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МЕТОДЫ МИКРОБИОЛОГИЧЕСКИХ ИССЛЕДОВАНИЙ

Рекомендовано учебно-методическим объединением
по высшему медицинскому, фармацевтическому образованию
в качестве учебно-методического пособия для студентов
учреждений высшего образования,
обучающихся по специальности 1-79 01 01 «Лечебное дело»

RESEARCH METHODS IN MICROBIOLOGY

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В данном учебно-методическом пособии представлены классические и совре-
менные методы микробиологических исследований, применяемые для диагности-
ки бактериальных, вирусных и грибковых инфекционных заболеваний. Материал
пособия позволит студентам более эффективно овладеть практическими навыка-
ми на учебно-практических занятиях по общей и частной микробиологии, общей
и частной вирусологии, а также теоретической и прикладной иммунологии. Мате-
риал пособия изложен в соответствии с действующими типовым учебным планом
и программой по микробиологии, вирусологии, иммунологии.

Предназначено для иностранных студентов 2–3 курсов учреждений высшего
медицинского образования, обучающихся на английском языке по специальности
1-79 01 01 «Лечебное дело».

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LIST OF ABBREVIATIONS

AFB	—	acid-fast bacteria
API	—	analytical profile index
ATCC	—	American collection of type cultures
BSS	—	balanced salt solution
CD	—	cluster of differentiation
CFT	—	complement fixation test
CFU	—	colony-forming unit
CIC	—	circulating immune complex
CLSI	—	Clinical and Laboratory Standards Institute, USA
CPE	—	cytopathic effect
DDM	—	differential-diagnostic media
dNTPs	—	deoxynucleotide triphosphates
DTH	—	delayed type of hypersensitivity
ELISA	—	enzyme immunosorbent assay method
EM	—	electron microscopy
EUCAST	—	European Committee on Antimicrobial Susceptibility Testing
FITC	—	fluoresceinisothiocyanate
HIV	—	human immunodeficiency virus
ICA	—	immunochromatographic analysis
IEM	—	immune electron microscopy
IFA	—	immunofluorescence assay
ITH	—	immediate type of hypersensitivity
KIA	—	Kligler iron agar
MALDI-TOF	—	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MIC	—	minimum inhibitory concentration
MPA	—	meat-peptone agar
MPB	—	meat-peptone broth
NK cells	—	natural killer
RA	—	reaction of agglutination
RHA	—	reaction of hemagglutination
RHads	—	reaction of haemadsorption
RIA	—	radioimmune assay
RIHA	—	reaction of indirect hemagglutination; reaction of inhibition of hemagglutination
RN	—	reaction of neutralization
RP	—	reaction of precipitation
RT-PCR	—	PCR with reverse transcription
PCR	—	polymerase chain reaction
TSA-agar	—	triple sugar iron agar

INTRODUCTION

Currently, microbiological studies have priority development, since they are in demand in almost all types of medical care. The tasks of microbiological studies are to identify microorganisms isolated from the studied material (which allows to make a microbiological diagnostics), as well as to estimate the antibiotic sensitivity of the selected microorganisms, which plays an important role in the selection of antimicrobial drugs for the etiotropic treatment of an infectious disease.

Despite the fact that the conduct of microbiological research falls under the competence of clinical microbiologists, whose professional training requires the obligatory development of practical laboratory skills, each clinician dealing with specific and non-specific infectious diseases must know how and when it is necessary to collect material for research what research to send it to and how to interpret the results. Thus, the formation of knowledge about the methods of laboratory diagnostics of infectious diseases and the development of practical skills in conducting microbiological studies is a necessary element in the training of medical students.

The educational-methodical manual was created to optimize the process of studying the subject when doing practical work in practical classes in the educational microbiological laboratory. In compiling the training manual, the author took into account the latest scientific achievements, used modern instructive and methodological materials, and, along with traditional methods for diagnosing infectious diseases, outlines modern automated methods of microbiological, immunobiological and molecular genetic studies using microbiological and immunological analyzers. The material of the manual is illustrated by diagrams, drawings and tables that allow students to gradually study the methodology of research and improve the absorption of knowledge and skills in practical classes.

The authors of the manual will be grateful for constructive comments and suggestions for improving the content of the material of this educational-methodical manual.

SECTION 1
MICROBIOLOGICAL LABORATORIES

The concept of microbiological laboratories

There are bacteriological, virological, immunological and specialized laboratories.

Bacteriological, virological and serological laboratories are part of the centers of hygiene and epidemiology, and are also organized in infectious hospitals, general hospitals, specialized hospitals, for example, tuberculosis and venereal diseases dispensaries as well as in some clinics [1].

In the laboratories of centers of hygiene and epidemiology, bacteriological, virological and serological studies of pathological materials obtained from patients and persons in contact with them are carried out, carriers are examined and sanitary-microbiological studies of water, air, soil, foodstuffs, etc. are carried out. In particular, virological laboratories diagnose viral infections (influenza, measles, rubella, viral hepatitis, tick-borne encephalitis, poliomyelitis, HIV infection, etc.) and diseases caused by chlamydia and rickettsia. When organizing and equipping virological laboratories, the specifics of working with viruses, tissue cultures, chicken embryos, which requires the strictest asepsis, is taken into account. Diagnostics of especially dangerous infections (plague, tularemia, brucellosis, cholera, anthrax, etc.) is carried out in specialized laboratories (laboratories of especially dangerous infections) [1].

In bacteriological and serological laboratories of hospitals, diagnostic tests are usually carried out for various infectious diseases of a bacterial nature (pyoseptic, airborne, intestinal, etc.), as well as studies to monitor the quality of disinfection and sterilization.

The organization of microbiological laboratory

The following rooms are provided in the microbiological laboratory:
1) rooms for bacteriological research;
2) a box for working with individual groups of bacteria or viruses under aseptic conditions;
3) a specially equipped room for sterilization — room with the autoclave: for sterilization of culture media, dishes, disinfection of waste infected material;
4) washing room, equipped for washing dishes;
5) equipped room for immunological research;
6) a room for the preparation of nutrient media;
7) the registry for receiving samples and issuing analysis results;
8) wardrobe for staff.

Before starting work, overalls are put on, which are stored in individual cabinets separately from outer clothing. The type of protective suit and the frequency of its change are determined depending on the nature of the work [1, 2].

Educational microbiological laboratory and rules for working in it

The following rules must be observed in the educational microbiological laboratory:

1. In the training laboratory, students should be in medical gowns, caps and replaceable shoes. It is forbidden to bring outerwear to the workshop and put bags, briefcases and other foreign objects on the working tables.

2. In the laboratory, it is strictly forbidden to eat, smoke.

3. Do not touch the biomaterial and microbial cultures with your hands. When working with biomaterials, it is necessary to use tools (loops, spatulas, and tweezers). Tools that have contact with infected material are flamed in the flame of a spirit lamp (alcohol burner), or completely immersed in containers with a disinfectant solution. Inoculations are carried out at the spirit lamp, flaming while the edges of the tubes, loops, spatulas.

4. It is forbidden to keep flammable materials and substances near working spirit lamps. It is also forbidden to leave unattended working spirit lamps. Before setting fire to the spirit lamp, you must make sure that its jar is in good condition, the wick is released to the desired height. The wick should fit tightly into the tube of the holder (otherwise, a flash of vapor inside the spirit lamp and an explosion is possible). A lighted spirit lamp must not be moved from place to place; one spirit lamp must not be lit from another. Extinguishing the spirit lamp must be done by covering the flame of the wick with a cap. Blowing out flames is prohibited.

5. Each student must work in the workplace assigned to him. While working in the laboratory, silence and order should be observed.

6. When performing a practical task, students are required to carefully follow the instructions of the teacher.

7. Students take everything they need to work in class (Petri dishes, test tubes, pipettes, bacteriological loops, etc.) at their work desk or special table, and after completing the work, they put everything in place.

8. When working with a microscope, the student needs to be careful, report all the deficiencies in the work of the microscope to the teacher.

9. In the event of contamination of the table surface and other objects with contagious material (in the event of an accident with dishes, spilling liquid infected material, etc.), the student must immediately notify the teacher and thoroughly disinfect the table surface and objects under the supervision of the teacher (at the scene of the accident apply a disinfectant, the exposure time is 5–10 minutes; then the disinfectant is wiped with a moisture-absorbing cloth, and antiseptic treatment and hand washing with soap) are performed. In case of contamination with infectious material of the skin of the hands and face, especially if the integrity of the skin is broken (injection, cut), the student, under the supervision of the teacher, must treat the damaged area (70 % with alcohol, or 5 % iodine, or 3 % hydrogen peroxide solution, or another antiseptic).

10. At the end of the lesson, the student is obliged to: put used glass slides in a special tray; tidy up the microscope and workplace; wash hands with soap or treat with antiseptic [1, 3].

SECTION 2
PRINCIPLES OF MICROBIOLOGICAL DIAGNOSTICS OF INFECTIOUS DISEASES

The most important place in laboratory diagnostics of infectious diseases is occupied by **specific microbiological diagnostics**, which is carried out in bacteriological, virological, immunological and other laboratories.

The purpose of microbiological diagnostics is to establish the presence or absence of the causative agent of the disease in the studied material and on the basis of this to decode the etiology of the disease and justify the appropriate treatment [1].

The tasks of microbiological studies are to identify microorganisms in the studied material, determine their species, morphological, biochemical, toxigenic and antigenic properties (which allows to make a microbiological diagnostics), as well as to investigate antibiotic sensitivity of isolated microorganisms, which plays an important role in the algorithm for choosing antimicrobial drugs for etiotropic therapy of infectious disease.

Microbiological diagnostics of infectious disease consists of three stages: preanalytical, analytical and postanalytical. The first stage of microbiological diagnostics is **preanalytical**, including the collection of material for research (further — pathological material). The **analytical** stage includes various methods of microbiological diagnostics. The **post-analytical** stage of microbiological diagnostics consists in the clinical interpretation of laboratory results [1].

Despite the fact that microbiological research is the responsibility of microbiologists, every doctor dealing with specific and nonspecific infectious diseases should know how and when to select pathological material for research, on which studies to send it and how to interpret the results. If the microbiological study gives a positive result, the preliminary clinical diagnosis is confirmed.

To establish the etiological role of *obligate pathogenic microbes*, it is enough to isolate them from a pathological focus (regardless of the number), detect antibodies in the patient's blood serum against specific antigens of those microbes and identify the correlation between the selected microbe and the clinical picture of the disease. Therefore, in this case, a positive result of microbiological research is an objective factual proof of the reliability of the clinical diagnosis.

In the case of isolation of *opportunistic pathogens*, the attending physician should correctly assess the etiological significance of microorganisms isolated from the patient's pathological material and adjust the antimicrobial chemotherapy carried out to the patient on the basis of microbiological data [4].

Material for microbiological studies

Any microbiological study begins with collection the studied material. The type of material studied depends on the purpose of the study. In the diagnostics of the disease, clinical material is taken from the patient or carrier. In this case, the material for the study is various pathological body fluids (blood, pus, sputum, cerebrospinal fluid, feces, etc.) and tissue-biopsy material from a living organism or autopsy from a corpse (sectional material). The choice of material for laboratory diagnostics of infectious diseases is determined by the localization of the pathological process, the peculiarities of the pathogenesis of the disease and the biological properties of the pathogen (table 1).

Table 1 — Material for microbiological studies

Type of pathological material	Examples of diseases or conditions in which this pathological material is taken
Blood	Sepsis, relapsing fever, in the early diagnostics of typhoid, hepatitis B, etc.; in case of the absence or obscurity of local infection foci
Feces	Intestinal infections (dysentery, salmonellosis, escherichiosis, rotaviral gastroenteritis, etc.)
Vomit	Cholera, botulism, food poisoning
Urine	Urinary tract infections (cystitis, pyelonephritis), leptospirosis
Sputum	Bronchitis, pneumonia, tuberculosis, legionellosis
Purulent contents (pus)	Infected wound, boil, carbuncle, abscess
Cerebrospinal fluid (CSF)	Meningitis, encephalitis
Contents from the nasopharynx, from the surface of the tonsils, from the pharynx, etc.	Enteroviral infections, meningococcal infection, tonsillitis, pharyngitis, diphtheria
Genital secretions (e.g., discharge from the urethra)	Gonorrhea

When conducting epidemiological analysis, samples from environmental objects (water, air, soil, food, swabs from objects, etc.) are additionally examined [1, 5].

General rules for the collection, storage and transportation of material for microbiological research

The diagnostic results of many microbial diseases largely depend on the correct choice of material and compliance with the following conditions for its collection, delivery, storage and processing.

1. The type of material is determined by the clinical picture of the disease, and it must correspond to the localization of the expected pathogen in the body at this stage of the pathogenesis of the disease. For example, in case of bronchopulmonary diseases, sputum is taken for research, in case of lesions of the

urinary system — urine, etc. In cases of absence or ambiguity of local foci, blood is taken for research.

2. A sufficient amount of material is collected (for example, in a blood test, 5–10 ml of blood is taken).

3. Material is taken whenever possible in the initial period of the disease, because it is during this period that pathogens stand out more often, there are more of them, and they have a more typical localization. An early etiological diagnosis implies an earlier and therefore more effective treatment and prevention of new cases of the disease.

4. Material sampling should be carried out before the start of antimicrobial therapy. If such therapy is started, then if necessary it should be interrupted for 1–2 days, and then sampling the material.

5. It is necessary to prevent possible contamination of the material with the patient's own normal microflora and environmental microbes. For this, the material should be obtained under aseptic conditions, in the treatment room, with sterile instruments, in sterile dishes. In addition, the pathways through which material is released or taken should be maximally freed from normal microflora.

6. The possibility of getting antimicrobial agents (disinfectants, antiseptics, antibiotics) into the material should be prevented; contact with metals, with cotton wool should be excluded.

7. Any clinical material should be regarded as potentially dangerous to humans. Therefore, during its collection, storage, delivery, processing in order to avoid infection, the same safety measures must be followed as in the microbiological laboratory.

8. After taking the material as soon as possible, it is necessary to deliver to the laboratory and begin its research. If it is not possible to quickly deliver it to the laboratory (for example, at night), it is placed in a household refrigerator (or a preservative is added); special transport media can be used.

9. Any material delivered to the laboratory must have an accompanying document —a referral to a microbiological examination, during the preparation of which it is necessary to indicate the name of the institution, patient data, iplocnary clinical diagnosis and the date of the disease, indications for examination; type of pathological material, date, time of its collection; purpose and name of the study; the name and signature of the doctor sending the material.

10. During the delivery process, the material should be protected from the effects of light, heat, cold, mechanical damage. It is best delivered in special metal containers that are convenient to clean and disinfect. Material cannot be sent with sick and random people.

11. After examination, the remnants of the material should be disposed of (preferably by autoclaving or incineration), and the dishes, containers, tools should be decontaminated [5, 6].

General concept of research methods in microbiology

Microbiological diagnostics includes the following main methods:

1. The **microscopic method** is based on the study of the morphological properties of microorganisms using various types of microscopy; in bacteriology, the microscopic method is called — bacterioscopic, in virology — virososcopic.

2. The **cultural method**: in bacteriology it was called — bacteriological (based on the isolation of pure cultures of bacteria and their further identification), in virology — virological, in mycology — mycological.

3. **Biological** (experimental or bioprobe) **method** is based on the infection of laboratory animals with the studied material in order to reproduce an infectious disease in them.

