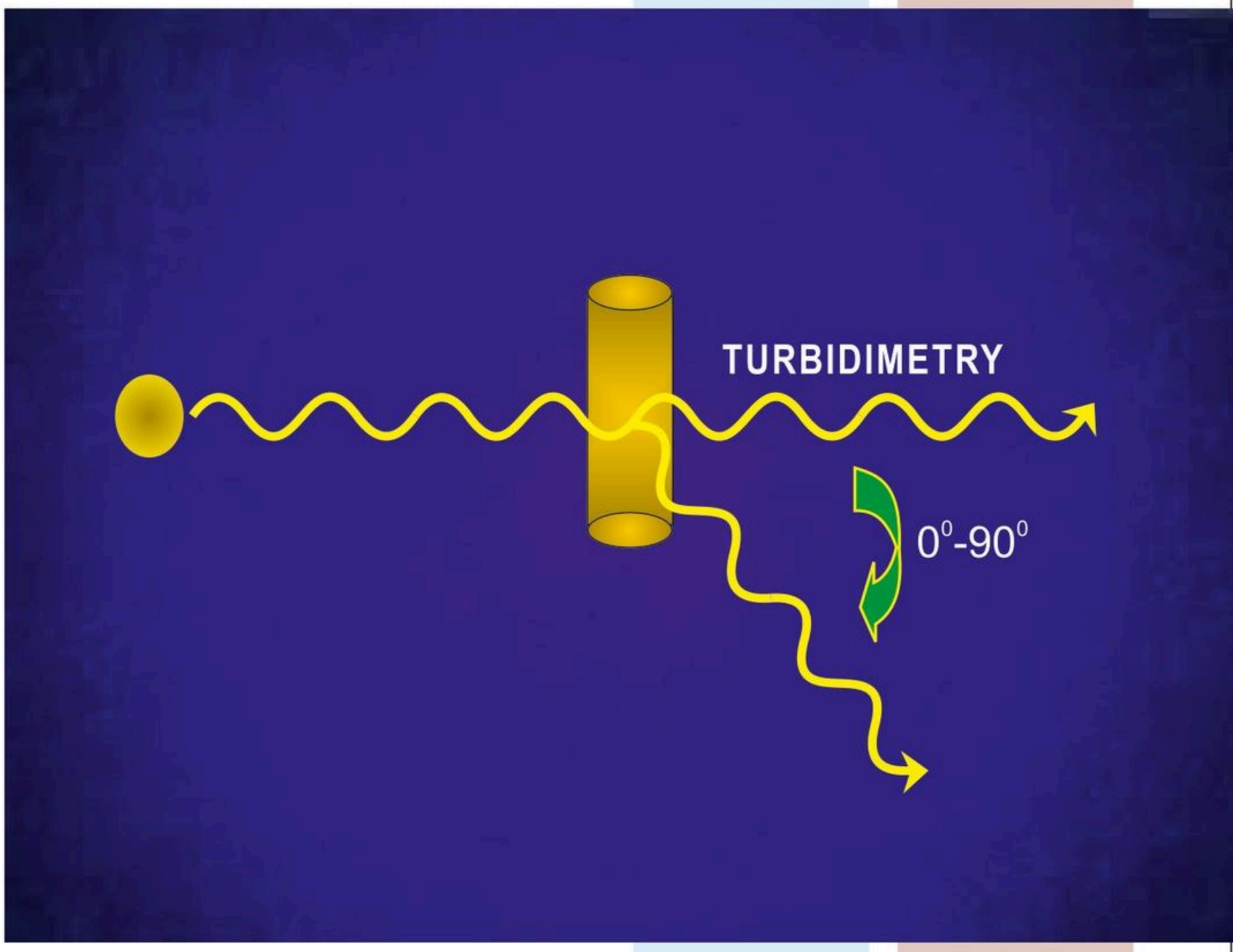
TURBIDIMETRY



- **Turbidimetry**
  - An Insight.....Pgs 23-42
- **Applications of Quantia Reagents**.....Pgs 43-51

KNOWLEDGE BOOK

For the Laboratorians

Turbidimetry

An Insight

Setting Trends...

Introduction

Diagnosis is a decision point. The decision is the intention to treat. It is the point at which sufficient evidence has been accumulated to state, beyond reasonable doubt, that the patient is or is not suffering from a particular disease.

Laboratory tests remain one of the mainstays on which the clinicians rely for diagnosis and management of the patient. Laboratory tests are indicated for;

1. **Detection:** The presence or absence of a particular substance e.g. testing for infectious diseases like VDRL, HBsAg.
2. **Quantification:** Accurately determining the concentration of a particular substance as an aid to diagnosis or differential diagnosis (e.g. Concentration of CRP in differential diagnosis of Viral and bacterial infections) and for establishing the extent of the Clinical condition (e.g. RF in measuring disease severity).
3. **Monitoring:** The course of clinical condition or response to therapy.
4. **Prognosis:** For predicting the probability of occurrence of a disease/disorder (e.g. Microalbuminuria for predicting Diabetic nephropathy) or predicting the outcome of a disease/disorder.

Microscopy, Biochemical assays, Microbiology procedures, and Immunoassays are various techniques that fulfil the requirements of routine laboratory tests to meet the needs of the clinicians.

Certain clinical analytes can be measured by specific techniques only, whereas for the measurement of certain analytes options exist for selecting the techniques of measurement. For example urinary albumin can be measured by biochemical methods such as pyrogallol red or coumassie blue. But for the diagnosis of Microalbuminuria, a condition where urinary excretion of albumin is in the range of 30-300 mg/L the accuracy of the measurements by biochemical methods is questionable because these methods also react with other proteins in addition to albumin which are frequently found to be present in urine. Immunochemical methods (Immunoassays) which are more sensitive and specific have a distinct advantage and hence are preferred.

Immunoassays

Immunoassays are assays that detect the presence of an antigen in the human body with the help of an antibody or detect the presence of an antibody with the help of an antigen. In this text for simplicity, all further information provided is based on considering antibody as a reagent to detect antigen in the human body fluids.
The first reported immunoassays were homogeneous. They are attributed to Kraus (1897), who coined the term '**precipitin**' for the precipitate formed upon mixing an antigen and an antibody.

Meyer in 1922 employed sheep erythrocytes to serve as a label and conjugated human immunoglobulin to them. Anti-immunoglobulin antibodies appearing in rheumatoid arthritis patients were shown to cause visible clumping of these erythrocytes. This method was known as **haemagglutination.**

Singer and Plotz replaced the erythrocytes with latex particles, which were easier to standardise, and these assays are popularly known as **latex agglutination tests**. High degree of sensitivity for wide variety of antigens/antibodies, which can be detected by these latex agglutination assays, has promoted their usage worldwide for screening since 1956 in clinical laboratories. The simplicity of performance and obviating the need for equipments, have made these assays extremely popular.

The need for quantitative estimation, and higher sensitivity led to the development of **radioimmunoassays** (RIA) first in 1959 by Berson and Rosalyn Yalow. The first RIA developed was used to detect and quantify insulin.

Since then immunoassays have been used to detect and quantify variety of molecules native to humans such as proteins, hormones as well as foreign molecules such as bacteria, viruses and parasites.

Qualitative Immunoassays

Qualitative Immunoassay techniques provide test results, which only help to identify or indicate the presence of analytes. Various techniques for qualitative detection of antigens have been in use, which include latex agglutination, passive gel diffusion, immunoelectrophoresis (IEP) and western blotting. These techniques at the best can give a semiquantitative or comparative information about analyte under assay.

Single immunodiffusion technique uses the diffusion of an antigen into agar impregnated with antibody. **Double immunodiffusion technique** allows the direct comparison of two or more test materials providing a simple and direct method for determining whether the antigens in the test specimens are identical, cross-reactive, or non-identical. **Immunoelectrophoresis** has been used over the years for detection of several antigens present in a common solution.

The latex agglutination assays though simple to use are subject to variations in results as the interpretation pattern between negative and weakly reactive samples may vary between laboratory to laboratory and person to person.

Lower sensitivity for many analytes & the need for correct quantification of analytes for;

- Effective monitoring of disease
- For differential diagnosis to aid correct therapy,
  have created the need for more sensitive and precise quantitative immunoassays.

Quantitative immunoassays:

Quantitative results of immunoassays are extremely useful in;

- Establishing the extent of severity of a disease
- Assessing the course and stage of clinical condition
- Differential diagnosis of many diseases
- Monitoring response to therapy
- Accurate prognosis of disease.

Various techniques have been used to develop quantitative methods that include radial immunodiffusion and electroimmunoassays, turbidimetric and nephelometric assays, and labelled immunochemical assays.

Radial immunodiffusion (RID) and electroimmunoassay (rocket electrophoresis) though reliable, are slow, relatively involvement intensive, and expensive. This limits their usage in routine laboratories. In many laboratories the gel-based techniques are restricted to qualitative studies or are used as reference methods.

During the last decade the gel techniques are increasingly being replaced by optical detection methods.

The various techniques by which quantitative immunoassays are performed can be broadly grouped as;

***Heterogeneous Immunoassays*:** These assay systems employ an antibody immobilized on a solid phase, which captures the corresponding antigen from the sample. A second labelled antibody specific to a different epitope of the antigen is used as a basis for signal generation. After the immunochemical reaction has taken place, the bound and unbound labelled antibody are separated. The concentration of antigen is then estimated by measuring bound or unbound-labelled antibodies through an appropriate signal generation and measurement system.

Heterogeneous immunoassays can be performed by various techniques such as;

- Radio immunoassays (RIA)
- Enzyme Immunoassays (EIA)
- Fluorescent Enzyme Immunoassay
- Chemiluminescent Enzyme Immunoassay

The difficulties associated with separation of bound and unbound-labelled antibodies, the need for dedicated instrumentation and labor intensive procedures has prompted the usage of heterogeneous assays in specialty laboratories mainly through use of expensive automation.

The need for simpler, affordable, user friendly assay techniques for detection of routinely encountered clinical analytes still remained to be explored. With the tremendous progress made in instrumentation technology, optics, and software, the face of quantitative estimation for routine parameters has changed dramatically in the recent years. Simultaneous development in purification techniques for polyclonal antibodies, emergence of monoclonal antibodies with high specificity and avidity have been instrumental in the development of homogeneous assay techniques which are simple to perform and easily adaptable for routine laboratory analysis.

Homogeneous Immunoassays: These assays require only the mixing of a sample (antigen) and the immunochemical reagents (antibody) followed by detection of signal. These assays do not require separation of free or bound labelled materials in the test system for the detection or measurement of the antigen. The immunochemical binding produces a detectable signal (agglutination, Absorbance, fluorescence etc.). The simplicity & flexibility associated with the performance of homogeneous assays has made their usage popular with laboratorians worldwide.

The homogeneous assays can be performed by different techniques such as;

- Turbidimetry
- Nephelometry
- Homogeneous Enzyme Immunoassays
- Enzyme–Multiplied Immunoassay Technique (EMIT)
- Enzyme Inhibitor Immunoassay
- Enzyme Complementation Immunoassay
- Substrate Linked Fluorescence Immunoassay (SLFIA)
- Scintillation Proximity Assay (SPA)
- Electrochemiluminescence (ECL)
- Luminescent Oxygen Channeling Immunoassay (LOCI)

The Clinical chemistry analyzers (photometers) were originally developed for colorimetric estimation of chemical or enzymatic reactions. Subsequently, it was shown that the visible scattered light in Kraus's precipitin reaction could be measured by turbidimetry & nephelometry on photometers, to quantitate the immunecomplex formation. These systems utilize the fast reaction between an antigen with their corresponding antibodies in a liquid phase.

The technique of quantitation by turbidimetry & nephelometry is apparently similar to the popular absorption spectrophotometry used in routine clinical laboratories and hence adaptable by high throughput as well as small and medium laboratories easily.

SPECTROPHOTOMETRY

Spectrophotometers work on the basis of the Beer's and Lambert's law.

Beer's law: when a colored solution is illuminated with a monochromatic light (light of a single wavelength), its absorbance is proportional to the concentration of the colored solution when the light path is constant.

i.e. $A \propto C$

Where A is the absorbance of light, C is the concentration of solution.

Lambert's law: When a colored solution is illuminated with a monochromatic light, its absorption is proportional to the length of the light path, when the concentration of the solution is constant.

i.e. $A \propto L$

Where A is the absorbance of light, L is the length of the light path.

Beer-Lambert's law

Combining the two laws together we have the Beer-Lambert's law, which states that when a colored solution is illuminated by a monochromatic light, its absorbance is proportional to the concentration of the solution and the length of the light path.

i.e. $A \propto C \propto L$

$$A = K \times C \times L \qquad \text{Equation (1)}$$

Where K is a constant

In all photometric estimations a reference standard whose concentration is known is used and its color intensity is compared with the color intensity of the test sample

i.e. $A_t = K \times C_t \times L$

$A_s = K \times C_s \times L$

Where A_t - Absorbance of test , C_t-Concentration of test , A_s-Absorbance of Standard, C_s-Concentration of the standard

Since the pathlength is constant in the spectrophotometer (1cm), L is constant, Concentration of the standard C_s is known, therefore

$$C_t = \frac{A_t}{A_s} \times C_s \qquad \text{Equation (2)}$$

It has been observed with most biochemistry analytes that as the concentration of analyte increases linearly, the absorbance also increases linearly within the pathophysiological concentration. When a graph of concentration Vs absorbance is plotted a straight-line graph is obtained (Fig 1A). A single standard method using a standard of known concentration or a factor method can be employed for calculating the concentration of the unknown.

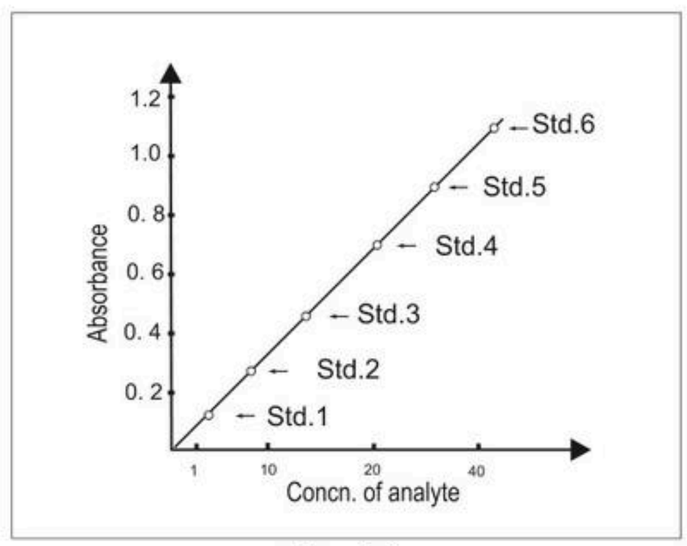


Fig. 1A

Fig. 1A. Illustration of a straight line graph obtained by plotting absorbance Vs concentration of analyte for reactions which obey Beer-Lambert's law.

Certain reactions however, may not follow the Beer-Lambert's law within the pathophysiological concentration for an analyte and hence do not provide a straight-line graph. For such analytes the unknown cannot be determined using a single standard. A graph using different concentration of standards Vs absorbance has to be plotted on a graph paper. The plotted curve is known as the **standard curve** (Fig. 1B).

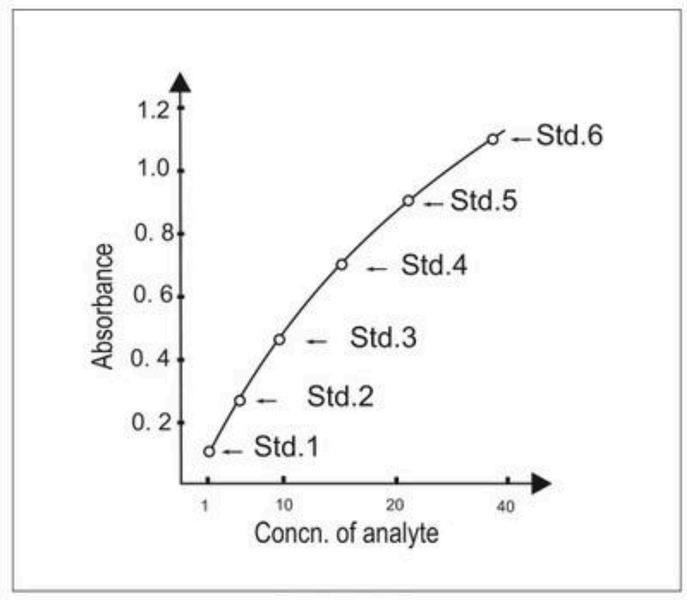


Fig. 1B

Fig. 1B. Illustration of a standard curve obtained by plotting absorbance Vs concentration of analyte for reactions which do not obey Beer-Lambert's law.

The concentration of the unknown can be interpolated from this standard curve.

Measuring principles in Biochemistry

● Criteria for wavelength selection

It has been established that when the wavelength of light used is complementary to the color of the chemical complex to be measured, peak absorbance is obtained. Thus selection of the wavelength depends on the color of the complex to be measured.

Complementary filters for measuring color complexes

Color of the complex	Wavelength/Color
Yellow	405 Violet
Red	505 Green
Blue-violet	546 Green
Green	630 Red

For the estimation of Haemoglobin using cynamethaemoglobin method, a red colored complex, which is formed during the reaction, is measured using a green filter.

● Reading methods

The measurements of biochemical reactions using enzymes, substrates or specific chemicals are read by methods mentioned below.

1. Equilibrium methods: Also known as end point methods. Here the absorbance of end product is measured when the reaction between the reagent and sample has virtually come to equilibrium (end) and all the substrate has been converted into a stable end product. The reaction ceases when equilibrium is reached. The concentration of the test specimen can be calculated by using the equation 2 as described earlier.

2. Kinetic methods: Also known as rate methods where the rate of change of absorbance (Δ A) produced in a fixed time interval is measured.

The kinetic measurements are of two types;

- ***Fixed time analysis*** - where the ΔA produced by the reaction between the reagent and the substrate is measured by stopping the reaction at a fixed time interval.
- ***Continuous monitoring*** **-** Where the ΔA produced is monitored continuously.

Results of the unknown are derived using a factor (K) in the kinetic methods, which is usually provided by the manufacturer or can be calculated as;

$$K = \frac{V_{total} \times 1000}{V_{sample} \times t \times \in \times d}$$

V_{total}- total volume of the reaction mixture, V_{sample}- Volume of sample, t -time, $\in$- molar extinction coefficient of the chromogen, d-length of the light path

● Standardisation of time interval for rate reactions

Determination of the reaction rate involves the measurement of the amount of change in absorbance (ΔA) produced in a defined time interval.
Depending on the reaction kinetics between a specific reagent and the substrate the time interval for reading the ΔA can be selected to measure the reaction rate. The different type of reaction curves, which can be obtained as the reaction progresses, typically follow the following patterns;

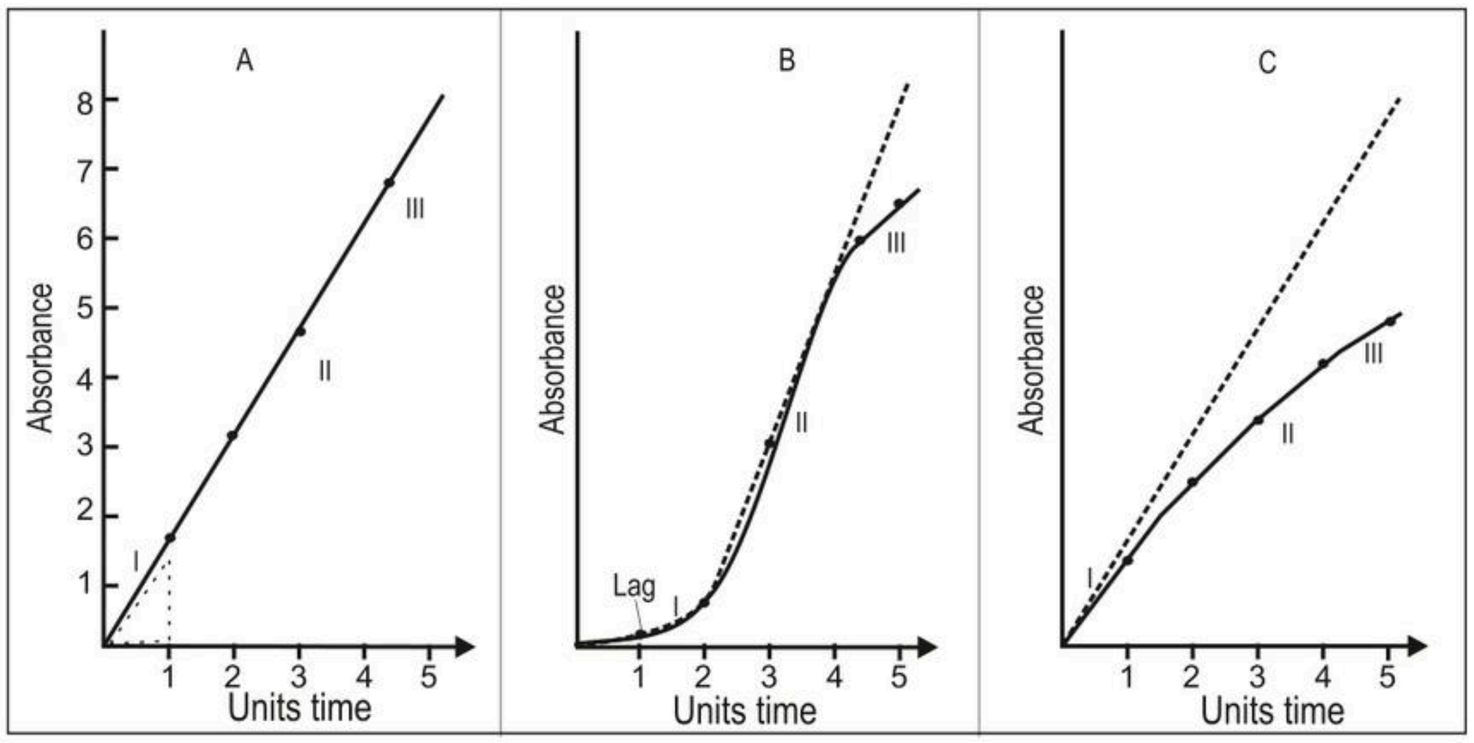


Fig. 2 . Forms of graph showing change in reaction rate as a function of time.
In A, the rate is constant in all the segments I, II, III. In B, a measurement at segment II will be representative of constant rate, but at segment I (lag period) and segment III it will be less. In C, the rate falls off continously.

Curve A: If a graph similar to 'curve a' is obtained then any time interval can be selected for reading reactions, as the rate of change is constant during the entire reaction run.

Curve B: Correct results can be obtained only if the rate is measured along segment II. Incorrect results are obtained if the ΔA is measured during the lag phase (I) or during the phase (III).

Curve C: deviates from linearity over its entire course and ΔA fall off with time. At no time does it give rate of constant changes. Such reaction curves are not suitable for measurements and the reagents have to be optimised to obtain better reaction curves.

Measurement of Immunecomplexes by Spectrophotometry

Unlike in classical biochemistry where the reactants are clear and endpoints are expressed as absorbances the behavior of light differs for solutions containing suspensions or particulates. Such insoluble immunecomplexes are formed as a reaction between antigens and antibodies takes place. When light of suitable wavelength is allowed to pass through a reaction solution containing antigens (analytes) and the initial absorbance is measured, the absorbance is minimum at this point.

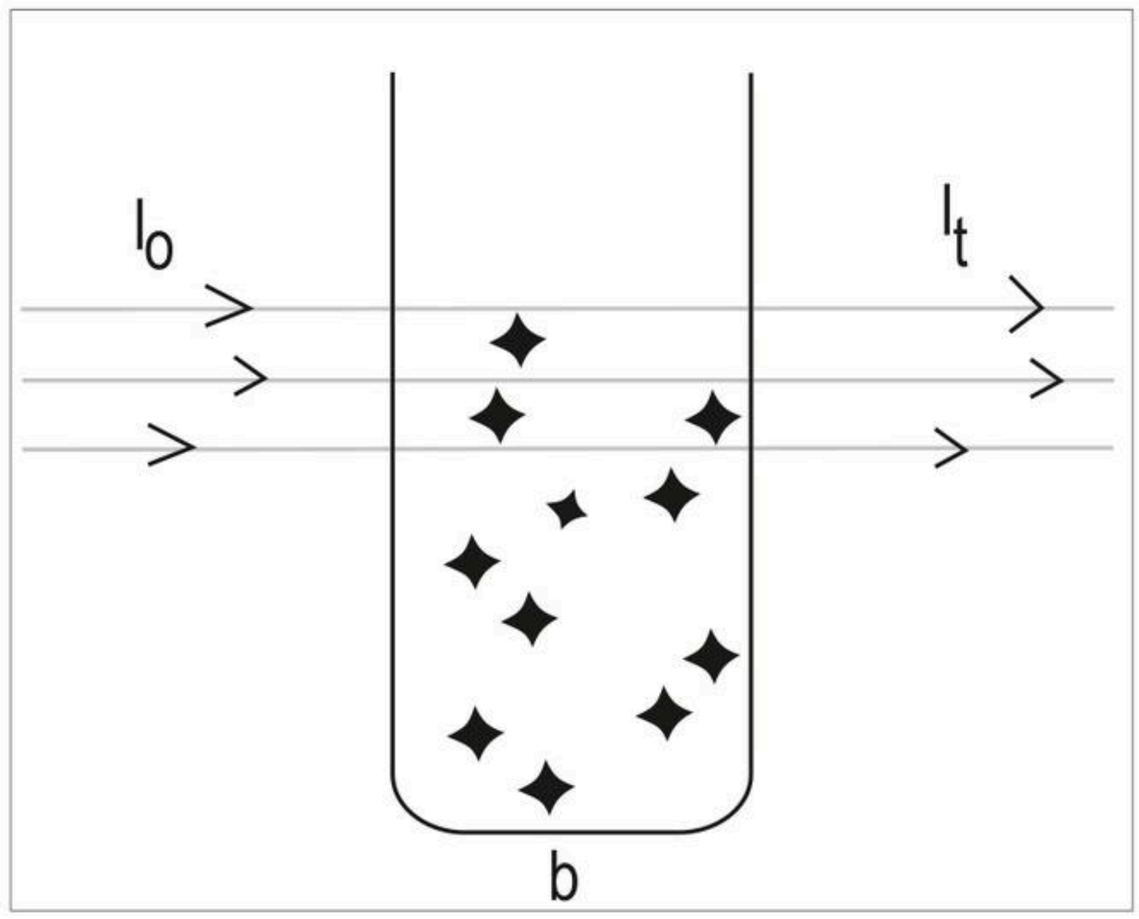


Fig.3 Behaviour of light in solution containing antigens

*where I_o is the intensity of incident light, I_t is the intensity of transmitted light and **'b'** is the cuvette containing antigens in the reaction solution.*

Subsequently the reagent containing antibody solution is then added to the antigen and allowed to react. An agglutination reaction takes place when a single molecule of antibody binds to at least two corresponding binding sites on different antigen particles. As the reaction proceeds the agglutinating particles aggregate and form immune complexes. Immune complexes increase in size, become larger, resulting in an increase in turbidity and the scattering of the incident light. Thus a decreasing part of the incident light is transmitted as the reaction proceeds. Spectrophotometers read this decrease in the intensity of the transmitted light as absorbance.

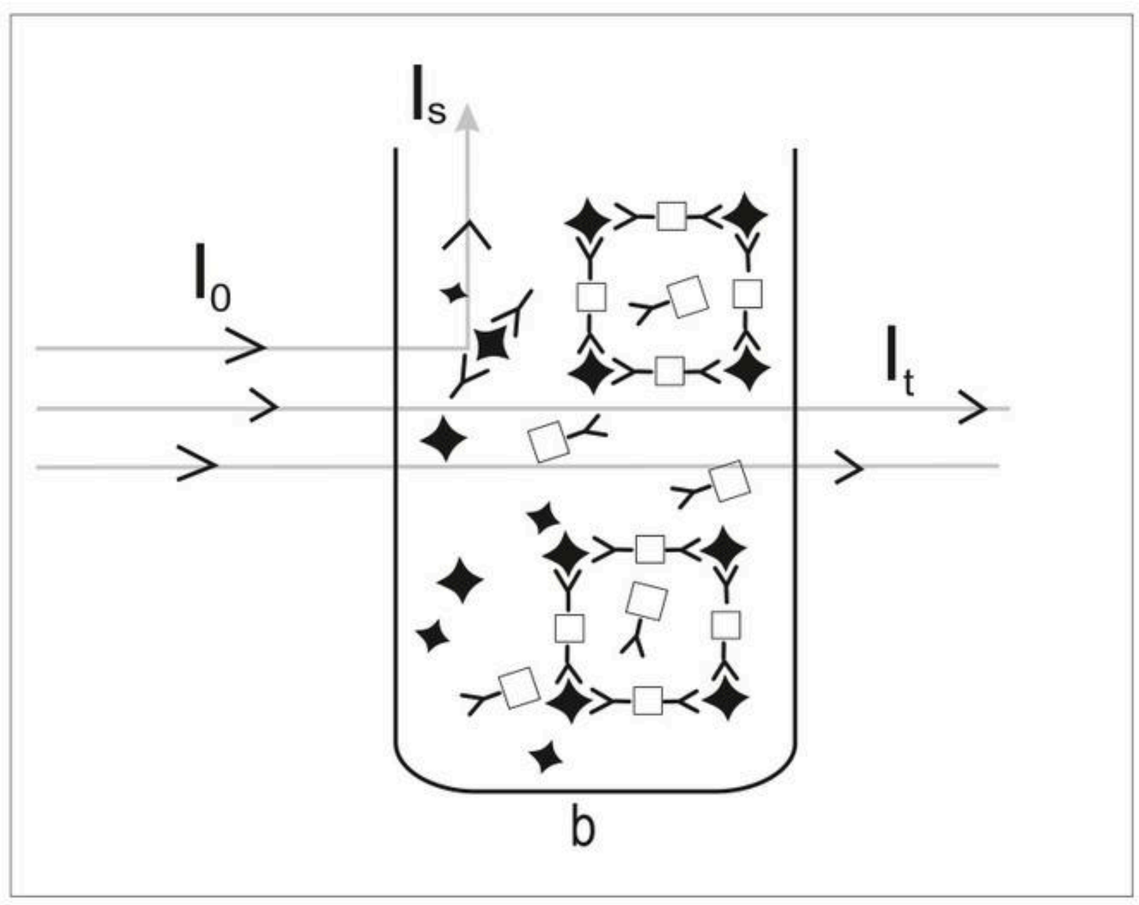


Fig.4 Behaviour of light in solution containing immunecomplexes(Ag-Ab)

*where I_o is the intensity of incident light, I_t is the intensity of transmitted light, Is is the intensity of scattered light and **'b'** is the cuvette containing immunecomplexes in the reaction solution.*

This measurement of reduction in the intensity of the transmitted light at 180° is defined as **Turbidimetry.** The turbidity is proportional to the analyte concentration, which inturn is proportional to the amount of agglutination. Based on this proportional relationship the amount of analyte in the sample causing the turbidity can be easily determined.

It should be noted that the nature of Immunochemical reaction is exactly the same in turbidimetry & and nephelometry. However it is the detection principle applied for measurement, which differentiates turbidimetry from nephelometry.

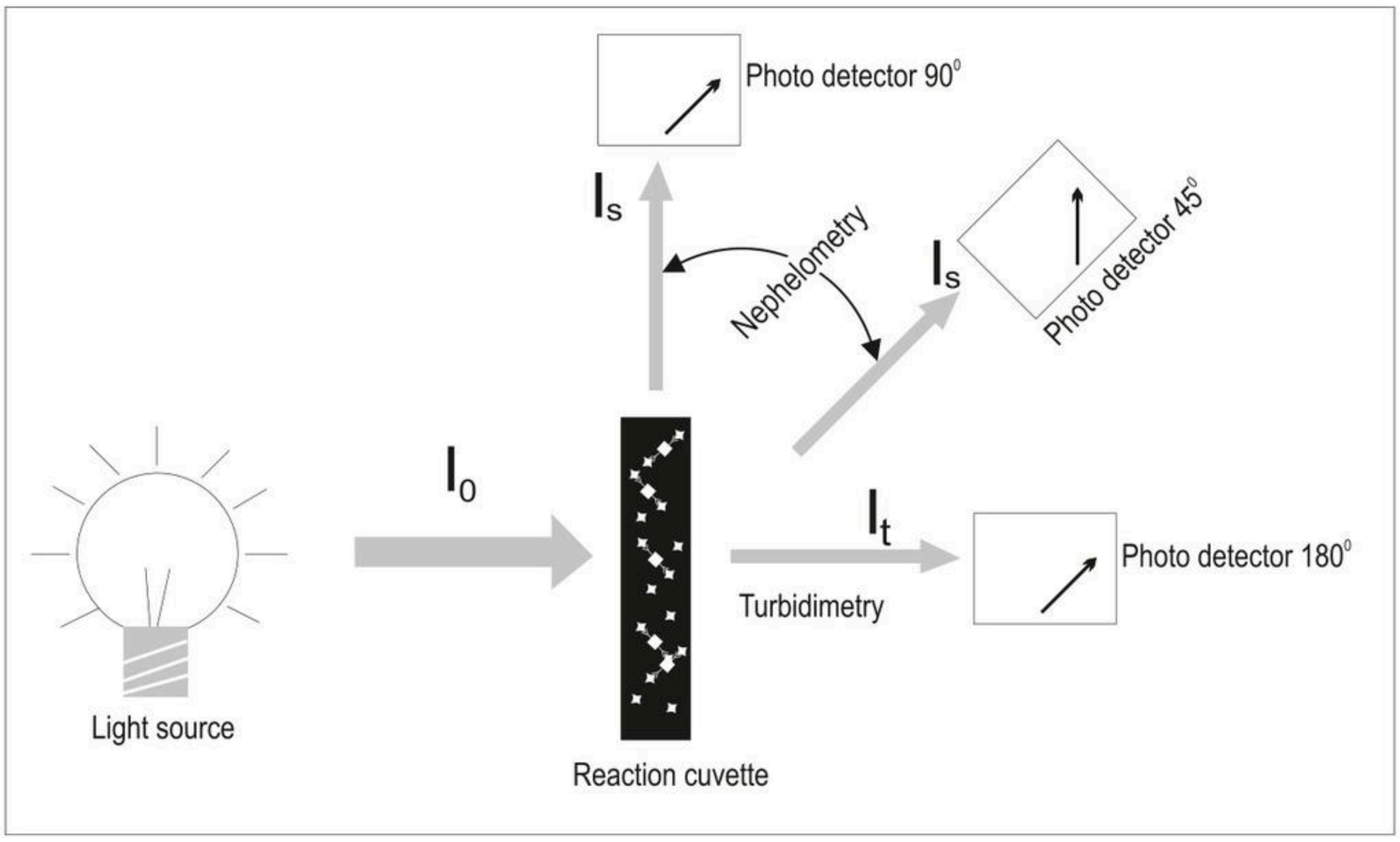


Fig.5 *Detection principles in turbidimetry and nephelometry where I_o is the intensity of incident light, 'b' is the reaction cuvette, I_t is the intensity of transmitted light measured by detector at* 180°, *I_s is the intensity of scattered light measured by detectors placed at* 90° *and at* 45°.

Nephelometry measures light scattered or reflected towards the detector, which is away from direct path of the transmitted light. Routine spectrophotometers cannot be used for nephelometry and hence nephelometers are required. Most nephelometers measure light scattering at a 90° angle. However in order to measure the forward scatter intensity caused by light scattering from large particles some nephelometers are designed to measure scattered light at an angle other than 90°.

- **Selection of wavelength for measuring Immunecomplexes:**

The optimum wavelength for optical measurement of immune complexes increases with the size of immune complex to be measured.
In general, if the size of the immune complex formed is less than I/10th the size of the wavelength of incident light, then the light scattering is relatively symmetrical (Fig.6) . This **uniform scattering** of light is known as **Rayleigh scattering.**

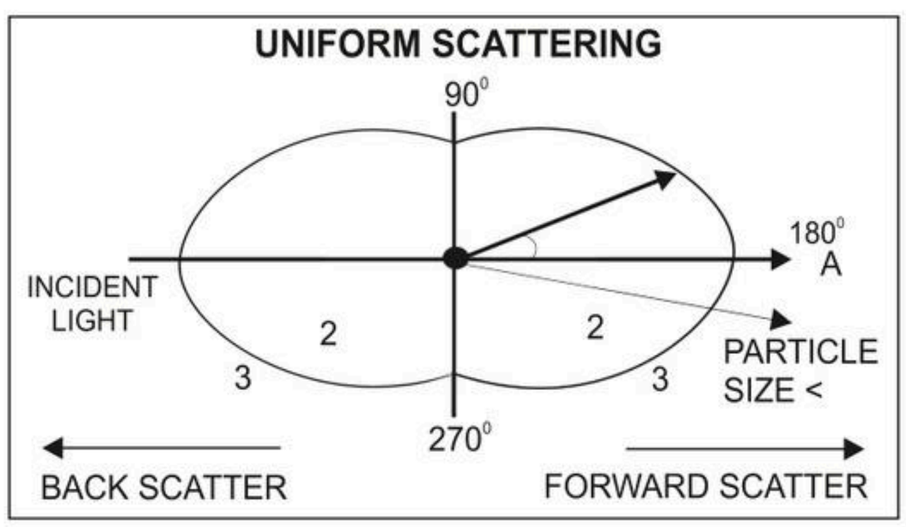


Fig.6 illustrating scattering of light for a immune complex with particle size <λ/10 of the wavelength of incidence light

On the otherhand when the size of the immunecomplex to be measured is more than 1/10th the size the wavelength of the incident light there is concentration of scattered light in **forward direction** at an angle of 45° or less, away from the axis of the incident light beam (Fig. 7). This type of scattering is referred as **Rayleigh-Debye scattering.**

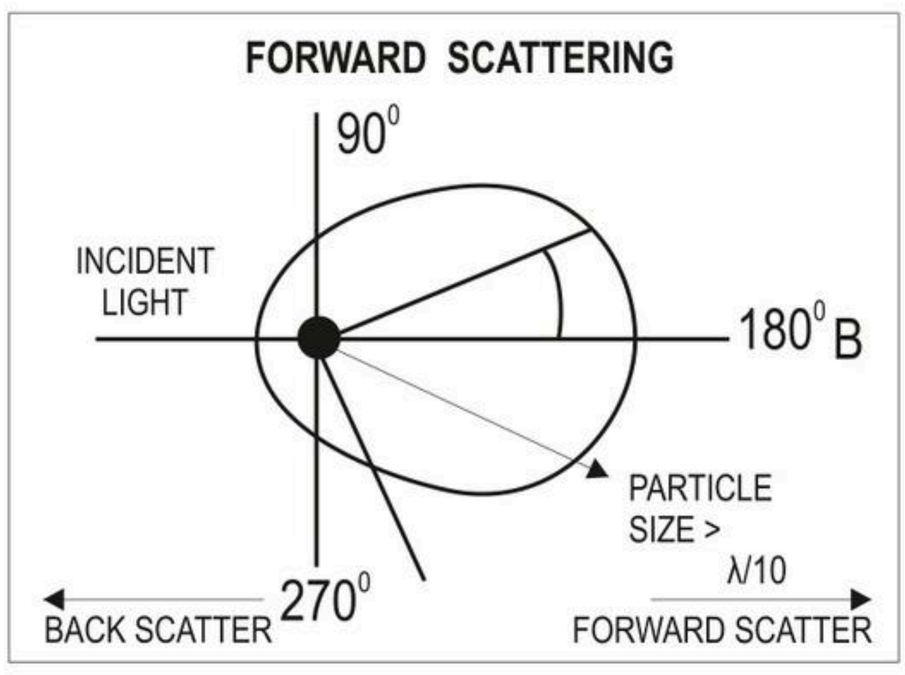


Fig.7 illustrating scattering of light for a immune complex with particle size > of the wavelength of incident light

Careful examination of both the figures (Fig. 6 & 7) show that the intensity of scattered light for forward and back scatter (0° and 180°) from small particles is equal but less at 90°(Rayleigh scattering). As the size of the particle becomes larger the angular dependence of light scattering becomes dissymetrical, increasing in forward scattering and decreasing in backward scattering.

The Rayleigh and Rayleigh-Debye expressions provide useful information about scattering of light by small and intermediate size particles and are important in the optimization of analytical instrumentation for measuring light using turbidimetric and nephelometric assays.

The upper limit on size of immune complexes exhibiting ***Rayleigh scattering*** is about 40 nm when a visible light at 400 nm is used. Many of the plasma proteins such as immunoglobulins, albumin etc. fall below this limit. As the immune complexes becomes larger in size from 40-400 nm, the angular dependence of scattered light at 400 nm loses the symmetry around the 90°axis, and shows an increase in forward scattering. Some plasma proteins of the IgM class, aggregating immunoglobulin/antigen complexes fall into this size category. For measuring such complexes a bigger wavelength of light depending on the size of the immune complexes formed, should be used. For latex based assays using a latex particle of ~200 - 300 nm, light of wavelength between 500 - 600 nm would be ideal for measuring the immune complexes formed.

Biochemical Measurements	Nephelometric measurements	Turbidimetric measurements
Measure absorbance of light at 180°, by colored complexes formed as a result of biochemical reactions	Measures light scattering at an angle away from the incident light due to formation of immune complexes	Measures reduction in intensity of light transmitted (as absorbance) at 180°due to the formation of immune complexes
Selection of wavelength of light is complementary to the color of the chemical complex to be measured.	Selection of wavelength of light depends on the size of the immunecomplex formed.	Selection of wavelength of light depends on the size of the immunecomplex formed.

The choice between turbidimetry & nephelometry will depend on application & the available instrumentation. Until recently it was assumed that for relatively clear solutions in which the transmission of light in the forward direction is greater than 95% small changes in absorption due to turbidity were difficult to measure with precision. The stability and resolution of modern microprocessor driven spectrophotometers and automated clinical chemistry analysers have greatly improved their ability to measure turbidity with dependable accuracy and precision. Turbidimetric methods have today become competitive in sensitivity with nephelometric methods for immunological quantitation of such solutions.

For some analytes the signal amplification and assay sensitivity requires the usage of conjugation chemistry to attach antibodies to inert and union latex particles. Such reagent systems are referred to as Particle enhanced turbidimetry (PET)

Particle enhanced turbidimetry reagents usually use latex of ~200-300 nm for conjugating antibodies to facilitate formation of larger immunecomplexes and thereby generate detectable signals. This leads to decrease in scattering of light at 90 and increase in forward scattering. Turbidimetric assays therefore have better precision for measuring larger immunecomplexes.

In the context of contemporary technology turbidimetric assays are gaining popularity over nephelometric determination due to their simplicity and overall consistency.

TURBIDIMETRY VS NEPHELOMETRY

TURBIDIMETERY	NEPHELOMETRY
Measures reduction in intensity of transmitted light at 180°C due to the formation of immune complexes	Measures scattering of light at an angle (usually 90°C) away from the incident light, due to the formation of immune complexes
Can be performed on most spectrophotometers	Requires dedicated Nephelometer
Sensitivity Competitive with nephelometeric measurements for small immune complexes such as serum proteins	Sensitive for measuring small immune complexes such as serum proteins
More precise for measuring large immune complexes	Less precise for measuring large immune complexes due to forward scattering of light
Blanking and reading reaction can be performed in the same measuring cuvette	Blanking has to be performed in separate measuring cuvette
Provides better precision due to slower reaction kinetics as blanking of reagent, sample & Immunochemical reaction can be monitored in a single cuvetteve	Because of the fast reaction kinetics it is difficult to obtain a sample and a reagent blank in case of Nephelometry

CONSIDERATIONS FOR MEASUREMENTS OF TURBIDIMETRIC IMMUNOASSAYS (TIA)

As far back in the year 1929 Heidelberger and Kendall have quantitatively described the formation of a precipitate when reacting an antigen with an antibody. They demonstrated that when an increasing amount of an antigen is added to a constant amount of corresponding antibody, the resulting degree of precipitate formed follows a bell shaped curve as shown in Fig. 8. To obtain the Heidelberger curve the antigen concentration is plotted against the absorbances obtained from measuring the Ag-Ab reaction.

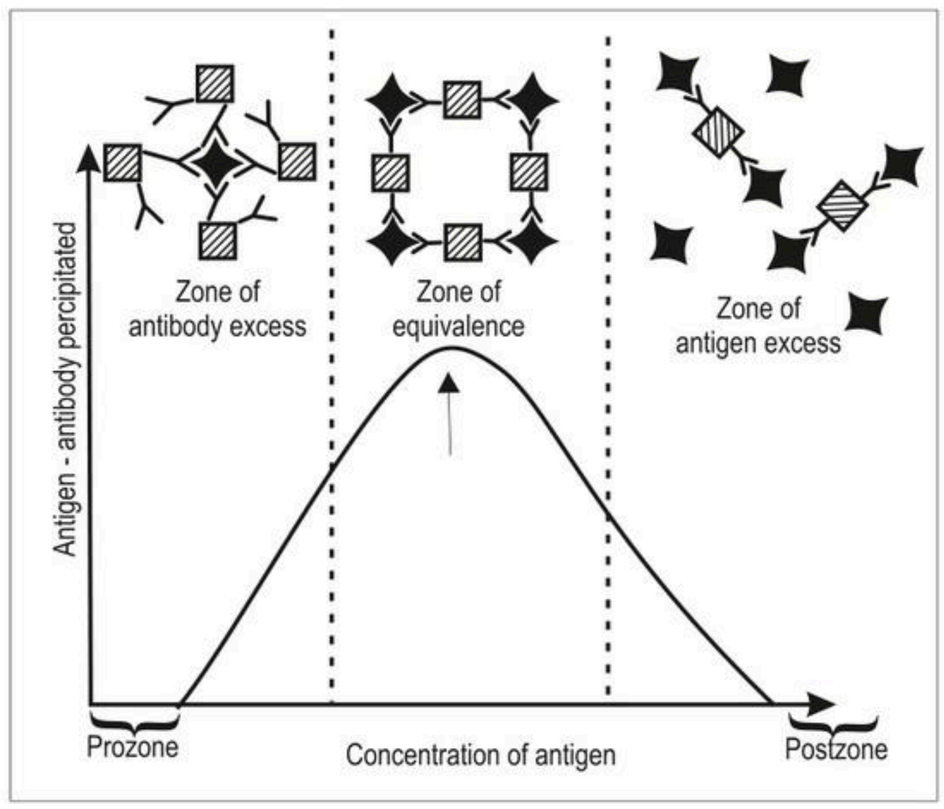


Fig. 8 The Quantitative immunoprecipitin curve (Heidelberger-Kendall Curve)

The Heidelberger-Kendall curve may be divided in three Zones;

1. **The antibody excess zone**

In the first stage of the reaction there is a large excess of binding sites in the reaction mixture available for the antigen to bind. First the antigen binding sites are quickly saturated by antibody before cross-linking begins to occur. This results in formation of small antigen-antibody complexes. **Here the absorbance increases proportionally to the analyte concentration.**

2. **Zone of equivalence**

In the second stage of reaction binding sites available for the antigen are proportionate to the antigen concentration. Here the probability of cross-linking is more likely resulting in formation of large immune complexes. As the saturation point is reached there is neither free antigen nor free antibody in the reaction mixture. **In this zone the absorbance increases with the analyte concentration, but does not increase proportionally.**

3. **The antigen excess Zone**

In the third stage of the reaction, the concentration of the antigen is so high that most of the binding sites are overcrowded, hindering the formation of real precipitate and favoring the formation of small immune complexes. This is called as the prozone effect or the hook effect. The term "prozone" is inappropriately used to describe "postzone" or "antigen excess" in day to day parlance. The existence of prozone effect will cause very high concentrations of antigen to produce signals, which are similar to the signals generated, by moderate concentrations of antigen. It is imperative to know, what concentration of analyte will cause a prozone effect in a turbidimetric immunoassay for a given antibody reagent system.

When an antigen-antibody reaction takes place and the formation of the immune complex is measured optically by turbidimetry than the absorbance and reaction kinetics in the three zones will be as follows:

Heidelberger - Kendall Curve	Absorbance
Antibody excess zone	Increases towards maximum
Equilibrium	Reaches Maximum
Antigen excess zone	Decreases below maximum

This quantitative immunoprecipitin curve forms the fundamental basis for all homogeneous antigen-antibody assays including turbidimetry and is usually referred to as the **dose response curve.**

For many analytes of diagnostic importance, the antigen-antibody reactions neither follow the Beer-Lambert's law nor provide a linear relationship between concentration and turbidity. **Estimating the concentrations of analytes using a single standard, as in biochemical analysis therefore results in inaccurate results near the zone of equivalence.** The ΔA is directly proportional to the concentration of analyte only in the initial region of the antibody excess zone. Use of single standard for calculating concentration of analyte may be acceptable only for lower analyte concentration. As the analyte concentration increases the error in measurement will start to magnify. Therefore for having a larger measuring range, the turbidimetric assays use that part of the dose response curve, which covers the maximum portion of the antibody excess region and demonstrates a linear reaction as the **standard curve**.

The standard curve is plotted using a number of different concentrations of standards (usually 5-6). The highest concentration of the standard is chosen in such a way that the analyte absorbance at that concentration will lie on the linear extreme of the standard curve. The lowest concentration of the analyte is usually selected below the reference values of the analyte of interest.

The linear range between the highest and lowest standards used for the preparation of standard curve is referred to as the **measuring range** of the assay.

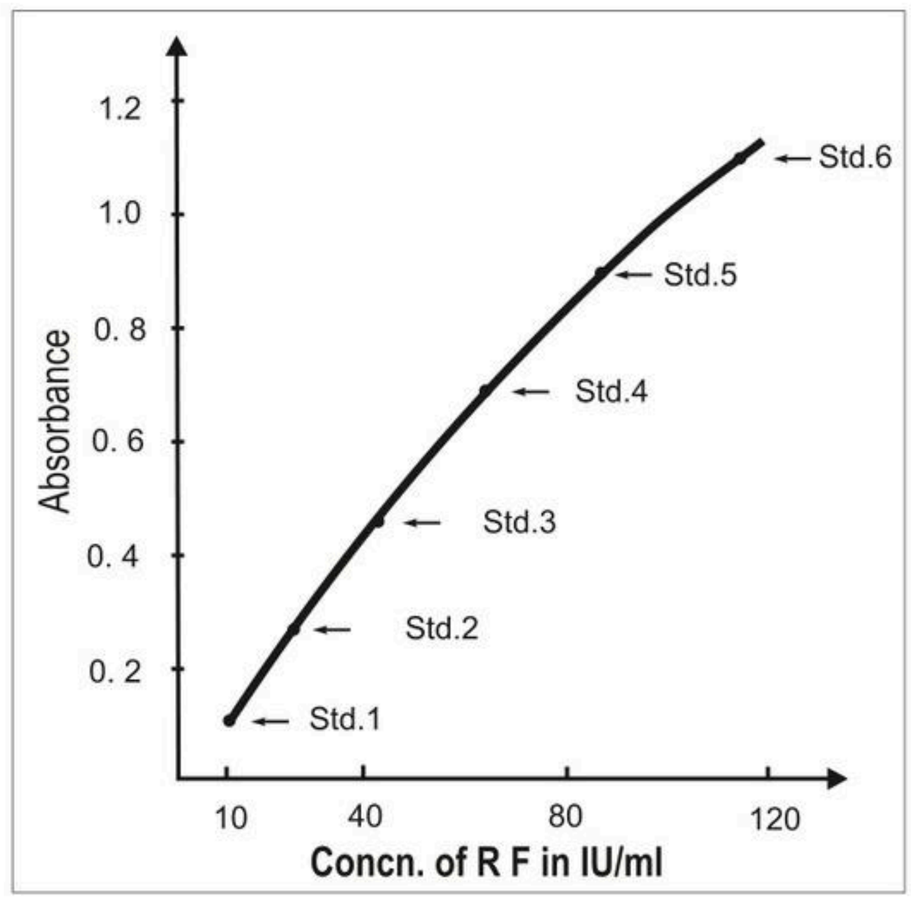


Fig . 9 Illustrating standard curve for RF using lowest standard as 7 IU/ml and highest standard as 120 IU/ml

Optimization, Standardization, and Quality Control of turbidimetric assays

To measure the antigen- antibody reactions reliably all the factors that affect the reaction rate, other than the concentration of the antibody, must be optimized and controlled. As the reaction velocity is at its maximum under optimal conditions, a larger analytical signal is obtained that can be more accurately and precisely measured as compared to a smaller signal obtained under suboptimal assay conditions.

Investigations of the factors affecting antigen-antibody reactions were extensively studied by Heidelberger and Kendall. In addition to the relative proportions of immune reactants, other conditions such as temperature, ionic strength of the medium, characteristics of the antibody such as avidity, and affinity are important for formation of Ag-Ab immune complex. These principles need to be applied to the reagent system optimisation for Immunoturbidimetry.

Ionic Strength: It has been observed that the antigen-antibody reactions are strongly influenced by the nature of ionic medium in which the reaction is carried out. The ionic strength of the reaction environment has a profound effect on the quantum and the rate of the Ag-Ab reaction. As the ionic strength increases, the depth of the electrical double layer that forms around the charged molecules is compressed, reducing the distance over which repulsive forces that keep the molecules apart can act. This in effect leads to the promotion of aggregation. The reduction in charge on the otherhand influences the electrostatic attraction between oppositely charged species, thereby reducing the specific binding between antigen and antibody.

pH: The reaction pH also influences the rate of aggregate formation. The rate of reaction is found to be fairly consistent at a pH of 6.0-8.0. Reduction in pH leads to some proteins having net positive charge (those with a pH above the reaction pH) leading to non specific agglutination with negatively charged proteins or particles.

Temperature: As is well known,temperature influences the rate of formation of immune complexes and it should be optimised to obtain accurate results. Assays are usually designed with an incubation temperature of 37°C because it is the most common temperature in the routine of clinical laboratories.

Enhancers: For enhancing the Ag-Ab reactions, polymeric compounds such as PEG, BSA may be included in the buffer system. These compounds facilitate the formation of immune complex and help in amplification of signals and improve the assay system sensitivity.

Interference: Many interfering factors such as bilirubin and lipids are normally present in the samples apart from the analyte of interest. High concentrations of some of these interfering factors are frequently encountered in clinical samples. They may influence signal generation and therefore can interfere in the assay result. Minimising the influence of these factors help provide accurate and precise assay results.

Turbidimetric assays usually employ a suitable buffer in the assay design to optimise assay conditions and the desired ionic strength, pH and enhancement for the reaction. In addition, the buffer is also useful in reducing the influence of interfering factors present in the sample. The buffer used in turbidimetric assays is generally referred to as **Activation buffer**.

Characteristics of antibody used as a reagent: Intrinsic characteristics of antibody employed as reagents have a profound effect on the antigen-antibody reactions. The specificity and affinity of the antibody to the antigenic sites affect the sensitivity of the assay and signal generation time. Usually

when high specificity and affinity antibodies are used a strong agglutination reaction will readily result. In contrast, antibodies with low affinity, even if highly specific, tend to react slowly and form a weak immunecomplex, thereby lowering the detectable signal. The avidity of antibody is also an important consideration in the formation of immunecomplexes. This characteristic of the antibody determines the degree of stability of the Ag-Ab complexes at the antigen binding sites. The tendency of the complexes to dissociate and disperse decreases substantially as the avidity of the antibody increases.

An antibody without cross reactivity and with a good titer is a prerequisite for a reliable turbidimetric assay utilising antibody as a principal reagent. In addition the antibody must be formulated as a clear solution to give a low reagent blank and should be free from particulate matter.

Standardization and Calibration:

During the course of treatment, individual patients are likely to have tests carried out for the same analyte by different methods, and to have results checked against reference intervals that were set elsewhere. To achieve agreement between different methods, a single recognized source of reference preparation is needed.
The reference preparation should;

- Have value assignment in meaningful units
- Be stable and identical to the analyte in the test samples
- Be free of interference from the test sample matrix.
- Be standardized by a reference method
- Demonstrate inter-method agreements.

Most International Reference Preparations (IRP's) & certified reference materials for immunoassay analytes can be obtained from the main custodians of international biological standards such as National Institute for Biological Standards and Controls (NIBSC) or W. H. O.

As the availability of international standards is limited, it is a practice to prepare sets of secondary standards, from which future lots of calibrators can be assigned values. The secondary standards act as an intermediate between IRP primary standard & future lots of calibrators for assay runs. The calibrator sets are made in bulk and values are assigned with reference to the secondary standards.

As discussed, the immunoturbidimetric assays require a set of five or six calibrators to obtain a standard curve. The quantitative values of unknown analyte obtained from the standard curve will be highly dependent on correct assignment of values to the calibrator used for preparing the standard curve.

Quality Control:

The tendency of most immunological reagents to produce changes in reactivity over time requires the application of quality control procedures to ensure the satisfactory analytical performance of immunometric assays on a day to day basis. Similarly in the case of turbidimetric immunoassays reagent stability within a defined usable time span is a prime requirement of the reagent systems, so is the need for accurate and stable controls to validate reagent functioning, precision and accuracy.

Reading principles in turbidimetry

For turbidimetric measurements both end point and rate measurements are applicable. However the factor method for calculating the concentration of the unknown is not preferred in the kinetic methods by turbidimetry due to the non-linear nature of relationship between absorbance and analyte concentrations.

Once the assay system has been designed the analyzers used for reading must be able to operate according to the principles mentioned below with respect to the addition of reagents and reading of signals (absorbance).

1. **Real sample blanking**

In this system first the Activation buffer (R1) is added to the sample cuvette (S). Then the sample is added, mixed and allowed to stabilize (preincubation period). The first reading (A1) is then taken at the end of preincubation period.

The antibody reagent (R2) is subsequently added, to the above mixture and mixed gently. Turbidity develops due to the reaction between the antigen and the antibody over a short period of time. A second reading is taken at the defined time interval (usually 2-10 minutes).

Table 1.

	Signals		
	First reading (A1)	**Second reading (A2)**	**ΔA_s (A2-A1)**
Sample cuvette (S)	Absorbance due to: ● Sample ● Buffer	Absorbance due to: ● Sample ● Buffer ● Reagent ● Immunecomplexes	Absorbance due to: ● Reagent ● Immunecomplexes

The difference ΔA_s (refer table 1) between the two readings represents the absorbance generated as a result of antigen-antibody reaction.

If required the absorbance due to the reagent ΔA_B can be measured by running in parallel a reagent blank in a separate cuvette (R) using saline in place of sample

Table 2.

	Signals		
	First reading (A1)	**Second reading (A2)**	**ΔA_R (A2-A1)**
Reagent cuvette (R)	Absorbance due to: ● Buffer ● Blank sample (saline)	Absorbance due to: ● Buffer ● Blank sample ● Reagent	Absorbance due to: ● Reagent

ΔA_R thus obtained of the reagent blank can be subtracted from ΔA_S of the sample to calculate the absorbance generated due to the Ag-Ab reaction in the sample.

The reagent blank facility may not be available in many semiautomated analyzers. However the reagent assay system can be optimized to provide a very low reagent blank in order to obviate the need for correcting the reagent blank signals which can contribute to the complete reaction absorbance.

The principle of taking a reading just before the addition of antibody solution (R2) is referred to as **'true sample blanking'** or **'real sample blanking'.**

2. Immediate mixed blanking:

In this system initially the activation buffer, sample, and the antibody reagent solution are all mixed simultaneously. Then as fast as possible usually 10 –20 seconds after mixing, the first reading A1 is taken. This 10-20 seconds delay time in taking a reading is referred to as **lag phase**. The reaction is allowed to proceed further and the second reading A2 is measured at the preselected time interval. The increase in absorbance ΔA (A2-A1) represents the signal generated due to the Ag-Ab reaction (refer table 3).

Table 3.

	Signals		
	First reading (A1)	**Second reading (A2)**	**ΔA_s (A2-A1)**
Sample cuvette (S)	Absorbance due to: ● Reagent ● Buffer ● Sample	Absorbance due to: ● Reagent ● Buffer ● Sample ● Ag - Ab reaction	Absorbance due to: ● Ag - Ab reaction

This method eliminates the need for determination of reagent blank as it measures the increase in absorbance after equilibration of the reagent and sample. Hence absorbance generated both due to interfering substances in the sample and the reagent would be blanked during the first reading.

In all Ag-Ab reactions in the initial contact phase the reaction kinetics do not follow a systematic pattern. As this initial chaotic phase settles the reaction pattern & the absorbances move proportionately. This pattern depends upon the intrinsic nature of the antibody such as affinity, avidity etc., and also the concentration of the analyte being measured.

Depending on the assay system requirements it is desirable that the initial chaotic phase is not included in the measurement of absorbance. Typically a lag phase would vary from ten to thirty seconds from analyte to analyte. It is therefore imperative to follow diligently the recommended time assigned for the lag phase for precise blanking in the **immediate mixed blanking** method.

Reaction kinetics & its effect on blanking:

The reaction kinetics of an antigen-antibody also guides as to the appropriateness of the blanking system. As the reaction kinetics is not the same for all Ag-Ab systems, for a system with slow reaction kinetics for e.g. RF, a first reading 10-20 seconds after mixing with the antibody is not very critical (Fig. 10).

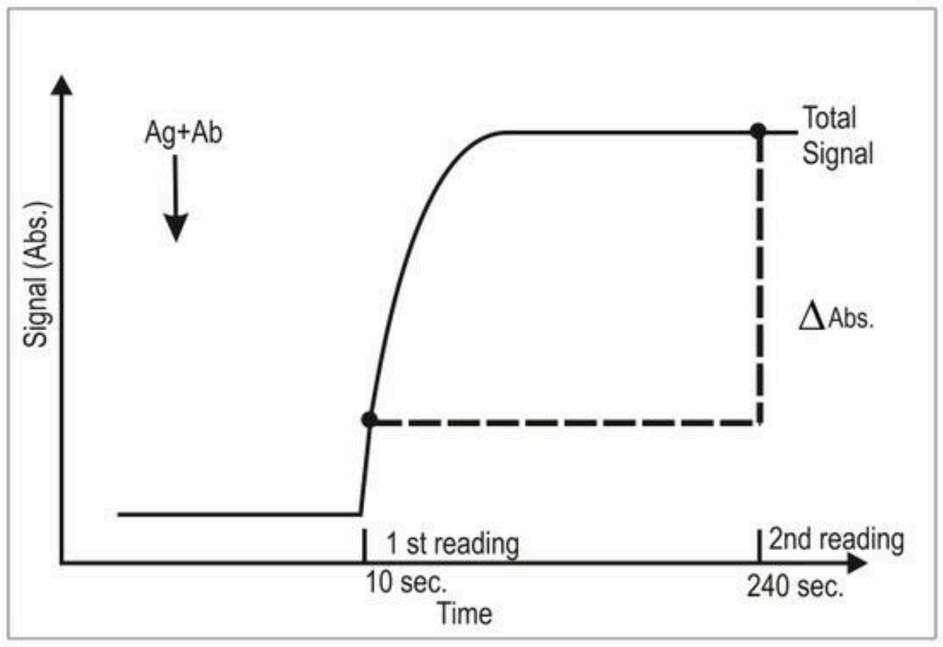


Fig. 10 Signal development as a function of time.
Fig. Illustrating 'Immediate mixed blanking' using RF as example.

However for a system with fast reaction kinetics for e.g. IgG (Fig. 11), half of the reaction would have taken place within 10-20 seconds when the first reading is taken. Here a poorly defined point for the first reading would be obtained.

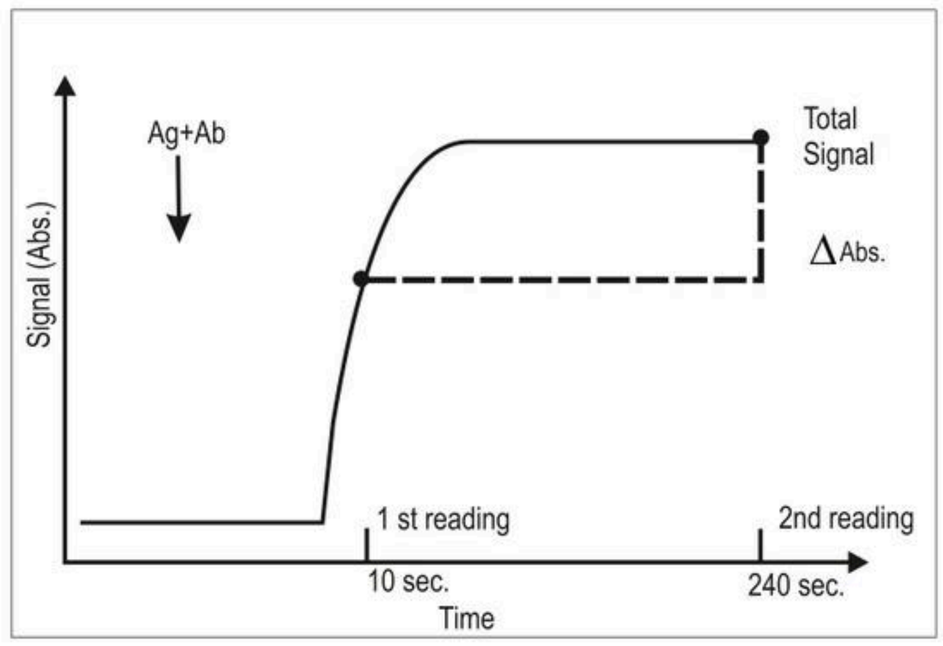


Fig. 11 Signal development as a function of time.
Fig. Illustrating 'Immediate mixed blanking' using IgG as example.

The implications of '**immediate mixed blanking'** can be demonstrated by comparing the standard curves obtained for the six calibrators of latex enhanced reagent system for measurement of RF (Fig. 12A) and IgG (Fig. 12B) at Zero seconds and 10 seconds respectively.

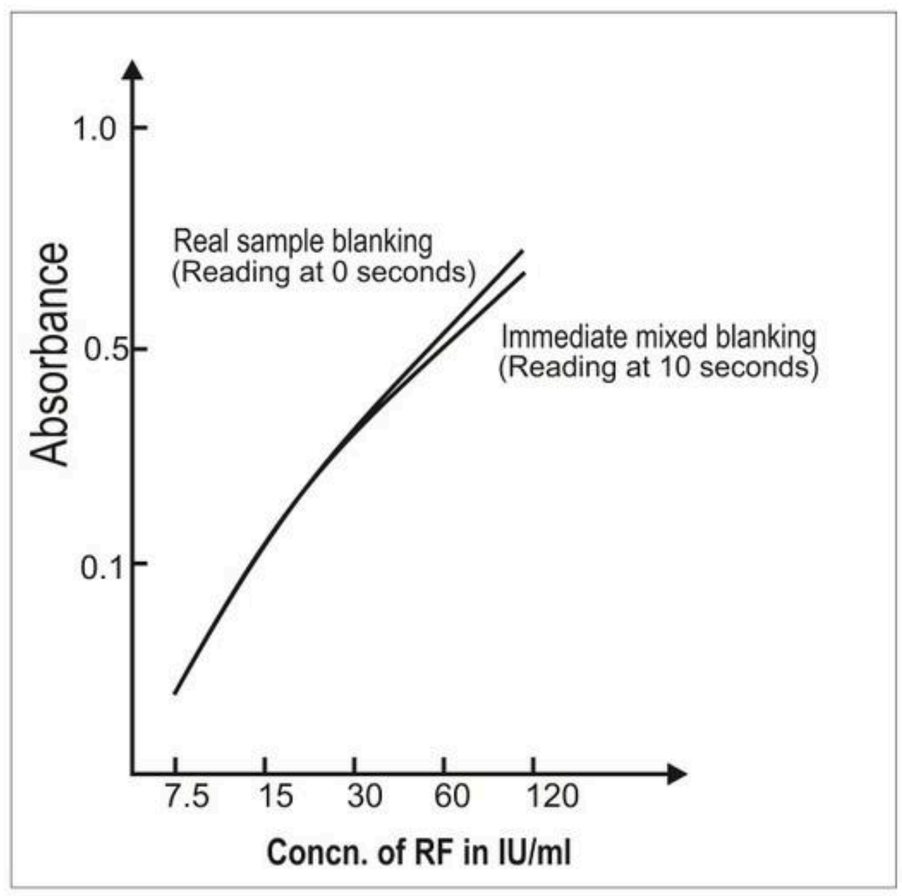


Fig. 12 A. Standard curves for RF obtained with 'real sample blanking' & 'immediate mixed blanking'.

The standard curve obtained for RF (Fig.12A) is practically not affected by the difference between the two ways of blanking indicating that a delay of 10 seconds is not very significant.

Whereas for a non-enhanced system with fast reaction kinetics for measurement of analytes such as IgG (Fig. 12 B), a delay of ten seconds becomes very critical.

There is considerable signal development during the first ten seconds. This results in decreased difference between A1 & A2 (Fig. 11). The loss of signal increases with increasing concentration of IgG in the calibrators.

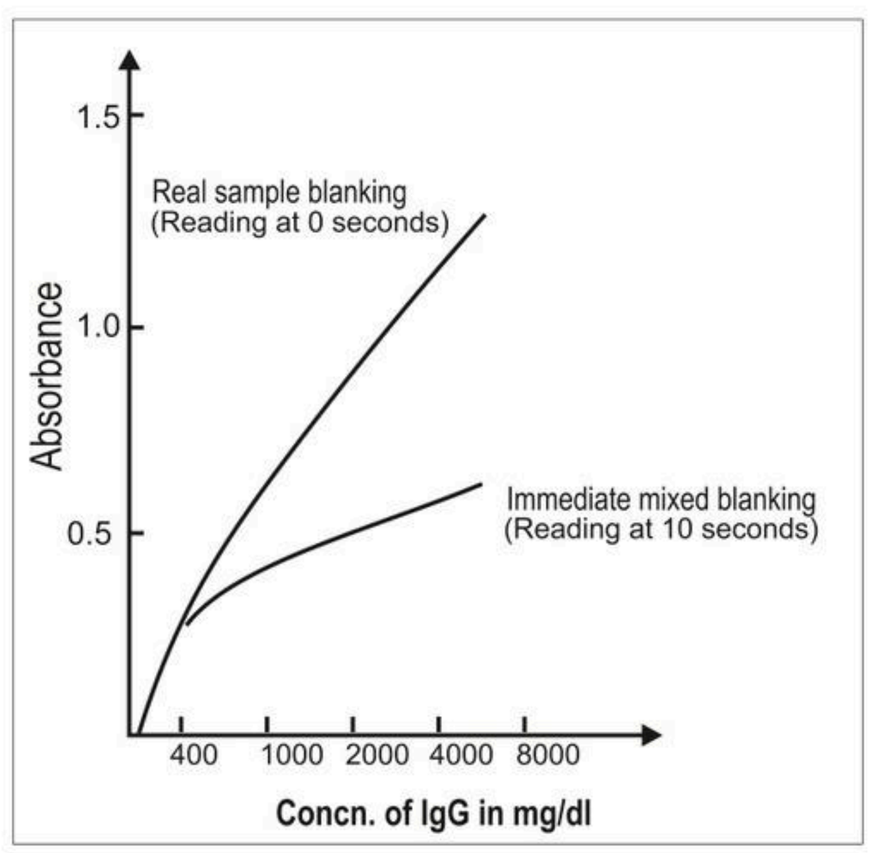


Fig. 12 B. Standard curves for IgG obtained with 'real sample blanking' & 'immediate mixed blanking'.

It can be observed from Fig.12 B that the curve for **"immediate mixed blanking"** tends to get flatter with the increasing concentration of IgG, resulting in a decrease in the precision of the analysis.

It would be desirable to optimize both slow reacting systems and assay systems based on particle enhanced Turbidimetry (latex based assays) where the reagent absorbance is very high, based on **"immediate mixed blanking"**

Whereas for systems with fast reaction kinetics such as IgG, the assays should be optimised using the **"real sample blanking"** principle as the sample blanking and the immonochemical reaction can be optimised separately.

CONCEPTS OF ASSAY OPTIMISATION

While optimizing reagent for Immunoturbidimetric assay system, it is important to optimize the dose-response curve **by iterating the amount of sample (antigen) and the antibody concentration** in the reagent until a dose response curve as shown in Figure 13 is obtained.

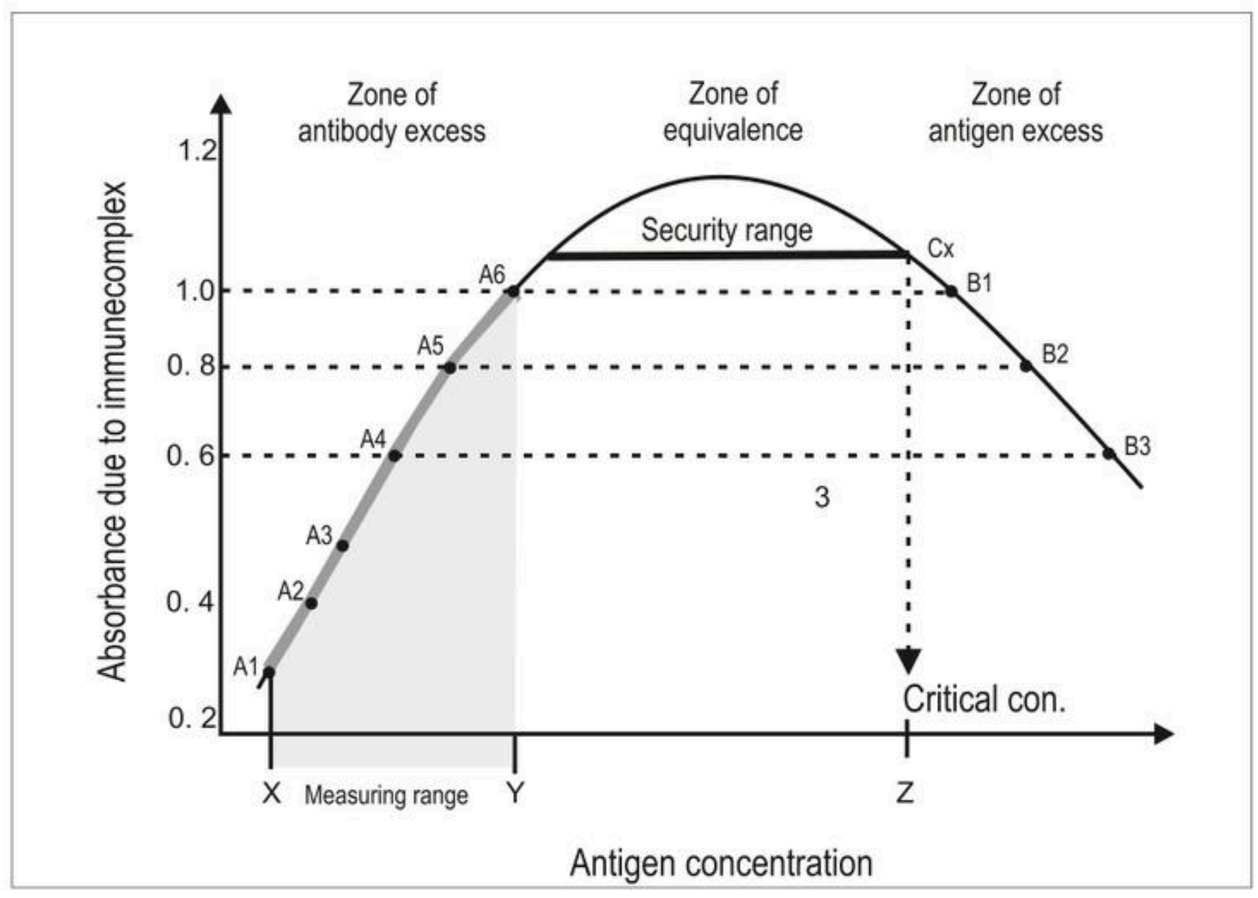


Fig. 13 Dose response curve, X-Y : Measuring range, z: Critical concentration, Abs val. 1.0 corresponds to concentration A1 and B1, Abs. Val. 0.8 Corresponds to concentration of A2 and B2, Abs. Val. 0.6 Corresponds to concentration of A3 and B3,

A portion of the **antibody excess** zone of dose-response curve is then selected as the "**range for the standard curve".**

Detection limit:

The lowest concentration of an antigen, which gives a detectable signal compared to the background noise, is defined as the **detection limit** or analytical sensitivity of the analysis. It is defined as the minimum concentration of analyte that is statistically unlikely to form part of the range of signals seen in absence of analyte. Usually the detection limit is set as the lowest signal where the standard deviation around that signal is less than one third of the signal itself. The lowest concentration selected for the calibration of the assay is usually above the detection limit.

Measuring range:

As long as the analyte signal is higher than the signal of the lowest calibrator and lower than the signal of the highest calibrator, the assay system will operate accurately for the said analyte and a concentration value of the sample can be interpolated.

The interval between the signal generated by the lowest calibrator to the signal generated by the highest calibrator, which gives proportionate and measurable signal , is referred to as **the measuring range** of the assay system.

Security range:

The **critical point** (C_x in Fig. 13), in the antigen excess zone of the dose-response curve corresponds to the maximum concentration value of analyte, which gives a signal value higher than the signal value of the calibrator of highest concentration, and just before the value (B_1 in Fig. 13) at which erroneous interpolation begins. The interval between the signal of highest standard and the signal of the critical concentration can be referred as the **security range** of the assay system for the analyte.

Reagent optimisation:

The risk of obtaining signals in the antigen excess zone are more relevant in analytes like c-reactive protein or immunoglobulins such as IgG where the concentration between normal and pathological sample can differ by manifolds. It is a necessary to make sure that the concentration values lying in the antigen excess zone (critical point) are beyond the concentrations which can be expected to occur in clinical samples during routine analysis.

The relation between the security range and measuring range is very important in optimizing assay systems. As mentioned earlier the shape of the dose response curve depends, on the ratio between the antigen and the antibody. At a constant antibody concentration an increase in the measuring range will result in a narrower security range, leading to antigen excess at a lower antigen concentration (refer Fig. 14).

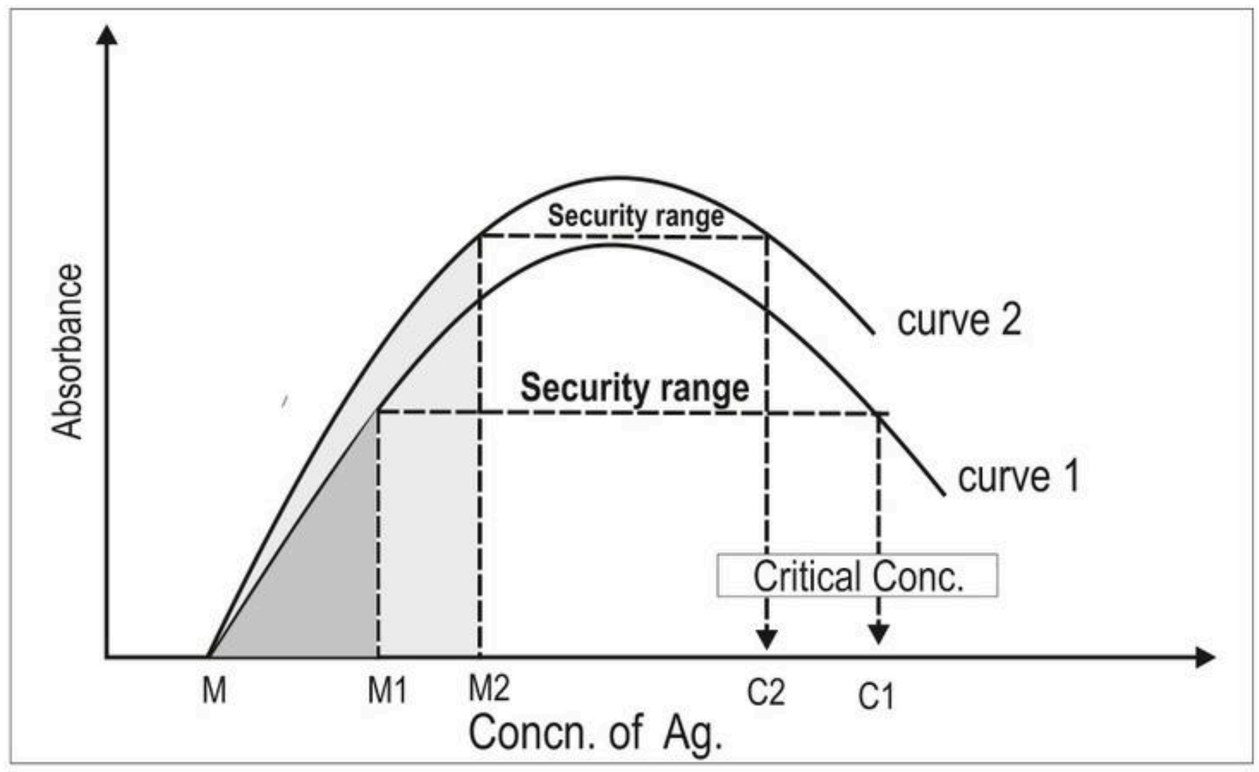


Fig. 14. Effect of increasing measuring range on security range.
M-M1: Measuring range of curve1 , C1: critical concentration for curve 1
M-M2 : Measuring range of curve 2, C2: critical concentration for curve 2

The implications of increasing the antibody reagent concentrations can be practically demonstrated using IgG as an analyte and Anti-human IgG as a reagent. The measuring range and the security range can be expanded by increasing the antibody concentration. However this expansion can only be done to a point where it is still possible to have the desired sensitivity for lower analyte concentration.

The Fig. 15 shows the effect of increasing the antibody concentration on the security ranges while keeping constant, the measuring range of the dose response curve.

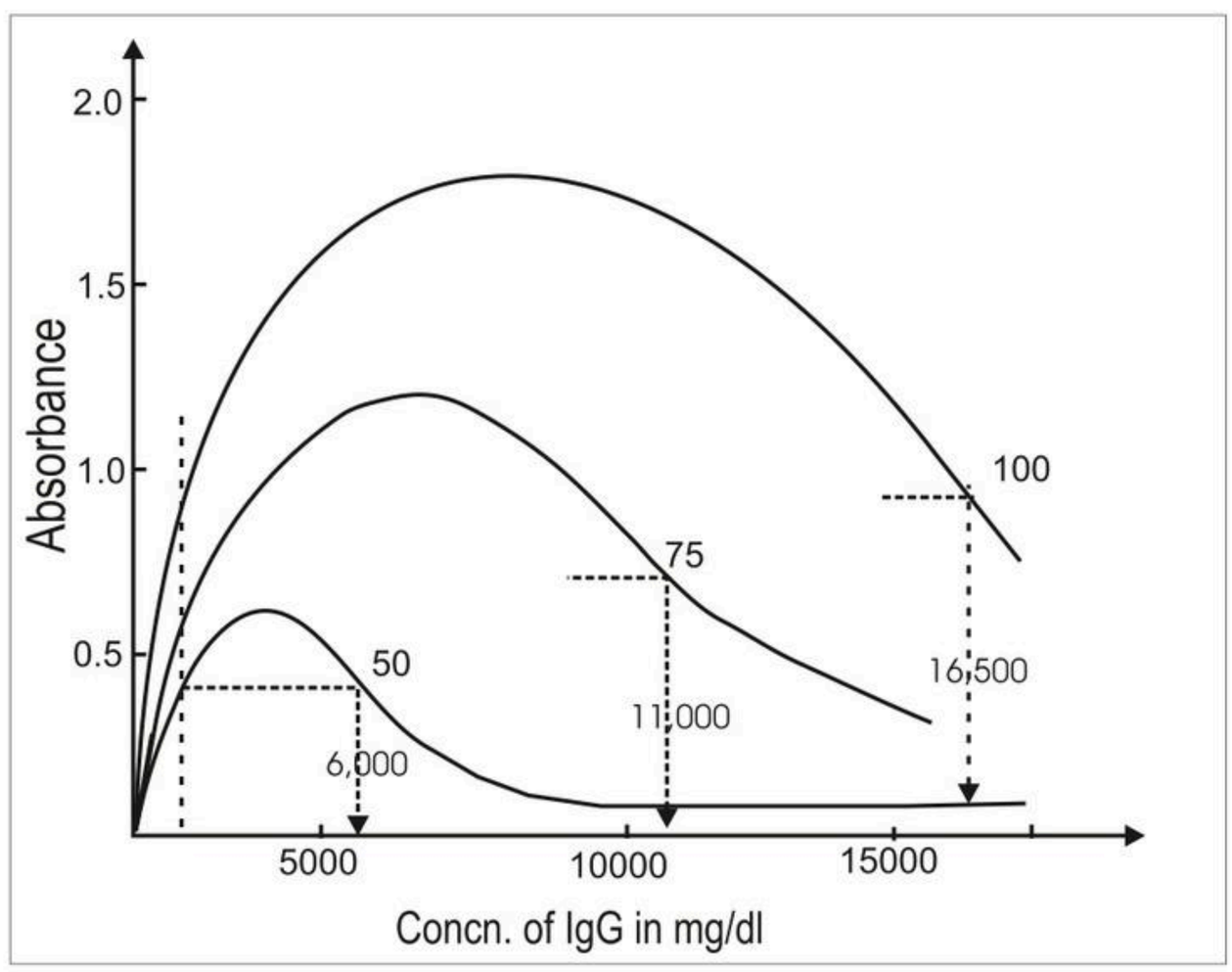


Fig. 15 Effect of increasing antibody concentration on security range.
Where 50, 75 and 100 are volumes of antibody. 6000, 11000 and 16500 mg/dl are critical concentrations.

When the volume of an Antibody solution of concentration 'X' used is 50 µl, the security range obtained is around 5000 mg/dl. As the volume of the same antibody concentration is increased to 75µl the security range increases to > 10,000 mg/dl. With a further increase in volume of the same antibody concentration to 100 µl, the security range shifts to >15,000 mg/dl. But this increase in measuring range is possible till a certain limit of increasing volume of antibody solution. If the volume of antibody solution is increased further there will be a decrease in the absorbance at the lowest concentration of the measuring range due to antibody excess, resulting in compromising with assay sensitivity.

By adjusting the sample dose and the antibody concentration, a measuring range 20 –25 times the lowest calibrator value can be possibly optimized, with a security range still giving a warning upto the pathophysiological concentration. A wide measuring range combined with a wide security range offer the advantage of a few reruns and maximum security against antigen excess problems.

Standard Curve

Once the dose-response curve for a reagent has been optimised, a standard curve can be obtained by using a number of dilutions of the calibrator (preferably 5-6) covering the optimal measuring range. The lowest calibrator should be chosen to give a signal significantly higher than the background noise. The highest calibrator should be selected to allow measurements for a reasonably wide range of analyte concentrations, and still leaving space for a fair security range.

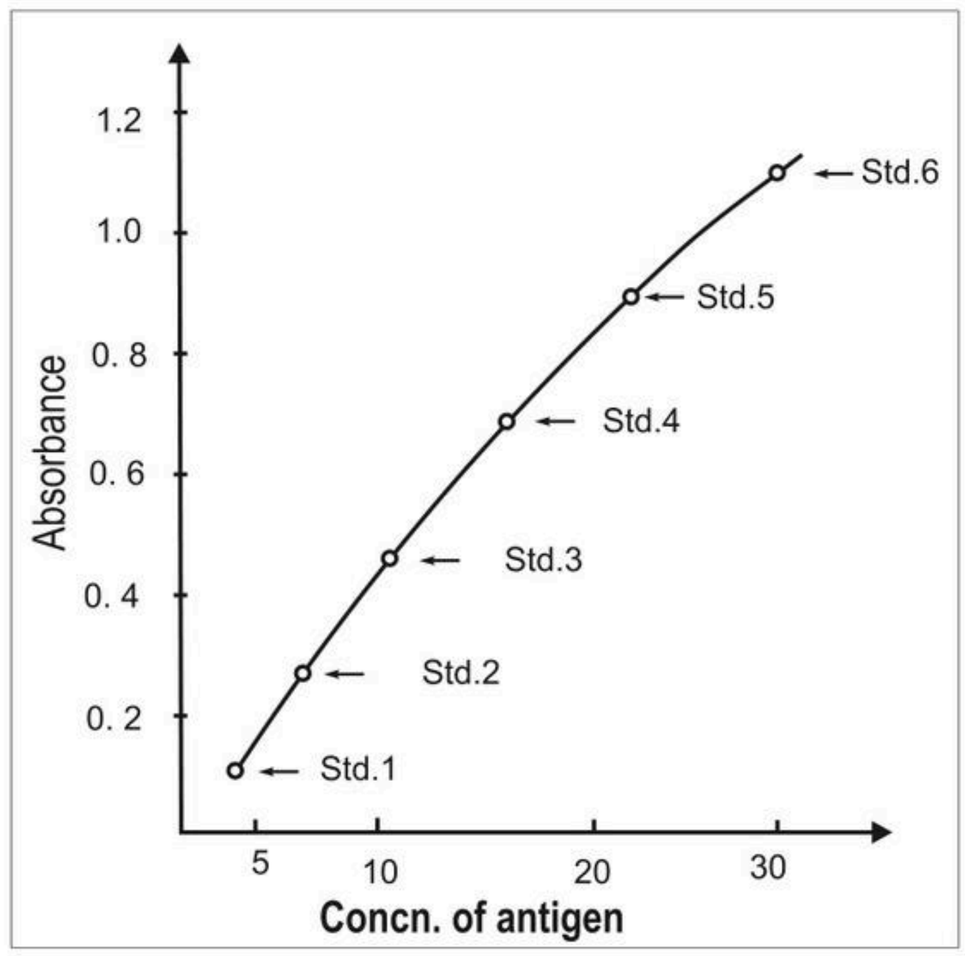


Fig. 16. Illustrating Standard Curve

A curve is fitted to the signals obtained for calibrator dilutions and can be stored in the memory of the instrument. Different curve fitting programs can be made available in instrument software.

Many of the instruments are equipped with a facility to give a 'warning' that indicate reruns of the test with dilution of the sample with high concentration values of analyte. This warning is given as long as the sample signal is higher than the signal of the highest calibrator.

The validity of the stored standard curve should be checked with known controls at regular time intervals.

INSTRUMENTATION FOR TURBIDIMETRY

The development of automated instruments for the clinical laboratory began in the 1950's at the same time as the demand for test such as RF, CRP, HBsAg escalated dramatically. One of the benefits of automation is a reduction in the variability of results and errors of analysis by eliminating tasks that are repetitive and monotonous for a human and that can lead to boredom or inattention. The significant improvement in quality of laboratory tests in recent years owes much to the combination of well designed instrumentation with good analytical methods.

The photometric requirements of turbidimetric analysis are no different from those of photometric biochemistry analysis. However the photometer used must be provided with means to maintain the contents of the cuvette at a constant temperature during the reaction along with compatible software to run the various steps of the reagent and reagent kinetics appropriately.

The chemistry analyzers (spectrophotometers) available utilize either or both of the two mode of measuring absorbance.

1. Aspiration mode:

In this mode the chemical reaction is carried out in a test tube/ cuvette. The instrument aspirates the reaction mixture from the test tube/cuvette, which enters into the flow cell (internally built reading chamber), where the absorbance is measured. After the absorbance is measured the reaction mixture is passed through a different outlet and is collected in a waste collecting bottle. As all the measurements are done in a single flow cell the flow cell has to be washed after each test. Improper washing may affect the test results of the subsequent tests. Especially if latex enhanced tests are used the latex has a tendency to stick and form a permanent coating on the internal walls of the flow cell resulting in variation in the wavelength of the incident light, and hence lead to erroneous results. Even non-enhanced antibody reagents are proteinacious and cleaning of the flow cell would remain a critical issue.

When Ag-Ab complexes are aspirated, because of the force of aspiration the formed immunecomplexes would be structurally disturbed and may break down into smaller complexes, which would result in lower absorbance values. Moreover the immune precipitates formed may block the aspiration tube or the flow cell itself.

Further for turbidimetric assays designed with real sample blanking reading principle, it would be inconvenient to run the test in the aspiration mode. Initially the sample mixed with the activation buffer would have to be aspirated first and absorbance A1 measured. Then again the activation buffer and the sample will have to be taken in another test tube and the principle reagent added and the reaction read after a fixed time interval. Hence for running one assay twice the amount of activation buffer and sample will have to be used.

2. **Cuvette mode:**
In this mode, the reaction of the reagent and sample takes place in a measuring cuvette, and absorbance is read in the same measuring cuvette itself. Hence assays using any of the reading principles can be conveniently read in cuvette mode without wastage of reagents.

Instruments applying this mode of measuring have an advantage. As the reactions are run in external cuvettes, the instruments are safe from the effects of reagents. Moreover availability of standardized optically clean disposable cuvettes eliminates the carryover effects of the previous tests.

Classification of Analyzers

There are several types of analyzers available in the market. They may be grouped in two categories.

- Semi-automated analyzers
- Automated analyzers

Semi automated analyzers
Instruments with an absorbance linearity of 2.0 are suitable for turbidimetric estimations of both particle enhanced and non-particle enhanced reagent systems. Most of the instruments with the above specification can be used for turbidimetric measurements using the absorbance mode. In the absorbance mode as the calibration curve cannot be stored it has to be drawn manually.

Among the instruments working on cuvette mode for measuring absorbance, very few instruments have software interface programmed with a facility to store the calibration curve utilising both the principles of reading i.e **'real sample blanking'** and **"immediate mixed blanking"** in the multistandard mode. Many instruments with cuvette modes are known to have programs in multistandard mode to store calibration curve for assay systems using the real sample blanking techniques only.

Automated analyzers
The automated analyzers can be grouped in two categories.
Centrifugal analyzers
Static instruments (non-centrifugal analyzers)

1. **Centrifugal analyzers**
In these analyzers the cuvettes are arranged in circle (rotor) that can be rotated at a velocity about 1000 rpm. The shape of each cuvette allows application of sample and reagent (reaction buffer, antibody) in separate compartments. When the rotor starts to spin, the contents of these compartments are mixed simultaneously and held in place in the cuvette by centrifugal force.
Readings of all the cuvettes are performed at essentially the same time (i.e when the rotating cuvettes are passing the optical measuring device). Two reading systems are used: either parallel to the length of the cuvette where the volume in the cuvette is proportional to the light path or perpendicular to the length of the cuvette where the width of the cuvette equals the light path.

2. **Static Instruments**
In these instruments the cuvettes are mostly arranged in a circle (rotor), and this is a slowly rotated in step at a fixed time interval (cycle time). Access to the cuvette is possible only at these intervals for sample or reagent application and reading. Mixing is in most cases performed with a mechanical stirring device. Modern instruments seem more and more to be based on these principles.

All instruments operate under software control. A part of this software is the user interface that makes it possible to program the instrument to perform analysis and calculation according to an optimized protocol. The analytical parameters available for user control vary from instrument to instrument.

Some instruments, however are "closed instruments" which implies that all parameter settings are read into the instruments by bar coded reagents. In this case the user cannot control the assay and will have to rely entirely on the manufacturer and their instructions.

Applications, reference values, of important Clinical Analytes

ANALYTE	DESCRIPTION	REFERENCE VALUES	APPLICATIONS
RF	Quantitation of Rheumatoid factors	<10 IU/ml	Detection of RA, Differential diagnosis of RA from Rheumatic fever and other rheumatic disorders
CRP	Quantitation of C-reactive protein	Adults and children < 0.6 mg/dl	Detection of inflammatory conditions Measuring the severity of conditions Differential diagnosis of bacterial and viral infections Monitoring the response to therapy
CRP US	Quantitation of ultrasensitive levels of C-reactive protein	<0.05 mg/dl	Prognostic cardiac marker
ASO	Quantitation of Antistreptolysin 'O'	Children: < 150 IU/ml Adults : < 200 IU/ml	Detection of Group A streptococcal infections such as sore throat, rheumatic fever, rheumatic heart disease
MA	Quantitation of urinary Albumin	< 20 mg/L	Detection of Microalbuminuria Monitoring the effect of ACE inhibitor or intervention strategies for reducing UAE
IgA	Quantitation of Immunoglobulin IgA	70 - 400 mg/dl	Chronic inflammatory diseases of GI & respiratory tract Deficiency of IgA myeloma
IgG	Quantitation of Immunoglobulin IgG	700 - 1600 mg/dl	IgG myeloma IgG deficiency Assessment of the progression and response to treatment of IgG myeloma
IgM	Quantitation of Immunoglobulin IgM	40 - 230 mg/dl	Monitoring patients with Waldenstrom's macroglobulinemia Estimating frequent, chronic and acute infections Screening for congenital infection
IgD	Quantitation of Immunoglobulin IgD	3 - 14 mg/dl	Monitoring IgD myeloma
IgE	Quantitation of Immunoglobulin IgE	Adult 3 - 423 IU/ml	Assessment of atopic diseases, dermatologic and parasitic infections
C3	Quantitation of complement component C3	90 - 180 mg/dl	C3 deficiency, recurrent infections, detection and monitoring of immune complex disorders such as SLE, vasculitis, glomerulonephritis, autoimmune hemolytic anemia
C4	Quantitation of complement component C4	10 - 40 mg/dl	Congenital deficiency in Lupus erythromatosus Hereditary angioneurotic oedema Recurrent infections
AT III	Quantitation of Antithrombin III	17 - 30 mg/dl	Evaluating patient at risk of developing thrombotic-embolic disease. In surgical patients receiving heparin assessment of thrombotic risk of contraceptive or estrogen therapy
Apo A-1	Quantitation of Apolipoprotein A-1	Males: 105 - 175 mg/dl Females:105 - 205 mg/dl	Independent risk factor for coronary artery disease
Apo B	Quantitation of Apolipoprotein B	Males: 60 - 140 mg/dl Females:55 - 130 mg/dl	Elevated Apo B levels are associated with atherosclerosis
Lp(a)	Quantitation of Lipoprotein(a)	< 300 mg/L	Risk factor for coronary heart disease that is independent of all other lipid parameters
B2-M	Quantitation of B2-Microglobulin	< 60 yrs.: 0.8 - 2.4 mg/L > 60 yrs.: < 3.0 mg/L	Prognosis of multiple myeloma Early detection of renal transplant rejection Differentiation of glomerular and tubular nephropathies Monitoring therapeutic response of patients with nonsecretory myeloma or light chain disease
Cp	Quantitation of Ceruloplasmin	20 - 60 mg/dl	Diagnosis of Wilson's disease, menkes disease, nutritional copper deficiency
Hp	Quantitation of haptoglobin	30 - 200 mg/dl	Diagnosis and of monitoring of hemolytic diseases

Note: The above reference values are for guidance on. As the reference values are related to age, geographical and methodological differences and vary widely each laboratory should define its own reference range for the relevant population.

SERUM PROTEINS IN DIFFERENT CLINICAL CONDITIONS

PROTEIN	INCREASED IN	DECREASED IN
Serum Albumin	Rare, usually associated with hemoconcentration	Acute phase response, severe liver disease, nephrotic syndrome, other renal disease, malnutrition, pregnancy, premature infants
Complement		
Complement C3	APR (infection, inflammation etc), biliary obstruction, obstructive jaundice, diabetes mellitus, gout, some connective diseases (excluding SLE)	Autoimmune diseases, immune complex diseases, mixed cryoglobulinaemia, serum sickness, chronic renal failure, thrombotic thrombocytopenic purpura, neonatal respiratory distress syndrome, genetic deficiency
Complement C4	APR, RA, SLE, rheumatic fever, ankylosing spondylitis, temporal arthritis, acute viral hepatitis, MI, DM, malignancies, thyroiditis, irritable bowel syndrome, pneumonia, pregnancy	**Acquired deficiencies resulting from;** ***Hypercatabolism:*** Disease in which circulating immunecomplexes are likely to lead to acquired hypocomplementemia, subacute bacterial endocarditis, mixed cryoglobulinaemia, hereditary angioneurotic edema, progressive glomerulonephritis ***Hyposynthesis:*** Protein calorie malnutrition, liver disease, sjogren's syndrome ***Congenital deficiencies:*** Associated with increased frequency of scleroderma, chronic hepatitis, auto immune hepatitis, Henoch-schonlein purpura.
Lipoproteins		
Apolipoprotein A-1	Familial hyper-alphalipoproteinemia, modest alcohol intake, estrogen use, exercise, thyroid hormones	Chronic renal failure, tangier disease, diabetes, hypertriglyceridemia, liver disease, familial and non-familial hypoalphalipoproteinemia, certain drugs, androgen use.
Apolipoprotein B	Premature atherosclerosis, familial defective Apo B_{100}, hyper-apobetalipoproteinemia, tendon xanthomata, hepatosplenomegaly, diabetes mellitus, chronic renal disease, tangier disease, hypothyroidism, nephrotic syndrome, familial hypercholesterolemia	Familial hypo-betalipoproteinemia, abetalipoproteinemia, neuromuscular degeneration, chronic anemia, exercise, liver disease, acute inflammation, certain drugs, neurologic disease.
Lipoprotein (a)	Coronary artery disease, cerebrovascular disease, peripheral vascular disease, nephrotic syndrome, DM (variable), cancer, gout, APR, familial hypercholesterolemia	Cirrhosis (particularly primary biliary cirrhosis), certain drugs (nicotinic acid, neomycin, oral estrogen), some steroids such as stanozolol.
Immunoglobulins		
IgA class	**Polyclonal:** Chronic liver disease, alcoholic and non-alcoholic cirrhosis, chronic respiratory infections, GI diseases, some immnodeficiency states, RA, ankylosing spondylitis, nephropathy **Oligoclonal:** May be observed in electrophoresis of IgA myeloma **Monoclonal:** Multiple myeloma (IgA type)	Infancy and early childhood, selective IgA deficiency, protein-losing syndromes, congenital rubella, macroglobulinemia or non-IgA multiple myeloma
IgG class	**Polyclonal:** Autoimmune diseases, chronic liver disease, chronic or recurrent infections, sarcoidosis, some parasitic infections, intrauterine contraceptive devices **Oligoclonal:** Lymphoid or non-lymphoid malignancies, various autoimmune disorders, infections **Monoclonal:** IgG myeloma, Lymphoma, Monogammopathies of unknown significance	Agammaglobulinemia, hypogammaglobulinemia, omenn's syndrome, X-linked hyper IgM syndrome, nephrotic syndrome, non-IgG myelomas, infancy, pregnancy
IgM class	**Polyclonal:** Viral infections(Hepatitis A, CMV), parasitic infections (filariasis, malaria), chronic liver disease, hyper-IgM dysgammaglobulinemia, collagen vascular disease, primary biliary cirrhosis, primary sclerosing cholangitis **Monoclonal:** Waldenstrom's macroglobulinemia, malignant lymphoma, reticulosis, cold agglutinin/hemolysin disease	Immnodeficiency states (Wiskott-Aldrich syndrome), non-IgM myeloma, infancy and early childhood, lymphoma

PROTEIN	INCREASED IN	DECREASED IN
IgD class	IgD myeloma, chronic infections (pyelonephritis), connective tissue disease, hodgkin's disease, some forms of liver disease.	Various hereditary and acquired deficiency syndromes
IgE class	IgE myeloma, allergic rhinitis, atopic dermatitis, bronchial asthma, hay fever, thymic dysplasia, selective IgA immnodeficiency, eosinophilic gastroenteritis, wiskott-aldrich syndrome, loffler's syndrome, hyper-IgE syndrome, active SLE nephritis, certain drugs (particularly gold compounds)	Some progressive neoplastic diseases, ataxia-telangiectasia, hypogammaglobulinemia, hypersensitivity
Acute phase reactants		
CRP	APR, bacterial infections, viral infections, rheumatic fever, active RA, vascular disorders, MI, crohn's disease, ulcerative colitis, renal transplant failure, early pregnancy, intrauterine devices, malignancies with widespread metastases	None described
Fibrinogen	APR, nephrotic syndrome, hemodialysis patients, pregnancy, estrogen therapy, contraceptives, acromegaly	Consumption coagulopathies, recurrent pulmonary embolism, recurrent stroke, DIC, incompatible blood transfusion reactions, obstetrical complications, inherited deficiency, prostatic carcinoma, liver disease, certain drugs (e.g. tamoxifen, anabolic steroids, nicotinic acid)
Haptoglobin (Hp)	APR, RA, biliary obstruction, nephritis, ulcerative colitis, aplastic anemia. Major depression, corticosteroid therapy, androgen use	Ineffective erythropoiesis (sickle cell anemia, folic acid deficiency), intravascular haemolysis, progressive tumors of liver and marrow, severe liver disease, pregnancy, estrogen therapy, newborns
Ceruloplasmin (Cp)	Bacterial infections, use of oral contraceptives or estrogen	Wilson's disease, menkes disease, nutritional copper deficiency
Proteinase inhibitor		
ATIII	APR, Vit K deficiency	Inherited deficiency, acute thrombosis, DIC, consumptive coagulopathies, certain chemotherapeutic drugs, severe liver disease, estrogen therapy, nephrotic syndrome, heparin therapy, some contraceptive medications
Other markers		
Serum β2 - Microglobulin	Decreased glomerular filtration, lymphoproliferative disorders, myeloma, RA, viral infections, anticancer drugs, newborns, ESRD, crohn's disease, sjogren's syndrome, dialysis related amyloidosis certain anti inflammatory drugs	None reported

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KNOWLEDGE BOOK

For the Laboratorians

Applications of Quantia Reagents

Setting Trends...

"Quantia" indigenous immunoturbidimetry reagents, have been standardized against the universally accepted reference preparations or secondary reference material to provide accurate results aligned to international standards. Quantia reagents have been designed with **user-friendly** assay procedures using ideal reagent and sample volumes to minimize procedural errors and thereby achieve high precision.

The **"Optimised turbidimetric assay system"** is designed to program immunoturbidimetry reagents in the **multistandard mode,** the suitable method for determining accurate measurements. It is one of the most ideal systems with a compatible **reagent-instrument** interface and can suffice the needs of routine laboratories.
The suggested applications of quantia reagents are discussed below:

RHEUMATOLOGY

Quantia RF: Turbidimetric immunoassay for determination of Rheumatoid Factor (RF)

Significant facts:

- Rheumatoid factor (RF) is the only serological marker suggested by ARA (American Rheumatism Association) for the diagnosis of Rheumatoid arthritis (RA).
- Rheumatoid Factor may be present, before the onset of clinical disease.
- RF positive persons have a > 70% probability of developing joint damages or erosions within two years of the onset of disease.
- Initial RF levels correlate well with radiologically determined joint damage upto 3 years.
- High RF levels at entry and persistent RF positivity during follow up indicates destructive disease in early RA.
- Initial RF level and serial measurement help prognosis of RF in distinguishing between progressive and non-progressive RA.

Suggested Applications

- Early diagnosis of RA.
- Identifying patients at risk of developing joint damages in early RA.
- Monitoring course of early RA.
- Identifying RA patients at risk of developing progressive disease.

