



AZERBAIJAN MEDICAL UNIVERSITY
DEPARTMENT OF MEDICAL MICROBIOLOGY and IMMUNOLOGY

Lesson 19.

Agglutination reaction and its variants (slide and tube) Hemagglutination reaction (HAR). Hemagglutination inhibition reaction (HAIR). Passive hemagglutination reactions (PHAR). Coombs reaction. Immobilization reaction of motile bacteria. Precipitation reaction and its variants (ring precipitation, gel diffusion, immune electrophoresis). Toxin neutralization reaction (TNR). Radial Immunodiffusion Reaction (RID)

FACULTY: General Medicine
SUBJECT: Medical microbiology - 1

Discussed questions:

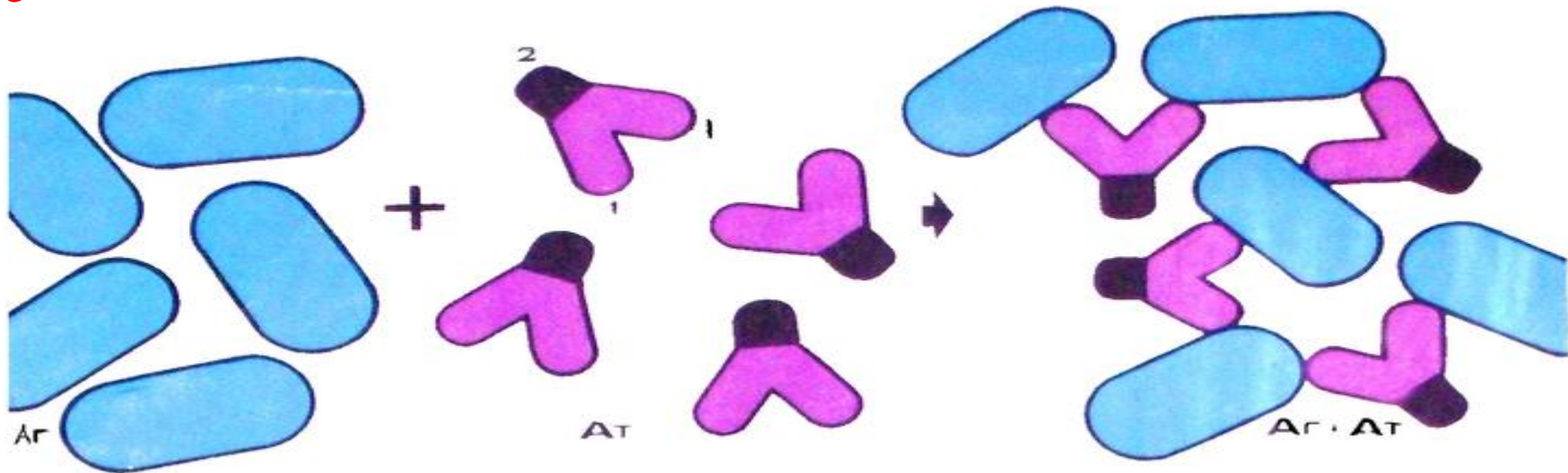
1. The essence of the agglutination reaction.
2. Mechanism of agglutination reaction.
3. Types of agglutination reaction: slide, tube. The concept of diagnostic titers.
4. HAR (hemagglutination reaction), HALR (hemagglutination inhibition reaction)
5. PHAR (passive hemagglutination reaction)
6. Coombs reaction (direct and indirect variants).
7. Immobilization reaction of motile bacteria.
8. The essence of the precipitation reaction.
9. Mechanism of precipitation reaction.
10. Variants for the precipitation reaction:
 - ring precipitation.
 - precipitation: double immunodiffusion (Ouchterlony method), radial immune diffusion.
 - immunoelectrophoresis.
 - counterimmunoelectrophoresis.
11. Neutralization reaction of the toxin, its essence, application.
12. Radial immunodiffusion reaction.

Purpose of the lesson:

- To acquaint students with the essence, mechanism and types of agglutination reaction and to teach methods of application. Explain HAR, HALR, PHAR, Coombs and immobilization reactions. To acquaint students with the essence, mechanism, types, methods of application and application of precipitation reactions. Explain the essence of the neutralization reaction of the toxin and the radial immunodiffusion reaction.

Agglutination reaction (mechanism)

- Agglutination (**lat. agglutinatio – clueing**) is sedimentation of microorganisms, erythrocytes, or other corpuscular antigens adhered together by antibodies (agglutinins). The reaction is based on specific combination of antigens and antibodies.
- Antibodies causing agglutination are called **agglutinins**, while agglutinated microorganisms – **agglutinogens**.



Agglutination reaction (application)

As all serological reactions agglutination allows detection of unknown antibody by known antigen, or conversely, detection of unknown antigen by known antibody.

- Serological identification of pure culture of pathogen obtained from patient
- Detection of specific antibodies in plasma, exp. Rayt, Heddelson, Widal etc.

Types of agglutination reaction

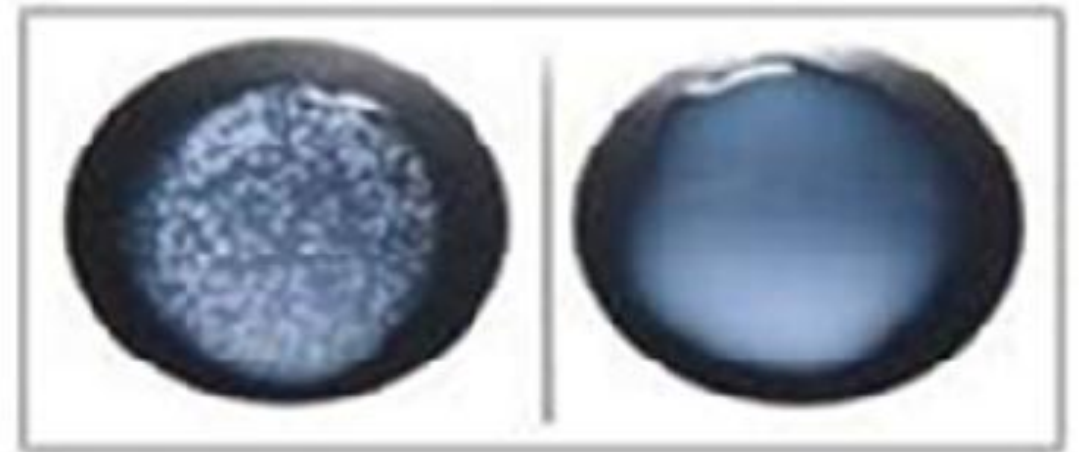
Agglutination

- **slide,**
- **tube,**
- **Passive etc.**

Slide agglutination reaction

Used for serological identification.