4. The **immunological method** is a set of methods, common to which is the use of immunological reactions for diagnostic purposes (variants of the method — serological identification of microorganisms, serological and iplocological methods).

5. The **molecular genetic method** is based on the detection of genome fragments (DNA, RNA) of the pathogen in biological material (molecular hybridization, polymerase chain reaction, sequencing, etc.).

When diagnosing an infectious disease, often one of these methods is the main one, and the others are additional [6].

Of particular importance are the methods of **express diagnostics**, which allow making microbiological diagnostics within a short period of time (from a few minutes to several hours) from the moment of delivery of the test material to the laboratory. Express methods include: immunofluorescence reaction, enzyme immunoassay, polymerase chain reaction, immune chromatography and others [1, 3, 6, 8].

SECTION 3
RESEARCH METHODS IN MICROBIOLOGY APPLIED FOR THE DIAGNOSTICS OF BACTERIAL INFECTIONS

Microbiological diagnostics of **bacterial infections** includes 5 main methods:

1. The **bacterioscopic method** is based on the study of morphological properties of microorganisms using different types of microscopy.

2. The **bacteriological method** is based on isolation of pure cultures of bacteria and their further identification.

3. The **biological method** is based on the infection of laboratory animals with the studied material in order to reproduce an infectious disease in them.

4. The **immunological method** is a set of methods, common to which is the use of immunological reactions for diagnostic purposes (variants of the method — serological identification of microorganisms, serological and iplocological methods).

5. The **molecular genetic method** is based on the detection of fragments of the genome (DNA) of the pathogen in biological material (molecular hybridization method, polymerase chain reaction, sequencing) [1, 3, 6, 8].

Microscopic (bacterioscopic) method

Microscopic method — a method based on the study of the morphological properties of microorganisms in the material under study (pathological material, laboratory culture) using different types of microscopy [1, 6, 8].

The *main purpose of the method* is to establish the etiology of infectious disease, as well as to determine the purity of the isolated pure culture.

Advantages of the method: fast results, technical and economic accessibility, simplicity.

Disadvantages of the method: low sensitivity (determined by about 10^5 or more bacteria per ml); relatively unsafe (working with living microorganisms); difficulty in recognizing similar morphology of microorganisms of different species and genera, due to the presence of microbes-twins; inability to identify due to changes in the characteristic morphology of microorganisms under the influence of a number of environmental factors, primarily antibiotics. These circumstances lead to the fact that only in the presence of specific morphological features of microorganisms and their typical localization in combination with the correctly chosen staining and type of microscopy of the smear can allow making a conclusion about the etiology of the disease. In other cases, the microscopic method is more often used as an indicative, preliminary method of investigation, and in some types of infection it is generally excluded. The bacteriologist, having received approximate information about the expected pathogen, its concen-

tration, combination with the accompanying microflora, determines the tactics of further isolation of the pure culture of the pathogen [1].

Morphological properties of microorganisms:

1. Microbial cell shape (figure 1).
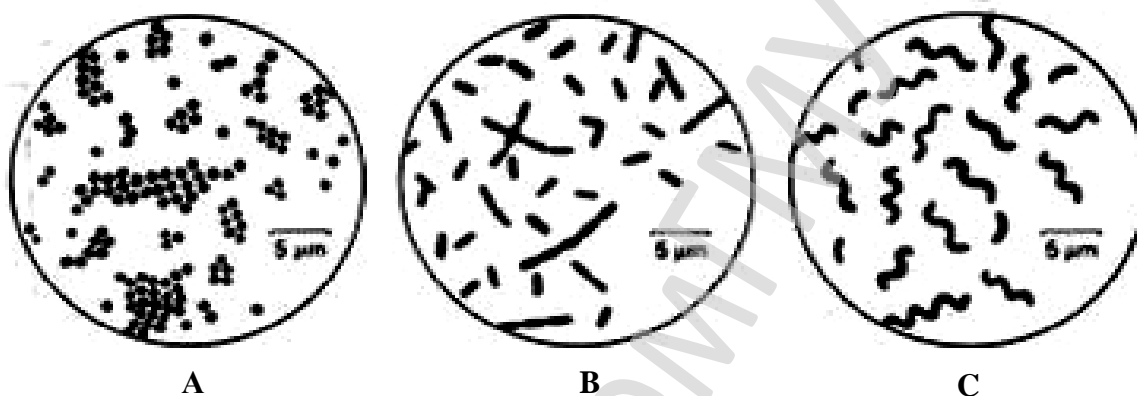
Figure 1 — Shapes of bacteria:
A — cocci; B — rods; C — spiral bacteria

2. The sizes of microbial cells.

3. Mutual arrangement of microbial cells in the smear (figure 2).

4. Protective (spore, capsule) and additional (inclusions, flagella, etc.) structural components of the microorganism.

5. Tinctorial properties (the ability of microorganisms to be stained with certain dyes) [7, 9, 10].
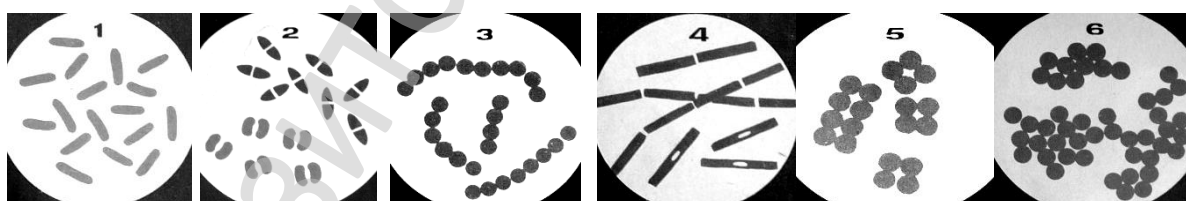
Figure 2 — The mutual arrangement of microbial cells in the smear (examples):
1 — Escherichia (chaotic arrangement); 2 — pneumococci, gonococci, meningococci
(iplococcic — located in pairs); 3 — streptococci (chains of cocci); 4 — streptobacteria
(chain of rods); 5 — sarcina (packets of cocci); 6 — staphylococci (form clusters
in the form of a bunch of grapes)

Stages of bacterioscopic method

1. Collection of the studied material.

2. Transporting it to the laboratory, preparing for the study.

3. Preparation of microscopic preparation (smear), it's staining.

4. Microscopy and study of morphological properties of microorganisms.

5. Evaluation of the result and conclusion [1].

Types of m,icroscopic preparations

In laboratory practice, the following types of microscopic preparations are used:
1) bacteriological preparation-smear or simply smear (fixed);
2) smear-print;
3) smears «twins»;
4) «hanging» drop and «crushed» drop;
5) thin blood smear;
6) thick drop of blood.

Bacteriological preparation-smear. Bacteriological smear is used most often. Stages of preparation of a preparation-smear: actually preparation of a smear, it's drying, fixing, and staining [2, 6].

Smear-print. Prepare from corpse organs in the next way: touch the surface incisions fulfilled by sterile scalpel by the slide glass, or cut out from body a small piece of tissue/organ and, taking over it by tweezers, several times touch, as print, to slide glass. Fix smear-print on the flame or, if it is necessary to preserve the structure of the cellular elements of the body, in a liquid fixative: a mixture of Nikiforov (equal volumes of ethyl alcohol and ether) — 15 minutes, methyl alcohol — 5 minutes. Stain with fuchsine (or methylene blue) and Gram staining [2, 6].

Twin smears. Prepare from the investigated material of viscous consistency (pus and sputum) as follows: the material by sterile tweezers or a loop is placed to the middle of the slide, and then tightly cover it with another slide. Taking the free ends of both glasses, push them apart with both hands: two uniform large smears are obtained (figure 3) [2, 6].
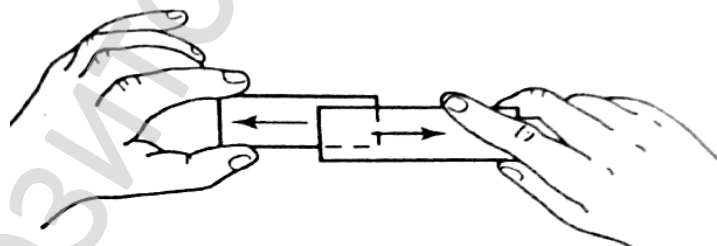
Figure 3 — Preparation of a smear from pus and sputum

«Hanging» drop and «crushed» drop. Used in the study of microorganisms in the living state, mainly to study the mobility of bacteria. Features of these smears: they are not fixed and not stained. A more convenient way to study «hanging» drop and «crushed» drop is represented by dark-field and phase-contrast microscopy [2, 6].

«Hanging» drop (figure 4). A drop of the test material is placed to the middle of the cover glass. Then take the second glass slide with a hole in the center, pre-lubricated with vaseline edge of the hole, and impose it on the cover glass so that the drop was against the center of the hole. The smear is quickly turned over with the cover glass up. The drop should hang freely in the hole, not in contact

with its bottom and edges. As a result, a hermetically closed wet chamber was formed, in which the drop does not dry for a long time [2, 6].

Figure 4 — Scheme of the «hanging drop» preparation

«Crushed» drop. A drop of the test material is placed to the middle of the glass slide with a sterile Pasteur pipette. Carefully cover («crush») a drop with a cover glass, imposing it with tweezers so that air bubbles do not form in the liquid. The drop should fill the entire space between the glasses and not protrude beyond the edges of the cover glass [2, 6].

Thin smear of blood. A drop of blood is placed to the edge of a glass slide. The smear is done with the second slide, mounted at an angle of 45º, moving it along the slide glass to the left (figure 5). It turns out a uniform thin smear, which is dried in air. The smear is fixed in a liquid fixative (a mixture of Nikiforov — 15 minutes, methyl alcohol — 3 minutes), but not on a flame. Further, the smear is stained, for example, according to Romanovsky-Giemsa [2, 6].
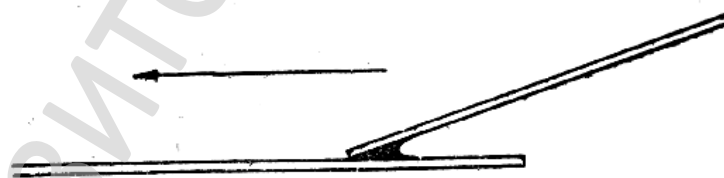
Figure 5 — Preparation of thin blood smear
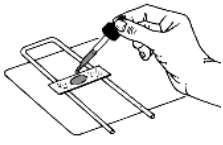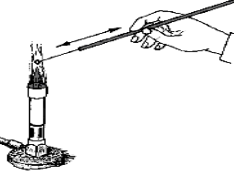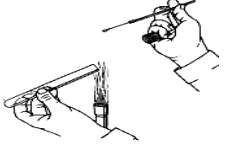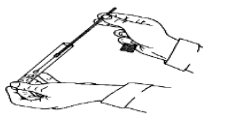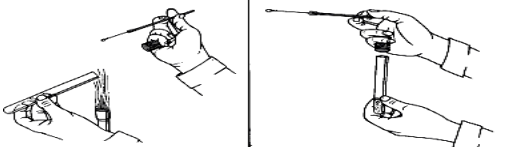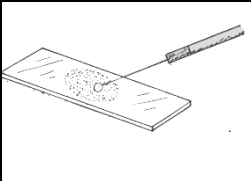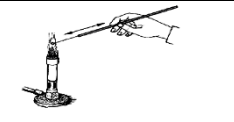
Thick drop of blood. On the glass slide, a large drop of blood or 2–3 ordinary drops are placed, which are mixed by a loop to get a flat blood spot with the size of a small coin. The smear is dried in the air, not fixed, and stained according to Romanovsky-Giemza.

Preparation of bacteriological smear

Stages of preparation of the smear: actual preparation of the smear (table 2), it's drying, fixation and staining [2, 6].

Table 2 — Technique of *smear preparation* of bacterial culture

Smear preparation steps	Stage diagram
1. When preparing a smear preparation from an agar culture of bacteria, apply a small drop of saline solution to the middle of a fat-free slide; when preparing a smear preparation from a broth culture of bacteria, saline solution is not used	
2. Take the bacteriological loop in the right hand, as a writing pen (left-handers — on the contrary), and sterilize it in a vertical position, using spirit lamp, until it turns red. Then move the loop to a horizontal position and hold it in the flame, including part of the loop holder	
3. Hold the test tube with agar culture with the thumb and forefinger of the left hand. Remove the cotton plug from the test tube with the little finger of the right hand, in which the loop is, and sterilize the edges of the test tube in the flame of the spirit lamp	
4. The sterilized loop is quickly inserted into the test tube, cool and touch the agar culture or immerse it in the broth culture	
5. Without touching the walls of the test tube, remove the loop, once again quickly sterilize the edges of the test tube and the plug over the spirit lamp, close the test tube and put it in a tripod	
6. Bring the taken material from the agar culture of bacteria into the drop of physiological solution with a bacterial loop, or apply a drop of the broth culture on a glass slide by a loop or a sterile Pasteur pipette, and evenly distribute the material in the form of a smear with a diameter of 1.0–1.5 cm by circular motions of the loop or pipette	
7. Sterilize the loop in the flame of a spirit lamp	

Drying of the smear. Dry the smear at room temperature. To speed up the drying the glass slide with the smear, dry over the flame of a spirit lamp in a stream of warm air [1, 6].

Fixation of the smear. It is produced for the purpose of:

1) killing bacteria and make it safe to continue handling them;

2) fixing the smear on the glass so that it does not wash off during further manipulations;

3) making microbes more susceptible to staining, since killed microbial cells are stained better than live ones [1, 6].

The ways of smear fixing:

• *Heat fixation*

It is the simplest, suitable for almost all objects and the most common method in microbiology. The principle of the method: glass with a smear facing

up take by the index and thumb fingers over the edge and hold in the flame 3–4 times for 5–6 s.

• *Chemical fixation*

It is used in the study of blood smears, preparations from sputum, pus, organs and a tissue for which fixation by heat is too rough and can change the structure of cells. In this case, the fixative is poured on a smear or the smear is immersed in a vessel with a fixing liquid for a certain time, and then dried [1, 6].

The following liquid fixatives are used in microbiology:
• ethyl alcohol for 10–15 min;
• methyl alcohol for 3–5 min;
• acetone for 5 min;
• Nikiforov's mixture (equal volumes of ethanol and ether) for 15 min.
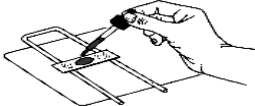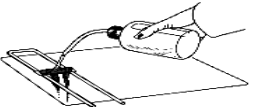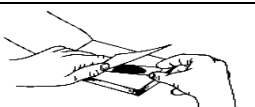
Smear staining. Methods of staining microorganisms are divided into simple and complex (differential). The ability of microorganisms to perceive dyes is called *tinctorial properties*.

Simple methods of staining

For simple staining, only one dye is used, for example, from the group of aniline dyes: **aqueous fuchsine** (Pfeiffer's fuchsine) — an exposure of 1–2 minutes or **methylene blue** (Loeffler's blue) — an exposure of 3–5 minutes (table 3). Simple staining methods allow determining the presence of bacteria in the preparation, their shape, size, and mutual arrangement of bacterial cells in a microscopic preparation [10, 11, 12].

