Ministry of education and science of the Republic of Kazakhstan

A. Baitursynov Kostanay Regional University

Department of Veterinary Sanitation

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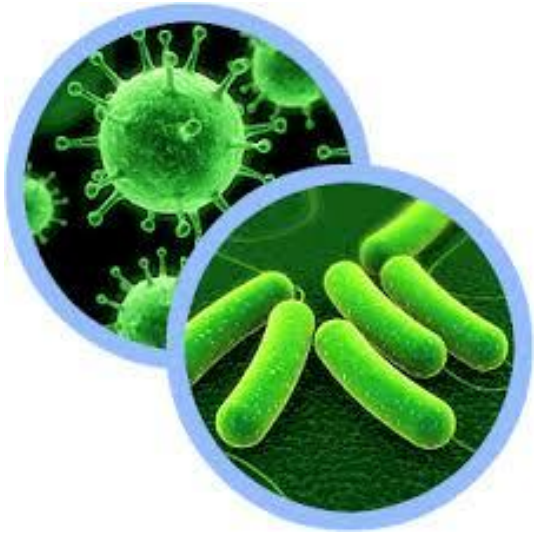
MICROBIOLOGY AND VIROLOGY
(Part 1)

Educational and methodical manual



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The tutorial outlines the structural organization of the bacterial cell, morphology, methods of cultivating bacteria on artificial nutrient media, the characteristics of viruses, their organization, methods of indication and identification. Modern methods of laboratory diagnosis of diseases of viral and bacterial etiology are described.

The tutorial contains: the name of the section and topics, the purpose of the lesson, equipment, questions for self-monitoring and assignments.

This tutorial is intended for implementation laboratory studies by students of veterinary and biological specialties.

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Contents

Introduction

The discipline "Microbiology and Virology" is assigned one of the leading roles in the preparation of biological and veterinary specialists. The knowledge, which was gained during the learning of this discipline, forms the basis for laboratory diagnosis of infectious diseases, microbiological assessment of the sanitary condition of environmental objects, as well as ensuring the safety of food and feed.

The proposed tutorial of microbiology and virology contains up-to-date information from the program for teaching microbiology and virology at veterinary, agricultural and biological faculties. The tutorial includes two sections: "General Microbiology" and "General Virology".

Information about modern diagnostic methods, which include the immunofluorescence reaction (RIF), enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction, significantly expanded and supplemented.

To achieve greater efficiency of self-training and knowledge control, most sections complete control questions, test tasks that can be used for computer control. The tutorial is designed for laboratory and practical classes based on theoretical knowledge.

The author, while compiling the tutorial, sought to maximize the use of the existing regulatory and technical documentation for the diagnosis of infectious diseases of farm animals. All practical comments and suggestions to the author will be accepted with appreciation and gratitude.

Section I. GENERAL MICROBIOLOGY

The main goal of studying general microbiology is to gain knowledge and acquire the skills of independent practical work, which are necessary for conducting microbiological studies of any orientation, laboratory diagnosis of infectious diseases, bacteriological studies of food, feed and other material.

Correct and timely diagnosis determines the basic conditions for organizing and conducting measures to eliminate infectious diseases, also defines the nature and effectiveness of measures to eradicate foci of infection, the success of specific prevention and therapy.

While diagnosing infectious diseases, a set of methods is used:

- epizootological, clinical, pathological and laboratory data.

In most cases, to confirm the final diagnosis laboratory diagnostics are organized. They include:

- depending on the type of pathogen (bacteria, viruses, microscopic fungi): bacteriological, virological, mycological;
- serological diagnosis;
- allergological diagnosis;
- molecular genetic diagnostics.

I. Bacteriological diagnosis consists of three stages (Application A):

1. Microscopy of stained preparations;
2. Plating on nutrient media with the release of a pure bacterial culture to establish the cultural and enzymatic properties of the pathogen;
3. Bioassay (infection of laboratory animals), to determine the virality of isolated bacteria.

II. Serological diagnostics includes the formulation of serological reactions in order to identify specific antibodies in the patient's serum.

III. Allergological diagnosis is based on the use of allergens.

IV. Molecular - genetic diagnostics is based on polymerase chain reaction (PCR), DNA hybridization, etc.

1.1 Veterinary laboratory, structure and equipment

The purpose of the lesson. To acquaint students with the organization and equipment of veterinary laboratories and safety measures at work. To master the general scheme of bacteriological diagnostics the technique of taking and preserving pathological material.

Material equipment. Equipment, organs from animal corpses. Tweezers, scalpels, scissors. A wooden box with sawdust, a metal box, cans, plastic bags, a 5% solution of carbolic acid, a 30% solution of glycerol, sodium chloride, distilled water.

Principles of organization and equipment of veterinary laboratories

Veterinary laboratories are public service institutions, their activities are aimed at ensuring well-being of animal husbandry, preventing and eliminating diseases and deaths of animals, and protecting the population from diseases common for both animals and humans.

Laboratory diagnostics are conducted by veterinary laboratories, which, according to the scale of their activities, are divided into republican, regional, district, and special ones at enterprises that process livestock products (meat, dairy, etc.).

The main task of veterinary laboratories is to establish an accurate diagnosis of infectious diseases (tuberculosis, brucellosis, anthrax, pig erysipelas, etc.) of farm animals (including birds), fur animals, fish and bees, as well as to conduct veterinary and sanitary examination of meat, milk and other food and feed.

The material in the laboratory is explored with bacteriological, serological, histological methods. Moreover, the necessary conditions must be created (specially allocated premises, equipment, microclimate, etc.).

Veterinary laboratories are placed in a separate building, away from roads, large cities, also are fenced and planted.

The laboratories include: an administrative annex, a reception department (currently it is a department of receiving samples and information), pathologist (opening), serological, bacteriological, virological and parasitological departments; PCR boxes; there is a food safety department (radiological, microbiology department, chemical and toxicological laboratory) in a separate annex; special rooms for thermostats and equipment for sterilization are also allocated.

Bacteriological laboratories are usually located in several rooms.

Each laboratory provides: a) boxes for working with individual groups of bacteria or viruses; b) premises for serological studies; c) preparation of nutrient media; d) sterilization, washing dishes; e) vivarium with boxes for healthy and experimental animals.

There should be tables, sinks, hot and cold water, a gas stove or electric stove, racks for washed dishes, a fume hood, enameled bathtubs, basins and other containers, a solution in glass vessels for disinfecting pipettes, glass slides and other utensils in the dishwashing room.

The following equipment is provided in the laboratory: a biological immersion microscope with additional devices (illuminator, phase-contrast device, dark-field condenser, etc.), a fluorescent microscope, thermostats, sterilization equipment

(autoclaves, drying cabinets), a pH meter, and a device for producing distilled water (distiller), centrifuges, technical, analytical and torsion scales; filtering equipment (Zeitz filters and bacterial suppositories), water baths, refrigerators, apparatus for the manufacture of cotton gauze plugs;

a set of tools - bacteriological loops, spatulas, needles, tweezers, etc.,

laboratory glassware (test tubes, flasks, Petri dishes, mattresses, bottles, ampoules, Pasteur pipettes graduated at 1, 2, 3, 4, 5 and 10 ml) and other equipment.

It is necessary to have a place for staining microscopic preparations in the laboratory, where dye solutions, spirit for decolorization, filter paper, etc. are located. Each workplace should be equipped with a gas burner (or spirit lamp) and a jar with a disinfectant solution. The laboratory must have the necessary nutrient mediums, chemical reagents, diagnostic serums and other materials for daily work.

Safety precautions while working in the laboratory

To enter and work in the laboratory only in special clothes - a dressing gown and a white hat (the dressing gown should be tightly fastened, the hair should be fitted under the hat).

Extraneous things, food should not be brought to the laboratory. Each student is assigs a workplace with a certain number, a microscope and other equipment.

A student can place only the necessary equipment for the work at the workplace. Usually this is a set of paints, a bottle of water, a drain cup, a cup with clean slides, a bacteriological loop, a jar with a disinfectant solution.

Students must not turn on electrical appliances and other equipment without the permission of the teacher or staff.

Each student must strictly observe accuracy in the work, keep the workplace and equipment clean.

While unpacking the material sent for research, the cans with the material must be wiped from the outside with a disinfectant solution and placed only on trays.

If the infected material accidentally hit the table while the work, it is immediately removed with a swab moistened with a disinfectant solution. If infected material gets on the skin, conjunctiva, or mouth, students should immediately tell a teacher.

At the end of the work, pathological material, used cultures of microorganisms, tools should be placed in disks for disinfection.

Before leaving the laboratory, special clothing must be taken off, hands thoroughly treated with a disinfectant solution and washed with soap.

Rules for taking, preserving and transporting pathological material

While selecting and processing pathological material for transmission to the laboratory, the following rules must be followed:

- take into account the pathogenesis of the disease and the tropism of the pathogen;
- use sterile instruments and utensils;
- deliver it as soon as possible;

- select material before treatment with antimicrobial agents.

The following can be sent to the laboratory: a) samples of material from sick and suspicious animals: blood, blood serum, feces, urine, milk, exudates, pus, scrapings of the skin, etc .; b) pathological material from animal corpses: aborted fetus, tubular bone, lymph nodes, muscles, brain, spinal cord, parenchymal organs, etc .; c) samples from environmental objects: water, soil, fodder; insects, rodents, etc.

It is better to send the corpses of small animals, as well as piglets, lambs, calves in a waterproof container.

Tubular bones sent for examination should be intact, with intact ends wrapped in gauze or cloth moistened with a disinfectant liquid (5% solution of carbolic acid).

Before milk is taken, the nipple of the udder is disinfected with 70 ° spirit, 100 - 150 ml of milk are poured into a jar of disinfectant, and subsequent 30 ml portions are transferred to sterile vessels.

Blood is taken in test tubes or flasks from the jugular vein in compliance with sterility. Feces are taken from the rectum in a glass tube with fused edges, one end of the tube is closed with a cotton-gauze stopper. After taking feces, the tube is lowered into a test tube with saline. Urine is taken by using a catheter.

Exudate is taken with Pasteur pipettes or sterile swabs.

Scrapes from the mucous membranes and skin are taken with a sterile scalpel in a sterile dish with a stopper.

The aborted fetus of the first half of pregnancy is sententirely. The fetus of the second half of pregnancy is sent entirely or only its parenchymal organs are taken

The heart of corpses is always taken entirely and with blood.

Pieces of the spleen, kidneys, liver with the gall bladder are taken using sterile instruments and sterile dishes. The stomach and intestines are ligated with a ligature at both ends and placed in a separate bowl. The entire brain is sent for research.

While packaging pathological material, it is necessary to exclude pollution by extraneous microflora from the external environment. Pathological material must be delivered to the laboratory within the first 24 hours after its taking out. If these conditions are difficult to fulfill, then the material must be preserved with a 30% aqueous solution of glycerol or a 10% aqueous solution of sodium chloride or a solution of glycerol with sodium chloride (glycerol - 250.0 ml; sodium chloride - 5.0 g; distilled water - 750.0 ml). A covering letter is drawn up to the taken pathological material sent to the laboratory.

Control questions:

1. The purpose of the study of general microbiology.
2. Methods of laboratory diagnosis of infectious diseases.
3. Veterinary laboratories - structure and tasks.
4. Equipment and safety while working in the laboratory.
5. The rules of taking pathological material for research.

1.2 General scheme for bacteriological diagnosis

The purpose of the lesson. To acquaint students with the features of conducting microbiological studies of biomaterial. To master the general scheme for the diagnosis of infectious diseases.

It is necessary to master the technique of isolation, typing and identification of microorganisms in a laboratory in order to study certain types of bacteria.

Bacteriological diagnosis begins with the taking, conservation and transportation of biomaterial, and includes three stages of research:

A. ***Microscopic researches*** of the source material make it possible to detect the presence of a pathogen in it, to study its morphological features and tinctorial properties.

B. ***Bacteriological studies*** are carried out in order to isolate the pure culture of the pathogen with the establishment of its morphological, tinctorial, cultural and biochemical properties, and in some cases - antigenic structure.

C. ***Biological studies (staging bioassays)*** are conducted by infection of laboratory animals, which allow us to determine the virulence of the pathogen, as well as isolate it in a pure culture.

D. Conducted researches allow us to determine the **species affiliation of the pathogen** and make a bacteriological diagnosis.

A. MICROSCOPIC RESEARCH METHOD

Microscopic examination (microscopy) is the simplest and most informative method of laboratory diagnosis. It lies in exporing the material under a microscope: optical, electronic, luminescent, etc.

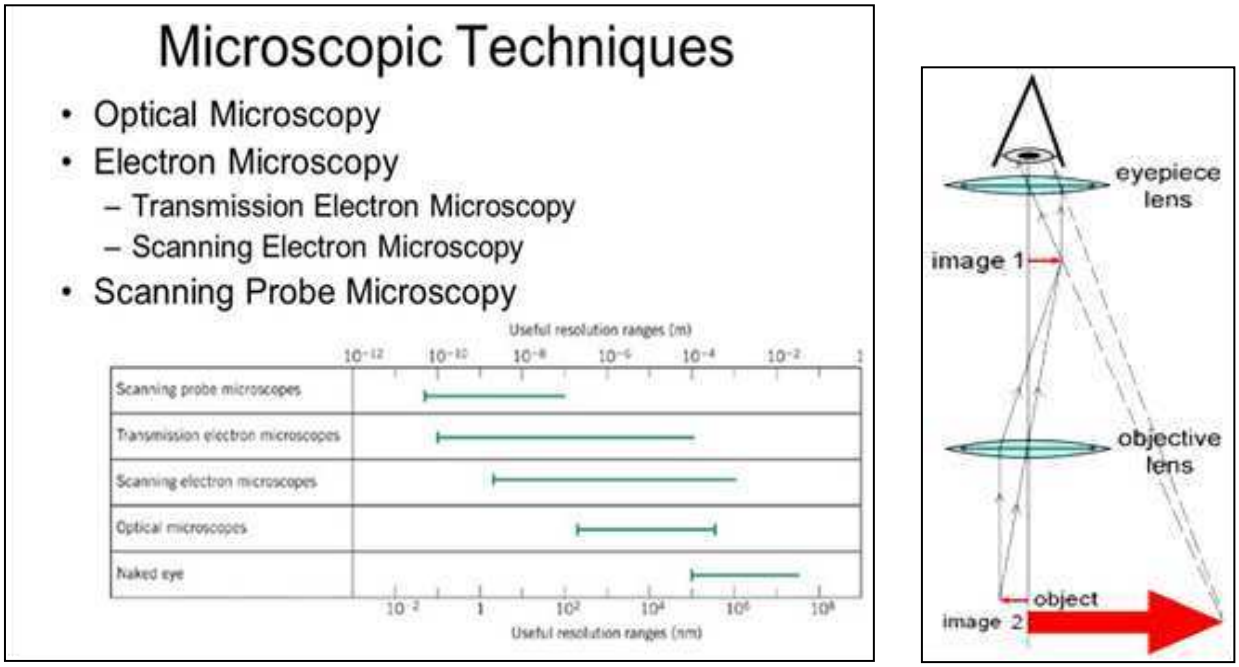


Figure 1 – Microscopy techniques

This method allows to detect microorganisms in the preparations, to study their **morphological features, mobility and tinctorial properties**.

To master the microscopic method, need to know:

- structure and types of microscopes;
- ultrastructure of the bacterial cell and morphological features;
- the chemical composition of the microbial cell;
- preparations for microscopy and methods for staining them;
- methods for determining the motility of bacteria.

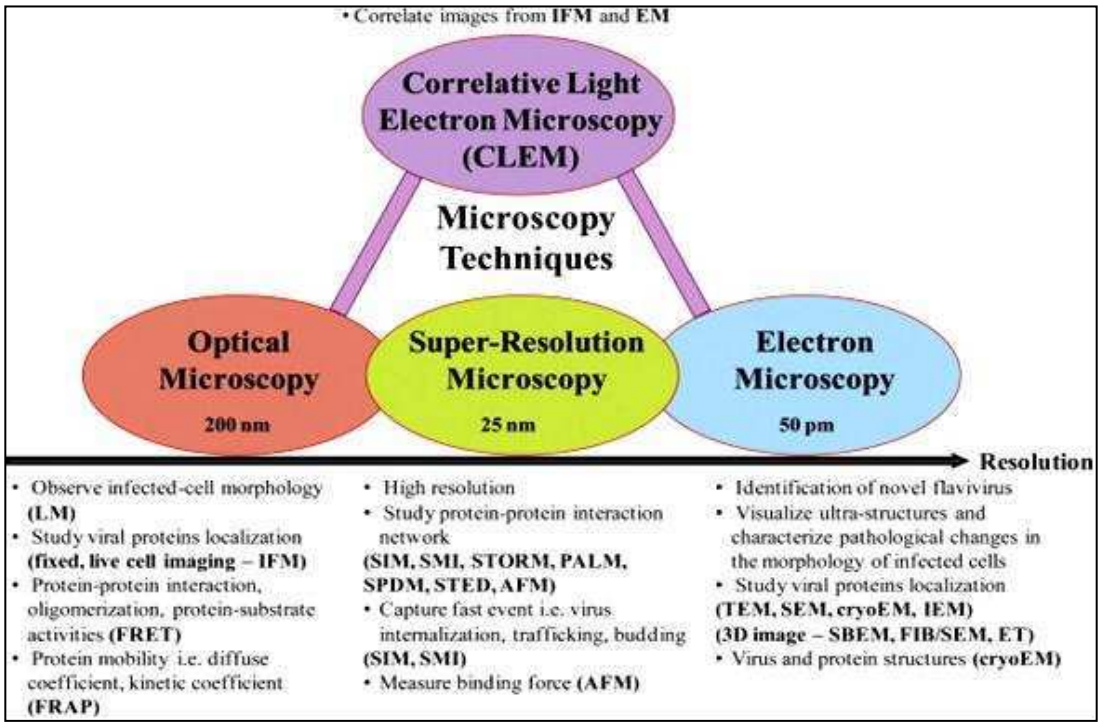


Figure 2 – Light electron microscopy

Control questions:

1. The basic scheme of bacteriological diagnosis.
2. The essence of the microscopic method of research.
3. Types of microscopy.
4. Eiap of bacteriological research.
5. for what purpose is a bacteriological study carried out ?

1.3 The structure of an optical microscope and the rules of working with it

The purpose of the lesson. To learn the structure of an optical microscope and the rules of working with it. To have a grasp of the types and purpose of various microscopy methods.

Equipment. Optical microscopes and a set of finished stained smear preparations. Immersion oil, illuminators.

Microorganisms are the smallest living organisms invisible to the naked eye. Their sizes vary widely - from 0.2 to 10 micrometers (microns) or more (1 micron is 1/1000 of a millimeter).

Bacterial cells are examined using microscopy, mostly - in a dead stained state, rarely - in a live unpainted form.

A light microscope is used to study stained bacterial preparations.

Microscopes allow us to examine objects in transmitted light. Such microscopes consist of mechanical and optical parts (Fig. 3 Structure of the optical microscope).

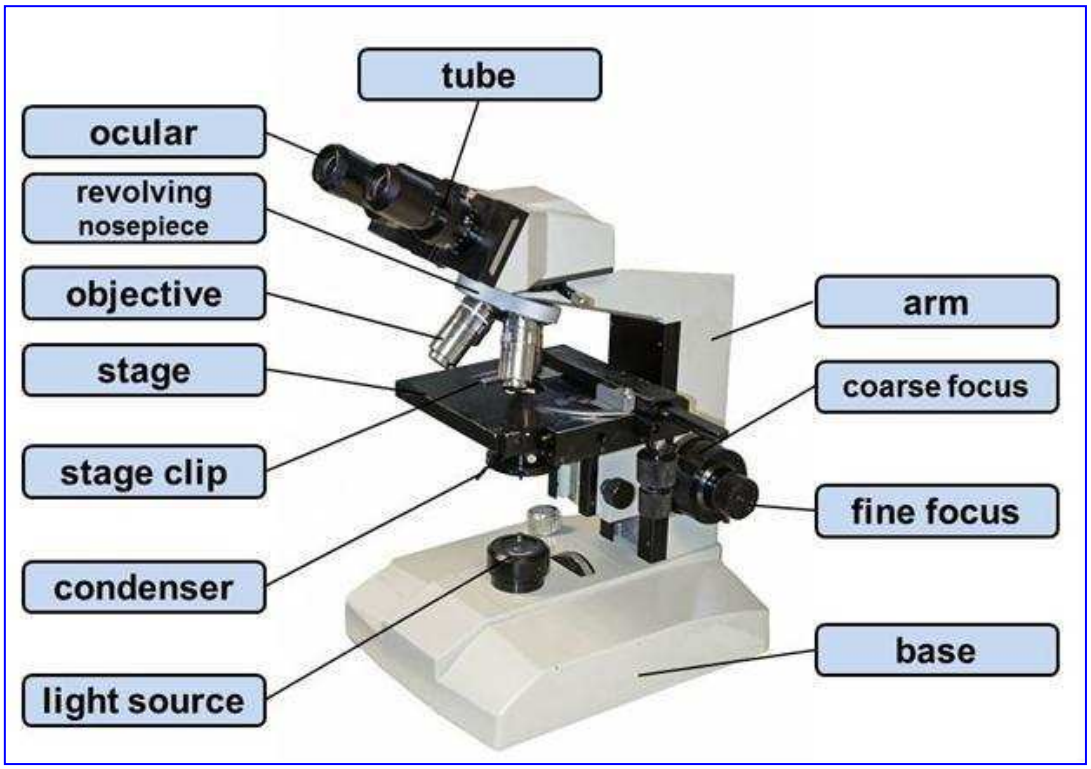


Figure 3 – Parts of an immersion microscope

The mechanical part of the optical microscope includes a tripod with an objective table and a tube. The objective table can be moved horizontally due to screws. It has two terminals securing the preparation. The upper part of the tripod - the tube holder - can be moved with the help of a macrometer screw and a micrometer screw, designed respectively for rough and accurate focusing of the preparation. The micrometer screw is one of the most fragile parts of the microscope and must be handled with particular care. A complete rotation of it moves the tube by 0.1 mm.

There is a revolver rotating around its axis in the upper part of the tube holder, lenses are screwed in the opening of it. An eyepiece is inserted into the upper end of the tube.

The optical part consists of lenses, eyepieces and a lighting apparatus.

The eyepiece is inserted into the upper end of the tube. It consists of two rimmed lenses. There are digital symbols on the eyepiece, showing the degree of image magnification by 7, 10, 18 times.

The objective is a system of optical lenses. There are signs on the objectives indicating the magnification given by the objective (x10, x40, x100). Dry and immersion objectives are distinguished. Objectives, which give an increase of 4 and 40 times, are called dry, because during the work between the objective and the preparation is a layer of air. If during the work a drop of immersion (cedar) oil is placed between the preparation and the objective, it calls an immersion objective. Immersion oil has an optical refractive index close to the refractive index of the glass, due to this light rays, do not deviate from their original direction, but fall on the lens of the objective.

The total magnification that the microscope gives is determined by the product of the magnification of the objective and the magnification of the eyepiece. The clarity of the final image depends on the resolution of the microscope - the minimum distance between two points, perceived separately. A light microscope has a resolution of about 0.2 microns when illuminated with visible light.

The lighting apparatus consists of a condenser, a mirror, an iris diaphragm. It is intended for the better illumination of the preparation. Rays of light emanating from a light source are directed into a condenser that concentrates the light in its focus. A condenser and an iris diaphragm are a system of optical lenses and serve for collection and direction light rays with the help of a screw. When lowering the condenser, the field of view is darkened, when raised, it is illuminated. The iris diaphragm is used to control the intensity of light, which is carried out using the lever to expand or narrow the hole that allows light to pass to the condenser.

Additional accessories for the microscope are also available, they allow using all its capabilities, facilitating working conditions and significantly expanding the range of applications.

The following devices are often used in microbiology:

1. The dark field capacitor.

2. KF-1, KF-4 phase-contrast device and other models.

3. A binocular nozzle that brings microscopy closer to the conditions of natural vision.

4. The eyepiece-micrometer and the object-micrometer, designed to measure microscopic objects.

5. A heating stage, which is installed instead of the microscope stage, to ensure a constant temperature of 37 ° C. It is used for long-term monitoring of living microorganisms.

6. Microphotography for photographing microscopic objects.

7. The microinstallation for time-lapse (intermittent) micro-filming, used in combination with phase-contrast microscopy, allows studying the dynamics of the development and reproduction of microorganisms, the influence of different factors on them, and many other issues [1, 2].

Rules of working with a light microscope:

1. In order to establish the best illumination of the field of view of the microscope:

- put the x8 objective 1-1.5 cm higher than the level of the microscope stage,

- raise the condenser to the level of the stage (open the diaphragm),

2. Install the preparation, strengthening the terminals.

3. Put a drop of immersion oil on the center of the preparation.

4. Replace the x8 objective on the x100.

5. Immerse the x100 objective in oil using a macroscrew.

6. Observe through the eyepiece and install an image with the help of a macroscrew.

7. Use a microscrewand rotat it half a turn in one direction or another to establish a clear image.

After finishing work, bring the microscope in order to storage:

1. Raise the microscope tube with the help of a macroscrew.

2. Remove the preparation.

3. Use a napkin to remove oil from the x100 objective and install the x4 objective.

4. Lower the condenser.

5. Place a tissue under the objective and lower the microscope tube.

Types of microscopy and their purpose

Various types of microscopy are used in microbiological studies,: light, luminescent, dark-field, phase-contrast, electronic.

The most common method is light (optical) microscopy. During microscopy, the morphology of microorganisms: their tinctorial properties (relation to dyes), as well as structural features (spores, capsules), motility, etc. is studied.

Microscopy and a phase contrast device. By using a phase-contrast device, the differences in the phase of light rays when they pass through transparent objects are converted into amplitude objects, as a result the objects become contrasted. The phase contrast method allows seeing transparent objects more clearly (in contrast), but not increasing the resolution of the microscope. The main value of this method is that it makes possible to observe living objects without fixing and staining them.

The phase-contrast device is an attachment to the microscope and consists of special phase objectives that give different magnifications; an auxiliary microscope; a condenser and a set of annular diaphragms, each of them corresponds to a specific objective. All phase objectives have the letter “F” on the rim.

Microscopy in a dark field. Microscopy in a dark field is based on the illumination of an object by oblique rays of light (Fig. 4). The rays do not fall on the lens and remain invisible to the eye, so the field of view looks completely black. If the preparation contains microorganisms, then oblique rays are reflected to a certain extent from their surface. So they deviate from their original direction and fall on the objective. In this case, dazzlingly bright luminous objects are visible on an intensely black field. Such lighting is achieved by using a special dark-field condenser having a darkened middle part. Therefore, the central rays of light coming from the mirror are delayed, and only the lateral rays reflected from the mirror surfaces located inside the condenser get on the plane of the preparation.

While microscopy in a dark field, objects outside the scope of a conventional microscope are visible. However, observing objects in a dark field makes it possible

to distinguish only their contours and does not allow considering the internal structure.

Luminescent microscopy. The luminescent microscope consists of a strong source of ultraviolet light, light filters and a biological microscope. A blue-violet filter is installed between the light source and the microscope mirror. Rays of light with a short wave fall on thepreparation and excite a glow in it. A yellow filter is placed on the eyepiece of the microscope, which cuts off the blue-violet rays and transmits visible to the eye the long-wavelength rays. The immunofluorescence method using specific luminescent sera has a great importance in the luminescent microscopy.

Their preparation is based on the ability of some fluorochromes, for example, fluorescein isocyanate, to enter into chemical bonds with whey of proteins without violating their immunological specificity.

Specific antibodies that bind to microbial antigens in the direct immunofluorescence method form complexes that glow upon fluorescence microscopy ofpreparations. Firstly, the antigen in an indirect method is treated with the help of homologous non-fluorescent antibodies, to detect formed antigen-antibody complex, a fluorescent antispecies serum corresponding to the type of animal producer of homologous antibodies is used. Anti-species sera are obtained by immunizing animals using animal globulins of those species that serve as producers of antimicrobial antibodies.

Electron microscopy. Electron microscopy makes it possible to observe objects with dimensions, which are beyond the resolution of a light microscope (0.2 μm). An electron microscope is used to study viruses, the fine structure of various elements of microorganisms, macromolecular structures, and other submicroscopic objects.

Light rays in electron microscopes are replaced by an electron flux having a wavelength of about 0.005 nm at certain accelerations, it is almost 100,000 times shorter than the wavelength of visible light. The high resolution of the electron microscope, is practically about 0.1-0.2 nm, and allows obtaining a total useful increase up to 1,000,000 times (Fig. 4).

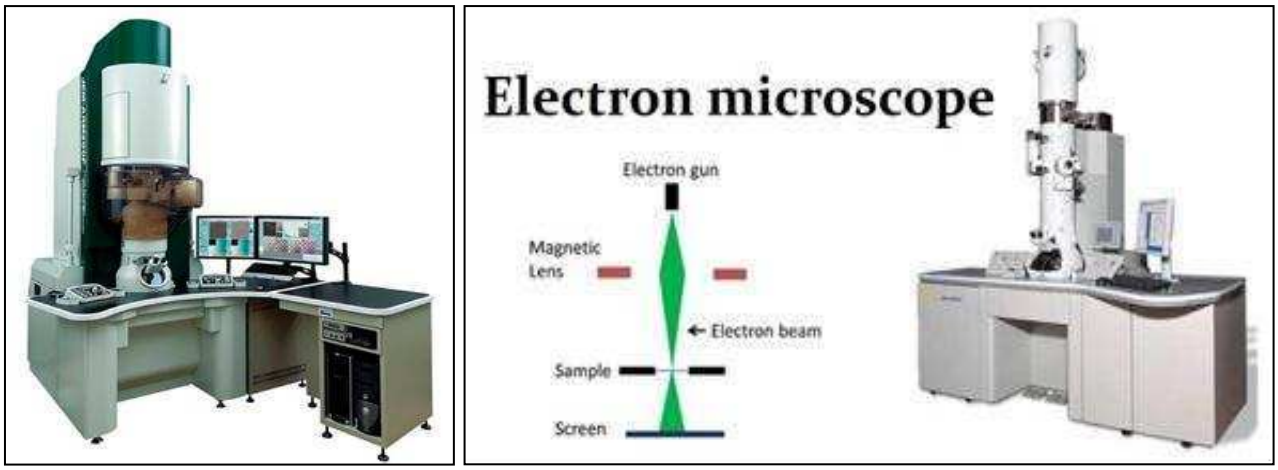


Figure 4 - Electron microscope

Side by side with devices of the "transmission" type, "scanning" electron microscopes are also used. They provide a relief image of the surface of the object. The resolution of these devices is much lower than the resolution of the electron microscopes of the "transmission" type.

Control questions:

1. To study the device of an optical microscope.
2. To learn the rules of work with a light microscope.
3. What relates to the mechanical part of the microscope.
4. The essence of luminescent microscopy.
5. Electron microscopy.

Questions for self-training and knowledge control:

1. The immersion oil objective differs from other objectives in:
   A. Black stripe.
   B. White stripe.
   C. Figure x100.
   D. Figure x40.
2. During microscopy of the preparation using x100 objective and a x10 eyepiece, a weak illumination of the visual field was revealed. Your actions to exclude this defect:
   A. Raise the condenser to the level of the stage.
   B. Check and open the condenser diaphragm.
   C. Apply immersion oil on the preparation.
3. When microscopy completed using an immersion system, it is necessary to do:
   A. Lift the tube from the oil using a macroscrew and remove the preparation.
   B. Remove the preparation, raise the microscope tube using a macroscrew.
   C. Lift the tube using a macroscrew, remove the preparation, remove the oil from the lens using a tissue, put the x8 objective, put a tissue under the objective, lower the tube and condenser.
4. It was found that staphylococci during microscopy have a diameter of 1.35 mm, in fact, this microorganism has a diameter of 1 μm. Determine which combination of objective and eyepiece was used for microscopy?
   A. Objective x40, eyepiece x15.
   B. Objective x90, eyepiece x7.
   C. Objective x90, eyepiece x10.
   D. Objective x90, eyepiece x15.
5. The microscope capacitor is designed for:
   A. Decreasing of a luminous flux.
   B. Focusing of light rays on the plane of the object under consideration.
   C. Magnification of the image of the object.
6. The microscope microscrew is designed for:
   A. Focusing when working with the x40 objective.
   B. Focusing when working with the x90 objective.

C. The movement of the preparation under microscopy when working with the x90 objective.

1.4 Brief systematics of microorganisms, basic concepts

The purpose of the lesson. To acquaint students with a brief modern classification of microorganisms.

Microorganisms are represented

precellular forms: viruses - the kingdom of *Vira*

and

cellular forms: bacteria, archaebacteria, fungi and protozoa.

The main groups of microorganisms are:

1. Bacteria
2. Mold fungi
3. Protozoa
4. Viruses.

According to modern classification, all living organisms are divided into 3 domains (suprasterness) on the basis of phylogenetic analysis of 16s-rRNA: «Bacteria», «Archaea» и «Eukarya»:

□ *domain «Bacteria»* — prokaryotes represented by the real bacteria (eubacteria);

□ *domain «Archaea»* — prokaryotes represented by archaebacteria;

□ *domain «Eukarya»* — eukaryotes, their cells have a nucleus with a nuclear membrane and nucleolus, the cytoplasm consists of highly organized organelles - mitochondria, Golgi apparatus, etc.

The domain of Eukarya includes:

- the kingdom of Fungi mushrooms;
- the kingdom of animals - Animalia (includes the simplest - the kingdom of Protozoa);
- The kingdom of plants - Plante.

According to the tutorial “Bacterial Identifier” by D. Bergey (2001), all bacteria are included in 2 domains:

1. "Bacteria"
2. "Archaea" (no pathogens).

There are 3 main phylums in the **Bacteria** domain:

• Gracilicutes (from lat. Gracilis-thin, cutes-skin) - bacteria with a thin cell wall, gram-negative.

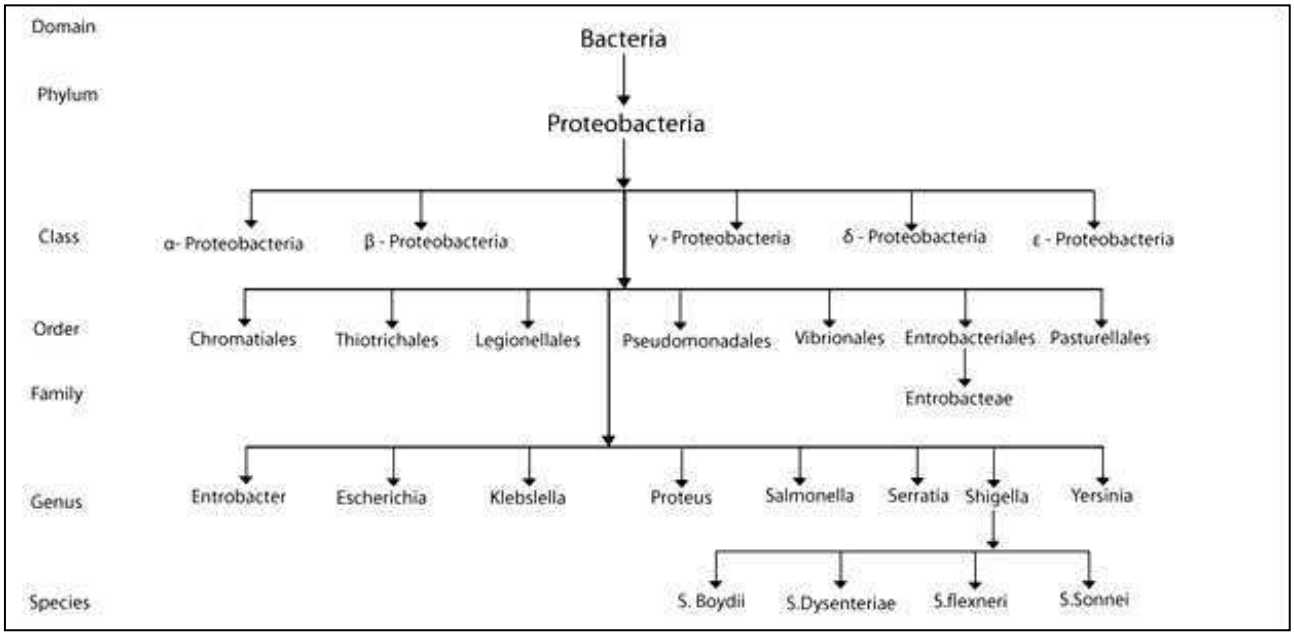
• Firmicutes (from lat. Firmus-durable) - bacteria with a thick cell wall, gram-positive.

• Tenericutes (from lat. Tener-soft, delicate) - bacteria without a cell wall (class Mollicutes - mycoplasmas)

The Archaea domain includes 1 phylum - Mendosicutes (mendosus-erroneous), the cell walls of these archaebacteria differ from the cell walls of prokaryotes. Archaebacteria do not contain peptidoglycan in the cell wall. They have special ribosomes and ribosomal RNAs (rRNAs).

The taxonomy of microorganisms is carried out according to the following scheme 1:

DOMAIN - KINGDOM – PHYLUM - SECTION - CLASS - ORDER - FAMILY – GENUS - SPECIES.



Scheme 1 - The taxonomy of microorganisms

The basis of the taxonomy of bacteria is the properties of microorganisms:

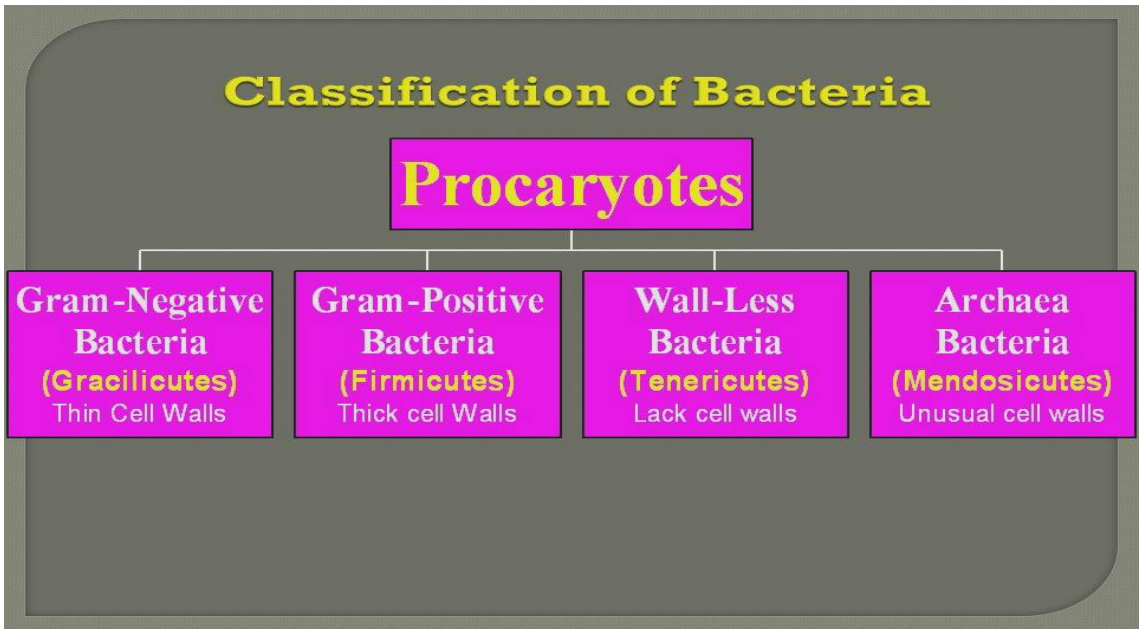
1. **Morphological** (what a microbe looks like under a microscope);
2. **Physiological** (how the microbe grows, on what it is grown, what are its properties in nutrient media, what substances it needs for growth);
3. **Biochemical** (which enzymes are produced by the microorganism, which substances (carbohydrates, proteins) are broken down);
4. **Molecular - biological** (the sequence of nucleotides in the genome, the genetic feature of individual microorganisms, this is the basis for modern methods of genetic diagnosis of microbes).

The main concepts used in the taxonomy of bacteria:

Species. One of the main taxonomic categories is the species. Species is a collection of individuals united by similar properties, but different from other representatives of the genus.

The binary nomenclature is recommended to name bacterial species. So the species name consists of two words. For example, the causative agent of syphilis is spelled as Treponema pallidum. The first word is the name of the **genus**

(Treponema) and is capitalized, the second word indicates the species and is written with a lowercase letter. When the species is mentioned again, the generic name is reduced to the initial letter, for example: *T.pallidum*.



Scheme 2 - Classification of bacteria

Pure culture. A set of homogeneous microorganisms isolated in a nutrient medium, characterized by similar morphological, tinctorial (relation to dyes), cultural, biochemical and antigenic properties, is called pure culture.

Strain. A pure culture of microorganisms isolated from a specific source and different from other representatives of the species is called a strain. A strain is a narrower concept than a species or subspecies.

Clone - is a collection of offspring grown from a single microbial cell.

To denote some sets of microorganisms that differ in certain properties, instead of the previously used type the suffix **var** (variety) is used.

Bacteria belong to prokaryotes, pre-nuclear organisms. They have a primitive nucleus without a shell, nucleolus, histones, and there are no highly organized organelles (mitochondria, Golgi apparatus, lysosomes, etc.) in the cytoplasm. Bacteria (prokaryotes) are significantly different from plant and animal cells (eukaryotes).

Bacteria (from the Greek. Βακτήριον - "stick") - small, invisible to the naked eye, unicellular prokaryotic living organisms [2, 3].

Differences of bacteria from other cells:

1. Bacteria belong to prokaryotes, they do not have a separate core.
2. The bacterial cell wall contains a special peptidoglycan - murein.
3. There is no Golgi apparatus, endoplasmic reticulum, mitochondria in the bacterial cell.
4. The role of mitochondria is performed by mesosomes - cytoplasmic membrane invagination.

5. There are many ribosomes in the bacterial cell.
6. Bacteria may have special organelles of movement - flagella.
7. The sizes of bacteria range from 0.3-0.5 to 5-10 microns.

Control questions:
1. Name the main groups of microorganisms.
2. To which domain do true bacteria belong?
3. What are the properties of microorganisms, which form the basis of the taxonomy of bacteria?
4. Give a definition of bacteria and name their features.
5. What is species?

1.5 Features of the bacterial cell structure

The purpose of the lesson. To acquaint students with the ultrastructure and important organelles of the bacterial cell.

The task:
1. Sketch the structure of the bacterial cell.
2. Know the permanent and additional components of the bacterial cell.
3. Know the functions of the organelles in the bacterial cell.

Topic content: Students must have an idea of the structure of the bacterial cell and be able to distinguish bacteria by morphological (form) characteristics in order to conduct the microscopic method of research and use the microscope correctly.

Ultrastructure of a bacterial cell

The ultrastructure of bacteria is studied by using electron microscopy, as well as by the method of microchemical studies.

All organelles of the bacteria are divided into 2 types: **basic /** permanent and **additional /** non-permanent.

The basic components are found in all bacteria: cell wall, cytoplasmic membrane, cytoplasm, nucleoid, ribosomes, mesosomes, plasmids and inclusions.

Additional components are inherent only to some bacteria: capsule, endospore, flagella and pilus.

The main components of a bacterial cell:

1. The cell wall of bacteria

The cell wall is an essential structural element of a bacterial cell. The exceptions are mycoplasmas and L-forms. The cell wall accounts from 5 to 50% of the dry matter of the cell. Functions - structural (determines the form of bacteria), protective, transport.

The main component of the cell wall of most bacteria is murein that is from the class of peptidoglycans. **Murein** is a heteropolymer built from chains of alternating acetylglucosamine and muramic acid.

The chemical composition and structure of the cell wall are constant for a

certain type of the bacteria and are important diagnostic feature that is used to identify bacteria.