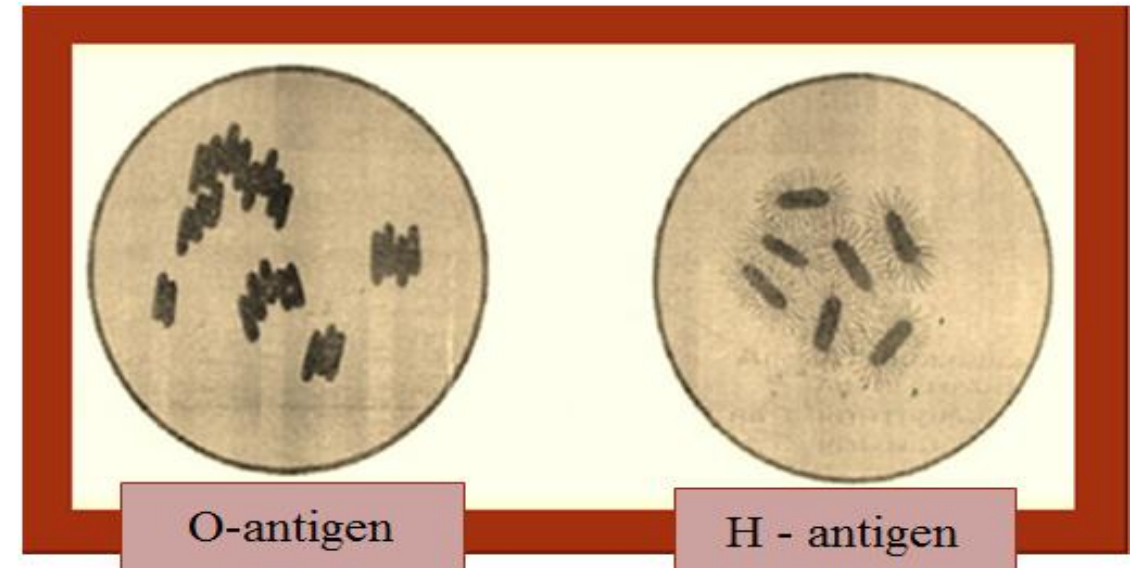
- A pure culture of microorganism is mixed with a drop of diagnostic agglutinating serum.
- Positive reaction is accompanied by clumping.



O- and H-agglutination

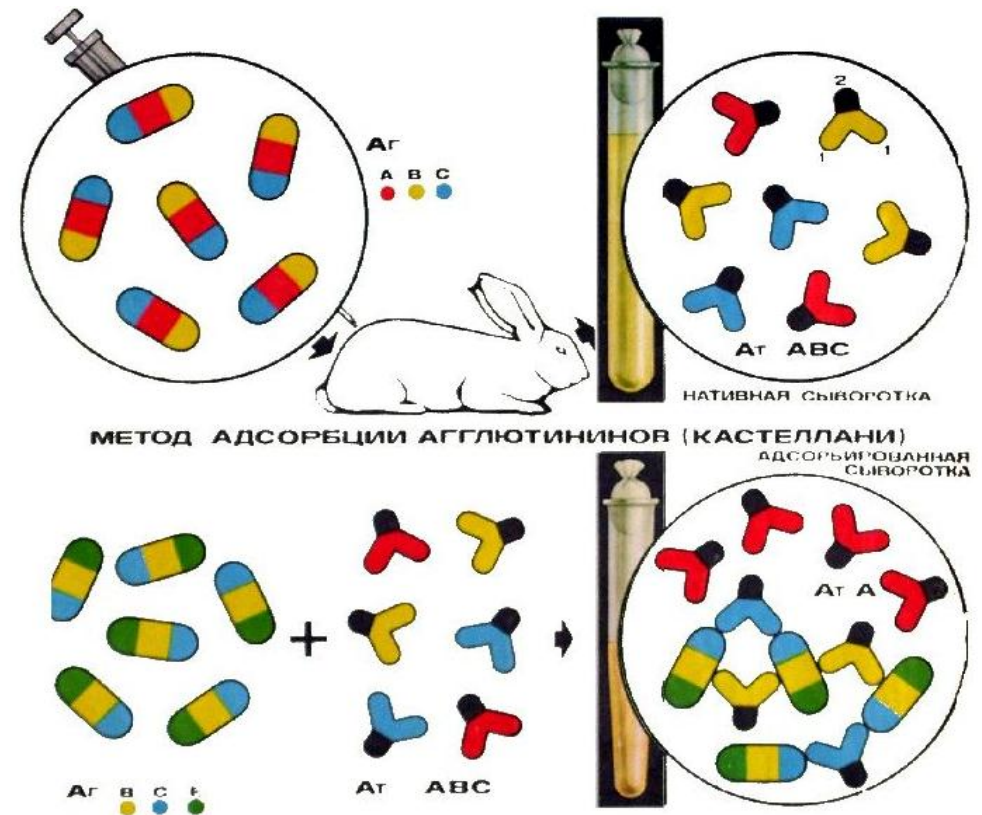
The character of agglutination varies depending on type of antigen.

- Bacteria without flagella form granular sedimentation (O-sedimentation),
- Bacteria with flagella form large cotton-like sedimentation (H-agglutination).



Obtaining of adsorbed agglutinating serum

- Some microorganism with antigenic similarities or cross reactive antigens can be agglutinated by the same agglutinating serum.
- In order to avoid this phenomenon **adsorbed agglutination serums** are used.
- The similar bacteria are added to serum (**Kastellani reaction**). As a result antibodies acting with cross reactive antigens are eliminated, while antibodies acting on specific microorganism remain in serum. Such serum is called **monoreceptor agglutination serum**.
- Recently, more specific monoclonal antibodies are used for this purpose.



Slide agglutination reaction allows to obtain preliminary results in serological diagnostics.

- Patient serum is mixed with diagnosticum.
- In case of positive result, tube agglutination is performed in order to evaluate antibody titer.

Tube agglutination

Tube agglutination detects titer of antibodies in patient plasma.

1:50, 1:100, 1:200, 1:400, 1:800 times diluted patient plasma is added in tubes/wells and mixed with diagnosticum. The tubes/wells are incubated for 1 hour at 37°C, and 1 day at room temperature.

Equipment and ingredients used in tube agglutination

- Examined blood serum
- Agar, broth culture of microorganism, diagnosticum (corpuscular antigen)
- Saline
- Pasteur pipette, sampler
- Test tubes

Obtaining serum for agglutination reaction

Blood is usually taken from an elbow vein or a finger and kept in test tubes for 1 hour and 10-15 minutes in incubator.

Clotted blood is separated from tube wall by Pasteur pipette. Tubes are kept in refrigerator until complete separation of plasma. Then, separated plasma is poured to another sterile tube.

If the plasma is turbid it should be centrifuged.

Diagnosticum preparation

- The microbial emulsion (antigen) can be obtained by washing the agar 18-24 hour culture with saline, or ready for use diagnosticum may be used.
- Saline is poured in agar slant.
- Test tube with culture is rotated between two hands which enables washing of culture.
- The resulted microbe emulsion is taken by pipette and poured to another sterile tube.

Dilution of blood plasma

- First, working dilution of examined plasma is prepared.
- 0.2 ml of plasma is mixed with 1.8 ml of saline, thus obtaining 1:10 dilution.

Tube agglutination

Reaction is performed in test tubes or dilution plates.

- 1 ml of saline is added to each test tube.
- 1 ml of working dilution is added to 1st tube and mixed. Then, 1 ml is transferred from 1st tube to 2nd, repeating the procedure for 3rd, 4th etc. tubes, thus making 1:20, 1:40, 1:80, 1:160, 1:320 plasma dilutions.
- 1 ml of working solution is poured in separate tube (plasma control), while 1 ml saline is used as antigen control.

Interpretation of agglutination reaction

The agglutination may with varying intensity. It is accepted to indicate the intensity of the reaction with (+).