Table 3 — Simple method of staining a smear using an aniline dye

The steps of staining a smear in a simple way	Stage diagram
1. Apply a few drops of the dye to a fixed smear; maintain the exposure time	
2. Wash off the dye with distilled water	
3. Dry the smear with filter paper	

Complex methods of staining

Complex staining methods involve the sequential use of multiple dyes. This makes it possible to identify certain structures of microbial cells and differentiate some types of microorganisms from others. Complex methods of staining include: Gram, Ziehl-Neelsen, Burri-Gins, Neisser, Zdrodovsky, Romanovsky-Giemza, and others (table 4) [1, 6].

Table 4 — Complex methods of staining for cell structures of bacteria and some microorganisms

The method of staining	Structure of bacteria	Some microorganisms
Gram	Cell wall	
Burri-Gins	Capsule	
Ziehl-Nielsen	Endospores	Acid-resistant bacteria (namely, mycobacteria)
Neisser	Inclusions (grains of volutin in corynebacteria)	
Romanovsky-Giemza	Nucleoid	Rickettsia, chlamydia, spirochetes, protozoa
Zdrodovsky		Rickettsia

Gram staining

It is used to determine the type of cell wall structure. This is the main method in bacteriology. Depending on the color after Gram staining, all bacteria are divided into gram-positive and gram-negative. Gram-positive bacteria turn blue-purple, and gram-negative bacteria turn red. The staining technique is shown in table 5 [1, 6, 10, 11].

Table 5 — Gram staining technique

Staining stage	Result at this stage
1. A fixed smear is stained with gentian violet: a strip of filter paper impregnated with a solution of **gentian violet** is placed on the smear and 2–3 drops of distilled water are applied. Exposure time — 1–2 min. Then the paper is removed	Both gram-positive (Gr$^+$) and gram-negative (Gr$^-$) bacteria are stained with this dye in a blue-purple color
2. Apply a **solution of Lugol** for 1 minute (the smear turns black), after exposure it is washed	Both Gr$^+$ and Gr$^-$ bacteria remain blue-purple at this stage
3. Discolor the smear with **96 % ethyl alcohol** for 20–30 seconds, shaking the smear, until the purple waves of dye stop flowing away. Then the smear is washed with water	In Gr$^+$ bacteria, due to the large amount of peptidoglycan in the cell wall, gentian violet and iodine form a strong complex that is retained when discolored with alcohol (Gr$^+$ bacteria remain blue-purple). And in Gr$^-$ bacteria, due to a thinner layer of peptidoglycan, the coloring complex is washed out with alcohol and, consequently, they will become colorless
4. Counterstain the smear with **aqueous fuchsine** (1–2 min), wash with water, dry with filter paper, and apply a drop of immersion oil and use microscopy.	Gr$^+$ bacteria remain blue-purple, and Gr$^-$ bacteria that were discolored at the previous stage are colored red.

Neisser staining

It is one of the methods for detecting volutin granules.

The technique of staining:

1. A fixed smear is stained with acetic acid and **Neisser's methylene blue solution** 1–2 minutes, and then the smear is washed with water.

2. Treatment with a **solution of Lugol** 20–30 s.

3. Stain the smear with **vesuvine** 0.5–1 min.; wash with water, dry with filter paper, apply immersion oil and use microscopy.

Volutin granules in the bacterial cell are inclusions, and their chemical structure is polyphosphates. The peculiarity of volutin is the ability to metachromasia, i.e. with complex staining; they adsorb other dyes than the cytoplasm, staining in a different color. In this regard, they are also called metachromatic granules.

Volutin granules are found, for example, in *Corynebacterium diphtheriae*, and when stained according to Neisser, the cytoplasm of bacteria is colored yellow, and the volutin granules are dark blue, almost black [2, 6, 12].

Ziehl-Neelsen staining

It is used to detect acid-resistant bacteria (or AFB — acid-fast bacteria) and endospores (table 6) [2, 6, 10, 12].

Table 6 — Ziehl-Neelsen staining technique

Staining stage	Result at this stage	
	Spore-forming bacteria (e.g., Bacilli, Clostridia)	Acid-resistant bacteria (e.g. *Mycobacterium tuberculosis*)
1. A filter paper is placed to a fixed smear, which is applied with **Ziehl's carbolic fuchsine**, and 3 times heated over the flame of an spirit lamp until vapors appear, adding dye. After the smear has cooled, the paper is removed	The vegetative bodies of bacteria and endospores turn red	Bacteria turn red
2. The smear is discolored with a **5 % solution of sulfuric acid**, immersed in an acid for a few seconds, after which the smear is washed with water	The vegetative bodies of bacteria are discolored, but the endospores are not, since the endospores are acid-resistant	Acid-resistant bacteria do not discolor with acid, they remain red
3. The smear is counterstained with **methylene blue** 3–5 minutes, washed with water, dried with filter paper, applied immersion oil, and examined under microscopy	The vegetative bodies of bacteria are colored blue, and the endospores remain red	Acid-resistant bacteria remain red

Due to the peculiarities of their chemical composition, endospores do not perceive aniline dyes in the normal mode of staining and remain colorless (figure 6) inside the colored vegetative bodies of bacteria (for example, when colored with water fuchsine, the vegetative bodies of bacteria will turn red, and the endospores — colorless).
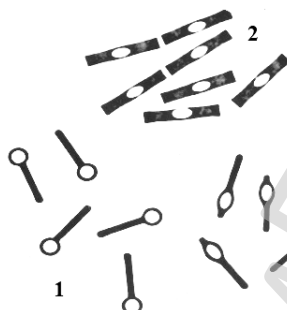
Figure 6 — Spore-forming bacteria:
1 — bacilli; 2 and 3 — clostridia

To stain spores, it is necessary to use a combination of three factors: exposure to concentrated dyes solutions, high temperature and acid treatment. All this is achieved by special complex methods of staining.

Burri-Gins staining

It is used to detect capsules in bacteria [2, 6, 12].

The technique of staining:

1. In a drop of **Indian ink**, diluted with water in 10 times, add the studied bacteria, mix evenly and use the glass to prepare the smear in the same way as a thin blood smear.

2. The smear is dried, fixed in a flame.

3. The smear is stained with **aqueous fuchsine** for 3–5 minutes, washed with water, dried, applied immersion oil and examined under microscopy.

In the smear stained by Burri-Gins capsules are visible as colorless light halos around red bacteria on a dark pink background (figure 7a).

When using a simple staining method (for example, methylene blue or aqueous fuchsine), which can be used to stain the capsules of microorganisms in smears from organs and tissue, a colorless halo is also found around the bacteria, since the capsule is not stained (figure 7b).
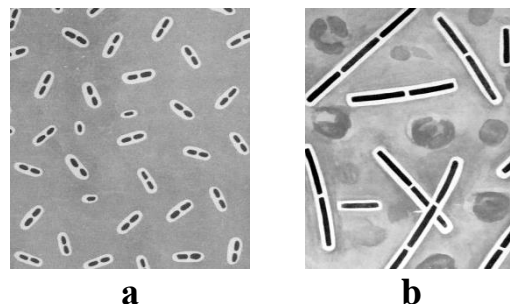
Figure 7 — Capsule bacteria
(the capsule is unpainted halo around the cell): a — Klebsiella in a pure culture;
b — anthrax Bacilli in organs

Zdrodovsky staining

It is used to detect rickettsia [2, 6].

The technique of staining:

1. A fixed smear is stained with diluted **carbolic fuchsine** — 5 min.

2. Immerse the product for a few seconds in a weak solution of **organic acid** (e.g. 1 % citric or acetic acid) and rinse with water.

3. Counterstain of the smear with **methylene blue** for 5 minutes. Wash the smear with water.

Rickettsia are colored in ruby-red color (due to relative acid resistance), and the remaining cells and elements of the smear — in blue.

Romanovsky-Giemza staining

The technique of staining:

1. Prepare a microscopic preparation and process it in a liquid fixative.

2. Place the fixed preparation on two glass rollers in a Petri dish down to the bottom and pour the freshly prepared dye diluted in 10–20 times into the slot on the side, which does not dry out for a long time in this closed chamber. The dye consists of **eosin, methylene blue**, and **azure** dissolved in a mixture of methanol and glycerine. Staining lasts from 30–60 minutes to several hours.

3. Smears are washed with water and dried in the air [2, 6].

This method of staining is one of the main methods in the study of the morphology of spirochetes, rickettsia, chlamydia, protozoa, as well as in the study of blood cells, and under the influence of a complex dye, they are colored in various colors. For example, spirochetes of different genera are colored according to Romanovsky-Giemza as follows: Treponema — in pale pink, Leptospira — in pink or red and Borrelia — in blue purple color.

Microscopy methods

Microscopes are used to study the morphology of microorganisms. The microscope is used to study small objects that are invisible to the naked eye. In Microbiology, a microscope is used to study both living and dead microbes in a stained or unstained form.

In microbiology, two types of microscopy are used — electron and light. Light microscopes are designed to study microorganisms that are at least 0.2 microns in size, and electron microscopes are designed to study smaller objects (viruses and microbial structures). Microscopy methods are presented in table 7 [8, 9, 10, 13].

Table 7 — Microscopy methods

Method		Characteristic of microscope	Principle of the method and purpose of application
Electron microscopy		Electron microscope	Instead of a light wave, a stream of electrons is used, which allows increasing the sensitivity of the method by several orders of magnitude. It used to study viruses, bacterial cell ultrastructure
Light microscopy	*basic light*	Conventional biological microscope, «dry» lens	The principle of the method is considered in the course of physics. It is rarely used in microbiology (for microscopy of the preparation «crushed drop» when determining the mobility of bacteria)
	immersion	A conventional biological microscope equipped with a special immersion lens	The lens is immersed in immersion oil (cedar, peach, vaseline), the refractive index of which is close to the refractive index of glass (1.52). Thanks to this, the light beam that goes beyond the scope of the slide is not scattered, and the rays, without changing direction, fall into the lens, which is why the best illumination of the smallest objects is achieved. It is used in bacteriology most often to study various microorganisms
	dark-field	A conventional biological microscope with a special dark-field condenser	All of the direct rays pass through the lens to the lens get only those refracted in the object on the human head. Therefore, microbes are seen as luminous objects on a dark background. It is used to detect spirochetes, motility of microorganisms
	phase-contrast	An ordinary biological microscope is equipped with a special attachment with a special set of lenses	The phase shift of the light wave oscillation, which occurs when it passes through transparent objects and is not perceived by the human eye, is converted by a phase-contrast device into a change in the amplitude of the light wave, which is perceived by our eye (the object becomes visible). They are used for studying living objects in unstained preparations, the internal structure of microbes
	luminescent	Special luminescent microscope	It is based on the ability of certain cells and fluorescent dyes to glow when they are exposed to ultraviolet and other short-wave light rays. To process the smears fluorochromes are used such as auramine, acridine orange, and fluoresceinisothiocyanate (FITC), etc. It is widely used in microbiology to detect certain types of bacteria and to assess immune fluorescent assay (IFA)

Rules for working with the immersion system of the microscope

Before working, check the serviceability of the microscope and the cleanliness of the optics.

1. The condenser using a special screw is moved up until it stops.

2. Open the diaphragm (for stained preparations).

3. Lower the lens of small magnification (for example, ×8) at a distance of 0.5 cm from the subject table and, rotating the mirror, adjust the illumination of the field of view (in low light, use a concave mirror, with sufficient — flat).

4. Turning the revolver, install an immersion lens (for example, with a magnification of ×90).

5. Place the preparation with a drop of immersion oil on the slide table and fix it with terminals.

6. Under visual control from the side, using a macrometric screw, the immersion lens is immersed in a drop of oil on the preparation.

7. Looking into the eyepiece, slowly raise the tube with a macrometric screw until the image appears in the field of view. Then, using a micrometric screw, a clear image of the object is set.

8. During microscopy, the macrometric screw is rotated with the right hand, and the smear is moved with the left hand.

9. After viewing the smear lift the microscope tube with a macrometric screw, remove the smear, and wipe with a soft cloth the front side of the immersion lens from the oil residues [1, 12].

Bacteriological (cultural) method

The **bacteriological (cultural) method** of diagnostics is based on the isolation of a pure culture of microorganisms from the pathological material on nutrient media and its further identification [1].

As a rule, a microbe isolated from a patient or from the environment cannot be quickly and accurately identified. For these purposes, it is necessary to use a variety of phenotypic and genotypic features that are accurately detected by certain tests (methods). The basic taxonomic unit for bacteria is the species [1].

A **species** is an evolutionarily formed population of microorganisms that have a common origin, ecological unity, a similar genotype and the closest possible phenotypic features and properties.

The genetic mechanisms underlying microbial variability provide only relative stability of traits that may vary within a species. Hence, there is an idea of the variants (varieties, types) of microorganisms that differ in individual traits from standard species.

Different types are distinguished depending on the nature of the differential characteristic. **Morphovars** (or **morphotypes**) are those strains that differ in morphological characteristics. **Phagovars** (or **phagotypes**) are those strains that differ in sensitivity to bacteriophages. **Serovars** (**serotypes**) are those strains that differ in antigenic structure. **Biovars** (**biotypes**) are those strains that differ in biochemical, biological or cultural properties. **Pathovars** are strains of bacteria of the same species with the same or similar pathogenic properties (virulence) [8, 10].

Identification is establishing the taxonomic position of microorganisms and, above all, their species. Determining the species is a crucial point in the bacteriological diagnostics of infectious diseases. Most often, to identify patho-

genic bacteria, their morphological, tinctorial, cultural, biochemical and antigenic properties are studied [10].

Microorganisms, with the exception of obligate intracellular parasites (rickettsia, chlamydia, viruses), can be cultivated on artificial nutrient media.

The concept of microorganism cultivation

Cultivation is the growing microorganisms on artificial nutrient media.

The purpose of cultivation:

1. Obtaining pure cultures of pathogenic microorganisms and their identification for the purpose of making a microbiological diagnostics of an infectious disease (i.e., cultivation is an integral part of the bacteriological method of research).

2. In sanitary microbiology to determine the sanitary important microbes which are indicators of environmental pollution.

3. Accumulation of biomass of biomass producers of biologically active substances (vitamins, amino acids, antibiotics, etc.).

4. Receiving diagnostic and prophylactic preparations (diagnosticums, vaccines).

5. Storage of reference cultures of microorganisms.

6. The study of various biological properties of microorganisms in research works [1].

A pure culture is a population of microorganisms of a single species grown on a nutrient medium. A mixed culture is a collection of bacterial populations of different species. The term "population" refers to a collection of bacteria of a single species, growing in a particular biotope or grown on an artificial nutrient medium from one or more cells.

Isolate — a population of bacterial cells in a pure culture, obtained in the laboratory from a single colony from the culture medium and identified to the level of the species [4, 10].

A **strain** is a pure culture of microorganisms of the same type, isolated from a certain source at a certain time (simultaneously), or from the same source at different times. Strains of the same species may differ insignificantly in their biochemical, genetic, serological, biological, and other properties, as well as in the place and time of isolation.

Each type of bacteria has a typical strain, which is a kind of its standard. Typical strains have the most complete biological characteristics and are stored in special collections. Collections of bacteria are available in many countries of the world. The most extensive of them are located in the United States (American collection of type cultures — ATCC), England, France, Germany, Japan, India, and Russia. There are collections of microorganisms in the Republic of Belarus. Appropriate standard test cultures should be used to control the accuracy and standardness of research (for example, when determining the sensitivity

of isolated pure cultures of bacteria to antibiotics, standard cultures with known antibiotic sensitivity are used as control strains, which are tested in parallel with testing of clinical isolates) [1].

