



AZERBAIJAN MEDICAL UNIVERSITY
DEPARTMENT OF MEDICAL MICROBIOLOGY and IMMUNOLOGY

Lesson 13.

Cultivation of viruses, rickettsia and chlamydia.

Methods of indication and identification of viruses.

Phages, obtaining, titration, application

FACULTY: General Medicine

SUBJECT: Medical microbiology - 1

Discussed questions:

- Viruses, rickettsiae and chlamydia - as obligate intracellular parasites.
- Reproduction of viruses. Reproductive properties of RNA and DNA viruses.
- Rickettsia and chlamydia reproductions.
- Cultivation of viruses, rickettsiae and chlamydia: susceptible animals, chicken embryos, cell cultures (single-layer, suspended, organ cultures).
- Techniques and types of preparation of single-layer cell culture (primary, transplanted, semi-transplanted).
- The contact methods of susceptible animals (abdominal, intravenous, intramuscular, intranasal, brain, etc.) for obtaining of intracellular microorganisms.
- The contact methods of chicken embryos (yolk sac, amnion and allantois cavity, chorionic membrane) for obtaining of obligate intracellular microorganisms.
- The contact methods of tissue culture for obtaining of obligate intracellular microorganisms
- Detection of obligate intracellular microorganisms in infected test systems or pathological materials: indication (CPE-cytopathic effect, hemagglutination, hemadsorption, intracellular additives, color test, formation of plaques, etc.) and identification (HAIR-hemagglutination inhibition reaction, CFT-complement fixation test, etc.).
- Phages, their structure, kinds and properties, their role in molecular genetics.
- Interaction of phages with bacterial cells and its types: virulent and temperate phages.
- Lysogenic bacteria and their detection.
- The obtaining of phages from different objects and titration methods (Appelman and Gracia methods).
- Application of phages:
 - phagodiagnosics: reaction of increased phage titer, detection of phage in pathological material, phagotyping
 - phage therapy
 - phage prophylaxis

Purpose of the lesson:

- To acquaint students with the reproduction of obligate intracellular parasitic microorganisms - virus, rickettsia and chlamydia, to inform them about the selection, preparation, infection methods of tissue culture, chicken embryo and sensitive animals for the cultivation of viruses and their indication and identification methods in infected test systems. To inform students about phages, their structure, types and properties, their role in molecular genetics, phages acquisition, titration methods and application (phagotherapy and phagoprophylaxis).

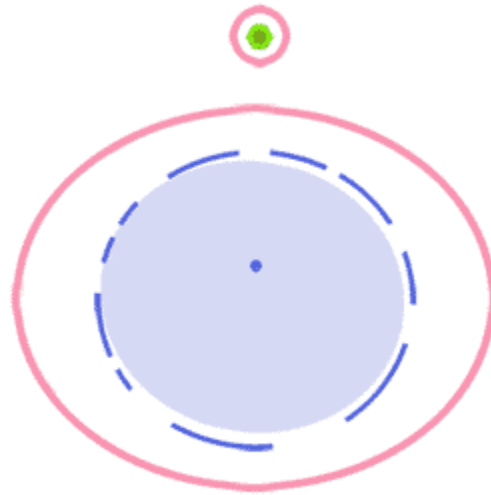
Viruses, Rickettsia and Chlamydiae – obligate intracellular parasites

- Viruses, Rickettsia and Chlamydiae are obligate intracellular parasites and not cultivated in artificial media
- Multiplication of Rickettsia occurs inside the host cell (nucleus and cytoplasm) by binary fission
- Multiplication of Chlamydiae occurs inside the host cell via complex development cycle
- Virus reproduction occurs by special way - replication.

Viruses - reproduction

- Viruses after entering the organism can not multiply in all cells – they infect only cells sensitive for particular virus.
- Mutual interaction between virus and sensitive cell occurs in several stages

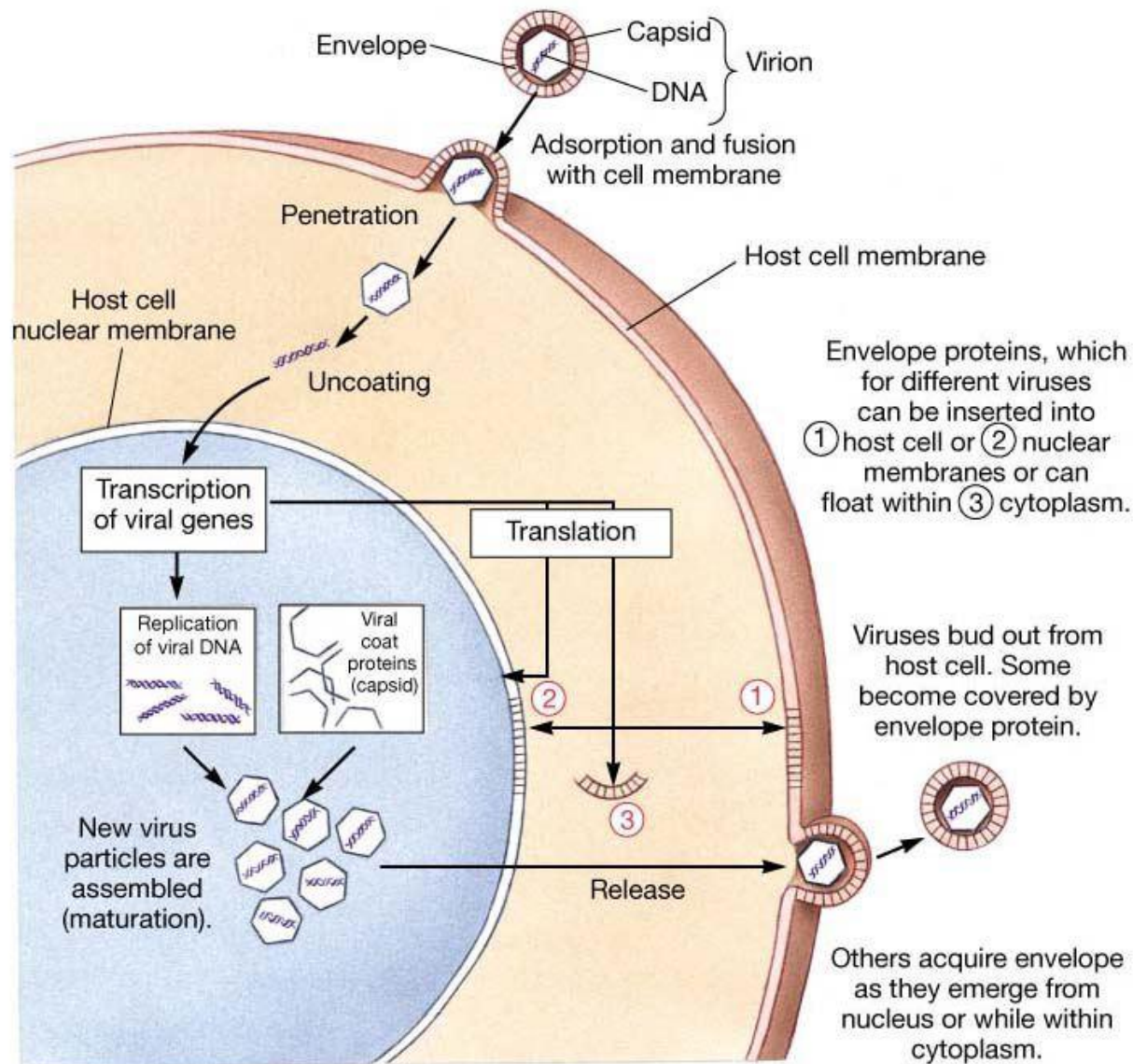
Viruses - reproduction



Reproduction stages

- **Virion attachment**
- **Penetration of virion inside the host cell** (*endocytosis–viropexis, fusion of cell membrane and viral envelope*)
- **Virion “uncoating”, disintegration or deproteinsation**
- **Replication of viral nucleic acids and synthesis of viral proteins**
- **Virion formation**
- **Release of viruses from the cell** (*lysis of host cell, «budding»*)

Reproduction of viruses



Reproductive features of DNA and RNA viruses

- **In DNA viruses:**

viral DNA → iRNA → synthesis of viral proteins

- **In positive-strand RNA viruses:**

viral RNA → synthesis of viral proteins

- **In negative-strand RNA viruses :**

viral RNA → iRNA → synthesis of viral proteins

- **In retroviruses:**

viral RNA → complementary DNA → iRNA →
synthesis of viral proteins

Types of virus-host interaction

- **Productive infection** - reproduction
- **Abortive infection**– noncomplete reproduction
- **Integrative infection**– integration (virogeny)

