

Med Chem 535P – Diagnostic Medicinal Chemistry

Immunoassays

I. The Immunoassays

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- B. Hemagglutination Inhibition
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- E. Gonorrhea Tests
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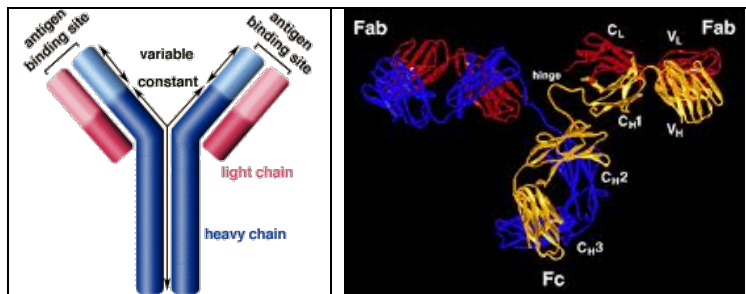
SEROLOGY AND IMMUNOASSAYS

Serology is the study of serum proteins, specifically the diagnostic detection of specific proteins and antibodies in a patient's blood.

Serological tests may be performed for diagnostic purposes when an infection is suspected, in rheumatic illnesses, and in many other situations, such as checking an individual's blood type.

Serology blood tests help to diagnose patients with certain immune deficiencies associated with the lack of antibodies. In such cases, tests for antibodies will be negative.

I. The Immunoassays comprise a variety of tests that are available to detect, both qualitatively and quantitatively, the presence of antibodies (Ab) and/or antigens (Ag) in serum.



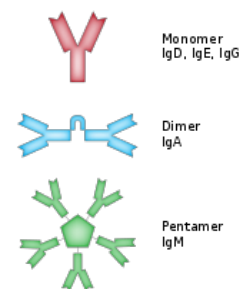
IgG is the major Ab found in plasma and provides the majority of antibody-based immunity against invading pathogens.

IgM is the first Ab to appear in the plasma after exposure to an antigen. It is a first line of defense, before sufficient IgG has been synthesized (~ 10 days).

IgA is the major class of Ab's present in mucosal secretions in the lungs, digestive and urogenital tract, where it protects against invading bacteria and viruses.

IgE molecules play a role in responses to **allergens** (substances that trigger allergies) and some parasites (e.g., helminthes).

IgD has no clear unique role.

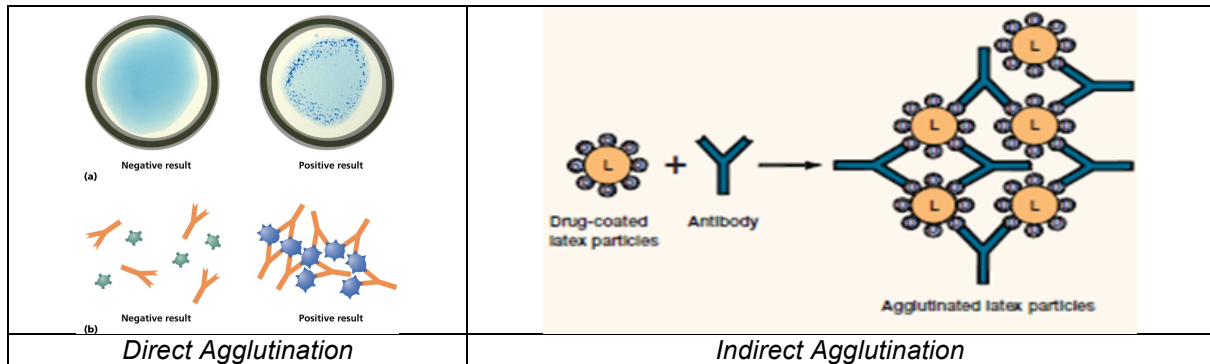


Immunoassay tests are available to detect a wide array of antigens; bacterial and viral proteins, rheumatoid factor (a protein), creatinine, and drug concentrations. A variety of approaches are available.