Reference values ≤10 IU/ml

Quantia ASO : Turbidimetric immunoassay for determination of Antistreptolysin 'O'

Infection with β-hemolytic group A streptococcal infection result in an Antistreptolysin 'O' (ASO) response. Due to frequent exposure to streptococcal enzymes practically all sera manifest some ASO titre depending on age, geographical circumstances and prevalence of streptococcal infections. Hence quantified ASO titres are of great significance in early and accurate diagnosis.
A positive throat culture does not necessarily prove an active infection by Group A streptococcus. It may reflect a chronic colonization of the pharynx by Group A streptococcus and, therefore, be unrelated to rheumatic fever. Consequently, an elevated or rising ASO antibody titer to Group A Streptococcus provides more reliable evidence of recent Group A streptococcus infection.

Significant facts:

- **Serial measurement resulting in an increase in ASO titre indicates recent infection.**
- Persons with rheumatic fever are at risk of progressing to complications like rheumatic heart disease until ASO titres remain constantly low, hence **monitoring** is required at an **interval of 2-4 weeks.**
- Collection of sample for diagnosis by culture is difficult in streptococcal complications such as glomerulonephritis, acute endocarditis, and streptococcal toxic shock syndrome.

Approximate ASO titres in various conditions:

Conditions	Usual ASO titres	
	In Todd Units	In IU/ml
Normal persons	12 - 166	~15 - 200
Active Rheumatic fever	500 - 5000	~600 - 6000
Inactive Rheumatic fever	12-250	~15 - 300
Rheumatoid arthritis	12 - 250	~15 - 300
Acute Glomerulonephritis	500 - 5000	~600 - 6000
Streptococcal URTI (upper respiratory tract infections)	100-333	~120 - 400
Collagen diseases	12 - 250	~15 - 300

Note: 166 Todd units = 200 IU/ml

Suggested Applications

- Correct classification of ASO titre for early diagnosis of recent streptococcal infection.
- Differential diagnosis of joint pains of rheumatic fever and rheumatoid arthritis.
- Monitoring patients with rheumatic fever.

Reference values: Adults 200 IU/ml. Children 150 IU/ml.

Quantia CRP: Turbidimetric immunoassay for measurement of CRP

The rise and fall of C-reactive protein (CRP) concentrations closely follows inflammatory processes of infectious and non-infectious geneis. An elevated CRP is always associated with pathological changes.

Significant Facts:

Rheumatoid arthritis (RA)

- CRP is elevated in RA but normal or moderately elevated in SLE, osteoarthritis, and systemic sclerosis. Increased CRP concentrations are found in more than 90% of adults with RA and in established disease, levels relate to severity. Values upto 5 mg/dl are associated with mild inflammation and values around 10 mg/dl indicate a more severe disease.

Differential diagnosis

- Bacterial infections elicit higher CRP response; parasitic infections moderate whereas viral infections give minor CRP response.
- Bacterial meningitis is associated with higher CRP levels than aseptic or viral meningitis in children. A cut off value of 2 mg/dl yielded a sensitivity of 97% and a specificity of 86% for diagnosis of bacterial meningitis for children younger than 6 years of age.
- Appropriate therapy for bacterial and tuberculous meningitis causes fall in CRP levels making it useful to **monitor therapeutic response** with advantages over **repeated lumbar punctures** especially in children.
- In children although fever is most often due to viral infection, it is difficult to distinguish it from bacterial infection and **antibiotics are prescribed unnecessarily.** It has been shown that CRP level > 4 mg/dl had a diagnostic sensitivity of 79% and specificity of 90% for diagnosis of bacterial infections in children who has been ill for more than 12 hours.
- In the diagnosis of pulmonary infections in the elderly where febrile response may be lost CRP levels above 10 mg/dl provide a strong indication of bacterial infection such as pneumonia or purulent bronchitis where as viral infections do not result in values above 5 mg/dl.
- Localization of infection with urinary tract influences decisions regarding choice and route of antimicrobial therapy and what follow up is needed. Clinical assessment cannot reliably distinguish cystitis from pyelonephritis in every case, particularly in young children. In general, it is observed that clinical symptoms of urinary tract infection associated with a high CRP level usually above 5 mg/dl indicates pyelonephritis, while a normal to slightly elevated CRP levels indicate uncomplicated lower urinary tract infections.

Neonatal sepsis

Bacterial sepsis is a common diagnostic challenge in newborn medicine. Diagnosis based on culture of blood, CSF, urine is usually reached only after a delay of a day or two. Initiation of antibiotic therapy is recommended before the availability of diagnostic results to decrease mortality. This empiric therapy may result in treatment of as many as 30 uninfected infants for everyone who may be infected.

- CRP levels are consistently elevated 24 to 48 hours after the onset of infection. It has been suggested that serial normal CRP levels may be useful in the diagnostic evaluation of neonates with suspected infection. The sensitivity, specificity, PPV and NPV of CRP with a cut off at 0.7 mg/dl were estimated to be 56%, 72%, 71%, and 57% for the screening of early sepsis in very low birth weight infants.
- CRP values greater than 1 mg/dl before the third day of life indicate infection with a clinical sensitivity of 20-60% and a specificity of 94 - 97% for the diagnosis of infections in newborns.
- Daily measurements of CRP were found to correlate with resolution of sepsis, specifically a **decrease in CRP by 25% or more from previous days level was a good indicator of resolution of sepsis,** with a sensitivity of 97%, specificity of 95% and predictive value of 97%.
- **Serial CRP levels** may be useful for early identification of infants for whom **antibiotic therapy can be safely discontinued.** Two CRP levels < 1 mg/dl, obtained 24 hours apart, 8 to 48 hours after presentation, indicate that bacterial infection is unlikely.

Postoperative complications

- Surgery of all types induces inflammation and CRP increases roughly in proportion to the extent of tissue damage. In uncomplicated cases CRP rises above 1 mg/dl by about 6 hours reaches peak rarely greater than 15 mg/dl and declines thereafter to baseline values by 7-10 days. In many cases of postoperative complications **raised CRP precedes the clinical diagnosis of complicating pathology** by upto 24 hours.

Burns

- CRP levels increase significantly in patients with burns. A second later peak of CRP develops if infection occurs as a late complication of burns.

CRP in Leukemia patients

- Fever in patients with leukemia and neutropenia, can be caused by bacterial or viral infections, the underlying disease process, administration of blood and blood products and cytotoxic therapy or other non-infectious causes. If levels of CRP is <4 mg/dl for 48 hours after the onset of fever, infection is unlikely, whereas level above 10 mg/dl indicate antibiotic treatment even in absence of bacteriological confirmation.

Causes related with chest pain

- CRP is elevated in pulmonary embolism, pleurisy, or pericarditis but normal in angina without infarction or invasive investigations.

Suggested Applications

- Differential diagnosis of;
  - Rheumatic diseases
  - Bacterial and viral pulmonary infections
  - Bacterial and aseptic or viral meningitis in children
  - Pyelonephritis and uncomplicated urinary tract infections in children
  - Causes related with chest pain
- Diagnosis of neonatal sepsis.
- Making decision to discontinue antimicrobial therapy in cases of neonatal sepsis.

- Monitoring post operative complications.
- Preventing unnecessary use of antibiotics in respiratory infections in children.
- Detecting late complications due to infection in cases of burns.
- Taking therapeutic decision for treatment of fever in acute leukemia patients and monitoring therapeutic response.

Reference values: ≤ 0.6 mg/dl.

OTHER MARKERS

Quantia MA : Turbidimetric immunoassay for determination of Microalbuminuria

Microalbuminuria is the earliest sign of diabetic nephropathy before it manifests as overt proteinuria, a condition where significant kidney damage has already occurred. Microalbuminuria is also a strong predictor of vascular complications.

Significant facts

- Early detection of Microalbuminuria can help reverse or control the progression of diabetic nephropathy.
- Early antihypertensive treatment with Ace inhibitors helps reduce/stabilize Microalbuminuria and preserves GFR.
- Researchers have observed an association of increased urinary albumin concentration and cardiovascular risk. The doubling of urinary albumin concentration has been found to associate a 1.20 fold increased risk of cardiovascular mortality and 1.2 fold increased risk of non-cardiovascular death.

Suggested Applications

Diabetic subjects

- IDDM: Annually in all patients suffering from diabetes for 5 years or more and over 12 years of age.
- NIDDM: As soon as diabetes is diagnosed, and at regular intervals thereafter incase of negative test results.
- Monitoring albumin excretion after initiation of intervention strategies in diabetic nephropathy.

Non Diabetic subjects

- Patients with potential involvement of the kidneys.
- Patients at risk of cardiovascular complications.

Reference values < 20 mg/L

CARDIOVASCULAR RISK MARKERS

Quantia CRP US: Turbidimetric immunoassay for determination of Ultra sensitive CRP levels

The baseline CRP levels of an individual remain stable over long period of time. Artherothrombosis, in addition to being a disease of lipid accumulation also represents a chronic inflammatory process. CRP is believed to be a mediator as well as a marker of atherothrombotic disease.

Significant facts:

- The Physicians health study observed that as the CRP levels in apparently healthy individuals increases from 0.055 mg/dl to 0.210 mg/dl the odds ratio for developing acute myocardial infarction increases from 1 to 2.9.
- Simultaneous measurement of ultra sensitive CRP, total and HDL cholesterol predict future cardiovascular risk better than lipid measurements alone.
- CRP levels may identify people at increased risk whose LDL cholesterol levels are not elevated.
- In individuals with LDL cholesterol above 160 mg/dl and for whom ATP III guidelines already call for therapeutic intervention, an elevated CRP level should aggressively encourage physicians and patients to **institute pharmacological therapy** in those instances where **none is currently being used** or where **compliance is poor.**
- For individuals with LDL levels below 130 mg/dl, the finding of **an elevated CRP implies substantially higher risk** than predicted on the basis of LDL alone.

Suggested Applications

- In outpatient settings, at the time of cholesterol screening.
- Monitoring patients with cardiovascular risk having low LDL levels along with the lipid profile.

Suggested Relative Risk Factor:

MALES		
CRP in mg/dl	Relative risk factor	
	Future MI	Future Stroke
> 0.211	2.9	1.9
0.115 - 0.210	2.6	1.9
0.056 - 0.114	1.7	1.7
< 0.055	1.0	1.0

FEMALES	
CRP in mg/dl	Relative risk factor
	Future MI or Stroke
> 0.73	5.5
0.38 - 0.73	3.5
0.15 - 0.37	2.7
< 0.15	1.0

Quantia Apo A-1: Turbidimetric immunoassay for measurement of Apolipoprotein A-I (Apo A-1)

Apo A is the major protein content of HDL. The two major components of apo A are apo A-I and apo A-II. HDL molecule **with apo A-1 is considered to be more protective than HDL molecule containing both apo A-1 and A-II.**

Significant Facts:

- The measurement of Apo A-1 directly represents measurement of anti-atherogenic HDL where precipitation of LDL is not required.
- In a study by Van Lennep et al., Apo A-1 showed a predictive value for CHD events similar to HDL-C value in women and was the **only significant predictor in men.**
- Kwiterovich et al. also found that **levels of apo A-1 but not the level of HDL - C, was an indicator of future CAD for men.**

Suggested Applications

Recommended for testing in:

- Patients with established atherosclerotic cardiovascular disease and a "normal" lipid profile on a routine analysis.
- Patients with a history of recurrent coronary artery stenosis.
- Patients on lipid lowering therapy with statins.
- Patients with common lipid abnormalities, but normal or low LDL-Cholesterol concentration.

Reference Values: 105 - 205 mg/dl

Quantia Apo B: Turbidimetric immunoassay for measurement of Apolipoprotein B (Apo B)

Apo B-100 is the major Apolipoprotein of normal LDL and it represents 90-95% of plasma apo B.

Significant facts:

- **Small dense LDL** particles are **more atherogenic** than are LDL particles of **normal composition.** Because these LDL particles are **cholesterol depleted** measurement of total and LDL cholesterol does not **accurately reflect their number** in plasma. Each of the atherogenic particles in plasma [VLDL, IDL, LDL and Lp (a)] contains one molecule of apo B therefore **apo B truly measures** the number of **atherogenic particles.**
- The three studies, Moss et al, the Air force/Texas coronary atherosclerosis prevention study and the study by Van Lennep et al., constitute strong evidence that for patients on statin therapy monitoring apo B is, in fact, superior to determining lipoprotein lipids.
- Although increased throughout the range of values of LDL cholesterol, apo B and apo A-1 might be of greatest value in diagnosis and treatment in patients **who have common lipid abnormalities, but have normal or low LDL cholesterol concentrations.**

Suggested Applications

Recommended for testing in;

- Patients with established artherosclerotic cardiovascular disease and a "normal" lipid profile on a routine analyis.
- Patients with a history of recurrent coronary artery stenosis.
- Patients on lipid lowering therapy with statins.
- Patients with common lipid abnormalities, but normal or low concentration of LDL - cholesterol.

Reference values: 55 - 140 mg/dl

Quantia Lp (a) : Turbidimetric immunoassay for measurement of Lipoprotein (a)

Lipoprotein (a) [Lp (a)] is the little known stepbrother of LDL. Lp (a) is a lipoprotein particle having Apolipoprotein B-100 as a protein moiety; disulphide linked to Apolipoprotein (a), a distinctive glycoprotein that is homologous to plasminogen. Lp (a) could promote cardiovascular disease in two ways. Its apo (a) moiety could promote thrombogenesis and its LDL moiety could promote Atherogenesis.

Significant facts:

- Lp (a) excess is the **most common inherited lipid disorder** in patients with **premature coronary heart disease.**
- Levels of Lp (a) correlate significantly with coronary artery disease documented by angiography.
- Lp (a) levels greater than 30 mg/dl may have a risk for myocardial infarction 1.75 times that of subjects with Lp (a) levels below this level.
- Excess Lp (a) concentrations are associated with increased risk of cardiac death in patients with restenosis after angioplasty and coronary bypass procedures.

Suggested Applications

For assessing and monitoring cardiovascular risk in:

- Patients with a personal/or family history of premature cardiovascular disease (<55 years)
- Patients with established atherosclerotic cardiovascular disease and a "normal" lipid profile on a routine analysis.
- Patients with a history of recurrent coronary artery stenosis.
- Family members of subjects found to have high plasma Lp (a).
- Suspect Lp (a) in patients with hypercholesterolemia that is refractory to the standard statin (drugs used for lowering cholesterol) therapy. Since the calculated value of LDL-C includes the LDL contained in Lp (a) and since the Lp (a) will not respond to statin therapy, significant hidden elevations may account for the treatment failure.

Quantia Fibrinogen: Turbidimetric immunoassay for measurement of Fibrinogen

Fibrinogen (Factor I) is a high molecular weight glycoprotein synthesized in the liver, which plays an important role in haemostasis. For normal haemostasis to occur in response to injury or tissue damage, a sufficient concentration of fibrinogen must be present in plasma.

Significant facts:

Low levels of fibrinogen are found in;

- Liver disease,
- Increase fibrinogen consumption due to the prolonged presence of disseminated intravascular coagulation.
- Hyperfibrinolysis in patients with neoplasia, acute promyeloctic leukemias & obstetric complications such as premature detachment of placenta or abruptio placentae, amniotic fluid embolism, retention of dead fetus.
- Dysfibrinogenemia "(functionally defective fibrinogen due to an abnormal molecular form, but the levels remain normal) found either congenitally or acquired in liver disease.

Thrombosis is recognized as a central mechanism of atherosclerotic complications. Fibrinogen may promote, together with other haemostatic factors, atherosclerotic changes and thrombotic effects in several ways:

I. Platelet aggregation.
II. Plasma viscosity
III. Fibrin formation

- The Prospective Cardiovacular Munster (PROCAM) study found that individuals who had **LDL and fibrinogen** levels in the highest tertile had a **6.1 fold increase** in **coronary risk** compared with those in **lowest tertile.**
- **Restenosis** remains the main problem associated with percutaneous transluminal coronary angioplasty (PTCA). Fibrinogen levels were found to be similar with or without restenosis when measured before or immediately after angioplasty. Patients with fibrinogen concentration **exceeding 3.5 g/L** had **higher restenosis** than patients with fibrinogen concentration **below 3.5 g/L** in patients on follow up **after angioplasty.**

Suggested Applications

- As an adjunct to the lipid profile for assessing global cardiovascular risk
- For monitoring patients for restenosis after PTCA.
- Detection of hypofibrinogenemia (except dysfibrinogenemia). It must be noted that normal fibrinogen levels may be obtained in cases of dysfibrinogenemia. A clot-based assay in suspected dysfibrinogenemia cases must be performed.
- Identification of dysfibrinogenemia when used in conjunction with clot based assay.

Reference values: 150 - 400 mg/dl

IMMUNOGLOBULINS

Increase in levels of immunoglobulins suggest invason by an antigen and low levels of immunoglobulins suggest deficiency which may be due to protein loss, failure to synthesize immunoglobulins or may be due to a primary congenital disorder.

Quantia IgA: Turbidimetric immunoassay for determination of serum IgA

IgA occur as serum IgA and Secretory IgA. Approximately half of serum IgA is located intravascularly. IgA does not cross the placental barrier and hence is absent in fetal blood. Serum IgA activates complements via and alternative pathway.

Significant facts:

- Chronic inflammatory diseases of the gastrointestinal tract, including the liver may cause polyclonal increases in serum IgA.
- One fourth of IgA deficient patients have anti IgA antibodies and are at risk of severe anaphylactic reactions to blood transfusions.
- Approximately 10-15 % of all myeloma are of IgA type.

Suggested Applications

- Detection of IgA deficiency.
- Monitoring progress of IgA myeloma.
- Chronic infections of gastrointestinal tract and respiratory tract.
- Cases of anaphylactic transusion reactions.

Interim reference values: 70 - 400 mg/dl

Quantia IgG: Turbidimetric immunoassay for measurement of IgG

In primary infections IgG are usually secondary antibodies and in repeat infection with the same organism, primary antibodies. IgG antibodies contain the major antiviral, antibacterial and antitoxin antibodies. It is the only immunoglobulin class to cross the placenta.

Significant Facts:

- Deficiency of IgG is associated with frequent and occasionally severe pyogenic infections.
- The diagnosis of deficiency state is important because gammaglobulin therapy can be provided.
- Approximately 70% of all myeloma are of IgG class.

Suggested Applications

- Detection of IgG myeloma.
- Diagnosis of IgG deficiency.
- Assessment of the progression and response to treatment of IgG myeloma.

Interim reference values: 700 - 1600 mg/dl

Quantia IgM: Turbidimetric immunoassay for measurement of IgM

IgM are immunoglobulins of primary response. 75-80% of IgM are located intravascularly. IgM antibodies predominate in certain humoral immune responses such as antibodies to blood group antigens, antibodies to typhoid "O" antigens.

Significant Facts:

- IgM is an indicator of acute B-cell response. A prolonged increase indicates infection. Monoclonal increase of IgM is seen in Waldenstrom's macroglobulinemia.
- Virus specific IgM in cord blood or neonatal serum is likely due to congenital infection.

Suggested Applications

- Persons with frequent acute and chronic infections.
- Screening of congenital infection in neonates.
- Monitoring patients with Waldenstrom's macroglobulinemia.

Interim reference values: 40 - 230 mg/dl

Complement components

Abnormal serum levels of complemen proteins may be due to either inherited or acquired diseases. Deficiency in each of the complement components has some association with susceptibility to infections. Complement is important in clearance of immune complexes from the circulation. Early complement component (C1, C4, C2) deficiency leads to improper handling of immune complexes. As a result these immune complexes accumulate in tissues such as kidney.

Quantia C3: Turbidimetric immunoassay for measurement of C3

Significant facts:

C3 determinates the rate of both classical and the alternate pathways.

- Decreased levels of C3 are indicative of complement activation, decreased synthesis, protein loss or consumption.
- Deficiency of C3 is associated with recurrent infections.

Suggested Applications

- Detection of C3 deficiency.
- Detection and monitoring of immunocomplex disorders: lupus erythromatosus, vasculitis, glomerulonephritis, cryoglobulinemia, autoimmune hemolytic anemia, alternative complement activation in allergic granulomatous angitis (C3 reduced, C4 normal).

Interim reference values: 90 - 180 mg/dl

Quantia C4: Turbidimetric Immunoassay for measurement of C4

Significant Facts:

- Decreased levels of C4 indicate the presence of classical pathway activation or incomplete genetic deficiency.
- Congenital C4 deficiency is common in lupus erythromatosus.
- In hereditary angioneurotic edema C4 is reduced, C3 is normal.
- In classical pathway C4 often shows marked drop than C3.

Suggested Applications

- Detection of C4 deficiency.
- Detection and monitoring of immunocomplex disorders: Lupus erythromatosus, vasculitis, glomerulonephritis, cryoglobulinemia, autoimmune hemolytic anemia, alternative complement activation in allergic granulomatous angitis (C3 reduced, C4 normal).

Interim reference values: 10 - 40 mg/dl

Coagulation inhibitors

Quantia AT III: Turbidimetric immunoassay for measurement of Antithrombin III

Antithrombin previously known as antithrombn III is a proteinase inhibitor. It is the most important inhibor of plasma coagulation system. The action of AT III is directed against thrombin, Xa, and IX leading to an effective inhibition of coagulation.

Significant Facts:

- Shortage of AT III affects normal coagulation process and can lead to excessive blood clothing.
- AT III deficiency is found in 1 in 2000-5000 persons.
- Patients with AT III deficiency may have thromboembolic problems that begin in early adulthood.
- In type I AT deficiency, the AT concentration is reduced.
- In type II AT deficiency, the AT concentration is normal but some of it does not function properly.

Suggested Applications

- Detection of suspected congenital or acquired AT III deficiency.
- Monitoring of AT replacement therapy (however functional levels of AT III in plasma must be measured by clotting assays).
- Evaluating patients at risk of developing thromboembolic disease.
- In surgical patients receiving heparin.
- Assessment of thrombotic risk of contraceptive or estrogen therapy.
- Detection of type II AT deficiency in conjuction with clot based assay for antithrombin.

Interim reference values: 17 - 30 mg/dl

Quantia Protein C: Turbidimetric immunoassay for determination of Protein C

Protein C is a Vitamin K dependant protein. After activation by thrombin which is accelerated in the presence of thrombomodulin, protein C complexes with phospholipids and Protein S (PS) to degrade factors Va and VIIIa down regulating coagulation. A deficiency or decrease in activity of protein C or the inability to activate the coagulation accelerators is associated with a significantly increased risk of thrombosis.
Protein C is lower in newborns than in older children or adults. Protein C levels slowly rise from birth, achieving normal adult evels only at adolescence.

Significant facts:

- Heterozygous Protein C deficiency is found in 2-5% of patients with thromboembolic diseases.
- The prevalence of protein C deficiency is about 5-8% in young patients with venous thrombosis.
- Inclusion of Protein C testing in general thrombophilia evaluation is suggested in laboratory evaluation of hypercoagulability of venous and arterial thrombosis.
- Homozygous protein C deficiency though rare is a significant cause of neonatal venous thrombosis, usually presenting as purpura fulminans neonatorum.
- Protein C antigen assay is a useful tool for distinguishing types I from type II protein C deficiency.
- Lower protein C levels are found to be common in patients with sepsis and are associated with severely negative clinical outcomes including increased mortaity of shock, as well as increased duration of hospitalization.

Suggested Applications

- Recurrent thromboembolism and deep venous thrombosis of unknown etiology especially in patients less than 40 years of age and in the case of a positive medical history.
- Differential diagnostic investigation of hemostatic defects, eg. In conjunction with DIC, severe liver disease, coumarin induced necrosis, hormone therapy (estrogen replacement therapy, oral contraceptives) or after major surgery.
- For distinguishing type I from type II protein C deficiency in conjunction with clot based assays.

Reference values: PC concentration: 70 - 140 % (3 - 6mg/L)

Quantia Protein S: Turbidimetric immunoassay for determination of free and total protein S.

Protein S (PS) is a Vitamin K dependant protein, which acts as a co-factor for activated protein C (APC). In plasma, 60% of the PS is bound to C4b-binding protein (C4bBP) and does not possess any APC co-factor activity, however the bound PS does have some other anticoagulant activities. The remaining 40% PS is free and available to interact with APC.

Significant Facts:

- A decrease or deficiency of PS is associated with increased risk of thrombosis.
- The clinical manifestations of inherited protein S deficiency include cerebral venous thrombosis, portal venous thrombosis, and purpura fulminans in additon to deep venous thrombosis and pulmonary embolism.
- Though some thromboembolic events have been recorded in first five years of life, most thrombotic events commence around puberty in PS deficient subjects.

Suggested Applications

- Recurrent thromboembolism and deep venous thrombosis of unknown etiology especially in patients less than 40 years of age and in the case of a positive medical history.
- Differential diagnostic investigation of hemostatic defects, eg. In conjunction with DIC, severe liver disease, coumarin induced necrosis, hormone therapy (estrogen replacement therapy, oral contraceptives) or after major surgery.
- For differentiating types, I, II and III protein S deficiencies in conjunction with clot based assays.

Reference values

Total protein S: 70-140%
Free Protein S: 70-140%

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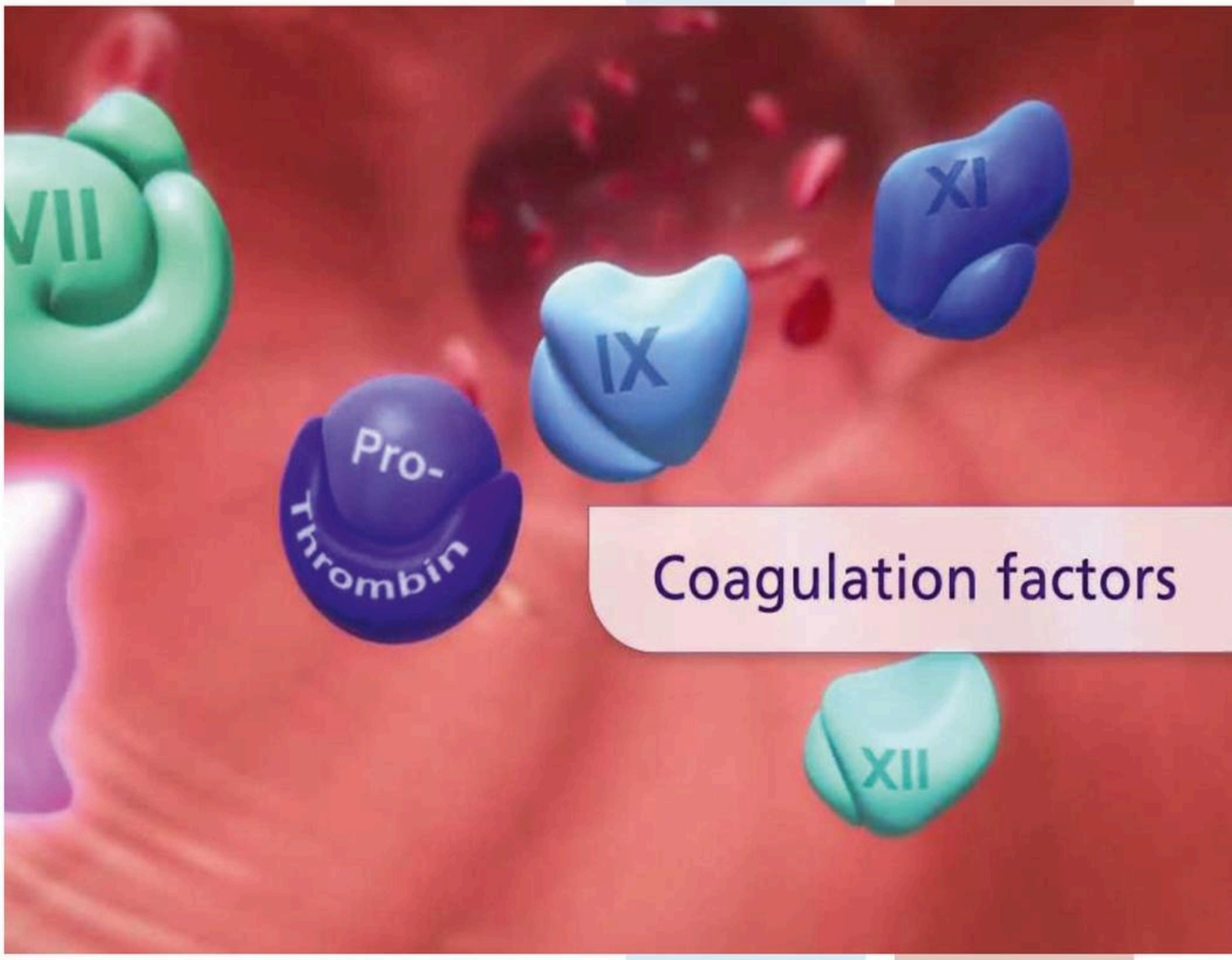
COAGULATION



- **Quality Assurance for routine Hemostasis Laboratory**..Pgs 52-57
- **Monitoring Oral Anticoagulant Therapy**
  - Concepts and Practices .....Pgs 58-66
- **Lupus Anticoagulants**
  - Basic Concepts and Laboratory Diagnosis .....Pgs 67-75

KNOWLEDGE BOOK

For the Laboratorians

Quality Assurance for routine Hemostasis Laboratory

Setting Trends...

Introduction

Coagulation tests for the routine assay parameters in laboratories are fairly simple to perform and master.

The performance of basic tests requires simple apparatus such as water bath, test tubes, pipettes and stop watch and / or automatic clot timer. And it's precisely for this reason that these techniques appear deceptively easy.

There are a number of pre test variables that effect the accuracy and precision of coagulation results. These may relate to collection techniques, processing of samples, selection and preparation of reagents. In order to achieve optimum and reproducible results the impact of variables needs to be understood and controlled so as to reduce variability and errors and improve accuracy and reproducibility. Various variables that impact the results are discussed, along with the basis that leads to such recommendation.

Preparation of patients

Although no special preparation of patients is required prior to approved techniques it is preferable that patients are not heavily exercised before blood collection. Fasting patients or patients on a light non-fatty meal are preferable.

- Patients who are fasting or on a light non-fatty meal prior to blood collection provide samples with desirable lower opacity this improves the sensitivity of clot detection especially when photo optical instruments are being used.

  Turbid, icteric, lipemic or grossly haemolysed samples generate erroneous results due to varying opacity.

Sample Collection techniques

(Phlebotomy)

- Blood should be withdrawn without undue venous stasis and without frothing into a plastic syringe with a short needle of 19 to 20 SWG.
- The venepuncture must be a 'clean' one and incase of difficulty with a new syringe and needle another vein should be tried. The tourniquet should not be placed too tightly or for extended lengths of time. Patting the venepuncture site should also be avoided.
- Distribute blood into test tubes (preferably plastic) after detaching the needle from the syringe. Do not delay mixing blood with anticoagulant by gentle inversion of the tube.
  - 'Clean' venepuncture is essential to avoid formation of microclots at the site of venepuncture and consumption of factors, which will lead to artificially prolonged results.
  - Usage of short bigger bore needle allows free flow of blood within the syringe and reducing blood contact with metal surface. With smaller bore longer needles blood will remain in contact with metal surface for longer time. This will lead to initiation of clotting or partial consumption of factors being assayed leading to erroneous results during test procedures.
  - Frothing when distributing the blood into anticoagulant tube should be avoided because frothing induces microclot formation.

Sample Preparation

- The anticoagulant of choice for most coagulation procedures is sodium citrate or preferably buffered sodium citrate.
  - Sodium citrate is an ideal anticoagulant since Factor V and Factor VII are more stable in citrate. These factors are more labile in sodium oxalate. Heparin neutralizes thrombins action on fibrinogen.
- The recommended molarity of sodium citrate for coagulation studies is 0.109M, which equates to 3.2% of tri-sodium citrate.
- Use of buffered sodium citrate is preferred over plain sodium citrate solution.
  - After collection of blood in citrate during centrifugation for preparation of PPP or PFP the pH of the solution shifts releasing carbondioxide (CO_2). This shift in pH affects the labile Factor V leading to erroneous results during test. Use of appropriately formulated buffered citrate overcomes this phenomenon.
  - When samples are collected in 3.8% citrate (129Mm) the prothrombin time of samples, especially with patients receiving oral anticoagulants give prolonged results. Also the ISI of thromboplastins is lowered. It is for this reason 3.2% citrate is recommended universally instead of 3.8% for increasing accuracy of test results.

- The optimum ratio of citrate to blood is 1 part of anticoagulant to 9 parts of blood.
  - When the molarity of citrate is accurate the anticoagulant supplied in this amount and ratio is sufficient to bind all the available calcium in the collected sample so as to prevent clotting. A shift in this ratio leads to erroneous results as follows:
  - More blood less citrate: The chelating activity of citrate will not be sufficient to bind the calcium present in the sample. This will lead to formation of clots, consumption of factors and subsequent prolongation of results during test.
  - More citrate less blood: Excess citrate remaining in the blood sample would consume the calcium from the reagents thereby giving prolonged results.

- The optimum concentration of Calcium chloride to be used for APTT test should be 0.02M.
  - The concentration of 0.02M $CaCl_2$ replaces the calcium necessary to activate the intrinsic coagulation cascade. This ultimately generates thrombin from prothrombin via the coagulation cascade.
  - Appropriate volumes of $CaCl_2$ should be aspirated for the days work. Prewarmed $CaCl_2$ should always be discarded at the end of the working day.

- The standard ratio of blood to anticoagulant of 9:1 is for normal haematocrit or PCV
  - For occasional patients with PCV less than 20% (e.g.: Microcytic hypochromic anemia) and greater than 55% (e.g.:Polycythemia vera) the anticoagulant to blood ratio must be readjusted using the following formula,

$$C = 1.85 \times 10\text{-}3\ (100\text{-}H)\ V$$

C = Volume of sodium citrate in ml.
V = Volume of whole blood - Sodium citrate in ml
H = Hematocrit in percentage

  - When the PCV is higher than 55% the patient blood contains so little plasma that excess unutilized anticoagulant remains and is available to bind reagent calcium leading to prolongation of test results.
  - On the other hand if the PCV is less than 20% the patient blood contains excess of plasma but less of anticoagulant and the chelating activity of citrate will not be sufficient to bind the calcium present in sample. This will lead to formation of clots invitro, consumption of factors and prolongation of results.

Sample Processing and Storage

- Containers for collection and processing of plasma should be ideally made out of plastic or siliconized glass tubes. They should be scrupulously clean and dry.
  - The containers should be ideally made out of plastic and not from glass as scratched glass surfaces can activate invitro the coagulation mechanism within the sample due to contact with silica. While plastic tubes overcome this problem they should be free from leavening chemicals used by the plastic industry during moulding. These chemicals usually have an inhibitory effect. Scrupulous washing and drying overcomes this problem.
  - All the containers used for collection, storage and test should be free from detergents, acids and alkalies. These chemicals have a varying effect on pH. Change in pH affects factor stability. Detergents inhibit reactive characteristics of the Sample/ Reagent mixture.
  - Ideally the cleaning of glassware used in coagulation tests should be the responsibility of one individual and should be handled separately from routine laboratory glassware. Alternatively disposable labware should be used.

- The specimen to be tested for coagulation studies must be used preferably immediately.
  - As most of coagulation factors are time as well as temperature labile it is of utmost importance that they should not be subjected to high temperatures and tests be performed as early as possible, preferably immediately.
  - If specimen are held at 22°C-24°C then they must be tested within 2 hours and if the specimen are held at 2°C-4°C then they must be tested within 3 hours.
  - Plasma samples held at 4°C-8°C for prolonged periods may undergo cold activation leading to erroneous results.
  - Samples obtained for factor assays and tests for fibrinolysis should be stored in crushed ice if a delay in testing is anticipated.
  - Citrated blood for platelet aggregation studies should remain in capped tubes at R.T. (20°C-25°C) before testing

- The samples collected must be stored tightly capped.
  - The samples collected must be stored tightly capped. If the tubes are not capped the samples will absorb atmospheric CO_2 leading to shift in pH to an unacceptable range. This hampers factor stability and accuracy of results.

- Centrifugation speed and time are of absolute importance in coagulation studies. The PT test uses PPP while the APTT test uses PFP.
  - Excessive centrifugation may destroy clotting factors due to the heat generated during centrifugation.
  - Under centrifugation would lead to the presence of platelets in the plasma sample which could lead to activation of clotting mechanism invitro which leads to erroneous results.
  - Normally centrifugation for 15 minutes at approximately 1500g yields PPP (platelet poor plasma) and centrifugation at approximately 2000g for 15 minutes yields PFP (platelet free plasma). The 'g' is a function of length of rotor head and RPM. It is for this reason each laboratory must calibrate its own equipment to achieve satisfactory samples depending on test performed and kind of plasma sample required.

Calibration of instruments / equipments

- Waterbaths or heating blocks calibrated and preset at 37°C + 0.5°C are an important requirement to achieve accuracy and reproducibility.
  - The whole process of the coagulation tests is based on a series of enzymatic reactions which are dependent on pH, ionic strength, and the temperature of the reaction process. A correct temperature at 37°C + 0.5°C is critical as most of the reagent systems are standardized at this temperature. Day to day shift in reaction temperature of equipment will introduce uncontrolled variation into test conditions.Therefore temperature of all equipments must be calibrated daily and diligently to avoid erroneous results and ensure accuracy and reproducibility.

- Sample/reagent dispensing mechanisms must be accurate and precise
  - Well-calibrated dispensing mechanisms are required for all coagulation-based tests to accurately dispense samples as well as reagents. Any shift in ratio or individual volumes of the sample and or reagent can lead to shortening or prolongation of results.
  - Straight 0.1 and 0.2ml glass pipettes are usually satisfactory, provided they are scrupulously clean and dry.
  - Automatic micro pipettes which are able to deliver the required volumes are replacing the glass pipettes, provided these pipettes are calibrated frequently. The use of clean disposable tips places this system at an advantage over the older mechanisms.

Storage of Reagents

- Usually reagent manufacturers recommend aspiration of adequate reagent for the days use in a thoroughly clean and dry tube instead of intermittent aspiration from reagent vials at the time of test.
  - Most coagulation reagents are extremely delicate reagents.
    For them to maintain their sensitivity and performance the reagent formulations must maintain reagent integrity over the usage period. Repeated intrusions into the reagent vial exponentially increases the chances of reagent contamination and destruction of reagent formulations and integrity. Undried and or contaminated pipettes, tips, glassware are usually the main culprits. Such contaminated reagents perform suboptimally.
  - The reagent vials must be immediately stored back to the recommended storage temperatures after the aspiration of the days requirement separately so that the remaining reagent remains at optimal temperature for future use. Keeping unused reagents at higher ambient temperatures during the day causes steady deterioration of the reagent due to thermal stress.

- The recommended storage temperature for reagents should be strictly complied to.
  - Most of the liquid stable or reconstituted reagents such as PT and APTT are colloidal suspensions of lipoproteins and or phospholipids. Subjecting them to elevated temperatures through repeated freeze-thaw cycles stresses the colloidal system. Especially detrimental are the effects of freezing (below 2°C). After freezing the reagent colloidal suspension undergoes an irreversible change and precipitates out or presents itself as a particulate mass. Such reagents give erroneous results.

- Bringing reagents/samples to room temperature should be a two step process.
  - When enough reagent is aspirated out for the days testing as recommended the reagent and samples stored at 2-8°C should be first allowed to attain room temperature (25°C-30°C) and then they should be subsequently brought to the optimal test temperature of 37°C + 0.5°C.
  - When reagents/samples from 2-8°C are directly brought to 37°C the required time of 3-5 minutes may not be sufficient for the reagent/samples to attain a homogeneous temperature of 37°C within the recommended time. This affects the reaction kinetics leading to erroneous results.

End Point Reading

- Reading of endpoint of clot based tests varies from user to user.
  - Usually when manual techniques are followed the definition of "end point" is important. Ideally the end point tests should be read "as soon as the first fibrin strand is visible and the gel clot formation begins".
  - When some users use a fully formed gel clot as an end point, there is a variation of 1-3 seconds between the end points as recorded by the ideal method and user based variation.
  - It is advisable to have well illuminated background for reading the clot based end points. Since user variations based on proficiency continue to influence results, it is advisable not to change personnel involved in coagulation tests off and on.

- Manufacturers instructions must be followed meticulously when instrument based or automatic clot detection systems are used.
  - Each clot detection system works on a different principle such as electro mechanical, turbidimetric or photo-optical. Each system of clot detection has its requirements for optimum functioning. Special care must be taken while using optical instruments for clot detection since reagent induced turbidity can influence the results dramatically. Usually low turbidity reagents are preferred for manual as well as instrument based clot detection.

Drugs/Clinical Conditions influencing patient results

- Drugs/clinical conditions influence results of patients coagulation studies
  - PT tests are influenced on administration of following drugs

PT may be shortened	PT may be Prolonged
DRUGS ● Antihistamines ● Butabarbital ● Phenobarbital ● Caffeine ● Oral contraceptives ● Vitamin K	**DRUGS** ● Corticosteroids ● EDTA ● Asparaginase ● Clofibrate ● Erythromycin ● Ethanol ● Tetracycline ● Aspirin ● Anticoagulants such as warfarin, heparin

- APTT tests are influenced on administration of following drugs

APTT may be shortened	APTT may be Prolonged
DRUGS ● Oral contraceptives ● Conjugated estrogen therapy	**DRUGS** ● Diphenylhydantoin ● Heparin ● Warfarin ● Naloxone ● Radiographic agents

- Thrombin Time Test is prolonged in the following clinical conditions

CLINICAL CONDITIONS	
● Normal newborn infant ● Systemic lupus erythematosus ● Macroglobulinemia ● Presence of exogenous/ endogenous circulating anticoagulants	● Hepatic diseases ● Toxemia of pregnancy ● Multiple myeloma

MNPT and INR

- MNPT is a critical requirement in the derivation of INR
  - MNPT is a critical requirement in the derivation of INR. Ideally each laboratory must derive its own MNPT from 20 or more normal patients for a given PT reagent and Lot under use. This corrects within laboratory test variables that influence PT results.

By definition INR represents the PT ratio which would have been obtained for a particular patient sample as if the WHO reference thromboplastin itself (ISI=1.0) had been used in the PT determination.

$$INR = [R]^{ISI}$$

$$INR = \left[\frac{\text{Patient PT in seconds}}{\text{Mean of the normal range}}\right]^{ISI}$$

A PT ratio is obtained by dividing the patient PT in seconds by the **"Mean of the normal range"** (MNPT). This ratio is then "normalized" by raising the results to the power of the ISI of the PT reagent used.

If "normal control plasmas" are used in place of patient plasma for arriving at the MNPT it can effect the evaluation of the patients level of anti coagulation.

For eg:

Reagent ISI=2.5	Test Day 1	Test Day 2	Test Day 3
Patient PT (sec)	16.0	16.0	16.0
Normal Control (10.4 - 12.3 sec)	11.5	10.4	12.3
INR Formula $[R]^{ISI}$	$\left[\frac{16}{11.5}\right]^{2.5}$	$\left[\frac{16}{10.4}\right]^{2.5}$	$\left[\frac{16}{12.3}\right]^{2.5}$
Resulting INR	2.27	2.89	1.92

If the control time is greater than the mean normal range (MNPT), the PT ratio for any patient PT will be smaller, potentially leading to over coagulation. If the control time is lesser than MNPT the ratio for any patient PT will be greater, leading to under coagulation.

On the other hand MNPT for a particular laboratory using the same combination of methodology, reagent and instrument would remain constant.

Quality Control Aspects

- Quality of water used for reconstituting lyophilized coagulation reagents must be good.
  - The water used for reconstitution of lyophilized coagulation reagents should be atleast distilled twice and kept separately labelled for "coagulation studies". The reagents employed for coagulation studies are extremely delicate and inability to use good quality distilled water could lead to incorporation of metallic impurities in the reagent formulation as well as change in pH.
    Such changes can alter reaction kinetics and overall stability and performance of reagents.

- Quality assurance for coagulation based reagents must be performed preferably on a daily basis.
  - Each laboratory should test coagulation reagents with normal and abnormal control plasma specimens at the beginning of each days work to verify instruments, temperature calibration and also reagent performance.
    If the control results fall within the stated limits, the test results are considered valid.
    But if the results fall outside the stated control limits then the reagents, control and equipments are checked and the problem should be corrected.
    Control results should be recorded and analyzed after regular intervals to ascertain the long term validity of results.

References and Suggested Readings

1. Human Blood Coagulation, Haemostasis and Thrombosis, Edited by R Biggs, Blackwell Scientific, First Edition, 1972.
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3. Practical Haematology, Sir John Dacie & S.M.Lewis, Churchill Livingstone, Eigth Edition, 1995.
4. WHO Expert Committee On Biological Standardisation, 33rd Report, Technical Report Series 687, WHO Geneva.
5. Effect of Concentration of Tri sodium Citrate anticoagulant on Calculation of the INR & ISI of thromboplastin; Duncan EM, Casey CR, Duncan BM, Lloyd JY, Thrombosis Haemostasis, 1994 July; 72(1): 84-88.

KNOWLEDGE BOOK

For the Laboratorians

Monitoring Oral Anticoagulant Therapy

Concepts and Practice

Setting Trends...

Introduction

Since the introduction of the one stage prothrombin time test by Dr. Armand Quick in 1935, there have been various attempts to standardize the methodology and reporting of results using the Prothrombin Time reagents.

Oral anticoagulant therapy with 4-hydroxy coumarin vitamin K antagonists, such as Warfarin are commonly used for the treatment and prevention of thromboembolic episodes. Most physicians also prefer to monitor the oral anticoagulant therapy using the Prothrombin time test.

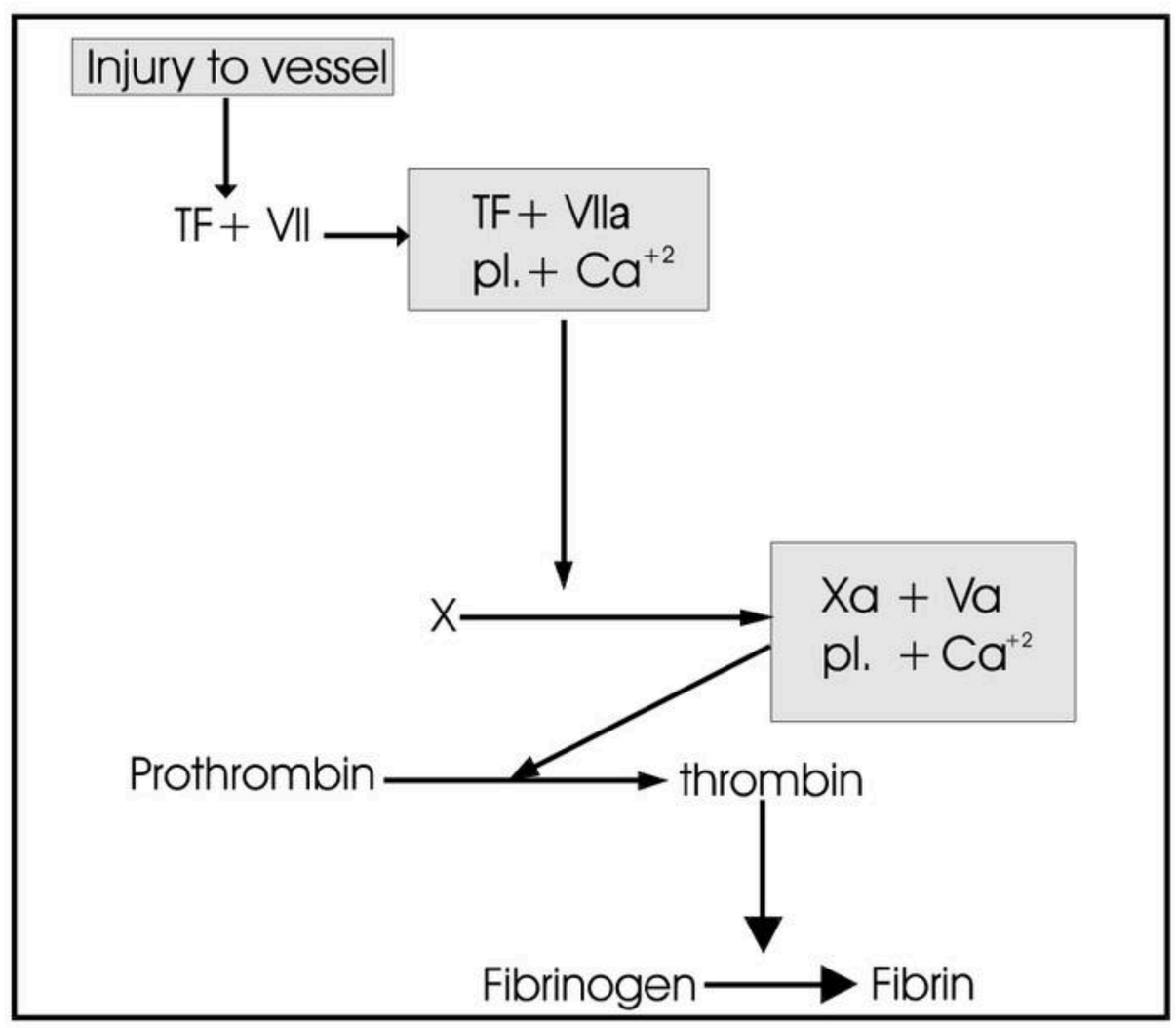
Since the individual thromboplastin reagent preparations differ significantly in their sensitivities to the deficiencies of the vitamin K dependent coagulation factors, the use of 'seconds' or 'PTR' (Ratio) as a reporting format has led to confusion amongst clinicians. In order to correctly dose deserving patients with oral anticoagulant drugs so as to achieve the dual goal of adequate anti-coagulation and reduce the risk of bleeding simultaneously, the WHO introduced a method for monitoring patients stabilized on oral anticoagulant therapy using International Normalized Ratio (INR).

The INR normalizes the PT ratio to an International reference preparation of thromboplastin. The therapeutic ranges expressed in terms of INR values for common clinical conditions requiring oral anticoagulant treatment and monitoring have been determined by double blind clinical trials.

The INR method of reporting PT results has helped to ensure that a uniform intensity of oral anticoagulant therapy is used world wide, independent of the influences of varying sensitivity of the thromboplastins used for obtaining the test result using PT reagents.

Relevance and use of the Prothrombin time test

The prothrombin time test is used to detect coagulation disorders and specifically the activity of the factors of the extrinsic pathway namely II, V, VII,X and Fibrinogen. The classical extrinsic pathway is initiated by the release of tissue thromboplastin (Tissue Factor) which is exposed to the blood when there is a damage to the blood vessel. Circulating factor VII forms a complex with the tissue thromboplastin and calcium in the presence of phospholipids. This complex activates factor X to Xa. Factor Xa catalyses prothrombin (Factor II) to thrombin (IIa). Thrombin converts fibrinogen (Factor I) to Fibrin.



Classical Extrinsic Pathway.

The factors of the extrinsic pathway namely II, V, VII and X are synthesized in the liver parenchymal cells from precursor proteins. These classical vitamin K dependent plasma clotting factors undergo a conformational change in the presence of Ca^{+2} ions. This is a necessary requirement for these vitamin K dependent factors (II, V, VII, X) for binding to their co-factors on phospholipid surfaces and exerting their coagulant activity.

Within the liver parenchymal cells the inactive precursor proteins of three key vitamin K dependent factors i.e. VII, IX and X are carboxylated. These carboxylated proteins then function as active factors with coagulant activity .

Oral Anticoagulant Agents

The clinical effectiveness of Oral anticoagulants has been well established based on results of numerous well designed trials for a variety of indications. Oral anticoagulants are effective and used for:

- Prevention of primary and secondary venous thromboembolism.
- Prevention of systemic arterial embolism in patients with mechanical prosthetic heart valves or with atrial fibrillation.
- Prevention of acute myocardial infarction in patients with peripheral arterial disease.
- Prevention of stroke and recurrent infarction.

Warfarin Sodium a 4-hydroxy coumarin derivative is the most common vitamin K antagonist used for oral anticoagulant therapy.

Acenocoumarol, phenprocoumon are the other derivatives, which are also used for achieving oral anticoagulation in patient groups. The use of indandione derivatives such as phenindione has been discontinued largely due to their relative hepatic toxicity.

All the 4-hydroxy coumarin derivatives, as far as known today, have the same mode of action, but exhibit different pharmacokinetics and are metabolized differently.

Pharmacology of Oral Anticoagulants

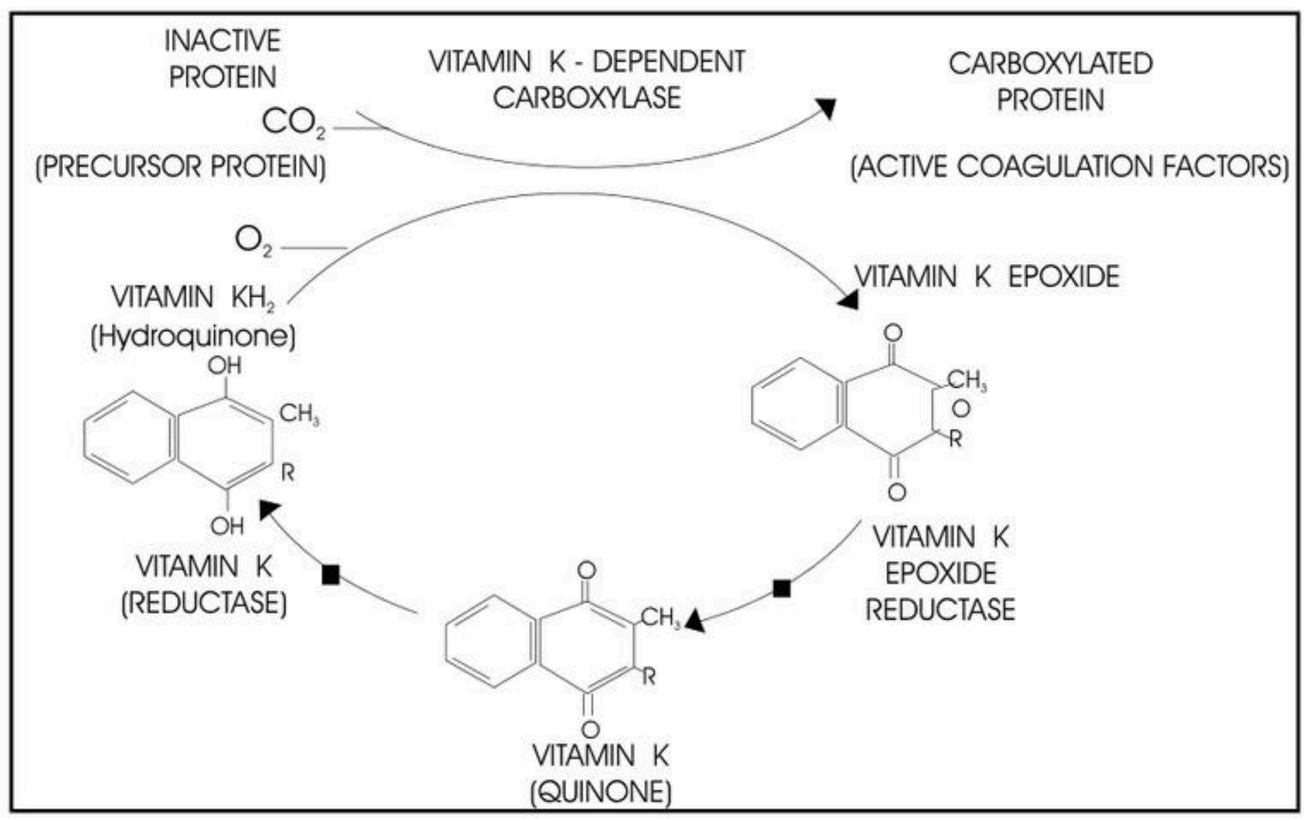
The oral anticoagulants such as Warfarin inhibit the process of carboxylation by interfering in the inter conversion of vitamin K epoxide.

This inhibition results in a decreased ability of the liver to effectively carry out posttranslational conversion of glutamyl residues (glu) on the inactive precursor proteins to g-carboxyglutamyl (gla) residues. The gla residues impart these proteins the ability to exert their coagulant action (as factors) in a Ca+2 dependent fashion.

It is now known that the fully functional carboxylated proteins i.e. factors, contain a normal complement of 10 to 13 gla residues.

It has been estimated that when the number of gla residues on the carboxylated proteins reduce from 10 - 13 to 9, it results in a 30% reduction in their coagulant activity, while reduction to less than 6 residues results in a loss of more than 95% of the coagulant activity of the factors.

This inhibition in the cyclic conversion of vitamin K also results in the production and secretion of proteins that are partially carboxylated or des-carboxylated. These are dysfunctional factors.

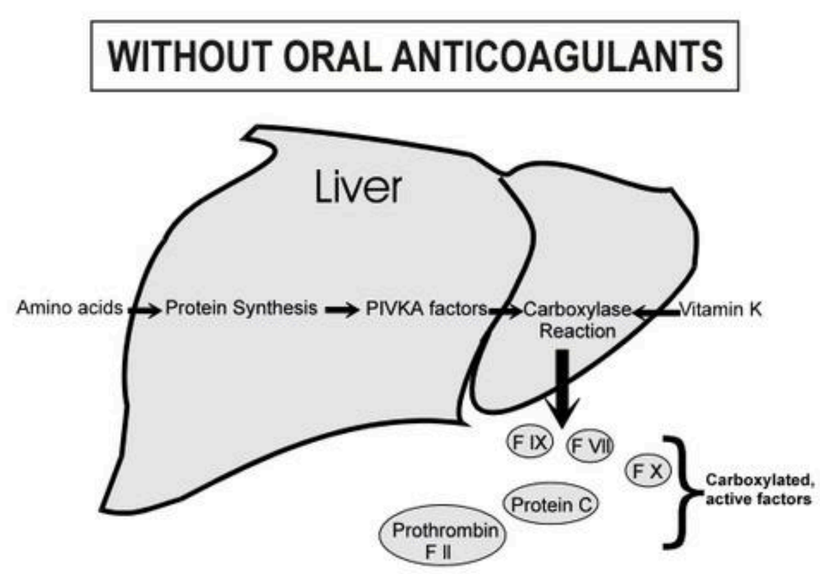


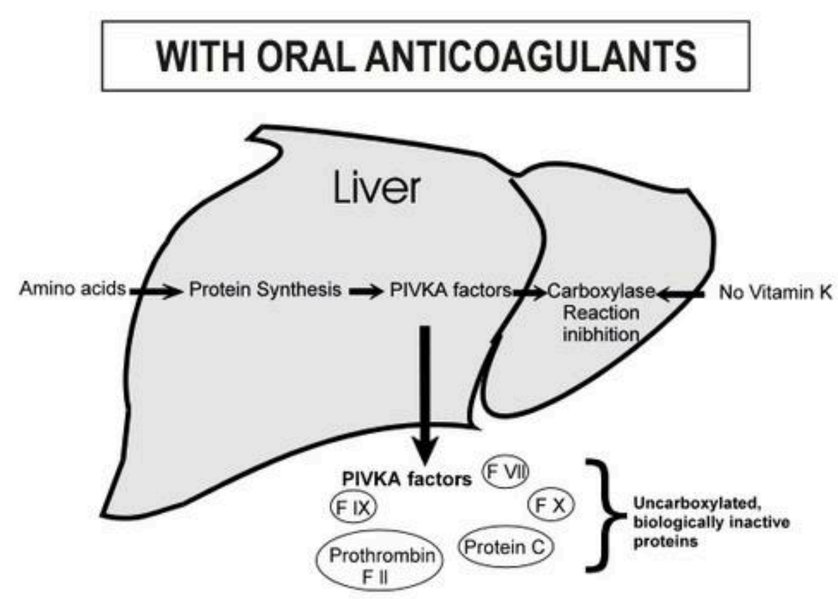
■ **INHIBITION OF CARBOXYLATION BY ORAL ANTICOAGULANTS**

PIVKA'S

As the effect of oral anticoagulants take effect, the process of conversion of the precursor proteins to active factors through carboxylation is inhibited. The partially carboxylated or des-carboxylated, biologically inactive proteins appear in plasma. On the other hand the plasma concentration of biologically active factors VII, IX, X decreases.

This pool of partially carboxylated proteins are as a group known as PIVKA's. (Protein induced by vitamin K absence / or antagonism). Since PIVKA's are biologically inactive, they do not participate in the haemostatic control mechanism in vivo.





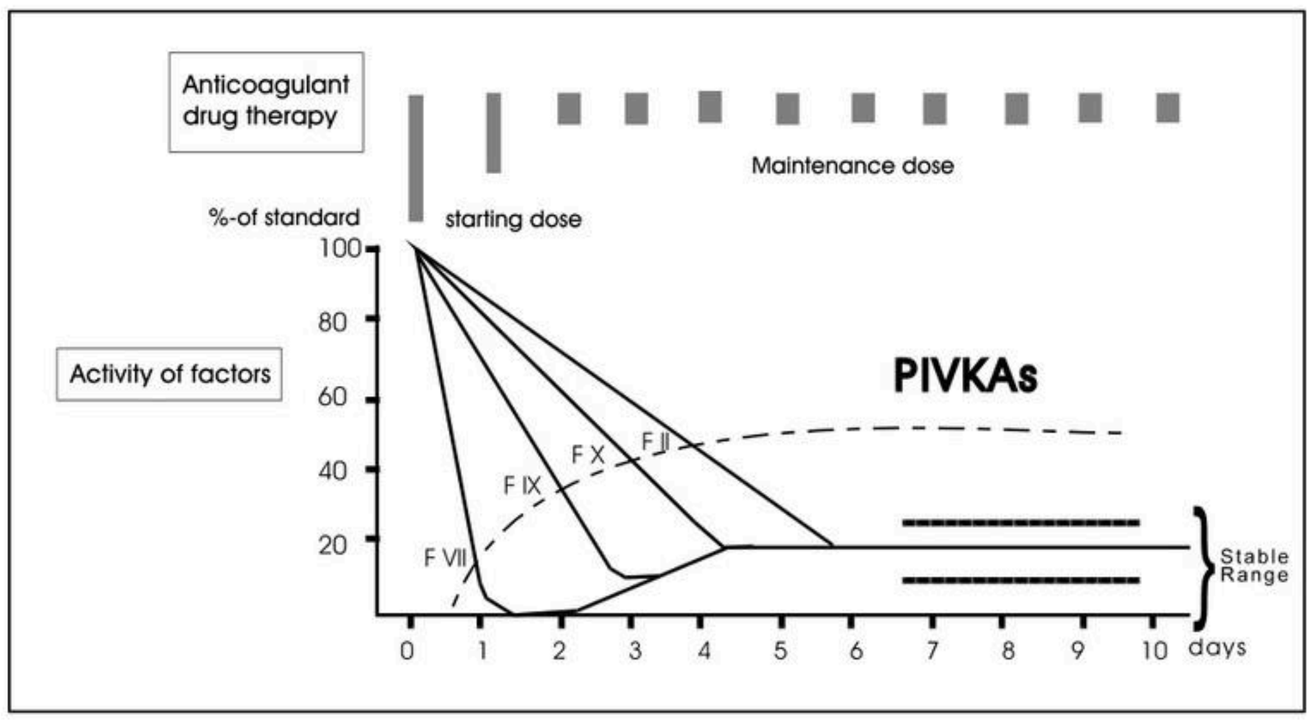
PIVKA'S also do not play a role in bleeding complications of anticoagulated patients.

However the most important practical consequence of the PIVKA effect is that it is an important confounding factor in the standardization of thromboplastins used for monitoring oral anticoagulant therapy.

PT reagents derived from rabbit or human tissues or from recombinant technology have varying sensitivity to PIVKA's.

The role of PT test in monitoring effects of oral anticoagulant therapy

The PT is the method of choice for monitoring oral anticoagulant therapy, as it is responsive to the depression of the vitamin K dependent clotting factors of the extrinsic pathway. Rate of depression of the individual clotting factors is determined by their biological half life. Factor VII falls the most rapidly followed by factors IX, X and II. As the active clotting factor levels begin to fall, the PT results start to prolong. Factor V and Fibrinogen are not affected by oral anticoagulants.



Course of Anticoagulant therapy

On the other hand upon withdrawal of the oral anticoagulant therapy (as may be required to review the effects of over anticoagulation) these factors return to normal levels in the reverse order and so do the PT results.

Factors Influencing Anticoagulant Effects of Warfarin

GUT

Anticoagulant Effect Potentiated
- Low vitamin K intake
- Reduced vitamin K absorption in fat malabsorption

Anticoagulant Effect Counteracted
- Increased vitamin K intake
- Reduced absorption of warfarin by cholestyramine

PLASMA

Anticoagulant Effect Unchanged
- Displacement of warfarin from albumin binding does not influence anticoagulant effect of coumarins

HAEMOSTATIC PLUG

Impaired Haemostatic Plug Formation
Impaired Coagulation:
- Reduced vitamin K dependent coagulation factors
- Reduction in concentration of other coagulation factors
- Other anticoagulants (heparin, ancrod)

Impaired Platelet Function:
- Thrombocytopenia
- Aspirin
- Other nonsteroidal anti inflammatory drugs
- Ticlopidine
- Moxalactam
- Carbencicillin and high doses of other penicillins

LIVER

Anticoagulant Effect Potentiated
- Drugs:

Phenylbutazone
Metronidazole
Sulfinpyrazone
Trimethoprim
Sulfamethoxazole
Disulfiram
Amiodarone
Erythromycin
Anabolic steroids
Clofibrate
Cimetidine
Omeprazle
Thyroxine Ketoconazole
Fluconazole
Isoniazid
Piroxicam
Tamoxifen
Quinidine
Vitamin E
(megadoses)
Phenytoin
Liver disease

Hypermetabolic States:
- Pyrexia
- Thyrotoxicosis

Anticoagulant Effect Counteracted
- Drugs:

Barbiturates
Rifampicin
Griseofulvin
Carbamazepine
Penicillin
Alcohol

Problems in monitoring Oral Anticoagulant therapy

Historically the Prothrombin time (PT) results are reported in "seconds" or as a percentage of coagulant activity or as ratio (PTR).
Physicians use sufficient oral anticoagulant drugs to maintain the PT ratio at 1.5 to 2.0 or 1.3-2.0 times the median value of the normal range. The problem with this approach is that it fails to take into account the variation in sensitivity or "responsiveness" of different PT reagents.

PT ratio (PTR)

For example: For a given reduction in the plasma levels of the clotting factors, more responsive PT reagents (A) results in greater prolongation of the PT in 'seconds' as compared to an unresponsive PT reagent (B).
Practically this translates into different PT times (seconds) or ratios for the same patient sample, when tested using reagent A or B.

For e.g.:

Lab.	Reagent	FNP Normal	Test	PTR	Therapeutic ratio
X	Reagent A	12.0	28	2.3	1.5-2.0
Y	Reagent B	12.0	18	1.5	1.5-2.0

This patient appears over anticoagulated when tested with reagent (A) and just about adequately anti coagulated when tested with reagent (B).

% Activity

The other method of reporting PT results is as a percentage of coagulation activity. First a dilution of normal plasma is made. Then the normal dilution curve is plotted using these dilutions. Then the patients clot time is converted to % activity of factors from the dilution curve.

Since the dilutions of normal plasma do not contain PIVKAS and Thromboplastins vary in their sensitivity to PIVKAS, the accuracy of the normal dilution curve has been rightly challenged.

Thromboplastins based on their tissue origin and method of preparation vary markedly in their responsiveness to depression of vitamin K dependent factors and PIVKAS. Therefore PT results reported using different reagents are not interchangeable between laboratories when the older system such as seconds, ratios or percentage activity are used to report PT results.

Standardization of Prothrombin time Reagents & Reporting System

In view of the aforesaid variations in the reagent sensitivities an attempt to standardize PT test results was initiated internationally. By mid 1970 a number of regulatory and advisory groups such as WHO (World Health Organization), ICTH (International Committee for Thrombosis and Haemostasis) and ICSH (International Committee for Standardization in Haematology) started work towards standardization of PT for monitoring oral anticoagulant therapy.

The first step was the preparation of IRP's (International Reference Preparations) of thromboplastins by the WHO. This was done to have an accurate reference reagent which could serve as a standard for calibrating responsiveness / sensitivity of commercial thromboplastin reagents.

The various IRP's were made from thromboplastins derived from various animal tissues in use for manufacture of commercial thromboplastin (PT) reagents. This was done in order to have more precise, like to like comparison.

Originally the following IRP's were made available:-

IRP's Tissue source	Code	Assigned ISI
Thromboplastin (Human combined)	67/40	1.0
Thromboplastin (Bovine combined)	68/434	1.0

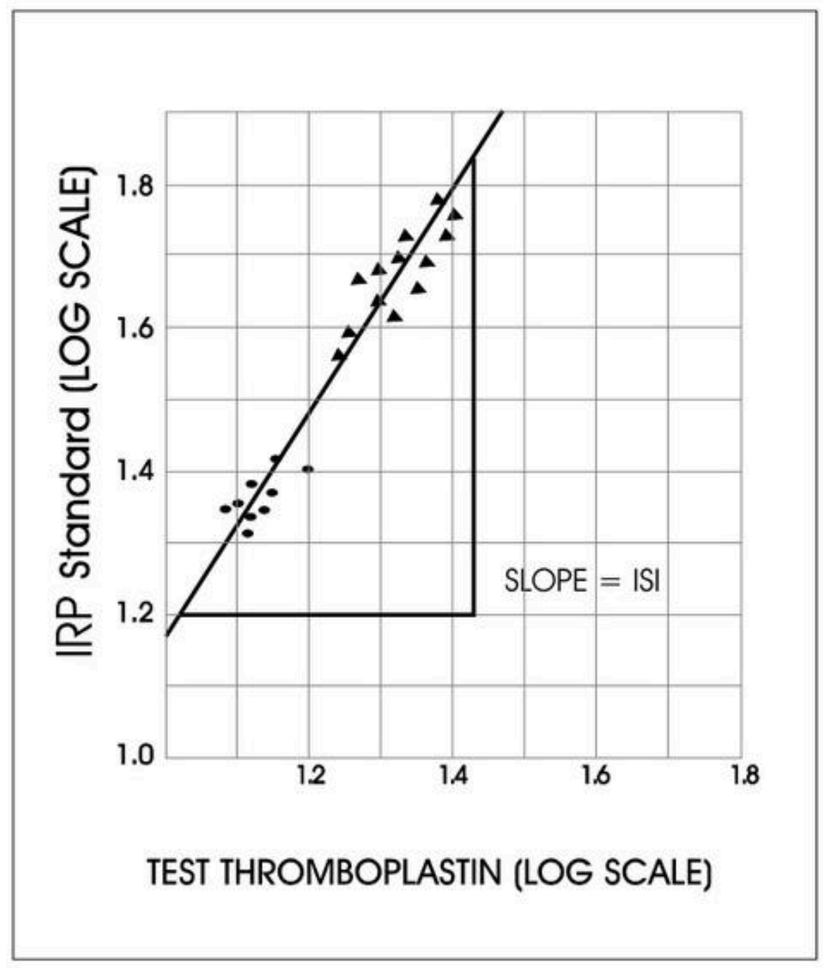
Subsequently after the stocks of primary IRP's were exhausted secondary IRP's have been made available under other code numbers. Till date however IRP's for recombinant thromboplastins are not available for primary calibration.

Basis of Calibration

This calibration system of individual commercial preparations vs. the reference preparations (IRP's) is based on the fact that when the commercial preparations of thromboplastin (test thromboplastin) and the reference thromboplastin (IRP's) of same tissue origin are tested on the same plasma samples, a linear relationship would exist between log of PT ratios obtained with reference and test thromboplastins.

Log of PT of reference thromboplastin is plotted on the Y-axis and the log of test PT are plotted on the X axis respectively on a log/ log paper.

The slope of the calibration line would then indicate the sensitivity of the test thromboplastin in comparison to the IRP.



▲ Samples from patients stabilised on oral anticoagulant for minimum 6 weeks.
● Normal patient population samples.

Calibration of Test Thromboplastin vs. WHO IRP

The ISI (International Sensitivity Index) indicates the sensitivity of test thromboplastin in comparison to the international reference preparation. When the calibration line has a slope =1.0, the test thromboplastin equates to the IRP in sensitivity or responsiveness.

The ISI in practical terms is a measure of the responsiveness (prolongation of PT values) of a given thromboplastin to the reduction of the vitamin K dependent coagulation factors. The lower the ISI (more close it is to 1.0) the more sensitive is the thromboplastin.

The recommended calibration methodology includes normal plasma samples as well as plasma from patients on oral anticoagulant therapy containing PIVKAS. Hence the assignment of sensitivity also corrects for thromboplastin sensitivity to PIVKAS.

This method for standardization of PT reagent sensitivity based on "ISI" was the first step towards eliminating reagent based variability in expression of PT results.

Manufacturers assign ISI value for each lot of thromboplastin by the WHO recommended methods to assist laboratorians in calculation of the INR (International Normalized Ratio).

The INR method of reporting results

By definition INR represents the PT ratio which would have been obtained for a particular patient sample as if the WHO reference thromboplastin itself (ISI=1.0) had been used in the PT determination.

$$INR = [R]^{ISI}$$

$$INR = \left[\frac{\text{Patient PT in seconds}}{\text{Mean of the normal range}}\right]^{ISI}$$

A PT ratio is obtained by dividing the patient PT in seconds by the **"Mean of the normal range"** (MNPT). This ratio is then "normalized" by raising the results to the power of the ISI of the PT reagent used.

Lower the ISI of the reagent used, closer will be the INR to the observed PT ratio.
Ideally when the ISI of the reagent is 1.0 then the INR is a simple PT ratio since $(R)^{1.0} = R$.
Currently many coagulation instruments are available that can perform this exponential calculation by entering the ISI of the reagent in use.

Alternatively a table is provided by reagent manufacturers for reading off "INR" directly for the given patient PT ratio, corresponding to the ISI value of the reagent used for e.g.

INR Conversion Table

PT RATIO	ISI 1.0	1.05	1.10	1.15	1.20	1.25	1.29
1.0	1.0	1.00	1.10	1.00	1.00	1.00	1.00
1.1	1.10	1.11	1.11	1.12	1.12	1.13	1.13
1.2	1.20	1.21	1.22	1.23	1.24	1.26	1.27
1.3	1.30	1.32	1.33	1.35	1.37	1.39	1.40
1.4	1.40	1.42	1.45	1.47	1.50	1.52	1.54
1.5	1.50	1.53	1.56	1.59	1.63	1.66	1.69
1.6	1.60	1.64	1.68	1.72	1.76	1.80	1.83
1.7	1.70	1.75	1.79	1.84	1.89	1.94	1.98
1.8	1.80	1.85	1.91	1.97	2.02	2.08	2.13
1.9	1.90	1.96	2.03	2.09	2.16	2.23	2.29
2.0	2.00	2.07	2.14	2.22	2.30	2.38	2.45
2.1	2.10	2.18	2.26	2.35	2.44	2.54	2.60
2.2	2.20	2.29	2.38	2.48	2.58	2.68	2.77
2.3	2.30	2.40	2.50	2.61	2.72	2.83	2.93
2.4	2.40	2.51	2.62	2.74	2.86	2.99	3.09
2.5	2.50	2.62	2.74	2.87	3.00	3.14	3.26

Recommended therapeutic ranges for oral anticoagulant therapy

Indications	INR	Intensity
• Prophylaxis of venous thrombosis (high risk surgery) • Treatment of venous thrombosis • Treatment of pulmonary embolism • Prevention of systemic embolism - Tissue heart valves - Acute myocardial infarction (to prevent systemic embolism) - Valvular heart disease • Atrial fibrillation	2.0 - 3.0	
• Mechanical prosthetic valves (high risk) • Prevention of recurrent myocardial infarction	2.5 - 3.5	

Other Factors influencing the INR

The variability in the responsiveness of the PT reagents, is corrected through the "ISI" calibration, however three additional technical factors influence the INR:

- Derivation of MNPT
- Magnitude of difference in the ISI value of test thromboplastin and IRP (ISI=1.0)
- Method of clot detection employed during PT test.

MNPT

MNPT is a critical requirement in the derivation of INR. Ideally each laboratory must derive its own MNPT from 20 or more normal patients for a given PT reagent and Lot under use. This corrects within laboratory test variables that influence PT results. If "normal control plasmas" are used in place of patient plasma for arriving at the MNPT it can effect the evaluation of the patients level of anticoagulation.

For eg:

Reagent ISI=2.5	Test Day 1	Test Day 2	Test Day 3
Patient PT (sec)	16.0	16.0	16.0
Normal Control (10.4 - 12.3 sec)	11.5	10.4	12.3
INR Formula $[R]^{ISI}$	$\left[\frac{16}{11.5}\right]^{2.5}$	$\left[\frac{16}{10.4}\right]^{2.5}$	$\left[\frac{16}{12.3}\right]^{2.5}$
Resulting INR	2.27	2.89	1.92

If the control time is greater than the mean normal range (MNPT), the PT ratio for any patient PT will be smaller, potentially leading to over coagulation. If the control time is lesser than MNPT the ratio for any patient PT will be greater, leading to under coagulation.

On the other hand MNPT for a particular laboratory using the same combination of methodology, reagent and instrument would remain constant.

ISI value of PT used and method of clot detection

INR loses some precision when comparisons are made with thromboplastins with markedly different ISI values as against the IRP (ISI=1.0) and different methods of clot detection e.g.: manual, mechanical,optical etc.

Therefore manufacturers must provide ISI values adapted to the method used for clot detection. Also the reagent used for reporting results should be ideally as close to 1.0 as possible.

Practical Considerations for Warfarin therapy

Oral administration of Warfarin results in a rapid absorption of the drug, however an observable anticoagulant effect is delayed. This delay is due to the time required for des-g-carboxylated (dysfunctional) vitamin K dependent factor to replace the normal clotting factors.

Depending on the dose the delay may range from 1 to 7 days.

The early anticoagulant effect is mainly caused by the loss of fully carboxylated procoagulant factor VII which has a half life of approximately 5 hours.

However these oral anticoagulant reagents also cause suppression in the synthesis of natural anticoagulant protein C and protein S. Due to this, in the early phase of initiation of oral anticoagulant therapy there is a potential for initial prothrombotic effect. This event underlines the syndrome of coumadin induced skin necrosis, especially in patients with hereditary deficiencies of protein C and protein S.

Therapy can begin with an anticipated maintenance dose (e.g:5mg/day). A small loading dose of about twice the average maintenance dose may also be used initially. This dosage achieves a steady state anticoagulant effect in 5-7 days. The use of a large loading dose (e.g.20-40 mg) has little benefit. Such dosing not only produces a marked factor VII deficiency (which alone may not protect against thrombosis) but also an acquired protein C deficiency, which could produce a prothrombotic state.

If the need for antithrombotic effect is more urgent, heparin should be given as indicated. Heparin is then discontinued when INR is in the therapeutic range.

Considerations for frequency of Laboratory tests for monitoring oral anticoagulant therapy

PT monitoring of patients initially should be performed daily for the first 5 days and 2 to 3 times a week for the first 1 to 2 weeks. Depending on the stability of the PT results from the third week onwards the frequency of monitoring may be further reduced to every 4-8 weeks.

While some patients on long term Warfarin therapy have unexpected fluctuations in dose response, some have unexplained requirement for increase in dosage.

The unexpected fluctuations in dose response could be due to: change in diet, undisclosed concomitant drug use, poor patient compliance, surreptitious self-medication, alcohol consumption, intermittent illness or unsuspected changes in the responsiveness of the PT reagent used to perform the PT test.

Management of patients with high INR values with or without bleeding complications

INR	Recommendations
INR> 3.5 but <6.0 patient not bleeding	Rapid reversal not indicated,omit Warfarin for a few doses and resume warfarin at lower dose when INR reaches therapeutic range
INR>6.0 but < 10.0 patient not bleeding	If rapid reversal required give 1-2 mg sc Vit.K ● reduction of INR will occur in 8 hrs ● back in therapeutic range in 24 hrs ● If INR remains high at 24 hrs additional dose of 0.5mg Vit.K may be given. ● Warfarin can be resumed at lower dose when INR returns to therapeutic range
INR > 10.0 patient not bleeding	Give 3 mg sc. Vit.K ● INR will be reduced substantially by 6 hrs ● Check INR at 6 hrs ● Vit.K can be repeated if necessary
INR > 20.0 or patient bleeding	Give 10 mg sc.Vit.K supplemented with FFP or prothrombin complex. ● Check INR every 6 hrs ● Administration of Vit.K inj. may be repeated every 12 hrs ● In case of life threatening bleeding or serious warfarin overdose replacement with prothrombin complex concentrate is indicated, supplemented with i.v. Vit.K 10mg; repeated as necessary depending on INR

Advantages of the INR system

- Major advantage of the INR system is that it helps alleviate confusion in the interpretation of PT results. Usually laboratory changes like change in thromboplastin and/ or equipments could go unnoticed by the attending physicians. The INR remains constant even with such changes.
- INR system affords comparison of PT results between laboratories.
- INR system provides a more accurate and convenient means of monitoring patients who travel extensively.
- INR therapeutic ranges for different clinical conditions are based · on inter national collaborative studies. Usage of standardized dosage reduces the risk of thrombotic episodes or secondary bleeding.

Disadvantages of the INR system

- The prothrombin time test is always a part of the preoperative screening panels. It is also frequently used to evaluate other haemostatic disorders such as liver disease, DIC, LA, hereditary factor deficiencies and acquired vitamin K deficiency.
- Since these disorders have been excluded from the derivation of the ISI, INR has a diagnostic and therapeutic value mainly applicable for patients stabilized on oral anticoagulants. Therefore laboratories may prefer to report both the INR and patients time in seconds depending on clinical application.
- The INR systems effectiveness would still depend on the calibration of the coagulation instruments as well as thromboplastin reagents used.
- Derivation of the correct MNPT and use of the mean normal range in each laboratory.
- Usage of thromboplastin reagents with ISI of preferably 1.0 or as close to 1.0 as possible.
- The correct use of the formula to compute the INR.
- Uniform understanding of the INR system by clinicians as well as laboratorians.

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KNOWLEDGE BOOK

For the Laboratorians

Lupus Anticoagulants

Basic Concepts and Laboratory Diagnosis

Setting Trends...

General Background

Lupus anticoagulants (LA) were first described in the year 1952 by Conley and Hartmann in two patients suffering from Systemic Lupus Erythematosus (SLE). It was given the name "Lupus" since it was initially recognized in patients with SLE and the name "Anticoagulant" because the two patients diagnosed as SLE developed bleeding complications. It is now clear that Lupus anticoagulant is also encountered in patients without SLE and is associated with thrombosis rather than abnormal bleeding.

Patients with Lupus anticoagulants but without SLE can be categorised as follows:

- Patients presenting with venous and arterial thrombotic events for which no underlying cause may be apparent
- Patients receiving drugs namely procainamide, chlorpromazine, quinidine, hydralazine, interferon
- Patients with a recent acute viral infection
- Patients with HIV infection
- Patients seeking medical attention for other disorders, discovered because of prolonged results with APTT in routine pre-operative evaluation of haemostasis status.

Though the terminology 'Lupus Anticoagulant' is a misnomer because LA invivo cause thrombosis rather than abnormal bleeding, it is still extremely popular and hence ISTH (International Society of Thrombosis and Haemostasis) has recommended the continued usage of the same. Thus LA is neither a syndrome nor a disease but a laboratory phenomenon detected by prolonged results with phospholipid dependent clot based assays.

Anti-phospholipid Antibody - Lupus Anticoagulants

Anti-phospholipid Antibody is an autoantibody directed against phospholipids, an important extracellular body constituent. Among the various types of Anti-phospholipid antibodies, clinically important are Lupus anticoagulants (LA) and Anti- cardiolipin antibodies (ACL). The clinical importance of LA and ACL is associated with their high frequency of occurrence in patients with unexplained thrombotic events in APS (Anti-phospholipid Syndrome).

Although LA and ACL may be found concurrently in patients with recurrent thromboemblic disease, they are not necessarily connected to each other. Recent reviews have suggested that LA and ACL are separate antibody subgroups with different phospholipid binding characteristics.

Lupus anticoagulant is an antibody (IgG or IgM or both) that prolongs phospholipid dependent coagulation assays by binding to the epitopes of the anionic phospholipid portion of the prothrombinase complex.

Prothrombinase is a complex of Factor Xa, Factor Va, anionic phospholipid and calcium, which converts prothrombin to thrombin in the coagulation pathway.

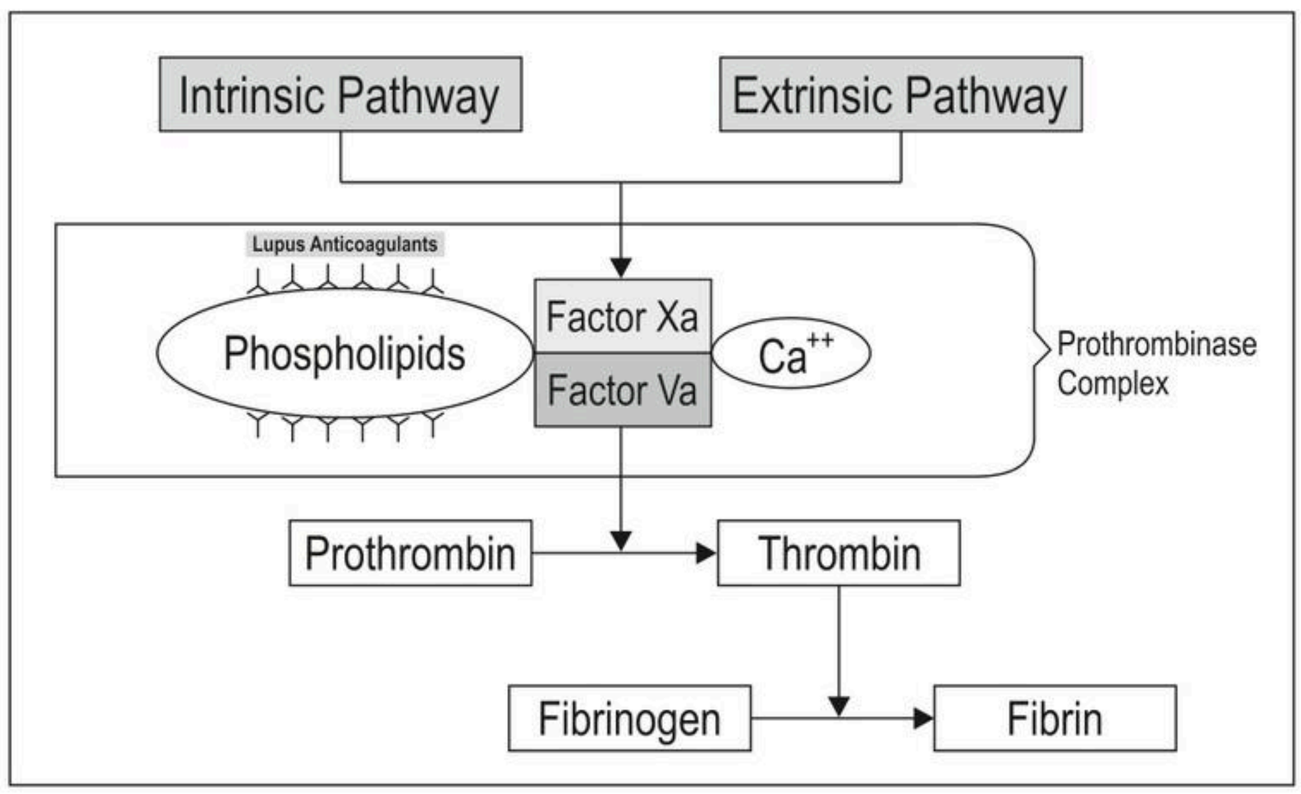

Fig. 1. LA action on Prothrombinase Complex

In vitro, Lupus anticoagulants prolong phospholipid dependent coagulation assays and exert their inhibitory effect by:

1. Binding to epitopes of anionic phospholipids on the prothrombinase complex
2. Blocking the calcium mediated binding of Factor Xa and Factor Va to the phospholipids
3. Reduce conversion of prothrombin to thrombin, which leads to prolongation assay results

On the other hand, **anti-cardiolipin antibodies** (ACL) are IgG or IgM or IgA class of antibodies directed against a complex consisting of β-2 Glycoprotein I (β-2 GPI) bound to anionic phospholipids, cardiolipin. The anti-cardiolipin antibodies are usually detected by the ELISA - based methods and employ cardiolipin and β-2 GPI (as a cofactor) on the solid phase.

Lupus anticoagulants V/s Anti cardiolipin antibodies

PARAMETERS	LUPUS ANTICOAGULANTS	ANTI-CARDIOLIPIN ANTIBODIES
Family	Anti-phospholipid	Anti-phospholipid
Autoantibody class	IgG or IgM	IgG or IgM or IgA
Autoantibody directed against	Anionic Phospholipids	Anionic Phospholipids
Phospholipid Binding Characteristics	Bind with anionic phospholipids on the prothrombinase complex	Bind to complex of anionic phospholipid, cardiolipin & β-2 GPI
Method of Detection	Phospholipid dependant coagulation assays	ELISA / Immunoassays
Diagnostic Relevance	Thrombosis, Recurrent fetal loss, Thrombocytopenia	Thrombosis, Recurrent fetal loss, Thrombocytopenia

APS (Anti-Phospholipid Syndrome)

The term "Anti-phospholipid syndrome" means that a patient has LA and/ or ACL in addition to typical clinical symptoms associated with these antibodies.

Both LA and ACL are associated with:

1. Arterial and venous thrombosis
2. Fetal death/ recurrent abortions
3. Thrombocytopenia (decreased platelet count)

In **"Primary anti-phospholipid syndrome"**, no underlying identifiable disease is present for the thrombotic condition. In primary APS, the antibodies persist in the body regardless of therapeutic intervention.

In **"Secondary anti-phospholipid syndrome"**, an underlying disease is present such as:

- An autoimmune disorder (e.g.: SLE)
- Malignancy
- Infection (bacterial or viral)
- Inflammation
- Drugs (e.g.: chlorpromazine, procainamide, quinidine, hydralazine, interferon)

In the secondary syndrome, the APA's usually decline with successful management of the underlying disease; especially in autoimmune disease such as SLE.

Clinical findings with LA

Patients with Lupus anticoagulants usually present one of these clinical symptoms:

CLINICAL MANIFESTATIONS	GENERAL FINDINGS
Venous thrombosis	• Deep venous thrombosis • Pulmonary embolism
Arterial thrombosis	• Myocardial infarction • Gangrene • Stroke
Thrombocytopenia	• Reduced Platelet count
Repeated abortions	• Intrauterine growth retardation • Intrauterine death of fetus
Neurologic symptoms	• Migrane • Chorea
Cutaneous symptoms	• Widespread cutaneous necrosis • Distal cutaneous ischaemia • Livedo reticularis • Leg ulcers

Clinically recognition of Lupus anticoagulants is important in the management of patients, with or without SLE, who experience unusual thrombotic events or thrombotic events at a younger age than expected.

In patients with SLE or related autoimmune disorders there is a definite increased incidence of thrombocytopenia associated with the presence of the Lupus anticoagulants/ anti-phospholipid antibodies.

Another major clinical manifestation associated with Lupus anticoagulant is fetal loss. Any patient with a history of recurrent first trimester abortion, second or third trimester intrauterine death, should be tested for Lupus anticoagulant. The probable mechanism by which fetal loss occurs can be attributed to thrombosis of placental vessels and placental infarction. The recurrent abortions can occur in patients with or without SLE.

In summary, the patient groups that need to be tested for LA are:

- Patients experiencing unexplained recurrent thrombotic events
- Patients presenting with thrombotic events at a younger age than expected
- Patients with SLE
- Pregnant patients with recurrent abortions or intrauterine death of fetus
- Patients seeking medical attention for other disorders, accidentally discovered by prolonged APTT time in routine pre-operative haemostasis status.

Pathophysiologic Mechanism of Thrombosis

A strong correlation exists between the occurrence of Lupus anticoagulants and high incidence of thrombotic disease. The exact pathophysiological mechanism accounting for the frequency of thrombosis in patients with Lupus anticoagulants is not clear but various data suggest that there is more than one mechanism causing thrombosis in LA patients.

The probable mechanisms of thrombosis can be attributed as follows:

1. Since a number of patients with Lupus anticoagulants and thrombosis have some degree of thrombocytopenia, platelet activation is thought to play an important role in arterial thrombosis. In patients with Lupus anticoagulants and concomittant Anti-thrombin III or Protein–C deficiency, there is an increased risk of venous thrombosis.
2. In patients with SLE, the mechanism of thrombosis is probably mediated by auto antibodies that affect the endothelial cell function.
3. In normal population, the vessel wall provides a non-thrombogenic surface, as PGI2 (Prostacyclin) secreted by the endothelial cells is a potent vasodilator and inhibits platelet aggregation. In some patients it has been observed that the Lupus anticoagulant interferes with the secretion of PGI2 from the vessel walls. Thus an impaired synthesis or deficient release of PGI2 from endothelial cells could account for an increased risk of thrombosis.
4. Other possible mechanisms by which Lupus anticoagulants might mediate thrombosis include,
   - Inhibition of Protein C activation thereby resulting in impaired degradation of Factor Va by activated Protein C
   - Decreased fibrinolytic Activity
   - Prekallikrein inhibition
   - Inhibition of Antithrombin III activity

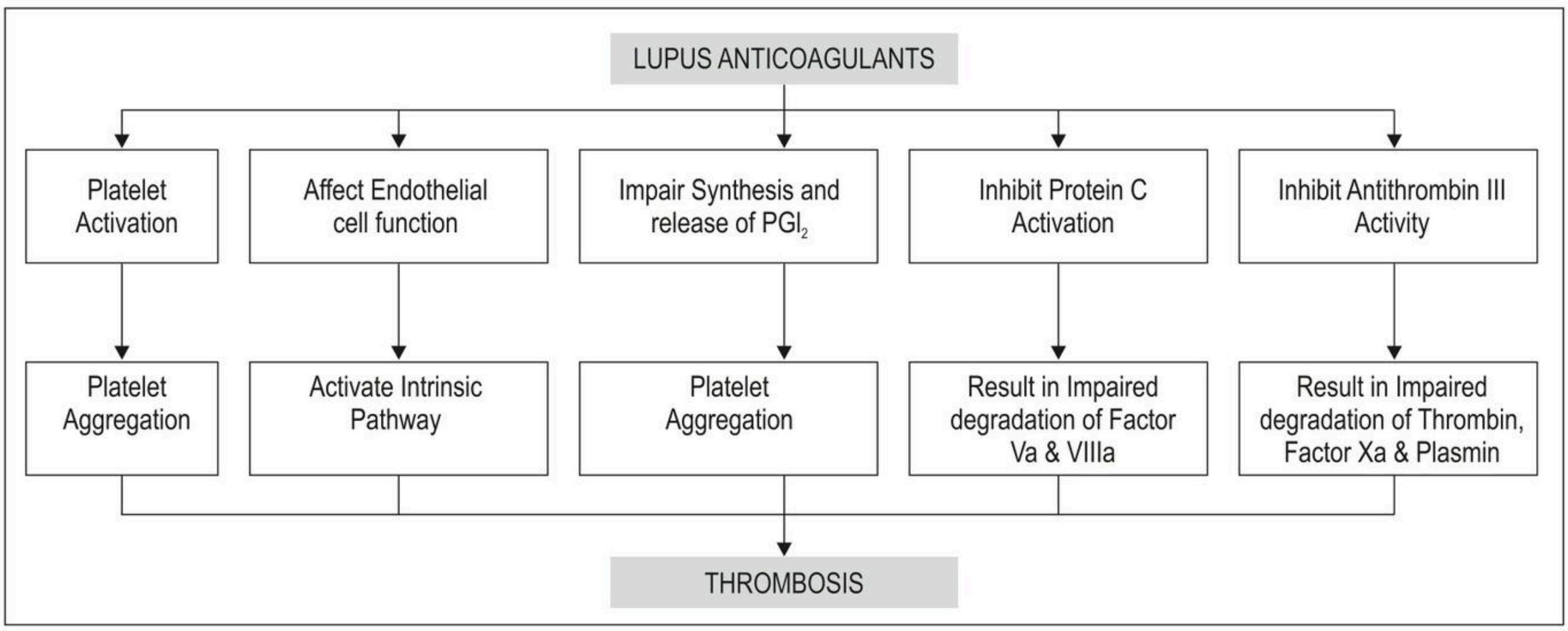

Fig. 2. LA and Thrombosis

Lupus Anticoagulants and Bleeding Complications

Bleeding complications in patients with Lupus anticoagulants is a rare phenomenon. But recent reviews indicate that a subset of patients with LA were associated, interestingly, with bleeding complications. A reason for this rare phenomenon remained obscure until some workers demonstrated that LA patients with bleeding complications also showed prothrombin deficiency.

As per their observations, the probable mechanism of LA interaction with prothrombin molecule can be summarized as follows,

1. Lupus anticoagulants bind to epitope of prothrombin molecule not actively involved in the coagulation pathway.
2. The LA – prothrombin complex so formed is cleared rapidly from the circulation giving rise to prothrombin deficiency, resulting in bleeding tendency.

The above mentioned phenomenon was coined as "Hypoprothrombinemia- Lupus anticoagulant" syndrome. Studies point out that clinically it may also be important to evaluate the prothrombin time when screening patients for LA.

Phospholipid dependence of Lupus Anticoagulants

It is now clear that Lupus anticoagulants are immunoglobulins of IgG class or IgM class or both that prolong phospholipid dependant clot based assays. The phospholipid dependence of Lupus anticoagulants in invitro assay systems has been extensively studied and convincing evidence of the same may be summarised as follows:

1. The Lupus anticoagulants react against negatively charged phospholipids and are able to inhibit the binding of Factor X and Prothrombin to the procoagulant phospholipid surface, thereby accounting for prolongation of phospholipid dependent clot based assays.
2. Reducing the phospholipid component of clotting mixture potentiates the Lupus anticoagulants effect. Thus the most sensitive tests for detecting the phospholipid inhibitor contain limited amount of phospholipids.
3. Increasing the procoagulant phospholipid in clotting mixture diminishes the effect of anticoagulant (neutralization effect) and corrects the abnormal clotting time so observed initially. This confirms the presence of phospholipid dependent Lupus anticoagulants.

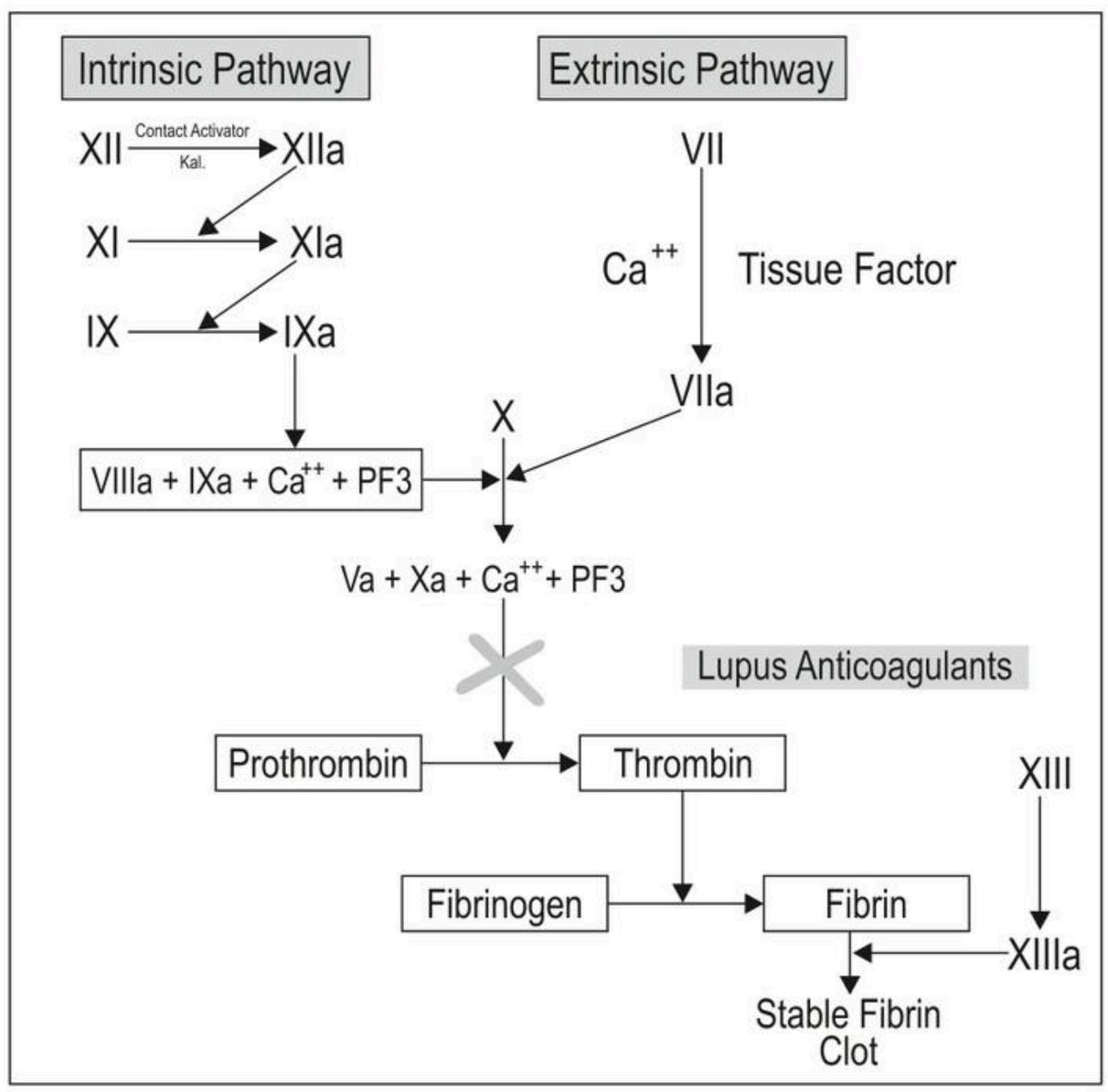

Fig. 3. In vitro LA action

Laboratory Diagnosis of Lupus Anticoagulants

In the past a number of different tests have been proposed and used for the diagnosis of Lupus anticoagulants.

The traditional screening methods used for the laboratory diagnosis of Lupus anticoagulants can be broadly classified as follows:

A. Immunological assays
B. Clot based assays incorporating phospholipids in the reagent sysytem

A. Immunological Assays

Individuals with Lupus anticoagulants may also show the presence of other anti phospholipid antibodies. The most frequent finding is the presence of Anti- cardiolipin antibodies. The commonly employed method is the ELISA technique where the solid phase is coated with cardiolipin and b-2 GPI (as a cofactor). The ELISA method detects IgM, IgG and IgA class of anti-cardiolipin antibodies. An important point to note is that Lupus anticoagulants and anti-cardiolipin antibodies differ in their phospholipid-binding characteristic hence detection of anti cardiolipin antibodies is not specific for the presence of Lupus anticoagulants though they may be present in the same patient.

B. Clot based assays

1. APTT (Activated Partial Thromboplastin Time)

Since Lupus anticoagulants bind to the phospholipid complex they do prolong phospholipid based coagulation assays. Logically the activated partial thromboplastin time (APTT) is prolonged and this property has been used for the detection of LA using APTT reagents.

In the context of LA detection, the APTT test has certain shortcomings:

- An important variable related to the suitability of APTT reagents in the detection of Lupus anticoagulants is the composition of phospholipids used in the reagent system. Different reagents have varying sensitivity for the presence of Lupus anticoagulants on the basis of their phospholipid composition.
- The APTT test is affected by the presence of inhibitors to Factor VIII, IX and XI. Inorder to rule out the presence of inhibitors ususally mixing studies are performed. In mixing studies a 1:1 or 1:4 (Normal plasma: Patient plasma) is used. Failure to correct the prolongation of clotting time using the mixing studies indicates the presence of Lupus anticoagulants.
- The APTT test is also the test of choice for monitoring heparin therapy. This reduces the specificity of the APTT to LA. The presence of heparin can be ruled out by using a thrombin time test. If thrombin time test shows normal values, then the sample does not contain heparin. But if the thrombin time test is abnormal, then heparin neutralization test is used which includes protamine sulphate. In this test, various concentrations of protamine sulphate are added to plasma before the addition of thrombin reagent. When protamine sulphate neutralizes all the heparin present, the clotting time reverts to normal value.

2. **TTI (Tissue Thromboplastin Inhibition test)**
   The Tissue Thromboplastin Inhibition test utilizes a diluted PT reagent. The results are expressed as ratio of patient values to normal control values.

   The TTI test is affected by numerous variables:
   - Species and tissue origin of thromboplastin can affect the test results as different sources of thromboplastin have varying sensitivity and responsiveness.
   - Choice of "Normal" reference plasma is the most critical variable, because depending on the laboratory the choice of reference plasma could be lyophilized plasma, a frozen plasma pool or fresh plasma. The ratio of patient to normal can therefore change according to the choice of "normal" plasma.
   - Some IgM Lupus anticoagulants do not prolong the TTI test.

3. **KCT (Kaolin Clotting Time)**
   KCT is similar to APTT, the difference being that KCT reagent is devoid of phospholipids and incorporates Kaolin as contact activator. The test is performed on a range of mixture of normal and patient's plasma. Different patterns of response are obtained indicating the presence of Lupus anticoagulants or the deficiency of one or more coagulation factors.

   The KCT test though sensitive is not specific for LA, additionally:
   - It cannot be automized
   - The test shows prolonged results with factor VIII, IX, XI & XII deficiency or corresponding inhibitors
   - The test is also highly sensitive for the presence of heparin.

4. **PNP (Platelet Neutralization procedure)**
   In PNP test platelets substitute the phospholipid extract of the APTT reagent. The platelets have the ability to adsorb the Lupus anticoagulant and this property is used in the PNP test.
   If a sample shows initial prolonged time with APTT reagent, then it is further tested with dilute APTT reagent incorporating platelet substitute. Since platelets have the ability to adsorb LA, a reduced clotting time with dilute APTT reagent indicates the presence of LA. But if an inhibitor is directed against specific coagulation factor, the clotting time continues to show prolonged values.

   The PNP test though useful did not gain wide usage :
   - Due to limited stability the platelet preparations loose their activities on storage hence do not show reproducible results.
   - They cannot differentiate between Lupus anticoagulants and Factor VIII inhibitors.

5. **Hexagonal Phase Phospholipids**
   The hexagonal phospholipid neutralization assay is a dilute APTT reagent, which contains phospholipids in hexagonal steric configuration. The principle of this method is same as PNP test.
   The major drawback of this test being hexagonal phospholipids do not occur *in-vivo* hence the authenticity of this test principle for detecting the presence of Lupus anticoagulants has been rightly challenged.