Main principles of viral cultivation

- Organism of laboratory animals
- Embryonated eggs
- Cell (tissue) culture

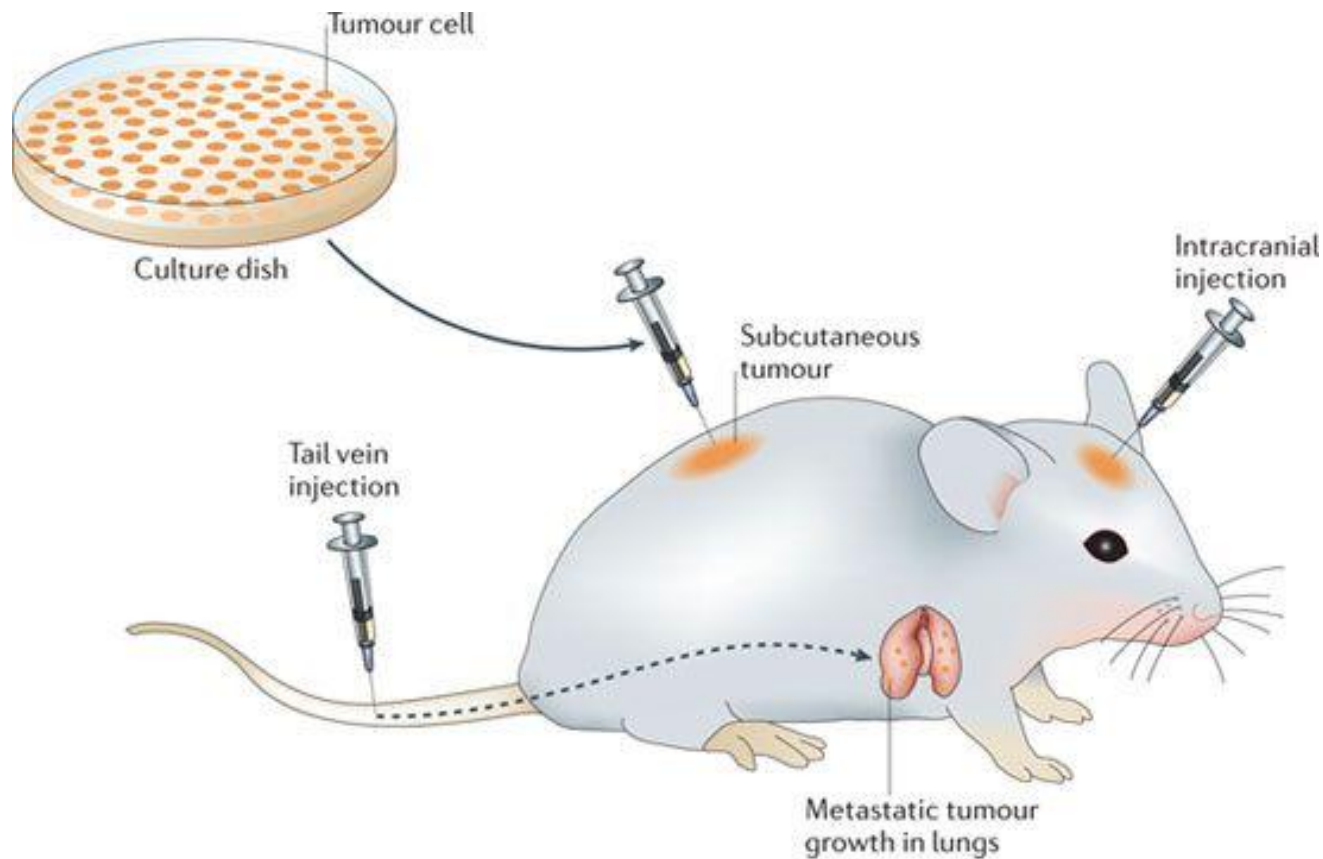
Cultivation of viruses in laboratory animals

organism

- Virological investigations commonly involves newborn laboratory animals (white mice, rats, monkeys, mountain mice etc.)
- Infection routes of laboratory animals (subcutaneous, intramuscular, intravenous, intranasal, intraperitoneal etc.) are selected in accordance with virus tropism.
- Currently application of this method is limited due to inability of human viruses to cause infection in animals, their contamination by microorganisms, ethical and economic issues.

Cultivation of viruses in laboratory animals

organism



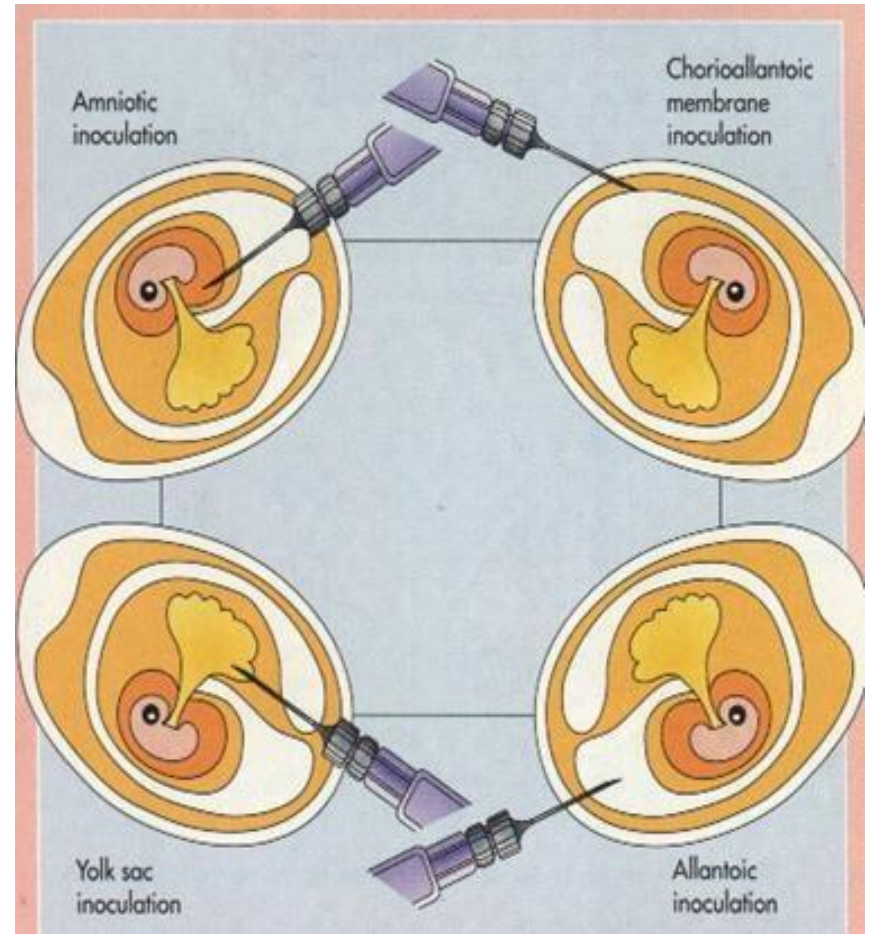
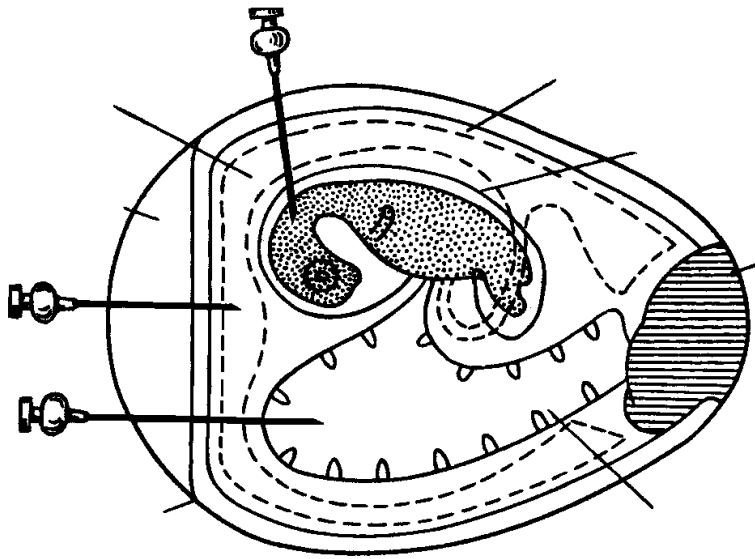
Cultivation of viruses in embryonated eggs

- Model using embryonated egg is convenient due to possibility to obtain high number of viruses, sterile object of investigation, simple technique etc.
- For this purpose 6-12 day chicken embryos grown in poultry farms or incubator are used
- However, there is possibility of latent infection or contamination of embryos by bacterial infection.

Infection of embryonated egg

- Large, sterile (unwashed), fertilized white eggs stored in refrigerator for no more than 10 days are used. Using ovoscope viability of embryo is checked. “Alive” embryo is motile, heartbeat is observed.
- Egg before infection is wiped by 70% ethyl alcohol, passed through a flame, wiped with iodine solution, then wiped again with alcohol and passed through a flame.
- Depending of investigated virus biological features examined specimen can be inoculated in chorio-allantoic membrane, allantoic, amniotic cavities or yolk sac.

Infection of chicken embryo



Infection of embrionated egg

- During allantoic inoculation small hole is made in egg shell over the air chamber (edges are premarked by pencil) using scissors or lancet. 0.1-0.2 ml of virus containing material is injected to are 2-3 mm below the air chamber using a syringe .
- The hole is covered with melted paraffine.
- Infected eggs are examined after 48-72 hours of incubation – time of maximum virus accumulation.

Examination of infected eggs

- After treatment with alcohol and 2% iodine solution, the shell is cut with scissors slightly above the boundary of the air chamber marked with a pencil, while the egg is bent so that the shell does not fall into the cavity.
- The shell of the egg is discarded, its membrane is carefully removed, and the chorionic-allantois membrane around the site of infection is examined for presence of lesions (hemorrhages, white foci).

Methods of viral indication in infected embryos

The growth of virus in infected chicken embryo is detected by

- Death of embryo,
- Necrotic areas made by some viruses in chorioallantoic membrane,
- Hemagglutination reaction with amniotic and allantoic fluids,

Changes in chorioallantoic membrane

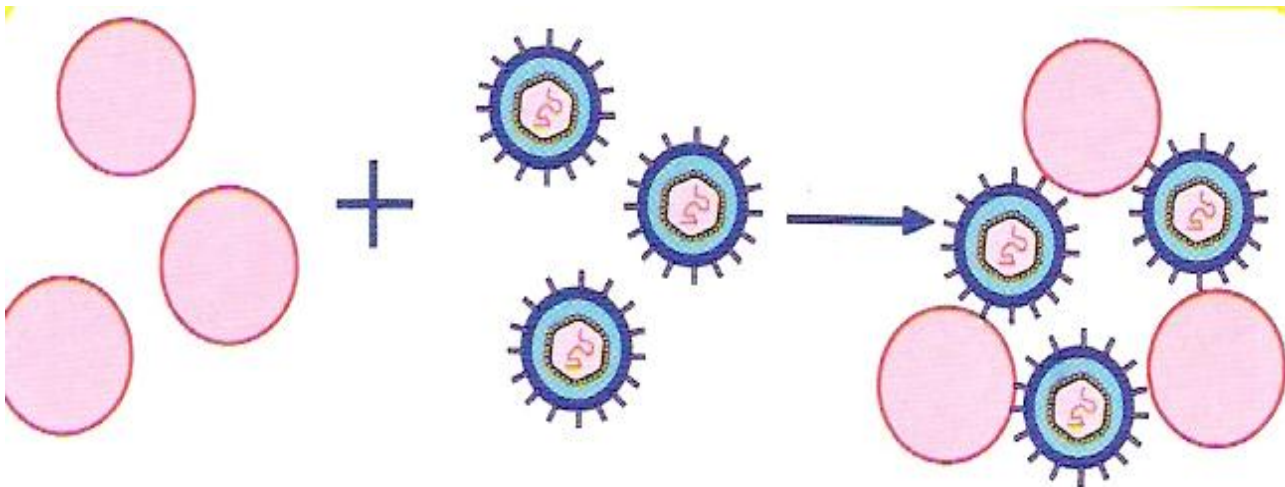
- Chorioallantoic membrane is cut and its content is poured into the Petri dish.
- Chorioallantoic membrane remains inside the shell. It is removed with tweezers, placed in a Petri dish containing saline, washed and the nature of the lesions is studied on a dark background.