A **clone** is a culture of microorganisms obtained by multiplying a single bacterial cell [4].

Nutrient media

The nutrient medium is used for the artificial growth of microorganisms. In particular, nutrient media are intended for the accumulation, isolation, study and preservation of microorganisms. By their nature, nutrient media are an artificial environment for microbes, so their composition takes into account both the needs of microorganisms for substances necessary for life, and the physical and chemical conditions in which microorganisms can carry out their normal activities.

Requirements for nutrient media

1) Sufficient content of nutrients necessary for the life of the microbial cell — the main organogens, minerals (ash minerals and microelements);

2) the presence of growth factors of various origins (amino acids, vitamins, purines, pyrimidines, etc.) — for auxotrophs;

3) the isotonicity of the medium for the microbial cell (for most microbes require 0.5 % NaCl; halotolerant staphylococci — 5–10 % NaCl);

4) optimal pH of the medium (most pathogenic bacteria grow mainly at a pH of 7.2–7.6; *alkaliphiles* (*Vibrio cholerae*) can grow on environments with an alkaline pH of 8.0–9.0; *acidophiles* grow on environments with an acidic pH of 4.0–6.0);

5) certain redox potential of the medium;

6) mandatory sterility of the media, since foreign microorganisms prevent the growth of the microbe under study and lead to confusion in the correct identification of the etiological pathogen of the disease;

7) sufficient humidity (at least 60 %) — for optimal process of nutrient diffusion into the cell;

8) certain consistency of the medium;

9) transparency of the medium [6, 9].

Preparation of nutrient media and their sterilization

Most of the nutrient media used in bacteriology are dried concentrates in factory packaging, which are weighed (take the weight of the medium according to the instructions on the label), dissolved in distilled water and boiled until the components are completely dissolved. Next, the nutrient media is sterilized and poured into sterile laboratory dishes. Therefore, laboratory utensils (glass test tubes, Petri dishes, etc.), as well as nutrient media, must be sterilized (table 8) [1].

Table 8 — Methods and modes of sterilization of nutrient media and glassware

Sterilizable objects	Appliance	Methods and modes of sterilization
Glass products (test tubes, Petri dishes)	Air sterilizer (hot air oven)	Hot air (or dry heat) sterilization (180 °C, 60 min)
Simple nutrient media (MPA, MPB)	Steam sterilizer (autoclave)	Steam (or moist heat) sterilization under pressure (121 °C, pressure 1 ATM., 20 min)
Media with carbohydrates	Steam sterilizer (autoclave)	Steam sterilization under pressure (112 °C, 0.5 ATM pressure, 15 min)
Complex nutrient media	Steam sterilizer (autoclave)	Fractional sterilization (100 °C, 0 ATM. «flowing steam», 3 days for 30 minutes)
Nutrient media containing proteins, serum, vitamins	Water bath →	Fractional sterilization (tindalization) — heating of media at 56–58 °C for 1 hour, 5–6 days
	Filters (deep, membrane) →	Sterilization by filtration

During dry heat sterilization, glass tubes closed with cotton plugs and Petri dishes are sterilized packed in special paper (paper bags), which allows to maintain sterility after sterilization for a certain period of time.

In the moist heat sterilization method, steel sterilization boxes are used, in which bottles or test tubes with nutrient media are placed, closed with cotton-gauze plugs and paper caps [2].

Packages with sterilized products must contain information about the date of sterilization.

Sterilization control involves checking the parameters of sterilization modes and evaluating its effectiveness.

Control of sterilization modes is carried out by the following methods:

• *physical control* is using measuring devices (thermometers, pressure gauges, etc.), monitor the temperature, steam pressure, time of sterilization exposure and other parameters;

• *chemical control* is using chemical indicators — thermotests (substances that change their color or physical state during sterilization because they are having different melting points);

• *biological control* is using biotests with heat-resistant spores of spore-forming test cultures, with subsequent assessment of the spore death by their inoculation on nutrient media) [4, 13].

The conclusion about the effective operation of sterilization equipment is made in the absence of growth of the test culture in the bacteriological study of all biotests in combination with satisfactory results of physical and chemical controls.

Classification of nutrient media

The nutritional requirements and environmental properties of different microorganisms are not the same. This eliminates the possibility of creating a single universal medium for all microbes. In addition, the choice of a particular

medium is influenced by the research goals. Currently, a huge number of different media have been proposed, the classification of which is based on various features presented in table 9 [6, 8, 10, 11].

Table 9 — Classification of nutrient media

Feature	Types of media
Origin	• **Natural media** are media made from natural products (milk, meat, eggs, potatoes, human and animal blood serum, etc.) • **Artificial media** are prepared artificially specifically for growing microorganisms; their composition is relatively constant, they are standard, and are widely used in diagnostic laboratories • **Synthetic media** have strictly defined composition of the ingredients included in them, created taking into account the nutritional needs of the microbe; used for growing microorganisms in the production of antibiotics, vaccines
Consistency	• **Solid media** (2–3 % agar-agar obtained from seaweed, gelatin, silicon dioxide are used for solidification of nutrient media) • **Liquid media** (without agar — broth, milk, peptone water). • **Semi-solid media** (contain 0.5 % agar-agar)
Composition	• **Simple media** — meat-peptone broth (MPB), meat-peptone agar (MPA), peptone water • **Complex media** are obtained by adding to simple media stimulating additives necessary for the reproduction of a particular microorganism: blood, serum, sugar, ascitic fluid. Examples of complex media: blood agar, serum agar and broth, sugar agar and broth, ascitic agar and broth, etc.
Application	• **Basic simple media** are designed for the cultivation of most undemanding microorganisms (MPA, MPB) • **Special media** are used for isolation and cultivation of microorganisms that do not grow on basic simple media. Examples of special media: blood agar, serum agar and broth, sugar agar and broth, and others • **Elective (selective) media** selectively promote the growth of one type of microbes, delaying or suppressing the growth of concomitant microorganisms. This effect is achieved by adding various microbial growth inhibitors (certain antibiotics, salts, bile), changing the pH value or composition of nutrients in the media, etc. Examples: salt agar for staphylococci, alkaline agar for *Vibrio cholerae* • **Differential-diagnostic media** (DDM) allow distinguishing (differentiate) one type of microbe from another, based on their growth, enzymatic activity and other characteristics. The composition of these media, in addition to nutrients, includes a substrate as a differentiating factor (for example, carbohydrate), and an indicator. The enzymatic cleavage of the substrate by microorganisms leads to the accumulation of cleavage products and a pH shift, which is accompanied by the staining of the medium and colonies in the color of the indicator. Examples: Endo, Levin, MacConkey, Hiss media, Kligler iron agar (for enterobacteria), etc. • **Differential-elective media** combine the properties of differential-diagnostic and elective environments. They contain, in addition to nutrients, a substrate as a differentiating factor, an elective chemical substrate

Feature	Types of media
	that prevents the growth of other types of bacteria, and, in some cases, an indicator. Examples: MacConkey agar medium for Shigella and Salmonella, yolk-salt agar (YSA) for staphylococci
	• **Chromogenic media** is a new type of media that has recently become widely used in the express bacteriological diagnostics of infectious diseases. On chromogenic media, the color of colonies can be used for preliminary identification of microorganisms. The composition of such media, in addition to growth and selective components, includes a chromogen-labeled substrate (substrates). When the substrate is decomposed by microorganisms of different species, colonies grow that are colored in different colors. Chromogenic media have been developed for isolation and preliminary identification of Listeria, Salmonella, Shigella, Candida and other microbes
	• **Enrichment media** are media for the reproduction and accumulation of certain types of bacteria and the suppression of the growth of concomitant microflora (for example, alkaline peptone water for *Vibrio cholerae*, salt MPB for *Staphylococcus aureus*, bile broth for Salmonella, etc.)
	• **Transport media** are media for temporary preservation of microorganisms after collection the test material and transporting it to the laboratory; the bacteria remain viable, but do not multiply in them, so the quantitative and qualitative composition of the microflora does not change (for example, the Amies transport medium — semi-solid agar with activated carbon)

Spilling nutrient media into sterile laboratory dishes

After preparation and sterilization of nutrient media, they are poured into sterile laboratory dishes (figure 8) [1].

Deep agar   Slant agar

A

Agar plate (agar layer thickness of 3–4 mm, distributed evenly throughout the bottom of the Petri dish)

B
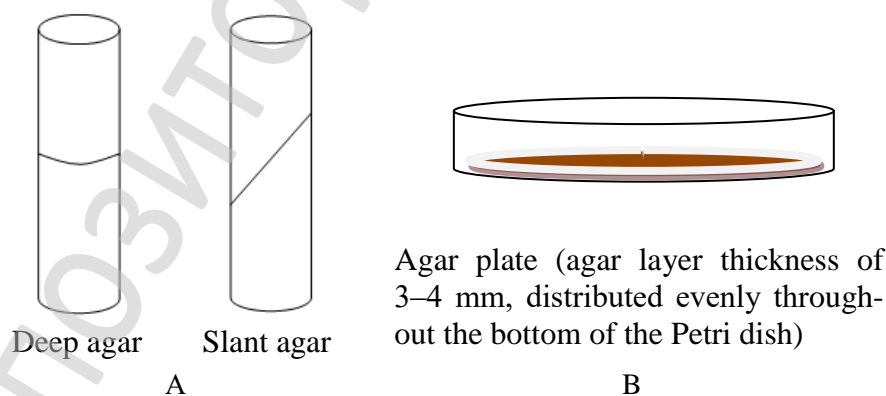
Figure 8 — Options for spilling dense nutrient medium (agar):
A — in a test tubes; B — in a Petri dish

Inoculation of microorganisms on nutrient media

Depending on the purpose of the study, the nature of the pathological material and the medium, different inoculation methods are used. All of them include a mandatory condition: protect the inoculated material from foreign microorganisms. Therefore, it is necessary to work quickly but without any sudden move-

ments, amplifying vibrations in the air. It is forbidden to talk while inoculating. It is better to make inoculation in a box.

Methods and techniques for inoculating pathological material on nutrient media

The inoculation technique depends on the nature of the material under study and the consistency of the nutrient medium.

When inoculating in a **liquid nutrient medium**, the loop with the material is immersed in the medium and the material is washed off with a light shake. The pipette is immersed in the medium and the material is drained [14].

Inoculation of the material on **agar plate media** in a Petri dish is carried out using a bacteriological loop, spatula or tampon. Inoculation with a *bacteriological loop* is carried out by the streak plate method on the surface of the agar. When inoculating with a *spatula*, the test material is applied to the surface of the medium with a loop or pipette, and then using a glass or metal spatula, the material is distributed over the agar surface in circular movements. After inoculation, the spatula is placed in a disinfectant solution. When inoculating with a *tampon*, the test material is distributed over the surface of the medium in circular movements while simultaneously rotating the tampon and the Petri dish [1, 14].

Lawn inoculation: 1 ml of the material (liquid broth culture or suspension of microbes in saline solution) is applied with a pipette to the surface of the agar and carefully distributed over the surface of the medium to get confluent growth of the culture. Another method: bacterial suspension with a sterile swab is applied to the surface of the medium with streak plate movements in three directions, turning the Petri dish by 60°.

Liquid material for inoculation is taken with a bacteriological loop or a sterile pipette. The bacteriological loop before taking the material and at the end of inoculation is sterilized by the flame of a spirit lamp. Pipettes after inoculation are immersed in a disinfectant solution [12, 14].

After inoculation, the Petri dishes are closed, turned upside down, signed and placed in the thermostat exactly upside down, to avoid the erosion of growing colonies on the medium by droplets of condensation water that accumulate on the inner surface of the lid in its normal position.

When inoculating (reinoculating) on a **slant agar** in a test tube, completely sterilized bacteriological loop is used and when opening and then closing the tube, carry its edges through the flame of a spirit lamp. Directly during inoculating, the loop with the bacterial culture is introduced into the test tube and, with a streaking movement from the bottom up, is distributed over the agar surface [12, 14].

Inoculation with a prick in the **deep agar** is carried out using a bacteriological loop (or needle) by piercing the column of the medium to the bottom.

The cultivation conditions of bacteria

In order to successfully cultivate bacteria on artificial nutrient media, it is necessary to take into account not only their nutritional needs, but also the conditions of cultivation: the temperature of cultivation and the aeration conditions necessary for a specific microbe.

According to the optimal culture temperature, bacteria are classified into three groups:

1. **Thermophiles** (thermophilic) — the optimal temperature of their cultivation is 50–60 °C.

2. **Mesophiles** — these include the vast majority of bacteria of medical significance; they grow best at the temperature of the human body (35–37 °C).

3. **Psychrophiles** («cold-loving») — these include a number of pathogenic human bacteria that grow best at lower temperatures (from 0 to 28–30 °C) [10].

To create an optimal temperature, the inoculated media are placed in a thermostat. A **thermostat** is a device in which a constant temperature is maintained by means of regulators [1].

According to the requirements for aeration conditions during cultivation, microorganisms can be divided into the following main groups (table 10) [7, 8, 10, 13].

Table 10 — Differentiation of microbes in relation to aeration conditions

Groups of microbes	Bacterial requirements for aeration conditions
Obligate aerobes	Require constant access of oxygen to the surface of the culture medium during cultivation
Obligate anaerobes	They are cultivated in conditions without oxygen access
Facultative anaerobes	Grow under any aeration conditions
Capnophiles	For their cultivation, they require an increased content of carbon dioxide (5–10 %)
Microaerophiles	Require reduced oxygen content (about 5 %)

Growing time of microorganisms

The growing time depends on the type of microorganism. Most bacteria are cultured at an optimal temperature for 18–24 hours, and, for example, the causative agent of whooping cough — for 2–4 days, *Mycobacterium tuberculosis* for about 3–6 weeks.

Character of bacterial growth on artificial nutrient media

The nature of growth on solid and liquid nutrient media is attributed to the cultural characteristics of microorganisms. The nature of bacterial growth depends primarily on which nutrient medium (liquid or solid) is used for cultivation (table 11) [1, 12, 14].

Table 11 — Character of bacterial growth on nutrient media

	Liquid media	Solid media
Character of bacterial growth on media	✓ **Diffuse turbidity** (most bacteria) ✓ **Film** on the surface of the broth (typical, for example, for *Vibrio cholerae*) ✓ **Bottom or wall growth** — sediment or small flakes at the test tube wall with a transparent broth (typical for streptococci)	✓ Growth in the form of **isolated colonies**. Colonies are visible clusters of microbial cells of the same species. Colonies can be differentiated into two main types based on their appearance (shape, edge character, surface character, size, consistency, transparency): **S-shape** («smooth») — with smooth edges, smooth surface, shiny, transparent or translucent **R-shape** («rough») — with uneven edges, rough surface, opaque ✓ Growth as «bacterial lawn» (confluent growth) on the surface of agar (if the inoculation density is high)

Isolation of microbial pure cultures

The **bacteriological (cultural) method** of diagnostics is based on the isolation of a pure culture of microorganisms from the pathological material on nutrient media and its further identification.

A **pure culture** is a population of microorganisms of a single species grown on a nutrient medium [10, 14].