Recommendations and Criteria for the Diagnosis of Lupus Anticoagulants

The Lupus Anticoagulant/ Anti-phospholipid Antibody sub-committee of ISTH (International Society of Thrombosis and Haemostasis) first published its recommendations and criteria for diagnosis of Lupus anticoagulants in 1991.

Subsequently, the sub-committee further reviewed:

- Important advances in the understanding of Lupus anticoagulants
- New assays for the identification of Lupus anticoagulants
- New information concerning the technical aspects of test performance.

In 1995, the sub-committee published the revised recommendations and criteria for the **laboratory diagnosis of Lupus anticoagulants.**

Current Recommendations of the ISTH

1. Both patient and normal plasma should be as platelet free as possible; the platelet count should be less than 10×10^9/l. This applies for testing on fresh plasmas, preparation of frozen samples and preparation of pooled normal plasma. Filtration appears to be an excellent method for the preparation of platelet free plasma for LA testing but may interfere with testing of other coagulation factors. Ultra- centrifugation must be performed carefully to avoid formation of platelet debris. Alternately atleast a two-step centrifugation of properly collected blood at the correct speed is also acceptable.
2. Two or more tests should be used to screen for diagnosis of LA. At least one of these tests should be based on a low phospholipid concentration,
   - KCT
   - dRVVT
   - dAPTT
   - dPT

   The assays should represent different assay principle for example, a dAPTT and a dRVVT.
3. Inhibitor activity should be documented by the effect of patient plasma on pooled normal plasma. This step can be incorporated into the initial screening procedure. The sensitivity and specificity of the ratio of patient to normal plasma should be established before it is used routinely.
4. A diagnosis of a LA should not be made on the basis of multiple abnormal screening assays and mixing studies alone. Confirmatory studies need to be performed to document the phospholipid dependence of the inhibitor.
5. Confirmatory assays should be based in the method giving an abnormal screening assay. For example if the dRVVT is abnormal, then a confirmatory assays based on the dRVVT should be used.
6. Routine clotting tests, such as PT and APTT, should be performed to evaluate the possibility of other coagulation disorders that may interfere with the LA methodology. If the chosen method for screening or confirmation is known to be sensitive to heparin, a thrombin time may be helpful in detecting its presence.
7. Solid phase assays for anti- phospholipid antibodies such as ELISA (e.g. anti- cardiolipin antibodies) although frequently positive in patients with LA's should not be considered as a confirmatory procedure for LA activity.
8. Factor assays should be performed whenever there is suspicion of a specific factor deficiency or inhibitor. Two or more dilutions of the patient plasma should be evaluated in any factor assay.
9. The term Lupus anticoagulant should be retained until the pathophysiology of these inhibitors is more fully delineated.

Based on these recommendations, a flow chart (Fig.: 4) was established thereby assisting the laboratorians to establish a routine approach in the diagnosis of LA's and improve testing standards in the detection of LA's.

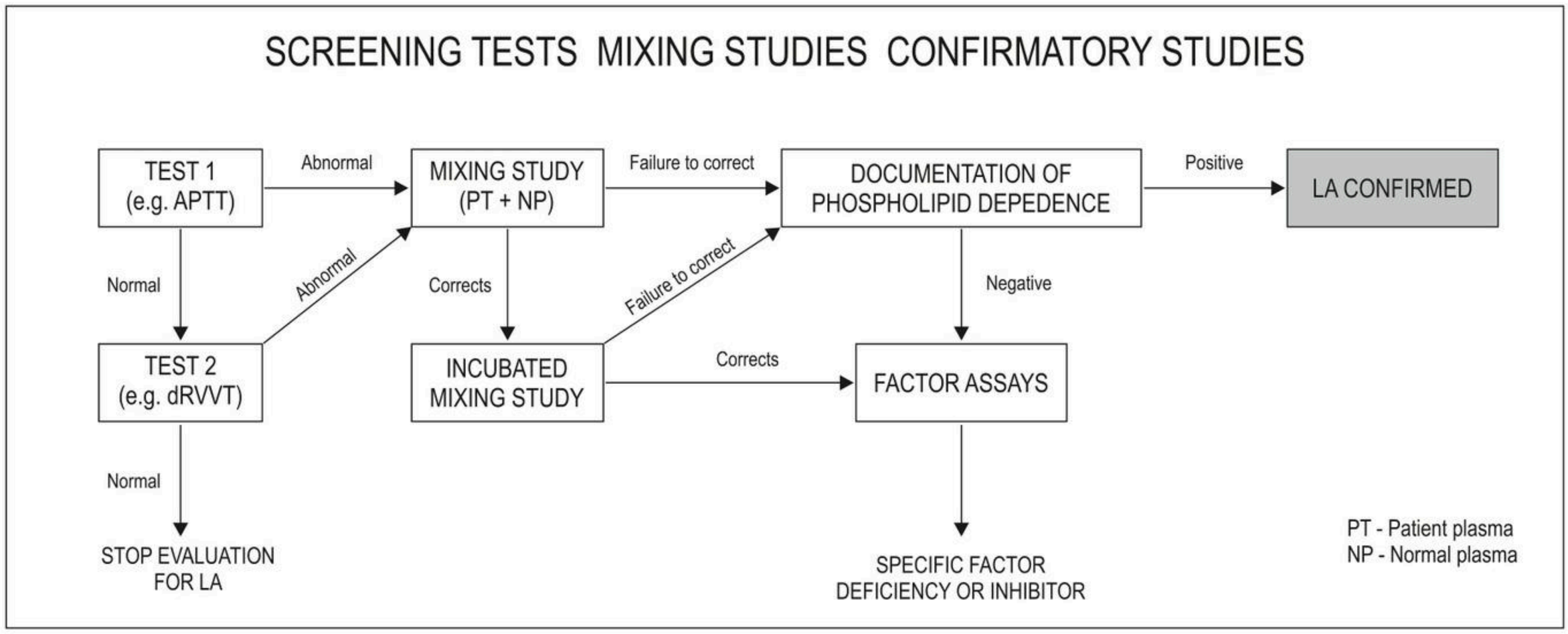

Fig. 4. Flow Chart for laboratory diagnosis of LA

Scientific Subcommittee criteria for the laboratory diagnosis of Lupus anticoagulants

In order to make a diagnosis of LA; sample should show each of the following:

1. Prolongation of at least one phospholipid – dependent clotting assay.
2. Evidence of inhibitory activity shown by the effect of patient plasma on pooled normal plasma.
3. Evidence that the inhibitory activity is dependent on concentration of phospholipid. This may be achieved by addition or alteration of phospholipids/ hexagonal phase phospholipids/ platelet/ platelet vesicles in the test system.
4. LA's must be carefully distinguished from other coagulopathies that may give similar laboratory results or may occur concurrently with LA's. Specific factor assays and the clinical history may be helpful in differentiating LA's from these other possibilities.

dRVVT : The test of choice for screening and confirmation of LA

The dilute Russell's viper venom time test (dRVVT) first became popular following publication by Thiagarajan et al in 1986. Dr. Exner further simplified and standardized this method subsequently.

In general dRVVT based tests comprise of:

- SCREENING REAGENT, containing limited amount of phospholipids with RVV (Russell's Viper Venom)
- CONFIRMATION REAGENT, containing additional phospholipids with same amount of Russell's Viper Venom, to confirm the presence of phospholipid dependent Lupus anticoagulants.

Principle of dRVVT for LA detection

Russell's Viper Venom directly activates Factor V and X in presence of phospholipid and calcium ions, bypassing Factor VII of the extrinsic pathway and the contact and antihaemophilic factors of the intrinsic pathway.

In normal plasma, in the absence of lupus anticoagulants, Factor V and X is directly activated by Russell's Viper Venom, which in presence of phospholipid and calcium ion leads to clot formation.

In patients with LA, autoantibodies bind the epitopes of reagent phospholipids thereby preventing **the activation of prothrombinase complex**. This results in a prolongation of clotting time with SCREENING reagent. The CONFIRMATION reagent incorporates additional phospholipids to neutralize the LA. Once LA are neutralized clot formation proceeds relatively uninterrupted achieving a lower clotting time, to prove the phospholipid dependence of the autoantibodies.

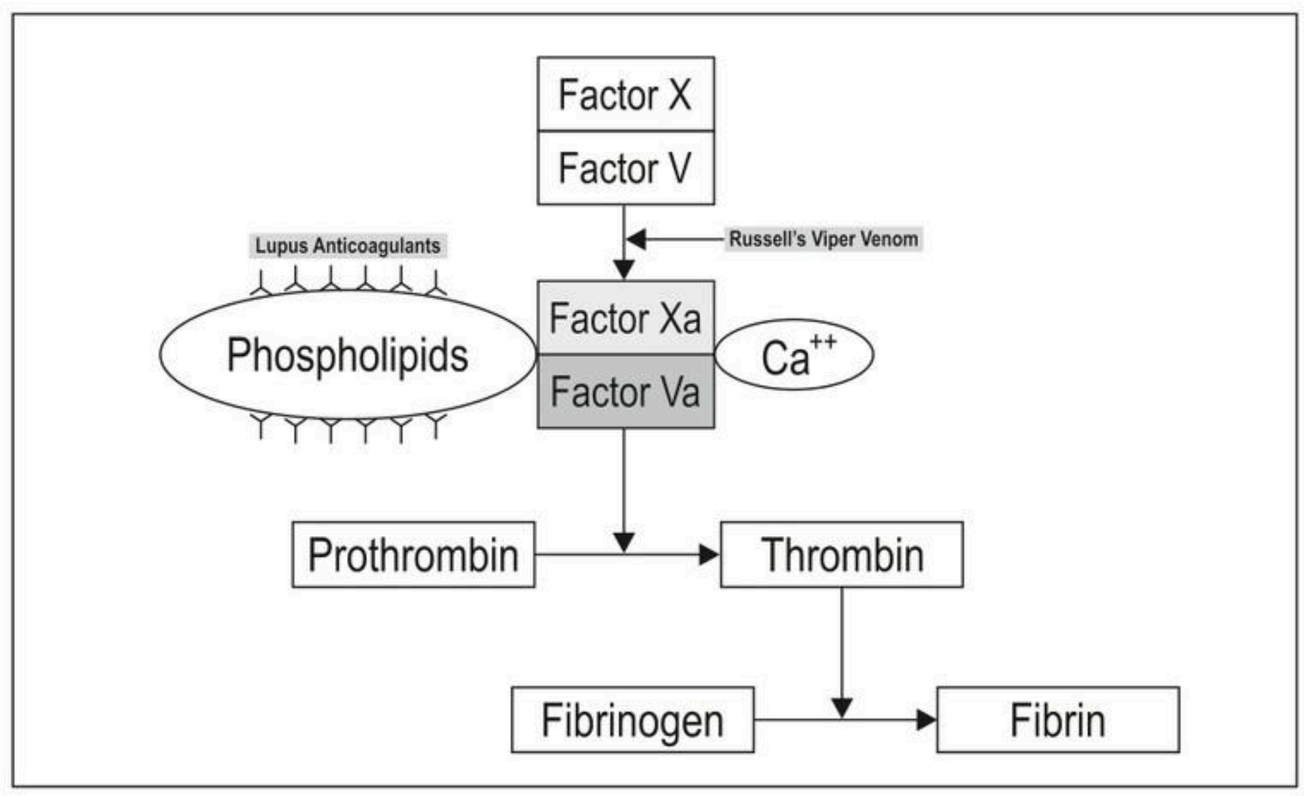

Fig. 5. Principle of the dRVVT Test

Interpretation of results with dRVVT test

If SCREEN TIME is prolonged, to confirm the presence of lupus anticoagulants the plasma sample is tested with CONFIRMATION REAGENT.

If CONFIRM TIME results shows a lower clotting time as compared to SCREEN TIME, it indicates the presence of phospholipid dependant Lupus Anticoagulants.

Also the results can be expressed as ratio,

$$\text{Ratio (R)} = \frac{\text{Mean Screen Time}}{\text{Mean Confirm Time}}$$

The results expressed, as ratio is further useful in classifying the patient as normal, moderate, high and very high LA.

If results of the ratio are borderline, mixing studies may be done further with the sample specimen. The mixing studies should be carried out with a 50:50 mixture of test plasma and normal plasma.

dRVVT as positive predictor of thrombotic events in APS

As suggested by ISTH in the criteria for the diagnosis of LA, the sample should show prolongation of results with phospholipid dependent clot based assays. Also, the inhibitory activity should be evident by altering the phospholipid concentration to confirm the phospholipid dependence of LA.

The dRVVT test incorporates the aforesaid ISTH recommendations and hence universally is being accepted as the coagulation assay of choice for LA detection.

The assay utilizes SCREENING and CONFIRMATION reagent thereby,

- Indicating the phospholipid dependence of LA
- Achieving maximum sensitivity for the presence of LA.

The clinical value of any diagnostic assay is directly proportional to its positive and negative predictive value. dRVVT in independent studies has proven to be 100% specific for presumed thrombotic events, miscarriage and pulmonary hypertension as compared to solid phase immunoassays which had lower clinical relevance (83%).

Therapeutic measures for patients with LA

For the therapeutic management of patients with Lupus anticoagulant, different regimens in uncontrolled trials has resulted some success in treatment. However no random prospective trial has been conducted in LA patients to determine the optimum therapeutic regimen.

Therapeutic measures for LA patients can be broadly classified as,

1. Management of patients with Primary Anti-phospholipid Antibody syndrome
2. Management of patients with Secondary Anti-phospholipid Antibody syndrome

1. Management of patients with Primary 'APS'

- When the Lupus anticoagulant is associated with evidence of severe prothrombin deficiencies or thrombocytopenia treatment with adrenal corticosteroids is indicated.
- Presence of Lupus anticoagulant and thrombosis is guided by the fact that recurrence is common. Hence for patients presenting with venous thrombosis long term anticoagulation (probably for life) with oral anticoagulants is indicated. For arterial thrombosis low dose aspirin is indicated.
- In management of pregnant patients with or without SLE and Lupus anticoagulants the exact regimen to be used till date is not clear. Initially some workers reported successful pregnancy out comes in patients with LA by treating with prednisolone and low dose aspirin. However long term, high dose corticosteroids during pregnancy are associated with significant side effects namely
  - severe preeclampsia
  - infections
  - gestational diabetes
  - osteoporosis.

  Recently some workers have suggested low dose aspirin and low dose heparin with some success in pregnancy outcomes. Thus, different therapeutic regimens in uncontrolled trials have resulted in some success, but no careful randomized trials have yet been conducted to arrive at an optimum and safe therapeutic regimen that can be universally adapted.

2. Management of patients with Secondary 'APS'

- When the Lupus anticoagulant is present in patients with an underlying autoimmune disease, management with appropriate immunosuppressive therapy is indicated.

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MALARIA

- **Malaria and its diagnosis**
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- **Malaria RDT's**
  - Performance Evaluation and Quality Assurance.....Pgs 91-96

KNOWLEDGE BOOK

For the Laboratorians

Malaria and its diagnosis

Rapid tests for Malaria detection

Setting Trends...

Malaria

Malaria is widespread, especially in hot, humid Tropical and Sub Tropical countries of the world. It is most common between 23.50 North latitude and 23.50 South latitude, in Africa, Asia, South America, Central America, the Caribbean and some isolated parts of North America.

Globally, approximately 300 million new cases occur every year, of which 2.7 million are fatal.

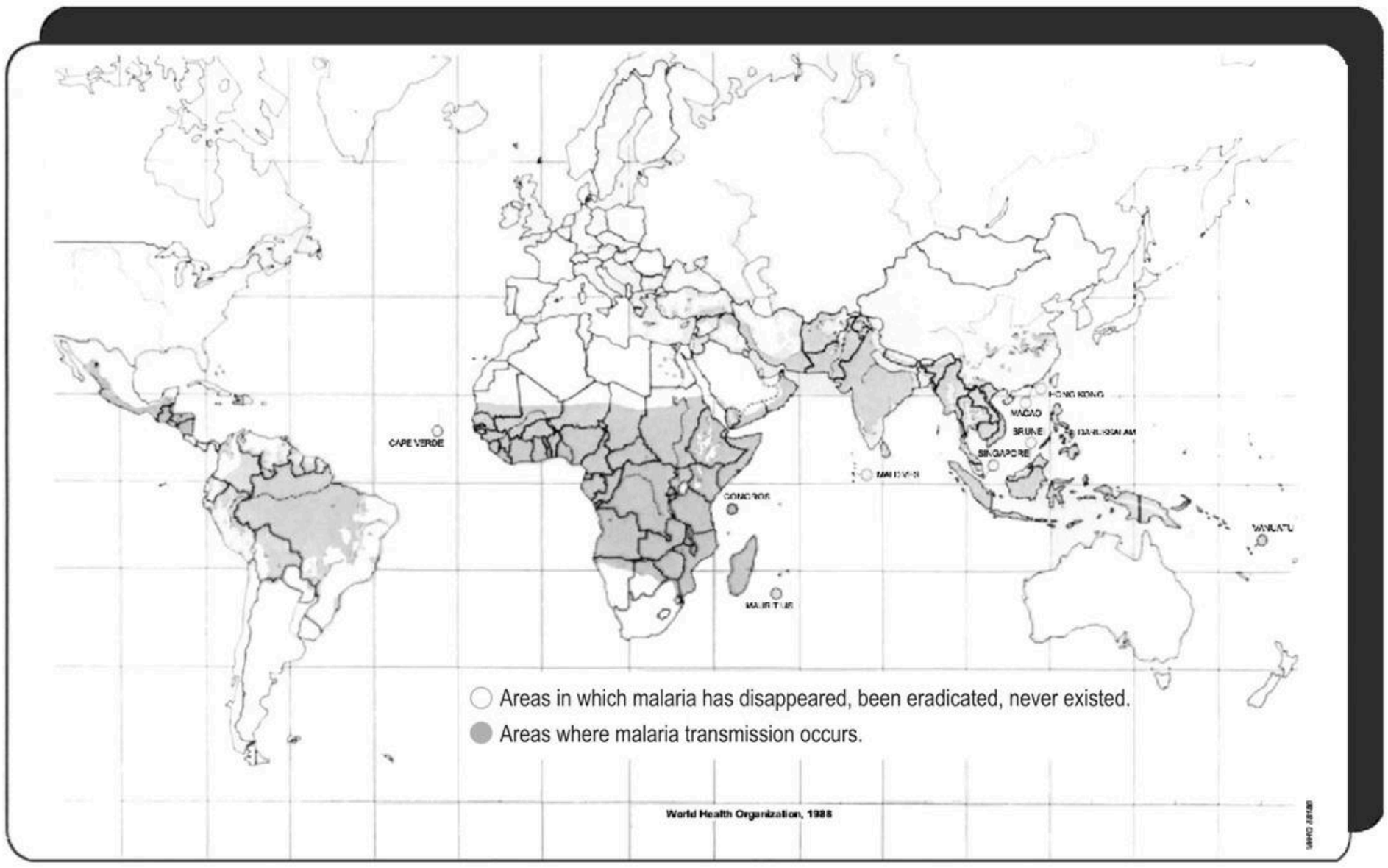


Fig. 1. Geographical Distribution of Malaria Infection

Malaria in humans is caused by the four species of the Plasmodium parasites; *P. vivax*, *P. falciparum*, *P. malariae* and *P. ovale*.
The tropical zone is the endemic home of all malaria parasites. *P. malariae* is a parasite of the sub tropic zone. *P. vivax* is the prevalent species of the temperate zone. The distribution of *P. ovale* has been mainly reported from east Africa, west Africa and from the Philippines in Asia.
Plasmodium falciparum is the most common species in tropical areas and is transmitted primarily during the rainy season. This species is the most dangerous, accounting for half of all clinical cases of malaria and 90 percent of deaths from the disease. *Plasmodium vivax* is the most widely distributed parasite, existing in temperate as well as tropical climates. *Plasmodium malariae* can also be found in temperate and tropical climates but is less common than *Plasmodium vivax*.
Plasmodium ovale is a relatively rare parasite, restricted to tropical climate and found primarily in eastern Africa.
Climatic changes, human migration and drug resistance are the key factors that affect the rapid spread of malaria all over the world.

Historical Perspectives

The word malaria (mal=bad, aria=air) is based on the association between the bad air of marshes (where the anopheles mosquito vector breeds) and human infection by malaria parasites, *Plasmodium* species. Malaria has afflicted humankind since millenia and continues to do so till today. Examination of the primate malarias suggests that human plasmodia of primates have evolved together with their hosts from the plasmodia of primates. This hypothesis is supported by the remarkable similarities between several primate malarial parasites (*Plasmodium reichenowi*, *P. cynomolgi* and *P. brasilianum*) and their counterparts (*P.falciparum*, *P.vivax* and *P. malariae* respectively).
In terms of recorded history, the earliest references to malaria are descriptions of splenomegaly with fever from China in the Nei Ching Canon of Medicine in 1700 BC and from Egypt in the Ebers Papyrus in 1570BC. Hippocrates clearly recognized the syndrome of malaria and its relationship to marshes. Literary references to malaria appear in Homer's illad and in the works of Chaucer and Shakespeare. Incidentally, that malarial parasite infects RBCs, was discovered by Ross (a British researcher) in India itself.
Today malaria remains an overwhelming problem in tropical developing countries (with 300 to 500 million cases and 2 to 3 million deaths per year) and a potentially life threatening complication for travellers to the tropics.

Malaria Life cycle

Plasmodium parasites undergo many stages of development, and their complete life cycle occurs in both humans and mosquitoes. The parasites are transmitted to humans by female mosquitoes of the genus Anopheles. About 60 of the 390 species of Anopheles mosquito transmit the malaria parasite. Of these only a dozen species are important in the transmission of malaria worldwide. Usually just one or two species play a role in malaria transmission in a particular region where the disease is prevalent.

Malaria transmission begins when a female mosquito bites a human already infected with the malaria parasite. The mosquito ingests blood containing immature male and female gametes (sex cells) of the malaria parasite. Inside the mosquito's stomach, the gametes quickly mature. A male gamete fuses with a female gamete to produce a cell known as zygote. The zygote enters the wall of the mosquito's gut and develops into an oocyst. The oocyst multiplies to produce thousands of cells known as sporozoites. The sporozoites leave the wall of the gut and migrate to the mosquito's salivary glands. The mosquito phase of the malaria parasite's life cycle is normally completed in 10 to 14 days. This development process occurs more slowly in areas with cooler temperatures. Sporozoite development of *Plasmodium falciparum* is slowed particularly by low temperatures, preventing transmission of this parasite in temperate climates except during summer.

When the infected mosquito bites another human, sporozoites in the mosquito's saliva transfer to the blood of the human. Sporozoites travel in the blood to the liver. In liver cells over the course of one to two weeks, the sporozoites divide repeatedly to form 30,000 to 40,000 merozoites. The merozoites leave the liver to enter the bloodstream, where they invade red blood cells. Inside these blood cells, the merozoites multiply rapidly until they force the red cells to burst, releasing into the bloodstream a new generation of merozoites that go to infect other red blood cells. This is the time the clinical symptoms of overt malaria in the infected individuals becomes apparent. Some merozoites divide to form gametocytes, immature male and female gametes. If another mosquito bites the human and ingests these gametocytes, the life cycle of the malaria parasite begins again.

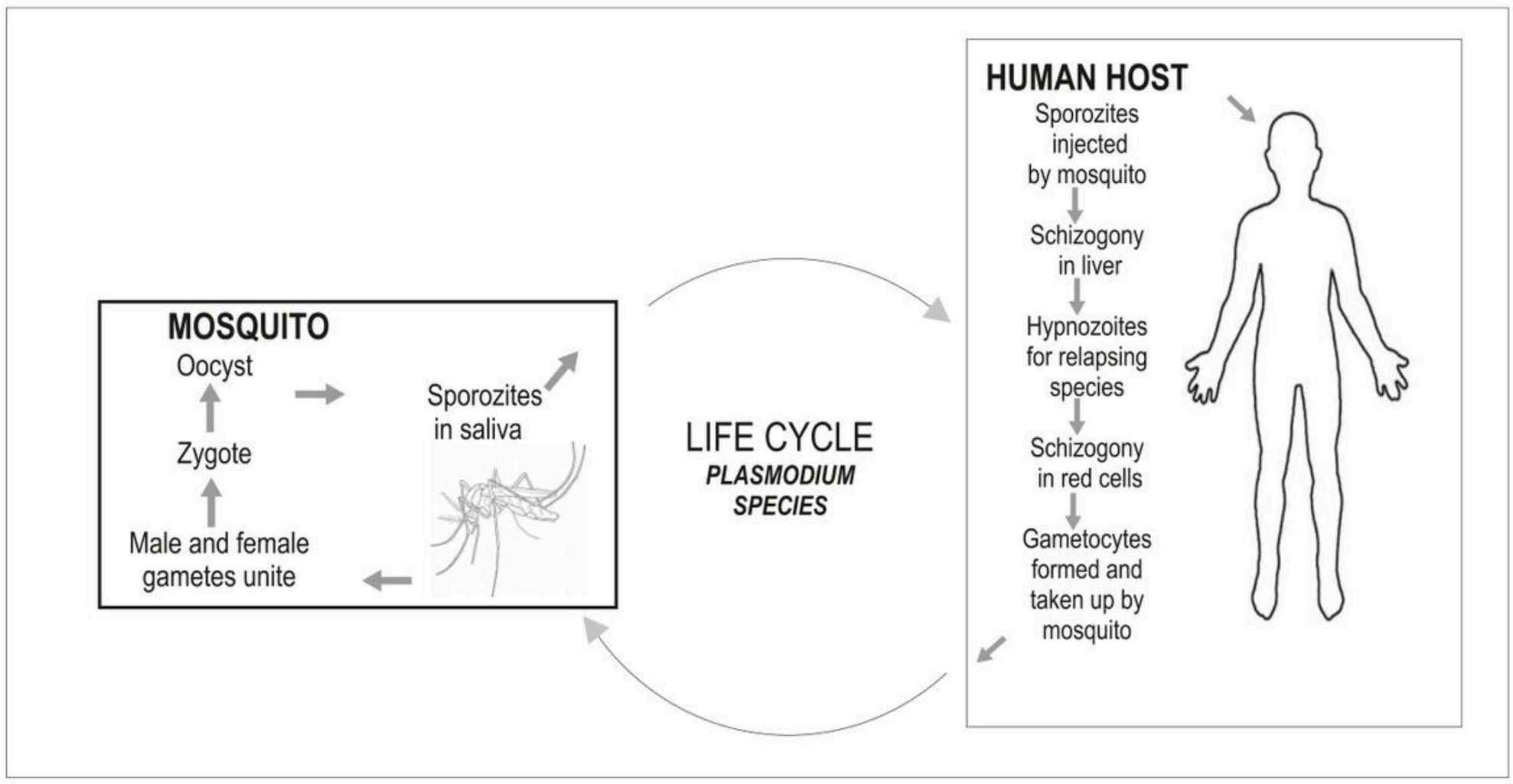


Fig. 2. Diagrammatic representation of *Plasmodium* life cycle.

Malaria and Plasmodium species

Out of the total occurrence of malaria cases globally, it is estimated that about 40% are caused by *P. falciparum*, about 50% caused by *P. vivax*, about 7 - 8% caused by *P. malariae* and the rest by *P. ovale*.

In India it is estimated that about 60% of the infections are due to *P. vivax* and 40% of the infections are due to *P. falciparum*. *P. malariae* in India is said to be responsible for less than 1% infection. In India it is estimated that about 4-8% of the malaria infection are reportedly mixed type, involving more than one parasite, usually the *P.vivax* and the *P. falciparum*.

The *P.ovale* has the exoerythrocytic cycle in the liver, similar to the *P.vivax* and the hypnozoites situated in the liver can cause a relapse.

P. malariae does not have an exoerythrocytic cycle like the *P.vivax* and *P.ovale* and in this sense is similar to *P. falciparum*. However malaria caused by *P. malariae* is benign type where as the one caused by *P. falciparum* is severe and malignant type.

P. falciparum malaria (due to the mortality and morbidity associated with it) and *P.vivax* (due to the morbidity and high infection and relapse rates) are still considered the "**Big two**" and logically have also become the target for prevention, eradication treatment and diagnostics.

Distribution of malarial disease by age and sex

South and East Asia and the Western Pacific

In most of this region, including the Mekong countries, China, Indonesia, the Philippines, India, and Sri Lanka, the incidence of malarial infection is spread throughout life. The incidence of *P. falciparum* tends to peak broadly between 10-40 years of age and that of *P. vivax* more sharply and earlier, when the patient is aged -2- 15 years. Point prevalence of either species in the age groups at highest risk tends to be ~0.3-3%. In the economically most productive age group of people ~ 15-50 years of age, the risk of malarial infection in boys and men relative to girls and women is between 1.3:1 and 2.5:1 and is due to differences in occupation that place men at a higher risk of exposure to infection.

A major exception to this generalization concerning malaria risk is the situation of pregnant women, who are at a generally higher risk of developing malarial disease and at 3 times the risk of developing severe malaria than non-pregnant women or men of the same age when they become infected with *P. falciparum*. The risk of acquiring even *P.vivax* has been found to be greater in the pregnant than non-pregnant state, although there is no associated risk of severe complications during pregnancy.

Within the Pacific region, the islands of Papua New Guinea, the Solomon Islands, and the Vanuatu islands have much higher levels of malaria transmission, and the above generalizations do not apply. Peak prevalences of *P.vivax* lie between 1-15 years, much as elsewhere in the region, whereas

peak prevalences of *P. falciparum* fall between 5- 20 years of age. Within the age groups at highest risk, however, the point prevalence of both *P. vivax* and *P. falciparum* are very much greater than elsewhere in the region, generally 5-50% for either species. Differences between sexes in risk of malaria are probably less marked than in the other countries of the region.

Eastern Mediterranean

Malaria transmission rates are, with the exception of those in the Sudan, relatively low throughout this region, and the incidence of malaria and risk of death from malaria can be expected to be spread throughout life, but mainly in early adulthood and middle age. For occupational reasons, the risks of malaria and of death from malaria can be expected to be several times greater in men than in women.

North and South America

Transmission intensities of malaria are generally low to moderate through this region, and incidence of malaria can be generally expected to be spread throughout life in most settings, with the peaks occurring in early adulthood. In a few areas, namely French Guiana, Guyana, and Suriname, transmission intensities are relatively very high (and mainly *P. falciparum*); the incidence of malaria can be expected to be highest in early childhood and low in adolescence and adulthood. Elsewhere, transmission intensities are low (and mainly *P. vivax*), and point prevalence is generally much less than 1%. Risk of malaria spreads throughout life, but young and middle-age adults are at the highest risk-especially men, who, for occupational reasons, may be at 2-3 times the risk of malaria infection than women.

Africa

Ninety percent of global mortality on account of malaria occurs in Africa, mainly in children and pregnant women.
Among the 4 species of malaria parasite that infect humans, *Plasmodium vivax* and *Plasmodium falciparum* vie for greatest prevalence in the world today. Of these 2 species, *P. falciparum* is justifiably regarded as the greater menace because of the high levels of mortality with which it is associated, its widespread resistance to antimalarial drugs, and its dominance on the world's most malarious continent. However, malaria due to *P.vivax* has also placed huge burdens on the health, longevity and general prosperity of large sections of the human population.

Clinical Malaria

Incubation time

The time interval between mosquito bite and development of malaria is 8-14 days except in *P. malariae* (35 days).

Typical Presentations

Initially the symptoms in patients resemble those of a minor viral illness such as; lack of sense of well being, headache, fatigue, abdominal discomfort, muscular aches followed by fever and nausea/ vomiting. These may be followed by typical malaria picture such as fever spikes (sudden rise and fall in temperature), chills and rigors.

Cold stage

As the temperature begins to rise, there is intense headache and muscular discomfort. The patient feels cold, clutches blankets and curls up shivering and uncommunicative. (The chill). Within minutes the limbs begin to shake and teeth chatter, and the temperature climbs rapidly to a peak. The rigor usually lasts for 10-30 minutes but can last upto 90 minutes.

Hot stage

By the end of rigor there is peripheral vasodilatation and the skin feels hot and dry. The temperature is high.

Sweating

Profuse sweat then breaks out. It lasts for 2-4 hours. The patient is soaked in sweat and the temperature falls. The blood pressure is relatively low. The patient feels exhausted and may sleep. Defervescence usually takes 4-8 hours. Fever is irregular at first with temperature exceeding 39 degrees centigrade. It may rise upto 40^0C.

If the infection is left untreated:

- Fever recurs every third day in *P. vivax* and *P. ovale* infection establishing a 2-day cycle (tertian).
- Spike occurs every three days (Quartan) in *P. malariae* infection i.e. fever every fourth day.
- The pattern of fever in *P. falciparum* infection is erratic. Paroxysms with rigors are more common in *P. vivax* and *P. ovale* than in *P. falciparum* and *P. malariae* malaria. True rigors are unusual in naturally acquired falciparum malaria.
- As the infection continues the spleen and liver enlarge and anemia develops. The patient loses weight. If no treatment is given the natural infection stabilizes for several weeks or months and then gradually resolves.

Relapse

Relapse is the return of a disease after its apparent cessation. Both *P. vivax* and *P. ovale* have a tendency to relapse after resolution of a primary infection. Relapses occur weeks or months after the primary infection.

Recrudescence

Recrudescence is the recurrence of symptoms after temporary abatement. The chief distinction between a recrudescence and relapse is the time interval, a recrudescence occurring after a few days or weeks, a relapse after some weeks or months. *P. falciparum* is the usual cause of recrudescence infection and these arise 2-4 weeks following treatment.

Complications in malaria

Severe falciparum malaria

Severe, life threatening malaria is nearly always caused by *P. falciparum*. The following conditions have been proposed as manifestations of severe falciparum malaria by a working group convened by the World Health Organization (WHO).

1. **Cerebral Malaria**
   The clinical symptoms of cerebral malaria are coma, convulsions, severe anaemia (particularly in children), muscle tone may be increase or decrease, fever, jaundice (adults), enlarged liver and spleen, retinal hemorrhage on opthalmoscopy. Untreated cerebral malaria is probably uniformly fatal. Overall mortality in treated cerebral malaria in reported studies average 15% in children and 20% in adults (but upto 50% in pregnant women).

2. **Hypoglycaemia**
   - May be asymptomatic
   - May present as further deterioration in the level of coma

3. **Pulmonary oedema**
   · Hyperventilation (respiratory distress)

4. **Acute renal failure**
   - Common complication of malaria in adults living in areas of low or unstable transmission
   - Oliguria or polyuria
   - Jaundice
   - Bleeding tendency

5. **Metabolic acidosis**
   - Hyperventilation with increased inspiratory effort
   - Hypotension

6. **Blackwater fever**
   Blackwater fever is a condition where:
   - After several bouts of falciparum malaria, particularly if there has been inadequate treatment, there is occasionally an abrupt onset of massive intravascular hemolysis with fever, chills and prostration.
   - The hemoglobin escapes into the urine turning it black, if the urine is acidic.

7. **Algid malaria**
   Algid malaria is a malarial condition where:
   - There may be subnormal temperature, weakness, prostration, feeling cold, vomiting, rapid respiratory and oliguria.
   - Death may occur but the patient is conscious till the end.
   - It could be due to adrenal crisis, absorption of endotoxin from the gut or cachectin-tumor necrosis factors from endotoxin activated macrophages.

8. **Malaria in pregnancy**
   - There is increased risk of severe falciparum malaria in the second and third trimester of pregnancy.
   - In areas of less transmission, it is an important cause of fetal death and results in high maternal morality.
   - In areas of intense transmission, it may be associated with low birth weight. The infected mothers may be asymptomatic.

9. **Malaria in children**
   - The majority of childhood malaria infections present with fever and malaise. In addition to the clinical features mentioned for adults, malaria in children may lead to convulsions, coma, hypoglycemia, metabolic acidosis and severe anaemia.

In highly endemic areas, the patient may be infected with one, two or even more species of the malaria parasite.
Tropical splenomegaly syndrome (TSS) is often the result of chronic malaria and has its own attendant complications.

Differentiating features of the" Big Two" (*P. vivax* and *P. falciparum* malaria)

Sr. No.	*P. vivax*	*P. falciparum*
1	*P. vivax* is relatively **benign** and rarely produces serious complications or death	*P. falciparum* on the other hand, is associated with **serious complications** e.g. cerebral malaria, jaundice, renal failure etc. including high mortality
2	In *P. vivax*, **relapse** occurs due to persistence of inactive forms (hypnozoites) in liver tissues which periodically invade blood stream producing clinical malaria	*P. falciparum* does not have any dormant form in liver and once infection is cured, there is no relapse
3	In *P. vivax* malaria, less than 1% of **RBC's** are parasitised	In *P. falciparum*, the number of RBC's involved may go up to 35%
4	Mainly the young RBC's are infected	Infects young and old erythrocytes alike
5	The **gametocytes** (sexual stage) – male and female mature in **peripheral blood** and are sucked up by the female Anopheles mosquito for completion of their life cycle	For maturation of gametocytes, *P. falciparum* **must invade deeper circulation**. The blood capillaries of internal organs get clogged with infected RBC's thus obstructing flow of blood Also some biochemical changes take place which damage the organ
6	Gametocytic stage persists in the peripheral blood for 2 days	Gametocytes persists in the blood for 30-60 days or more

Fig. 3. Difference Between the 'Big Two'

Drug resistant malaria

- Literally speaking, drug resistant malaria means malaria caused by a *Plasmodium* species resistant to usual antimalarial drugs.
- Although chloroquine resistant strains of *P. vivax* have been described, drug resistance poses a serious clinical problem only with *P. falciparum,* which accounts for over 70% of cases and much of the mortality of human malaria.

Indian scenario of drug resistance malaria:

- Of the two plasmodia which causes malaria in India, incidence of drug resistance is more common with *P. falciparum*.
- Occasionally, *P. vivax* may also be drug resistant and this occurs specially as a result of improper treatment and inadequate dosage.
- Originally, both the *Plasmodia – vivax* and *falciparum* – were sensitive to chloroquine, but, in recent years, more and more *P. falciparum* are developing resistance against chloroquine.
- To overcome this problem of chloroquine resistance, sulphadoxine, and pyrimetamine combinations was used. But very soon, some strains of falciparum developed resistance to this combination also.
- *P. falciparum* resistant to traditional drugs like Quinine have also been reported.
- Incidence in India will be difficult to know because in may cases it may not be recorded.
- Resurgence of *P. falciparum* resistant to chloroquine has been noticed in several regions of India. Earlier reports indicated chloroquine resistance to *P. falciparum* in North Eastern parts of the county with new foci of drug resistance being added gradually.

  However, for all practical purposes, drug resistant malaria in the Indian context means malaria caused by strains of *P. falciparum* which are resistance to chloroquine.

Clinical and Laboratory Diagnosis of Malaria

Several approaches have been adopted to detect malaria

- **Clinical diagnosis**

  Clinical diagnosis is still the most widely used approach in the developing world, particularly in rural areas and in the peripheral health care system that lack laboratory facilities. Inhabitants of endemic areas are sometimes familiar with the symptomology and frequently, self diagnose malaria. Though pure clinical diagnosis is inexpensive to perform, since the malaria symptoms are non specific and mimic other febrile illness, diagnosis and treatment of malaria on clinical basis alone is unreliable and should be supported by laboratory test results as far as possible.

- **Microscopic diagnosis**

  Microscopy of thin and thick blood smears for detecting malaria parasites with non specific chemical stains is still the frontline and established method for the laboratory confirmation of malaria.

  Well prepared and well stained blood films read by careful and experienced microscopists is still considered as the gold standard for detecting and identifying malaria parasites. In the hands of an expert and skilled technician microscopy can detect as low as 10 parasites per µl of blood. Apart from being helpful in speciation, morphological alterations can be read to assess the parasitaemia, as well as efficacy and response to chemotherapy. While microscopy is relatively inexpensive it does suffer from a few important short comings, such as;

  - In field conditions, typical microscopic procedures may not be able to detect parasitaemia under 100/µl of blood.
  - Microscopy is labor intensive and each slide may take about 60 minutes from blood collection to being read and may involve delay in providing results.
  - Reliable microscopy results are dependent absolutely on individual microscopist's skills, techniques, microscopes and reagents which vary inherently especially in the rural and peripheral areas, where the aforesaid variables may not be controlled well.
  - Paradoxically, microscopy which is a 'Gold Standard' can be an unreliable tool that may lead to doubtful results.

  Delay in providing results, inherent variability of the method and risk of unreliability may in fact revert the clinicians back to pure clinical diagnosis of the disease.

  In view of the limitations of pure clinical diagnosis and the microscopic methods, a need has been felt for specific, simpler and rapid tests for laboratory diagnosis of malaria. Preferably tests that are versatile enough to be used in:

  - A cross section of laboratory settings
  - Field conditions
  - Simple to perform
  - Independent of technician skill
  - Rapid enough to accord the benefit of malaria laboratory results for diagnosis and treatment.

Key Factors affecting Diagnosis and treatment of Malaria

- Microscopic detection suffers from variability assay arising out of microscopist skill, techniques, reagents equipment
- At least one hour needs to be spent by the expert microscopist to rule out negative smear results
- Issues of due diligence vs urgency.
- Ability to speciate correctly.

- Patient history/ especially travel details
- Knowledge of the malaria species endemic to the area of travel and where the infection was contracted
- Clinical picture of the patient
- Rule out overlapping febrile illness

- Drug regimen required for adequate effective treatment
- Drug regimen required to prevent relapse/ recrudescence
- Geographical/ drug resistance patterns
- Follow up of patient to assess clearance of parasitaemia, rule out drug resistance, establish efficacy of treatment.

Microscopy Interpretation Chart for Malaria Diseases

Stage (or period of infection)	*P. vivax*	*P. malariae*	*P. falciparum*	*P. ovale*
Early trophozoites	1/3 diameter of RBC; prominent vacuole; heavy chromatin	Single chromatin dot; vacuole less common than other species; cytoplasm "heavy"	1/5 diameter of RBC; small chromatin; marginal forms frequent	Similar to *P. vivax* and *P. malariae*
Late trophozoites	Large amount of chromatin; hemozoin almost fills cell	Cytoplasm dense, round oval or band shape; nearly fills cell	Not usually seen in peripheral blood	Compact cytoplasm; small (if any vacuole)
Hemozoin	Short rods, scattered irregularly; yellowish brown on color	Rounded; larger, darker than in *P. vivax*; often peripheral	Granular; coarse in gametocytes	Lighter than in *P. malariae* ;similar to *P. vivax*
Erythrocytes	Larger than normal, irregular shaped; Schüffner's dots apparent in all but earliest stages; multiple infections common	About normal; stippling and multiple infections are rare	Normal size; Maurer's dots often in late trophozoites (late trophs rarely seen in peripheral blood	Schüffner's dots often present; red cell often enlarged and shaped irregularly
Schizont	12-24 merozoites; hemozoin clumped; often fills cell	8-10 merozoites in a rossette or cluster	8-24 merozoites (rarely seen in peripheral blood)	4-16 merozoites
Microgametocytes (usually smaller and less common than macrogametocytes	Rounded or oval; almost fills cell; hemozoin evenly distributed; chromatin clumped; minimal cytoplasm; no vacuoles	Similar to *P. vivax* but smaller; pigment more conspicuous	Crescent shaped; about 50% larger than blood cell; chromatin diffuse; hemozoin central; pale blue cytoplasm	Similar to *P. vivax* but smaller
Macrogametocytes	Similar to microgametocyte but cytoplasm darker blue; chromatin more compact and red	Prominent pigment; round, dark brown granules; course than in *P. vivax*	Similar in size and shape to microgametocyte; chromatin red, more compact; hemozoin concentration	---
Exoerythrocytic cycle	8 days	13 days	6 days	9 days
Prepatent period (minimum)	11-13 days	15-16 days	9-10 days	10-14 days
Schizogonic cycle	48 hours (tertian)	72 hours (quarten)	36-48 hours (tertian)	48 hours (tertian)
Development in mosquito	10 days	25-28 days	10-12 days	14 days

Some characteristics of Plasmodium species infecting human (Modified after Roberts. L.S. and Janovy J.Jr. (1996) Foundation of Parasitology, 5th Edition, Wm C. Brown Publishers)

Rapid Diagnostic Tests for Malaria

Considering the expertise and skill that is required to detect and speciate malaria microscopically, there has been a pressing need to have sensitive and specific diagnostic tests to serve as simple and rapid tools for onsite diagnosis. Research efforts have been targeted specifically at this task. Advancements in protein purification, monoclonal antibody production as well as nitrocellulose membrane technology have lead to design and development of specific diagnostic platforms fulfilling this aim, in the form of Rapid Diagnostic Tests, also popularly known as **RDT's**.

Isolation of Malaria Parasite Antigen

The discovery and isolation of many specific malarial parasite antigens from the blood of infected individuals and the development of corresponding antibodies raised and directed against these target parasite antigens has led to the development of several diagnostic platforms for the specific and rapid detection of malaria infection.

The two most important antigenic markers targeted for detection by the rapid diagnostic tests are

- Histidine rich protein II (HRP-II) specific to the *P. falciparum* malaria.
- Parasite Lactate dehydrogenase (pLDH).

Antibodies to The Antigen Markers of Malaria

To date, the following antibodies are being used to detect malarial specific antigens in human blood:

- Antibodies for HRP-II (Histidine rich protein 2) specific for *P. falciparum*
- Antibody to pLDH (parasite lactate dehydrogenase) specific to *P. falciparum* malaria
- Antibody to pLDH (parasite lactate dehydrogenase) specific to *P. vivax* malaria
- Antibodies to pan malaria pLDH (parasite lactate dehydrogenase) co-specific to all four malarial species *P. vivax P. falciparum*, *P. ovale* and *P. malariae*.

While antibodies specific to parasitic Aldolase have also been isolated, however due to the poor sensitivity, the use of Aldolase has not found favor with manufacturers or users lately.

Antibodies to HRP –II (*P.falciparum* specific antigens) and antibodies to *P.falciparum* specific pLDH and antibodies to pan pLDH (co-specific to pLDH for all malarial species) have been, in recent times the back bone for assay design of all malaria rapid test being used currently. Recently, development of monoclonal antibodies specific to *P.vivax* specific pLDH have opened up a new avenue for a better and more advanced assay design for the immunodiagnosis of malaria RDTs. The malaria RDTs are grouped in terms of 'Generations" from the point of view of their ability to speciate between the different malarial infections.

First Generation Rapid Diagnostic Tests For Malaria

P. falciparum specific Histidine Rich Protein II (Pf. HRP II) based assays

HRP II is a water-soluble protein produced by trophozoites and young (but not mature) gametocytes of *P. falciparum.*

The amount of P.f HRP II increases throughout the intraerythrocytic cycle of the infection, with the largest amount being released during schizont rupture. On an average 9-12 days after the mosquito bite P.f HRP II is found in the circulation coinciding with the overt clinical malarial symptoms.

P.f HRP II based assays which are well formulated achieve sensitivity and specificity for *P. falciparum* malaria detection well in excess of 99% as compared to expert microscopy.

The test runs on a 5 µl whole blood sample. Results are obtained within 15 minutes. These 'first generation' tests are in intensive use by various International organizations for RBM programs, NGO's and laboratorians globally.

These tests are available in a cassette as well as a dipstick format.

Principle of P.f HRP II based assay

Principle of P.f HRP II based assay P.f HRP II utilizes the principle of immunochromatography. As the test sample flows through the membrane assembly of the dipstick or device after placing the clearing buffer, the colored anti P.f HRP II antisera-colloidal gold conjugate (monoclonal) complexes the P.f HRP II in the lysed sample.

This complex moves further on the membrane to the test region where it is immobilized by the anti P.f HRP II (monoclonal) antisera coated on the membrane leading to formation of a pink colored band which confirms a positive test result.

Absence of this colored band in the test region indicates a negative test result. The unreacted conjugate and unbound complex if any, moves further on the membrane and are subsequently immobilized by anti rabbit antibodies coated on the membrane at the test region, forming a pink band. This control band serves to validate the test performance.

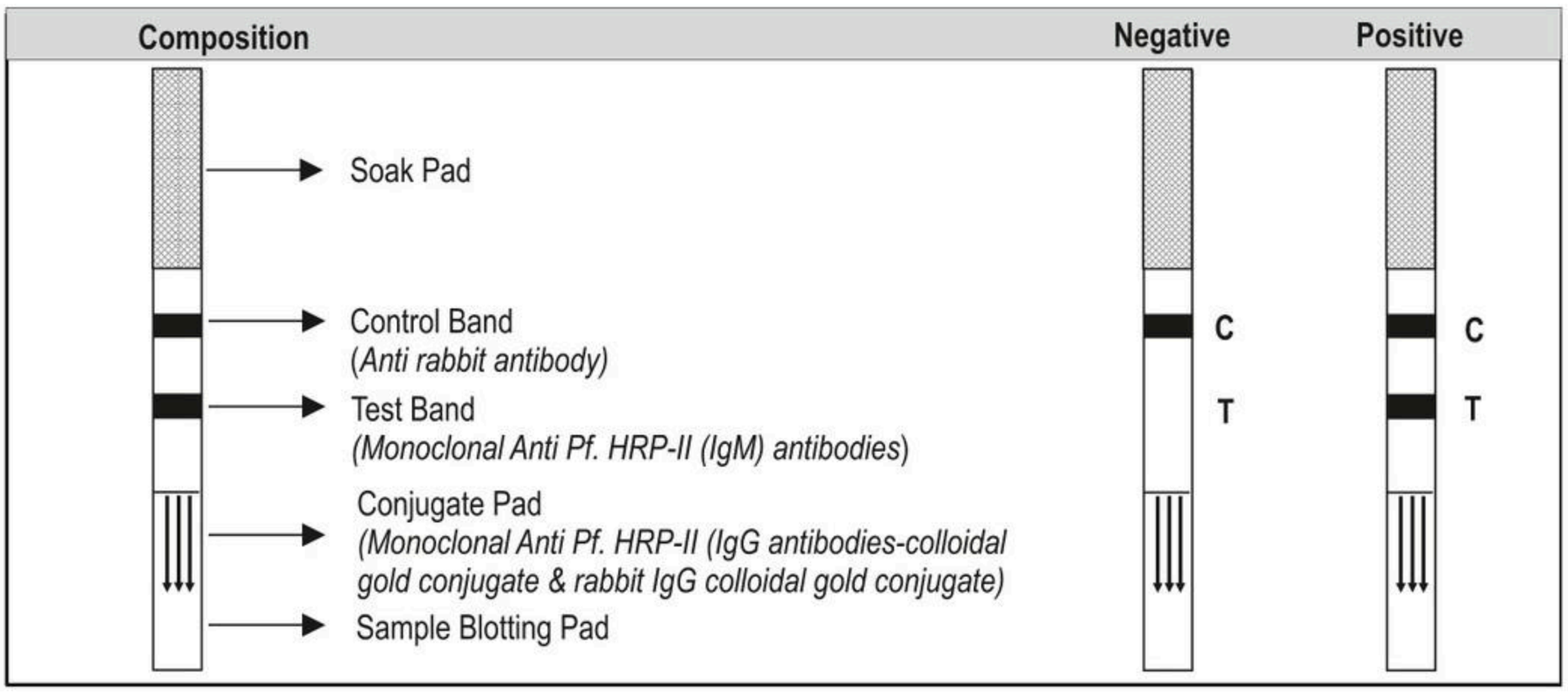


Fig. 4. Principle of HRP-II RDT

Plasmodium Lactate Dehydrogenase (pLDH)

pLDH is a soluble intracellular metabolic glycolytic enzyme that is expressed at high levels in blood stage parasites. All the four human malaria parasites produce a specific and unique pLDH activity as well as co-specific pLDH activity that follows the level of parasitaemia in patient plasma as measured by microscopy.

pLDH is a good antigenic marker for active malarial infection. A panel of monoclonal antibodies raised against the pLDH; when used in the right combination are capable of distinguishing and binding specifically pLDH isoforms from either *P. falciparum* or *P. vivax*, allowing pLDH based malaria detection assays to correctly speciate *P. falciparum* from non *P. falciparum* malaria in lysed whole blood samples. Antibody to pan malaria antigen, that is co-specific to pLDH from all malarial parasites (*P.vivax*, *P.falciparum*, *P. malariae* and *P.ovale*) has also being utilized in the development of RDT's for malaria.

Principle of pLDH based assay

1. HRP-II + pan pLDH combination RDT

HRP-II + pan pLDH combination RDT utilizes the principle of immunochromatography. As the test sample flows through the membrane assembly of the dipstick/device after addition of the clearing buffer, the colored anti HRP-II/ anti pan specific pLDH colloidal gold conjugate (monoclonal) antisera complexes the HRPII/ pLDH in the lysed sample.

This complex moves further on the membrane to the test region where it is immobilised by the monoclonal anti HRPII/ anti pan specific pLDH antibody coated on the membrane leading to the formation of purple colored band/s which confirms a positive test result. While both bands appear at the test region in falciparum positive samples, only one band appears in non-falciparum malaria positive samples.

Absence of this colored band/s in the test region indicates a negative test result. The unreacted conjugate and unbound complex, if any, move further on the membrane and are subsequently immobilised by anti rabbit antibodies coated on the membrane at the control region, forming a purple band. This control band serves to validate the test performance.

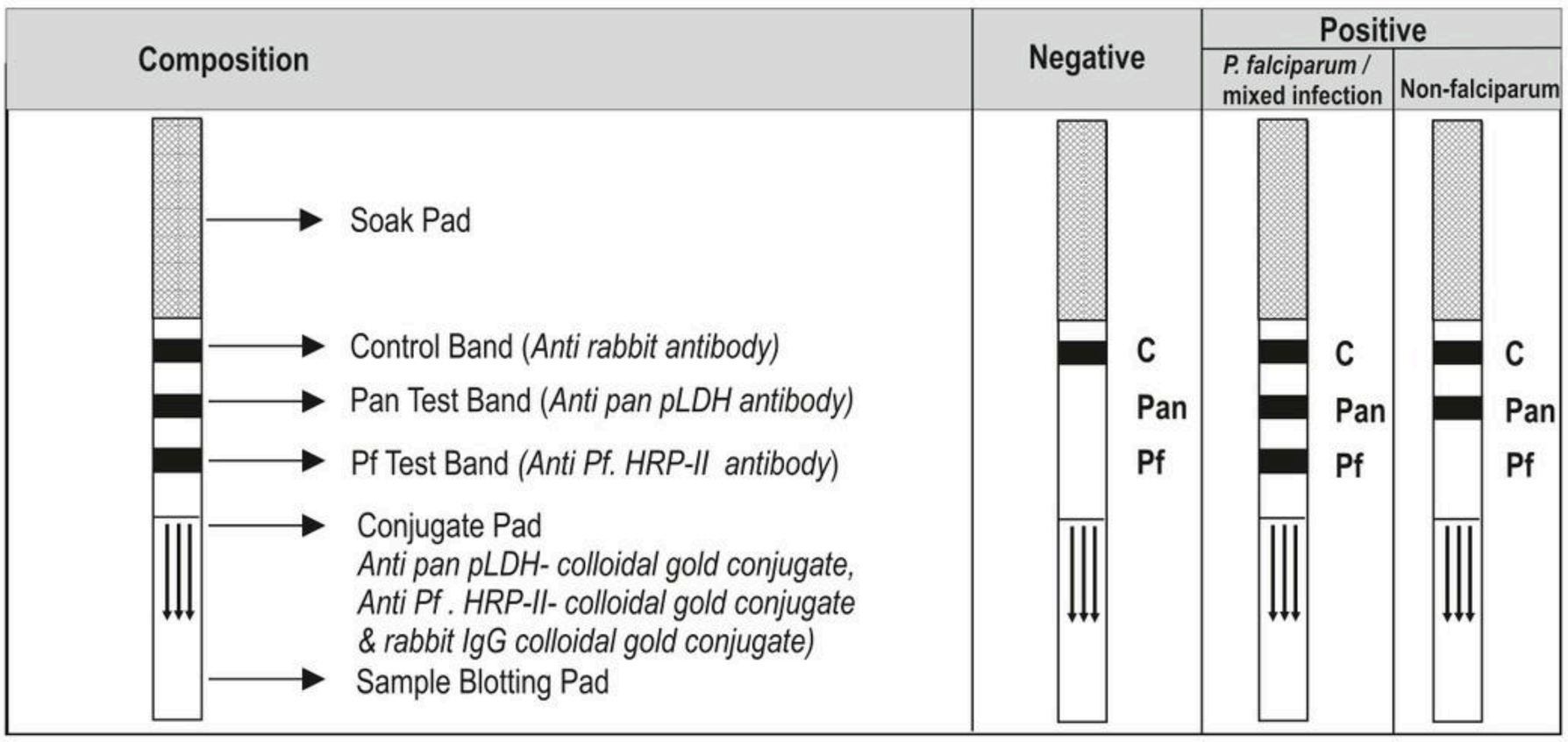


Fig 5: Principle of HRP II + pan pLDH RDT

2. Pf. pLDH + pan pLDH combination assay

Pf. pLDH + pan specific pLDH combination are usually two-component systems comprising of a conjugate well and a test membrane dipstick, is based on the principle of immunochromatography. The conjugate well contains an indicator-tagged monoclonal antibody to pLDH, dried onto its surface. The anti pLDH antibody is specific to all the isoenzymes LDH of the genus Plasmodium (pan pLDH)

The membrane assembly contains three regions each coated with monoclonal anti-pLDH specific to *P. falciparum* monoclonal anti-pan pLDH and anti-rabbit antibodies respectively.

The blood sample is added to the conjugate wells along with the lysing buffer, pLDH if present in the sample reacts with the anti-pLDH conjugate in the well and rises up the membrane dipstick where it is captured by anti Pf. Specific pLDH and /or anti-pan specific pLDH, causing the appearance of a colored band at the respective regions.

Absence of band(s) at this region indicates a negative test result. The unreacted conjugate reacts with the anti rabbit antibodies at the control region and serves to validate the test performance.

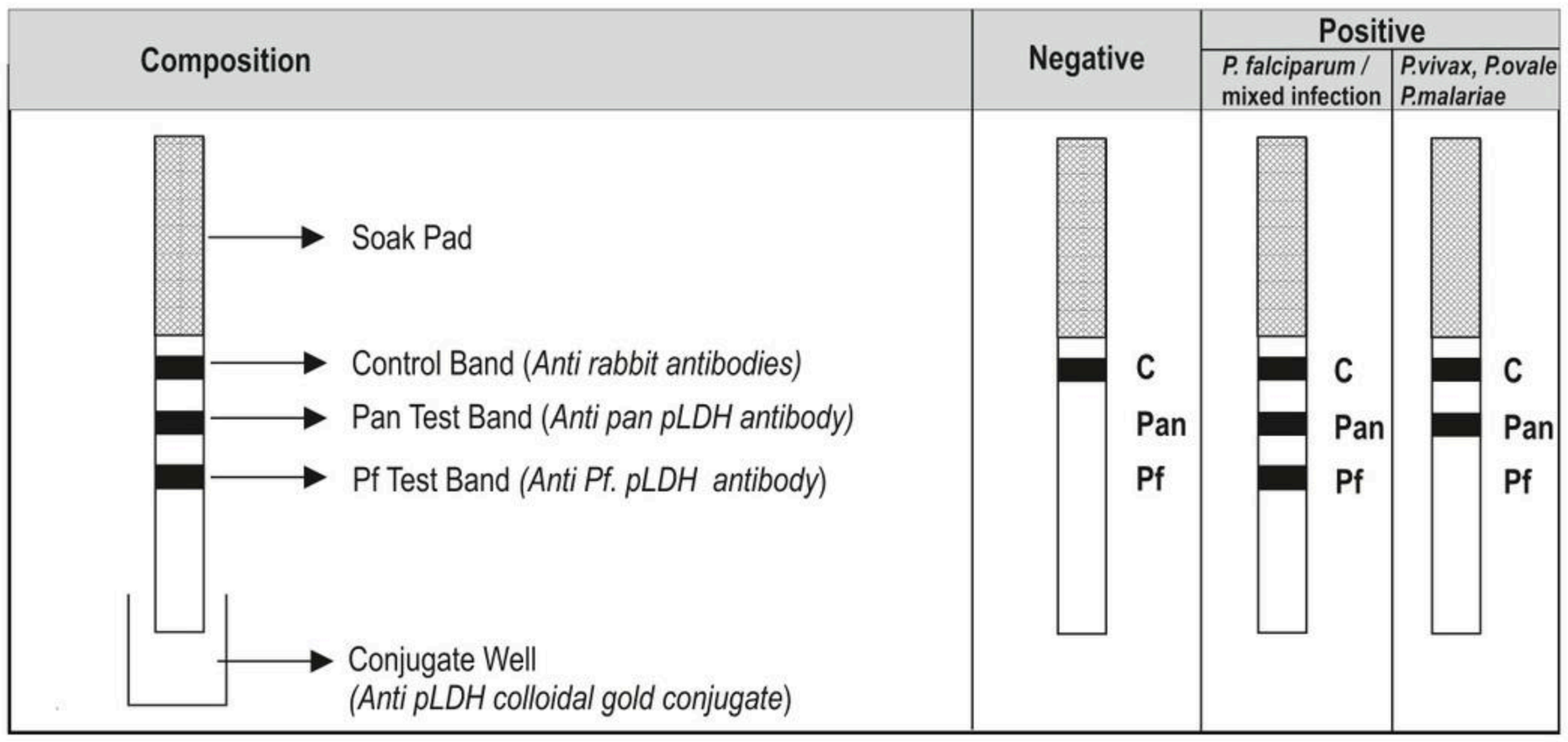


Fig 6: Principle of Pf pLDH + pan pLDH RDT

Limitations of First Generation Rapid Diagnostic Tests For Malaria

The stand alone rapid tests for *P.falciparum* malaria test based on HRP –II detection achieve excellent sensitivity and specificity for the detection of *P. falciparum* malaria. However, they do suffer from the following limitations:

- The detection is limited to *P. falciparum* malaria only
- These tests remain positive for 7-14 days following successful antimalarial therapy, even though these patients no longer have clinical symptoms or parasitaemia.
- Since HRP-2 is secreted only in asexual erythrocytic schiozogony stage, the immunochromatographic test that detect the presence of Pf. HRP-II would give a negative reaction if mature gametocytes are present due to the absence of the antigenemia Pf. HRP-II.

On the other hand, tests for the detection of *P. falciparum* and differentiation of nonfalciparum malaria test based on *P. falciparum* specific pLDH and pan malaria co-specific pLDH have the following limitations:

When the patient specimen is positive for *P.falciparum* malaria, the pan malarial band is also positive.

- Differentiation between *P. falciparum* infection and mixed infection is not possible.
- Speciation under microscopy must still be done for correct therapy especially in hyper endemic areas where mixed infection rates are high

When *P. falciparum* band is negative and the pan band is positive

The correct interpretation is "non – falciparum malaria".

- In the absence of speciation under microscope the presence of pan band has to be interpreted in the context of prevalence and infection rates for the other non falciparum species.
- *P.falciparum* negative samples still need to be speciated under microscopy for correct therapeutic decision to pinpoint as to whether the positivity of the pan band is due to *P.vivax*, *P.ovale* or *P.malariae* .

Experience with HRP-II and pLDH for the detection of *P. falciparum* malaria

Currently first generation malaria test based on stand alone detection of HRP-II for *P.falciparum* malaria and pLDH. *P. falciparum* and pan pLDH (non falciparum) combined alike have been used extensively for the detection of malarial globally. Experience of users in field settings as well as in reference labs have bought out a few important characteristics of antibodies to HRP-II via a vis pLDH for *P.falciparum* detection as well as for the detection of *P.vivax* malaria through pLDH based systems.

The general conclusion being that:

- Tests for *P. falciparum* malaria based on detection of HRP-II have on an overall found to be superior to *P. falciparum* specific pLDH.
- The detectability of *P. falciparum* infections with lower parasitaemia, is better with HRP-II based systems as compared to pLDH based system.
- The tests utilize pLDH specific to *P. vivax* have a relatively poor sensitivity for *P. vivax* infection.
- Test using similar antibodies to the malarial antigen may yet have different sensitivities and specificities probability due to over all system design competence amongst manufacturers.
- The heterophile antibody interference in the assay system have lead to an unacceptable level of false positives with some assays
- At very high parasitemia , some assays show ' Hook or prozone" effect leading to false positives.
- The test must have robustness to withstand the rigors of field conditions in terms of storage conditions and requirements of additional material for performance of the tests.

Assay design considerations for Second Generation rapid test for malaria

As discussed earlier in this text *P. falciparum* malaria is no doubt the most morbid of all malarial associated with significant mortality and drug resistance . Also P. vivax malaria, though not as severe as *P. falciparum* in mortality is associated with significant morbidity. Together 90% of the malarial infections are caused by these two parasites leading to tremendous economic burden due to lost mandays and cost of therpeutics.

Thus it is imperative that any advancements in rapid diagnosis of malaria should ideally speciate all four malarial species and if not , at least the "**Big two**" that is *P. falciparum* and *P. vivax* .

Thus second generation test for malaria should:

- Speciate and differentiate between *P.falciparum* and *P.vivax* malaria
- Should have good clinical sensitivity for P.*falciparum* and *P.vivax* malaria
- Should also be able to monitor success of anti-malarial therapy
- Should be stable at ambient temperatures, without the need for additional materials for field testing adaptibility
- Should be free from 'hook' effect
- Should be free from interference from Rheumtoid factors and heterophile antibodies

It is imperative that the second generation RDTs for malaria should meet all the assay design requirements and help speciate between the '**Big Two'.** Third generation tests likely to be available in the not-too distant future would be able to conserve all the advantages of the second generation tests but would be able to conserve all the advantages of the second generation tests but would also improve upon the same by being able to speciate clearly between all the malarial species namely; *P.falciparum, P.vivax, P.ovale* and *P. malariae.*

Until such time, the choice and use of available format of rapid diagnostic tests for malaria detection should be based on adequate evaluation of the existing health delivery systems as well as the species prevalence rates. Employment of the type of laboratory diagnosis of malaria, including the use of RDTs should be based on the following criteria:

- Prevalence rates of various species.
- Areas of endemic malaria and in situation which call for rapid confirmation of infection.
- Inadequate laboratory services (inexperienced and /or non-expertise laboratory technicians
- Excessive time lag for diagnosis
- Mobile clinics/ on-field health workers
- Severe and complicated cases in which peripheral parasitemia may be absent but wherein the rapid tests may be expected to provide evidence of antigenemia

In malaria patients, a prompt and accurate diagnosis is the key to effective disease management. The two diagnostic approaches currently used most often, however, do not allow a satisfactory diagnosis of malaria. Clinical diagnosis, the most widely used approach, is unreliable because the symptoms of malaria are very non-specific. Microscopic diagnosis, the established method for laboratory confirmatory of malaria, presents technical and personnel requirements that cannot be met, particularly in facilities at the periphery of the healthcare system. In addition, delays in the provision of the microscopy results to the clinician means that decisions on treatment may be taken without the benefit of the results.

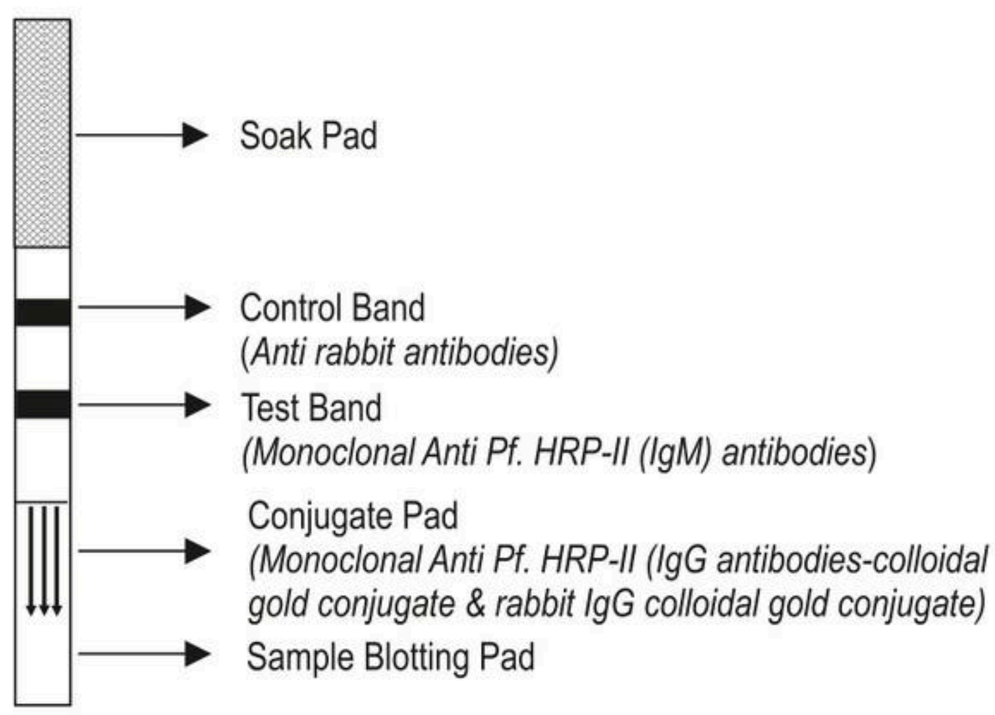
Thus, the recent introduction of rapid diagnostic tests (RDTs) for malaria is of considerable interest.

It is expected that with the availability of second generation and third generation RDTs, detection, speciation and diagnosis of malaria would be simpler and easier, leading to swift detection and treatment of malaria globally.

Rapid Diagnostic Tests for Malaria; Formats- Pros and Cons

First Generation

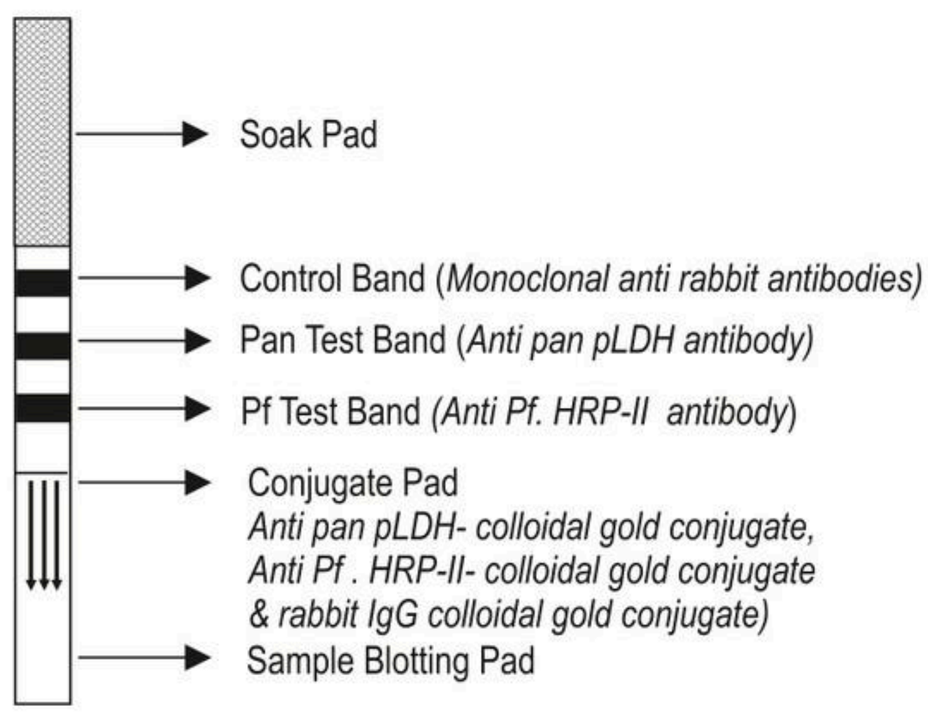
Pf. HRP-II stand alone RDT



Pros
- Sensitivity and specificity >98% for *P. falciparum* malaria
- Results comparable with Gold standard
- No cross reactivity with other species
- Correlates with on-set of clinical symptoms

Cons
- Stand alone test for *P.falciparum*
- Cannot be used for monitoring therapy
- Cannot identify' Carriers' due to absence of HRP-II in gametocytes

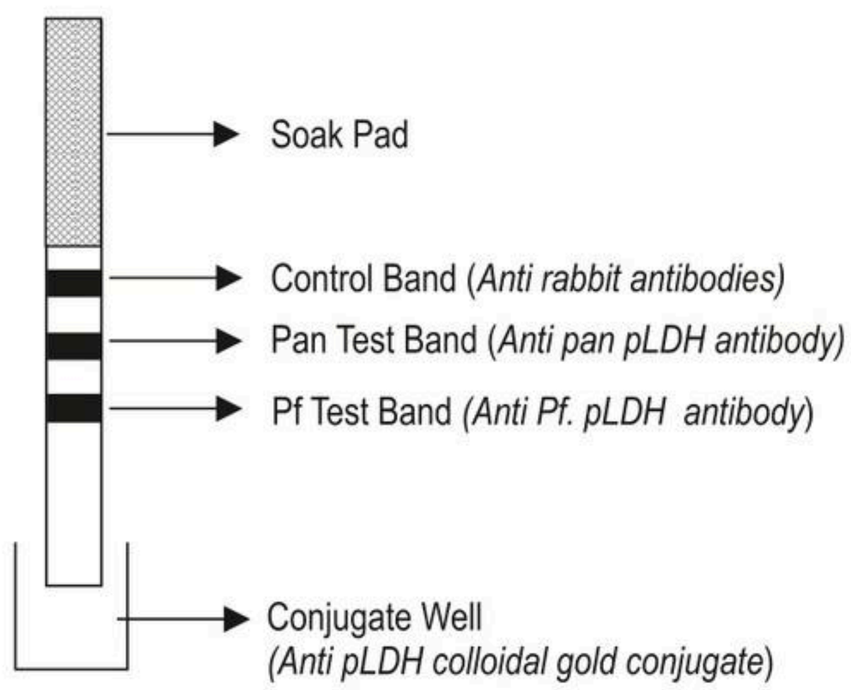
Pf.HRP-II/ Pan pLDH combination RDT



Pros
- Pan band specific for all 4 malarial species
- High sensitivity for *P. falciparum*

Cons
- Differentiation between stand alone *P. falciparum* & mixed infections is not possible
- In endemic areas of high prevalence of mixed infection, need for speciation under microscopy for correct therapy
- Presumption fo causative species based on the presence of pan band and species prevalence rate

Pf. pLDH /Pan pLDH combination RDT

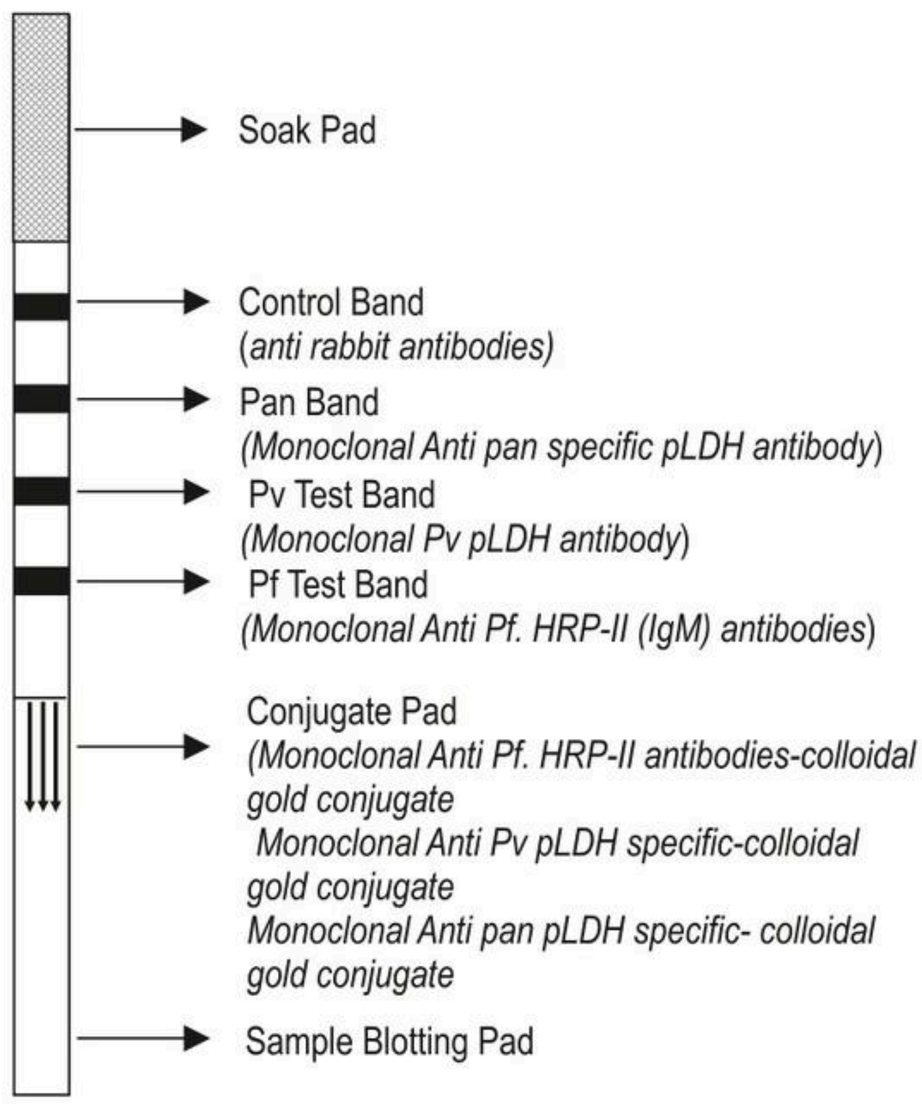


Pros
- Pan band specific for all 4 malarial species

Cons
- Relatively lower clinical sensitivity for *P. falciparum* and *P.vivax* malaria
- Differentiation between stand alone *P.falciparum* and mixed infection
- In endemic areas of high prevalence of mixed infection, need for speciation under microscopy for correct therapy
- Presumption of causative species based on the presence of pan band and species prevalence rate

Second Generation

Pf. HRP-II/ Pv pLDH/Pan pLDH combination RDT



Pros

- Can speciate and differentiate between the " **Big Two**"
- Good clinical sensitivity and specificity >98% for *P. falciparum* malaria, due to Anti HRP-II based detection
- Able to monitor success of anti-malarial therapy, through pan pLDH band
- Overcomes limitations associated with stand alone HRP-II based assays

Cons

- Cannot speciate and differentiate *P. malariae* and *P. ovale*

Management of Malaria

Management of Clinical Malaria (Febrile Episode)		Prevention of Relapse (*for P.vivax*)
Uncomplicated	Complicated	Primaquine
P. vivax/ P. falciparum	*P. falciparum*	
Chloroquine Sulphadoxine + Pyrimetamine Quinine sulphate (Tab)	Quinine Diydrochloride inj. Halofantrine tabs Artemether/Artesunate/ Arteether /Mefloquine	

Management of Drug resistant malaria

Patients Not Seriously ill (Can take oral medications)	Patients Critically ill (Cannot take medications)
Sulphadoxine/ Pyrimetamine combination Quinine Artemisinin and related compound Mefloquine Amodiaquine (use limited because of severe hepatotoxicity)	Quinine Dihydrochloride inj. Artemether injection

General Prophylactic drugs used
Chloroquine Chloroquine + Proguanil Mefloquine Doxycyline

Drugs in various combination are used for treatment and prevention of further transmission of parasites. Many vaccines are also under clinical trials, to impart immunity against malarial infections.

KNOWLEDGE BOOK

For the Laboratorians

Malaria RDT's

Performance Evaluation and Quality Assurance

Setting Trends...

Background

Early rapid diagnosis of malaria is crucial to the healthcare programmes in endemic countries. Their increasing importance is in response to increasing drug costs and recognition of the importance of early and correct treatment to attain reduction in malaria morbidity and mortality.

Malaria RDT's in the last decade have played a vital role in this regard in reaching objective treatment to affected populations even in resource poor settings, where the traditional microscopic diagnosis is either impractical or impossible.

In the last decade there has been an increase in the operational use of RDT's as well as an increase in number of companies manufacturing or dealing in malaria RDT's.

Wide range of field and laboratory trials have been conducted in the last decade to assess the accuracy and effectiveness of various products to establish their performance, quality and appropriateness for use in the endemic areas.

These trials have given the users and decision makers a huge database to review. In addition, local testing in country of use works to further supplement and validate this performance data for decision making.

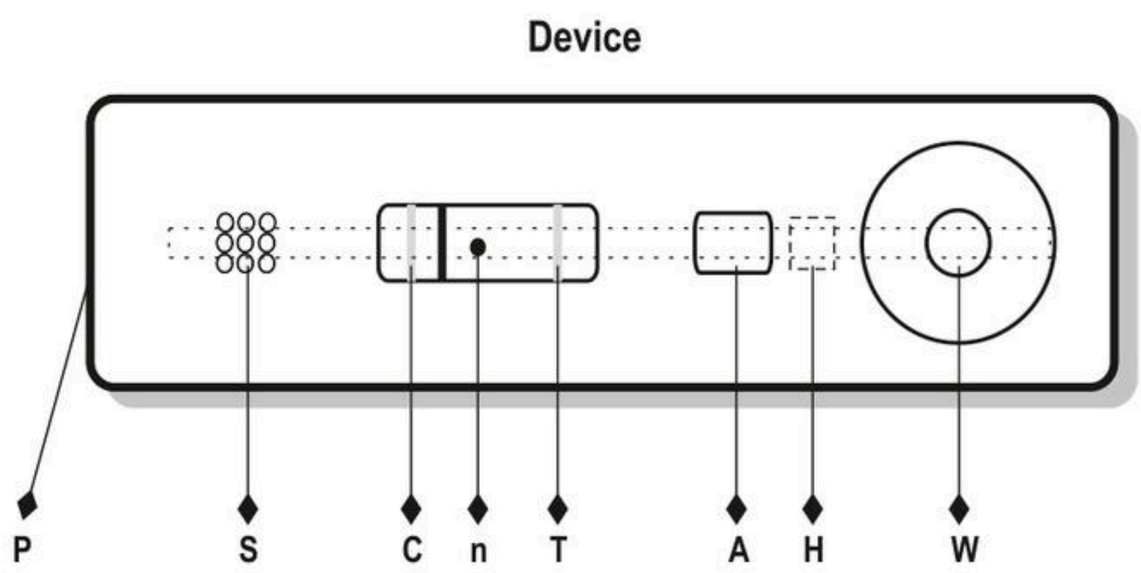
Published field trials of Malaria RDT's have however shown high variability in performance due to the following reasons:

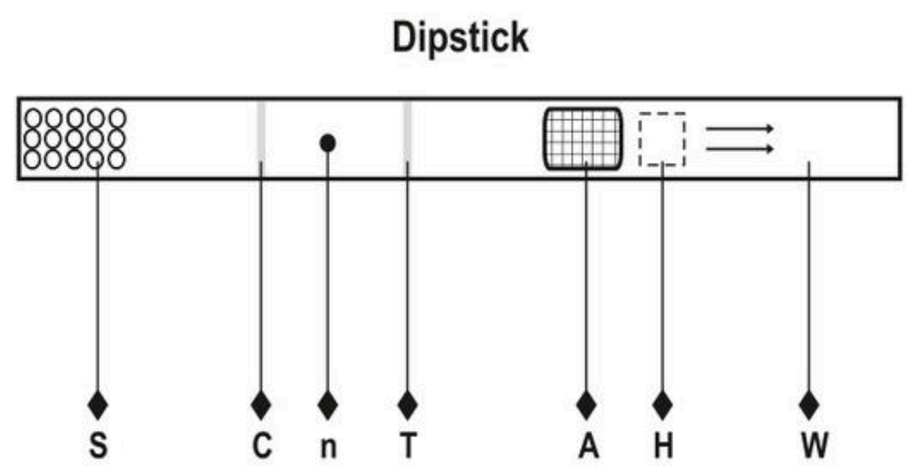
- Poor study methods, analysis & reporting.
- Inexperienced manpower.
- Incorrect handling.
- Poor preparation and interpretation.

Attempts have been made to undertake larger centralized trials to evaluate the performance of Malaria RDT's.

Before discussing the alternative approaches to the performance evaluation of Malaria RDT's, it would be useful to understand the architecture and functioning of Malaria RDT's.

Typical Architecture and Design of Malaria RDT's





The Essential Components of A Malaria RDT:

W = Wicking area for sample / buffer.
A = Conjugate pad containing the gold conjugate to target antigen specific antibody.
C = Control line striped with relevant antibody to give a test run validation.
S = Soak pad that absorbs the unreacted sample post test conclusion.
H = HAMA Blocking Reagents embedded on sample pad.
T = Test line striped to target antigen specific antibody.
n = Nitrocellulose membrane.
P = Plastic housing (for device).

Components to be Used with RDTs:

Desiccant with indicator to ensure moisture free pack.

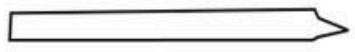
Buffer bottle for test run.

Alcohol swab.

Glass / plastic test tube (for dipstick).

Sample dispensing loop.

Sample dispensing straw.


Lancet for obtaining finger prick blood.

Malaria RDT's: Immunological Considerations

RDT Combinations	Possible Detection Systems	Detects
Pf only	Monoclonal Anti Pf HRP-II specific	*P. falciparum* infection
Pf only	Monoclonal Anti Pf pLDH specific	*P. falciparum* infection
Pf and Pan	Monoclonal Anti Pf HRP-II specific + Monoclonal Anti Pan pLDH specific (co-specific to *P. falciparum, P. vivax, P. ovale* and *P. malariae)*	*P. falciparum* infection & Differentiate *P. falciparum* infection and non *P. falciparum* infection
Pf and Pan	Monoclonal Anti Pf HRP-II specific + Monoclonal Anti Pan Aldolase specific (co-specific to *P. falciparum, P. vivax, P. ovale* and *P. malariae)*	*P. falciparum* infection & Differentiate *P. falciparum* infection and non *P. falciparum* infection
Pf and Pan	Monoclonal Anti Pf pLDH specific + Monoclonal Anti Pan Aldolase specific (co-specific to *P. falciparum, P. vivax, P. ovale* and *P. malariae)*	*P. falciparum* infection & Differentiate *P. falciparum* infection and non *P. falciparum* infection
Pf and Pan	Monoclonal Anti Pf pLDH specific + Monoclonal Anti pan pLDH specific (co-specific to *P. falciparum, P. vivax, P. ovale* and *P. malariae)*	*P. falciparum* infection & Differentiate *P. falciparum* infection and non *P. falciparum* infection
Pf and Pv	Monoclonal Anti Pf HRP-II specific + Monoclonal Anti *P. vivax* pLDH specific	*Speciates P. falciparum* infection & *P. vivax* infection
Pan only	Monoclonal Anti pan pLDH specific (co-specific to *P. falciparum,* P. vivax, P. ovale and *P. malariae)*	All malaria parasite infection
Pf, Pv and Pan	Monoclonal Anti Pf HRP-II specific + Monoclonal Anti *P. vivax* pLDH specific + Monoclonal Anti pan pLDH specific (co-specific to *P. falciparum, P. vivax, P. ovale* and *P. malariae)*	*Speciates P. falciparum* infection, P. vivax infection, Detects non *P. falciparum* and *P. vivax* infections

General Comments:

- All HRP-II detection tests, have a better analytical sensitivity and a lower limit of detection for *P. falciparum* malaria as compared to pLDH and Aldolase based assays.
- Since HRP-II persists in the blood circulation 2-3 weeks even after successful treatment testing within the said "hang over"period will give "false positive results". Hangover period of pLDH assays is much shorter than for HRP-II based assays.
- HRP-II detection systems by and large do display better thermal stability over other detection systems based on pLDH and aldolase. However, product to product the stability may vary.
- pLDH based detection assays have lower analytical sensitivity as compared to HRP-II based assays for detection of *P. falciparum* infections.
- pLDH and Aldolase based assays also have lower thermal stability as compared to HRP-II assays.

Similarities and Differences in Malaria RDTs

Though the various RDT's appear to be similar, they vary considerably in their functioning due to the intrinsic character of the critical components employed. The performance of each RDT is based on the optimization of all these functional components, which vary from manufacturer to manufacturer in terms of their basic characteristics and processing methods employed during manufacture.

Component	Typical function	Impact on RDT
Sample Pad	Usually contains HAMA blocking agents.	Improves specificity by blocking RF and heterophilic antibodies.
Buffer	Brings about lysis of the red cells, especially of the sequestered parasite and release of target antigens.	The lysing efficiency of the buffer • dictates the revelation of the target antigen from the parasitized red cells. • Formulation effectiveness impacts sensitivity of test.
Gold Conjugate	Binds to the Target antigen as the lysed blood flows through the conjugate pad.	Primary binding agent to the target antigen, it influences sensitivity, specificity and binding affinity to target antigen.
Nitrocellulose Membrane	Serves as a migration template /platform for the reaction and directs reactants and reaction kinetics of each test.	**Large porosity membranes:** Reactants move fast. Better background clearance, however, leads to loss of test sensitivity since accords less time for reagents to react and bind. **Small porosity membranes:** Reactants move slowly, slower background clearance, improves test sensitivity as reagents have more time to react and bind.
Test lines	Capture antibody directed towards the specific malaria antigen in the sample.	Secondary binding agent in tandem with gold conjugate; drives sensitivity, specificity and affinity of binding and signal intensity.
Control line	Reacts with excess / unreacted conjugate to validate test run.	Ideally should be the weakest visible intensity to serve as a practical comparator for test intensity.
Soak pad	Absorbs excess reactants at test culmination.	Absorbance capacity impacts back flow of reactants post test completion.

The final optimization of each product will then have an impact on its functioning and the resultant

- Migration and Reaction Kinetics
- Sensitivity
- Specificity
- Thermal stability
- Ease of use

Problems associated with the usage of Malaria RDT's

- RDT's retrieved from cold storage (2-8°C) must be allowed to come to room temperature (ambient temperature, in case of field conditions) before the pouches are opened and the test used. When specimen is added to a cold device, it attracts a 'moisture rush' thereby altering the migration properties of the membrane. If specimen is added to 'cold' devices the blood flow is usually slowed down affecting the background clearance and visualization of test results: especially in specimen containing low target antigen concentration the test result affected. At lower temperatures the antigen-antibody binding is less than optimum, leading to loss in sensitivity and resultant signals.
- Use of specimen transfer devices such as loops and straws are extremely simple to use. However, to ensure accuracy and precision of sample delivery, user training has to be imparted to build usage competence with actual users.
- The end point of a Malaria RDT reaction is qualitative. Its interpretation has an element of personal subjectivity, that introduces variability. What appears as a weak positive to one reader may well be negative for another. Usually “tie break”method or “best of 3” is the best way to resolve interpretative subjectivity. User training, hands on experience in reading and interpreting RDT end points must be assessed for consistency as a prerequisite. This is especially relevant when samples used during evaluation are low analyte concentrations, nearing the detection limits of RDT's are being used that generate very low signal intensities.

Alternative approach to Laboratory Quality Assurance of Malaria RDT's

For large scale comparative trials of malaria RDT's, availability of standardized, fresh and well characterized clinical specimen is essential. However, achieving this has posed significant challenges due to variability linked to specimen collection, preservation and logistics involved.

To overcome these challenges, centralized evaluations with regards to the performance evaluation of malaria RDT's have used alternative methods. These methods have involved alternate materials such as cultured *P. falciparum* parasites at different parasitic densities, wild type *P. falciparum* and *P. vivax* specimen etc. These samples have been diluted and cryo-preserved before use. During evaluation the panels so prepared have been thawed and used to assess the performance of the malaria RDT's with regards to their performance especially towards sensitivity, specificity and other characteristics.

Effect of Clinical Specimen Quality on Performance of Malaria RDT's

Malaria RDT's are designed and standardized primarily for testing specimen obtained from fresh capillary whole blood in field conditions, and blood correctly collected through veni puncture in laboratory settings. Venous blood collected in appropriate anticoagulants, when stored at 2-8^0 C may be stable for up to 72 hours provided they are not contaminated.

Unhindered sample flow is at the heart of the performance of a malaria RDT. When the sample does not flow on the Malaria RDT, blood clearance on the nitrocellulose membrane is affected. Poor clearance affects visualization of the test results and also the sensitivity and specificity of the RDT.

The following aspects of sample quality affect the functioning of malaria RDT's.

- Cold blood samples can flow differently from those stored at R.T.
- Stored blood can lose target antigen activity.
- Early lysis, protein coagulation, presence of artefacts inhibit the flow of blood specimen on RDT's.
- Freeze thaw accelerates target antigen denaturation and alters blood flow properties on malaria RDT's, hampering their performance.
- In some instances, for some products improvement in RDT sensitivity has been reported, owing to target antigen release through parasite lysis.

Performance of RDT's using venous blood specimen and archived blood specimen can differ from performance obtained with fresh finger prick blood.

Effect of Prepared whole blood controls on performance of Malaria RDT's

Though Malaria RDT's are essentially designed and optimized to work with capillary finger prick whole blood samples, for large scale laboratory based trials it may become necessary to procure well characterized veni puncture blood and use diluted or archived whole blood for longitudinal studies.

Preparation of serial parasite dilution necessitates mixing parasitized blood into well characterized parasite negative whole blood. The preparation of serial dilution can affect quality of the diluted blood due to:

- Inadequate mixing.
- Blood type incompatibility.
- Freeze thawing during storage and use.
- Cell lysis due to use of small bore pipettes for dispensing samples.

The method of preparation of whole blood controls can affect the blood flow on the RDT's, greatly influencing on the threshhold of sensitivity, specificity and the validity of the Malaria RDT's.

Effect of Artificial panels and reference materials on the performance of RDT's

It is well known that the production of target antigen varies with the stage of the parasite development. Yet at present there is insufficient information on the relationship of target antigen concentration and parasite density. In vivo, during the malarial infection as the parasitaemia increases, there is a sustained build up of the target antigens in the blood. On the other hand in vitro, when the parasite is grown in culture systems the dynamics of the production of the target antigen is completely different. This is probably due to the dissimilar environment for growth between the cultured and the wild parasites.
Thus, the relationship between parasite density and target antigen activity varies significantly between wild type parasites and cultured parasites.
This fact introduces a disconnect between "parasitaemia" and "Target antigen concentration". When reference material from these two systems are used for assessing performance of RDT's, the reference material prepared from the "cultured parasites"would not accurately reflect the target antigen concentrations as may be present in the wild type parasites, even for synchronous stage of parasitaemia or stage of development.

Importance of Clinical Samples

Target antigen sequences vary from region to region for the Malaria parasites. Especially for Pf HRP-II, wide diversity in Pf HRP-II sequences both within and between countries have been reported. Variation in the number and combination of repeats within the Pf HRP II sequences have been shown to affect the sensitivity of Malaria RDT's. Additionally various isolates also differ in terms of:

- Antigen expression.
- Structural variations.
- Variations in parasite density versus target antigen concentration.

The above differences introduce variations in apparent RDT sensitivities from region to region. These factors also make setting lower detection limits for Malaria RDT's in terms of Parasite density practically and biologically incredible. In fact it makes strong case for well designed and executed local level performance evaluation of products even stronger.

Commutability of Reference Materials & Standards

The fundamental goal of laboratory medicine remains that "results for patient samples should be comparable independent of the medical laboratory that produced the results" Cummutability of a Control material is its ability to have inter assay properties comparable to properties demonstrated by authentic clinical samples. Commutability must be validated and demonstrated amongst all the methods that will use the material including the reference test procedure.

Until commutability of a reference material is not established, results from various methods cannot be legitimately compared.

Non commutability of reference materials can be caused by Matrix alteration or a on native analyte. The Matrix effect or Matrix bias is caused by the differences in sample Matrix of the reference material and the native clinical samples.

Non native forms of the target analytes, can produce a different measurement signal than expected from the native forms of analyte.

Ideally the sample matrix includes all components of a material system except the analyte itself.

Test calibration with reference materials that are not commutable can cause poorer rather then improved agreement of results among methods for native samples. Poor commutability of reference materials cause grave difference in apparent product performance and introduce bias in measurement of analytes, compromising comparability of product performance.

Setting Standards for Sensitivity and Performance

Since Malaria RDT's detect the appropriate 'target antigens' and not the 'parasite' per se, "parasite antigen concentrations" could be a more appropriate benchmark to be used to set the lower limits of detection for Malaria RDT's. Determination of the relationship between target antigen concentration to parasite density would be a critical step towards this goal.

There is an urgent need to make available commutable reference panels that are well characterized in terms of target antigen concentration and their antigenic structure for the objective evaluation of the performance of malaria RDT's and for their unbiased comparability.

Summary

Various methods have been devised and are in the process of evolution with regards to validating the sensitivity, specificity and performance of Malaria RDT's. Each method has the potential to significantly alter the specimen characteristics in ways that is detrimental to the performance characteristics of malaria RDT's in unpredictable ways.

Preparation and availability of commutable Reference material and their wide availability to manufacturers, regulators and users, by setting a common and consistent "reference Benchmark" could lead to the desired outcome for improvement in quality, consistency, performance and deliverance of "better Malaria RDT's" to the users.

Till such time:

- Well designed and executed field / laboratory evaluations of Malaria RDT's.
- Performed by well trained personnel.
- Using clinical / well characterized specimen bank.
- At the user level.

Perhaps remains the most appropriate, effective and practical method for assessing the performance of Malaria RDT's and their appropriateness for use in each setting.

Do's & Don'ts of Malaria RDT Assay

Do's	Don'ts
1. Once opened use the device immediately.	1. Do not use blood specimen, stored for more than 72 hours.
2. Always check the colour of desiccant immediately upon opening the pouch. It should be blue in colour. If the desiccant has turned colourless or pink, discard the test device and use another device.	2. In case the test device pouches have been stored at 4-8°C, do not open the pouches immediately after retrieving from 4-8°C storage. Cold devices will attract 'moisture rush' and thereby alter migration properties of the membrane leading to erroneous results.
3. In case of anti-coagulated venous blood, ratio of blood & anticoagulant should be accurate as recommended.	3. Do not use cold whole blood specimen (stored at < 25°C) as test specimen. Cold blood can flow differently and may effect the clearance of test window besides, the sensitivity and specificity of the Malaria RDT's.
4. In case the test device pouches have been stored at 4-8°C, they must be allowed to come to ambient temperature before opening/testing.	4. Do not use clotted, partially clotted, lysed, or contaminated whole blood specimen for testing.
5. Always evenly mix anti-coagulated blood specimen gently before testing.	5. Do not reuse the sample applicator/ loop.
6. Always make sure that the blood from specimen applicator/ loop has been completely transferred to the sample pad.	6. Do not intermix the buffer and devices of different lots.
7. Stored cold whole blood specimen must be allowed to come to ambient temperature before testing.	7. Do not dispense less / excess number of buffer drops in the buffer port other than those recommended in test procedure. This could effect the reaction kinetics between target antigen and capture elements of the test system.
8. Read the result only at the end of recommended reading time.	8. Do not move the device during assay. It could effect the buffer flow.
9. Always lay the test device on a flat surface horizontally before testing.	9. Do not compare test band intensity with control band intensity.
10. Users interpreting the test result must be well-trained to read the test band signals especially with low analyte concentration specimen.	10. Do not repeatedly freeze and thaw test specimen. Repeated freeze-thaw cycles accelerate target antigen denaturation and alter blood flow properties on the RDT's, hampering their performance.

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SYPHILIS

- **Syphilis Diagnosis** .....Pgs 97-108

KNOWLEDGE BOOK

For the Laboratorians

Syphilis Diagnosis

Setting Trends...

INTRODUCTION

Syphilis, which is also called *lues* (from a Latin word meaning plague), has been a major public health problem since the sixteenth century. The disease was treated with mercury or other ineffective remedies until World War I, when effective treatments based on arsenic or bismuth were introduced. Antibiotics succeeded these after World War II. It is estimated that about 50 million persons worldwide need treatment for syphilis with 12 million new cases added every year.

MODE OF TRANSMISSION

Syphilis is caused by *Treponema pallidum,* a spirochete. It is a thin spiral or coil-shaped bacterium that enters the body through the mucous membranes or breaks in the skin. In around 90% of cases, the spirochete is transmitted by sexual contact.

Causative organisms and characteristics of different pathogenic treponemal infections

	Syphillis	Bejel - endemic syphilis	Yaws	Pinta
Causative organism	*T. pallidum* subsp. pallidum	*T. pallidum* subsp. Endimicium	*T. pallidum* subsp. pertenue	*T. carateum*
Geographic Distribution	Worldwide	Mid. East, Africa	Africa, Pacific	Central & South America
Preferred climate	All	Subtropical	Tropical	Warm
Typical age of infection in yrs	15-40	1-10	1-15	10-30
Main mode of transmission	sexual, congenital	skin contact	skin contact	skin contact
Late complications				
- skin / mucosa	+	+	+	+
- bone / cartilage	+	+	+	-
- CNS	+	-	-	-
- heart / circulation	+	-	-	-

Other means of transmission include passage through the placenta (vertical transmission), kissing, transfusion of fresh blood, and direct inoculation such as by needlestick. Transmission by blood transfusion is rare, not only because blood products are screened for the disease, but also because the spirochetes do not survive more than 3 days in stored blood. However, there have been some recent reports, that the spirochete can survive upto 5 days, at 4°C, in stored blood. So it is mandatory to screen all blood samples before transfusion. To avoid vertical transmission, all pregnant women should also be routinely screened.

CLINICAL MANIFESTATIONS

Primary syphilis

Primary syphilis is the stage of the organism's entry into the body. The first signs of infection are not always noticed. After an incubation period ranging between 10 - 90 days, the patient develops a chancre, which is a small blister-like sore about 0.5 inches (13 mm) in size. Most chancres are on the genitals, but may also develop in or on the mouth or on the breasts. Rectal chancres are common in male homosexuals. Chancres in women are sometimes overlooked if they develop in the vagina or on the cervix. The chancres are not painful and disappear in 3-6 weeks even without treatment. They resemble the ulcers of lymphogranuloma venereum, herpes simplex virus, or skin tumors.

About 70% of patients with primary syphilis also develop swollen lymph nodes near the chancre. The nodes may have a firm or rubbery feel when touched but are not usually painful.

Secondary syphilis

Syphilis enters its secondary stage between 6 weeks - 6 months after contracting the infection. Chancres may still be present but are usually self-healing. Secondary syphilis is a systemic infection marked by the eruption of skin rashes and ulcers in the mucous membranes.

The skin rash may mimic a number of other skin disorders such as drug reactions, rubella, ringworm, mononucleosis, and pityriasis rosea. Characteristics that point to syphilis include:

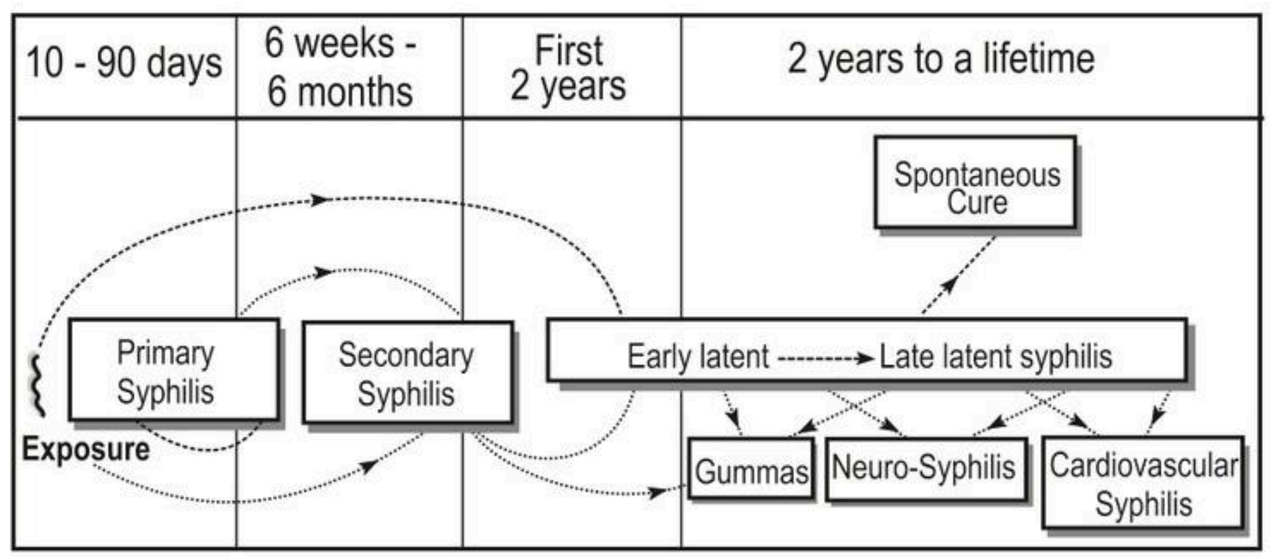
- A coppery color skin rash
- Absence of pain or itching
- Occurrence of rash on the palms of hands and soles of feet.

The skin eruption may resolve in a few weeks or may last as long as a year. The patient may also develop condylomata lata, which are weepy pinkish or grey areas of flattened skin in the moist areas of the body. *The skin rashes, oral and genital ulcers, and condylomata lata are all highly infectious.*
About 50% of patients with secondary syphilis develop swollen lymph nodes in the armpits, groin, and neck areas; about 10% develop inflammations of the eyes, kidney, liver, spleen, bones, joints, or the meninges (membranes covering the brain and spinal cord). Patients may also have flu like general illness with a low fever, chills, loss of appetite, headaches, runny nose, sore throat, and aching joints.

Latent syphilis

Latent syphilis is a phase of the disease characterized by relative absence of clinical symptoms. The term latent does not mean that the disease is not progressing or that the patient cannot infect others. For example, *pregnant women can transmit syphilis to their unborn children during the latency period of the infection.*
The latent phase is sometimes divided into *early latency* (less than two years after infection) and *late latency.* During early latency, patients are at risk for spontaneous relapses marked by recurrence of the ulcers and skin rashes of secondary syphilis. In late latency, these recurrences are less likely. Late latency may either resolve spontaneously or continue for the rest of the patient's life.

Course of untreated syphilis



Tertiary syphilis

Untreated syphilis progresses to a third or tertiary stage in about 35-40% of patients. *Patients with tertiary syphilis cannot infect others with the disease.* It is thought that the symptoms of this stage are a delayed hypersensitivity reaction to the spirochetes. Some patients develop so-called benign late syphilis, which begins between 3 - 10 years after infection and is characterized by the development of gummas. Gummas are rubbery tumor-like growths that are most likely to involve the skin or long bones but may also develop in the eyes, mucous membranes, throat, liver, or stomach lining. Gummas are increasingly uncommon since the introduction of antibiotics for treating syphilis. Benign late syphilis is usually rapid in onset and responds well to treatment.

Cardiovascular syphilis

Cardiovascular syphilis occurs in 10-15% of patients who have progressed to tertiary syphilis. It develops between 10 and 25 years after infection and often occurs together with neurosyphilis. Cardiovascular syphilis usually begins as an inflammation of the arteries leading from the heart and causes heart attacks, scarring of the aortic valves, congestive heart failure, or the formation of an aortic aneurysm.