During complete agglutination (++++), the solution becomes transparent, the sediment at the bottom of the bottle is well visible;

during incomplete agglutination (+++) the solution becomes weakly turbid, the sediment is visible;

during partial agglutination (++) the solution becomes cloudy, sediment is small;

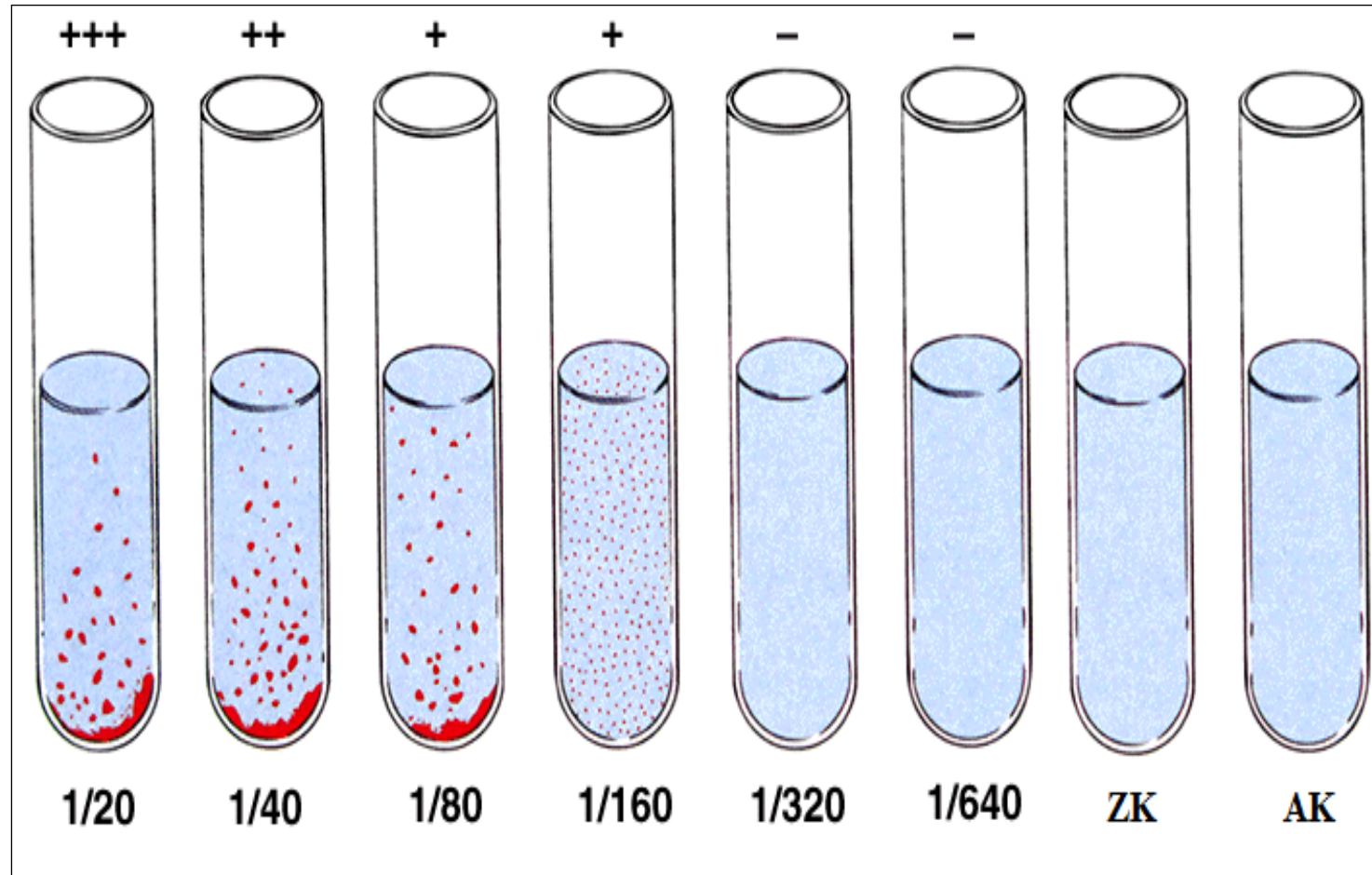
During weak agglutination (+) the solution becomes cloudy, very little sediment is observed.

In the presence of turbidity in the solution, without sediment, the agglutination reaction did not take place, ie the reaction is negative.

Titer of agglutination reaction

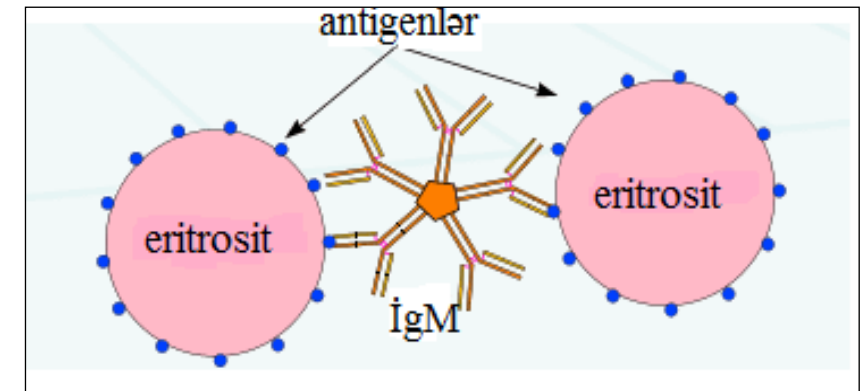
- The reaction is considered positive if there is visible agglutination (++) in the test tubes and no agglutination in the control test tube. The highest dilution of plasma with agglutination is called a *titer of agglutination reaction*.
- Detection of titer has diagnostic significance. Normal antibodies of plasma may cause agglutination in low dilutions. Thus, “diagnostic titer” for each disease should be detected. Presence of agglutination at a certain dilution (or higher) - *diagnostic titer*, indicates disease.

Tube agglutination (ZK-plasma control, AK-antigen control)



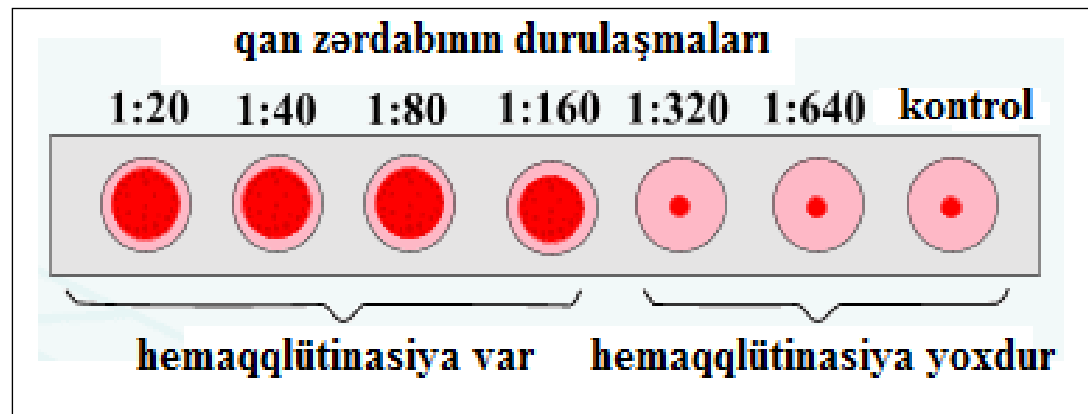
Indirect (passive) hemagglutination

- At some cases, high dispersion of antigen makes impossible to observe by naked eyes antigen-antibody complex.
- Thus, such antigens are adsorbed on large particles – erythrocytes, latex etc.
- As a result, the reaction manifests itself as agglutination of these particles. Agglutination occurs when adsorbed antigens combine with appropriate antibodies.
- Depending on the type of adsorbent, indirect (passive) hemagglutination reaction (IHAR, or PHAR), latex agglutination reaction (LAR), etc. available.