L-forms of bacteria are bacteria that are partially or completely devoid of the cell wall, but retain the ability to develop. The letter L is the first letter of the name of the Lister Institute in London, where for the first time was paid attention to the development of unusual cells in a bacterial culture.

The following organelles are distinguished in the structure of a bacterial cell (Figure 5): capsule - 1, cell wall - 2, cytoplasmic membrane (CPM) - 3, cytoplasm - 4, nucleoid - 5, flagella - 6, ribosomes - 7, mesosomes - 8, plasmid - 9, endospore - 10, the periplasmic space, inclusions, villi (pilus).

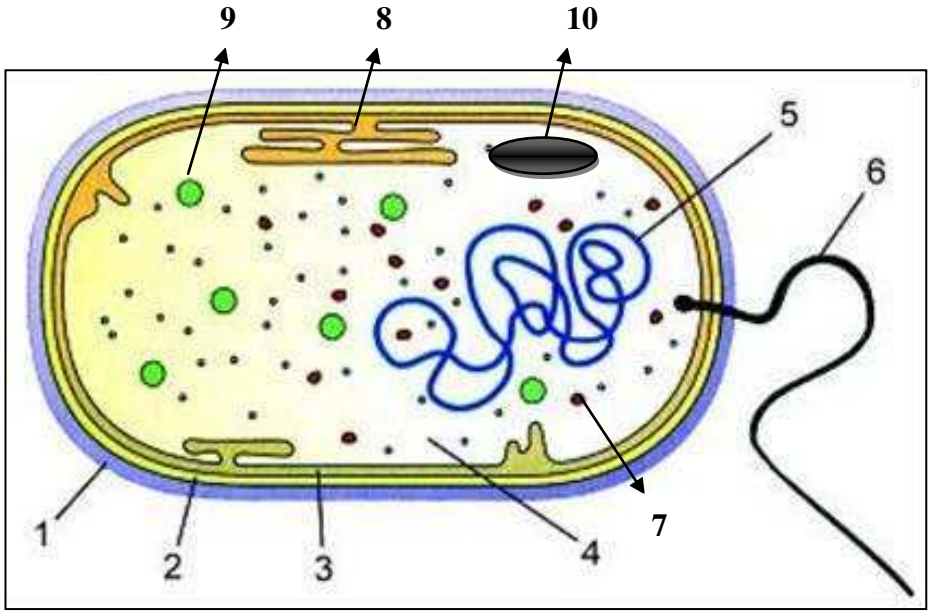


Figure 5 - Ultrastructure of a bacterial cell

2. Cytoplasmic membrane (CPM)

Cytoplasmic membrane - is a three-layer membrane (2.5 nm thick): a double protein layer and a phospholipid layer. Its chemical composition is represented by a protein-lipid complex, where proteins account for 50–75%, lipids - for 15–50%.

The CPM performs a number of **essential functions** in the cell:

• maintaining the internal constancy of the cell cytoplasm. A feature of the CPM is that it is semi-permeable. It is permeable for water and low molecular weight substances, but not permeable for macromolecules and other compounds.

• the function of substances transport into the cell and their removal out is related to the semipermeability of the CPM;

• the electron transport chain and oxidative phosphorylation enzymes are localized in the CPM;

• the cytoplasmic membrane is related to the synthesis of the cell wall and capsule;

• Flagella is fixed in the cytoplasmic membrane. The energy support of the flagella is related to the cytoplasmic membrane.

If there is excessive growth, the cytoplasmic membrane forms invaginates - invaginations in the form of complexly twisted membrane structures called

mesosomes. Mesosomes of bacteria are diverse in shape, size and localization in the cell. Three main types of mesosomes are distinguished: ***lamellar***, ***vesicular*** (having the form of bubbles) and ***tubular***.

Between the outer and cytoplasmic membranes there is the periplasmic space, or periplasm, containing enzymes (proteases, lipases, phosphatases, nucleases, beta-lactomas) and components of transport systems.

3. The cytoplasm

The cytoplasm is the colloidal content of a cell surrounded by a cytoplasmic membrane. The cytoplasm consists of soluble proteins, RNA, intracytoplasmic inclusions in the form of numerous small granules - glycogen, polysaccharides, beta-hydroxybutyric acid and polyphosphates (volutin). They are reserve substances for the nutrition and energy needs of bacteria.

4. Ribosomes

Ribosomes are intracellular organelles of a cell that look like granules in the cytoplasm. The function of ribosomes is synthesis of protein (biosynthesis).

Ribosomes are composed of protein and three types of RNA: informational (mRNA), transport (tRNA) and ribosomal (rRNA). Ribosomes are formed by two subunits - 30S and 50S. The smaller 30S subunit contains a 16S rRNA molecule and one protein molecule of 21 types. The 50S subunit consists of two types of rRNA molecules (23S and 5S) and about 35 molecules of various proteins. Bacterial ribosomes have a sedimentation constant of 70S.

Based on the study of 16S rRNA, a modern bacterial taxonomy has been compiled, it allows to assess the degree of microorganism affinity.

The bacterial cell contains from 5 to 50 thousand ribosomes, their number becomes more when the cell growth rate is also great.

5. The nucleoid

A nucleoid is the genetic material of prokaryotes. The bacterial cell contains one chromosome, represented by a double-stranded DNA molecule, closed in a ring and tightly laid in the form of a coil, which does not have its own nuclear membrane. It is localized in the cytoplasm. Functions are - storage and transmission of hereditary information, participation in cell division.

6. Plasmids

Plasmids are extrachromosomal genetic elements located in the cell cytoplasm. They are double-stranded supercoiled circular DNA molecules. Bacteria have F-type, Col-type, D-plasmid, R-type, and other plasmids. Currently, many types of plasmids have not been studied.

Plasmids give various signs to bacterial cells:

1) resistance to antibiotics, heavy metal ions, mutagens (R-plasmids);

2) the ability to cause biodegradation of camphor, xylene, naphthalene, salicylate, toluene, n-alkanes and other unnatural and natural compounds (xenobiotics). They called biodegradation plasmids or D plasmids;

3) the ability to synthesize antibiotics, bacteriocins, pigments, insecticides, hemolysins, toxins, fibrinolysins, hydrogen sulfide, surface antigens;

4) the ability to use various carbohydrates and unusual amino acids as a carbon source;

5) the ability to cause the formation of tumors in plants (Ti plasmids);
6) the ability to modify DNA, etc.

7. Inclusions

They are divided into actively functioning structures and products of cellular metabolism, which do not stand out, but are deposited inside the cell. The following species were found: aerosomes / gas vacuoles, magnetosomes (in water bacteria), chlorosomes (in cyanobacteria), polyhedral bodies, storage substances - polyphosphates (in the form of volutein grains), polysaccharides, fats (in the form of granules) and sulfur (in purple sulfur bacteria - in the form of balls) [4, 8, 9].

Additional components of the bacterial cell:

1. A microcapsule or capsule is the superficial mucous structure of a bacterial cell that forms around the cell wall. It is important for the life of the cell.

Capsule Functions:

- protective - protect the bacterial cell from adverse environmental factors (mechanical damage, drying, etc.) and the internal environment (phagocytosis process);
- create an additional osmotic barrier;
- virulence factor in some bacteria (for example, Streptococcus pneumoniae);
- serve as a barrier to bacteriophages, preventing their adsorption on bacterial cells;
- are a source of reserve nutrients;
- unite cells in chains, microcolonies;
- ensure the attachment of cells to the surface of the substrate.

Two layers are distinguished in the capsule structure: internal and external. The inner layer is a part of the outer layer of the cell cytoplasm, and the outer layer is the result of the secretory function of the bacterium. There are 2 types of capsules: Microcapsules. The mucus layer is less than 0.2 microns. It is detected only due to electron microscope. Macrocapsules having walls thicker than 0.2 microns. They can already be examined by a conventional microscope, if they are stained.

2. Bacterial endospores

Endospore is a special type of resting cells (in a state of suspended animation), mainly of gram-positive bacteria. Endospores form endogenously, inside a maternal cell called sporangium. The *bacterial endospore* differs from the *vegetative cell,* because it has resistance to heat, high temperatures, ultraviolet rays, antibiotics and other factors. The spores of some bacteria can withstand boiling for two hours, they can also remain for a long time (up to 50 years or more) in a resting state.

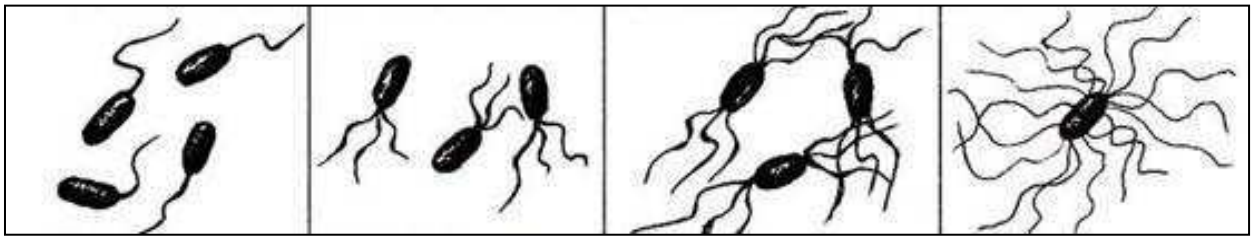
3. Flagella and bacteria movement

Flagella are the organ of movement in most bacteria. It is possible to consider flagella only in an electron microscope. Without special processing methods, individual flagella are not visible in a light microscope.

According to the location and number of flagella on the surface of the cell, there are ***4 groups of bacteria:***

1. **Monotrichous** have one flagellum (for example: Vibrio cholerae, Campylobacter spp);
2. **Lofotrichous** have a bundle of flagella at one or both poles of the cell (for

example, bacteria of the genus Pseudomonas, Chromatium);
3. **Amphitrichous flagella** - at both poles of the cell (for example, bacteria of the genus Spirillum);
4. **Peritrichous** have a large number of flagella located over the entire surface of the cell (for example, bacteria of the E.coli species and the Erwinia genus) (Fig. 6).



1 – monotrichi *2 – lofotrichi* *3 – amphitrichi* *4 -peritrichi*

Figure 6 - Location of flagella on the surface of a bacterial cell

Flagella are spirally twisted filaments consisting of a specific flagellin protein. Flagellum diameter is about 12–20 nm, length is 10–80 μm; there are also long strands of flagellin protein in thestructure.

4. Pili (fimbriae, villi) bacteria

Pili - threadlike formations, thinner and shorter (width 3-10 nm x long 0.3-10 microns) than flagella. The pili depart from the surface of the cell and consist of a pilin protein with antigenic activity.

Pili are divided: pili responsible for adhesion, it means pili responsible for the attachment of bacteria to the affected cell; pili responsible for nutrition, water-salt metabolism and sexual (F-pili) or conjugated pili. Pili are numerous - a few hundred pili for a cell. However, genital pili are usually 1-3 for a cell. A distinctive feature of genital pili is its interaction with special "male" spherical bacteriophages, which are intensively adsorbed on genital pili [1, 2, 4, 8].

Control questions:
1. Name the main components (organelles) of bacterial cells.
2. The function of the bacterial cell wall.
3. Types of plasmids and the main properties they give to a bacterial cell.
4. List the main types of bacteria according to the presence of flagella.
5. Name the difference between endospores and capsules in bacteria, their functions.

1.6 Bacterial morphology

The purpose of the lesson. To study the morphological features of bacteria, to distinguish bacteria according to the shape.

Goals:
1 Examine the shape and location of bacteria.
2 Draw a microscopic picture of the preparations.
3 Examine stained preparations under an immersion microscope system.

Bacteria according to the forms of cells are divided into **the main 3 groups**: spherical (cocci), rod-shaped and convoluted bacteria (Figure 7).

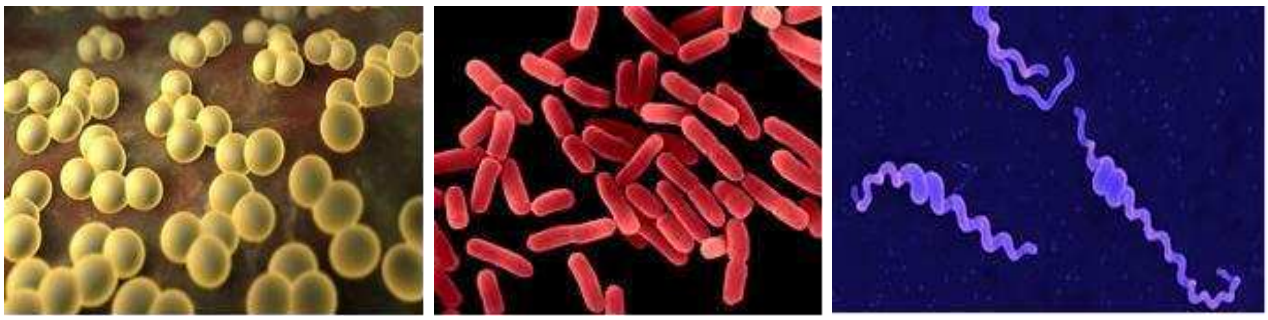


Figure 7 - The main forms of bacteria

Cocci (from the Greek. *kokkos* - grapes, lat. *coccus* - berry) – are bacteria of a spherical form. Among the spherical forms, there are ***6 types*** depending on the location of the cells after their division:
- *staphylococci* (randomly located cells, sometimes form clusters in the form of a "bunch of grapes");
- *diplococci* (located in pairs);
- *tetracocci* (as a result of cell division in two mutually perpendicular directions - four cocci are saved together);
- *streptococci* (cells located in the form of chains of various lengths);
- *sarcinomas* (accumulations of cocci after their division in the form of "bales", "packets" of 8 or 32 cells) (Figure 8).

Spherical bacteria:
a) Streptococcus;
b) Diplococcus;
c) Tetracoccus;
d) Sarcinia;
e) Staphylococcus;

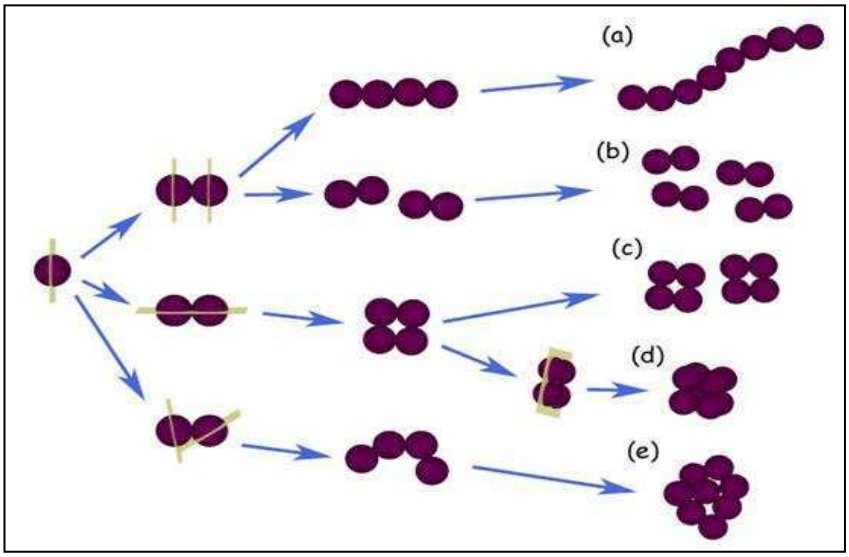


Figure 8 - Location of spherical bacteria after cell division

Rod-shaped bacteria - have the shape of a cylinder, with rounded or chopped ends. Rod-shaped bacteria are divided into ***two groups***: ***the actual bacteria and bacilli.***

Bacilli are rod-shaped bacteria with endospores in their structure. For example,

bacilli of anthrax, tetanus, hay bacillus, etc.

This is a stable form of the existence of bacteria in the environment; it forms only in certain living conditions (drying, high temperature, nutrient deficiency, etc.). For example, bacteria of the genus Bacillus have spores not exceeding the diameter of the cell.

Bacilli, forming a spore with the diameter larger than the bacillary (vegetative) cell, because of their spindle shape are called **clostridia**. For example, bacteria of the genus Clostridium (lat. Clostridium - spindle).

Spores can be located in the center of the microbial cell (centrally - 1.4), closer to the end of the cell (subterminally - 6) or at the end of the microbial cell (terminally - 2.3.5) (Fig. 9).

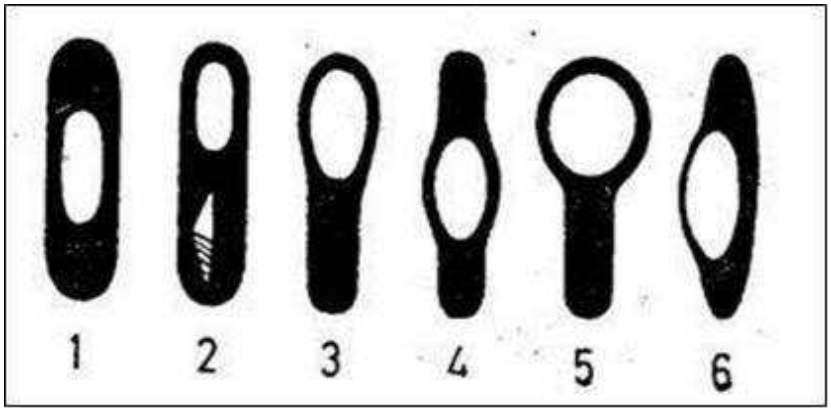
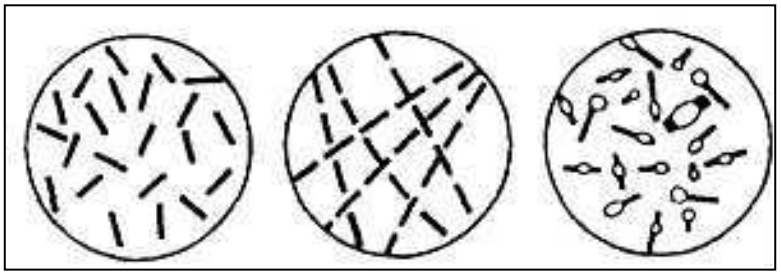


Figure 9 - Location of endospores in bacilli and clostridia

Fusobacteria and corynebacteria are also found among rod-shaped bacteria.

Actual bacteria are nonspore-forming rod-shaped bacteria.

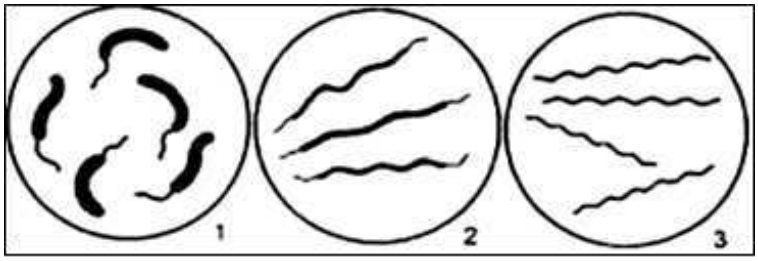
Rod-shaped bacteria of various species are located singly, randomly or in the form of chains, threads (streptobacteria, streptobacilli). Among rod-shaped bacteria, there are bacteria that acquire a branched form in old cultures, for example, mycobacteria (causative agents of tuberculosis, paratuberculosis). Actinomycetes are also referred to unicellular branching bacteria. Typically, these microorganisms have a dipteroid shape (with a thickening at the ends) or a V-, Y-, T-shape.



Rod-shaped bacteria:
a) Actual bacteria;
b) Streptobacteria;
c) Clostridium;

Spiral bacteria – are bacteria with one or more curls. Spiral bacteria are heterogeneous. There are **3 types** of spiral bacteria: *vibrio bacteria*, the body of which is one incomplete curl, in the form of a comma (cholera vibrio); *spirilla*

bacteria are microorganisms with several large curls (3-5 in a spiral). The most specific representative of this group is the causative agent of Sodoku disease (common in Southeast Asia); *spirochetes bacteria*, their body is represented by many spiral-shaped curls along the axial thread. The genus Leptospira belongs to the group of spiral microorganisms.



Spiral bacteria:
а) vibrio (1);
б) spirilla (2);
в) spirochetes (3).

The shape of cells (morphology) is a constant property for a certain type of bacteria. At the same time, some bacteria are characterized by ***pleomorphism*** – the ability to change morphological properties [1, 2, 3].

Control questions:
1. What groups of spherical bacteria are distinguished by their location?
2. What is the basis for the division of bacteria into the bacilli and clostridia?
3. What morphological groups are there among spiral forms?
4. Match the names of the main forms of bacteria to the pictures (Fig. 10):

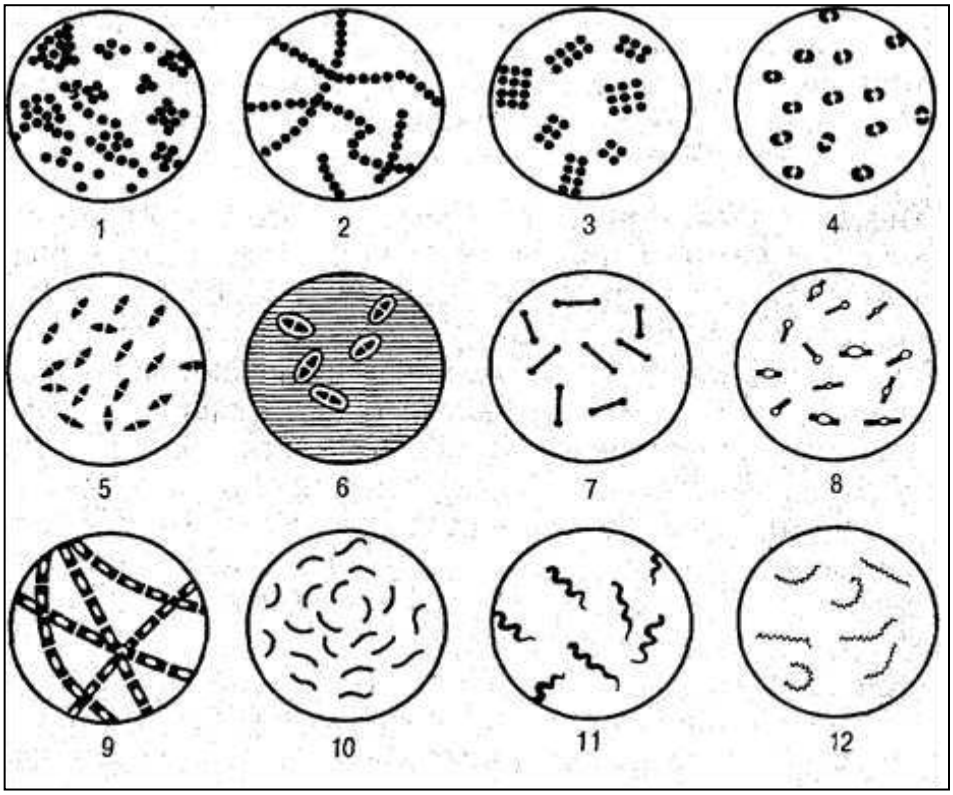


Figure 10 - The main forms of bacteria

1.7 The chemical composition of the bacterial cell

The purpose of the lesson. To familiarize students with the features of the bacterial cell's chemical composition.

Goals:

1 Study the general characteristics of microorganisms.

2 Know the basic chemical composition of the bacterial cell.

3 To assimilate the functions of the microbial cell's organic substances.

General characteristics of microorganisms

A specific feature of microorganisms is the very small size of an individual. The diameter of most bacteria is not more than 0.001 mm. The unit of measurement of bacteria is a ***micrometer or micron (μm),*** 1 μm = 10^{-3} mm. The bacterial cell ultrastructures are measured in nanometers (nm), 1 nm = 10^{-3} μm = 10^{-6} mm, and the smallest unit of measurement is ***angstrom (A):***

1 mm = 1000 μm = 1 000 000 nm = 10 000 000 A (Fig. 11).

For example, the diameter of the mold hyphae reaches 100 microns; bacteria - 5 microns; spirochete - 0.14; mycoplasmas - 0.1; rickettsia - 0.05 microns.

The total sizes of bacterial cells are measured from 0,2 μm to 10 μm. Due to its small size, microorganisms are easily carried with the flow of air, water and quickly spread in the environment.

One of the important properties of microorganisms is the ability to rapidly reproduce. Some types of bacteria are able to divide every 8-10 minutes. For example, from one bacterial cell weighing 2.5 • 10-12 g, biomass up to 10 tons can form in 2-4 days under favorable conditions.

Another feature of bacteria is the diversity of their physiological and biochemical properties. Some bacteria remain viable under extreme conditions (minus 196^{0}C - liquid nitrogen temperature and above + 80^{0}C). There are types of microorganisms that are resistant to increased hydrostatic pressure (in the thickness of the ocean and seas), ultraviolet rays, etc.

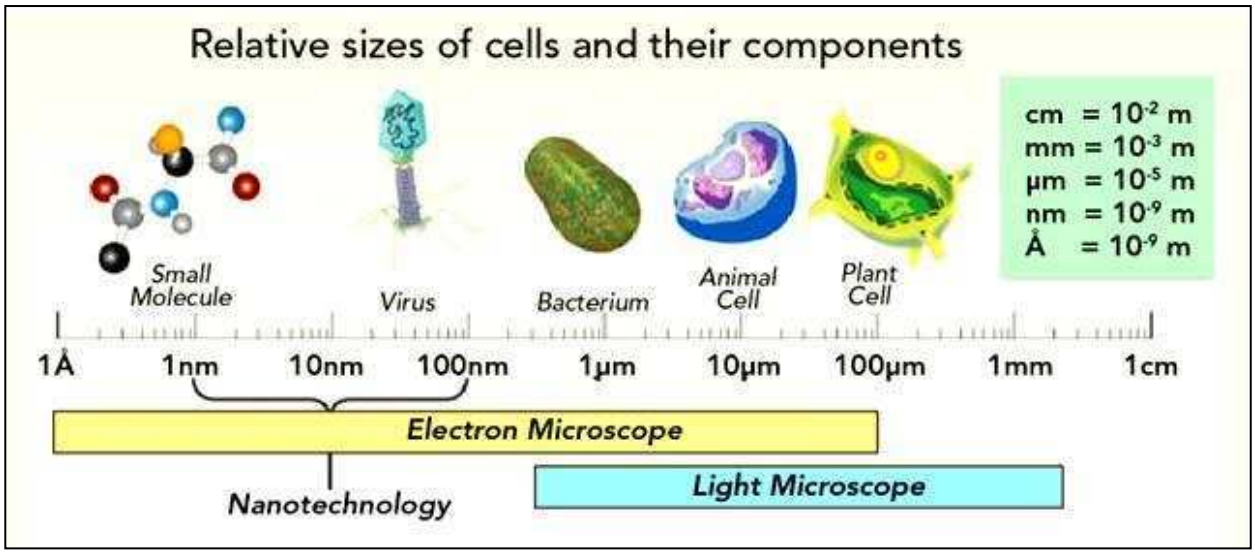


Figure 11 - Comparative characteristic of units

The chemical composition of a bacterial cell

The chemical composition of a prokaryotic cell is similar in composition to a eukaryotic cell. A bacterial cell consists of water and solids.

Water (H_2O) - accounts for 75-85% of the total mass of the bacterial cell. The younger the cell, the more water it contains. Bacterial spores contain significantly less water than vegetative cells. Water serves as a medium for numerous diverse biochemical processes, and also takes part in such reactions as hydrolysis and oxidation. Water performs transport functions: it provides the flow of nutrients into the cell and the withdrawal of waste products from it.

Water is in a *free* and *bound* state in the cell. *Free water* serves as a dispersed medium for colloids, a solvent for crystalline substances, and a source of hydrogen and hydroxyl ions. Part of the water in the cells is *related to colloids* and enters into the composition of intracellular organelles. Bound water is a structural solvent.

Removing water from the cell and drying stop the metabolic processes. Microorganisms do not multiply, if there is a lack of water. Drying in a vacuum from a frozen state (lyophilization) stops reproduction and contributes to the long-term preservation of microbial individuals.

Dry matter (15-25%) - consists of organic substances and mineral elements. Important chemical organogenes are carbon, nitrogen, oxygen, hydrogen. They are used to build proteins, carbohydrates and lipids. Organogenes as a percentage of dry matter account for: C - 45-55%; N_2 - 8-15%, O_2 - 31%, H - 6-8%.

Organic substances (90-92% of the total mass of solids) are proteins, nucleic acids, carbohydrates and lipids.

Mineral elements (5-15% of the total mass of dry substances) are macro- (S, P, K, Mg, Ca, Fe) and trace elements (Cu, Mn, Zn, Mo).

Organic matter:

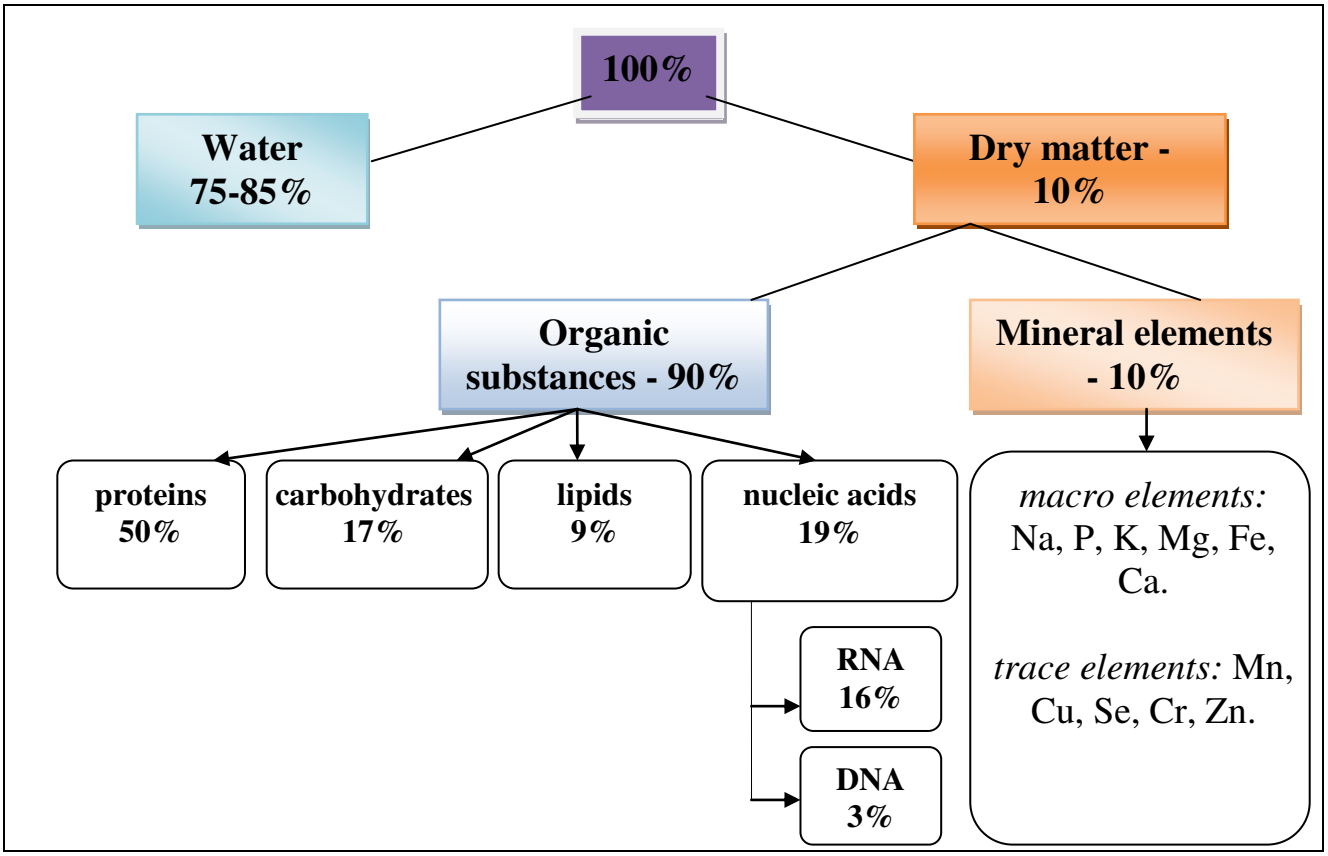
Proteins are the vital element of a microbial cell. Proteins make up 40-80% of dry matter of bacteria; 15-40% of microscopic fungi, 60% of yeast.

The cells of microorganisms include simple (*proteins*) and complex (*proteids*) proteins. Proteins perform 2 main functions: firstly, they are part of all membranes; secondly, they are biochemical catalysts - *enzymes*. Their role is giving the antigenic structure to bacteria, performing transport, protective, nutritional, construction, and motor functions. Hazardous substances - *toxins* - are also found among proteins.

There are 2 types (10-30%) of ***nucleic acids*** in the composition of the cell: DNA and RNA. RNA is found in the nucleus and cytoplasm of bacteria, and DNA is part of the nucleoid. *Functions:* DNA performs a hereditary function; RNA (there are 3 types: mRNA-*informational* or matrix, tRNA-*transport* and rRNA-*ribosomal*) - participate in synthesis of protein (biosynthesis).

Carbohydrates (make up 12-18% of dry matter) - are represented by *simple* substances (mono- and disaccharides - glucose, fructose, ribose, ramnose, sucrose, galactose, etc.) and *complex* compounds (polysaccharides - inulin, starch, glycogen , dextrin, cellulose, etc.). As well as polyhydric spirits, sorbitol, mannitol, dulcite. Polysaccharides are often found in capsules. Carbohydrates play an energetic role in the cell, and are reserve nutrients.

The chemical composition of the bacterial cell is presented in the following diagram:



Scheme 3 - The chemical composition of the bacterial cell

Lipids (fats, lipoids) - make up 3-8% of dry matter. They are part of the cell wall, cytoplasmic membrane and its derivatives in the cell. Bacterial lipids are represented by phospholipids, fatty acids and glycerides. Lipids are found in a free state (reserve nutrients) and in a bound state (as a part of proteins and carbohydrates). Rickettsia, yeast, mycobacteria contain up to 40% lipids.

The amount of protein, carbohydrate and lipid in a bacterial cell depends on the type of bacteria and nutrition.

Minerals (5-15% of dry matter). They meet in the form of macro- and microelements in the structure of cells. *Microelements* are iron, copper, manganese, molybdenum, cobalt, sulfur, zinc. *Macroelements* are phosphorus, sodium, potassium, magnesium and others. Phosphorus plays an important role among the ash elements. It is part of nucleic acids, phospholipids, many enzymes, and ATP (adenosine triphosphoric acid), which is an accumulator of energy in the cell, and is involved in energy metabolism reactions. Sodium is involved in maintaining the osmotic pressure in the cell. Iron is found in respiratory enzymes. Magnesium is a part of magnesium ribonucleate, which is localized in the wall of gram-positive bacteria. Thus, minerals play an important role in regulating the osmotic pressure and maintaining the colloidal state of the cytoplasm, they are part of the structural components of the microbial cell.

Moreover, acids and their salts, spirits, pigments and vitamins are found in the

composition of the microbial cell.

Pigments (dyes) are specific photoreceptor molecules, secondary metabolites that are formed on the light and give bacteria a color. The ability of pigmentation is expressed in the genus Sarcina, Micrococcus, Staphylococcus, Corynebacterium, Mycobacterium, Nocardia, etc.

The value of pigments:

- protection against the action of visible light and UV rays;
- neutralize toxic oxygen radicals;
- participate in the synthesis of vitamins;
- have antibiotic properties;
- The color of the pigment is used in the identification of bacteria [4, 8].

Table 1 - Classification of pigments by chemical composition and color

№	Chemical composition	Colour	Pigment-forming microorganisms
1	Quinone	Yellow	Mycobacteria
2	Azachinone (Indigoidin)	Blue	Corineacteria, pseudomon, arthrobacteria
3	Carotenoids	Red, orange, yellow, white	Sarcinomas, actinomycetes, staphylococci, micrococci, corynebacteria, yeast
4	Melanin	Black, brown	Bacteroids, porphyromones
5	Pyrrole (prodigiosin)	Bright red	Serrations
6	Phenosin (pyocyanin)	Blue-green (alkaline medium) or red (acid medium)	Pseudomonas aeruginosa
7	Pyrazine (pulcherrimin)	Dark red	Candida

Control questions:

1. Characterize the features of microorganisms.
2. What is the basic chemical composition of a bacterial cell?
3. The role of organic substances in the microbial cell, types of minerals
4. The dimensions of the bacteria are measured in micrometers (microns), one micron is:

A. 1/10 mm.
B. 1/100 mm.
C. 1/1000 mm.

5. Structural formations of a bacterial cell are measured in nanometers (nm), one nm is:

A. 1/10 microns.
B. 1/100 microns.
C. 1/1000 microns.

1.8 Aniline dyes, their use in laboratory practice. The technique of preparing a bacteriological preparation

The purpose of the lesson. To familiarize students with the main types of aniline dyes used in laboratory practice. Master the technique of arrangement preparations for microscopy.

Goals:

1 To know the types of aniline craters used in bacteriology.

2 To master the technique of making smear preparations and prints.

3 To make preparations with a suspension of broth and agar culture.

Equipment. Petri dishes with bacterial cultures. A set of paint solutions - diluted fuchsin, alkaline methylene blue; slides, spirit lamps, bacteriological loops, microscopes.

Aniline dyes - are artificial dyes. They are organic compounds formed during the oxidation of aniline or its salts. The main substance is initially obtained from natural indigo and subsequently undergoes chemical synthesis. Due to the substance, it becomes possible to make bright colors of paints. Dyes are widely used in laboratory practice, many of them have a bactericidal and some carcinogenic effects.

The most widely spread is aniline dyes of a basic and neutral nature. Acidic paints are not suitable for staining bacteria (because of the chemical composition of the microbial cell).

There is a limited set of dyes in clinical laboratory diagnostics used to stain smears:

1) red (different shades) –basic fuchsin, neutral-neutral red, safranin;

2) blue - Leffler's blue, methylene blue;

3) violet - gentian violet, crystal violet, methyl violet;

4) green - diamond green, malachite green.

All dyes are delivered to the laboratory in dry powder or crystalline form.Such dyes as basic fuchsin, gentian violet, methylene blue are used to prepare *saturated spirit solutions*, but they are not suitable for direct coloring. Then, due to adding distilled water *working spirit-water solutions of dyes* are prepared from saturated spiritic solutions of dyes, they are less stable than saturated (purely spiritic) ones, they can be used for a long time. In some cases, *aqueous solutions of dyes* (their dry paints) are prepared, although they are not stable and are used only in freshly prepared form.

To enhance the action of spirit-water solutions, mordant (physical and chemical) is used.

Physical mordant includes heating. To do this, a hot dye solution is used or a paint solution is poured directly on the preparation and heated over the flame of the burner until vapor appears.

In chemical mordant, chemicals (phenol, KOH, H2SO4) are added to the working solutions of the dyes, or the smear preparation is first treated with a solution of hydrochloric, sulfuric or chromic acid before staining, then the mordant is washed off with water and stained.

Among the spirit-water solutions of dyes, carbolic fuchsin (Tsil fuchsin, Pfeiffer fuchsin), gentian violet carbene, methylene blue (Pfeyffer alkaline solution) and aqueous solutions of malachite (or diamond) green are widely used.

Technique of making preparation for microscopy

To study the properties of bacteria under a microscope, they are examined in a *living* and *non-living* state.

To study *the morphology* of bacteria (form and structural elements), and *tinctorial features* (the ability to perceive a certain type of dye) in laboratory practice, staining of bacteria in a **non-living state** is used.

The study of bacteria in a **living state** has found practical application in determining *the mobility of microorganisms.*

Unpainted bacteria under transmitted light in a microscope almost merge with the general background and become invisible. Bacteria stain better in a non-living state. Various staining methods that require dyes of different colors are used for microcopying.

Microscopy preparations are made on glass slides. For work, it is necessary to have specially prepared slides and coverslips, which should be clean and degreased. A drop of water, applied to well-degreased glass, spreads evenly. Water breaks up into small drops on insufficiently defatted glass,.

Depending on the nature of the test material, a bacteriological loop or a Pasteur pipette is used when taking the material. The bacteriological loop is made of platinum or nichrome wire 5-6 cm long and fixed in a loop holder (Fig. 12). A loop is made at the end of the wire. The loop is held like a pencil. The working part of the loop is sterilized in the flame of the burner in an upright position, first the end of the loop, then the loop holder.

There are **2 types** of bacteriological preparation: *imprint preparation* and *smear preparation*. The imprint preparation is made mainly from biomaterial sent to the laboratory for research (pieces of parenchymal organs - liver, lungs, heart, etc.). The smear preparation is made from liquid materials (blood, pus, exudate, milk, etc.), and from ready-made bacterial cultures.

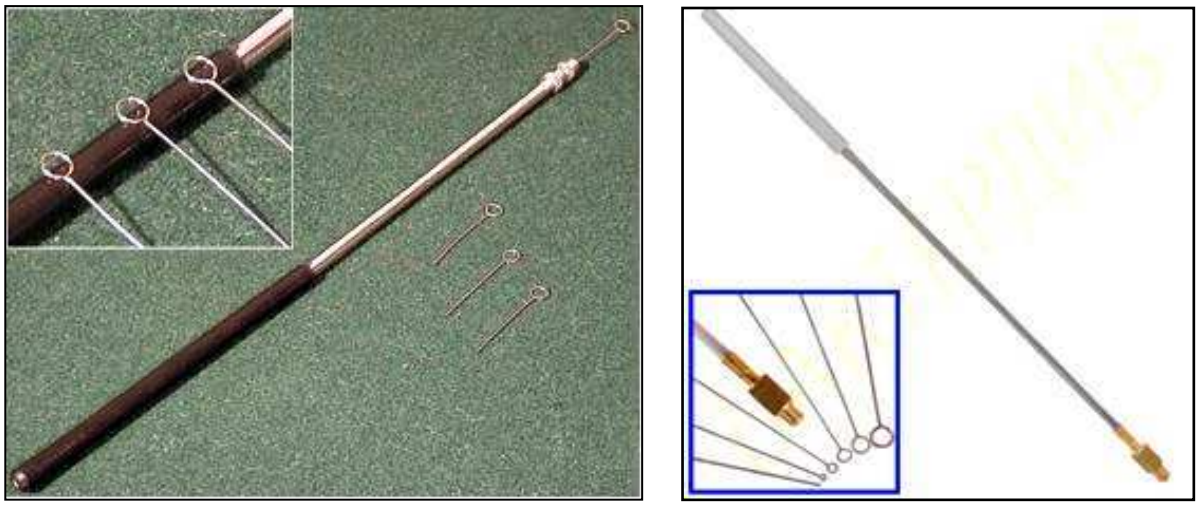
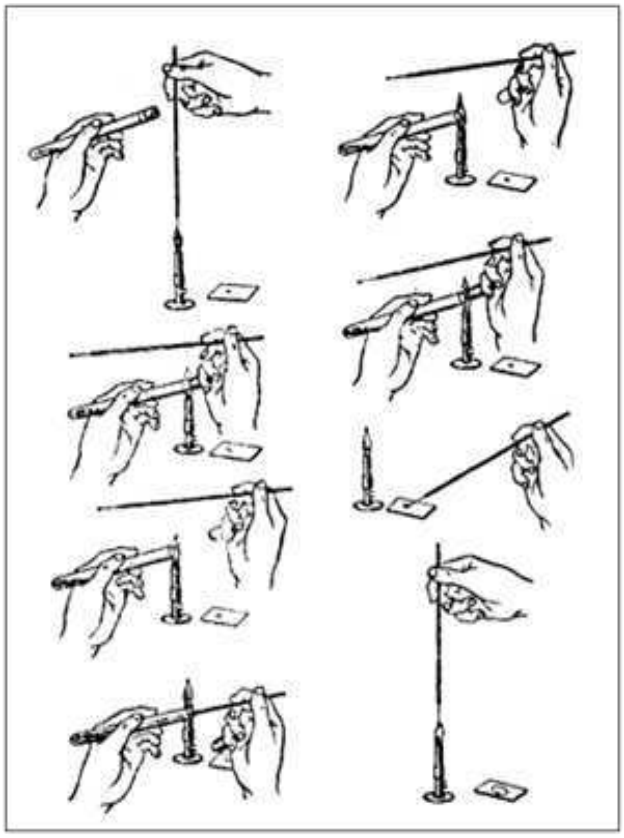


Figure 12 - Bacteriological loop and loop holder

Making a preparation for staining consists of several stages (Fig. 13): 1) preparation; 2) drying; 3) fixation; 4) coloring.