Neurosyphilis

About 8% of patients with untreated syphilis will develop symptoms in the central nervous system that include both physical and psychiatric symptoms. Neurosyphilis can appear at any time, from 5-35 years after the onset of primary syphilis. Neurosyphilis is classified into four categories:

1. **Asymptomatic.** In this form of neurosyphilis, the patient's spinal fluid gives abnormal test results but there are no symptoms affecting the central nervous system.
2. **Meningovascular.** This type of neurosyphilis is marked by changes in the blood vessels of the brain or inflammation of the meninges (the tissue layers covering the brain and spinal cord). The patient develops headaches, irritability, and visual problems. If the spinal cord is involved, the patient may experience weakness of the shoulders and upper arm muscles.
3. **Tabes dorsalis.** Tabes dorsalis is a progressive degeneration of the spinal cord and nerve roots. Patients lose the sense of perception of their body position and orientation in space (proprioception), resulting in difficulties in walking and loss of muscle reflexes. They may also have shooting pains in the legs and periodic episodes of pain in the abdomen, throat, bladder, or rectum. Tabes dorsalis is sometimes called locomotor ataxia.
4. **General paresis.** General paresis refers to the effects of neurosyphilis on the cortex of the brain. The patient has a slow but progressive loss of memory, ability to concentrate, and interest in self-care. Personality changes may include irresponsible behavior, depression, delusions of grandeur, or complete psychosis. General paresis is sometimes called dementia paralytica, and is most common in patients over 40 years of age.

CLINICAL MANIFESTATION IN SPECIAL POPULATIONS

Congenital syphilis

Almost all infants born to mothers with untreated primary or secondary syphilis will be infected, whereas the infection rate drops to 40% if the mother is in the early latent stage and 6-14% if she has late latent syphilis.

The prognosis for early congenital syphilis is poor: about 54% of infected fetuses die before or shortly after birth. Those who survive may look normal at birth but show signs of infection between three and eight weeks later.

Diagnostic classification of congenital syphilis

Laboratory criteria for diagnosis

Demonstration of *Treponema pallidium* by darkfield microscopy, fluorescent antibody, or other specific stains in specimens from lesions, placenta, umbilical cord, or autopsy material.

Case classification

Probable: A condition affecting an infant whose mother had untreated or inadequately treated* syphilis at delivery, regardless of signs in the infant, or an infant or child who had a reactive treponemal test for syphilis and any one of the following:

- Any evidence of congenital syphilis on physical examination
- Any evidence of congenital syphilis on radiographs of long bones
- A reactive CSF VDRL Test
- An elevated CSF cell count or protein (without other cause)
- A reactive fluorescent treponemal antibody absorbed 19S class-IgM antibody test or IgM enzyme-linked immunosorbent assay

Confirmed: A case that is laboratory confirmed

Also includes mother with serologic evidence of relapse or reinfection after treatment or with a documented history of treatment of insufficient serologic follow-up after appropriate treatment.
From " Case Definitions for Infectious Conditions Under Public Health Surveillance."
Morbidity and Mortality Weekly Report , Vol.46, No.RR-10. Pp.37-38
Centers for Disease Control and Prevention. U.S. Public Health Service
U.S. Department of Health and Human Services, 1997.

Infants with **early congenital syphilis** have systemic symptoms that resemble those of adults with secondary syphilis. There is a 40-60% chance that the child's central nervous system will be infected. These infants may have symptoms ranging from jaundice, enlargement of the spleen and liver, anemia, skin rashes, condylomata lata, inflammation of the lungs, "snuffles" (a persistent runny nose), and swollen lymph nodes.

Late congenital syphilis

Children who develop symptoms after the age of two years are said to have late congenital syphilis. The characteristic symptoms include facial deformities (saddle nose), Hutchinson's teeth (abnormal upper incisors), saber shins, dislocated joints, deafness, mental retardation, paralysis, and seizure disorders.

As far as possible, all pregnant women should be tested for syphilis at their *first prenatal visit.* It should be *repeated in the third trimester and at delivery* if the patient is at high risk or in communities or populations with high prevalence of syphilis.

Sero-titers may increase slightly when serofast women who were previously adequately treated for syphilis become pregnant. It is generally less than a fourfold increase. Serologic response to treatment is the same as for non-pregnant women. As far as possible, no infant should be discharged from the hospital without the serological status of the mother being known.

HIV patients

Syphilis has been closely associated with HIV infection since the late 1980s. Syphilis sometimes mimics the symptoms of AIDS. Conversely, AIDS appears to increase the severity of syphilis in patients suffering from both diseases. AIDS also speeds up the development or appearance of neurosyphilis. Although standard serologic tests for syphilis are considered a relatively reliable method of diagnosis in patients with HIV infection, false-negative treponemal and nontreponemal tests occur somewhat more frequently in patients who have HIV infection. If suspicious lesions are present in a patient with HIV infection, but serologic tests are negative, biopsy of the lesion should be performed for microscopic examination to identify spirochetes.

Ocular syphilis

Syphilis is the commonest bacterial intraocular infection in HIV-infected patients. Vision loss in patients with syphilis occurs most frequently as a result of either uveitis or optic nerve disease, which may manifest itself as papillitis, perineuritis or retrobulbar optic neuropathy, although uveitis appears to be the most common complication.

IMMUNOLOGY OF SYPHILIS INFECTION

Immune response to the first infection

The activation of the cellular & humoral immune system takes place during the incubation period. Approximately 4 days after invasion by the organism, *T.pallidum*-specific antibodies of IgM class are synthesized in the host. At 10-21 days post infection these antibodies reach titers which can be detected using appropriate IgM antibody assays. IgG antibodies are detectable a few days later. It has not been entirely elucidated why symptoms of secondary syphilis occur about 3-6 weeks after the primary complex, at a time when the patient appears to have immunological control over the local infection. During the stage of the infection hematogenous spread of the treponemes throughout the body, eruption of skin rashes and the development of systemic immunity occur. Although the body is protected against reinfection during this stage, it is unable to eliminate the causative organism. The manifestations of the secondary stage, such as recurrent skin eruptions lasting from weeks to months, seem to be related to the development of systemic immunity.

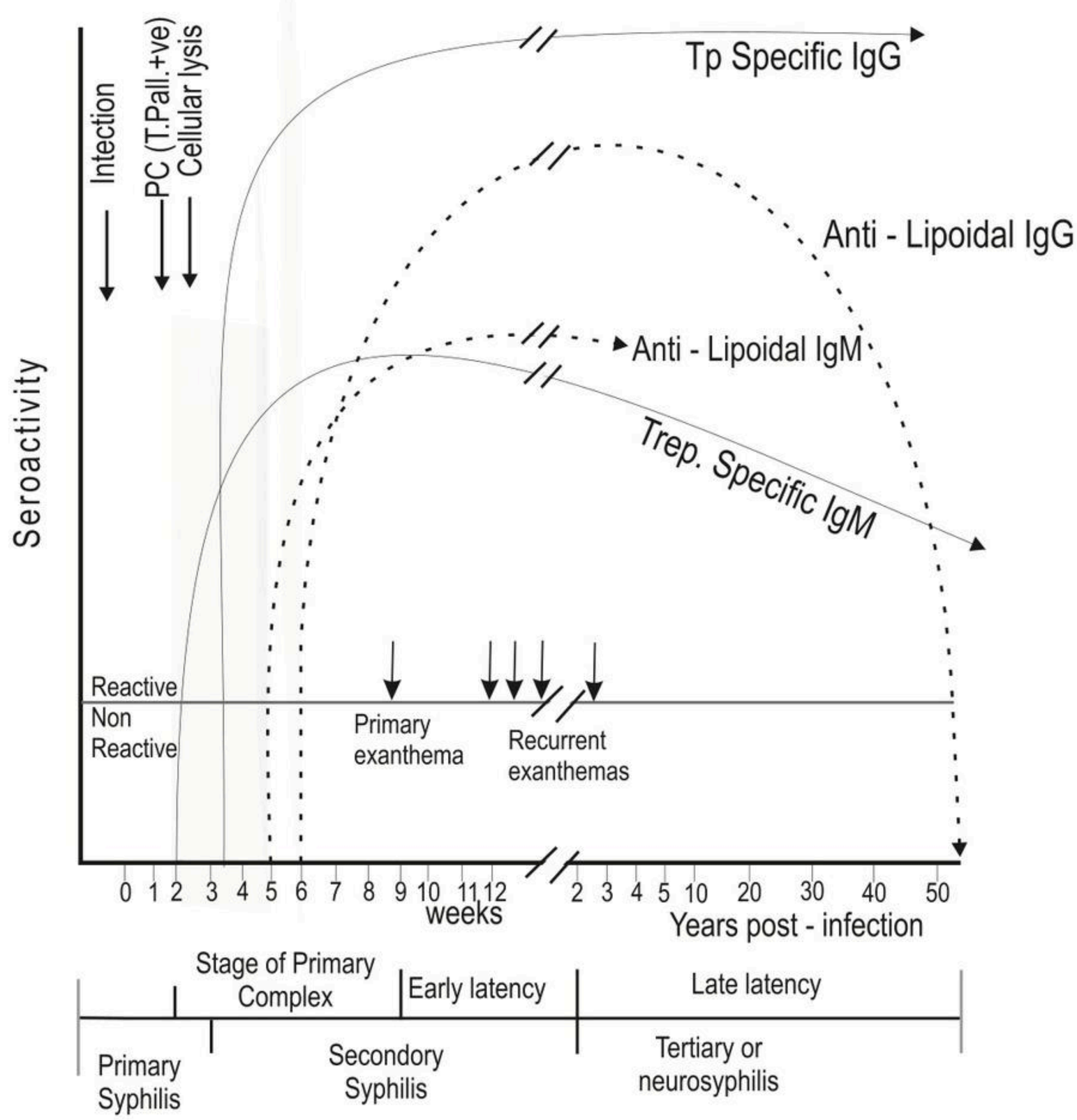
If the host has immunological control over the *T.pallidum* infection to the point where no further clinically apparent eruptions occur, the period of late latency is said to be present. During this phase a delayed type of hypersensitivity reaction against the causative organism is detectable. This reaction, together with the humoral immune response, is considered to be crucial for the suppression of infection. The elimination of treponema from the host is not linked to this and the mechanism of *T.pallidum* persistence in the infected patient has not been elucidated. It is possible that the causative organism survives within so-called "immunological niches", such as CNS.

After another latency period, which may last more than 20 yrs, clinical manifestations of tertiary syphilis may occur. The immunological mechanisms leading to this are unknown.

Immune response after specific therapy

Curative treatment of the infection results in the disappearance of *T.pallidum*-specific IgM antibodies from patient's serum usually within 3-12 months. The reduction of IgG antibodies depends on the time interval between infection & first antibiotic treatment. If this interval is short the infection may resolve without "immunological scars" i.e., all serological tests may again have negative results. If the time interval amounts to several months or even years many clones of memory cells will have developed, containing the information for the production of *T.pallidum*-specific IgG antibodies. Under these circumstances, it is possible that the antibody concentration will not decrease below the lower limit of detection in some assays. In such cases there is a residual, so-called, immunological IgG scar, i.e., persistence of IgG, which may remain detectable for life.

Sero-response during various stages of syphilis infection



Immune response after the second or multiple reinfections

In the case of second or multiple reinfections different pathophysiological mechanisms control the synthesis of antibodies. Second or multiple contacts with the antigens boost production of pre-formed IgG antibodies. Immediately after infection these antibodies show a steep rise in titer. IgG synthesis simultaneously leads to the *in vivo* inhibition or suppression of the synthesis of specific IgM antibodies. These IgM antibodies are either not detectable in serum of patients, or only after a time delay of 2-4 weeks.

DIAGNOSIS

Patient history and physical diagnosis

The diagnosis of syphilis is often delayed because of the variety of early symptoms, the varying length of the incubation period, and the possibility of not noticing the initial chancre. Patients do not always connect their symptoms with recent sexual contact. Women may be diagnosed in the course of a gynecological checkup. Definite diagnosis, however, depends on the results of laboratory blood tests.

Direct visualization of the spirochetes

T.pallidum is too thin to be observed by light microscopy. Therefore, *dark field microscopy* or electron microscopy is required to visualize the organism. No effective method exists for culturing *T. pallidum* in the routine clinical laboratory setting. It has been cultured *in vivo* most commonly by inoculating rabbits (RIT: rabbit infectivity testing). This experimental model for detection through propagation in rabbits is impractical, expensive and requires too much time to be practically useful.

The presence of characteristic organisms in a specimen obtained from a typical lesion of very early syphilis is diagnostic, even in the absence of positive serologic tests. Dark-field microscopy of material from a genital lesion demonstrates corkscrew-like organisms moving with a spiraling motion. Oral and anal lesions cannot be used as sources for specimens because of the presence of nonpathogenic treponemes.

SEROLOGICAL TESTS

Serological tests for syphilis may be classified into two distinct groups - *Non-treponemal & Treponemal* tests. Serology cannot distinguish between the different treponematoses (syphilis, yaws, pinta, and bejel). In practice, serological tests for syphilis are used for:

- Screening asymptomatic individuals with no history suggestive of syphilis, such as pregnant women.
- Screening genitourinary medicine clinic attenders at recent risk of acquiring a sexually transmitted infection.
- Screening blood and organ/tissue donors.
- Detecting or excluding current or past syphilis in patients with HIV infection.
- Testing patients whose history or clinical signs are consistent with syphilis – for example, genital ulceration or chronic neurological illness.
- Confirmatory testing of specimens reactive in screening tests for syphilis.
- Assessment of the stage of infection and monitoring the therapeutic response.

Performance characteristics of non treponemal tests

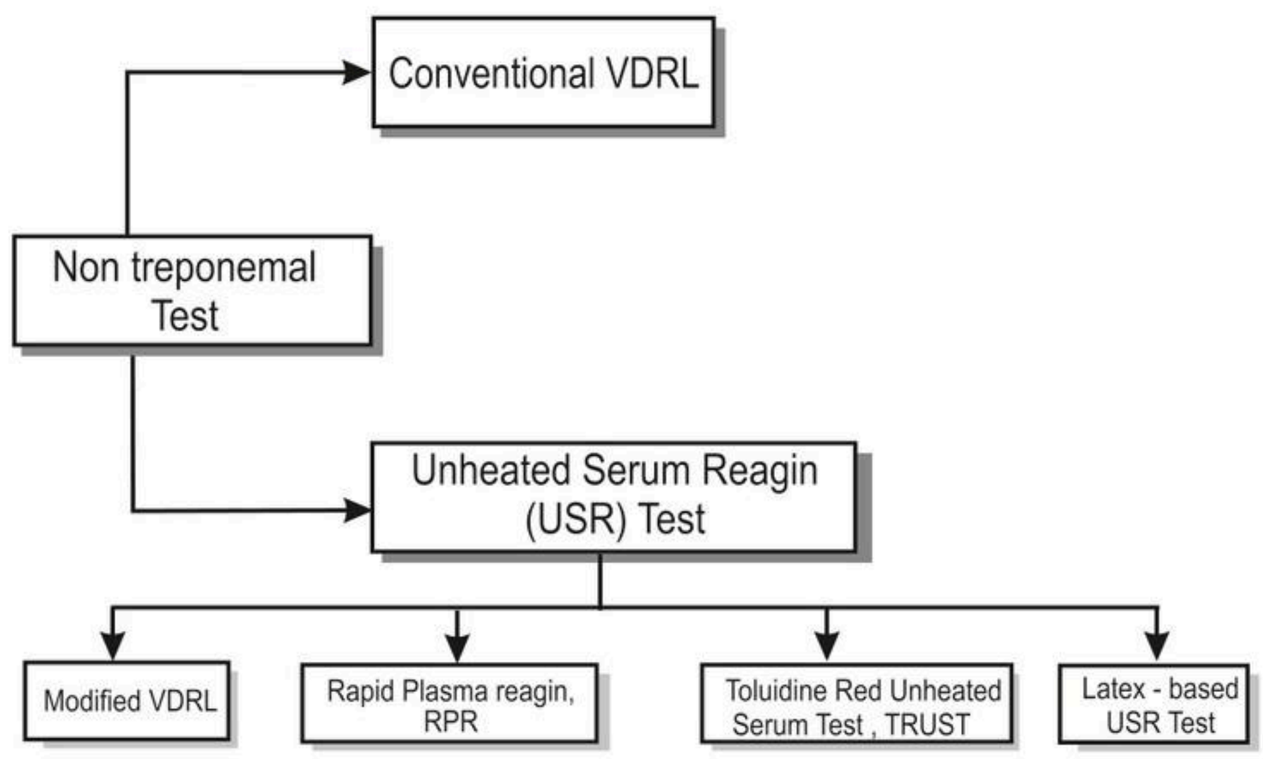
Parameter	Conventional VDRL	Modified VDRL	RPR	TRUST	Latex
Sample used	Serum /Plasma/CSF	Serum /Plasma/CSF	Serum /Plasma	Serum /Plasma	Serum /Plasma
Sample Inactivation	Required @ 56^0 C	Not Required	Not Required	Not Required	Not Required
Test Done On	Glass Concavity Slide	Glass Concavity Slide	Disposable Plastic Cards	Disposable Plastic Cards	Disposable Plastic Cards
Reagents	•Cardiolipin Antigen •Buffer	Mod. VDRL Reagent	Carbon Antigen	Toluidine Red Antigen	Latex Reagent
Stability	Reconstituted Stability: 24 Hrs.	•Ready to use •Stability > 1 yr.	•Ready to use •Stability > 1 yr.	•Ready to use •Stability > 1 yr.	•Ready to use •Stability > 1 yr.
Test duration	4 Minutes	4 Minutes	8 Minutes	8 Minutes	6 Minutes
Additional material provided with the kit	None	•Pos. Control •Mod.VDRL reagent •Sample dropper	•Pos. Control •Neg. Control •Dispensing pipettes •Mixing sticks, •Reaction Cards	•Pos. Control •Neg. Control •Dispensing pipettes •Mixing sticks, •Reaction Cards	•Pos. Control •Neg. Control •Dispensing pipettes •Mixing sticks, •Reaction Cards
Relative sensitivity in different stages of Syphilis	Primary- 70% Secondary- 99% Late - 1%	Primary- 70% Secondary- 99% Late - 1%	Primary - 80% Secondary-99% Late - 0%	Primary - 80% Secondary-99% Late - 0%	Primary - 80% Secondary-99% Late - 0%
Final reaction	Opaque floccules against clear background	White/Opaque floccules against clear background	Black aggregates against white background	Red/Pink aggregates against white background	White aggregates against black background
Reading	Macroscopic + Microscopic	• Macroscopic + Microscopic	•Macroscopic only	Macroscopic only	Macroscopic only
Reaction grading	•Non-reactive •Weakly reactive •Reactive	•Non-reactive •Weakly reactive •Reactive	•Non-reactive •Weakly eactive •Reactive	•Non-reactive •Weakly reactive •Reactive	•Non-reactive •Weakly reactive •Reactive

Nontreponemal tests

Nontreponemal antigen tests are used for screening as well as monitoring the patient's response to treatment. They measure the presence of reaginic antibodies, which are a group of anti-lipoidal antibodies. They detect both IgM and IgG antibodies (reagin) directed against the cardiolipin-lecithin-cholesterol antigen. The reaction may be microscopic (Venereal Disease Research Laboratory test e.g. VDRL) or macroscopic (Rapid Plasma Reagin e.g. RPR). They are not specific for *T. pallidum* and their sensitivity varies with stage of infection. A positive nontreponemal test must be confirmed with a treponemal test for a diagnosis of syphilis. After adequate treatment, 97% of patients with primary syphilis should have a negative RPR/VDRL within one year. The greater the duration of untreated disease the greater the time needed to achieve seronegativity.

In the **VDRL test,** a sample of the patient's serum is mixed with the antigen (cardiolipin-lecithin- cholesterol antigen). If the mixture forms clumps (flocculation), it is reactive or positive. The serum sample can be diluted several times to determine the concentration of reagin in the patient's blood. One of the major disadvantages of conventional VDRL test is that the serum needs to be decomplemented by heating prior to testing to reduce interference from complements.

Different format for Non - treponemal Tests



Unheated Serum Reagin (USR) tests solve this problem. Here the reagent is ready-to-use and no serum heating is required. *Chelating agents* are added to neutralize the interference due to complements. Also, a buffer is added to stabilize the emulsion. Several types of USR tests are available. These types of tests show a high incidence (8-10%) of false negatives due to the prozone phenomenon. For this reason its preferable to run the tests at two dilutions.

The **Rapid Plasma Reagin (RPR)** test works on the same principle as the VDRL. Here charcoal particles are used to visualize the results.

TRUST utilizes toluidine Red dye instead of charcoal.

Another format is using cardiolipin-coated polystyrene **LATEX** particles for visualization. Of late the LATEX-based tests are becoming popular owing to their good readability.

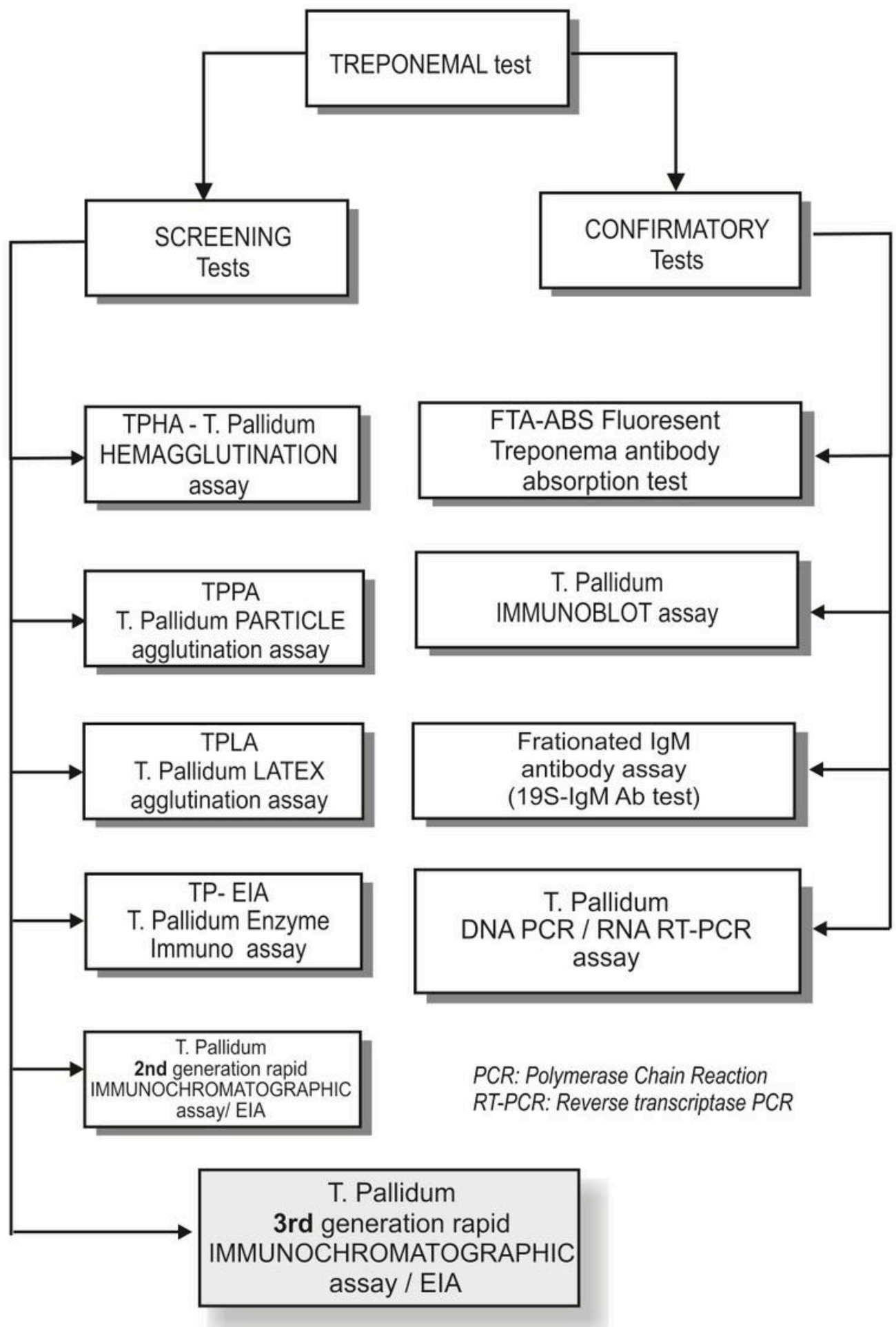
Nontreponemal tests can yield both false-negative and false-positive results. False-positive results can be caused by other infectious diseases, including mononucleosis, malaria, leprosy, rheumatoid arthritis, and lupus. False-negatives can occur when patients are tested too soon after exposure to *Treponema pallidum*; it takes about 14-21 days after infection for reaginic antibodies to appear in the blood.

Treponemal tests

The period between latent syphilis and the development of late syphilis, which can either be benign or involve the cardiovascular / central nervous system, is unpredictable. The fact that it is unpredictable is important because it is not known which patients will develop the late manifestations. *Treatment, at this stage, prevents the late manifestations.*

Non-treponemal tests like VDRL or RPR lacks sensitivity for late disease. Thus it is preferable to screen patients with treponemal tests. Recently in the US, treponemal tests have been introduced for **screening in blood banks** and their use has been also been advocated for **screening psychiatric patients** because of their greater ability to detect late stage infection. Treponemal tests measure the presence of antibodies that are specific for *T. pallidum.* The various types of treponemal tests are:

- **TPHA (Treponema pallidum hemoagglutination assay):** In the TPHA tests, mammalian/avian red blood cells are coated with *T. pallidum* antigen. The RBCs agglutinate if the patient's serum contains antibodies for syphilis. There are reports of inconsistent results due to improper antigen uptake on the cell wall of the RBCs. Also, the use of erythrocytes of animal origin leads to non-specific reactions with some human sera. This may be due to the presence of heterophilic antibodies found in some human sera. Also, TPHA has a variable sensitivity in the early stage of infection.
- **Fluorescent treponemal antibody absorption (FTA-ABS) tests:** In the FTA-ABS, the patient's serum is mixed with a preparation that prevents interference from antibodies to other treponemal infections. The test serum is added to a slide containing *T. pallidum*. In a positive reaction, syphilitic antibodies in the sample coat the spirochetes on the slide. The slide is then stained with fluorescein, which causes the coated spirochetes to fluoresce when the slide is viewed under ultraviolet (UV) light. The FTA-ABS is still generally regarded as the 'gold standard', but it has a number of limitations. It is a subjective test and difficult to standardise. Both false positive & false negative results has been reported in FTA-ABS. Moreover, this is not a practical test to be carried out in blood banks & pathological laboratories.

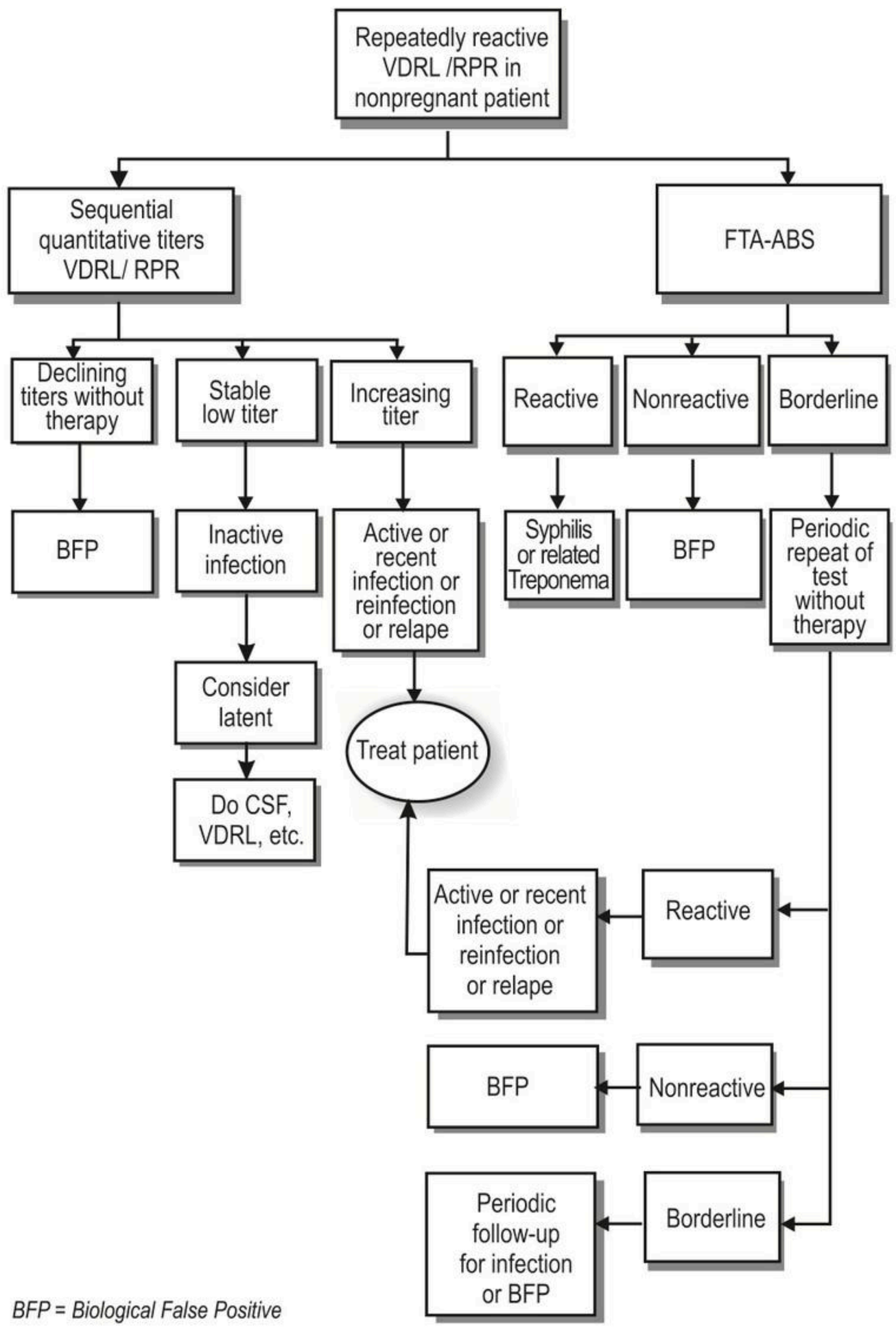


- **Treponema pallidum (Tp) Immunoblot assay:** The Tp proteins are separated by SDS-PAGE (Sodium dodecyl sulphate – PolyAcrylamide Gel Electrophoresis) and IgG and IgM antibodies are detected by from HRP (horse radish peroxidase) – labeled antisera. Although immunoblotting has been suggested as a possible confirmatory test, further evaluation is needed in order to define its precise role.
- **Fractionated IgM Antibody assay (19S IgM Ab test):** Here IgM & IgG antibodies in patient's serum is separated by column chromatography or ultracentrifugation. Then they are detected by means of indirect immunofluorescence This test is sensitive, but very laborious. It cannot be automated and only a limited number of samples can be processed at one time. It requires highly skilled technologists in order to obtain reproducible "readings".
- **Treponema pallidum Enzyme immunoassay (Tp-EIA):** In this treponemal assay, *T. pallidum*-specific antibodies are detected. Several methodologies are available from different manufacturers.

  For example, in the **Differential Conjugate System** in an IgM assay, microtitre wells coated with *T. pallidum* antigens are exposed to test specimens, which may contain specific antibodies. After an incubation period, unbound components in the test sample are washed away. Now an anti-human IgM (μ chain specific) conjugate is added. Following a second wash cycle, specifically bound enzyme conjugate is detected by reaction with a chromogenic substrate. The assay is measured spectrophotometrically to indicate the presence or absence of IgM treponemal antibodies. The drawback of this system is the interference caused by co-specific IgG (in specimen) that compete with specific IgM antibodies. They block or "mask" the reaction sites (epitopes) and cause false negative reactions.

  The IgM assays with some manufacturers use the **sample pre-treatment (absorption) method.** Here the test specimen is incubated with anti-human IgG for neutralising IgG class Anti-Tp antibodies. IgG is bound & removed from the mixture by centrifugation. The supernatant contains unbound/un-neutralised IgM, which is assayed with specific Tp-antigens bound to the solid phase, specific Anti-human IgM enzyme-conjugate & chromogenic substrate. The disadvantage in this system is that there may be non-uniform /inadequate antibody absorption during the pre-treatment phase. This may lead to false negative results.

Flow chart for positive serologic test for syphilis



BFP = Biological False Positive

The interference of co-specific IgG can be overcome in the **μ-capture IgM assay**. Antibodies against human IgM (μ chain specific) are coated on the inner surfaces of microtitre wells. Patient serum is dispensed into the wells and, during incubation; a proportion of the total serum IgM is "captured" on the coated plastic. Unbound serum components are washed away. Surface-bound IgM antibodies to *T. pallidum* are subsequently traced by incubation with the Tp antigen-conjugate reagent. Unbound conjugate is washed away and surface-bound conjugate is detected by reaction with a chromogenic substrate.

But, again, as a screening test, only IgM assay may not provide adequate information to take effective clinical decisions e.g., in a blood bank, a pre-natal clinic or in an HIV patient. At the same time, it is not practical to screen all negatives (over 90% in general population are negative) once again for presence of IgG antibodies. Thus, ideally, a screening test should detect both IgG & IgM antibodies. This would make re-testing of IgM negatives redundant.

The latest double antigen sandwich **3rd Generation EIA** uses specific treponemal antigens at both solid phase and conjugate (also refer 3rd generation Immunochromatographic assay). Both IgG & IgM antibodies can be detected by this method. However, Tp-EIA requires skilled personnel & automation to consistently deliver reproducible results.

- **T. pallidum 2nd Generation Rapid Immunochromatographic assay:** Immunochromatographic assays are rapid, simple and easy-to-perform tests where investment in skilled manpower & automation is greatly reduced. The 2nd Generation Immunochromatographic assays use a Tp-recombinant antigen, at one site i.e., the solid phase, bound to the membrane. The conjugate pad contains immunoglobulin-binding protein like **Protein-A** (derived from *Staphylococcus aureus*) or **anti-human immunoglobulins** to Tp (raised in goat/rabbit) conjugated with a **dye** (instead of colloidal gold). Protein A binds to the F_c fragment of most mammalian IgGs. Each Protein A molecule can bind 2- 4 IgG molecules. Also, anti-human IgG, as used in some 2nd generation tests, binds to human IgG only. Thus, the 2nd Generation Immunochromatographic assays detect only total IgG and not IgM in the sample. False negatives can occur due to prozone effect because the total IgG titre in sera is much higher than Tp-specific IgG alone. This is the reason, these tests are recommended to be performed with diluted sample & not with neat sera.

- **T. pallidum 3rd Generation Rapid Immunochromatographic assay:** For maximum sensitivity at all stages of syphilis, it is important that tests should detect both types of anti-treponemal antibodies i.e., IgG & IgM. The problems faced in 2nd Generation assays, has been overcome after the introduction of The 3rd Generation Immunochromatographic assays.

Causes of False - Positive Serologic Tests for Syphils

Non treponemal Tests

Acute condition(<6 months)	*Chronic condition(>6 months)*
1. Pneumonia	1. Liver disease
2. Viral	2. Malignancy
3. Pneumococcal	3. Intravenous drug use
4. Mycoplasma	4. Aging
5. Hepatitis	5. Connective tissue disorders
6. Tuberculosis	6. Multiple Blood transfusions
7. Mononucleosis	
8. Chancroid	
9. Chickenpox	
10. HIV infection	
11. Measles	
12. Malaria	
13. Immunizations	
14. Pregnancy	
15. Laboratory error	

Treponemal Tests

Acute condition(<6 months)	*Chronic condition(>6 months)*
1. Mononucleosis	1. Systemic lupus erythematosus
2. Lyme disease	
3. Leprosy	
4.Malaria	

They detect both IgG & IgM types of antibodies and can be used for both screening & confirmation. It is a double antigen sandwich immunoassay, where a cocktail of **two** specific Tp recombinant antigens (double antigen sandwich) are used at two sites – one in the conjugate pad (conjugated to **colloidal gold**) and the other immobilised on the membrane at the test line. These antigens are recombinant (not purified from Tp as in some 2nd generation assays) and isolated in such a way that the most immunodominant epitopes are expressed. The recombinant Tp antigen-colloidal gold conjugate forms a complex with Tp specific antibodies in the sample that flows on the membrane. This complex moves further on the membrane to the test region where it is immobilized by the specific recombinant Tp antigens leading to the formation of a coloured band. As in the 2nd Generation assay, these tests are simple & rapid – takes 15 mins. Due to the use of specific recombinant Tp-antigens at two sites (sandwich assay), both IgG & IgM antibodies are detected. The problem of prozone effect is also overcome. The sample need not be diluted and can be tested neat. Thus, The 3rd Generation Immunochromatographic assays are currently the best available for rapid diagnosis of syphilis.

THE PROZONE PHENOMENON – Diagnostic implications in syphilis

The Prozone phenomenon exhibited by some sera, which give effective agglutination reactions when diluted several fold, but do not visibly react with the antigen when undiluted. The phenomenon is not simply due to antibody excess, but often involves a special class of antibodies (blocking or incomplete), which react with the corresponding antigen in an anomalous manner. The bound antibody not only fails to elicit agglutination, but also actively inhibits it. The prozone phenomenon can occur in pregnancy, and request for dilution of sera should be made.

Prozone phenomenon is often encountered in non-treponemal tests. The non-treponemal tests like VDRL or RPR are economical & simple for early syphilis. Other than Biological False Positives (BFP), the main limitations of non-treponemal tests are:

- Poor sensitivity in late stage infection, and
- False negatives due to *Prozone phenomenon* in early infection

Due to *Prozone phenomenon* the reaction may show "roughness" instead of clear-cut positive or negative. So it is recommended to dilute the serum and run in duplicate along with the neat sample. The *Prozone phenomenon* is a greatly underestimated limitation of this type of tests. It has been described that prozone were more likely in HIV positive patients due to high antibody levels resulting from B-cell dysregulation. There are reports of prozone in women during pre-natal screening and congenitally infected infants resulting from this misdiagnosis. The prozone reaction can be as high as 8% in secondary & 10% in early latent syphilis.

Alogarithm for Syphilis Diagnosis in different patient groups

	Initially use	If positive, use
Pre - natal / Ante- natal	Treponemal test	Non-treponemal test for titre
Blood transfusion	Non-treponemal test	Treponemal test to rule out BFP
Random Screening	Treponemal test	
R_X monitoring	.....Non-treponemal test for titre.....	
Organ transplantation	Treponemal test	Non-treponemal test for titre
Patients @ VD clinics	Treponemal test	Non-treponemal test for titre
Psychiatric patients (Neurosyphilis)	Treponemal test	Non-treponemal test for titre
HIV Patients	Treponemal test	Non-treponemal test for titre

TREATMENT / MEDICATION

Syphilis is treated with antibiotics given either intramuscularly (benzathine penicillin G or ceftriaxone) or orally (doxycycline, minocycline, tetracycline, or azithromycin). Neurosyphilis is treated with a combination of aqueous crystalline penicillin G, benzathine penicillin G, or doxycycline. It is important to keep the levels of penicillin in the patient's tissues at sufficiently high levels over a period of days or weeks because the spirochetes have a relatively long reproduction time. Penicillin is more effective in treating the early stages of syphilis than the later stages. Separate medications for the skin rashes or ulcers of secondary syphilis are not usually prescribed. The patient is advised to keep them clean and dry, and to avoid exposing others to fluid or discharges from condylomata lata.

Pregnant women should be treated as early in pregnancy as possible. Infected fetuses can be cured if the mother is treated during the second and third trimesters of pregnancy. Infants with proven or suspected congenital syphilis are treated with either aqueous crystalline penicillin G or aqueous procaine penicillin G. Children who acquire syphilis after birth are treated with benzathine penicillin G.

Jarisch-Herxheimer reaction

The Jarisch-Herxheimer reaction, first described in 1895, is a reaction to penicillin treatment that may occur during the late primary, secondary, or early latent stages. The patient develops chills, fever, headache, and muscle pains within two to six hours after the penicillin is injected. The chancre or rash gets temporarily worse. The Jarisch-Herxheimer reaction, which lasts about a day, is thought to be an allergic reaction to toxins released when the penicillin kills massive numbers of spirochetes.

GLOSSARY OF TERMS

Aortic aneurysm
Aortic aneurysm is an abnormal bulging or swelling of a portion of the aorta. It occurs when a weakness develops in the part of the wall of the aorta.

Chancre
The initial skin ulcer of primary syphilis, consisting of an open sore with a firm or hard base.

Condylomata lata
Highly infectious patches of weepy pink or gray skin that appear in the moist areas of the body during secondary syphilis.

Dark field
A technique of microscope examination in which light is directed at an oblique angle through the slide so that organisms look bright against a dark background.

General paresis
A form of neurosyphilis in which the patient's personality, as well as his or her control of movement, is affected. The patient may develop convulsions or partial paralysis.

Gumma
A symptom that is sometimes seen in tertiary syphilis, characterized by a rubbery swelling or tumor that heals slowly and leaves a scar.

Lues maligna
A skin disorder of secondary syphilis in which areas of ulcerated and dying tissue are formed. It occurs most frequently in HIV-positive patients.

Lymphogranuloma venereum
Lymphogranuloma venereum (LGV) is a sexually transmitted disease that primarily infects the lymphatics. LGV synonyms include lymphopathia venerea, tropical bubo, climatic bubo, strumous bubo, poradenitis inguinales, Durand-Nicolas-Favre disease, and lymphogranuloma inguinale. LGV is caused by *Chlamydia trachomatis.*

Mononucleosis
Infectious mononucleosis (IM) is a clinical syndrome. IM represents the immunopathologic expression that occurs under a specific set of circumstances and in response to infection with the Epstein-Barr virus (EBV).

Pityriasis Rosea
Pityriasis rosea (PR) is a common benign papulosquamous disease that was originally described in 1860 by Camille Melchoir Gibert. Pityriasis denotes fine scales and rosea translates as rose colored or pink. PR can have a number of clinical variations. Its diagnosis is important, as it may resemble secondary syphilis. PR has often been considered to be a viral disease. Till date, no single virus has been proven to cause the disease.

Serofast
It is expected that the non-treponemal test will eventually become non-reactive after treatment. However, in some patients, non-treponemal antibodies can persist at a low titre for a long period, sometimes for the remainder of their lives. This response is referred to as serofast.
Spirochete
A type of bacterium with a long, slender, coiled shape. Syphilis is caused by a spirochete.
Tabes dorsalis
A progressive deterioration of the spinal cord and spinal nerves associated with tertiary syphilis.
Uveitis
Uveitis is defined as inflammation of one or all parts of the uveal tract of the eye. Components of the uveal tract include the iris, the ciliary body, and the choroid. The exact pathophysiology of uveitis is unknown. In general, uveitis is caused by an immune reaction.

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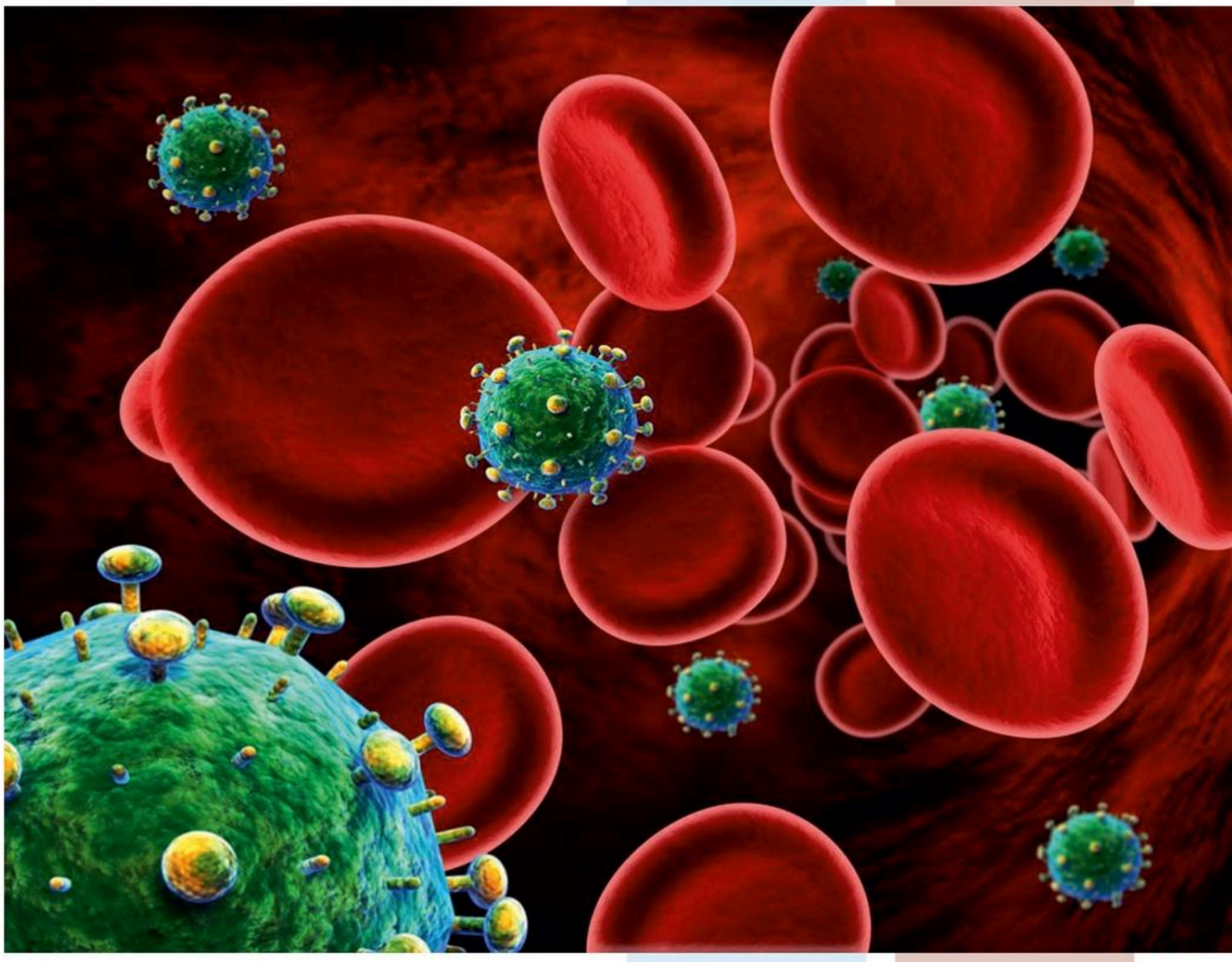
HUMAN IMMUNODEFICIENCY VIRUS



- **Human Immunodeficiency Virus**
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KNOWLEDGE BOOK

For the Laboratorians

Human Immunodeficiency Virus

An Perspective

Setting Trends...

Background

Since its recognition in 1981, the HIV pandemic has spread unabated across continents. The catastrophic potential of the pandemic may still not have been fully realized as it continues to exact enormous toll throughout the world in both human and economic terms.

The demographic characteristics of those affected by the epidemic has changed drastically since the first cases were reported in the year 1981 in the USA comprising overwhelmingly of gay population. Today new cases of HIV infection originate predominantly through heterosexual contacts and injecting drug users. The infection rates amongst women, adolescent girls and children have increased dramatically. It is estimated that in the year 2001 a total of 40 million people worldwide were living with HIV/AIDS and in the year 2001 it is estimated that at least 5 million people were infected with HIV and at least 3 million people perished due to the disease.

The epidemic has negated decades worth of work on controlling diseases, improving nutrition and in many African countries it has become an unprecedented emergency with approximately 25% of the most productive age group between 15 years to 49 years being infected.

It is estimated that in sub-Saharan Africa the life expectancy would reduce from average 64 years to 47 years. If the present warning of epidemiologists go unheeded, South Asia, South East Asia and China may follow the disastrous course of Sub-Saharan Africa. The battle against AIDS has to be fought on multiple fronts such as education, counselling, prevention of infection, voluntary testing, treatment and vaccination programmes.

People infected with HIV in 2001	**Total**	**5 Million**
	Adults	4.3 Million
	Women	1.8 Million
	Children <15 years	800,000
	Total	**40 Million**
No. of people living with HIV/AIDS	Adults	37.2 Million
	Women	17.6 Million
	Children <15 years	2.7 Million
	Total	**3 Million**
AIDS deaths in 2001	Adults	2.4 Million
	Women	1.1 Million
	Children <15 years	580,000
	Total	**21.8 Million**
Total no. of AIDS deaths since the beginning of the epidemic until 2001	Adults	17.5 Million
	Women	9 Million
	Children <15 years	4.3 Million
Total no. of AIDS orphans since the beginning of the epidemic until 2001	**Total**	**13.2 Million**

TABLE 1: Summary of the HIV / AIDS epidemic, December 2001.

Region	Epidemic started	Adults and children living infected with HIV/AIDS (million)	Adults and children newly infected with HIV (million)	Percent Adult prevalence rate*	Percent of HIV-positive adults who are women	Main mode (s) of transmission for adults living with HIV/AIDS
Sub Saharan Africa	Late 70's Early 80's	28.1	3.4	8.4	55	HTS
North Africa and the middle east	Late 80's	0.44	0.080	0.2	40	HTS, IDU
South and South East Asia	Late 80's	6.1	0.8	0.6	35	HTS, IDU
East Asia and Pacific	Late 80's	1.0	0.27	0.1	20	IDU, HTS, HMS
Latin America	Late 70's early 80's	1.4	0.13	0.5	30	IDU, HMS, HTS
Caribbean	Late 70's early 80's	0.42	0.06	2.2	50	HTS, HMS
Eastern Europe & Central Asia	Early 90's	1.0	0.25	0.5	20	IDU
Western Europe	Late 70's early 80's	0.56	0.03	0.3	25	HMS, IDU
North America	Late 70's early 80's	0.94	0.045	0.6	20	IDU, HMS, HTS
Australia and New Zealand	Late 70's early 80's	15,000	500	0.1	10	HMS
Total		**40**	**5**	**1.2**	**48**	

HTS = Heterosexual, HMS = Homosexual, IDU = Injection Drug Use

TABLE 2: Regional HIV / AIDS Statistics, December 2000

The HIV Virus

The HIV-1 and HIV-2 belong to the retrovirus family, sub family lentivirinae. Both retroviruses are so named because one step of their replication cycle is the creation of DNA copy of their RNA genome. The genetic information of a retrovirus therefore flows from RNA to DNA at this step, which is in the opposite direction of the flow in most biological systems. HIV is a lipid enveloped positive stranded RNA virus like all Retroviruses.

RNA viruses are distinguished by the possession of an enzyme viral reverse-transcriptase that transcribes the viral RNA into provirus DNA which is integrated into the host cell genome.

Origin of HIV

Lentiviruses other than HIV have been found in a wide range of non human primates. As a result of molecular analysis and analogy , it is now generally thought that the HIV-2 is similar to SIVsm (Sooty mangabey monkey) and the HIV-1 is similar to the SIVcpz (Pantroglodytes troglodytes chimpanzees), indigenous to the West and West Central Africa.

It is postulated that through Zoonosis these viruses at some point of time crossed over to the humans.

The HIV Virion

The mature HIV-1 virion measures approximately 100 nm in diameter. The HIV-1 genome consists of two identical, noncomplementary sense (+) strands of RNA, each approximately 9.2 kb long, packaged in a cone-shaped protein core, composed of 24kDa capsid (CA) proteins (p24), surrounded by a unique bilayer envelope derived from the host cell membrane. The viral enzymes, Reverse Transcriptase, integrase, and protease, are packaged with the RNA in the core. The surface (SU) and transmembrane (TM) glycoproteins, gp 120 and gp 41, respectively, form a noncovalent complex. Also incorporated into the envelope are various host proteins including HLA class I and II DR molecules.

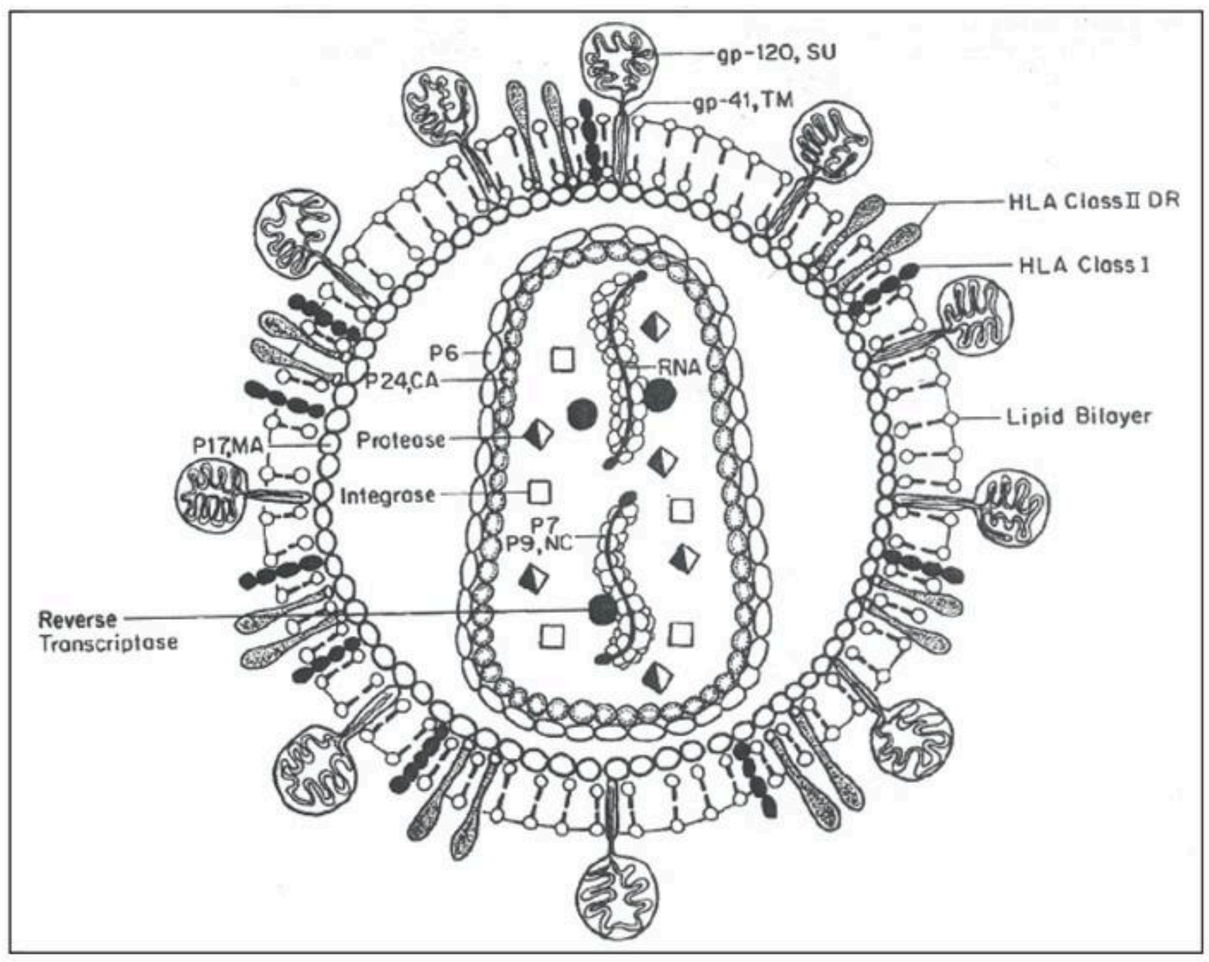


FIGURE 1: The HIV genome.

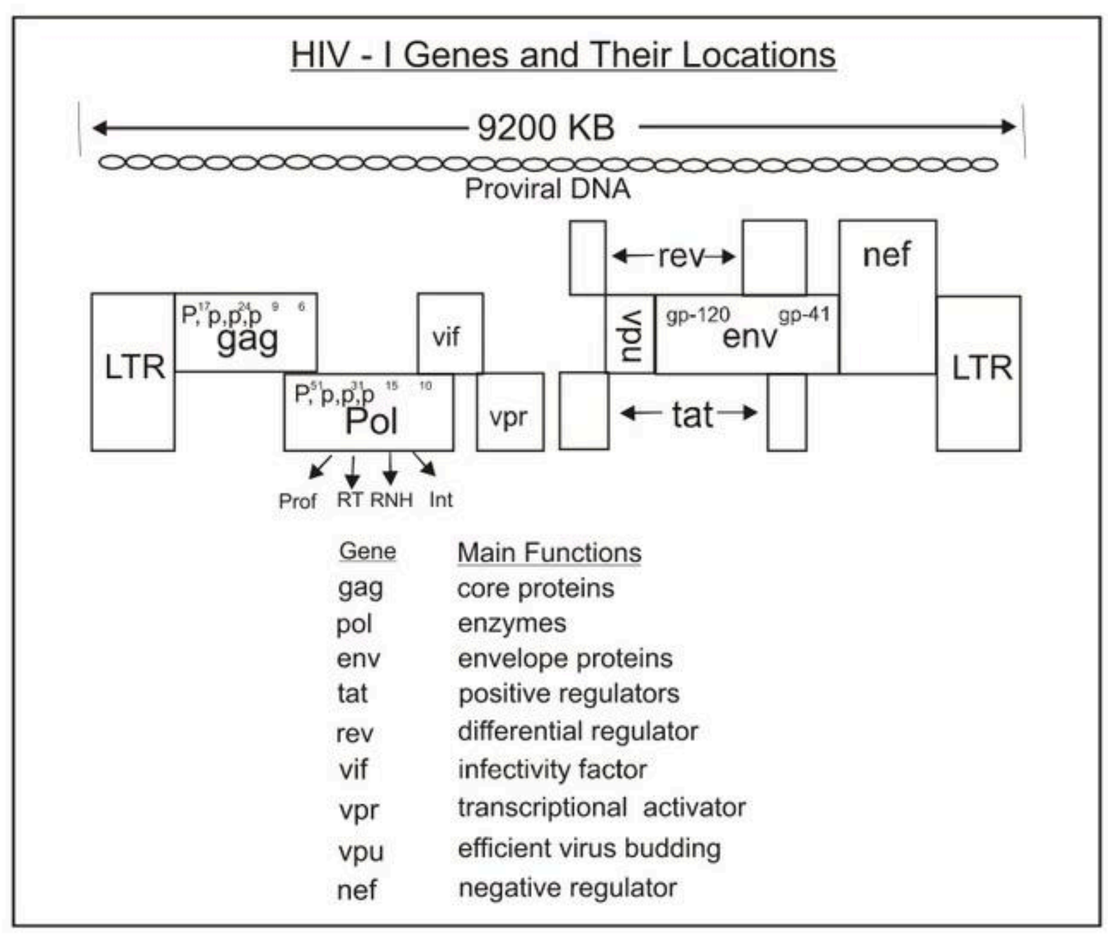


FIGURE 2: Genetic Map of HIV.

Biologically HIV-2 is very similar to the HIV-1, with the genome exhibiting about 60% homology in the gag and pol genes and about 30-40% homology in the other viral genes. It is recognized that HIV-2 is less easily transmitted than HIV-1 and the period between infection and illness is relatively longer in the case of HIV-2. While the HIV-1 is widespread globally, the HIV-2 is primarily found in West Africa.

HIV Types & variants

The HIV virus is a term used to refer collectively to the HIV-1 and HIV-2 retroviruses which are distinct from one another.

The HIV-1 genome is heterogeneous and the envelope gene with its 5 hyper variable region V3 of the surface glycoprotein gp 120, encoding a central loop structure contains the most important site for antibody binding. The phylogenetic analysis of V3 sequences have lead to the identification of three major groups of HIV-1: M (major), O (outlier) and the most recently described group N (New).

Within the major group M, there are eight subtypes A through to J. As the virus continues to mutate and the global epidemic continues, new subtypes are expected to develop and be isolated.

Sr.	HIV-1 Sub Types	Geographical Distribution
1	B	USA, Japan, Australia
2	A,D	Sub Saharan Africa
3	C	Africa, India
4	E	Central African Republic, Thailand, South East Asia
5	F	Brazil, Romania
6	G,H	Russia, Central Africa
7	I	Cyprus
8	O	Cameroon

TABLE 3: Geographic Distribution of HIV -1 Sub types

The group O, of HIV-1 shares 55% to 70% homology with the major group M of HIV-1.

In Africa, most subtypes are found, although, subtype B is relatively less prevalent.

Life Cycle of the HIV

Like all lipid enveloped viruses, the HIV must remain moist to be infectious and it is easily inactivated. For this reason, HIV is primarily transmitted sexually, by exchange of blood, or from mother to child perinatally or by breast-feeding or through sharing infected needles during injecting drug use.

After initial contact and attachment to the hosts immune system (e.g. lymphocytes, monocytes) post infection a cascade of intracellular events within the host are unraveled, the end product being the production of new viral particles, death of the infected cells and the ultimate devastation of the hosts immune system.The main steps in the HIV replication cycle are as follows:

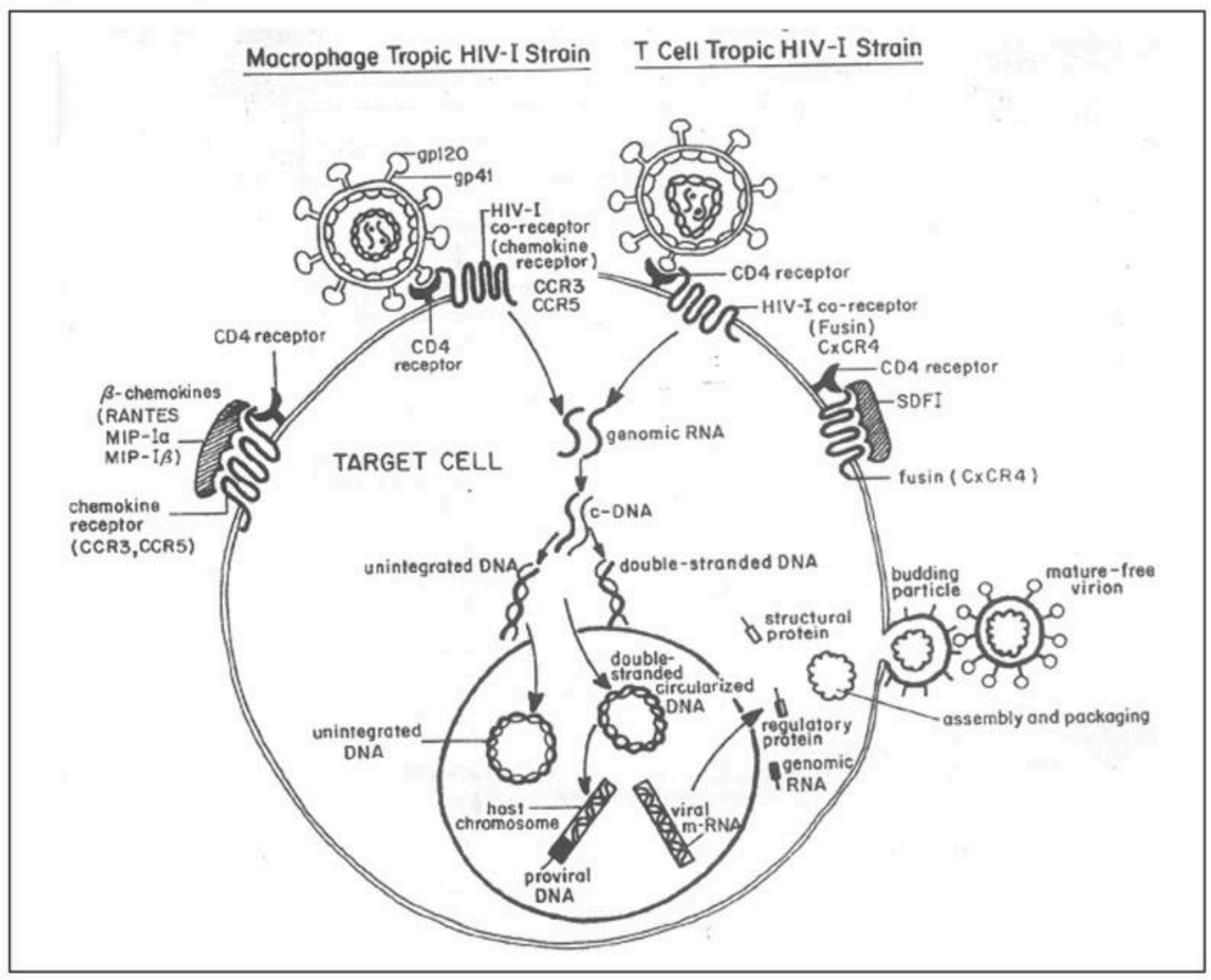


FIGURE 3: The HIV Replication Cycle.

Functions of the HIV Gene

HIV Gene	Protein Product	Function in Life Cycle of HIV
gag	Gag/core protein precursor	Viral core proteins: p24:major capsid protein p17: matrix protein p9: binds to viral RNA p7: binds to viral RNA
env	Env./ envelope proteins	Viral envelope proteins: gp 120: major envelope protein, mediates virion binding to cell surface receptor (CD4) gp 41: mediates fusion of viral envelope and cell membrane
pol	Reverse transcriptase	Converts single-stranded viral RNA into viral DNA duplex
	Integrase	Integrates viral DNA duplex into host cell genome as provirus DNA
	Protease	Cleaves core precursor polyprotein into functional core proteins
tat	Tat protein	Essential regulatory protein, trans -activates expression of all viral genes
rev	Rev protein	Regulatory protein, a ctivates expression of HIV structural and enzymatic genes
vif	Vif protein	Role in viral budding and infectivity of free virions
vpu	Vpu protein	Promotes release of budding virions from host cell
nef	Nef protein	Regulatory protein (essential for pathogenicity of SIV)
vpr	Vpr protein	Regulatory protein, role uncertain

TABLE 4: Functions of the HIV Gene.

Attachment to the Lymphocyte Membrane: On the surface membrane of all living cells are complex protein structures called "receptors". A receptor is often compared to a lock into which a specific key or "ligand" will fit. There are at least two receptors on T-lymphocytes to which the human immunodeficiency virus (HIV) locks on to. The primary receptor is called "CD4". But a second receptor that loops through the cell membrane seven times is critical for infection to occur.

HIV infection of a lymphocyte requires attachment of the virus to the cell membrane through both of these "ligand receptor" links. In cells whose "7-transmembrane receptor" is different, the HIV "key" no longer matches the lymphocyte "lock" and attachment is incomplete. Such cells may avoid infection with HIV.

Entry of the Viral RNA: Tight attachment of the viral particle to receptors on the lymphocyte membrane enables fusion with the cell membrane. The viral contents including viral RNA then empty into the cell's cytoplasm, like the other viruses.

Reverse Transcription: Converting viral RNA into DNA
An enzyme that is part of the human immunodeficiency virus reads the sequence of viral RNA nucleic acids that have entered the host cell and transcribes the sequence into a complementary DNA sequence. This enzyme is called "Reverse transcriptase". Without reverse transcriptase, the viral genome cannot be incorporated into the host cell, and cannot reproduce.

Reverse transcriptase sometimes makes mistakes reading the RNA sequence. The result is that not all viruses produced in a single infected cell are alike. Instead, they end up with a variety of subtle molecular differences in their surface coat and enzymes. Thus through each replication cycle HIV surface molecules are continually changing.

Integration of Viral DNA:
Once the viral RNA has been reverse-transcribed into a strand of DNA, the DNA can then be integrated (inserted) into the DNA of the lymphocyte. The virus has its own enzyme called "integrase" that facilitates incorporation of the viral DNA into the host cells DNA. The integrated DNA is called a provirus.

Transcription: Back to DNA
As long as the lymphocyte is not activated or "turned-on", nothing happens to the viral DNA. But if the lymphocyte is activated, transcription of the viral DNA begins, resulting in the production of multiple copies of viral RNA. This RNA codes for the production of the viral proteins and enzymes (translation) and will also be packaged later as new viruses.

Translation: RNA -> Proteins
There are nine genes in the HIV RNA. Those genes have the code necessary to produce structural proteins such as the viral envelope, core and the enzymes like reverse transcriptase, integrase, and another crucial enzyme called protease.

Viral Protease: When viral RNA is translated into a polypeptide sequence, that sequence is assembled in a long chain that includes several individual proteins (reverse transcriptase, protease, integrase). Before these enzymes become functional, they must be cut off from the longer polypeptide chain. Viral protease cuts the long chain into its individual enzyme components, which then facilitates the production of new viruses.

Assembly and Budding: Finally, viral RNA and associated proteins are packaged and released from the lymphocyte surface, taking with them a swatch of lymphocyte membrane containing viral surface proteins. These proteins will then bind to the receptors on other immune cells facilitating continued infection and propagation.

Budding viruses are often exactly like the original particle that initially infected the host, however, the resulting viruses also exhibit a range of variations.

During its replication cycle, the HIV is capable of mutating at a rate of approximately one nucleotide substitution per genome per replication cycle; making the virus capable of wide genetic diversity and flexibility to respond to a wide array of selection pressures.

As a consequence drug resistance and immunologic escape mutants are rapidly generated in infected persons through all stages of infection.

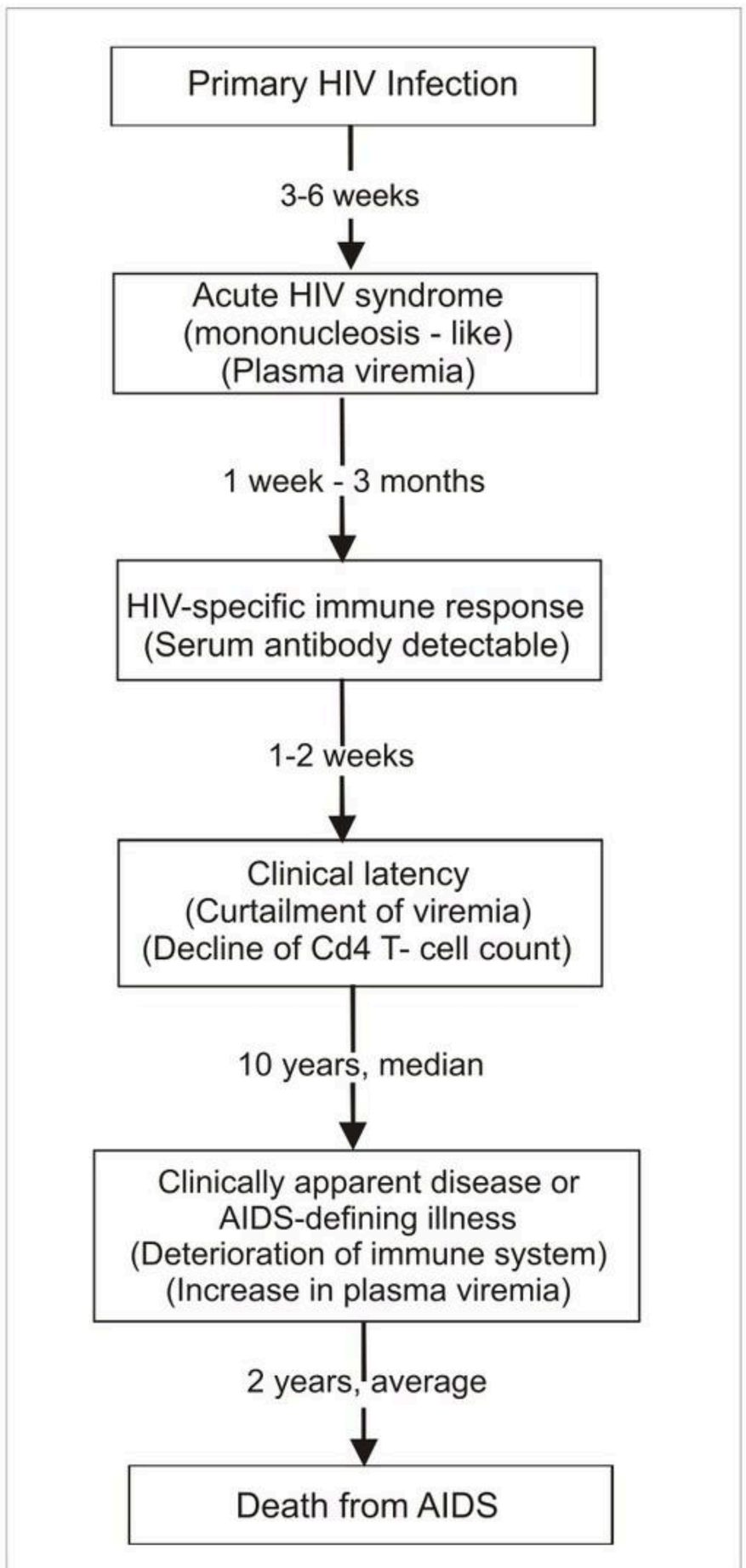


FIGURE 4: Natural History of HIV infection

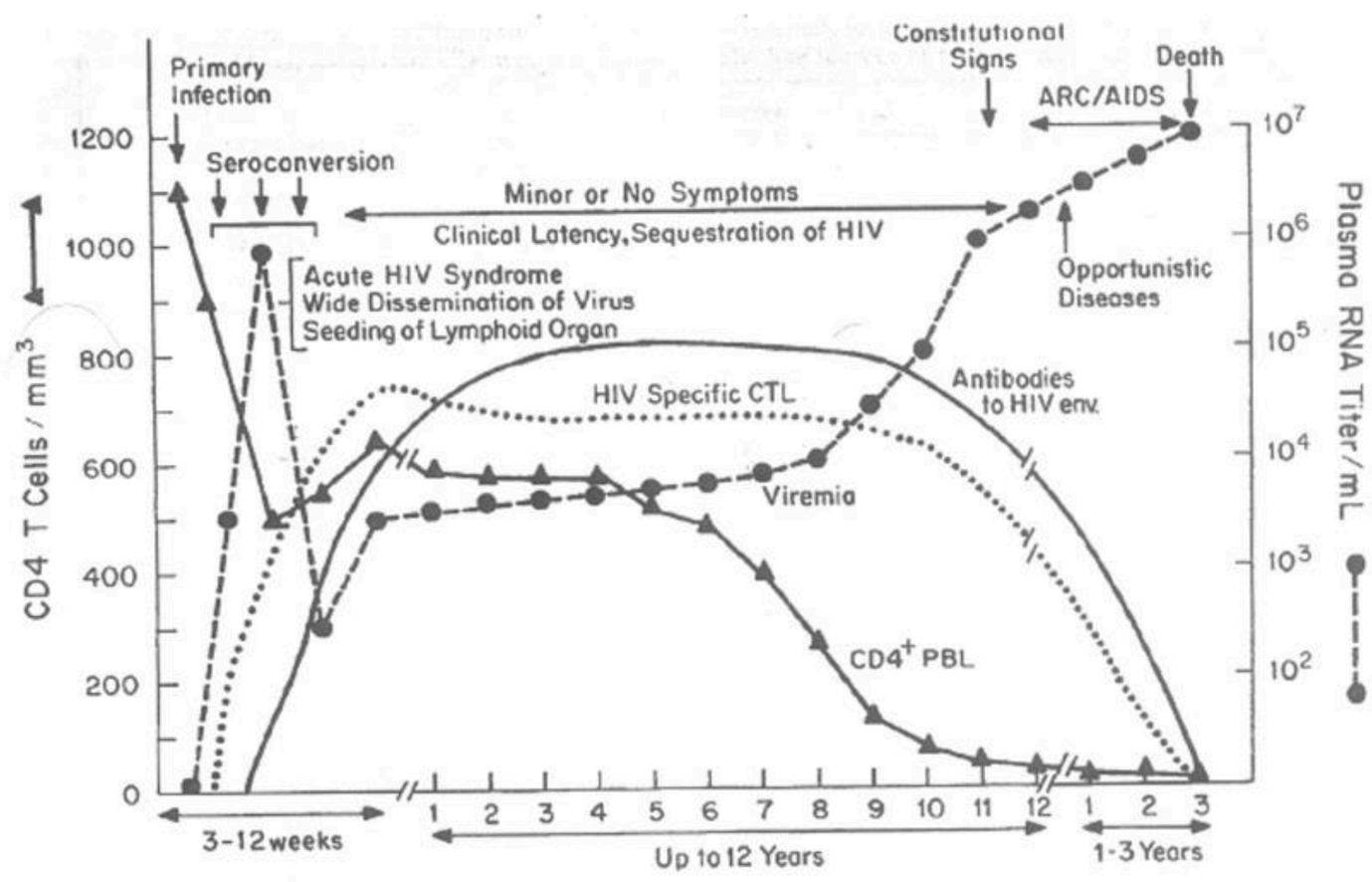


FIGURE 5: Immuno Pathogenesis of HIV infection

The duration of clinical latency following primary HIV infection varies widely among individual persons depending upon many factors; such as exposure categories (blood, sexual contact, mother-to-child), portal of entry, viral pathogenicity and mutation, host resistance, intrinsic and specific immune responsiveness, new treatment strategies with combinations of potent antiretroviral drug therapy etc.

There are also some HIV-seropositive, long-term "non-progressors" who have continued to be healthy without evidence of disease or immune defects for ten or more years after primary HIV infection.

Although reliable clinical data are available for only the past 15 years or so, it is predicted that approximately 95% of HIV-1-infected patients will progressively develop AIDS within 15 years of infection. The first few weeks after primary infection are characterized by an acute mononucleosis or flu-like illness. During this stage seroconversion usually occurs and is associated with a rapid increase in circulating viral titers. This is followed by virus dissemination to lymphoid organs. Adaptive host immunity, including neutralizing antibodies and cytotoxic T lymphocytes (CTL) against virus-infected cells, subsequently develop. While these responses may temporarily control the infection, generally they are unable to eliminate the virus. The patient then enters the stage of clinical latency or the asymptomatic period. A progressive decrease in CD4+ peripheral blood lymphocytes (PBL) continues during the latency stage. When CD4 counts fall below 200/mm^3, the disease usually enters a symptomatic phase, characterized by opportunistic infections and other AIDS-defining conditions in the host.

Clinically apparent disease

This clinically apparent, symptomatic stage is a consequence (secondary manifestation) of the progressive and profound deterioration of the immune system that occurs over time in most patients with HIV infection. The CD4 T-cell count continues downward to the range of 200-400/ cubic mm. Plasma viremia and p24 antigenemia approach high levels such as seen in the primary infection. Some of the constitutional symptoms, opportunistic infections, and other manifestations of advanced symptomatic HIV disease are given in the following table.

	(CD4 T-cell count 200-400/ cubic mm).
1	**Constitutional symptoms**: • Fever • Weight loss • Fatigue • Night Sweats • Diarrhoea • Persistent generalized lymphadenopathy
2	**Infections:** • Oral or vaginal candidiasis • Oral hairy leukoplakia • Herpes zoster (shingles) • Herpes simplex • Listeriosis
3	**Other manifestations:** • Cervical dysplasia • Cervical carcinoma in situ • Idiopathic thrombocytopenic purpura (ITP) • Neuropathy • Seborrhea

TABLE 5: Manifestations of Advanced Symptomatic Disease.

As the CD4 T-cell count continues to fall, sometimes precipitiously, and reaches levels that define AIDS (CD4 T-cell count less than 200/cubic mm.), predisposition to AIDS-defining conditions, such as opportunistic infections caused by viruses, bacteria, fungi and protozoa, neoplastic disease, HIV encephalopathy, wasting syndrome, and progressive multifocal leukoencephalopathy that complicate the clinical course and are often the cause of death.

	List of conditions in the 1993 AIDS Surveillance Case Definition issued by the Centers for Disease Control and Prevention
1	**CD4 T-cell count < 200/cubic mm.**
2	**Opportunistic Infection:** • Candidiasis of bronchi, trachea, or lungs • Candidiasis, esophageal • Coccidioidomycosis, disseminated or extrapulmonary • Cryptococcosis, extrapulmonary • Cryptosporidiosis disease (other than liver, spleen, or nodes) • Cytomegalovirus retinitis (with loss of vision) • Herpes simplex: chronic ulcer (s)(>1 month duration); or bronchitis,] pneumonitis, or esophagitis. • Histoplasmosis, disseminated or extrapulmonary • Isopsoriasis, chronic intestinal (>1 month duration) • Mycobacterium avium complex or M. kansasii, disseminated or extrapulmonary • Mycobacterium tuberculosis, any site • Mycobacterium, other species or unidentified species, disseminated or extrapulmonary • Pneumocystis carinii pneumonia • Pneumonia, recurrent • Salmonella septicemia, recurrent • Toxoplasmosis of brain
3	**Neoplastic disease:** • Cervical carcinoma, invasive • Kaposi's sarcoma • Lymphoma, Burkitt's (or equivalent term) • Lymphoma, immunoblastic (or equivalent term) • Lymphoma, primary in brain)
4	**HIV encephalopathy (AIDS dementia complex)**
5	**Wasting syndrome due to HIV**
6	**Progressive multifocal leukoencephalopathy (PML)**

TABLE 6: List of CDC conditions.

HIV SPECIFIC PROGNOSTIC MARKERS

Several HIV specific markers can be used for disease prognosis. Of these declining levels of antibody to p24 (anti-p24) and increasing concentrations of HIV antigen, especially the p24 antigen have been most widely used.

Levels of antibody/antigen however can vary widely between individuals and therefore sequential tests are usually necessary to confirm a trend. Indeed some patients are not measurably antigenaemic until later on in the disease progression, where as others may be antigenaemic for many years. There is still a debate as to the mechanism of these changes. A decline in anti p24 levels could lead to reduced formation of antigen – antibody complexes and hence allow detection of the p24 antigen. Alternatively, the developing p24 antigenaemia may reflect increased virus replication. As the infection progresses, the amount of viral RNA and infectious virus in plasma increases and the proportion of peripheral blood mononuclear cells carrying the provirus also increases.

The prognostic significance of p24 antigenaemia is indicated by a four-fold increase to the progression to AIDS within 3 years, in those who show circulating p24 antigen. However only about 50 per cent of patients with ARC and approximately two-thirds of patients with AIDS will be antigenaemic.

A decrease in titre of anti p24 antibody is also associated with disease progression and has been shown to occur before the rise in p24 antigen levels. The decline in titre of anti p24 antibody may therefore be an early indicator of disease progression.

NON SPECIFIC MARKERS OF HIV PROGRESSION

Markers other than HIV specific antigens and antibodies can be of use in predicting and assessing progression to AIDS.

At present the most commonly used marker is the CD4 cell count, expressed either as an absolute value (normal adult range 600-1700/mm^3 or as a CD4:CD8 ratio (normal adult ration 1.2 to 3.5).

Similarly, when CD4 cell counts fall to 200/mm3 or less, prophylaxis against pneumono cystic carnii is generally initiated.

Measurements of cell counts are, however, subject to potentially large biological and laboratory variation and interpretation should be based on more than one result. A recent prospective study has suggested that combining measurements of the levels of either serum β-microglobulin or neopterin along with the CD4 cell count improves its prognostic significance. It is likely that a combination of markers rather than a single marker will prove useful for the monitoring of disease progression. The precise role and practicality of this approach requires further evaluation.

* CD4 Cell Count
* CD4/CD8 Ratio
CD8 Cell Count
Serum interferon
Serum IgA
Serum IgE
* Serum β2-microglobulin
Serum/ urine neopterin

* Denotes more commonly used marker

TABLE 7: Non-specific markers of HIV Progression

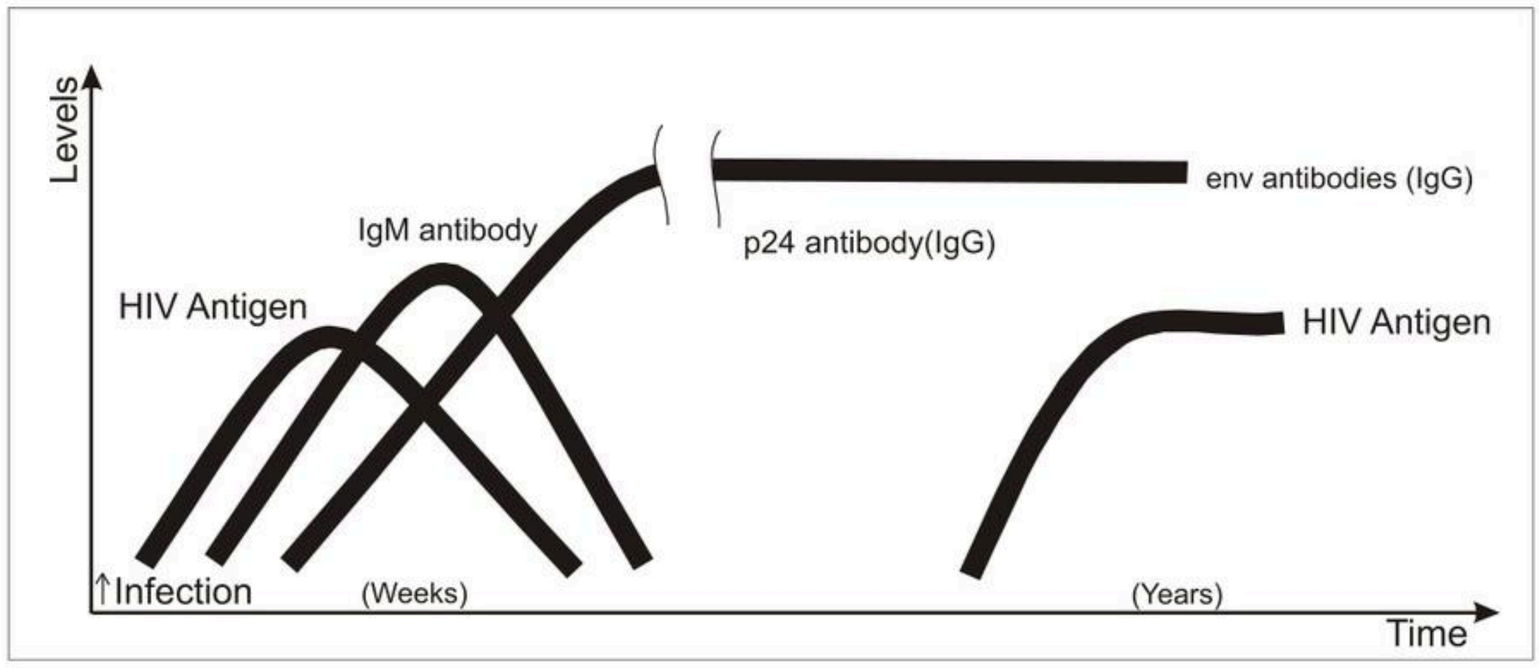


Figure represents the idealized sequence of serological events during HIV infection progressing to AIDS.

FIGURE 6: Serological Profile of HIV Infection

Following infection there is evidence of active viral replication as virus can be isolated from cultures of peripheral blood mononuclear cells and p24 antigen can be detected in the serum by immunoassays and viral RNA based assays. This antigenaemia may only last for a week or two and disappears when the antibody response develops following seroconversion. The timing of collection of any blood samples is crucial as prior to or at seroconversion antigen is detected in less than 50% of samples. Seroconversion occurs in most patients by 4-8 weeks after exposure although in some cases antibody may not appear until 6-9 months later. This delay between infection and appearance of antibodies is termed as the "Window Period". At seroconversion, antibody of the IgM class can be detected. Analysis of the reactivity of the IgG response by immunoblotting or enzyme immunoassay based on recombinant proteins shows that antibodies develop rapidly to the principal structural proteins e.g. the envelope glycoproteins gp 160, gp 120 and gp 41 and the core proteins p18, p24. Antibody to the polygene proteins can also be detected. Antibodies to the envelope glycoproteins gp 160, gp 120 and gp 41 incase of HIV-1 and gp 36 in case of HIV-2 infections may appear before antibodies to the core proteins although the time difference is generally only a matter of days or a few weeks.

After seroconversion there is evidence of continued virus replication as virus can be cultured from the plasma as well as detected by PCR based RNA assays. Indirect evidence is provided by the progressive increase in the level of antibodies to all the major virus components during this time.

In neonates the serological profile is complicated by the presence of the maternal anti-HIV antibody.

For some years after seroconversion the patient may remain well or develop persistent generalized lymphadenopathy. Throughout this period viraemia may increase. A reduction in the level of antibody to the p24 core protein and the appearance of p24 Ag are associated with progression to clinically overt disease. In the later stages of AIDS, as the immune system is destroyed, antibody may not be detectable in some patients.

HIV Diagnostic Testing techniques

As has been discussed that virtually all individuals who become infected with HIV mount an IgG antibody response to the virus which current immunoassays will detect by 4 to 8 weeks after primary infection and in the majority of cases for the remainder of the individuals life. Screening and diagnostic tests thus rely upon the detection of antibodies to HIV. During part of the window period IgM anti-HIV and HIV Ag may be present, but specific screening for them though desirable is not cost effective in low prevalence populations.

During the search for the causative agent of AIDS, tests for anti-HIV detection were developed. Initial testing was by Western Blot analysis. However, the magnitude of the HIV epidemic and the need for extensive screening were not fully understood. By 1983 and 1984, it had become apparent that rapid, simple and inexpensive assays would be required. Research assays were developed and in 1985 several manufacturers introduced enzyme immunoassays for blood donor screening. In the ensuing years these assays have evolved rapidly by utilizing products of modern biotechnology, for screening and confirmation of HIV infection.

Enzyme Immunoassays (EIA)

The diversity of EIA formats makes a simple, meaningful definition difficult to formulate, but EIA's can be described as a binding assays that depend upon the antigen-antibody reaction as the basis and the enzyme reaction as a marker for the proof of reaction. Anti-HIV EIA kits in various formats are available. Obtaining high quality results not only depends upon the quality of the kit but also on strict adherence to kit protocols, well-maintained equipments and good performance assurance procedures. Under these conditions many assays currently available will achieve>99% sensitivity and specificity. Typically, a batch of tests takes 1.5-3 hours to complete. Since the EIA's detect Anti-HIV antibodies, the solid phase on the microwell is coated with HIV specific proteins.

Generation of Assays

The terms first, second and third generation have been applied to anti-HIV assays according to the source of the HIV Ag used on the solid phase as opposed to sequential improvements.

Generations of Anti HIV Assays	
First Generation	Native viral antigens from lymphocyte culture
Second Generation	Expression products of recombinant HIV cDNA
Third Generation	Chemically synthesized oligopeptides (approximately 15-40 amino acids) Double antigen sandwich assays

TABLE 8: Generations of Anti HIV Assays

First generation assays may lack specificity because of reactions with contaminating cell proteins and lack sensitivity due to the high sample dilution needed to attain acceptable specificity. For second and third generation assays, the identification and sequencing of important antigenic sites, the expression of proteins of precisely targeted portions of HIV cDNA, and the synthesis of oligopeptides containing immunodominant epitopes has provided new antigens for use in the assay systems. When incorporated into EIAs these recombinant proteins and peptides have led to substantial improvements in sensitivity and specificity and have the added advantage of being safer, easier and more reproducible in performance.

EIA Assay Formats

There are four basic assay formats for the detection of antibodies to HIV

Type 1: Indirect or Antiglobulin

A relatively crude native HIV antigen preparation is immunoabsorbed to the solid phase by pre-coating with HIV antibody. Anti human globulin conjugated to enzymes is used as a tracer, with a compatible substrate as a chromogenic marker.

Type 2: Competitive Assay

In Competitive assays the sample need not be diluted. These assays exhibit high sensitivity. To ensure good specificity and sensitivity the spectrum of anti-HIV detected is predominantly against env. determinants, but because antibodies to env antigens of HIV-1 and HIV-2 show only limited cross reaction the competitive format is unsuitable for combined anti-HIV-1/ HIV-2 screening.

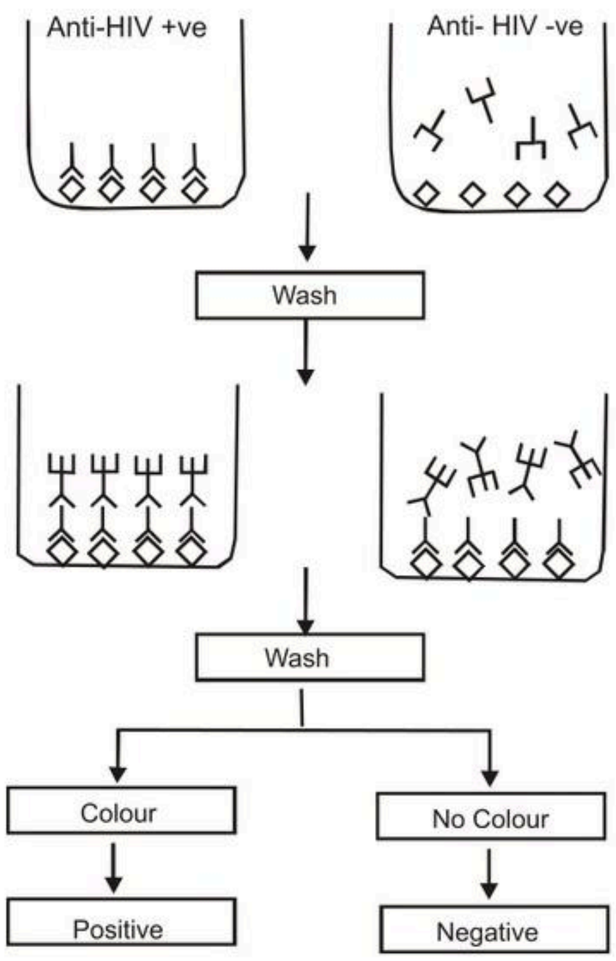


FIGURE 7: Indirect or Antiglobulin assays

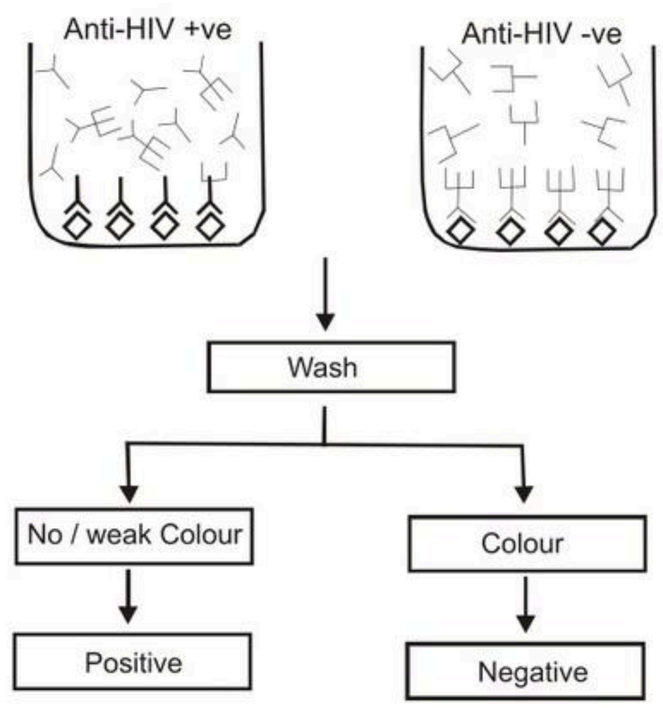


FIGURE 8: Competitive assays

Type 3: Class Specific Antibody Capture Assay

Class Specific Antibody Capture assays are only available as a research tool. They have proved to be highly accurate for detecting anti-HIV in specimen such as saliva and urine and find important applications in epidemiological research and other situations where venipuncture is impractical.

Type 4: Double Antigen Sandwich or Immunometric Assay

A commercially available example of this assay detects anti-HIV-1 & anti-HIV-2 by using recombinant HIV-1 antigens and synthetic oligopeptide antigens from the HIV-2.

These assays are sensitive and specific, an added advantage being that they do react with IgM anti HIV antibodies and they are reported to detect seroconversion earlier.

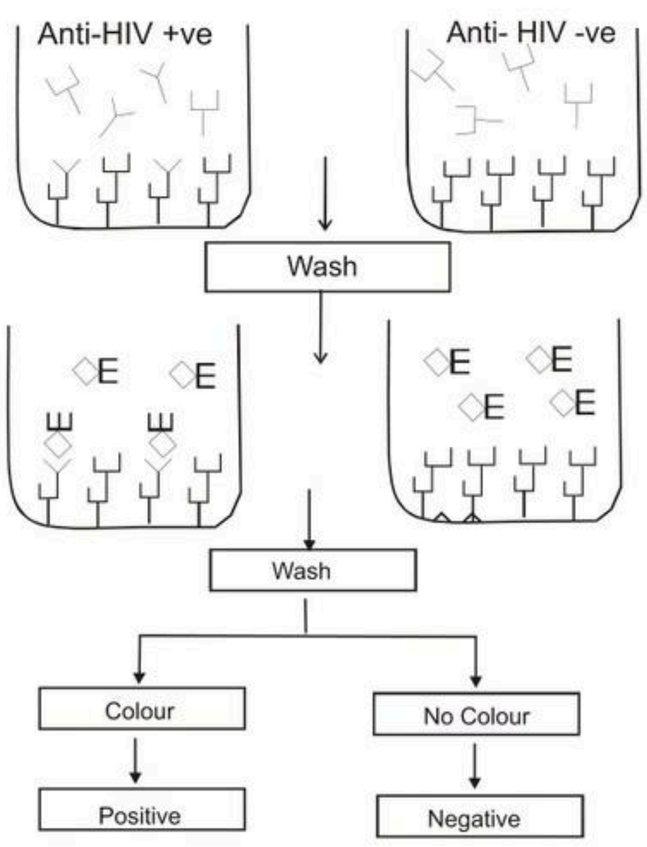


FIGURE 9: Class Specific Antibody Capture Assays

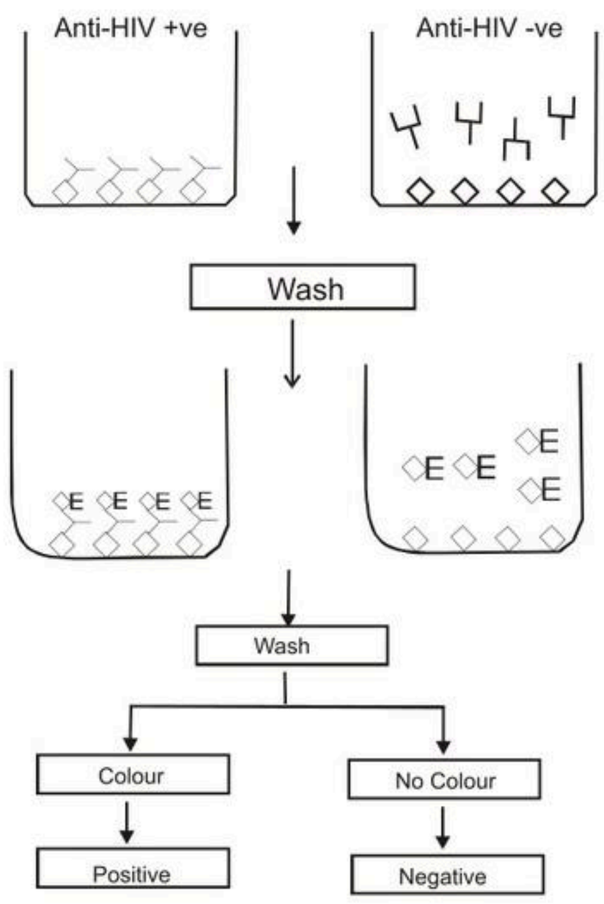


FIGURE 10: Immunometric Assays

HIV Antigen EIAs

HIV Antigen EIAs have been developed primarily for the purpose of monitoring HIV-infected individuals for antigenaemia, but are also used to monitor growth of HIV in lymphocyte cultures. The methods used are in general modifications of antibody sandwich assays described earlier.

Anti-HIV prepared from the serum of HIV infected individuals, animal antisera raised against purified viral or recombinant antigens and monoclonal antibodies have all been used in various combinations as capture and detector antibody as well as a variety of enzymes and substrates.

The sensitivity of HIV Ag assays can be below 50 pg/ml. It should be noted, however, that standards do vary from one commercial kit to another reflecting the unsuitability of standardizing by weight of a complexed antigen, which will be, measured immunometrically.

Estimations of HIV antigen level can, therefore, be regarded as 'semi quantitative'. Most manufacturers do provide HIV antigen standards, which can be included in assay runs in order to produce a standard curve from which specimen HIV antigen levels can be extrapolated. Despite the 'semi quantitative' nature of the estimation, sequential monitoring of titres may be informative regarding disease progression or efficacy of antiviral therapy.

Confirmation of positive findings is recommended and some manufacturers have reagents available for this purpose, usually a blocking antibody.

Confirmatory / Supplementary Assays

Confirmatory tests are used to confirm whether specimens found reactive with a particular screening test contain antibodies specific to HIV. The most commonly used confirmatory test until recently, was the Western Blot (WB). However, its use has proven to be very expensive and prone to producing large numbers of indeterminate results. The differences in criteria for the interpretation of Western Blot also has lead to classification difficulties of indeterminate samples. Now, studies have shown that combinations of ELISAs or Simple/Rapid assays can provide results as reliable as the WB at a much lower cost.

1. Western Blot

The Western Blot was developed to detect antibodies to HIV and to qualitatively determine to which viral proteins the antibody response had been raised. The assay is based on the detection of the reaction of specific antibodies in the sample with viral antigens that have been electrophoretically separated and transferred ("blotted") onto a nitrocellulose sheet on a backing. Antibodies, which react with antigens on the membrane, are visualized with an enzyme labelled anti-human immunoglobulin and an enzyme substrate, which gives an insoluble coloured product at the site of reaction.

Although WB allows the humoral immune response to HIV to be mapped, there are several problems: Not all sera from infected individuals react with all HIV proteins. Sera from some uninfected individuals may react with one or more HIV proteins or with contaminating residual lymphocyte cell proteins. Large differences between batches and particularly between manufacturers occur in the number of proteins and their relative concentrations and positions on the strips. The assay is read visually. To counter these difficulties, some standardization has been attempted, particularly in terms of interpretation of the reactivity observed. Several sets of criteria have been recommended which differ in details, but commonly require an antibody to at least one env. gene product, and at least one band from gag and /or pol for classifying a positive test result.

Criteria for Interpretation of Western Blot

Organization	Criteria	
	Positive (minimum of:)	Negative
World Health Organization	Two env. bands	No HIV specific bands
C.D.C.	Any two of p24; gp41;gp 120/160	No bands at all
Consortium for Retrovirus serology standardisation	P24 or p32 and gp 41 or gp120/160	No bands at all
American Red Cross	At least one from each of gag, pol and env	No bands at all
Food and Drug Administration (USA)	P24, p32 and gp 41 or gp 160	No bands at all

TABLE 9: Criteria for Interpretation of Western Blot Assay

Results which neither satisfy 'Positive' nor 'Negative' criteria are termed as 'Indeterminates'.
Recently, immunoblots, which resemble WB but utilize a selection of synthetic HIV antigens applied to membrane strips as discrete bands have been developed (LIA / RIBA). These are reportedly comparable to the electrophoterically manufactured traditional blots, with reduction in the proportion of indeterminate results. However these are equally expensive.

2. Indirect immunofluorescence:

IF has been commonly employed to detect anti-HIV antibodies. Acetone fixed HIV infected lymphocytes are incubated with sample, followed by a fluorescein conjugated anti-human IgG. The cells are then examined using fluorescent microscopy. If anti-HIV is present a typical pattern of fluorescent staining is seen in the cells. IF is usually reserved for confirmation as it is time-consuming and requires highly-skilled operators and an expensive, well-maintained fluorescence microscope.

3. Radioimmunoprecipitation assay:

RIPA is used by very few laboratories. Initially, HIV Ag is metabolically labelled by substituting one or more S tagged amino acids in HIV culture medium. The labelled cells are then disrupted to release the labelled proteins and the resulting suspension is clarified. The labelled proteins are used for testing the sample.Containment facilities for handling live HIV and radioisotopes are required along with expensive equipment and consumables for immunochemistry, limiting its usage to few reference laboratories.

Virus Detection Methods

Virus Isolation has traditionally been achieved by co-cultivation of gradient purified patient's peripheral blood lymphocytes with stimulated lymphocytes from an uninfected individual. To seek evidence of viral replication the cultured cells may be examined by microscopy for cytopathic changes and by HIV immunofluorescence. The culture medium may also be assayed for HIV p24 Ag or reverse transcriptase activity. Isolation of HIV indicates beyond any doubt that the subject is infected with HIV. However, it has a very restricted role to play in confirmatory testing because of its relatively low sensitivity and the cost and complexity of the technique. HIV can sometimes be isolated from fresh or properly stored plasma, but usually a fresh blood sample will be required to provide peripheral blood mononuclear cells for co-cultivation. Very few laboratories claim nearly 100% HIV isolation from all anti-HIV positive subjects, but most achieve 60-80% success. Virus isolation is generally reserved for research purposes due to its expense, low sensitivity, long incubation time and need for high containment facilities.

p24 Antigen and Nucleic Acid Technologies

A number of other assays have been introduced in recent years which assist in the establishment of the diagnosis of HIV infection and may also be used to monitor the progress of the infection and the response to therapy. These include assays that detect virus particles e.g. the HIV p24 antigen ELISA, or the presence of HIV viral nucleic acid sequences (RNA or DNA) by means of nucleic acid amplification techniques. NAT assays have made it possible to detect minute amounts of viral material - as little as a single viral genome (the detection limit for most assays is around 300 copies/ml. These sensitive procedures are well suited for early diagnosis of mother-to-child transmission and for monitoring the viral load of patients who are on antiretroviral therapy. However, in routine practice this technique can have limited specificity, needs sophisticated equipment, rigorous laboratory conditions and highly trained staff, and is still very expensive.

Rapid tests for the detection of anti-HIV antibodies

Advances in technology have now made it possible to design rapid HIV antibody assays based on agglutination, immunodot, immunochromatographic and immunoconcentration / filtration techniques, the assays have comparable sensitivity to equivalent generation EIAs.

The assays are easy to perform and ideal for low through put laboratories. Since same day results are offered through the use of these tests, they help in timely detection and treatment intervention of HIV infection.

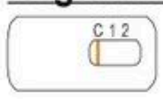
The overall considerations in rapid test assay development are as follows:

1. The tests should pick up antibody response to HIV-1 & HIV-2.
2. The test when used in areas endemic with variants of HIV 1 such as O & M (Cameroon, Gabon) should be reactive with the antibodies to the variants.
3. The test should increase the sensitivity of detection during the "window period" shortening it as much as possible.
4. The antigenic configuration of the test should preferably use recombinant antigens or peptides from the conserved immunodominant regions of the HIV-1 and HIV-2 genome.
5. The screening test should have sensitivity and specificity in excess of 99%.