A. Agglutination is the “clumping” of particles and it is one of the simplest and cheapest immunoassays available.

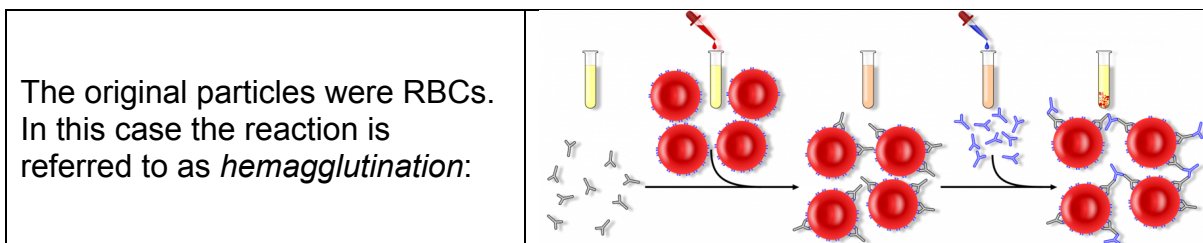
1. *Direct Agglutination*. Binding of an Ab to its Ag can result in “cross-linking” of the material; remember, an antibody has two Ag binding sites.

Under appropriate conditions, this forms clumps (*agglutination*) that can be detected visually. This is a *direct* agglutination test.



The test can be modified to detect Ags or Abs in the sample. It can be used as a qualitative test for the presence of an antigen or an antibody (yes or no answer).

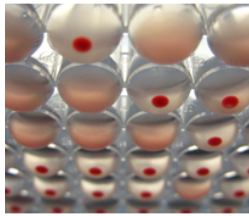
2. *Indirect Agglutination*. In some cases, the Ab-Ag interaction does not result in agglutination. Indirect agglutination involves putting the Ag on the surface of a particle (e.g., a cell or latex bead). Ab-induced cross-linking results in agglutination of the particles.



A *semi-quantitative* approach is *serial dilution*; the maximum dilution that gives visible agglutination is called the *titer*, which corresponds to the highest dilution factor that still yields a positive reading.

For example, positive readings in the first 9 serial twofold dilutions translate into a titer of 1:512 (i.e., 2^{-9}).

Titers are sometimes expressed by the denominator only; i.e., 1:512 is written 512.



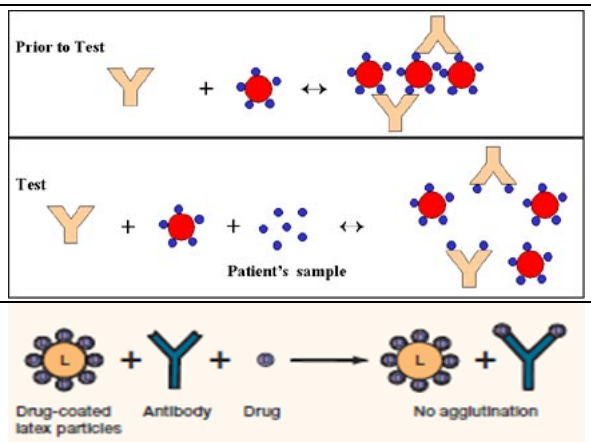
Patient	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	Pos.	Neg.	Titer
1	+	+	+	+	+	+	+	+	+	+	+	+	64
2	+	+	+	+	+	+	+	+	+	+	+	+	8
3	+	+	+	+	+	+	+	+	+	+	+	+	512
4	+	+	+	+	+	+	+	+	+	+	+	+	<2
5	+	+	+	+	+	+	+	+	+	+	+	+	32
6	+	+	+	+	+	+	+	+	+	+	+	+	128
7	+	+	+	+	+	+	+	+	+	+	+	+	32
8	+	+	+	+	+	+	+	+	+	+	+	+	4

B. Hemagglutination Inhibition Assay (HAI).

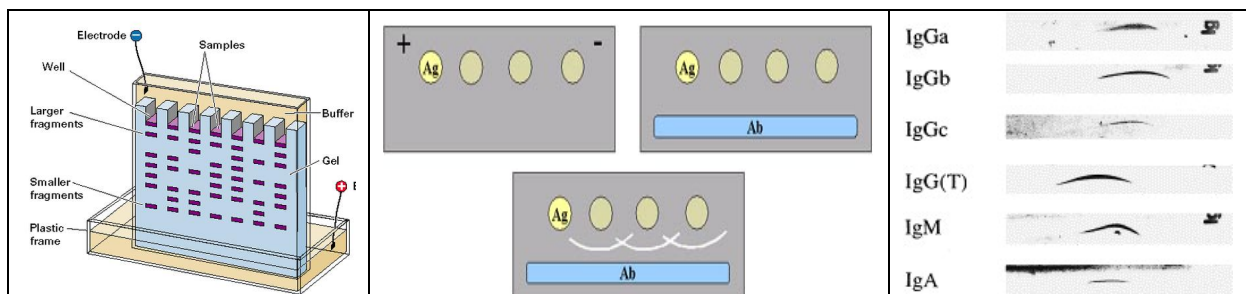
RBCs (e.g., rabbit) are coated with an Ag (e.g., virus protein) that binds to a specific Ab resulting in hemagglutination.