It is known that pathogens of infectious diseases in the human body, animals and the environment are mainly mixed with other microorganisms (including opportunistic bacteria of normal flora and saprophytes). Isolation of a pure culture makes it possible to identify its morphological, cultural, biochemical and other features, which together determine the *species of the pathogen*, i.e., carry out its **identification**.

Advantages of the method: relatively high sensitivity and accuracy, the ability to determine the number of microbes in the investigated material, in some cases to prove the pathogenicity of isolated microorganisms, as well as to determine the sensitivity to antibiotics.

Disadvantages of the method: duration, the method is expensive [1].

Methods of isolation of pure cultures of microorganisms

Methods for separating pure cultures from microbial mixtures can be divided into several groups:

1. *Methods of mechanical separation of microorganisms*: inoculation of the material on the surface of a solid agar plate medium with a spatula or a streaking

plate method using a bacterial loop; separation based on the mobility of microbes [1, 6].

2. Methods based on the selective sensitivity of microorganisms to external *factors*: for example, treatment with acid or alkali to isolate resistant bacteria; heating a mixture of microbes, where under the influence of temperature, spore-forming microbes survive, and non-spore-forming microbes die; when inoculating a mixture of microbes on the medium with the addition of a certain antibiotic, sensitive microbes are destroyed and insensitive ones grow; creating anaerobic conditions allows to separate a group of anaerobic microbes from obligate aerobes [1, 6].

Isolation and identification of pure cultures of aerobic and facultative anaerobic bacteria

► **1st stage: 1 day**

a) Collection of material for research;

b) Transportation, storage, preparation for research;

c) Preparation of smears from pathological material, Gram staining and microscopy;

d) Inoculation of pathological material, if necessary, into the enrichment medium. It is carried out if the test material contains a small number of bacteria, for example, when the hemoculture is isolated. To do this, the blood taken at the height of fever is inoculated in the medium in a ratio of 1:10 (to overcome the action of bactericidal factors in the blood); the inoculated enrichment medium is incubated at a temperature of 37°C, 18-24 hours [6, 12, 14].

In the main, immediately after the microscopy of the smear, the pathological material is inoculated with a bacteriological loop on agar plate nutrient media *to obtain isolated colonies that are pure cultures*. The inoculation technique is presented in the figure 9.
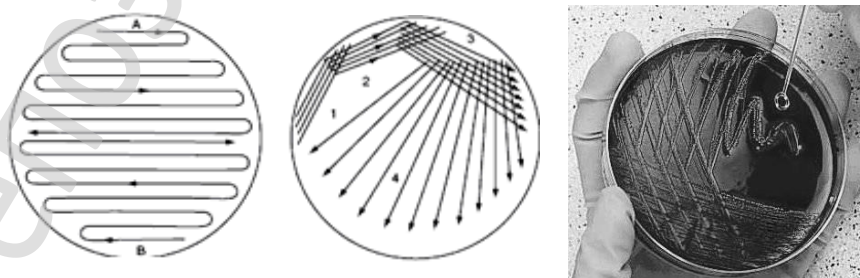
Figure 9 — Inoculation technique of the investigated material on a agar plate nutrient medium

In the case of infections caused by opportunistic microorganisms, where the number of microbes present in the pathological material matters, a quantitative inoculation of the material is made, for which 10-fold dilutions (10^{-1}–10^{-4})

of the material are prepared in a sterile isotonic solution of sodium chloride, 0.05 ml of which is inoculated on the sectors of media.

For cultivating inoculated media, conditions corresponding to the particular microbe are created (cultivation temperature and aeration conditions). This is followed by incubation in a thermostat, mainly at a temperature of 37 °C, since most pathogenic microbes are mesophiles. Most bacteria are cultured at an optimal temperature for 18–24 hours [1].

► **2nd stage: day 2**

a) Study of the growth pattern of isolated colonies grown on media, i.e. determine the **cultural properties** for the purpose of selecting suspicious colonies.

A *colony* is a population of microbial cells of a single species formed as a result of the division of a single microbial cell under cultivation on a solid nutrient medium at an optimal temperature.

The growth of culture is studied visually through the eyes or with a magnifier, as well as under a small magnification of the microscope. The size and shape of the colonies, the edges and transparency are studied in passing light, viewing the Petri dishes from the bottom. In the reflected light (from the side of the lid), the surface character and color of the colony are investigated (figure 10). The consistency is studied by touching the loop to the surface of the colony [6, 12, 14].
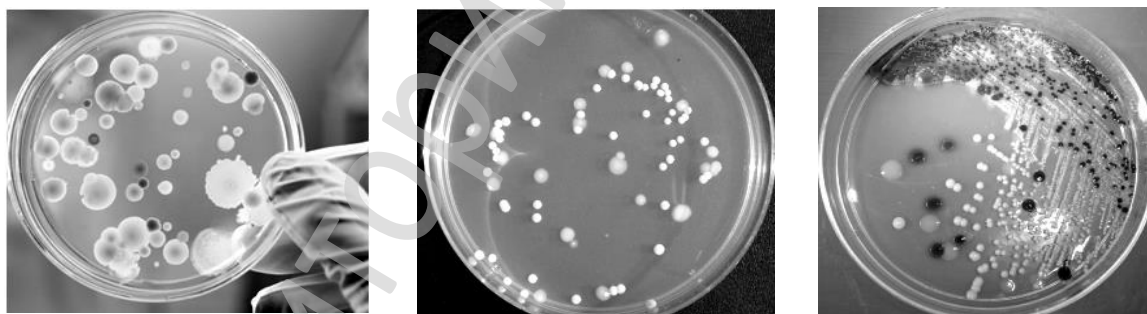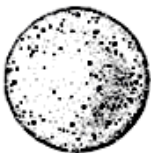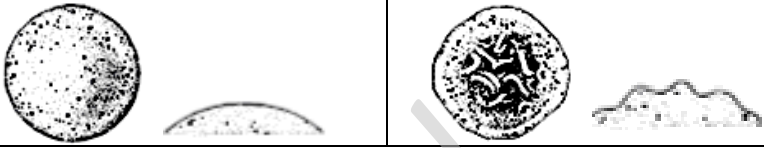
Figure 10 — Study of growing isolated colonies

The cultural properties of colonies are shown in table 12 [1].

Table 12 — Cultural properties

Cultural property	Characteristics of the colonies
Form of the colony	Regular rounded, irregular, flat, rising above the surface of the medium
Size of the colony	Large, small, and dotted (measure the diameter of the colony with a millimeter ruler from the bottom of the Petri dish)
The edge of the colony	Smooth, uneven, fringed

Cultural property	Characteristics of the colonies	
The surface of the colony	Smooth, shiny, wrinkled, rough, matte	
Color of the colony	Colorless, pigmented (colored)	
Consistency of the colony	Dry, viscous, moist, mucous	

b) Microscopy of a smear prepared from a suspicious isolated colony and stained by Gram (to clarify the purity of the isolated bacterial culture). When microscopy of the smear, all microbes must have the same morphological properties (figure 11) [1, 6].

Figure 11 — Examples of a microscopic picture of smears

c) Reinoculation of the remaining part of the isolated suspicious colony on the slant agar (figure 12) for isolation and accumulation of pure culture; create conditions for cultivation (incubation in a thermostat).
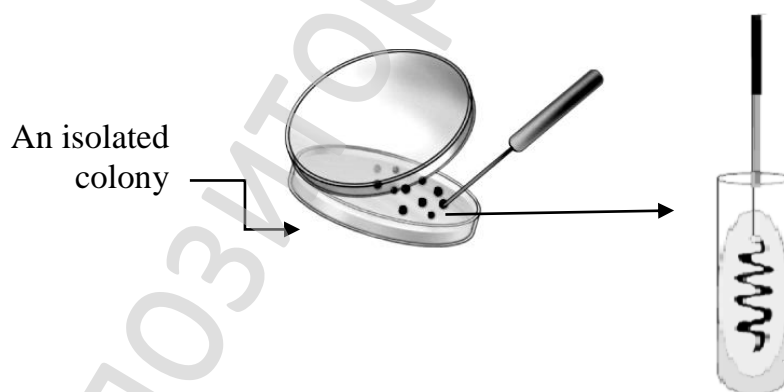
Figure 12 — The reinoculation of an isolated colony on the slant agar

► **Stage 3: day 3**
Identification of selected pure culture is carried out on the following properties:

a) morphological and tinctorial properties (preparation of a smear from the slant agar, Gram staining, if it is necessary ― other methods of staining);

b) cultural properties (properties of grown colonies are studied);

c) biochemical properties (determination of the enzymatic activity of the studied culture of microorganisms);

d) antigenic properties (study of the microbial antigenic structure and determination of its serovar (serotype), i.e. serotyping is determination of an unknown antigen of the isolated microbial culture using a known diagnostic antiserum) [6, 12, 14].

Also, in some cases, the **sensitivity of the isolated culture of microorganisms to antibiotics** is determined with the receipt of an antibioticogram, the results of which are important, for example, for a clinician in the etiotropic therapy of an infectious disease. Thus, the microbiological principle of rational antibiotic therapy must be observed, which requires the use of antibacterial drugs based on the results of an antibioticogram. To determine the sensitivity of microbial culture to antibiotics, for example, disco-diffusion or automated methods for determining antibiotic sensitivity are used, which are described in the following material [1].

► Conclusion: the species of isolated microorganism is determined, and if necessary, the results on the sensitivity of the isolated culture to antibacterial drugs are indicated (an antibioticogram). When isolating opportunistic microorganisms, their concentration in the test material (CFU/ml) is additionally indicated.

The general scheme of isolation and identification of pure cultures of aerobic and facultative anaerobic bacteria is shown in figure 13 [6, 12, 14].

Stage I (examined pathological material):
▪ Microscopy of Gram-stained smear.
▪ Inoculation of the pathological material on agar plate nutrient media — to obtain isolated colonies; creating conditions for cultivation.

↓

Stage II (isolated colonies):
▪ Study of the cultural properties of grown colonies (shape, size of the colony, color, edge character, surface character, transparency, consistency).
▪ Microscopy of a smear prepared from an isolated colony and stained by Gram.
▪ Reinoculation of the isolated colony under study on slant agar — to isolate and accumulate pure crop; creating conditions for cultivation.

↓

Stage III (isolated pure culture):
▪ Identification of the isolated pure culture by the following properties: morphological, cultural, biochemical, antigenic, phagotyping, etc.
▪ Determination of the sensitivity of pure culture to antibiotics.

**Figure 13 — Stages of pure culture isolation and identification
of aerobic and facultative anaerobic bacteria**

Additionally, in some cases (for example, in staphylococcal infections), **phagotyping** (or **phage typing**) is performed, which is of great epidemiological significance and used to identify the source of infection by detecting different or same strains of bacteria (**phagotypes**) within a single species with help of typical standard bacteriophages. Thus, phagotyping can be used for **intraspecies differentiation of bacteria** [2, 12].

The principle of phagotyping is as follows:

1. The studied strain of bacteria is inoculated as a lawn on an agar plate media (MPA).

2. Then, on the inoculated surface of MPA, typical standard bacteriophages are also applied (each in its own region, marked in advance on the bottom of the Petri dish). A grid is drawn on the base of the Petri dish to mark out different regions Inoculated MPA is incubated in a thermostat.

3. The result of the experiment is estimated on the basis of the appearance of sterile spots (negative colonies or «plaques») — the place of absence of bacterial culture growth at the place of application of the bacteriophage drop, to which this bacterial strain is sensitive to given typical bacteriophage.

An example of staphylococcal phagotyping is shown in figure 14. On *S. aureus* strains inoculated as the lawn, drops of known typical standard staphylococcal phages are applied in squares — 21 phages with the corresponding numbers (80, 79, 52A, 29, 71, 55, 3C, 3B, 3A, 53, 47, 42E, 7, 6, 42D, 77, 75, 83A, 54, 81, 187). When estimating the results, the phagotype is indicated by listing the typical phages that can cause lysis of this strain. Different phagotypes of staphylococcal cultures indicate different sources of infection, the same — one source. For example, the coincidence of the phagotype of staphylococci isolated from a patient's postoperative wound and from the hands of a nurse performing wound dressing indicates a single source of infection [6, 12].
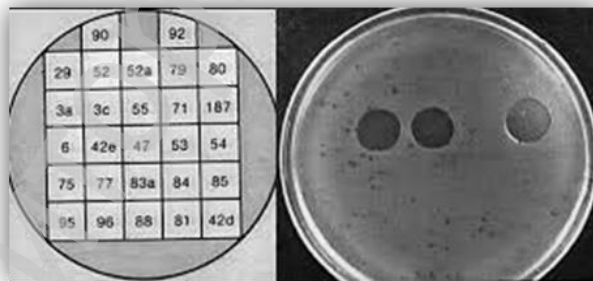
Figure 14 — Staphylococcus phagotyping (negative colonies are in 52, 52a and 80 regions, hence phagotype of studied strain of Staphylococcus is 52/52a/80)

Cultivation and isolation of pure cultures of obligate anaerobic bacteria

The biological features of obligate anaerobes require the use of special cultivation methods that differ from those used when working with aerobic and facultative anaerobic microorganisms.

An important condition that must be observed at all stages of isolation and identification of anaerobes is the protection of these microorganisms from the toxic effects of molecular oxygen. The time between taking the material and inoculation it on nutrient media should be as short as possible. To protect the obligate anaerobes contained in the pathological material from the effects of atmospheric ox-

ygen, special transport media are used. Anaerobic bacteria can only be cultured on nutrient media with low redox potential. To control the degree of saturation of these media with oxygen, special redox indicators (resazurin, methylene blue) are used, the reduced forms of which are colorless. When the redox potential increases, resazurin, for example, turns the media pink, which indicates the unsuitability of such nutrient media for the cultivation of obligate anaerobes [5, 6, 14].

Examples of special culture media for the cultivation of obligate anaerobic microorganisms are presented in table 13.

Anaerobic type of respiration in many times less productive than aerobic, so the nutrient media for anaerobes must be significantly richer in nutrient substrates and meet the complex nutritional needs of anaerobes, and must also contain various reducing agents that reduce redox potential (for example, sodium thioglycolate) [9, 10, 14].

Table 13 — Culture media for anaerobic bacteria

Name of the culture medium	Main composition of the nutrient medium	Notation
Thioglycolate culture medium (culture medium for control of sterility)	MPB, glucose, thioglycolate sodium, an indicator of oxygen resazurin	The medium is designed to control the sterility of the biomaterial. Due to the oxygen indicator resazurin it controls anaerobic conditions for the appearance of pink staining of the upper part of the medium
Anaerobic blood sugar agar (Zeissler agar)	MPA, glucose, defibrinated blood	It is used for obtaining isolated colonies of anaerobic microorganisms, as well as for studying the hemolytic activity of bacteria (for example, the hemolysis zone around the colony)
Wilson-Blair culture medium	MPA, glucose, sodium sulfite, iron chloride	Anaerobic bacteria grow in the depth of the medium and form colonies of black color, since during cultivation, sodium sulfate is formed, which reacts with iron chloride, resulting in the formation of iron sulfide, which has a black color

Conditions for cultivation of obligate anaerobic microorganisms

Cultivation of obligate anaerobes is performed in oxygen-free conditions. Anaerobiosis conditions are created by physical, chemical, biological and mixed methods.