Well designed rapid tests based on protein A based tracers that pick up IgG class anti-HIV antibodies bound to the solid phase, in general achieve such specifications. However since protein A based assays necessarily map only IgG class anti-HIV antibodies, they tend to be less sensitive than systems having the ability to pick up IgG and IgM class of Anti-HIV antibodies. Third generation rapid tests now utilize the principle of **double antigen sandwich immuno assays**. In the double antigen sandwich system recombinant proteins from the immunodominant regions of the HIV genome are used on the capture as well as on the tracer reagent. Antibodies to HIV are thus specifically captured and sandwiched during the test specifically between the solid phase and tracer antigens. This third generation system not only improves specificity but also improves sensitivity of the assay since not only IgG but also IgM antibodies react in the system, improving the test sensitivity in the early seroconversion phase of the HIV infection. Such improved third generation rapid tests are being made available now.

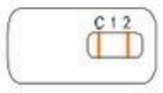
Flow Through Technology

Negative Test result:

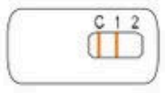


Appearance of only one control band corresponding to control region 'C'.

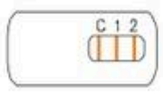
Positive Test results:



In addition to the control band 'C', appearance of reactive band at test region '1': specimen positive for Antibodies to HIV 1.



In addition to the control band 'C', appearance of reactive band at test region '2': specimen positive for Antibodies to HIV 2.



In addition to the control band 'C', appearance of reactive bands at test region '1'and test region '2': specimen positive for Antibodies to HIV 1and HIV 2.

Invalid Test results: The test should be considered invalid if neither the test band nor the control band appears.

Lateral Flow Technology

Negative test result:

Appearance of only one coloured band at the control region 'C'.

Positive test result:

Appearance of a coloured band at the test region 'T' in addition to the band at control region 'C'.

Invalid Test results:

The test should be considered invalid if neither the test band nor the control band appears.

Flow Through v/s Lateral Flow Technology

Rapid test currently available are based either on the second generation protein A based flow through systems or the third generation lateral flow based double antigen system. The flow though technology based tests are multicomponent, multistep based systems that require cold storage. These tests also require higher degree of laboratarian involvement and can only use serum or plasma as a sample.Since the membrane needs to be rehydrated before use there is a limit on the number of tests that can be run at a time simultaneously.

On the other hand lateral flow technology based double antigen sandwich assays are single step / two step and easier to use. Suitability of whole blood as a sample and room temperature storage in addition to higher clinical sensitivity due to anti-HIV IgM detection capability make these tests a method of choice for most settings. These assays have an overall advantage over flow through technology based tests.

Application of HIV Testing

The donor screening tests for anti HIV have been also adopted for clinical diagnosis and for epidemiological prevalence and monitoring studies. Because the consequences of a positive diagnosis of HIV infection are so serious it is generally considered that people who are tested should be asked to give consent to testing only after counselling, which in simple and sensitive terms explains the background to the screening test, the implications of a positive finding and advice on risk avoidance. Follow-up clinical and psychological support for those found to be infected is also essential. Of equal importance is a mechanism for distinguishing which of those samples that react in screening assays are genuine, or true positives.

Screening tests for anti-HIV-I will not detect all HIV-2 infections and the discovery of HIV-2 infections outside of its recognized endemic regions in West Africa has influenced the decision by many countries to introduce tests which can detect antibody to HIV-2 reliably. There are now many 'combined' HIV-1/HIV-2 EIAs and rapid tests that have become the screening method of choice. Simultaneously test that can in addition detect the antibody to subgroup 'O' are considered "complete" as a screening test.

Screening

Anti-HIV screening assays were primarily developed for blood bank screening with the objective of protecting the recipients of blood or its products from infection with HIV. In addition to screening, vetting of donors and phasing out of inducements to donate are now strongly recommended by W.H.O. However, in regions where HIV has spread primarily by heterosexual contacts, vetting of donors is difficult as 'high-risk" groups are not easily recognized and more reliance is therefore placed on anti-HIV screening. Nevertheless, anti-HIV screening alone is insufficient to ensure that the blood supply is free from HIV contamination. Even if the assays were 100% sensitive, technical and clerical errors can lead to reporting errors.

Many considerations affect the choice of screening assay including sensitivity, specificity, ease of performance and cost. Poor sensitivity may jeopardize the safety of recipients of blood or other tissues while, on the other hand, poor specificity is costly in terms of unnecessarily wasted donations, the cost of extra confirmatory testing and the problems of management of donors whose samples are reactive. Limited resources have led some transfusion services to consider the screening test simply as a means to protect recipients. Many however, are also able to assume responsibility for their donors, ensuring that screen-reactive samples are confirmed before counselling the donor and offering referral to an expert HIV clinic.

Worldwide application of anti-HIV screening assays outside the strictly clinical arena are increasing: many life insurance companies require anti-HIV screening before offering cover to applicants, and some authorities require HIV testing before issuing marriage licences, immigration documents or admitting recruits to armed services. Some controversy surrounds these applications of HIV screening with respect to their moral, ethical and legal justification. If carried out, confirmation of a screen reactive finding is an absolute requirement, especially as these tests are applied to populations in which HIV prevalence is generally low and therefore false positive reactions are common relative to genuine positive reactions.

Diagnosis

When screening tests for anti-HIV first made it possible to identify HIV-infected individuals who did not manifest the classical signs and symptoms of AIDS, there was some debate whether testing was worthwhile for them. However, improvements in predicting the progression of disease by use of prognostic markers and continuous improvements in treatment, both with Antiretroviral and prophylactic treatment of opportunistic infections, now favour the policy of early identification of all HIV-infected individuals.

There are three main diagnostic applications of anti HIV testing:

(a) To confirm that a patient presenting with symptoms associated with HIV infection is infected with HIV

(b) To investigate a person who has been exposed to infection because of exposure to body fluids from a known HIV-infected individual, perhaps by sexual intercourse or by inoculation

(c) And to test those who belong to one of the recognized risk groups, are healthy and have no definite history of exposure, but are anxious and present themselves for testing in the hope of gaining the reassurance of a negative result.

Many diagnostic laboratories now take the precaution of running two screening tests (usually different manufacturers kits) on each sample to decrease the possibility of false negative findings, whether due to a technical or clerical error or some inherent fault in an assay.

Approach to Confirmatory Supplementary Tests

The best of the currently available anti-HIV screening assays will give rise to less than 1 in 1,000 repeatable false positive reactions. Although this represents excellent specificity, when such a test is applied to a low prevalence population only a small proportion of reactive samples will be genuine. To avoid the potentially serious consequences of a false diagnosis, several strategies have been devised for further testing to determine the authenticity of a positive screening reaction. This is referred to as confirmatory or supplementary testing. It is generally accepted that only positive and unexpected negative results in screening assays need to be confirmed. So that unnecessary confirmatory testing is not undertaken, most laboratories first retest reactive samples twice or more in the screening assay. Unrepeatable reactions are reported as negative. Whatever confirmatory strategy is employed, it is good practice to seek a follow-up sample from any individuals found to be reactive. This is to primarily safeguard against a false diagnosis due to technical or clerical errors, but also to check that an uncomfirmable positive reaction on a screening assay is not a result of sampling too early during seroconversion. Cases of dual infectivity with HIV-1 and HIV-2 have been reported and thus in some instances this may require confirmation.

Western Blot (WB)

The most widely accepted 'confirmatory' test is Western Blot. A reaction in a screening assay simply indicates that antibody from the specimen is bound to the solid phase: this can be an HIV-specific or non-specific reaction. WB provides additional information about specificity, in terms of reactivity with particular HIV proteins. The reaction pattern obtained may be interpreted as positive, negative or indeterminate according to the application of one of several sets of accepted criteria. The disadvantages of WB are its cost, subjectivity and high rate of indeterminate reactions. Stringent criteria, while achieving high specificity, lead to low sensitivity. Thus, some sera from infected subjects only give indeterminate results. Conversely, low stringency criteria lead to some false positive diagnosis by WB. A further disadvantage is that criteria have usually been determined using a particular manufacturer's product. When these are applied to a different WB they may not be valid.

Line immunoassays resembling WB and 'dot-immunoblots', each using several synthetic antigens derived from different HIV genes are now being widely used. They are usually more rapid and at least as sensitive as WB. Interpretative criteria vary but are generally similar to WB, with the same three possible outcomes.

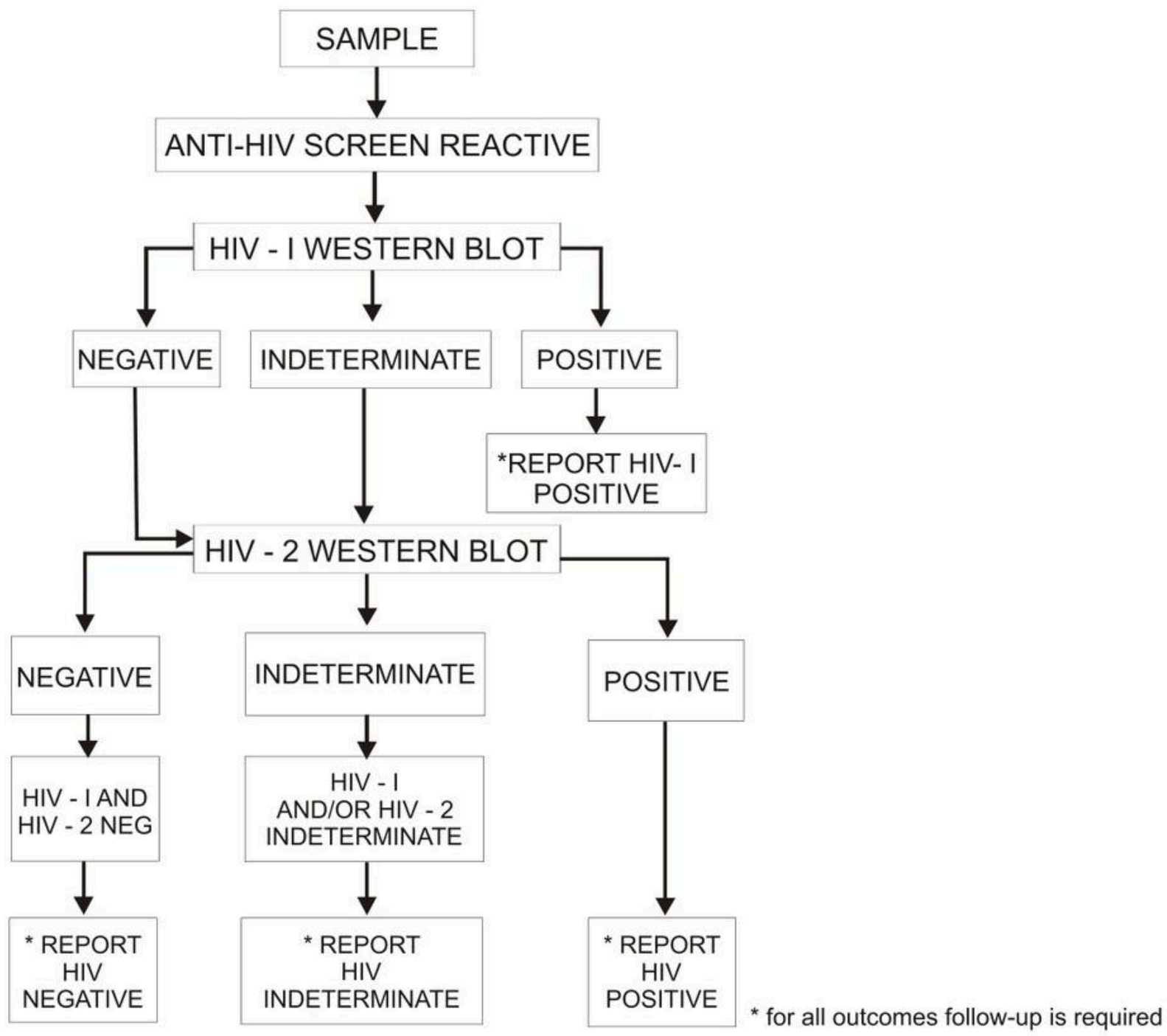


FIGURE 11: Approach to Confirmatory tests.

NAT-PCR based Methods

The NAT-PCR based assays detect the nucleic acid of HIV directly in an infected patient's sample and is employed as a confirmatory method. The expense and technical demands of PCR will, for the foreseeable future restrict its use to carefully selected cases and to providing support to investigations of simpler, less expensive confirmatory strategies.

Alternative Approach to Confirmatory Testing

This approach seeks to determine if a sample reaction in a screening test will also give a positive signal in at least two further 'independent' screening assays. Assays were originally defined as 'independent' if they were each of a different type. But this concept has recently been broadened to incorporate assays using antigens from different sources, eg. viral, recombinant DNA expression products and synthetic peptides. Just as there have to be trials of WB to develop accurate interpretive criteria, particular combinations of assays must be examined to demonstrate that they are generally independent of each other, i.e. they are not susceptible to the same non-specific effects.

It has recently been shown that a combination of rapid screening assays can also give diagnosis as accurate as WB, and more cheaply. This option should be particularly useful for anti-HIV confirmation especially in the developing world, as a cost effective solution.

WHO / UNAIDS strategies for HIV antibody screening

These three strategies take into account the objective of testing (i.e. whether for transfusion safety, surveillance or diagnosis) and the local seroprevalence rate. Depending on these factors, three different algorithms for repeat and confirmatory testing have been proposed to confirm initial screening results.

They avoid the use of the Western Blot for confirmation of positive screening test result; although Western Blot confirmation is still the rule in the USA, Germany and other countries, it can be replaced by more economical testing algorithms using ELISA-type assays and / or rapid / simple test devices.

Strategy 1 requires only one test. It is to be used for:

- **Transfusion screening** (if no results are to be provided)
- **Surveillance** (if prevalence>10% and no results are to be provided)
- **Diagnosis** (only if patient is symptomatic and population HIV prevalence>30%)
  NB: This is not normally adequate for diagnosis!

Strategy 2 requires up to two tests and is to be used for:

- **Surveillance** (if prevalence ≤ 10% and no results are to be provided)
- **Diagnosis** if patient symptomatic and population prevalence ≤ 30%, or if patient asymptomatic and population prevalence > 10%.

Strategy 3 requires up to three tests for:

- Diagnosis if patient asymptomatic and population HIV prevalence £ 10%.

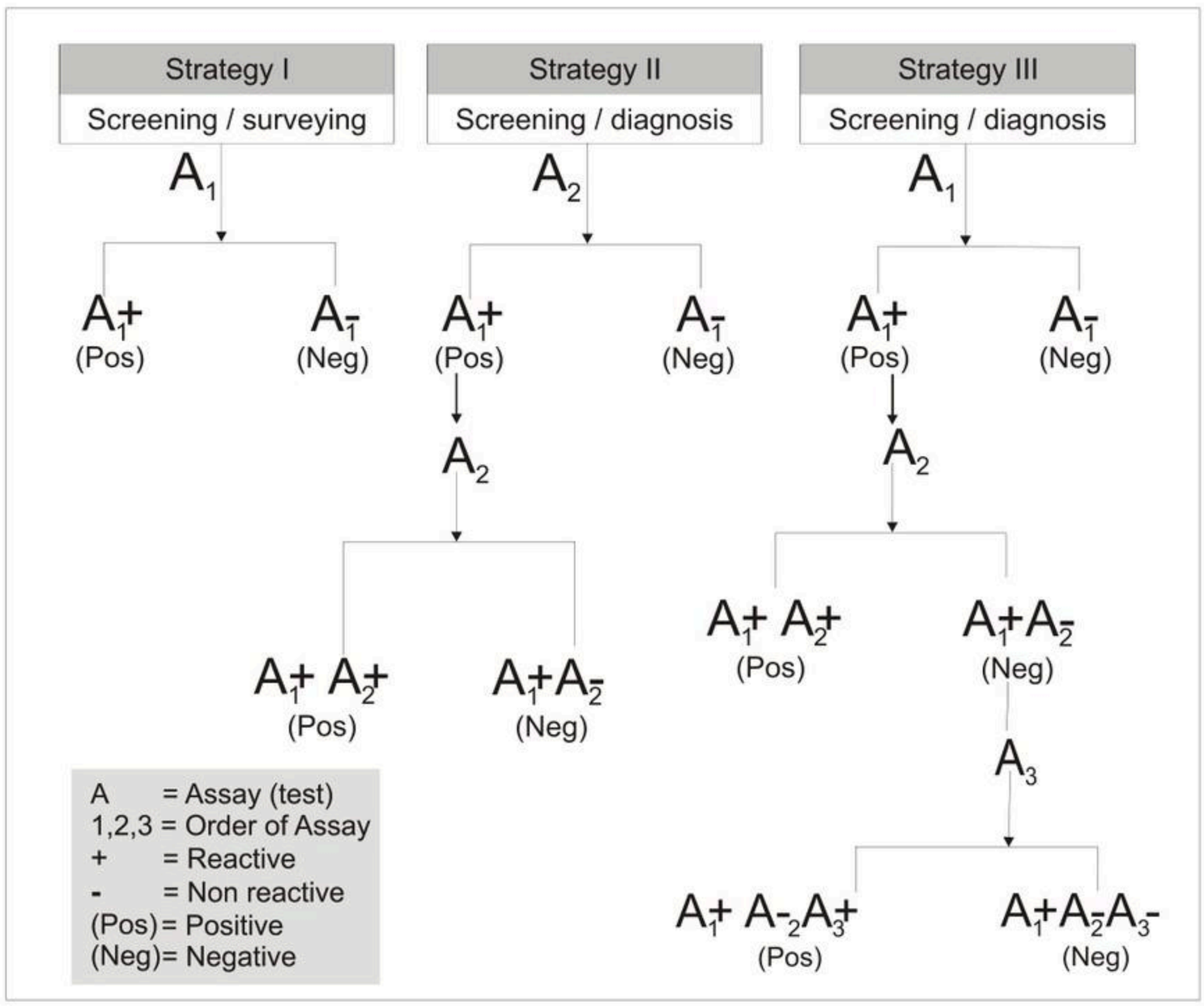


FIGURE 12: WHO/ UNAIDS Strategies for HIV Antibodies Screening

The successful confirmation using these strategies requires the user to distinguish strictly between assay results (non-reactive or reactive) and patient diagnosis ("Positive" or " Negative")!
For all strategies, the first (screening) test (Test 1) should be chosen for its very high sensitivity (>99%) (to avoid false-negative results), while the confirmatory tests (Tests2 and 3) must have a high specificity (>99%). No test performance characteristic must be <95%. Furthermore, the different tests used for each strategy must employ different formats.

Discrimination Between HIV-1 and HIV-2 Infection

Tests that claim to distinguish HIV-1 from HIV-2 infection cannot necessarily be regarded as confirmatory tests. Some of them rely on a reaction with a single short length oligopeptide and may lack sensitivity for antibody from all individuals infected by the homologous HIV type. However, most of the confirmatory methods already mentioned will discriminate to some degree between HIV-1 and HIV-2. Cross reactions, between env and gag antigens in WB make it less efficient than other approaches such as utilizing a pair of type-specific competitive EIAs or a line immunoassay employing HIV-1 and HIV-2 specific synthetic antigens. Also, the cost of two WB strips or PCR for HIV-1 and HIV-2 is high and it is doubtful whether such expenditure should be committed to making a differential diagnosis for the small proportion of cases in which more simple and inexpensive approaches fail.

Paediatric HIV Infection

Since the first report of an infant with AIDS in November 1982, the incidence of HIV infection in the pediatric population has increased exponentially. By the year 2000, it is estimated that HIV pandemic was responsible for the deaths of 3 million women and more than 2.5 million children worldwide. Additionally about 5.5 million children might have been orphaned because of premature death of their parents.

It has been proposed that when maternal infection rates reach 2-3% globally, HIV infection acquired by the perinatal route could become the most common cause of death in children. Of the reported children, younger than 13 years old in the USA, 90% were born to mothers with HIV or HIV risk factors, 5% received blood transfusions or tissue transplants, 3% were hemophiliacs and 1% had no identified risk factor.

Transmission of HIV from mother to infants

Transmission of HIV from mother to infants is not absolute but relatively efficient. Prospective studies have reported mother to infant HIV transmission in 13% to 39% of infants born to HIV infected mothers. Perinatal transmission may occur before, during or after birth. Many characteristics of the HIV infected mothers may be associated with increased risk of perinatal HIV transmission; women with symptomatic HIV infections or AIDS may have an increased risk of transmitting infection to their infants as compared to asymptomatic pregnant mothers.

Higher transmission rates have been reported for infants delivered vaginally than those by cesarean section. In some studies presence of other STD's during pregnancy, such as syphilis, appear to increase the likelihood of HIV transmission possibly due to placentitis associated with *Treponema pallidum* coinfection. Other ulcerative genital infections may also increase the transmission rates.

Perinatal feeding practices can also affect vertical HIV transmission as HIV infection does occur via breastfeeding. Type of milk ingested (colostrum versus later milk) duration of breastfeeding, duration of mother's HIV infection, her viral load and the antibody content in her milk influences mother to infant transmission rates.

The working definition of intra uterine infection as suggested by experts is a positive PCR or HIV culture or a p24 antigen test on the infants blood within 48 hours of life. Perinatal infection is defined as negative assays shortly after birth and positive results occurring later. Voluntary testing for HIV to recognize HIV infected mothers can benefit through antiretroviral therapies, early prophylaxis of genital infections, providing passive HIV antibody to pregnant women, fetus and the newborn and providing active or passive enhancement of fetal and neonatal anti HIV specific immunity.

Diagnostic Challenges: Child born to a HIV infected mother

Diagnosis of HIV infection in children born to HIV infected mothers is complicated by the presence, in almost all cases, of the maternal anti HIV antibody which crosses the placenta to the fetus. Over all less than one third of the children are actually infected. Since HIV antibody assays such as EIA, immunoblots primarily detect IgG class of antibodies and in infants, this class of antibody may persist from upto 9-18 months, conventional antibody tests cannot be used to reliably predict the HIV infection in children under 18 months of age. In the first year of life, HIV infection can be reliably diagnosed by PCR and viral culture, since they can correctly identify upto 50% of the infected infants at birth and nearly 100% of the infected infants by 6 months of age. It must be however recognized that false positive PCR results occur in the first week of life and the standard p24 antigen assay is less sensitive than culture or PCR as it fails to detect immuno complexed p24 antigen.

The additional potentially useful methods currently under development and evaluation are:
a) Test based on in vitro production of virus specific antibodies by patients lymphocytes.
b) Detection of patient specific IgA antibodies to HIV, since the IgA does not cross the placenta.

Anti Retroviral therapy

The treatment of HIV infection requires an understanding of viral replication, dynamics, antiviral potency, pharmacokinetics and toxicities of individual drugs, interactions between drugs when used in combination and the limitations imposed by failure of one drug on the future use of same and related drugs.

Most of the anti retroviral drugs available today exert their anti retroviral action by disrupting the viral replication cycle of the HIV within the infected host. Broadly these drugs can be classified into:

- Inhibitors of viral reverse transcriptase that block the conversion of the viral RT dependent genomic RNA into double stranded DNA notable amongst those being:
  - Non nucleoside RT inhibitors
  - Nucleoside analogue RT inhibitors, and
  - Nucleotide analogue RT inhibitors.
- Inhibitors of the viral protease enzyme to prevent the cleavage of poly proteins & conversion of viral particle into an mature infectious virion (protease inhibitors).

The inhibitors of viral transcriptase compete with natural enzyme substrates in vivo, leading to perinatal DNA chain termination of the HIV life cycle.
On the other hand, action of the protease inhibitors does not let the viral particles mature and remain replication defective.

Since HIV infection is a dynamic process with high rates of replication and great potential for genetic mutation, a major determinant of anti retroviral drug failure is viral genotypic variation conferring drug resistance.

When a patient starts a drug regime, the regime selects for pre existing mutation as well as additional mutants that may appear in different genetic backgrounds. Mutations continue to appear unless rate of replication is curtailed enough to retard genetic variation.

Use of highly active retroviral therapy (HAART)

Combination regimes that produce more potent inhibition of viral replication may delay or prevent resistance from arising by limiting the new mutations from occurring or accumulating. Because of high cost and limited availability HAART is beyond reach of most infected individuals or individuals at risk for HIV infection.

The recent widespread use of plasma HIV-RNA levels to monitor infection and therapy has transformed clinical management of infected individuals. Therapy for persons with HIV infection is based upon CD4 cell counts and HIV-RNA plasma levels.

CD4 cells/ml	Plasma HIV-1 RNA levels (copies/ml)		
	<5000	5000-30000	>30000
<350	Recommend therapy	Recommend therapy	Recommend therapy
350-500	Consider therapy	Recommend therapy	Recommend therapy
>500	Defer therapy	Recommend therapy	Recommend therapy

TABLE 10: Plasma HIV RNA levels.

The level of HIV-RNA reflects the magnitude of viral replication. It correlates with the rate of CD4 T cell decline, the relative risk of clinical disease progression and the time to death.

Baseline RNA levels are measured before the anti retroviral therapy is started and during drug therapy to guide subsequent steps.
A consistent increase in viral load of more than 0.5 log10 usually suggests a failing anti retroviral regimen. The RNA viral load test is currently available for HIV 1 infection only.

The CD4 T cell count on the other hand reflects the extent of immune damage that has already occurred because of HIV infection. CD4 cell counts correlate with the risk of developing opportunistic infections, neoplasms and with time to death. Base line CD4 T cell counts are measured before anti retroviral therapy is initiated to assess prognosis and guide prophylaxis of opportunistic infections.

Vaccines for HIV infection

It is clear, that attempts to modify high risk behaviour amongst population has met with variable success. Because of the high cost and limited availability, HAART is beyond the reach of most individuals at risk for HIV infection. Effective vaccination probably offers the best hope for containing the HIV epidemic.

The development of vaccines against HIV poses a formidable, scientific, logistic and social challenge and the most important problems are:

- Lack of understanding of the critical parameters of immunity that would protect against the infection / disease or both.
- Substantial genotypic variations occur among the infective HIV strain along with high level of mutation and recombination in the replication of individual isolates.
- The relationship of these genotypic variations to the expression of antigens that might induce protective immunity remains poorly defined.
- An effective vaccine would have to stimulate broadly directed immune response so as to be able to inhibit diverse strains of HIV

A large body of information continues to be generated concerning the immune response to HIV infection as various phase I, II, and III efficacy trials are under way in addition to the development of vaccines based on diverse approaches.

It is hoped that an effective vaccine is closer than never before, to contain the pandemic of HIV and AIDS.

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KNOWLEDGE BOOK

For the Laboratorians

HIV Testing

Evolution & Practical Issues

Setting Trends...

OBJECTIVE OF THIS TECHNICAL SERIES

Over the past two decades, HIV diagnostics have been essential in detecting and monitoring infection, and continue to play a major role in saving lives throughout the world. As technology evolved, screening, confirmatory, and HIV monitoring assays have been improved and offer better alternatives to address blood screening, surveillance, diagnosis, and patient management.

Molecular methods are critical in detecting early infection and for managing patients on anti-retroviral therapy whose viral infection may become resistant to therapy. Many of the newly evolving technologies are essential for use in resource-limited settings because they can address cost issues, limited infrastructure, and a lack of formally trained personnel. Newer rapid HIV kits can be stored in a wide range of temperatures (2-30°C) to address cold-chain issues, can be performed using easily collected fingerstick blood and also other body fluids like saliva, and have one-step procedures that are relatively foolproof.

Despite all the advancement, developing countries like India face another daunting task; that of correct scientific information available to laboratory professionals involved in HIV testing. Terminologies like "generations of HIV test", "window period", "false positives", "p24 antigen", "HIV-1 group-O", "indeterminate results" etc. have become common laboratory jargon. But, what do they actually mean? This publication is an attempt to answer these questions in the correct scientific perspective. The purpose of this technical series is as follows:

1. ***To break myths and remove confusion in HIV testing***
2. ***To understand problems associated with HIV testing***
3. ***To throw light on how to report a positive & negative test result***

BACKGROUND

Human immunodeficiency virus (HIV) is a lymphotropic retrovirus that primarily infects and destroys $CD4^+$ lymphocytes. These cells are crucial for the induction and regulation of the immune response. Their progressive depletion by the virus causes irreversible disruption of normal immune function, leading to immunosuppression and the subsequent development of AIDS. Early medical intervention reduces the risk of transmission and delays progression to AIDS. This necessitates early detection of the infection, which may be asymptomatic for a prolonged time.

HIV is believed to have originated in West-Central Africa and jumped species (zoonosis) from primates to humans. HIV-1 evolved from a Simian Immunodeficiency Virus (SIV_{cpz}) found in the chimpanzee subspecies *Pan troglodytes*. HIV-2 crossed species from a different strain of SIV, this one found in sooty mangabeys. The earliest documented HIV-1 infection dates from 1959, and was discovered in the preserved blood sample of a man from Kinshasa in the Democratic Republic of the Congo.

CHRONOLOGY

1981 - 5 cases of pneumocystis carinii pneumonia (PCP) in Los Angeles
1983 - Pasteur Institute isolates retrovirus (HIV-1) from lymph node
1984 - Gallo's group confirms and extended findings to AIDS
1986 - HIV-2 was isolated

THE GRIM PICTURE

Infection in humans is now pandemic. As of January 2006, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimate that AIDS has killed more than 25 million people since it was first recognized in 1981, making it one of the most destructive pandemics in recorded history. In 2005 alone, AIDS claimed an estimated 2.4-3.3 million lives, of which more than 570,000 were children. A third of these deaths are occurring in sub-Saharan Africa, retarding economic growth by destroying human capital. Current estimates state that HIV is set to infect 90 million people in Africa, resulting in a minimum estimate of 18 million orphans. It's a similar situation in India as well. As of April 2006, National AIDS Control Organisation (NACO) reported an estimated 5.21 million HIV-positive adults in India.

ROUTES OF TRANSMISSION

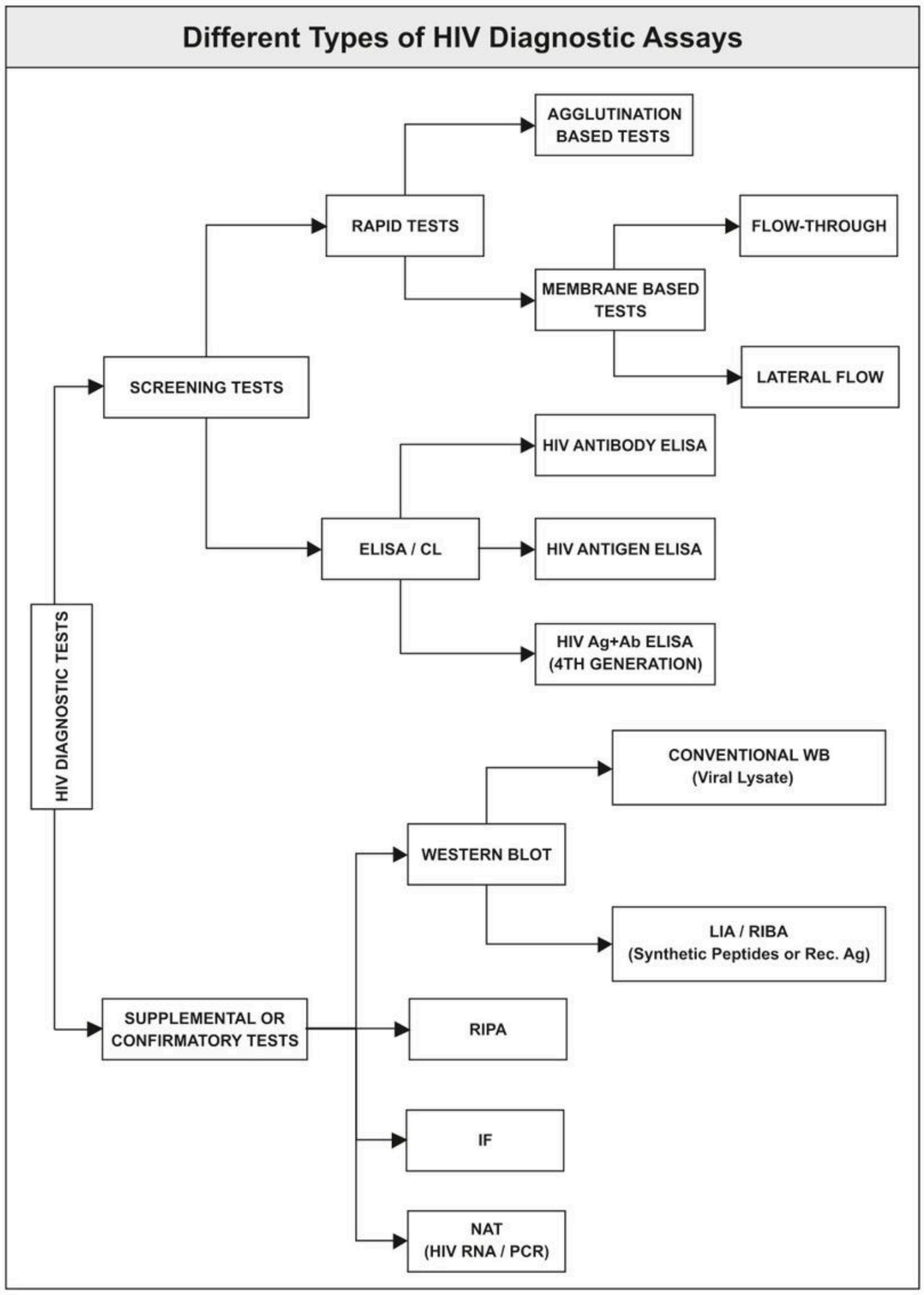
The following chart shows estimated HIV infections through different routes. It is clear that blood transfusions & mother-to-child infections dominate HIV infections. Another interesting point to note is that needle- stick injuries also result in a significant number of HIV infections. This means the healthcare workers; particularly laboratory professionals and surgeons are at increased risk of exposure to HIV.

Exposure Route	Estimated infections per 10,000 exposures to an infected source
Blood Transfusion	9000
Childbirth	2500
Needle-sharing injection drug use	67
Sexual transmission*	73
Percutaneous needle stick	30

* Assuming no condom use

DIAGNOSIS OF HIV

HIV infection can be detected by a variety of tests. The following chart illustrates the different type of HIV tests:



ELISAs or EIA/CL

The most commonly used type of test for screening is the ELISA (enzyme-linked immunosorbent assay) or EIA (Enzyme Immunoassay). When immunoassay is performed using chemiluminescent label instead of enzyme (as in ELISA), it is known as Chemiluminescent Assay (CL). ELISAs / CL are probably the most efficient tests for testing large numbers of samples per day, as in large blood banks or for surveillance studies.

Since ELISAs require skilled technical staff, equipment maintenance and a steady power supply, they are less suitable for smaller or more isolated hospitals, clinics or laboratories. Regular maintenance of the ELISA equipment is crucial to obtaining reliable results.

The original ELISAs involved the use of viral lysate, and positive specimens were usually confirmed by means of Western Blot technology, which is technically difficult, time consuming and expensive. Second-generation and third-generation ELISAs were developed on the basis of recombinant proteins and synthetic peptides, which increased sensitivity and specificity and considerably shortened the interval between the time of infection and the ability to detect HIV antibodies, i.e. the window period. This period has been further reduced by means of combined antigen- antibody ELISAs, comprising the fourth generation of such tests.

RAPID TESTS

The conventional ELISAs available for the detection of antibodies to HIV require instrumentation (i.e., incubators and mechanical washing and optical reading devices) and generally take 2 to 4 hrs to produce a result. The need for simpler, faster, less expensive, and easier-to-perform tests has become more acute as the HIV pandemic has expanded; thus, a variety of rapid test formats continue to be evaluated worldwide. Rapid tests for the detection of HIV (HIV rapid tests) which provide results concurrent with the patient's visit were preferred and resulted in significant improvement in the delivery of counseling without increasing the cost or decreasing the effectiveness of testing.

Advances in technology have led to the development of a wide variety of rapid HIV tests, including agglutination assays, dipstick assays, flow- through membrane assays, and lateral flow membrane assays. Many of these tests are presented as strips or cartridges incorporating the reagents and not requiring additional equipment. They are suitable for the performance of single tests, are easy to use and can be carried out by any health care worker who has received appropriate training. Most rapid HIV test kits can be stored at room temperatures of up to +20 to +30ºC. Furthermore; the diagnostic performance of high-quality rapid tests is comparable to that of traditional ELISAs. WHO has developed testing algorithms showing that sequential combinations of two or three antibody tests (ELISAs and/or rapid tests) can be reliably used to confirm HIV test results.

The lateral-flow tests are currently the most advanced in rapid HIV assays because of its simplicity, convenience, advanced generation, option of whole blood as sample, longer shelf life and storability at room temperature. However, earlier generation flow-through assays that are less sensitive, are still used in some developing countries like India owing to lack of access to advanced technology.

COMMONLY ENCOUNTERED PROBLEMS IN RAPID TESTS

The following section deals with the commonly encountered problems in rapid tests and their solutions.

High Background

Background formation is dependant to a large extent on the quality of sample used. Because of the quality of sample used, there is negligible / no background with some samples whereas some show a background colour. Samples that are:

- turbid,
- lipemic or
- contaminated;

show background colour in membrane based assays. In addition, aged samples i.e., stored samples that have undergone repeated freeze thaw cycles also show background colour. In such cases, samples should be spun hard at 3500 rpm for 15 minutes to pellet the suspended matter. Only clear supernatant should be used for testing. However, as explained in "background due to artifact factor" section, some samples show background despite being centrifuged due to presence of certain artifact.

Background due to the pH factor: Dark background may be due to abnormally high pH of the sample > 7.45. At high pH the colloidal gold conjugate in rapid assays may be precipitated on the membrane and show a strong background colour. The range of blood pH in the first few hours of life is 7.09-7.50 but thereafter is 7.35-7.45. The unusually high pH in some patients may be due to:

- ***Metabolic alkalosis -*** This is a result of increased bicarbonate in blood due to excessive alkali administration or loss of Cl (-) as gastric HCl due to prolonged vomiting or intracellular K (+) depletion.
- ***Respiratory alkalosis -*** Respiratory alkalosis is one of many acid-base disorders found among critically ill patients. Hyperventilation in hysteria and stimulation of the respiratory center are the usual causes. Patients have fever if respiratory alkalosis is the result of an infectious disorder. Hyperthermia (fever) of any origin may in turn result in respiratory alkalosis.
- ***Pre-analytical error -*** Any pH above 7.45 is considered to be alkalemia. In Metabolic or Respiratory alkalosis, usually the pH goes up maximum to 7.8. Above pH 7.8 indicates a fatal consequence. This error may take place during sample collection (contamination of sample vial by detergents).
- ***Low pH -*** Similarly low pH below 7 renders the sample acidic. This too causes the colloidal gold conjugate in rapid assays to precipitate / aggregate and show a dark background. Therefore, for obtaining proper reactivity and valid results, the ideal pH of the sample should be between 7-8.

Background due to the artifact factor: Background colour may also occur due to the presence of a high molecular weight molecule (or artifact) in sera that blocks the pores of the membrane. As a result the conjugate may not flow adequately leading to its precipitation on the membrane surface. For example, in some cancer patients there may be presence of artifacts (immunoglobulins, high molecular weight proteins like cell debris etc.) in the serum. These artifacts may block the pores of the membrane or interact with gold conjugate to precipitate the same. These samples show background colour even after centrifugation. Therefore, such samples should be tested with other assays like ELISAs and not with membrane based rapid assays.

Improper sample flow in rapid assays

Due to inherent variability of sample, problems related to flow of sample on membrane may not be totally eliminated. That is why all rapid assay manufacturers indicate the possibility of "invalid results" in their pack insert. However, despite all precautions, the following errors may occur with rapid assays:

- ***Dispensing error -*** The sample / buffer / reagents may be added in incorrect quantities. Low volume of reactants may cause inadequate hydration of membrane and lead to improper sample flow. On the other hand, excess liquids may result in overflow and show invalid or variable results.
- ***Reaction time -*** Sometimes reading is taken before reaction time is complete. This error commonly occurs with lateral flow rapid HIV assays. The technologist may not wait till the full reaction time of 15-30 minutes is complete. This may be apparently interpreted as "flow-problem", because sufficient clearance time has not been allowed.
- ***Damaged foil pouch -*** The flow properties of the nitrocellulose membrane may be affected if the foil pouch containing the device or dipstick is damaged. To avoid this, most manufacturers incorporate a desiccant in the pouch. Therefore, the pouch should be checked for pinholes and the desiccant should be observed for any colour change.

  This is mentioned by the manufacturers in the product pack insert and should be routinely followed as part of GLP (Good Laboratory Practice).

NOTE: ***Rapid assays need to be discarded after the reaction time is over.*** *A membrane-based rapid assay should not be read after the specified reaction time. This is because some sera contain certain non- specific artifact that do not react at first contact with antigen or antibody in the membrane but may do so after the reaction time is over. This happens due to evaporation (in flow-through) and back-flow (for lateral-flow assays) of the reactants. Again due to evaporation / back-flow, gold conjugate returning from the absorbing pad is very likely to dry out at the capture region because during drying the capture element becomes very hydrophobic. This hydrophobicity causes charged gold particles to bind non-specifically to the Test region and show a false positive result. Therefore, if future documentation of test results is absolutely required, the device / dipstick may be photographed / scanned at the end of reaction timeand the image stored.*

CONFIRMATORY & SUPPLEMENTAL ASSAYS

Due to the inherent variability of HIV and its serological response, it is difficult to consider a particular assay as confirmatory. WESTERN BLOT & HIV-RNA detection by PCR may be at the most termed as supplemental assays; not confirmatory assays. This is because both false positives & negatives have been reported in various studies with these tests. Virus isolation & culture is considered to be the most sensitive & specific test. However, false-negative HIV cultures sometimes are observed, particularly during the newborn period, and either false negative or false positive PCR test results may be noted occasionally. Therefore, for purposes of clinical decision-making, any positive test result should be confirmed with a second HIV culture or PCR test performed on a separate blood specimen.

Therefore, Confirmatory or supplemental assays are designed to offer a greater specificity than screening assays, and therefore are the methods of choice to verify that reactive results from screening assays represent HIV infection. They do not, however, verify that screening test negative results are from non-infected persons, nor do they verify that negative or indeterminate results from these tests are from a person who is not infected. The purpose of supplemental tests is to rule out false positive results by screening tests not to confirm that a person is unequivocally infected with HIV or to confirm that a person is negative for HIV. They are highly effective in great majority of persons.

Western Blot

Western Blot (WB) detects antibodies to HIV qualitatively to viral proteins. Viral antigens are electrophoretically separated and transferred ("blotted") onto a nitrocellulose sheet. Antibodies, which react with antigens on the membrane, are visualized with an enzyme labeled anti-human IgG and an enzyme substrate. WB shows an insoluble coloured product at the site of reaction. Therefore, WB detects only IgG antibodies and is less sensitive than 3rd / 4th Generation assays. However, as mentioned earlier, like any other supplemental test, WB has higher specificity than screening assays. Despite this, as per some reports, WB tests are indeterminate in 20 to 40% of healthy blood donors who are negative on the ELISA test. To improve sensitivity & specificity, there have been some modifications of the conventional WB that uses viral lysate as antigens. Currently, Western Blots using synthetic peptides (Line Immunoassay, LIA) and recombinant antigens (Recombinant Immunoblot Assay, RIBA) have been developed and marketed.

Radioimmunoprecipitation Assay (RIPA)

In this method, HIV Ag is metabolically labeled in HIV culture medium. The labeled cells are then disrupted to release the labeled proteins. The resulting suspension is clarified to obtain the proteins. These labelled proteins are used for testing the sample. The method is not popular because of the use of live HIV particles. Therefore, containment facilities for handling live HIV are critical. Also special facilities for handling radioisotopes and necessary regulatory clearance are essential. Moreover, the need of expensive equipment & skilled personnel makes RIPA one of the least practical assays for diagnosis of HIV infection.

Indirect Immunofluorescence (IF)

Like WB, IF also detects anti-HIV antibodies. Acetone fixed HIV infected lymphocytes are incubated with sample and a fluorescein conjugated anti-human IgG. The results are examined using fluorescent microscopy. If anti-HIV is present a typical pattern of fluorescent staining is seen in the cells. Similar to WB, IF also detects only IgG antibodies. Therefore, as mentioned earlier, its sensitivity is also less than 3rd/4th Generation HIV assays. IF requires expensive fluorescence microscope and highly skilled operator. It is also time-consuming to perform. This is the reason, like RIPA, IF is not a popular TOC (Test of Choice) for HIV diagnosis.

Nucleic Acid Tests (NAT)

In this method viremia is determined by measurement of HIV RNA in sample. The basic idea is that the viral genome copies must stem from virus particles (virions) as free blood nucleic acid is split by nucleases. The number of copies correlates therefore with the amount of the virus, one HIV particle containing two genome copies.

Several NAT technologies are available. All molecular methods have three steps in common, including: (i) a front end step that includes sample preparation and/ or viral nucleic acid extraction; (ii) a middle step consisting of target nucleic acid sequence amplification or amplification of the signal generated from the detection of target viral RNA; and (iii) a back end step that allows detection and/or quantification of the amplified products. The three major methods are:

(i) the reverse transcription polymerase chain reaction (RT-PCR),
(ii) nucleic acid sequence based amplification (NASBA) / transcription mediated amplification (TMA), and
(iii) branched chain DNA (bDNA).

SOME FAQs IN HIV TESTING

What are "Generations" of HIV Immunoassays?

Since the eighties, HIV immunoassays have constantly evolved. Depending upon the sensitivity and specificity, the assays are grouped into "generations". The following chart shows the different generations of HIV immunoassays.

Nomenclature	Antigen type	What is detected
1st Generation	Uses viral lysate as antigen	Detects only IgG antibody to HIV
2nd Generation	Uses recombinant antigens as antigen	Detects only IgG antibody to HIV
3rd Generation	Uses recombinant antigens and / or synthetic peptidesas antigen	Detects all HIV antibody isoptypes e.g., IgG, IgM, Iga, etc
4th Generation *	Uses recombinant antigens and / or synthetic peptides as antigen and monoclonal and / or polyclonal anti-p24 antibody	Detects all HIV antibody isoptypes and also p24 antigen

**Not available in rapid test format.*

Therefore, from the above it is clear that the most sensitive test is the 4th generation immunoassay. Unfortunately, no 4th generation rapid assay has been developed for testing in resource-poor settings.

What is "Window Period"?

Window period is also known, as SEROCONVERSION PERIOD. Seroconversion refers to the development of detectable antibodies to HIV in the blood as a result of HIV infection. A patient who goes from being HIV negative to HIV positive is said to have seroconverted or is a seroconverter. The time of seroconversion depends upon:

- The host-virus interaction.
- Immune status of the host.
- Route of infection.
- Type / subtype of infecting HIV strain.
- Viral inoculum at the time of exposure.

Therefore, there is no fixed time of the window period. The following chart shows how different bodies have defined window period:

Authorised body	Duration of window period
CDC, USA	2 weeks-1 year; Average: 3-6 months
The Canadian Medical Association	Within 6 months, 95% seroconvert within 3 months
WHO	6 weeks-3 months, unusual cases: 6 months or longer
NACO, India	3 weeks-3 months on an average, can be longer sometimes

The length of the window period varies not only on the factors mentioned above but also on the "generation" of test used. For example, as explained in the subsequent section, the length of the window period, if HIV RNA is detected, is just 1 week. However, the length is increased to 6-12 weeks if the test used is a 1st /2nd generation HIV assay.

What is the detectability of different HIV tests?

As mentioned earlier, several factors influence the time of seroresponse & the titer of antibodies produced. Moreover, the detection of HIV antibodies based on the test sensitivity (Generation) also determines the length / duration of the window period. The following chart illustrate the detectability of different types of HIV assays.

1st & 2nd Generation assays	6-12 weeks after exposure
3rd Generation assays	3-4 weeks after exposure
4th Generation assays	2-3 weeks after exposure
p24 antigen ELISA	2 weeks after exposure
NAT * (HIV RNA detection)	1 week after exposure

** NAT - Nucleic Acid Tests*

What are false positive reactions and why do they occur?

False positive reactions are those that show positive in a HIV immunoassay despite the patient being a non-infected subject. This happens with all HIV diagnostic assays including supplemental tests like HIV RNA by PCR. The reasons for such occurrences are debatable and not well understood.

As we are aware, the antigen (lysate or synthetic peptide or recombinant) used in any immunoassay is actually a small portion of the microorganism whether it is a virus or bacteria or protozoa etc. They are proteins molecules with a particular sequence of amino acids the "building blocks" of life on this planet. These amino acids are found in all organisms on earth. That is why cross-reacting epitopes are often found even in apparently unrelated microorganisms.

In case of HIV, the following chart shows how the antigens used in HIV assays worldwide including gp120, gp41, gp160, p18 & p24, shows some degree of cross reactivity with certain sera.

Reference	HIV antigen implicated
Tomiyama T et al. Recognition of human immunodeficiency virus glycoproteins by natural anti carbohydrate antibodies in human serum. Biochem Biophys Res Commun. 1991 May 31; 177(1):279-85.	gp160, gp120 and gp41
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ShivRaj L et al. Antibodies to HIV-1 in sera from patients with mycobacterial infections. Int J Lepr Other Mycobact Dis. 1988 Dec; 56(4): 546-51.	p24
Healey DS & Bolton WV. Apparent HIV-1 glycoprotein reactivity on western blot in uninfected blood donors. AIDS. 1993 May; 7(5): 655-8.	gp41
Sayre KR et al. False-positive human immunodeficiency gp41 virus type 1 western blot tests in noninfected blood donors. Transfusion. 1996 Jan; 36(1): 45-52.	gp41

Therefore, all HIV immunoassays are prone to false positivity because all of them use at least one of the "implicated" antigens.

Apart from the above, the following is the list of conditions other than HIV that can cause positive results in serological assays as reported in various studies.

Conditions that cause false positives in HIV tests

1. Renal failure
2. Alpha interferon therapy in hemodialysis patients
3. Flu
4. Flu vaccination
5. Herpes simplex I
6. Herpes simplex II
7. Upper respiratory tract infection
8. Recent viral infection or exposure to viral vaccines
9. Pregnancy in multiparous women
10. Malaria
11. Rheumatoid arthritis
12. Hepatitis B vaccination
13. Tetanus vaccination
14. Organ transplantation
15. Renal transplantation
16. Anti-lymphocyte antibodies
17. Anti-collagen antibodies
18. Autoimmune diseases
19. Malignant neoplasms
20. Alcoholic hepatitis/alcoholic liver disease
21. Antibodies with a high affinity for polystyrene
22. Blood transfusions, multiple blood transfusions Ss
23. Multiple myeloma
24. Anti-smooth muscle antibody
25. Anti-parietal cell antibody
26. Anti-hepatitis A IgM
27. Anti-Hbc IgM
28. Haemophilia
29. Stevens-Johnson syndrome
30. Heat-treated specimens
31. Lipemic serum
32. Haemolyzed serum
33. Hyperbilirubinemia
34. Healthy individuals as a result of poorly understood cross-reactions
35. Normal human ribonucleoproteins
36. Anti-mitochondrial antibodies
37. Anti-nuclear antibodies
38. Anti-microsomal antibodies
39. Epstein-Barr virus
40. Visceral leishmaniasis
41. Receptive anal sex
42. Tuberculosis
43. *Mycobacterium avium*

Do more false positives occur in some geographic areas?

Yes, it is reported that more false positive results occur in certain geographic areas. More false positives have been reported in **malaria & dengue endemic areas (like India)**. Normally, diagnostic tests are evaluated in developed countries by using samples locally obtained from well-defined populations in which major parasitic infections are absent. On the other hand, in developing countries, parasitic infections are frequent and lead, in conjunction with poor nutrition to **increased polyclonal antibody stimulation** in the affected individual that can remain throughout life. The increase in nonspecific antibody titers can interfere with the performance of any antibody detection assay like HIV. In an Indian study (a malaria-endemic area) it has been reported that a significant proportion of population shows non-specific false positive serology with various tests like RPR, HIV, WIDAL, RF and DCT in acute other phase of malaria. Studies indicate that false positive HIV serology may occur in low HIV-infection prevalence areas.

What does indeterminate (equivocal) test results mean?

Indeterminate samples are those, which can neither be termed as positive nor as negative. These samples are usually found reactive with initial HIV screening assays (ELISAs or Rapid Tests) and subsequently show indeterminate band patterns in Western Blot. Also, as per WHO/UNAIDS testing strategies, a sample that shows discordant results with the three tests suggested in the algorithm, is also termed as indeterminate or equivocal.

The proportion of repeatedly reactive samples by ELISA but with an indeterminate WB pattern varies 13 to 48% of samples from blood donors in the United States. This proportion seems to be even higher in some regions of Africa. As mentioned in the earlier section, the observation of ELISA-negative samples that render indeterminate WB patterns is surprising, with reports that this result is found for as much as 20% of the ELISA-negative blood donors at the National Institutes of Health (USA).

The long-term outcome and conditions associated with persons identified as indeterminate WB after being repeatedly reactive by screening ELISA are not well characterized and may vary from one geographical region to the other.

A vast number of conditions have been reported by different authors as being associated with indeterminate WB results. They include: systemic lupus erythematosus, presence of rheumatoid factor and polyclonal gamopathy, antibodies to DR-HLA, cross-reactivity to core proteins of other retroviruses like Bovine Immunodeficiency Virus, Mycobacterium leprae infection, heat inactivation of serum samples, in vitro hemolysis, elevated bilirrubin levels and tetanus vaccination.

The significance of the majority of the cases of donors with an indeterminate WB results remains to be determined. Prolonged clinical and laboratorial follow-up of these individuals in reference centers, with addition of molecular tests may be necessary to answer these questions and to improve donor selection and notification strategies.

What are false negative reactions and why do they occur?

Recombinant and synthetic antigen based immunoassays are very sensitive and specific for the HIV subtypes found in the United States and Europe (mostly HIV-1 subtype B). However, some react poorly with sera from other geographical regions where non-B subtypes are prevalent. (e.g., in India subtype-C is more prevalent). Also it was reported that some diagnostic kits could not detect the highly divergent HIV-1 subtype O all the time in African sera. This may be due to an inadequate amount or inappropriate sequence of the antigen used, low-affinity antibody in the sera tested, low antibody titer, or inherent feature of the assay system. Therefore, in order to reduce the number of false-negative results it is recommended that manufacturers include in the assay additional peptides from the gag (e.g., p24 antigen) apart from the env region that are also immunodominant and highly conserved. In addition, manufacturers should test sera derived from different geographical locations.

The different causes of false negatives are described in the following chart:

Cause of false-negative results
1. Performance and technical errors ● Pipetting error ● Mislabeling of samples or wells ● Variability in test kits ● Decreased intensity of EIA reaction by Powder from powdered gloves Storing of samples in serum separator tubes 2. Biologic, pathologic, and pharmacologic determinants ● Window (preseroconversion) period ● Delayed antibody synthesis in infants ● Diminished immune response because of HIV-1-related immune dysfunction Immunosuppressive therapy Concurrent infection with Epstein-Barr virus or cytomegalovirus 3. Congenital or drug-induced hypogammaglobulinemia ● Formation of antigen-antibody complexes 4. Sensitivity and specificity of the assay ● Infection by HIV-1 subtype O (if test kit do not use subtype O-specific antigen) ● Sampling prior to immunoglobulin M to immunoglobuli G class switching (only if 2nd generation assay is used for testing) ● Hemodilution ● Nondenatured antigenic epitopes ● Limited antigenic determinants

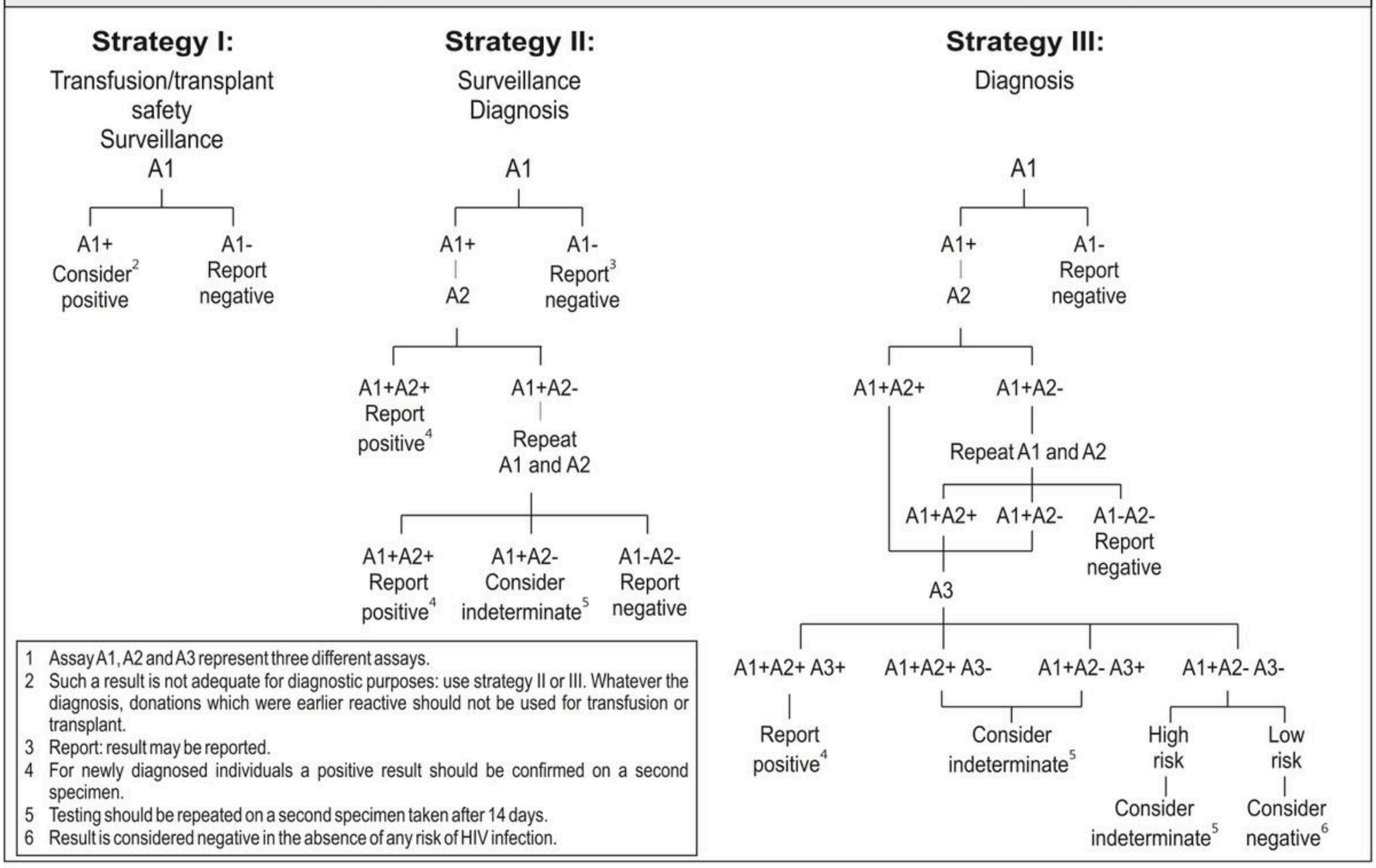
What are the different strategies for confirmation of HIV?

As described earlier, because of endemicity of different pathogenic infections in most developing countries, the probability of false positive reactions are high. In fact, the following quote from WHO/UNAIDS document is pertinent in this context - *"When a single screening assay is used for testing in a population with a very low prevalence of HIV infection, the probability that a person is infected when a positive test result is obtained (i.e., the positive predictive value) is very low, since the majority of people with positive results are not infected. This problem occurs even when a test with high specificity is used."*

Therefore, because of the natural cross-reactivity that exists between HIV antigens and other proteins present in the human serum, all immunoassys are considered to be screening tests. This is where the role of supplemental or confirmatory tests becomes important. Results of screening tests in HIV that arouse suspicion, based on clinical symptoms, should be investigated by other appropriate supplemental or confirmatory tests.

To avoid the potentially serious consequences of a false diagnosis, several strategies have been devised for further testing to determine the authenticity of a positive screening reaction. This is referred to as confirmatory or supplementary testing. WHO / UNAIDS has recommended three strategies for HIV antibody screening. These three strategies take into account the objective of testing (i.e. whether for transfusion safety, surveillance or diagnosis) and the local seroprevalence rate. Depending on these factors, three different algorithms for repeat and confirmatory testing have been proposed to confirm initial screening results.

Schematic representation of the UNAIDS and WHO HIV testing strategies



1 Assay A1, A2 and A3 represent three different assays.
2 Such a result is not adequate for diagnostic purposes: use strategy II or III. Whatever the diagnosis, donations which were earlier reactive should not be used for transfusion or transplant.
3 Report: result may be reported.
4 For newly diagnosed individuals a positive result should be confirmed on a second specimen.
5 Testing should be repeated on a second specimen taken after 14 days.
6 Result is considered negative in the absence of any risk of HIV infection.

Which strategy to follow?

UNAIDS / WHO also recommends for HIV testing strategies according to test objective and prevalence of infection in the sample population. The following chart explains that:

Objective of testing	Prevalence of infection in the category to which the patient belongs (percentage)	Testing strategy applicable
Screening of blood and blood products	-	I
Surveillance	> 10 < 10	I II
Diagnosis: With clinical signs/ symptoms	> 30 < 30	I II
Asymptomatic	> 10 < 10	II III

For example, in India, HIV prevalence is < 10%. Therefore, as per the above guideline, strategy to be followed in India for "Diagnosis of Asymptomatic Individual" is Strategy-III. On the other hand, for "surveillance" purpose it should be Strategy-II.

How should HIV test results be reported?

As per WHO/UNAIDS recommendations, the following chart elaborates how to report HIV samples that may be of three categories viz., positive, negative and indeterminate or equivocal.

Report	**Negative -** if initial/screening test shows non-reactive result. **Positive -** if the sample shows reactive results concordantly by the three screening tests. **Indeterminate (equivocal) -** if the sample shows discordant results by the three screening tests, the follow up samples are required to retest at two weeks and at three, six and 12 months before the final status of the test results should be conveyed. If the status remains indeterminate after one year, the person is considered to be HIV antibody negative.
Special considerations	Pre-test, post-test counselling service should be provided and confidentiality should be maintained.

From the above chart it is clear that indeterminate results are to be followed up upto at least 1 year after. If still indeterminate then the person should be considered as HIV antibody negative.

HIV TESTING & COUNSELLING

HIV testing and counseling have been recognized as necessarily linked since the first HIV enzyme-linked immunosorbent assay (ELISA) tests became available for the identification of HIV infection in the mid-1980s. Pre-test and post-test counseling were seen as crucial for the testing process because of the seriousness of the news of HIV infection for people receiving a positive result. Additionally, the process of pre-test counseling was designed to ensure that those tested were sufficiently informed about the testing process and the potential consequences: counseling made informed consent possible and ensured that people were not tested in a coercive manner. People with HIV needed the support of post-test counseling in order to manage disclosure and cope with living with HIV. This counseling included the provision of information on preventing the infection of partners and families in the future and on decision-making about pregnancy.

UNDERSTANDING HIV TESTING OBJECTIVES

The main objectives for HIV tests are:

1. Screening of donated blood to reduce transmission of the virus through transfusions.
2. To prevent mother to child transmission.
3. To detect and prevent sexual transmission.
4. Surveillance of HIV prevalence or trends over time in a given population, through unlinked testing of serum (anonymous testing for statistical purposes only).
5. Diagnosis of infection in individuals.

Tests with high sensitivity should be used when the objective is to minimize the number of false negative results, such as in screening of donated blood. When the objective is to minimize false-positives, such as in confirming whether an individual is HIV-infected, tests with high specificity should be used. In areas where HIV prevalence is low (like in India), most positive results in initial screening tests are in fact false- positives, so supplemental tests should always be performed if the objective is diagnosis. Today's standards require HIV tests to have a sensitivity of >99% and a specificity of >98%.(Note that the specificity of test kits may vary according to the geographical origin of the serum samples.)

There is no single test suitable for all objectives in all settings. For this reason, different types of tests based on different technologies are often used in combination, depending on the testing objective and the assays cost-effectiveness under local conditions.

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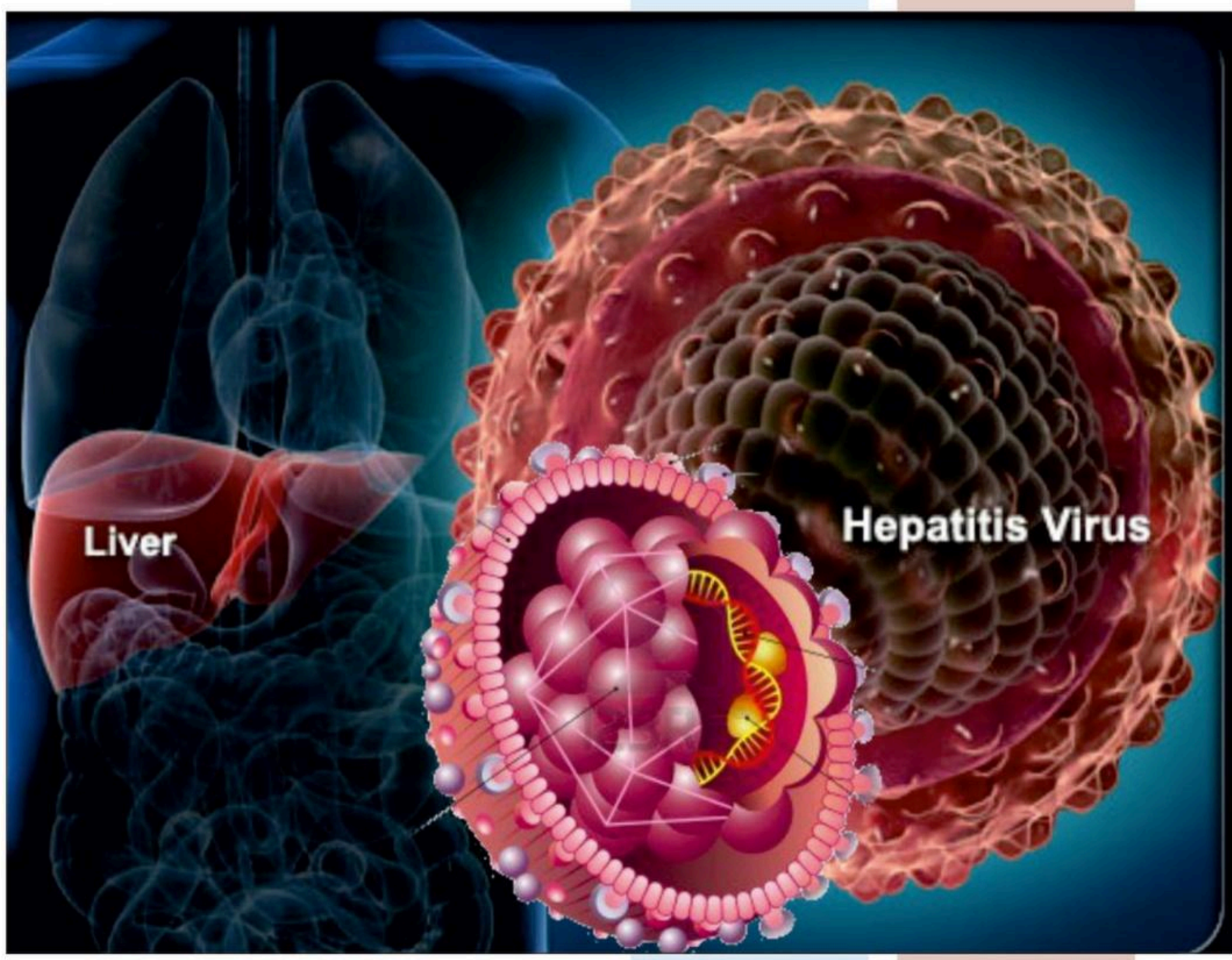
HEPATITIS C



- **Hepatitis C Screening**
  - Overcoming Challenges.....Pgs 138-146

KNOWLEDGE BOOK

For the Laboratorians

Hepatitis C Screening

Overcoming Challenges

Setting Trends...

HEPATITIS C VIRUS (HCV)

Hepatitis C is an infectious disease caused by Hepatitis C Virus (HCV) which primarily affects the liver.
The Hepatitis C virus is the major cause of the disease formerly known as non-A non-B post transfusion hepatitis. The virus can cause both acute and chronic hepatitis, ranging in severity from a mild illness lasting a few weeks to a serious, lifelong illness.
HCV, a single stranded RNA virus, is a member of the family Flaviviridae. Six major genotypes (1-6) and a series of subtypes of HCV have been identified. Genotypes 1-3 show a worldwide distribution while genotypes 4 and 5 appear predominantly in Africa and genotype 6 in Asia.

GENOTYPES & SUBTYPES

In addition to the impressive heterogeneity that often exists amongst HCV sequences present within an infected individual, (quasispecies variation) there is also a remarkable genetic heterogeneity and divergence amongst the sequences that have been recovered from different individuals (strain and genotype variations). Based on HCV sequences recovered from multiple geographical regions of the world, there are at least six major genotypes of the HCV that have been identified, and many more are in the process of being characterised. At the second International Conference of HCV and related viruses, a consensus nomenclature system was proposed for the future studies of the HCV genotypes and subtypes.
According to this system the HCV is classified on the basis of their nucleotide sequence into major genetic groups designated as “genotypes” and are assigned a number (Arabic numerals) in the order of their discovery. The more closely related HCV strains within same genotypes are designated “subtypes” and are assigned lower case letters (in alphabetical order) in the order of their discovery. The complex of genetic variants found within an infected individual's isolate are termed as the “quasi species”. It is hypothesized that the distinct viral quasispecies play a role in the pathogenesis and progressive HCV infection. Due to the sequence variability of the quasispecies post infection, HCV is present in patients as a pool of viruses representing different epitopes. Modification of both B and T epitope patterns during HCV infection have been observed and clones contribute to HCV evasion from the immune system.

Terminology used in studies related to the HCV genomic heterogeneity

Terminology	Definition	% Nucleotide* similarity
Genotype	Genetic heterogeneity among different HCV Isolates	65.7-68.9
Subtype	Closely related isolates within each of the major genotypes	76.9 - 80.1
Quasispecies	Complex of genetic variants within individual isolates	90.8 -99.0
* % Nucleotide Similarity refers to the nucleotide sequence identities of the full - length sequences of the HCV genome.		

Comparative sequence analysis amongst HCV subtypes

Comparative sequence analysis among HCV subtypes of a 222-nucleotide segment derived from the viral NS5 region*											
% Similarity to											
Subtype	1a	1b	1c	2a	2b	2c	3a	3b	4a	5a	6a
1a	100	81	85	65	66	63	67	66	68	69	64
1b		100	77	64	67	64	67	71	64	70	65
1c			100	68	70	67	65	70	64	61	61
2a				100	82	77	67	67	66	66	68
2b					100	81	64	69	65	67	66
2c						100	64	65	65	66	65
3a							100	79	65	67	64
3b								100	66	68	61
4a									100	66	66
5a										100	68
6a											100
*Nucleotide position 7975 to 8196 of the prototype virus.											

The phylogenetic grouping of HCV strain appears to be independent of the segment of genome that is analysed.
Substantial regional differences appear to exist in the distribution of HCV genotypes. Although genotypes 1, 2 and 3 appear to have a worldwide distribution, their relative prevalence varies from one geographical area to another.
HCV subtypes 1a and 1b are most common in USA and also predominant in Europe. Subtype 1b is responsible for upto 73% of the HCV infections in Japan. Subtype 2c is found commonly in north Italy whereas subtype 3a is particularly prevalent in IV drug users in Europe and North America. The genotype 4 is prevalent in North Africa and the Middle East and 5 and 6 seems to be confined to South Asia and Hong Kong respectively. Genotype 7, 8, 9 have been identified in Vietnamese patients and 10 and 11 have been identified in Indonesia.
In India, genotype 1 and 3 are common to all parts of India. Genotype 3 is predominant (63.85%) followed by Genotype 1 (25.72%). Genotype 3 is most common in northern, eastern & western parts. Genotype 1 is common in southern part. An increasing trend in prevalence of 4 & 6 Genotypes is observed in India. 4 is found in south India patients from Andhra Pradesh & Tamil Nadu & 6 is found in north eastern patients. Genotype 2 is rarely reported &Genotype 5 not yet reported in India.

The presence of numerous genotypes may provide clues about the historical origin of the HCV as well as proof that HCV has been endemic in these areas for a long time.

TRANSMISSION

HCV is a blood borne virus and the most common modes of infection are through exposure to small quantities of blood. This may happen through injection drug use, unsafe infection practises, unsafe health care, and the transfusion of unscreened bold and blood products.
Some modes of transmission of hepatitis C virus are well documented and widely accepted; others are less well defined and require further study. It is clear that HCV is most frequently transmitted through large or repeated direct percutaneous exposures to infected blood. The two most common exposures associated with transmission of HCV are blood transfusion and injection drug use.

- **Blood Transfusion / Receipt of Blood Products**
  Early case-control studies of patients with newly acquired, symptomatic non-A, non-B hepatitis found a significant association between disease acquisition and a history six months prior to illness of blood transfusions, injection drug use, health care employment with frequent exposure to blood, personal contact with others who had hepatitis, multiple sexual partners or low socioeconomic status.

- **Injection Drug Use**
  Injection drug use has been the principal mode of transmission of HCV since the 1970's. In comparison to other viral infections, HCV is more rapidly acquired after initiation of intravenous drug use. In addition, rates of HCV among young injecting drug-users are four times higher than HIV infection. Studies of injection drug users have demonstrated that the prevalence of HCV infection in them is extremely high, with up to 90% having been exposed. In addition, the incidence of new infections is also high, with seroconversion rates of 10-20 percent per year of injecting. Duration of injecting is the strongest single predictor of risk of HCV infection among injection drug users.

- **Sexual Transmission**
  The topic of sexual transmission of HCV has been controversial. It is believed that HCV can be transmitted sexually, but that it is inefficient -- meaning, it is not easy or likely to pass the virus during sex. On the other hand, HCV infection is very efficient when it is passed from the blood of one person to the blood of another person, such as when people share needles for drug use. The frequency of HCV transmission between monogamous sex partners is very low according to most studies. However, the likelihood of sexual transmission of HCV is increased under any of the following circumstances:
  - Having multiple lifetime sex partners.
  - Engaging in rough sex such as anal sex.
  - Having a history of a sexually transmitted disease.
  - Having HIV
  - Having sex with a prostitute or intravenous drug user
  - Having sex during menstruation or whenever blood is present

Other Modes of Transmission

- **Household Transmission**
  The prevalence of HCV among household contacts of people with HCV infection is low. Care providers need only advise patients to take "common sense" precautions such as not sharing items that may have blood on them (e.g., razorblades, toothbrushes) and properly covering open cuts or wounds.
  The study of HCV transmission among household contacts is complicated by the difficulty of ruling out other possible modes of acquisition.

- **Occupational Exposures**
  Health care workers who have exposure to blood are at risk of infection with HCV and other blood borne pathogens. The prevalence of HCV infection, however, is no greater in health care workers, including surgeons, than for the general population. According to the CDC, the average rate of anti-HCV seroconversion after unintentional needle sticks or sharps exposure from an HCV-positive source is 1.8% (range 0%-7%).

SYMPTOMS

Hepatitis C infection is mostly asymptomatic. Approximately 70%–80% of people with acute Hepatitis C do not have any symptoms. Some people, however, can have mild to severe symptoms soon after being infected, including:

- Fever
- Fatigue
- Loss of appetite
- Nausea
- Vomiting
- Abdominal pain
- Dark urine
- Clay-colored bowel movements
- Joint pain
- Jaundice (yellow colour in the skin or eyes)

SCREENING AND DIAGNOSIS OF HCV INFECTION

Due to the fact that acute HCV infection is usually asymptomatic, few people are diagnosed during the acute phase. In those people who go on to develop chronic HCV infection, the infection is also often undiagnosed because the infection remains asymptomatic until decades after infection when symptoms develop secondary to serious liver damage.

HCV infection is diagnosed in 2 steps:

- Screening for anti-HCV antibodies with a serological test identifies people who have been infected with the virus.
- If the test is positive for anti-HCV antibodies, a nucleic acid test for HCV ribonucleic acid (RNA) is needed to confirm chronic infection because about 30% of people infected with HCV spontaneously clear the infection by a strong immune response without the need for treatment. Although no longer infected, they will still test positive for anti-HCV antibodies.

After a person has been diagnosed with chronic hepatitis C infection, they should have an assessment of the degree of liver damage (fibrosis and cirrhosis). This can be done by liver biopsy or through a variety of non-invasive tests.

In addition, these people should have a laboratory test to identify the genotype of the hepatitis C strain. There are 6 genotypes of the HCV and they respond differently to treatment. Furthermore, it is possible for a person to be infected with more than 1 genotype. The degree of liver damage and virus genotype are used to guide treatment decisions and management of the disease.

LABORATORY ASPECTS OF HCV TESTING

The laboratory diagnosis of HCV infection is usually made on the basis of the detection of circulating antibodies. Serological tests for detecting antibodies to HCV are generally classified as screening tests or confirmatory tests. Screening tests provide the presumptive identification of antibody-positive specimens, while confirmatory tests are used to confirm that specimens found reactive with a particular screening test contain antibodies specific to HCV. Several screening tests may be used in a testing algorithm to determine a final sero-status. These second and/or third line tests are generally referred to as supplemental tests.

The most widely used anti-HCV screening tests are ELISAs as they are the most appropriate for screening large numbers of specimens on a daily basis, as is the case in blood transfusion services in industrialized countries. Currently, more sensitive tests such as Chemiluminescence are also developed which are able to detect the analyte at a very low concentration thereby further reducing the diagnostic window period and ensuring early diagnosis of HCV infection. However, many blood transfusion services in resource limited countries process only limited numbers of specimens. Hence, individual tests would be more appropriate.

Several simple, instrument and electricity-free screening tests have been developed including agglutination, immunofiltration (flow through) and immunochromatographic (lateral flow) membrane tests. A positive result is indicated by the appearance of a coloured dot or line, or the presence of an agglutination pattern. While most of these tests can be performed in less than 10 minutes, other simple tests are less rapid and their performance requires 30 minutes to 2 hours. The results are read visually. In general, these simple/rapid (S/R) tests are most suitable for use in laboratories that have limited facilities and/or process low numbers of specimens daily.

Confirmatory assays that are commercially available for the diagnosis of HCV include Molecular HCV RNA tests based on Nucleic Acid Amplification Technologies (NAT). This assays are available both as qualitative and quantitative test. The quantitative test are capable of detecting HCV RNA down to 100 copies/ml.

- **GENERATIONS OF HCV TEST**

  Following the discovery of HCV and the sequencing of its genome in 1989, the first generation of anti-HCV ELISAs was produced using, as antigens, recombinant proteins complementary to the NS4 region of the HCV genome. These assays showed limited sensitivity and specificity. Second generation tests, which included recombinant or synthetic antigens from the putative core and non-structural regions NS3 and NS4 resulted in a marked improvement in sensitivity and specificity. The third generation tests include antigens from the NS5 region of the genome, in addition to those used in second generation assays. Third generation tests have improved sensitivity, though this has been shown to be more likely due to the improvements to the core and NS3 antigens rather than the inclusion of the NS5 antigen. However, despite these improvements, the time between infection with HCV and the appearance of detectable antibodies (window period) is generally more than 40 days (Schreiber et al, 1996; Barrera et al, 1995). It is anticipated that test kits will undergo further improvement in the future.

  The term generation is used to show the development of the kits over time by the manufacturers in their effort to increase the specificity and sensitivity of the assays and each generation does not denote a specific format or configuration of the assay. Some manufacturers use the term generation in their kit title which does not mean a specific format but is indicative of further developments in their assay.

Scenario of HCV testing in India.

Hepatitis C virus (HCV) causes both acute and chronic infection. Acute HCV infection is usually asymptomatic, and is only very rarely (if ever) associated with life-threatening disease. About 15–45% of infected persons spontaneously clear the virus within 6 months of infection without any treatment. The remaining 60–80% of persons will develop chronic HCV infection. Of those with chronic HCV infection, the risk of cirrhosis of the liver is between 15–30% within 20 years.

The above clearly suggest that Hepatitis C infection is dangerous and could be life-threatening. Still HCV screening is not performed routinely in many laboratories as clinicians are not aware about the increasing prevalence of HCV infection in India.

Most of the laboratories perform HIV and HBsAg screening of all the patients as prescribed by the clinicians. Rural India remains unaware about the rising threat to Humanity. Due to this, the health care workers become vulnerable to HCV as they are in repeated contact with Human blood or blood products.

HCV screening is mostly performed in Dialysis centres. Even Blood banks have to test each blood component for HCV following the guidelines of Transfusion Transmitted Infections. (TTI).

Considering the rising cases of HCV infections in India, the need is to create awareness about HCV infection and incorporate HCV screening in routine profiles such as Antenatal Profile, Pre-operative cases, routine Health check-ups and also during admission of patients in the hospital.

Challenges in HCV testing

HCV screening (diagnosis) is associated with high number of false positivity in the areas with low prevalence of HCV infection. This means that out of the total number of samples which are reactive with antibody test, the probability of samples being true positives decreases. According to Centre for Disease Control & Prevention (CDC), the false positive results averages approximately 35-40 % in case of areas with low prevalence for HCV infection.

Currently, majority of laboratories report positive anti-HCV results based on a positive screening assay alone. When a single screening test is used for testing in a population with a very low prevalence of HCV infection, the probability that a person is infected when a reactive test result is obtained (i.e. the positive predictive value) is low, since the majority of people with reactive results are not infected (i.e. the positive results are false). This problem occurs even when a test of excellent quality and having high specificity is used.

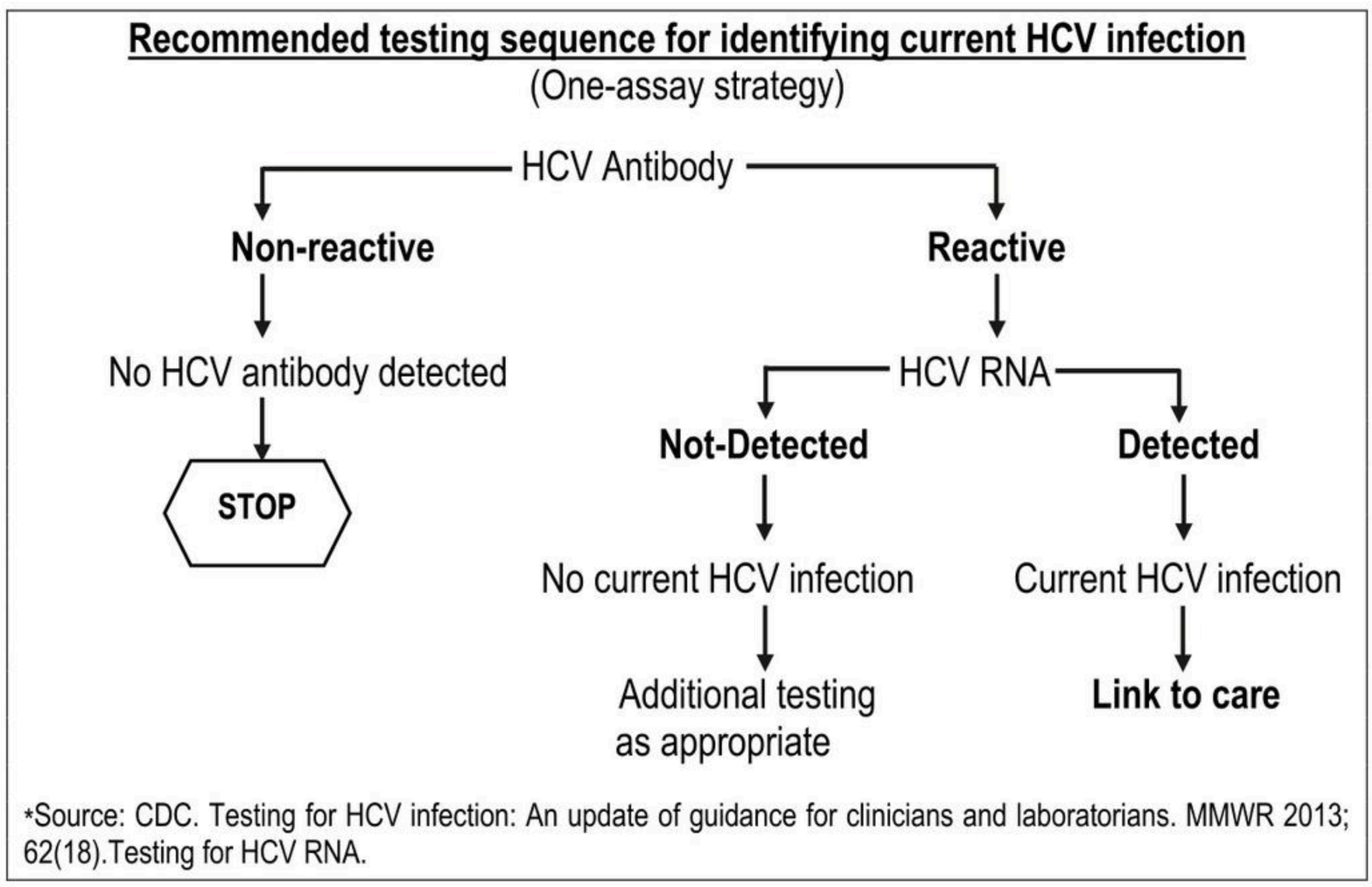
The current trends in testing also leads to lack of differentiation between current and resolved infection. This is important in HCV infection as 15-25% of new infections spontaneously resolve. Those who resolve the infection may still test positive in the antibody assays for many years following the clearance of the virus.

Accuracy can be improved if additional supplemental or confirmatory test(s), of equal or higher specificity, are used to retest all those samples found reactive by the screening test. Screening and supplemental tests, to be used in an HCV confirmatory strategy, must be selected carefully to ensure that common false reactivity between these assays does not occur.

The discontinuation of Immunoblot assays as supplemental / confirmatory tests has further increased the difficulties in HCV testing as majority of the people are not aware about the new testing algorithm. By creating the awareness and educating laboratories on the recommended HCV testing algorithm, laboratories will overcome the challenges faced in Hepatitis C testing.

RECOMMENDED HCV TESTING ALGORITHM

Testing for HCV infection begins with either a rapid or ELISA / CLIA assay for HCV antibody in serum or plasma. An FDA-approved test for HCV antibody should be used. In order to appropriately identify anyone with active HCV infection, two laboratory tests must be conducted. The first is a test that screens for HCV antibodies. If this initial HCV antibody test is reactive, it should be immediately followed with an HCV RNA test. If HCV RNA is detected in serum or plasma, active HCV infection is confirmed. (See HCV testing algorithm).



*Source: CDC. Testing for HCV infection: An update of guidance for clinicians and laboratorians. MMWR 2013; 62(18).Testing for HCV RNA.

- **Testing for HCV RNA.**

  An FDA-approved NAT assay intended for detection of HCV RNA in serum or plasma from blood of at-risk patients who test reactive for HCV antibody should be used. There are several possible operational steps toward NAT after initial testing for HCV antibody:

  1. Blood from a subsequent venepuncture is submitted for HCV NAT if the blood sample collected is reactive for HCV antibody during initial testing.
  2. From a single venepuncture, two specimens are collected in separate tubes: one tube for initial HCV antibody testing; and a second tube for HCV NAT if the HCV antibody test is reactive.
  3. The same sample of venepuncture blood used for initial HCV antibody testing, if reactive, is reflexed to HCV NAT without another blood draw for NAT.
  4. A separate venepuncture blood sample is submitted for HCV NAT HCV if the antibody test for initial testing of HCV antibody has used finger stick blood.

- **Interpretation of results of tests for hepatitis c virus (HCV) infection**

TABLE. Interpretation of results of tests for hepatitis c virus (HCV) infection and further actions.

Test Outcome	Interpretation	Further action
HCV antibody non-reactive	No HCV antibody detected	Sample can be reported a non-reactive. No further action required. If recent HCV exposure in person tested is suspected, test for HCV RNA.*
HCV antibody reactive	Presumptive HCV infection	A repeatedly reactive result is consistent with current HCV infection, or past HCV Infection that has resolved, or biologic false positivity for HCV antibody. Test for HCV RNA to identify current infection.
HCV antibody reactive HCV RNA detected	Current HCV infection	Provide person tested with appropriate counselling and link person tested to medical care and treatment. †
HCV antibody reactive HCV RNA not detected	No current HCV infection	No further action required in most cases. If distinction between true positivity and biologic false positivity for HCV antibody is desired, and if sample is repeatedly reactive in the initial test, test with another HCV antibody assay. In certain situations follow up with HCV RNA testing and appropriate counselling.§

* If HCV RNA testing is not feasible and person tested is not immunocompromised, do follow-up testing for HCV antibody to demonstrate seroconversion. If the person tested is immunocompromised, consider testing for HCV RNA.
† It is recommended before initiating antiviral therapy to retest for HCV RNA in a subsequent blood sample to confirm HCV RNA positivity.
§ If the person tested is suspected of having HCV exposure within the past 6 months, or has clinical evidence of HCV disease, or if there is concern regarding the handling or storage of the test specimen.

CDC has recommended that a person be considered to have serologic evidence of HCV infection only after an anti-HCV screening-test-positive result has been verified by a more specific serologic test or a Nucleic Acid Test (NAT). This more specific, supplemental testing is necessary, particularly in populations with a lower prevalence of disease, to identify and exclude false positive screening test results.

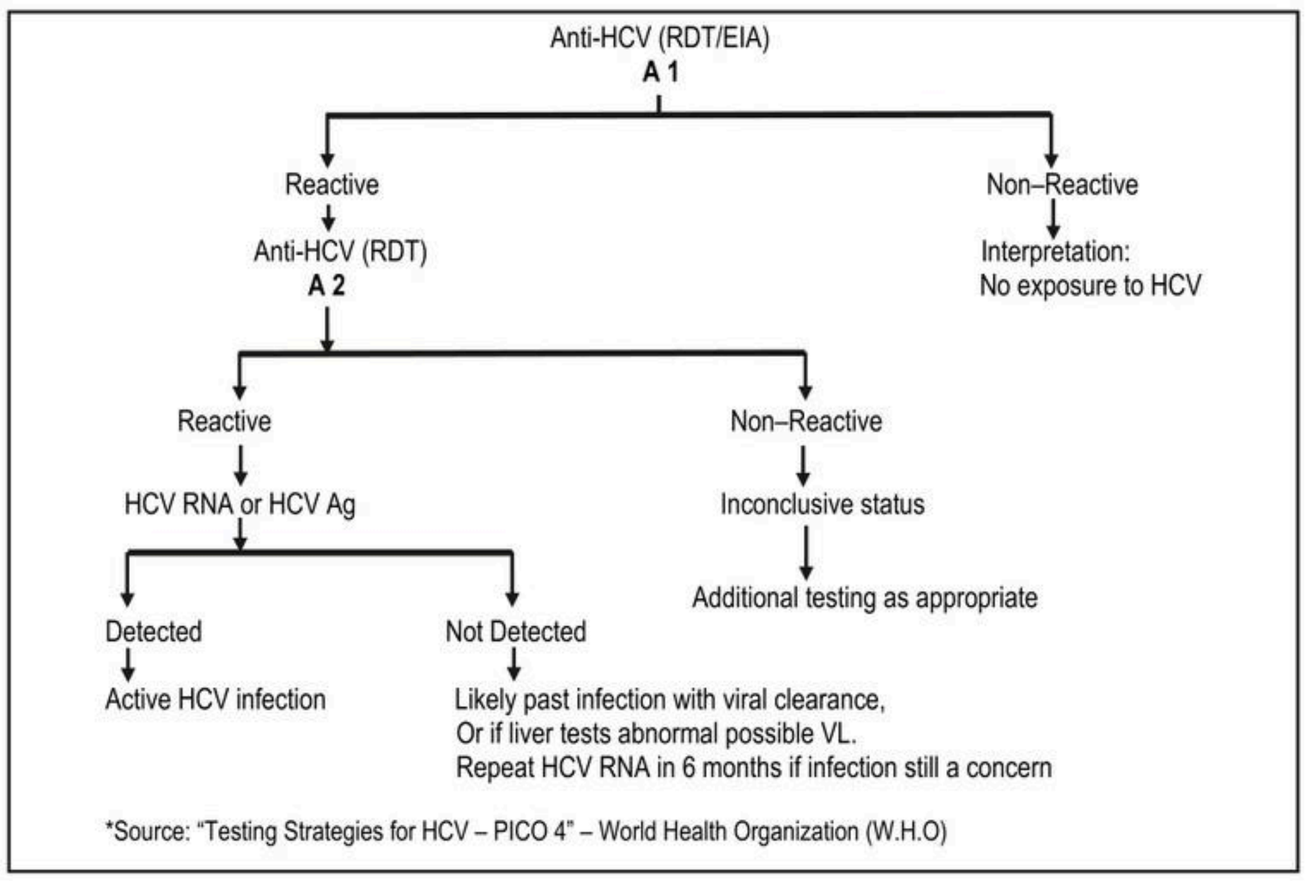
BIOCHEMICAL TEST PANEL FOR DIAGNOSING HCV IN CONJUGATION WITH IMMUNOASSAYS

Biochemical liver panel play an important role as a supplementary to Immunoassays for diagnosing HCV infection. These test measures the activity in the liver. During HCV infection, the levels of 2 enzymes i.e. Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) in the patient sample provides a useful insight on the condition of liver.
When HCV infects liver, the hepatocytes produces higher than normal ALT levels, indicating inflammation of liver. In acute phase, ALT may increase to 10 times the normal levels. When HCV becomes chronic, ALT drops to a relatively low level, but remains persistently high.
AST is also a liver enzyme, which is often elevated in chronic phase. AST levels are usually lower than ALT but during cirrhosis, AST levels may increase than ALT indicating worsening damage to the liver.

STANDARDISED TESTING STRATEGIES FOR HCV

World Health Organization (W.H.O) recommends standardized testing strategies to maximize accuracy while minimizing cost and increasing simplicity. A testing strategy describes a testing sequence for a specific testing objective, taking into consideration the presumed prevalence of the analyte to be tested in the population. In both high and low prevalence settings, more than one serological assay may be required to establish exposure to HCV.
Antibody testing is an important first step, but antibody detection by using a single assay is not sufficient to identify someone with an active HCV infection. To ensure the complete and timely diagnosis of HCV, HCV reflex testing is recommended to ensure that the HCV RNA test is performed following all reactive HCV antibody screening assays.

- **Supplemental / Reflex testing**
  Reflex testing simply means a laboratory will automatically perform a second test, or refer the specimen for the second test. When the result of the first and second test meets specific criteria, then the laboratory will perform an HCV RNA test on the specimen as it is repeatedly reactive for HCV antibody.



Challenges in following the recommended HCV testing algorithm & strategies in India

The recommended testing algorithm will help in overcoming the challenges faced in HCV testing but the most critical issue is the accessibility and affordability to HCV RNA test. HCV RNA test are still not available in most parts of India. The laboratories situated in rural parts have to rely on reference centres which are mostly situated in cities. The financial resources of such laboratories are also limited. Moreover, it becomes difficult to appoint technicians who are skilled and technically sound to perform RNA testing. The accessibility to HCV RNA is limited also in urban areas.

Even the laboratories in interiors parts of India lack financial and other resources for upgraded / latest technologies such as ELISA or CLIA which are more sensitive screening tests. Unskilled workers

Follow-up testing is also a challenge in India. It becomes difficult to follow-up patients for requirement of repeat / fresh sampling, monitoring and post treatment follow-up due to lack of awareness and information about HCV infection amongst the people. Further testing an antibody positive patient with HCV RNA is generally not possible considering the paying capacity of the patients.

Moreover, the testing strategy designed by World Health Organization (W.H.O) suggest of using two different assays. When the result of two assays are reactive, the sample is termed as reactive for HCV. But in case of discrepancy, wherein one assay is reactive and the other is non-reactive, it is suggested to report the status of the sample as inconclusive and recommends further testing, which is to test with HCV RNA.

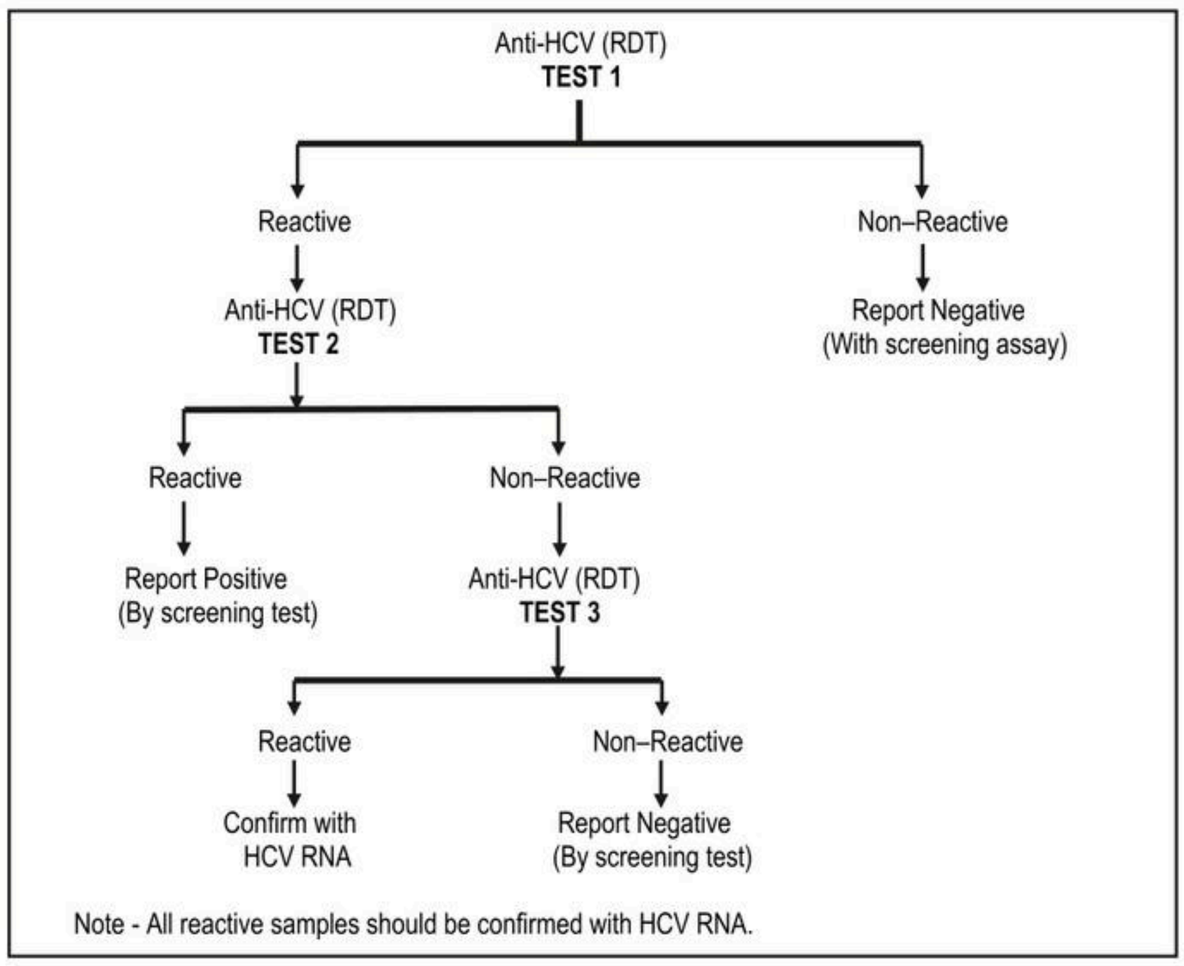
As the disease prevalence in India is low, the probability of producing inconclusive results is high. The inconclusive results will further add to the challenge of reporting and as mentioned earlier the availability of HCV RNA PCR is also subjective. To overcome such situation, a third alternate assay can be incorporated in the same strategy. The results of the third assay will help the laboratories in reporting the status of the patients and recommend them for further confirmatory testing.

ALTERNATE REFLEX TESTING

Considering the challenges faced by the laboratories in India, the following alternate reflex testing can be adopted to report the status of HCV infection of a patient and reduce the cost of HCV RNA testing for each antibody reactive sample.

- **Alternate Reflex testing**
  - A. Rapid assay reflex testing
  - B. ELISA / CLIA reflex testing

A. Rapid assay reflex testing



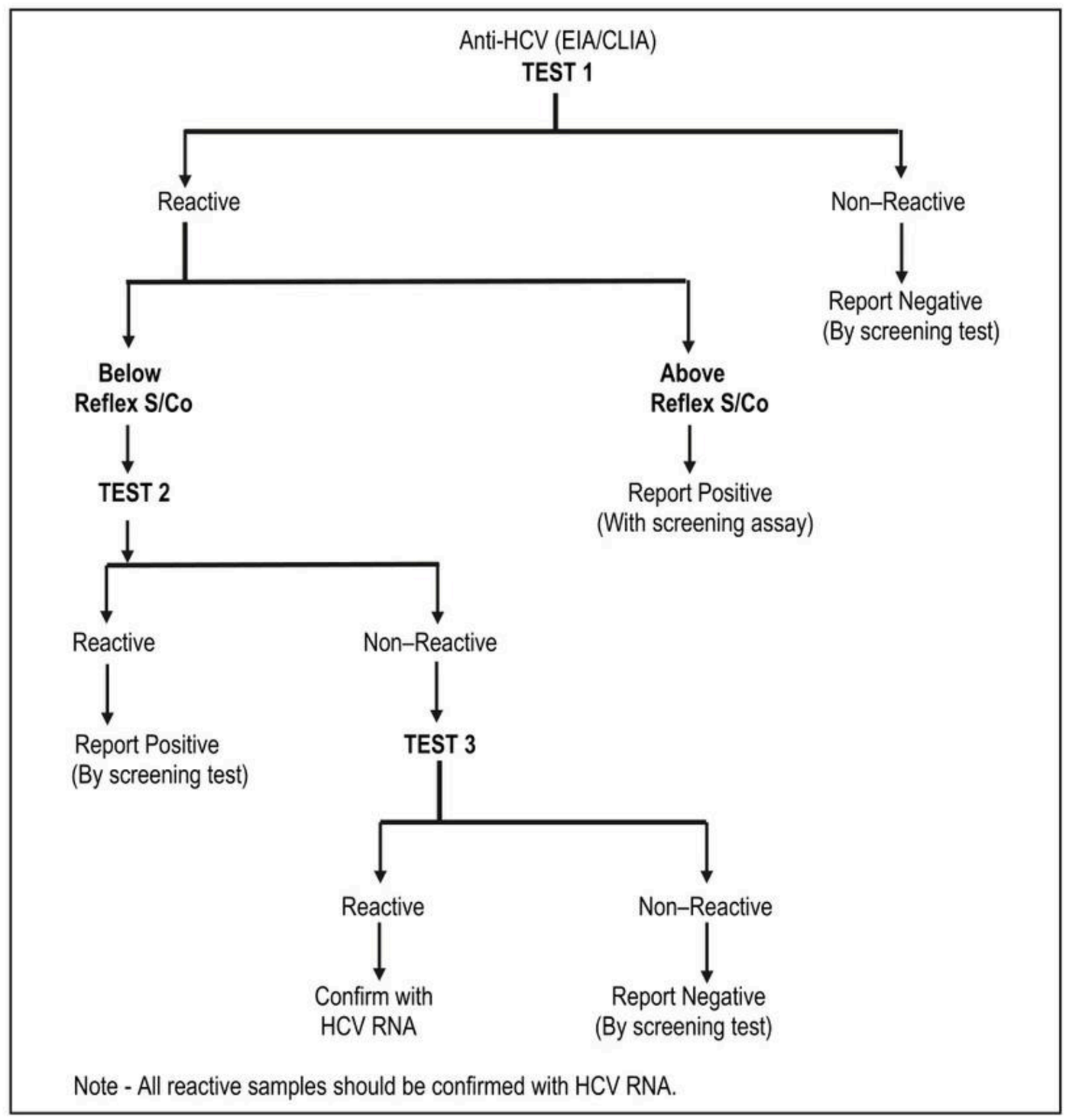
B. **ELISA / CLIA reflex testing**

Use of Signal-to-Cut–Off Ratio.

The recommended anti-HCV testing algorithm has been expanded to include an option that uses the signal-to-cut–off (s/co) ratios of screening-test--positive results. This can serve as an alternative to a supplemental test in some circumstances, minimizing the number of specimens that require supplemental testing and providing a result that has a high probability of reflecting the person's true antibody status.

Setup Reflex signal to Cut-off Ratio

(Specific S/Co ratio that would predict a true antibody positive result ≥ 95% time, through comparative testing on HCV RNA)



- **Establishing Reflex S/Co ratio**

Signal-to-cut–off ratio is calculated by dividing the optical density (OD) value of the sample being tested by the OD value of the assay cut-off for that run. Analysis of enzyme immunoassay and Chemiluminescence assay data indicates that s/co ratios can be used to predict supplemental test-positive results. A specific s/co ratio can be identified for each test that would predict a true antibody-positive result (as defined by the results of supplemental testing) ≥95% of the time, regardless of the anti-HCV prevalence or characteristics of the population being tested).

1. Select "N" number of samples repeatedly reactive with HCV antibody ELISA / CLIA screening test.
2. Supplemental testing of these samples with HCV RNA/ Western Blot / RIBA.
3. Analyse & compare the supplemental test results with S/Co ratio of ELISA /CLIA.
4. Calculate the average S/Co ratio for – False positive results (ELISA /CLIA) and True Positive results (by Supplemental test).
5. Determine the specific S/Co ratio that predicts a true antibody-positive result >95% of the time.
6. Establish as Reflex S/Co ratio.

ROLE OF BIOCHEMICAL TEST PANEL

The liver panel test i.e. Alanine aminotransferase (ALT or SGPT) and Aspartate aminotransferase (AST or SGOT) provide a useful insight and are important marker in conjugation with Immunoassays for the diagnosis of HCV infection.

Almost all laboratories today are equipped with biochemistry analysers which are semi-automated. These analysers are efficient to perform all type of biochemical tests and are open systems i.e. reagents from different manufacturer can be used.

Moreover, the biochemical tests are easy to perform and may not require skilled manpower. Also, factors such as availability, affordability of the laboratories and paying capacity of the patients are not limited.

The laboratories should consider these biochemical reactions in conjugation with Immunoassays before reporting the results of HCV status.

PREVENTION OF HCV INFECTION

The key to reducing the incidence of HCV is by decreasing exposure to contaminated blood and reducing post transfusion HCV infection rates. This can be achieved by screening all patients for HCV and utilizing high sensitivity screening test, as per World Health Organization (W.H.O) recommendation, which ensures early detection of HCV infection.

Nosocomial HCV transmission can be controlled through adherence to universal precautions and infection control protocols diligently.