1. Smear preparation

From a culture grown in a liquid nutrient medium (from a test tube), a drop of material is taken with a sterile loop and distributed evenly on a dry glass slide.

From cultures grown in a dense medium (in a Petri dish), a small amount of material is taken from the surface with a loop, then mixed in a drop of distilled water or physiological saline that was previously applied on a glass slide. The resulting suspension (suspension) is smeared evenly over an area of 1-2 cm with a loop, possibly a thinner layer, so that the smear dries faster after its preparation.

Figure 13– Making the smear preparation

Imprints are made of dense pathological material (liver, kidneys, spleen, etc.). For this, a piece of an organ is cut with sterile scissors and applied on a glass slide, making one or more prints.Then allowed to dry.

After making smears, the loop is necessarily burnt (*flambered*) over the upper part of the burner flame, first the end of the loop, then the loop holder.

The place of the smear is indicated with wax pencil on a slide on the reverse side of the preparation. Its diameter is about 2-3 cm.

2. Drying the smear

Smears are dried on the air at room temperature or in a thermostat.

3. Fixation of the smear

Smears are fixed after complete drying in order to:

1) fix the smear on the glass so that it does not wash off during further work;
2) kill the bacteria and make safe their further handling;
3) killed bacteria better perceive color.

Fixation of smears is carried out in one of *two ways*: physical and chemical. **The physical method** includes three – times - conducting the opposite side of the smear through the flame of an spirit burner, holding it in the flame for 1-2 seconds.

In the chemical method, a smear is immersed in a liquid:

a) ethanol 96 ° for 10-15 minutes, or

b) a mixture of equal volumes of spirit and ether for 10-15 minutes, or

c) acetone - 5 minutes.

4. Staining of the smear

Bacteria are differently responsive to dyes, these properties are called tinctorial.

Control questions:

1. List the main types of aniline dyes used in laboratory practice.
2. What is the use of mordant preparations, types of mordant?
3. Stages of making the smear preparation.
4. Methods of fixing the smear preparation.
5. Technique for making a bacteriological preparation.

Assignment for self-training and knowledge control 1.

1. Prokaryotes differ from eukaryotes in the following ways:

A. Have a core.
B. Have ribosomes.
C. Have mesosomes.
D. Have a nucleotide.

2. Match names of the organelles with their functional properties:

1. Mesosomes
2. Ribosomes
3. The nucleotide
4. Cytoplasmic membrane

A. Carries out protein synthesis.
B. Participates in the transmission of hereditary traits.
C. Performs the function of active transport of various substances into a bacterial cell.
D. Are centers of respiratory activity.

3. Set the correct sequence of steps when making microscopy preparations:

A. Making a smear, fixing, drying, painting.
B. Making a smear, drying, fixing, coloring.
C. Making a smear, fixing, drying.

4. Fixation of smear preparation is carried out with the aim of:

A. Attach a smear to a glass slide.
B. Kill the germs that are in the material.
B. Identify the internal structure of bacteria.
G. Fix flagella.

5. When staining bacteria, you need to know the colors of the paints. Determine which of the following colors is red:

A. Gentian violet.
B. Fuchsin.
V. Safranin.
G. Crystal violet.

6. For a simple method of staining preparations, the following dye solutions are used:

A. Methylene blue up to 5 minutes.
B. Lugol's solution up to 3 min.

B. Diluted fuchsin for 2-3 minutes.
G. Carbolic fuchsin up to 5 minutes.

7. Microscopy of preparations made from biomaterial or bacterial cultures stained with a simple method allows to establish:
A. The form of bacteria.
B. Flagella.
B. Absence or presence of bacteria.
G. Capsule.
D. Mobility.

1.9 Methods of staining bacteria. Gram method. The Ziehl-Neelsen method. Coloring spores and capsules.

The purpose of the lesson. To study the basic methods of staining bacteria and some structural elements of a bacterial cell.

Goals:

1 Master simple methods of stainingpreparations.

2 Stain the bacteria with the Gram method.

3 Know the technique of staining spores and capsules of bacteria.

Equipment: prepared bacterial cultures (staphylococci, E. coli); dye solutions - gentian violet, fuchsin, methylene blue; Lugol's solution, spirit, bacteriological loops, spirits, glass slides, microscopes.

Microbial staining is not a mechanical process for the penetration of paint into a microbial cell. The mechanism of staining microbes should be considered as a physicochemical process, which depends on the chemical composition of the cytoplasm of the microbial cell.

This method allows detecting microorganisms in the preparations, studying their ***morphological features, mobility and tinctorial properties.***

The preparations are stained on a specially equipped table covered with plastic or glass. On the table there should be a vessel with distilled water, a container for draining the dyes, a stand of two glass tubes connected on both sides by rubber tubes (Fig. 14), ink bottles with pipettes and rubber cans.

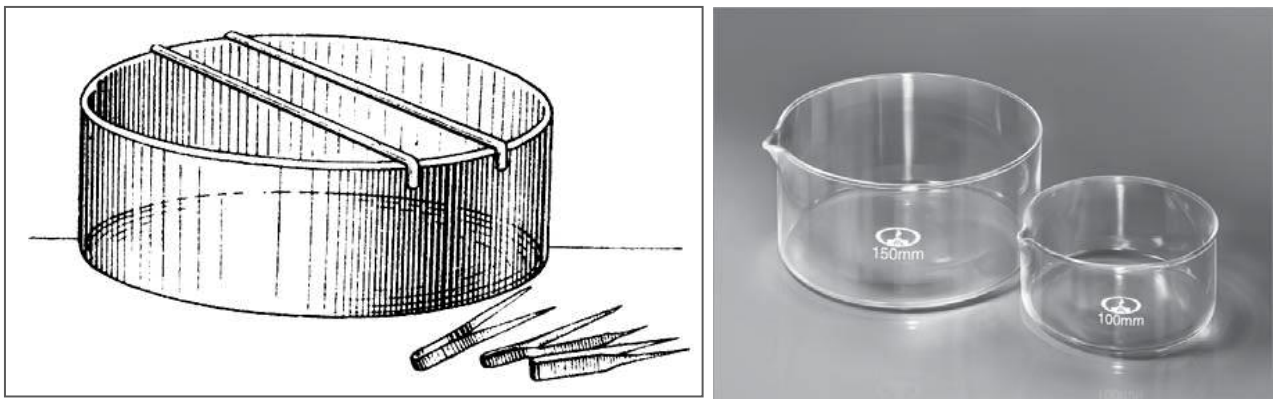


Figure 14 - Bowl with stand for coloring preparations

Methods for staining microorganisms

Methods for staining microbes are divided into *simple and complex* (or differentiated).

Only one dye is used in a ***simple coloring method***: mostly red - fuchsin or blue - methylene blue.

Smear is stained with diluted fuchsin for 1-2 minutes; with Leffler alkaline methylene blue - for 3-5 minutes.

A simple method of staining the preparations allows establishing the presence or absence of bacteria in the test material, studying their morphology (shape, location), as well as getting acquainted with the general morphology of fungi.

Course of work: a fixed preparation is placed as the smear on a stand. Dye is applied on the smear with a pipette so that it is completely covered with the solution. After the required time, the dye is drained, the preparation is washed off with distilled water and dried on the air. The water remained on the wall is carefully removed with filter paper. A drop of immersion oil is applied on a dry smear and the preparation is observed with an immersion microscope lens. The stained smear should be completely dry, because the remainder of the water and the cedar oil applied on the smear form an emulsion that interferes with microscopy.

Complex (differential) staining methods make it possible to detect bacteria in the test material, to study their morphological and tinctorial features. These methods allow identifying differences in the chemical composition of a bacterial cell (Gram stain), as well as establishing its structural features - the presence of spores, capsules, etc. All these signs are taken into account in laboratory practice when determining the type of bacteria.

Sophisticated staining methods consist of using several reagents and dye solutions.

One of the universal methods of a complex staining is Gram staining. It was proposed in 1884 by the Danish scientist H. Gram.

Gram staining

This is a universal differential diagnostic staining method. All bacteria in their reaction to coloring in this way are divided into two groups:

- *gram-positive (G +)*
- *gram-negative (G -)*

Gram stain is such an important identifying attribute of bacteria that it is necessarily mentioned in their characterization.

Example:

Gram-positive bacteria include: *Bacillus subtilis, Sarcina ventriculi, Streptococcus lactis, Staphylococcus aureus, Clostridium perfringens, Micrococcus luteus*, etc.

Gram-negative bacteria are: *Escherichia coli, Salmonella enterica, Proteus vulgaris, Yersinia pestis, Pseudomonas aeruginosa*, etc.

The essence of the method:

This method is based on the ability of bacteria to retain dyes (crystalliolet or gentian violet) in the cell wall, which in turn depends on the chemical composition

and ultrastructure of the bacterial cell wall. According to the structure of the bacterial cell wall, bacteria are divided into two large groups: gram-positive and gram-negative.

The cell wall of gram-positive bacteria - is a homogeneous dense layer with a thickness of 20-80 nm. The main component is a multilayer peptidoglycan - murein, which accounts for 50-90% of the cell wall mass. Such polymers as teichoic acids are also associated with murein. Additionally, the cell wall contains a small amount of polysaccharides, lipids, proteins.

The cell wall of gram-negative bacteria is multilayer, its thickness is 14-17 nm. The inner layer of the cell wall is represented by murein, which accounts for 1-10% of its dry mass. The structural microfibrils of the peptidoglycan are less compactly stitched, therefore, the pores in peptidoglycan layer are much wider than in the framework of gram-positive bacteria. One of the distinguishing features of gram-negative bacteria is the absence *of teichoic acids* in their cell wall. Moreover, the structure of the cell wall of gram-negative bacteria contains many proteins, phospholipids and lipopolysaccharides.

The staining technique contains (Fig. 15):

Gram's method

1) A small piece of filter paper impregnated with a solution of gentian-violet (a modification of A.V.Sinev) is applied on a fixed smear and moistened with distilled water. Coloring lasts for 2 minutes.
2) The filter paper with a gentian violet is removed and a few drops of Lugol's solution are applied on the smear for 2 minutes (the smear turns black).
3) Lugol's solution is drained and 96 ° spirit is acting for 30 seconds.
4) The smear is washed off with water.
5) Additionally, stained with diluted fuchsin for 1 minute.
6) Washed off with water and dried between filter papers.

A drop of immersion oil is applied on the dried smear and microscopied under an immersion lens (× 100, × 90) in a light microscope.

Microscopic picture:

Gram-positive bacteria turn ***dark purple.***
Gram-negative bacteria turn ***pink (can be red).***

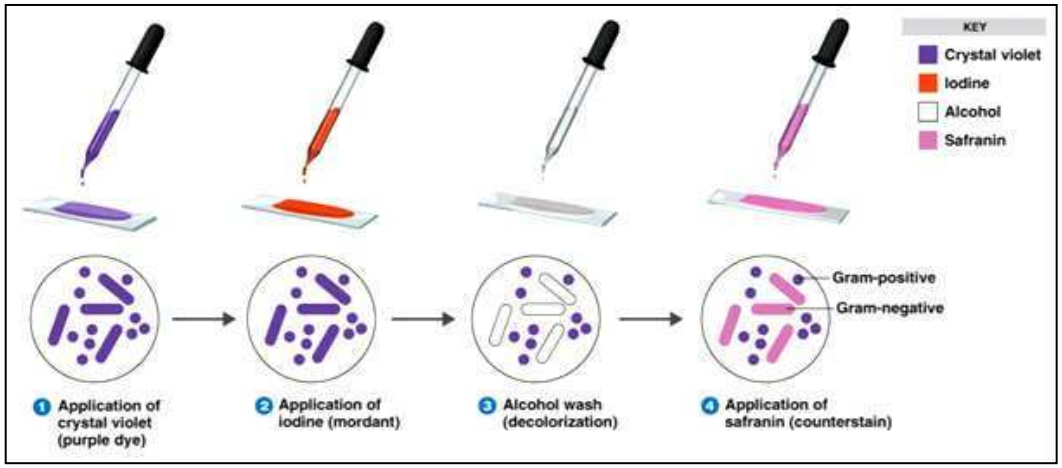


Figure 15 - Gram-staining techniques

Staining mechanism: gram-positive bacteria contain a significant amount of the magnesium salt of ribonucleoprotein (proteins connected to RNA in the cytoplasm) and peptidoglycan in the cell wall. Therefore, when the preparation is treated with a gentian violet and Lugol's solution, such bacteria form a strong combination of dye and iodine - an insoluble complex. When treated with spirit, this compound is retained and does not decolorize with spirit, the violet paint remains (microbes are painted in the color of a gentian violet - purple).

The other types of bacteria that do not contain the magnesium salt of RNA, do not form a strong connection with gentian violet, the cells are easily discolored with 96% spirit, and easily stain pink-red (gram-negative) when additionally stained with Fuchsin Pfeiffer's solution.

The spherical forms of microorganisms are mostly Gram-colored positively, spiral forms - negatively, and rod-shaped forms are found both gram-positive and gram-negative [4, 8].

The method of staining acid-resistant bacteria

To stain a group of acid-resistant bacteria a special staining method is used in laboratory practice - Ziehl-Neelsen's method.

The Ziehl-Neelsen method

This method is designed to differentiate acid-resistant bacteria (*Mycobacterium tuberculosis, M. bovis, M.avium, and leprosy pathogens*) from non-acid-resistant bacteria.

1) Ziehl's carbolic fuchsin (*the main dye*) is poured onto a fixed smear and heated over the burner flame for 3-5 minutes until fumes appear.
2) When the preparation has cooled, the dye is drained and washed off with water.
3) The preparation is bleached by immersing in a 5% solution of sulfuric acid (*differentiating substance*) for 1-2 minutes.
3) Rinsed thoroughly with water.
4) Cross-stained with methylene blue (*additional dye*) for 3-5 minutes.
5) Washed off with water, dried and microscopied.

Microscopic picture:

- *Acid-resistant bacteria turn red.*
- *Non-acid-resistant bacteria turn blue.*

Staining mechanism: the cell wall of acid-resistant bacteria is characterized by a high content of lipids (up to 40%), wax and hydroxy acids. Such microorganisms are poorly stained with diluted dye solutions. To facilitate the penetration of the dye into the bacterial cell, it is heated above the burner flame. The main dye is retained when the smear is discolored with acid.

Non-acid-resistant bacteria are easily stained, and then easily bleached with acid and stained with an additional dye.

Methods for painting capsules of bacteria

Some types of pathogenic bacteria in the infected body are able to form a mucous layer around themselve, which is called a capsule. The causative agents of anthrax, diplococcal septicemia can form capsule.

Capsule of bacteria is surrounded with a mucous layer. Mucus is a product of

the vital activity of the cytoplasm and is secreted out through the cell wall. If the mucous layer is thick and strong enough, has a certain shape and concentrates around a bacterial cell, it is called a capsule.

The physiological significance of the capsule in pathogenic microbes is defined as protection against the influence of factors of the body's immunity. Bacteria that surrounded by a capsule are weaker phagocytized and stay resistant to the action of antibodies. The capsule of pathogenic microbial species forms in the infected body. Detection of a capsule in a bacterium is a species trait. It has the particular importance in the bacteriological diagnosis of anthrax - according to the results of such study a preliminary answer is immediately given.

To study the presence of a capsule in bacteria, smears, fingerprints, are prepared from the test material (pieces of organs, blood of a fallen animal).

There are several methods for **staining bacterial capsules**: Mikhin's method, Burri-Gins, Romanovsky-Giemsa method, Olta's safranin staining method, etc.

• Mikhin's method for coloring capsules

1) Loffler methylene blue is poured onto a fixed smear (blood, smears-prints from tissues). (Loffler's methylene blue - 30 ml of a saturated spirit solution of methylene blue is dissolved in 100 ml of water, 1 ml of a 1% NaOH solution is added to it, and incubated for 1 month in an thermostat);
2) After the dye is drained the smear is heated over the flame of the burner for 5-7 minutes.
3) Washed off thoroughly with water, dried with filter paper and microscopied.

Microscopic picture:

- The capsule is pink.
- The bacterial cell is blue.

• Burri-Gins Method

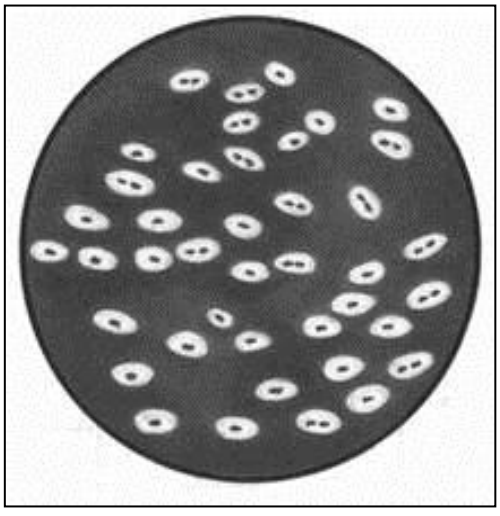


Figure 16 - Coloring of capsules by the Burri-Gins method

It is a negative method of staining: not the bacteria themselve, but the background is stained. To do this, mascara is used.

1) A drop of black mascara is applied on the middle of the slide and mixed with a drop of bacteria culture using a loop (*according to Burri*);
2) A blood smear is made with the edge of another glass slide. Dried on the air and fixed above the flame of the burner;
3) Stained for 5-10 minutes with carbolic fuchsin (*according to Gins*);
4) Gently washed off with water, dried, microscopied.

Microscopic picture:
Bacteria turn red; unpainted capsules stand out on the dark background of the preparation (Fig. 16).

- **Romanowsky-Giemsa method**

1) A fixed smear-imprint is placed with a smear down in a Petri dish, at the bottom of which stands (matches) are installed. Giemsa paint is poured under the preparation. The paint is pre-diluted with distilled water 1:10.
2) Stained with diluted Giemsa paint for 50-60 minutes.
3) Washed off with water, dried and microscopied.
Microscopic picture:

- ***The capsule*** is pale pink.
- ***The bacterial cell*** is blue.

Methods for staining bacterial endospores

Some types of microorganisms under adverse conditions of their existence form a *spore*. The process of spore formation is accompanied by a significant loss of free water by the bacterial cell, and compaction of the cytoplasm. The endospore forms inside the bacterial cell. The endospore membrane is impregnated with special resinous substances, which significantly reduce its permeability. Inside one microbial cell there is only one endospore, and only one bacterial (vegetative) cell develops from it. *Spore formation of bacteria is not a method of reproduction, but has a protective character - a form of preserving their appearance.* Endospores are resistant to external environmental factors (physical, chemical).

Due to the tightness of the shell, the spores do not perceive simple staining. However, the shell loses its stability when exposed to hydrochloric acid. Therefore the paint penetrates into the spores. To stain spores, special strong coloring solutions or preliminary chemical etching of spore shells are used.

Spore staining is used rare in laboratory practice. Usually there is only detection of non-stainable round or oval formations inside bacterial cells when stained by a simple or by Gram method.

There are several ***methods for staining the endospores*** of bacteria, the most common methods are : methods of Meller, Orzhesko, Peshkov, etc.

• Meller method

1) A fixed smear is etched with 5% chromic acid for 2-3 minutes (to improve the absorption of the dye), then washed off with water;
2) Dried with filter paper, stained with carbol fuchsin. In order to avoid paint residue, a sheet of filter paper is preliminarily applied on the smear, then paint is applied on it.
3) The preparation is heated from below until vapor appears, then stained for 7-8 minutes;
4) The paint with a piece of paper is poured without rinsing with water, treated with 5% sulfuric acid for 5-7 seconds, then washed off with water;
5) Additionally stained with methylene blue for 4-5 minutes, washed off again with

water, dried with the help of filter paper, microscopied.
Microscopic picture:
Endospores turn pink-red color,
Vegetative cells turn blue.

• method of Ozheshko

1) A non-fixed smear is immersed for etching for 2-3 minutes in a cup of 0.5% hydrochloric acid and heated on a burner flame.
2) After this, the smear is washed off with water, dried, fixed over a flame and dyed with Ziel's carbol fuchsin through a filter paper with heating for 3 minutes.
3) The cooled paper-free preparation is discolored with 5% sulfuric acid, washed off with water
4) It is stained with methylene blue for 3-5 minutes, then washed off with water and dried.

Microscopic picture:

- ***Endospores*** *become* a bright red color of fuchsin.
- ***Vegetative body*** of bacteria turns blue.

• Peshkov method

M.A. Peshkov suggested staining the spores with Leffler blue. Moreover, he used boiling to change the stability of the shell.
1. Leffler methylene blue is applied on a fixed smear and boiled for 15-20 seconds on a flame of an spirit burner.
2. The cooled preparation is washed off with water and stained with a 0.5% neutralroth aqueous solution for 30 seconds.
3. Washed off with distilled water and dried on the air.
Microscopic picture:
Endospores - painted in blue / blue.
The protoplasm of the vegetative body of bacteria becomes pink-red

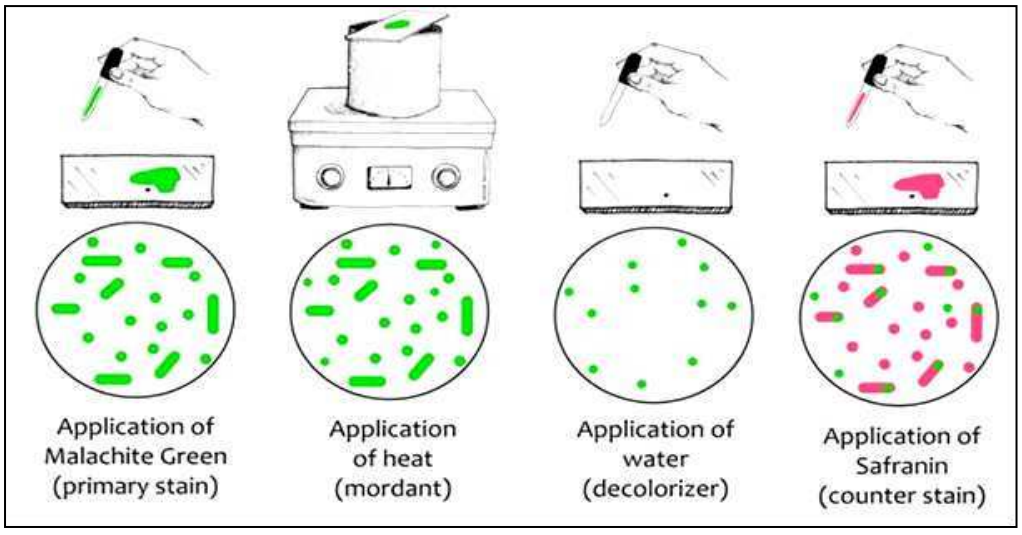


Figure 17 - Bacterial endospore staining

1.10 Questions for self-training and knowledge control 1:

1. Bacteria stain differently by the Gram method (G + and G–), because:
A) Have a capsule.
B) They differ in the shape of the cells.
C) They differ in the structure and chemical composition of the cell wall.
D) They have a different composition of the chemical cytoplasm.
2. What structural and chemical features does the cell wall of gram-positive bacteria have in contrast to gram-negative?
A) Contains phospholipids.
B) Contains lipoproteins.
C) Has peptidoglycan up to 90% and numerous convoluted tubules.
3. Select the correct sequence of paints and reagents needed to stain the preparation according to Gram method:
A) Gentian violet, discoloration with spirit, washing off with water, Lugol's solution, diluted fuchsin.
B) Diluted fuchsin, Lugol's solution, gentian violet, discoloration with spirit, washing off with water.
C) Gentian violet, Lugol's solution, discoloration with spirit, washing off with water, diluted fuchsin.
D) Decolorization with spirit, washing off with water, Lugol's solution, gentian violet, diluted fuchsin.
4. When the preparation made from a known culture of gram-positive bacteria was staining according to Gram method, one inaccuracy was allowed, as a result microscopy revealed bacteria stained in **red.** Find the probable cause of malformed bacteria:
A) Long-term coloring with a gentian violet.
B) Prolonged discoloration with spirit.
C) Short-term staining with diluted fuchsin.
D) Long-term treatment with Lugol's solution.
5. When the preparation made from a known culture of gram-positive bacteria was staining according to Gram method, one inaccuracy was allowed, as a result microscopy revealed bacteria stained in **purple.** Find the probable cause of malformed bacteria:
A) Long-term coloring with a gentian violet.
B) Prolonged discoloration with spirit.
C) Short-term staining with diluted fuchsin.
D) Long-term treatment with Lugol's solution.
6. While examining the material in Gram stain preparations, purple cocci were found. What is the reason of the difference in the color of microorganisms according to the Gram method?
A) The ratio of DNA to RNA
B) Features of nuclear substance
C) Bacterial morphology
D) Isoelectric point

E) The presence of magnesium salt of RNA

7. Bacteria are unicellular organisms capable of autonomous existence. What are the bacterial structures, which play a major role in protein synthesis?

A) Mesosomes
B) Ribosomes
C) Cytoplasm
D) Cytoplasmic membrane
E) Cytoplasmic inclusion

8. Microscopy of smears from the patient's sputum revealed diplococci of a blue-violet color. What method was used to stain the smears?

A) Burri Ginsa
B) Meller
C) Morozov
D) Gram
E) Ozheshko

9. The bacteriologist found large blue sticks with a terminal spore of red color in the preparation made from the content of the wound. What staining method did the bacteriologist use?

A) Mikhin
B) Ozheshko
C) Burri Ginsa
D) Gram
E) Ziel-Nielsen

10. Gram-positive spherical bacteria located in the form of chains are identified in a smear prepared from the patient's pus. What bacteria can be the causative agent of the disease?

A) Diplococci
B) Streptococcus
C) Staphylococci
D) Sarcin
E) Tetracocci.

Questions for self-training and knowledge control 2:

1. Why do bacteria have spores:

A) Reproduction.
B) Preservation of the species in adverse conditions.
C) Movement
D) Adhesion.

2. Choose the correct sequence of paints and reagents necessary for staining spores according to the Zlatogorov method:

A) Methylene blue, discoloration with a solution of sulfuric acid, washing off with water, carbolic fuchsin and heating.

B) Carbolic fuchsin and heating, washing off with water, discoloration with a solution of sulfuric acid, methylene blue.

C) Carbolic fuchsin and heating, decolorization with sulfuric acid solution, washing off with water, methylene blue.
D) Methylene blue and heating, discoloration with a solution of sulfuric acid, washing off with water, carbolic fuchsin.
3. Microscopy of a preparation made from a spore culture of hay bacillus and stained according to the Zlatogorov method revealed: vegetative cells are colored blue, spores are colorless. Find the mistake that was made while staining the preparation:
A) Short-term staining with methylene blue.
B) Short-term discoloration with a solution of sulfuric acid.
C) Prolonged discoloration with sulfuric acid
D) Long-term staining with carbol fuchsin.
4. The macrocapsule in pathogenic bacteria serves to:
A) Protection against phagocytosis.
B) Preservation of hereditary information.
C) Protection against bactericidal factors of blood.
D) Adhesion.
5. Which staining methods are used to identify capsules in bacteria,:
A) Gram.
B) Zlatogorov.
C) Peshkov.
D) Mikhin.
6. Determine the correctness of the tinctorial signs in the capsule-forming microbe of the causative agent of anthrax stained by the Olt method (the preparation is made from the blood of an animal that has died from anthrax):
A) The capsule is yellow, the vegetative cell is brick red.
B) The capsule is brick red, the vegetative cell is yellow.
C) Capsule is pink, vegetative cell is brick red.
7. What is the physical method of fixation?
A) Smear treatment with 5% sulfuric acid
B) Drying the smear on the air
C) Dipping a smear in spirit
D) Passing the smear through the flame of the burner

B. BACTERIOLOGICAL METHOD OF RESEARCH

The bacteriological method is the most important metod in the practical activities of any microbiological laboratory. Bacteriological studies are carried out in order to isolate the pure culture of the pathogen with the establishment of its morphological, tinctorial, cultural and biochemical properties, and in some cases antigenic structure, to identify the type of bacteria by serotype and serogroups.

To master the bacteriological method, it is necessary to know:
- classification and types of nutrient media;
- the technique of preparation and sterilization of nutrient media;
- the technique of plating microorganisms in solid and liquid media;
- the allocation of pure cultures of bacteria (aerobes and anaerobes);
- the study of the cultural properties of bacteria in liquid and solid nutrient media;
- the study of biochemical (enzymatic) properties of bacteria;
- determination of the sensitivity of bacteria to antibiotics.
- the study of bacteria for mobility in such preparations as "hanging" or "crushed" drop, made from pure cultures of isolated bacteria [1, 2, 4, 8].

1.11 Nutrient media, classification and their application in bacteriology. The technique of plating microorganisms

The purpose of the lesson. To get acquainted with the types of nutrient media, their purpose and use. Learn the technique of plating microorganisms.

Goals:
1. Know the basic nutrient medium.
2. Methods of preparation of artificial nutrient media.
3. Know the basic requirements for nutrient media.

Equipment: dry powdery nutrient media - MPA, MPB, the environment of Endo and Levine; scales, distilled water, filter paper, sterile Petri dishes, flasks, autoclave.

For the cultivation of microorganisms in the laboratory, such special substrates as nutrient media are needed. There bacteria carry out all life processes (feed, breathe, multiply, etc.).

Nutrient media is a substrate used in bacteriological laboratories for the cultivation of microorganisms in artificial conditions.

Microorganisms grown on nutrient media are called - ***bacterial culture***, the process of growing bacteria under artificial conditions (in vitro) on nutrient media is called - ***cultivation***.

Nutrient media are necessary for the isolation, accumulation, preservation, study of the cultural and biochemical properties of microorganisms. They should include all the components necessary for the structural and energy processes of the bacterial cell, must have sources of carbon, nitrogen, oxygen, hydrogen, macro- and micronutrients, “growth factors" (vitamins).

The following basic conditions are necessary for cultivation: the optimum temperature regime, taking into account which group the studied bacterial species (mesophylls, thermophylls, psychrophiles) belongs to, and the corresponding nutrient media.

Nutrient media classification:

- Accordint to the consistency (density):

a) liquid;
b) semi-liquid;
c) dense / solid.

In bacteriological practice, dense and semi-liquid media are prepared from liquid, to obtain a medium of the necessary consistency, usually such substances are added - *agar-agar or gelatin.*

Agar-agar – polysaccharide (gelatinous substance) obtained by extraction from red and brown algae growing in the Black Sea, White Sea and the Pacific Ocean. It forms a dense jelly in aqueous solutions. It is not a nutrient for microorganisms, it only gives the nutrient medium a seal.

Agar melts at 80-100^{0}C in water, solidifies at 40-45^{0}C.

Gelatin is an animal protein. Gelatinous media melt at 25–30°C; therefore, cultures on them are usually grown at room temperature. The density of these media at a pH below 6.0 and above 7.0 decreases, and they do not solidify well.

Some microorganisms use gelatin as a nutrient - as they grow, the medium liquefies.

In addition, cohesive blood serum, coiled eggs, potatoes, and media with silica gel are used as dense media.

• According to the origin (source components):

a) natural media - prepared from products of animal and vegetable origin (meat, fodder yeast, milk, vegetable juice, MPA, MPB);
b) synthetic media - prepared from certain chemically pure organic and inorganic compounds taken in precisely specified concentrations and dissolved in twice distilled water. An important advantage of these media is that their composition is constant.

- According to the composition:
- ***a) simple -*** meat-peptone agar (MPA), meat-peptone broth (MPB), nutritive gelatin (MPG), Hottinger broth and agar, and peptone water;
- ***b) complex -*** multicomponent media that may contain amino acids, vitamins, microelements, serum and other substances.
- According to the use:

a) basic / universal media - serve for the cultivation of most microorganisms, for example, MPA, MPB, Hottinger broth;
b) special - serve for the isolation and cultivation of microorganisms not growing on simple nutrient media. For example, for the cultivation of tubercle bacillus, Levenshtein-Jensen medium is used, for the cultivation of streptococcus – sugar, for pneumo- and meningococci - blood serum, for the causative agent of pertussis – blood is added to the media;

c) elective (selective) media - serve to isolate a specific type of microbes, the growth of which they favor by inhibiting the growth of related microorganisms. Such media are prepared by adding certain antibiotics, salts, and changing pH. Liquid elective media are called cumulative media. For example, bile salts, inhibiting the growth of Escherichia coli, make the environment elective for the causative agent of typhoid fever; Müller's medium, selenite broth - used to isolate salmonella, for enterobacteria lard — Rapoport, etc.

d) selective media - on which microorganisms of a certain type grow faster and more intensively, also go ahead of other types of bacteria in their development. For example, 1% alkaline peptone water is a selective medium for cholera vibrios; Palcam agar - for Listeria, Leffler's medium for the isolation of colibacilli, etc.

e) differential diagnostic media - they allow to distinguish (differentiate) one type of microbes from another by enzymatic activity, and determine the species and generic characteristics of bacteria. For example, Giss media with carbohydrates and an indicator - while the growth of microorganisms that break down carbohydrates, the color of the medium changes; milk and breast cancer - while the growth of bacteria that break down proteins, the medium coagulates or liquefies, etc.

f) transport (or preserving) media - intended for the initial plating and transportation of the test material. For example, a glycerin mixture is used to transport biomaterial, in order to detect a number of intestinal bacteria.

Nutrient media should comply with the following requirements:

1) To be nutritious, to contain all the substances necessary to satisfy nutritional and energy needs in an easily digestible form. When cultivating a number of microorganisms, growth factors — vitamins, some amino acids that the cell cannot synthesize — are contributed to the medium;

Microorganisms need a lot of water.

2) To have an optimal concentration of hydrogen ions - pH, because only while an optimal reaction of the medium affecting the permeability of the shell, microorganisms can absorb nutrients.

For most pathogenic bacteria, a slightly alkaline media is optimal (pH 7.2-7.4). There are exceptions, for example, cholera vibrio - its optimum in the alkaline zone (pH 8.5-9.0), the causative agent of tuberculosis - slightly acidic pH 6.2-6.8.

During the growth of microorganisms, to prevent acidic or alkaline products of their vital activity changing the pH, the media ***must have buffering,*** it means contain substances that neutralize metabolic products;

3) To be isotonic for a microbial cell; it means the osmotic pressure in the medium should be the same as inside the cell. For most bacteria, the optimal medium corresponds to a 0.5% sodium chloride solution;

4) To be sterile, since extraneous microbes interfere with the growth of the studied microbe and determining its properties, change the properties of the medium;

5) Dense media should be moist and have an optimal consistency for microorganisms;

6) To be standardized, to contain constant amounts of individual ingredients. Thus, cultivation media for most pathogenic bacteria should contain 0.8-1.2 g / l of amine nitrogen NH2; 2.5-3.0 g / l of total nitrogen N; 0.5% sodium chloride; 1% peptone.

It is desirable that the media is transparent - it is more convenient to monitor the growth of crops and easier to notice environmental pollution by extraneous microorganisms.

Figure 18 - Types of nutrient media

Preparation of nutrient media

Prepared ***dry media*** are widely used in modern practice. They are produced by industrial biofactories. Dry media are used in different purposes: simple, elective, differential - diagnostic, special. These are powders in vials with caps (Fig. 18). It is better to store dry media in a dark place, tightly closed, they are hygroscopic. In the laboratory, powders are used to prepare media due to writing on the label (preparation method).

The advantages of dry media in comparison with media made directly from natural components, - standard (they are produced in large batches), ease of preparation, stability, economy.

Sometimes, in laboratories, the media is prepared directly from raw materials. The raw materials for the preparation of most media are products of animal or vegetable origin: meat and its substitutes, milk, eggs, potatoes, soy, corn, yeast, etc. The main nutrient broths are prepared on meat water or on various digests obtained by acid or enzymatic hydrolysis of feedstock. Broths from digests are 5-10 times more economical than from meat water. Medium on digestion is richer in amino acids, therefore, more nutritious; have greater buffering, have a more stable pH value. In addition, overcooks can be prepared from meat substitutes (blood clots, placenta, casein, etc.).

Stages of media preparation: 1) boiling; 2) establishing the optimal pH; 3) clarification; 4) filtration; 5) spill; 6) sterilization; 7) control.

The technique of plating microorganisms

An important stage of bacteriological research is plating.

Crops are carried out both from the material sent to the laboratory, and from existing bacterial cultures.

Depending on the purpose of the study, the nature of the seed and the media, different methods of plating are used. All of them include a mandatory goal: to protect the plating from extraneous microbes. Therefore, it is important to work quickly, but without sudden movements, which amplify air vibrations. Talkng during plating is inhibited. Plating is better done in boxing and always above the flame of the burner.

Platings from samples of material received at the laboratory are carried out with a Pasteur pipette, from the bacterial mass with a bacteriological loop (Fig. 19). On the seeded Petri dishes, flasks or test tubes is written the examination number under which the received material is registered.

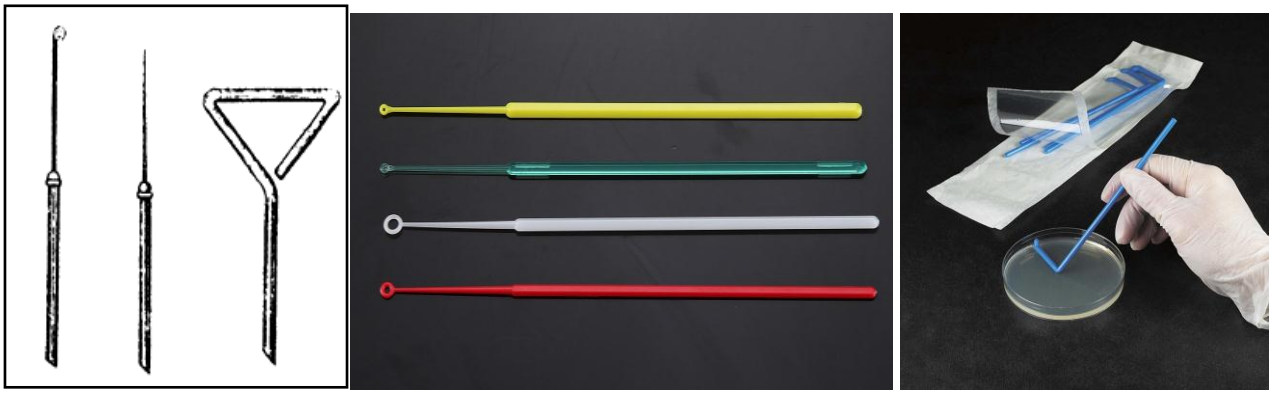


Figure 19 - Instruments for plating microorganisms

The importance of this method is explained by the fact that in many cases doctors deal with microbial associations, then it is important to establish the role of each of the microbes in the occurrence of the disease.

Therefore, before mastering the basic principles and methods for the isolation of pure cultures, it is necessary to master the technique of plating and reseeding bacteria in liquid and solid nutrient media.

The technique for plating microorganisms into liquid and solid nutrient media has its own characteristics, which are presented in the next section.

Control questions:

1. What are criteria for the classification of nutrient media?
2. At what temperature do agar media melt and solidify?
3. What are definitions for bacterial culture and cultivation?
4. Prepare Endo, EMC, Ploskirev from dry powders and pour them into Petri dishes.
5. Prepare mowed agar.

1.12 Methods for the cultivation of microorganisms (aerobic and anaerobic bacteria). Technique for plating bacteria in liquid and solid nutrient media

The purpose of the lesson: To familiarize students with the technique of plating bacteria on nutrient media, to learn the features of the cultivation of anaerobes and aerobes

Goals:

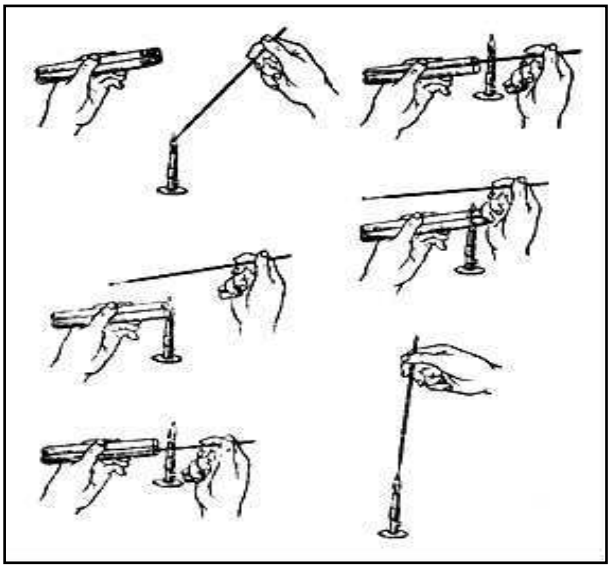
1. To master the technique of plating bacteria in liquid nutrient media.
2. To master the technique of plating bacteria on solid nutrient media.
3. To master the technique of plating anaerobic bacteria on Kitta-Tarotsi medium.
4. To learn how to cultivate bacteria.

Equipment: prepared nutrient media - MPA, MPB; beveled MPA in test tubes, MPB in flasks; vials with dry media; culture of staphylococci, hay bacillus; sterile tubes with MPB and MPA, bacteriological loops, spatulas, sterile Petri dishes and Pasteur pipettes, desiccator, thermostat.

Technique for plating aerobic bacteria

Inoculation from test tube to test tube. The bacterial culture tube and the medium tube are held slightly slanted in the left hand between the thumb and forefinger so that the edges of the tubes are at the same level and their bases are on top of the brush. Typically, the seed tube is held closer to itself. In the right hand, a bacterial loop is hold like a writing pen, and sterilized vertically in the flame of the burner. With the little finger and edge of the palm of the right hand, both plugs are removed at the same time. Corks are not removed jerkily, but smoothly - with light screw movements. When the plugs are taken out, the edges of the tubes are fired in the burner flame. The calcined loop is input through the burner flame into a test tube with seed, cooled and, when a bit material is collected, carefully transferred into a test tube with medium (Fig. 20).

While inoculation into a liquid medium, the plating material is triturated on the wall of the tube above the liquid and washed off with medium.



Then carefully taken out, closed the tubes with stoppers, burnt the loop on the flame of the burner (pasteur pipette is lowered into a jar with a disinfectant solution - phenol, formalin). During operation, it is important to make sure that the jams are not wet.

When plating on liquid media with a *tampon*, it is immersed in the medium and rinsed in it for 3-5 seconds. When plating on a dense medium, the material is rubbed into its surface by rotating the tampon, after which the tampon is disinfected.

Figure 20 – Technique for test-tube plating

Attention! Make sure that the medium does not spill out and dampen the cork.

While *plating on agar*, the material is usually ground with a loop on the surface of the medium in zigzag movements from the bottom up, starting from the boundary of the condensate.

When plating on solid media, the material is poured into test tubes in a column, with a loop of inoculums the column is pierced, making the ingection plating.

After plating the loop is removed from the test tube, the edges of the test tubes are fired and after passing the stoppers through the flame of the burner, the tubes are closed, and then the loop is calcined. Plating liquid material can be done with sterile pipettes (Pasteur or graduated). After plating the pipettes are immersed in a disinfectant.

Test tubes are closed, the loop is burnt. Inoculated culture tubes are labeled and placed in a thermostat for cultivation; after 16-18 or 24-48 hours, the result is taken into account.