1. Physical methods

• Use of an *anaerobic chamber* (box), anaerobic conditions in which are achieved by creating a vacuum followed by the introduction of a special gas mixture: N_2 (85–90 %) + CO_2 (5–10 %) + H_2 (5 %). The anaerobic box is shown in figure 15 [9].

• Use of an *anaerostat*, which is a cylindrical, hermetically sealed metal or plastic container in which mechanical air pumping is performed (figure 16) [8, 14].

• *Pre-boiling* of liquid nutrient media (regeneration of the nutrient media) followed by coating the media with vaseline oil to reduce oxygen access.

Figure 15 — Anaerobic box   **Figure 16 — Anaerostat**

• Use of *hermetically sealed bottles* and test tubes, use of tightly closed desiccators with a burning candle.

• Seeding in a high column of agar or *deep agar* (in the depth of the medium favorable conditions are created for the growth of obligate anaerobes).

2. Chemical method — use of chemical oxygen «scavengers».

• Inoculated media are placed in a desiccator — a glass container with lapped edges of the lid. A *chemical oxygen «scavenger»* is added to the bottom of the *desiccator*: sodium hyposulfite or pyrogallol, as well as sodium carbon dioxide [6].

• Chemical absorption of air oxygen occurs when, for example, an alkaline solution of pyrogallol is added to special devices, an example of which is the *Omeliansky candle* — a glass device in the form of a candle [1].

• Adding reducing substances to the nutrient media that bind oxygen residues in the nutrient media (glucose, cysteine, thioglycolic acid, pyruvic acid, etc.

Use of anaerobic gas generators to create anaerobic conditions in hermetically sealed vessels (figure 17).

• Application of anaerobic packages (figure 18). An anaerobic bag is a transparent, hermetically sealed plastic bag designed for 1–2 Petri dishes. Anaerobic conditions in it are created by chemical binding of oxygen to an anhydrous reaction system. Anaerobic bags are convenient for transporting material, cultures of anaerobes, in the field, as well as in the laboratory with a low diagnostic load [1, 6].

Anaerobic gas generators
(«AnaeroGas Pack»,
«Generbox anaer»)

Hermetically closed vessels
(for example, anaerostats, desiccators)
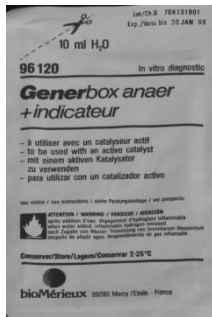
**Figure 17 — Application of anaerobic gas generators
for creation of anaerobic conditions in hermetically sealed vessels**

Figure 18 — Anaerobic package

3. Biological method

• Cooperative cultivation of strict aerobes and anaerobes (aerobes absorb oxygen and create conditions for anaerobes to reproduce). Currently not used [6, 14].

Stages of isolation of pure cultures of obligate anaerobic bacteria

► **1st stage: 1 day**

a) Collection of material for research;

b) Transportation, storage, preparation for research;

c) Preparation of smears from pathological material, Gram staining and microscopy;

d) Inoculation of pathological material in liquid media for anaerobes (e.g., thioglycolate medium). Incubation in a thermostat under anaerobic conditions (anaerostat) 24–48 hours.

► **2nd stage: day 2**

a) Study of bacterial growth in liquid media, preparation of smears, Gram staining, microscopy;

b) Reinoculation of grown material from a liquid nutrient medium to an agar plate nutrient medium (e.g., anaerobic blood agar) in order to obtain isolated colonies. Incubation in a thermostat under anaerobic conditions (anaerostat) 24–48 hours [2, 5, 6, 14].

► **Stage 3: day 3**

a) study of the growth pattern of isolated colonies on agar plate media (cultural characteristics) in order to select suspicious colonies, preparation of smears from suspicious colonies, Gram staining, microscopy;

b) Reinoculation the remaining part of the colony into a liquid nutrient medium in order to accumulate a pure culture. Incubation in a thermostat under anaerobic conditions (anaerostat) 24–48 hours.

► **Stage 4:**

Identification of isolated pure culture is carried out on the following properties:

a) morphological and tinctorial properties (smear preparation, Gram staining);

b) cultural properties (cultural properties of grown colonies are studied);

c) biochemical properties (determination of the enzymatic activity of the studied culture of microorganisms);

d) antigenic properties (determine the unknown antigen of the isolated microorganism using a known diagnostic antiserum);

e) determination of toxigenicity (the ability of microorganisms to produce exotoxins) by use of a reaction of exotoxin neutralization with antitoxic serum in an experiment on laboratory animals, for example, on white mice.

Also, in some cases, the sensitivity of the isolated culture of microorganisms to antibacterial drugs is carried out [2, 5, 6, 14].

► **Conclusion**: the species of isolated pure culture is determined.

Study of biochemical properties of microorganisms

The biochemical properties of microorganisms are based on their enzymatic activity. **Microbial enzymes** are biological catalysts that determine the metabolic processes occurring in microbial cells [10, 12].

The method for determining the biochemical properties of microbes involves studying the enzymatic degradation of various substrates (carbohydrates, proteins and amino acids, urea, hydrogen peroxide, etc.) with the formation of intermediate and final products. Given that different types of microbes often differ in the set of enzymes that they are able to synthesize, therefore, the isolated pure culture of microorganisms can be identified by their biochemical (enzymatic) activity.

Determination of enzymes that is important for identifying microorganisms by their biochemical properties

Detection of enzymes that decompose carbohydrates allows determine the **saccharolytic properties** of microbes. The following media are used for this purpose:

a) **Hiss media** — monocarbon liquid or semi-solid media (MPA), which include peptone water, substrate such as carbohydrate (mono- and disaccharides — glucose, lactose, maltose, sucrose; polysaccharides — starch, glycogen, etc.), polyatomic alcohols (glycerol, mannitol, etc.), and pH indicator (Andrade's indicator or bromothymol blue). To detect gas formation, a float (a glass tube, the upper end of which is sealed) is inserted into the liquid media [10, 12].

Principle of operation. Under the action of the acid formed during the decomposition of the carbohydrate, the indicator changes the color of the medium; in the absence of enzymatic activity of the microbe, the color of the medium is not observed. Thus, differences in color in several test tubes with a Hiss medium when different enzymatic activity of the microbe is shown allowed us to call it a «*motley Hiss row*». Gas formation is determined by the presence of gas bubbles in the semi-solid media or, if the medium is liquid, the float floats to the surface of the medium [1].

The composition of the short variant of "motley Hiss row" includes lactose, glucose, mannitol, maltose, and sucrose. If necessary, determine the ability of the studied culture to ferment larger number of substrates (monosaccharides, polysaccharides, alcohols).

b) **Other differential diagnostic media** (Endo, Levine, MacConkey), which include lactose and the corresponding indicator; these media are used for enterobacteria. If microorganisms decompose lactose to acid (lactose-positive), the color of the colony changes according to the indicator, i.e. colored colonies are formed, if the bacteria do not decompose lactose (lactose-negative) — colorless colonies [11, 14].

c) **multicarbohydrate media** (Kligler iron agar/double sugar agar — KIA, triple sugar iron agar — TSA-agar). KIA composed of MPA, glucose, lactose, reagent for H_2S, and phenol red is added as the indicator.

Principle of operation of KIA. The medium in the tube (slant MPA) has a solid, poorly oxygenated area on the bottom, called the *butt*, and an angled, well-oxygenated area on the top, called the *slant*. The investigated culture is inoculated into the butt and across the surface of the slant. If the microbe ferments glucose, a large amount of acid is produced, which turns indicator becomes yellow in the butt. If the microbe ferments lactose (*lactose fermenter,* e.g. *Escherichia coli*), a large amount of acid is produced, which turns indicator becomes yellow on the slant. Hence, if lactose is not fermented (*lactose nonfermenter*, e.g. *Salmonella, Shigella, Yersinia*), but glucose is, the butt will be yellow, and the slant will be red; if neither lactose nor glucose is fermented, both the butt and the slant will remain red. The presence of black FeS in the butt indicates

H$_2$S production (e.g. *Salmonella*). Gas production is demonstrated by the presence of bubbles or cracks in the medium [1, 13].

By determining proteases (enzymes that break down proteins), the **proteolytic properties** of microbes are revealed. The following media and tests are used for this purpose:

a) The **breakdown of substrate proteins** by microbial culture can lead to the formation of peptone, albumoses, and amino acids (due to enzymes — proteinases and peptidases). To identify these enzymes, the investigated culture is inoculated on a number of media:

• milk (there is a liquefaction of casein clots to form peptones in the milk which takes the form of whey) [6];

• coagulated serum (liquefaction of the serum in the presence of proteolytic activity of microbes) [6];

• gelatin stab method (when inoculating with a prick by a sterile loop or needle in the gelatin column in a positive case, the gelatin liquefies, and different microbes — differently, for example, layered, funnel-shaped, in the form of a nail, «inverted fir tree», and other variants — figure 19) [2].

•
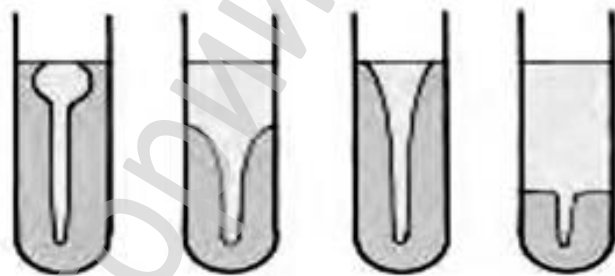
Figure 19 — Gelatin stab method

b) **At deeper splitting of proteins** (fermentation of peptone, amino acids) by microbes indole, hydrogen sulfide, ammonia and other compounds are formed, which are detected by the following tests:

• *Indole test*: when the amino acid tryptophan is cleaved (due to the action of the enzyme tryptophanase), indole is formed, which is detected using an indicator paper moistened with oxalic acid and fixed under the stopper in a test tube over a liquid culture medium with seeding. In the presence of indole, the paper turns red [2].

• *Hydrogen sulfide test*: when splitting sulfur-containing amino acids by desulfurase enzymes, H$_2$S is formed, which is detected using an indicator paper impregnated with a solution of lead acetic acid and fixed under the plug over the nutrient medium; in positive cases, the paper turns black. The presence of H$_2$S is also detected in the Kligler medium (when H$_2$S is isolated, the medium is blackened) [2].

• *Ammonia test*: apply a litmus paper fixed under the plug over the nutrient medium; in the presence of ammonia, the paper turns blue.

Redox enzymes (oxidoreductases)

To determine catalase, the investigated culture is introduced by a loop into a drop of 3 % hydrogen peroxide solution on a slide. When a positive reaction occurs, rapid formation of gas bubbles occurs. This test is used, for example, for differentiation of *Staphylococcus spp.* (catalase-positive) and *Streptococcus spp.* (catalase-negative) [6].

Oxidase test: a daily culture of bacteria is applied with a bacterial loop to the surface of filter paper moistened with a special reagent (tetramethylpara-phenylenediamine). With a positive test, after 1–2 minutes, purple staining appears. This test is used, for example, for differentiation of *Pseudomonas* spp. (oxidase-positive) and *Escherichia coli* (oxidase-negative) [6].

Determination of invasive/aggressive enzymes and hemolysins

*The principle of **fibrinolysin** determination method*: an investigated culture is introduced into a test tube with fibrin (a blood clot washed from red blood cells). After incubation in the thermostat, if the result is positive, the clot dissolves [14].

*Principle of **hyaluronidase** determination method*: an investigated culture is added to the test tube with hyaluronic acid. After incubation in the thermostat, if the result is positive (due to coagulation of the hyaluronic acid), no clot is formed [14].

*Principle of **plasmocoagulase** determination method*: an investigated culture is introduced into a tube containing rabbit citrate plasma. After incubation in the thermostat, if the result is positive, the plasma coagulates.

*Principle of **lecithinase** determination method*: isolated pure culture of *Staphylococcus* is inoculated on yolk-salt agar (YSA), which contains 7.5% sodium chloride and a suspension of egg yolk. If the result is positive, an iridescent halo is formed around the colonies of virulent staphylococci due to the splitting of lecithin contained in the yolk of a chicken egg [14].

Hemolysins are enzymes that break down the membranes of red blood cells, cause hemolysis. They are detected by inoculating a culture on 5–10% blood agar. Variants of hemolysis: α-hemolysis is incomplete hemolysis with the formation of a zone of incomplete enlightenment around the colonies of the medium, which within 2–5 days becomes greenish-brown; β-hemolysis is complete hemolysis with the formation of a zone of complete enlightenment around the colonies; γ-hemolysis is invisible hemolysis to the eye [14].

Microplate method (miniaturized multitest systems, API test-systems)

In recent years, in bacteriological laboratories, API test-systems (analytical profile index — API) have been used for rapid identification of isolated pure culture of microorganisms by their biochemical activity. These are test-systems with lyophilized substrates and indicators in microwells, table 14 [1, 9, 13].

Table 14 — Miniaturized multitest systems (API test systems)

Principle of operation	Registering the results of bacterial biochemical activity	
From an isolated colony grown on a nutrient medium in a Petri dish, or a pure culture *in vitro*, a suspension of microorganisms in physiological solution is prepared, which is introduced into the wells of the test system; incubation in a thermostat 4–24 hours	*Visual interpretation*: when the substrate is decomposed and acid is formed, the color of the indicator in the well changes and the result is considered as positive; if the color of the indicator does not change, then the result is considered as negative. All positive and negative test results are compiled to obtain a *profile number*, which is then compared with profile numbers in a commercial codebook to determine the identification of the bacterial species	*Automatic interpretation*: using automatic analyzers (e.g. ATB-expression, VITEK, WalkAway, etc.) for reading results of special API strips APIWEB is a software product containing all of the API databases for a reliable automated interpretation of API strip results. The software provides a detailed report that is displayed on the screen and can be printed.
	API test-system	

Automation of microbiological research

Performing bacteriological studies in a modern laboratory requires the following conditions to be considered:
• increase in the volume of researches;
• expansion of the spectrum of the studied pathogens;
• an increase in the frequency of occurrence of antibiotic-resistant strains.

Under these conditions, standardization and automation of all stages of the bacteriological analysis is the optimal solution.

Many modern laboratories are equipped with automatic identification of bacterial cultures, such as BACTEC blood culture machines, PHOENIX, VI-TEK, ATB-expression microbiological analyzers and others. In this case, the

work of the bacteriologist is much easier; he is exempted from many uniform and time-consuming procedures. However, even in this case, the principle of conducting the cultural method of research coincides with that described in this section, only the second and third stages of bacteriological method are carried out without human intervention — they are almost completely automated. But it should be remembered that a computer can only work with the information that a person "put" into it, so if any kind of microorganism is not included in the automatic analyzer database, the work on its isolation and identification is carried out by the classical algorithm [1, 13].

Hemocultivators

Hemocultivators (figure 20) are designed to isolate microorganisms from the blood and other sterile fluids.

Hemocultivator Bact/Alert   Hemocultivator BACTEC   Bottle with medium

Figure 20 — Hemocultivators and an example of a bottle with a special medium

The principle of their operation is based on automated detection of microbial growth in blood samples placed in a bottle with a special medium. Indication of microbial growth in bottles is carried out using fluorescence or colorimetrically.