TREATMENT

Hepatitis C does not always require treatment as the immune response in some people will clear the infection, and some people with chronic infection do not develop liver damage. When treatment is necessary, the goal of hepatitis C treatment is cure. The cure rate depends on several factors including the strain of the virus and the type of treatment given.

The standard of care for hepatitis C is changing rapidly. Sofosbuvir, daclatasvir and the sofosbuvir/ledipasvir combination are part of the preferred regimens in the WHO guidelines, and can achieve cure rates above 95%. These medicines are much more effective, safer and better-tolerated than the older therapies. Therapy with DAAs can cure most persons with HCV infection and treatment is shorter (usually 12 weeks). WHO is currently updating its treatment guidelines to include pan genotypic DAA regimens and simplified laboratory monitoring. Meanwhile, there remains a very limited role for PEGylated interferon and ribavirin in certain scenarios.

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ELISA

- **ELISA**
  -Troubleshooting Aspects .....Pgs 147-155

KNOWLEDGE BOOK

For the Laboratorians

ELISA

Troubleshooting Aspects

Setting Trends...

INTRODUCTION

ELISAs have emerged as the mainstay for diagnosis of various human diseases in a modern clinical laboratory. Despite being an extremely sensitive & specific assay format, several pre-analytical and analytical factors may affect the performance of ELISAs. Thus it is imperative that laboratory professionals be aware of the problems so that he/she has more control over the final assay results. Many errors can be avoided if the protocol is read and fully understood before starting the assay.

- On identifying assay failure, check the expiration dates of the individual reagents and ensure that all the reagents have been stored as indicated on the product label.
- Once this has been established, check for signs of instability or deterioration in reagent solutions, (e.g., precipitation or discoloration).
- All substrate solutions should be colorless.
- Use clean plastic disposable pipettes, tips, and containers for reagent preparation and storage.
- Avoid cross-contamination of kit reagents by changing pipette tips between addition of each calibrator, sample and reagent.
- Ensure that specified incubation times and temperatures have been adhered to and that no substitution of kit reagents has occurred.
- To improve accuracy, it is recommended that samples and standards be run in duplicate.

In this technical series we present various problems commonly encountered in ELISAs and the necessary corrective action.

Problem: High negative control value or high background

Possible Causes	Corrective Action
• Contamination of negative control wells by positive control.	• When washing, do not allow wells to overflow.
• Contamination of negative control vial.	• Check pipette barrel for residual fluid of dried material. Remove if present. • Always format negative control wells before positive control. • Use new pipette tip for each sample. • Check that pipette tips are long enough to provide air space between top of tip and the barrel. • Re-run with fresh reagents.
• Insufficient washing or contamination of negative control by conjugate.	• Make sure wells are completely filled. While washing ensure residual conjugate is removed form well. • Pipette all specimen and reagents in the center bottom of the micro well. Avoid contact with inner wall and rim. • Re-wash
• Non specific attachment of antibodies.	• Unsuitable blocking buffer or omission of blocking buffer. • Wells not pre-processed to prevent non specific attachment of antibodies.
• Antispecies conjugate reacts with reagent coated on plate.	• Set-up controls to assess whether any reagent binds unexpectedly to any reagent.

Problem: Low positive control value or low absorbance

Possible Causes	Corrective Action
• Reagent not at room temperature.	• Make certain all kit components are at RT (22-28°C).
• Test volume low.	• Ensure pipette tips are fitted correctly/ tightly. • Check pipette barrels for obstructions. • Check calibration of pipettes.
• Substrate A & B not freshly combined or incorrectly prepared (in case of 2-reagent substrate system).	• Prepare substrate immediately before use. • Follow working reagent preparation.
• Contamination of substrate with or bacterial contamination of positive control.	• Re-run the assay with fresh reagents.
• Incubation time too short.	• Check calibration of timers. • Record time of incubation.
• Moisture in pouches.	• Check whether deccicant in pouch is in working condition. • Seal unused wells in pouches. • Date pouches when first opened.
• Improper incubation temperature.	• Check incubator temperature/ Room Temperature (22-28°C).
• Room temperature too low for substrate incubation.	• Check temperature of the working area.
• Washing step too vigorous.	• Reduce pressure in wash system.
• Reagent not mixed before using.	• Mix the reagents before use.
• Wells allowed to dry after assay has started.	• Complete all assay steps without interruption.
• Failure to add stop solution.	• Addition of stop solution increases intensity of colour reaction and stabilizes final colour reaction.
• Insufficient conjugate concentrate added in preparing working stock.	• Prepare conjugate accurately. • Follow working reagent preparation as described by the manufacture.

Problem: Entire plate gives positive OD or colour all over plate

Possible Causes	Corrective Action
• Inadequate wash volume or contamination of substrate by residual conjugate left in well.	• When washing, fill the wells to the rim and ensure no overflow
• Too strong conjugate.	• Check dilution.
• Antispecies antibodies react with absorbed antigen.	• Check suitable controls.
• Serum factors in heated sera.	• Do not heat sera.
• Substrate solution contaminated by conjugate.	• Check pipette barrels for residual fluids or dried material, remove if present. • Pipette tips should be long enough to provide air space between top of tip and pipette barrel. • For automated system, make sure regent lines are in proper position. Do not switch lines.
• Substrate solution is not fresh.	• Do not hold substrate solution longer than Manufacturer claims.
• Failure to stop reaction. • Acid not added.	• Check bottle before use. • Check assay procedure.
• Plate sat idle too long before reading.	• Read within 30 minutes of adding stop solution.
• Chromogen may not be working.	• Use fresh chromogen.
• Substrate solution container is dirty.	• Do not add fresh substrate to reagent bottle containing old substrate. • Clean old solution bottle with acid and thoroughly rinse with distilled water.
• Plate exposed to light during substrate incubation	• Place plates in dark immediately after addition of substrate solution (Check Product Insert)

Problem: False positive reactions

Possible Causes	Corrective Action
• Inadequate washing.	• Check washer before use to determine they are working properly. • Perform routine maintenance.
• Clogged cannulas in washer.	
• Contamination of wells by conjugate.	• Carefully add conjugate to wells. Pipette reagent to center bottom of microwell. • Avoid contact with sides and rims of wells. • Check alignment and delivery of automated systems. • Check pipette barrels for residual fluid or dried material. Remove if present. • Check pipette tips are long enough to provide air space between top of tip & pipette barrel.
• Splashing of conjugate on rims of wells during conjugate addition.	
• Contamination of substrate solution by conjugate.	
• RBCs in test sample.	• Centrifuge before use.
• Evaporation of sample of conjugate during the 37°C incubation (if 37°C is a must, not applicable in RT incubation).	• Place the covered test plate in a pre-warmed (37°C) moist incubation box inside the incubator (dry or humidified).
• Mold in incubation and / or wash buffer bottle.	• Visually check incubation boxes & wash buffer bottles. • Clean any moldy containers. • Be sure all containers are free of cleaning agents before using. • Set up routine cleaning schedule.
• Too much conjugate concentrate used in preparing working stock.	• Prepare fresh working conjugate. • Follow conjugate preparation.
• Incubation temperature too high.	• Check room temperature, whether at 22-28°C / Check AC.

Problem: Poor reproducibility or bad duplication

Possible Causes	Corrective Action
• Bubbles in wells.	• Use pin or needle to burst. Use separate pin for each well.
• Dispensing error.	• Check dispensing instrument.
• Finger tips on plates.	• Clean bottom surface of plate with wash buffer, blot to dry.
• Mis-aligned wells in plate.	• Re-align wells.
• Improper washing technique.	• Be certain to wash the specified no. of times. Fill each well to the rim with wash buffer. Do not allow well to overflow. Blot plate dry at end of wash.

Problem: Poor sensitivity

Possible Causes	Corrective Action
• Usage of non human serum based calibrators (for quantitative assays)	• Re-run with human serum based calibrators (Primary Standards)
• Insufficient conjugate concentration added in preparing working stock.	• Prepare fresh working conjugate, follow working procedure.
• Error in pipetting working conjugate.	• Check calibration of pipettes.
• First incubation time insufficient.	• Repeat run using proper incubation time.
• Temperature more than suggested by manufacturer. • Plates being held too long after first incubation before further processing.	• Process plate continuously throughout entire assay procedure.

Problem: High absorbance of calibrator

Possible Causes	Corrective Action
• Plates being held too long after first incubation at a higher temperature than what is recommended by the manufacturer before further processing.	• Process plate continuously throughout entire assay procedure
• Insufficient sample volume added.	• Check calibration of pipettes.

Problem: Specimen absorbance out of range of calibrators

Possible Causes	Corrective Action
• Concentration in specimen is too high.	• Dilute with '0' calibrator & re-assay.

Problem: Overall low absorbance

Possible Causes	Corrective Action
• Temperature of room < 20°C.	• Increase time of reaction between enzyme/ substrate (Check with manufacturer). • It is recommended to maintain 22-28°C ambient temperature in the laboratory.

Problem: Controls out of range

Possible Causes	Corrective Action
• Contamination of controls.	• Re-run assay with new controls.
• Contamination of calibrators.	• Re-run assay with new calibrators.

Problem: Strips slip from holder

Possible Causes	Corrective Action
• Improper handling.	• Grasp holder on grip marks when tapping.

Problem: Strips do not fit in holder

Possible Causes	Corrective Action
• Improper alignment or incorrect holder.	• Rotate strip 180° & re-insert or use correct holder.

Problem: Substrate A is blue

Possible Causes	Corrective Action
• Contaminated.	• Obtain fresh Substrate A.

Problem: Substrates A and B turn blue when mixed

Possible Causes	Corrective Action
• Contaminated.	• Obtain Fresh substrate A & B.

Problem: Stop solution yellow

Possible Causes	Corrective Action
• Contamination.	• Obtain fresh stop solution.

Problem: Waited over 30 minutes before measuring plate

Possible Causes	Corrective Action
• End product of enzyme reaction may precipitate and cause error.	• Re-run the essay.

Problem: No colour even after 30 minutes incubation with substrate

Possible Causes	Corrective Action
• Improper mixing of Substrate A & B.	• Re-mix the substrates.
• Substrate not working.	• Contact manufacturer.

Problem: Colour develops very quickly

Possible Causes	Corrective Action
• Contaminated enzymes.	• Common in wells, pre-treatment may be necessary. • Make sure all reservoirs are clean.

Problem: Colour develops too slowly

Possible Causes	Corrective Action
• Sample not at room temperature.	• Bring samples to room temperature before assay run
• Conjugate too weak	• Check dilutions & time when diluted.
• Contamination inhibits activity of enzyme e.g. sodium azide on peroxidase.	• Avoid wrong preservatives.
• Low temperature of laboratory or substrate solution.	• Makes sure temperature of substrate is correct.

TECHNICAL TIPS

Washing

The purpose of washing is to separate bound and unbound (free / unwanted) reagents/serum components. This involves the emptying of microwells of reagents followed by the addition of liquid into the wells. Such a process is performed at least 3-6 times for every well. The liquid used to wash wells is usually buffered (PBS) in order to maintain isotonicity, since most Ag-Ab reactions are optimal under such conditions. Tap water is not recommended, since tap water varies greatly in composition (pH, molarity, and so on). Generally, the mechanical action of flooding wells with a solution is enough to wash wells of unbound reagents. Some workers leave washing solution for a short time (soak time) after each addition (1-5 minutes). Sometimes detergents, notably Tween-20 (0.05%) are added to washing buffers. This can cause problems where excessive frothing takes place producing poor washing conditions, since air is trapped and prevents the washing solution from contacting the well surface. For most cases, this addition does not contribute significantly to the washing procedure. When using detergents, care has to be taken that they do not affect reagents adversely (denature Ag), and greater care is needed to prevent frothing in the wells.

Normal Washing

In washing plate manually, the most important factor is that each well receives the washing solution so that, no air bubbles are trapped in the well or a thumb is not placed over corner wells.

Strip / Plate Washers

- Various washing cycles can be programmed.
- Careful maintenance is essential, since they are prone to machine errors, such as having a particular nozzle being blocked.

Washing Tips

- Follow procedure for preparation of wash buffer.
- Check washer alignment daily as part of routine instrument start-up procedures.
- Ensure that the plate is leveled.
- Make certain well is completely filled, when washing, to ensure residual conjugate is removed.
- Examine the fill volume (a slight dome should be observed at the top of the well).
- When washing do not allow wells to overflow.
- Reduce pressure in wash system.
- Check washers before use to determine they are working properly. Perform routine maintenance.
- Be certain to wash the specified number of times.
- Allow approximately 20 seconds soak-time between the addition of wash solution and subsequent aspiration (if soak-time is not indicated in the assay pack insert).
- Examine the wells for complete aspiration of contents.
- Upon completion of wash cycle, blot to remove residual fluid.

Pipetting Tips

- Calibrate pipettes regularly according to manufacture's instructions.
- Avoid touching side wall of well with tips.
- Avoid splashing of sample and reagents.
- Avoid blowing out tip contents.
- Use a new tip for each sample/control/reagent addition.

- New tips should be used on the multichannel pipettes for each reagent to be added.
- Reverse pipette when using the multichannel pipettes to add conjugate and substrate solution.
- Forward pipette when using the multichannel pipettes to add stop solution.
- Check pipette tips are long enough to provide air space between top of tip and pipette barrel.
- Check pipette barrel for residual fluid of dried material, remove if present.
- Ensure pipettes tips are fitted tightly.

Microplates

- Bring microplate pouches to room temperature before opening.
- Level microwells evenly in microplate frame as the individual breakaway wells have very flexible plate frames leading to bowing off wells and yield poor washes.
- Place plates in dark immediately after addition of substrate solution, provided the substrate is sensitive to light.
- Grasp holder on grip marks when tapping to avoid strips slipping from holder.
- Rotate strips 180° and re-insert or use correct holder if strips do not fit in holder.
- Seal unused wells in purchase along with the dessicant.
- Date the pouches when first opened.
- Clean bottom surface of plates with wash buffer to remove fingerprints.
- Make sure microwells are at level during washing, reagent addition and plate/strip reading.
- Wipe the bottom the plate with a lint-free cloth/ towel before reading.
- Do not allow microwells to become dry once the assay has begun.

Substrate Preparation

- Use freshly prepared substrate A and substrate B (in 2-reagent substrate systems)
- Do not hold substrate solution longer then 1 hour.
- Follow procedure of working substrate solution.
- The temperature of solution is important because it effect s rate of colour reaction.
- Do not add fresh substrate to reagent bottle containing old substrate.
- Clean old substrate solution bottle with H_2SO_4 and thoroughly rinse with distilled water.

Conjugates

- Store at recommended temperature.
- Never store excessively diluted conjugate for use at some later time.
- Always make up the working dilution of conjugate just before you need it.
- Never leave conjugate on the bench for excessive time.

Addition of Samples

Problems caused by :

- Failure to put sample into buffer in well, leaving it on the side of the plate.

Stopping Reagents

Stopping reagents are added to prevent further enzyme reaction in ELISA. The stopping is usually made at a time when the relationship among the enzyme-substrate product is in the linear phase. Molar concentration of strong acids or strong bases stops enzyme activity by quickly denaturing enzymes. Some stopping reagents are enzyme-specific.

Sodium azide is a potent inhibitor of HRPO, whereas EDTA inhibits Alkaline phosphatase by the chelation of metal ion cofactors. Since addition of stopping agents may alter the absorption spectrum of the product, the absorption peak must be known.

Temperature

- Bring test reagents to room temperature (22-28°C) approximately 30 minutes prior to use.
- Maintain proper incubation temperature :
  - Lower temperature can decrease OD values.
  - Higher temperatures can increase OD values.
  - Evaporation in wells can cause edging effect.
- The optimal temperature for incubation is 22-28°C
- Check temperature against calibrated thermometer.
- Strict adherence to time must be maintained :
- Check calibration of timers.
- Record time of incubation.
- Read plate with specified time limits of adding stop solution

Rotation of plates while incubating reagents

In certain ELISA systems, the plates are rotated during incubation for better antigen-antibody reaction. The effect of rotating plates is to mix the reactants completely during the incubation step. Since the solid-phase limits the surface area of the absorbed reactant, the mixing ensures that, potentially reactive molecules are continuously coming into contact with the solid-phase.

During stationary incubation, mixing only takes place because of diffusion of reagents. Thus, to allow maximum reaction from reagents in stationary conditions, greater times of incubation may be required, than if they are rotated.

Rotation also allow ELISA to be performed independent of temperature conditions. The interaction of antigen & antibodies relies on their closeness, and the kinetic energy provided to the system, which is encouraged with the mixing during rotation. Stationary incubation relies on the diffusion of molecules & thus is dependent on temperature.

Laboratory conditions
The laboratory should be devoid of any acid fumes.

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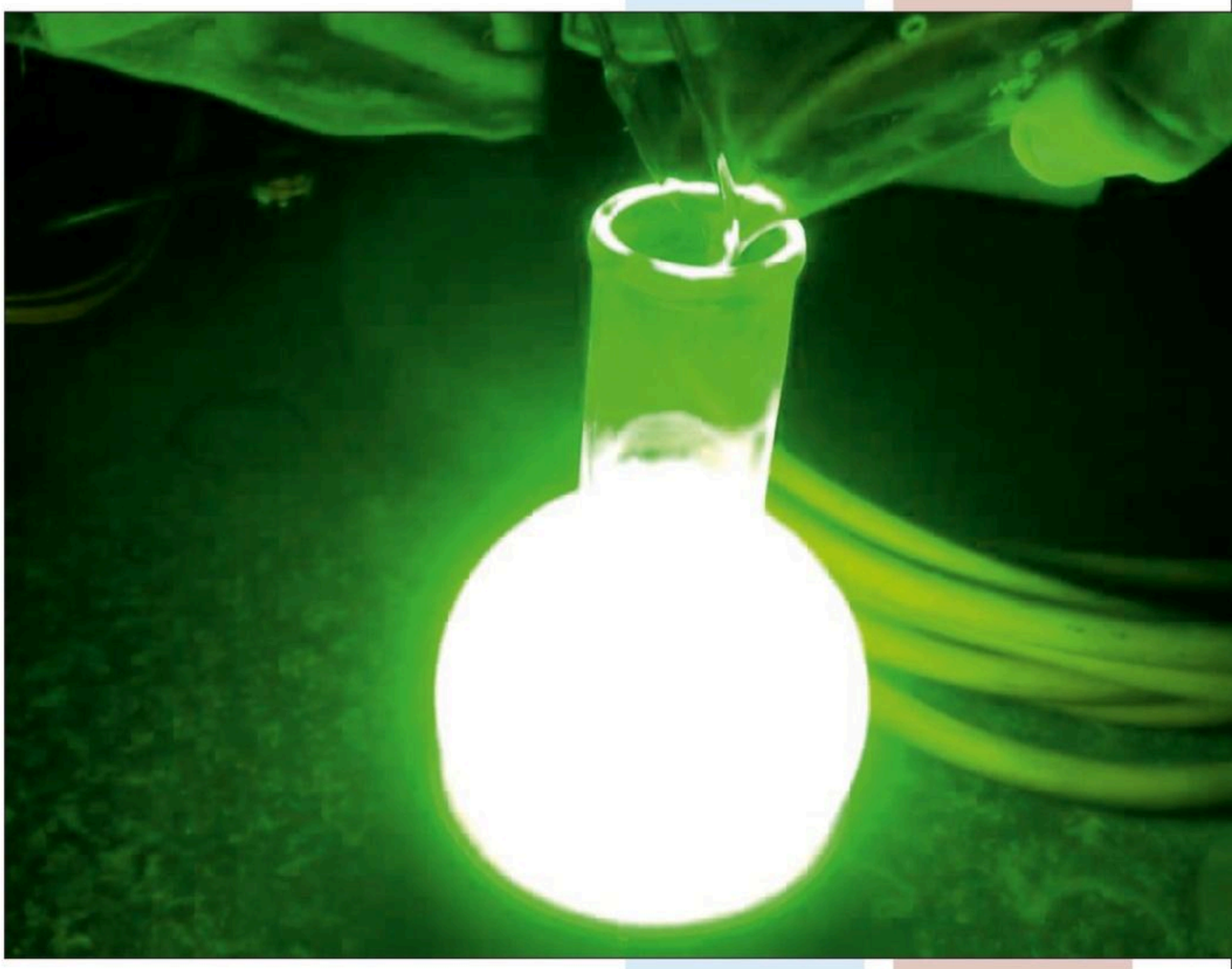
CHEMILUMINESCENCE



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KNOWLEDGE BOOK

For the Laboratorians

Chemiluminescence

Principle & Troubleshooting Aspects

Setting Trends...

Introduction

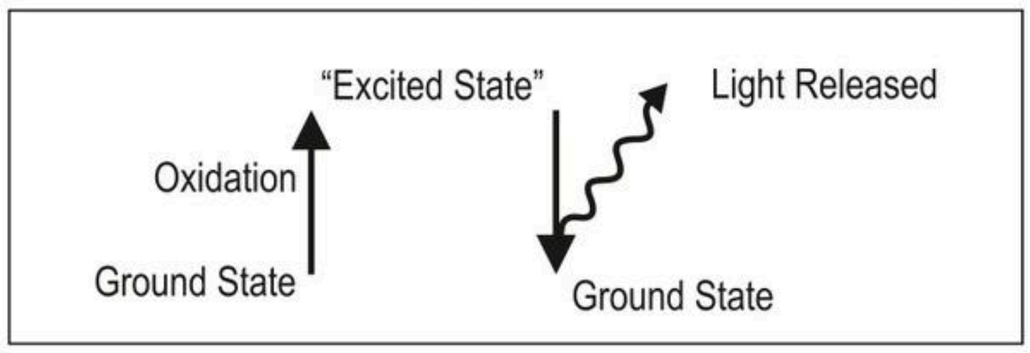
In recent years, chemiluminescence immunoassay (CLIA) has gained increasing attention in different fields, including life science, clinical diagnosis, environmental monitoring, food safety and pharmaceutical analysis because of its high sensitivity, good specificity, wide range of applications, simple equipment and wide linear range. Traditional immunoassay always needs a long incubation time, which in turn causes the whole analysis to take several hours for completion, so the throughput and application range are largely limited. Researchers have designed different methods to shorten the analysis time by improving mass transport and reaction kinetics. The immunoassay has expanded the applications of CLIA.

Chemiluminescence immunoassays are generally considered as 4th generation assays when compared to 3rd generation EIA's.This is because CLIA has significantly improved sensitivity & high signal to noise ratio.

What is Chemiluminescence ?

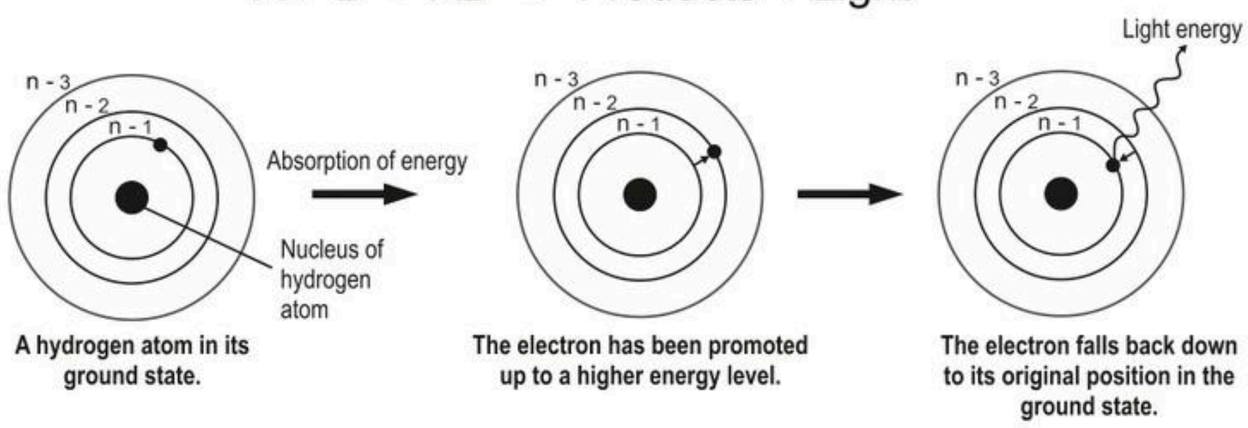
The term" Chemiluminesence" was first coined by Eilhardt Weidemann (1888). Chemiluminescence, which is the phenomenon observed when the vibronically excited product of an exoergic reaction relaxes to its ground state with emission of photons, can be defined in simplistic terms: chemical reactions that emit light. The chemical reaction produces energy in suficient amount (approximately 300 kJ mol 1 for blue light emission and 150 kJ mol 1 for red light emission) to induce the transition of an electron from its ground state to an excited electronic state. This electronic transition is often accompanied by vibrational and rotational changes in the molecule.

In organic molecules, transitions from a π bonding to a π* anti-bonding orbital (π→π*) or from a non-bonding to an anti-bonding orbital (n→π*) are most frequently encountered. Return of the electron to the ground state with emission of a photon is thus called chemiluminescence. The ex-cited molecule can also lose energy by undergoing chemical reactions, by collisional deactivation, internal conversion or inter-system crossing. These radiations less processes are undesirable from an analytical point of view when they compete with chemiluminescence. (see Figure below).



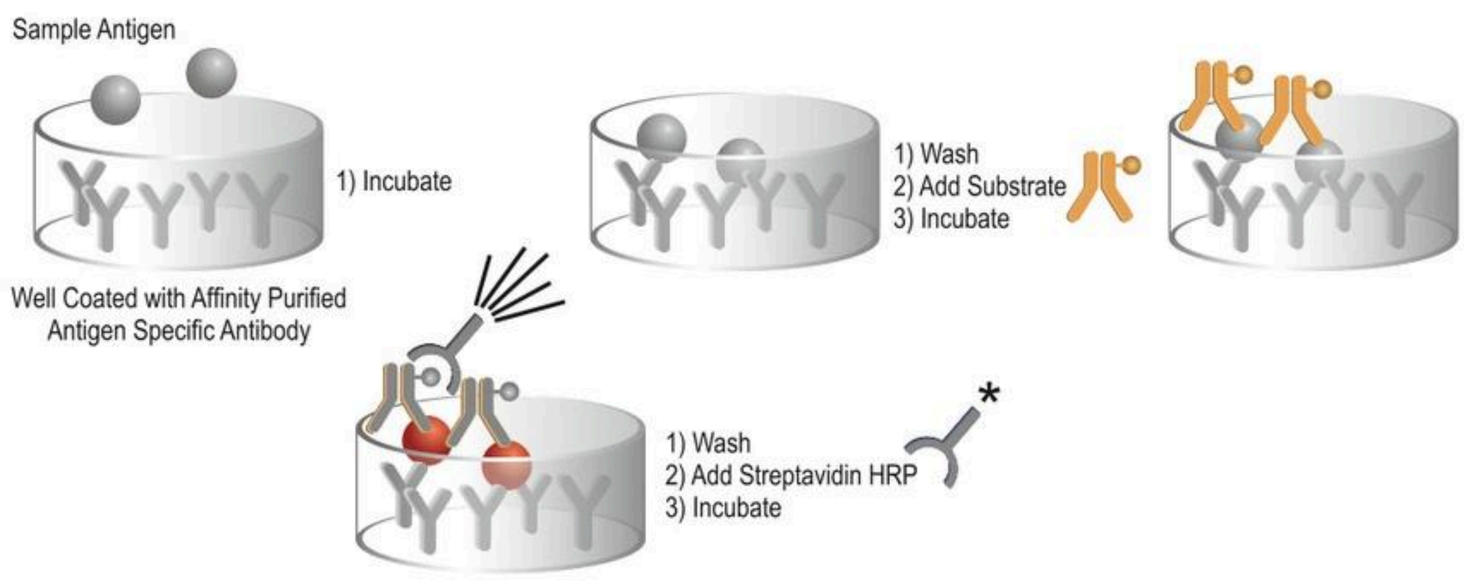
When two molecules react chemically so that there is a release of energy (an exothermic reaction), that energy sometimes manifests itself not as heat but as light. This occurs because the energy excites the product molecules into which it has been funneled. A molecule in this excited state either relaxes to the ground state, with the direct emission of light, or transfers its energy to a second molecule, which becomes the light emitter. This process is referred to as chemiluminescence. (see Figure below).

A + B -> AB* -> Products + Light



Principle of Chemiluminescence Immunoassay (CLIA)

In the presence of complimentary antigen and antibody, the paratope of the antibody binds to the epitope of the antigen to form an antigen-antibody or an immune complex. Estimating the levels of such immune complex by use of labeled antibodies form the basis of CLIA. It involves use of stationary solid phase coated either with the antigen or antibody of interest. This results in generation of light, the intensity of which is directly proportional to the amount of labeled complexes present and which indirectly aids in quantification of the analyte of interest. The intensity of light is measured in terms of Relative Light Units (RLU). (see Figure below).

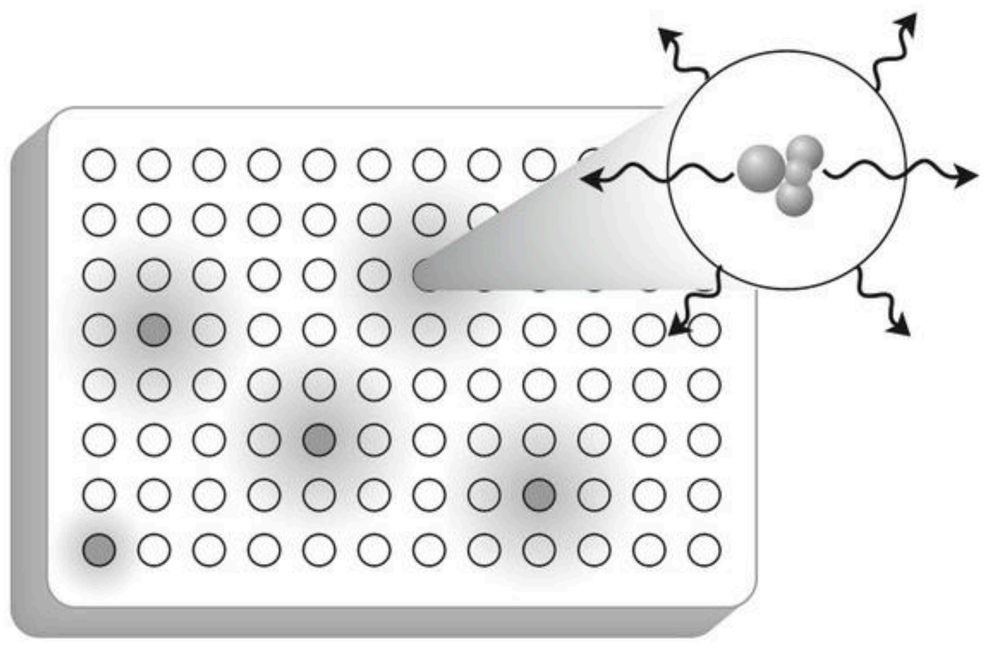


The main advantage of this technology includes sensitivity and its ability to be unaffected by background signals. Also, the analyzers working under this principle are simple in design and operation.

Basic Components & Prerequisites for CLIA

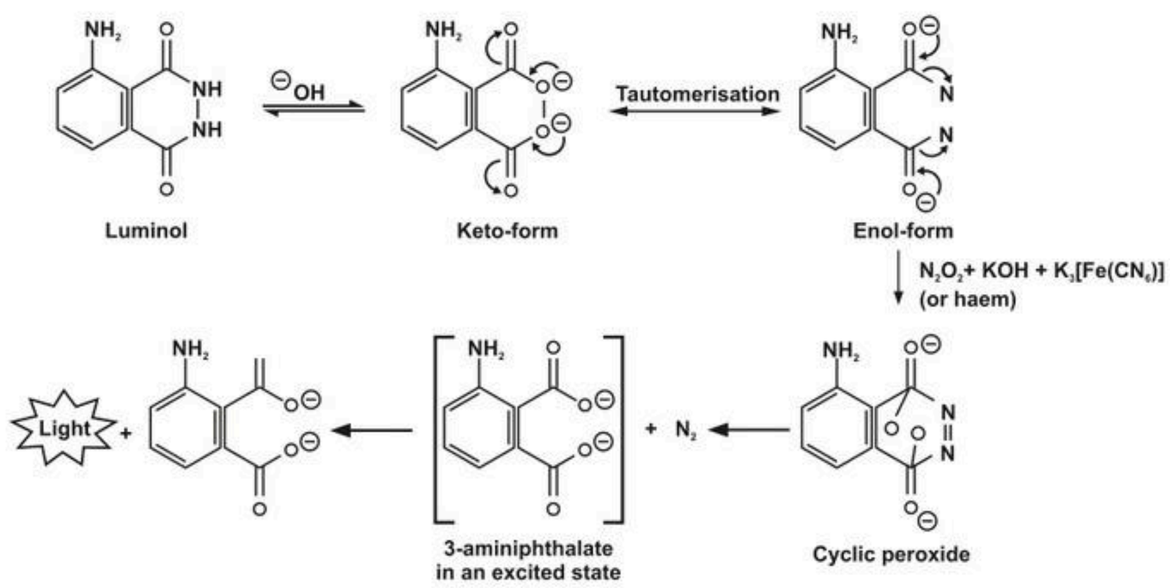
The basic components for a CLIA reaction and the corresponding prerequisites are:

1. **Solid Phase:** Where all reactions including the Ag-Ab and all subsequent reactions (till the generation of light) take place.
   Prerequisite:
   (a) Light emitted in CL reactions is isotropic- it is emitted equally in all directions. If CL reactions are conducted in wells of a transparent plate, light radiates out not only vertically in the direction of the detector, but also laterally to other wells. Light is easily transmitted through the plate material itself, a phenomenon termed light piping. **For this reason, CLIA should never be performed in clear plates.** Instead, opaque plates must be used. Two kinds of opaque plates are available: Black & White. There is usually a 10-fold decrease in signal owing to light absorption, when using black plates.
   The best option is to use white plates of uniform opacity. A recent breakthrough to minimize light piping and the resultant cross-talk between reaction cells is to coat the reaction cells with a layer of **Titanium. Titanium, which has a complex mesh work structure cuts down light piping and cross-talk drastically.** (see Figure below)



   (b) The solid phase must allow efficient adsorption of Ag or Ab as capture.
   (c) Optimum concentration of capture is critical: Prevents Hook Effect & ensures Sensitivity.
   (d) High Specificity of capture prevents false positivity.
2. **Conjugate :** It is an enzyme linked secondary Ab as a in immunoassays and serves to locate the primary Ag-Ab complex. The tagged enzyme catalyses the reaction whereby the signal produces light.
3. **Signal Reagent :** The Signal Reagent is the actual Chemiluminescent compound (e.g. Luminol, 1, 2-dioxetane, Acridinium ester). Produces light upon oxidation by the enzyme in the conjugate. (May require the presence of an additional molecule like H_20_2)
   Prerequisite : The Signal reagent must have a combination of these compounds
   (a) The Cl-Substrate Luminol I 1, 2-dioxetane (and also H_20_2 if using luminol) to produce light.
   (b) The Enhancer to enhance heighten the light produced.
4. **CLIA Substrate :** This is a critical factor in determining the CLIA efficacy. The CL- substrate, upon oxidation by the enzyme tagged to the tracer yields light. Quantum of light yield depends on the kind of substrate used. Several kinds of CL substrates are available. We take a look at two of the widely used CL substrates & the reactions involved therein:
   Luminol
   In luminol-based assays oxidizer (OX:H_20_2) & light-emitting material (LEM: Luminol) is mixed in liquid phase under a desired pH value. In presence of H_20_2, Luminol is oxidized & decomposed into anIntermediate Compound-I, in the presence of the enzyme Peroxidase. Intermediate Compound-I reacts with an Enhancer (Iodophenol Compounds are strong enhancers that intensify luminol CL by 1000 times) to generate Intermediate Compound-II, which in turn is converted into 3- aminophthalate dianion with the concomitant emission of light.

$$\text{LUMINOI} \xrightarrow[\text{Peroxidase}]{H_2O_2} \text{INTERMEDIATE-1} \rightarrow \text{INTERMEDIATE-2} \xrightarrow{\text{Enhancer}} \text{3-AMINOPHTHALATE+ CL}$$



Higher the signal generated in this step (light in CL) better the sensitivity. But, increase in signal should not be accompanied by increase in the noise. Discriminatory measurements add to the accuracy of results. Ideally the signal is stable for 10-15 mins.

4. **The Enhancer :** Enhancers are molecules, the addition of which increases the light output by several orders of magnitude. For example, HRPO, in the presence of H_20_2, causes oxidation of luminol with the emission of light. This light output increases more than 1000- fold in the presence of 4-iodophenol. Most enhancers are substituted phenols & naphthols.

Enhancers improve the signal-to-noise ratio.

Enhanced CL is a glow rather than a flash of light which persists for hours, although reading is normally taken within 10-15 mins.
Examples of enhancers: Iodophenol, Phenothiazine.

Luminescence the ultimate result of the CL reaction

The ultimate result of a CL reaction is LIGHT. The intensity of all CL reactions is usually high (as enhancers are used). Depending on the duration of the light, CL can be of 2 types:
I- "FLASH"- type wherein addition of reagent(s) causes the immediate emission of light, which lasts for a few milliseconds.
II-"GLOW"- type where the emission of light builds up slowly and reaches a maximum after a substantial incubation time.

Measuring Luminescence

Light consists of billions of tiny packets of energy called photons. Light or Photons emitted from the reaction need to be measured or counted. Light from CL reactions are measured by an instrument:
Luminometer. The choice of a luminometer is dictated by the required dynamic range, sensitivity, reproducibility, temperature control, on-board software for data analysis.

Luminometer

All CL detection systems consist of the following components:

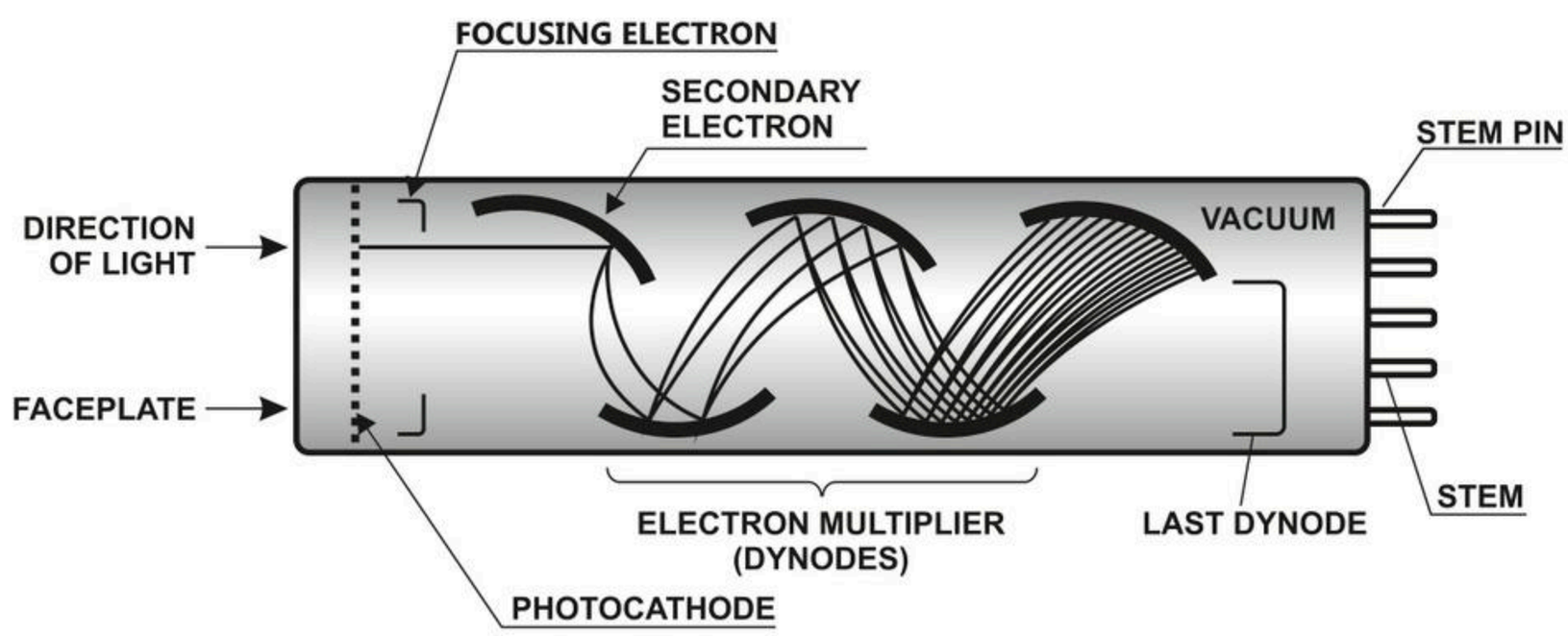
A. **The Sample Chamber**
B. **The Detector**
C. **Single Processing Method & Signal Output Display**

A. The Sample Chamber:-

a. Presents the luminescent sample to detector for measuring the quantity of light.
b. Must be sealed from ambient light in order to minimize potential interferences.
c. Must be positioned as close to the detector as possible to maximize optional efficiency.
(High Efficiency is desirable for an optional signal-to-noise ratio, which allows rapid, which allows rapid and precise measurements).

B. The Detector :-

The photo-multiplier tube (or PMT) is a versatile device that provides extremely high sensitivity and ultra-fast response. A typical photo-multiplier tube consists of a photo emissive cathode (photocathode) followed by focusing electrodes, an electron multiplier and an electron collector (anode) in a vacuum tube.
When light enters the photocathode, the photocathode emits photoelectrons into the vacuum. These photoelectrons are then directed by the focusing electrode voltages towards the electron multiplier where electrons are multiplied by the process of secondary emission. The multiplied electrons are collected by the anode as an output signal.
Because of secondary-emission multiplication, photomultiplier tubes provide extremely high sensitivity and exceptionally low noise among the photosensitive devices currently used to detect radiant energy in the ultraviolet, visible, and near infrared regions. The photomultiplier tube also features fast time response, low noise and a choice of large photosensitive areas.
This section describes the prime features of photomultiplier tube construction and basic operating characteristics.



A Photo Multiplier Tube

Process: Light which enters a PMT induces an output signal by the following process:

1. Light (Photons) enters through the input window and strikes the photocathode.
2. The Photocathode emits photoelectrons into the vacuum (due to photoelectric effect). The Photocathode is normally kept at a very high negative voltage, typically -500 to -1500 volts.
3. These photoelectrons are then directed by the focusing electrode voltages towards the electron multiplier (dynodes) where the electrons are multiplied (normally 106 to 107) by a process of secondary emission. This secondary emission is repeated at each of the process of secondary emission. This secondary emission is repeated at each of the successive dynodes.
4. The multiplied electrons emitted from the last dynode are collected by the anode. Because of the secondary-emission multiplication, PMTs provide extremely high sensitivity & exceptionally low noise.

Construction:- The photomultiplier tube generally has a photocathode in either a side-on or a head-on configuration. The side-on type receives incident light through the side of the glass bulb, while in the head-on type, it is received through the end of the glass bulb. The head-on type (or the end-on type) has a semitransparent photocathode (transmission-mode photocathode) deposited upon the inner surface of the entrance window. The head-on type provides better spatial uniformity than the side-on type having a reflection-mode photocathode.
Other features of head-on types include a choice of photosensitive areas from tens of square millimeters to hundreds of square centimeters.
Variants of the head-on type having a large-diameter hemi-spherical window have been developed for high energy physics experiments where good angular light acceptability is important.

Figure : External Appearance

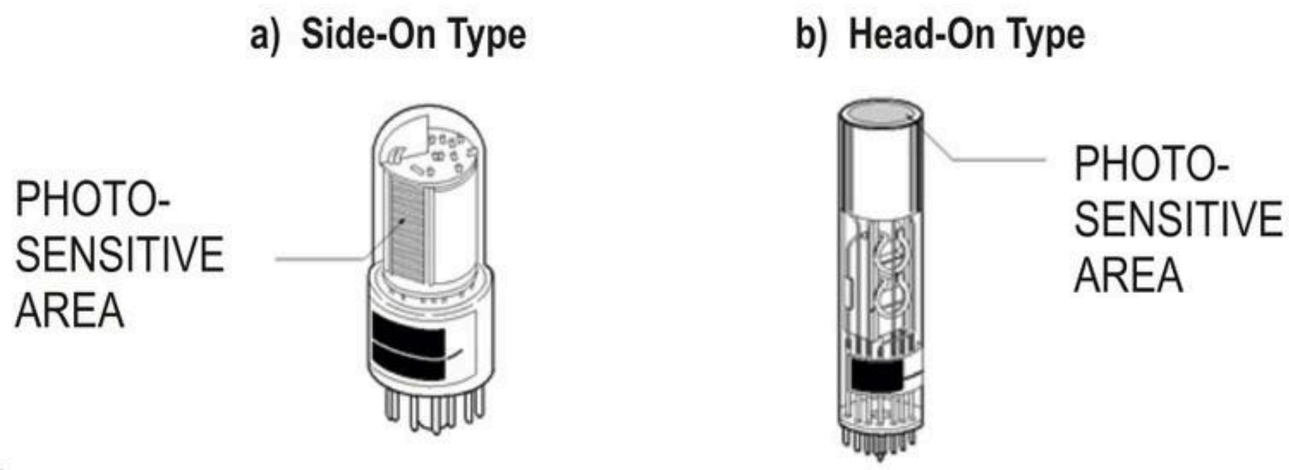
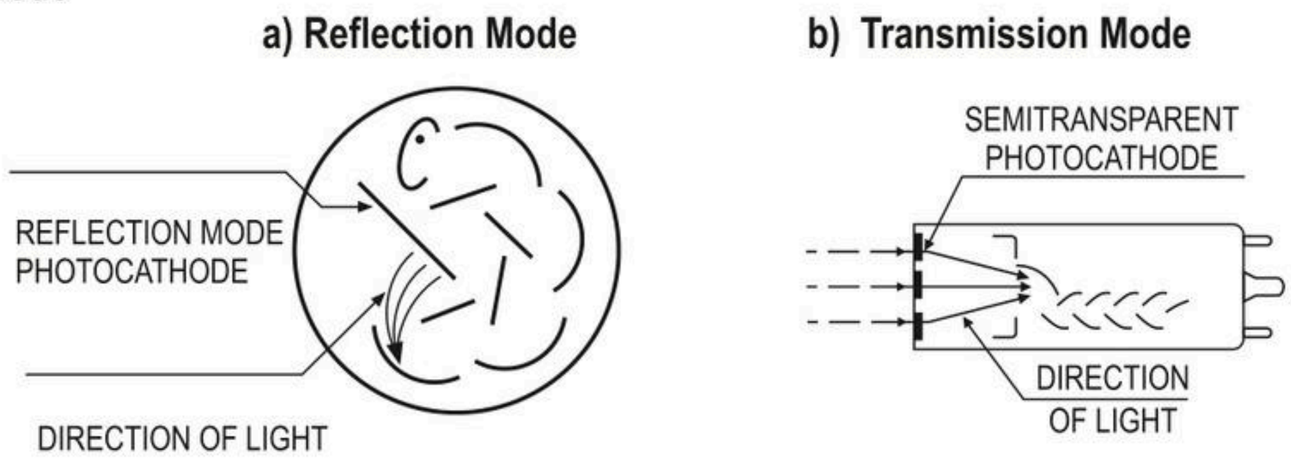


Figure: Types of Photocathode



The superior sensitivity (high current amplification and high S/N ratio) of photomultiplier tubes is due to the use of a low-noise electron multiplier which amplifies electrons by a cascade secondary electron emission process.

C. Signal Processing Method & Signal Output Display.

In the signal processing method it is important to underline two very critical facts:

1. All electrons generated from the photocathode will not follow the same trajectory and hit the dynode. Some may deflect and become a source of noise.
2. Many times, output pulses are present even if no light falls on the PMT (dark current) owing to radiation & cosmic rays. This again contributes to high noise.

Advantages of Chemiluminescence:-

Rated as the best detection technology, Chemiluminescence, has many advantages. We take a look.

1. **High Sensitivity:** The most important parameter that differentiates one detection technology from another is the analytical sensitivity. It is a well known fact, that higher the sensitivity, the better the technology.
   Enhanced Chemiluminescence is upto 100,000 times more sensitive than absorption spectroscopy & at least 1000 times more sensitive than fluorometry.
2. **High Linearity :**
   A higher linear range is limited in ELISA readers as they cannot read O.D.s higher than 3.0, thus compelling dilution.
   In Chemiluminescence Immunoassays, samples of larger concentration ranges can be analyzed without dilution. Chemiluminescence offers linear measurement over a dynamic range of 10· or 10'. Furthermore, the higher dynamic range in CLIA enhances the resolution, making discrimination between positives and negatives easy. This is particularly useful in the diagnosis of infectious disease, where the discrimination between positives and negatives is absolutely essential for proper treatment.

3. **High Signal To Noise Ratio :**
In Chemiluminescence, there is better discrimination of signal from noise. This is possible by the use of enhancer, pulse height discriminator and photon counting.

4. **Simplicity :**
The attractiveness of ECL as an analytical tool is the simplicity of detection. Measurement of lightintensity is relatively simple, requiring only a photomultiplier or photodiode & the associated electronics to convert and record the signals.

5. **Reproducibility :**
Chemiluminescent assays are extremely robust and reproducible.

6. **No Quenching:**
Lower-than-expected quantum yield occurs in Fluorescence assays. This happens when amacromolecule such as an antibody heavily labeled with a fluorophor (FITC, fluorescein), is excited, and being spatially very close, radiation less energy transfer occurs. Also in aqueous environments quenching occurs due to collision. Inclusion of Enhancers excludes water from the site of CL production and increases emission efficiency.

7. **No Inner Filter Effect :**
Unlike in fluorescence, where the linear relationship between concentration and fluorescence emission holds as long as solutions are used that absorb less than 2% of the exciting light, in chemiluminescence, relationship between light emission and concentration is linear as no exiting light is required.
In fluorescence, as the absorbance of the solution increases above **this** amount the relationship becomes non-linear. The effect is called the inner filter effect and is caused by a loss of excitation intensity across the cuvet path length as the excitation light is absorbed by the fluorophor. Chemiluminescence is free from this error.

8. **No Light Scattering Effects:**
Rayleigh and Raman scattering common in Fluorescent measurements is not seen in Chemiluminescence as there is no excitation light source involved.

9. **No Material & Solvent Effects:**
In Fluorescence assays quartz glass and plastic materials containing ultraviolet absorbers may emit fluorescence and add to the background noise. This possibility is completely done away with as in Chemiluminescence, the light comes only from the reaction.
Also, some solvents like ethanol can cause appreciable fluorescence, but no chemiluminescence.

10. **No Photodecomposition Effects :**
Photodecomposition of analyte due to usage of intense light sources, which occurs in conventional and laser-induced fluorometry, does not occur in chemiluminescence.

11. **No Dependence On Oxygen Concentration:**
Electrochemiluminescence, a variant of chemiluminescence (wherein reactive species at the surface of an electrode emit light from stable precursors) is heavily dependent on oxygen availability.
The reaction usually involves oxidation-reduction type reaction with ruthenium (II) tris (bipyridyl) and triptopylamine. Chemiluminescence on the other hand, has no such dependence on oxygen availability as simple single-step reactions are involved in the generation of light.

12. **No Pseudo-base Formation:**
Pseudo-base formation is an inherent drawback in electro chemiluminscence, dictated by the complex chemistry involved.
Chemiluminescence, on the other hand has no such complex chemical intermediate formation, as the basic reaction involved is very simple. Also, flexibility to include enhancers in system does not affect overall Ag-Ab reaction.

13. **Not Overly Prone To Interferences:**
High background intensity observed in fluorescence and electro chemiluminecscence is not an issue when it comes to Chemiluminescence. Whatever little interferences arise in the PMT from stray electrons and radiations from the environment, can be taken care of, by the appropriate use pulse height discrimination mechanisms is a good luminometer.

14. **Greater shelf-life:**
Chemiluminescence substrates have a shelf-life of about a year, whereas those of fluorescence (containing fluorescin molecule) last only for a week.

Conclusion:

Chemiluminescence immunoassays are generally considered as 4th generation assays when compared to 3rd generation EIA's.This is because CLIA has significantly improved sensitivity & high signal to noise ratio. With so many advantages that it offers (as proven by numerous scientific studies), Chemiluminescence, definitely dominates over all other detection technologies. But the fact of the matter is, in India, Chemiluminescence is still a technoloqy accessible to a select few clinical laboratories. This marvel of a technology can do wonders if it becomes accessible to laboratories in all corners of the country. Near-patient testing and doorstep, high quality immunoassay results generated by Chemiluminescence will redefine immunosassays and benefit laboratories and patients alike.

In general, Chemiluminescent immunoassays are superior (or atleast comparable) to the most commonly used radioimmunoassay with respect to sensitivity, speed of analysis, cost & stability (H.A.H.Rongen et.al)

Trouble shooting guidelines for CLIA

1. High RLU values of negative control	
Possible Causes	**Corrective Action**
• Contamination of negative control cells by preceding cell or positive control.	• When washing do not allow wells to overflow.
• Contamination of negative control vial.	• Check pipette barrel for residual fluid or dried material. Remove if present. • Use new pipette tip for each sample. • Always format negative control wells before positive control. • Re-run with fresh reagents.
• Insufficient washing.	• Make sure cells are completely filled. While washing ensure residual conjugate is removed from well. • Pipette all specimen and reagents in the center bottom of the microcell. Avoid contact with inner wall and rim. • Re-wash.
• Wash buffer prepared incorrectly or not freshly prepared.	• Rerun the assay with fresh wash buffer. • Check the pH of the distilled water used for the preparation of wash buffer.

2. Overall high RLU values	
Possible Causes	**Corrective Action**
• Insufficient wash step.	• Repeat wash step. Ensure wells are filled completely but do not overflow.
• Contamination of reagents.	• Check pipette barrel for residual fluid or dried material. Remove if present. • Check that pipette tips are long enough to provide air space between top of tip and the barrel. • Use new pipette tip for each sample. • Always format negative control wells before positive control. • Re-run with fresh reagents.
• Wash buffer prepared incorrectly or buffer not freshly prepared.	• Rerun the assay with fresh wash. • Check the pH of the distilled water used for the preparation of wash buffer.

3. Low RLU values for positive control	
Possible Causes	**Corrective Action**
• Reagent not at room temperature.	• Make certain all kit components are at R.T. (20 – 25°C).
• Test volume low.	• Ensure pipette tips are fitted correctly/ tightly. • Check pipette barrels for obstructions. • Check calibration of pipettes.
• Substrate A & B not freshly prepared or incorrectly prepared.	• Prepare substrate immediately before use. • Follow working reagent preparation chart.
• Contamination of signal reagent.	• Re-run the assay with fresh reagents.
• Incubation time too short.	• Check calibration of timers. • Record time of incubation.
• Moisture in pouches.	• Check whether desiccant in pouch is in working condition. • Seal unused wells in pouches. • Date pouches when first opened.
• Improper incubation temperature.	• Check incubator temperature/ Room Temperature (20 – 25°C).
• Room temperature too low for substrate incubation.	• Check temperature of the working area.

3. Low RLU values for positive control	
Possible Causes	**Corrective Action**
• Washing step too vigorous.	• Reduce pressure in wash system.
• Reagent not mixed before using.	• Mix the reagents before use.
• Cells allowed to dry after assay has started.	• Complete all assay steps without interruption.
• Insufficient tracer concentrate added in preparing working stock.	• Prepare conjugate accurately. • Follow working reagent preparation as described by the manufacturer.

4. Substrate "A" developing yellowish or brownish coloration	
Possible Causes	**Corrective Action**
• The substrate is contaminated.	• Obtain fresh Substrate "A".

5. Poor assay reproducibility	
Possible Causes	**Corrective Action**
• Incorrect volume of reagent(s) added. Insufficient wash step.	• Be sure that hand wash technique is consistent. If using an automatic plate washer, perform regular Maintenance to ensure the machine is running properly and is clean.
• Incubation temperature.	• Try to maintain a consistent environment especially in regards to temperature. Refer to protocol for optimum reaction conditions.
• Protocol Variations.	• Be sure to follow the written protocol as closely as possible to limit the variation that may be introduced by the environment, equipment, and technician.
• Dispensing/ pipetting error.	• Ensure pipette tips are fitted correctly / tightly. • Check pipette barrels for obstructions. • Check calibration of pipettes.
• Improper wash step.	• Ensure all wash equipment is working properly and wells are filled completely but do not overflow.

6. Edge Effect	
Possible Causes	**Corrective Action**
• Incubation chamber providing uneven temperature.	• Maintain a consistent environment around the reaction vessel paying close attention to the temperature.

7. Assay drift	
Possible Causes	**Corrective Action**
• Interruption(s) during assay set-up.	• Set-up of assays should be free of interruptions and be done in the shortest time possible while maintaining good technique. To prevent any delays/ interruptions while setting up an assay prepare all reagents and samples prior to beginning.
• Reagents/samples are not at room temperature.	• Dispense samples/reagents only when all are at room temperature to prevent variation of temperature throughout the plate wells.

8. Automation results are different than manual method	
Possible Causes	**Corrective Action**
• Automation application is not up to.	• Set-up of assays should be free of interruptions and be done in the shortest time possible while maintaining good technique. To prevent any delays/interruptions while setting up an assay prepare all reagents and samples prior to beginning.
• Reagents/samples are not at room temperature.	• Dispense samples/reagents only when all are at room temperature to prevent variation of temperature throughout the plate wells.

TIPS FOR GOOD LABORATORY TECHNIQUES

Light reaction plates

The Light reaction plates are the platform on which the assay reaction proceeds so the correct type of plate is vital. Do not open and use any plate until it has reached room temperature. Once the pouch is opened, date the package, remove desired amount of strips, and seal remaining strips in the dated microplate pouch. Place the strips in the correct holder and place on a level surface for the remaining steps in assay setup. The strips should be inspected for any defects.

Micro-pipetting

The volume used in assays is very small so it is extremely important to calibrate the pipettes accurately and in accordance with the time frame suggested by the manufacturer. In addition to calibration of the pipetting instrument, be sure to use the correct tips required for the specific pipette; the tips should consistently draw/dispense the same volume and fit properly on the end of the pipette. To prevent contamination of any reagents used, change the pipette tip between dispensing of different samples/reagents. Further precaution can be taken by transferring only the amount of a sample/reagent needed to a separate container before dispensing.

Washing

The washing step is performed in order to remove the excess reagents from the well that now contains bound components. This step can either be done manually or with an automatic plate washer. The end result should be the same irrespective of the wash method used. The wash solution should flood each well 3-5 times and left to soak for 5-30 seconds between each wash, depending on the assay and the equipment being used. Many factors are taken into consideration when a wash solution's components are chosen so be sure to follow the suggested preparation. Here are some additional things to consider depending on the wash method of choice:

Manual – Fill each strip with the same volume of washing solution and keep the time it takes for each strip to be washed the same. No air bubbles should be trapped in the wells and the wells should not be overflowed with wash solution.

Plate Washers – Program the desired wash cycles and soak times for reproducible wash steps. Perform regular maintenance on the washers to ensure accurate dispensing volumes and that the Washing heads are clean.

Substrate

Always check the substrate for any coloration prior to use. Incubation time of the signal a vital point in the assay and time constraints should be kept according to those indicated in the protocol.

Reading environment

To ensure reproducible results run all assays in a stable environment. An ideal stable temperature is one with little to no air drafts. In tropical or cold climates, it is recommended that labs regulate the room temperature (20 - 25 °C) to ensure proper reactions. Vibrations/rotations will also affect the test so these should be avoided. The surrounding environment is not the only of concern; the immediate environment contained in the plate holder is also critical. All plates, samples/controls, conjugates, and other reagents should only be used when at room temperature. This will minimize any variations in temperature across the plate. The main goal is to keep the environment as stable and consistent as possible.

CARDIAC MARKERS

- **Cardiac Troponin I (cTnI)**
  - New generation cardiac marker of choice.....Pgs 165-181
- **CK and its isoenzymes**
  -The time tested biomarker for diagnosis and monitoring of MI.....Pgs 182-195

KNOWLEDGE BOOK

For the Laboratorians

Cardiac Troponin I (cTnI)

New generation cardiac marker of choice

Setting Trends...

Introduction

Coronary artery disease (CAD) is the most important cause of morbidity and mortality in the industrialized world. In western countries approximately 15 million people are affected by heart failure. The W.H.O.-MONICA project on myocardial infarction and coronary death in 38 populations from 21 countries in 4 continents revealed that with regards to both morbidity and mortality significant regional differences exist. The risk of coronary artery disease is significantly higher in Northern Europe than in Central and Southern Europe. This is the so-called **'French Paradox'** (low rate of coronary artery disease with high caloric nutrition), which can be best explained by differences in drinking, eating patterns and genetic factors. The risk of coronary artery disease is especially high in Eastern Europe, whereas Canada, USA and Australia show a midlevel risk. Also the risk of CAD is comparatively higher in males as compared to females.

In the more recently reported SHARE study the overall prevalence of coronary artery disease was 10.7% among South Asians as against 4.6% in Europeans and 1.7% in Chinese population. Projections based on global burden of disease estimate that by year 2020, the burden of atherothrombotic cardiovascular disease in India would surpass that in any other region in the world.

Hence during preselection of patients for further cardiological examinations sensitive and specific laboratory tests can play an important role in diagnosing acute and chronic heart diseases.

Triaging of patients with or without AMI - A diagnostic challenge

According to W.H.O. criteria, diagnosis of Acute Myocardial infarction (AMI) is based on the detection of at least two out of three infarction specific findings:

- Chest pain > 20 minutes, resistant to nitro derivatives.
- Infarction specific ECG changes (ST segment elevation, development of abnormal Q wave) in at least two leads of the standard 12 lead ECG within the same vascular area.
- Serial enzyme changes (cardiac markers) with initial rise and subsequent reduction in level of concentration.

The triaging of patients presenting in emergency department is the major diagnostic challenge the physicians face today.

If an ECG reveals ST segment elevation or abnormal Q wave, the probability of acute myocardial infarction is high and further management is well established.

However, the sensitivity of ECG may be as low as 50% and even less in patients with an evolving myocardial infarction i.e. in Non-ST segment elevated myocardial infarction and Non-Q wave myocardial infarction. Though these patients may present with chest pain because of the absence of diagnostically specific ECG changes they could be discharged undiagnosed.

On the other hand, many patients with symptoms of chest pain suffer from severe unstable angina pectoris. Also in these patients ECG changes may be less specific and fail to provide conclusive diagnostic information.

Besides a subset of patients with absence of chest pain but with myocardial infarction i.e. silent infarction is especially common in diabetic patients. With advancing age the course of AMI is often atypical (without chest pain) but presenting with shortness of breath.

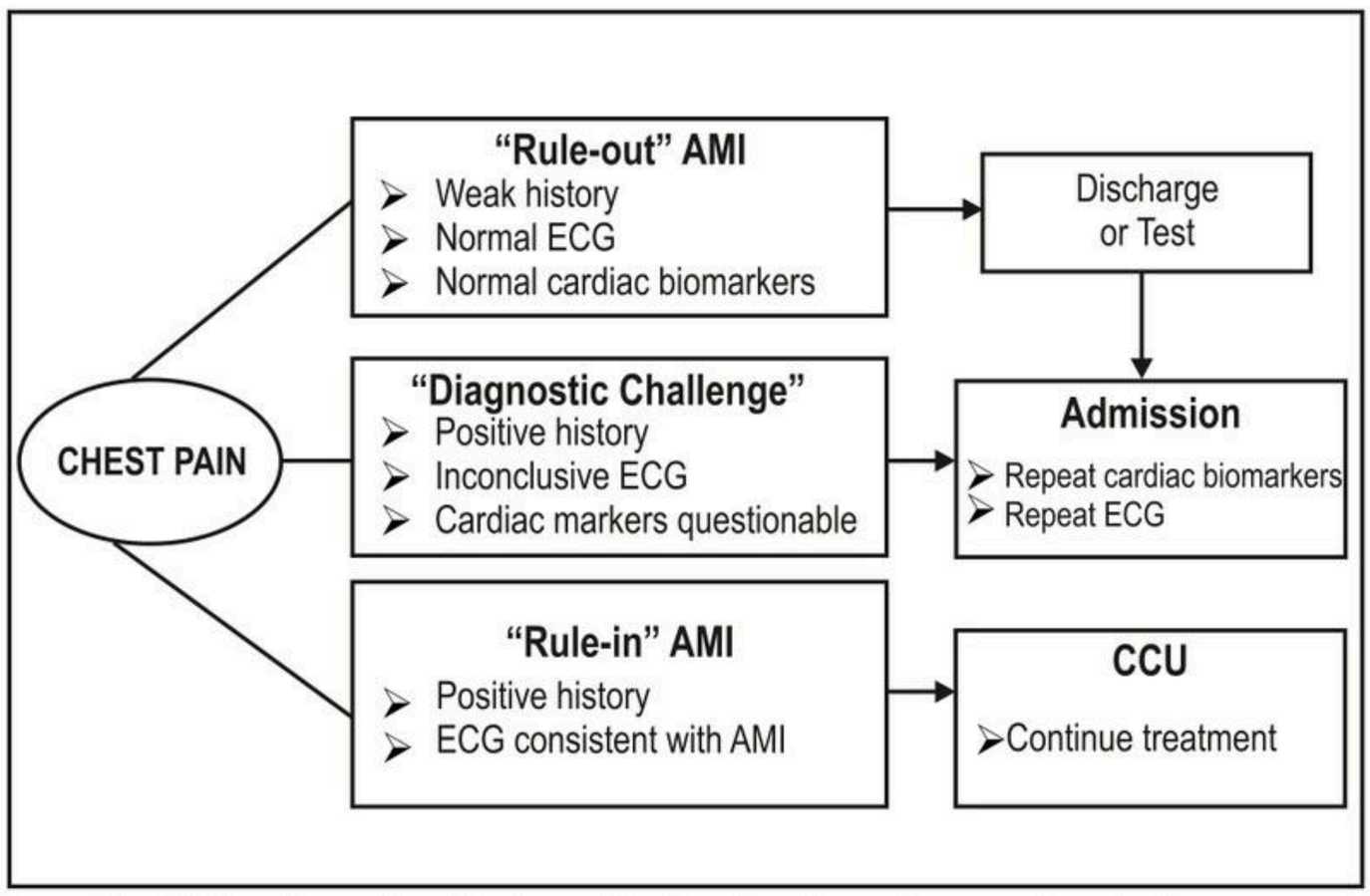


Fig.1:Triaging of patients with chest pain-a diagnostic challenge

Importance of accurate triaging

Coronary ischaemia is the root cause of acute myocardial infarction, hence early and reliable detection of myocardial ischaemia is a prerequisite for appropriate triage decision in emergency room so as to initiate the right therapy. It has been observed that only 10-15% of patients presenting in emergency room with the cardinal symptom of chest pain develop acute myocardial infarction. **Hence early diagnosis of AMI has to be so sensitive that all suitable patients with myocardial infarction can be treated with thrombolytic therapy and yet so specific that patients with chest pain but without myocardial infarction are not unnecessarily exposed to the risks of such therapy.** This is the most important decision the clinicians have to take when the patients present in the emergency rooms.

Role of cardiac markers

Following are the important applications of cardiac markers for management of patients with acute coronary syndrome:

- Confirm the diagnosis of AMI in the presence of diagnostically specific ECG changes.
- Diagnosis of AMI in the absence of unequivocal ECG changes (NSTEMI and Non-Q wave MI).
- Identification of high-risk patients with unstable angina pectoris in the absence of unequivocal ECG changes.
- For monitoring patients with AMI undergoing thrombolytic therapy i.e. success of therapy for reperfusion.

From a clinical point of view, an ideal cardiac marker that detects myocardial injury should satisfy the following properties:

- It should be present in myocardium in high concentration and absent in other tissues thereby ensuring high cardiac specificity.
- It should be released rapidly in blood stream after myocardial injury, so as to achieve optimal sensitivity in early phase after the onset of myocardial injury.
- It should remain abnormal for several days thereby offering wide diagnostic window time.
- It should be assayed with a rapid turnaround time.

Cardiac Markers

Creatine Kinase (CK) / Creatine Kinase MB (CK-MB) activity

Three different isoenzymes of CK exist namely CK-MM, CK-MB and CK-BB. Skeletal muscle approximately consists of CK-MM (97-99%) and CK-MB (1-3%). The cardiac muscle approximately contains CK-MM (95%) and CK-MB (5%). CK-BB is found primarily in brain and contributes very little to the total CK level.

After myocardial infarction CK and CK-MB levels rise after 2-6 hours; peak levels are observed at 12-24 hours. CK returns to normal levels after 3-4 days where as CK-MB because of shorter half-life returns to normal level after 2-3 days.

The calculation of CK-MB/CK ratio improves the specificity of CK-MB for acute myocardial infarction in patients accompanying with skeletal muscle damage. CK-MB returns to normal levels within 2-3 days after myocardial infarction, hence it is useful in detecting reinfarction.

Limitation:

Though CK-MB/CK ratio improves specificity of CK-MB, however small myocardial necrosis may be missed (unstable angina pectoris may show the presence of micro infarcts / minor myocardial injury).

CK-MB isoforms

In an attempt to improve sensitivity of CK-MB, high voltage electrophoresis technique was developed to separate CK-MB into its two isoforms: CK-MB2 and CK-MB1. In serum of healthy individuals CK-MB2/CK-MB1 ratio of approximately 1 is present. The reference range of this ratio is 1.5. A higher ratio indicates acute myocardial infarction.

Limitation:

- This technique is labour intensive.
- Requires high level of technical skill.
- Long delay in reporting of results.

CK-MB mass

Here CK-MB is detected immunologically by using a combination of CK-B and CK-M specific monoclonal antibodies or with CK-MB specific monoclonal antibodies.

Limitation:

- Interference in these assays is observed because of CK-MM, CK-BB, and CK-B autoantibodies.

CK-MB immunoinhibition method

The theoretical basis for the clinical application of immunoinhibition method is the assumption that only CK-MM and CK-MB are released into the blood stream after muscle damage. The reagent contains anti CK-M antibodies, which completely inhibit all CK-M activity i.e. both M subunits in CK-MM and the single M subunit in CK-MB. The remaining non CK-M activity corresponding to the CK-B activity of CK-MB is measured. Since only CK-B of the dimeric CK-MB molecule is measured, multiplication by a factor of 2 gives the CK-MB activity in the specimen.

Limitation:

- In case of macro CK, which contains no CK-M subunits immunoinhibition cannot take place.

Common limitations of CK-MB assays:

- As CK-MB is also present in skeletal muscle it is not absolutely specific to cardiac muscle damage.
- Evaluation of CK-MB levels may present problems in conditions such as extensive skeletal muscle injury with small infarction, chronic skeletal muscle injury and myocardial infarction after coronary artery bypass graft.
- Determination of CK and CK-MB activity alone is not suitable for assessment of risk in patients with unstable angina pectoris (minor myocardial damage).

Lactate Dehydrogenase

Lactate Dehydrogenase is also an enzyme released by ischaemic heart muscle. Out of the 5 isoenzymes only two of them LD1 and LD2 are useful in the diagnosis of AMI. Usually in normal healthy individuals the amount of LD2 in blood is higher than LD1 but patients with AMI show more of LD1 than LD2.

Limitation:

- LD1 and LD2 are not cardiospecific markers
- Elevated levels of LD1 and LD2 are observed in leukemia, renal and hemolytic diseases.

Myoglobin

Myoglobin, the oxygen binding haem protein constitutes about 2% in both skeletal and cardiac muscle. The low molecular weight of Myoglobin (17.8 kDa) facilitates its rapid release in circulation and is the first marker to exhibit rising levels after AMI. The advantages of Myoglobin in early diagnosis of myocardial infarction are its high early sensitivity and the possibility of rapidly assessing the success of thrombolytic therapy.

Limitation:

- Since Myoglobin is also present in skeletal muscle it is not a cardiospecific marker.
- The extremely short biological half life (10-20 minutes) restricts the usage of Myoglobin to detect unstable angina pectoris (minor myocardial injury or micro infarcts).

Cardiac Marker	M.Wt (kDa)	Half life (hours)	Increase (hours)	Peak* (hours)	Normalization (days)
LD - 1	135	110	6 - 12	48 - 144	7 - 14
CK	86	17	3 - 12	12 - 24	3 - 4
CK-MB	86	13	3 - 12	12 - 24	2 - 3
CK-MB mass	86	13	2 - 6	12 - 24	3
Myoglobin	17.8	0.25	2 - 6	6 - 12	1

* Strongly dependent on the timing of reperfusion of the infarct-related blood vessel

Fig.2: Typical characteristics of cardiac markers

Cardiac Marker	Hours after onset of pain		
	0 - 2	3 - 4	5 - 6
CK activity	15	35	70
CK-MB activity	10	25	55
CK-MB mass	30	70	90
CK-MB isoform ratio	25	60	90
Myoglobin	35	80	95

Fig.3: Average diagnostic sensitivities (%) of cardiac markers during early phase of AMI

Cardiac Troponins (cTn)-emerging cardiac marker of choice

Limitations of existing cardiac markers led to the search for markers uniquely expressed by the myocardium. The cardiac troponins T and I (cTnT and cTnI) have excellent sensitivity and specificity and are superior to CK-MB in indicating minor myocardial injury.

The advent of cardiac Troponins T and I, unarguably the most sensitive and specific markers encompass all the requirements that physicians and laboratarians require for accurate triaging and better risk stratification of patients with acute coronary syndrome.

Role of Troponins in muscle contraction

The contractile apparatus of striated muscle fiber is composed of thick and thin filaments. The thick filament is composed mainly of myosin. Actin, tropomyosin and Troponin comprise of thin filament. Muscle contraction occurs when thick and thin filament slide past each other. The interaction

between thick and thin filament is regulated by Troponin complex found on thin filaments. The Troponin complex is composed of three protein subunits: Troponin I (TnI), Troponin T (TnT) and Troponin C (TnC). The calcium-mediated contraction of striated muscle (fast-skeletal, slow-skeletal and cardiac muscle) is regulated by the Troponin complex. Contraction of smooth muscle is regulated by calmodulin (intracellular protein that combines with calcium and is involved in smooth muscle contraction).

Troponins are proteins that are integral to the functioning of striated muscle. They exist as a complex with actin and tropomyosin on thin filament of the contractile apparatus. The Troponin complex consists of three protein subunits:

- Troponin C, binds with calcium and regulates activation of thin filaments during contraction.
- Troponin T, binds the Troponin complex to tropomyosin.
- Troponin I, prevents the contraction of muscle in the absence of calcium and Troponin C.

During the functioning of the contractile apparatus depolarization of muscle leads to intracellular release of calcium, which binds with Troponin C. A conformational change occurs in Troponin-Tropomyosin complex in such a way that actin molecules can then interact with myosin, resulting in muscle contraction.

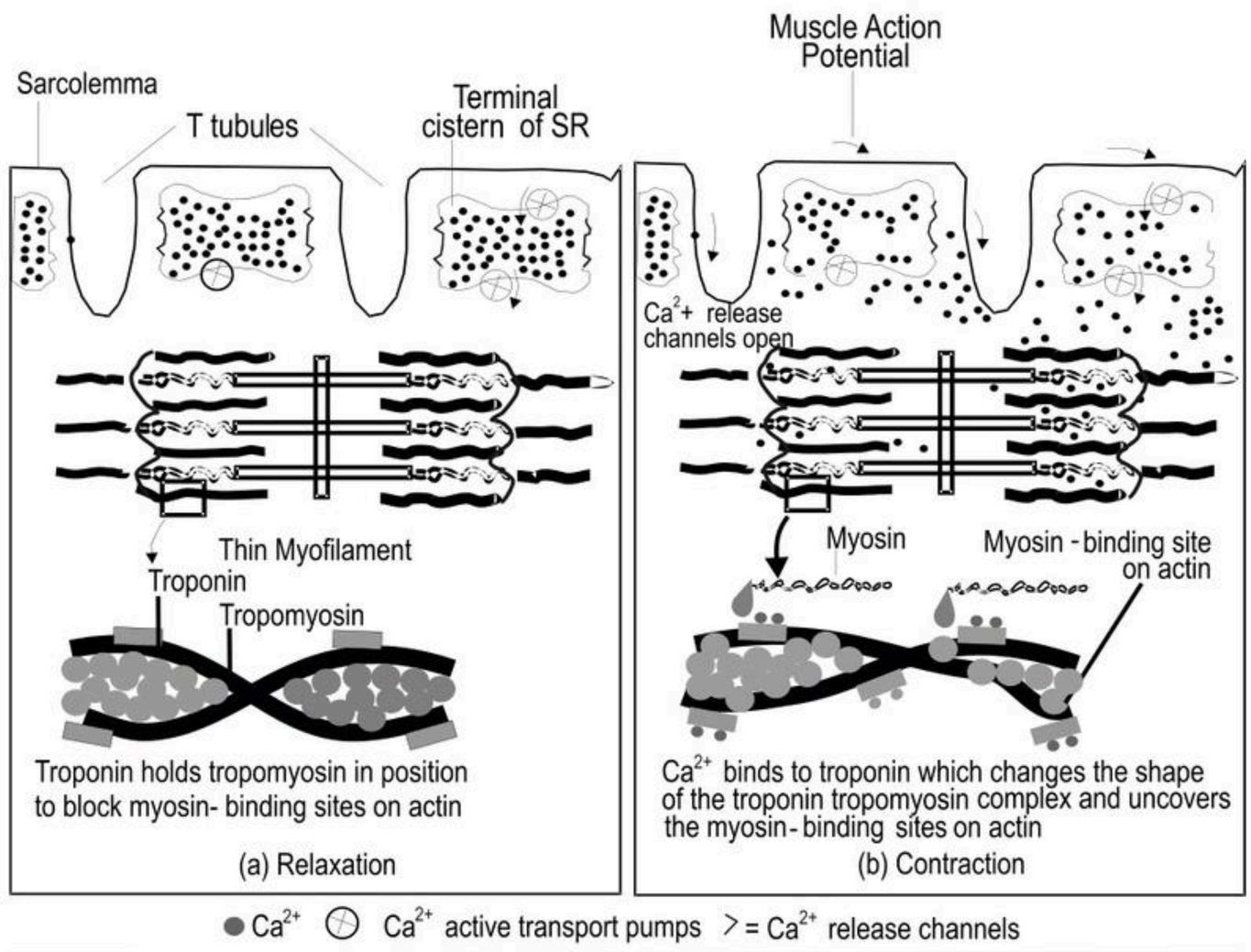


Fig.4 : Diagram of muscle contraction depicting Troponin involvement

Types of Cardiac Troponins

- Troponin C exists as two isoforms, fast and slow. The fast isoform is found only in skeletal muscle, but the slow isoform is found both in skeletal and cardiac muscles. The molecular weight of cardiac isoform (cTnC) is 18 kDa.

- Troponin T is also found in fast and slow skeletal muscle, cardiac muscle. Troponin T present in skeletal muscle exists as a slightly different subform. The cardiac isoform (cTnT) has a molecular weight of 37 kDa.

- Three isoforms of Troponin I have been identified, one each in fast and slow skeletal muscles and one isoform in cardiac muscle. The cardiac isoform of Troponin I (cTnI) has a molecular weight of 22.5kDa. cTnI has an extra 30 amino acid sequence at the N terminal portion of molecule making it absolutely specific to cardiac muscle. cTnI is mostly bound to contractile apparatus in myocardium, but about 8% is found free in cytoplasm.

Cardiac Troponin	M.Wt. (kDa)	Half Life (hours)	Increase (hours)	Peak* (hours)	Normalization (days)
cTnI	22.5	2 - 4	3 - 8	12 - 24	7 - 10
cTnT	37	2 - 4	3 - 8	12 - 96	7 - 14

* Strongly dependent on the timing of reperfusion of the infarct-related blood vessel

Fig.5: Characteristics of cardiac troponin (I and T) in AMI

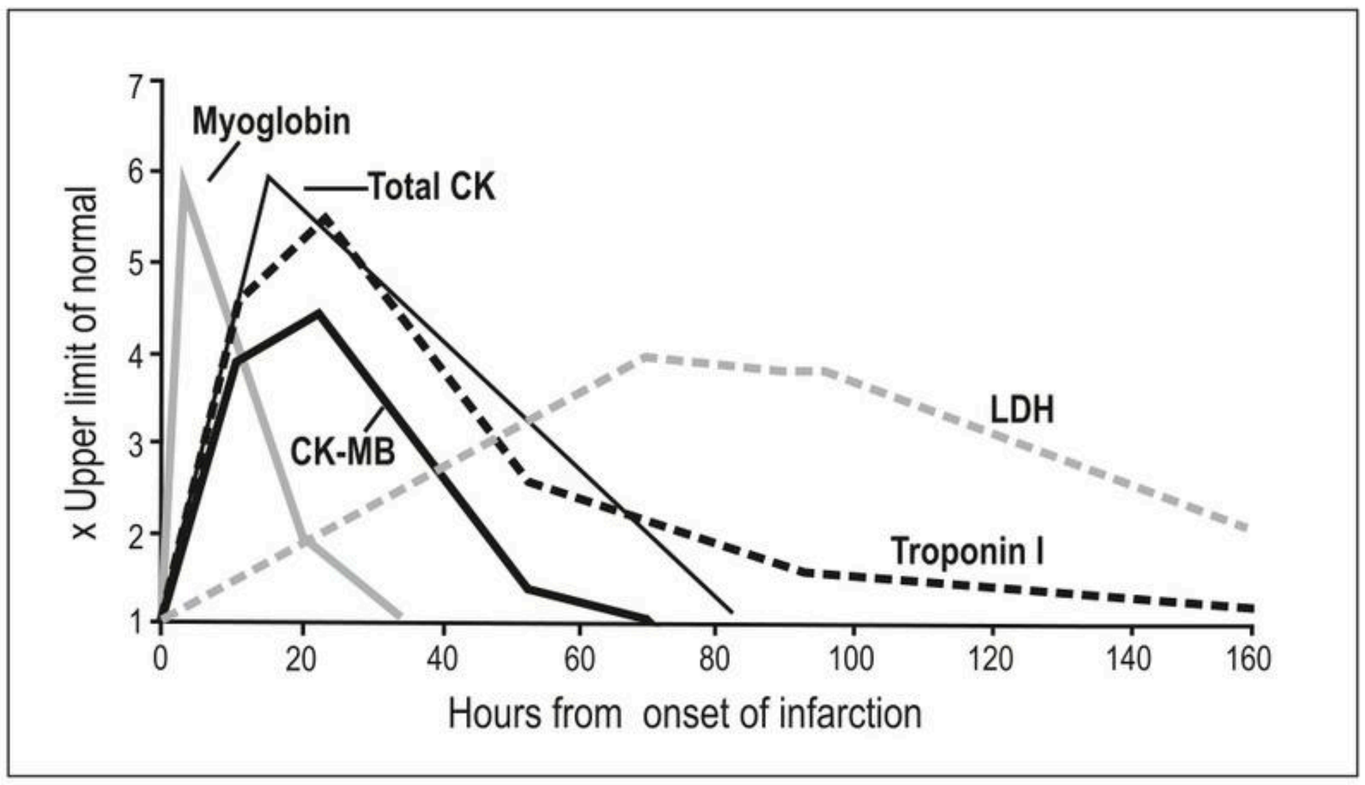


Fig.6: Graphical representation-Levels of cardiac markers in AMI

Cardiac Troponins (cTnI and cTnT) - sensitivity & specificity

Any damage or injury to myocardial cells results in the release of cardiac Troponins into the circulation. Concentration of cTnI and cTnT in the circulation initially increases with the number of hours after the onset of chest pain and decreases as the enzymes are cleared from the circulation. The most important take home message is that sensitivity of cardiac Troponin tests, like any other cardiac marker is dependant on the number of hours after the onset of chest pain.

Internationally a lot of scientific research work has been done to evaluate important parameters of sensitivity, specificity and predictive values of cTnI and cTnT in clinical settings.

The comparative data and evaluation report along with the study defined are summarized below.

- Sensitivity: Proportion of patients with AMI with abnormal cardiac Troponin test results.
- Specificity: Proportion of patients without AMI with normal cardiac Troponin test results.
- The positive and negative likelihood ratios were calculated using the following equations,
- Positive likelihood ratio=Sensitivity/(100-Specificity)
- Negative likelihood ratio=(100-Sensitivity)/Specificity

The positive and negative likelihood ratios correspond to the clinical concepts of ruling in and ruling out disease. A higher positive likelihood ratio means that a test result is better for ruling in the disease when test is positive. A lower negative likelihood ratio means that a test result is better for ruling out disease when the test result is negative.

Summary of data for cardiac Troponin T and I tests for diagnosing AMI

	Hours from Onset of Chest Pain	Sensitivity	Specificity	LR+	LR-
Cardiac Troponin T > 0.1ng/ml	1	0.47	0.87	3.7	0.6
	2	0.53	0.87	3.9	0.5
	3	0.58	0.86	4.1	0.5
	4	0.64	0.85	4.2	0.4
	6	0.74	0.83	4.4	0.3
Cardiac Troponin I > 0.1ng/ml	1	0.13	0.95	2.7	0.9
	2	0.34	0.95	6.8	0.7
	3	0.52	0.95	10	0.5
	4	0.67	0.95	13	0.34
	5	0.80	0.95	16	0.23
	6	0.90	0.95	18	0.1

Adapted from 'The Journal of Family Practice', 2000, 49:550-556

Fig.7: Summary of data for cardiacTroponin T and I Tests for Diagnosing AMI

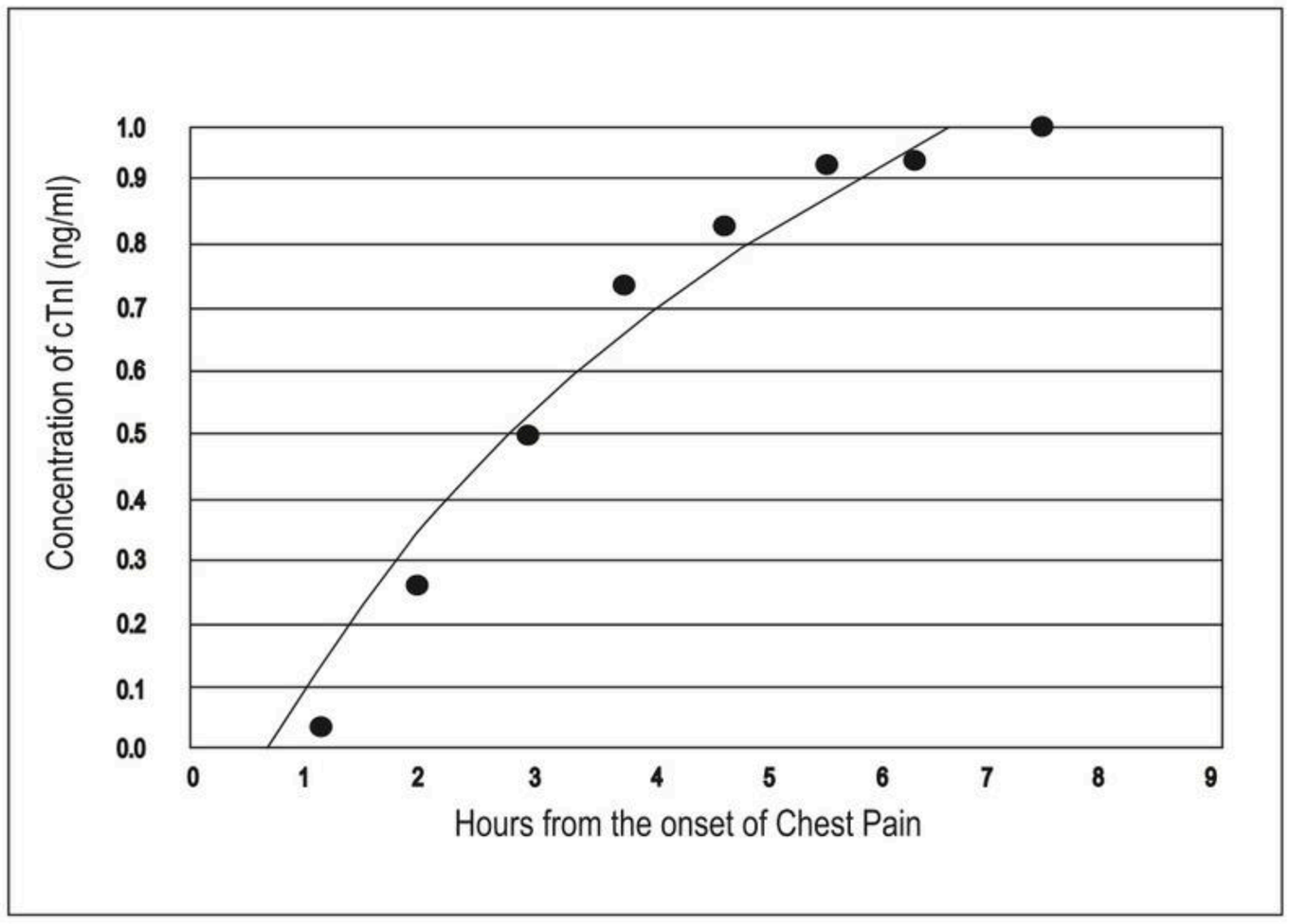


Fig.8: Sensitvity of cTnI viz a viz number of hours of onset of chest pain in AMI

Inferences from the study:

- Sensitivity of cardiac Troponin tests (cTnI and cTnT) is dependant on the number of hours since the onset of chest pain.
- **cTnI appears to be better at ruling in MI than cTnT. A positive cTnT value is only moderately useful at ruling in AMI at 6 hours from the onset of chest pain where as cTnI values appears to be very useful at ruling in AMI at 6 hours from onset of chest pain.**
- cTnI and cTnT are very useful at ruling out AMI when the value is negative at 10 or more hours from the onset of chest pain.
- A normal cTnT or cTnI level at 8 or more hours after the onset of chest pain is strong evidence against the presence of AMI.
- Abnormal values of cTnT and cTnI at 8 or more hours after the onset of chest pain are moderately strong evidence in favor of presence of AMI.

cTnI versus cTnT

The debate continues as to which of the two, cTnI or cTnT is better for management of patients with acute coronary syndromes. Since assays of cTnT were commercially available few years before cTnI, more peer-reviewed publications on the clinical utility of cTnT might have appeared in the past.

However, recent studies have questioned the diagnostic specificity of cTnT assays in patients with myocardial injury and chronic renal failure, muscular dystrophies and skeletal muscle damage. cTnI indeed scores over cTnT in the specificity aspect because cTnI is the only Troponin I expressed in myocardial cells during postnatal development. cTnI is not expressed in normal skeletal muscle at any time including,during postnatal development.

Thus cTnI determination promises higher diagnostic efficacy because of the following unique characteristics,

- Wide diagnostic window time with early appearance and prolonged presence in circulation.
- Allows detection of minor myocardial injury because cTnI levels is almost absent in normal healthy individuals.
- No cross reactivity with skeletal muscle isoforms
- Virtually absent in skeletal muscle tissue.

Inferences from international reports highlighting the superior diagnostic efficacy of cTnI

- `cTnI values of less than 0.4 ng/ml are associated with a 42 day mortality of 1% and this risk increases progressively to a mortality of 7.5% at values of 9.0 ng/ml or more. Patients presenting with cardiac chest pain and ECG changes can be classified as Troponin positive or negative acute coronary syndromes, with consequent prognostic and therapeutic implications'.
  -BMJ, 2002; 34.

- `The current study demonstrates that ACS patients who have increased cTnI measured on a point of care whole blood assay show a significant increase in risk over 30-180 days for all cause death, cardiac death and cardiac events in the presence or absence of ST-elevation. These findings add to the evidence based metaanalyses which demonstrates that increased cTnI predicts the risk of adverse outcomes in ACS'.
  - Clinical Chemistry, 2002; 48.

- 'Cardiac Troponin T, but not cardiac Troponin I, has been found to be re expressed in skeletal muscles of patients with renal failure and muscular dystrophy. cTnI appears to be more specific than cTnT, with discordant results more often being cTnT(+) /cTnI(-). cTnI is more specific for cardiac injury in settings of renal and muscle disease.'
  - The Journal of Emergency Medicine, Vol.23, Jan. 2002.

- 'In patients with clinically documented acute coronary syndrome who are treated with glycoprotein IIb/IIIa inhibitors, even small elevations in cTnI identify high risk patients who derive a large clinical benefit from an early invasive strategy'.
  - JAMA, Vol. 286 No.19, November 21, 2001.

- 'The prognostic power of cTnI testing in combination with ECG improves efficiency of low risk patient management and improved patient risk stratification. This study adds to the evidence favoring cTnI evaluation as part of the management of acute coronary syndromes'.
  - Q. J. Med. 2001; 94.

- 'In the management of acute coronary syndromes and acute MI in clinical practice, cTnI is comparable in diagnostic and prognostic efficacy to cTnT. In renal impairment even against second generation cTnT assays, cTnI is superior'.
  - Heart, 2000; 83.

- 'Early studies have questioned the clinical specificity of cTnT assays in patients with chronic renal failure. With the development of second generation assay for cTnT, the frequency of positive results in these patients is lower than first generation, although still higher than for cTnI'.
  - Clinical Chemistry, 45 No.7, 1999.

- 'The goal of this prospective study was to assess whether cTnI could replace CK-MB mass as the serum biomarker for detection of AMI. Findings have strongly supported our clinical implementation of cTnI, replacing CK-MB mass as the preferred marker for detection of AMI'.
  - American Heart Journal, 1999 Feb.; 137 (2).

- 'The first generation of cTnT assay lacked absolute specificity for the cardiac isoform and allowed interference by skeletal muscle Troponin T. However, cTnT is still detectable in many cases of end stage renal failure, to a lesser extent than first generation but to an extent far more frequent than cTnI'.
  - Clinical Chemistry, 44:7, 1998.

- 'The cTnI determination is expected to reveal absolute myocardial specificity because cTnI is not expressed in fetal and healthy or diseased adult human skeletal muscle tissue, promising no false positive test results in patients with substantial skeletal muscle damage or renal failure. In conclusion the use of cTnI rapid device could improve efficacy and safety of decision making in patients with chest pain that might produce more cost effective use of intensive care facilities'.
  - Clinical Chemistry, 44, No.9, 1998.

- 'Questions about the cardiac specificity of cTnT remain. Some subjects with musculoskeletal or renal disease have elevated levels of cTnT, thus this marker may not be as sensitive as cTnI for detection of myocardial injury'.
  - American Journal of Critical Care, Vol.7, No.6, Nov.1998.

- 'cTnI is part of a new generation of biochemical markers that provide an additional clinical tool for assessment of acute coronary syndromes, a term that describes the continuum of myocardial injury ranging from angina, or so called reversible ischaemia to Q-wave MI and definite tissue necrosis. Studies have indicated that cTnI is a more specific marker in cases involving skeletal muscle injury and renal failure. Therefore cTnI may have an important role in real time strategies for evaluating acute coronary syndrome patients, an area that has been of intense interest, discussion and study over recent years'.
  - Clinical Chemistry, 44:1, 1998.

- `Routine use of cTnI bed side test in the emergency room improves decision making and is highly cost effective. cTnI values provide additional prognostic information over and above the level of critical illness in patients presenting to the emergency room'.
  - CAP, February 1998.

- `The slightly higher sensitivity of cTnI test as compared with the cTnT may be related to different release kinetics. The findings of false positive results for cTnT but not cTnI, in patients with renal failure may, however, represent a true difference between the two test'.
  - The New England Journal of Medicine, Dec.4, 1997

- 'In conclusion, the results of our prospective study provide evidence that cTnI is an indicator of adverse outcome in patients with severe unstable angina. Our results are keeping in mind with the recent retrospective analysis of patients enrolled in the TIMI III B study. The use of cTnI in the immediate triage of patients with unstable angina appears warranted to identify those at greater risk for cardiac events'.
  - Circulation, 1997; 95.

- 'The study compared the diagnostic accuracy of measurement of serum cTnI with CK-MB mass in patients with minor myocardial injury whose measured total CK activity did not exceed twice the upper reference limit. The clinical sensitivity of myocardial injury for cTnI was 100% compared with 81.8% for CK-MB. Thus, cTnI was more sensitive than CK-MB mass for detection of myocardial injury in patients with small increases of total CK'.
  - Clinical Chemistry, 1997; 43.

- 'cTnI was as sensitive and specific for AMI as was CK-MB in ED patients who presented within 24 hours of symptom onset. However, cTnI was more sensitive in patients who presented at 24 hours after symptom onset'.
  - Academic Emergency Medicine, Vol.4, 1997.

- 'cTnI could replace CK-MB and would facilitate the rapid and effective triage of patients with chest pain in the emergency department'.
  - American Journal of Clinical Pathology, 1997 Nov., 108 (5).

- 'In patients with acute coronary syndromes, cardiac Troponin I levels provide useful prognostic information and permit early identification of patients with an increased risk of death'.
  - The New England Journal of Medicine, Vol. 335, No.18, 1996.

- `Elevations of cTnI are highly specific for myocardial injury. Use of cTnI should facilitate distinguishing whether elevations of CK-MB are due to myocardial or skeletal muscle injury'.
  - Circulation, Vol.88, 1993.

cTnI - Cut off levels

The National academy of Clinical Biochemistry (NACB), USA and International Federation of Clinical Chemistry (IFCC), Germany have recommended the use of two decision cut off limits for cardiac Troponins. A low limit that establishes the presence of myocardial injury and a high limit that establishes injury to the extent that qualifies as AMI.

Based on literature data and clinical assessments cTnI levels greater than 0.1 ng/ml places a patient with unstable angina in the high-risk category for short-term risk of death or non-fatal MI. The cut off for the definition of AMI is taken to be greater than 1.2 ng/ml. Thus cTnI levels with a cut off of 0.1 ng/ml identify patients at higher risk for very early adverse outcomes.

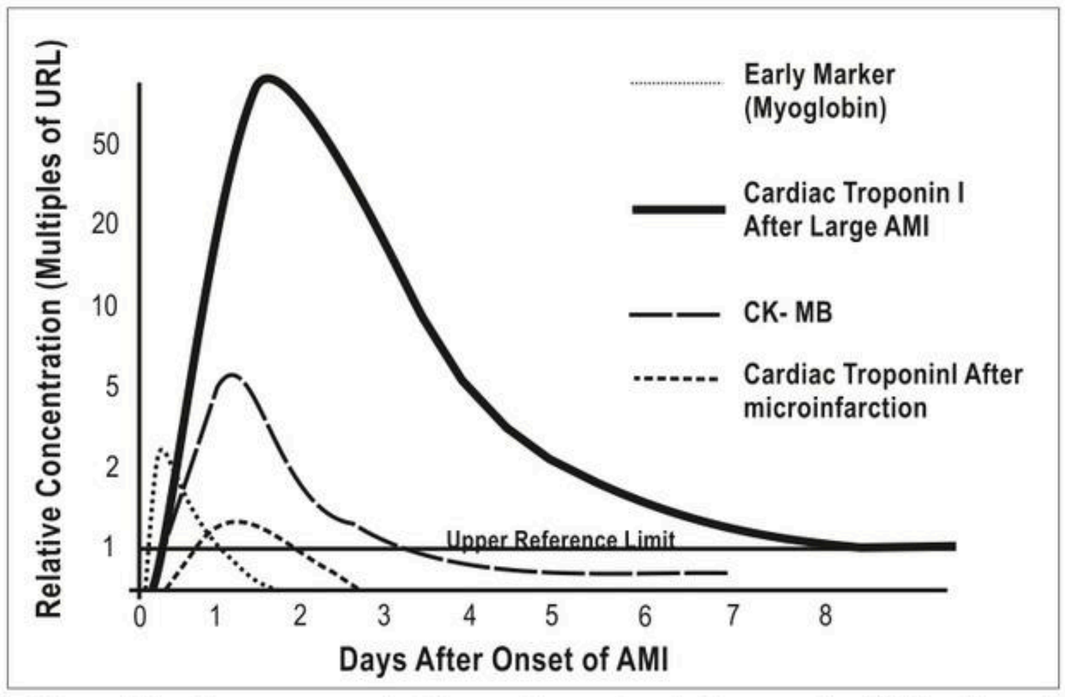


Fig.9: Graphical representation - two decision cut off limits of cTnI

cTn I - Standardization

In 1998 the American Association for Clinical Chemistry (AACC) appointed a troponin I Standardization Subcommittee to address the critical need for standardization between different cardiac Troponin I tests. Working with diagnostic companies and National Institute of Standards (NIST) this group was performing complex technical studies that involved cardiac Troponin I calibration materials from several sources to help improve the clinical utility of this very important test by identifying a single reference material that all assays can use to assign cardiac troponin I values.

AACC Troponin I Standardization Subcommittee recently conducted a second round robin study. Liquid native Troponin I-C-T complex from Hytest has been selected as committee's choice of cTnI reference standard. The Standard Reference material - 2921, issued in April, 2004 is now available widely for assay standardization.

Applications of cTnI test

Following are the important applications of cTnI test in management of patients with acute coronary syndrome:

- Patient with ECG specific findings and elevated cTnI test confirms the diagnosis of AMI.

- An elevated cTnI level is of immense diagnostic value in patients with symptoms of chest pain, absence of diagnostically significant ECG change and normal CK-MB level. cTnI test value 1 ng/ml classifies these patients under NSTEMI. A negative cTnI test classifies the patient as UAP.

- Also elevated cTnI value provides prognostic value in identifying patients with unstable angina pectoris. Symptoms of chest pain, absence of diagnostically significant ECG change, normal CK-MB level and cTnI value >0.1 ng/ml are classified under UAP. If the test is negative retesting probably at 12 hours after post onset of chest pain is important to rule out diagnosis of acute coronary syndrome.

- Patients presenting with chest pain after trauma or surgery and elevated CK-MB assay value (to rule out true elevation of CK-MB).

- Patients presenting with chest pain 2 to 6 days prior to admission may have sustained acute myocardial infarction but CK-MB would have returned to normal levels.

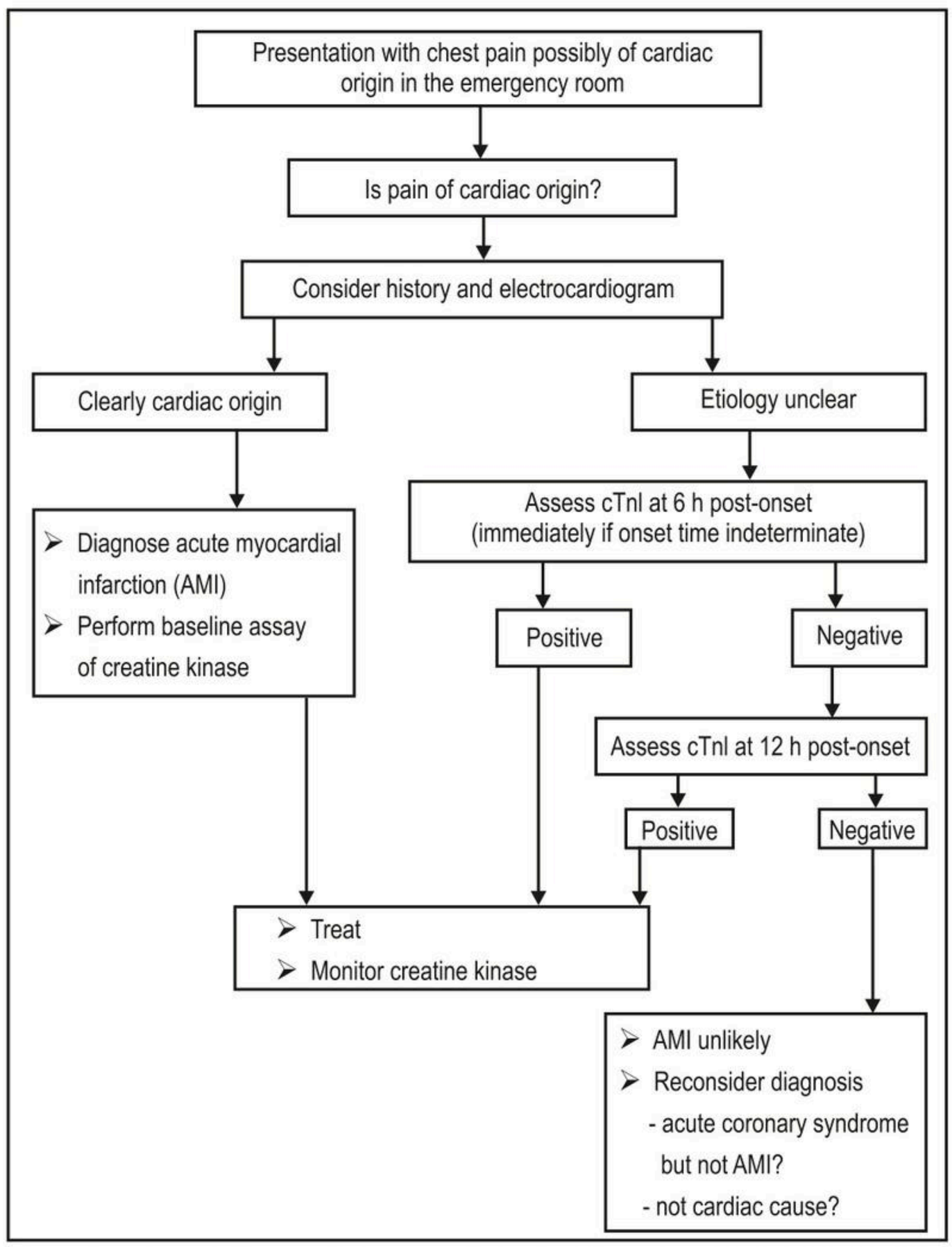
Superior diagnostic efficacy of cTnI over CK-MB in detecting microinfarcts

Patients with normal CK-MB levels and elevated cTnI levels could be attributed probably to the low sensitivity and specificity of CK-MB in detecting micro infarction. Since CK-MB is present in skeletal muscle and normal healthy individuals, diagnostic cut off values are typically set above the upper limit of reference range for CK-MB assay. Cardiac troponin I is not present in normal healthy individuals and is approximately 13 times more abundant in myocardium than CK-MB on a weight basis. Hence the signal to noise ratio (increased sensitivity) associated with cTnI is more favorable for detection of micro infarction (NSTEMI and UAP).

Limitations

- cTnI levels remain elevated for about 7 days hence for serial monitoring of patients undergoing thrombolytic therapy cardiac markers such as CK-MB and Myoglobin may be used for successful reperfusion. Also in cases of reinfarction markers with shorter half-life such as CK-MB should be used for accurate diagnosis.

