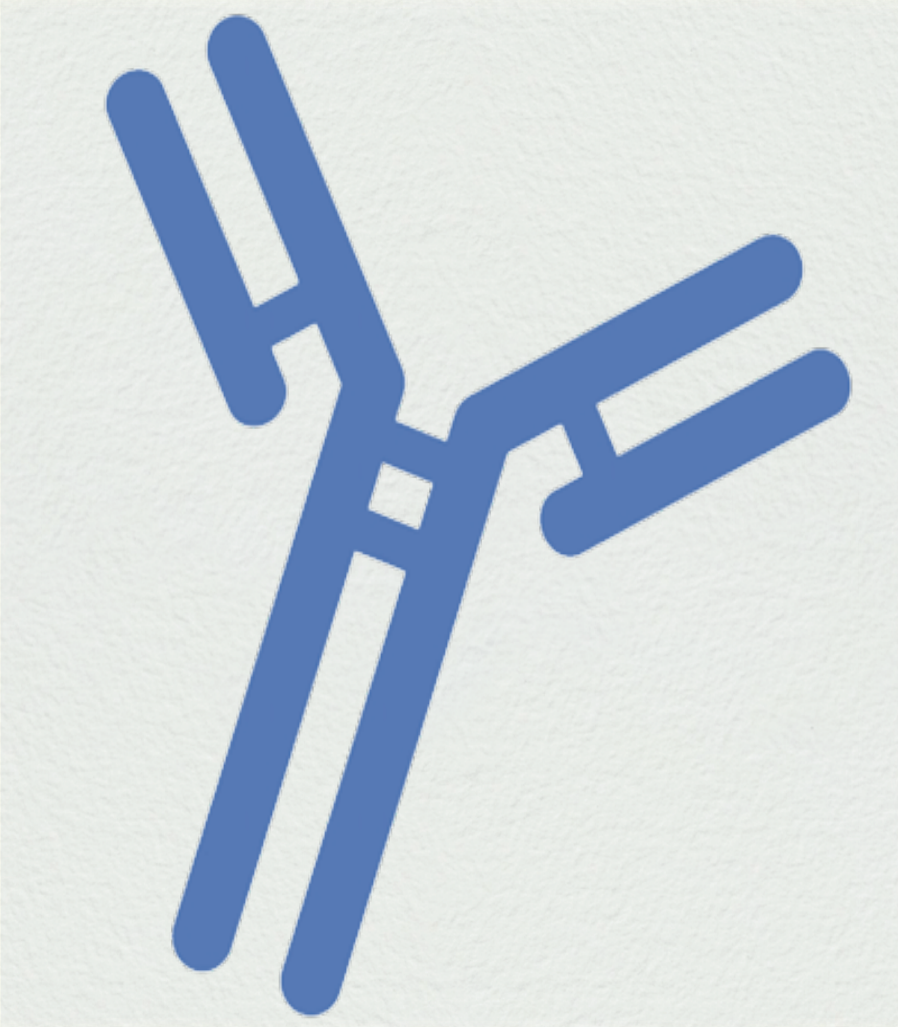
Komar University of Science and Technology
Medical Laboratory Science Department

Immunology Laboratory Manual

MLS3425C



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Fall 2017

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Immunology Lab

Introductory

Resistance to disease depends on the **innate mechanisms** and adaptive or **acquired immunity**. Acquired immune mechanisms act in a specific manner and function to supplement the important nonspecific or natural resistance mechanisms such as physical barriers, granulocytes, macrophages, and chemical barriers (lysozymes, etc.).

The specific immune mechanism constitutes a combination of less specific factors, including the activation of macrophages, complement, and necrosis factors; the early recognition of invaders by cells exhibiting a low level of specificity.

The Immunology laboratory course covers the vast majority of immunological and serological procedures used to provide valuable knowledge in the process of diagnosis (diseases and infections). Thus, special emphasis is placed on correlating of laboratory results with the patient's possible condition.

This manual will cover essential needs of immunological experiments necessary for undergraduate students. Principles of serial dilution, total and differential counts of white blood cells, hemagglutination and latex agglutination tests will be covered. From this lab manual immunochromatography, techniques of blood transfusion and ELISA techniques will also be covered once students are familiarized with essential needs.

WEEK 1. Sample Handling

The first step in the accurate diagnosis of infectious diseases is the proper collection and handling of specimens. The pre-analytical, analytical, and post-analytical factors are essential for quality assessment in the medical laboratories.

Specimen handling involves the following steps:

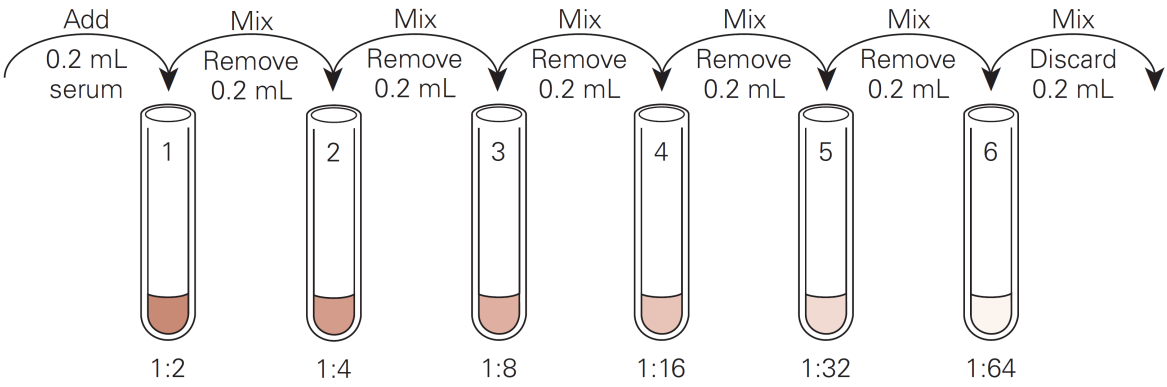
1. Correct identification of the patient.
2. Proper specimen collection.
3. Appropriate use of suitable specimen containers.
4. Proper labeling of forms and containers.
5. Timeliness of specimen transport.
6. Proper identification of special procedures based on suspected pathogens.
7. Proper handling in the laboratory on the selection of growth media, isolates, incubation.
8. Times and temperatures.
9. Accurate reporting of results.
10. Right time for specimen collection – blood cultures, etc.
11. Correct identification of special handling – ice, pre-chilled tubes, spins immediately, etc.

Serial Dilution

Serial dilution is a method of decreasing the strength of an antibody solution by using the same dilution factor for each step.

Serial dilutions are a set of dilutions in which the dilution factor is the same at each step. These are used to make high dilutions with a small number of test tubes and a minimal amount of the diluent. It is commonly used to determine the strength or the titer of a particular antibody in the patient's serum as a part of the diagnosis of a disease or infection.

Traditionally, serological pipettes have been used in this process, but now it is more common to employ micropipettes for the same purpose.



Each tube contains 0.2 mL of the diluent. Patient's serum (0.2 mL) is added to the first tube. After mixing carefully, 0.2 mL is withdrawn and added to the second tube, and the process continued until the last tube. The sample is mixed, and 0.2 mL is discarded.

The serum sample must be diluted to perform quantitative tests.

The **titer** can be calculated as follows:

$$New\ Dilution = \frac{Vol.\ of\ sample}{Vol.\ of\ sample + Vol\ of\ Diluent} * Previous\ Dilution \text{ (Concentration)}$$

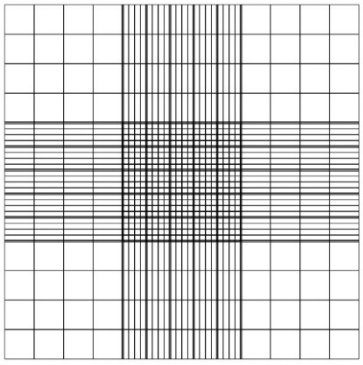
Th titer is the last dilution of the serum sample in which the reaction is positive.

WEEK 2. Total and Differential WBC Counts

2.1. Total WBC Counts

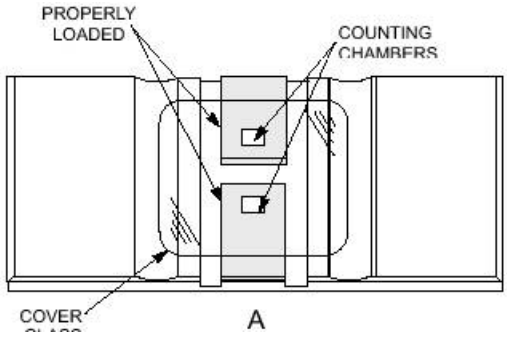
2.1.1. Background:

Total White Blood Count: A WBC count, also called a leukocyte count, is part of a complete blood count. It is counting the number of different types of leucocytes in one cubic millimeter. The total number of leukocytes may increase or decrease in certain diseases, but it's diagnostically useful only when the **patient's white cell differential** & **clinical status** are considered.



2.1.2. Purpose:

- Determining the occurrence of any abnormalities due to infections and inflammations
- Explaining if there is a need for further tests such as bone marrow biopsy
- Monitoring response to chemotherapy



2.1.3. Material:

- Glacial acetic acid
- Fresh blood sample (anticoagulated)
- Neubauer Chamber
- Microscope

2.1.4. Procedure:

- Dilute the blood sample 20 times with glacial acetic acid by adding 0.02 ml of blood to 0.38 ml glacial acetic acid.
- Enumerate the numbers of WBC in **4 corners of the chamber** and multiply by 50.

Calculation:

$$Total\ WBC\ No.\ = No. \times \frac{Dilution\ Factor}{Volume}$$

$$Total\ WBC\ No.\ = No. \times \frac{20}{0.4}$$

$$Total\ WBC\ No.\ = No. \times 50$$

Results:

Write the name, gender, age, occupation, health status and WBC count result for your sample.

Name:	
Gender:	
Age:	
Occupation:	
Health Status:	
WBC count:	
Extra Notes:	

2.2. Differential WBC Counts

2.2.1. Background:

The differential WBC count is used to evaluate the distribution and morphology of WBCs, providing more accurate information about the patient's immune system than a total WBC counting alone. WBCs are classified into five major types [neutrophils, eosinophils, basophils, lymphocytes, and monocytes]. Through this test the percentage and the proportion of each type can be determined.

The total number of each WBC type can be calculated by multiplying its percentage to the total WBC count. Elevated leukocytes are associated with various health conditions, for example high eosinophil counts are related to allergic diseases and reactions to parasites. An eosinophil count is sometimes ordered as a follow-up test when an elevated or depressed eosinophil level is reported.

Differential WBC Counts: The differential counts measures the percentage of each type of white blood cell (WBC) found in blood. It also reveals if there are any abnormal or immature cells. The normal ranges are:

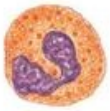
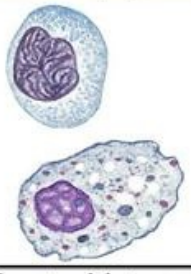
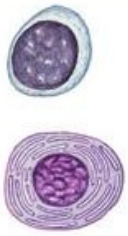
~ 60% neutrophils (50% - 70%)

~ 3% eosinophils (>0% - 5%)

~ 0.5% basophils (>0% - 2%)

~ 5% monocytes (1% - 9%)

~ 30% lymphocytes (20% - 40%) These cells play both an immediate and delayed role in response to infection or inflammation.