Inoculation in test tubes from a Petri dish. After studying the nature of the growth of culture on a cup, the site necessary for plating is marked on the bottom side with wax pencil. A cup with the material is placed with the lid up. With the left hand, the lid is opened and a burnt loop is input under it. When a loop is cooled, material from the marked site is gaqned. A loop is taken out, the cup is closed and a test tube with medium is taken in the left hand. Plating is carried out in the same way as from a test tube into a test tube. After plating, the cup is turned upside down (Fig. 21, c).

Plating on agar in Petri dishes. Plating with a spatula (Fig. 21, b). A spatula is a glass or metal tube, the end of which is bent in the form of a triangle. A spatula can be made from a Pasteur pipette by bending at an angle its thin end, previously heated in the burner flame.

With the left hand, the lid is slightly opened, holding it with thumb and forefinger. Plating the material is applied on the surface of the medium with a loop or pipette or glass rod, carefully rubbed with circular movements of the spatula until the spatula ceases to slide freely on the surface of the medium, holding the lid with the left hand and rotating the cup at the same time. At the end of plating, the spatula is removed from the cup and the lid is closed. A glass spatula is placed in a disinfectant solution, and a metal spatula is calcined in a burner flame.

Plating with a tampon. The tampon with the material is input into the slightly opened cup and in a circular motion rubbed its content on the surface of the medium, rotating the swab and cup.

Plating the lawn. About 1 ml (20 drops) of the liquid culture (if the culture is from a dense medium, it is emulsified in a sterile isotonic solution or broth) is applied on the surface of the agar and the liquid is carefully distributed over the surface of the medium. The cup is slightly tilted and an excess of culture is sucked off with a pipette, pouring it into a disinfectant solution. A pipette is also placed there.

Plating in the thickness of agar. A culture grown in liquid medium or emulsified material is input into a vessel with agar molten and cooled to 45 ° C, mixed and poured into a sterile Petri dish. It is possible to make the material in an empty cup and pour 15-20 ml of agar cooled to 45 ° C. To mix the contents of the

cup, it is shaken slightly and rotated. The cups are left on the table until the medium solidifies.

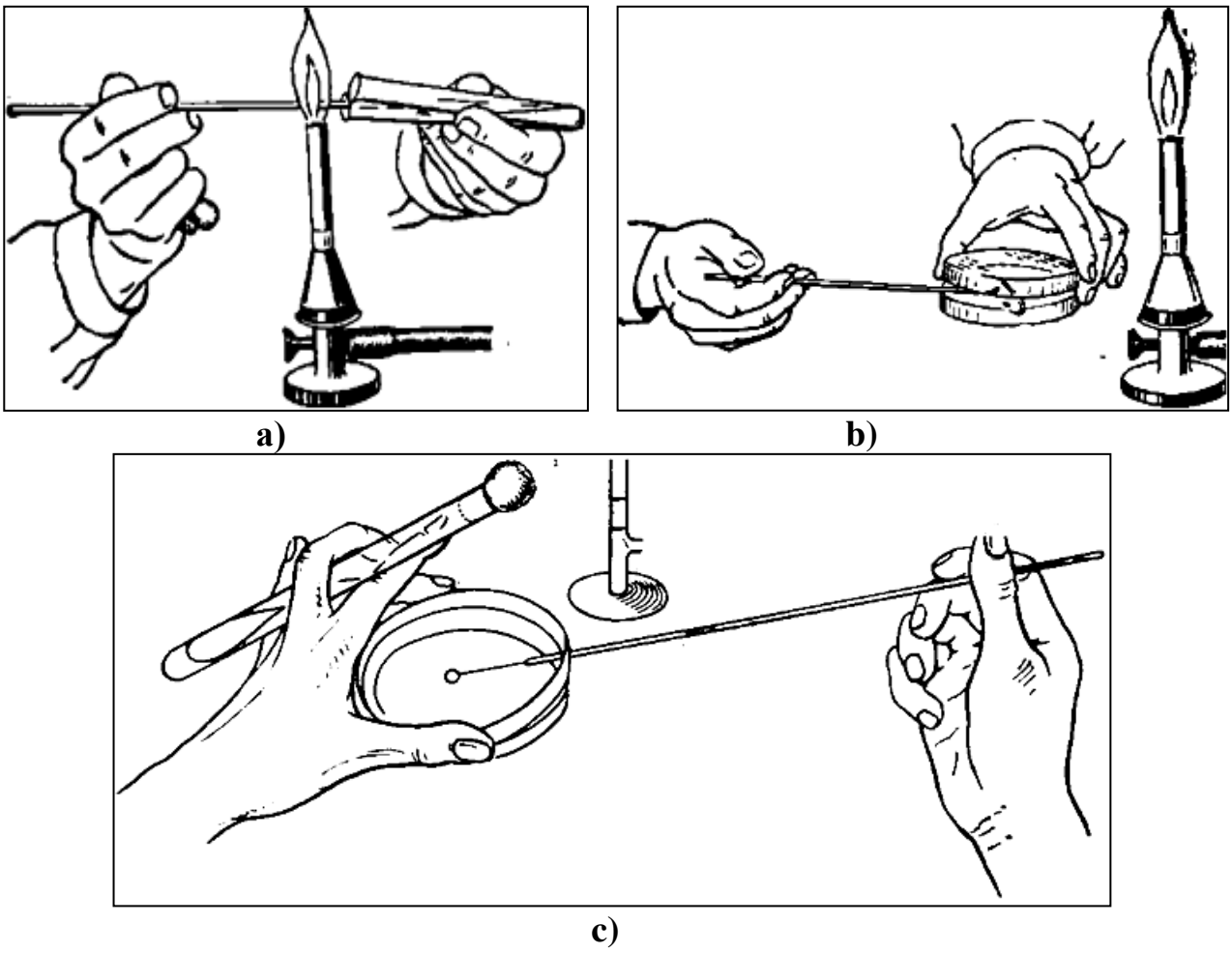


Figure 21 - Technique for plating bacteria on a nutrient medium:
a) in a liquid medium with a bacteriological loop; b) with a spatula on agar;
c) with a bacteriological loop from the broth onto a dense agar medium

Cups with the material are signed from the bottom and placed in the thermostat upside down.

For plating bacteria on the surface of a dense medium (MPA, mowed agar, etc.) the method of ***streak*** with a bacteriological loop is used (Figure 22).

Loop plating. A small amount of the plating material (sometimes it is pre-emulsified in sterile isotonic solution or broth) is rubbed with a loop into the surface of the medium at the edge of the cup, several times looping it from side to side. Then, at the place where the strokes ended, the agar is pierced with a loop, removing the excess material. The material remained on the loop is distributed in zigzag motions over the entire surface of the medium. At the end of plating, the cup is closed and the loop is burnt.

Loop plating on sectors. The bottom of the cup is lined into sectors. Plating is done in zigzag movements from the edge of the cup to the center. It is necessary to prevent strokes from entering the neighboring sector.

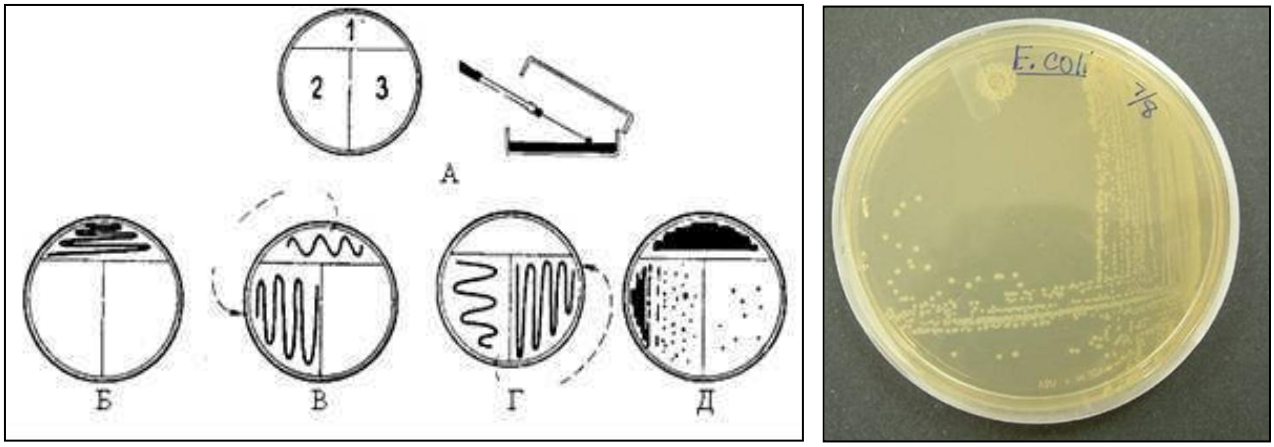


Figure 22 - Technique for plating bacteria by the streak method

Cultivation methods of aerobes

For the successful cultivation of aerobic bacteria, the following conditions are necessary:

- choosing the right environment and correct plating;
- creating optimal conditions: temperature, humidity, aeration (air supply).

Temperature. For the cultivation of microbes in laboratory conditions, a special apparatus is used - ***a thermostat***, which maintains a constant and optimal temperature (37° C) for the cultivation of most pathogenic microorganisms (Fig. 23). This is a double-walled device, between walls there is air or water, heated by electricity. It is equipped with a temperature controller that maintains the necessary temperature, and a thermometer to monitor the temperature.

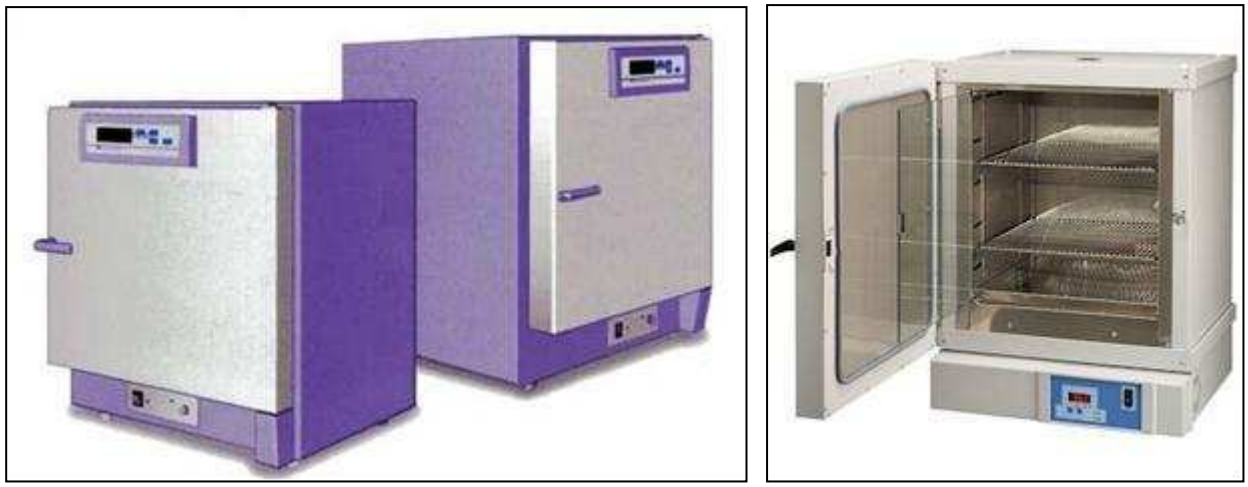


Figure 23 - Thermostat, for the cultivation of microorganisms

Test tubes with the plating material in tripods, wire mesh or banks are installed on the shelves of the thermostat. Petri dishes with cultures in the thermostat should stand upside down.

The shelves in the thermostat are made with slots and do not load tightly for free circulation of air in the thermostat and uniform heating. In order not to cool the

material, the thermostat is not left open for long.

Light - most microbes (all pathogens belong to them) do not need light - they are cultivated in the dark. However, to study pigmentation, which occurs more actively in ambient light, the cultures after the thermostat can withstand 2-3 days at room lighting.

It is important to avoid direct sunlight, which is harmful to the material.

Humidity. Life of microbes is impossible without moisture - nutrients penetrate into the cell only in dissolved form. It must be taken into account when cultivating on solid media: pouring the material into cups and mowing in tubes is better to do on the day of plating. When microbes especially sensitive to the absence of moisture are cultivated, for example gonococci, an open vessel with water is placed in a thermostat.

Aeration. According to the need of microbes in free oxygen, they are divided into aerobes and anaerobes. Both groups require different cultivation conditions.

The oxygen supply necessary for the cultivation of aerobes and facultative anaerobes is carried out with passive and active aeration.

Passive aeration is cultivation on solid and liquid media in vessels covered with cotton or cotton-gauze plugs, or in Petri dishes. In such cultivation, microbes consume oxygen dissolved in the medium, located in a vessel above the medium and entering through the cork. Passively aerated cultures may be grown on the surface or in a thin layer of the medium where air oxygen penetrates.

Active aeration is used in the deep cultivation of microbes, when they are grown in large volumes of the medium. In order to sufficiently supply oxygen to such cultures, they are placed in special rocking chairs - the constant mixing of the culture ensures that it is in contact with air. While culturing in volumes of liquid, reaching tens and hundreds of liters, carried out in devices called reactors or fermenters, air is blown through the culture using special devices.

Duration of cultivation. Mostly bacteria grow 18-24 hours, but there are species that grow slowly (up to 4-6 weeks). To retain moisture in them, after plating cotton plugs are replaced with sterile rubber caps or rubber caps are put on them.

Methods for the cultivation of anaerobes

This is more difficult than growing aerobes, as the contact of microorganisms with molecular oxygen should be minimized here. Various methods are used to create anaerobic conditions.

They are divided into *physical, chemical and biological*. All of them are based on the fact that microorganisms are cultivated in some kind of closed space.

Physical methods for creating anaerobic conditions include growing microorganisms in microaerostats - vacuum metal chambers equipped with a manometer. An anaerostat can be a regular glass desiccator with a ground lid. Air is pumped out of the anaerostat, and then it is filled with a gas mixture consisting of 80–90% nitrogen and 10–20% carbon dioxide, with a pressure of about 500 mm Hg. Art.

Chemical methods for creating anaerobic conditions include the use of *oxygen-absorbing chemicals.* In laboratory practice, an alkaline pyrogallol solution, sodium dithionite (Na2S2O4), metallic iron, monovalent copper chloride and some other

reagents are used as oxygen scavengers. Absorbers are placed at the bottom of a chemical desiccator, into which anaerobic microorganisms are also placed, seeded in test tubes, flasks or Petri dishes. The desiccator is closed with a ground lid. In this method of creating anaerobic conditions, it is necessary to take into account the absorption capacity of the substances used and the amount of confined space the culture is grown in.

The biological method of creating anaerobic conditions includes the next. The nutrient medium in the Petri dish is divided by a groove into two halves, obligate aerobic bacteria are sown on one half, and obligate anaerobic bacteria are sown on the other. The cup is closed, the gap between the bottom and the lid is filled with paraffin or wax and placed in a thermostat with an optimum temperature for the growth of microorganisms. Initially, growth of aerobic microorganisms is observed (before depletion of free oxygen), after that the proliferation of anaerobic cells begins.

For the cultivation of anaerobic bacteria, **other methods** that restrict air access to a growing culture are used:

• cultivation in a high layer of the environment;
• cultivation in the thickness of a dense medium;
• cultivation in viscous media in which the diffusion of molecular oxygen into a liquid decreases with an increase in its viscosity;
• pouring medium with inoculation with a high layer of sterile paraffin oil or paraffin.

Plating for the cultivation of anaerobes

The plating technique for culturing anaerobes is the same as for anaerobes, but it is important to create conditions for *anaerobiosis*, special media are used for this: meat-peptone liver broth (Kitta-Tarozzi medium) - pieces of boiled liver are placed on the bottom of the tube, the medium is filled with a layer liquid paraffin (1-2 cm). Before plating, this medium needs to be *regenerated* - for this, test tubes with the medium are boiled in a water bath, then quickly cooled under a stream of cold (tap) water. When boiling, the oxygen of the air from the medium is removed and the liquid paraffin protects against the penetration of air from the outside. Pieces of the liver provide anaerobes with bound (atomic) oxygen due to the *"biosystem"* of liver cells.

Anaerobes can also be cultured in semi-liquid MPA with a high column. To do this, plating is carried out deep into the medium or anaerobiosis is created asin the next example: the test material is seeded, for example, on a special Wilson-Blair molten medium, after the test material is input and mixed well by rotating the flask (tube) between two palms, then the content is carefully poured into a sterile Petri dish and allowed to congeal with an even layer. When the medium cools down, molten MPA is poured on its surface with an even layer. After cooling (medium compaction), the cups are placed in a thermostat.

The cultivation of actinomycetes, fungi, mycoplasmas, L-forms, spirochetes and protozoa. The cultivation of these microorganisms is fundamentally similar to the cultivation of bacteria. Special media are developed and modes that correspond to their needs are selected.

The cultivation of rickettsia. Rickettsie are obligate parasites, so they can develop only in living cells. They are cultivated in tissue cultures, the body of experimental animals, developing chicken embryos.

Control questions:

1. What should be understood by the name "cultivation" of bacteria?
2. Name the main conditions and methods of cultivation.
3. The main methods for creating anaerobiosis.
4. What is the basis for the use of the Kitta-Tarozzi medium in the cultivation of anaerobes?
5. Features of the cultivation of rickettsia.

1.13 Methods for isolating a pure bacterial culture

The purpose of the lesson. To assimilate the diagnostic value of isolating a pure culture and master the methods used to obtain a pure culture of microorganisms

Goals:

1. To isolate a pure culture of bacteria according to the Koch method.
2. To isolate a pure culture of staphylococcus by the Drigalski method.
3. To isolate a pure culture of anaerobes.
4. To isolate a pure culture of bacteria by chemical method.

Equipment: sterile test tubes with 10 ml saline, sterile MPA in test tubes (9 ml each), Petri dishes with agar, graduated pipettes, Pasteur pipettes, test tubes with suspensions of bacteria - staphylococci, salmonella.

Microbes isolated from the external environment, from the organism of animals or people and propagated on nutrient media, are called cultures.

In many cases, in the test material there is a mixture of two or more types of bacteria. Isolation from a mixture of one type of microorganism (for example, the causative agent of a particular disease) is called the isolation of pure culture. This is the main task when conducting bacteriological diagnostics.

A pure culture is a culture consisting of microorganisms of only one species, which is determined by the totality of its properties.

In this purpose, the growth of bacteria in separate colonies (on solid nutrient media) is achieved by special growth methods.

A colony is an accumulation of bacteria visible to the naked eye on the surface or in the bulk of a dense nutrient medium. Each colony is formed from the descendants of one microbial cell (clones), therefore their composition is always homogeneous.

The culture of microorganisms isolated from a specific source (animal organism, air, soil, etc.) is called a ***strain***.

If the isolated bacterial culture of one species differs in some ways, then it is called - ***option***.

Pure bacterial cultures make it possible to establish a bacteriological diagnosis. They are important in the diagnosis of infectious diseases to determine the species and type of microbes, in research to obtain the vital products of microbes (toxins, antibiotics, vaccines, etc.).

Methods of isolating a pure culture of microorganisms from the studied material can be divided into two groups - ***mechanical and biological***.

The mechanical principle of isolating a pure bacterial culture

1. The method of serial dilutions, proposed by L. Pasteur, was one of the first methods, which was used for the mechanical separation of microorganisms. It consists of the serial dilution of material containing bacteria in a sterile liquid nutrient medium. To do this, a number of tubes with sterile MPB (9-10 ml each) is taken, the test material is input with pipette into the tube, mixed, then a small amount (0.1 ml) of it is transfered into the second tube, after mixing transfered into the third, etc. (up to 10 test tubes).

This technique is painstaking and imperfect in work, because it does not allow controlling the number of microbial cells that enter the tubes during dilutions.

L. Pasteur suggested that in the last test tube one kind of microbe can grow, which was unlikely, and at present Pasteur's serial dilution method is used only as an auxiliary method for other methods.

2. Koch method (plate dilution method).

R. Koch used solid nutrient media based on gelatin or agar-agar. The essence of the Koch method is that, using the Pasteur method, the test material (with the association of different types of bacteria) is diluted in 4-5 tubes with melted and cooled to 45-500 MPA in 10-15 ml, then the contents of the tube are carefully poured into sterile Petri dish and the medium is distributed with a thin layer. The cup is closed and when the agar hardens, turned upside down, placed in a thermostat for 18-24 or 48 hours. After that, bacterial colonies grow on the MPA surface, but in the area where the concentration of microbes was lower, isolated colonies grow. On the reverse side (along the bottom of the cup), the necessary colony is marked with a pencil on the glass. Then the lid of the cup is opened and one (marked) colony is removed with a bacteriological loop without touching the others and transferred to test tubes with sterile MPB and MPA, the latter are placed in a thermostat. The growth of pure culture is got (Fig. 24).

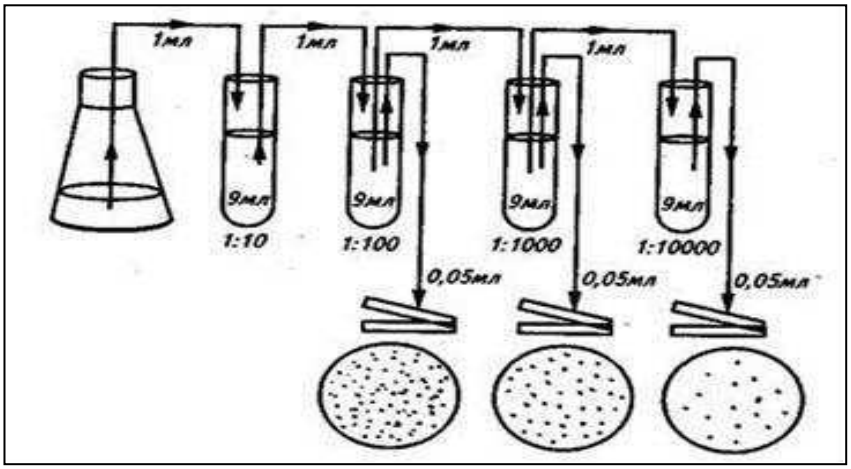


Figure 24 - Koch Method

3. Drigalski method (plastic plating method) - is a more advanced method that is widely used in everyday microbiological practice (Fig. 25).

To do this, 4-5 sterile Petri dishes with a dense agarized medium are prepared. A drop of the test material is applied on the surface of the agar with a bacterial loop, and then carefully rubbed into the medium using a spatula of Drigalski. The cup is kept open during seeding and carefully rotated for even distribution of the material. Without sterilizing the staple, it is rubbed on the surface of the medium in the second cup, then sequentially in the third. Only after that the spatula is dipped in a disinfectant solution or fry in the burner flame.

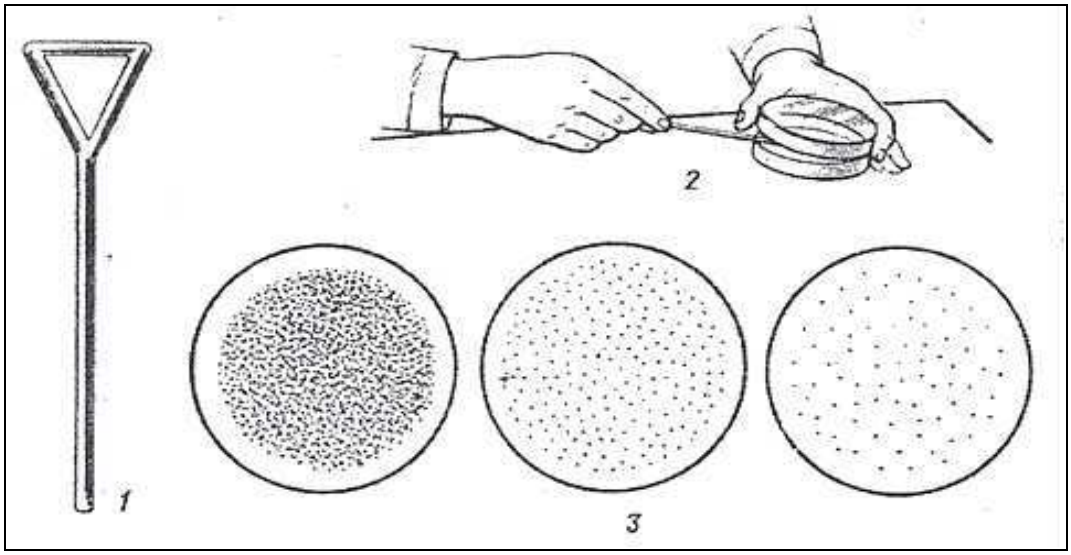


Figure 25 - Drigalski Method

On the surface of the medium in the first cup, as a rule, continuous growth of bacteria is observed, in the second - dense growth, and in the third - growth in the form of isolated colonies (Fig. 26).

Next, necessary colony is noted, taken in MPB and MPA, placed in a thermostat for cultivation and further identification.

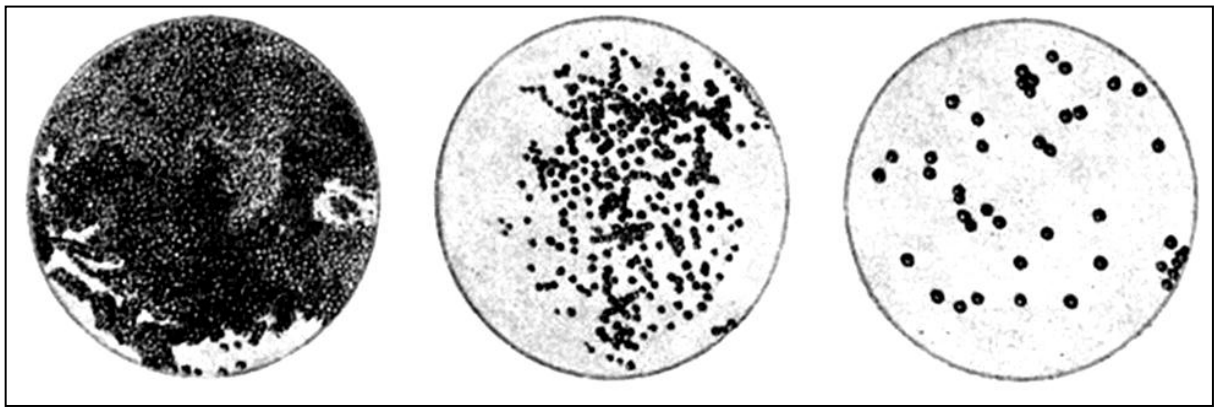


Figure 26 - Colonies by the Drigalski method

4. The streak - plating method is often used today in microbiological laboratories. Material that contains a mixture of microorganisms is collected with a bacteriological loop and applied on the surface of the nutrient medium near the edge of the cup. The excess material is removed and held in parallel strokes from edge to edge of the cup. After a day of incubation of the plating material at the optimum temperature, isolated microbial colonies grow on the surface of the plate (Fig. 27).

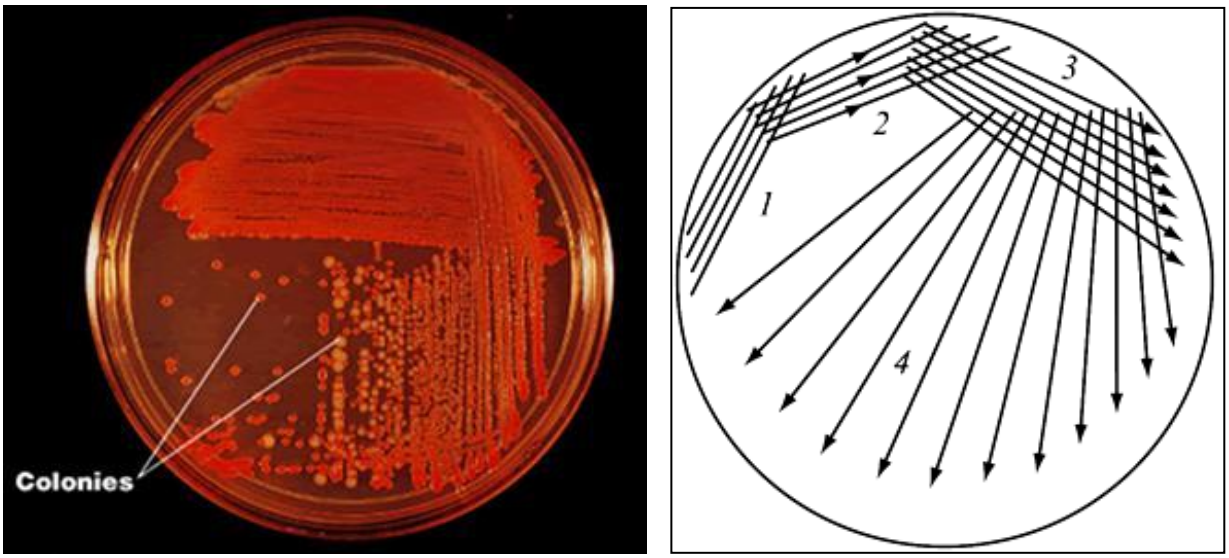


Figure 27 - Streak Method

Pure culture isolation methods based on the biological principle

They are based on taking into account certain biological characteristics of the bacteria released into a pure culture.

The most common methods are:

1. Isolation of spore-forming microorganisms

When pure cultures are isolated from the test material containing spore forms of microbes, it is heated for 10 minutes at 80 °C or 2-3 minutes at 100 °C in the expectation that less resistant non-spore forms will die at this temperature. In this case, spore forms will remain viable.

From the heated material, plating is carried out on nutrient media on which spore-forming microorganisms grow.

If it is necessary to separate the spore forms of bacteria from non-spore forming species, a suspension of the test material is prepared and heated in a water bath at 800 ° C for 30-40 minutes, vegetative forms of microorganisms die, spores remain viable. Then the heated suspension is sown according to the method of Drigalsky or Koch.

2. Isolation of acid-resistant microorganisms.

A chemical method is used to isolate a pure culture of the causative agents of tuberculosis and paratuberculosis from pathological material. In this purpose, the searched material after its homogenization is treated with a 6% solution of sulfuric acid for 5-7 minutes, adding it in an equal volume. Due to this, microbes of concomitant microflora die and acid-resistant ones remain viable.

After neutralizing the acid solution, plating is carried out on special nutrient media, where acid-resistant microbes grow.

3. Isolation of motile microorganisms (Shukevich method)

The examined material, from which the mobile microbe is released, is seeded into the condensated water of the mowed MPA. While plating, it is necessary to ensure that the loop with the material does not touch the surface of the medium above condensation water. Bacteria with active mobility will grow not only in condensation water, but also outside it, on the surface of MPA. From the upper part of the growth, re-plating in the condensate of fresh nutrient medium is performed. By doing several passages in this way, a pure culture of motile bacteria is eventually got.

4. Isolation of pathogenic microorganisms

Pure cultures of pathogenic bacteria are isolated by infection with the test material of laboratory animals that are most susceptible to a particular pathogen (biological method).

After the death of the animal, the material taken from the blood and organs of the corpse is sown on nutrient medium, where a pure culture of the pathogen grows (this method is described in detail in the section "Infection of laboratory animals").

Getting a pure culture of anaerobes

The principle remains the same as when working with aerobes, but special media are used: using the Drigalski method plating is carried out on glucose-blood agar in Petri dishes, which are then placed under anaerobiosis conditions (anaerostat device). The Wilson-Blair culturing method is also used when individual black colonies grow, the colonies are transplanted into the Kitta-Tarozzi environment, thereby obtaining a pure culture. A biological sample can serve the same purpose: the susceptible animal is infected with the test material. After its death plating in the medium of Kitta-Tarozzi, semi-liquid agar, as mentioned above is performed. There are other methods, which are described in the description of certain types of anaerobes.

Thus, the isolation of pure cultures of pathogens has several methods, as well as the sequence of actions (steps).

Stages for isolation of aerobic bacteria's pure cultures:

1st stage of the study - pathological material is taken into sterile dishes (test tube, flask, vial).

Plating is carried out with a bacteriological loop and a spatula on solid nutrient media according to the Drigalski method. The cups are turned upside down, signed with a special pencil and placed in a thermostat at the optimum temperature (37 ° C) for 18-48 hours.

The purpose of the stage is to obtain isolated colonies of microorganisms.

2nd stage of the study - on the second day isolated colonies that grew on the surface of the agar are examined. The needed colony is isolated with a pencil on the back of the Petri dish and re-plated on mowed agar to obtain a pure culture. Suspicious colonies are used to prepare smears, to do Gram stain, and to study the morphological and tinctorial properties of pathogens.

3rd stage of the study - on the third day the nature of the growth of microorganisms' pure culture is examined and its identification is carried out (studying the biochemical, cultural and antigenic properties of bacteria);

4) The conclusion about the isolated culture.

Order in the identification of bacteria:

I **–** Identification of the main species characteristics of bacteria:

1. The morphology of the microbial cell;
2. Tinctorial properties;
3. Cultural properties - features of bacterial growth on media;
4. Biochemical properties - the presence of enzymes in the bacteria.

II **–**Determination of additional features in the identification of bacteria:

1. The presence of species-specific antigens;
2. Species sensitivity to bacteriophages;
3. Species resistance to a specific antibiotic;
4. For pathogenic bacteria - the production of a specific virulent factor (toxin, enzyme, etc.).

In recent years modern biochemical methods have been widely used for ***molecular - biological identification*** of microorganisms: chemo-identification, nucleic acid analysis, restriction analysis, hybridization, polymerase chain reaction (PCR), ribotyping, etc.

Control questions:

1. What is the basis for the principle of obtaining a pure culture of bacteria according to the method of Koch, Drigalsky?
2. What is the essence of the biological method for isolation of pure culture?
3. Name the principles of the chemical method of obtaining a pure culture.
4. Methods of obtaining a pure culture of anaerobes.
5. What are the main species features of bacteria?

Questions for self-training and knowledge control.

1) Match the definitions with the appropriate terms:

1. A population of microbes, consisting of individuals of one species.
2. Culture of microorganisms obtained from one individual (unicellular culture).
3. The culture of microbes of one species isolated from a specific source (animal organism, environment).
4. The culture of microorganisms of one species, differing in some ways (within the characteristics of the species):

A) Clone. C) Option.
B) Strain. D) Pure culture.

2) The veterinary laboratory received the test material after the slaughter of an animal suspected in tuberculosis. What treatment should be subjected to material before plating for isolation the causative agents of tuberculosis?

A) Warm up at 80 ° for 2-3 minutes.
B) Treat with a 6% solution of sulfuric acid in a ratio of 1: 6.

C) Sow without treatment.
3) The veterinary laboratory received stale pathological material (pieces of muscle) after the death of a cow suspected in emphysematous carbuncle. What treatment is necessary to expose the material before plating in order to isolate the pathogen?
A) Warm up at 80 ° for 2-3 minutes.
B) Treat with a 6% solution of sulfuric acid in a ratio of 1: 6.
C) Sow without treatment.
4) The veterinary laboratory received meat samples after the forced slaughter of the animal for examination. It is necessary to exclude the presence of a vulgar protea in them, which can cause mass poisoning of people. What method do you use to isolate the pure culture of this microbe?
A) Plating on MPA in Petri dishes.
B) Warming up the material with subsequent plating at MPA.
C) Plating on the MPB.
D) Plating on the beveled MPA into the condensate.
5) The veterinary laboratory received the test material (ear) from the fallen heifers suspected in anthrax. What method do you use to isolate a pure culture of the pathogen?
A) Plating on MPA and MPB.
B) Plating on Endo medium.
C) Infection of a laboratory animal.
D) Inoculation on mowed agar in condensate.

1.14 Cultural properties of microorganisms

The purpose of the lesson. To acquaint students with the basic cultural properties of microorganisms - the nature of growth in liquid and solid nutrient media. To assimilate the significance of determining cultural properties in bacteriological diagnostics (in identifying bacteria).

Goals:

1. To describe the cultural properties of hay bacillus on MPB.
2. To describe the cultural properties of hay bacillus on MPA.
3. To study the cultural properties of anthrax bacillus (vaccine strain) in the breast cancer.

Equipment: plating from the previous lesson, Cultures (for demonstration): B. mucoides, hay bacillus (B. Subtilis) on MPA and hay bacillus on MPB, anthrax bacillus (vaccine strain) in MPF. Sterile culture media in test tubes (MPA, MPB), beveled MPA. Magnifiers, bacteriological loops, spirit lamps, glass slides, a set of paints for Gram staining. Microscopes.

Using the results of plating on the last lesson - to describe the growth of bacteria in a dense and liquid medium under the guidance of a teacher.

The cultural properties of bacteria are the nature of their growth in dense and liquid nutrient media.

The study of the cultural properties of bacteria (with other properties) is necessary while conducting bacteriological diagnostics in order to identify isolated bacteria from the studied material.

Identification of a pure bacterial culture is determination of the type of the microorganism, its morphological, cultural, biochemical (enzymatic), serological (antigenic) and pathogenic properties.

Bacterial growth in liquid nutrient media

The growth of microorganisms in liquid nutrient media is not diverse so much.

The growth of bacteria in liquid nutrient media is characterized by the following features:

A macroscopic examination (with the naked eye) indicates the ***nature and degree of turbidity of the environment:***

- steady (diffuse),
- intensive,
- moderate, weak and in the form of opalescence.

Without shaking the contents of the test tube with the plating material, first attention is paid to the surface growth, which can:

- be in the form of parietal ring, or
- pellicles over the entire surface of the medium,
- rise (zigzag) onto the walls of the tube,
- cover only part of the surface of the medium, not reaching the wall of the vessel.

According to its nature, a pellicle can be:

by thickness - thin, thick, tender or rough;
by the nature of the surface - folded, wrinkled, smooth, mesh, fluffy or even*;*
by consistency - fragile, mucous, greasy.
by color, shade - bluish, yellow, gray, white.

Many bacteria in liquid nutrient media form ***sediment*** at the bottom of the tube, which is detected by slightly shaking the tube. It can be:

- plentiful and insignificant;
- dense (compact), loose, granular, in the form of lumps of cotton wool, flocculent, tiny, mucous.
- by color - white, yellow, dull, green, gray, etc.

While shaking, the sediment either breaks up, creating a uniform turbidity of the medium, or large or small flakes and clumps are formed; the mucous sediment can rise up in the form of a “pigtail” in a turbidmedia or it remains transparent.

A number of bacteria form ***water-soluble pigments*** that evenly color the nutrient medium in blue-green, bright red and yellow.

The growth of culture in a liquid medium can be ***near-wall***. It is accompanied by the attachment and propagation of microbes on glass (on the walls of the tubes) with the formation of a representative matte coating, small flakes, grains.

Bacterial cultures of some species may possess not only one but several signs of growth in a liquid medium, for example, cause turbidity of the medium with the formation of sediment, wall ring, etc. (Fig. 28).

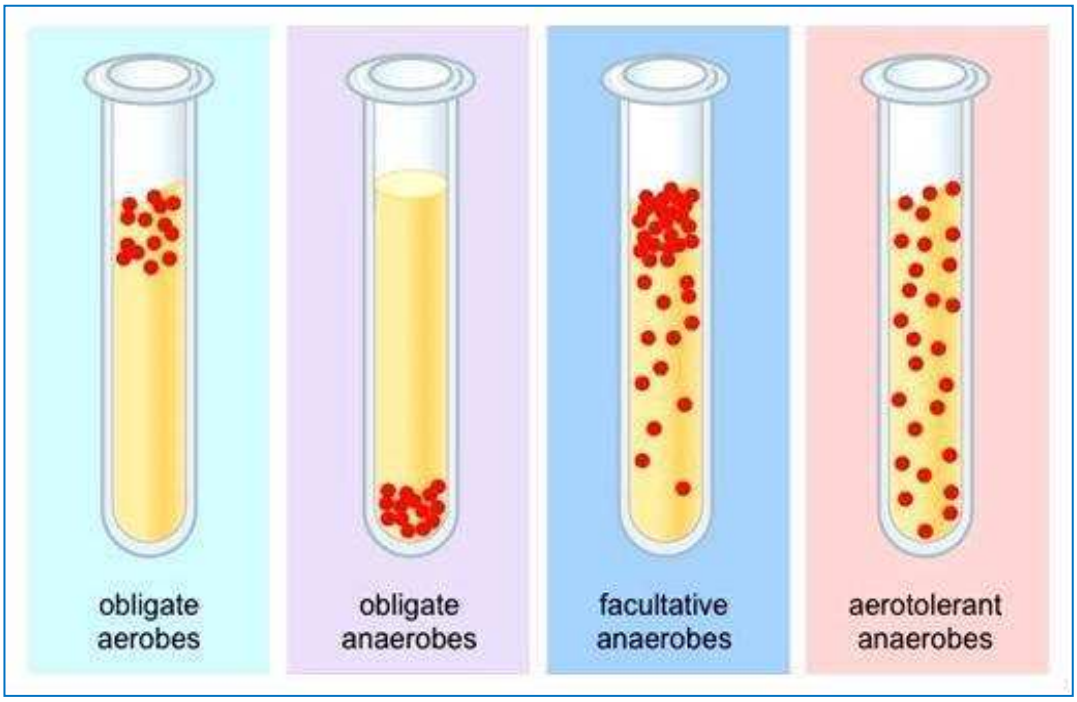


Figure 28 - Features of bacterial growth in liquid nutrient media

Growth of microorganisms in dense nutrient media

On dense media (MPA, blood agar, etc.), bacteria grow in the form of colonies, which are then studied (Fig. 29).

Colonies are the accumulations of microorganisms formed in the result of the reproduction of one bacterial cell.

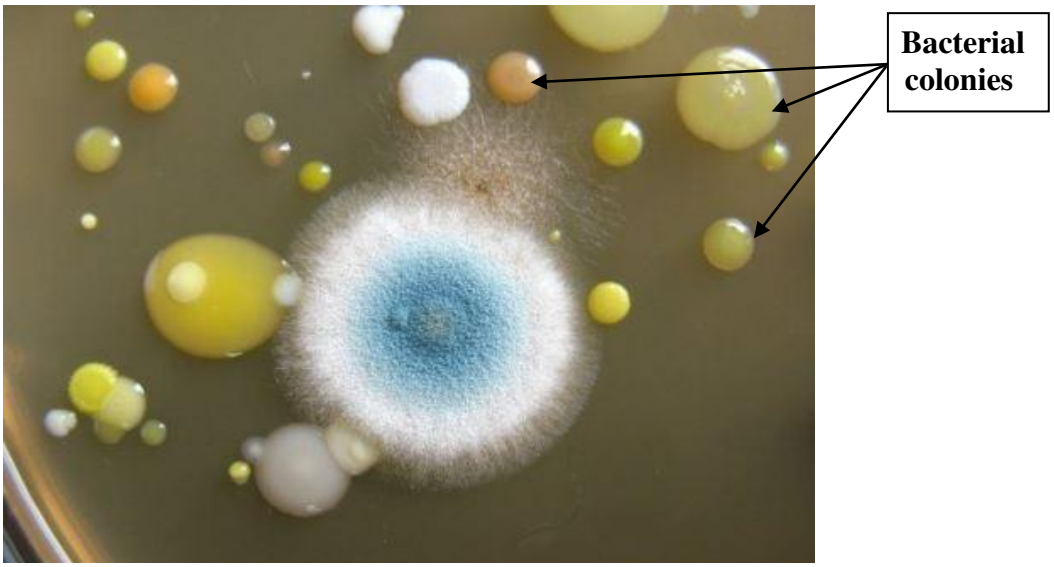


Figure 29 - Types of bacterial colonies in a dense media

A macroscopic study of the colonies in transmitted light is carried out with the naked eye, either under a microscope (x8 lens) or with a magnifier. The cup is turned upside down at a distance of better visibility. If there are two different types of colonies, they are numbered and described individually. The ***following features*** are taken into account:

1) The growth of colonies is described by great diversity, they can be ***isolated and merged***.