The sample (blood/urine) is then added to the reaction. If the sample contains the Ag it competes for Ab binding and hemagglutination is inhibited.

Ag-coated latex beads are also available for several tests.



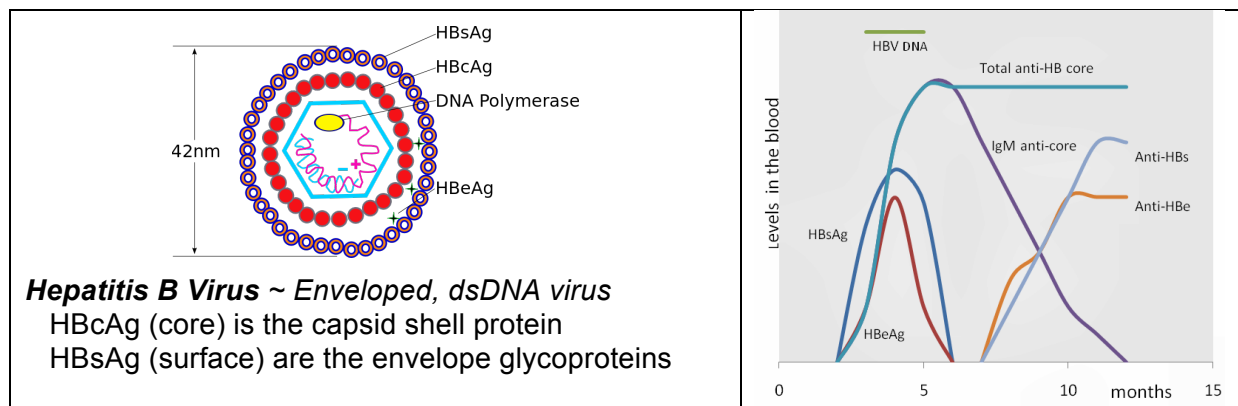
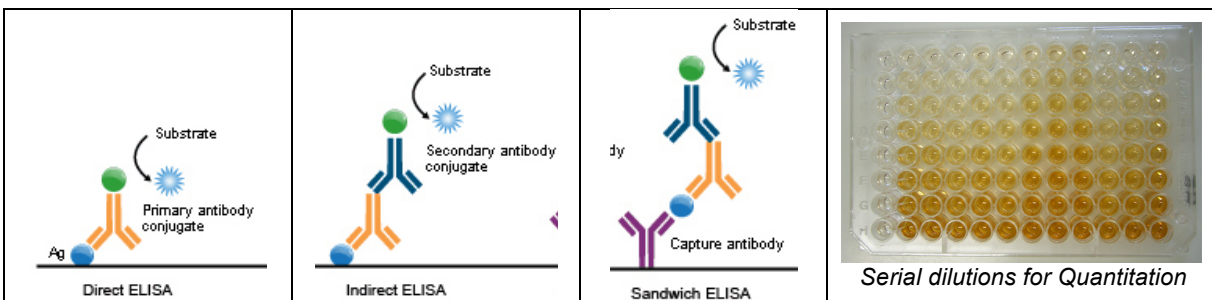
C. Immunoelectrophoresis. Proteins migrate in an electrical field based on their size and charge (electrophoresis).



An Ab solution is then layered next to the fractionated proteins; diffusion allows for migration of the two species, which precipitate when they encounter each other in the gel. This is a specific technique but it is time consuming and costly.

D. **Enzyme Linked ImmunoSorbent Assay (ELISA)**. This assay uses multiple antibodies and enzyme-linked detection systems.

1. **Direct ELISA**. In this version, an antigen (e.g., virus) is directly affixed to the plastic surface of a reaction well. A *primary* antibody is then incubated with the antigen to form a complex. This antibody is covalently linked to an enzyme that converts a substrate to a colorimetric product (*enzyme-linked antibody*) and interaction is read *directly*.
2. **Indirect ELISA**. In this version, the primary antibody does not carry the detection system. Rather, a *secondary* antibody, which recognizes the Fc portion of the primary antibody is covalently linked to the enzyme detection system.
3. **Sandwich ELISA**. In this version, a *capture* antibody is affixed to the plastic support. The antigen is added to the well, which “captures” the antigen. This is followed by a secondary antibody that also recognizes the antigen. Since both antibodies recognize the antigen, they form an antibody “sandwich”. In some cases, the second antibody carries the detection system (i.e., enzyme-linked). In others, a third antibody that recognizes the Fc portion of the secondary antibody is for detection.