	Basophils and mast cells	*Neutrophils*	*Eosinophils*	*Monocytes and macrophages*	*Lymphocytes and plasma cells*	*Dendritic cells*
						
Primary function(s)	Release chemicals that mediate inflammation and allergic responses	Ingest and destroy invaders	Destroy invaders, particularly antibody-coated parasites	Ingest and destroy invaders Antigen presentation	Specific responses to invaders, including antibody production	Recognize pathogens and activate other immune cells by antigen presentation

Lymphocytes:

These cells are variable in size; the smallest lymphocytes may be smaller than erythrocytes (down to ~ 5 µm in diameter) while the largest may reach the size of large granulocytes (up to 15 µm in diameter).

Lymphocytes Functions:

Most lymphocytes in the blood stream belong to either the group of B-lymphocytes (~5%) or the group of T-lymphocytes (~90%).

Unless they become activated, the two groups cannot easily be distinguished using standard light or electron microscopy. Upon exposure to antigens by antigen-presenting cells (e.g. macrophages) B-lymphocytes differentiate into antibody-producing plasma cells and memory B-cells. The amount of cytoplasm increases and RER fills a large portion of the cytoplasm of plasma cells.

T-lymphocytes represent the "cellular arm -cell mediated-" of the immune response (cytotoxic T cells) and may attack foreign bodies, cancer cells, and viral infected cells.

2.2.2. Purpose:

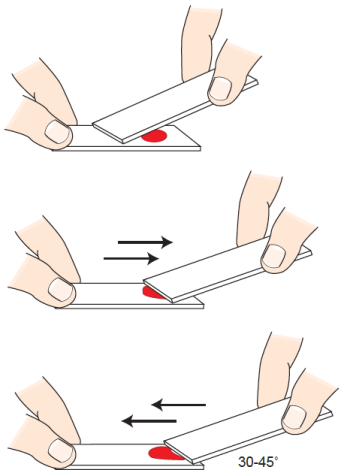
- To evaluate the body's capacity to resist and overcome infections.
- To detect and identify various types of leukemia.
- To determine the stage and severity of an infection.
- To detect allergic reactions and parasitic infections and assess their severity (eosinophil count)
- To distinguish viral from bacterial infections.
- Detect any morphologic changes of leukocytes.

2.2.3. Material:

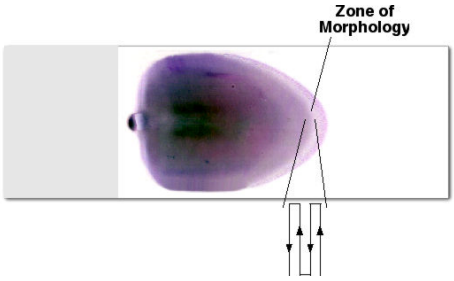
Leishman's Stain:

It is used for staining of blood and bone marrow, and to differentiate and identify leucocytes, malaria parasites and Trypanosomes (unicellular parasitic protozoa). This stain is based on a mixture of methylene blue and eosin.

2.2.4. **Procedure:** *See reagent product insert to verify the method.*

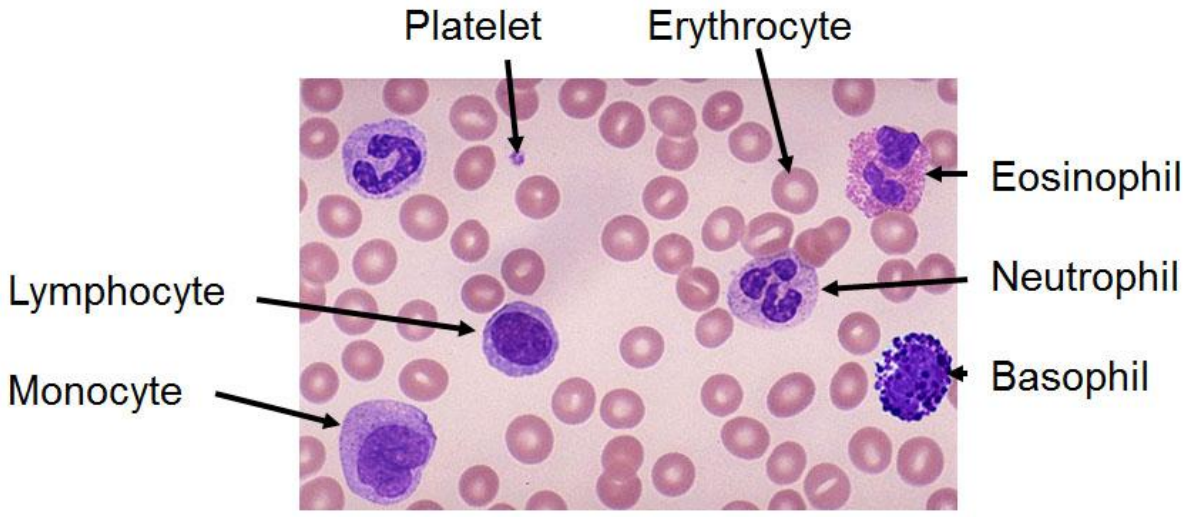


1. Use smears that are as thin as possible and air-dried. Fully cover the smears with Leishman's Stain solution. Let for about 2 minutes.
2. Add twice the amount of distilled water and mix by swirling. Incubate for at least 10 min.
3. Rinse thoroughly with distilled water.
4. Dry the slides using blotting paper and air-dry.



Below points help you in identifying the cells:

- Erythrocytes: light pink to brown
- **Cores of lymphocytes: deep, dark blue to blue-violet**
- **Cytoplasm of lymphocytes: light blue**
- Nuclear lobes of neutrophil, polymorphonuclear leukocytes: a dark blue to blue-violet
- Granules of neutrophilic polymorphonuclear leukocytes: red
- Cores of eosinophil leukocytes: blue violet
- Granules of eosinophilic leukocytes: dark red
- Cores of basophilic leukocytes: blue violet



2.2.5. Result:

Total WBC Count = _____ Cell/mm^3

White Blood Cell Types	Counted Number	Percentage	Real Number
NEUTROPHIL		_____ %	_____ cell/mm^3
BASOPHIL		_____ %	_____ cell/mm^3
EOSINOPHIL		_____ %	_____ cell/mm^3
MONOCYTE		_____ %	_____ cell/mm^3
LYMPHOCYTE		_____ %	_____ cell/mm^3
Total	_____ Cells	100%	Same as Total WBC

2.2.6. Review Questions:

Could Total and differential WBC counts be used as a specific indicator to any bacterial infection?

If yes, How?

If no, Why?

WEEK 3. Phagocytosis

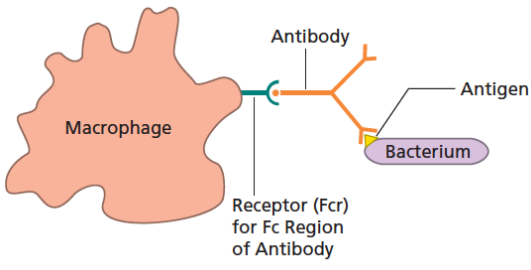
3.1. Background:

Phagocytosis is the process of engulfment and digestion of foreign bodies including microorganisms. Phagocytosis is such an interesting cell behavior, which could be an excellent topic for student exploration.

In many eukaryotic cells, substances taken into the cell start their journey at the cell surface, when a membrane invagination encloses the material being ingested. The invagination pinches off to form a membrane vesicle containing the ingested material. This process is called endocytosis. The membrane vesicle travels within the cell and ultimately fuses with a lysosome. There, the ingested substance can be digested. One type of endocytosis is phagocytosis in which large (>250 nm) particles are "eaten" by cells. This dramatic cell behavior is characteristic of many protozoa whereas, in multicellular organisms, it is a behavior seen only in certain specialized cells (for example, macrophages). In protozoa, phagocytosis is a feeding mechanism. Particles are brought into the cell in the major endocytic vesicles called phagosomes, which fuse with lysosomes and digestion of the ingested particles occurs.

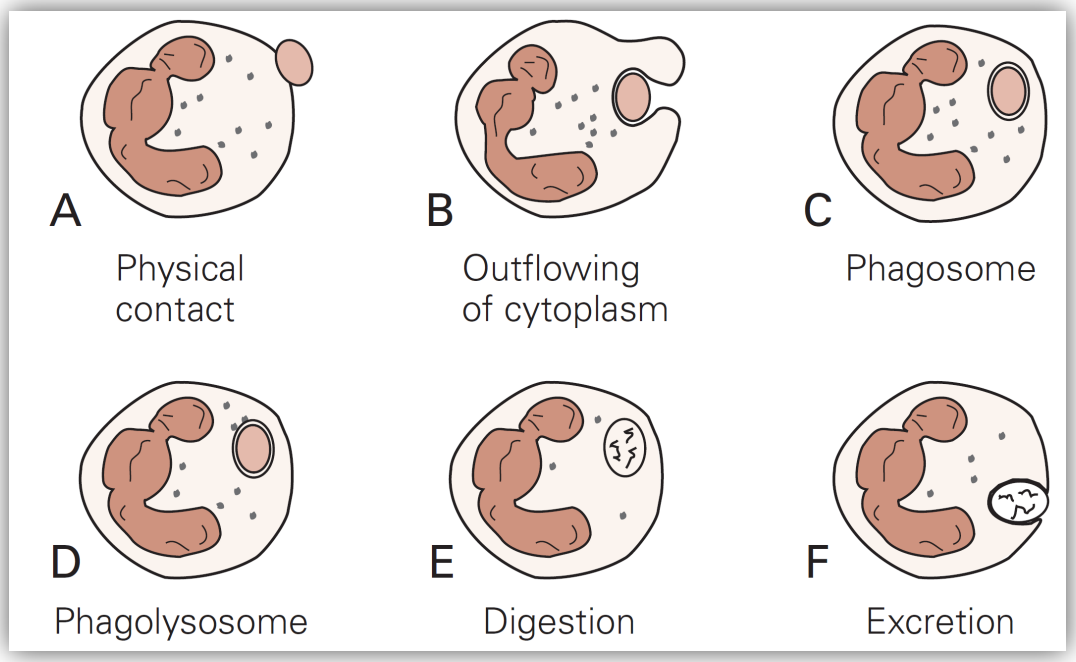
- Phagocytosis is a two-stage process in which particles are first bound to the cell surface and then ingested.
- The process of binding and ingesting particles is a critical part of the immune response.
- *In vitro,* it is important to distinguish these two processes.

Steps Involved in Phagocytosis:



1. Activation and Adherence: physical contact between the phagocytic cell and the microorganism occurs, aided by opsonins.
2. Outflowing of cytoplasm (pseudopod formation) to surround the organism (prey).
3. Formation of phagosome: through membrane extensions the phagocytic cell surrounds the prey, and ingests it in a sac called phagosome.
4. Formation of the phagolysosome: cytoplasmic granules fuse with the membrane of the phagosome, emptying contents into this membrane-bound space.

5. Digestion of the microorganism by hydrolytic enzymes.
6. Excretion of the digested (contents of phagolysosome) to the outside by exocytosis.



3.2. Principle:

White Blood cells are responsible for clearing human body from foreign bodies such as microbes. In vivo WBCs eliminate any foreign particles, including microbes, by the process of phagocytosis. In vitro this process can be observed by introducing the foreign body to freshly taken WBC from an individual.