Indirect (passive) hemagglutination reaction technique

- This reaction is commonly used to detect specific antibodies in the blood serum. During reaction, antigens adsorbed on the surface as erythrocytes - erythrocyte diagnosticums are used.
- Series of sequential dilutions of serum are prepared in the wells of polystyrene plates. 0.5 ml of positive serum is added to penultimate well and 0.5 ml of saline (control) is added to the last well. Then 0.1 ml of dissolved erythrocyte diagnosticum is added to all wells, mixed and kept in a incubator for 2 hours.
- If reaction is positive erythrocytes form “inverted umbrella”, if negative – sedimentation of erythrocytes is observed.



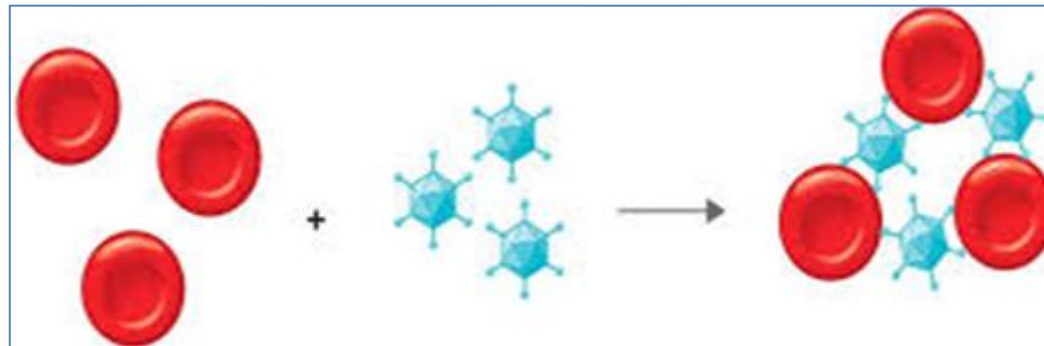
Hemagglutination reaction

Hemagglutination – agglutination of erythrocytes. There are two types of hemagglutination: serological and non-serological.

- *Serological hemagglutination* is based on interaction of erythrocyte antigens (hemagglutinogens) and antibodies (hemagglutinin) and used for blood typing.
- *Non-serological hemagglutination* is based on ability of viral hemagglutinins to cause agglutination of animal erythrocytes and used in indication of viruses.

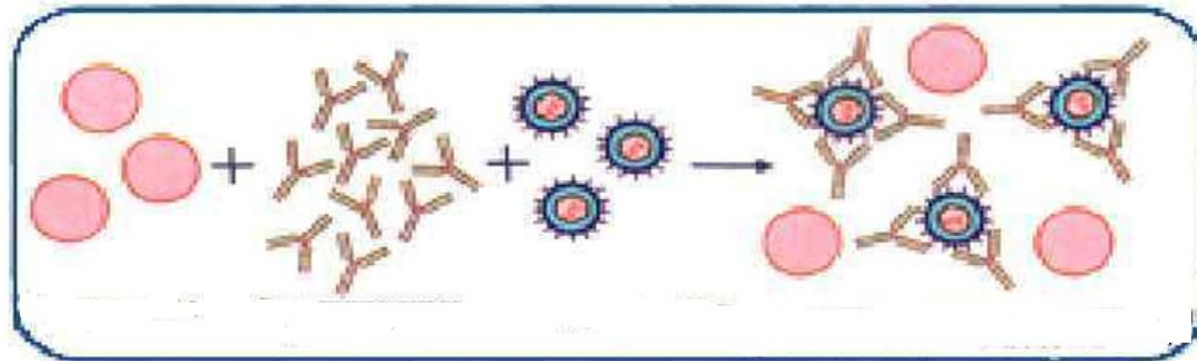
Non-serological hemagglutination technique

- 0.5 ml of infected allantoic fluid is taken and twofold dilution series polystyrol plates is prepared.
- 0.5 ml of allontoic fluid from an uninfected chicken embryo is added to a separate hole in the tablet (control). 0.5 ml of 1% erythrocyte suspension is added in all wells. The result interpretation is performed 40 minutes after the sedimentation of erythrocytes in control well.
- If reaction is positive erythrocytes form “inverted umbrella”, if negative – sedimentation of erythrocytes is observed. Absence of hemagglutination in the control well, the presence of hemagglutination in the experimental wells indicates the presence of the virus in the examined fluid.



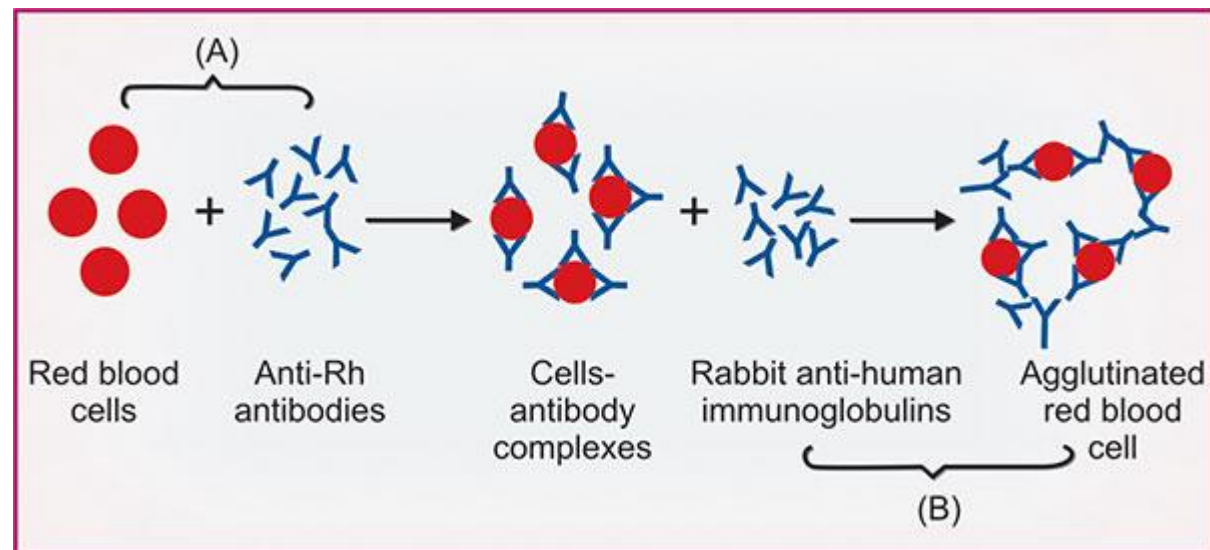
Hemagglutination inhibition reaction.

- It is used for identification of some viruses (influenza, measles, tick-borne encephalitis, etc.).
- To identify the type of virus in examined specimen, serums containing antibodies against certain viruses are added.
- If there are virions in the specimen, due to the action of antibodies, they lose the ability to agglutinate erythrocytes and the titer of the reaction is significantly reduced.