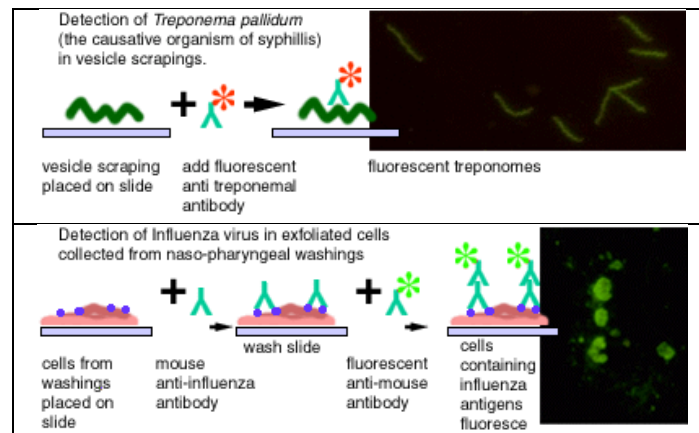
ELISAs are used to detect *Mycobacterium*, rotavirus (feces), west nile virus (serum, CSF), *E. coli* enterotoxin (feces), HIV (blood), etc.

ELISAs are also used in drug screening (benzodiazepines, cannabinoids, cocaine, opioids, etc.) and the food industry (food allergens; peanuts, eggs, etc.)

E. Fluorescent Antibody Tests

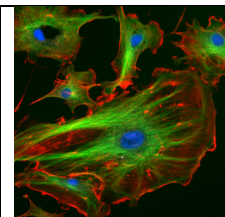
1. *Direct Immunofluorescence* uses an antibody that is tagged with a fluorescent dye (Ab*) and that is directed against a particular protein (Ag) to be detected in the patient's serum.

The patient's serum is heat-affixed to a slide to which is added a solution of the Ab*. If the patient's serum contains the Ag, it will react with Ab* resulting in a fluorescence signal emitted from the slide.



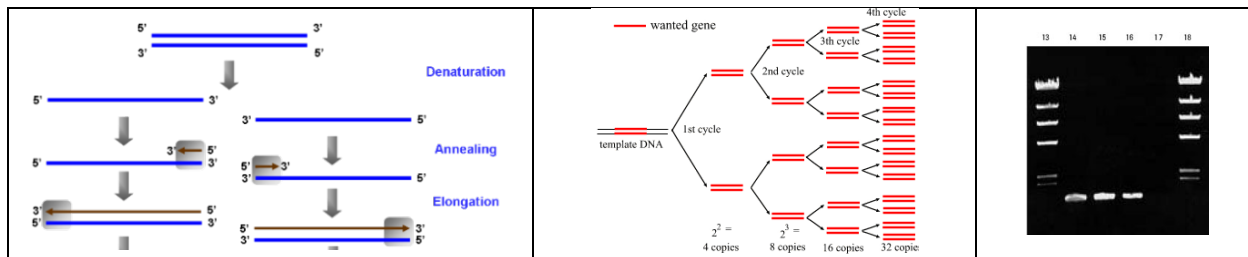
2. *Indirect Fluorescence Antibody Test*. Similar to above except that a secondary Ab* is used.
3. *Fluorescence Microscopy*. Fluorescently labeled antibodies are added to the sample, which is then examined under a fluorescence microscope. Multiple antibodies to different proteins, each tagged with a differently colored fluorophore can be used with striking effects.

Bovine pulmonary artery endothelial cells under the microscope. Nuclei are stained blue with DAPI, microtubules are marked green by an antibody bound to FITC, and actin filaments are labeled red with phalloidin bound to TRITC.



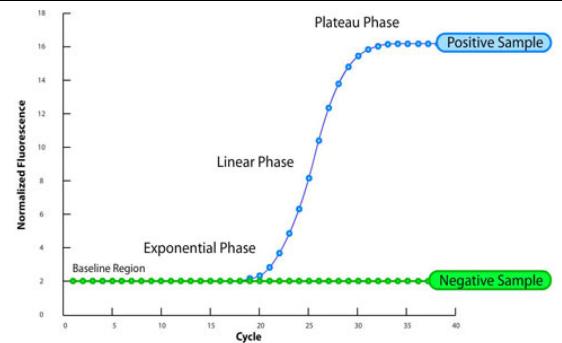
F. Polymerase Chain Reaction (PCR) is a technique that allows detection of DNA and RNA with exceptional sensitivity and specificity.