3.3. Material:

- Active broth cultures of Bacteria and Yeast
- Anticoagulated Blood
- Water-bath
- Leishman's stain
- Slide, cover-slip & Microscope
- Timer

3.4. Procedure:

1. Collect fresh venous blood sample into anticoagulant tube
2. Centrifuge at 1500*g* force for 15 minutes [about 4000rpm]
    - G-force = 0.000001118 x R x RPM²
    - R: radius of centrifuge in millimeters [80mm]
3. Withdraw the WBC rich buffy coat from the blood sample and transfer into a clean test tube contains 0.5 normal saline
   [1dorp of fresh blood + 1drop of resuspended culture can be used as well]
4. Mix gently to homogenize the cells in the test tube before each use.
5. Take small amount of bacteria and yeast cultures into a clean tube and centrifuge.
6. Discard the supernatant and resuspend by vortex mixing in 3 to 4 ml normal saline.
7. Prepare a mixture by mixing 0.5 ml of bacterial suspension with 0.25 of WBCs in a new clean tub
8. Incubate the mixture in a water bath (37^{o}C) and examine under the microscope at 5-minute time intervals. [i.e.; 0, 5, 10, 15, etc.]
9. Once phagocytosis takes place or visualized, prepare a smear using Leishman's stain.

3.5. Result:

Blood Sample	Foreign Body	Phagocytosis	Your Notes
1	Bacteria	Seen or not	
2	Yeast	Seen or not	

3.6. Review Questions?

- Why do we prefer to discard the culture media from the bacteria and yeast before mixing them with white blood cells?
- What is the importance of water bath in this procedure?

WEEK 4. Antibacterial Activity of Serum

4.1. Background:

Serum:

Within the body, the liquid portion of blood is called plasma, but if a blood is allowed to clot, the collected liquid portion is called serum. Thus, the serum is plasma that no longer contains clotting factors. However, serum contains two major types of protein; globulins and the albumins and it is an excellent source of immunoglobulins. Especially serum IgG, as it is more abundant than IgM, IgA, IgD or IgE.

4.2. Principle:

The serum can act as an antibacterial agent as it contains some biologically active proteins that serves in protecting the host.

4.3. Materials:

- Fresh serum (should be divided into two equal parts)
- Sterile D.W.
- Bacterial growth (One or more than one type of bacteria)
- Gel (can be prepared by adding 2-3% agar to the nutrient broth)
- Water bath
- Different test tubes and glass wares
- Incubator
- Centrifuge
- Petri dishes

4.4. Procedure:

1. Collect sufficient amount of serum (at least from 2-3 persons)
2. Divide the collected sera into two equal parts (part A and B)
3. Heat one part (A) by putting in a water bath (56^{o}C for 15 minutes) – this deactivates the complement proteins.
4. Prepare a gel (If no ready to use gel is available, it can be prepared in the lab by adding 3% of agar into the nutrient broth and dispensing into Petri dishes.

5. Make wells in the gel after bacterial cultivation and labeling (at least three well in each plate- one for D.W, one for fresh serum and the other for the heated serum)
6. Incubate overnight (after adding D.W and the two serum samples into the wells)
7. Try to record inhibitory zones around each well and compare the obtained results and discuss

4.5. Result:

Contents of the wells	Clear zone diameter (In millimeter)
Distilled Water	_____ mm
Heated Serum	_____ mm
Unheated Serum	_____ mm

4.6. Review Questions:

- What is the effect of heating on the antibacterial activity of serum?
- What is the aim of using D.W.?

WEEK 5. Blood Group [ABO Slide Agglutination Test]

5.1. Background:

Direct agglutination occurs when antigens are found naturally in a particulate form. One of the best examples of direct agglutination testing involves known bacterial antigens used to check for the presence or absence of unknown antibodies in the patient's sample. Typically, the patient serum is diluted into a series of tubes or wells on a slide and reacted with bacterial antigens [specific for the suspected disease]. Detection of antibodies primarily used in the diagnosis of diseases for which the bacterial agents are difficult to cultivate. One such example is the Widal test, a rapid screening test or the possibility of typhoid fever. The antigens used in this procedure include *Salmonella* O (somatic) and H (flagellar) antigens.

A significant finding is a four-fold increase in the antibody titer over time when paired dilutions of serum samples are tested with any of these antigens. This test is still considered useful in diagnosing typhoid fever in developing countries, if while more accurate tests are now available, and it remains in use in many areas throughout the world.

When an agglutination reaction involves red blood cells, then it is called hemagglutination. The best example of this occurs in ABO blood group typing of human red blood cells. Antisera of the IgM type can be used to determine the presence or absence of the A and B antigens, and this reaction is usually performed at room temperature without the need for any enhancement techniques. This type of agglutination reaction is simple to implement, it is relatively sensitive, and is easy to read. A titer that yields semi-quantitative results can be performed in test tubes or microtiter plates by making serial dilutions of the antibody. The reciprocal of the last dilution still exhibiting a visible reaction is the titer, indicating the antibody's strength.

5.2. Principle:

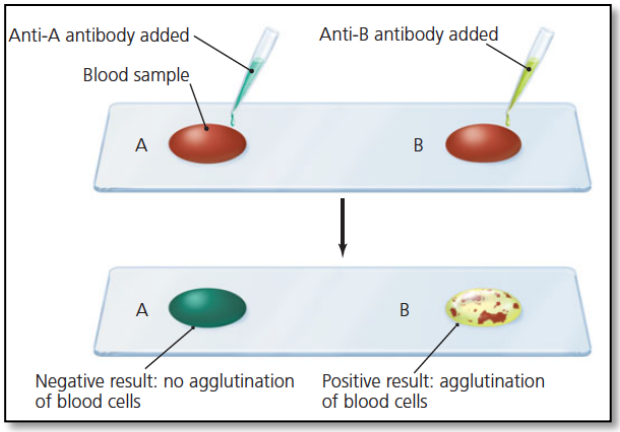
When red cells mixed with various reagent antisera (antibody), agglutination will occur on the slides containing cells positive for (possessing the antigen) the corresponding antigen. No agglutination will happen when the red cells do not include the corresponding antigen. Blood typing is an application of this principle.

Using the slide agglutination method, blood groups can be determined with 100% accuracy through using certain reagent antisera (anti-A, anti-B).

	Group A	Group B	Group AB	Group O
Red blood cell type	A	B	AB	O
Antibodies in Plasma	Anti-B	Anti-A	None	Anti-A and Anti-B
Antigens in Red Blood Cell	A antigen	B antigen	A and B antigens	None

5.3. Material:

- Antibody A (Anti-A reagent)
- Antibody B (Anti-B reagent)
- Red blood cells (labeled; more than two samples)
- Slides
- Applicator sticks
- Pipets



5.4. Procedure:

See reagent product insert to verify the method.

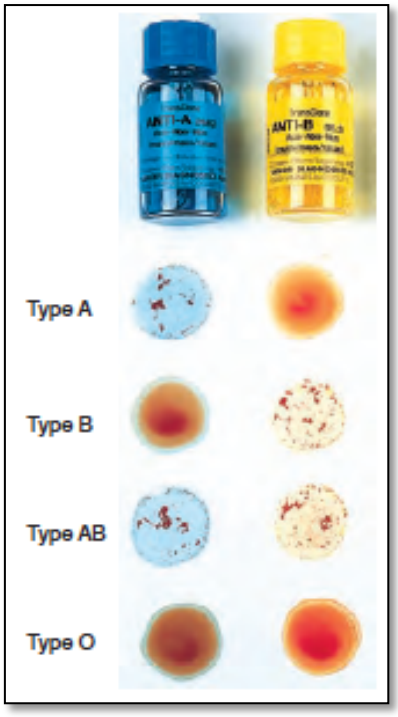
1. On the section of slide labeled anti-A place one drop of antibody A.
2. On the part of slide labeled anti-B place one drop of antibody B.
3. Place one drop of cells in each antibody containing a circle.
4. Carefully mix each solution with a separate applicator stick.
5. Tilt slowly for one minute.
6. Record results.

5.4.1. Limitations of the Procedure:

1. Carefully dispense the red cell suspension and antibody in separate areas of the slide. Adding red blood cells directly to the drop of antisera may cause splash back, resulting in contamination of the red blood cells.
2. When mixing the antibody with the red blood cells be sure the mixture covers the entire bottom of the testing area. Inadequate spreading of the mixture will make interpretation difficult and may result in misinterpretation of results.
3. It is critical to read the results at the end of the designated time. Allowing the reaction to go beyond the allotted time may result in the drying of the reactants, resulting in a false positive.

5.4.2. Interpretation:

Agglutination (clumping) of the red blood cells means positive result. No agglutination means negative test. It is critical to read the results immediately as false positives can occur when the mixture begins to dry on the side.



Blood Group	anti-A	anti-B
A	+	-
B	-	+
O	-	-
AB	+	+

5.5. Results

Record your reactions for sample 1 and 2 in the boxes below. Write “aggt” if agglutination observed, write “no aggt” if no agglutination occurred. Use the table on the previous page to interpret your results.

Sample	**Anti-A**	**Anti-B**	**Interpretation**

5.6. Review Questions:

1. Describe the process of agglutination as you understand it.
2. Explain why agglutination occurred with one antiserum but not with another.

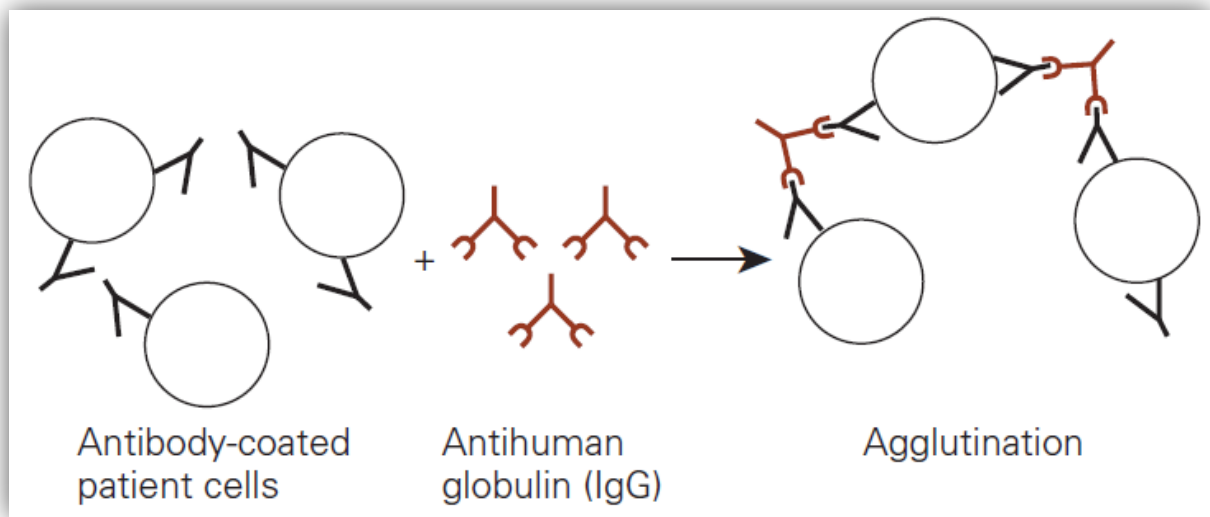
WEEK 6. Coombs' test

6.1. Background:

The anti-human globulin test, also known as the Coombs' test, is a technique that detects nonagglutinating antibody using a coupling with a second antibody. It remains one of the most widely used procedures in blood banking. The key component of the test is an antibody to human globulin that is produced in experimental animals or using hybridoma techniques. Such antibody will react with the FC portion of the human antibody attached to red blood cells. Agglutination takes place because the anti-human globulin can bridge the distance between cells that IgG alone cannot do. The strength of the reaction is proportional to the amount of antibody coating the red blood cells. The Coombs' test either direct or indirect, each of which has a different purpose.