Coombs reaction

- In some infections, the detection of incomplete antibodies in the serum is of diagnostic value. Incomplete antibodies are antibodies that for some reason do not have one of its active centers. Although such antibodies combine with the corresponding antigens to form an antigen-antibody complex, it is not possible to observe it. Thus, incomplete antibodies bind to the antigen through only one active center, as if blocking it ("blocking" antibodies).
- Therefore, the Coombs reaction is used to determine these antibodies: after incubation of the test serum with diagnosticum, antibodies to human immunoglobulins (antiglobulin) are added to the mixture. If the test serum contains the appropriate antibodies, they combine with the diagnosticum to form an antigen-antibody complex. The antibodies in this complex, in turn, combine with the antibodies against human globulins to form a visible precipitate



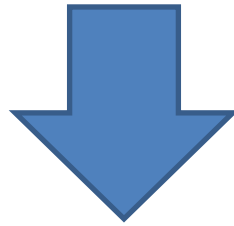
Coombs reaction

Coombs reaction is also used in the diagnosis of rhesus conflict, as antibodies to rhesus antigen are also incomplete antibodies.

- If a rhesus-positive fetus develops in a rhesus-negative mother, sometimes anti-rhesus antibodies are formed in the mother's blood. Anti-rhesus antibodies are formed as a result of the introduction of rhesus antigens into the mother's blood during a previous birth or when there are defects in the placenta. These antibodies enter the fetus through the transplacental route causing hemolytic disease and death of newborns.
- Rhesus-positive erythrocytes and then anti-globulin serum (antibodies against human immunoglobulins) are added to the mother's blood serum to detect anti-rhesus antibodies. When the reaction is positive, agglutination of erythrocytes is observed

Immobilization reaction

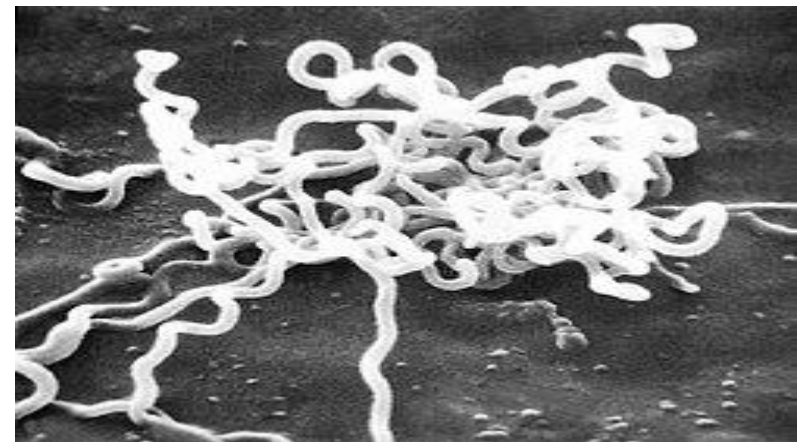
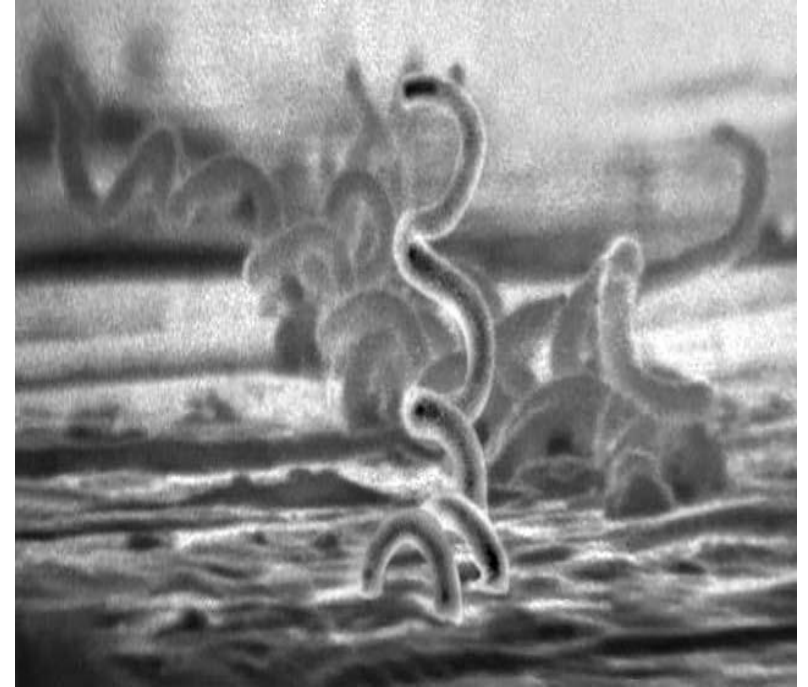
Motile bacteria +antibodies+complement



Immobilization of bacteria

Immobilization of motile bacteria

- Immobilization of motile bacteria by immune serum is related to specific antibodies in presence of complement.
- These antibodies are detected in syphilis, cholera etc.
- Immobilization of *T.pallidum* is used in syphilis diagnosis. This test has high sensitivity and specificity.



Immobilization of *Treponema pallidum*

Ingredients (ml)	Test					Ingredient controls			
	Numbers of test tubes								
	1 test	2 control	3	4	5	6	7	8	9
Inactivated examined blood plasma	0,05	0,05							
Active complement	0,15		0,15		0,15		0,15		
Inactive complement		0,15		0,15		0,15		0,15	
Antigen	0,35	0,35	0,35	0,35	0,35	0,35	0,35	0,35	0,35
Inactivated positive plasma			0,05	0,05					
Inactivated negative plasma					0,05	0,05			

Precipitation reaction

- Interaction of corpuscular antigens with antibodies results in agglutination. Interaction of soluble antigens (*precipitinogens*) with antibodies (*precipitins*) results in ***precipitation reaction***. It is manifested with formation of turbidity called precipitate (*lat., praecipito* – sediment). When an equivalent amount of antigen and antibody is mixed, the precipitate is formed more rapidly and the turbidity intensity is higher.

Precipitation reaction

- Depending on the environment in which the serological reaction takes place different manifestations of precipitation phenomenon are observed.
- In liquid media it manifests as turbidity, while in solid – as ring precipitate.
- Thus, there different precipitation reaction variants

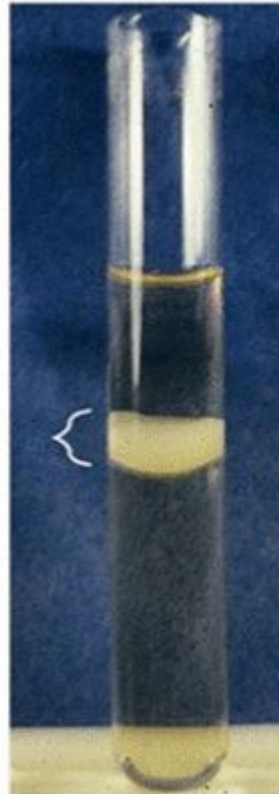
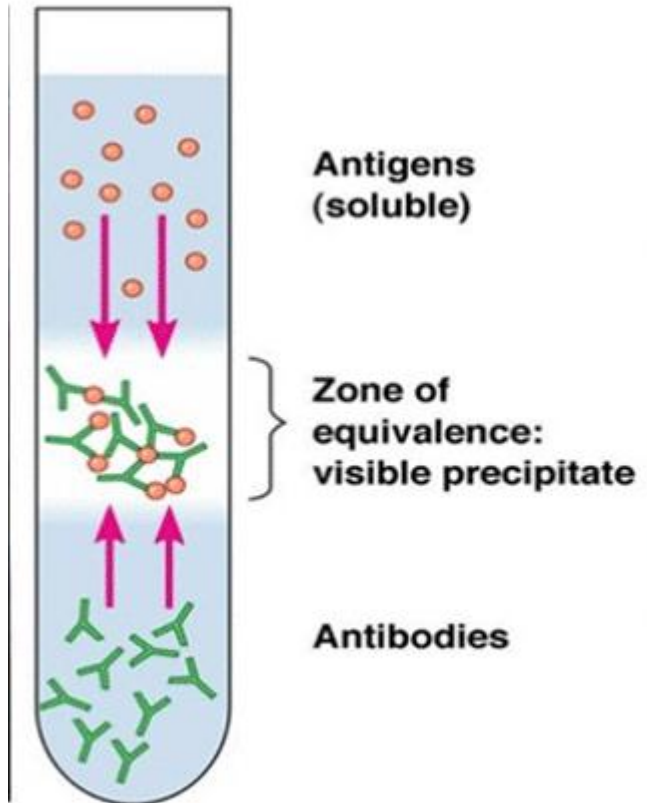
Ring precipitation