2) The ***features of growth*** - abundant, moderate, meager, then the uniformity of the forms of colonies (or heterogeneity);

3) ***The size*** of the colonies (large - 4-5 mm in diameter or more, medium - 2-4 mm, small - 1-2 mm, dwarf - not more than 1 mm);

4) ***The shape*** of the colonies is regular (round, oval) and irregular (rosette, rhizoid, etc.);

5) ***The degree of transparency and luster*** (is viewed in transmitted light) - dense - opaque, translucent, transparent, cloudy, thick, shiny, fluorescent colony;

6) ***The edges*** of the colony are smooth (S-shape), rough (R-shape), wavy, fringed, serrated, curl-shaped, rugged;

7) ***The color of the colony*** (is viewed in reflected light from the side of the lid without opening it) - grayish-white, colorless, cream, orange, green, bluish, golden, lemon yellow, red, blue, black, etc.

The color of the bacterial culture colonies depends on the color of the pigment they produce. In addition to the color (coloration) of the colonies, the ability of microorganisms to release pigment in the thickness of the medium (soluble) or on the surface, staining only the colony, and not the medium (insoluble pigment), is also noted;

8) ***The profile (relief)*** of the colonies is crater-shaped, convex, flat, conical, with a roller around the circumference;

9) ***The surface*** - smooth, bumpy, wrinkled, folded, furrowed, with concentric circles;

10) ***The consistency*** (is determined by touching the surface of the colony with a bacteriological loop) - dense (easily removed from the agar or grows into the thickness of the medium), tiny, brittle, mucous, viscous (sticks to the loop), pasty, oily;

11) ***The structure -*** homogeneous, fibrous, membranous, granular;

12) ***The position of the colony in a nutrient medium*** — towering above the medium, immersed in the medium, at the level of the medium — flat, tightly adjacent to the medium — flattened.

Thus, when studying the culturative properties of bacteria in liquid media, the above features are taken into account (Fig. 30), and the result is entered into the study protocol.

When viewing colonies under a microscope in a Petri dish, they are placed upside down on an object table, and tubes with an agar culture are placed on the sloping agar surface.

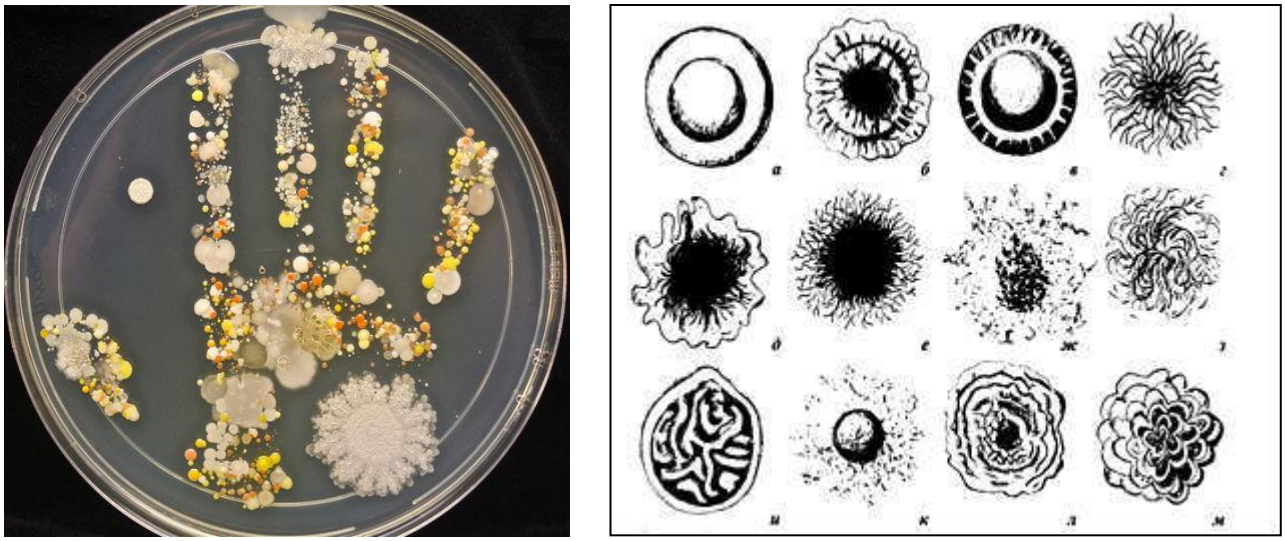


Figure 30 - Types of bacterial colonies

Control questions:

1. What is included in the concept of "cultural properties of bacteria"?
2. What signs are taken into account when describing the growth of bacteria in dense nutrient media?
3. Define a “colony of bacteria”.
4. Name the principle for identification of microorganisms.
5. Describe th bacterial growth in liquid culture media.

1.15 Enzymatic (biochemical) properties of microorganisms

The purpose of the lesson. To familiarize students with the types of enzymatic activity of bacteria. To master laboratory methods for determining the biochemical properties of microorganisms.

Goals:

1. To determine the saccharolytic properties, sow microbial cultures on media with carbohydrates (semi-liquid MPA with glucose, lactose, sucrose, on Endo agar).
2. To determine the proteolytic properties, sow in the MPF, in test tubes with MPB, fix indicator papers with tubes to determine indole, hydrogen sulfide, ammonia.
3. To determine the reducing properties of the microbes of their culture, sow in a medium with methylene blue and litmus.

Equipment: tubes with cultures from the previous lesson, tubes with pure cultures of Staphylococcus aureus, Salmonella, Escherichia, sterile Pasteur pipettes, spirit, 5% hydrogen peroxide solution, sterile forceps, sterile culture media: semi-liquid MPA with glucose, lactose, sucrose and BP indicators (tubes with liquid Gissa media - with Andreede indicator, with carbohydrates and floats), milk with methylene blue, milk with litmus, MPB, MPJ one tube, Endo (or Levine) agar and blood agar, indicator papers for determining hydrogen, indole, ammonia.

The enzymatic (biochemical) activity of microorganisms is rich and diverse due to the presence of specific enzymes in bacteria and environmental conditions.

Enzymes have a significant role in the life of microorganisms - they participate in various biochemical reactions that underlie the nutrition, growth, and reproduction of microorganisms.

Different types of microorganisms have a different set of enzymes. Under optimal conditions, bacteria produce permanent enzymes ***(constitutive enzymes)*** or a group of enzymes depending on the type of substrate ***(inducible enzymes).***

Certain types of microorganisms have the ability to break down only proteins and carbohydrates with the formation of final and by-products of decay, and some can oxidize or reduce various substrates. This circumstance served as the basis for the inclusion of the enzymatic properties of bacteria in the group of features for their *identification.*

Consider the basic enzymatic properties of bacteria and methods for their qualitative determination.

The biochemical (enzymatic) properties of bacteria include:

1. Saccharolytic properties.
2. Proteolytic properties.
3. Hemolytic properties.
4. Reducing / restoring properties.
5. Catalase activity, the presence of oxidase enzymes, plasmocoagulase, etc.

In laboratory microbiological practice, studying the biochemical properties of bacteria is one of the most important differential diagnostic methods for accurate recognizing the causative agent of an infectious disease. According to the enzymatic activity, it is possible to establish not only the species and type of the microbe, but also determine its variants (biovars).

Identification / correspondence is the studying of a certain complex of phenotypic traits of a microorganism, determination of the species composition of microorganisms.

Methods for determination of enzymatic (biochemical) properties:

1. Saccharolytic properties (saccharolytic activity of bacteria) - determination of carbohydrate fermentation.

This is the ability to break down sugars (carbohydrates) and polyhydric spirits to form acid or acid and gas.

A pure culture of bacteria is seeded on *differential - diagnostic media*, which include various carbohydrates and indicators. After incubation in a thermostat, the result of carbohydrate fermentation is taken into account (Fig. 31).

Depending on the genus and species of studied bacteria, media with appropriate mono- and disaccharides (glucose, lactose, maltose, sucrose, etc.), polysaccharides (starch, glycogen, insulin, etc.), higher spirits (glycerin, mannitol, etc.) are chosen.

While the process of fermentation bacteria form the final products: aldehydes, acids and gaseous products (CO_2, H_2, CH_4, H_2S).

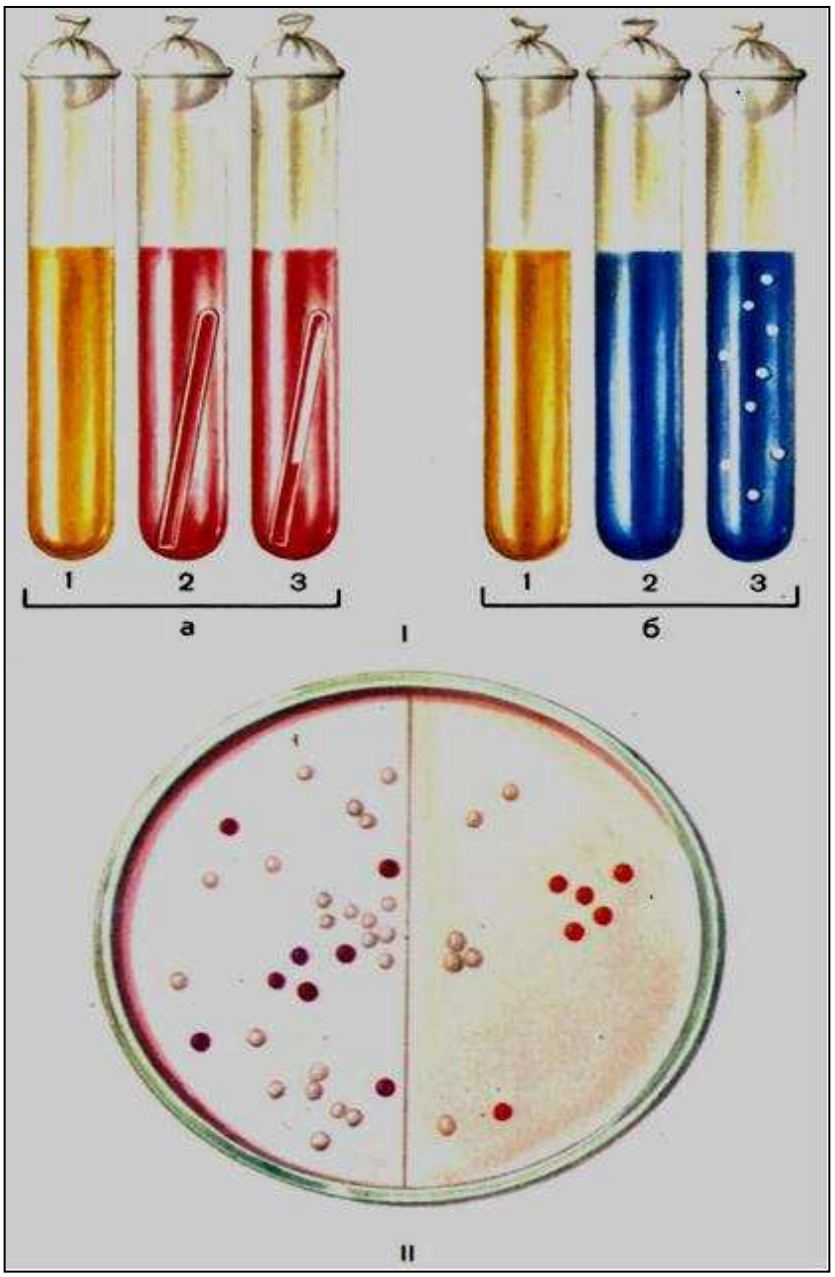


Figure 31 - Study of the saccharolytic activity of microorganisms.

I - *'motley row': a - liquid medium with carbohydrates and Andrede indicator; b - semi-liquid medium with BP indicator: 1 - microorganisms do not ferment carbohydrates; 2 - microorganisms ferment carbohydrate with the formation of acid; 3 - microorganisms ferment carbohydrate with the formation of acid and gas;*

II *- colonies of microorganisms that do not decompose (colorless) and decompose lactose (purple on the EMC medium on the left, red on the Endo medium on the right)*

Nutrient media are used to determine the enzymatic properties: semi-liquid with a BP indicator, liquid (Hiss medium) and dense (Endo, Levin, Ploskirev, etc.) media.

In semi-liquid media with the BP indicator, inoculation is performed by a loop dropped to the bottom of the tube. As a result of carbohydrate fermentation, the

indicator changes its color, gas bubbles are distributed in the nutrient medium and due to its viscosity, can remain for some time in the medium.

In liquid nutrient media - Gissa medium with the Andrede indicator (can be used: bromothymolblow, bromcresol purple, litmus, etc.). Because of the action of the acid formed during the cleavage of carbohydrate, the indicator changes the color of the medium. If there is no fermentation, the color of the medium does not change. Since bacteria do not ferment everything, only some of the carbohydrates that make up the Giss medium, a rather varied picture is observed. Therefore, a set of media with carbohydrates and a color indicator was called the "motley" row. Breakdown of carbohydrates is also detected by gas formation. The presence of gas is determined by the formation of bubbles in agar media or by its accumulation in a "float" on liquid media. ***"Float"*** is a narrow glass tube with a sealed end facing up, which is placed upside down in a test tube with medium before sterilization.

In dense differential - diagnostic media, for example: Endo medium, Ploskirev bactoagar, Levin medium, which include carbohydrates and an indicator, colonies grow in different colors.

For example, Escherichia coli fermenting lactose forms bright red colonies on Endo medium; Salmonella, not fermenting lactose, form colorless or light pink colonies on this medium. This is explained by the fact that bacteria, fermenting milk sugar (lactose) to acid, form colored colonies - the acid changes the color of the indicator in the medium. Colonies of microbes that do not ferment lactose are colorless.

The growth of microorganisms formed amylase on media with soluble starch lead to its splitting. It can be detected by adding a few drops of Lugol's solution to the culture - the color of the medium does not change. Uncleaved starch gives a blue color with this solution.

Milk, while the growth of microbes that ferment lactose, coagulates.

Thus, microbes that do not ferment a specific type of carbohydrate grow on media without changing its color and without the formation of gas.

2. Proteoletic properties - is the ability of bacteria to break down proteins, polypeptides.

To determine the *proteolytic* ability of microorganisms, the studied culture is seeded in MPG, milk, sometimes clotted horse blood serum, chicken's coagulated egg white.

Mostly for the detection of proteolytic enzymes, a pure culture of bacteria is inoculated by an injection in a column of meat-peptone gelatin, by immersing a loop in the interior of the medium to the bottom of the tube. The plating material is kept at room temperature (20-22°C) for several days. Moreover, not only the presence of liquefaction, but also its nature is recorded.

In those test tubes where proteolysis of gelatin occurs under the action of enzymes, the medium liquefies. The nature of the dilution caused by different microbes is different (Fig. 32).

Liquefaction can be layered (typical for Pseudomonas aeruginosa - Pseudomonas aeruginosa), staphylococcus - in *the form of a "stocking".* The nature

of gelatin dilution by the causative agent of anthrax resembles an *inverted Christmas tree*, also in *the form of a funnel* for aerobic bacteria.

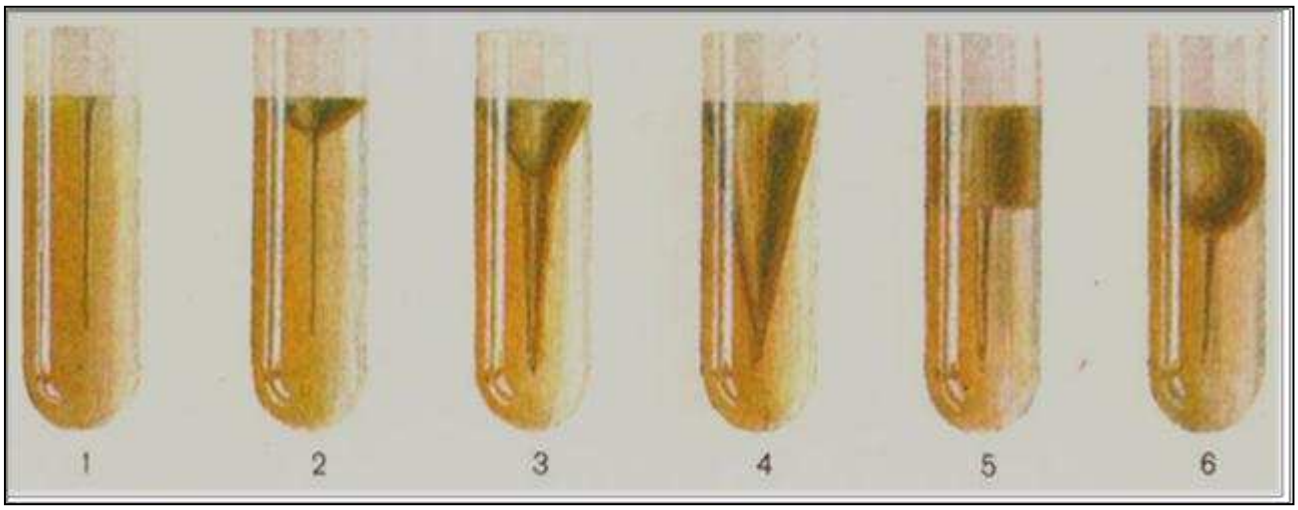


Figure 32 - Types of gelatin dilution under the action of bacterial enzymes

Microbes *that break down casein* (milk protein) cause peptonization of milk - it takes the form of whey. When peptones are cleaved, gaseous substances can be released - indole-C_8H_7N, hydrogen sulfide-H_2S, ammonia-NH_3.

The ability of microorganisms to hydrolyze casein is determined on *Aikman's milk agar*: 3 ml of sterile skim milk is added to 10 ml of melted in the water bath sterile MPA, mixed in Petri dishes and cooled. Plating is carried out with a loop and a spatula over the entire surface of the medium to obtain isolated colonies. They are kept in the thermostat for 24-48 hours. Proteolysis is manifested by casein peptonization - a clear zone of milk agar's enlightenment is formed around the colonies.

While inoculation into milk, proteolysis is expressed by the enlightenment of a column of milk, the appearance of loose or mucous sediment at the bottom of the tube.

The degree of proteolysis and the depth of protein breakdown in different types of bacteria is determined by the formation of the final decay products (indole, hydrogen sulfide, ammonia, etc.). Their formation is established using indicator papers (Fig. 33).

Reaction for indole. 1) The filter paper is impregnated with a hot saturated aqueous 12% solution of oxalic acid, dried on the air, cut into strips (10x12 cm) and stored in a special jar with a ground lid. To detect the formation of indole, the studied bacterial culture is inoculated into a test tube with MPB or Hottinger broth, where an indicator paper is inserted, pressed on the end with a cotton stopper (the lower edge of the paper should not touch the nutrient medium). It is maintained in the thermostat for 1-3 days at a temperature of 37 ^{0}C. If there is the *indole formation*, the lower part of the indicator paper turns *pink*.

2) Ehrlich method: filter paper is impregnated with a warm mixture of Ehrlich reagent, dried and placed in a test tube with a pure culture of bacteria. If there

is the *indole formation,* the lower end of the paper (attached, as in the pre-drying method) is painted *from dark pink to intense crimson* (other color, not taken into account).

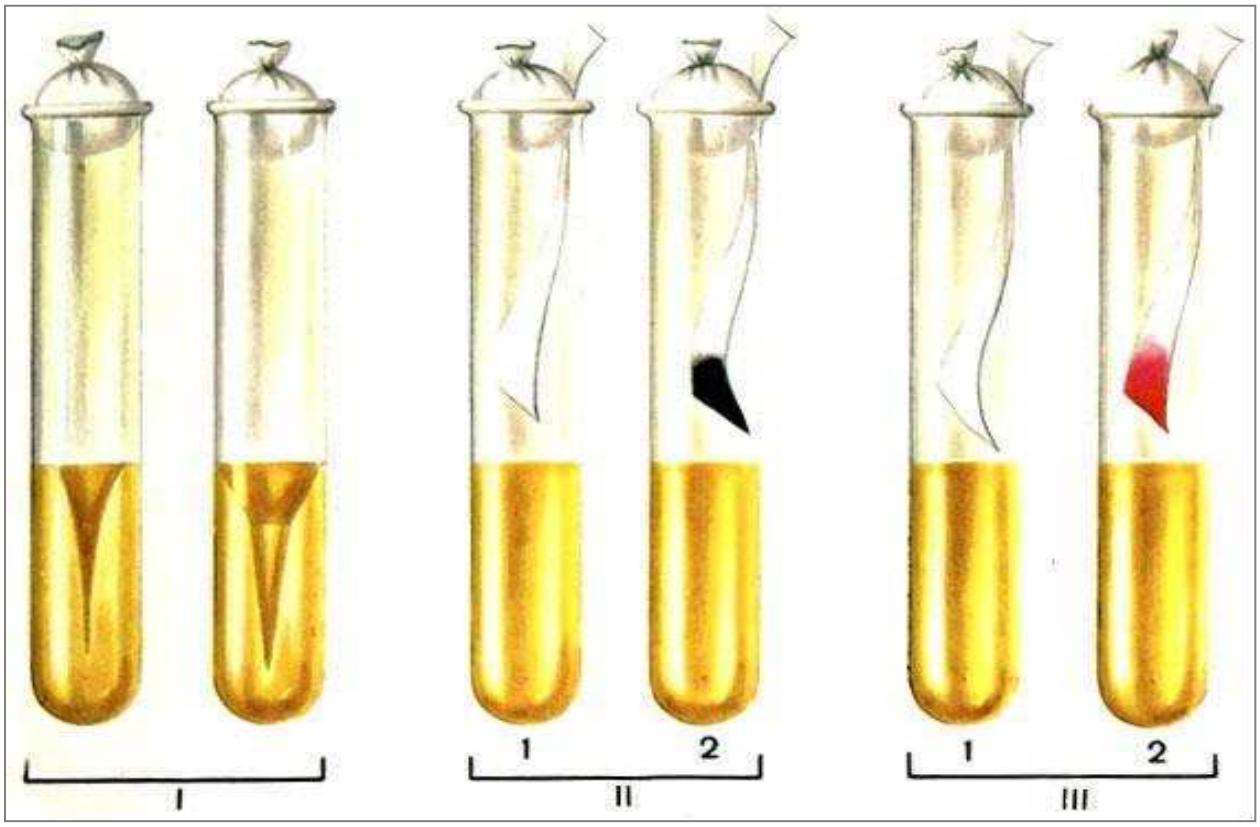


Figure 33 - Proteolytic properties of bacteria.

I - forms of dilution of gelatin; II - determination of hydrogen sulfide (the paper turns black); III - determination of indole: 1 - negative result (paper is unchanged); 2 - a positive result (staining in pink color of indicator paper).

Determination of sulfuretted hydrogen. 1) By using a special dense medium with the addition of iron sulfate and an indicator - phenolroth. The medium is poured into sterile tubes and cooled in the inclined position of the tube. Plating is done on the sloping surface of the agar, then with an injection into the lower part of the medium column. If there is *the sulfuretted hydrogen* under the influence of a growing bacterial culture, the medium column *turns red*, the lower part *turns black.*

2) By using a liquid medium (peptone water). A strip of filter paper, soaked in 10% solution of acetous plumbum or ironic sulphate, is placed in a test tube. If there is the sulfuretted hydrogen, a strip of filter paper turns *black* (sulfur plumbum or ironic sulfide of black color is formed).

Reaction for ammonia. 1) In a test tube with a plating bacterial culture, a pink litmus test is fixed between the wall of the test tube and the cork. Coloring a piece of paper in blue indicates the presence of ammonia.

2) A drop of broth culture of microbes is poured into a porcelain cup by pipette and mixed with a drop of Nessler's reagent. If there is ammonia (depending on its

quantity), the culture with the indicator acquires *yellow or brown souring*.

Additionally, the proteolytic effect of microorganisms can be observed during inoculation on ***coagulated blood serum***; recesses and dilution zones appear around the colonies.

3. Hemolytic properties (the ability to destroy red blood cells).

The hemolytic activity of bacteria is an important indicator of their virulence, therefore, the determination of hemolytic properties in laboratories is a mandatory search.

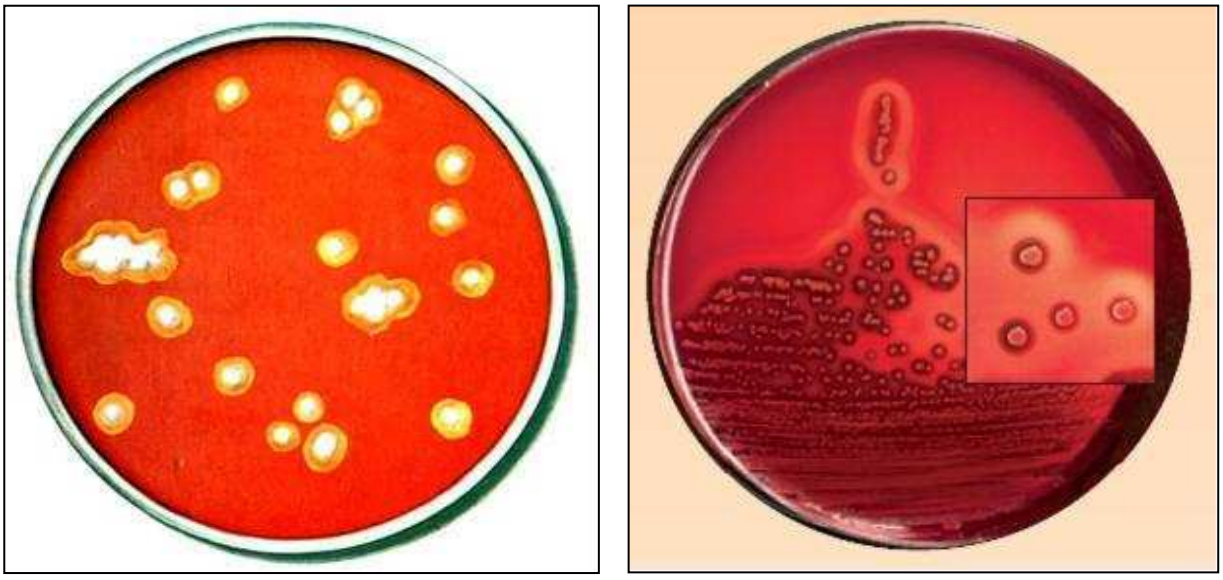
Bacteria of certain species in the process of life produce special substances - ***hemotoxins (hemolysins)*** - that have a lysing effect on red blood cells. These substances of a protein nature destroy the membrane of red blood cells.

To determine the hemolytic ability of a bacterial culture, nutrient media containing 5% defibrinated blood - blood of MPA is used. Plating is carried out according to a generally recognized technique.

While the growth of microorganisms with hemolytic properties, a transparent zone (colorless or light colored) — ***a hemolysis zone*** (Fig. 34 a, b) —is formed around the colony as a result of erythrocyte lysis.

Depending on the color of the hemolysis zone (colorless, green), it is possible to determine the type of hemolysin (alpha, beta, gamma, delta, etc.).

The medium becomes transparent (red lacquered blood) in liquid media during hemolysis.



a) **b)**

Figure 34 - Hemolysis around colonies growing on agar with blood

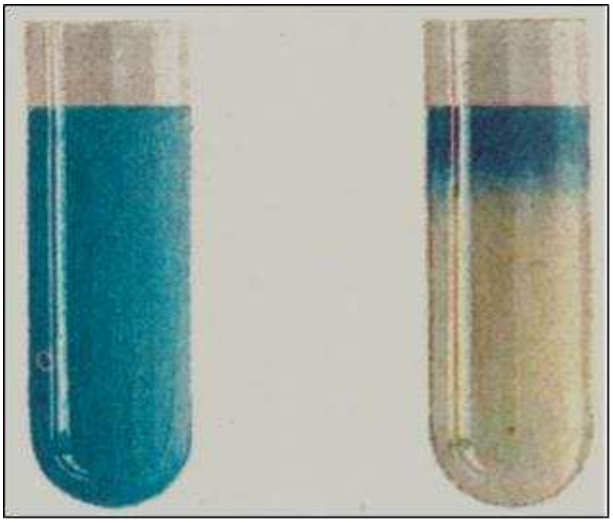
4. Reducing properties – are determined on the basis of a change in the color of organic paint input into the nutrient medium (often in milk).

To determine the reducing (restoring) ability, dyes are used - methylene blue, thionine, litmus, neutral red, etc. A solution of one of these dyes is added to a MPB or sterile milk. The loop of the studied culture is sown on medium with a dye, incubated in a thermostat for 24 hours. Under the action of microbial enzymes, the dye is restored, it becomes discolored or the original color changes.

Determination of reducing properties on Minkevich's medium (milk with litmus tincture). The plating material is put in a thermostat for 10 days, tubes are scanned daily. Litmus reduction is manifested by complete discoloration of milk. Acid and alkali formation are detected on the same medium. In the first case, litmus milk turns pink, in the second it turns blue (Fig. 35).

When taking into account the time of indicator bleaching, it is possible to assess the degree of reducing (restoring) ability of microorganisms.

At present, Rotberger medium (MPA with 1% glucose and a few drops of a saturated solution of neutral red) is also widely used. In a positive reaction the red color of the medium turns into yellow.



a) b)

Figure 35 - Reducing properties of bacteria in milk

5. Determination of catalase activity. Catalase decomposes peroxide of hydrogen into water and molecular oxygen. 1) To detect catalase, 1-2 ml of a 1% hydrogen peroxidase solution is poured onto the surface of a 24-hour culture on a beveled MPA. The appearance of gas bubbles at an inclined position of the tube is recorded as a positive reaction. As a control, a culture known to contain catalase should be investigated in parallel.

2) It is necessary to apply 1 drop of 1-3% hydrogen peroxidase to a glass slide and add a bacterial culture with the bacterial loop. The appearance of gas bubbles indicates the presence of the enzyme catalase - a positive catalase reaction (Fig. 36).

Figure 36 - Positive reaction to catalase

6. Determination of plasma coagulation - under the influence of the pathogenicity enzyme of bacteria - coagulase, coagulation of blood plasma occurs.

It is one of the most reliable tests for detecting the pathogenicity of staphylococci. For its production, 1-2 ml of sterile plasma is poured, a bacterial culture is input with the bacteriological loop and placed in a thermostat. It is necessary to check the results after 30 minutes, 2, 4 hours and the next day. Coagulation occurs (coagulation of plasma) in a positive reaction.

7. Determination of the DNAse enzyme.

The sample of DNA is added to the molten MPA in a rate of 2 mg per 1 ml of medium. The plating material is carried out in the form of strips and placed in a thermostat for 18-24 hours. 5-6 ml of HCl is poured on the grown staphylococcus. The presence in the culture of an enzyme that breaks down DNA is detected by the formation of transparent zones around staphylococcus colonies.

In recent years, microbiological laboratories begin to use **special test systems** for determining the biochemical properties of bacteria: "Roche", "API", "Enterotest", biochemical plates and disks. They are convenient to use, reliable, allow identifying 12-20 and more various signs, facilitating the identification of microbes.

In order to establish the ***species affiliation of bacteria***, their **antigenic structure** is studied by identification of their antigenic properties. Each microorganism has different antigenic substances. In particular, representatives of the Enterobacteriaceae family (Escherichia, Salmonella, Shigeli) contain shell - O-antigen, flagellum - H-antigen and capsular - K-antigen. They are heterogeneous in their chemical composition, therefore exist in many versions. They can be determined by using specific agglutinating sera. Such determination of the type of bacteria is called **serological identification** (section III). It is attributed to one of the main methods of establishing the type of isolated culture.

Sometimes bacteria are identified by ***infecting laboratory animals*** with a pure culture and observing the changes caused by pathogens in the body (tuberculosis, botulism, tetanus, salmonella and the like). Such method is called identification by biological properties (paragraph 1.19).

The final conclusion about the identification of bacteria is based on the study of morphological, cultural, biochemical, antigenic, biological and other properties of microbes. For example: "Escherichia coli is isolated " or "Isolated pathogen belongs to the species Staphylococcus epidermidis".

Control questions:

1. Name methods for the determination of saccharolytic properties.
2. Methods for the determination of indole, sulfuretted hydrogen, ammonia.
3. How to determine the reducing properties of microbes?
4. What does determination of hemolytic properties of bacteria include?
5. What is the essence of the plasma coagulation reaction?

1.16 Methods for determining the motility of bacteria

The purpose of the lesson. To master the methods of studying bacteria without staining, but in a living state.

Goals:

1. To learn the “hanging drop” method.
2. To learn the “crushed drop” method.

The mobility of the bacteria is determined on a liquid nutrient medium by isolating a pure culture. Motility or its absence in bacteria is one of features to determine their type.

The organs of movement in some types of bacteria are flagella. They consist of protein substances that are different from the proteins in the cell. Flagella are very thin formations, their diameter is about 0.02-0.05 microns. They are longer than bacteria. The movement of bacteria is associated with the spiral movements of the flagella.

It is impossible to examine flagella of bacteria under a conventional light microscope, because their small sizes lie beyond the resolution of the microscope. Flagella of bacteria are detected in various ways. Their structure and other details are studied by electron and phase-contrast microscopy. The location and number of flagella can be examined under a light microscope by using the “super dye” according to the Leffler method. The complexity and inaccessibility of these methods precludes their use in practical laboratories. According to the location of the flagella, motile microbes are divided into 4 groups:

1. Monotrichi - bacteria with one flagellum at the end.
2. Lofotrichi - bacteria with a bundle of flagella at one end.
3. Peritrichi - bacteria with flagella all over the surface of the body.
4. Amphitrichi – bacteria with flagella at both poles of the cell.

In practice, the study of microbes in a living state is used to determine mobility, it is indirect confirmation of the flagella presence. The movement of microbes can be observed in such preparations as *“crushed drop” or “hanging drop”*. These preparations can be microscopied with a dry or immersion lens. The best results are obtained by microscopy in a dark field of view.

The "Hanging Drop"preparation of microorganisms’ living cells.

A drop of suspension from microorganisms and physiological saline is looped on a cover slip. Then it is turned upside down and placed on a special glass slide with a recess (hole) in the center. The edges of the wells are pre-lubricated with petroleum jelly, due to it the cover slip adheres to the subject glass. The drop should hang freely, without touching the edges and bottom of the hole. The drop is sealed in a humid chamber, it makes possible to observe the object for many days. Sterile glasses are used and a suspension of microorganisms is prepared in a liquid nutrient medium for long-term observations.

Installing a hanging drop under a microscope requires certain rules to be followed, otherwise the cover slip will be crushed by the lens. Firstly, at low magnification, (with the condenser lowered and the diaphragm half closed), the edge

of the drop placed in the center of the field of view is found. The glass is fixed with clamps in this position. After that, a drop of cedar oil is applied on the coverslip, into which, under the control of the eyes, the immersion lens is lowered until it comes into contact with the glass, then, be raising the condenser slowly and increasing the illumination by partially opening the diaphragm, the focus is set. The edge of the drop allows quick setting of the immersion lens into focus.

It should be borne in mind that each active movement with the help of flagella makes bacteria cross the entire field of view and make circular movements.

The "Crushed drop" preparation.

A crushed drop is prepared by covering with a coverslip a drop, made from a mixture of saline and microbes, placed on a glass slide. Microorganisms grown in a solid nutrient medium are transferred to a drop of water with a bacteriological loop, grown in a liquid medium with a sterile pipette. In this case, a drop of water on the glass slide can not be applied. A drop of the test material should be so small that after pressing it with a coverslip there will not be an excess of liquid protruding under it. Otherwise, excess liquid must be removed with filter paper.

A drop of cedar oil is applied on the coverslip and the preparation is examined under an immersion microscope system with a slightly darkened condenser.

The disadvantage of the "crushed drop" method is the quick drying of the drug. It is recommended to lubricate the edges of the coverslip with Vaseline, if it is prolonged microscopy of the preparation.

In order to identify large microorganisms visible with small and medium microscope lenses, the preparations are viewed without coverslips. The prepared smear is allowed to dry at room temperature. Before the test, a drop of solution is applied, then, it is immediately shaken off on filter paper. The solution layer remained on the surface replaces the coverslip.

Control questions:

1. Describe the technique of preparation of the "Crushed drop" drug.
2. Describe the preparation of the "hanging drop".
3. What are the disadvantages of drugs in determining the motility of bacteria?
4. What are the functions of flagella in bacteria?
5. Bacterial groups according to the number of flagella

1.17 Sterilization methods

The purpose of the lesson. To familiarize students with the basic sterilization methods, their purpose and practical use, the devices for each method. To learn the rules of preparation laboratory glassware and instruments for sterilization.

Goals:

1. To prepare dishes for sterilization - cut paper into strips and wrap graduated pipettes into it, make cotton tubes for test tubes, wrap tubes and Pasteur pipettes in paper in 10-15 pieces, put paper caps on cotton caps and tie them with twine. To sterilize prepared material in a drying cupboard.

2. To filter the dye solutions to demonstrate the phenomenon of adsorption during filtration.

3. To know the device of the autoclave, Koch boiler and other sterilizing devices.

Equipment: syringes, needles, tweezers, scalpels, scissors, etc ; Koch apparatus, water bath, drying cupboard (Pasteur oven), autoclave for demonstration; bactericidal ultraviolet lamps, Seitz bacteriological filters; cotton wool, clean pipettes, graduated and Pasteur, flasks, Petri dishes, test tubes, paper, bix.

Sterilization is a process aimed at the complete destruction of all living (pathogenic and non-pathogenic) microorganisms and their forms in sterilized objects, both in laboratories and in industrial conditions.

In bacteriological laboratories, nutrient media, glassware (pipettes, tubes, Petri dishes), instruments, cotton and gauze swabs, and others are sterilized. For special conditions of aseptic work, air and necessary items are sterilized in boxes.

Asepsis is a set of measures aimed at preventing the ingress of microorganisms into the tissues and cavities of the human and animal body from the external environment during medical or diagnostic manipulations, as well as the sterilization of instruments and cultures during laboratory research. Asepsis ensures the observance of basic sanitary and hygienic measures, clean hands of staff, sterilization of materials and tools.

Antisepsis – is a set of therapeutic and preventive measures aimed at the destruction of microorganisms that cause an infectious process in damaged areas of the skin or tissues; decrease intoxication in body, caused by microbial infection of the wound, and increase in the protective forces of the animal.

Disinfection is a set of measures for the destruction of pathogenic microorganisms in the environment by chemical methods.

The main purpose of sterilization in microbiological and immunological studies is to prevent the penetration of microbial cells into the macroorganism and the contamination of nutrient media.

There are physical, chemical and mechanical sterilization methods.

Physical sterilization methods include the action of high temperature, ultraviolet rays, ionizing radiation, ultrasound, etc. *Chemical methods* are used to treat vaccines, serums, and other objects preserved by various antiseptics. *The mechanical method* is filtering liquids through bacterial filters. By using this method, the liquid is cleaned from the bacteria. Filterable forms of bacteria and viruses pass through such filters.

Their mechanisms of action are not the same, but when choosing any of them, two basic requirements must be satisfied: achieving full provisioning and maintaining the physicochemical properties of the sterilized material.

Physical sterilization methods include:

1. Sterilization by dry heat - flaming, dry heated air;
2. Sterilization by wet heat:
- boiling;
- flowing steam at 100 ° C;
- fractional sterilization at a temperature below 100 ° C;

- steam sterilization under pressure with a temperature above 100 ° C;
- pasteurization;

3. Irradiation by electromagnetic waves:
- ultraviolet rays;
- ultrasound;
- gamma and x-ray irradiation.

1. Dry heat sterilization methods

Flaming (from lat. *flamma* - flame and french *flambé* - burn) – is sterilization by calcining small metal or glass objects in a flame. Bacteriological loops, metal tweezers, glass spatulas and tubes, glass slides, etc. are sterilized due to this method. The flame temperature is about 1000ºC. During annealing all microorganisms burn down (vegetative and spore forms). This is a quick and reliable way to sterilize.

Dry heat sterilization.

It is produced by hot air in a Pasteur oven or drying cupboard. Pasteur's oven is a double-walled closet covered with asbestos from the outside for thermal insulation. Inside the cabinet, there are metal shelves with holes, where the sterilizable material should be placed (Fig. 37).

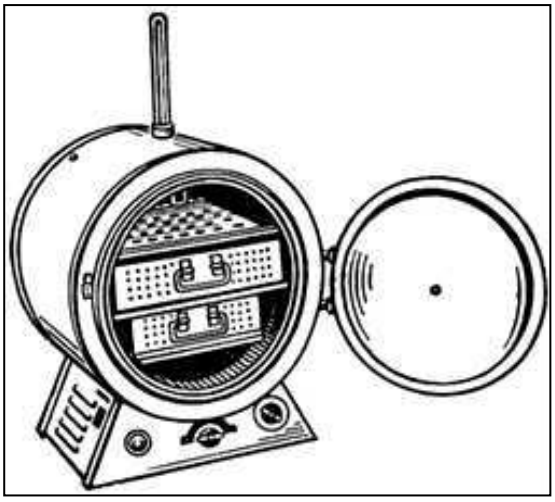


Figure 37 – drying cupboard

Electric heating. Sterilization is carried out at a temperature of 160-180ºC, within 1-2 hours from the moment of reaching these temperatures (controlled by a thermometer).

Dry glass heat mainly sterilizes glassware - Petri dishes, pipettes, spatulas (wrapped in paper), as well as test tubes and flasks. This technique is reliable - non-spore and spore forms of microorganisms die. Wrapped in paper and sterilized material can be stored.

2. Wet heat sterilization methods

Boiling is one of the easiest methods of sterilization.

It is carried out in a sterilizer - a metal rectangular box with a lid and a net at the bottom for sterilizable objects. Water is poured into it and heated to a boiling point (electric heating or on fire). Boiling can last from 15-30 minutes to 2 hours, at a temperature of about 100ºC (Fig. 38).

Small metal or glass objects are sterilized - syringes, needles, glass tubes, etc. In this case, vegetative forms of microorganisms and part of the spores die.

The scalding of the material to be sterilized with boiling water is also practiced.

Figure 38 - Sterilizers for boiling

Flowing steam sterilization. It is carried out by hot humid air in the *Koch apparatus*, which is widely used in laboratory practice.

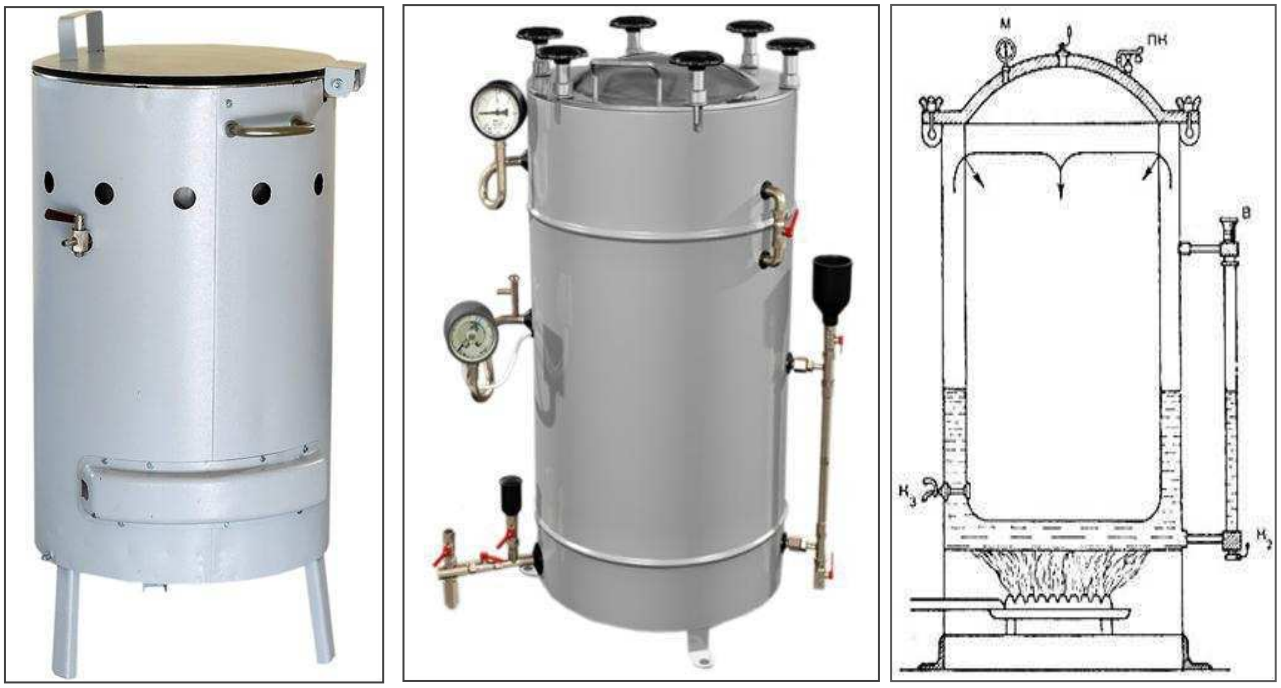
The Koch apparatus (boiler) is a metal cylinder coated with a heat-insulating material. In the conical cover of the apparatus there is an opening for the exit of steam. In the lower part there are a crane and a water measuring tube. The stand for sterilizable materials is inside. Water is poured into the bottom of the apparatus, which should not come into contact with the material being sterilized. The device is tightly closed with a conical lid with a hole and heated over a fire. When the water boils, hot water vapor with a temperature of about 100 ºC will "flow" as a strong stream from the opening of the lid. From this moment, the beginning of sterilization is noted (Fig. 39, a).