Direct Antiglobulin Test (Direct Coombs' test)

The direct antiglobulin test is used to demonstrate *in vivo* attachment of antibody or complement to an individual's red blood cells. This test serves as an indicator of autoimmune hemolytic anemia, hemolytic disease of the newborn, sensitization of red blood cells caused by the presence of drugs, or a transfusion reaction. The test is called direct, because red blood cells are tested directly as they come from the body. A blood sample is obtained from the patient, the red blood cells are washed to remove any antibody that is not specifically attached, and then cells are tested directly with antibody to IgG or complement components. If IgG or complement is present on the red blood cells, the antihuman globulin (Coombs' reagent) is able to bridge the gap between red blood cells and cause a visible agglutination. Polyspecific antiserum will react with IgG and with complement component C3b. Antibodies to C3b, C4b, or C4a may also be present. If such a reaction is positive, then monospecific antibody, which will react with only one component, is used. This will differentiate between IgG and individual complement components on the patient's red blood cells. A positive test indicates that an immune reaction is taking place in that individual.

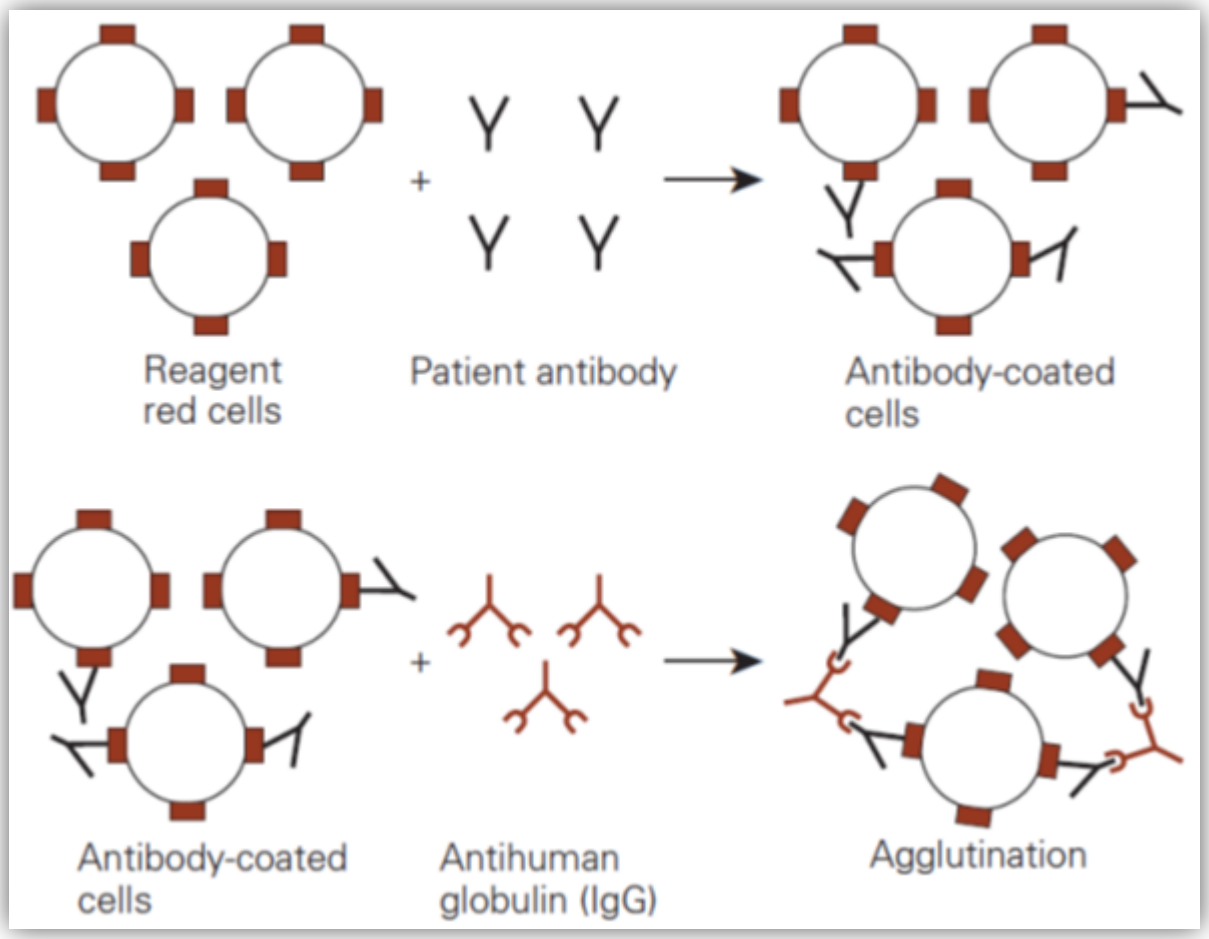


Indirect Antiglobulin Test (Indirect Coombs' test)

The indirect antiglobulin test, or indirect Coombs' test, is used to determine the presence of a particular antibody in the patient's serum, or it can be used to type patient red blood cells for specific blood group antigens. It is a two-step process, in which washed red blood cells and antibody are allowed to combine at 37°C, and the cells are then carefully washed again to remove any unbound antibody. When antihuman globulin is added, a visible reaction occurs where the antibody has been specifically bound.

This test is most often used to check for the presence of clinically significant alloantibody in patient serum when performing compatibility testing for a blood transfusion. In this case, patient serum is used to combine with reagent red blood cells of known antigenicity. All reactions are run at 37°C to detect clinically significant antibodies. Cells are then washed, and antihuman globulin is added. Tubes are centrifuged and read for agglutination.

Possible sources of error in performing the Coombs' test include failure to wash cells, improper centrifugation, inability to add test serum or antihuman globulin, and use of expired reagents or those that have not been properly stored. Also, an incorrect concentration of red cells may alter the results. Too heavy a red cell concentration may mask agglutination while too light level will make the reaction hard to read. Thus, it is important to use quality controls and to interpret results carefully.



6.2. Principle:

Red cells coated with complement or IgG antibodies do not agglutinate directly when centrifuged. These cells are said to be sensitized with IgG or complement. For agglutination to occur an additional antibody, which reacts with the Fc portion of the IgG antibody, or with the complement component, must be added to the system. This will form a "bridge" between the antibodies or complement coating the red cells, causing agglutination.

6.3. Materials:

1. Patient's Blood sample.
2. Coomb's reagent.
3. Test tubes, syringes, dropper.

6.4. Procedure:

6.4.1. Direct (Direct Coombs' test):

1. Prepare a 5 % suspension in isotonic saline of the red blood cells to be tested.
2. With clean pipette add one drop of the prepared cell suspension to a small tube.
3. Wash three times with normal saline to remove all the traces of serum.
4. Decant completely after the last washing.
5. Add two drops of Anti-human serum (globulin).
6. Mix well and centrifuge for one minute at 1500 RPM.
7. Resuspend the cells by gentle agitation and examine macroscopically and microscopically for agglutination.

6.4.2. Indirect (Indirect Coombs' test)

1. Label three test tubes as T (test serum) PC (Positive control) and NC (negative control).
2. In the tube labeled as 'T', add two drops of Anti-D serum.
3. In the tube 'PC' add one drop of saline.
4. Add one drop of 5 % saline suspension of the pooled 'O' Rho (D) positive cells in each tube.
5. Incubate all the three tubes for one hour at 37°C.
6. Wash the cells three times with normal saline to remove excess serum with no free antibodies, (in the case of inadequate washings of the red cells, negative results may be obtained).
7. Add two drops of Coombs serum (anti-human globulins) to each tube.
8. Keep for 5 minutes and then centrifuge at 1,500 RPM for one minute.
9. Resuspend the cells and examine macroscopically as well as microscopically.

6.5. Review Questions:

In which of the following circumstances would the indirect Coombs' test be employed?

a. Identification of the ABO blood groups

b. Identification of cold-reacting antibody

c. Identification of an unexpected IgG antibody

d. Identification of hemolytic disease of the newb

WEEK 7. Antistreptolysin O (ASO) Testing

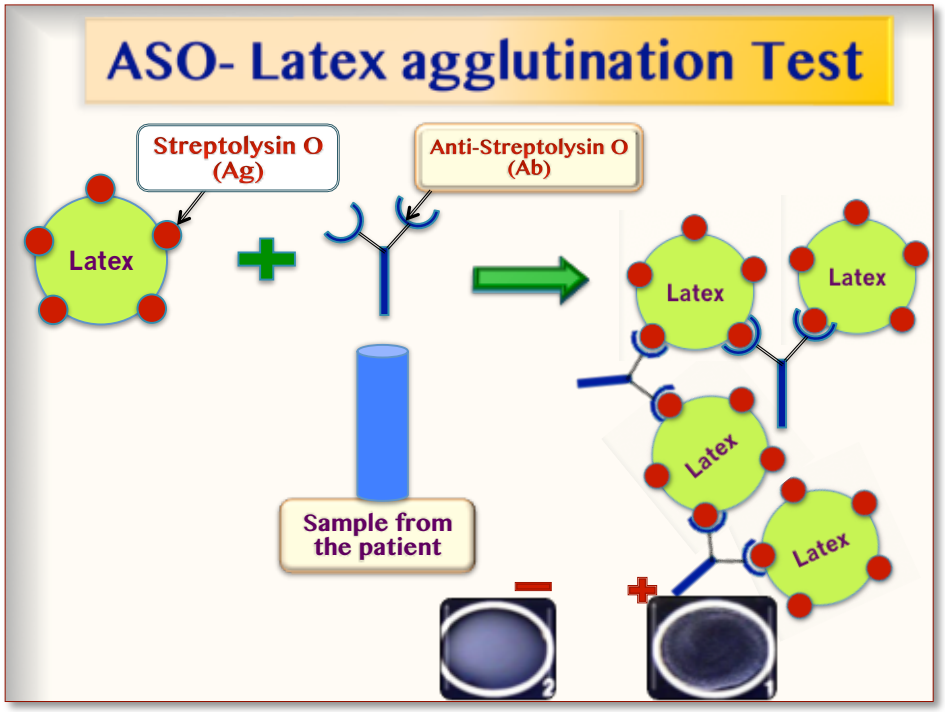
7.1. Background:

ASO tests detect antibodies to the streptolysin O enzyme produced by group A *Streptococci*, which can lyse red blood cells. The presence of antibodies to streptolysin O indicates recent streptococcal infection in patients suspected with acute rheumatic fever or post-streptococcal glomerulonephritis following throat infection.

The classic hemolytic method for determining the ASO titer was the first test developed to measure streptococcal antibodies. This test based on the ability of antibodies in the patient's serum to neutralize the hemolytic activity of streptolysin O.

The traditional ASO titer involves dilution of the patient serum, to which a measured amount of streptolysin O reagent is added. These are allowed to combine during an incubation period after which reagent (red blood cells) are added as an indicator. If enough antibodies are present, the streptolysin O is neutralized, and no hemolysis occurs. The titer is reported as the reciprocal of the highest dilution demonstrating no hemolysis.