Amplified genetic material is then identified by gel electrophoresis.



PCR is most often used to detect pathogen nucleic acid (HIV, HSV, mycobacteria, chlamydia), but is increasingly being used to detect genetic diseases (cystic fibrosis, sickle cell anemia, von Willebrand's disease).

“Real-Time” PCR allows rapid detection of the amplified DNA product by a reporter fluorophore that is included in the reaction mixture.



II. Clinical Tests

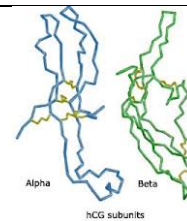
A. **Pregnancy Test, 1350 BC Egypt:** A papyrus describes a test in which a woman who might be pregnant could urinate on wheat and barley seeds over the course of several days: "If the barley grows, it means a male child. If the wheat grows, it means a female child. If neither grow, she will not bear at all." This method was tested in 1963; the urine of pregnant women promoted growth 70% of the time, while the urine of non-pregnant women and men did not.

All modern methods test for the presence of Human Chorionic Gonadotropin (HCG), which is released from the trophoblast in early pregnancy and later by the placenta.

Note: choriocarcinomas of the ovaries and testes also secrete HCG.

All tests use monoclonal antibodies (MAbs) to peptides derived from the α - and β -chains of HCG protein.

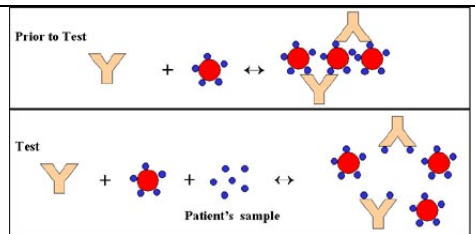
There are three different kinds of tests.



1. **Hemagglutination Inhibitor (HAI) Test.** This was the original EPT test.

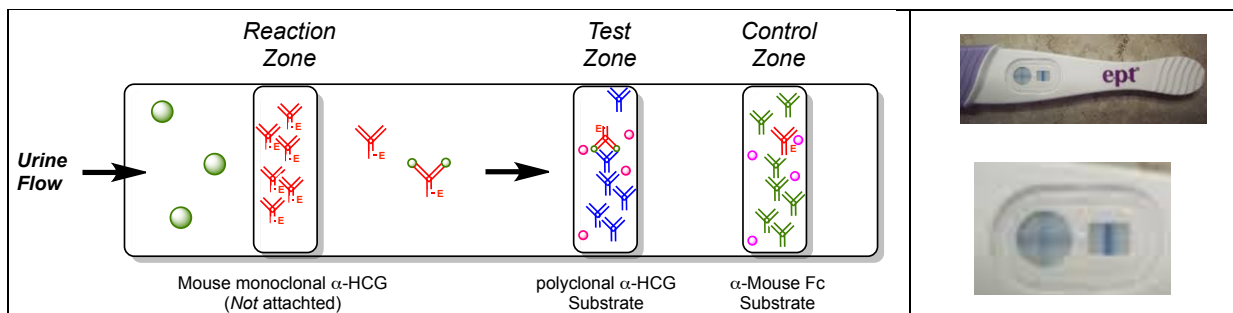
It contains sheep RBCs coated with HCG and an anti-HCG antibody.

The ability of the patient's urine to *inhibit* hemagglutination indicates that it contains HCG.

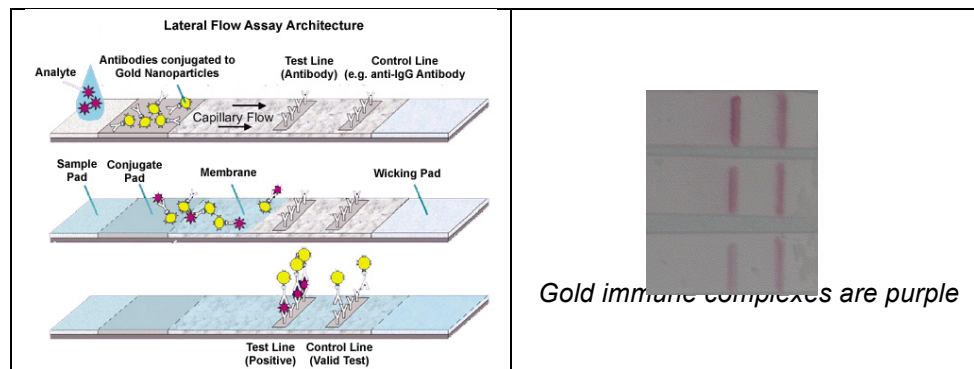


The test is not very sensitive and does not detect HCG until at least 9 days after a missed period. The test takes 2-3 hours to develop, has up to 25% false negative and 5% false positive results.