Flowing steam sterilization lasts from 45 minutes to 1.5 hours, depending on the volume of material being sterilized. During sterilization at 100 ° C for 30-60 minutes, only vegetative forms of microorganisms completely die, but spores persist, and germinate at room temperature in a day. This sterilization method is not completely reliable.

To achieve complete sterilization of the media, *fractional sterilization* is used in the Koch apparatus. Repeated exposure by steam neutralizes vegetative forms. A third warming up kills all other bacilli that develop from spores.

This method (fractionally) sterilizes nutrient media that cannot be heated above 100 ° C, and other materials that change their properties at temperatures above 100 ° C: milk, gelatin, potato media and media with carbohydrates. For example, meat-peptone gelatin is sterilized fractionally, because the gelatin liquefies and does not freeze at temperatures above 100 ° C.

Steam sterilization can be autoclaved with the unscrewed cap and the opened outlet valve. Serum, egg and other media are sterilized with fluid steam and compacted at the same time. Coagulation of serum occurs at 80-90 ° for an hour, in a special double-walled coagulant coated with heat-insulating material.



a) b) c)

Figure 39 - **a)** Koch apparatus; **b)** Autoclave; **c)** Autoclave structure

Flowing steam sterilization under pressure (autoclaving) is carried out by saturated water vapor in an autoclave.

Autoclaves come in a variety of designs. *The vertical autoclave* is a massive double-walled boiler, surrounded outside by a metal casing, with a heavy hinged lid, tightly screwed to the boiler with bolts. At the bottom of the boiler there is a funnel and a tap through which water is poured into the free space between the internal boiler and the casing. The material to be sterilized is laid on the bottom of the boiler, the lid is tightly screwed, the electrical heating is turned on. When the water boils, steam passes into the internal boiler. The pressure in the autoclave rises during the increase in temperature. When the pressure gauge shows that the pressure in the boiler has reached the set value, the start of sterilization is noted. The pressure is maintained at a certain level by using a safety valve (Fig. 39- b, c).

Typically, in laboratory practice, sterilization under pressure is carried out at 1.5-2 atm and a temperature of about 115-120 ºC for 20-30 minutes. After the time of sterilization has passed, the heating is stopped, the steam valve is opened and the vapor releases.

At a pressure of 0.5 atm. the temperature in the autoclave is about 110-112 ° C, at 1 atm - 120-121 ° C; at 1.5 atm - 124-126 ° C; at 2 atm - 132-133 ° C.

These nutrient media are sterilized in an autoclave - MPB, MPA, glassware with water, bandages, surgical instruments, etc.

Autoclaving is a fast and reliable method of sterilization, in which all forms of microorganisms die, even the most resistant spores.

Fractional sterilization is based on the processing of sterilized material in several stages. Boiling or sterilization with fluid steam in a Koch apparatus can be fractional. Sterilization is usually carried out for 3 days for 30 minutes everyday by

one of the indicated methods, and at breaks the medium is left at room temperature. This is done to provoke the growth of spores in vegetative forms and their destruction during subsequent processing, which increases the reliability of these techniques.

Tyndalization is a type of fractional sterilization (fractional pasteurization). The method is named after the English scientist D. Tyndall who proposed it. It is carried out in a water bath at a temperature of 56-58ºC for 1 hour with 5-7-fold repetition after 24 hours. The material is kept at room temperature in the intervals between heating.

The multiplicity of heating depends on used temperatures: at 70-80 ° C for 3 days, 60-65 ° C for 5 days, 56 -58 ° C for 6-7 days.

Tyndalization at 56-58 ° C is subjected to materials that break down at a higher temperature (colloidal solutions, blood serum, etc.), protein-containing substances. As a result of tindalization, complete destruction of microorganisms is achieved.

Pasteurization (partial sterilization). This method was proposed by the French scientist L. Pasteur and named after him. The method is based on the preservation of the nutritional value of a food product (milk, meat, fish and canned vegetables), which decreases when boiled.

The method is aimed at the destruction of indisputable / vegetative forms, of pathogenic species while spores remain viable.

The pasteurization method consists in the fact that various products are neutralized by a single heating to 65-70° C for 1 hour or up to 70-80° C for 5-10 minutes, then they are cooled rapidly (to 4-8° C). Sudden cooling and subsequent storage at low temperature (4-5° C) prevents the subsequent germination of spores.

It is used for liquid media and materials that change their physicochemical properties at high temperatures.

Such products are pasteurized: milk, cream, wine, beer, juices, etc. Milk is pasteurized in order to free it from lactic acid and pathogenic bacteria (pathogens of tuberculosis, brucellosis, staphylococcosis, etc.). During pasteurization of beer, wine, fruit juices, pathogens of diseases of these drinks are destroyed.

At the same time, vitamins and palatability of the products are preserved. Pasteurized products are not subject to long-term storage, because due to this method of sterilization only vegetative forms of microorganisms die and spores remain. Pasteurized products are stored in a refrigerator.

В лабораторной практике этим способом пользуются главным образом для отделения спорообразующих видов от неспорообразующих.

3. Methods of exposure by electromagnetic waves:

Irradiation with electromagnetic waves is used for disinfection and sterilization of thermolabile materials.

- Ultraviolet (UV) rays – the length of wave is about 250 and 270 nm. Bactericidal lamps are widely used for air purification in boxes, operating rooms and other rooms.

Vegetative forms are more sensitive to radiation than spores, which are 3-10 times more stable.

- Gamma and X-ray irradiation are widely used for sterilization of antibiotics, hormones, plastic products of single use (syringes, Petri dishes, pipettes, etc.).

- Ultrasound can be used to disinfect milk, water, and other food products.

Chemical sterilization methods

The application of these methods is used for the prevention of microbial contamination of nutrient media, vaccines, diagnostic and therapeutic serums by including various chemical preservatives in their composition.

Nutrient media can be preserved with chloroform, toluene, ether. Before the plating such media are heated at 56 ° C to release these substances.

In the biological industry, vaccines and treatment sera are preserved with carbolic acid, bringing the concentration to 0,25-0,5%, formalin to 0,05%, and thiolate to 1:5000 - 1:10000.

Serum canned by boric acid, toluene or glycerin.

Various chemicals have been widely used for disinfection in laboratory practice. For this purpose, solutions of carbolic acid (3-5%), chloramine (1-3%), ethyl spirit (70°), etc. are used.

Chemical methods include *sterilization by gases*. The sterilizing gases are: formaldehyde, ethylene oxide and propiolactone. The advantage of gases as sterilizing substances is that their use does not require heating; in addition, they can be used to sterilize large volumes, such as rooms. However, great care must be taken while using gases, because they have a toxic effect on animals and humans.

Mechanical sterilization methods – are based on the filtration of liquids through fine-porous bacterial filters that trap microorganisms visible under the microscope. Viruses and mycoplasmas freely pass through them, therefore these methods should be recognized *as "partial" sterilization*.

1. Filtration is one of the very convenient methods for sterilizing those liquids that cannot be sterilized by other methods, for example, blood serum, nutrient media, etc.

Candle filters and membrane filters are widely used in microbiological practice.

Candle filters - have the form of a cylinder with thick walls and a cavity inside. One group of candles (Berkefeld) is made from ciliates. Larger pores in decreasing order are denoted by the letters W, N, V.

Membrane filters – are characterized by a sieve mechanism and a constant pore size. They are widely used and made from asbestos, paper, collodion, cellulose acetate. Typically, these filters are disks of various diameters. Such discs are penetrated by countless tiny cylindrical holes. Depending on the method of manufacturing the filters, the diameter of these holes can be from 1 μm to less than 0.005 μm.

Filtration of solutions through membrane filters is carried out under vacuum or under pressure. The effectiveness of sterilization is checked by direct plating of the filter sample on a nutrient medium.

In laboratory practice, membrane filters are mounted in a *Seitz device*, which consists of a glass (funnel) and a Bunsen flask; a rubber vacuum hose with a cotton filter is put on the tube of this flask (Fig. 40).

The liquid to be sterilized is poured into the funnel of the device, inside which a vacuum is formed and the liquid is filtered, so it becomesfree from bacteria.
The assembled device is wrapped in paper and sterilized by autoclaving.

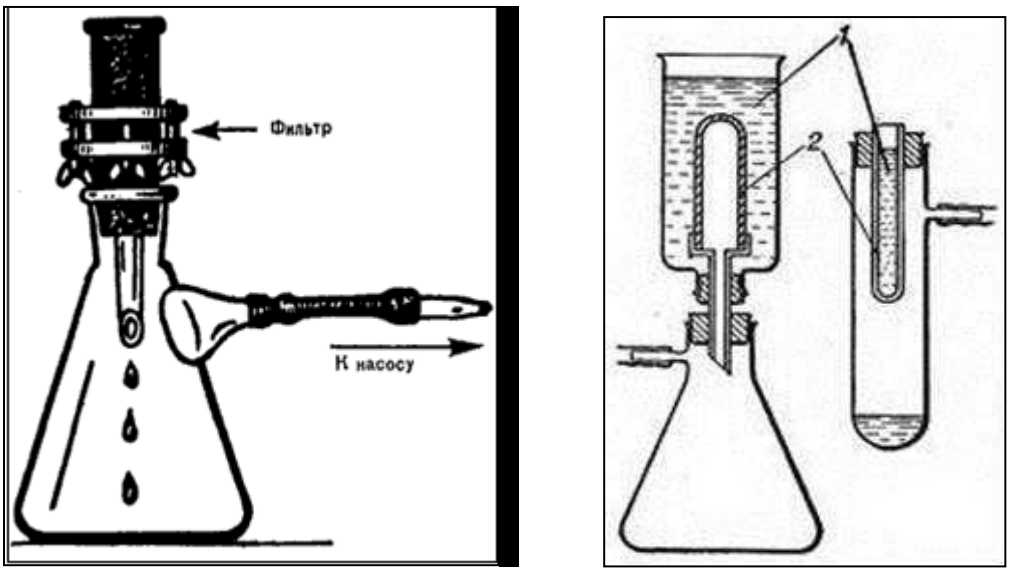


Figure 40 - Seitz device

Thus, it is necessary to wash and dry laboratory glassware for high-quality sterilization. Test tubes, vials, bottles, flasks are closed with cotton-gauze plugs. On top of the test tube, a paper cap is put on each vessel. The cups are sterilized in 2-4 pieces wrapped in paper.

To sterilize the pipettes, a piece of cotton wool is put into the top of each of them and then the pipettes are wrapped in thick paper, cut in strips of 2-2.5 cm wide and 50-70 cm long. The volume of the wrapped pipette is written on paper. Pipettes can also be sterilized in special cases.

Control questions:

1. What are the methods of sterilization?
2. What is the autoclave and what are its structure and purpose?
3. What is the essence of the steam sterilization method, when should it be used?
4. Describe Bacteriological filters, the principle and technique of filtration, verification of filtration results.
5. What is flaming and what is its application?

Tasks for self-training and knowledge control

1. Sterilization is:

A. Destruction of pathogenic microbes in objects or in the environment.

B. A set of measures aimed at preventing the penetration of microbes on (in) any object.

C. Complete destruction of all viable microbes in the object and their spores.

2. Physical sterilization methods include:

A. Gamma rays.

B. Gas sterilization.

C. High temperature.

D. Filtration through bacterial filters.

E. Ultraviolet rays.

3. What is sterilized in an autoclave at 1.0 atm (120 °) for 15 minutes?

A. Simple nutrient media (MPA and MPB).

B. Serum media.

C. Saline solution.

D. Nutrient media with carbohydrates.

E. Processed material (microbial cultures, corpses of laboratory animals, etc.)

4. What is sterilized with dry heat in drying cupboard?

A. Glassware.

B. Simple nutrient media (MPA and MPB).

C. Wadding, gauze.

D. Heat-resistant powdered medicinal substances.

5. Bacillus spores die while:

A. Autoclaving.

B. Pasteurization.

C. Sterilization by dry heat.

D. Prolonged drying.

6. Which of the following sterilization methods will be applied if the following mode of operation was used: fractional sterilization at a temperature of 56-58 °C for 5-6 days?

A. Autoclaving.

B. Pasteurization,

C. Tyndalization.

D. Boiling.

7. Which of the following sterilization methods will be applied if the following operating mode was used: 1 atm - 120 ° for 20 minutes?

A. Boiling.

B. Pasteurization.

C. Autoclaving.

D. Fractional steam sterilization.

8. Which method is the most suitable for killing bacterial cultures?

A. Pasteurization.

B. Autoclaving.

C. Filtration through bacterial filters.

D. Tyndalization.
9. Which of the following sterilization methods will be applied if the following mode of operation was used: temperature of 100 ° C for three consecutive days, for 30 minutes everyday?
A. Boiling.
B. Autoclaving.
C. Pasteurization.
D. Fractional steam sterilization.
10. What is the operating mode used for pasteurization:
A. 100 ° - 30 min.
B. 60 ° - 20 min.
C. 80 ° - 10 min.
D. 80 ° - 30 min.
11. What is the autoclave's normal state of working while sterilizing infected material:
A. 80 ° at a pressure of 1.0 atm.
B. 56 ° at a pressure of 0.5 atm.
C. 126 ° at a pressure of 1.5 atm.
D. 126 ° at a pressure of 2.0 atm.
12. Which of the following methods should be used to sterilize nutrient media with carbohydrates?
A. Fractional steam sterilization.
B. Boiling.
C. Pasteurization.
D. Autoclaving.
13. What method can be used to pasteurize blood serum:
A. Boiling.
B. The tindalization method.
C. Pasteurization.
D. Filtration through bacterial filters.
14. Disinfectants are:
A. Chloramine.
B. Erythrin.
C. Streptomycin.
D. Phenol.
E. Diamond greens.

1.18 Methods for determining the antibiotic susceptibility of bacteria

The purpose of the lesson. To master the methods for determining the antibiotics sensitivity and resistance of bacteria.

Goals:

1. To inoculate bacterial cultures on MPA Petri dishes.
2. To place standard discs with four or five antibiotics on the surface of the agar with the colonies of bacteria.
3. To master the technique of determining the zone of growth inhibition of bacteria around the disk (on the demonstration cups).

Equipment: for independent work for a group (2-3 people) of students it is necessary to have two sterile Petri dishes with MPA (pH 7.2 - 7.4), tests tube with a pure culture of Staphylococcus aureus and Escherichia (or Salmonella), hatched under numbers 1 and 2; two measure sterile pipettes for 2.0, sterile tweezers, standard discs with different antibiotics, sterile Pasteur pipettes, a rubber bulb, a ruler (or a strip of graph paper of 5 cm). For demonstration in the laboratory there should be tables, two Petri dishes with daily agar culture of Staphylococcus aureus (test microbe) with paper disks soaked in penicillin solutions in different concentrations; two Petri dishes with MPA: in one of dishes - daily (or 18-hour) culture of E. coli or Salmonella, in the other - Staphylococcus aureus with discs of diluted antibiotics; tubes with BCH containing serial diluted penicillin and Staphylococcus aureus culture.

While isolating a pure bacterial culture, it is important to determine their antibiotic sensitivity in order to select the optimal drug for subsequent treatment.

Antibiotics are specific vital products of bacteria, fungi, plants (phytoncides) and animals, possessing activity against microorganisms of certain groups, capable of inhibiting their growth *(bacteriostatic effect)* or completely suppressing their vital activity (*bactericidal effect*). The mechanism of the antagonistic action of antibiotics leads to disruption of metabolic processes in the microbial cell.

The sources of antibiotics are microscopic fungi (penicillin), actinomycetes (streptomycin, tetracycline), bacteria (gramicidin, polymyxin). Antibiotic substances are also extracted from plant cells (volatile onion, garlic) and animal tissues (lysozyme, ecmoline).

Antibiotics are drugs with antimicrobial effects. They have been widely used for the treatment of many diseases of animals and humans. Every year the arsenal of antibiotic drugs manufactured by the industry expands.

The creation of a large number of various antibiotics is caused, on the one hand, by the search for increasingly effective therapeutic agents, and on the other, by the fact that, because of the widespread use of antibiotics, their therapeutic effect is reduced due to the emergence of *resistant forms* of microbes.

Antibiotics are prepared industrially in the form of sodium, potassium, calcium salts and are released in special packages. With the release of an antibiotic (industrial or laboratory), control of its activity is mandatory.

The biological activity of antibiotics is expressed in ***units of action*** (UA) contained in 1 ml of solution (UA / ml) or in 1 mg of the drug (UA / mg).

For one unit of action the minimum amount of antibiotic, which inhibits the growth of a standard test - microbe in a strictly defined volume of the nutrient medium, is taken.

While determining the activity of an antibiotic, a test microbe, which has the highest sensitivity to this antibiotic, is used. For example, for penicillin the test-microbe is Staphylococcus aureus, 209-P strain, for tetracycline and streptomycin - B. subtilis strains, for chloramphenicol - E. coli biomycin.

It was found that most antibiotics in 1 ml of the substance contain 1000 UA. The UA of biological activity for different antibiotics is different: 1 unit of penicillin is equivalent to 0.6 μg; streptomycin - 1 mcg; neomycin - 3.3 μg of pure substance. The amount of antibiotic equivalent to 1 UA is called the *international unit of action* (IUA).

The effectiveness of the use of antibiotics largely depends on the sensitivity of the pathogen to the antibiotic, so there was a need to determine the sensitivity of microorganisms to these drugs.

In laboratory practice, the **following methods** are used to determine the sensitivity of bacteria to antibiotics:

1) The method of serial dilutions;

2) Method of diffusion in agar (disc-diffusion method or standard paper disc method).

Serial dilution method

It includes the following main stages: the choice of nutrient media, the preparation of antibiotic solutions, the preparation of microbial cultures for research and the accounting of results.

The nutrient medium should ensure optimal growth of the culture of the microorganism (pathogen).

For aerobic bacteria, mainly:

- Liquid nutrient media - BCH (pH 7.2-7.4) and Hottinger broth with a content of 180-200 mg% of amine nitrogen (pH 7.4-7.6);
- Dense nutrient media - 2% MPA or 2% agar on a Hottinger digest with a content of 120-140 mg% of amine nitrogen; 2% MPA supplemented with 5% blood or blood serum is used.

For *anaerobic microorganisms*, Kitta-Tarozzi medium without pieces of the liver with the addition of 0.5% glucose (pH 7.4-7.6) is used.

Working process. In a liquid medium, to determine the sensitivity of one strain of bacteria to the antibiotic, it is necessary to have six test tubes with 2 ml of medium in each (for serial dilution of the antibiotic), two test tubes of 9-10 ml of medium for diluting the culture of bacteria and a flask with nutrient medium for preparing the working solution antibiotic (Fig. 41).

Firstly, its main solution is prepared (using also the standards of antibiotics of a certain activity) at the rate of 1000 μg of antibiotic in 1 ml of solvent (distilled water, buffer solution). Working solutions are prepared on a nutrient medium from the main solution by the method of successive two-fold dilutions, so that in 1 ml the antibiotic

concentration is, respectively, starting from the first tube – 0,25; 0,12; 0,06; 0,03; 0,015; 0,07 mcg.

Figure 41 - The method of serial dilutions in a liquid medium

In a dense medium, working antibiotic dilutions are prepared in six tubes, so that the amount of the drug in the first tube was 400, in the second - 200, in the third - 100, in the fourth - 50, in the fifth - 25, in the sixth - 12.5 μg / ml . From each tube, 1 ml of the diluted antibiotic is transferred into a Petri dish with a sterile pipette and 19 ml of molten (and cooled to 55 ° C) MPA are added into it by rotational movements, mixed and left on the table until the medium hardens. The concentration of the drug in Petri dishes becomes 20 times lower than in test tubes (20; 10; 5; 2.5; 1.25 and 0.6 μg / ml, respectively).

A clean 16-18 hour microbe broth culture, isolated from the test material, diluted to the desired concentration in accordance with the turbidity standard, is plated in the test tubes or Petri dishes with a nutrient medium containing a diluted antibiotic. If the culture is grown on a solid medium, it is washed away, adjusted to the desired concentration of 500 ml of microbial bodies in 1 ml and seeded, as described above. After 16-18 hours of incubation of crops in an incubator at 37 ° C, the result is taken into account. A tube (or cup), where is no growth, is noted. The amount of antibiotic in it and the subsequent test tube (cup), where the growth of bacteria is observed, is added up, the arithmetic mean is derived, which shows the sensitivity of the microorganism to this antibiotic. The presence of bacterial growth indicates their resistance to this drug, and the lack of growth is an indicator of the high sensitivity of bacteria to the antibiotic.

Agar diffusion method (disk diffusion method)

The simplest and most accessible method for determining the sensitivity of bacteria is using paper disks saturated with antibiotics (Fig. 42).

To do this, 15 ml of MPA or other nutrient mediumis poured in sterile Petri dishes located on a horizontal surface. The medium is dried in a thermostat and inoculated with the test material (bacterial culture) using the "lawn" method.

For plating, the suspension of pathological material (feces, milk, pus, urine, etc.) or an isolated pure culture of the pathogen is used. The bacterial suspension is evenly

distributed over the surface of a dense nutrient medium using a spatula and a Petri dish, and dried again in a thermostat.

On the surface of seeded agar, paper discs soaked with various antibiotics are laid out with tweezers - 5-6 discs per cup at a distance of 25 mm from the center of the cup. Crops are incubated in an incubator at an optimum temperature of 37 ° C for 16-18 hours, after the results of the experiment are taken into account. ***The zone of microbial growth inhibition*** around antibiotic discs is measured, including the diameter of the disc itself. The size of the zones depends on the degree of sensitivity of the pathogen to this antibiotic: *sensitive bacteria, moderately resistant or resistant strains.*

With a zone of diameter up to 10 mm, the strain is regarded as stable;
11-15 mm - as unstable (moderately resistant);
15-25 mm - as sensitive.
Zones exceeding 25 mm indicate a high sensitivity of the microorganism to this antibiotic (Fig. 43).

Figure 42 - Standard paper discs with antibiotics

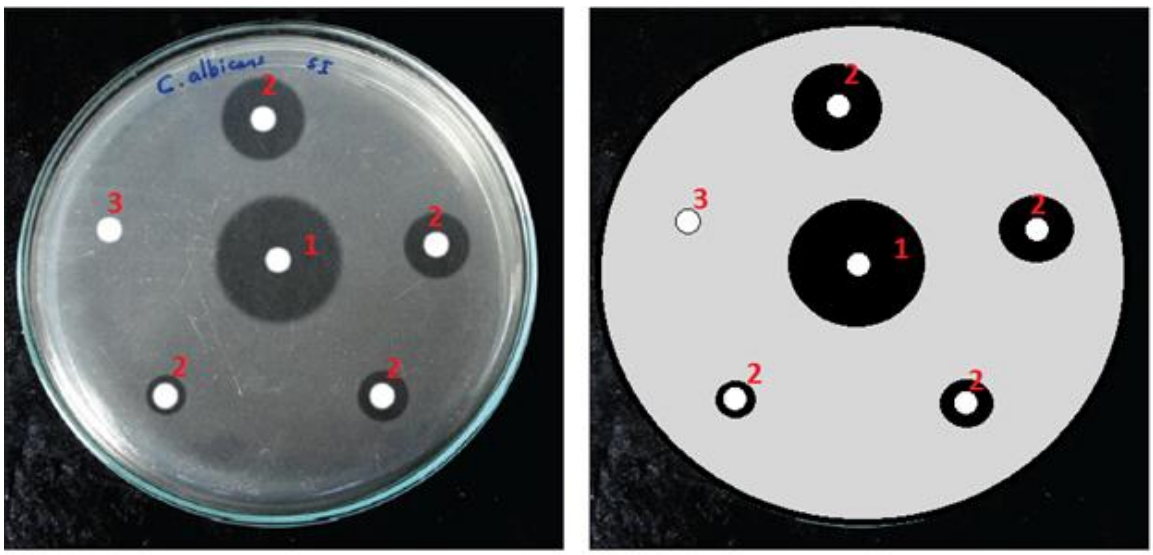


Figure 43 - The diffusion method in agar:

1 - high sensitivity of bacteria to antibiotic;
2 - moderate sensitivity of bacteria to antibiotic;
3 - resistance / resistance of bacteria to antibiotic.

Control questions:
1. Define antibiotics.
2. Units for measuring the activity of antibiotics.
3. Methods for determining the activity of antibiotics.
4. The method of serial dilutions.
5. The essence of the disk diffusion method.

Assignment for self-training and knowledge control
1. What is the bacteriostatic effect of antibiotics on bacteria in nutrient media?
A. Inhibition of bacterial growth.
B. The death of bacteria.
C. Stimulating their growth.
2. In the studied culture, after the addition of the antibiotic, growth retardation was observed after 24 hours (MPB transparent), with further cultivation in vitro, turbidity appeared. What is the effect of the antibiotic?
A. Bactericidal action.
B. Bacteriostatic effect.
3. When determining the sensitivity of staphylococcus culture to neomycin by the method of serial dilutions in BCH, working dilutions of the antibiotic were prepared:
1. 8 UA / ml; **2.** 4 UA / ml; **3.** 2 UA / ml; **4.** 1 UA / ml;
5. 0.5 UA/ ml; **6.** 0.25 UA / ml; **7.** 0.125 UA / ml.
After plating the culture and daily growth, the absence of growth was found in the first four tubes. Determine the bacteriostatic dose of the antibiotic:

A. 8.	B. 4.	C. 2.	D. 1.	E. 0,5.	F. 0,25.	G. 0,125 UA/ml.

4. When determining the sensitivity of bacteria to penicillin by diffusion in MPA, it was found that the diameter of the zone of delay of bacteria is 8 mm. What is the degree of bacteria sensitivity to antibiotic?
A. Sustainable.
B. Insensitive.
C. Sensitive.
D. High sensitive.
5. When examining feces from a calf with a colibacteriosis patient for the sensitivity of microflora to antibiotics by diffusion in MPA, it was found that the zone of growth inhibition of bacteria with penicillin was 8 mm, levomycetin - 23 mm, biomycin - 32 mm. What antibiotic can be recommended for treatment?
A. Penicillin.
B. Levomycetin.
C. Biomycin.

C. BIOLOGICAL METHOD OF RESEARCH

1.19 Laboratory animals. Methods of infection the animals

The purpose of the lesson. To master the methods of infection the laboratory animals, methods for determining the absolute lethal dose for microorganisms and bacteriological studies of animal corpses.

Goals:

1. To master the method of transdermal infection of laboratory animals.
2. To master the method of intramuscular infection in the rabbit and pigeon.
3. To master the method of intraperitoneal infection of a white mouse.
4. To master the method of intravenous infection of a rabbit.
5. To master the method of intranasal infection of guinea pig.

Equipment: laboratory animals (white mice, guinea pigs, rabbits, pigeons), bacterial culture, test tubes, sterile saline, injection needles and sterile syringes, cotton swabs, spirit.

In the laboratory diagnosis of infectious diseases, one of the important methods is the **biological method or bioassay** (infection of sensitive laboratory animals).

Experimental infection of laboratory animals is carried out with ***the aim of***:

- isolating the causative agent of the pathogen from the test material of the pure culture;
- determination the type of bacteria in the disease diagnosis, that is the identification;
- determination the virulence of bacteria;
- tests for the effectiveness of vaccines and treatment sera.

Infection of animals in order to isolate a pure culture of the pathogenic microorganism that caused the disease is carried out if the test material contains extraneous microflora that suppresses the growth of the pathogen on nutrient media. For example, to examine stale pathological material or environmental objects for the presence of anthrax pathogens, white mice or guinea pigs are infected.

For infection, laboratory animals of various species are used — mice, white rats, guinea pigs, rabbits, pigeons, cats, dogs, chickens, etc. Animals of the same species, age, and weight are taken into the experiment.

Laboratory animals are used depending on the sensitivity to the type of microorganism under study, and in some cases the infection of naturally susceptible animals (pigs, cattle, sheep, etc.) is carried out.

If the isolated culture of a microorganism is studied, then the 18-24 hour agar or broth culture of bacteria is taken for infection. When animals are infected with such material (organ tissue, pus, mucus, blood, etc.), the latter is ground in a mortar with sterile saline. In infected animals, septicemia - the reproduction of microbes in the blood, occurs. Infected animals die in 1-3 days. A pure culture of the pathogen is secreted by plating on culture media of blood from the heart and internal organs.

Infection of animals is also carried out to study material containing a small number of microbes or their filter forms that cannot be isolated during cultivation on

nutrient media. So, for example, if microscopic examination of sputum or urine sediment fails to detect mycobacterium tuberculosis, guinea pigs are infected with this material. An experimental animal develops a generalized infectious process after 4-6 weeks. At autopsy, tubercles (tubercles) are found in all internal organs, microscopic examination of which emit a large number of tuberculous mycobacteria.

Experimental infection is carried out in the study of diseases caused by viruses and rickettsia, in cases where pathogens cannot be detected in other ways. Reproduction of a typical disease in an animal confirms the presence of the virus in the test material.

In addition, *infection of animals is used to determine the virulence of microbes isolated from the test material.*

In microbiological work, animals are also used to obtain immune serums and vaccines, to study their effectiveness and harmlessness (control of biological preparations), to take the blood necessary for serological reactions, and to prepare special nutrient media.

Before infection, animals are prepared. *Tag*: rabbits and guinea pigs - with metal ear numbers; mice and rats with a solution of paint (fuchsin, methylene blue, etc.). For convenience and safety, animals are *fixed*. For this purpose, special machines, fixing boards, boxes are used. The assistant places the rabbits and guinea pigs with their back on the table, holds hind limbs with one hand, and grabs the chest with the other, puting fingers into the armpits.

The mouse is taken with one hand by the tip of the tail, with the other by the skin fold of the back of the head, firmly pressed the head to the table surface, with the other hand hold the tail and turn into a position convenient for infection, pulled the head with a forceps.

To take blood from the heart of animals, they are placed on their back, stretching their forelimbs to the sides and slightly upwards. For intravenous injections, it is most convenient to wrap the rabbit in a towel, tightly pressing the limbs to the body.

Methods of infection the laboratory animals

- cutaneous - cutaneous (scarification), intradermal, subcutaneous;
- intramuscular;
- intraperitoneal;
- intranasal (through the nasal cavity);
- oral (through the oral cavity);
- intracerebral;
- intracardial;
- into the anterior chamber of the eye.

Instruments for infection must be sterile (syringes, needles, lancets, tweezers, etc.).

Scarification - small incisions of the skin (notches) are made with a scalpel and the studied material or bacterial culture is rubbed into them with a stiff brush. Wool at the site of infection is pre-cut and the skin is disinfected.

Intradermal infection - is used less frequently. The skin is stretched with two fingers of the left hand or pulled on a finger like a glove. The needle is inserted at an

acute angle with the hole upward into the surface layer of the epidermis so that the end of the needle is visible. With the introduction of the liquid, a bubble appears - a small swelling the size of a pea, which does not disappear within 5 minutes. This is an indicator of the correct introduction. Material is injected intradermally in a volume of 0,1 - 0,2 ml.

Subcutaneous infection - with the fingers of the left hand the skin is pululed away, and a syringe needle is inserted into the formed "pocket", then its contents. The place of infection in rabbits is from the back, in white mice and rats - from the back to the base of the tail. The volume of the introduced substance is not more than 0,1 – 0,2 mm for mice, for rats, guinea pigs should not exceed -1 ml, for rabbits - 10 ml.

Intramuscular infection - the material is injected into the muscle from the inner surface of the thigh. Pigeons and chickens are also infected in the pectoral muscle. The volume of input material for mice is 0.5 ml, guinea pigs and rats - 5-8 ml, large doses should be administered fractionally in 2-3 places.

Intraperitoneal infection - the animal is fixed with its head down, the syringe needle is inserted into the lower third of the abdomen, slightly departing from the white line. The dose should not exceed 0.1-0.2 ml. The material under investigation is injected into rabbits in the marginal vein of the ear, in mice and rats in the tail vein. Before infection, the injection site is wiped with a swab moistened with xylene or warm water to cause blood vessels to fill.

Intracerebral infection is carried out on animals fixed in the dorsal position. In rabbits, for this purpose, the skull is trepanized in the area between the brow angle and the cranial crest. After the operation, the skin is cut and disinfected, fingers of the left hand are stretched over the orbit parallel to the cranial crest and dissected (the edges are pulled apart), the cuticle is cut crosswise, the cranial bone is carefully pinned, the disc is carefully rotated, and this small piece of bone is removed. 0.2 ml of test material is injected with a syringe. After this, the edges of the periosteum are connected, the skin wound is closed with a tampon and filled with collodion. Trepanation is not done in mice and rats, but the tip of a thin needle is injected with a light puncture of the bone tissue of the skull and material is injected.

Intranasal infection is carried out by the drip method using an eye dropper. Previously, the animal is slightly anesthetized, applying cotton wool moistened with ether to the nose.

In case of ***oral infection***, the test material is added to food, water or through a small probe.

Methods of infection - *subdural* (under the dura mater), *intracerebral* (inside the brain), *intraocular* (in the anterior chamber of the eye), *oral* (through the digestive tract), infections are produced into the respiratory tract in cases where a different route of injections does not cause typical infectious process in experimental animals.

Bacteriological examination of an animal corpse

The **main purpose** of a bacteriological research of a corpse is to detect the microbe that caused the death of the animal, to isolate it in a pure culture and determine the location of the pathogen

At autopsy of an animal, a ***number of conditions*** must be observed.

1. An autopsy should be performed as soon as possible after the death of the

animal, baecuse the intestinal flora quickly penetrates into tissues, blood, organs. At room temperature, this happens after 10-18 hours, and at the temperature of the refrigerator - after 20-22 hours. The corpse is kept in the cold until autopsy.

2. Autopsy, taking material for research is carried out in compliance with aseptic rules. Only sterile instruments are used and changed when opening each cavity or each organ.

3. It is necessary to exclude the possibility of infection of workers and pollution of surrounding objects. Before opening, the corpses of small animals are immersed in a disinfectant solution; in larger animals, the wool is moistened with this solution. Workers open the corpses on a well-planed painted board placed in a metal bath or a cuvette with a disinfectant solution. After the autopsy, the corpse is destroyed.

4. All autopsy data must be logged. Records should be detailed and clear. The protocol indicates the date of infection and a description of the material that produced the infection.

The procedure of opening. The body of the laboratory animal is fixed in a dorsal position on a board or paraffin cuvette. Paws are stretched to the sides and fixed with dissecting needles or sharp nails.

The skin and coat are disinfected with a 5% solution of phenol or lysol. The skin is cut along the white line from the perineum to the sternoclavicular joint. Then the skin is separated from the muscles, transverse incisions are made and the skin flaps are aside. The xiphoid process is captured with forceps, muscles are cut under it, ribs are cut with scissors on both sides, the sternum is folded up. Initially, the *chest cavity* is opened. The pathoanatomical picture is taken into account and data is recorded in the examination journal.

The surface of the heart, lungs, and lymph nodes is burned with a hot staple, an organ is calcined in this place with a Pasteur pipette, the small amount of blood (tissue pulp) is sucked in and sown on nutrient media, being careful.

Then the *abdominal cavity* is opened. The abdominal wall is pulled up with tweezers and cut with scissors from the diaphragm to the anus (the intestines should not be damaged!). The organs of the abdominal cavity are studied, the size, color and texture of the parenchymal organs, the condition of the intestine, the presence of exudate in the abdominal cavity and its nature are noted. Moreover, after cauterization of the surface, platings are made from the liver, kidneys, spleen, lymph nodes, and, if necessary, from the contents of the intestine and other organs.

At the same time, fingerprint smears are prepared from the tissues of the organs for microscopic examination: a piece of the organ is cut with sterile scissors and a glass slide is applied several times to the cut surface in separate sections; the drug is dried on the air, fixed, stained, microscopied.

According to a similar scheme, the bodies of naturally susceptible animals are examined, as well as particular organs of animal(pathological material) entering the laboratory for research. All work with animal corpses is carried out, observing measures to prevent the spread of the pathogen. After the study of the corpse, the cuvette, the board, the desktop are disinfected. Tools are sterilized. Corpses of animals and individual organs are neutralized by autoclaving or burned in an incinerator [1, 2, 3].

Control questions:
1. What is the purpose of experimental infection of animals?
2. Types of laboratory animals used for infection.
3. Methods of infection of laboratory animals.
4. The method of bacteriological research of the corpse of an animal.
5. The procedure for opening.

1.20 Methods for determining the pathogenicity of microorganisms

The purpose of the lesson. To study methods for research the pathogenic properties of bacteria and determining the absolute lethal dose of microorganisms.

Goals:

1. To know the concepts of pathogenicity and virulence of microorganisms.
2. To be able to determine the lethal and infectious dose.

The concept of pathogenicity and virulence of bacteria

In laboratory animals, the course of the infectious process can be created by artificial infection.

An infection or an infectious process (from lat. Infectio - to infect, pollute) is a combination of phenomena that arise and develop in a macroorganism during the introduction and propagation of pathogens in it.

The extreme degree of severity of the infectious process is **an infectious disease**.

Pathogenicity (from Latin *pathos* - suffering, *genos* - birth) – is the ability of microorganisms to cause pathological processes in a macroorganism, that is an infectious disease. Microorganisms with this ability are called *pathogens*. This is a genetically determined species trait.

Most of pathogenic microorganisms have **specificity** - this is the ability of a particular type of microbe to cause a specific disease. For example, cholera causes cholera vibrio, gonorrhea - gonococcus, etc.

Different strains of the same bacterial species may have a different pathogenicity effect. The degree or measure of pathogenicity is called *virulence*.

Virulence is an individual trait, the measure of its pathogenicity, which is specific for each type of microbe. The ability of bacteria to cause an infectious disease under spontaneous conditions is possible due to the *presence of certain virulence factors*: toxins, enzymes, adhesion (adherence), colonization (reproduction), invasion (penetration into the tissues and cells of a macroorganism) and the suppression of phagocytosis.

Virulence can be enhanced by the passage (re-introduction) of microorganisms through animals, which are sensitive to them.

If the microbe does not have virulence, then it will not be able to cause an infectious process.

Determination of the pathogenicity of microorganisms

While studying the properties of pathogenic microbes, in some cases, their virulence is also determined. This is necessary to identify microbes isolated from patients, carriers or from the external environment, characteristics of the strength of the vaccine, the degree of immunity in animals, etc.

In such cases, the virulence (toxicity) of the microorganism is measured in special arbitrary units:

- **DLm** ***(dosis letalis minima)*** – the minimum lethal dose, that is, the minimum number of microbes that cause the death of a certain type of animal;
- **LD_{50}** – *the average lethal dose* causing the death of 50% of infected animals;
- **DcL*(dosis certae letales)*** – *absolute lethal dose* - causes the death of 100% of infected animals.

LD_{50} is the most accurate indicator, since it reflects the sensitivity to the pathogen (or its toxin)of most of the animals taken in the experiment, but DcL and DLm show the sensitivity of the most stable individuals.

Considering the methodology for determining DLm.

For this purpose, cultures grown on solid or liquid nutrient media are used. Cultures on solid nutrient media are washed off with a 0.85% solution of sodium chloride and the certain amount of microbial bodies is set in 1 ml using the optical turbidity standard. 1.8 ml of physiological saline solution is pipetted into the first row of tubes. Then, 0.2 ml from the main dilution of the microbial culture is added to the first test tube, obtaining a dilution of 500 million microbial bodies. Then the dilution of 250, 125, 62.5 million / ml, etc., each dilution is prepared with a separate sterile pipette. The contents of each tube are injected in a volume of 0.5 ml subcutaneously to white mice weighing 18-20 g.

Animals are observed for 10 days, noting dead animals. The minimum number of microbes that caused the death of a white mouse is taken as MLD. The obtained data are summarized in table form.

The determination of the virulence of a microorganism in the experiment with infection of laboratory animals

The amount of suspension of bacteria introduced into the animal	The number of animals in the group	Results of infection
500 ml	4	4/4
250 ml	4	3/4
125 ml	4	2/4
62,5 ml	4	0/4

Note: The numerator is the number of dead animals, the denominator is the number of animals in the group.

In the above example, DcL was 500 million microbial cells, LD_{50} - 125 million microbial cells. However, in most cases, the experimental results do not give an

unambiguous answer about the LD_{50} value and its calculation according to the ***Kerbek formula*** is required: $IgLd_{50}$ = Ig Dn - S (Σ Li - 0.5), in which S is the logarithm of the ratio of each subsequent dose to the previous one; Li is the ratio of the number of dead animals to their total number in the group; Li is the sum of the Li values found for all doses; DN is the maximum of the tested doses.

Cultures grown on liquid nutrient media are bred several times.

The minimum lethal dose can vary dramatically depending on the type and strain of the microbe, as well as the type of animal, injection site and other factors. *For example*, to determine the virulence of the causative agent of pasteurellosis, an 18-hour agar culture of P. multocida bacteria is washed off with physiological saline and the microbial content is determined according to the optical standard to 1 billion in 1 ml.

Determiningof LD50. This method of determining the virulence of microbes is more reliable and less dependent on the individual sensitivity of the animal.

Tenfold dilutions are made from a culture of bacteria or viruses. Each breeding is injected to several animals.

It is very difficult to pick a breeding that would cause the death of 50% of the animals, therefore, the method of statistical accounting and calculation of LD_{50} proposed by L. Reed and H. Mench is currently being applied.

In the study of material diluted from 10-1 to 10-8, 8 groups of animals are required. After termination of the observation, the number of dead animals in each group is noted and the LD_{50} is determined using special tables.

Conducting a dermonecrotic test.

It is used to detect necrotoxin contained in the filtrate of the broth culture.

To do this, in a white rabbit, a skin area is shaved on the lateral surface and disinfected, 0.2 ml of the test material is injected intradermally. if it is a positive test, firstly hyperemia is noted, then edema, and lastly necrosis (usually after 2 to 3 days).

Control questions:

1. What is the purpose of infection of laboratory animals?
2. What types of laboratory animals are used for experimental infection?
3. What methods are used to infect animals?
4. In what arbitrary units is virulence of microorganisms measured?
5. Reveal the essence of the method of bacteriological research of an animal's corpse.

Section II. General Virology

2.1 Rules of work in the virology laboratory

The purpose of the lesson. To familiarize with the operating mode in the virological laboratory, with safety measures while working with virus-containing material.

Goals:

1. To learn basic safety precautions.
2. To acquire skills of the preparation of laboratory glassware and material for virological research

Equipment: table box, luminescent microscope, centrifuge, refrigerators, magnetic stirrers, thermostats, spirits, glassware (pears, tubes under rubber stoppers, bottles, test tubes, mattresses, Petri dishes, pipettes). The register of students who received safety training while working in the virus laboratory; laboratory documentation and basic approved instructions for the rules of work in veterinary institutions.