7.2. Principle



The ASO-latex is a slide agglutination test for the qualitative and semiquantitative detection of ASO in human serum. Semiquantitative detection of ASO is done by adding a specified quantity of streptolysin O (bacterial produced antigen) to progressively decreasing amounts of patient serum by two-fold dilution. In the tubes where patient antistreptolysin O antibody is present in sufficient amount to form a complex with the antigen agglutination will occur. This agglutination should be examined on a slide.

7.3. Materials:

- ASO Latex Reagent: Latex particles coated with streptolysin O
- ASO Positive Control
- ASO Negative Control
- Pipette-Stirrers
- Disposable agglutination Slides.
- Patient serum specimen
- Timer
- Small glass/plastic test tubes.
- Serological pipettes
- Graduated container
- 9 g/L saline solution

7.4. Procedure:

See reagent product insert to verify the method.

1. Allow the reagents and samples to reach room temperature. The sensitivity of the test may be reduced at low temperatures.
2. Place 50 µL of the sample and one drop of each Positive and Negative controls into separate circles on the slide test.
3. Swirl the ASO-latex Reagent gently before using and add one drop (50 µL) next to the sample to be tested.
4. Mix the drops with a stirrer, spreading them over the entire surface of the circle. Use different stirrers for each sample.

5. Swirl the slide gently and after 2 minutes read the results macroscopically. False positive results could appear if the test is read after more than two minutes.

7.4.1. Semi-quantitative Method:

1. The semi-quantitative test can be performed in the same way as the quantitative technique using dilutions of the serum in 9 g/L saline solution.
2. Make doubling dilutions of specimen as follows:

Dilution	Serum	Saline
1/2	100 µl undiluted serum	100 µl
1/4	100 µl 1/2 diluted serum	100 µl
1/8	100 µl 1/4 diluted serum	100 µl

3. Test the sample dilutions in the same way as for the quantitative technique above.
4. Agglutination of the sera indicates:

Dilution	ASO Levels (I.U. / ml)
1/2	400 (200 x 2)
1/4	800 (200 x 4)
1/8	1600 (200 x 8)

5. Normal range of ASO in adults is < 200 I.U/ml.

7.5. Review Questions:

- What two toxins are produced by the *Streptococcus sp.*? Which one is oxygen labile?

WEEK 8. Slide agglutination test for the detection of rheumatoid factor (RF)

8.1. Background:

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting the joints and periarticular tissues primarily. For many years, it has been known that several abnormal proteins circulate in the blood of patients with RA. These proteins, because of their apparent correlation with the disease, became known as rheumatoid factor (RF). Researches on these proteins have characterized them as usually being a group of IgM class immunoglobulins that interact with antigenic determinants on human IgG molecules (i.e., they are anti-antibodies). Rheumatoid factor is detected in 60-80% of cases of diagnosed rheumatoid arthritis.

8.2. Principle:

Rheumatoid factor (RF) is an anti-antibody, which invitro, is identified by its ability to agglutinate latex particles (or red blood cells) coated with human IgG. If RF is present in patient sample, it will attach to the IgG coating the latex particles. Agglutination of the latex particles is a positive result indicating the presence of RF.

8.3. Materials:

- Rheumatoid factor test kit(s).
- Patient and control serum specimens.
- Timer
- Other materials as directed by reagent product insert(s).

8.4. Procedure:

See reagent product insert to verify the method.

1. Bring all reagents and test samples to room temperature before use.
2. Fill a capillary to the mark with patient serum, and empty it into the center of the middle section of the slide.

3. Place a drop of positive control in the left part of the slide and 1 drop of negative control in the right section.
4. Add a drop of well-shaken reagent to each section of the slide.
5. Mix with a disposable stirrer, spreading each mixture over the entire section. Use a clean disposable stirrer for each mix.
6. Rock the slide gently with a rotary motion for 2 minutes and immediately observe for agglutination. If the test is positive for agglutination, dilute the specimen 1:10 with distilled water or isotonic saline, and repeat the test.

8.4.1. Semi-quantitative Method:

If a titer is desired, the following procedure can be used:

1. Label eight test tubes 1 to 8 and place in a test tube rack.
2. Add 1.8 mL of saline to tube 1 and 1.0 mL of saline to tubes 2 through 8.
3. Add 0.2 mL of the specimen to tube 1, mix, and transfer 1.0 mL of this mixture to tube 2. Mix the contents of tube 2 and transfer 1.0 mL of this mixture to tube
4. Continue serially diluting in this manner through tube 8. Starting with a 1:10 dilution in the first tube, the dilution in the last tube will be 1:1280.
5. Test each dilution as described in the qualitative procedure. The final dilution to show positive agglutination is reported as the titer.

8.5. Result:

Agglutination of latex particles is considered as a positive reaction, indicating the presence of rheumatoid factor at a significant and detectable level. Although the diagnosis of rheumatoid arthritis is based largely on clinical findings, the demonstration of the presence of rheumatoid factor is useful to support the diagnosis, as well as to evaluate the severity and course of the disease.

8.5.1. Limitations:

6. RF can not be detected in all patients diagnosed with RA.
7. RF may be detected in increased amounts in patients with infectious mononucleosis, sarcoidosis, systemic lupus erythematosus, Sjogren.'s syndrome, TB or leprosy, and

other conditions of acute or chronic immune response. The significance of a positive result should be interpreted with caution. Testing should be done to confirm the diagnosis of RA.

8. The procedure must be followed carefully and results read at the appropriate time. Reading after the specified time can lead to misinterpretation due to drying of specimen/reagents.
9. Some products may produce questionable results from hemolyzed, lipemic or contaminated samples. Consult individual reagent product inserts for information.
10. Avoid contamination of reagent or reagent dispensing dropper.

8.6. Review Questions:

- According to the specific product insert, why are plasma specimens not acceptable?
- Describe “rheumatoid factor”.

WEEK 9. C- reactive protein (CRP Test)

9.1. Background:

This is a latex agglutination test to screen for elevated levels of CRP in serum. A level of 0.6 mg/dL or higher gives a positive result with the undiluted specimen. The Normal range are (0.1 to 0.5 mg/dL) in newborns and adults respectively. Usually, with the onset of a substantial inflammatory stimulus, such as infection, myocardial infarction, or surgical procedures, the CRP level increases very significantly (>tenfold) above the value reported for healthy individuals. Following surgery, CRP levels rise sharply and usually peak between 48 and 72 hours. The levels decrease after the third postoperative day and should return to near normal between the fifth and seventh postoperative day. Thus, CRP levels can be used to monitor the outcome of surgery. CRP testing can also be used to monitor graft rejection, drug therapy with anti-inflammatory agents, and recurrence of malignancies. For patients with rheumatoid arthritis, elevated CRP can be used as an indicator of the active stage of the disease. In most situations, however, it is desirable to have more than one determination so that base levels can be established.

9.2. Principle

Latex particles coated with antibody to CRP can react with patient serum. The CRP is acting as the antigen. If CRP is present above average threshold levels, the antigen–antibody combination will result in a visible agglutination reaction. An elevated CRP level is a sensitive, although nonspecific, indicator of inflammation.

9.3. Material:

(Kit from any manufacturer)

- CRP latex reagent, which contains a 1% suspension of polystyrene latex particles coated with anti–human CRP produced in goats or rabbits
- Positive human serum control with a concentration of approximately 20 mg/dL of CRP
- Negative human serum control

- Disposable sampling pipettes
- Disposable test slides
- Not in kit but needed:
- Timer; disposable stirrers; serological pipettes; test tubes

Specimen Collection:

1. Collect blood aseptically by venipuncture into a clean, dry, sterile tube and allow it to clot.
2. Separate the serum without transferring any cellular elements. Do not use grossly hemolyzed, excessively lipemic, or bacterially contaminated specimens. Fresh non-heated inactivated serum is recommended for the test. However, if the test cannot be performed immediately, serum may be stored at 2°C and 8°C for up to 2 days. If there is any additional delay, freeze the serum at –20°C or below.

9.4. Procedure:

Qualitative Slide Test:

See reagent product insert to verify the procedure.

Before starting e the reagents and specimens should be at room temperature.

1. Using one of the pipettes provided, fill it about two-thirds full with undiluted serum.
2. While holding the pipette perpendicular to the slide, deliver one free-falling drop to the center of one oval on the slide. If a calibrated pipette is used instead of the pipettes provided, adjust the pipette to deliver 0.05 mL (50 µL).
3. Using the squeeze dropper vials provided, add one drop of positive control and one drop of negative control to separate ovals on the slide. Note: A positive and a negative control should be run with each test.
4. Re-suspend the latex reagent by gently mixing the vial until the suspension is homogeneous. Place one drop of CRP latex reagent next to each serum specimen and each control.
5. Using stirrers mix each sample and control until the entire area of each oval is filled.
6. Tilt the slide back and forth, slowly and evenly, for 2 minutes. Place the slide on a flat surface and observe for agglutination using a direct light source.

7. The CRP positive control serum must show specific agglutination, and the negative control must be nonreactive. If the reagent fails to agglutinate with the positive control or does agglutinate with the negative control, it should be discarded.

Semiquantitative Slide Test:

1. If a positive reaction is obtained, the specimen may be serially diluted with a glycine-saline buffer to get a semiquantitative estimate of the CRP level.
2. Begin with a 1:2 dilution of patient serum obtained by mixing equal parts specimen and glycine-saline buffer. Blend the tube contents thoroughly.
3. Add 0.1 mL of buffer to the desired number of test tubes. Add 0.1 mL of 1:2 dilution to the first tube; mix and transfer 0.1 mL to the next additional tube. Continue until all tubes are diluted.
4. Perform a slide agglutination test on each dilution by repeating the above procedure (steps 3 - 7), and look for agglutination.

9.5. Results:

1. A positive reaction is reported when the specimen shows agglutination, indicating the presence of CRP in the serum at a level equal to or greater than 0.6 mg/dL.
2. The titer is represented by the last dilution that shows a positive reaction.
3. A negative reaction is characterized by a lack of visible agglutination in the undiluted specimen.

9.5.1. Limitations:

1. Specimens with markedly high CRP may demonstrate the post zone (antigen excess) effect. Therefore, some manufacturers recommend diluting the sample before testing. Consult product literature.
2. A quantitative titration procedure on positive samples is required to observe increasing or decreasing levels. Consult product literature.
3. Patients with high titers of rheumatoid factors may give positive results. It is recommended that patients suspected with RA be tested for the presence of rheumatoid factors.

4. The procedure must be followed carefully and results read at the appropriate time. Reading after the specified time can lead to misinterpretation due to drying of specimen/reagents.
5. Avoid contamination of reagent or reagent dispensing dropper.

9.6. Review Questions:

1. Measurement of CRP levels can be used for all of the following except
    A. Monitoring drug therapy with anti-inflammatory agents.
    B. Tracking the normal progress of surgery
    C. Diagnosis of a particular bacterial infection.
    D. Determining active phases of rheumatoid arthritis.
2. State three advantages that CRP test has over ESR.
3. Why do most manufacturers recommend testing specimens diluted and undiluted?