2. **ELISA-Based Test.** This is the most widely used method due to its simplicity, sensitivity (can detect 1 day after a missed period), and accuracy (1% false negative; 0.5% false positive). Test results are available in 5 minutes.



3. Colloidal Gold Test. Same advantages as ELISA



B. Ovulation Prediction Tests are used to detect the optimal time of fertility. These tests detect the surge of *lutinizing hormone* (LH) that occurs 24-48 hours prior to ovulation.

All of these tests are ELISA based, they use MAbs to LH, and are 95% accurate.

They are available as 5, 7, and 9 day tests. The 5 day test is usually sufficient unless the menstrual period is irregular.

Some tests also include estrone detection.

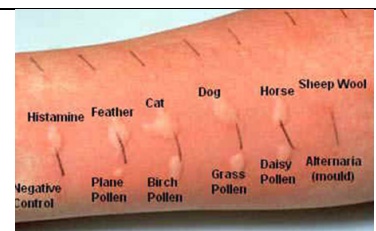


C. A Tuberculin Test (the Mantoux test) is available for detecting exposure to *Mycobacterium tuberculosis*. The test involves the intradermal injection of *tuberculin purified protein derivative* (PPD), which is a precipitate of species-nonspecific molecules obtained from filtrates of sterilized, concentrated *M. tuberculosis* cultures.

If the patient has been exposed to *M. tuberculosis* they will have antibodies to the PPD, which results in edema and erythema at the injection site in about 48 hours.

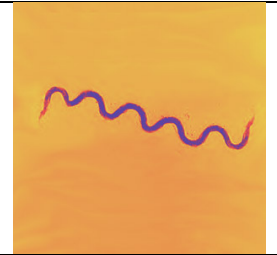


A similar test is available to detect exposure to mumps, hay fever, and allergens.



D. Syphilis is a sexually transmitted disease (STD) caused by infection with *Treponema pallidum*.

There are ~ 50,000 cases of syphilis reported annually. The time between infection and appearance of the *primary* symptom (chancre) is ~21 days. The organism can be isolated from sores in this “primary” stage. The sores resolve but the infection continues and progresses into the *secondary* stage, which presents as a diffuse rash, often on the palms of the hands and soles of the feet. A long period of latency ensues, which can last up to 30 years. Tertiary syphilis can result in damage to the internal organs, including the brain, nerves, eyes, heart, blood vessels, liver, bones, and joints.



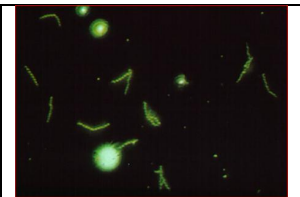
The bacteria can be isolated from the primary chancre and visualized microscopically (gram stain).

Screening tests are important in diagnosing infection and in monitoring therapy, especially in the latter stages. These include two basic types:

Non-treponemal tests are used initially. Infection elicits non-specific antibodies that react to cardiolipin (a lipid found in the bacterial membrane). The Venereal Disease Research Laboratory (**VRDL**) and Rapid Plasma Reagin (**RPR**) tests are “flocculation” tests that look for Ab aggregation in the presence of cardiolipin (ox heart extract).

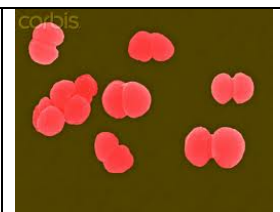
These tests are rapid and inexpensive but these are only qualitative. Some viruses (EBV), rheumatoid diseases, and leprosy (*M. leprae*) can yield false positives.

Treponemal tests are used to confirm the infection. These include the Fluorescent Treponemal Antibody-absorption (FTA-ABS) test, the *T. pallidum* particle agglutination assay (TPPA), and the *T. pallidum* hemagglutination assay (TPHA). These assays probe for specific *M. pallidum* antigens microscopically, on gelatin beads, or on RBCs, respectively.