Obtaining amniotic and allantoic fluids

- The chorio-allantoic membrane is punctured with Pasteur pipette in free of vessels area, the allantoic fluid is aspirated. For sterility control it is inoculated in sugar or meat-peptone broths. Viral indication is performed by hemagglutination and examined specimen is stored at -4°C
- When obtaining amniotic fluid allantoic fluid is aspirated, then holding amniotic membrane amniotic fluid is aspirated with Pasteur pipette.

Hemagglutination reaction with amniotic and allantoic fluids

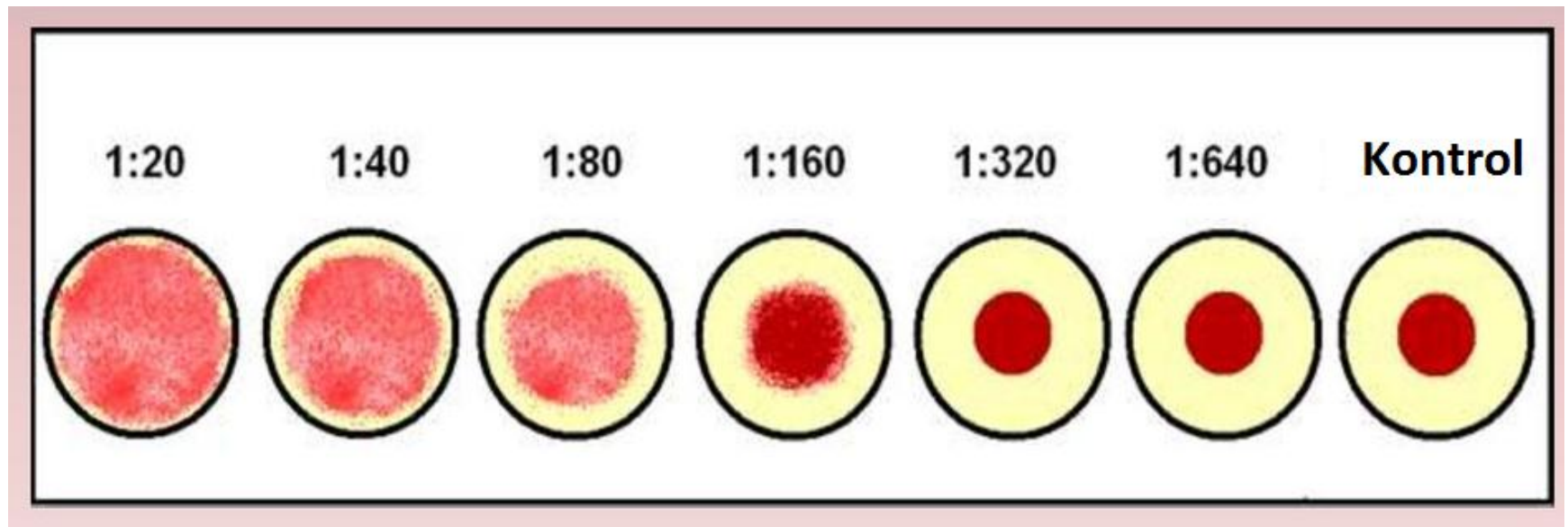
- The presence of the virus in the allantoic and amniotic fluid of an infected embryo is detected by a hemagglutination reaction.
- This reaction is based on the ability of viral antigens called hemagglutinins to agglutinate erythrocytes of various animals and used to indicate viruses.



Hemagglutination reaction technique

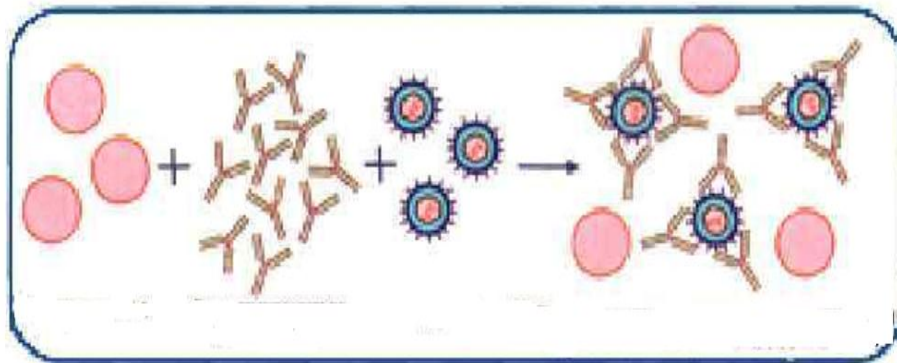
- 0.5 ml of amniotic and allantois fluid is poured into test tubes or wells of plexiglass plates (0.5 ml of the same fluid of an uninfected embryo is taken for control).
- 0,2 ml of 1% suspension of washed hen erythrocytes are added and kept at room temperature.
- The results of the reaction are recorded 40 minutes after sedimentation of erythrocytes;
- (++++) – strong hemagglutination – a thin membrane of erythrocytes adhering to the bottom of the test tube;
- (+++) - the presence of pores in the membrane;
- (++) - the presence of a membrane with wrinkled edges, consisting of adherent erythrocytes;
- (+) – sedimented erythrocytes surrounded by zones of agglutinated erythrocytes;
- -- sedimented erythrocytes which do not differ from control tube.
- In case of absence of control vials, the presence of hemagglutination in the test vials indicates the presence of the virus in the tested fluid.

Hemagglutination reaction technique

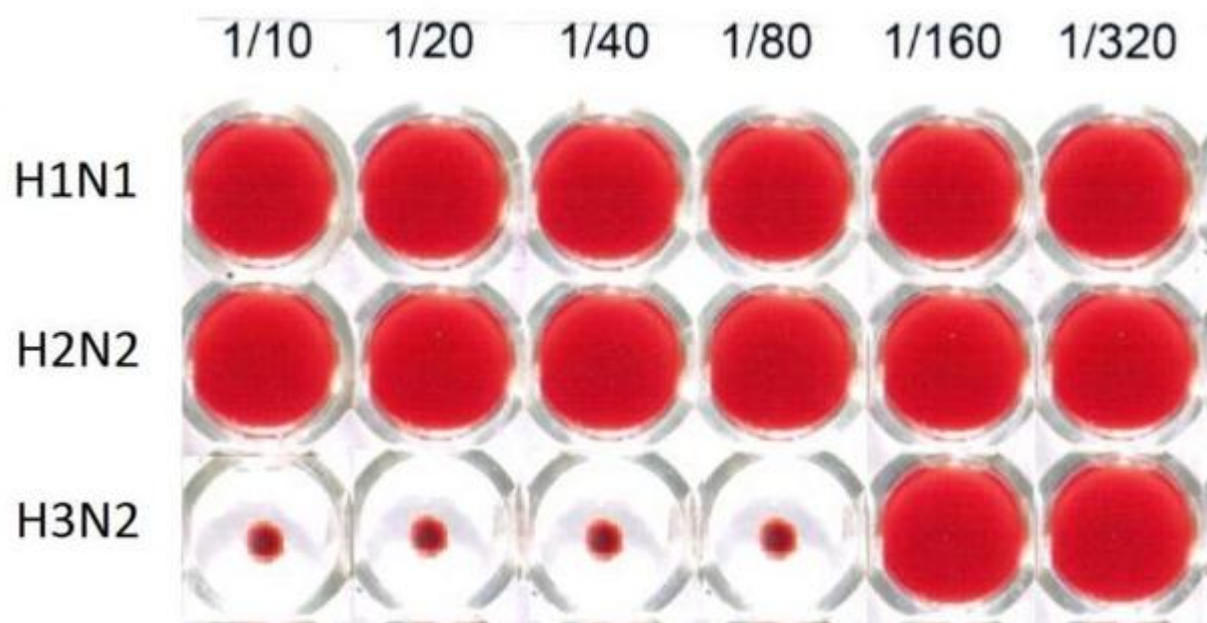


Hemagglutination inhibition

- This reaction is used for identification of some viruses (influenza, measles, tick-borne encephalitis, etc.).
- Serum containing antibodies against particular virus is added on examined specimen in order to detect virus.
- In case of presence of virus in specimen antibodies reacting with them cause reduction of their ability to agglutinate erythrocytes which results in reduction of reaction titer.