Virology laboratory is designed to carry out research, production or educational work. The laboratory has 5-6 rooms: a reception room, where pathological material is recorded; room - where material is pre-treated; room - for conducting virological studies, where are boxes. Boxing rooms should be well lit, equipped with bactericidal lamps. The room is autoclaved, for sterilizing dishes, neutralizing infectious material. Washing room - for washing dishes, apparatus, appliances. Vivarium - a room for keeping laboratory animals.

While working with virus-containing material, the *following requirements* must be satisfied:

- prevent the dispersion of viruses in the environment;
- prevent contamination of virus-containing material by extraneous microflora;
- provide personal safety.

To fulfill these requirements, the following rules of work should be observed: you must be very careful, collected and accurate, enter the laboratory room and leave it only in a dressing gown, dressing in a wardrobe, work in a dressing gown and a gauze mask, there should be no extraneous objects, it is forbidden to smoke and eat food. Use only sterile instruments and utensils, open and close the vessels at the burner flame, do not take pipettes into your mouth, put the used tools into the sterilizer for subsequent disposal and boiling. Collect used pipettes into a vessel with a disinfectant solution, and collect solid and liquid waste in specially adapted containers for subsequent sterilization. It is forbidden to pour or dump waste into toilets and sinks.

Before and after work, the workplace is disinfected.

In the virological laboratory there should be *documentation*:

1 The inventory book of museum strains of viruses.
2 The journal of accounting for the movement of virus-containing material in the laboratory.

3 The journal of accounting for the movement of virus-containing material in the laboratory.
4 The register of infected experimental animals.
5 The register of isolated viruses.

Laboratory glassware should be clean and sterile. It is washed with ruffs, soap, powder and soda, rinsed with running water and distilled water, then dried in drying closets or in a room, dry dishes are wrapped in parchment paper and sterilized by autoclaving [5, 6, 7].

The main methods for laboratory diagnosis of viral diseases

The laboratory diagnosis of viral infections is based on 3 groups of methods:

1st Group - Detection of the virus or its components directly in the clinical material taken from the patient, and receiving a response after a few hours (quick; rapid diagnosis). Rapid diagnostic methods for the most common viral infections:

- electron microscopy,

2nd group of methods - Isolation of the virus from clinical material, its indication and identification (virological diagnosis). This group of methods requires a long time and is laborious, often retrospective.

3rd group of methods - Serological diagnosis of viral infections.

A single serological examination only in rare cases can diagnose a viral disease (for example, HIV infection). In most cases, serological diagnosis requires paired sera taken in the acute phase of the disease and after 2-4 weeks. The detection of a fourfold or more increase in antibody titer is considered to be a diagnostic sign of acute viral infection.

The main methods of laboratory diagnosis of infectious diseases of viral etiology (virosis):

1. Viroscopic method;
2. Virologic diagnosis;
3. Biological method;
4. Serological method;
5. The molecular - genetic method.

I. Viroscopic method

The study of viruses using a microscope. In virology, the following types of *microsocpies* are mainly used: optical (light), electronic and luminescent.

Using histological methods, the detection of intracellular Taurus inclusions (for example, Babesh-Negri body - in case of rabies, Paschen body - in case of smallpox, etc.)

RIF (immunofluorescence reaction) - luminescent microscopy.

II. Virological method

This method involves the cultivation and accumulation of the virus; for this, chicken embryos, cell cultures, and laboratory animals are used in virological practice.

The study is carried out in 2 stages:

• **virus indication,** detection of the virus in the material;
•**virus identification,** determination of the phylum and species of virus.

Indication is carried out due to:

- detection of specific changes in the chicken embryo;
- phenomenon of hemagglutination;
- cytopathogenic effect of the virus on the cell;
- the formation of specific inclusions in the cell;
- phenomenon of hemadsorption.

Viruses are identified by using specific immunological reactions: pH - neutralization reaction, HA – hemagglutination assay, complement fixation test, etc.

III. Biological method

Bioassay - is based on the use of sensitive laboratory animals for the experiment. Animals are used for the cultivation, indication and identification of viruses.

IV. Serological method

Serological diagnosis is based on immunological reactions. According to them specific antiviral antibodies are detected in the blood serum of a sick animal. In virology, ELISA (enzyme-linked immunosorbent assay), HAI (hemagglutination inhibition test) and others are more often used.

V. Molecular genetic method

One of the specific methods. For detection in a biomaterial, a fragment of the virus genome is used - PCR (polymerase chain reaction), DNA hybridization, and others.

Control questions:

1. Safety rules while working with virus-containing material.
2. The device of virological laboratory.
3. Methods for laboratory diagnosis of viral infections.
4. What is the indication and identification of the virus?
5. Explain the documentation in the virology laboratory.

2.2 Morphology and chemical composition of viruses

The purpose of the lesson. To familiarize students with the structure of the virion and its chemical composition.

Goals:

1. Know the types of symmetry and shapes of viruses.
2. Know the difference between a simple and complex virus.

Viruses are intracellular parasites of animals, humans, plants, insects, bacteria, fungi, protozoa, and other living creatures.

Viruses are ultramicroscopic non-cellular life forms, obligate intracellular parasites of a living organism (Fig. 44).

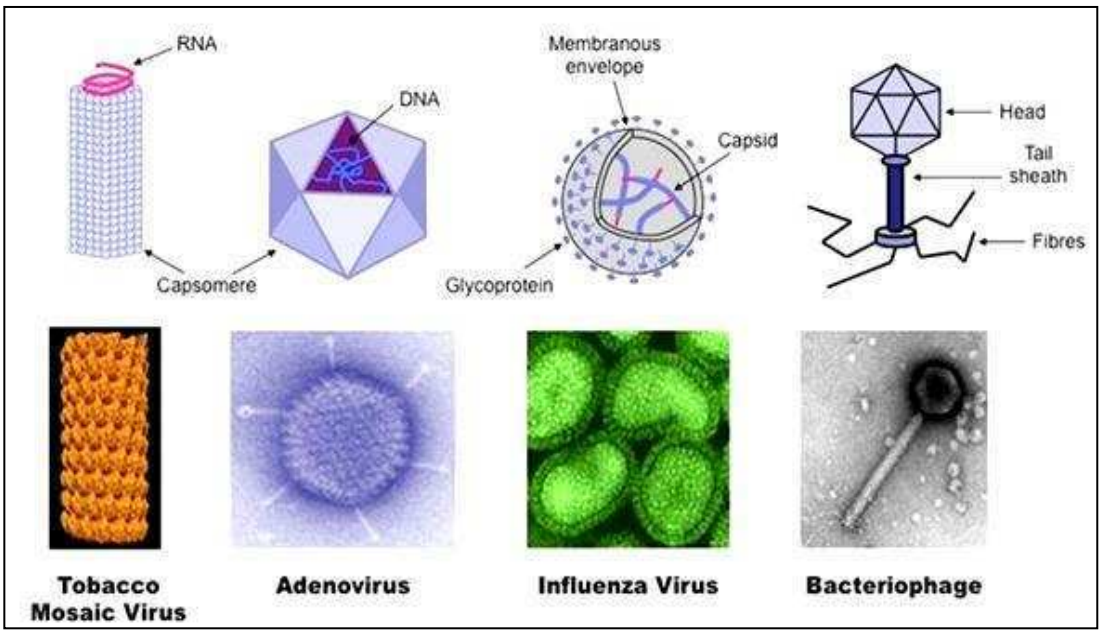


Figure 44 - Morphological diversity of viruses

In nature, a virus exists **in the form of**:

1) Virion - extracellular or resting form.

2) Intracellular, multiplying (reproducing) form. The synonym is “virus-cell complex”.

3) Provirus - the genome of a virus, is a particle of the cellular genome.

4) DIPs - defective interfering particles that have lost a portion of the parent genome (virus mutants).

Viruses exhibit a wide variety of forms and biological properties, but all of them have common structural features. Mature virus particles are called virions.

Virions are distinguished **by their shape** - spherical (round), icosahedral (20-faceted), rod-shaped, oval, filiform, sperm (Fig. 45).

All viruses are classified as those which infect humans, animals, insects, bacteria and plants.

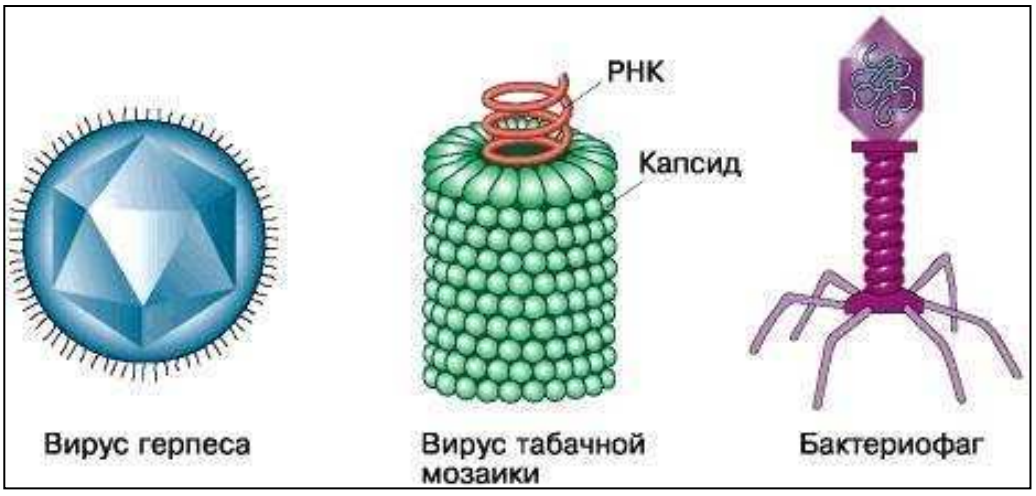


Figure 45 - Forms of virions (cubic, rod-shaped, sperm)

The structure / morphology of viruses

simple virus (Fig. 46)

complex virus (Fig.47)

Simple viruses – there is **the genome (1)** in the structure of the virion- is represented by one type of nucleic acid (either DNA or RNA), which is covered on top with a protein shell called - **capsid (2)** (from the Greek. Capsa - case). Virion capsids are formed by protein subunits arranged in a strictly defined way, called **capsomeres (3).** Nucleic acid and capsid are called - **nucleocapsid (4)** (Fig. 46).

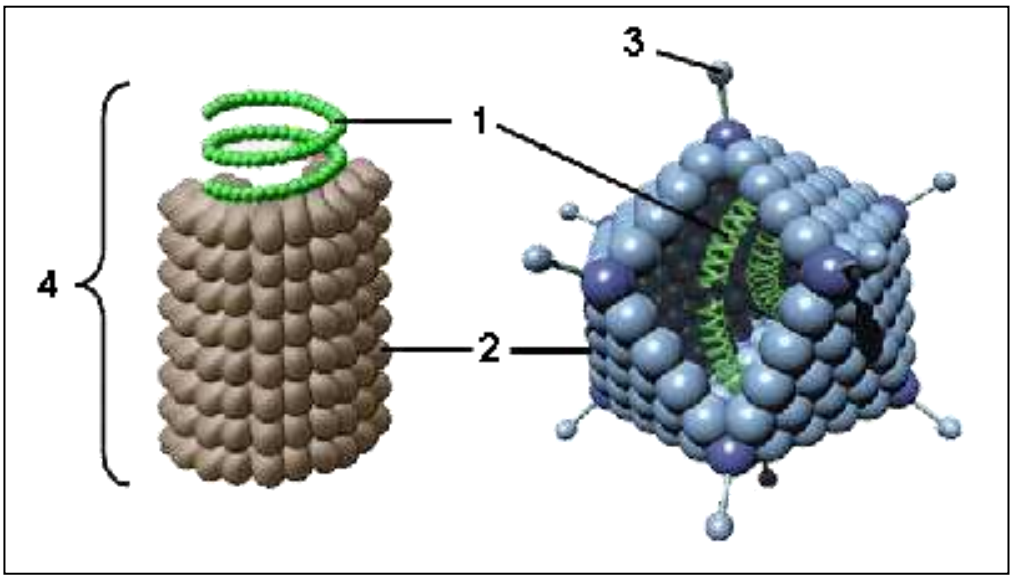


Figure 46 - The structure of a simple virus

Capsid performs a **protective function**. It protects the nucleic acid of the virus from various physical and chemical influences, primarily from the action of numerous nucleases.

The second function of the capsid is the presence in its composition of a receptor corresponding to the receptor of the infected cell. In other words, the capsid determines the adsorption of the virus on the surface of the host cell.

In the process of evolution, a unique ***selectivity*** of viruses has developed - to infect a strictly defined group of hosts, and in the host's organism a certain type of cells. This phenomenon is called **the tropism of the virus** [5, 6].

Complex virus - in the structure of the virion there is also 1 type of nucleic acid covered with a capsid. The feature of the structure is the presence of a second shell - the lipoprotein upper cover - supercapsid or peplos. It is represented by a double layer of lipids, in which molecules of specific proteins are immersed. In some viruses, supercapsid protrudes processes called peplomeres. In the structure of viruses, there are also enzymes (Fig. 47).

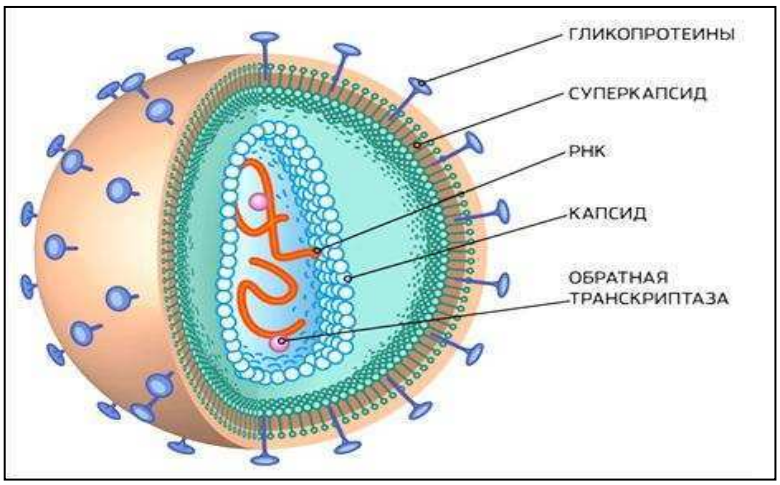


Figure 47 - The structure of a complex virus

Depending on the location of capsomeres, there are **3 types of symmetry** of the capsid among viruses: *spiral, cubic, mixed.*

Spiral symmetry: capsomeres are spiral arranged (along the helix of nucleic acid) and interact with the nucleic acid of the virus. To release NA, it is necessary to destroy the entire capsid.

Spiral symmetry of capsid is observed in plant viruses, in RNA-containing viruses of the family coronavirus, orthomyxvirus, paramyxovirus, rhabdoviruses, etc.

Cubic symmetry is observed in DNA-containing viruses (adenoviruses, herpesvirus, papovavirus, parvoviruses), some RNA-containing viruses (picornaviruses, togaviruses, etc.)

Cubic symmetry: capsomeres in viruses with an icosahedral shape, are located on the vertices, edges, facets of triangles by two (dimer), three (trimer), five (pentamer), six (hexamer). Because of such arrangement of capsomeres, their interaction with NA is not observed. Each capsomer in various viruses consists of a certain number of chemical, morphological units. Therefore, the size of capsomeres in viruses will be different.

The number of capsomeres in viruses is a species trait. For example, the herpes virus has 162 capsomeres.

Viruses are divided by size into **3 groups**: large, medium, small viruses.

Large viruses - have a size of 150-450 nm. These include poxiviruses and herpes viruses.

Medium viruses - have a size of 100-150 nm. These are otromyxoviruses, rhabdoviruses, coronaviruses and others.

Small viruses (parbo-, picorno-viruses) are 20-40 nm in size.

The molecular weight of virions is measured **in daltons** (D).

1 D – is mass of 1 hydrogen atom - 1.67 g.

1000 D - 1 kilodalton (KD).

1,000,000 D - 1 megadalton (MD).

The chemical composition of viruses

Simple (naked) viruses consist of a nucleic acid and *a protein cover* - a capsid. The structural unit of the capsid is - capsomer.

Complex viruses, except the nucleocapsid, have a supercapsid (or peplos) —this is the outer *lipoprotein membrane*, which includes *proteins, lipids, and carbohydrates*. Complex capsid and supercapsid proteins have antigenic properties.

Genetic information about the encoded polypeptides of the virus is embedded in the genome of the virus (its nucleic acid).

Unlike all living things in nature, viruses have only one nucleic acid; either DNA or RNA.

The features of DNA and RNA viruses are that they have no different structures: linear, single and double stranded forms of DNA / RNA, and there are fragmented, circular. The genome of small simple viruses is represented by several genes that encode several proteins. The genome of large complex viruses can have hundreds of genes that encode a large number of polypeptides (structural and non-structural proteins).

Non-structural proteins - enzymes are involved in protein, nucleic and carbohydrate metabolism, in all stages of the reproduction of viruses.

Such *enzymes* in viruses are distinguished:

1. Virus-specific - are part of viruses (DNA polymerase, RNA polymerase, transcriptase, reverse transcriptase).

2. Virus-induced - are synthesized in an infected cell after virus penetration.

Proteins of viruses are different in chemical composition and therefore in function. The variety of proteins is determined by the number and sequence of amino acids. Proteins are built from 20 amino acids and are polymers where the amino acid is the monomer.

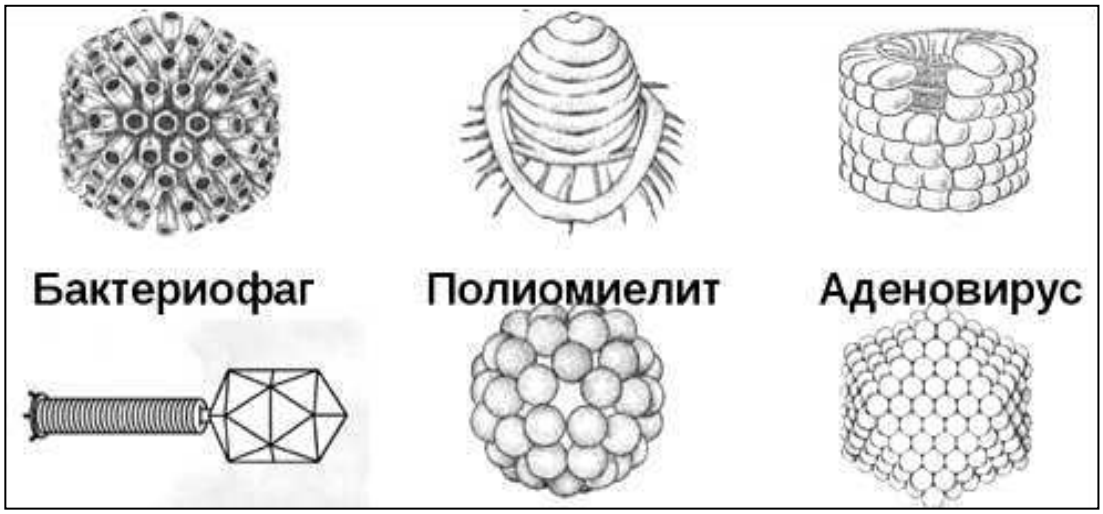
In proteins, the types of bonds are distinguished: *peptide* (CO-NH2), between amino acids (side chains are not involved); *hydrogen bonds* between hydrogen and oxygen atoms (weak bond); *disulfide bonds* between sulfur atoms.Thus, proteins of primary, secondary, tertiary and quaternary structures are distinguished.

DNA and RNA of viruses are also polymers where the monomer is a nucleotide. In the synthesis of DNA and RNA strands, each subsequent nucleotide is attached (sewn) by enzymes.

A chemical study of DNA in cells of various origins revealed the following patterns: the amount of A = T, G = C.

A complementary arrangement of nitrogen bases in two strands of DNA was established. These discoveries, as well as the DNA X-ray patterns obtained by English scientists (M. Wilkins, R. Franklin), contributed to the decoding of the DNA structure by scientists D. Watson and F. Crick. Watson and Crick not only decoded the DNA structure (double helix), but also proposed a scheme for its replication (reproduction), transmission of hereditary traits to offspring [7].

Task for the student's independent work: Sketch simple and complex viruses. Consider the physical structure of viruses. Determine the shape of the virions in the figure:



Control questions:
1. Forms of the viruses existence. Types of capsid symmetry.
2. The structure of a simple and complex virus.
3. Virus units.
4. The main chemical elements of the virion.
5. Virus enzymes, their functions.

2.3 Preparation of virus-containing material for research

The purpose of the lesson. To acquaint with the technique of taking and preparing pathological material, safety rules while working with virus-containing material.

Goals*:*

1. To master the requirements of the taking, transportation, storage and preparation of material for virological research.
2. To acquire skills in preparing laboratory glassware for virological research.

The diagnosis of viral infections often needs to be *confirm*ed, clarified in the laboratory by *virological studies*.

For this purpose, it is necessary to be able to select correctly the pathological material for research. Material from diseased, dead, or involuntarily killed animals should be taken as soon as possible after the appearance of clear signs of the disease, or no later than 2-3 hours after clinical death or slaughter. Because of the fact that straight away after the disease or for the first 1-2 days, the intestinal barrier role is significantly weakened, which, side by side with the increased permeability of blood vessels, contributes to the dissemination / spread of the intestinal flora.

The pathogenesis of the studied infection is very important in taking material for virus isolation. So, for respiratory infections nasopharyngeal swabs are taken, swabs from the nose and throat, scrapings of the trachea and pieces of lungs of corpses; in the case of enterovirus - feces; neurotropic - pieces of the brain or spinal cord; dermatotropic infections - fresh skin lesions, etc., it means that the material which contains the highest concentration of the virus is selected.

The material for the isolation of the virus can be various excreta and secretions, exudate, pieces of organs, blood, lymph, etc.

Washings from the conjunctiva, from the nasal mucosa, from the back of the pharynx, rectum and cesspools of birds are taken with *sterile cotton swabs* and immersed in penicillin vials or tubes containing 3-5 ml of Hanks solution or medium for cell culture with antibiotic (penicillin and streptomycin at 500 UA and nystatin at 20 UA per 1 ml of medium) and a protein stabilizer, for example, 0.5% gelatin solution or 0.5-1% albumin solution of bovine serum.

The *device designed by Thomas and Stock* can be used in taking the material from the nasopharynx. It consists of a tube with a diameter of 9 mm and a length of 30 cm, inside which a second thin tube with a stainless steel rod ending with a nylon brush is placed. The device is inserted deep into the nasal passages or into the throat through the nasal passage, extending the brush, and then, again pushing it into the tube, before removing the device from the organ. The brush is thoroughly washed from mucus and cells in 2 ml of liquid.

It makes sense to take saliva if there are the signs of damage to the oral cavity or salivary glands. Leaking saliva from the mouth can be collected directly in a test tube and closed with a rubber bung.

Urine is collected in sterile dishes by using a catheter.

Feces are taken from the rectum with a spatula or stick and placed in a sterile test tube or penicillin vial.

Vesicular fluid can be collected with a syringe or a Pasteur pipette into a sterile tube.

Aft walls, crusts from the surface of the skin are removed with tweezers.

Cerebrospinal fluid is rarely used. It is taken aseptically by conventional puncture.

Samples should be by 10-20g. into sterile vials or test tubes with a label indicating the type of animal, animal number, organ.

The accompanying document indicates the name of the farm, clinical signs of sick animals, and the preliminary diagnosis.

Pathological material is delivered to the laboratory urgently to keep the virus in an active state. For this purpose, the material is preserved by cooling, freezing in a thermos, in a solution of glycerol.

In the laboratory, the resulting material is freed from the preservative, thawed, washed from glycerin, and weighed. Part of the material is taken for virological studies. The rest is stored in the refrigerator in case of additional studies.

The virus must be released from the cells of organs and tissues. To do this, the material is thoroughly crushed with scissors and rubbed with sterile quartz sand to obtain a paste-like mass. A 1:10 sterile physiological solution is added, transferred

into centrifuge tubes and centrifuged for 15 minutes at 1000 turn/min. The supernatant, after checking for bacteriological contamination, becomes the material for virological research. It is sucked off into sterile bottles and freed from microflora, treated with broad-spectrum antibiotics (penicillin, streptomycin, nystatin, tetracycline, etc.). Doses of antibiotics vary from 100 to 1-2 thousand UA or more per 1 ml, depending on the nature of the test material.

The suspension with antibiotics is exposured for at least 30-60 minutes at room temperature, then the material is exposed to bacteriological control for the presence of bacteria, fungi by plating on MPA, MPB, MPLB. After receiving a negative result, the virus-containing material is used to infect laboratory animals, chicken embryos or cell cultures. In the case of a positive control, the suspension of the virus is exposed to additional antibiotic treatment and re-control. The suspension is stored at minus 20-70 ° C [3, 5].

Control questions:
1. The technique of taking pathological material from sick animals.
2. Technique for taking pathological material from corpses or slaughtered animals.
3. Preservation of the virus in the material and its delivery to the laboratory.
4. How to prevent contamination of pathological material by extraneous microflora?
5. Rules for the selection of material from the nasopharynx.

2.4 Morphological methods for the indication of viruses in pathological material

The purpose of the lesson. To acquaint with the basic methods for indicating viruses in pathological material by detecting viral inclusion bodies and virions in the diagnosis of viral infections.

Goals:

1. To study the morphological methods for detection of specific inclusion bodies in the material.

2. To learn the nature of viral inclusion bodies and their role in the diagnosis of viral diseases.

Equipment: histological preparations, which contain cytoplasmic inclusion bodies; stained preparations, which contain intranuclear inclusion bodies, smears of smallpox vesicles (or follicles) stained according to Morozov; microscopes with illuminators; immersion oil, electron micrographs of diffirent virions.

Morphological methods for indicating viral infections are based on light, electron and luminescent microscopy.

Because of the low resolution of the light microscope, its use in virological research is limited.

The **light microscopy** is used in the **following cases:**
1. To detect viral inclusion bodies in cells;
2. For detection of large virions in preparations;

3. To study the cytopathogenic effect of the virus on cell culture.

1. Detection of viral inclusion bodies in cells

During the reproduction of many viruses in cells, intracellular ***viral inclusion bodies*** are formed. They can be *cytoplasmic* (RNA viruses) and *intranuclear* (DNA viruses).

<u>According to the nature inclusion bodies can be:</u>

- the accumulation of thousands of virions remained in the cell; or
- cellular material that has changed under the influence of virion reproduction;
- an excess of viral proteins not included in the virions;
- a combination of these elements.

The size of inclusion bodies is barely noticeable from the size of the cell nucleus, and the number ranges from 1-2 to 10-12 pieces per cell.

Inclusion bodies formed in cells by certain viruses have received special names. For example, inclusion bodies formed by the rabies virus in the cytoplasm of nerve cells are called *Babesh-Negri bodies*, the bodies formed in the cytoplasm of epithelial cells by smallpox viruses are called the *bodies of Bollinger*, and mammalian smallpox - *Guarnieri bodies*, the plague virus of carnivores - *bodies of Lenta,* infectious laryngotracheitis virus - *Seyfried bodies*.

As a rule, RNA-containing viruses form cytoplasmic viruses, and DNA-containing viruses form intranuclear inclusion bodies.

The ability to stain with certain dyes, sizes, shape, structure and location in the cell of inclusion bodies formed by different viruses are different, but specific for each virus. Therefore, the detection of intracellular inclusion bodies with certain characteristics in the material from sick animals allows judging which virus they are formed from, and therefore the presence of this virus in the material under study.

To detect inclusion bodies, smears or prints (posthumously or intravitally) are prepared, which are exposed to special staining methods with subsequent microscopy. For inclusion bodies formed by different viruses, color methods are different (Fig. 48).

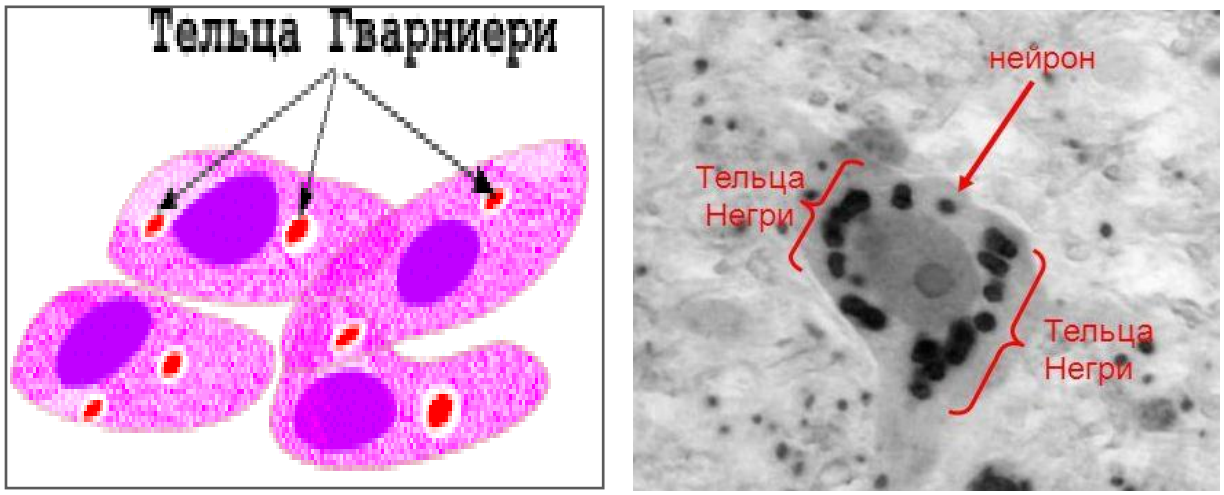


Figure 48 - Intracellular viral inclusion bodies

<u>For example,</u> in diagnosing rabies, drugs are prepared from all parts of the brain

of dead or killed animals and stained according 4o Muromtsev. In positive cases, Babesh-Negri bodies are found. They are sharply1 outlined, purple with a pink tint, with well-pronounced lobation, the background and cytoplasm of the cells are pale blue, the nuclei are blue, red blood cells are orange-red. For coloring drugs, it is possible to use the methods of Sellers, Turevich, Mann, etc.

2 Detection of large virions in preparations

Only *viruses* that are larger than 200-350 nm can be detected due to light microscope. For this purpose, special staining methods are used that artificially increase the viral particle and increase the contrast of the micro picture. The best method is staining the preparation with *ammonia silver according to Morozov*.

Only the virions of smallpox viruses can be seen in a light microscope, since they are giants among the virions of other viruses, reaching 300-400 nm. A method for detecting smallpox virions by using light microscopy is called viroscopy. Drugs are prepared from smallpox skin lesions. In the positive case, very small, round-oval-shaped dark brown bodies are found on a yellow background, lyed in groups, rows or clusters, but not along.

3 The study of the cytopathogenic effect of the virus on cell culture

The characteristic of this method of indicating viral inclusion bodies is shown in paragraph 2.10 of this manual [3, 6, 7].

Control questions:

1. The main methods of microscopy of viral infections.
2. What are viral inclusion bodies?
3. Detection of virions in pathological material by light microscopy.
4. What are the types of viral bodies?
5. Detection of large virions.

2.5 Electron - microscopic examination of viruses

The purpose of the lesson. To get acquainted with the structure of the microscope, the basic principles of the electron microscope and the technique of preparation.

The discovery of electrons, their wave nature, the ability to deviate from their initial motion when pass through magnetic and electrostatic fields allowed creating an electron microscope (Fig. 49).

Electronic microscopy makes it possible to study the morphology of viruses (structure, forms) at the molecular and submolecular level, the morphogenesis of viruses, and the changes they cause in the affected cell.

Because of this, electronic microscopy is one *of the most important methods for identifying viruses* in the diagnosis of viral infections, and is an express diagnostic method.

Figure 49 - Types of electron microscopes

An indicator of the qualitative characteristics of any microscope is its useful increase, it means the resolution is the smallest distance between points, which is perceived separately. Theoretically the resolution of the microscope is approximately half the wavelength of the ray. The wavelength of the electrons varies from 0.055-0.039 A ° and the resolution of the microscope should be 0.02 A ° (this is theoretically, in fact, in electron microscopes up to 8A °).

In an electron microscope column, air is removed *by two pumps*: mechanical and diffusional. Firstly, a preliminary vacuum is created (10 mmHg), then a pressure up to 10 mmHg is created. In electronic microscope magnetic lenses are used. *Magnetic lenses* are a magnetic field that is created by a wire coil while transmitting current. Electrons in a magnetic field do not move directly, but in a spiral way.

In an electron microscope, an electron beam is emitted by a cathode (a tungsten filament) and rushes down the column, focused by a condenser lens on the object.

As a result of different electron densities of the parts of the object, electrons are scattered from their common electron beam and their uniformity changes. This modified electron beam is focused and magnified a second time by an objective lens. Then the image is enlarged once again by a magnetic projection lens and hits the fluorescent screen.

Control questions:

1. The importance of electron microscopy in virology.
2. The resolution of electron microscopes.
3. The principle of operation of the electron microscope.
4. What is the resolution of a microscope?
5. Types of electron microscopes.

2.6 Laboratory animals and their use in virology

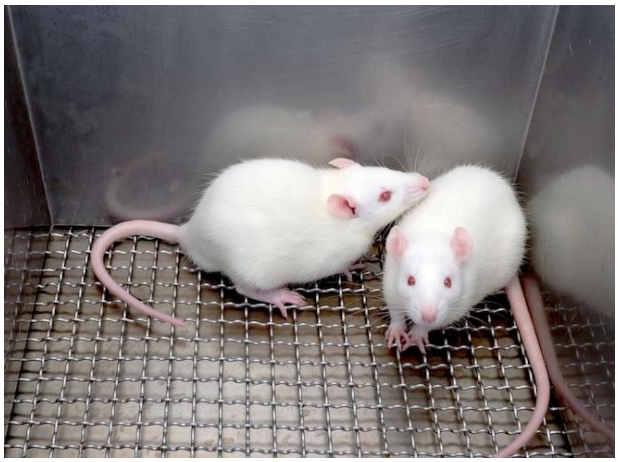
The purpose of the lesson. To study methods of fixation, labeling, blood collection techniques from various laboratory animals, as well as methods of infection of laboratory animals.

Goals:

1. Master the technique of fixing and marking animals.
2. Master the obtaining of blood components.
3. Work out methods of infection of laboratory animals.

Equipment: rabbits, guinea pigs, white mice, scissors, sterile syringes with needles, spirit, cotton swabs, animal stands, paints, sterile Pasteur pipettes, clean tubes, centrifuge tubes, citric acid sodium, cones with glass beads, a centrifuge.

Laboratory animals are various types of animals that are specially bred in nurseries or in laboratory conditions for experimental purposes. Laboratory animals are used to diagnose infectious diseases. Simulations of various pathological conditions, the study of therapeutic and prophylactic preparations, diagnostic preparations, serums, vaccines, cell cultures, etc. In total, up to 250 species of animals are used in biological studies. Laboratory animals are divided into invertebrates and vertebrates. Vertebrate laboratory animals have been studied for cognitive purposes since ancient days, initially experiments were conducted on domestic animals. In the 4th century, white mice, rats, and guinea pigs became famous. But the concept of laboratory animals developed by the end of the XIX century. Of all laboratory animals, these are widely used in virological studies: white mice, guinea pigs, rabbits, hamsters, dogs, monkeys, chickens. But the choice of an animal depends on the type of virus used. Life expectancy of laboratory animals is different. So, white mice live 1.5 - 2 years, rats - 2-2.5 years, guinea pigs - 6-8 years, rabbits - 4-9 years (Fig. 50) [3, 4, 6, 7].



a) white mice

b) golden hamsters

c) rabbits

d) guinea pigs

Figure 50 - Types of laboratory animals

While isolating the virus from pathological material, at least three blind passages are carried out in virological practice, and a biological test is performed on laboratory animals. After that, the isolated virus is identified by using serological reactions.

The purpose of using laboratory animals in virology:

- For virus detection - bioassay.
- To isolate the virus and obtain virus-containing material from a positive bioassay.
- To maintain the virus in the laboratory, that means to keep it in active form for many years. This is achieved by alternating passages and storaging under conservative conditions. **Passage** - infection of a living object with the subsequent receipt of a new virus population.
- For the accumulation of viral mass in order to study the virus and obtain vaccines.
- For titration of viruses.
- As a test object in the neutralization reaction.

After infection, in laboratory animals are noted:

1. Death - in the time period characteristic for the virus.
2. Characteristic pathological changes.
3. Characteristic clinical signs.

Purposes of the autopsy:

- detect changes in internal organs characteristic for this viral pathology.
- take material for subsequent passages (material is taken depending on the tropism of the virus).

Keeping animals. Laboratory animals are kept **in vivariums**, there should be rooms for animals, washing rooms. The kitchen for preparing feed, the pantry for the staff, and other rooms for animals must be bright, with good ventilation and exceptionally clean. At the same time, walls and floors should be easily disinfected. Animals infected with various viruses are kept separately. Dishes for water and feed should be disinfected daily with a 3% solution of chloramine and then thoroughly washed and rinsed. The premises are treated with 1% sodium hydroxide solution.

In the vivarium, zoohygienic rules for keeping and feeding animals must be observed, and each room should have a thermometer and a hygrometer. In vivariums,

laboratory animals are kept in metal cages, glass flasks, and in cages made of artificial materials.

Feeding laboratory animals. For all types of animals, the daily norms of protein, carbohydrates, vitamins, mineral ions, microelements are established. Recently, balanced feeds have been used in the form of feed tablets, pellets that can be accurately metered, take up little space and are cheaper.

Fixation and marking.

The white mouse is taken with the hand, forceps or anatomical tweezers by the tail and fixed to the back of the head with the index fingers.

Guinea pigs are very agile and nimble. They are caught by hand, stroking the back, lowering their hands to the chest and then holding their heads up and supporting the back of the body with the other hand.

The rabbit is held by the dorsal part of the skin and lifted to the top, holding the back of the body with the other hand. The rabbit taken this way will not scratch (to hold the rabbit by the ears or legs means to torment him). You can wrap a rabbit in a blanket and so fix it.

Chicken is fixed with hands: legs are held with one hand, wings are arched.

Animals are marked in various methods:

1) Color marking - stains or numbers are applied with paints (mice and rats).
2) Ear tattoo (rabbit, dogs).
3) Numbering by ear tags (guinea pigs, rabbits).
4) Cutting out wool (hamsters, monkeys).
5) Amputation of fingers (mice) with small sharp scissors cut off the fingers.

Preparation for the experience. Animals must be healthy, well-fed. For the experiment, animals of the same age, weight or same sex are used. Young mice are more susceptible to many viruses.

According to the genetic qualities laboratory animals can be divided into **4 groups:**

1. Animals of mixed origin are heterogeneous animals obtained from different livestock breeders.

2. Bred animals - obtained from one parent, but genetically these animals are variable.

3. Inbred lines - this is the number of animals that arose by closely related mating: brother - sister, parents - children for 20 generations. The high degree of homozygosity is achieved due to this dilution method. For example, the following mouse strains are known: the inbred mouse strain of SZN, which is highly sensitive to the breast cancer virus.

4. Homogeneous hybrids - these mice are genetically uniform, which correspond to the degree of homozygosity of the parental lines. These animals are less variable than their parent lines.

To conduct virological studies on laboratory animals, it is necessary to consider that laboratory animals *can be hidden virus carriers*, and because of artificial infection, these latent infections can intensify. In addition, as a result of cross-immunity, there may be errors in serological studies. Because of this, some studies should use gnotobiots.

Gnotobiot is a germfree, sterile animal. The gnotobiotic animals are highly sensitive to viral infection. Sterile animals are kept in absolutely sterile rooms, there are no contact with living microorganisms. Air, water, feed should be completely sterile. Among gnotobiots animals that are free only from pathogenic microbes are very important. Non-pathogenic microflora and viruses are found in the body of such animals. These animals in a number of countries served as the nucleus for the creation of breeding commodity firms free of infectious diseases.

In virological practice, laboratory animals are used for various studies. Most viruses of different taxonomic groups can be differentiated from each other based on their pathogenicity for different animal species or for different age categories of one animal species.

Therefore, the choice of an animal and its age for infection is determined by the virus we are working with or which we are going to isolate. The most commonly used laboratory animals in virology are: white mice, hamsters, rats, rabbits, guinea pigs, chickens. To study one infection, animals of various species, which have different sensitivity to this virus, are used. It helps to differentiate viruses that cause a clinically similar picture of the disease (vesicular stomatitis, foot and mouth disease, swine vesicular disease).

Selection of laboratory animals. The entire group of homogeneous animals should have similar initial indicators (weight, age, physiological state, temperature), so animals are specially selected into the group.

To a large extent, success in isolation, titration and passivation of the virus depends on the correct selection of animals; an even number of animals are taken to the infection group so that ID50 and LD50 (an infectious dose of the virus that causes infection in 50% of the tested animals or lethal dose, which cause the death of 50% of infected animals) can be determined.

Methods of infection the animals. There are several ways to infect laboratory animals. The choice of method for infection depends on the type of virus and its tropism. Before parenteral infection, the test material is treated; exempt from preservative (thawed and washed), the fabric is crushed with scissors, rubbed in a mortar with quartz sand. A 10% suspension is prepared from the resulting mass in a Hanks solution and centrifuged for 15 or 20 minutes at 1500 - 3000 turn/min, the supernatant is aspirated into sterile vials and freed from microflora, either by passing through bacterial filters or treating with broad-spectrum antibiotics (penicillin, streptomycin, nystatin, etc.). Before the injection of the test material, depending on the method of infection, the animals are anesthetized. Anesthesia technique: the animal is planted under a cap and there is should be a tampon moistened with a narcotic substance (ether, chloroform). After a short period of excitement, the animal falls asleep.

Methods for infection the laboratory animals:

1. Oral injection (per os - through the mouth). The material is mixed with ordinary feed or drinking water. It is possible to infect through a pharyngeal probe, which is inserted through the hole of a gag inserted into the mouth or an elastic catheter reaching the stomach.

2. Intronasal injection (through the nose). After anesthetizing the animal, the

infectious material is injected dropwise into the nostrils by using a thin capillary pipette or round cannula. To protect against the spread of material, it is necessary to work in a mask and goggles.

3. Intravenous injection. In the case of mice and rats, injections are made into the tail vein after immersion of the tail in warm water: rabbits - into the ear vein; chickens - into the vein of the wing; a guinea pig - in the heart. Before injection, the hair is cut off at the injection site, treated with a cotton swab, which was dipped in spirit or iodine tincture. Filling a blood vessel is carried out by holding it with the hand. The needle is injected along the vessel through the blood stream.

4. Intracardial injection (in the heart). Laboratory animals are fixed under anesthesia on their backs, chickens –are fixed in a position on the side. The injection site is: in the case of guinea pig - the second intercostal space, 2 mm from the edge of the sternum: the rabbits - the third intercostal space along the shoulder-joint and caudal ring of the sternum. The needle is inserted perpendicular to the surface of the body through the skin until a pulsation of the heart is felt and until blood appears in the space of the needle. The injection is carried out slowly.

5. Intercerebral (in the brain). The injection is carried out on the side of the midline in the middle between the upper edge of the eye cavity and the external auditory canal. Before the injection, hair on the forehead is cut off and the surgical field is disinfected. The needle is inserted for 2 mm (mouse), the material is introduced slowly and in small doses. After the injection, they are treated with iodine tincture or spirit.

6. Intraperitoneal injection (intraperitoneal). Animals are fixed by lifting the hind limbs down. Due to this this fixation, the internal organs go down. This creates free space for the needle and reduces the risk of damage to certain sections of the intestine. The puncture site is located between the fart and the symphysis away from the midline (in the groin). Infected animals are monitored daily.

7. Subcutaneous - area on the back.

8. Intradermal - more often into the heel.

9. Intramuscularly - into the inner thigh.

The outcome of infection may be different:

a) the animal dies; b) the animal is ill, but does not die.