WEEK 10. Enzyme Linked ImmunoSorbent Assay (ELISA)

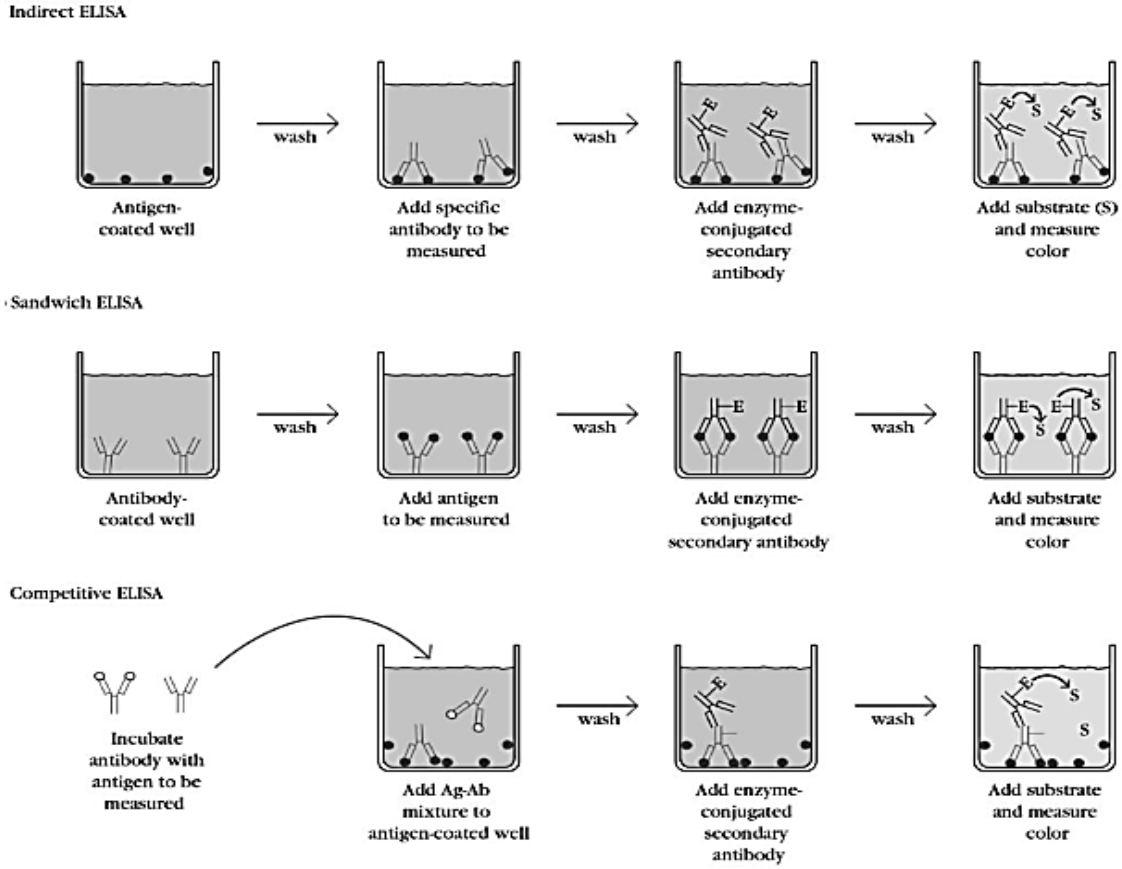
10.1. Background:

This is an important technique with a high specifity and sensitivity, and quantitative method. It is a multipurpose technique used in different fields of diagnosis. A set of components are included;

- Antigens or antibodies curried on solid phases in a micro-titer plate (mostly 96 wells).
- An indicator system which mostly composed of an enzyme (Horse radish peroxidase) with its specific substrate (H2O2),
- Stop solution and
- Washing buffers.

Types of ELISA

There are many different types of ELISA, it can be typically performed as direct or indirect methods, or can be performed as sandwich method as well as competitive assay.



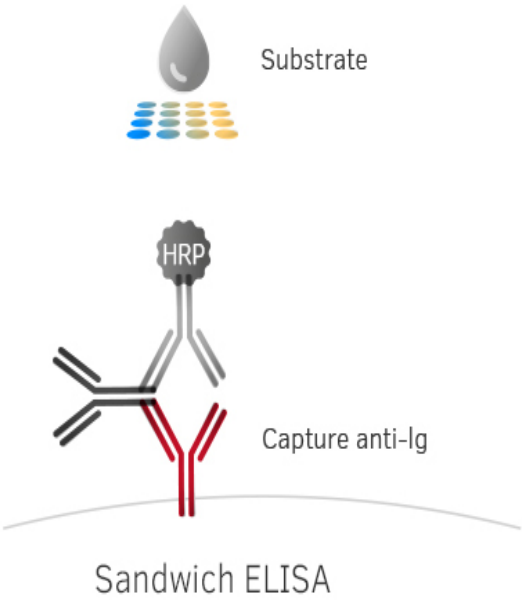
10.2. Total Human Immunoglobulin G ELISA:

10.2.1. Purpose:

This kit is intended for use in quantifying very low concentrations of total human immunoglobulin G (hIgG).

10.2.2. Principle:

The human IgG (hIgG) assay is a two-site immunoenzymetric assay. Samples containing hIgG are reacted in microtiter strips coated with an affinity purified capture antibody. A second, horseradish peroxidase (HRP) enzyme labeled anti-hIgG antibody, is reacted simultaneously resulting in the formation of a sandwich complex of solid phase antibody- hIgG -enzyme labeled antibody. The microtiter strips are washed to remove any unbound reactants. The substrate, tetramethyl benzidine (TMB) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of hIgG present.



10.2.3. Materials:

1. Kit components:

1. Anti-hIgG labeled with HRP (conjugate)
2. Anti-hIgG coated microtiter strips
3. hIgG standards
4. TMB substrate
5. Stop Solution
6. Washing solution concentrate (20X)

2. Materials required but not provided:

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450 nm, 620-630 nm
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Rotating mixer
- Distilled water
- Disposable tubes
- Timer

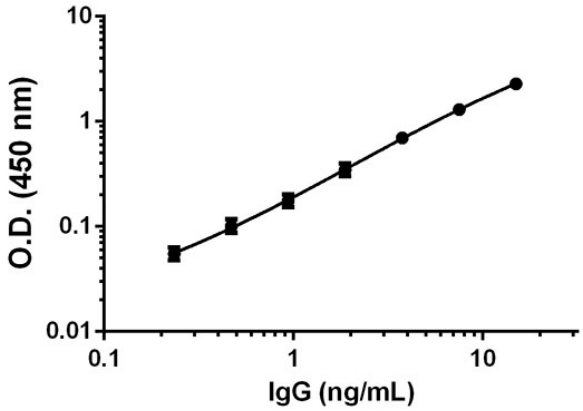
10.2.4. Procedure: (Please read the test protocol carefully before performing the assay)

1. Pipette 25µL of standards, controls and samples into wells indicated on work list.
2. Pipette 100µL of anti-hIgG:HRP into each well.
3. Cover & incubate for 1 hour at room temperature, 24°C+4°.
4. Dump contents of wells into waste or gently aspirate with a pipettor. Blot and vigorously bang out residual liquid over absorbent paper. Fill wells generously with diluted wash solution by flooding well from a squirt bottle or by pipetting in ~350 µL. Dump and bang again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding TMB substrate.
5. Pipette 100µL of TMB substrate.
6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.
7. Pipette 100µL of Stop Solution.
8. Read absorbance at 450/650nm blanking on the Zero standard.

10.2.5. Result analysis

The standards are used to construct a standard curve with values reported in ng/mL. The absorbance values of the standard on the y-axis versus concentration on the x-axis and drawing a smooth point-to-point line, or through computer methods using curve fitting

routines such as point-to-point, spline, or 4 parameter logistic fit. Absorbances of samples are then interpolated from this standard curve.



10.2.6. Attention

1. Samples and test kit need to be used under room temperature
2. Shake reagents before using
3. Slowly draw and discharge samples with micropippettor
4. Change tips between solutions
5. Filter paper and tape can't be reused
6. Wash wells carefully to prevent overflow
7. Plate needs to be read within 10 minutes after adding the termination solution

WEEK 11. Pregnancy Test: Beta-HCG ELISA

11.1.1. Background:

Serology pregnancy tests are designed to detect human chorionic gonadotropin (hCG) which is a glycoprotein hormone secreted by the developing placenta shortly after fertilization. During pregnancy, the hCG hormone can be found in detectable amounts in serum and urine of pregnant women 7-10 days following fertilization. The concentration of hCG rises rapidly, frequently exceeding 100 mlU/ml by the first missed menstrual period and peaks in the range of 30,000- 200,000 mlU/ml by 8-10 weeks into pregnancy. The appearance of hCG in urine soon after conception and its subsequent rise in concentration during early gestational growth make it an excellent marker for the early detection of pregnancy.

During early weeks of pregnancy, hCG level doubles every 36 - 48 hours until a peak level, which is achieved sometime during the tenth to twelfth week (end of the first trimester). During the second and third trimesters, the hCG levels fall considerably to plateau at about 1/6 of the peak level. Following delivery, the hCG hormone is rapidly cleared from the blood and pregnancy tests become negative in three to four days.

While confirmation to diagnose pregnancy is the most common reason a pregnancy test is ordered there are many other conditions which may warrant this test:

- Suspected choriocarcinoma
- Suspected hydatidiform mole
- Testicular tumors
- Prostatic cancer
- Breast cancer
- Lung cancer
- Pre-surgical procedures
- Pre-X-ray and Radioisotopic procedures

11.1.2. Purpose:

Immunoenzymatic colorimetric method (ELISA) is used for quantitative determination of beta-HCG in human serum or plasma.

11.1.3. Principle:

The beta-HCG assay is based on the simultaneous capture of HCG by a monoclonal antibody immobilized on the microplate and directed against the β-HCG fraction, and another monoclonal antibody conjugated with peroxidase horseradish (HRP) and directed against the α-HCG fraction. After the incubation, the bound/free separation is performed by a simple solid-phase washing. The enzyme HRP in the bound-fraction reacts with the substrate (H2O2) and the TMB substrate and develops a blue color that changes into yellow when the stop solution (H2SO4) is added. The color intensity is proportional to the β-HCG concentration in the samples. The β-HCG concentration in the sample is calculated based on a calibration curve.

11.1.4. Materials:

Reagents supplied

- **Anti-β-HCG IgG Coated Wells:** 12 breakapart 8-well snap-off strips coated monoclonal antibody direct against β- HCG subunit, in resealable aluminium foil.
- **Stop Solution:** 1 bottle containing (avoid any skin contact).
- **Conjugate (conc.):** 1 bottle containing 1 ml of monoclonal antibody direct against α-HCG subunit conjugated with horseradish peroxidase (HRP)
- **Incubation buffer:** 1 bottle containing phosphate buffer salin.
- **TMB Substrate Solution:** 1 bottle containing 15 ml 3, 3′, 5, 5′-tetramethylbenzidine (H2O2-TMB 0.26g/l) (avoid any skin contact).
- **Wash solution 50x conc.:** 1 bottle containing 20 ml washing buffer
- **β-HCG Control:** 1 bottle containing 1 ml of a lot-specific, ready to use control. The concentration is mentioned on the label.
- **β -HCG Standards:** 6 bottles, 1 ml each. The standards have the following concentrations:
    - Standard 0: (0 mIU/ml0), Standard 1: (1 mIU/ml), Standard 2: (5 mIU/ml), Standard 3: (20 mIU/ml), Standard 4: (100 mIU/ml) and Standard 5: (400 mIU/ml).

Materials and Equipment needed

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450 nm, 620-630 nm
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Rotating mixer
- Distilled water
- Disposable tubes
- Timer

Specimen collection and preparation

- Use human serum or plasma samples with this assay. If the assay is performed within 48 hours after sample collection, the specimens should be kept at 2-8°C; otherwise they should be aliquoted and stored deep-frozen (-20 to -70°C). Samples with concentration of β-HCG over 400 mIU/ml have to be diluted with Incubation buffer.