E. Gonorrhea (a.k.a., “The Clap”, derived from clavier, Old French for brothel) is a common STD caused by infection with *Neisseria gonorrhoeae*, a gram negative diplococcus.

The organism is often detected by culture and microscopic examination. Direct fluorescent antibody, ELISA and PCR assays are now available.

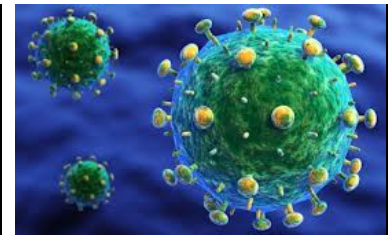


F. Rubella (a.k.a., German Measles) is caused by infection with the rubella virus, an enveloped, negative sense, single stranded RNA virus. Infection is particularly dangerous to a fetus, so pregnant women are routinely tested for antibodies to rubella antigens. This is an ELISA based assay that detects either IgM or IgG antibodies using rubella antigens adsorbed to the plate.



G. The Human Immunodeficiency Virus (HIV, a retrovirus) is responsible for Acquired Immune Deficiency Syndrome (AIDS).

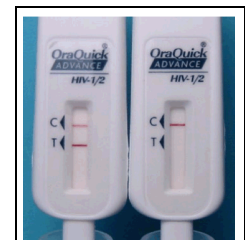
There are an estimated 34 million people infected with HIV worldwide; 1.7 million have died from AIDS.



There are about 1.2 million cases of HIV infection in the US with ~ 50,000 new infections annually; more than half of these among those under 25 years of age. The CDC recommends HIV screening for all individuals 13 - 64 years of age.

Early detection is important for treatment and control of the spread of the disease. Unfortunately, *a major problem is that there is a 2-12 week latency (window) period from the time of infection to antibody detection. It can be as long as 6 months in some cases.*

An ELISA assay, which detects serum antibodies to HIV, was the first widely used screening test. There are now several kits that are available as point of care (POC) devices for health care providers. They can use blood or saliva, are highly sensitive and accurate.

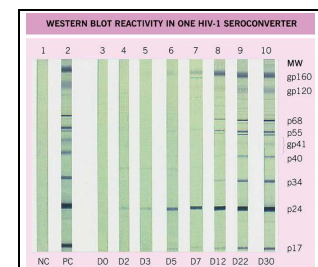


OraQuick is now available as a home test kit that uses saliva.

The ELISA tests are highly sensitive with few false positives and even fewer false negatives. False positives can be observed with syphilis (*T. pallidum*), lyme disease (*Borrelia burgdorferi*), and lupus erythematosus (anti-nuclear antibody).

A positive HIV ELISA can be repeated to confirm the result; however, HIV infection is confirmed by *Western blot analysis*.

Once HIV infection has been confirmed, treatment is followed by blood analysis for CD4+ counts and by PCR amplification of HIV RNA to assess viral load.



H. Others

Group A β -hemolytic streptococci ~ throat swab and ELISA test for Strep antigen

Epstein Barr Virus (mononucleosis) ~ fluorescence and ELISA for EBV antigen

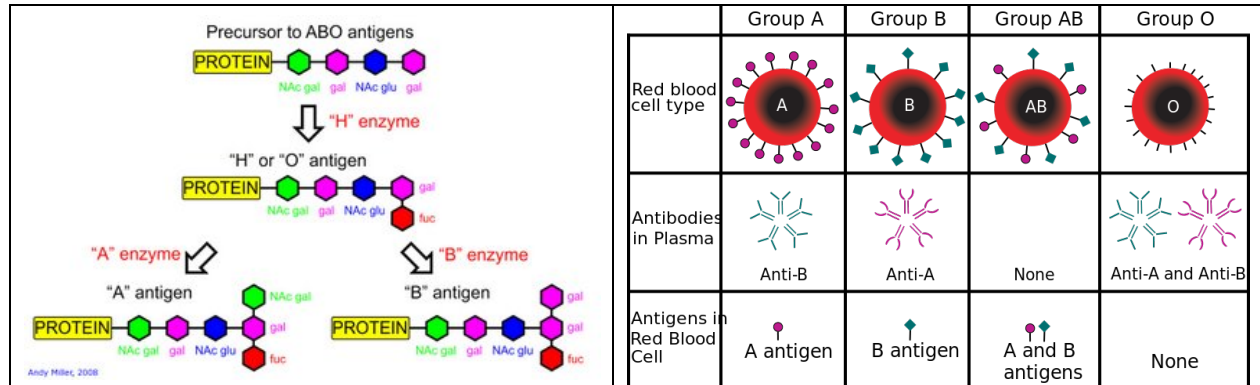
Rheumatoid arthritis ~ ELISA for Rheumatoid factor (antibody)

Helicobacter pylori ~ ELISA urine test for anti-helicobacter antibodies

III. Blood Banking

A. The ABO System

Blood groups, or types, are based on the absence or presence of specific glycoproteins and glycolipids presented on their surface. A total of 30 human blood group systems are now recognized; here we consider only the major four (the ABO system).