- Careful addition of soluble antigen in test tube with immune serum results in formation of *turbid precipitate ring* in border between two solutions
- Antigen and immune serum should not be mixed as it will result in diffuse turbidity.
- The reaction is used to detect thermostabile antigens of anthrax pathogen (Ascoli reaction).

Ring precipitation technique

- The ring precipitation is performed in narrow test tubes with a small diameter (0.5 cm). 0.3-0.5 ml of precipitating serum is added to the test tubes. Then, holding the test tube in an inclined position, the same amount of antigen solution is added by a pipette. Antigen and immune serum should not be mixed.
- In the control test tube certain amount of saline added to immune serum. The test tubes are carefully placed on a vertical position without mixing the liquids.
- The results of the reaction are interpreted after 5-10 minutes, 1-2 hours or 20-24 hours, depending on the type of antigen and antibody. In case of a positive reaction a *white ring precipitate* appears on the border of the serum and the antigen solution (extract).

Ring precipitation



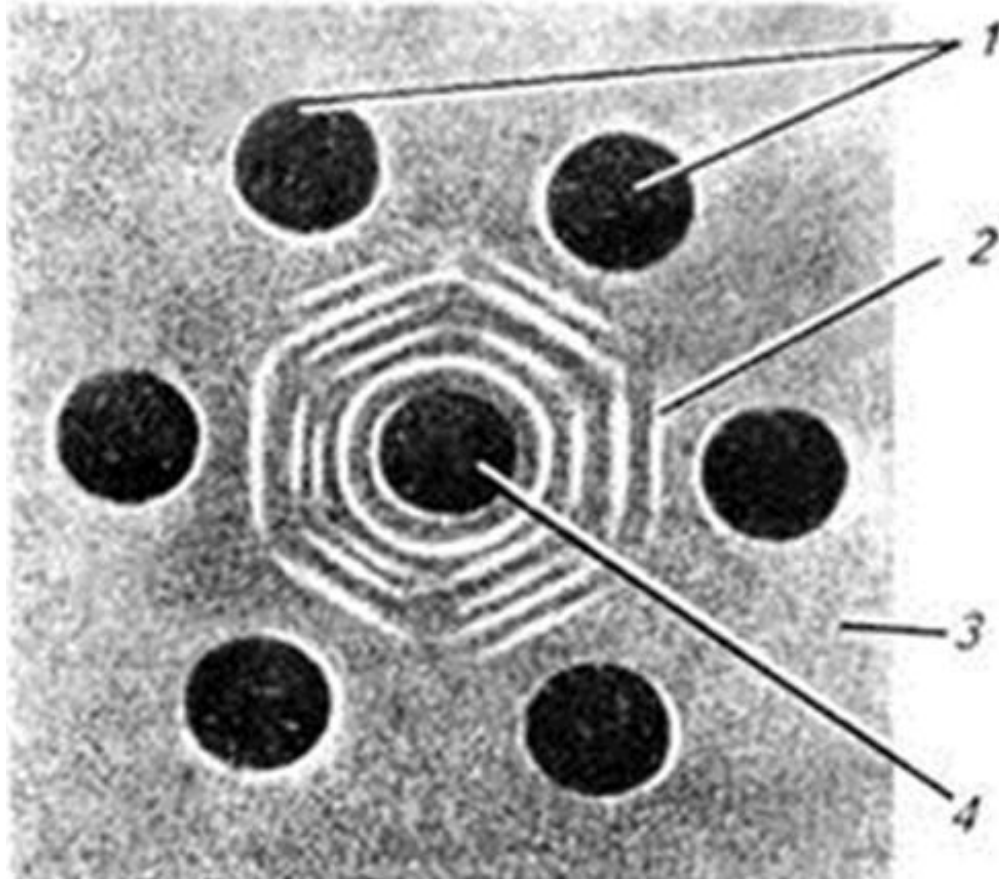
Agar (gel) precipitation

- The reaction is performed in agar or gel (solid substances).
- In contrast to ring precipitation, antigen and antibody interact after diffusion into solid phase.
- As a result, precipitation line appear in the relevant area. *Double immunodiffusion, radial immunodiffusion and immunoelectrophoresis* are the most commonly used methods.

Double immunodiffusion (Ouchterlony method)

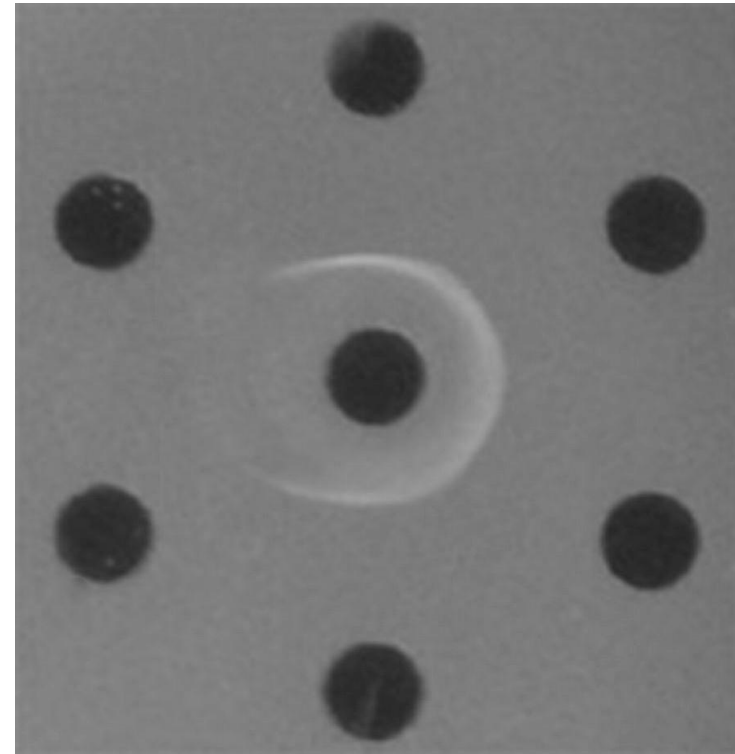
- The reaction is performed on transparent agar layer placed on glass.
- Soluble Ag and soluble Ab are placed in separate small wells punched into agar that has solidified on a slide or glass plate, the Ag and the Ab will diffuse through the agar.
- The precipitin band appears at place of antigen antibody interaction.