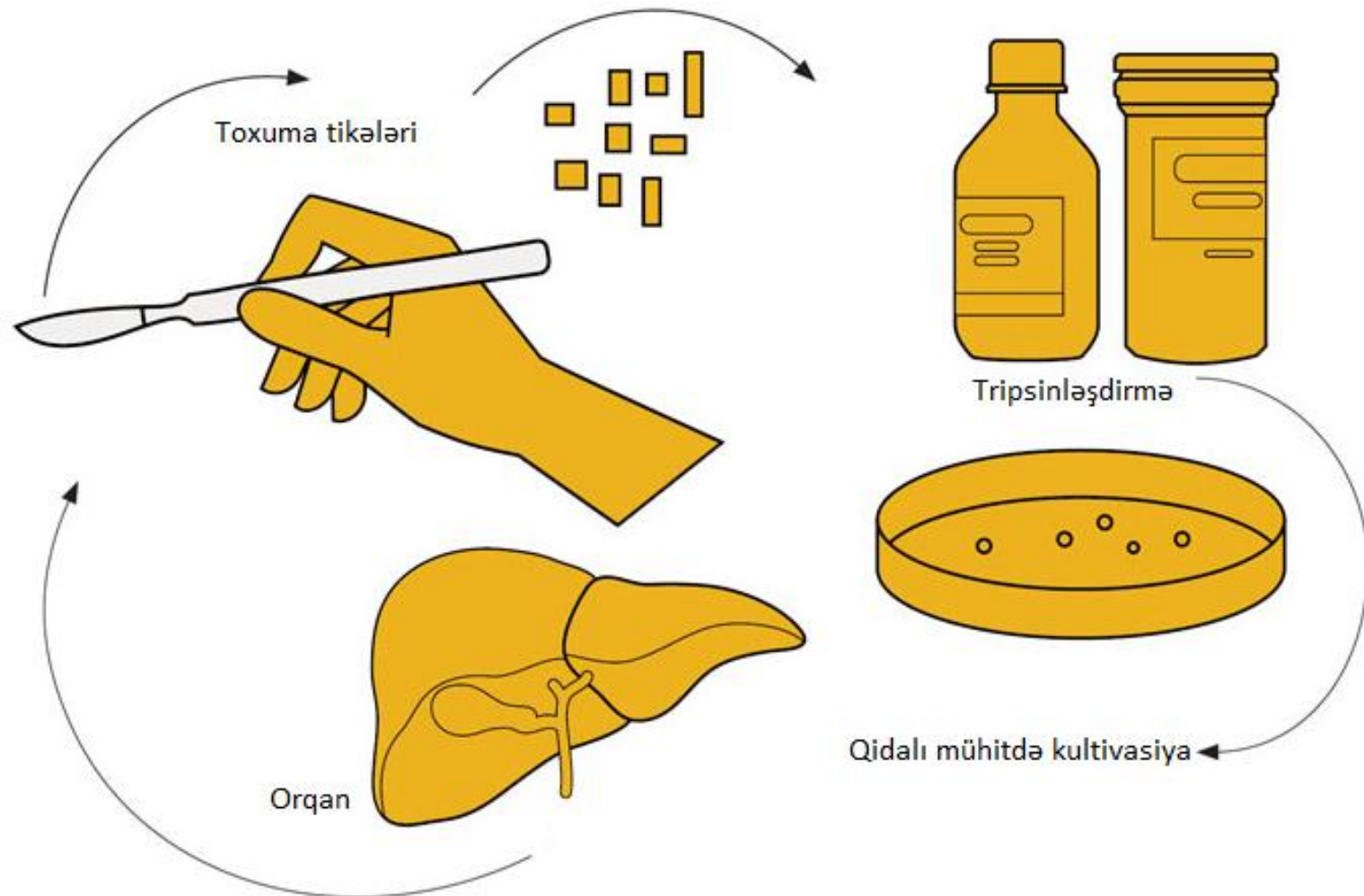
Hemagglutination inhibition technique



Cultivation of viruses in cell cultures

- Cell(tissue) culture consists of organ, tissue parts or separated cells able to survive and grow in nutrient media.
- For this purpose cells obtained from human, bird tissues and organs are cultivated in nutrient media

Obtaining cell culture

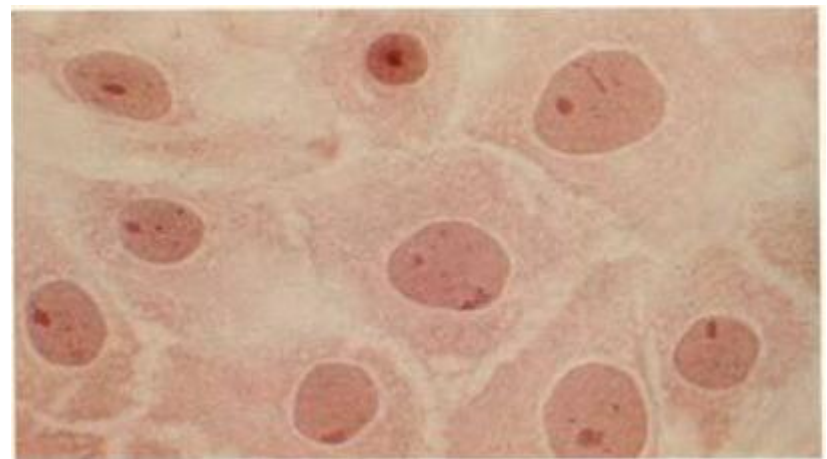
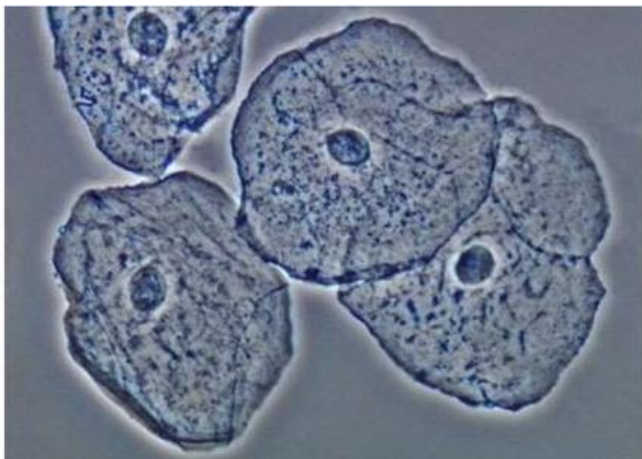


Cultivation of viruses in cell lines

- **Cell lines:**
 1. Monolayer
 2. Suspended
 3. Organ culture
- **One layer cell lines:**
 1. Primary cell lines
 2. Continuous cell lines
 3. Semicontinuous cell lines

Primary cell lines

- Primary cell lines are obtained directly from animal or human tissue via breakdown of intercellular substance by proteolytic enzymes (trypsin, collagenase).
- Cells disaggregated in nutrient media adhere to surface of culture plate, form a layer and grow.
- It is possible to transfer cell lines from one container to another using trypsin or version. Due to the fact that the primary cultures are derived from highly differentiated cells, their ability to divide and multiply is limited, and it is only possible to transfer them 5-10 times.

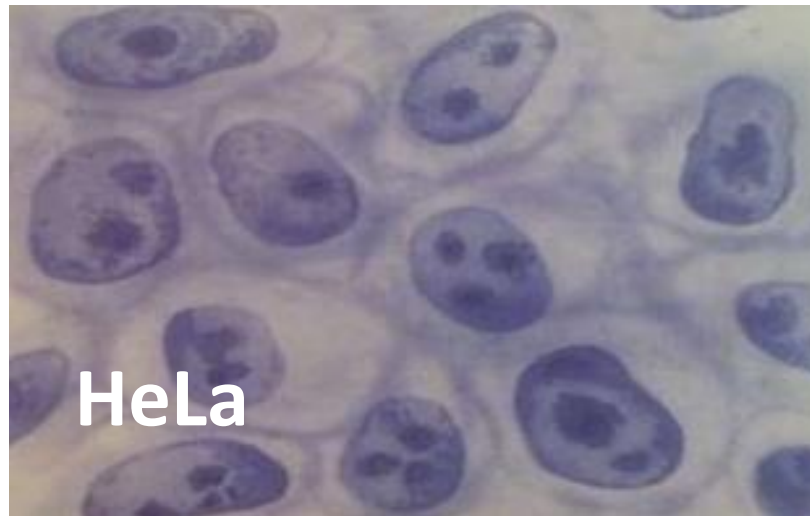


Primary cell lines

- Primary cell lines are prepared from embryonic tissues of animals and human due to their ability for good growth and multiplication.
- Commonly cell lines are prepared from mixture of tissues (exp., bone, skin, and muscle tissue).
- Using this method human embryonic fibroblasts(HEF) and hen fibroblasts(HF), human kidney cells(HKC) etc., are obtained. Human embryonic tissue (when pregnancy is terminated) as well as 8-12-day-old chicken embryos are used to obtain cell cultures.
- Cell cultivation is carried out in glass or plastic containers of various shapes and sizes and in strict accordance with aseptic rules.

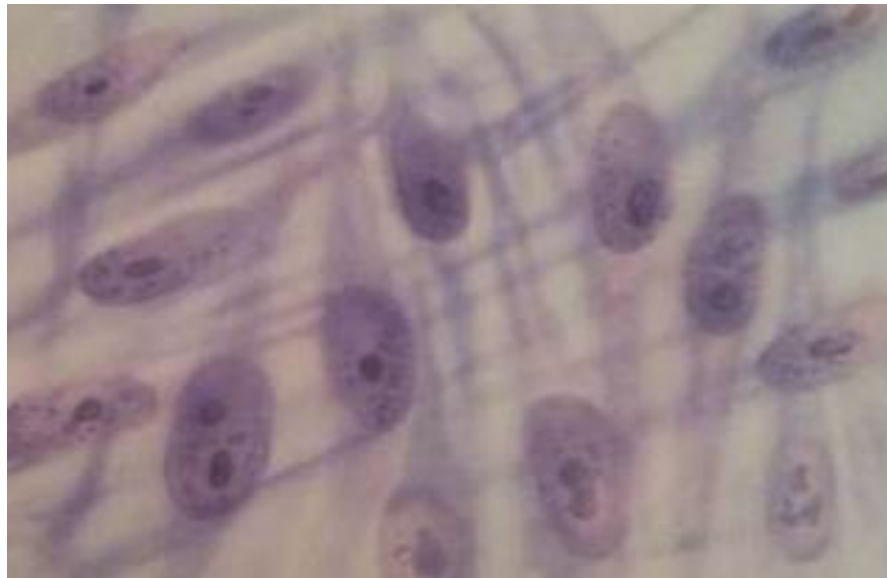
Continious cell lines

- Transplanted (continuous, stable, permanent) cell lines are able to withstand an unlimited number of passages.
- They are derived from tumor cells which have lost their differentiation and have no growth restriction.
- Continious cell lines were obtained from various normal and tumor human tissues: amniotic fluid (A-0, A-1, FL), kidney (Rh, PPC), cervical carcinoma (HeLa), laryngeal cancer (Hep-2), lung cancer. from the patient's bone marrow (Detroit-6), rhabdomyosarcoma of the human embryo (RD), etc.