The use of automatic systems increases the sensitivity of the method (100 microbial cells in 1 ml) and guarantees the absence of external contamination by microorganisms (the bottle is not opened during the monitoring). The system is controlled by a computer. The investigated blood sample is inserted into the bottle with the medium with a syringe. The bottle is placed in an individual cell of the incubation unit (60 cells in one unit), the further process is automatic. Bottles are incubated at a temperature of 37°C, every 10–15 minutes of incubation; automatic reading of signs of microbial growth in the bottle is performed (by changing the color of the indicator disk at the bottom of the bottle). When the growth of microorganisms occurs, a sound and light indication is triggered, the bottle is removed from the device for further work — the isolation of a pure culture and its identification [13].

Microbiological (bacteriological) analyzers

Microbiological (bacteriological) analyzers are designed to identify microorganisms and determine their sensitivity to antibiotics. Currently, the bacteriological market in Belarus includes automatic bacteriological analyzers BD Phoenix (Becton Dickinson, USA), VITEK and VITEK2 (bioMerieux, France) and WalkAway (Dade Behring, USA), as well as semi-automatic bacteriological analyzers, for example, ATB-Expression (bioMerieux, France), figure 21.

PHOENIX   ATB Expression   VITEK 2 Compact
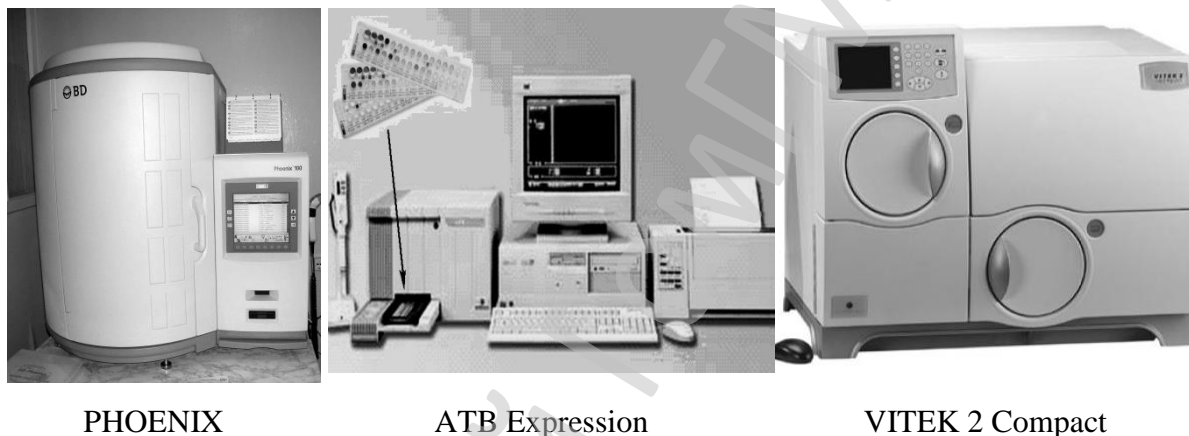
Figure 21 — Examples of microbiological analyzers

The microbiologist's activity when using a microbiological analyzer includes obtaining a pure culture, setting up indicative tests, microscopy and Gram staining, preparing a suspension of investigated microorganism, filling in API test-systems or cards for identification and sensitivity, setting test-systems/cards in the device, entering patient data. In automated systems, incubation, reading of the result and its processing take place without the participation of a laboratory assistant [1, 13].

The **VITEK** bacteriological analyzer, controlled by a computer with a special program, consists of two blocks — devices for filling and sealing cards, as well as an incubator and reader. Tests are performed in plastic cards. After preparing a suspension from a pure culture, the turbidity of which (i.e. the number of microorganisms per unit of liquid) is necessarily estimated on the nephelometer included in the device, the card is filled automatically using a vacuum, which avoids contamination, after which the card is hermetically sealed. Moving the card for filling, sealing and incubation requires manual actions of the laboratory assistant; then, until the result is received, the analyzer works in automatic mode. After use, the cards are removed from the device manually. Cards for identification of pathogens contain 30 wells with lyophilized biochemical substrates and necessary reagents. The result of interaction of the microorganism with reagents is recorded by the device and evaluated by a computer program. Cards for determining antibiotic sensitivity consist of 45 holes filled with various concentrations of antibiotics. The time of identification of microorganisms

and determination of sensitivity to antibiotics using the VITEK analyzer for most microorganisms does not exceed 4–6 hours [1, 5].

More advanced is the **VITEK2** bacteriological analyzer, in which the number of manual manipulations at the stage of preparation and preparation of the suspension of microorganisms is reduced compared to the VITEK device. The device can hold up to 60 cards at a time, and the cards are also deleted automatically. Tests are also performed in plastic cards, separately for identification, separately for determining sensitivity, however, the number of cells in VITEK2 cards has been increased to 64, which in turn speeds up identification by using more biochemical substrates. The device uses a fluorescent indicator, and in the case of enzyme tests, a labeled substrate is used.

Automated bacteriological analyzer **Phoenix** has high performance (up to 200 tests at a time) and is designed for large laboratories. The main unit of the device consists of an incubation module for 100 places and a reader. The device is controlled by a computer integrated with the monitor in the analyzer unit. All analyses are performed on specially designed test-systems with 45 biochemical substrates for identification and special test-systems for determining the sensitivity of microorganisms to antibiotics. The results are registered by color change during the fermentative activity of the bacterium or the presence of turbidity indicating microbial growth, using the fluorescence method [5, 8].

Another system for automated bacteriological research is **WalkAway**, which allows identifying microorganisms in 3–4 hours and determining their sensitivity to antibacterial drugs in 4–24 hours. The analyzer uses a modern fluorescent detection method. The device allows performing only identification or drug sensitivity assessment if necessary, as well as simultaneously identifying microorganisms and determining their antibiotic sensitivity on a single test-system [1, 8].

The **ATB-Expression** device (or its equivalent miniAPI) is a semi-automatic bacteriological analyzer. It is a reader with a built-in monitor, keyboard and densitometer for automatic determination of the turbidity of bacterial suspension by McFarland. Identification strips are developed based on an API technology that is well known in bacteriology. Identification test-systems are divided into classic (incubation up to 24 hours) and fast (4–5 hours). Identification strips can be read either automatically or manually. Strips for determining antibiotic sensitivity are developed with an expanded set of antibiotics [13].

The analyzer of the mass spectra of protein molecules

Mass spectrometry (MS) is a physical method for measuring the ratio of the mass of charged matter particles (ions) to their charge. Devices that implement this method are called *mass spectrometers*. Mass spectrometer is a vacuum device that determines the mass of atoms (molecules), using the physical laws of motion of charged particles in magnetic and electric fields, necessary for obtaining the mass spectrum. Examples of mass spectrometers: VITEK MS, MALDI-

TOF (matrix laser desorption time-of-flight mass spectrometer) and the MALDI Biotyper system.

The greatest effectiveness and clinical significance of mass spectrometry is achieved in the analysis of nucleic acids (DNA/RNA) and peptides (proteins). *Protein profiling* is a method of direct mass spectrometric analysis of the protein fraction, which allows obtaining mass spectra unique to the object under study. A large number of resulting peaks on the mass spectrum is a representative phenotypic characteristic in the study of microorganisms, plasma proteins, and tissues, comparable to a fingerprint.

The MALDI Biotyper system allows identifying microorganisms by ribosomal proteins that are unique to any microorganisms. For research a small amount of pure culture is used, which is applied to a disposable slide. With the help of a matrix solution, the cell wall of the microorganism is destroyed. Under the influence of a laser, the mass distribution spectrum of ribosomal proteins is extracted in a vacuum tube. Bioinformatics software allows reliable and accurate species identification of any microorganisms by comparing the obtained bacterial mass spectra with an extensive database (MALDI Biotyper contains a library of mass spectra containing data on strains of several thousand microorganisms, which are constantly updated with the participation of laboratories around the world). Fully reproducible identification results are obtained within 1–2 minutes.

Mass spectrometric analysis of biological macromolecules allows solving a wide range of diagnostic problems of practical medicine; it can be successfully used both in routine practice of clinical and bacteriological laboratories, and for the implementation of sanitary and epidemiological studies [1, 9, 13].

Determination of bacterial susceptibility to antibiotics

For etiotropic therapy of the infectious process, the clinician prescribes antibacterial drugs to patients.

In the algorithm for selecting antibacterial drugs for etiotropic therapy of the infectious process, the following main stages are distinguished.

An empirical choice is based on the idea of the etiology of the pathological process, the specific antibiotic sensitivity of the suspected pathogen, and the experience of etiotropic therapy of this nosological form. This information is summarized in numerous reference books, manuals, and instructions for use. In addition, it takes into account the experience of using individual antibiotics accumulated in a given region or in a specific medical institution, taking into account the characteristics of antibiotic resistance of strains circulating in a particular area [1].

However, due to the formation of resistance in microorganisms, empirically prescribed antibiotic therapy may be ineffective. Therefore, it is important to correct the empirical choice, i.e., **the etiotropic administration of antibiotics**, observing the microbiological principle of rational antibiotic therapy (use anti-

biotics taking into account the results of determining resistance to antibacterial drugs (antibioticograms) of strains isolated from this patient); comparison with the clinical effectiveness of the therapy in the treatment of this patient [1].

Determination of antibiotic resistance is carried out in accordance with approved documents-standards or orders that regulate the procedure for determining antibiotic resistance of microorganisms. In Belarus, there is an order from the Ministry of Health to determine antibiotic resistance; in Europe and America, recommendations were developed by EUCAST (European Committee on Antimicrobial Susceptibility Testing), CLSI (Clinical and Laboratory Standards Institute, USA) [1, 14].

Methods for determining the susceptibility
of microorganisms to antibiotics

Disk diffusion method (standard disc method, Kirby-Bauer test)

Principle of the method: the surface of a solid nutrient medium (Muller-Hinton agar) is inoculated with a continuous lawn of investigated pure culture, a suspension of which is prepared in a 0.9 % sodium chloride solution to an optical density of 0.5 according to McFarland ($1{,}5 \times 10^8$ CFU/ml). Not later than 10 minutes after inoculation using sterile forceps, apply no more than 6 discs, impregnated with antibiotics. This process can be semi-automated by applying a dispenser for disks with antibiotics, which is designed to simultaneously lay 8 standard disks with antibiotics on the surface of inoculated agar [5, 6, 14].

The results are registered after 18–24 hours of incubation in a thermostat along the diameter of the *zone of growth inhibition* around antibiotic discs (figure 22). The presence of growth around the disk indicates the insensitivity (resistance) of this microbe to the antibiotic.
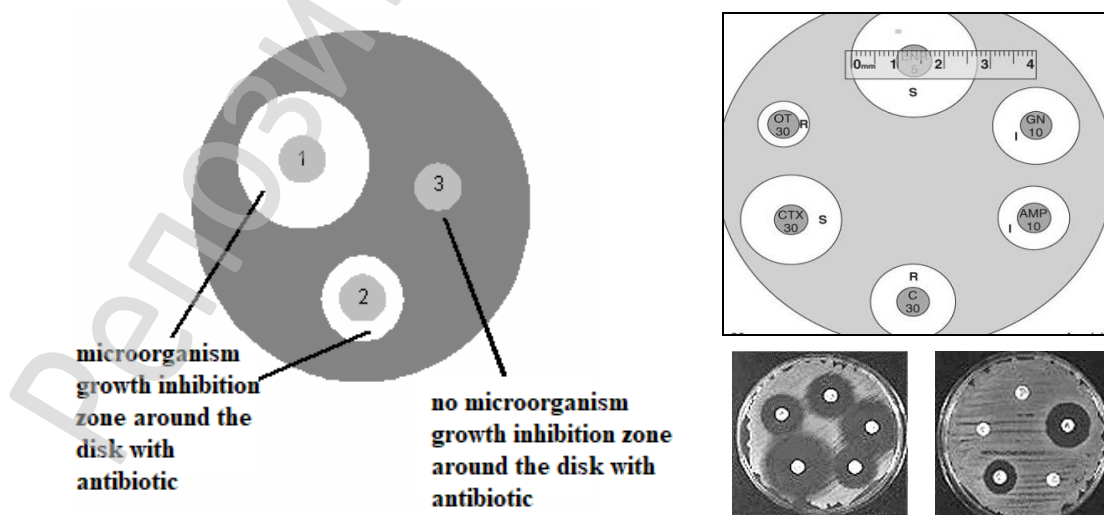
Figure 22 — Determination of the sensitivity of microorganisms to antibiotics by the disk diffusion method: 1 — the microorganism is sensitive to the antibiotic (S); 2 — the microorganism is intermediate resistant to the antibiotic (I); 3 — the microorganism is resistant to antibiotic (R)

To interpret the results, special tables are used.

When characterizing microorganisms, generally accepted indicators are used — *sensitive* (S) to an antibiotic; moderately resistant (I) to the antibiotic (*intermediate*); *resistant* (R) to an antibiotic.

In parallel with the testing of clinical isolates, the control strain is tested. If the zones of growth inhibition of the control strain correspond to its passport characteristic, the experimental conditions are considered standard, and the results of determining the antibiotic sensitivity of clinical isolates are reliable.

Advantages of the method: easily reproducible; sensitivity to several antibiotics can be determined simultaneously.

Disadvantages of the method: a qualitative method, it is not possible to determine the minimum inhibitory concentration of the drug, is unacceptable for testing slowly growing microorganisms (*Mycobacterium tuberculosis*) [1, 5].