Double immunodiffusion(Ouchterlony method)



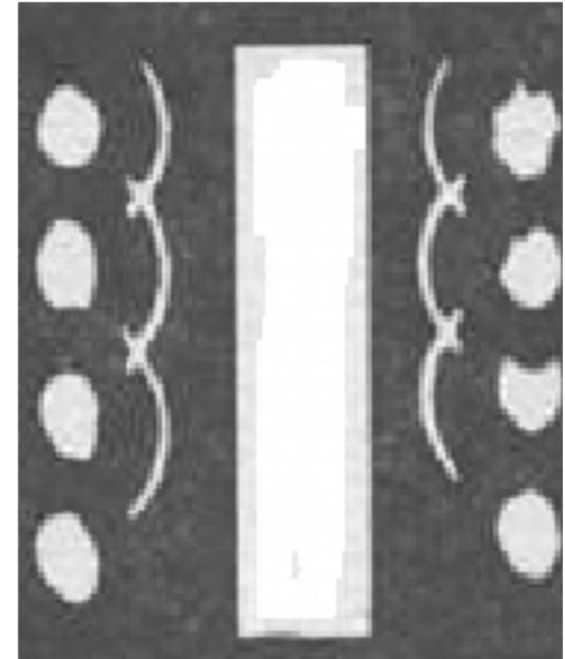
1. Wells with examined antigens
2. Precipitation area
3. A slide with agar gel
4. The well with immune serum

Double immunodiffusion (Ouchterlony method)



Detection of diphtheria toxin by agar precipitation.

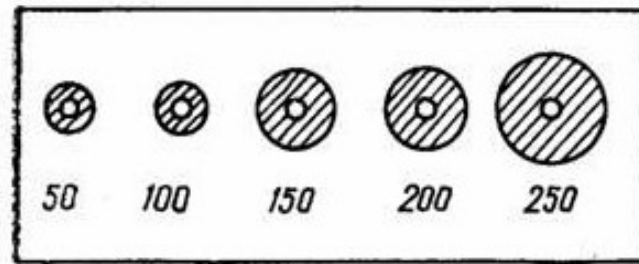
- This technique called *Elec* method is used to detect diphtheria toxin.
- A filter paper with impregnated antitoxin is placed on agar. After drying, microorganism cultures are inoculated at a distance of 1 cm from the edge of the paper strip. 3-10 cultures may be inoculated simultaneously.
- A nontoxigenic culture is used as control.
- The Petri dishes are incubated at 37°C for 24-48-72 hours. A precipitate band appears at some distance from paper in case of positive result.



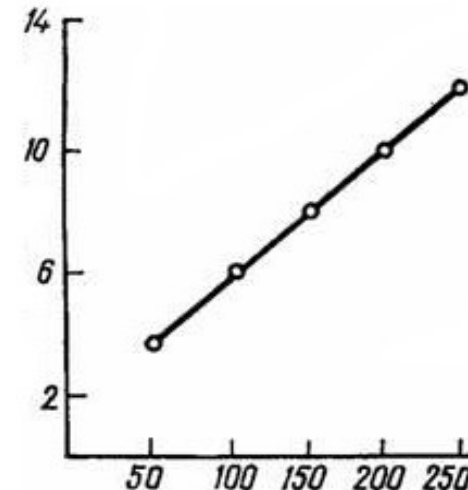
Elec test

Radial immunodiffusion

- Immune serum is mixed with agar gel cooled to 40°C.
- Agar is poured on glass. Then, different antigen dilutions are added to holes punched in solidified agar.
- Antigen diffusing to agar and bind with antibodies forming precipitation bands around holes.
- The diameter of precipitate correlates with antigen concentration. Using known concentration of the Ag in question, a standard curve can be prepared by plotting the diameter of the precipitin ring versus Ag concentration.
- This reaction is used to detect immunoglobulin levels in plasma (Manchini method).



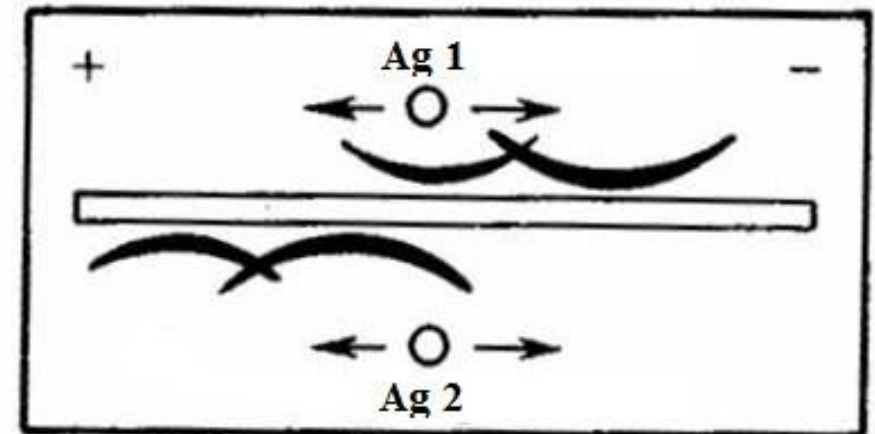
A



B

Immunelectrophoresis

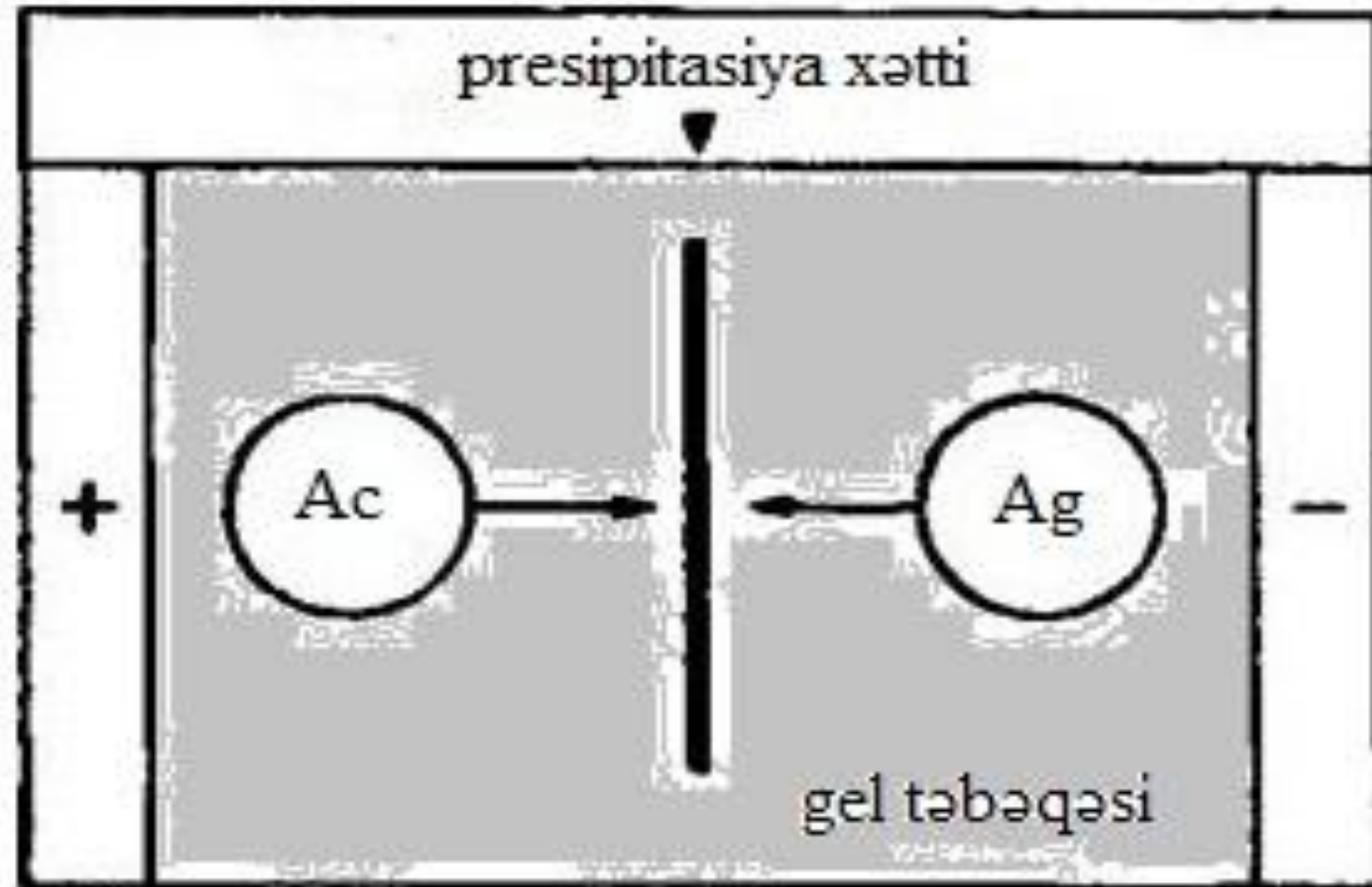
- *Immunelectrophoresis* is used to examine antigenic structure of complex substances. First, antigen is separated into different fractions by agar electrophoresis. Then, separated antigens are visualized by precipitation.
- Antigen mixture is poured in hole punched in agar and separated by electrophoresis.
- Finally, immune serum is added on channels parallel to electrophoresis line. Precipitation bands are observed in area of Ag-Ab interaction.