Diploid (semicontinuous) cell line

- Diploid cell line - a cell line in which more than 75% of cells have the karyotype of normal cells.
- Some of them can maintain diploid status for 50-80 and more divisions.
- Fibroblast cells derived from human and animal embryonic tissue are used to obtain a diploid culture of cells.



Nutrient media for cell lines

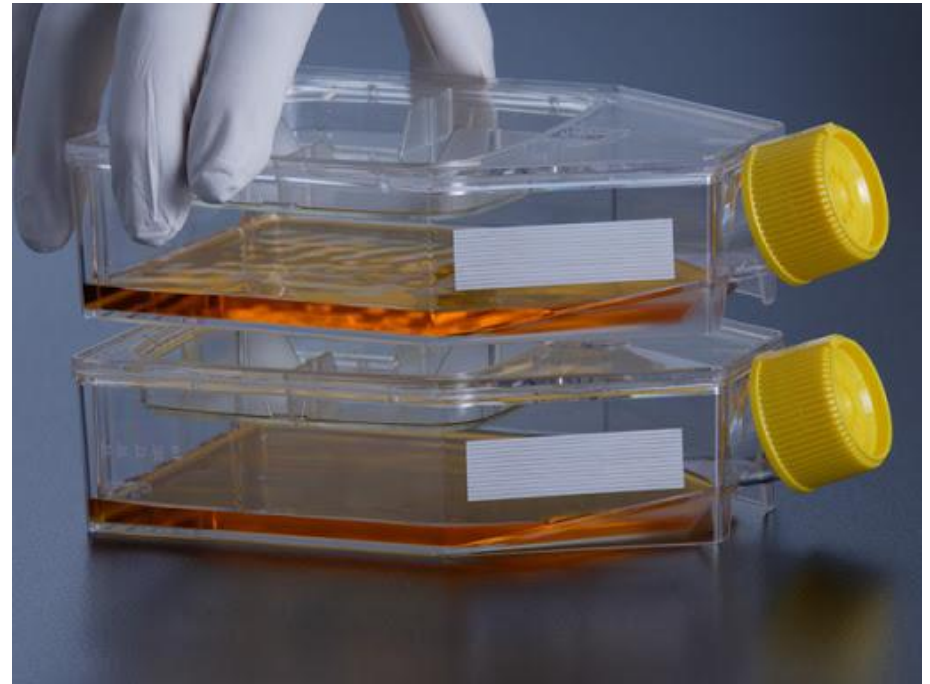
- These media contain a full set of amino acids, vitamins and growth factors.
- In addition to dry media and individual components, ready-made liquid media (199, Igla, lactalbumin hydrolyzate, dry media and concentrates) are also produced.
- Cultural media are divided into growth and conservation media. For the cultivation of cell cultures, growth media enriched with animal and human serum (eg, ox serum, fetal cow serum, etc.) are used. The amount of serum in the nutrient medium is usually 2-30%, depending on the characteristics of the cell culture and the composition of the medium.

Nutrient media for cell lines

Phenol red is added to nutrient media which obtain yellow color in acidic and red color in alkalic environment



Cultivation containers



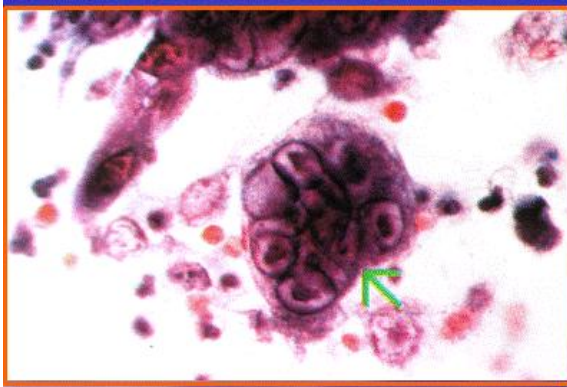
Cultivation containers



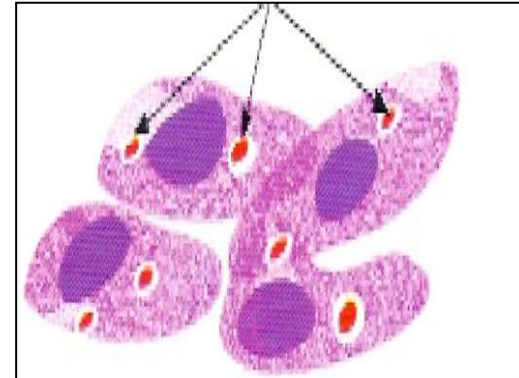
Methods of viral indication in cell lines

- It is not always possible to observe growth of viruses after inoculation of virus containing specimen in cell lines.
- In order to detect growth of virus *effects on* developing in cell lines are taken into account.

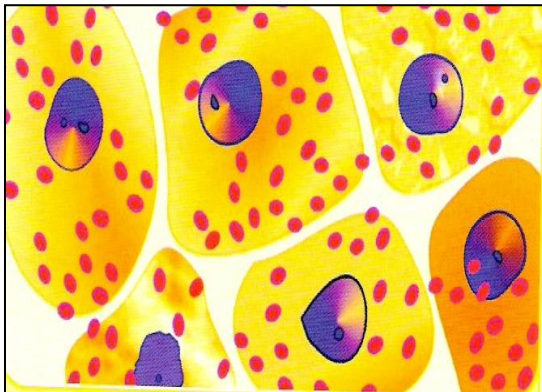
Methods of viral indication in cell lines



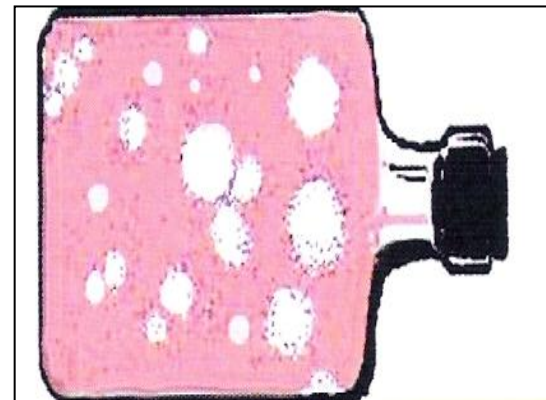
Cytopathic effect (CPE)



intracellular inclusions



hemadsorption phenomenon

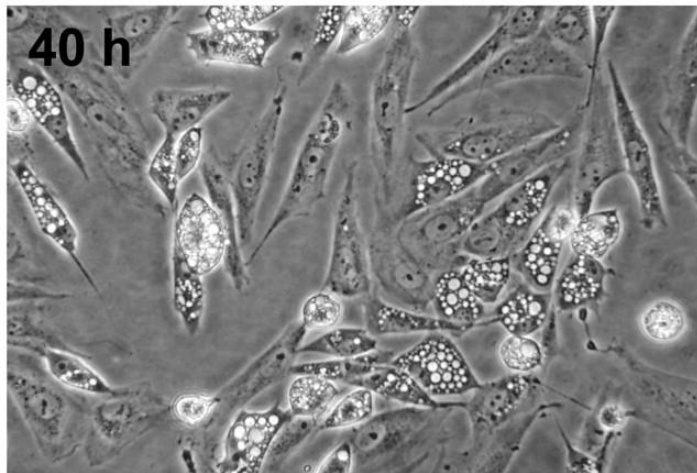
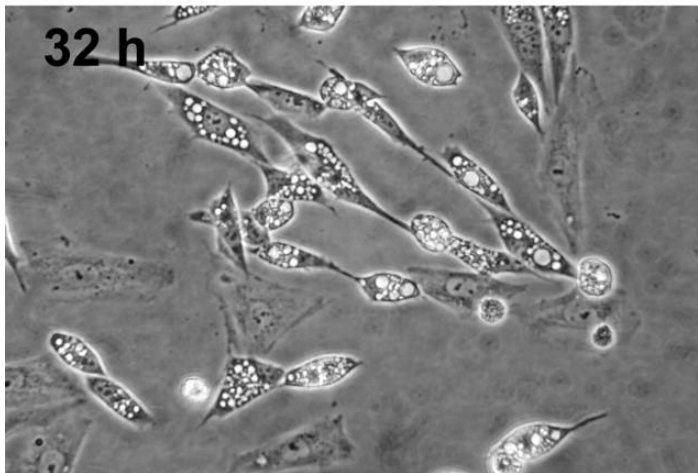
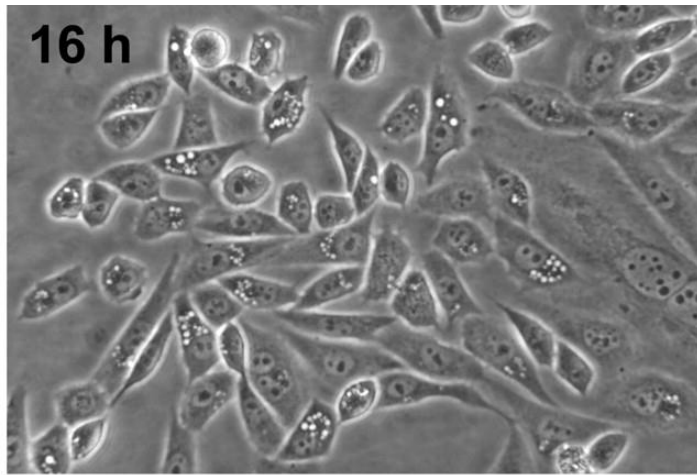
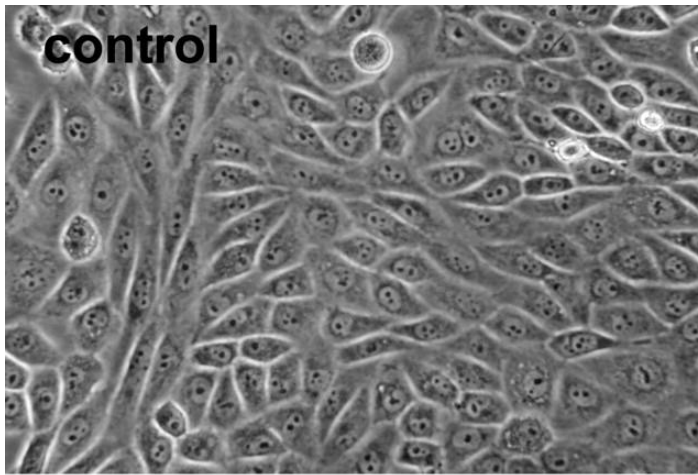