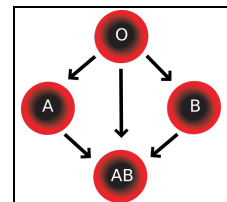


1. Blood Group O (~ 45% of the population). These patients have O-antigens that are very weak and therefore essentially non-antigenic. Thus, these patients are “*universal donors*”.

However, this group must receive Type O blood because they have antibodies to A and B antigens in their serum.

2. Blood Group A (~ 35% of the population). These patients have A-antigens on their RBCs and antibodies to B-antigens in their serum.
3. Blood Group B (~ 15% of the population). These patients have B-antigens on their RBCs and antibodies to A-antigens in their serum.
4. Blood Group AB (~ 5% of the population). These patients have both A- and B-antigens on their RBCs but do not have antibodies to either the A- or B-antigens in their serum. They are “*universal recipients*”.

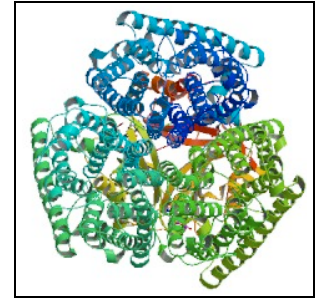
If a unit of incompatible blood is transfused between a donor and recipient, a severe acute hemolytic reaction can occur, leading to renal failure, shock, and possibly death.



B. Rh System. The Rhesus factor (Rh factor; a.k.a., D factor) was discovered in Rhesus monkeys and is a separate set of antigens on RBC surfaces. The most important is the Rh factor and 85% of the US population is Rh positive.

The protein is an ammonia transporter found in the RBC.

anti-Rh antibodies in a Rh⁻ patient are not expressed well under normal conditions, so their concentration is very low.



Hemolytic Disease of the Newborn (HDN) arises when a mother is Rh negative and the fetus is Rh positive due to inheritance from the father. This is not usually a problem for the first birth; however, it is not uncommon for the mother to be exposed to the baby's blood during this birth.

If the mother has previously been exposed to Rh positive blood from a previous pregnancy or a transfusion, this can lead to a significant increase in the mother's anti-Rh factor antibodies. These IgG antibodies can cross the placenta, which can lead to destruction of RBCs in the Rh positive fetus.

Blood typing is done on the mother and father, and if possible on the fetus in subsequent pregnancies. If warranted, Rho-GAM (Rh-immune globulin, anti-Rh antibodies) is administered IM at 28 weeks of pregnancy and again within 72 hours of delivery.

Immunoassays Study Guide

Terms You Need to Know

- Agglutination
- Antigen
- Epitope
- Hapten
- Serial dilution
- Titer

Be prepared to describe the five different human antibodies and their basic biological roles.

Be prepared to describe the basic principles behind the immunoassays:

- Agglutination (direct, indirect, inhibition; how do they differ?)
- Direct Immunofluorescence
- ELISA (direct, indirect, sandwich)
- Hemagglutination
- Immunoelectrophoresis
- Western blot

and the Polymerase Chain Reaction (normal vs. real time).

Be prepared to describe the basic principle behind a urine “stick” test.

Be prepared to describe the basic principle behind pregnancy and ovulation tests. What proteins are detected in each test?

Be prepared to describe the basic principle behind the Mantoux TB test and the allergen skin test. What is actually being measured in these tests?

Be prepared to discuss the difference between the non-treponemal syphilis tests (e.g., VRDL) and the treponemal tests. What does each test actually measure?

You should know the microorganisms responsible for syphilis, gonorrhea, tuberculosis, rubella, and AIDS.

Be prepared to discuss the ABO and Rh blood antigens, what they are and why they are important. Know which antibodies are associated with each blood type and why.

Be prepared to counsel a patient about the use of Rho-GAM ... what is this drug, why is it used, and how does it work ... in a manner that would be understandable to a *patient*.