Competitive electrophoresis

- This method is based on the formation of precipitation lines as a result of the co-diffusion of antigens and antibodies under the electric field in agar gel.
- Antigen and serum are added in two holes punched in transparent agar gel.
- Hole with antigen is placed near anode, while hole with antibody – near cathode.
- The positive reaction is manifested by the formation of precipitate lines between the holes to with the antigen and serum.

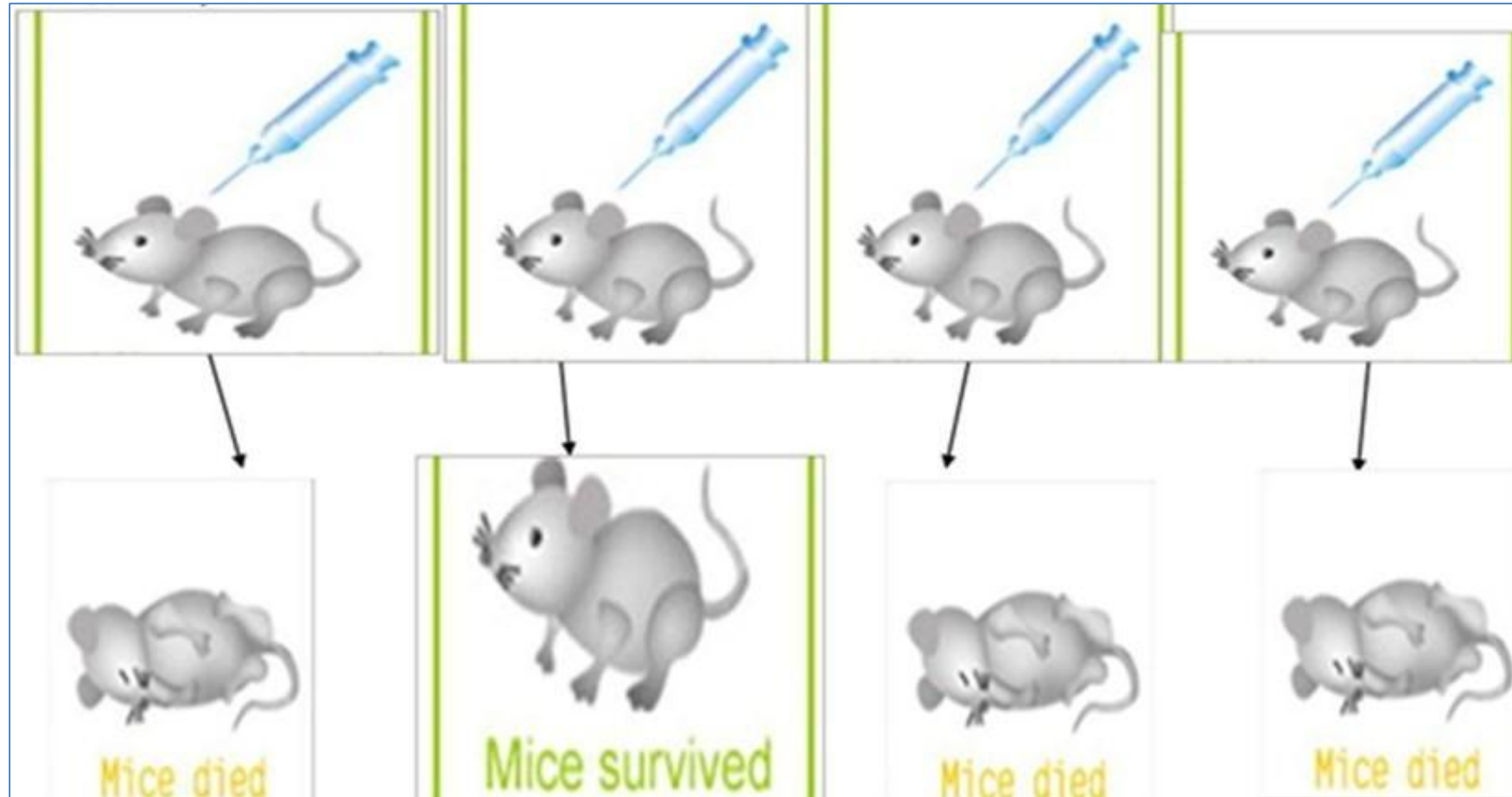
Competitive electrophoresis



Neutralization reactions

- Antibodies can eliminate microorganisms and their toxins in sensitive biological objects – animals, cell and tissue cultures, chicken embryo. This phenomenon is based on neutralization of antigens by antibodies.
- During virus neutralization viruses can not cause disease in experimental animal, do not have *cytopathic effect (CPE)* in cell and tissue cultures, do not reproduce in chicken embryo.
- During *toxin neutralization reaction* a suspected for toxin specimen is mixed with antitoxic serum, incubated and injected to experimental animal. If the toxin is neutralized, the laboratory animals survive, otherwise they die.

In vivo toxin neutralization reaction



Flocculation reaction

- *The flocculation reaction* is neutralization of a toxin or anatoxin with an antitoxin in a test tube (in vitro).
- This reaction is used to determine the activity of an antitoxic serum, toxin or anatoxin. It is possible to determine the activity of a toxin based on an antitoxic serum whose activity is known in advance, or vice versa.
- For example, a certain amount of the tested toxin (anatoxin) is added to various dilutions of antitoxic serum with known activity. In this case, a precipitation reaction (turbidity) is observed faster (initial flocculation) in a test tube with an equivalent amount of antigen and antibody. Based on this, the activity of the toxin is determined.