«negative colonies»

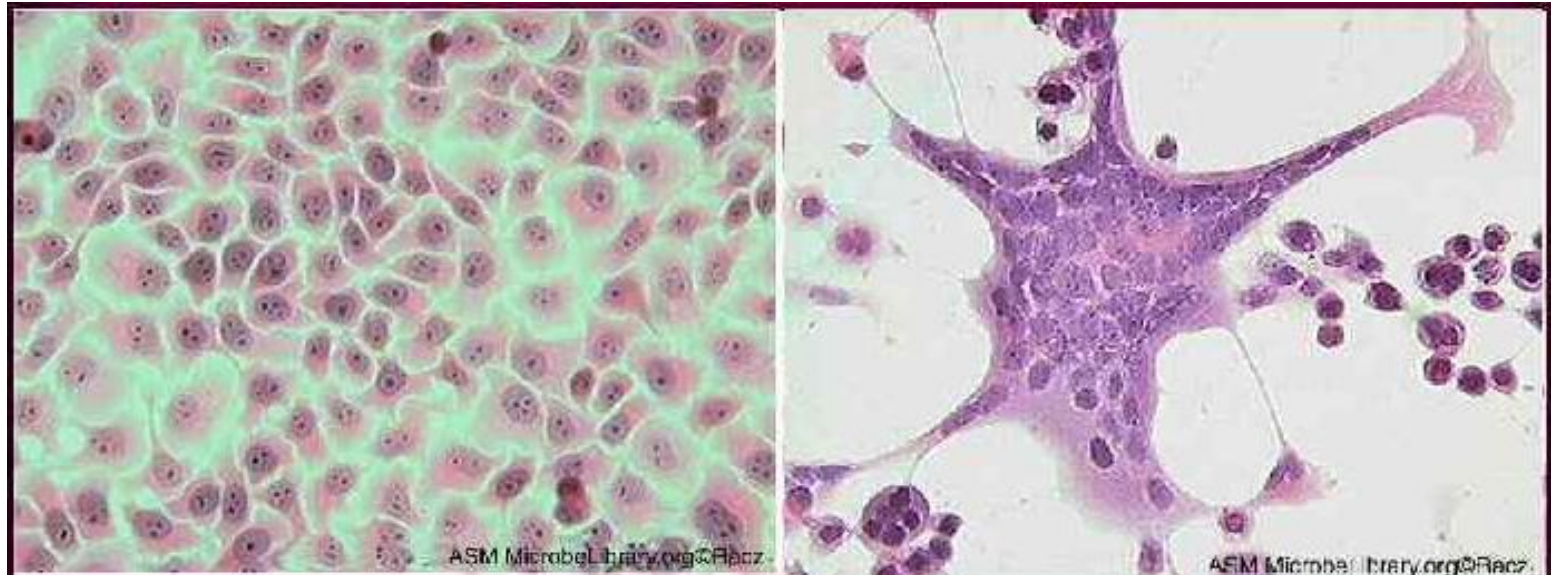
Cytopathic effect(CPE)

- During reproduction some viruses cause degeneration of cell lines (cytopathic effect).
- CPE is evaluated dynamically by observation under the microscope of cell lines. CPE is one of the methods of viral indication and identification.
- Different viruses cause different CPE.

Cytopathic effect

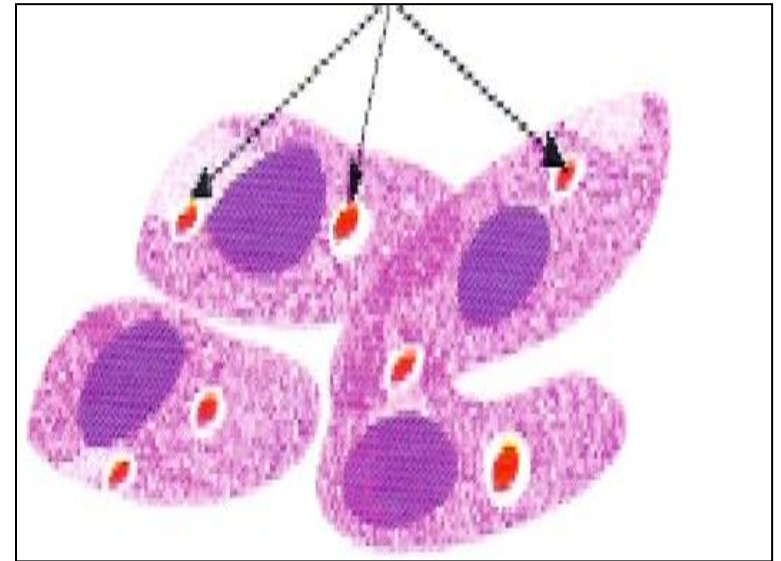


CPE of Herpes simplex virus



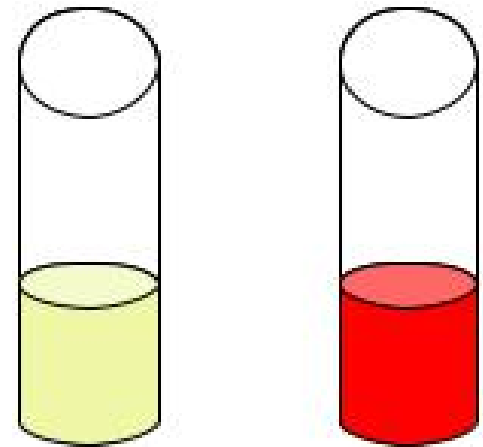
Intracellular inclusions (bodies)

- Some viruses can be detected and identified by observation of their inclusions in cytoplasm and nucleus.
- Inclusion forms are different and range in size from 0.25 μm to 25 μm .
- They represent the sites of accumulation of viral particles, and detected by Giemsa staining or fluorochrome staining



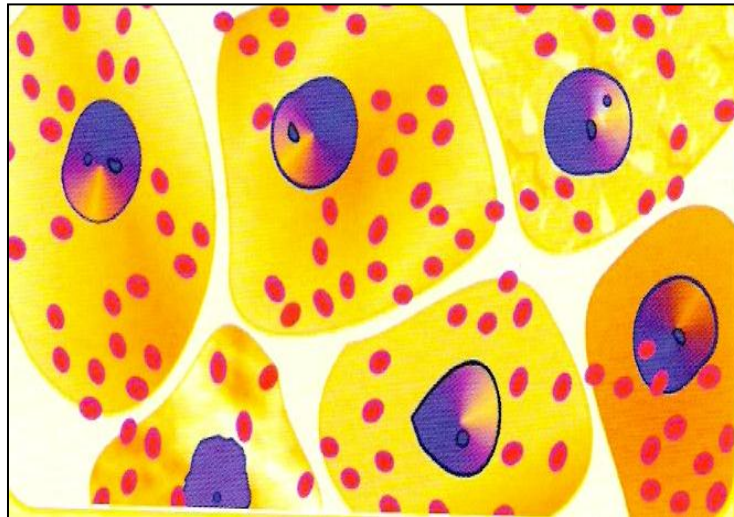
«Color test»

- Growth of viruses in cell lines can be detected by «**color test**». For this purpose cell lines growing in nutrition media with indicator (exp., methyl red) are used.
- Virus reproducing in cells causes their death. Thus, the color of medium remains unchanged.
- In case of viral growth absence metabolic products of cells cause change of medium color.



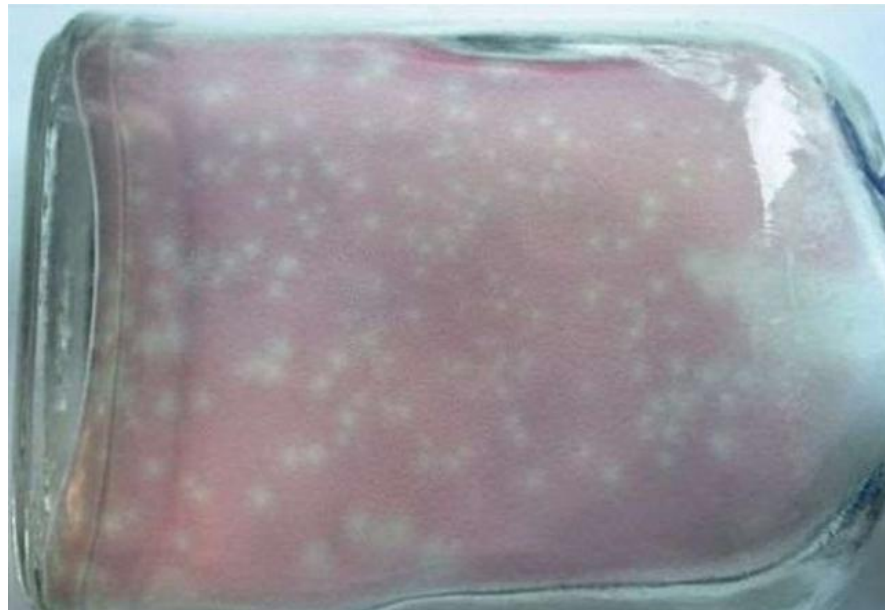
Hemadsorption phenomenon

- Hemadsorption is one of the phenomena used for indication of viruses in cell lines. Cells infected with some viruses become able to adsorb erythrocytes on their surfaces. The reason for this is existence of hemagglutinins in viruses (orthomyxoviruses, paramyxoviruses etc.). Viruses reproducing inside the host cell are transported to cell surface and attach to erythrocytes causing hemadsorption. Thus, this phenomenon is one of the variant of hemagglutination.