11.1.5. Test Procedure:

Please read the test protocol carefully before performing the assay. Prepare reagents as described in the protocol.

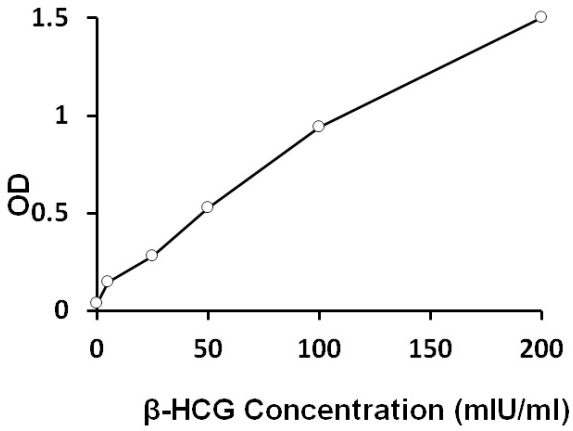
1. Dispense 25 µl standards, control and samples into their respective wells. Add 100 µl diluted conjugate to each well. Leave well A1 for substrate blank.
2. Cover wells with the foil supplied in the kit.
3. **Incubate for 1 hour at room temperature.**
4. When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300 µl diluted wash solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be >5sec. During each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbing paper towel. At the end

carefully remove remaining fluid by tapping strips on tissue paper prior to the next step! (if you use automated equipment, wash the wells at least 5 times). *Note:.Washing is critical! Insufficient washing results in poor precision and falsely elevated absorbance values.* Dispense 100 µl TMB Substrate Solution into all wells.

5. **Incubate for exactly 15 min at room temperature in the dark.**
6. Dispense 100 µl Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution. Shake the microplate gently. *Any blue colour developed during the incubation turns into yellow.*
7. Read the absorbance at 450 nm against a reference wavelength of 620 -630 nm or against blank within 5 minutes.

11.1.6. Results:

- Adjust the ELISA Microwell Plate Reader **to zero** using the **substrate blank in well A1**.
- **Measure the absorbance** of all wells at **450 nm** and record the absorbance values for each standard and patient sample in the distribution and identification plan.
- Calculate the mean absorbance for each point of the standard curve and each sample. Plot the mean value of absorbance of the standards against concentration. Draw the best-fit curve through the plotted points.
- Interpolate the values of the samples on the standard curve to obtain the corresponding values of the concentrations expressed in mIU/ml.



WEEK 12. Rose Bengal & Anti-Brucella (IgM or IgG) ELISA

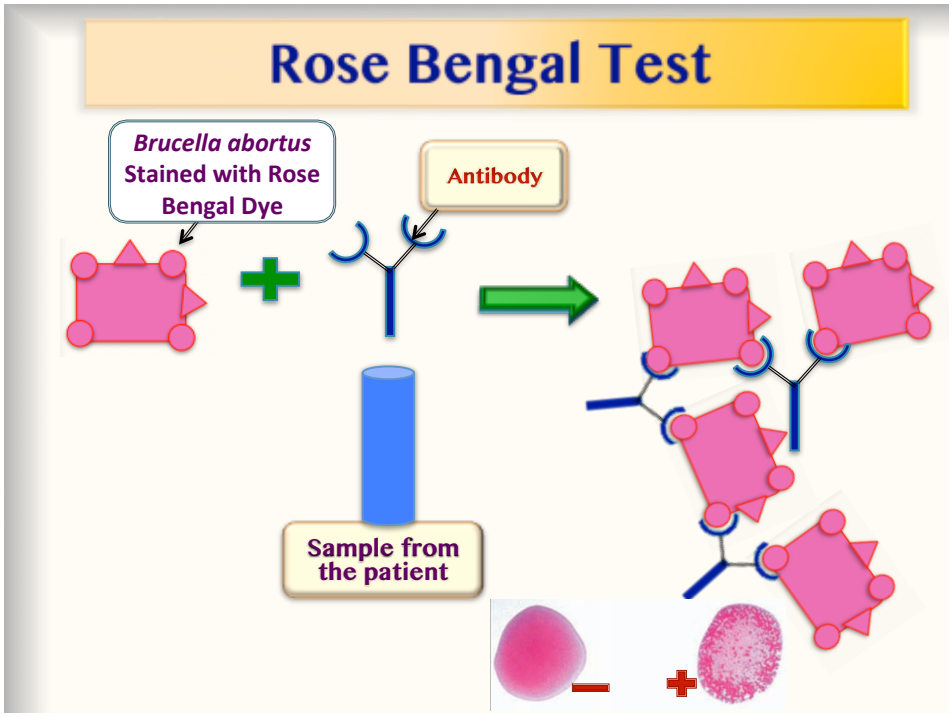
12.1. Rose Bengal

12.1.1. Background:

The Rose Bengal test (RBT) is a simple, rapid slide-type agglutination assay performed with a stained *Brucella abortus* suspension at pH 3.6–3.7 with the tested serum. Although the overall sensitivity reported for RBT varies widely, with the use of high-quality antigens made by experienced or reference laboratories, the sensitivity of RBT can increase. It is often used as a screening test in human brucellosis and would be optimal for small laboratories with limited means. False-negative reactions occur especially in the early stages of acute infection.

12.1.2. Principle:

When viable bacteria introduced into a susceptible host, an immune response can occur, which results in the production of antibodies called agglutinins. The principle of the test is an immunological reaction (agglutination) between the antibodies produced against the viable bacteria (agglutinins) and the corresponding bacterial antigen.



12.1.3. Material:

- Serum sample
- Rose Bengal Test kit.
- Test tubes

12.1.4. Procedure:

1. Mix (0.03 ml) of the serum sample with an equal volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter.
2. Agitate the mixture gently for four minutes at ambient temperature and then observed for agglutination.
3. Any visible reaction is considered to be positive.

12.2. Anti-Brucella (IgM & IgG) Human ELISA:

12.2.1. Purpose:

Anti-Brucella (IgM & IgG) Human in vitro ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate qualitative and quantitative measurement of IgM OR IgG antibodies against Brucella in Human serum and plasma. The monitoring of antibodies can serve as a usual indication of the status of infection. During the first days, IgM is the only immunoglobulin that appears. As the disease progresses, IgM recedes quantitatively and IgG becomes predominant. In chronic brucellosis, IgG may be produced for an extended period.

12.2.2. Principle:

A 96-well plate has been precoated with Brucella antigens to bind cognate antibodies. Controls or test samples are added to the wells and incubated. Following washing, a horseradish peroxidase (HRP) labelled anti-Human (IgM OR IgG) conjugate is added to the wells, which binds to the immobilized Brucella-specific antibodies. TMB is then catalyzed by the HRP to produce a blue color product that changes to yellow after adding an acidic stop solution. The density of yellow coloration is directly proportional to the amount of Brucella (IgM OR IgG) sample captured in plate.

12.2.3. Materials:

The kit:

- Brucella antigen coated microplate (12X8 wells)
- 20X washing solution
- Sample diluent
- Brucella Anti-(IgM OR IgG) + HRP conjugate
- TMB Substrate
- Stop Solution
- Controls and standards (Negative control, positive control and Cut-OFF Standard)

Materials required but not provided with the kit:

- 5 µ L-, 100 µ L- and 500 µ L micro- and multichannel pipets
- Microtiter Plate Reader (450 nm)
- Microtiter Plate Washer
- Reagent tubes for the serum dilution
- Deionized Water
- Timer
- Incubator at 37°C

PLEASE READ THE INSERT CAREFULLY BEFORE RUNNING THE TEST

12.2.4. Assay Procedure

- Strict adherence to the protocol is advised for reliable performance. Any changes or modifications are the responsibility of the user.
- All reagents and samples must be brought to room temperature before use, but should not be left at this temperature longer than necessary.
- Standards and samples should be assayed in duplicates.
- A standard curve should be established with each assay.
- Return the unused microtiter strips to the plastic bag and store them dry at 2-8°C.

Specimen Collection and Handling:

Principally serum or plasma (EDTA, heparin) can be used for the determination. Serum is separated from the blood, which is aseptically drawn by venipuncture, after clotting and centrifugation. The serum or plasma samples can be stored refrigerated (2-8°C) for up to 48 hours, for a longer storage they should be kept at -20 °C. The samples should not be frozen and thawed repeatedly. Lipemic, hemolytic or bacterially contaminated samples can cause false positive or false negative results.

For the performance of the test the samples (not the standards) have to be diluted 1:100 with ready-to-use sample diluent.

Preparation of Reagents

Washing Solution: dilute it with distilled water, If during the cold storage crystals precipitate, the concentrate should be warmed up at 37°C for 15 minutes.

Assay Steps:

1. Prepare a sufficient amount of microtiter wells for the standards, controls and samples in duplicate as well as for a substrate blank.
2. Pipet 100 µL each of the **diluted** (1:101) samples and the **ready-to-use** standards and controls respectively into the wells. Leave one well empty for the substrate blank.
3. Cover plate with the enclosed foil and incubate at room temperature for 60 minutes.
4. Empty the wells of the plate (dump or aspirate) and add 300 µL of diluted washing solution. This procedure is repeated totally three times. Rests of the washing buffer are afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.
5. Pipet 100 µL each of ready-to-use conjugate into the wells. Leave one well empty for the substrate blank.
6. Cover plate with the enclosed foil and incubate at room temperature for 30 minutes.
7. Empty the wells of the plate (dump or aspirate) and add 300 µL of diluted washing solution. This procedure is repeated totally three times. Rests of the washing buffer are afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.
8. Pipet 100 µL each of the ready-to-use substrate into the wells. This time also the substrate blank is pipetted.

9. Cover plate with the enclosed foil and incubate at room temperature for 20 minutes in the dark.
10. To terminate the substrate reaction, pipet 100 µL each of the ready-to-use stop solution into the wells. Pipet also the substrate blank.
11. After thorough mixing and wiping the bottom of the plate, perform the reading of the absorption at 450 nm (optionally reference wavelength of 620 nm). The color is stable for at least 60 minutes.

12.2.5. Result analysis:

1. Qualitative Evaluation

The calculated absorptions for the patient sera are compared with the value for the cut- off standard.

- **Positive result:** The value of the sample is higher.
- **Negative result:** The value of the sample is below the cut-off standard.
- **Grey zone (Intermediate):** A range of +/-20 % around the value of the cut-off. In such a case the repetition of the test with the same serum or with a new sample of the same patient, taken after 2-4 weeks, is recommended. Both samples should be measured in parallel in the same run.

2. Quantitative Evaluation

The ready-to-use standards and controls of the Brucella IgG antibody kit are defined and expressed in arbitrary units (U/mL). This results in an exact and reproducible quantitative evaluation.

For a quantitative evaluation the absorptions of the standards and controls are graphically drawn *point to point* against their concentrations. From the resulting reference curve the concentration values for each patient sample can then be extracted in relation to their absorptions. It is also possible to use automatic computer programs. As curve fit *point-to-point* has to be chosen.

12.2.6. Review Questions:

- How long does antibody formation against Brucellosis take?
- How to differentiate acute infection from past infection?

WEEK 13. Precipitation reaction (Immuno-precipitation)

13.1. Background:

These reactions can occur when the antigens are in a soluble form. Corresponding antibodies can bind to soluble antigens and cause precipitation, although it's hard to detect the precipitation visually and sometimes other means may be necessary to decide it the test is positive or negative.