- Since cardiac Troponins (both cTnI and cTnT) are sensitive markers for myocardial damage, they are detected in many other cardiac conditions such as acute pericarditis, acute myocarditis, congestive heart failure (CHF), perioperative myocardial infarction and cardiac contusion



(Adapted from: Troponin testing: an audit in three metropolitan hospitals, Richard X Davey, MJA, Vol.179, 21 July 2003)

Fig.10 :Protocol for use of cardiac troponin I test

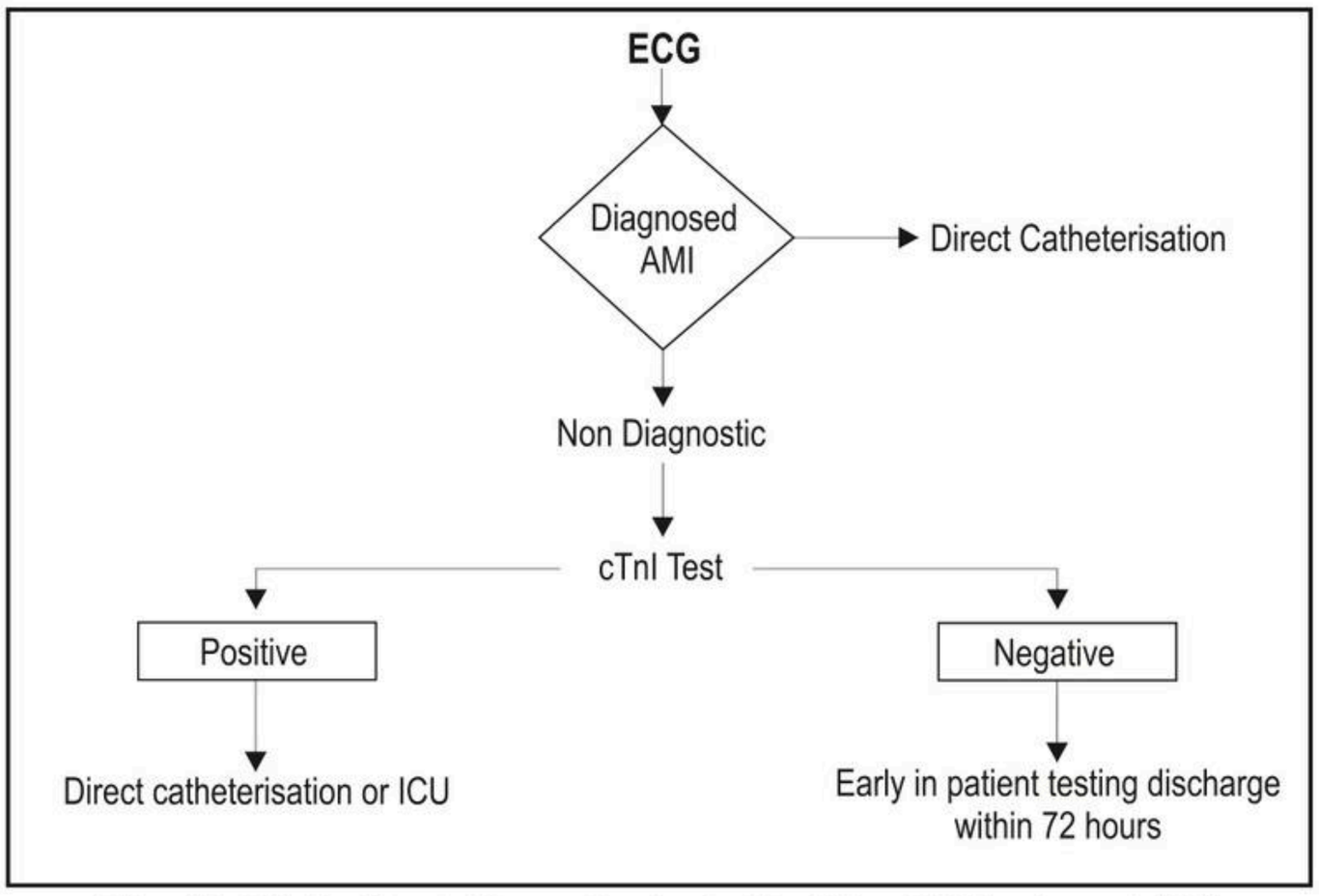


Fig. 11: Clinically stable patient admitted for UAP/ rule out AMI

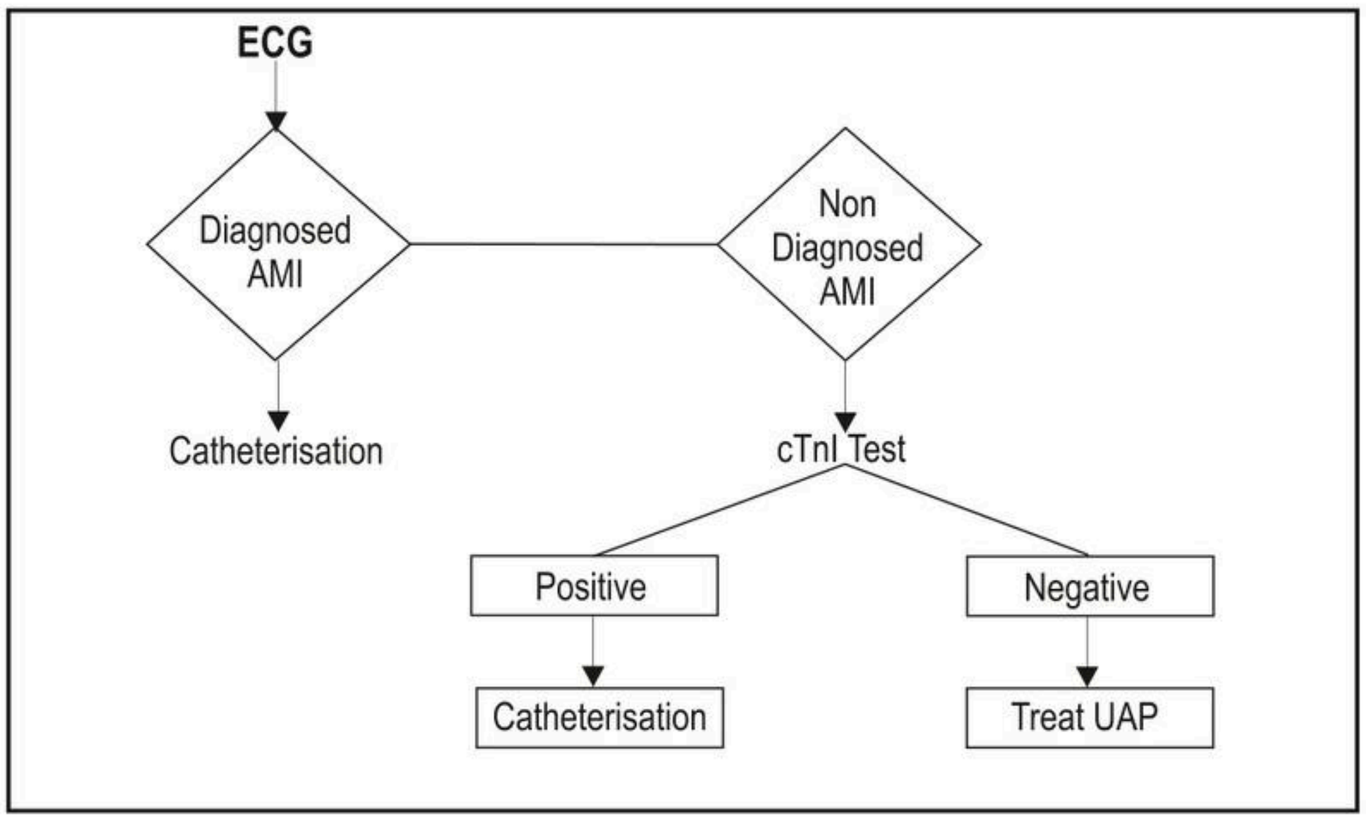


Fig. 12: Patient with chest pain: UAP or AMI?

Critical parameters for correct usage of cTnI test

Rising levels of cardiac markers are dependant on time elapsed since the onset of myocardial necrosis. This is true regardless of whichever cardiac marker being used for ruling in and ruling out AMI. Since patients present at varying times for testing following the onset of chest pain in a cardiac event, **it is necessary to perform sequential testing of cTnI levels for optimal diagnostic accuracy.**

Following are the important points to be considered regarding cTnI test when triaging patients for acute coronary syndrome:

- First test must be done preferably within onset of symptoms at baseline or within 3 hours of onset of chest pain along with ECG test. A single cTnI negative test within 3 hours cannot safely rule out AMI.
- If the first test is negative, second test for cTnI must be done at 6 hours from the onset of chest pain accompanied by ECG test and if negative should be repeated between 6-12 hours after the onset of chest pain. A third test at 8-9 hours after onset of chest pain represents the best time for assessment of cTnI level and is the most predictive for ruling in or ruling out acute coronary syndrome.
- If 6 hour cTnI test and ECG is normal it is unlikely that the patient will have an adverse outcome in the next 30 days (1% chance).
- An elevated level of cTnI (positive cTnI test) in patients with normal ECG, but with UAP or NSTEMI or Non Q wave MI identifies patient group at greater risk of death.
- Any elevated level of cTnI is indicative of an increased short-term risk of death or nonfatal myocardial infarction.
- The half life of cTnI is approximately 2-4 hours hence samples should be tested preferably immediately. In case of delay in testing the samples may be tested within 2 hours of blood collection.

ANNEXURE-I

The heart and how it works

The normal human heart is a strong muscular pump little larger than a fist. Each day an average heart beats 1,00,000 times and pumps about 2000 gallons of blood. In a 70-year lifetime, an average human heart beats more than 2.5 billion times.

The heart pumps blood continuously through the circulatory system. The circulatory system is the network of elastic blood vessels that carries blood throughout the body. The circulatory system comprises of heart, arteries, arterioles, veins and capillaries. The arteries are the blood vessels which carry oxygen and nutrient rich blood to all parts of the body. The veins and capillaries are the blood vessels that carry oxygen and nutrient depleted blood back to heart and lungs. If all these blood vessels were laid end to end they would extend for about 60,000 miles.

The circulating blood brings oxygen and nutrients to all the body's organ and tissues, including the heart. It also picks up waste products from the body's cells. These waste products are removed as they are filtered through the kidney, liver and lungs.

Structure of heart

Heart is a hollow, muscular, contractile organ, and the center of circulatory system. It provides the propulsive force for circulating blood throughout the vascular system. The heart wall is composed of three layers, the outer epicardium, a serous layer, the middle myocardium, composed of cardiac muscle, and the inner endocardium, a layer that lines the four chambers of the heart and covers the valves. The heart is enclosed in a fibrous sac, the pericardium. The space between pericardium and the epicardium is the pericardial cavity.

Heart has four chambers through which blood is pumped. The upper two are the right and left atria. The lower two are right and left ventricles. Four valves open and close only in one direction when the heart beats.

- The tricuspid valve is between the right atrium and right ventricle.
- The pulmonary valve is between the right ventricle and the pulmonary artery.
- The mitrial valve is between the left atrium and left ventricle.
- The aortic valve is between the left ventricle and aorta.

Each valve has a set of flaps, also known as leaflets or cusps. The mitral valve has two flaps. The other valves have three flaps. Under normal conditions, these valves allow blood flow in one direction. Blood flow occurs only when there is a difference in pressure across the valves that cause them to open.

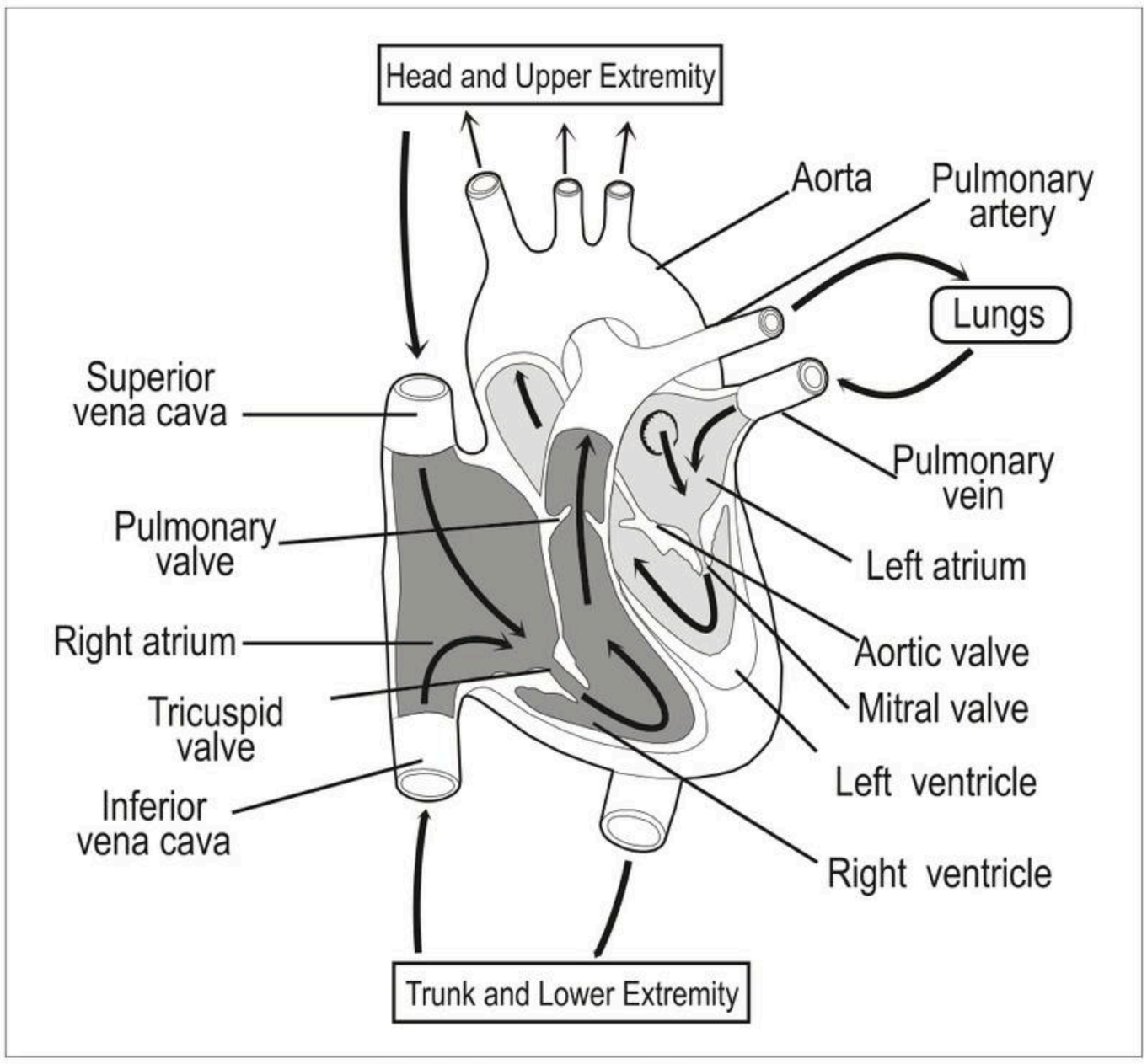


Fig.1: Structure of heart

ANNEXURE-II

Atherosclerosis: major cause of cardio vascular disease

Atherosclerosis comes from the Greek word athero (meaning gruel or paste) and sclerosis (meaning hardness). The inner lining of blood vessels namely the arteries contains deposits of fatty substances, cholesterol, cellular waste products and calcium. This build up is known as plaque.

Plaque formation results in luminal obstruction, abnormalities of blood flow, diminished oxygen supply to target organs.

Atherosclerosis begins with damage to innermost layer of blood vessels namely the endothelium. The probable cause of endothelial injury includes oxidized LDL cholesterol, by products of cigarette smoking, hyperglycemia and hyperhomocystinaemia.

Circulating monocytes infiltrate the intima of the vessel wall and the tissue macrophages act as scavenger cells forming the characteristic foam cell of early atherosclerosis. These activated macrophages produce numerous factors that are injurious to endothelium.

Injury to endothelium leads to increased platelet adhesion, increased tissue factor release, increased plasminogen activator inhibitor, decreased plasminogen activator, decreased thrombomodulin and alterations in heparan sulfate. Thus the sequence of events results in procoagulant milieu and enhanced thrombus or clot formation.

Atherosclerotic plaques characteristically occur in regions of branching and marked curvature at areas of geometric irregularity and where blood undergoes sudden changes in velocity and direction of flow. Decreased shear stress and turbulence may promote atherogenesis at these important sites within the coronary arteries, the major branches of the thoracic and abdominal aorta and vessels of lower extremities of our body.

Generally plaques are static, but they can also become unstable and rupture. Those that rupture can initiate thrombus formation and can totally block blood flow (occlusion) in the artery. A blood clot that breaks off and travels to another part of the body is known as emboli.

If a clot blocks a blood vessel, that supplies blood to heart it causes heart attack. If it blocks a blood vessel supplying blood to brain, it causes stroke. And if blood supply to the arms or legs is reduced, it can cause gangrene.

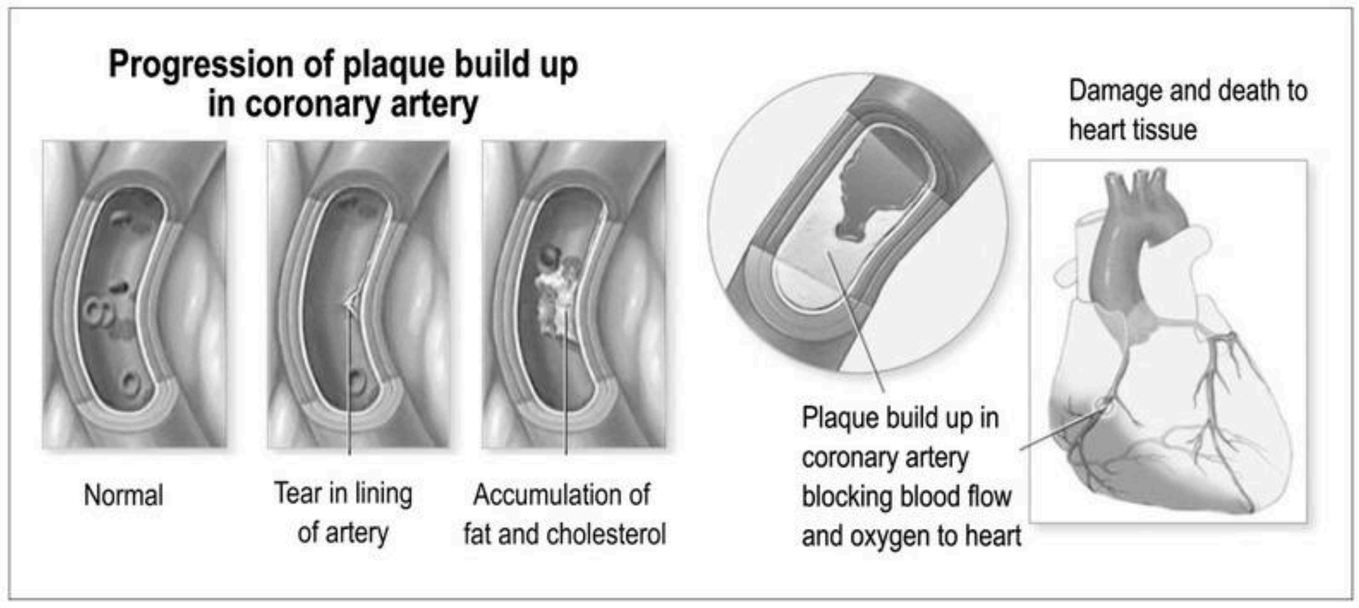


Fig.2: Depiction of Atherosclerosis

Atherosclerosis - Risk factors

Epidemiological studies indicates the following risk factors that potentiate atherosclerosis,

- Hyperlipidaemia
- Hypertension
- Cigarette habituation
- Diabetes mellitus
- Age
- Sex

Signs and symptoms associated with atherosclerosis

The signs and symptoms of atherosclerosis are highly variable, but mainly present as follows:

- Unstable angina pectoris
- Acute Myocardial Infarction
- Transient ischaemic attack
- Stroke
- Peripheral vascular disease
- Mesenteric angina
- Abdominal aortic aneurysm
- Atheroembolism

Risk Factors	Signs
Hyperlipidaemia	Xanthelasma, Tendon Xanthomata
Coronary artery disease	Fourth heart sound, Tachycardia, Hypotension, Hypertension
Cerebrovascular disease	Diminished carotid pulses, carotid artery bruits, Focal neurological deficits
Peripheral vascular disease	Decreased peripheral pulses, peripheral artery bruits, pallor, peripheral cyanosis, gangrene, ulceration
Abdominal aortic aneurysm	Pulsatile abdominal mass, peripheral embolism, circulatory collapse
Atheroembolism	Livedo reticularis, gangrene, cyanosis, ulceration

Fig4.: Risk factors and associated signs in Atherosclerosis

Angina pectoris

Angina pectoris is the medical term for chest pain or discomfort due to coronary heart disease. Angina is a symptom of condition known as myocardial ischaemia. It occurs when the heart muscle (myocardium) is deprived of required amount of blood it needs for performing normal function. Myocardial ischaemia (insufficient blood supply to myocardium) occurs due to narrowing or occlusion (blockage) of one or more arteries that supply blood to heart.

Typical angina is characterized by uncomfortable pressure, fullness, squeezing pain in center of chest. The discomfort also may be felt in the neck, jaw, and shoulder.

Angina is a sign that someone is at high risk of heart attack, cardiac arrest and sudden cardiac death.

Stable angina pectoris

People with stable angina pectoris have episodes of chest pain that are usually predictable. The chest pain episode occurs with exertion or mental or emotional stress. Normally the chest discomfort is relieved with rest and/or sublingual nitroglycerin administration.

Unstable angina pectoris

People with unstable angina pectoris have unexpected chest pain that usually occurs while even at rest. The discomfort may be more severe and prolonged than typical angina.

People with unstable angina pectoris should be treated as an emergency because they are at increased risk for acute myocardial infarction, severe cardiac arrhythmias and cardiac arrest leading to sudden death.

Variant angina pectoris (prinzmetal angina pectoris)

Unlike typical angina, it nearly always occurs when a person is at rest. It does not follow a period of physical exertion or emotional stress. Attacks can be very painful and usually occur between midnight and 8 a.m. morning.

Ischaemic Heart Disease

Ischaemia is a condition where the flow of blood, and therefore oxygen, to a part of body is restricted. Cardiac ischaemia refers to lack of blood flow and oxygen to heart muscle.

Ischaemic heart disease refers to heart problems caused by narrowing down of arteries that supply blood to heart. When arteries are narrowed, less blood and oxygen reaches the heart. This condition is also known as coronary artery disease or coronary heart disease.

Acute Myocardial infarction

Acute myocardial infarction is defined as death or necrosis of myocardial cells. It is the end diagnosis of myocardial ischaemia. Myocardial infarction occurs when myocardial ischaemia exceeds a critical threshold and overwhelms myocardial cellular repair mechanisms that are designed to maintain normal operating function.

Critical myocardial ischaemia may occur as a result of increased myocardial metabolic demand, decreased supply of oxygen and nutrients to myocardium via the coronary circulation. An interruption in the supply of myocardial oxygen and nutrients occurs when a thrombus is superimposed on an unstable atherosclerotic plaque and results in coronary occlusion. Conditions associated with increased myocardial metabolic demand include extremes of physical exertion, severe hypertension and severe aortic valve stenosis.

Mechanism of Myocardial damage

The severity of myocardial infarction is dependant on three factors:

- Level of occlusion in coronary artery
- Length of time of occlusion
- Presence or absence of collateral circulation

Generally, more proximal the coronary occlusion, more extensive is the amount of myocardial necrosis. Larger the myocardial infarct, greater is the chance of death due to mechanical complication. Longer the time period of vessel occlusion, greater the chances of irreversible myocardial damage distal to the occlusion.

The death of myocardial cells first occurs in the area of myocardium that is most distal to arterial blood supply, the endocardium. As the duration of occlusion increases, the area of myocardial cell death enlarges, extending from the endocardium to the myocardium ultimately to the epicardium. Thus the extent of myocardial cell death defines the magnitude of myocardial infarction.

The human body often creates small blood vessels known as collaterals to help compensate for reduced blood flow. Collateral vessels normally are not open and normal healthy individuals do show the presence of collateral vessels but in microscopic form. But in people suffering from coronary artery disease or any other blood vessel disease these collateral vessels grow and enlarge. When a collateral vessel enlarges, it allows flow of blood from an open artery to either an adjacent artery or further downstream on the same artery. Thus collateral vessels grow and form a detour around a blocked blood vessel.

Types of Myocardial Infarction

Myocardial infarction can be subcategorized on the basis of anatomic, morphologic and diagnostic clinical information.

From an anatomic or morphologic standpoint, the two types of myocardial infarction are as follows,

Transmural Myocardial infarction:
In transmural myocardial infarction the ischaemic necrosis affects muscle segment extending from the endocardium through the myocardium to epicardium.

Non-Transmural Myocardial infarction:
In non-transmural myocardial infarction the area of ischaemic necrosis does not extend through the full thickness of myocardial wall segments. The area of ischaemic necrosis is limited to either endocardium or endocardium and myocardium. It is the endocardial and subendocardial zones of myocardial wall segment that are least perfused regions of heart and are most vulnerable to conditions of ischaemia.

An old sub classification of myocardial infarction based on clinical diagnostic criteria is determined by the presence or absence of Q wave on ECG. But a more accepted clinical diagnostic scheme based on ECG findings is the presence of ST segment elevation.

The presence of Q wave or ST segment elevation is associated with high early mortality and morbidity. However the absence of these two findings does not necessarily confer better long-term mortality and morbidity.

Signs and symptoms of AMI

Acute myocardial infarction may have unique presentations in individual patients. The degree of symptoms range from none to sudden cardiac death. Asymptomatic myocardial infarction is not necessarily less severe than a symptomatic event, but patients who experience asymptomatic myocardial infarction are more likely to be diabetic. Despite the diverse presenting symptoms of myocardial infarction, there are some characteristic typical symptoms,

- Chest pain described as pressure sensation, fullness or squeezing in the midportion of thorax
- Chest pain radiating to jaw, teeth, shoulder, arm and back
- Associated dyspnoea or shortness of breath
- Associated epigastric discomfort with or without nausea and vomiting
- Associated diaphoresis or sweating
- Syncope or near syncope without other cause

Acute myocardial infarction may occur at any time of the day, but most appear to be clustered around the early hours of morning and are associated with demanding physical activity. Approximately 50% of patients have some warning symptoms prior to infarction.

Diagnosis of Myocardial Infarction

W.H.O. criteria for diagnosis of AMI

Twenty years ago, W.H.O. defined the diagnosis of AMI as a triad, two of which atleast must be present for diagnosis,

- Typical history of severe and prolonged chest pain greater than 20 minutes resistant to nitroglycerin
- Unequivocal electrocardiographic changes, with ST segment elevation and development of abnormal Q wave.
- Serial enzyme changes (cardiac markers) with initial rise and subsequent fall of catalytic concentrations.

Electrocardiogram (ECG)

ECG is usually the first diagnostic test performed. Diagnostic specificity is approximately 100%, and a positive tracing, signaled by an elevated ST segment, essentially confirms diagnosis of AMI. The diagnostic sensitivity however has been estimated to range from 63-82%.

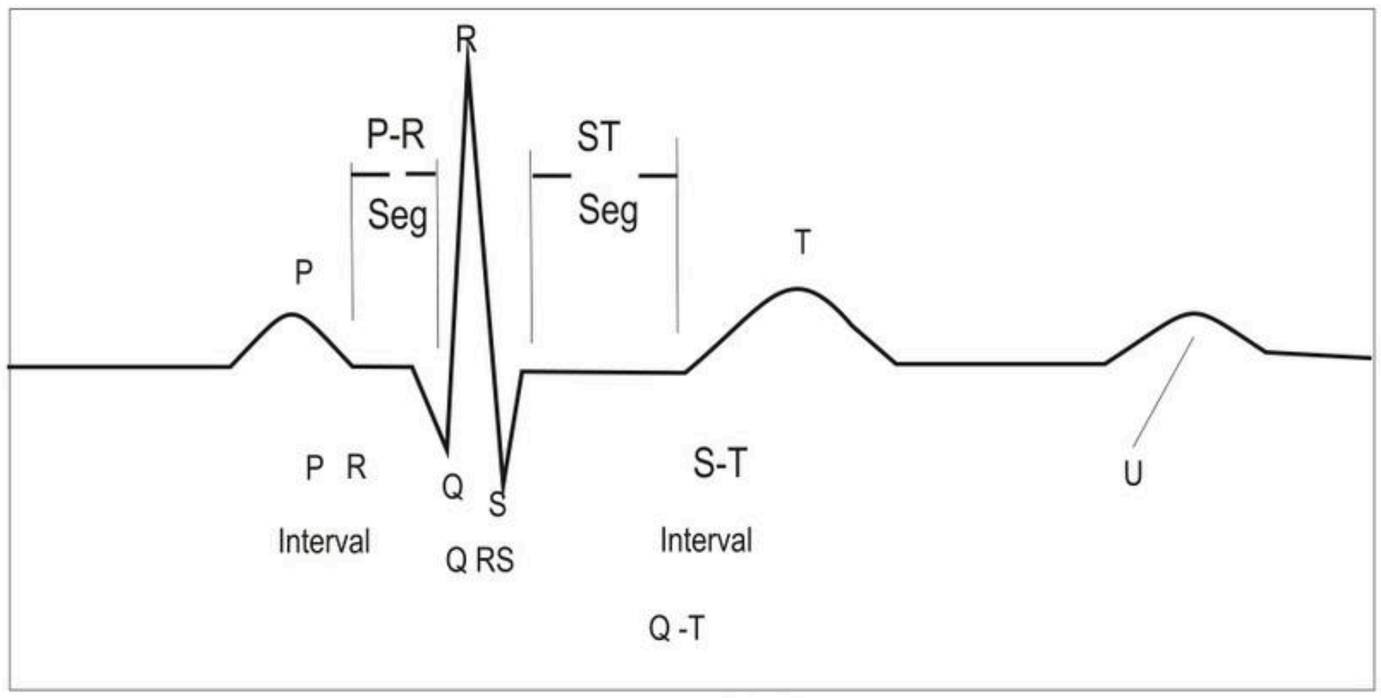


Fig.4: Normal ECG tracing

Fig.5: ST segment elevation ECG tracing in AMI

ECG tracings are indeterminate in a substantial fraction of patients with chest pain at rest but no ST segment elevation (Non ST segment elevation myocardial infarction - NSTEMI), severe unstable angina pectoris, non-Q wave myocardial infarction. Testing for elevated levels of serum cardiac markers that indicate myocardial necrosis when non Q-wave myocardial infarction or NSTEMI is present, usually makes the discrimination between these conditions.

Imaging

Imaging techniques have been used to assist in:

- Ruling out or confirming the presence of acute infarction or ischaemia
- Identifying non ischaemic conditions causing chest pain
- Identifying mechanical complications of acute infarction
- Defining short term and long term prognosis

Imaging methods that are used:

- Cross sectional echocardiography
- Radio nuclide angiography
- Myocardial single photon emission computed tomographic (SPECT) perfusion imaging

Radionuclide techniques enable the physician to assess perfusion at the time of patient presentation. This can be performed with immediate tracer injection because image acquisition can be delayed for 60 to 90 minutes. Quantitative analysis is an advantage of this technique, but accuracy of the studies is high when interpreted by skilled observers.

Some of the limitations of imaging being,

- Requirement of expensive equipment and skilled personnel
- Injury involving greater than 20% of myocardial wall thickness is required before an abnormality can be detected. Also greater than 10 g of myocardial tissue must be injured before a radionuclide perfusion defect can be resolved.

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KNOWLEDGE BOOK

For the Laboratorians

CK and its isoenzymes

The time tested biomarker for diagnosis and monitoring of MI

cardiac

MARKERS

Setting Trends...

INTRODUCTION

Myocardial Infarction (MI) is the gross necrosis of the myocardium due to interruption of blood supply to the area.

In over 90% of patients with acute MI, an acute thrombus, often associated with plaque rupture, occludes the arteries (usually it is already partially obstructed by an atherosclerotic plaque) that supply the damaged area. Altered platelet function induced by endothelial change in the atherosclerotic plaque presumably contributes to thrombogenesis. Spontaneous thrombolysis occurs in about two thirds of the patients so that, 24 hrs later, thrombotic occlusion is found in only about 30% of the patients.

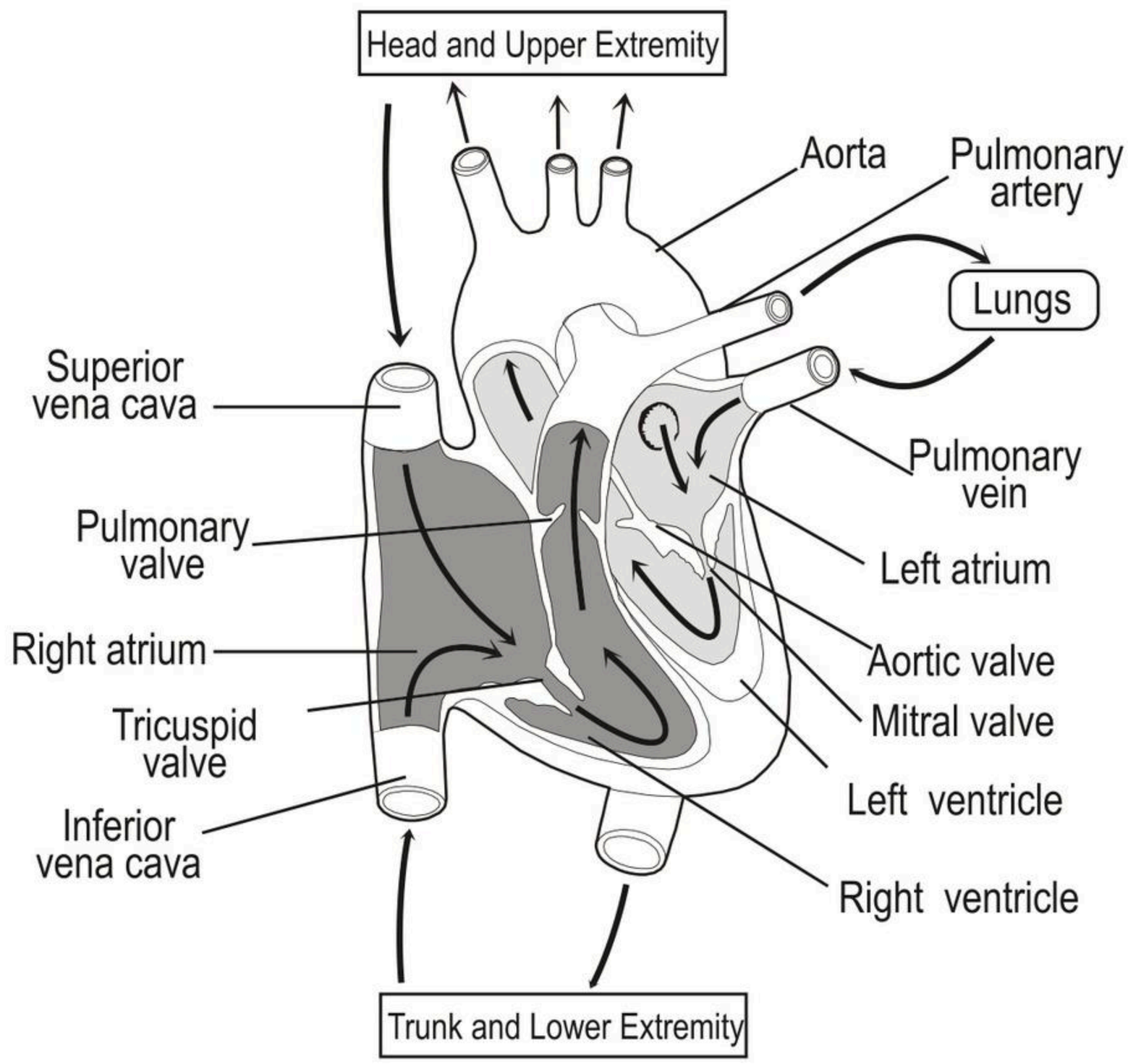
MI is rarely caused by arterial embolization for example in mitral or aortic stenosis, infective endocarditis, and marantic endocarditis. MI has been reported in patients with coronary spasm and otherwise normal coronary arteries. Cocaine causes intense coronary arterial spasm, and users may present with cocaine-induced angina or MI. Autopsy studies and coronary angiography have shown that cocaine-induced coronary thrombosis may occur in normal coronary arteries or be superimposed on pre-existing atheroma.

MI is predominantly a disease of the left ventricle (LV), but damage may extend into the right ventricle (RV) or the atria. RV infarction usually results from occlusion of the right coronary or a dominant left circumflex artery and is characterized by high RV filling pressure, often with severe tricuspid regurgitation and reduced cardiac output. Some degree of RV dysfunction occurs in about half of patients with an inferior-posterior infarction, producing hemodynamic abnormality in 10 to 15%. RV dysfunction should be considered in any patient with inferior-posterior infarction and elevated jugular venous pressure with hypotension or shock.

The ability of the heart to continue functioning as a pump relates directly to the extent of myocardial damage. Patients who die of cardiogenic shock usually have an infarct, or a combination of scar and new infarct, of about 50% of LV mass. Anterior infarcts tend to be larger and have a worse prognosis than inferior-posterior infarcts. They are usually due to occlusion in the left coronary arterial tree, especially the anterior descending artery, whereas inferior-posterior infarcts reflect right coronary occlusion or occlusion of a dominant left circumflex artery.

Transmural infarcts involve the whole thickness of myocardium from epicardium to endocardium and are usually characterized by abnormal Q waves on ECG. Nontransmural or subendocardial infarcts do not extend through the ventricular wall and cause only ST segment and T-wave abnormalities. Subendocardial infarcts usually involve the inner one third of the myocardium where wall tension is highest and myocardial blood flow is most vulnerable to circulatory changes. They may also follow prolonged hypotension. Because the transmural depth of necrosis cannot be precisely determined clinically, infarcts are better classified by ECG as Q wave and non-Q wave.

The Human Heart



CLINICAL PRESENTATION

It is accepted that the term MI reflects a loss of cardiac myocytes (necrosis) caused by prolonged ischemia. Ischemia is the result of a perfusion-dependent imbalance between supply and demand. Ischemia in a clinical setting can be identified from the patient's history and from the ECG. Possible ischemic symptoms include chest, epigastric, arm, wrist or jaw discomfort with exertion or at rest. The discomfort associated with acute MI usually lasts at least 20 min, but may be shorter in duration. The discomfort may develop in the central or left chest and then radiate to the arm, jaw, back or shoulder. The discomfort is usually not sharp or highly localized and may be associated with dyspnea, diaphoresis, nausea, vomiting or light-headedness. The discomfort can develop in the epigastrium, which is often confused with indigestion, arm, shoulder, wrist, jaw or back, without occurring in the chest, but such a pattern is atypical. The discomfort is not affected by moving the muscles of the region where the discomfort is localized, nor is it worsened by deep inspiration. The discomfort is not positional in nature. Symptoms can also include unexplained nausea and vomiting, persistent shortness of breath secondary to left ventricular failure and unexplained weak-ness, dizziness, lightheadedness or syncope, or a combination of these. These symptoms may be noted in association with chest discomfort or they may occur in the absence of chest symptoms. Although many patients have symptoms such as those just described, these complaints may go unrecognized or may be erroneously labeled as another disease entity, such as indigestion or a viral syndrome. Myocardial necrosis may also occur without symptoms.

DIAGNOSIS OF MI

Electrocardiogram (ECG)

An electrocardiogram is usually the first diagnostic test performed. Diagnostic specificity is approximately 100 percent, and a positive tracing, signalled by an elevated ST segment, essentially confirms a diagnosis of AMI. The diagnostic sensitivity, however, has been estimated to range from only 63-82 %. ECG tracings are therefore indeterminate in a substantial fraction of MI patients. One of the major advantages of ECG is that it allows assessment of most nonischemic causes of acute chest pain, such as perimyocarditis, valvular heart disease (aortic stenosis), pulmonary embolism and aortopathies (aortic dissection).

This sensitivity limitation necessitates reliance on biochemical serum cardiac markers for triaging patients with indeterminate ECGs.

Imaging

Imaging techniques have been used to assist in:

- Ruling out or confirming the presence of acute infarction or ischemia in the Emergency Departments
- Identifying nonischemic conditions causing chest pain
- Defining short- and long-term prognosis
- Identifying mechanical complications of acute infarction

The rationale of imaging using echocardiographic or nuclear techniques in patients suspected of having acute ischemia is that ischemia results in regional myocardial hypoperfusion, leading to a cascade of events that can include myocardial dysfunction and ultimately cell death. Some imaging methods are –

- Cross-sectional Echocardiography
- Radionuclide Angiography
- Myocardial Single Photon Emission Computed Tomographic (SPECT) perfusion imaging

Radionuclide techniques enable the physician to assess perfusion at the time of patient presentation; this can be performed with immediate tracer injection, because image acquisition can be delayed for 60 to 90 minutes. Quantitative analysis is an advantage of this technique. The accuracy of the studies is high when interpreted by skilled observers. These studies also provide simultaneous information on myocardial perfusion and function.

Biomarkers are more sensitive, more specific and less costly than imaging techniques for the diagnosis of myocardial necrosis. Injury involving >20% of myocardial wall thickness is required before a segmental wall motion abnormality can be detected by Echocardiography. In general, >10 g of myocardial tissue must be injured before a radionuclide perfusion defect can be resolved. Neither technique can distinguish ischemia from infarction.

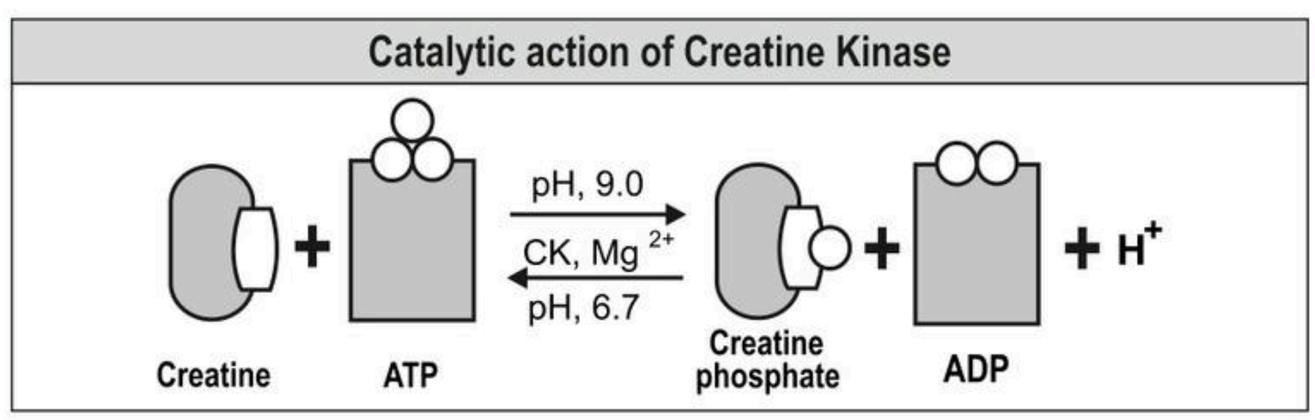
Thus the sensitivity limitation in ECG, requirement of expensive equipment and skilled personnel for imaging, necessitates reliance on biochemical serum cardiac markers for routine diagnosis and follow-up of MI.

Biochemical markers of myocardial necrosis

Myocardial necrosis results in and can be recognized by the appearance in the blood of different markers released into the circulation due to the damaged myocytes: Creatine Kinase (CK), Myoglobin, cardiac Troponin T and Troponin I, Lactate dehydrogenase (LDH), SGOT as well as many others. Myocardial infarction is diagnosed when blood levels of sensitive and specific biomarkers are increased in the clinical setting of acute ischemia. These biomarkers reflect myocardial damage but do not indicate its mechanism. Thus, an elevated value in the absence of clinical evidence of ischemia should prompt a search for other causes of cardiac damage, such as myocarditis.

CK and its isoenzymes – The time-tested biomarker

Creatine kinase (CK), also called as creatine phosphokinase (CPK), catalyses the reversible phosphorylation of creatine by ATP, as shown in the following equation:



The optimum pH values for the forward and reverse reactions are 9.0 and 6.7 respectively. The reverse reaction is 2-6 times faster than the forward reaction, depending upon the reaction conditions. Mg^{2+} is the activating ion. The optimal concentration range of Mg^{2+} is quite narrow, and excess Mg^{2+} is inhibitory. Several metal ions and other agents inhibit CK enzyme activity. Some common inhibitors are shown in following figure:

Some common inhibitors of CK catalytic reaction		
• Mn^{2+} ions	• Excess ADP	• Iodide
• Zn^{2+} ions	• Citrate	• Bromide
• Ca^{2+} ions	• Fluoride	• Chloride
• Cu^{2+} ions	• Nitrate	• Malonate
• Iodoacetate	• Sulphate	• L-thyroxine
• Sulfhydryl-binding reagents	• Acetate	• Urate
	• Adenylate Kinase enzyme	

Diagnostic applications of CK and its isoenzymes

- Suspected cardiac muscle disease in patients with clinical and ECG signs typical of myocardial infarction.
- In patients in whom an intervention is contraindicated.
- For evaluation of thrombolytic therapy.
- For risk stratifications in patients with angina.
- Myocarditis.
- Suspected skeletal muscle damage.
- Monitoring cardiac and skeletal muscle disease.

Special findings

Serum CK is particularly valuable for the following reasons:

- Serial total CK has sensitivity close to 100% early in course of Myocardial Infarction (MI).
- It allows early diagnosis because increased levels appear within 3-6 hours after onset of MI and reaches its peak at 24-36 hours.
- It is more sensitive indicator than other enzymes because an increased CK level shows a larger amplitude of change (6-12 times normal).

Isoforms of CK

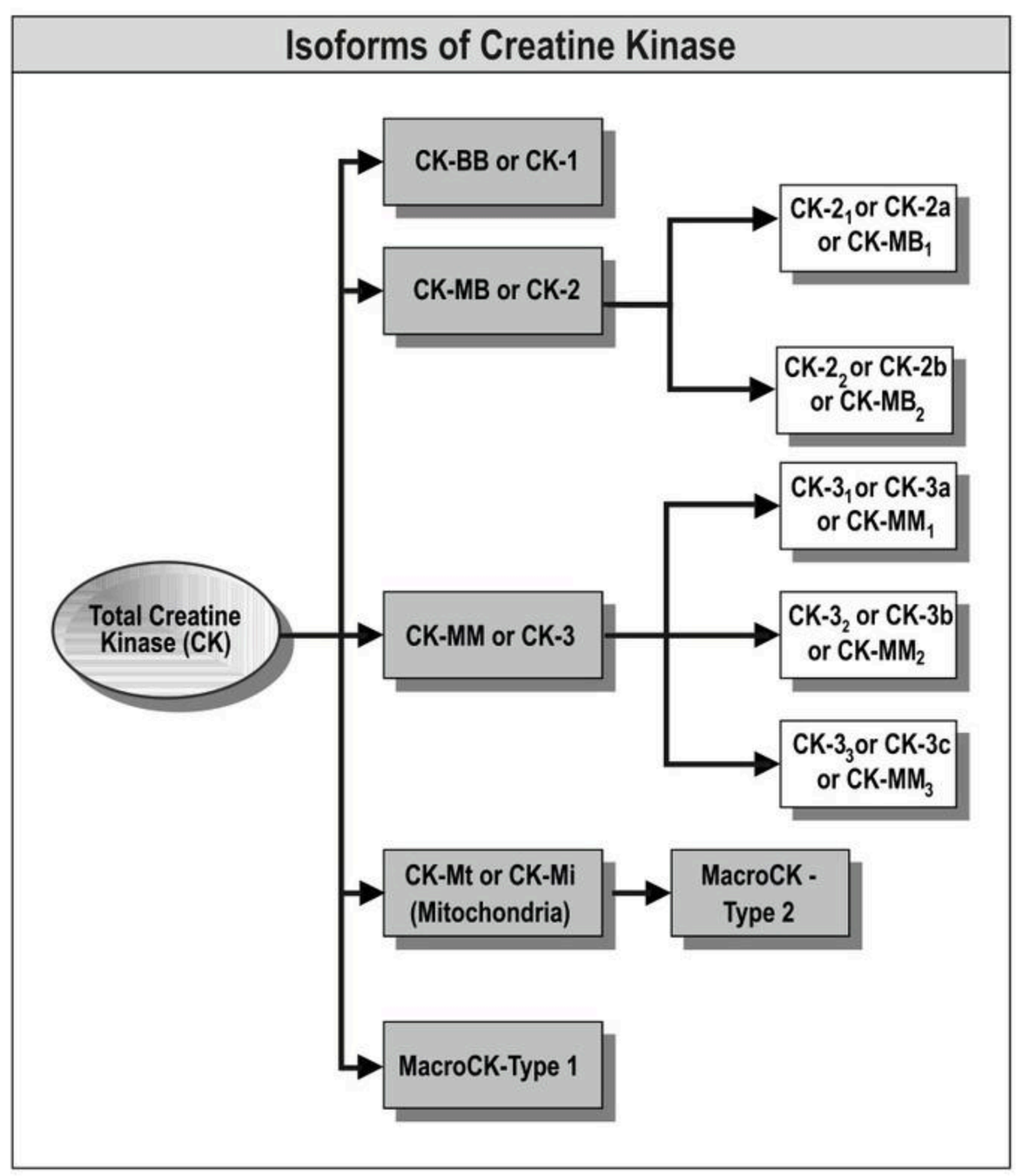
CK is a dimer composed of two subunits, each with a molecular weight of about 40000 Daltons. These subunits – B (brain) and M (muscle), are the products of the loci on chromosomes 14 and 19 respectively. Only three different pairs of subunits exist – CK-BB, CK-MB and CK-MM. Although CK-MB is not an isoenzyme in the true sense but a hybrid molecule consisting of one CK-M and one CK-B subunit, this term is, nevertheless, widely used.

There exists a fourth form of CK that differs from the others based on both immunologic and electrophoretic mobility. This isoenzyme, CK-Mt (or CK-Mi), is located between the inner and the outer membranes of mitochondria. Its structure is determined by a locus on chromosome 15. Unlike other subunits, no hybridisation (e.g CK-MB) between subunits of CK-Mt has been found.

CK activity may also be found in macromolecular form – macro CK. Macro-CK is sometimes found in hospitalised patients in small proportions. It exists in two forms MacroCK-Type 1 and MacroCK-Type 2. MacroCK-Type 1 is a complex of CK-BB+IgG or CK-MM+IgA. It is associated with gastrointestinal diseases, adenoma, carcinoma, myocardial / vascular diseases and other life-threatening conditions. It often occurs in women older than 50 yrs.

MacroCK-Type 2 is oligomeric mitochondrial CK. It is found predominantly in adults who are severely ill with malignancies / liver disease or in children with myocardial disease.

Both the M and B subunits have a C-terminal lysine residue, but only the former can be hydrolysed by the action of carboxypeptidases normally present in blood. Based on this hydrolysis, several isoforms are possible with different nomenclatures.



Biochemistry and Physiology

The two enzymes CK and Adenylate kinase (AK) play a decisive role in the synthesis of ATP, the immediate energy source of the muscle, the CNS and many proliferating tissues. CK is involved in providing energy for the muscle in two ways. In mitochondria, the site of energy production, CK-Mt catalyses the synthesis of Creatine phosphate (CrP) from ATP. The energy-rich CrP is carried in the CrP shuttle from the mitochondria to the cytoplasm where it is converted back into ATP by CK at the sites of energy consumption. Some of the intracellular CK is present in free form, some attached to the respective cell structure.

After release of CK into the blood (post-injury/infarct/damage), the enzyme undergoes the so-called postsynthetic modifications producing the forms known as CK-variants. Variants with normal molecularmasses of about 80kDA occur when carboxypeptidase successively removes the C-terminal lysine of both M-chains. The tissue form CK-MM3 becomes first CK-MM2 and finally CK-MM1. The analogous action on the tissue form CK-MB2 results in CK-MB1.

As described earlier, variants with higher molecular mass > 200kDA can occur when CK is bound by specific immunoglobulins (macro CK type 1) or when CK-Mt occurs in preferred oligomeric form (macro CK type 2).

The total CK activity measurable in serum is composed of the activities of the cytoplasmic dimeric isoenzymes CK-MM, CK-MB, CK-BB and their post-synthetically modified forms and of the activities of the macro CK. In healthy individuals the total activity consists mainly of CK-MM while the other CK isoenzymes and variants are only present in trace amounts or are undetectable. If the total activity of CK or of a particular isoenzyme is increased, the isoenzyme pattern can provide information on the underlying organ damage.

Tissue distribution of CK and its isoenzymes

Tissue	CK activity U/gm wet Weight	CK - MM %	CK - MB %	CK - BB %
Skeletal muscle	2500-3000	95-99	1-5	<0.1
Myocardium	500-700			
-Normal		90	5	
-Pathologically altered		70-80	20-30	
Brain	200-300			100
Gastrointestinal tract	120-150			100
Bladder	85			100
Uterus				
-Non Pregnant	165			100
-Pregnant	245		6	94
Placenta	250	19	1	80
Prostate gland	85			100
Lung	15	0-20		80-100

CK activity- SKELETAL MUSCLE DISEASE

Serum CK activity is greatly elevated, at some time during the course of the disease in all types of muscular dystrophy like Duchenne Muscular Dystrophy (DMD) and Becker Muscular Dystrophy (BMD). Levels up to 50 times the upper limit of normal may be observed.

In progressive muscular dystrophies like DMD/BMD, serum CK activity is the highest at 7-10 yrs of age and falls, as the patient gets older. 50-80% of asymptomatic female carriers of DMD show a 3-6-fold rise in CK activity, but values may be normal if specimens are obtained after patients have experienced a period of physical inactivity.

High to very high values of CK are noted in viral myositis, polymyositis, acute rhabdomyolysis and malignant hyperthermia. Hence attention needs to be paid when interpreting the results with the clinical findings.

Fructose-bisphosphate aldolase (FBPA) is also present in high concentrations in skeletal muscle and thus could be used to support the conclusions reached from the assay of CK. However, FBPA is also present in many other tissues, though not at such high concentrations. Particularly important in this respect is the high FBPA content of red blood cells. It is therefore essential that a sample of serum to be assayed for CK does not contain lysed red blood cells i.e., the serum should be free from haemolysis.

Apparently, CK-MB replaces part of the CK-MM form in the muscles, resulting in impaired storage of Creatine Phosphate in the muscle. In dystrophies and myopathies, usually only CK-MM is present in serum; but if total CK activity is high, some CK-MB can also be detected (<6%) because diseased skeletal muscle may contain a significant proportion of this isoenzyme owing to the phenomenon of fetal reversion, in which fetal pattern of protein synthesis reappear.

In polymyositis, CK-MM isoform pattern correlates well with the course of the disease. Total CK activity can also be elevated by direct trauma to muscle, including IM injections and surgical interventions, hypo or hyperthermia seizures, neuroleptic malignant syndrome, sepsis, exposure to certain drugs and chronic endocrinopathies.

Activity of Total CK and CK-MB as a result of ACUTE skeletal muscle damage		
Disease		**Assessment**
Acute damage (general)		● Total CK can increase to well above 5000U/L (37°C) in proportion to the damage ● The CK-MB activity is < 3% of the total CK. ● CK-MB concentration shows the same behaviour.
Physical activity	**Work / sport**	● All strenuous exercise increases CK activity. ● There is no limit, which permits a distinction between a normal reaction and pathological muscle damage. ● High activity is observed after some forms of exercise (like running downhill) and rhabdomyolysis. ● Excessive training (like marathon runners) leads to CK-MB activity > 3%.
	Diseases	● Increased CK activity is observed in diseases like tetany, cramps, Parkinson's disease, cough attacks, status asthmaticus, psychosis and delirium tremens.
Intramuscular injections		● Depending upon the substance, volume, concentration and osmolality, IM injections increases CK activity and can interfere with diagnosis of infarction ● Some drugs like chlorpromazine, diazepam, lidocaine, pentazocine, pethidine, phenobarbital, procainamide and promethazine are known to cause muscle damage that causes a rise in CK activity.
Surgery, trauma, arterial embolism		● Surgery or trauma (also burns, electrical injuries and pressure necroses) leads to an increase in CK activity proportional to the severity of damage. ● CK-MB increase after chest injuries is thought to indicate myocardial damage
Polymyositis, viral myositis, bacterial and parasitic myositides.		In acute and sub acute myositides, the CK activity > 20000U/L (37°C)
Medications and drugs of abuse, chemicals		● No dynamic changes in CK activity observed with pharmacological doses. ● Drugs of intoxication (abuse) like amphetamines, barbiturates, heroin etc. can result in very high CK activities. ● In extreme cases of rhabdomyolysis or malignant hyperthermia CK activities >20000U/L (37°C) can occur

Activity of Total CK and CK-MB as a result of CHRONIC skeletal muscle damage	
Disease	**Assessment**
Chronic damage (general)	● CK is elevated depending upon the severity of the disease ● The CK-MB activity may be > 10% of the total CK due to isoenzyme synthesis of the chronically stressed muscle. Thus an elevated CK-MB, in this case, is not due to myocardial involvement.
Muscular dystrophy - DMD and BMD.	● Disease with X-linked recessive inheritance and extreme CK activities > 25000U/L (37°C), particularly in the initial phase. ● The CK-MB activity is also increased. This does not confirm myocardial involvement. ● In other type of dystrophies like limb-girdle type, ocular type, myotonic dystrophy, CK activities are sometimes within reference range and rarely >2000U/L (37°C).
Myotonia congenita, Glycogenosis type V, myasthenia gravis, spinal muscular atrophies	These primary myopathies also have CK activities, which are within the reference range or only slightly increased depending on the phase of the disease.
Polymyositis, dermatomyositis, ocular myositis.	The CK activities in the chronic forms of myositis are considerably lower than in the acute forms. Activities within the reference are also possible.
Secondary myopathies.	Secondary increase in CK activity may bee seen in endocrine diseases, intoxications, seizure disorders, paralysis, lupus erythematosus multiple sclerosis and taking certain drugs. The CK activity returns to the reference range with improvement in underlying conditions.
Medications and drugs of abuse.	● Pharmacological doses of anti-arrhythmic drugs, beta-blockers, Potassium lowering drugs, lithium, halothane etc lead to increased CK activity. ● Prednisone and related steroids and chemotherapy agents lead to lower CK activity.

CK activity- HEART DISEASE

Total CK activity shows an increase following MI in which it is increased earlier than other enzymes, beginning at 4-6 hrs, peaking on an average at 24 hrs and returning to normal within 2-3 days. The area under the peak and slope of the initial rise are proportional to the size of the infarct. False negatives are rare and are usually due to technical errors. False positives may occur, and for accurate diagnosis of MI CK-MB activity should be measured. If the Total CK- activity is raised and CK-MB contributes more than 6% of the activity then MI is considered highly probable.

After MI the maximum CK activity rarely exceeds 7500U/L (37°C). Higher CK activities suggest concomitant skeletal muscle damage. Early reperfusion is marked by a particularly rapid rise and early peak of the CK-MB. A characteristic second peak of CK-MB indicates a second trauma or reinfarction.

As CK-MB also occurs in skeletal muscles, it is, therefore, not absolutely cardiospecific; a diagnostic strategy for careful preselection of patients based on clinical criteria is necessary. It is also necessary, that, the **ratio of total CK to CK-MB activities** be measured. This helps in differential diagnosis of MI and skeletal muscle damage. If this is done, the clinical questions can be answered in most cases.

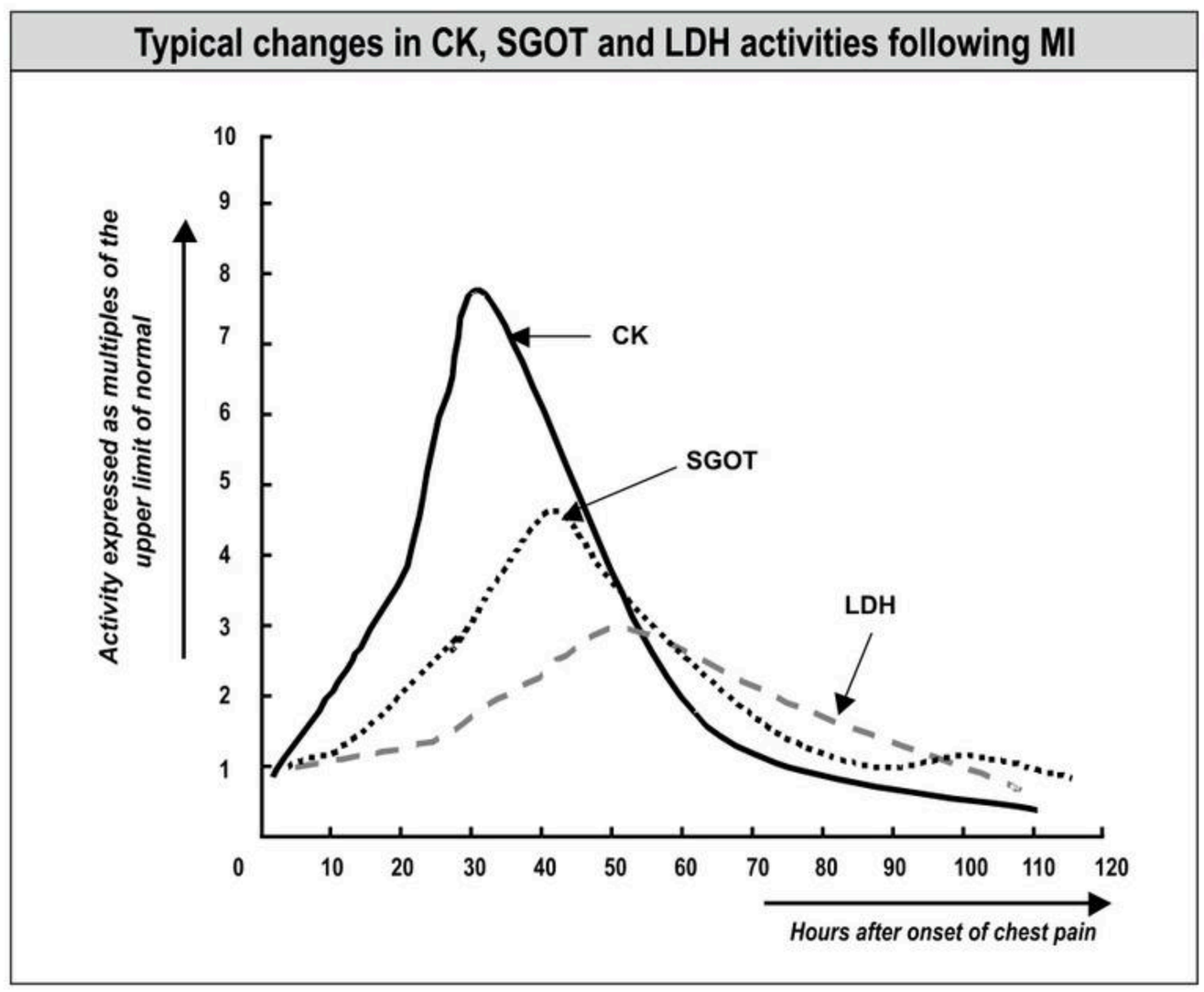
Activity of Total CK and CK-MB as a result of CARDIAC muscle damage	
Disease	**Assessment**
Acute myocardial damage	● The CK increases in proportion to the damage; the CK - MB activity is more than 6% of the total CK. ● Total CK activities > 7500U/L (37^{0}C) indicate additional sleletal muscle trauma, e.g. as a result of resuscitation. ● CK-MB activities > 25% after immunoinhibition require determination of CK variants.
Myocarditis, Endocarditis and Pericardities	Elevated total CK and CK-MB Levels are typical, particularly in myocarditis; activity-time curves resembling infarction are possible.
Angina pectoris	● No increases in CK-MB are observed in statle angina with immunoinhibition method; unstable angina can show small increases in CK-MB concentration, which are within the reference interval. ● Uncomplicated cardiac catheterisation and coronary angiography do not lead to significant increase in CK-MB. ● Resuscitation, defibrillation and chest trauma lead to increased CK activities, which correspond to the extent of skeletal muscle damage; if there is myocardial involvement the CK-MB increases.
Diagnostic and therapeutic interventions, trauma	● Surgical procedures involving the myocardium release CK-MB. ● CK-MB activities > 50 U/L (37^{0}C) after coronary artery bypass grafting suggest infarction. ● Valve replacement and transplantation lead to increases in CK and CK-MB ● Stress tests (exercise etc) do not lead to pathological concentrations.
Tachycardia, heart failure and valve	These conditions do not usually lead to a significant increase in activity.

CLINICAL ENZYMOLOGY IN MI – A BRIEF ANALYSIS

Enzymological methods have been particularly useful in providing supporting evidence for myocardial infarction (MI) and also monitoring the course of the infarct.

In MI, a coronary artery becomes obstructed and this leads to irreversible damage and necrosis of the heart tissue. Enzymes are released from the necrotic tissue into the plasma; the three enzymes most commonly assayed are creatine kinase (CK), aspartate aminotransferase (SGOT) and lactate dehydrogenase (LDH). Each enzyme shows a different time course for release into the plasma and subsequent disappearance. These differences depend upon the concentration of each present in the cardiac tissue, their rate of release and their subsequent rate of clearance or degradation. Their **half-life** in the plasma is:

- CK: » 15h
- SGOT: » 17h
- LDH: » 110h



CK is the earliest to be detectable, rising 4-6h after onset of pain, reaching a peak at 24-36h and then rapidly declining. SGOT reaches a peak between 48-60h and LDH between 48-72h, the latter declines more slowly than the former. The prognosis and progress can be assessed by the increase in the activities of these enzymes. CK, which does not occur in the liver unlike SGOT and LDH, can therefore be used to differentiate heart disease from liver disease. Thus, CK can be used in the early detection and prognosis of myocardial infarction.

Comparison of sensitivity and specificity of various test in MI

TEST	SENSITIVITY %	SPECIFICITY %
ECG	63 - 84	100
Increased SGOT	89 - 97	48 - 88
Increased LDH	87	88
LDH1 >LDH2 (on 3rd day after chest pain)		
Increased Total CK	93 -100	57- 88
Increased CK-MB	94 -100	93 -100

Determination of CK and CK-MB activity alone is not suitable for assessment of the risk of MI. It can be particularly useful in interpretation, if its estimation is accompanied by determination of the isoenzyme (CK-MB), serial tests (enzyme-time curves), or ECG under exercise testing. The CK-MB results can be interpreted on the basis of the 6% rule, a limit of 12U/L or preferably the dynamic activity time curve of an enzymogram.

The 6% Rule: The decision criterion is an increase in the total CK activity to > 100 U/L (25°C) within the diagnostic time window and a simultaneous increase in CK-MB activity. A CK-MB fraction more than 6% of the total activity is regarded as diagnostic for MI. A fraction less than 6% indicates skeletal muscle damage. The clinical specificity of this rule is high as the number of false positive results caused by the presence of extracardiac CK-MB is small. This high clinical specificity is of course obtained at the expense of some loss of sensitivity in the detection of smaller infarctions.

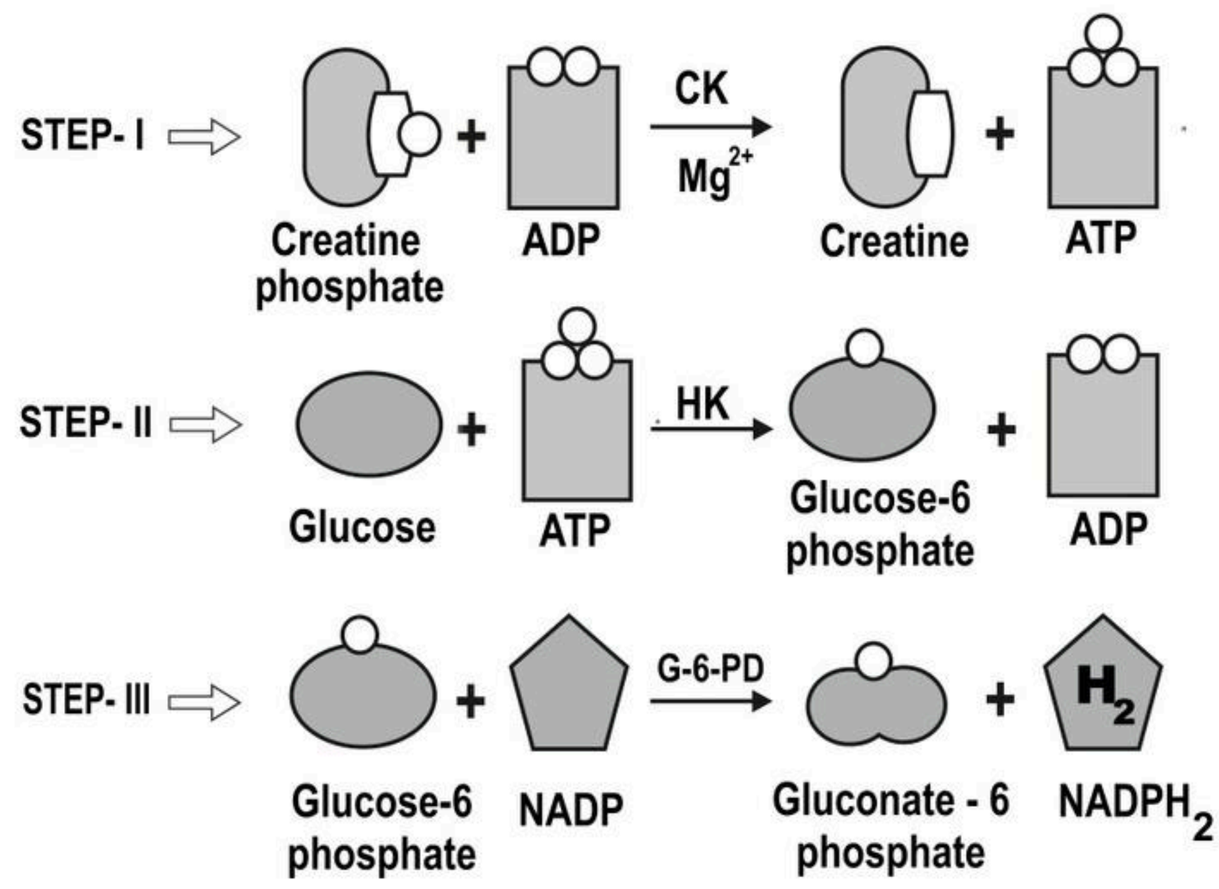
The 12U/L limit: The decision criterion is – increased total CK activity during diagnostic time window and a simultaneous increase in CK-MB activity to > 10U/L (25°C) / 15U/L (30°C) / 24U/L (37°C). This is equivalent to 12U/L CK-B activity (25°C). This strategy is characterised by a higher clinical sensitivity, which also permits identification of small infarctions. This high diagnostic sensitivity is obtained at the expense of some loss of specificity if the prevalence of infarction is low.

Dynamic activity-time curves (cardioenzymogram): If an acute MI is suspected serial determination of total CK and CK-MB activity are carried out so that the changes in the enzyme activities observed in this dynamic condition can be taken into account in the diagnosis and monitoring of treatment. This strategy is characterised by high levels of diagnostic sensitivity, specificity and flexibility as regards to medical and logistic requirements.

METHOD OF DETERMINATION – Total CK

The methodology recommended by International Federation of Clinical Chemistry (IFCC) is a photometric rate assay. The reaction conditions are so robust that the measurements can be performed without problems at reaction temperatures of 37°C, 30°C or 25°C under optimal reaction conditions. The principle of the IFCC method can be depicted as per the following equation

Typical changes in CK, SGOT and LDH activities following MI



CK: Creatine Kinase
HK: Hexokinase
G-6PD: Glucose 6-phosphate dehydrogenase

METHOD OF DETERMINATION – CK isoenzymes

At present only determination of the isoenzyme CK-MB is of clinical significance.

Immunoinhibition method

This is currently the most economical, practical and widely used method in clinical laboratories worldwide. The theoretical basis for the clinical application of the Immunoinhibition method is the assumption that only CK-MM and CK-MB are released into the blood stream after muscle damage. The reagent used contain anti CK-M antibodies, which completely inhibit all CK-M activity i.e., both M subunits in CK-MM and the single M subunit in CK-MB. The remaining non-CK-M activity corresponding to the CK-B activity of CK-MB is measured. Since, only CK-B activity of the dimeric CK-MB molecule is measured, multiplication by a factor of 2 gives the CK-MB activity in the specimen.

Isoenzyme electrophoresis method

The CK isoenzymes are separated on cellulose acetate strips or agarose gel and the activity bands then visualised fluorometrically using the total CK reagent. The fluorescence of the $NAPH_2$ formed in the reaction permits quantitative analysis.
This method is useful only to exactly differentiate between isoenzymes. It is labour intensive, complicated and hence expensive. Moreover, the interference due to AK makes the interpretation difficult. Not practical for routine use in a clinical laboratory.

Immunoassy for determination of CK-MB mass (concentration)

Here CK-MB is determined with a combination of CK-B and CK-M specific antibodies or with CK-MB specific antibodies. The first generation assays use the double antibody technique with CK-M and CK-B specific antibodies. The second-generation assays use CK-MB specific monoclonal antibodies, in some cases, in combination with CK-M and CK-B specific antibodies. The assay format may be EIA, Fluoroscent immunoassay, Luminiscent immunoassay, radio immunoassay etc.
Interferences have been described in these assays too.

- Apart from interference typical of immunoassays (like HAMA-human anti-mouse antibody), specific interference by high concentrations of CK-MM and CK-BB or by CK-B binding autoantibodies has been demonstrated.
- Lack of a universal standardisation has influenced the non-comparability of results.
- The data on reference ranges, decision limit and specific activities are therefore not uniform but vary from reagent-to-reagent.

CRITICAL DETERMINANTS OF CK AND CK-MB REAGENT SYSTEM DESIGN

Appropriate activator

During storage, CK is rapidly inactivated by the oxidation of the sulphydryl groups at the active site of the enzyme. The need to reactivate the enzyme has been demonstrated and the various sulphydryl reagents like cysteine, dithiothreitol, glutathione, 2-mercaptoethanol and N- acetylcysteine have been used as an activator in the reagent. But:

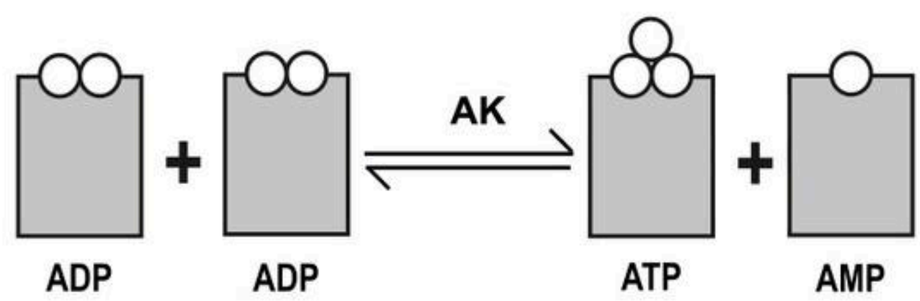
- Cysteine deteriorates rapidly in solution.
- Dithiothreitol causes turbidity by precipitation of albumin.
- Glutathione is unstable since it allows interference by glutathione reductase released from red cells in haemolysed specimens.
- 2-mercaptoethanol cause turbidity and its decomposition products inhibits CK.

N- acetylcysteine (NAC) suffers none of these shortcomings and is accepted to be the best thiol (sulphydryl) activator for maintenance and reactivation of CK activity in serum. The optimum concentration of NAC is of utmost importance. A small imbalance in its concentration may cause free cysteine to be released. This may cause the reagent system to behave erroneously.

Adenylate Kinase (AK) interference

AK is present in many tissues and cells including skeletal and cardiac muscle, liver, brain, and erythrocytes. The enzyme is also present in normal serum and may be markedly raised in haemolysed specimens. Adenylate kinase deficiency in the erythrocytes, a rare genetic disorder, is associated with haemolytic anaemia. AK catalyses the reaction as shown in figure.

Adenylate Kinase catalytic action



ATP : Adenosine Tri-phosphate
ADP : Adenosine Di-phosphate
AMP : Adenosine Mono-Phosphate

The ATP generated in this catalytic action of AK enters the reaction of the CK assay and leads to an apparent increase or false positive values of CK.
AK activity can be inhibited by adding:

- Fluoride or
- AMP or
- Diadenosine pentaphosphate (DAPP)

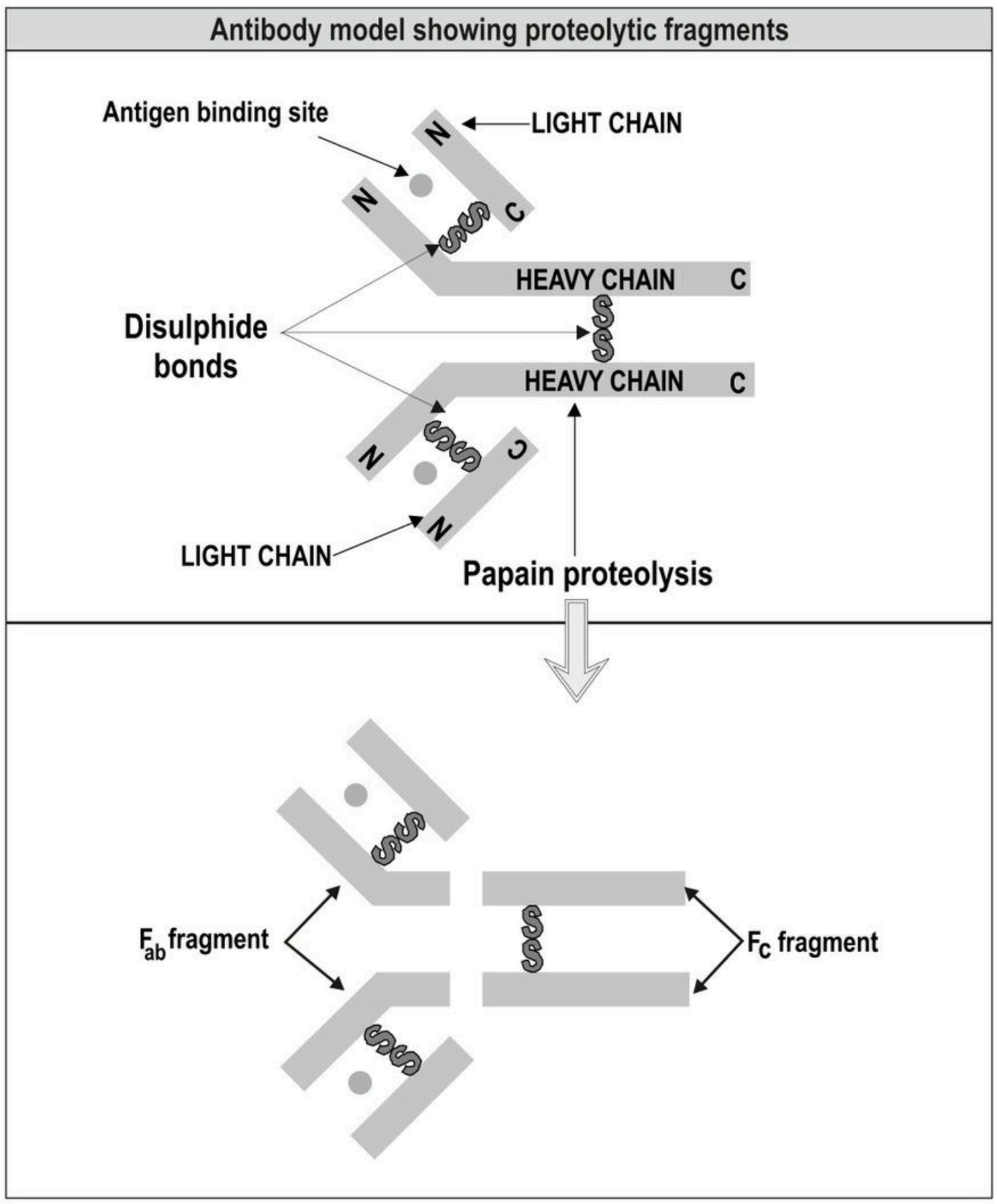
Although Fluoride is an effective inhibitor, it may form insoluable magnesium fluoride with magnesium ion in CK assay. AMP alone can competitively inhibit product formation (2ADP $\rightleftharpoons$ ATP+AMP). Thus, to adequately inhibit AK from red cell and muscle and to ensure better results, an **optimised blend** of AMP and DAPP should be used. DAPP competitively inhibits the AK of muscle and erythrocytes while AMP inhibits AK derived from liver and kidney.
Fildes and Harris (1966) found electrophoretic variation in red cells and defined 3 phenotypes, designated AK1, AK2-1 and AK2. In India, in majority of the populations the AK1 gene occurs in high frequencies, approaching 90% and above. Thus, this demographic pattern also indicates that, in India, to get accurate results, a CK assay systems should contain optimised blend of AK inhibitor.

Cation inhibitor

CK is readily inhibited by polyvalent cations such as Ca^{2+} . This can be prevented by addition of EDTA. EDTA also helps in the protection of the thiol reagent from oxidation by metal ions.

Anti CK-M antibody used in Immunoinhibition method

The anti CK-M antibodies quantitatively inhibit all M subunits of the sample within the incubation time. These antibodies should have high specificity and corresponding affinity.
Since, the specificity of the antibody used as anti-CK-M reagent is of utmost importance, a brief discussion on antibody structure would be worthwhile.
The antibody molecule is made up of two identical heavy and two identical light chains held together by interchain disulphide bonds. These chains can be separated by reduction of the S-S bonds and acidifications. The exposed hinge region is extended in structure due to high proline content and therefore vulnerable to proteolytic attack. The molecule is split by papain to yield two identical fragments, each with a single combining site for antigen, called F_{ab} fragment (Fragment antigen binding). A third fragment, known as F_c fragment, is also formed. The F_c fragment lacks the ability to bind to antigens.
The F_{ab} fragment of the "Y–shaped" antibody carries the paratopes, the binding sites that bind to the epitopes on the CK-M antigen. In an intact antibody molecule F_{ab} is present along with the F_c fragment.
Because the F_c fragment binds to complements, certain bacterial proteins (like staphylococcal Protein A) and other non-specific molecules such as heterophillic antibodies, the reagent for inhibition should preferably be devoid of the F_c fragment. Since, the F_{ab} fragment is specific with high antigen binding capacity, to achieve adequate inhibition of CK-M activity without interference, a reagent system should preferably use the purified F_{ab} fragment in the anti-CK-M reagent.



Liquid reagent system

In the CK /CK-MB assay, the calculations to arrive at the results involve a multiplication step with a relatively high value of factor - over 6000 for CK-MB and over 8000 for total CK. This means a small change in the optical density (OD) would result in a significant change in the final results of CK /CK-MB activities.

In a dry powder–based assay system, the reagent powder is reconstituted with deionised water or buffer to make the working reagent. This reconstituted reagent may contain suspended microparticles, though apparently, it may seem to be **optically clean**. These microparticles are heterogeneous molecular aggregates derived from the lyophilised reagent. During the course of photometric measurement, the passage of light through the solution cause "excitation" of these microparticles leading to erroneous OD readings. This error is compounded by multiplication with the high factor. Consequently, the final results of CK /CK-MB activities may show unexpected variation leading to erroneous/inadequate clinical decisions.

This problem can be solved by filtration of the reconstituted reagent through a 0.22 micron sterile filter. But, in a routine clinical laboratory this may not be a practical option. Thus, the best option is to use ready-made liquid reagent system available with some manufacturers. These reagents are optically clean and do not cause the interference from suspended microparticles.

OTHER BIOMARKERS OF MI

Myoglobin

Myoglobin, a relatively small (17.8 kDa) heme protein that transports oxygen within muscle cells, constitutes about 2 percent of muscle protein in both skeletal and cardiac muscle. Thus, the marker is not cardiospecific. Because of its low molecular weight, myoglobin is rapidly released into the circulation and is the first marker to exhibit rising levels after an AMI. The advantages of myoglobin in the early diagnosis of MI are the high early sensitivity and the possibility of rapidly assessing the success of thrombolytic therapy.

The biologic half-life of myoglobin is extremely short (10-20 min) vis-à-vis CK (approx.15 hrs) and CK-MB (approx. 12 hrs). Thus the diagnostic window available is small compared to CK or CK-MB.

Cardiac Troponin-I (cTnI)

Troponin is a complex consisting of three single-chain polypeptides:

- Troponin-I, which prevents muscle contraction in the absence of calcium
- Troponin-T, which connects the troponin complex to tropomyosin
- Troponin-C, which binds calcium

Together with tropomyosin and under the influence of calcium, troponin regulates muscle contraction. The cardiac muscle-specific isoform cTnI (24 kDa) exhibits approximately 60 percent homology with the skeletal isoforms (sTnI), and has a unique 31 amino acid extension of the N-terminus. After myocardial infarction, elevated cTnI levels appear within 3 to 6 hours. Levels peak within 14 to 20 hours, and return to normal after 5 to 7 days. Cardiac Troponin - I is 100% cardiospecific.

Unfortunately, the standardization of cardiac troponin - I assays is problematic. Up to 20-fold variation of serum cTnI mass determinations may be observed for a given patient sample when measured by different assay systems. As a result, significant ambiguity often exists in the clinical interpretation of serum cTnI concentrations. Recent efforts have been directed toward the biochemical standardization of cTnI assays. However, the heterogeneous nature and biochemical complexity of the serum forms of cTnI and differences of the epitope recognition by the various methods have hindered the harmonization of serum cTnI assays.

GLOSSARY OF TERMS

Angina pectoris

Angina pectoris is the medical term used to describe chest pain caused by poor blood flow to certain areas of the heart muscle. Often, the name is shortened to angina.

Angiography

It is a description of blood vessels and lyphatics. The relative inaccessibility of the blood vessels and lyphatics for physical examination requires investigation with other means, primarily radiologic examinations that include lung scintigraphy, conventional or digital subtraction pulmonary angiography (DSPA), pulmonary computed tomography angiography (PCTA), and pulmonary magnetic resonance angiography (PMRA).

Aortic stenosis

Aortic stenosis (AS) is the obstruction of blood flow across the aortic valve. AS has several etiologies: congenital unicuspid or bicuspid valve, rheumatic fever, and degenerative calcific changes of the valve.

Arterial embolism

Embolism is the obstruction of a blood vessel by foreign substances or a blood clot. If the blood vessel concerned is an artery it is called arterial embolism. Obstruction in the pulmonary artery is called pulmonary embolism (PE). In PE the blood clot is in the lung. PE is usually caused by an embolus from thrombosis in lower extremities, pelvis, arms, or heart. When a clot forms in the legs or arms, it is referred to as a deep venous thrombosis (DVT).

Atheroma

Fatty degeneration or thickening of the walls of the larger arteries occurring in atherosclerosis.

Atria

A chamber or cavity communicating with another structure.

Defibrillation

Fibrillation is a small, local, involuntary, muscular contraction, due to spontaneous activation of single muscle cells or muscle fibers. Defibrillation is the termination of atrial or ventricular fibrillation, usually by electroshock.

Delirium tremens

Delirium tremens (DTs) is a severe manifestation of alcohol withdrawal. Pearson first described it in 1813 as an acute psychosis following abstinence from alcohol. Although it only occurs in a relatively small number of patients who undergo alcohol withdrawal, it can be fatal. DT is a medical emergency that requires prompt recognition and treatment.

Diaphoresis

Profuse sweating.

Dyspnea

Air hunger resulting in laboured or difficult breathing, sometimes accompanied by pain.

Endocarditis

Endocarditis refers to an infection of a heart valve. Although several different types of organisms can cause endocarditis, it is usually caused by a bacterial infection.

Endocardium

Serous lining membrane of inner surface and cavities of the heart. It is continuous with the intima or interior coat of arteries.

Epicardium

The inner or visceral layer of the pericardium, which forms a serous membrane forming the outermost layer of the wall of the heart.

Epigastrium

Region over the pit of the stomach.

Glycogenosis type V

Also known as McArdle disease. It is caused by a deficiency of myophosphorylase (alpha-1,4-glucan orthophosphate glycosyl transferase), which normally initiates glycogen breakdown by removing 1,4-glucosyl groups from the glycogen molecule with release of glucose-1-phosphate.

Malignant hyperthermia

Malignant hyperthermia (MH) is a genetic disease, which may result in rhabdomyolysis. In this disease, episodes of hyperthermia and rhabdomyolysis are triggered by exposure to volatile anaesthetics such as halothane, or succinylcholine, a depolarising muscle relaxant. MH appears to be an autosomal dominant condition.

Marantic endocarditis
Endocarditis is the inflammation of the lining membrane of the heart. It is usually confined to the external lining of the valve, sometimes to the lining membrane of its chambers. May be due to invasion of microorganisms or an abnormal immunological reaction. Marantic endocarditis, also known as Nonbacterial Thrombotic Endocarditis (NBTE), is the endocarditis due to non-bacterial causes.
Muscular Dystrophy
Muscular dystrophy (MD) is a collective group of inherited noninflammatory but progressive muscle disorders without a central or peripheral nerve abnormality. The disease affects the muscles, with definite fiber degeneration but without evidence for morphologic aberrations. Dystrophin was found to be at the root of the disorder. Defects in the genetic code for this 427-kDa-muscle protein resulted in the various manifestations commonly associated with MD.
Myasthenia gravis
Myasthenia gravis (MG) is an acquired autoimmune disorder characterized clinically by weakness of skeletal muscles and fatigability on exertion. Thomas Willis reported the first clinical description in 1672.
Myocarditis
Myocarditis is an inflammatory disease of the myocardium with a wide range of clinical presentation, from subtle to devastating. It usually manifests in an otherwise healthy person and can result in rapidly progressive (and often fatal) heart failure and arrhythmia.
Myocardium
The middle layer of the walls of the heart composed of cardiac muscle.
Myocytes
Muscular tissue cells.
Myositis
Inflammation of muscle tissue especially voluntary muscles.
Myotonia congenita
A benign disease characterised by tonic spasms of the muscles induced by voluntary movements; usually congenital and transmitted from one generation to another.
Ocular myositis
Inflammation of muscle tissues of the eye.
Parkinson's disease
Parkinson disease (PD) is a progressive neurodegenerative disorder associated with a loss of dopaminergic nigrostriatal neurons. It is named after James Parkinson, the English physician who described the shaking palsy in 1817.
Pericarditis
Pericarditis is the inflammation of the lining of the heart. Pericarditis is a commonly found syndrome in the hospital setting, particularly in the coronary care unit. It most often affects men aged between 20-50 yrs, usually following respiratory infections.
Polymyositis
Polymyositis (PM) is an inflammatory muscle disease of unknown etiology. PM, dermatomyositis (DM), and inclusion body myositis are the major members of a group of syndromes called the idiopathic inflammatory myopathies. In much of the literature, PM and DM are grouped as one disease.
Regurgitation
A backward flowing, as in the return of solids or fluids from stomach or the backward flow of blood through a defective heart valve.
Resuscitation
Restoration to life of one apparently dead. Also may be used for cardiopulmonary resuscitation (CPR). CPR is the re-establishing of heart and lung action by artificial ventilation and closed-chest cardiac massage, after cardiac arrest or apparent sudden death resulting from electric shock, drowning, respiratory arrest, and other causes.
Rhabdomyolysis
Rhabdomyolysis is the breakdown of muscle fibers with leakage of potentially toxic cellular contents into the systemic circulation. The final common pathway of rhabdomyolysis may be a disturbance in myocyte calcium homeostasis.
Spinal muscular atrophy
The spinal muscular atrophies (SMAs) are a clinically and genetically heterogeneous group of disorders. They are characterized by primary degeneration of the anterior horn cells of the spinal cord and often of the bulbar motor nuclei without evidence of primary peripheral nerve or long-tract involvement.
Status asthmaticus
Status asthmaticus is a medical emergency in which asthma symptoms are refractory to initial bronchodilator therapy in the emergency department. Patients complain of chest tightness, rapidly progressive shortness of breath, dry cough, and wheezing. Typically, patients present a few days after the onset of a viral respiratory illness or following exposure to a potent allergen or irritant.
Syncope
Syncope (fainting) is a sudden fall of blood pressure resulting in loss of consciousness.
Tachycardia
Abnormally rapid heart rate.
Tetany
A syndrome manifested by sharp flexion of the wrist and ankle joints, muscle twitching, cramps and convulsions due to abnormal calcium metabolism and occurring in parathyroid hypofunction, vitamin D deficiency, alkalosis and as a result of alkaline salts.
Thrombogenesis
The formation of a blood clot.
Transmural
Across the wall of an organ or structure, as in transmural myocardial thrombosis, in which the tissue in the entire thickness of the portion of the cardiac wall is affected.

Triage

Triage is a French word that literally means, "to separate". Medical Triage refers to the classification of patients or casualties to determine priority of need and proper place of treatment.

Viral myositis

Viral myositis is an acute, subacute, or chronic infection of skeletal muscle. Once considered a tropical disease, it is now seen in temperate climates as well, particularly with the emergence of HIV infection. In addition to HIV, other viruses, bacteria, mycobacteria, fungi, and parasites can cause myositis.

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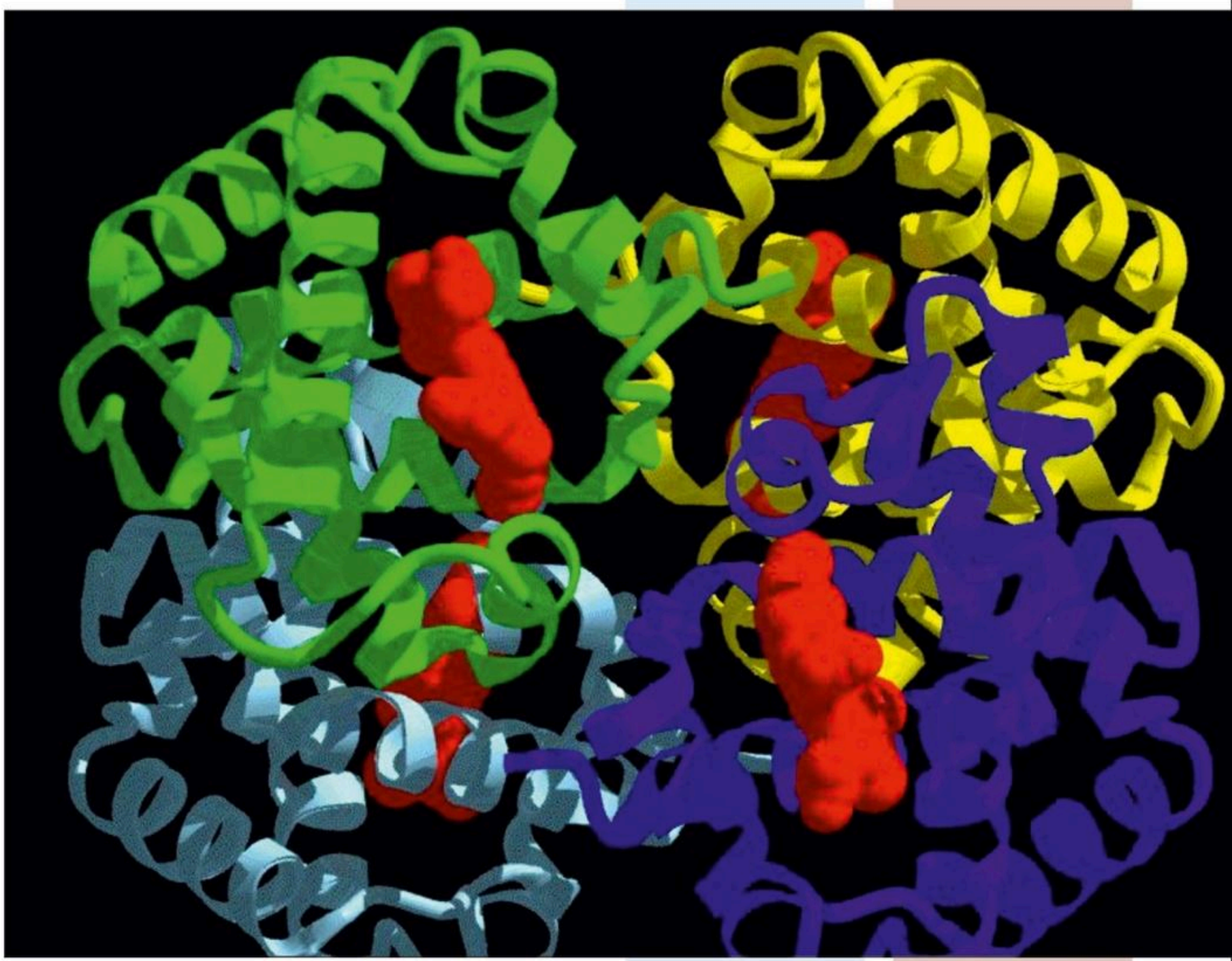
GLYCATED HEMOGLOBIN



- **Glycated Hemoglobin (Ghb)**
  - The marker for retrospective glycemic control.....Pgs 196-207

KNOWLEDGE BOOK

For the Laboratorians

Glycated Hemoglobin

The marker for retrospective glycemic control

Setting Trends...

INTRODUCTION

Worldwide projections suggest that more than **220 million people** would be affected by Diabetes Mellitus **(DM)** by the year 2010. **In the U.S.A.** alone, health care costs due to Diabetes Mellitus would be approximately **14% of the healthcare budget**.

For reasons of genetics & lifestyles, **Indians form the world's largest diabetic population.** Today, **India has 25 million diabetic patients, more than any other country** & the number is expected to rise to 35 million by 2010 & to 57 million by 2025.

It is reported that **in urban India 12% of the adult population suffers from Diabetes**, compared to 6% in the U.S.A. & the U.K. Strangely though, **in the rural** Indian population (comprising about 70% of the total population) , **a mere 2-3% suffer from Diabetes.**

WHAT IS DIABETES MELLITUS (DM)?

Diabetes Mellitus is a group of metabolic disorders, connected by raised / elevated levels of plasma / serum, glucose concentration, either when fasting, or after ingestion of carbohydrates, often accompanied by the presence of glucose in urine, arising from a disturbance of glucose metabolism.

During Diabetes, glucose is under utilized resulting in hyperglycemia. This under utilization of blood glucose is caused by a relative or absolute deficiency of insulin. It occurs with different degrees of severity & in its severest form causes keto-acidosis, coma & untimely death if not managed appropriately.

CLASSIFICATION OF DIABETES MELLITUS

The World Health Organization **(WHO**) classification of DM is as follows:

- TYPE 1 Diabetes Mellitus (TYPE-1 DM)
  Also known as Insulin dependent diabetes mellitus (IDDM) & / or Juvenile Onset diabetes mellitus, this is characterized by the autoimmune / viral / chemical toxins, induced destruction of the beta cells of the pancreas. The patients require insulin to avoid keto-acidosis. This type of diabetics constitutes roughly 10 - 20 % of the total diabetic population
- TYPE 2 Diabetes Mellitus (TYPE-2 DM)
  Also known as non insulin dependent diabetes mellitus (NIDDM), adult onset diabetes, it comprises between 80-90% of the diabetic population. In this case, the pancreas retains some beta cell capacity, resulting in insulin levels that vary, but in all cases are less than that required to maintain glucose homeostasis.
  TYPE-2 DM is frequently accompanied by target organ insulin resistance, which results in a decreased responsiveness to both exogenous & endogenous insulin, which in some cases can be due to a decreased number of insulin receptors on the target cells.
  The occurrence of TYPE-2 DM, is almost completely determined by the genetic factors. There appears to be very little, if at all, involvement of viruses or autoimmune antibodies. The metabolic alterations observed are milder than those for the TYPE-1 DM.

Other forms of DM:

- Genetic defects of insulin action
- Diseases of the exocrine pancreas

LONG TERM COMPLICATIONS OF UNCONTROLLED DM

Elevated Fasting plasma glucose (FPG) is considered to be a basic indicator of DM. Once the condition is established, regular & appropriate control / monitoring / assessment of the patient condition is critical to decrease morbidity & mortality due to DM, with its associated debilitating complications.

Vascular complications in DM may be classified as micro-vascular predominantly, affecting the retina, kidney & the nerves and macro-vascular which, predominantly affect the coronary artery, cerebrovascular and peripheral arterial circulation.

Uncontrolled DM, leads to **acute complications** such as:

- Hypoglycemia, occurring in more than 90% cases of TYPE-1 DM.
- Keto-acidosis develops where there is an absolute or relative insulin deficiency.
- Hyperosmolar non-acidotic diabetes, of which there is no satisfactory evidence differentiating it from diabetic keto-acidosis.
- Somogyi effect, which is a unique combination of hypoglycemia during night with a rebound hyperglycemia in the morning. This condition is more common in TYPE-1 DM and more so in children.
- Dawn phenomenon, where there is a early morning rise in blood glucose levels with no hypoglycemic episodes in the night.
- Infections, where a variety of factors predisposes the diabetic patient to an increased incidence, or increased severity of infections.

Chronic complications resulting from long term sequelae of unmonitored DM are:
Retinopathy
Neuropathy
Angiopathy
Diabetic Nephropathy (DN)
Thrombosis
Atherosclerosis / Coronary Artery Disease (CAD).
Diabetic Nephropathy (DN) & Coronary Artery Diseases (CAD), account for more than two thirds of all deaths among diabetics.

LABORATORY DIAGNOSIS OF DM

The three routine laboratory diagnostic tests used for establishing DM in a patient are:

- Random plasma / serum glucose test **(RPG)**
- Fasting plasma / serum glucose test **(FPG)**
- Oral glucose tolerance test **(OGTT)**

Diagnostic algorithm for DM

Test	Range	Diagnosis
RPG	⩾ 200 mg/dl	DM
	⩽ 99 mg/dl	DM excluded
	101 - 198 mg/dl	FPG suggested
FPG	⩾ 126 mg/dl	DM
	⩽ 110 mg/dl	DM excluded, but suggested OGTT to diagnose **Impaired glucose Tolerance (IGT)** in Absence of IFG.
	110 - 125 mg/dl	**Impaired Fasting Glucose (IFG)** but suggested OGTT
OGTT (2 hour glucose)	⩾ 200 mg/dl	DM
	⩽ 140 mg/dl	No IGT
	140 - 199 mg/dl	IGT

Adapted from the WHO & International Diabetes Federation guidelines. All glucose concentrations refer to venous plasma.

In the absence of classical symptoms (thirst, polyurea, unexplained weight loss) RPG is the recommended first test & at least two results on different days are essential. If results are discordant, 2-hour OGTT result takes precedence, but one should consider re-testing after an interval.

UTILITY OF THE CLINICAL TESTS IN ESTABLISHING & MONITORING DM

While all the tests (RPG / FPG / OGTT) measuring the mean plasma glucose, are currently used for the diagnosis of the condition , **the tests themselves are subject to inaccuracies & variations** due to various factors such as :

- short term illness of the patient
- stress
- non-compliance by the patient, such as irregular & uncontrolled eating habits otherwise, but a strict regimen of diet & medication prior to the test being performed, leading to a "misleading" glycemic status.

All the above tests reflect the body's metabolic state at the time of the test, at best giving an indication to the immediate glycemic status of the body (2-4 hours prior to sampling , at the most) & hence are not suitable as a marker for long term monitoring of the condition (DM).

As is evident, not only is the establishment of the condition (DM) important, **it is equally if not more important to monitor the Glycemic status of a patient,** once the condition has been established & confirmed.

GLYCATED HEMOGLOBIN (glycosylated hemoglobin - GHb)

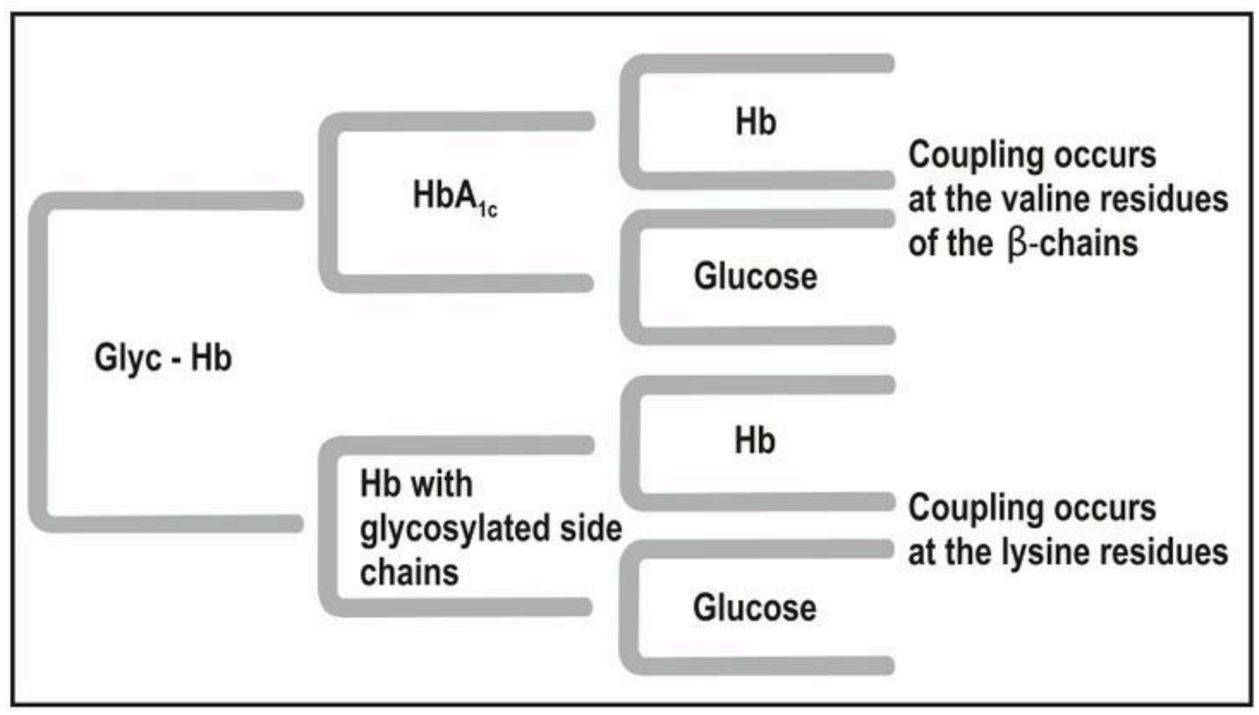
The extensively researched marker, currently in use for the long term monitoring of the Glycemic status among diabetics, is the Glycated hemoglobin test.

Whereas all the other tests measuring glucose indicates only the immediate Glycemic status, **Glycated hemoglobin (GHb) is the only test that gives the measure of mean blood glucose (MBG) "round the clock" for the last 6 - 8 weeks.**

Given the importance of long term monitoring & benefits of appropriately managing DM, the testing, for levels of Glycated hemoglobin (GHb- as being the only such long term marker), assumes a critical position in the overall management of DM. GHb values provide an extremely useful clinical tool in quantitating glucose levels over the previous 6 - 8 weeks.