«Negative colonies»

- Growth of some viruses causes death of cells. Detection of these area without cells (negative colonies) makes possible indication of viruses.
- Addition of agar on infected cell lines limits enhancement of reproduction zones.
- As a result, the necrotic centers caused by viruses are observed as discrete zones.

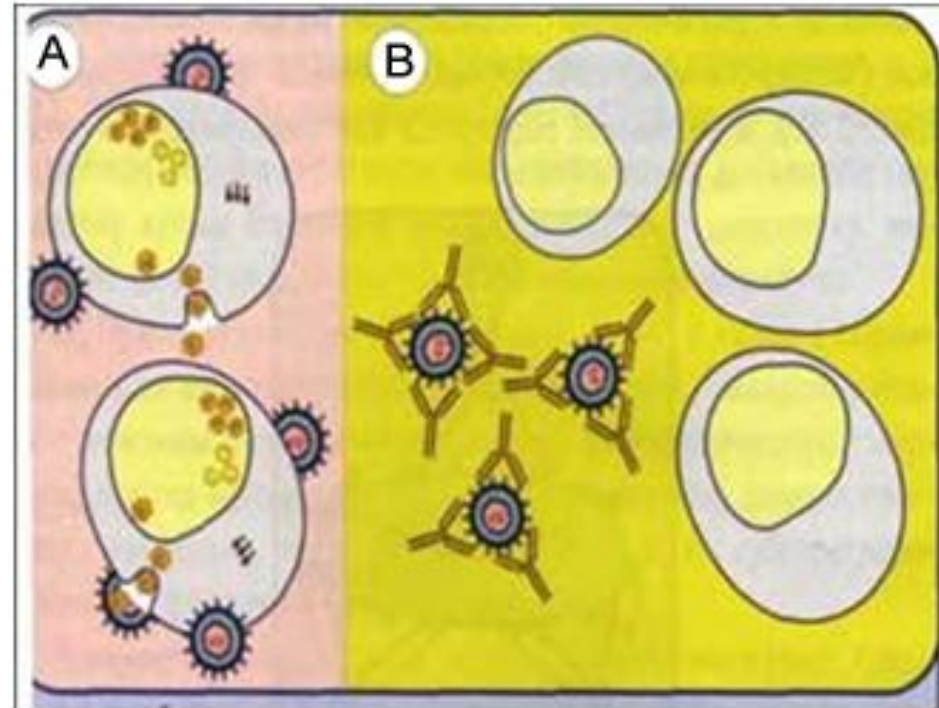


Interference phenomenon

- For detection of viruses which do not cause CPE interference phenomenon is used. This phenomenon is represented by resistance of cell infected with one virus to other viruses.
- For example, measles virus does not cause CPE. This virus can be detected in primary cell lines by interference phenomenon.
- Cell lines infected with measles virus are coinfecting with indicator virus causing CPE (e.g. virus of vesicular stomatitis). Growth of measles inhibits reproduction of indicator virus which results with absence of CPE. If there is no reproduction of measles virus CPE will be observed in cell culture.

Neutralization reaction of viruses

- **Neutralization reaction**(biological neutralization reaction) makes possible indication of viruses.
- Impact of antibodies on viruses results with absence of CPE in cell lines, inability of viruses to infect laboratory animals and chicken embryo.



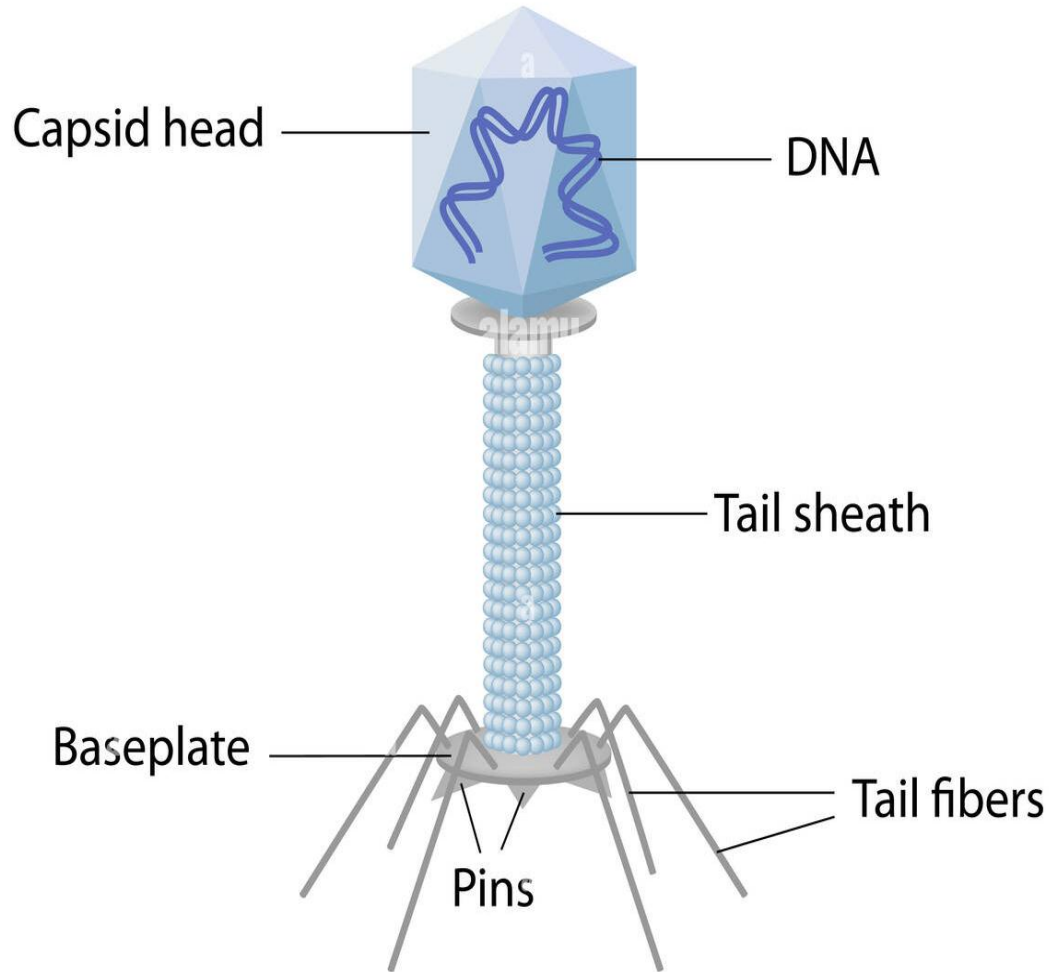
Bacteriophages

- Reproduce in bacteria and other microorganisms and in special conditions cause their lysis.
- First was observed in 1917 F.D'Erell when he detected lysis of pathogen obtained from patient with dysentery by filtrate obtained from stool specimen of the same patient.
- D'Erell concluded that factor causing the lysis is a virus which can pass through bacterial filter He called this virus as ***bacteriophage***(«eating bacteria»), and phenomenon - as bacteriophagy.

Structure of bacteriophage

- Phage sizes are similar to other viruses and vary between 20-800 nm. They have thread, cube and spermatozoid like morphology. E.coli phages have been (T phages) studied well. T (*type*) group phages are represented by 7 members, 4 of which single (T1, T3, T5, T7) and paired 3 (T2, T4, T6).
- Paired T phages, especially T2 have complex structure

Bacteriophage structural scheme



Interaction with bacterial cell

- Due to character of interaction with bacterial cell phages divided to **virulent** and **temperate** one.
- **Virulent** phages enter and reproduce in bacterial cell causing its death – **lysis**. It is represented with loss of turbidity of microorganism broth culture -phage lysis. In solid nutrition media they visible by eyes zones of lysis – **phage negative colonies**.

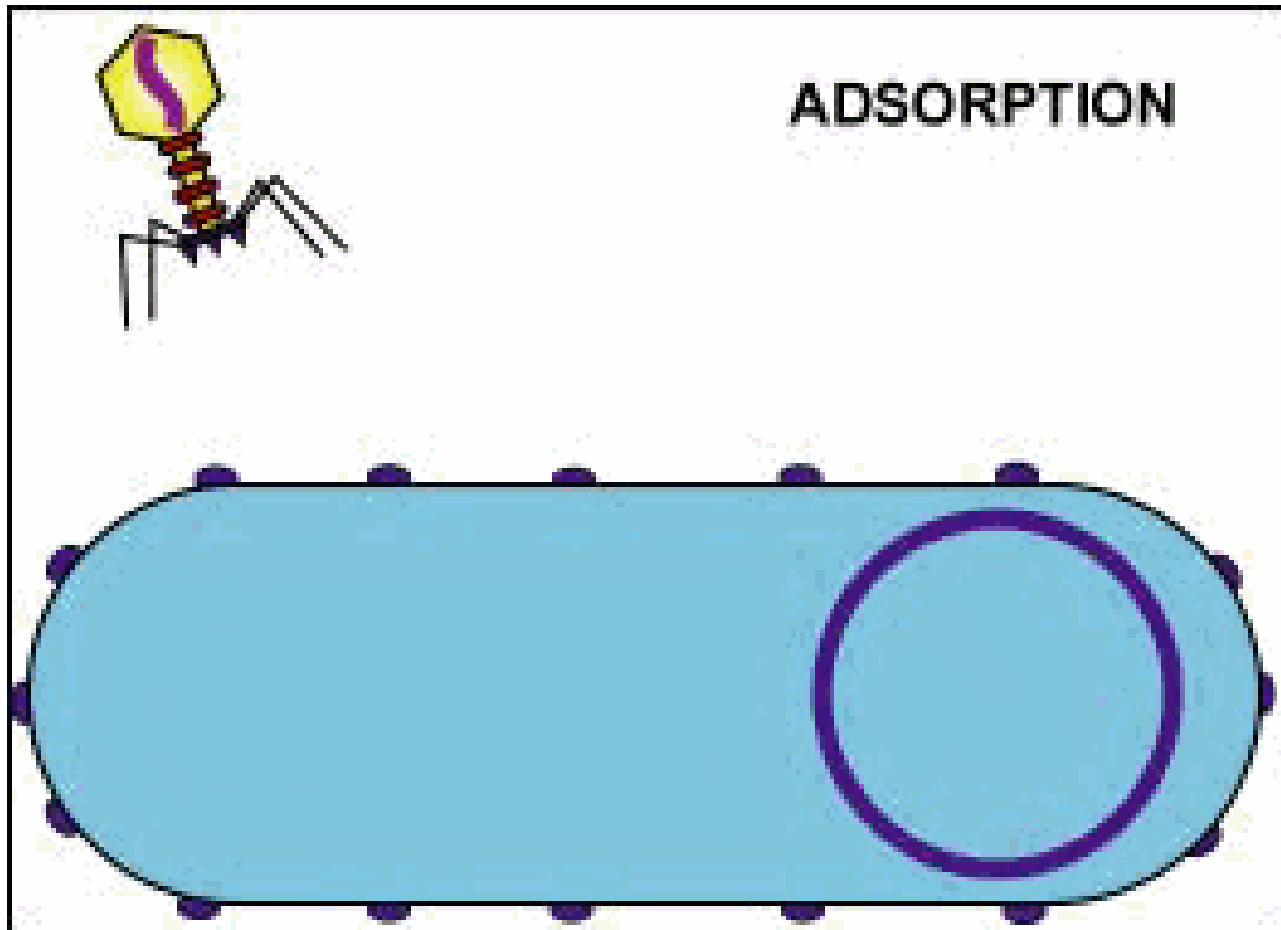
Interaction of virulent phage with bacterial cell