According to the outcome of the infection, animal studies are subsequently carried out differently. If the animals have died, then it is necessary to isolate the pure source virus from the organs or detect it or the virus antigen under a microscope. If the animals were ill, but did not die, it is essential either to isolate virus or to detect its antibodies in the blood serum of an ill animal in serological reactions.

Infection of animals in diagnostic purposes has disadvantages:

Animals can be infected before the experiment and get sick latently, that is, the absence of marked clinical symptoms, which can distort the results.

Gnotobiotic animals are also expensive and inaccessible. In addition, after infection, the virus is isolated and identified by using other research methods, which complicate the work.

Preparation of laboratory animals (for example, white mouse):

- The skin is lubricated with a disinfectant.
- An incision is made along linea alba / white line.
- Opening of the sternum - the lungs are taken and placed in flask №1.
- Opening the abdominal cavity - the liver, spleen, kidney are taken and placed in the flask №2.
- An autopsy of the cranium is performed. The brain is taken, sections of 4 layers are made, pieces are placed on filter paper and fingerprints are made on glass.

Taking blood and getting its components

Mice's blood is taken from the heart, femoral, caudal veins, retrobulbar plexus and axillary bundle. The most convenient method is taking blood from the retrobulbar plexus. For this purpose, an anesthetized white mouse is fixed between the thumb and forefinger in the head and shoulder girdle. By pressing on the neck of the animal there is a delay in blood circulation in the vessels of the head, as a result, the eyes bulge out of the orbit. A capillary is inserted into the medial corner of the eye with a Pasteur pipette and directed towards the larynx. In case of damage to the venous plexus, blood enters the pipette.

Taking blood from axillary fascia

Fix the anesthetized mouse in an upright position with the thumb and forefinger in the neck. Pull out the front foot and make an incision in the skin. Into the formed pocket, insert the capillary of the Pasteur pipette in the direction of the head. Blood is drawn into the capillary. The resulting blood is introduced into test tubes with a solution of sodium citrate or in a cone with glass beads and defibrinated.

Guinea pigs' blood is taken from the heart, external jugular vein. From the femoral, ear veins. The pig is fixed in the decommissioned position. The assistant fixes the hind and front legs of the animal with his hands, treats the puncture site with spirit. The operator fumbles with the fingers of his left hand for the location of the heart and with his right hand inserts the needle into heart, then gently pulls the plunger of the syringe and draws 2-3 ml. blood.

Rabbits' blood is taken from the heart, external jugular, ear vein.

Taking blood from a rabbit's ear vein

The assistant fixes the rabbit, treats the puncture site with spirit and compresses the ear vein at the base of the ear. An operator inserts a needle into a vein or punctures a vein with a scalpel and collects blood. In the case of chickens, blood is taken from the axillary vein, blood is taken from the crest by cutting a tooth.

Obtaining blood components

Getting washed red blood cells. Blood is defibrinated, introduced into special tubes and centrifuged for 5 minutes at 1000 rpm. Then the supernatant liquid is extracted, a physiological solution of sodium chloride is added, mixed with red blood cells several times, and centrifuged for 10 minutes at 1000 rpm. It is repeated 3-4 times till saline becomes colorless. The supernatant is taken away and the red blood cells are used for further work.

Getting blood serum

The blood obtained from animals is introduced into a clean dry test tube and placed in a thermostat for 20-30 minutes, then is circled with a glass rod from the test tube wall and left in a cool place. Blood coagulates, serum forms over a blood clot,

and it is sucked off in a clean tube.

Unrestricted work of students.

A group of 2-3 people takes various laboratory animals, fixes and markes them according to the method described above, takes blood and its components (receives red blood cells).

They should take one white mouse, work out the techniques (infect subcutaneously, intramuscularly, intraperitoneally, intracerebrally and into the tail vein). The dose of the material varies from 0.1 to 0.5, depending on the route of injection.

Control questions:

1. The importance of laboratory animals in the diagnosis of viral diseases.
2. The purpose of infection of laboratory animals, methods of infection
3. What are the genetic qualities of laboratory animals for virological research?
4. Gntobiotic animals, their importance in the diagnosis of viral infections.
5. The technique of taking blood.

2.7 Chicken embryos, their use in virology

The purpose of the lesson. To study modern methods for indicating viruses by bioassay on chicken embryos

Goals:

1. To know the structure of a 7-12 day old chicken embryo, sketch it.
2. To learn the purpose of application in virology.
3. To know the basic methods of cultivating the virus in a chicken embryo.

Equipment: ovoscope, chicken embryos, sterile needles for piercing the shell, injection needles, syringes. Tripods for chicken embryos. Test tubes with iodinated spirit. Spiritlamps and test material. Paraffin sticks, pencils. Tampons. Tables with a schematic image of the chicken embryo's structure and methods of infection.

Chicken embryo (CE) is an embryo of a chicken egg that is at different stages of embryonic development.

Chicken embryos as a living system entered virology in the 30s of the twentieth century. In virology, *mostly 5-7-day-old chicken embryos are used, less often - 10-12-day-old ones.*

A large number of different viruses can multiply in a chicken embryo. This is possible due to the fact that the chicken embryo contains various structures suitable for the propagation of viruses (Fig. 51) [3, 5, 7, 8].

Purpose of using chicken embryos. In virology, chicken embryos are used:

1) to detect bioassay in the biological material of an active virus;
2) for the primary isolation of the virus and its cultivation;
3) to maintain viruses in the laboratory in an active state;
4) for titration of viruses;

5) for the accumulation of a large mass of viruses for laboratory research in the production of vaccines and diagnostic test systems;

6) as a test object in the neutralization reaction.

7) to obtain cell cultures.

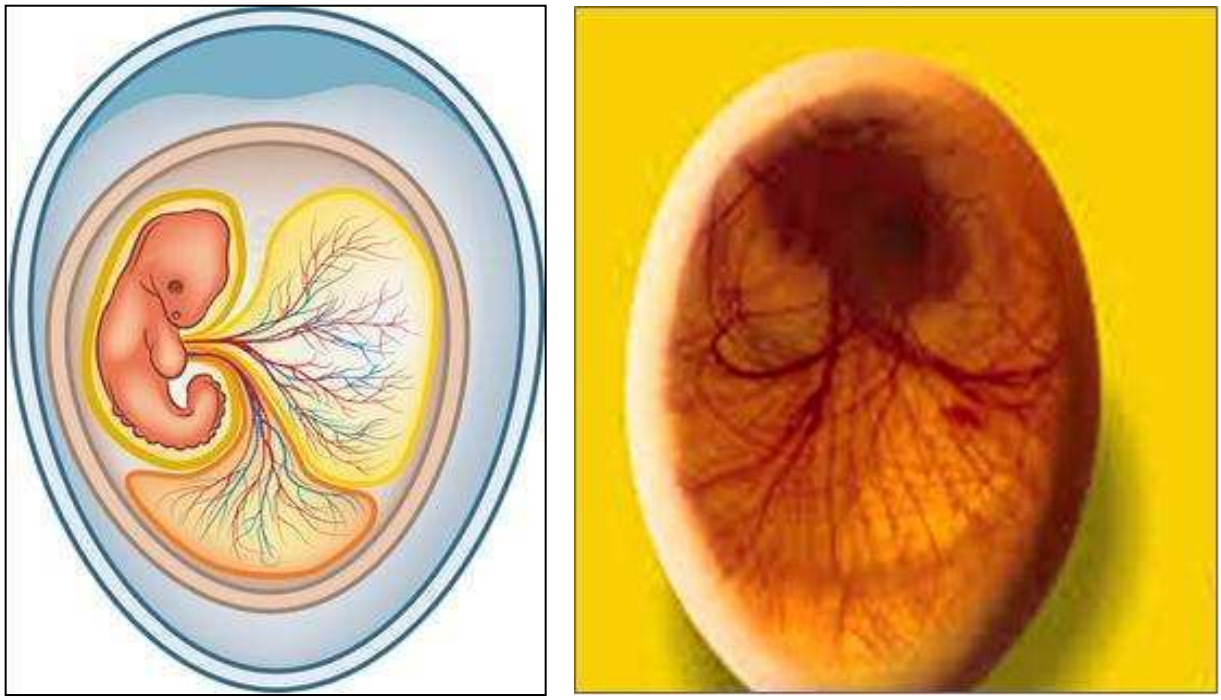


Figure 51 - Chicken embryo

Advantages of a chicken embryo over laboratory animals:

1. High sensitivity to a wide range of viruses (due to insufficient development of the immune system, they do not form antibodies in response to infection, which ensures high sensitivity of the embryo to viruses).

2. Easily accessible facility (a large number of poultry farms and hatcheries).

3. Sterility (shell and shell membrane reliably protect embryos from bacterial infection from the external environment). However, the content of pathogenic agents (viral bronchitis of hens, flu, leukemia, Newcastle disease, etc.) cannot be completely ruled out.

4. Economical (low cost, because they do not require care, feeding).

5. The simplicity of the experiment (a simple technique of infection and obtaining virus-containing material).

Requirements to chicken embryo

In selecting chicken embryos for infection with virus-containing material, the following requirements are imposed to them:

1. Chicken embryos must be *healthy*, and obtained from poultry farms that are safe from infectious diseases.

2. The eggshell must be *unpigmented, clean* (they must not be washed).

3. *The age of the embryo* should correspond to the chosen method of infection.

4. *Viability of the embryo.* The signs of a living embryo are: active movements of the embryo, blood vessels of the CAM are clearly visible. Signs of a fallen

embryo: lack of active movement of the embryo, dull, dying blood vessels.

The structure of the chicken embryo

Usually, a chicken lays a fertilized egg, where the embryo is at the blastula or early gastrula stage. When the egg is heated to a temperature close to the temperature of the chicken body, the embryo develops further. During the period ***from the 5th to the 12th day of incubation***, chicken embryos can be used to be infected by viruses. The following **elements** are distinguished in the embryo (Fig. 52):

- *shell* – is a hard porous membrane that covers the outside of an egg with a developing chicken embryo;
- the *inner shell membrane* – is a thin membrane that hermetically fits to the shell;
- the *allantoic cavity* – is located under the inner shell membrane, covers the amnion and yolk sac, and metabolic products are collected in it;
- the *chorion-allantoic membrane (CAM)* - borders the allantoic cavity, formed as a result of fusion of the cavity and chorion, is rich in blood vessels that supply the embryo with oxygen - a function of the respiratory organ;
- the *air chamber* – is an area at the blunt end of an egg filled with air. It is formed by dividing the inner shell membrane into two leaves.
- *the body of the embryo* (embryo) - lies in the egg eccentrically, with its back closer to the shell, the head is directed towards the air chamber;
- *amnion* – is the cavity in where the embryo lies in the amniotic fluid;
- *yolk sac* – is the supply of nutrients, is connected to the embryo with the umbilical cord, is also located eccentrically and relative to the embryo, on the other side of the longitudinal axis;
- *protein* – is located at the sharp end of the egg.

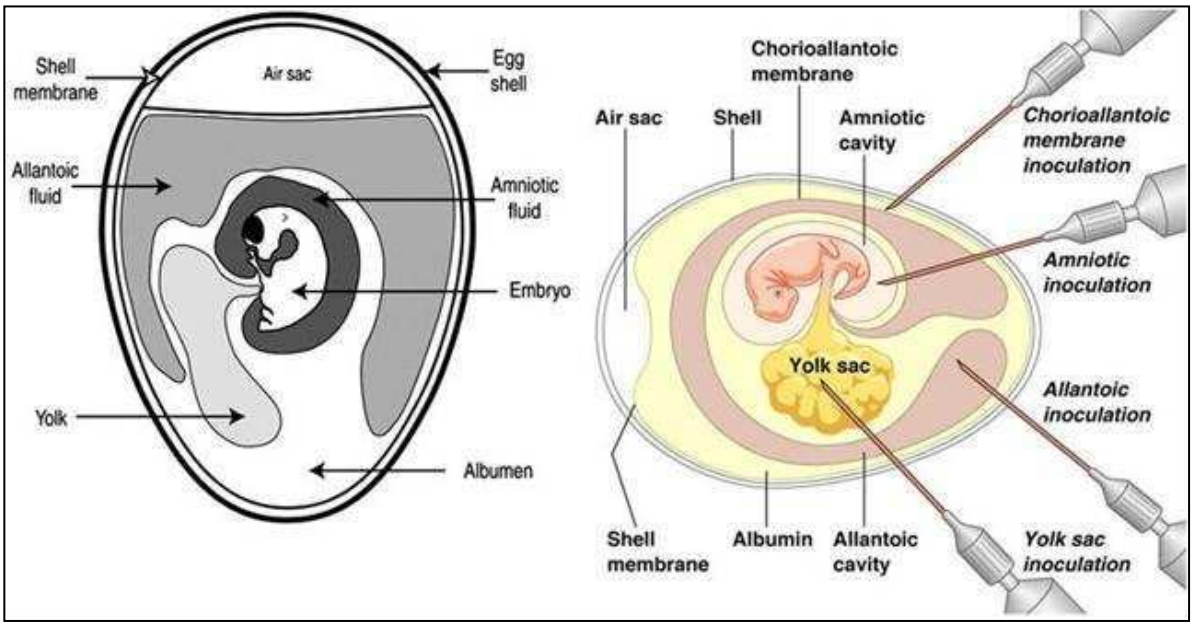


Figure 52 - The structure of the chicken embryo

The accumulation of viruses occurs in all structures of the embryo with a cellular structure (embryo, CAM, yolk sac). Many viruses can accumulate in allantoic

and amniotic fluids, and form an almost ready-made suspension of viruses.
Infection in one or another part of the embryo is carried out during the period of its maximum development, when the number of sensitive cells will be the greatest.

Topography of the chicken embryo (longitudinal section, 12 days):

1. Shell
2. The inner shell membrane
3. Air chamber
4. Allantoic cavity
5. Yolk sac
6. Albumin bag / protein
7. CAM - chorion-allantoic membrane
8. Amniotic cavity
9. Embryo
10. Cord (connection of the yolk sac with the umbilical cord)

Chicken embryo infection methods

For infection, healthy embryos are chosen. Before infection, the chicken embryo is *ovoscopied* - viewed by using an ovoscope in order to determine the viability and position of the embryo. Notes are made with a pencil on the shell - the boundaries of the air chamber and the location of the embryo should be marked. Then the shell is disinfected by processing it with a tampon with iodized spirit, and they are flamed. After disinfection, the chicken embryo is infected with virus-containing material.

In practice, ***infection*** is often made into the allantoic cavity and on the CAM, less often into the yolk sac and amnion, and even less often into the body of the embryo and blood vessels. The volume of the infecting dose is about 0.1 - 0.2 ml.

The method of infection depends on the tropism of the injected virus and according to the method, the age of the embryo is chosen:

- allantoic cavity infection, ***9-12-day-old*** embryos are used;
- the amnion infection - ***6-10 days***;
- CAM infection - ***10-12 days***;
- the yolk sac infection - ***5-7-days***.

Chicken embryos are incubated in an incubator or thermostat at a temperature of 37-300C, at a relative humidity of 63-65% and periodic ventilation.

Chicken embryo infection technique

Allantoic cavity infection. The embryo is fixed vertically with a blunt end up and in the shell on the side of the embryo, retreating 5-6 mm from the edge of the air chamber to its middle, by making a hole a syringe needle is inserted through it to a depth of 10-12 mm and the virus-containing material is injected. The needle is taken away, and the hole in the shell is closed with a drop of molten paraffin.

Chorion-allantoic membrane (CAM) infection.

The infection of the CAM is carried out through a natural air chamber. For this purpose, the chicken embryo is fixed vertically with a blunt end up and with pointed scissors in the shell, a hole with a diameter of 15-20 mm is cut out against the center of the air chamber. The shell is carefully removed with tweezers, and virus-containing material is applied to the exposed CAM. The hole is closed with a plaster.

The yolk sac infection. Chicken embryo is fixed vertically in a tripod with a blunt end up. In the shell, in the center of the air chamber, a hole with a diameter of 1-3 mm is made and a syringe needle is inserted to a depth of 40-45 mm at an angle of 45^0 in the direction of the opposite side, on which the embryo is located and injected. The hole in the shell is covered with a drop of molten paraffin.

The amnion infection. The embryo is strengthened vertically with a blunt end up. In the shell above the air chamber, a hole is made and a blunt long needle is inserted in the direction of the embryo. After this, the needle must be carefully removed. If the embryo repeats the movement, then the needle has fallen into the amnion. Then an injection is made. The hole in the shell is covered with a drop of molten paraffin.

After ***infection*** of the chicken embryo, an inscription (about when and what the embryo is infected with) is made on the shell with a pencil.

Infected chicken embryos are placed in a thermostat for further incubation, during which the introduced viruses are reproduced and accumulated in the corresponding structures.

Independent work. Students take CE, ovoscopy it, disinfect and make the allantoic cavity infection. An inscription is made on the shell of a chicken embryo with a pencil. It should contain the information about what the embryo is infected with, and name and group of students. Then, the infected chicken embryos are handed over to the attendant, who places the embryos in thermostat for further incubation. Used tools are also handed over to the attendant [5, 6].

Control questions:

1. The structure of the chicken embryo (CE).
2. The use of CE in virology.
3. Requirements, submitted to CE.
4. Advantages of CE over other laboratory animals.
5. Methods of infection and what it depends on.

2.8 Dissection of the chicken embryo, indication of the virus and obtaining virus-containing material

The purpose of the lesson. To master the technique of opening a chicken embryo

Goals:

1. To now the pathological changes in the chicken embryo.
2. To be able to open the embryo and identify the virus.

Equipment: stand for chicken embryos, spirit lamp, sterile ophthalmic scissors and tweezers, Petri dishes, test tubes with physiological solution, empty tubes, 5-10 ml pipettes, cans for pipettes, ovoscope; disinfectant solution; Nutrient media - MPA, MPB, Kitta-Tarroci medium.

The presence and propagation of the virus in the chicken embryo is identified by the death of the embryo and by the pathological changes in the embryonic structures.

Indication of the virus in the chicken embryo:

1. *The death of the chicken embryo* in the time period, which is characteristic for the virus. The death of embryos on the first day is not specific. Embryos that died at a later date are transferred to the refrigerator (+ 4^0C). All embryos are taken away from the thermostat at the time of the virus maximum accumulation. The term for each virus is determined by reference data.

2. Dissection of the embryo and *analysis of pathological changes*:

- Edematous CAM and white nodules of necrosis (smallpox).
- Turbidity or discoloration of allantoic and amniotic fluids. The reddish color of the fluids is the result of hemolysis.
- Embryo changes: dwarfism, hemorrhage, mummification.
- The organs of the embryo - necrosis, hemorrhage.

3. The presence of *hemagglutination* - while conducting a drip reaction (allantoic fluid + 5% of chicken erythrocyte solution).

The death of infected chicken embryos is established by daily ovoscopy. After the incubation period determined for each virus, infected chicken embryos are opened to analyze pathological changes and to take virus-containing material for further research. Before opening, the chicken embryo is cooled by keeping them for at least two hours in the refrigerator at a temperature of + 40C.

Depending on the tropism of the virus, allantoic and amniotic fluids, CAM, yolk sac, and the body of the embryo can be taken *as a virus-containing material*.

For opening the chicken embryo is fixed in that position, where the air chamber is upward, the shell is disinfected and shears are cut off above the air chamber with scissors. Tweezers are removed under the shell. Then, by slightly inclining the embryo, the CAM is punctured with a petal and the allantoic fluid (5-6 ml) is aspirated. The fluid may be cloudy and bloody. The presence of hemagglutinating viruses is determined by the hemagglutination reaction (Fig. 53).

Taking amniotic fluid. After suction of the allantoic fluid, we begin to take the amniotic fluid. To do this, we need to capture the embryo with tweezers and a pipette, puncture the amniotic membrane, and aspirate the liquid. The amount of it, is about 0.5-1.5 ml. Usually the liquid is cloudy, sometimes it has a bluish tint.

Taking the yolk sac. After suction of the allantoic and amniotic fluid, contents of the embryo are extracted into a sterile Petri dish. By using two tweezers, we take a yolk bag, carefully squeeze its contents, and the bag itself is placed in another Petri dish, because the virus accumulates in the membrane of the yolk sac. The yolk sac is ground in a mortar and a suspension is prepared.

The capture of the chorionallantoic membrane. CAM is removed at last turn and placed in a Petri dish, where should be a sterile physiological solution, under which a piece of black paper is placed. The shell is washed, straightened and examined macroscopically in order to establish edema, hemorrhages, the presence of smallpox and other lesions. Pathological changes are clearly visible on a dark background. For histological examination, cross sections of the membrane are

prepared. For stating IFR (immunofluorescence reaction) prints from it are taken. A suspension of CAM is prepared for subsequent infection.

By using all the taken material, plating on nutrient media is done to ***exclude bacterial infection*** and to make smear preparations.

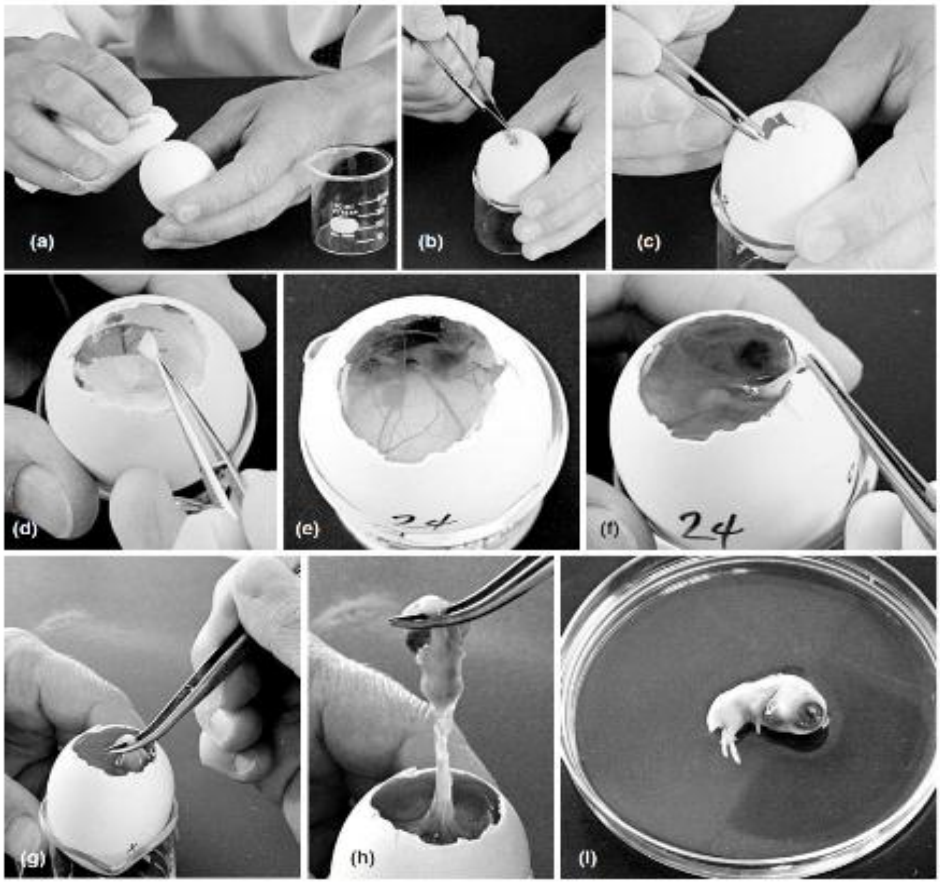


Figure 53 - Stages of opening a chicken embryo

REMEMBER:

The main methods of infection: amnion, allantoic cavity, chorion-allantoic membrane (CAM) and yolk sac infections.

- the chorion-allantoic membrane (CAM)
- the allantoic cavity
- the cavity of the yolk sac
- the cavity of the amnion
- the body of the embryo

Virus indication:

- death of the embryo
- morphological changes of the embryo / membranes
- Hemagglutination test with fluid from the cavities of the chicken embryo

Virus identification:

- HAI
- Complement binding reaction

Control questions:
1. The importance of CE in the cultivation of viruses.
2. Incubation of CE after infection
3. Specific and non-specific death of CE.
4. What are the criteria for identifying the presence and propagation of a virus in CE?
5. Indication of the virus in the embryonic structures.

2.9 Cell cultures and their use in virology

The purpose of the lesson. To introduce the general information about cell cultures used in virological studies.

Goals:
1. To know the main types of cell cultures.
2. To learn methods for indicating the virus in cell culture.

Equipment: nutrient media - No. 199, needle; blood serum, lactoalbumin hydrolyzate, saline solutions of Hanks, Earle, Tyrode, trypsin, versene solutions, test tubes, bottles, mattresses, panels. Magnetic stirrer, centrifuge, flask with magnet, graduated pipettes of 1.2.5.10 ml; test tubes, centrifuge tubes, funnels with two-layer gauze, rubber hose with a pear, rubber tubes, scissors, tweezers, spirit lamp, Petri dishes, micro-scopes, Goryaev's camera, indicator paper, 7.5% NaHCO solution 5% - HCI solution, penicillin**,** streptomycin, physiological solution, 0.2% trypan blue solution.

The modern development of virology is possible due to the inculcation of cell cultures into practice [5, 6, 7].

Cell cultures are cells of a *multicellular organism* that grow and propagate outside the body *("in vitro" —in test-tube*).

The method of cultivating viruses on cell cultures was developed and put into practice by American virologists John Enders, Frederick Robbins and Thomas Weller in 1948-1955.

Currently, cell culture is widely used in virology:

1. To study the biology of the virus (the mechanism of reproduction, the relationship between the virus and the cell).

2. For the quantitative study of viruses and virus-neutralizing antibodies.

3. For diagnostic purposes - isolation of the virus from the primary material.

4.For the production of therapeutic and diagnostic products (vaccines, diagnosticums, interferon).

Advantages: the cell culture method is feasible, economical.

Deficiencies in the use of cell culture, especially in the production of vaccines: this is often the contamination of the original tissue material with latent viruses, mycoplasmas and other microorganisms.

The massive use of tissue and cell cultures in the laboratories of the world and the use of this technique for various purposes has led to terminological confusion. On June 3, 1966 in San Francisco, at the annual meeting of the Association of Tissue Cultures, a unified terminology was proposed for animal tissue cultures.

The **basic terms** in abbreviated form:

Tissue culture is a collective concept, which is denoting a system, where the cells, tissues or organs, taken from an animal's body, retain viability or ability to reproduce *in vitro* for more than 24 hours.

According to the type of biological object, what should be supported to grow - cells, tissues or organs are distinguished as:

a) **cell culture** - cells growing in vitro without tissue formation;

b) **tissue or organ culture** - the maintenance or growth of tissues and the whole organ (or a piece of it) in vitro, while maintaining their differentiation, structure and function.

An explant is an isolated piece of tissue or organ used for cultivation.

Monolayer – cells, which grow in one layer on a specific surface.

Suspension culture is a type of cell culture, in which cells multiply in suspension on a nutrient medium.

Primary culture is a culture derived from cells, tissues or organs, which were taken directly from the body. Culture is considered as primary until it is subcultured.

Primary cultures are obtained directly from normal or tumor tissues of humans or animals by the method of explantation or trypsinization of tissues with growth potentials (embryonic: musculoskeletal tissue, amnion, lung, etc.; adults: skin, renal parenchyma, etc.) . In this case, mostly cultures of epithelial cells or fibroblasts are received.

The cell line is obtained from the primary culture after the first passage, consists of heterogeneous cells presented in the primary culture.

A stable (transplantable) cell line is a cell line capable of subculturing in vitro to infinity. It is considered as stable when it has passed at least 70 passages at three-day intervals.

A cell strain is a population of homogeneous cells (according to one or more of the signs - markers). They are got from the primary culture or from the cell line, in the same time the markers must be preserved in the process of subsequent cultivation.

A clone is a population of cells that originates from a single cell through mitosis. The clone is not always homogeneous.

Cell cultures used in virological practice can be divided into single-layer and suspension.

1. Single-layer cell cultures, in which cells grow and multiply, adhere tightly to the surface of the glass (tubes, mattresses) and locate in a single-cell layer.

Single-layer cell cultures are divided into 3 types:

a) Primary cell culture – is obtained directly from animal tissue. They possess a diploid set of chromosomes and are not capable of prolonged inoculations (passage).

b) Diploid cell cultures – are obtained from primary cells, they have a diploid set of chromosomes, and are capable of large (50-70 passages), but a limited number of inoculations.

c) Transplantable cell cultures – are obtained from primary culture, they lost diploid set of chromosomes (it became heteroploid), and are capable of an unlimited number of cell transplantations.

2. Suspension cell cultures - cells live and multiply, being weighed in a liquid nutrient medium.

Only some of the transplantable and diploid cell cultures succumb to suspension culture (Fig. 54).

Figure 54 - Tablets (mattresses) for the cultivation of cell cultures

Preparation of dishes for cell culture

Dishes (test tubes, flasks, vials, mattresses, bottles, pipettes, rubber tubes, corks) for cell culture should be absolutely clean and sterile, made from neutral glass. Improper washing can cause cell detachment from glass or rapid degeneration of the cell monolayer.

The dishes are soaked in a warm powder solution for a day, washed with ruffs, rinsed with tap water for 6-8 times, shaken, washed with distilled water, dried and sterilized in an autoclave. New dishes are additionally treated with solutions of potassium dichromate in sulfuric acid (chromic peak) or hydrochloric and sulfuric acid.

Rubber products are also kept in a solution of detergents, washed in them, then washed with warm tap water, and boiled in enameled tanks for 1 hour in a solution of 0.3% bicarbonate salt, washed again, boiled in a 2% hydrochloric acid solution for 30 minutes, then washed repeatedly with tap and distilled water, dried, mounted and sterilized.

Nutrient media used for cell cultures

Nutrient media that contain everything necessary for cell life are used for breeding cells *in vitro*.

Nutrient media can be *natural and synthetic*:

Natural nutrient media include:

1. Blood serum of cattle, which is obtained from the blood of clinically healthy animals.

2. Enzymatic hydrolysates of various protein products. They include hydrolysates of lactoalbumin, casein, hemohydrolyzate and others.

Synthetic nutrient media – are obtained by adding all known amino acids, vitamins, precursors of nucleic acids, growth factors, lipid sources to saline solutions. The most widely used in virology are *199 medium, Igl's medium*.

Nutrient media for cell cultures are also divided into:

- ***supporting*** (do not contain blood serum) - to maintain cell viability, while the cells do not multiply;

- ***growth*** (contain up to 5-10% of blood serum) - provide cell viability and reproduction.

Nutrient synthetic media are prepared on saline isotonic solutions. Saline solutions are balanced by ions, osmotic pressure and other indicators. The following saline solutions are used: Tyrode, Earl, Hanks, which consist of salts of Ca, Na, K, Mg. In addition, ***dispersing solutions*** of trypsin, pancreatin, hyaluronidase, collagenase are used to disaggregate tissues.

Obtaining primary trypsinized cell cultures

In sterile conditions, the tissue is crushed, washed and placed in an enzyme solution on a magnetic stirrer. The resulting cell suspension is centrifuged, and the cell pellet is diluted with growth medium to the optimal concentration. After plating in culture flasks, the cells attach to the glass and adapt to in vitro conditions (stabilization phase, lag phase), then they begin to actively proliferate (logarithmic growth phase, log phase), then enter stationary phase, and form a monolayer.

Control questions:

1. The importance of cell cultures in virological research.
2. Types of cell cultures.
3. What are the advantages of cell cultures over other laboratory systems?
4. The terminology used in the culture of cells and tissues.
5. Types of nutrient media and saline solutions.

2.10 Indication of viruses in cell culture by cytopathogenic effect

The purpose of the lesson. To study the cytopathogenic effect (CPE) of the virus on cell culture.

Goals:

1. To know methods for indicating the virus in cell culture.
2. To study the cytopathogenic effect of the virus.

Equipment: microscopes, illuminators, cell cultures, preparations from fixed stained cell cultures, related tables.

Indication of the virus in cell culture is carried out by the following ***methods***:
1. Identification of cytopathogenic effect (CPE);
2. Positive hemadsorption;
3. The phenomenon of plaque formation;
4. The presence of specific inclusion bodies;
5. Suppress of cell metabolism (color test);
6. Detecting a specific antigen by the method of fluorescent antibodies.

1. Many viruses, when propagated in cells, have a ***cytopathogenic effect*** (CPE), which is appearing by morphological changes in the cells.

The nature of the CPE, the time of its development depends on the type and dose of the inoculated virus. However, it is necessary to distinguish the viral CPE from the "age-related" degenerative changes in old cultures and from cell degeneration caused by contaminating viruses.

The assessment of the intensity of the CPE is carried out according to the cross system:
++++ is the destruction of all cells in a monolayer;
+++ - destruction of 3/4 cells;
++ - the destruction of 1/2 cells;
+ - destruction of 1/4 cells.

The CPE of the cells manifests in the form of:
1) rounding of the cells - when the cells lose their ability to attach to the glass (usually should be flattened) and become spherical, they pass into the culture fluid and die;
2) cell fragmentation - is the destruction of cells into fragments;
3) symplastogenesis - due to lysis of cell membranes, cytoplasms of adjacent cells merge into one and form symplasts with many nuclei.

According to these destructive changes, it is possible to tentatively identify the group of the virus. The final diagnosis is confirmed by typing specific serum in the reactions.

2. The reaction of haemadsorption – is a method for detecting the virus.

Its essence lies in the fact that a 0.5% suspension of washed red blood cells is added to the culture of cells infected with the virus. Red blood cells after contact with the virus are adsorbed on the surface of the cells. Only those viruses that cause hemagglutination of these red blood cells cause haemadsorption.

3. The formation of plaques - this method can also be used to identify the propagation of viruses. In virological practice, this method is used in the titration of viruses and antibodies.

The method of plaques contains such steps as : in mattresses with a monolayer of cells the growth medium istaken, a suspension of the virus is added, and left for 1-2 hours in a thermostat for adsorption / attachment of the virus. Then the cell layer is coated with expanded agar with nutrient medium. Mattresses are placed in the thermostat with the cells up. The virus as a result of reproduction causes cell death, and enters neighboring cells, which also die. Thus, islands of *dead cells appear in the*

monolayer of animal cells, over which *the color of the agar does not change*, and remain light yellow, while above *living cells*, *the color of the agar changes* as a result of acidification by the products of cell metabolism.

These bright spots on a pink-red background are called ***plaques***. The titer of the virus is determined by the formula 1:

$$A = \frac{a \times b}{V} \quad (1),$$ where

A - the number of PFU (plaque forming unit);
a - the average number of plaques in 1flask;
b - virus dilution;
V - the volume of virus injected into the mattress.

4. The detection of specific inclusion bodies in preparations from infected cell cultures is also a method for identifying certain viruses.

To do this, cell cultures are bred on glasses, infected with a virus, then the drug is fixed, stained and under the light or electron microscope, the inclusion bodies, which are specific for a particular virus, are detected

For example, inclusion bodies form cattle adenoviruses in the cell nucleus; smallpox and contagious ectima viruses in the cytoplasm of cells.

5. Color test – is based on a change in the color of the nutrient medium, which contains a pH indicator - phenol red.

When cell cultures are infected with a cytopathogenic virus, their metabolism is suppressed, and the cells gradually die. As a result, acidification is delayed in the culture fluid and the color of the medium does not change (does not turn yellow), remains red or turns yellow slowly. Thus, the cytopathogenic virus can be detected by the color test method.

6. The method of fluorescent antibodies (MFA) – due to this method, viral antigens localized on the surface of cell culture, can be detected. Suspension cells, which, after appropriate treatment, are combined with antibodies stained with fluorescent dyes, are used for this method.

Preparations made from these cells are examined under *a luminescent microscope and specific fluorescence is observed.*

Control questions:

1. What is the CPE of the virus?
2. The difference between the CPE and non-specific “age-related” cell degeneration.
3. Forms of manifestation of the CPE. Evaluation of the virus CPE.
4. What is PFU?
5. Methods for indicating the virus in cell culture.

2.11 Bacteriophages. Identification of phage in bacterial culture

The purpose of the lesson. To familiarize students with bacteriophages and their use in virological practice.

Goals:

1. To define bacteriophages
2. To determine the activity of phage in bacterial culture

Equipment: sterile Petri dishes, sterile Pasteur or graduated pipettes, bacteriological loops, 1.5% MPA, culture of bacteria, homologous and heterologous phages, pencils on glass, tables.

Bacteriophages are viruses of bacteria. They are able to infect a bacterial cell, reproduce in it, form numerous offspring, and cause cell lysis, accompanied by the release of phage particles into the habitat of bacteria.

Phages are widely found in soil, water, in the intestines of humans, animals, birds, in their excrement, in milk, in all pathogenic and many non-pathogenic microbes.

The phages were first identified in 1917 by the French scientist D'Herelle, from the feces of people with dysentery [5, 6].

Phage Classification:

1. According to the type of nucleic acid, phages are divided: DNA and RNA-containing.

2. According to the interaction with a bacterial cell: moderate and virulent.

Virulent phages cause lysis of the bacterial cell.

Moderate phages establish a symbiotic relationship with the cell. Bacteria, which are bearing a moderate phage, are called *lysogenic*, and the form of a bacteriophage in a lysogenic bacterium is called a prophage.

3. According to the relation of phages to sexual differentiation of bacteria:

a) f-1, f-2, QB, MS-2 - attack only male bacteria.
b) T-3, T-7, F-1 1 - interact with female cells.
c) Indifferent to sexual differentiation of cells.

4. According to the morphology:

1) Large - virulent coli phages (T group)

T group is divided into:

a) even and odd: T-1, T-2, T-3, T-4, T-5, T-6, T-7.
b) moderate intestinal phages: X, P-1, P-2, P-22.

2) Small - RNA-containing phages: f-2, MS-2, M-12.
DNA-containing phages: ΦX-174.

T-even phages of E. coli (T-2, T-4, T-b) are more complicated; some of the phages of *Bac.subtilis* are SPO-1, SP-8, SP-82, etc. These phages have an elongated hexagonal head, tail spike and an attached contractile sheath.

a) Tail - 100 nm, diameter of 2.5 nm.

b) T-1, T-5 intestinal phages, X moderate phage has a hexagonal head and tail.

c) the phages have a large hexagonal head and a short tail spike, which contain T-3, T-7 phages.

d) tailless DNA - contains small phage and polygonal capsid structure.

d) RNA-containing phages are characterized by small sizes. For example, they are intestinal phages (f-2, f-7, M-2, R-17).

f) filamentous intestinal phages, which contain single-stranded DNA, length up to 800 im, diameter bnm. Most of these phages interact with male strains.

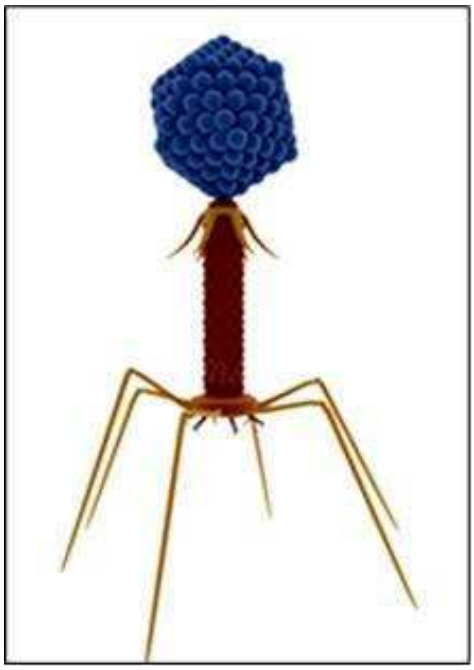


Figure 55 – Bacteriophage

Bacteriophagy – is the interaction of phage with bacterial cells.

The process of interaction of the bacteriophage with bacteria consists of 7 stages:

1. Adsorption through the interaction of phage receptors located in the tail of the phage, with the surface of the microbial cell.
2. Dissolution of the membrane of the microbial cell by using the lysozyme.
3. Injection of NK phage through the canal of the tail spike and the hole formed in the cell membrane in the cytoplasm.
4. Blocking of NK and inhibitors (early proteins) of the phage activity of the cell nucleus.
5. Reproduction of phage's nucleic acid molecules.
6. Synthesis of phage protein (late protein).
7. The assembly of phage particles and their exit from the cell, which is accompanied by lysis (virulent phages) or lysogeny (moderate phages) of the bacterial cell.

Bacteriophages **are used:**

- for identification of bacteria (phagotyping);
- for diagnostics (phagodiagnosis, reaction of increasing phage titer),
- for prevention and treatment of infectious diseases.

Identification of phages:

1. By electron microscopy.
2. Detection of the phages action on a population of sensitive bacteria:

a) the addition of a virulent phage to a suspension of bacteria and subsequent incubation at t = 37-38 ° C - this leads to the lysis of bacteria, which is accompanied by the enlightenment of the nutrient medium, which contain bacteria.

The enlightenment rate of the medium depends on the number of bacteriophages contained in it.

If the phagolizing culture is incubated for a longer time, then secondary growth may appear due to the multiplication of resistant mutants selected in the bacterial population.

If bacteria are infected with a moderate phage, a moderate turbidity of the

culture is formed, and then turbidity increases due to the growth and reproduction of lysogenized bacteria.

b) The number of viable phages is determined on a solid nutrient medium (by the Grazia method). On lawn-sown sensitive bacteria, areas of culture lysis, which are called phage plaques or negative colonies, or sterile spots, are determined.

Virulent phages form transparent negative colonies.

Moderate phages form turbid colonies.

The number of colonies or plaque-forming units (PFU) is multiplied by the dilution of the initial suspension of phages, which gives the value of the phage titer, that is, the number of PFU in 1 ml of the original drug.

Students' independent work

1.5% molten meat peptone agar is poured into a petri dish. After hardening agar with a pencil on glass, the bottom of the Petri dish is divided into two sectors. A 16-18 hour culture is plated with a thick lawn on agar in Petri dishes. 5-10 minutes after plating, homologous and heterologous phages (in different sectors) are applied dropwise to the dried surface of the nutrient medium. Then the cups are dried, turned upside down and put in a thermostat at a temperature of 37 C for 18-24 hours.

Experiment accounting is carried out by:

1) the complete absence of culture growth at the site of application of a drop of phage (active bacteriophage).

2) by the presence of small sterile spots - colonies of a bacteriophage (bacteriophage of weak activity).

Control questions:

1. The morphology of the bacteriophage. Types of bacteriophages.
2. The chemical composition of the bacteriophage.
3. Features of the interaction of the phage with the cell.
4. Methods for detecting phage.
5. The practical use of phage.

Bibliography

1. Ветеринарная микробиология и микология: учебное пособие / составители Г. П. Тихонова [и др.]. — Чебоксары: ЧГСХА, 2017. — 104 с.

2. Ермаков, В. В. Ветеринарная микробиология и микология: учебное пособие / В. В. Ермаков. — Самара : СамГАУ, 2018. — 262 с. — ISBN 978-5-88575-496-5.

3. Колычев, Н. М. Ветеринарная микробиология и микология: учебник / Н. М. Колычев, Р. Г. Госманов. — 3-е изд., стер. — Санкт-Петербург: Лань, 2019. — 624 с. — ISBN 978-5-8114-4735-0.

4. Кисленко В.Н., Колычев Н. М. Ветеринарная микробиология и иммунология. Ч.2 – М.: КолосС, 2007г. - 224с.

5. Госманов Р.Г., Колычев Н.М. – Ветеринарная вирусология – М.: Колос, 2006 – 304 с.

6. Сюрин В. Н. Диагностика вирусных болезней животных. – М.: Колос, 1991. - 435с.

7. Белоусова Р. В. Практикум по ветеринарной вирусологии. М.: Колос, 2006. - 248с.

8. Лысак В.В. Микробиология. – Минск, БГУ, 2007. – 403 с.

9. Никитина Е.В.– Микробиология - СПб.: «ГиоРд», 2008 -368 с