The test is simple, provides reproducible results & quantitative help in adjusting the therapeutic approach.

The ultimate goal in Diabetes management is to provide the patient with skills & ways to manage their chronic illness as well to provide motivation to sustain the efforts to do so. GHb measurements are helpful in achieving both these goals.



The "Elements" - Glucose & Hemoglobin:

Glucose

Considered to be nature's fuel, glucose is a ready source of energy, since its carbon atoms are easily burnt (oxidized) to form carbon dioxide, releasing energy in the process. The importance of glucose can be well understood by the fact that an average human adult has roughly 5-6 grams (1 teaspoon) of glucose circulating in the blood, which will supply the body's energy needs for roughly 15 minutes. Thereafter the levels must be replenished from internal reserves or external sources.

Structurally glucose exists in both the ring (pyranose) & the linear forms. While it is relatively inactive in the ring form, the free aldehyde linear form can react at a number of sites, especially the amino groups of proteins.

The erythrocytes (RBC) being freely permeable to glucose (not insulin dependent), an elevation of the plasma glucose level leads to a simultaneous elevation of the glucose level within the RBC's.

Hemoglobin

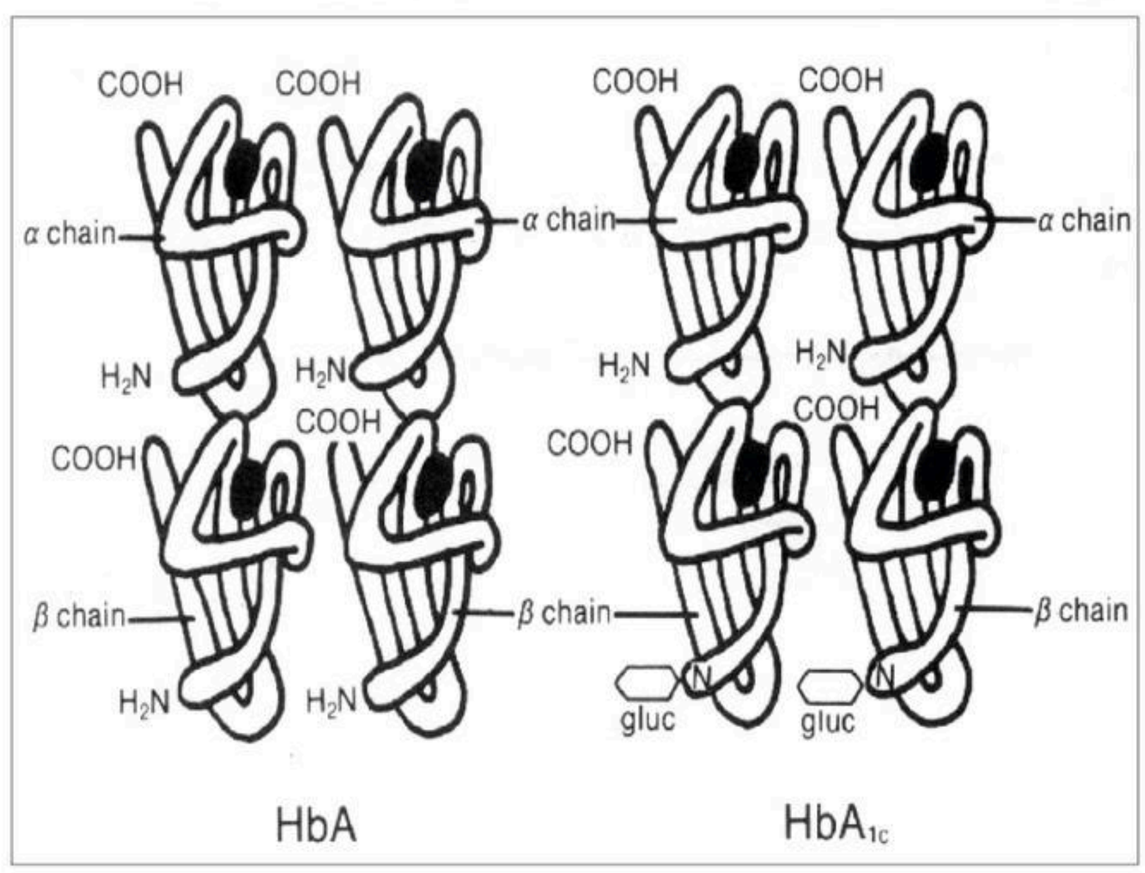
Circulating within the erythrocytes (RBC-average life span of 120 days), hemoglobin (Hb) with a molecular weight of 64,000 daltons, is the primary oxygen carrying vehicle (protein) in the human body.

Structurally the Hb molecule consists of two α & two non α chains, of which, the following variations are found in the healthy adult hemoglobin :

Fetal Hb	-	$2\alpha + 2\gamma$; < 1%	(designated HbF)
Adult Hb	-	$2\alpha + 2\beta$; > 97%	(designated **HbA**)
		$2\alpha + 2\delta$; < 2.5%	(designated HbA2)

The 2 α & 2 β chains of normal **adult hemoglobin (HbA)** are structurally similar, with each chain containing a heme molecule & a series of amino acids (141 & 146 for alpha & beta chain respectively).

Due to the unique molecular structure, the hemoglobin molecule presents a number of reactive sites on the N-terminal valine as well as several lysine residues on both the alpha & the beta chains.



Schematic representation of the hemoglobin with 2 alpha and 2 beta chains

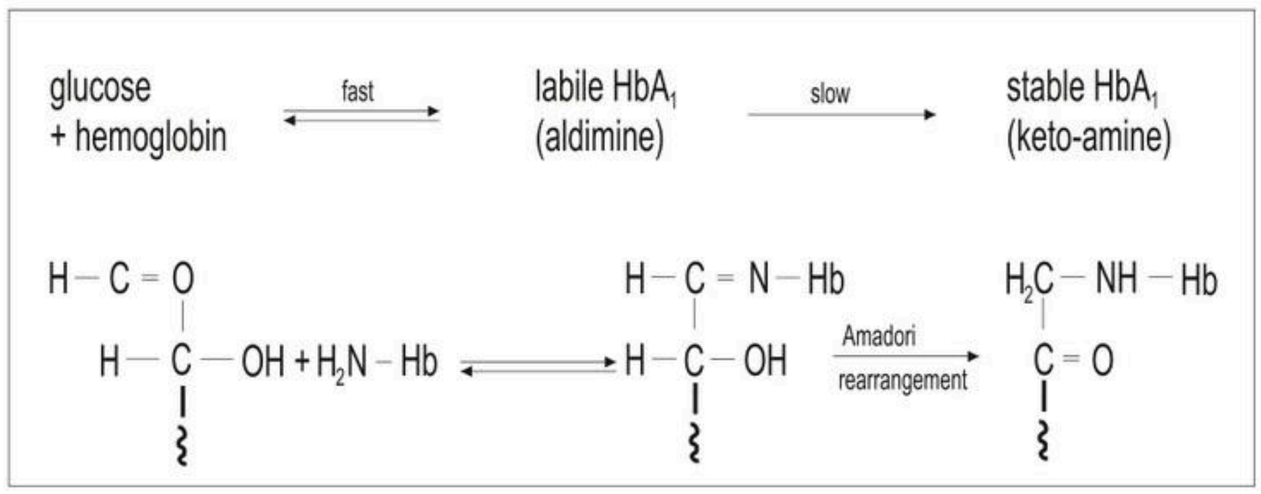
The Process of Glycation

The term GLYCATION is suggested & currently accepted by the IFCC / IUPAC for all such nonenzymatic reactions that link a sugar to a protein or peptide. The product of glycation is a glycoprotein, or, in the special case of the reaction with hemoglobin, glycohemoglobin, or Glycated hemoglobin's **& as such the term Glycated hemoglobin is used throughout in this text.**

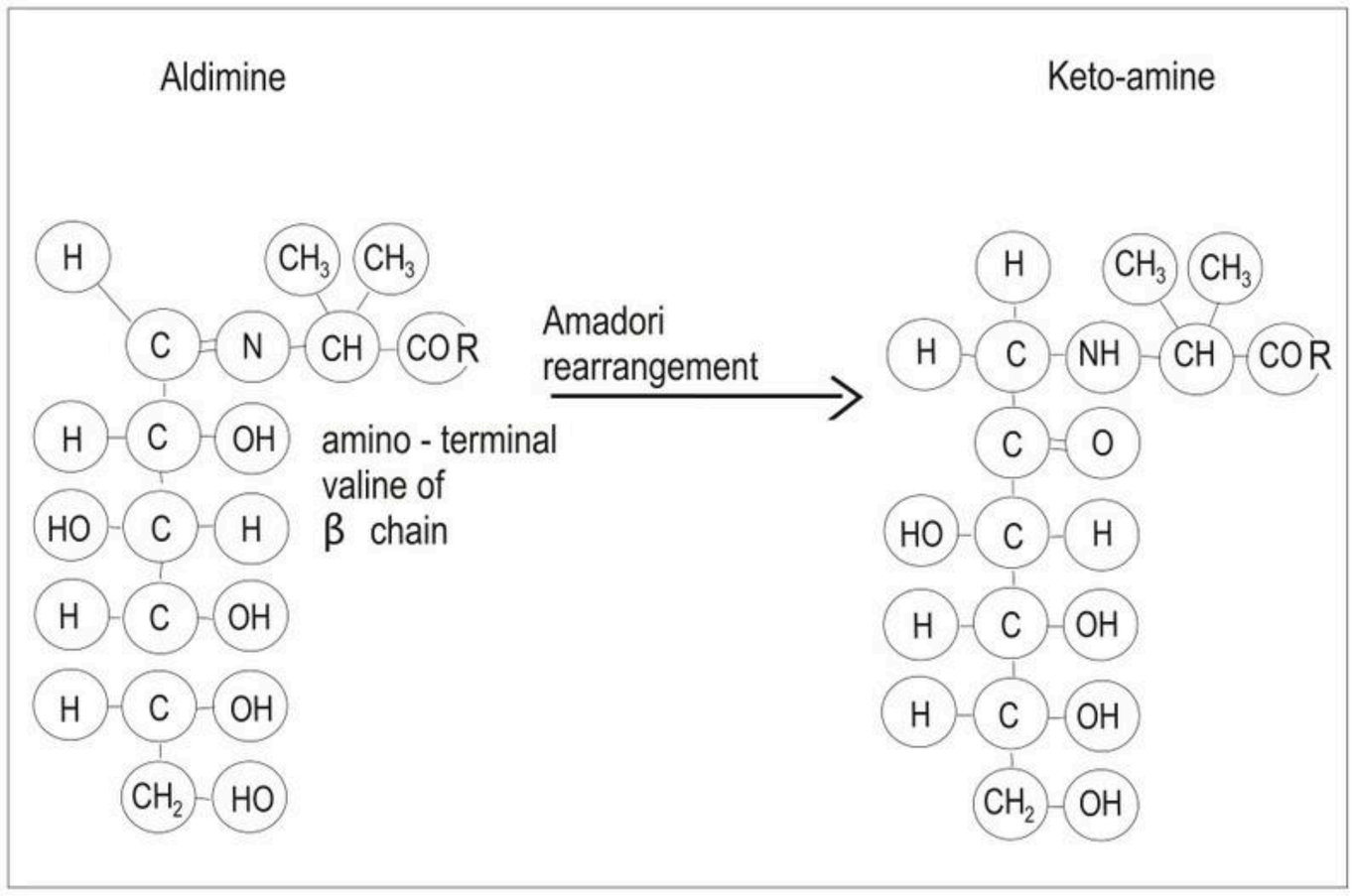
In a normal person glucose circulates in the blood. The erythrocytes are freely permeable to glucose and the concentration in the cell is approximately the same as in the plasma. When the glucose levels are elevated in plasma, they are proportionately elevated in the erythrocytes.

When the glucose is in its free aldehyde form, it can react with the hemoglobin molecule present in the erythrocytes. The free aldehyde group of the glucose attaches to one or both of the N-terminal valine of the β chain or the free amino group of lysine of the hemoglobin molecule to form a Schiff's base or an aldimine. This process is relatively fast and reversible. It depends on the glucose concentration in the erythrocytes. As the glucose molecule has a hydroxyl group at carbon-2, it can undergo a shift in the double bond to carbon-2 via what is known as an Amadori rearrangement to form a keto -amine.

This is a slow process and once formed is relatively irreversible. The stable ketoamine form, with the glucose molecule attached to the hemoglobin remains for the life span of the erythrocyte. This process is called **Glycation**.



The Glycated Hemoglobin (GHb) is formed continuously by the adduction of glucose by covalent bonding to the amino terminal valine of the hemoglobin chain progressively and irreversibly over a period of time and is stable till the life of the erythrocyte. This process is slow, non-enzymatic and is dependent on the average blood glucose concentration over a period of time.



Reaction of glucose with hemoglobin at the amino terminal valine of hemoglobin to form labile and stable adducts.

TYPES OF GLYCATED HEMOGLOBIN

Of the normal Hemoglobin, HbA, the glycated hemoglobin is also referred to as Hemoglobin A1 (HbA1) and the non glycated portion as HbAo. HbA1 comprises of several fractions depending on sugar, which has glycated the hemoglobin.

HbA1a	
HbA1a1	Glycation with Fructose 1, 6 diphosphate
HbA1a2	Glycation with Glucose 6 phosphate
HbA1b	Glycation with an unknown reaction partner
HbA1c	Glycation with D glucose
I HbA1c	denotes the labile HbA1c, or the aldimine fraction
S HbA1c	denotes the stable HbA1c, or the ketoamine fraction

As the Glycated hemoglobin remains in the erythrocytes till their life span of around 120 days and its formation is unaffected by diet, insulin or exercise on the day of testing, it reflects the averaged blood glucose levels over the last several weeks. Thus, GHb reflects on the long term control and status of the carbohydrate metabolism of the past 6-8 weeks.

Methods for the determination of glycated hemoglobin.
These can be broadly classified into the following categories:

- Methods based on charge difference
- Methods based on chemical reactivity
- Methods based on structural differences
- Methods based on immunochemical techniques

1. **Methods based on charge differences**
   These are mainly of two types:
   A. Cation exchange chromatography
   B. Electrophoresis

A. Cation exchange Chromatography
These are mainly of three types:
i. Columns
ii. High Performance Liquid Chromatography (HPLC)
iii. Resin slurry

Glycated Hemoglobin is less positively charged at a neutral pH than HbAo and binds less strongly to a negatively charged resin. Hence it is eluted first and then followed by the HbAo to separate the same. Usually this is done in three ways:

- Columns / micro columns: the hemolysate is applied to a column filled with resin and the elute is collected. The less positively charged HbA1a, HbA1b and HbA1c are eluted before the HbAo. Then their percent to the total Hemoglobin can be determined. The results are affected by temperature, pH, ionic strength and column size.
- HPLC: These systems use a column and elute the different fractions, HbA1a, HbA1b, HbA1c and HbAo successively by using different buffers having different ionic strength and pH. These methods show excellent precision and permit rapid separation of HbA1c. The cost of equipment and its running can only be done by some large laboratories or research institutions.
- Resin slurry method: Here the HbAo is bound to the resin and the HbA1 remains in the supernatant buffer. The resin bound with the HbAo is separated using a resin separator and the absorbance of the HbA1 is measured. The absorbance of the total Hemoglobin is measured separately. The results are calculated by taking the ratio of the sample against a control or standard.

B. Electrophoretic methods
Glycated hemoglobin can also be measured by electrophoresis, or isoelectric focusing. The various hemoglobin components are separated according to their charge differences. These methods are time consuming, require specialized equipment for electrophoresis, densitometer, etc. In the separation of the hemoglobin there is interference by aldimine and HbF.

2. **Methods based on chemical reactivity**
   These are colorimetric methods using thiobarbituric acid. The sugar attached to the hemoglobin is converted to hydroxymethylfurfural (HMF). The process requires treatment with heat and acid and is quantified by the color formed with thiobarbituric acid. The method is time consuming and as the free glucose of the sample interferes it has to be first removed before testing. Results are expressed as nanomoles of HMF / mg of Hb or fructose equivalent /mg of Hb. Colorimetric analysis gives rise to falsely elevated values unless specimens are handled appropriately. The presence of free glucose with hemoglobin also adversely affects the assay.

3. **Methods based on structural differences**
   In these methods the determination of GHb is done by affinity chromatography. The test is normally done with micro columns which have a resin/gel charged with M-aminophenylboronic acid. The sugar portion of the glycosylated hemoglobin has an affinity for the boronic acid and binds with the same. The HbAo is first eluted using a buffer and then the HbA1 is eluted with a buffer containing sorbitol which has a greater affinity to the boronic acid and displaces the HbA1, which then elutes out. Some of these methods also have buffers which only displace out the HbA1c. These methods are sensitive to gel properties such as ligand concentration.

4. **Methods based on Immunochemical techniques**
   These are recent methods being used on fully automatic systems based on the immunoturbidimetric principles. A hemolysate of the sample is added to the reagent containing latex particles coated with an antibody to HbA1c and the amount of turbidity formed due to the binding of the antibody and the antigen (HbA1c) is measured colorimetrically at around 550nm. In some tests a further reagent is added which contains other antibodies or an agglutinator which binds all the hemoglobin's and further rise in turbidity is measured. The ratio of HbA1c to total hemoglobin is then calculated.

Glycated hemoglobin determination is useful in monitoring the long term status of the carbohydrate metabolism or the glucose level in the body. It helps the clinician in monitoring the status of the patient especially in the maintenance programme of improved glycemic control. The GHb test can also be used as a diagnostic assay for the detection of TYPE-1 DM, as normally these patients are hyperglycemic and their GHb levels are usually high. (Though it must be kept in mind that the **GTT is a more definitive test.)**

CLINICAL IMPORTANCE OF THE GHb DETERMINATION

- Evaluation of widely varying blood sugar values

- Evaluation of glucose values in doubtful cases:
  - checks performed by the patient are unsatisfactory
  - unreliable patient non compliance
  - diagnosis of diabetics without any previous clinical symptoms
  - short term illness of the patient

- Support & control of the checks performed by the patient
  - encouragement towards achieving the glycemic targets
  - it is a parameter for the therapeutic monitoring for the patient
  - The measurement excludes incorrect interpretations etc.
  - it is a long term retrospective control of metabolic measurement independent of the actual blood sugar concentration.

The only factors on which the test is dependent are
- the circulating levels of blood glucose &
- the life of the erythrocytes

CLINICAL INTERPRETATION OF GHb TEST:

* Subjects with normal metabolism - 5 8% GHb
* Suspect range - 8 - 10% GHb
* Unsatisfactory control - > 10% GHb

Relation Of GHb Values to that of the mean blood glucose

Degree of Glucose Control	GHbA1	HbA1c	MBG/dl
Normal (non diabetic)	⩽ 7 %	< 6 %	<114
Near (Borderline) Normoglycemic but targets	7 - 8 %	6 - 7 %	114 - 147
Diabetics, but, in good control	8 - 9 %	7 - 8 %	147 - 180
Actions suggested	9 - 11 %	8 - 9 %	180 - 214
Diabetics & not at all in control	> 11 %	> 9 %	> 214

The MBG (mean blood glucose) values above are arrived at by using the formula used in the study done by **Nathan et al.**

MBG in mg/dl = 33.3 x HbA1c value - 86
It is however to be noted that the relationship in the above formula is linear in the range of HbA1c values falling between 6.5 - 13%.

Significance of GHb measurement

- A single fasting blood glucose measurement only gives an indication of the patients immediate past (last 2-4 hours at most) condition & is dependent on many factors. It may not necessarily represent the true status of the blood glucose regulation.

- In contrast the level of glycated hemoglobin (GHb) is directly related to the average plasma glucose concentration and the life span of the RBC's and thus hemoglobin in the circulation and is independent of any other factor.

Conclusive evidence towards the reduction of chronic risks of unmonitored DM, through regular GHb estimation

The Projects

Study	DM type	No/Subjects	Mean Age	Period of Assessment
DCCT	TYPE-1	1441	27 years	10 years
UKPDS	TYPE-2	3867	54 years	5 years

Objectives

The above two multicenter randomized clinical trials were designed to compare the benefits / risk reduction of long term chronic complications of DM, with intensive therapy, compared to those given the conventional management of TYPE-1 & TYPE-2 DM, respectively.

RESULTS

Estimating GHb values regularly & maintaining them in the normoglycemic range in those receiving the intensive therapy showed dramatic results as under:

Relative risk reduction for chronic diabetic complications

RISK	DCCT	UKPDS
Retinopathy	reduced by 63%	reduced by 21%
Proteinuria	reduced by 54%	reduced by 34%
Neuropathy	reduced by 60%	reduced by 40%
Coronary Artery Disease	reduced by 41%	reduced by 16%

Frequency (need based assessment) of GHb testing:

Nature of Patient	Recommended GHb testing frequency
Controlled diabetic patient	once every three months
Diabetic pregnant woman	once a month
Patient whose diabetes regimen has been altered to improve control or in whom evidence is present that intercurrent events may have altered a previously satisfactory level of control	once every 1 - 2 months
For uncontrolled TYPE-1 / TYPE 2 diabetics	more than four times a year

(Source; American Association of Clinical Endocrinologists guidelines for the management of DM)

Factors that interfere with GHb Test results

- **Hemoglobin Variants and Derivatives**
  Genetic variants (e.g. HbS trait, HbC trait) and chemically modified derivatives of hemoglobin (e.g. carbamylated Hb in patients with renal failure, acetylated Hb in patients taking large amounts of aspirin) can affect the accuracy of GHb measurements. The effects vary depending on the specific Hb variant or derivative and the specific GHb method. When selecting an assay method, laboratories should take into consideration characteristics of the patient population served, (i.e., high prevalence of hemoglobinopathies or renal failure).

- **Shortened Erythrocyte Survival**
  Any condition that shortens erythrocyte survival or decreases mean erythrocyte age (e.g., recovery from acute blood loss, hemolytic anemia) will falsely lower GHb test results regardless of the assay method used.

GHb results from patients with HbSS, HbCC, and HbSC must be interpreted with caution given the pathological processes, including anemia, increased red cell turnover, transfusion requirements, that adversely impact GHb as a marker of long-term glycemic control. Alternative forms of testing such as glycated serum protein (fructosamine) should be considered for these patients.

In patients with renal failure, GHb has been reported to be elevated despite the fact that RBC survival rate may be decreased in patients with uremia. Carbamylation reaction appears to account for some of the change in the physical properties of the hemoglobin in uremia. Cyanate, a breakdown product of urea, can react with amino groups in an irreversible fashion & thus change chromatographic properties.

It is now known that in a diabetic population, there are a proportion of people who appear to glycate hemoglobin at a faster or slower rate than most others. What it means is that **two individuals with the same degree of glucose tolerance might well have their glycated hemoglobin values differing by 2%, which is substantial.** Originally, it was thought that differences in inter-individual tissue glycation was the reason, but recent studies have data that establishes that **the high glycators seem to have red blood cells that survive longer than the low glycators. The changes in the GHb levels appear to be related to the red blood cell life rather than glycemia or glycation rates.**

- **Alcohol consumption**
  Acetaldehyde, the major breakdown product of alcohol, has been shown to change the chromatographic pattern of hemoglobin. Thus **patients consuming large amounts of alcohol may have falsely elevated levels of GHb.**

- **Other factors**
  Vitamins C and E are reported to falsely lower test results, possibly by inhibiting glycation of hemoglobin; vitamin C may increase values with some assays. Iron-deficiency anemia is reported to increase test results. Hypertriglyceridemia, hyperbilirubinemia, uremia, chronic alcoholism, chronic ingestion of salicylates, and opiate addiction are reported to interfere with some assay methods, falsely elevating the results.

Other Glycated Proteins

Serum proteins with shorter half lives (2-3 weeks) such as **albumin** also become heavily glycated in the presence of persistently elevated glucose levels. Thus total glycated serum proteins (GSPs) & glycated serum albumin (GSA) can provide an estimation of **glycemic control over the 14 - 20 days.**

Fructosamine Test

Fructosamine is the generic name for plasma protein ketoamines. The name refers to the structure of the ketoamine rearrangement product formed by the interaction of glucose with the epsilon amino group on the lysine residues of the albumin. Circulating half-life for albumin being roughly 20 days, the concentration of glycated albumin reflects glucose control over a period of 2 - 3 weeks. Although the assay can be automated, gives good precision, there is till date a lack of consensus on its clinical utility due to:

- Apparent lack of specificity for glycated proteins
- Lack of standardization among laboratories
- Difficulty in calibrating the assay
- Interferences by urates & hyperlipidaemia

As per the recommendations of the National Medicare Programme for Diabetes (USA), Glycated protein testing may be used in place of glycated hemoglobin in the management of diabetic patients who have abnormalities of erythrocytes such as hemolytic anemia or hemoglobinopathies. In such cases it is conceivable that a patient will have both a GHb and GSP ordered on the same day. However this should be limited to the initial assay of GHb, with subsequent exclusive use of GSP. Moreover, clinicians should be aware of two important points concerning the use of GSP's & GSA as measures of long-term glycemic control:

- These tests assess the degree of glycemic control over a period of 2 weeks as opposed to 2 - 3 months with GHb.
- Neither test has been correlated with the development of long-term complications from diabetes mellitus as was shown with GHb in the DCCT and with the UKPDS.

GUIDELINES FOR LONG TERM GLYCEMIC CONTROL

In light of the substantial evidence now available in support of tighter glycemic control it is today possible to optimize reduction of microvascular & macrovascular complications of diabetes. Surprisingly though, despite the widespread use of self monitoring of blood glucose, **patients do not know their diabetes status & goals. It has also been found and established that in a vast majority of populations, diabetes was present long before diagnosis was made.**

As a consequence of the enormity & gravity of the situation, the following issues were raised, discussed & clarified, as being the **global guidelines of long term Glycemic control:**

1) what is the goal of diabetes management?
2) to what extent glycemic control attains that goal?
3) what factors should be used to assess glycemic control?
4) what are the guidelines for the attainment for glycemic control?
5) what further recommendations are needed regarding glycemic control & reduction of complications of diabetes?

The goal of diabetes management is the prevention of acute & chronic complications of diabetes mellitus. Traditional complications of DM are viewed as microvascular complications of diabetes including retinopathy, nephropathy, & neuropathy.

Reductions in hyperglycemia significantly decrease the microvascular complications of diabetes. Prevention of eye and kidney damage by intensive diabetes management in the DCCT & Kumamoto study reduced the incidence of these complications by as much as 50 - 70 %. All trials demonstrated a 30 - 35 % reduction in microvascular complications per 1% absolute reduction of glycated hemoglobin.

It was the consensus that glycemic control be assessed by the periodic measurement of glycated hemoglobin.

Fasting glycemia (FPG) can measure the glycemic burden only at a single point in time & does not accurately reflect the overall glycemic control. Of course, with the advent of self monitoring technology, assessments of fasting & pre-prandial plasma glucose levels have evolved into an important element of day to day decision making in the routine management of diabetes.

Post-prandial hyperglycemia is a key component of the total glycemic burden in patient with diabetes; it is an important contributor to the Glycated Hemoglobin levels, which can be viewed as reflecting the summation of both preprandial & postprandial glycemia. Thus for maximum reduction of Glycated Hemoglobin levels, assessment of both pre-prandial & post-prandial glucose levels are necessary as part of diabetes management.

Studies from the DCCT & UKPDS analysis of data have implicated the association of high Glycated Hemoglobin levels with the development of complications of diabetes, especially atherosclerosis. It has thus been recommended that **Glycated Hemoglobin be universally adopted as the primary method of assessment of glycemic control.**

It is also **recommended that the Glycated Hemoglobin estimations be performed at least twice a year in patients who are a target**. Assessments should be made quarterly, or more frequently in patients who are above target, who are undergoing a change in therapy, or both. Patients with diabetes should be identified as early as possible in their illness. Current recommendations are towards targeted screening for populations at high risk for the development of diabetes. Risk factors include:

- Family history of diabetes
- Cardiovascular disease
- Overweight
- Sedentary lifestyle
- Latin/Hispanic/African/ Asian ethnicity
- Previously identified impaired GTT, or IFG
- Hypertension
- Increased levels of triglycerides, low concentrations of HDLC
- History of gestational diabetes
- Delivery of a baby weighing more than 4 kilograms
- Polycystic ovary syndromes.

The high frequency of complications at the time of diagnosis of diabetes makes earlier diagnosis imperative. It is also seen that within certain ethnic groups the onset of diabetes is at a much younger age within the general population. Thus, it is recommended that targeted case finding in high risk persons 30 years of age or older is undertaken.

CONCLUSION

In conclusion, it may be argued that even though glycemic targets set are perhaps "unrealistic" & not quite achievable by most, it however contradicts the clinical judgement that **patients who achieve ANY reduction in their levels of Glycated Hemoglobin, significantly decrease the risk for complications of diabetes regardless of their ability to achieve the specific glycemic targets** recommended by the various panels.

Therefore, it is now accepted that setting of clinical standards should reflect the best estimate of maximal benefit that may accrue from that care & should not depend on the ease or convenience of attaining such targets for either patients or clinicians.

Most importantly it is now sincerely believed that establishing glycemic targets would assist & encourage patients & clinicians strive towards achieving normoglycemia; that in itself would result in improved health, augmented longevity & an enhanced quality of life.

These, after all, are the goals of diabetes management.

GLOSSARY

Diabetes Insipidus
Condition leading to polyuria & polydipsia caused by the deficiency of Anti Diuretic Hormone (ADH), usually due to trauma or tumour involving posterior pituitary.

Gestational Diabetes Mellitus (GDM)
Defined as any degree of glucose intolerance with onset or first recognition of pregnancy. In majority of the cases of GDM, the glucose levels will return to normal levels after delivery.GDM complicates only around 4% of all pregnancy cases.

Impaired Glucose Tolerance (IGT)
Identifies a group of individuals whose response to the ingestion of a glucose load lies between normal & diabetic responses. A portion of these subjects eventually becomes diabetic, but some remain stable & few revert back to normal.

Glycation
The term currently accepted by the IFCC & IUPAC for any non enzymatic attachment of a carbohydrate moiety to a protein molecule.

HbA
The major form of adult hemoglobin comprising between 95-97% of the total hemoglobin.

HbAo
The major component of normal adult hemoglobin, which is the labile non Glycated portion , separated out during the GHb assay.

HbA1
More negatively charged, post- translationally modified, Glycated form of HBA,
Comprising of the following chromatographically distinct stable fractions: HBA1a / HBA1a2 / HBA1b / HBA1c

HbA1c
The IFCC defines HBA1c as the stable ketoamine form (adduct) of gluose to N-terminal valine of the β chain of the hemoglobin.

Variant
The normal genetic composition of the Hb tetratmer is $\alpha 2\beta 2$ & to a lesser extent (3-4%) $\alpha 2\delta 2$ (HBA2),and $\alpha 2\gamma 2$ (HbF) <1%. Any hemoglobin types other than these are called variants. Majority of variants arise from point mutations of either α , β, γ or the δ Hb chains. Till date more than 850 variants have been identified.

DCCT
Diabetes control & complications trial; a landmark multicenter evaluation of Glycemic control carried out over a period of ten years in the United States.

UKPDS
United Kingdom Prospective Diabetes Study; another landmark evaluation of long term Glycemic control, carried out in U.K. over a period of five years.

DN
Characterized by marked hyaline thickening of the glomerular basement membrane as a result of deposition of proteins or glycoproteins from plasma, Diabetic Nephropathy, refers to damage of the glomerulus (filtering apparatus of the nephron) & capillaries associated with the nephron, leading to a reduction in the filtering capability of the kidneys & presenting a clinical picture of marked Proteinuria (> 3.5gm/day),red blood cell cast,oedema, & hypertension. DN develops in 30-40% cases of TYPE-1, & 10 - 20% of cases with TYPE-2 DM, and is the single, most common cause of end stage renal disease (ESRD). There is a correlation between diffuse diabetic glomerulosclerosis & deterioration of renal function determined by increased Diastolic blood pressure, proteinuria, blood urea, & creatinine with corresponding decrease in serum albumin, urea & creatinine clearances.

CAD
The pathogenesis of Coronary Artery Diseases (CAD) in DM is not entirely clear & conventional risk factors such as smoking, obesity, blood pressure, serum lipids fail to fully explain this. Important features in the pathogenesis of CAD appear to include vascular endothelial injury, platelet adhesion and activation, fibrin deposition, cellular proliferation & LDL cholesterol accumulation. Therefore disturbances of haemostasis due to DM leading to accelerated fibrin formation (hypercoagubality), & delayed fibrin removal (impaired fibrinolysis) may contribute to CAD. Hyperactive platelets, hypercoagubility & impaired fibrinolysis also promote thrombosis formation at the site of a ruptured atherosclerotic lesion leading to the final occlusion event in the progression of CAD.

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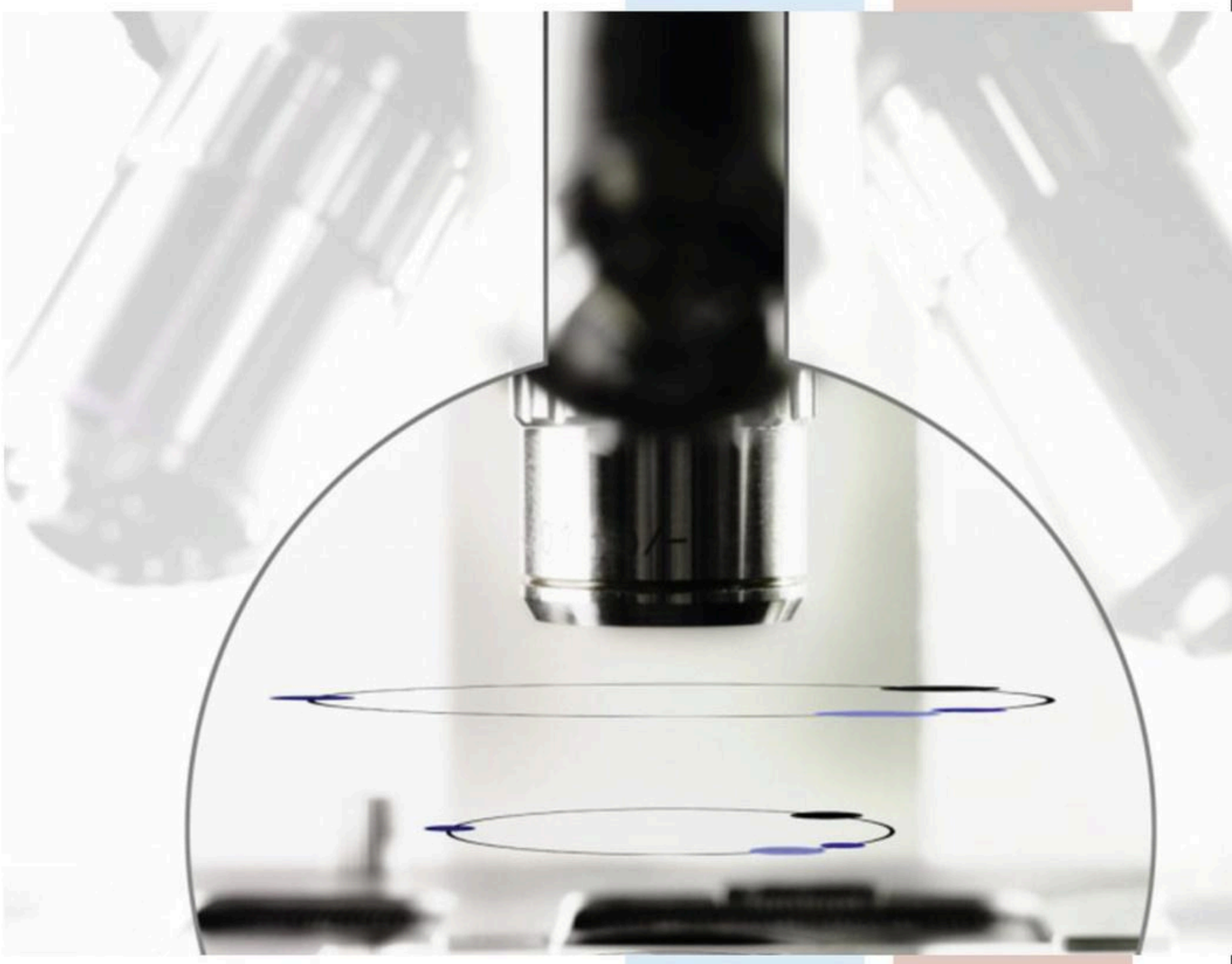
MICROSCOPY STAINS



- **Microkrom Microscopy Stains**
  -Basic Guidelines for
  Quality Staining with
  Romanowsky Stains .....Pgs 208-219

KNOWLEDGE BOOK

For the Laboratorians

Microkrom Microscopy Stains

Basic Guideline for Quality Staining with Romanowsky Stains

Setting Trends...

Introduction

In microscopic examination of blood smears for blood cell morphology or parasite examination there are a few important parameters that essentially play vital role in blood smear staining.

These parameters being,

- Clean and grease free glass slide
- Good microscope
- Technique for preparing the smears
- Quality of the stains used
- Maintaining appropriate pH by using buffered water to attain a good staining reaction and better differentiation
- Care of glassware and microscope
- Expertise of the person in evaluating the results

All the above mentioned parameters have to be optimized so that the quality of smears, so achieved will lead to better staining and differentiation.

Cleaning and storing microscopic slides

Microscope slides are usually supplied in boxes. They may be described on the box as "washed" or" pre-cleaned", but they need to be properly washed, dried and wrapped. It is not possible to make good quality films on dirty microscope slides.

Blood films made on dirty or greasy slides will wash off easily during staining

It is therefore best to discard slides that,

- Have rainbow like colour or appear white or opaque
- Are not properly cleaned
- Are old, with surface scratches or chipped edges

Cleaning slides

In order to clean the slides, the following items are suggested.

- A large plastic basin
- Gauze or cotton wool
- A good quality detergent
- Clean cotton cloth
- Clean water

Cleaning new slides

- All new slides should be washed with detergent and clean water
- After being soaked for 30 minutes to 60 minutes, the slides should be rinsed under running tap water or in several changes of clean water
- Each slide should be individually wiped dry and polished with clean, dry cotton.

Cleaned slides should be handled only by the edges to avoid finger marks or grease being deposited on the slide

Cleaning of used slides

- Used, dirty slides should be soaked for a day or two in water containing detergent (warm water should be used whenever possible)
- After soaking, the slides should be cleaned one by one with a small piece of gauze or cotton wool

All traces of blood film and oil (used during microscopy) should be removed from the slides

- ***Caution: Do not leave the slides in detergent for too long, soaking should be for a few days only, not weeks. If slides are left in the detergent solution for too long period, water will evaporate, leaving a deposit on exposed slides that is impossible to remove.***
- After cleaning, the slides should be transferred to a fresh solution of detergent and later rinsed under running water or in several changes of water
- Each slide should be individually wiped dry and polished with clean, dry cotton cloth

Wrapping cleaned slides

Items required for wrapping cleaned slides

- Sheets of thin, clean paper
- Empty cardboard slide boxes
- Rubber bands or adhesive tapes

  Cleaned slides may be wrapped in packs of 10
  Each pack may be secured with adhesive tape or rubber band
  Packs may then be placed in the cardboard slide boxes

Storage of clean slides

- Slides should be stored in a dry place
- If stored at room temperature with high humidity, the slides will stick together after a few weeks. As a result they cannot be used unless they are re-washed and dried.

Care of the microscope

The microscope is an expensive instrument, which if properly maintained and taken care will be useful for many years.
The following are the important points that need to be considered,

Remove dust and grease

1. When not in use during the day, the microscope should be kept covered with a clean cloth or plastic cover to protect the lenses from dust that settles out of the air.
2. When the microscope is to remain unused for long periods or to be stored overnight, it should be placed inside the box with door tightly closed. To protect the objective lenses, the x4 objective should be rotated to line up with the ocular.
3. Oil and grease from eyelashes and fingers are easily deposited on the lenses and oculars. These parts should be cleaned with very soft cotton cloth.
4. The oil immersion objective should be cleaned after use. If it is not cleaned, the oil will harden and make the objective ineffective. A soft cotton cloth is usually sufficient for cleaning. However the same cloth should never be used to clean the other objectives, the oculars or the mirror, otherwise oil will be transferred to these components. From time to time, dried oil should be removed from the oil immersion objective using xylene. Oil can be washed off the slides with a small amount of xylene, if no xylene is available, the oil smear can be carefully removed by using absorbent paper.

Preventing the growth of fungus

In warm, humid climates it is easy for fungal growths to be established on lenses and prisms. These growths can cause problems and may even become so bad that the microscope can no longer be used.
Fungus cannot grow on glass when the atmosphere is dry, and every effort should be made to store the microscope in a dry atmosphere when it is not being used.
The following are preventive measures that can be adopted,

1. Keep the microscope in a continuously air conditioned room.
2. Place the microscope in a "warm cupboard" (air tight cupboard) in which one or two 25 - watt bulbs are constantly glowing.
3. Keep all the lenses and prism heads in airtight box or dessicator where the air is kept dry by means of silica gel.

Preparation of Blood films or Smears

Examining a stained blood smear or film is a routine part of the technologist to view the elements of blood or components of the blood.
The procedure of studying the blood film involves,

- Preparing a blood film on a microscope slide
- Drying the film (to prevent distortion and washing away of cells)
- Fixing the film
- Staining the film
- Finally the cellular components of blood are observed under microscope

The main objectives of fixation are,

- To kill the cells suddenly and uniformly so that they retain the same appearance which they possessed in living condition
- To preserve the cells by the inhibition of autolytic changes
- To set and hold the intracellular components, by precipitation in the position which they occupied in living condition. Hence facilitating the closest possible study of the cells
- To render the cells and its components resistant to subsequent process such as dehydration, embedding, clearing prior to examination under microscope
- To facilitate differentiation in the refractive indices of certain cell elements which would otherwise be invisible owing to the narrow margin between the refractive index of one type of cell with other type
- To facilitate proper staining

Techniques for estimation of Blood films

Examination of a fixed and stained blood film is an essential part of a haematological investigation. Ideally the blood films must be well spread, well fixed and well stained for microscopic observation.
The films or smears are of two types,

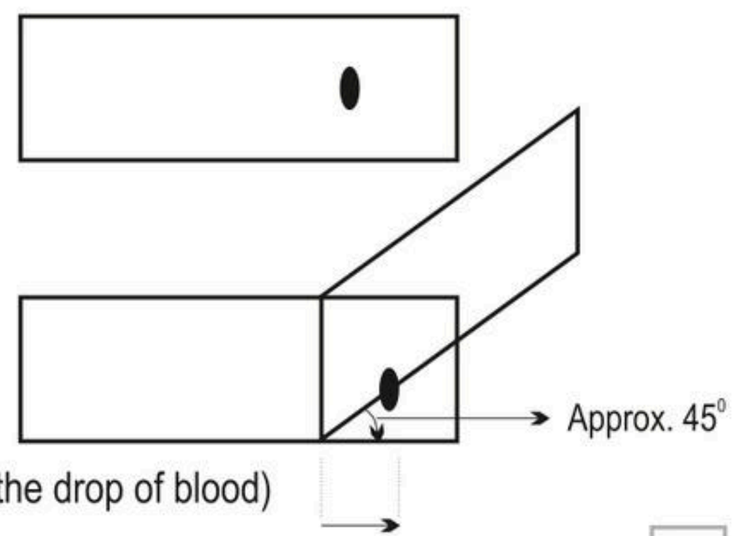
- Thin blood film or smear
- Thick blood film or smear

Preparation of thin blood films

Use clean, grease and moisture free glass slides for accurate diagnosis

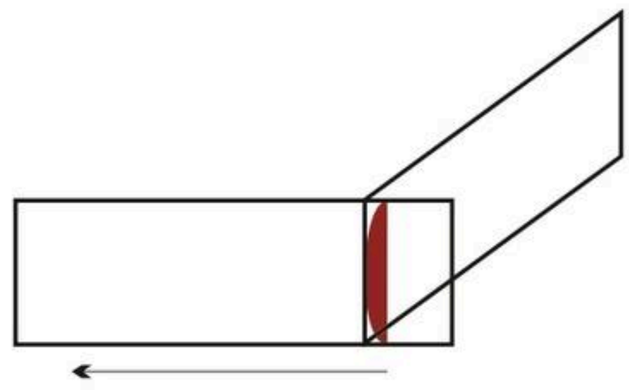
- Place a drop of blood, approximately 3 mm diameter on the slide one-third distance away from the labelled end.

- Without delay, place a glass slide or spreader at an angle of 45^0 to the slide and move it back to make contact with the drop. The drop should spread out quickly along the line of contact of the spreader with the slide.

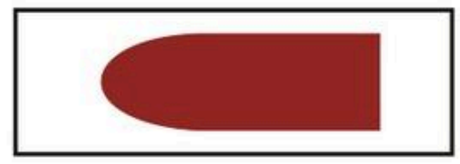


(Move the spreader till it touches the drop of blood)

- The moment this occurs spread the film by a rapid, smooth, forward movement of the spreader. (Spread the film)



- A thin film appears as follows



- Air-dry the film thoroughly.

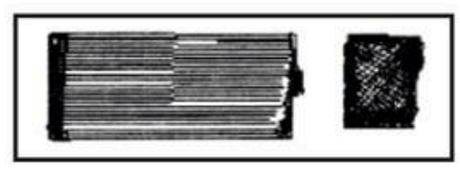
Preparation of thick blood films or smears

- Add approximately 5 µl of blood on a clean slide
- With the end of applicator stick or a corner of slide, quickly distribute the blood to make a even thick film about 1cm^2
- Allow the thick film to air dry

Common faults in making thin and thick blood films

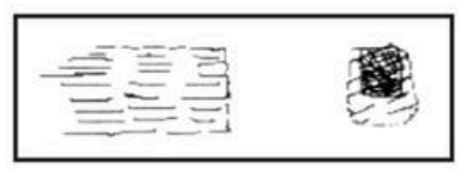
- *Badly positioned blood films*
  Care should be taken that the blood films are correctly sited on the slide for thick or thin film. Improper positioning of the blood drop will lead to difficulty in examination of the film.

- *Too much blood*
  After staining films made with too much blood, the background of the thick film will be excessively blue in colour thereby leading to improper estimation especially in identification of parasites. If the thin film is too thick, red blood cells should be on top of one another and it will be impossible to examine them properly after fixation.

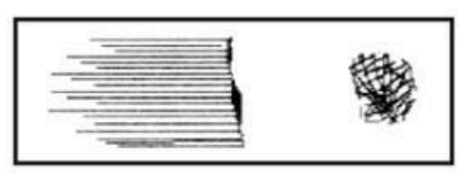


- *Too little blood*
  If too little blood is used to make the films (for thin film preparation the drop of blood should be approximately 3 mm in diameter and for thick film approximately 5 µl of blood) there will not be enough white cells in the thick film leading to improper examination. Also the thin film may be too small for identification.

- *Blood films spread on greasy slides*
  The blood films will spread unevenly on a greasy slide, which makes examination very difficult. Some of the thick film will probably come off the slide during staining process.



- *Edge of spreader slide chipped*
  When the edge of the spreader slide is chipped, the thin film spreads unevenly and has many "tails". The spreading to the thick film may also be uneven.

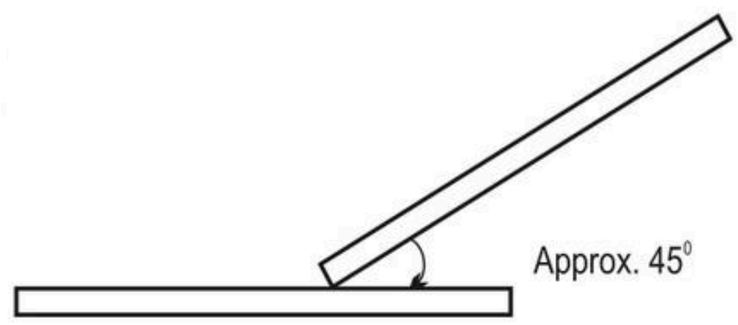


Other common faults

- Blood films are made on badly scratched slides
- The thick film is allowed to dry unevenly.
- Auto fixation of thick film occurs with the passage of time through exposure to heat, and then staining becomes difficult or unsatisfactory
- Slides are wrapped together before all the thick films are properly dried, and slides stick to one another.

Thus the necessary precautions that need to be ascertained inorder to attain a good smear are,

- The glass slide must be clean, grease and moisture free
- Usage of appropriate drop size for preparing the respective smears
- Make the smear immediately after putting a drop of blood on the slide (a delay will cause uneven distribution of white cells on the smear)
- The spreader or the spreader slide must be moved steadily, without any jerky movements. Since loss of contact between the spreader slide and blood will lead to poor smears
- The angle of holding the spreader slide essentially determines the thickness of the smear. The approximate angle should be around 45^0. A greater angle will result in too thick a smear, too thin if a smaller angle is employed



- The blood smear should be made preferably immediately after blood collection but certainly not later than four hours of blood collection. Inability to do the smear within four hours will result in distortion of red blood cells and cause hemolysis.
- As far as possible films should be stained as soon as they are air- dried. They certainly should not be left unfixed. If the films are left unfixed at room temperature for a day or more, it may be found that, in addition to distortion of morphology, the background of dried plasma stains a pale blue colour that is impossible to remove thereby spoiling the staining of the blood cells.

Commonly used Romanowsky stains

Stains are primarily chemicals that react with blood components (cellular) at a particular pH and aid in identification of the blood dyscrasias and parasite estimation thereby helping the clinician to diagnose disease or underlying pathology, initiate a therapy and also help in prognosis.
The mechanism by which certain component of a cell structure stain with particular dye and other component fail to do so depends upon,

1. The chemical composition of various cellular organelles.
2. The binding of the dye molecule and chemical components of the cell organelles.

Generally, the acidic groups of the nucleic acid, proteins of the cell nuclei react with the basic component of the dye. Similarly the granules in the cytoplasm are alkaline in nature and react with the acidic component of the dye.
For any chemical reaction to occur one of the most important factor is pH. A pH of 6.8 is usually recommended for general use. When looking for malaria parasites a pH of 7.2 is recommended inorder to see Schuffner's dots.
Inorder that the staining is attained properly buffered water is used which maintains a correct pH to ensure proper staining reaction and blood films can be seen clearly under the microscope with good differentiation.
Romanosky stains are universally employed for staining blood films. Giemsa and Leishman stains are types of Romanosky stain, which is utilized for blood smear staining.
Field stain is used for rapid identification especially for thick film in malarial parasite estimation.

Staining procedure of thin blood films

With Giemsa stain

- Fix the blood smear with methanol for 3-5 min
- Prepare a 10% solution of Giemsa stain with buffered water
- Stain the slides in diluted stain for 30-45 minutes
- Rinse the slide in buffered water
- Blot dry, or air dry in a vertical position

Freshly diluted stains should be used for each day's work. Prepare sufficient amount of one day's use of diluted stain, since the diluted stains are not stable overnight.

With Leishman's stain

- Prepare a thin film and air dry the film
- Flood the slide with Leishman stain (e.g. x ml) on the air dried smear and spread over the film, which acts as a initial fixative
- Add double the volume of buffered (e.g. 2 x ml) and stain the film for approximately 5-7 min. Take care not to allow the stain to dry up during the time
- Then wash it in a stream of buffered water until it has acquired a pinkish tinge (upto 2 min)
- And dry the slide in a vertical position

Washing is done with buffered water because pH must be carefully maintained in Leishman stain inorder to attain a proper staining reaction.

With Field's stain

- Prepare a thin blood film
- Fix blood film in methanol for one minute
- Wash off methanol with tap water
- Cover the blood film with Field's stain B
- Immediately add an equal volume of Field's stain A
- Mix well and stain for one minute
- Rinse the slide with tap water, and place upright to drain and dry

Staining procedure of thick blood films

With Giemsa stain

- Prepare a thick blood film
- Prepare a 10% solution of Giemsa stain in buffered water
- Apply the stain to the unfixed thick smear and stain for 30 minutes
- Remove the slides and wash in buffered water
- Air dry the film

With Field's stain

Requirements

- One staining dish filled with Field's stain A
- One staining dish filled with Field's stain B
- Two dishes filled with neutral Distilled water

Method

1. Dip the unfixed air-dried slide into Field's stain A for 3 seconds
2. Wash gently by dipping (once) into neutral distilled water
3. Dip into Field's stain B for 3 seconds
4. Wash as in step 2
5. Place the slide upright in a draining rack to air dry

Depending upon the moisture/humidity in each laboratory the staining time may be required to be fine tuned further to optimize results.

Artefacts

Blood films may contain many features that can cause confusion and problems in diagnosis. Such features are known as artefacts.

- Fungus will show up artefacts on blood films. The best way to prevent fungal growths on slides is to stain blood films as soon as possible after making and drying them **within 48 hours at most**
- Dust particles floating in air will settle on blood films while they are drying, either before or after staining. Specks of dirt may be transferred from a patient's finger when the blood sample is taken, or the original slide may not be clean. **Hence it is always advisable to take a clean slide and hold the slide by its edges**
- Precipitated stain suspensions super-imposed upon red blood cells may cause confusion
- Debris from the patient's skin, also may produce a source of confusion
- Algae and other organisms may grow in staining solutions which have become contaminated and may be introduced in a well produced film in the staining stage.

Colour responses of blood cells to Romanowsky staining in thin blood films*

Cellular component	Colour
Nuclei	
Chromatin	Purple
Nucleoli	Light blue
Cytoplasm	
Erythrocytes	Dark blue
Reticulocytes	Pink
Lymphocytes	Blue
Monocytes	Grey blue
Neutrophils	Pink
Basophils	Blue
Granules	
Basophil	Purple black
Eosinophil	Red orange
Neutrophil	Purple
Platelet	Purple

*Table from book Practical Hematology by Sir John V. Dacie / S.M. Lewis, 8th Edition, 1995

Basic precautions to be taken in handling and storage of stain solutions

What you should do

- When the bottle of the stain solution is not being used, keep the screw capped tightly to prevent the evaporation of solvent and oxidation of the stain
- Keep the stain bottle away from sunlight
- Store the solution in cool dry place at all times

What you should not do

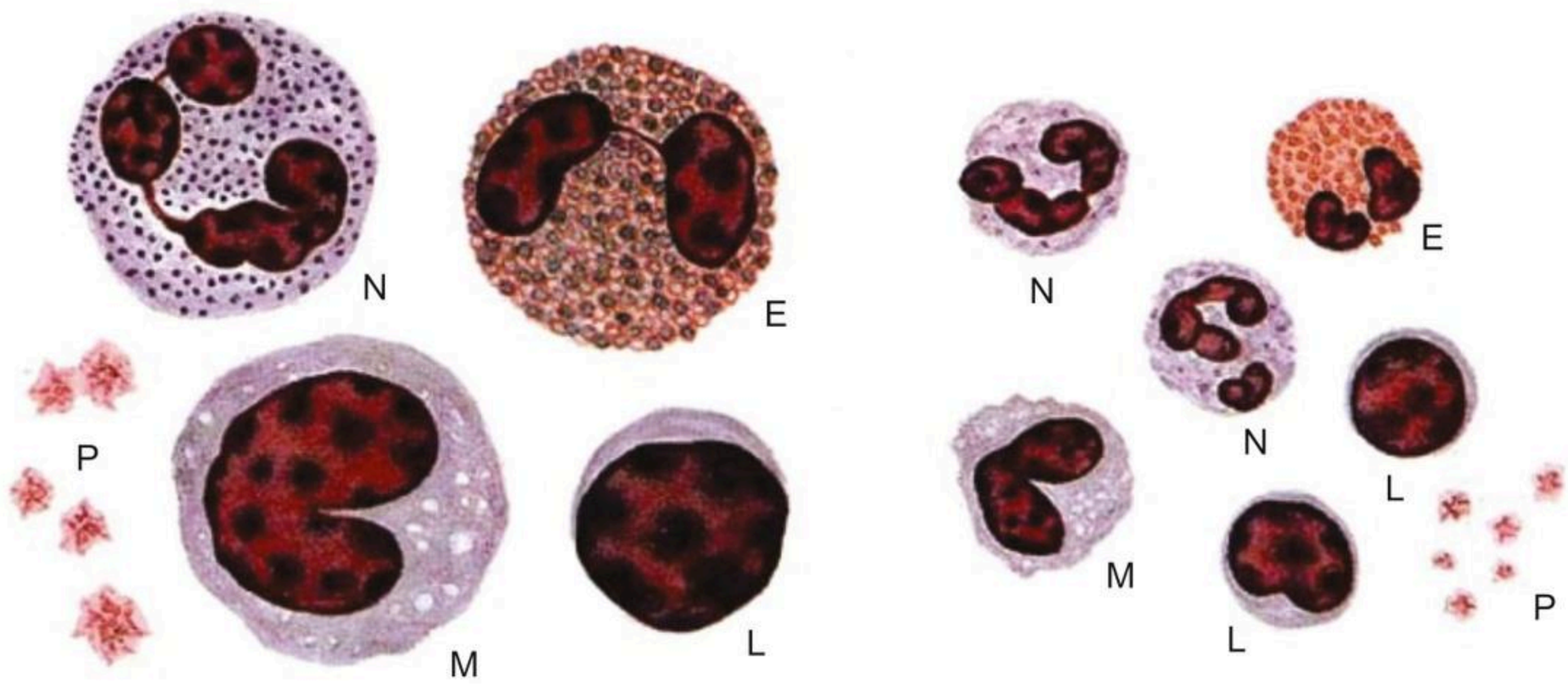
- Never add water to the stain stock solution, the smallest amount of water will cause deterioration of the solution
- Do not shake the stain bottle before use. This can lead to resuspending of very small undissolved crystals of stain, which can settle on the blood films during staining and cause problems in microscopic examination
- Never return unused stain to the stock solution bottle, it is better to measure out a small quantity for one or two day's use

Care of glassware

- Glassware such as measuring cylinders, pipettes and staining troughs must always be clean and dry before use
- Any glassware that has been used for staining purpose should be rinsed in clean water immediately after use to remove as much stain as possible
- The glassware should then be soaked for some time, preferably overnight in a detergent solution
- Then they should be washed with clean water and dried. Washing glassware in detergent gives satisfactory results provided that you rinse it thoroughly with clean water. Deposits of the detergent left on the glassware can upset the pH of buffered water and spoil the staining

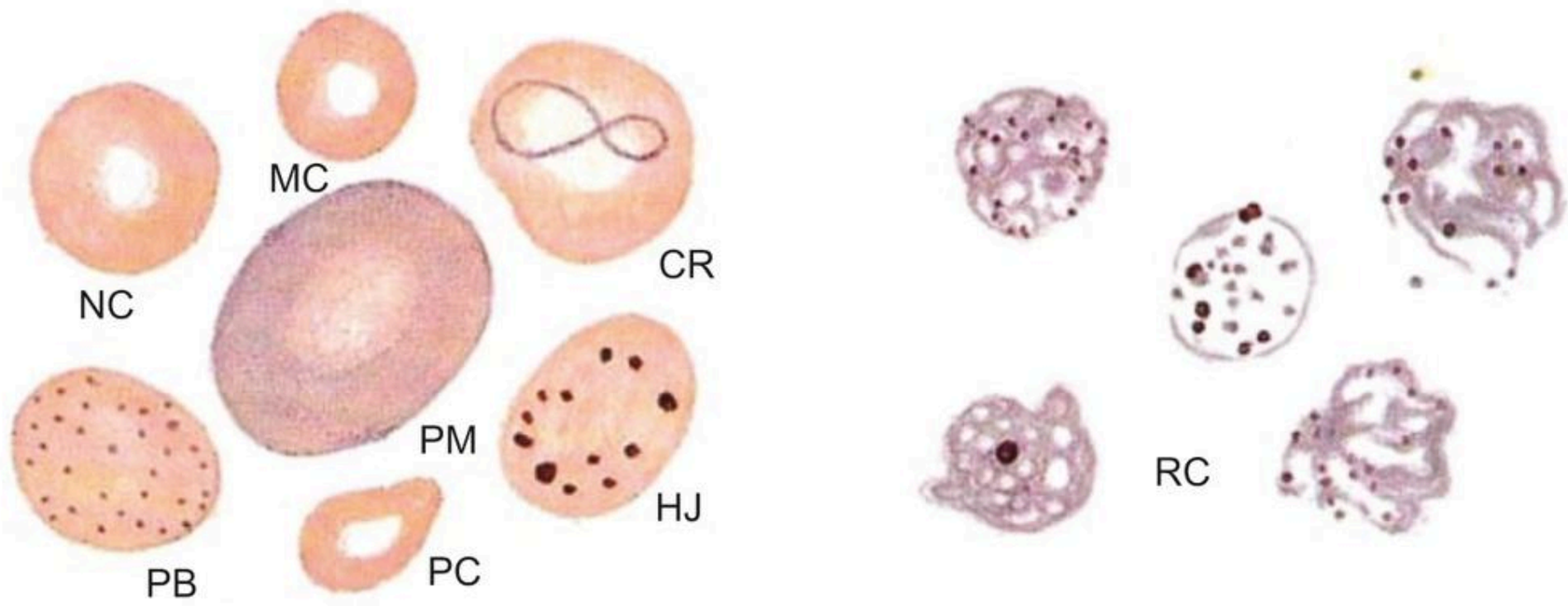
Any stain deposits that are allowed to dry on the glassware will become difficult to remove and may spoil the staining of subsequent films. In such a case soaking the glassware in methanol and then washing with detergent in the normal way can remove them.

Components of Blood



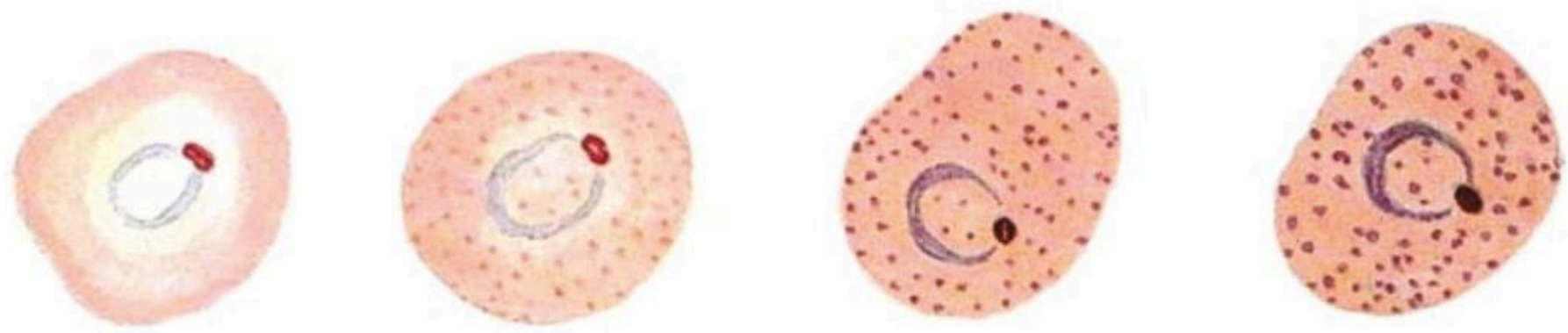
Thin Film LEUKOCYTES Thick Film

N = Neutrophil, E = Eosinophil, M = Monocyte, L = Lymphocyte, P = Platelets



Thin Film ERYTHROCYTES Thick Film

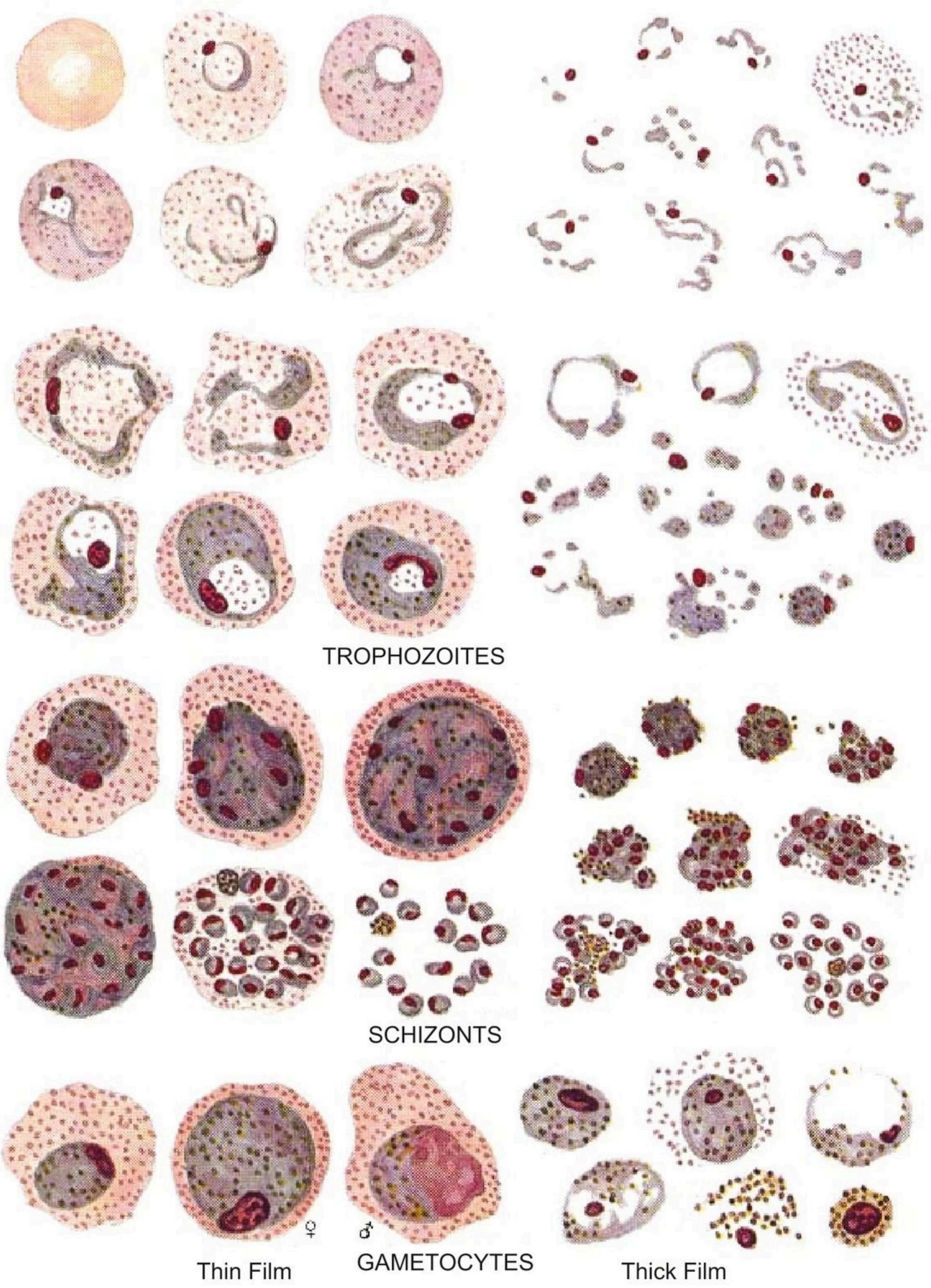
NC = Normocytes, MC = Microcyte, PM = Polychromatic macrocyte, PC = Poikilocyte, PB = Punctate basophilia, CR = Cabot's ring, HJ = Howell-Jolly bodies, RC = Reticular 'clouds' and chromatoid bodies in severe anaemia



MALARIA STAINING AND pH

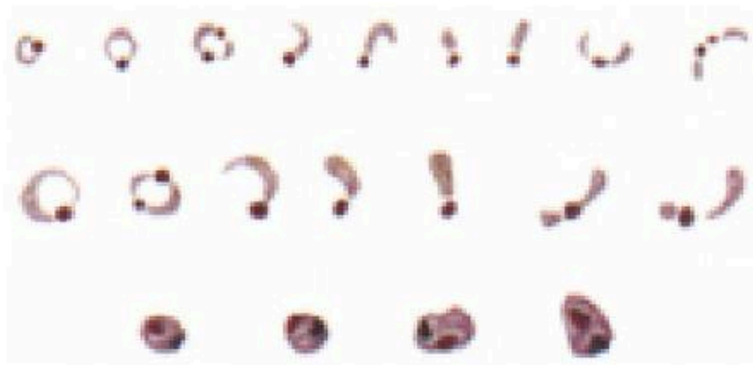
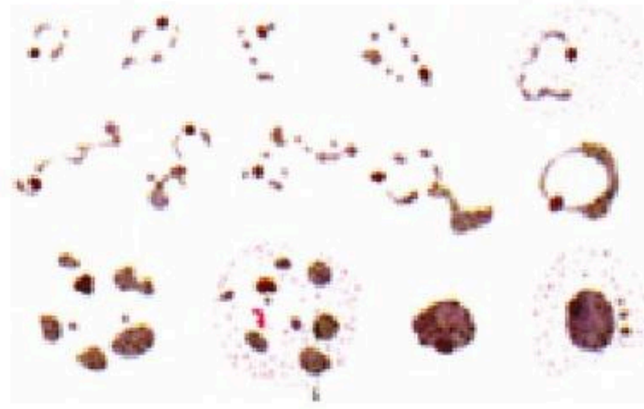
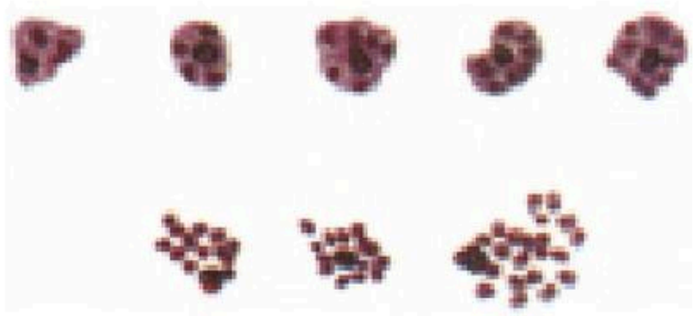
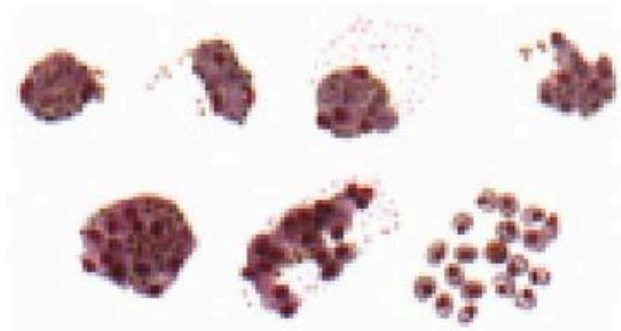
Appearance of *Plasmodium falciparum* stages in Giemsa Stained Thin and Thick films

Appearance of *Plasmodium vivax* stages in Giemsa Stained Thin and Thick films

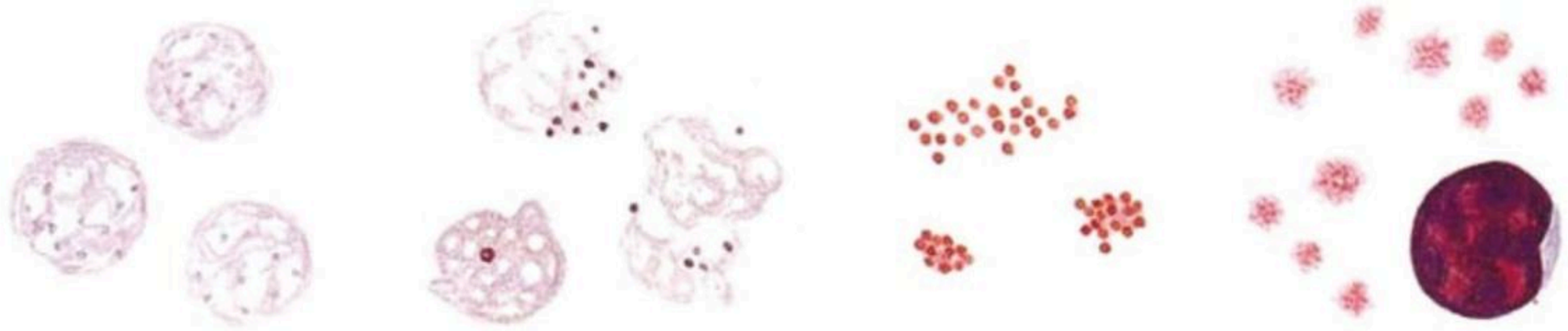


Species identification of malaria parasites in Giemsa stained Thick Blood films

Stage of parasite in peripheral blood

	Plasmodium falciparum Young, growing trophozoites and/or mature gametocytes usually seen	*Plasmodium vivax* All stages seen, Schuffner's stippling in "ghost' of host red cells, especially at film edge
Trophozoite		
	Size: small to medium, *number:* often numerous, *shape:* ring and comma forms common; *chromatin:* often two dots: *cytoplasm:* regular, fine to fleshy; *mature forms:* sometimes present in severe malaria, compact with *pigment:* as few coarse grains or a mass.	*Size:* small to large, *number:* few to moderate, *shape:* broken ring to irregular forms common; *chromatin:* single, occasionally two; *cytoplasm:* irregular or fragmented; *mature forms:* compact dense; *pigment:* scattered, fine.
Schizont		
	Usually associated with many young ring forms. *Size:* small, compact, *number:* few, uncommon, usually in severe malaria; *mature forms:* 12-30 or more merozoites in compact cluster; *pigment:* single dark mass.	*Size:* large, *number:* few to moderate, *mature forms:* 12-30 merozoites, usually 16, in irregular cluster, *pigment:* loose mass.
Gametocyte		
	Immature pointed-end forms uncommon. *Mature forms:* banana-shaped or rounded; *chromatin:* single, well defined; *pigment:* scattered, coarse, rice-grain-like; pink extrusion body sometimes present. Eroded forms with only chromatin and pigment often seen.	Immature forms difficult to distinguish from mature trophozoites. *Mature forms:* round, large; *chromatin:* single, well defined; *pigment:* scattered, fine. Eroded forms with scanty or no cytoplasm and only chromatin and pigment present.

Artefacts that may cause confusion in diagnosis



'Clouds' and chromatoid debris derived from immature erythrocytes in severe anemia

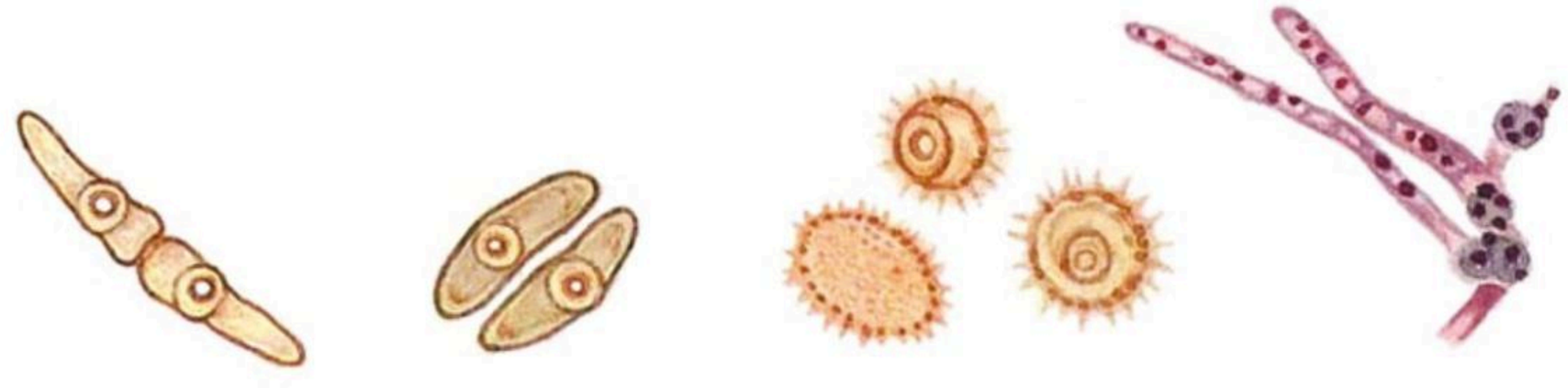
Isolated groups of eosinophilic granules

Blood platelets
Lymphocyte for comparison of size

BLOOD ELEMENTS

BACTERIA

SPORES



VEGETABLE CELLS

Hyphae and spores
FUNGUS

Dust particles

Giemsa stain crystals

Herring-bone scratches in glass slide

Crystalline 'pits' in devitrified slide

VARIOUS SOURCES

References

1. Basic Malaria Microscopy, Part I Learner's Guide, WHO, Geneva, 1991.
2. Practical Haematology, Sir John V. Dacie / S.M. Lewis, Eighth edition, Published by Churchill Livingstone, 1995.
3. Medical Parasitology- A Practical Approach, S.H, Gillespie / P.M. Hawkey, Published by OIRL Press at Oxford University Press, 1994.
4. A Practical Manual of Medicine And Biological Staining Techniques, Edward Gurr, Published by Leonard Hill Books Ltd, 1956.

ANTIBIOGRAM

- **Antimicrobial Susceptibility Testing**
  - Practical Issues.....Pgs 220-228

KNOWLEDGE BOOK

For the Laboratorians

Antimicrobial Susceptibility Testing

Practical issues

Setting Trends...

Background

Antimicrobial Susceptibility Testing (AST) is performed on bacterial isolates in clinical laboratories. The development and improvement of accurate, efficient methods of rapid antimicrobial susceptibility testing is important for public health. Antimicrobial susceptibility information about pathogens may significantly reduce morbidity and mortality, cost of treatment, and duration of hospitalization if this information can be provided to clinicians in a rapid and timely fashion.

Many different clinical laboratories perform Antimicrobial Susceptibility Testing (AST). It is very important that all laboratories use methods that are comparable, for uniform reporting of results and better patient management.

For years, there has been a professional organization, CLSI (Clinical and Laboratory Standards Institute) that was formerly known as NCCLS (National Committee on Clinical Laboratory Standards) that created standards for performance of Antimicrobial Susceptibility Testing (AST). All clinical laboratories performing tests for antimicrobial susceptibility should have access to current CLSI guidelines. This assures that clinicians use appropriate antibiotic therapy for better patient prognosis.

In view of the above we have discussed the following points in our technical series :

1. Features of Mueller Hinton Agar medium used for Antimicrobial Susceptibility Testing.
2. Troubleshooting aspects in Antimicrobial Susceptibility Testing.

Antibiotics : Mechanism of Action

Antibiotics are low molecular weight substances that interfere with specific activities in certain types of organisms. These effects could be cidal (killing) and/or static (inhibitory). If an antibiotic has a widespread effect on Gram-positive and Gram-negative bacteria it is said to be a **broad spectrum antibiotic**. **A narrow spectrum antibiotic** will only act on either Gram-positive or Gram-negative bacterial strains. Antibiotics are found throughout nature and are used by organisms like moulds and soil inhabitants to gain advantages over their competitors. The early antibiotics were isolated from these natural sources, however today many are genetically engineered to be even more effective than their natural counterparts. For an antibiotic to be useful to humans it must have the ability to destroy pathogens while being relatively non-toxic to the host organism. It should be chemically stable and be able to reach the part of the host organism in which the infection persists.

Antibiotics work in a variety of ways, most of which attack Bacterial cell components and their synthesis. Some broad-spectrum antibiotics use several of the following modes of cellular attack:

1. Cell Wall Synthesis Inhibitors
2. Cell Membrane Inhibitors
3. Protein Synthesis Inhibitors
4. Nucleic Acid Effectors
5. Competitive Inhibitors

1. **Cell Wall Synthesis Inhibitors :** These antibiotics are particularly selective because they typically target the formation of peptidoglycan cell walls which are only found in prokaryotic cells. Cell Wall Synthesis Inhibitors do not impact eukaryotic cells of humans due to the lack of peptidoglycan. These types of antibiotics are referred to as Beta lactam antibiotics. Some common Beta lactam antibiotics are Ampicillin, Cephalothin and Penicillin.

2. **Cell Membrane Inhibitors :** These are less common than other types of antibiotic inhibiting mechanisms. Cell membrane inhibitors attack integrity of the bacterial membranes. An example of these is Polymyxin, which binds to membrane phospholipids. Once antibiotic has disrupted the membrane, the cell loses its integrity and will die. However a problem arises when there is a similarity between phospholipids in bacterial and eukaryotic cell membranes. These drugs can therefore be very dangerous to the patient due to the lack of selectivity in target cells and thus have only topical application.

3. **Protein Synthesis Inhibitors :** These types of antibiotics have numerous ways of attacking protein synthesis in bacterial cells and will usually target activities occurring at the ribosome. These drugs affect the ribosome and donot bind to any other components of the protein synthesis process. Examples of these are Erythromycin, Streptomycin, Gentamicin and Tetracycline.

4. **Nucleic Acid Effectors :** Some antibiotics attack the DNA or RNA of a cell. These chemotherapeutic agents affect the synthesis of the DNA (in some case RNA). This serves to block the natural growth of the cell and will lead to death without replication.
   One group is called Quinolones, and an example of this type is Nalidixic acid. Nalidixic acid binds itself to the enzyme topoisomerase, which uncoils supercoiled DNA before replication. The drug also inhibits the enzyme DNA gyrase, which returns the DNA to its supercoiled state after replication. This widely utilized nucleic acid effector possesses it's own caveats and has been known to affect animal cells in addition to bacterial DNA. A new class of drugs, called Rifamycins (e.g. rifampin), solves the specificity problem of the quinolones. These only attack the eubacterial RNA polymerase, which is essential to mRNA synthesis. It is inactive towards RNA polymerase found in eukaryotic cells.

5. **Noncompetitive and Competitive Inhibitors :** A competitive inhibitor blocks an enzyme from performing its normal function by mimicking the substrate and binding to the enzyme's active site. A noncompetitive inhibitor will bind to the enzyme at a different location (the allosteric site), which will change the structure of the enzyme affecting the rate at which it can perform a task.

   The Sulfonamides are a class of competitive inhibitors. They inhibit Dihydropteroate Synthase (DHPS) an enzyme present exclusively in bacterial cells. DHPS enzyme is required in a series of reactions to synthesize folic acid. Inhibition of DHPS will results in many damaging effects including failure in biosynthesis of Purine and Thymylidate nucleotides and eventually inhibiting DNA synthesis.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing measure the ability of an antibiotic or other antimicrobial agent to inhibit bacterial growth *in vitro*. This ability may be estimated by either dilution method or diffusion method.

1. **The dilution method**
   For quantitative estimates of antibiotic activity, dilutions of the antibiotic may be incorporated into broth or agar medium, which is then inoculated with the test organism. The lowest concentration that prevents growth after overnight incubation is known as the minimum inhibitory concentration (MIC) of the agent. The MIC value is then compared with known concentrations of the drug obtainable in the serum and in other body fluids to assess the likely clinical response.

2. **The diffusion method**
   Paper discs impregnated with a defined quantity of antimicrobial agent are placed on agar medium uniformly seeded with the test organism. A concentration gradient of the antibiotic forms by diffusion from the disc and the growth of the test organism is inhibited at a distance from the disc that is related among other factors to the susceptibility of the organism.

The recommended method for intermediate and peripheral laboratories is the modified Kirby-Bauer method. **This method has been recommended by National Committee on Clinical Laboratory Services (NCCLS-USA) Subcommittee on Antimicrobial Susceptibility Testing.** This is the most thoroughly described disc diffusion method for which interpretive standards have been developed and which is supported by laboratory and clinical data.

Kirby-Bauer Disk Diffusion Method

To ascertain antibiotic efficacy, degree of antibiotic resistance or sensitivity to bacterium, is the objective of Kirby-Bauer Test.
To carry out Kirby-Bauer Test following components are used :

1. Mueller Hinton Agar - Culture Media
2. Antimicrobial Susceptibility Discs
3. Standard Strains for Quality Control
4. Turbidity Standard (McFarland standards)
5. Swabs

Mueller-Hinton Agar : Mueller-Hinton Agar is considered the best medium to use for routine susceptibility testing of nonfastidious bacteria for the following reasons:

- It shows acceptable batch-to-batch reproducibility for susceptibility testing
- It is low in sulfonamide, trimethoprim, and tetracycline inhibitors
- It supports satisfactory growth of most nonfastidious pathogens
- A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

Please note that the use of media other than Mueller-Hinton Agar may result in erroneous results. Aerobic or facultative bacteria grow well on unsupplemented Mueller-Hinton Agar. Fastidious organisms require Mueller-Hinton Agar to be supplemented with additional nutrients.

1. Mueller-Hinton Agar should be prepared from a dehydrated base or purchased as Mueller Hinton Agar ready prepared plates. Follow the manufacturer's recommendation for storage of prepared plates. Be sure to prepare the media according to the manufacturer's directions. The medium should be such that control zone sizes within the standard limits are produced. It is important not to overheat the medium.
2. If you prepare Mueller-Hinton Agar plates from dehydrated media, **the plates must be poured to a depth of 4 mm**. Plates that are too shallow will produce false susceptible results as the antimicrobial compound will diffuse further, creating larger zones of inhibition. Conversely, plates poured to a depth more than 4 mm will result in false resistant results.
3. While pouring media into plates medium temperature should be in between 45-50ºC.
4. Dry the plates at 35-37ºC in the incubator in an upright position for immediate use.
5. Any unused plates may be stored in a plastic bag, which should be sealed and placed in the refrigerator. Plates stored this way can be kept for 2 weeks.
6. pH of the Mueller-Hinton Agar should fall between 7.2 and 7.4 at room temperature after solidification and should be tested when the media is first prepared. If the pH is less than 7.2 certain drugs will appear to lose potency (aminoglycosides, quinolones, macrolides), while other agents may appear to have excessive activity (tetracycline). If the pH is more than 7.4, the opposite results may occur.
7. Excessive thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim resulting in smaller and less distinct zones of inhibition, or no zones at all.
8. The incorrect concentration of divalent cations (calcium and magnesium) will affect the results of aminoglycoside and tetracycline tests against *Pseudomonas aeruginosa*. Excess cation concentration will result in reduced zone sizes and low concentration will increase zone sizes. Excess calcium will increase the zone size of *P. aeruginosa* against daptomycin. Excess zinc ions may reduce the zone size of carbapenems against *P. aeruginosa.*
9. Mueller-Hinton Agar should be tested with known strains of organism at least weekly in order to verify that the media and discs are working as expected.

Mueller-Hinton Agar Physical Parameters

PARAMETERS	SPECIFICATIONS
Dehydrated powder appearance	Yellow coloured, homogenous, free flowing powder
Final pH at 25ºC	7.3 ±0.1
Gelling temperature	Gel is formed at 32ºC
Colour and clarity of prepared media	Light amber coloured, clear gel

Cultural Characteristics Observed with Mueller-Hinton Agar after incubation for 18 to 24 hours at 30 - 35ºC

ORGANISM	TYPE CULTURE	GROWTH
Escherichia coli	ATCC (8739)	Luxuriant
Streptococcus faecalis	ATCC (11420)	Luxuriant
Neisseria gonorrhoeae	ATCC (49226)	Luxuriant
Pseudomonas aeruginosa	ATCC (9027)	Luxuriant
Staphylococcus aureus	ATCC (6538)	Luxuriant
Escherichia coli	ATCC (25922)	Luxuriant
Staphylococcus aureus	ATCC (25923)	Luxuriant
Pseudomonas aeruginosa	ATCC (27853)	Luxuriant
Enterococcus faecalis	ATCC (29212)	Luxuriant

Antimicrobial Susceptibility Discs : Antimicrobial susceptibility discs with proper diameter and potency should be used. Discs should be made of an absorbent material, usually paper, which has no interfering effect either on bacterial growth or on the action of the antibiotic. It must be capable of absorbing moisture rapidly and the antibiotic should be evenly distributed in it. Sealed cartridges containing commercially prepared paper discs should be stored at either 8°C or frozen at -14°C in a non-self-defrosting freezer. A small working supply of discs can be kept in the refrigerator for one week. On removal from the refrigerator, the containers should be left at room temperature for about 1 hour to allow the temperature to equilibrate. **Once opened, store the cartridges in a storage container containing desiccant for not more than 1 week.**

Standard Strains for quality control : The quality control should use standard reference strains of bacteria that are tested in parallel with the clinical culture. They should preferably be run every week, or with every fifth batch of tests, and in addition, every time that a new batch of Mueller Hinton agar or a new batch of discs is used.

Standard Strains :
Staphylococcus aureus (ATCC 25923)
Escherichia coli (ATCC 25922)
Pseudomonas aeruginosa (ATCC 27853)
Culture for day-to-day use should be grown on slants of Nutrient Agar or Tryptic Soya Agar and stored in the refrigerator. These should be subcultured onto fresh slants after every 2 weeks.

Turbidity Standard (McFarland standard) : McFarland standard may be prepared in-house as described below :

1. Add a 0.5-ml aliquot of a 0.048 mol/liter $BaCl_2$ (1.175% wt/vol $BaCl_2 \cdot 2H_20$) to 99.5 ml of 0.18 mol/liter H_2SO_4 (1% vol/vol) with constant stirring to maintain a suspension.
2. Verify the correct density of the turbidity standard by measuring absorbance using a spectrophotometer with a 1-cm light path and matched cuvette. The absorbance at 625nm should be 0.08 to 0.13 for the 0.5 McFarland standard.
3. Transfer the barium sulfate suspension in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in standardizing the bacterial inoculums.
4. Tightly seal the tubes and store in the dark at room temperature.

Use of McFarland standard in the Kirby-Bauer procedure :

1. Prior to use, vigorously agitate the barium sulfate standard on a mechanical vortex mixer and inspect for a uniformly turbid appearance. Replace the standard if large particles appear. If using a standard composed of latex particles, mix by inverting gently, not on a vortex mixer.
2. While adding bacterial colonies to saline in the "preparation of the inoculum" step of the procedure, compare the resulting suspension to the McFarland standard. This is done by holding both the standard and the inoculum tube side by side and no more than 1 inch from the face of the Wickerham card (with adequate light present) and comparing the appearance of the lines through both suspensions. If the bacterial suspension appears lighter than the 0.5 McFarland standard, more organisms should be added to the tube from the culture plate. If the suspension appears denser than the 0.5 McFarland standard, additional saline should be added to the inoculum tube in order to dilute the suspension to the appropriate density. In some cases it may be easier to start over rather than to continue to dilute a bacterial suspension that is too dense for use.

Swabs : Sterilized cotton swab stick can used or cotton swabs sticks can be sterilized in tins, culture tubes, or on paper, either in the autoclave or by dry heat.

Procedure to Perform Kirby-Bauer Disc Diffusion Test

- To prepare the inoculum from a primary culture plate, touch with a loop the top of each of 3-5 colonies of similar appearance of the organism to be tested (Fig.1).

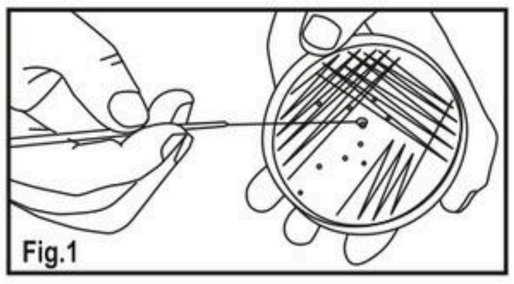


Fig.1

- When the inoculum has to be made from a pure culture, a loopful of the confluent growth is similarly suspended in saline (Fig. 2).

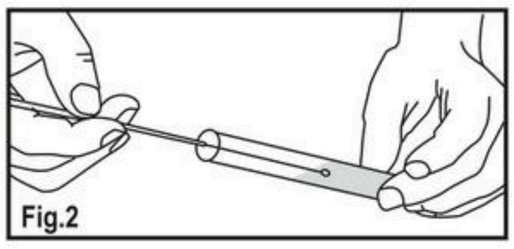


Fig.2

- Compare the tube with turbidity standard and adjust the density of test suspension to that of the standard by adding more bacteria or more sterile saline. Proper adjustment to the turbidity of the inoculum is essential to ensure that the resulting lawn of growth is confluent or almost confluent. Use this suspension within 15 minutes of preparation (Fig.3).

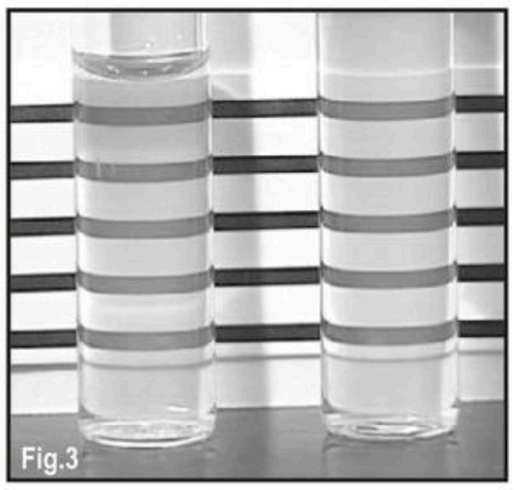


Fig.3

- Inoculate the plates by dipping a sterile swab into the inoculum. Remove excess inoculum by pressing and rotating the swabs firmly against the side of the tube above the level of the liquid (Fig. 4).

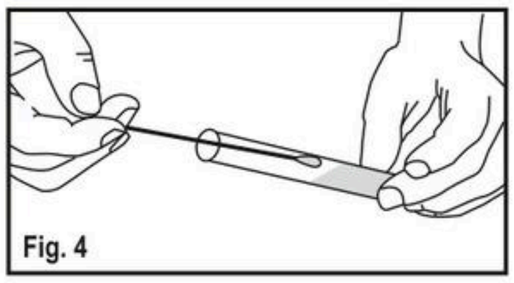


Fig. 4

- Streak the swab all over the surface of the medium three times, rotating the plate through an angle of 60º after each application. Finally, pass the swab round the edge of the agar surface. Leave the inoculum to dry for a few minutes at room temperature with the lid closed (Fig. 5).

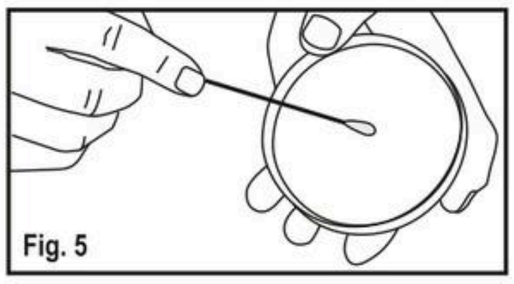


Fig. 5

- The antibiotic discs may be placed on the inoculated plates using a pair of sterile forceps or a sterile needle tip. Alternatively, an antibiotic disc dispenser can be used to apply the discs to the inoculated plate (Fig. 6).

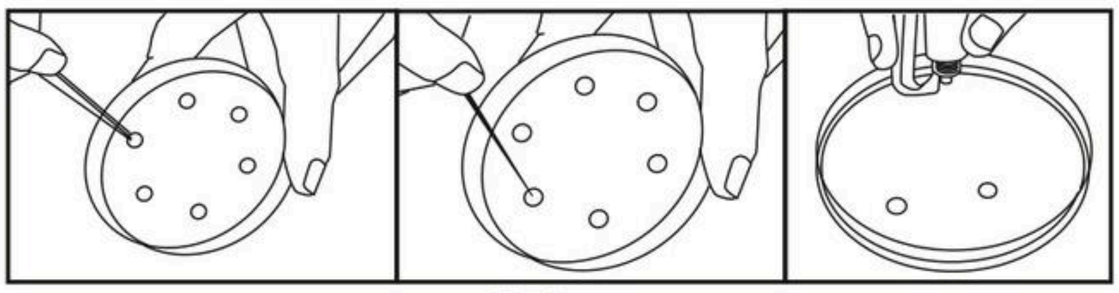


Fig. 6

- A maximum of 4-5 discs can be placed in a 90-100 mm diameter petridish.
- The plates should be placed in an incubator at 35-37ºC within 30 minutes of preparation.
- Incubate fastidious organism in an atmosphere of carbon dioxide.
- After overnight incubation, the diameter of each zone (including the diameter of the disc) should be measured and recorded in mm. The results should then be interpreted according to the critical diameters by comparing them with standard tables (Fig. 7).

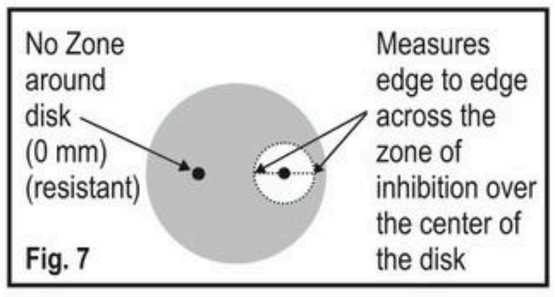


Fig. 7

- The measurements can be made with a ruler on the under surface of the plate without opening the lid (Fig. 8).

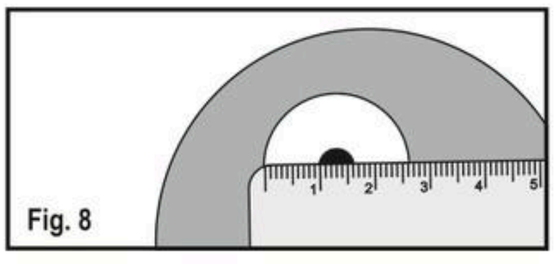


Fig. 8

- The endpoint of inhibition is judged by the naked eye at the edge where growth starts, but there are three exceptions,
  - ☞ With sulfonamides and co-trimoxazole, slight growth occurs within the inhibition zone; such growth should be ignored.
  - ☞ When β-lactamase producing staphylococci are tested against penicillin, zones of inhibition are produced with a heaped-up, clearly defined edge; these are readily recognizable when compared with the sensitive control and regardless of the size of the zone of inhibition, they should be reported as resistant.
  - ☞ Certain *Proteus* species may swarm into the area of inhibition around some antibiotics, but the zone of inhibition is usually clearly outlined and the thin layer of swarming growth should be ignored.

Clinical Definition of Terms Resistant and Susceptible

The result of the susceptibility test, as reported to the clinician, is the classification of the microorganism in one of two or more categories of susceptibility. The simplest system comprises only two categories, susceptible and resistant. This classification, although offering many advantages for statistical and epidemiological purposes, is too inflexible for the clinician to use. Therefore, a **three-category classification** is often adopted. The Kirby-Bauer method recognizes three categories of susceptibility and it is important that both the clinician and the laboritarian understand the exact definitions and the clinical significance of these categories.

Susceptible : An organism is called "susceptible" to a drug when the infection caused by it is likely to respond to treatment with this drug at the recommended dosage.

Intermediate susceptibility : This term covers two situations. It is applicable to strains that are "moderately susceptible" to an antibiotic that can be used for treatment at a higher dosage because of its low toxicity or because the antibiotic is concentrated in the focus of infection (e.g. urine). The term also applies to those strains that are susceptible to a more toxic antibiotic that cannot be used at a higher dosage. In this situation this category serves as a buffer zone between susceptible and resistant.

Resistant : This term implies that the organism is expected not to respond to a given drug, irrespective of the dosage and of the location of the infection.

Quality Assurance in Antimicrobial Susceptibility Discs

Antimicrobial Susceptibility Testing has become a very essential step for properly treating infectious diseases and monitoring antimicrobial resistance in various pathogens. The choice of antimicrobial needs to be made taking into consideration the susceptibility profile of the pathogen, pharmacology of the antimicrobial, the need for antimicrobial therapy, and its cost effectiveness.

Factors Influencing Zone Size and Common Problems Encountered in Performing Susceptibility Testing

FACTORS	IMPACT ON ZONE SIZE
Inoculum density	Larger zones with light inoculum and smaller zone with heavy inoculum
Timing of disc application	If after application of disc, the plate is kept for longer time at room temperature, small zones may form
Temperature of incubation	Larger zones are seen with temperatures < 35°C
Incubation time	Ideal 16-18 hours; less time does not give reliable results
Depth of the agar medium	Thin media results excessively large inhibition zones and very thick media results small inhibition zone
Proper spacing of the discs	Avoids overlapping of zones
Potency of antibiotic discs	Deterioration in contents leads to reduced size
Composition of medium	Affects rate of growth, diffusion of antibiotics and activity of antibiotics
Addition of thymidine to medium	Decreases activity of trimethoprim
Acidic pH of medium	Tetracycline, novobiocin, methicillin zones are larger
Alkaline pH of medium	Aminoglycosides, erythromycin zones are larger
Incubation in the presence of CO_2	Increases zone size of tetracycline and methicillin
Reading of zones	Subjective errors in determining the clear edge
Chelating agents such as calcium, magnesium and iron	Decreases diffusion of tetracycline, gentamicin
On chocolate agar	Decreased activity of Sulfonamides, trimethoprim, aminoglycosides
Addition of defibrinated blood	Decreases activity of sulfonamides

Troubleshooting Guide for Disc Diffusion Test in Antimicrobial Susceptibility Testing

ERRONEOUS RESULT	PROBABLE CAUSE	CORRECTIVE ACTION
Tetracycline zone too large and clindamycin zone too small with *E. coli* or *S. aureus* control strains.	pH of medium too low.	Adjust pH 7.2 to 7.4 before pouring media. Commercial media should not have pH problems. Report to manufacturer.
Tetracycline zone too small and clindamycin zone too large with *S. aureus* or *E. coli* control strain.	pH of medium too high.	Get a new lot. (Incubation in CO_2 may alter agar surface pH.)
Aminoglycoside zone too small with *P. aeruginosa,* Acinetobacter control strain.	Calcium ion and/or Magnesium ion too high in medium.	Acquire a new lot of agar medium that will meet QC criteria.
Aminoglycoside zone too large with *P. aeruginosa* control strain.	Calcium ion and/or Magnesium ion too low in medium.	Acquire a new lot of agar medium that will meet QC criteria.
Zones universally too large on control plates.	Inoculum too light.	Adjust inoculum to a McFarland 0.5 turbidity standard.
	Nutritionally poor medium.	Use only Mueller Hinton Agar medium.
	Slow-growing organism. (not seen with controls)	Use minimum inhibtory concentration (MIC) procedure only.
	Improper medium depth. (too thin)	Use 4-5mm depth.
Zones universally too small on control plates.	Inoculum too heavy.	Adjust inoculum to a McFarland 0.5 turbidity standard.
	Agar depth too thick. (minor)	Use 4-5mm depth.
Methicillin zone decreasing over days or weeks with control organisms.	Methicillin degrading during refrigerator storage	Change methicillin discs or use oxacillin or nafcillin as the routine disc.
Methicillin zone indeterminate in disc test.	Methicillin being degraded by strong beta-lactamase producing staphylococci.	Change methicillin discs or use oxacillin or nafcillin as the routine disc.
Carbenicillin zone disappears with *Pseudomonas* control.	Resistant mutant has been selected for testing.	Change *Pseudomonas* control strain every two weeks and whenever resistant mutants appear within the carbenicillin zone.
S.aureus from a patient was resistant to methicillin one day and sensitive the next.	May be two different organisms. Temperature shift from 35-37ºC can dramatically alter the zone size in this case.	Check testing temperature. Test must be performed at 35ºC or 37ºC. for methicillin (oxacillin or nafcillin) and *S.aureus.*
A single disc result above or below the control limit.	Error in reading. Fuzzy zone edge. Transcription error. Bad disc.	Note error. Recheck error and ask for a second opinion.
	Disc may not be pressed firmly onto the agar surface. (Bad discs usually demonstrate a trend toward being out of control.)	Statistically, one may expect an occasional out-of-range result. Values usually fall within range on retesting.
Colonies within zone of inhibition.	Mixed culture.	Isolate, identify and retest pure cultures only.
	Resistant mutants within zone.	Gram stain or do another test to rule out contamination.
Very large zones with anaerobes.	--	Do not use disc agar diffusion procedure to test anaerobes.
With colistin, growth seen immediately adjacent to disc, then larger zone at endpoint (Occurs with colistin when testing *Serratia* spp. and some *Enterobacter* spp.).	"Prozone-like" phenomenon.	Confirm with MIC.

ERRONEOUS RESULT	PROBABLE CAUSE	CORRECTIVE ACTION
The methicillin disc test shows "resistant" but an MIC shows "sensitive" for *S.aureus.*	Mueller Hinton Broth is inadequate in this case. A modified broth used in some commercial MIC systems frequently eliminates this problem.	No action necessary with disc test. To be expected if Mueller Hinton Broth is used in MIC test. Use broth with 2% NaCl if MIC testing is necessary.
	Low methicillin content in disc.	Use new discs
Zones overlap.	Discs too close together.	Use no more than 12 discs on a 150mm plate and 4 to 5 discs on a 100mm plate. Place discs no closer than 15mm from the edge of the plate.
"Zone within a zone"	Swarming / movement of *Proteus* spp.	Read the wide distinct zone and disregard the growth that swarmed over.
	Feather edges of zones around penicillin or ampicillin discs usually occur with beta-lactamase-negative strains of *S. aureus.*	Take half the distance from the inner zone to outermost zone as measure mark.
	Sulfonamides	Disregard growth from disc margin to the major inner zone.
	Beta-lactamase-positive *Haemophilus influenzae* with penicillin or ampicillin.	Use inside zone.
Zones indistinct (hazy) with single colonies noted on the plate.	Poorly streaked plate. Inadequate inoculum.	Use properly adjusted inoculum and repeat test.
Indistinct zones with sulfamethoxazole with or without trimethoprim or with trimethoprimalone.	Thymidine in medium inhibits the action of these antimicrobics.	Use commercial thymidine-free plates. Disregard small amount of growth within the zone as with sulfonamides.

Salient Features of Quality Control in Antimicrobial Susceptibility Testing are Summarized below

- Use antibiotic discs of 6 mm diameter.
- Use correct content of antimicrobial agent per disc.
- Store supply of antimicrobial discs at -20ºC.
- Refrigerate the containers at 8ºC or below, or freeze at -14ºC or below, in a non frost-free freezer until needed.
- Sealed packages of discs that contain drugs from the ß(Beta)-lactam class should be stored frozen, except for a small working supply, which may be refrigerated for at most one week.
- Some labile agents (e.g., imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.
- The unopened disc containers should be removed from the refrigerator or freezer one to two hours before use, so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold discs.
- **Once a cartridge of discs has been removed from its sealed package, it should be placed in a tightly sealed, desiccated container.**
- Use Mueller-Hinton medium for antimicrobial sensitivity determination.
- Use appropriate control cultures.
- Use standard methodology for the test.
- Use coded strains from time to time for internal quality control.
- Incubate the sensitivity plates for 16-18 hours before reporting.
- Incubate the sensitivity plates at 35-37ºC.
- Space the antibiotic discs properly to avoid overlapping of inhibition zone.
- Use inoculum size that produces 'near confluent' growth.
- Ensure even contact of the antibiotic disc with the inoculated medium.
- Measure zone sizes precisely.
- Interpret zone sizes by referring to standard charts.
- Only those discs that have not reached the manufacturer's expiration date stated on the label may be used. Discs should be discarded on the expiration date.

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