13.2. Principle:

Known antibodies are mixed with soluble antigens in a tested sample, after a period of incubation, the antigen-antibody reaction will happen if corresponding antigens (or antibodies) are found the tested sample, this can result in Ag-Ab precipitation either in a test tube or gel.

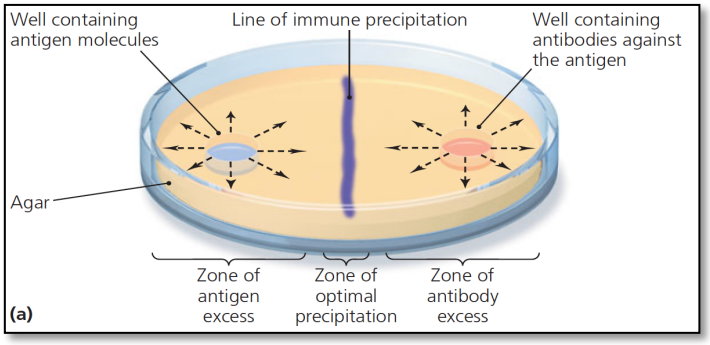
Antibodies + soluble antigens (in a sample) —Incubation→ Precipitation (+ve)
No precipitation (-ve)

This reaction can be done either using test tubes (Tube precipitation) or through using gel (Immunodiffusion technique). This can be achieved by two methods:

1. Double immunodiffusion technique
2. Single immunodiffusion technique (Radial immunodiffusion)

First: Double immunodiffusion:

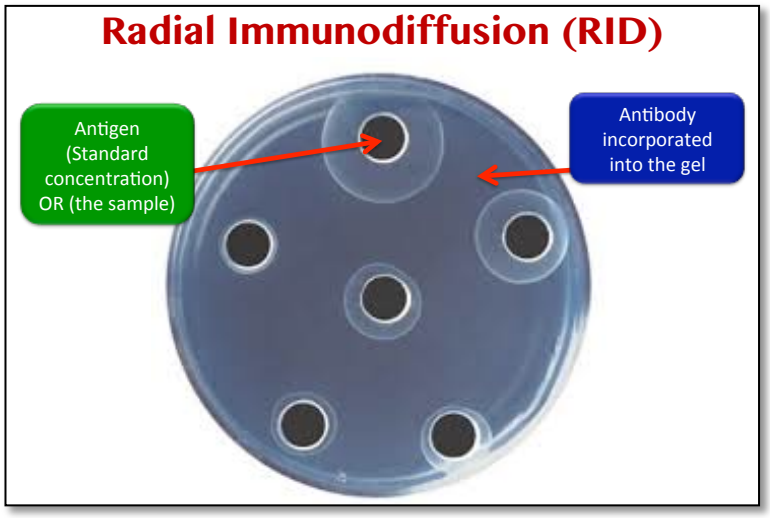
In this technique both antigens in the tested sample and the known antibodies will move through a gel and when they met each other a line of precipitation will form which easily can be seen.



Second: Single diffusion technique:

In this case, one (either antigen or antibody) will incorporate into the gel, wells can be cut, and the corresponding Ag or Ab should be adding into the wells. The Ag or the Ab in the well begins to diffuse through the gel radially, and a ring of precipitation will appear if the test is positive. The higher the concentration, the wider the ring diameter

This technique can be used to determine the concentration of the studied Ag or Ab semi-quantitatively.



13.3. Materials:

1. Agarose gel
2. Known Antibody or Antigen (depending on the test type)
3. Petri dishes
4. Tested sample
5. Well cutting pipettes or devices
6. Ruler

13.4. Procedure:

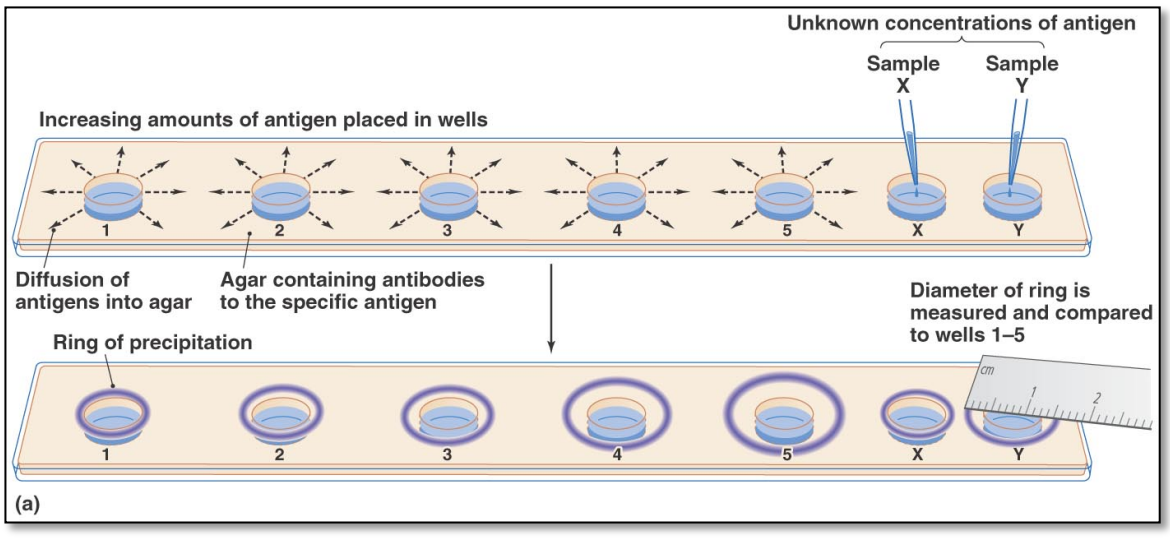
13.4.1. Double diffusion:

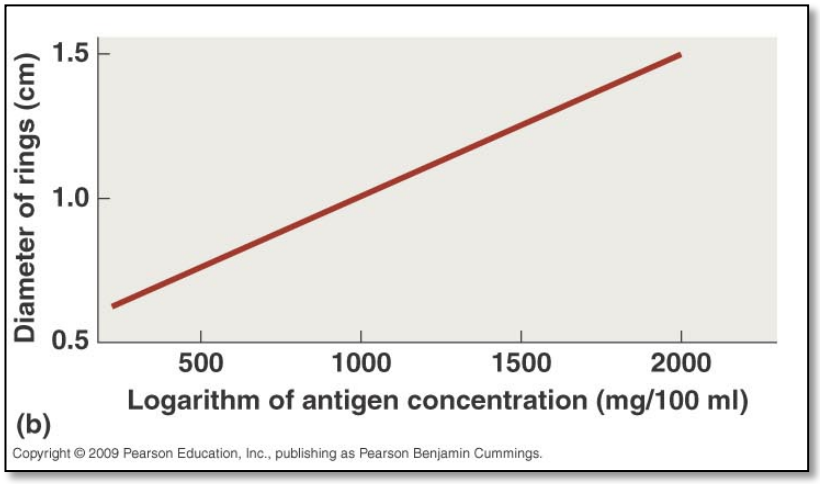
1. Prepare a gel and after sterilization dispense into sterile Petri dishes
2. Make at least 2-4 opposite wells.
3. Add little amount of each antigen and antibodies to opposite wells (the amount should be equal)
4. Incubateb2-3 hours at room temperature.

5. Look for precipitation line between the opposite wells
6. Appearing of precipitation line indicates positive results

13.4.2. Single diffusion technique:

1. From the known antibody prepare different concentrations in sterile test tubes.
2. Prepare a gel as previously mentioned
3. Before dispensing the gel into the petri dishes incorporate the known (corresponding antigen) into the gel and then dispense after mixing well.
4. Make different wells as mentioned before and labe each well
5. Add similar amount of previously prepared Ab concentrations into the wells as labeled.
6. Incubate 2-3 hours and try to measure the precipitations ring diameters around each well
7. Try to plot the dimeters against the concentrations to prepare a standard curve (calibration curve)
8. It is possible to determine the concentration of any tested Ag or Ab depending on the standard curve.





13.5. Review Questions:

1. Why this method is called semi-quantitative method?
2. In double diffusion method, the precipitation line can be near to the antigen or the antibody? How you explain it?
3. Why single diffusion technique is called radial diffusion? Why the antigen or the antibody must be incorporated into the gel?

WEEK 14. Typhoid Fever

14.1. Background:

Widal test is an agglutination test, which detects the presence of serum agglutinins – antibodies- against (H and O) antigens in patients' serum suffering from typhoid and paratyphoid fever. When facilities for culturing are not available, the Widal test is reliable and can be of value in the diagnosis of typhoid fevers in endemic areas.

The patient's serum is tested for anti-O and ant-H antibodies (agglutinins) against the following antigen suspensions (usually stained suspensions):

- *S. Typhi* 0 antigen suspension
- S. *Typhi* H antigen suspension
- S. Paratyphi A 0 antigen suspension
- S. Paratyphi AH antigen suspension
- S. Paratyphi B 0 antigen suspension
- S. Paratyphi B H antigen suspension

Salmonella antibodies begin to appear in the serum at the end of the first week and rise sharply during the 3rd week of endemic fever. In acute typhoid fever, O agglutinins can usually be detected 6–8 days after the onset of fever and H agglutinins after 10–12 days.

It is preferable to test a pair of specimens of sera at an interval of 7 to 10 days to demonstrate a rising antibody titer. *Salmonella* antigen suspensions can be used as slide and tube techniques.

14.2. Principle:

The bacterial suspension, which carries antigen will agglutinate on exposure to antibodies to *Salmonella* organisms. Patients suffering from enteric fever would possess antibodies in their sera, which can react and agglutinate serial doubling dilutions of killed, colored *Salmonella* antigens in agglutination test.

The principle of the Widal test is that if the homologous antibody is present in patient's serum, it will react with respective antigen in the reagent and gives visible clumping on the test card and agglutination in the tube. The antigens used in the test are "H" and "O" antigens of *Salmonella Typhi* and "H" antigen of *S. Paratyphi*. The paratyphoid "O" antigen are not

employed as they cross react with typhoid "O" antigen due to the sharing of factor 12. "O" antigen is a somatic antigen, and "H" antigen is a flagellar antigen.

14.3. Material:

- Widal Test Kit
- Serum sample
- Test tubes

14.4. Procedure:

14.4.1. SLIDE TEST

1. Place one drop of positive control on one reaction circles of the slide.
2. Pipette one drop of isotonic saline on the next reaction circle. (-ve Control).
3. Pipette one drop of the patient's serum onto the remaining four reaction circles.
4. Add one drop of Widal test antigen suspension 'H' to the first two reaction circles. (PC & NC).
5. Add one drop each of 'O,' 'H,' 'AH' and 'BH' antigens to the remaining four reaction circles.
6. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
7. Rock the slide, gently back and forth and observe for agglutination macroscopically within one minute.

14.4.2. SEMI-QUANTITATIVE METHOD

1. Pipette one drop of isotonic saline into the first reaction circle and then place 5, 10, 20, 40, 80 µl of the test sample on the remaining circles.
2. Add to each reaction circle, a drop of the antigen which showed agglutination with the test sample in the screening method.
3. Using separate mixing sticks mix the contents of each circle uniformly over the reaction circles.
4. Rock the slide gently back and forth, then look for agglutination macroscopically within one minute.

14.5. Review Questions:

Explain cross-reaction in immunological tests