1. Adsorption of phage to bacterial cell
2. Entrance of phage nucleic acid inside the bacteria
3. Reproduction of phage nucleic acid and protein synthesis
4. Assembly of phage
5. Release of phage from the cell

Interaction of temperate phage with bacterial cell

- After entering the bacterial cell nucleic acid of temperate phage **integrate** with bacterial cell chromosome. It does not cause lysis of bacterial cell.
- Nucleic acid of phage connected to chromosome is called **prophage**.
- Symbiosis of bacterial cell with phage is called **lysogeny** while bacteria is called lysogenic bacteria.
- Prophage of lysogenic bacteria is able to disintegrate from chromosome and become virulent phage. At this circumstance phage causes lysis of bacteria.
- The process of conversion prophage to virulent is triggered by various factors, especially by radioactive rays.

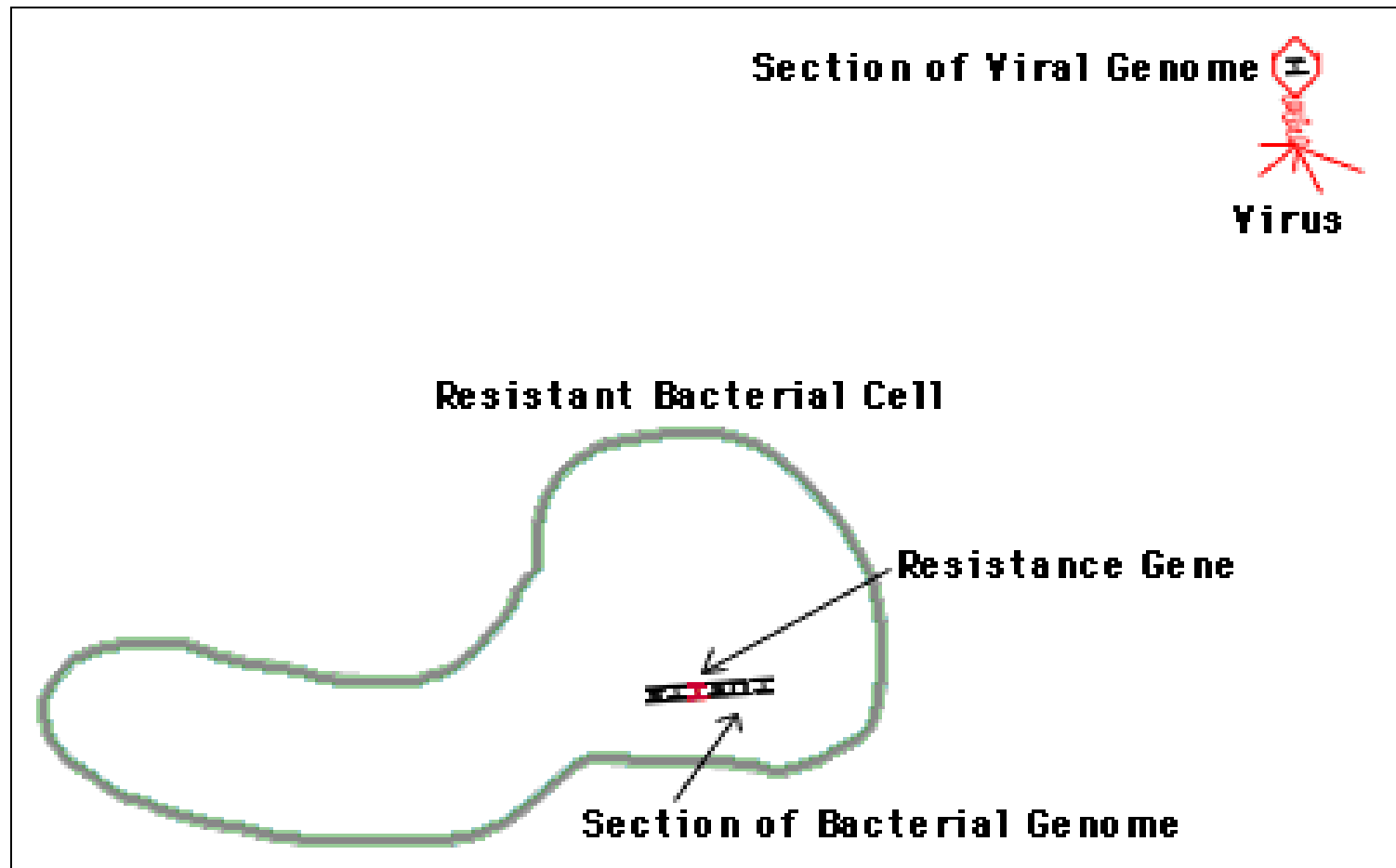
Interaction of phages with bacterial cells



Defective phages

- During lysogeny with **defective phage** possessing genes responsible for some features lysogenic bacteria obtain new features.
- Defective phages temperate phage which are unable to carry out complete infectious cycle.
- Using this way bacteria can obtain ability to produce toxins, new antigens, morphological features, etc. It is called **phage conversion** or **lysogenic conversion**.
- They are used in genetic engineering as transductive phages.

Defective phages



Obtaining bacteriophages

- Examined material (water, stool, wound) is suspended and filtered. Filtrate and homologous test culture are inoculated in broth and incubated at 37°C for 18-24 hours.
- Incubated material is centrifuged and filtered in order to sort out bacteria
- Both filtrate and test-culture are inoculated in nutrient agar and incubated. During growth of bacterial culture round spots (negative colonies) are formed.
- Material obtained from negative colonies is placed to broth, then test culture is added and this mixture is incubated. Phages growing in bacteria cause their lysis and phage lysate is obtained containing high number of phages.
- Bacteria are completely removed from phage lysate

Detection of bacteria susceptibility to phages by “flowing drop” method

Susceptibility to phages is based on the high specificity of their effect.

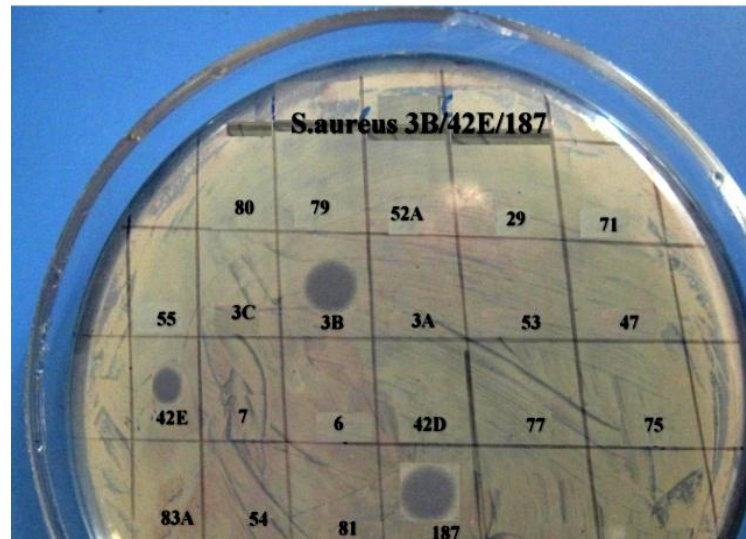
- It is possible to identify unknown microbe culture using known diagnostic phages.
- The examined culture of the bacterium is inoculated very thickly (with a lawn) on the surface of the solid nutrient medium in the Petri dish. Then a drop of known phage suspension is placed on the surface of the agar, lifting one side of the plate to allow the drop to flow.
- After incubation the lack of growth in the area where the phage was added indicates susceptibility of the microorganism to phage.



Phage typing

In order to find out infection source phage typing is conducted

- Bacterial culture is inoculated (lawn) to Petri dishes (with divided squares) with solid nutrient medium.
- Then drops of different type-specific phages is added to each square on the surface of the agar.
- After incubation, lysis of bacteria is observed in the squares corresponding to the phage type



- They titrate the bacteriophage to know its activity.
- Bacteriophage titer is determined by Appelman and Gracia methods.

Practical use of phages

- Phage specificity is a base of phage diagnostics
- - It is possible to identify unknown microbe culture using known diagnostic phages.
 - Phage typing makes possible to detect source of infection.
- ***Phage prophylaxis and therapy is based on ability of phages to kill sensitive bacteria in patient organism.*** For this purpose phages are prepared in drug forms

Phagodiagnosics