E-test method

Principle of the method: the determination of the sensitivity of a microorganism is carried out similarly to testing by the disk diffusion method. The difference is that instead of a disk with an antibiotic, use an E-test strip containing a gradient of antibiotic concentrations from maximum to minimum. At the intersection of the ellipsoidal growth suppression zone with the E-test strip, the *minimum inhibitory concentration* (MIC) value is obtained. An example of the E-test method is shown in figure 23 [5, 6, 14].
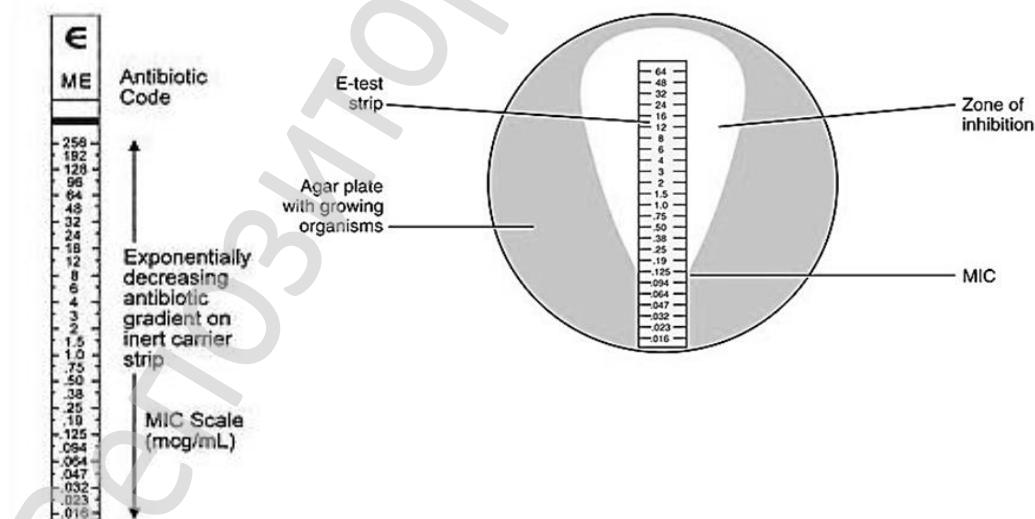
Figure 23 — Determination of bacterial sensitivity to antibiotics using E-test

Serial dilution method in broth

Principle of the method: in test tubes containing 1 ml of Muller-Hinton broth, serial two-fold dilutions of an antibiotic are prepared, for example 100 μg/ml — 1st, 50 μg/ml — 2nd, 25 μg/ml — 3rd, 12,5 μg/ml — 4th, etc. Then,

0.1 ml of the tested bacterial suspension is added to each tube. At the same time put a control of growth (1 ml Muller-Hinton broth and 0.1 ml of a suspension of bacteria). Inoculated media are incubated at 37 °C for 18–24 hours, after which results are registered. The inhibition of turbidity indicates a growth retardation of bacteria in the presence of a given concentration of the antibiotic (figure 24) [5, 6, 14].
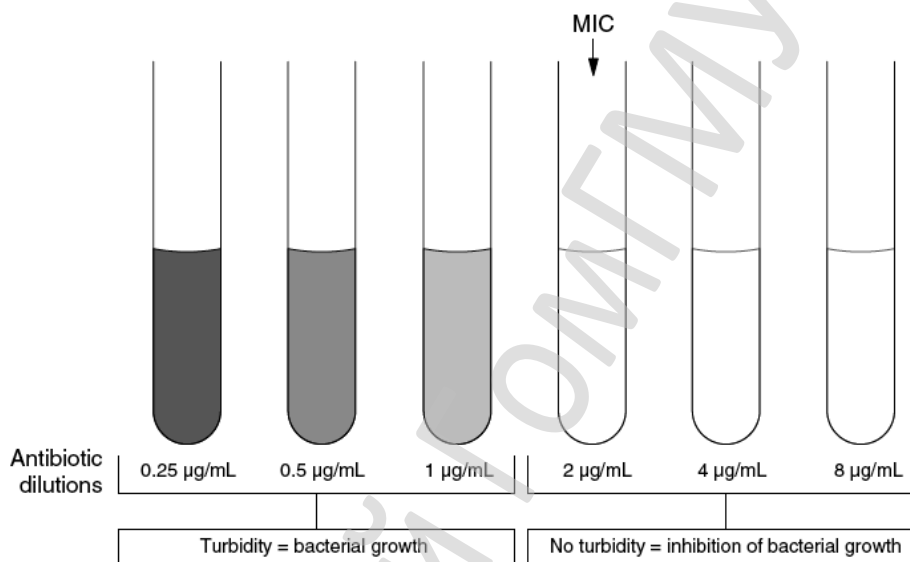
Figure 24 — Determination of the minimum inhibitory concentration dilution method in a liquid nutrient medium (MIC = 2 µg/ml)

The minimum inhibitory concentration (MIC) is the lowest antibiotic concentration (in µg/ml or mg/l), which *in vitro* completely inhibits the visible growth of bacteria.

Advantages of the method: quantitative, allows to determine the MIC of the antibiotic.

Disadvantages of the method: more material and time-consuming compared to the method of standard disks [1].

Agar series dilution method

The principle of the method. Daily agar cultures of microorganisms are diluted in sterile isotonic sodium chloride solution to a standard turbidity of 0.5 according to McFarland and used for plating on Mueller-Hinton agar with different concentrations of antibiotics. The dilution range of antibiotics can be, for example, 0.25–128 µg/ml. After autoclaving, flasks with a nutrient medium are placed in a water bath at 48–50 ºC, after which antibiotic working solutions are aseptically added (1 part of the antibiotic working solution to 9 parts of molten agar). Then the medium is thoroughly mixed and poured into Petri dishes. Next, the bacterial suspension is placed on the surface of the agar in a Petri dish; inoculated media are incubated in the thermostat for 18–24 hours. The presence of microorganism growth on the surface of the agar indicates that this antibiotic

concentration is insufficient to suppress its viability. As the antibiotic concentration increases, the growth of the microbe decreases. MIC is the first lowest concentration of antibiotic (from a series of serial dilutions) at which bacterial growth is not visually determined. MIC is measured in mg/l or μg/ml [5, 6, 14].

Automated methods for determining the sensitivity of microorganisms to antibiotics

In mass studies, automated methods for determining sensitivity to antibiotics are used. This allows simplifying and speeding up the study. For this, *microbiological (bacteriological) analyzers* are used, for example, VITEK, ATB-expression, Phoenix (information about them is presented in the above mentioned material), which are intended not only to identify microorganisms, but also to determine their sensitivity to antimicrobial preparations [8].

Molecular genetic method

In recent years, a *polymerase chain reaction* has begun to be used in practice to identify specific genes in microorganisms that are responsible for the formation of drug resistance (genoindication of antibiotic-resistant cultures of microorganisms) [1, 8].

Immunological method of research. Immunological reactions

Immunological (serological) is the **research method**, which is based on the immunological reaction. An immunological reaction is a reaction of a specific interaction of an antibody (Ab) with an antigen (Ag), proceeding in vitro. This reaction is also called serological, since serum containing antibodies is used to formulate it [1, 10].

Immunological reactions proceed in two phases:

1) Specific — the interaction phase in which a complementary connection of the active centers of antibodies and epitopes of the anti-gene occurs; usually this phase lasts a few seconds or minutes;

2) Non-specific — the phase of manifestation, characterized by external signs of the formation of immune complexes; can develop from a few minutes to several hours (on average — 30 minutes) [8].

The reaction «antigen-antibody» in the in vitro system may be accompanied by the appearance of several phenomena — agglutination, precipitation, neutralization, and lysis. External manifestations of the reaction depend on the physicochemical properties of the antigen (particle size, physical condition), the class and type of antibodies (complete and incomplete), as well as the experimental conditions (medium consistency, salt concentration, pH, temperature). The optimal specific interaction of antibodies with an antigen occurs in an isotonic solution with a pH close to neutral at a temperature of +37 ºC [6].

Immunological reactions are used for diagnostic purposes in two ways (table 15) [1], to identify:

1. Antigen of a microorganism (serological identification);
2. Antibodies in the patient's blood serum (serodiagnostics).

Table 15 — The use of immunological reactions

Direction of research	Purpose of research	Antigen (Ag)	Antibodies (Ab)
Serological identification (serotyping, express diagnostics)	Establishment of the microorganism genus and species names	Unknown (pure culture or test material containing microorganisms)	Known (immune diagnostic antiserum)
Serodiagnostics	Detection of serum antibodies	Known (antigenic diagnosticum)	Unknown (blood serum of a patient)

Thus, in immunological reactions, one of the two main components (Ag or Ab) must always be known in order to find another unknown component.

Serological identification of microorganisms

Serotyping is the identification of an unknown microbial antigen in an isolated by the bacteriological method pure culture using a known diagnostic serum [10, 11].

Express diagnostics is the determination of an unknown microbial antigen directly in the investigated pathological material using a known diagnostic serum [1].

Diagnostic serum — an immune serum containing antibodies of known specificity in a known titer. Known diagnostic immune antisera are obtained by hyperimmunization (multiple immunization) of animals (rabbits, horses) with the corresponding antigens. Immune sera contain known antibodies distinguished by their immunological activity: agglutinins, precipitins, bacteriolysins, opsonins, antitoxins. Agglutinins cause adhesion of microbes, give an agglutination reaction. Precipitins cause precipitation of antigen — precipitation. Bacteriolysins cause the lysis of bacteria - bacteriolysis. Opsonins contribute to phagocytosis. Antitoxins neutralize the action of microbial exotoxin [2, 6].

Monoclonal antibodies are antibodies produced by a single clone of plasma cells. C. Milstein and G. Köhler in 1975 developed a method for producing monoclonal antibodies by creating immortal clones of B-cells called hybridomas. Hybridomas are obtained by fusion of antibody-producing cells (plasmocytes) normal in their life cycle to tumor (immortal) myeloma cells that are not capable of secreting antibodies. Then, hybridoma clone B-cells selected and propagated by breeding are cultured on nutrient media or inoculated into an abdominal cavity of an ascites tumor mouse, where (in the exudate of the abdominal cavity) numerous monoclonal antibodies of the same specificity appear.

Using hybridomas, we can get an unlimited number of antibodies that retain their high specificity and sensitivity [9, 11, 13].

The obtained monoclonal antibodies are widely used in clinical diagnostic practice in the form of monoclonal diagnostic antisera.

Serodiagnostics of infectious diseases

Serodiagnostics is the determination of unknown antibodies in the blood serum of a patient using a known antigenic diagnosticum. Antibodies to the causative agent of the disease in the patient appear, as a rule, by the end of the 1st week of the disease [1].

Diagnosticum — a suspension of bacteria killed by heat or chemicals or extracted from them Ag. Types of diagnosticum: O-, H-, Vi-diagnosticum, erythrocyte antigenic diagnosticum, etc.

Serodiagnostics can be carried out both to detect the presence of antibodies (qualitative reactions) and to determine the amount of antibodies (titer increase). The **titer of serum** is the maximum (last) dilution of the patient's blood serum, in which a positive reaction result is still observed. A positive result of a serological reaction indicates the presence of antibodies in the blood serum of the examined patient that are homologous to the antigen used; a negative result indicates a lack of above mentioned antibodies [1, 12].

Criteria for serodiagnostics

1. A **diagnostic titer** is an antibody titer indicating a disease, i.e. in this titre; the reaction is positive only in patients and negative in healthy ones. If the obtained serum titer of the patient is the same as the diagnostic titer or higher, therefore, the patient is given a serological diagnosis of the corresponding disease; if the obtained serum titer is lower than the diagnostic titer, then at this time stage the serological diagnosis of the disease is not confirmed [1, 2, 16].

2. Reliable results are obtained in the study of **paired blood sera** of a patient: one — taken by the end of the 1st week of the disease, and the second — after 7–14 days, when the antibody titer rises under the influence of a pathogenic microorganism in his body. In this case, it is possible to observe the dynamics of the increase in antibody titer. When establishing a fresh case of bacterial disease or with viral infections, a 4-fold increase in antibody titer in paired sera is of diagnostic value. However, in the presence of additional clinical and epidemiological data, a 2-fold increase in antibody titer is sufficient to establish a diagnosis. In acute infectious diseases, the detection of antibodies is often a retrospective diagnosis, because they appear in sufficient titers by 7–8 days from the onset of the disease, and by this time the disease may end [1, 2].

3. **Determination of various antibody classes**

With the introduction of the enzyme immunosorbent assay method (ELISA) in laboratories, it became possible to determine antibodies belonging to dif-

ferent classes of immunoglobulins (IgM and IgG) in the blood of patients, which significantly increased the informativeness of serological diagnostic methods. In the primary immune response, when the human immune system interacts with the infectious agent for the first time, IgM antibodies are synthesized. Consequently, the detection of specific IgM indicates a fresh case of the disease. Only later, on days 8–12 after the antigen enters the body, IgG antibodies begin to accumulate in the blood. Thus, the detection of specific IgG indicates a later period of the disease, or is it an anamnestic reaction: post-infectious (antibody titer higher) or post-vaccination (antibody titer below) [1, 15].

Upon repeated contact with the antigen (secondary immune response), from the first hours of the development of the immune response, the number of serum IgG antibodies against this infectious disease agent exceeds the number of IgM class antibodies. Therefore, the quantitative determination in the blood of patients of antibodies of the IgM and IgG classes to the corresponding antigen allows not only to judge the presence of the disease, but also to evaluate whether this infection is primary or secondary [1].

In the immune response to infectious antigens, IgA antibodies are also produced, which play an important role in protecting against infectious agents of the skin and mucous membranes.

General classification of immunological reactions

Immunological reactions include:
• serological reactions — reactions between antigens and antibodies in vitro;
• allergological tests — detection of hypersensitivity;
• cellular reactions — with the participation of immunocompetent cells [1].
General classification of serological reactions:
a) *Simple reactions* (2 components: Ag + Ab):
• agglutination reactions (RA) — with a corpuscular antigen;
• precipitation reactions (RP) — with a soluble antigen;
• neutralization reactions (RN) of exotoxin with antitoxins.
b) *Complex reactions* (3-component: Ag + Ab + complement):
• reaction of immune bacteriolysis;
• immune hemolysis reaction;
• complement fixation reaction (CFT).
c) *Labeled reactions*:
• enzyme linked immunosorbent assay (ELISA);
• immunofluorescence reaction (IFA);
• radioimmune method (RIA);
• immunoblotting [14, 17].

Agglutination reactions

The **agglutination reaction** (RA) is the binding of corpuscular antigens (bacteria, red blood cells, etc.) with specific antibodies in the presence of an electrolyte, resulting in an agglutinate [17, 18].

The reaction proceeds in two phases:

1. Specific phase ― the binding of the determinant group (epitope) of the antigen to the paratope of immunoglobulin (invisible phase);

2. Non-specific (visible) phase ― the resulting immune complex (Ag + Ab) loses its solubility and agglutinates in the form of flakes.

Classification of agglutination reactions (figure 25) [1]:
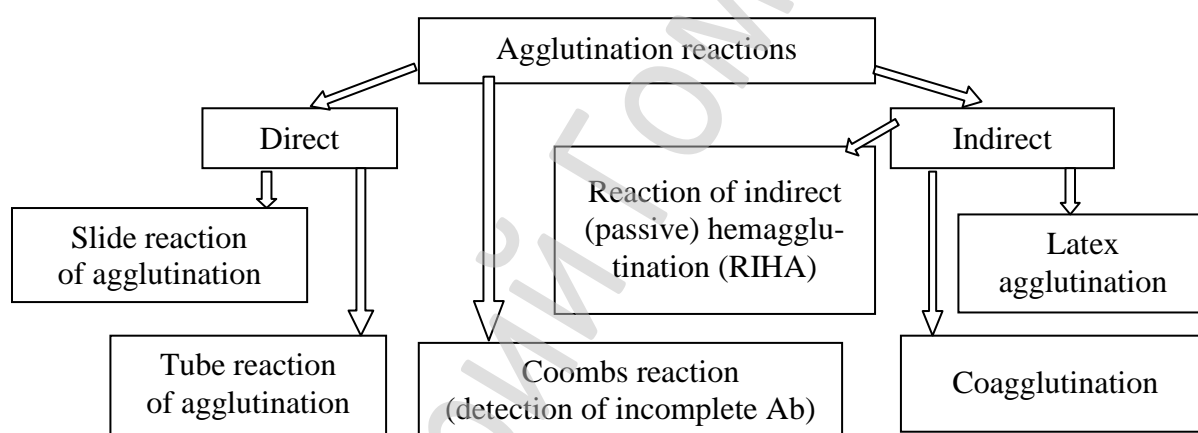
Figure 25 ― Classification of agglutination reaction

Reaction components (figure 26):

1) Ag — antigen (*agglutinogen*) in the form of a suspension of cells (bacteria, red blood cells, etc.);

2) Ab — antibodies (*agglutinins*) in the form of immune serum;

3) Electrolyte (0.85 % sodium chloride solution).

RA can be used to detect specific antibodies in the patient's blood serum (serodiagnostics) and, conversely, isolated microbes can be identified using standard agglutinating serum (serotyping).

Agglutinating serums are used to formulate an agglutination reaction and contain antibodies — agglutinins. Agglutinating sera are obtained by hyperimmunization of rabbits: they are administered 5–7 times parenterally with an interval between injections of 2–7 days in increasing amounts of suspension of killed and, at the end, 2–3 times of live bacteria [6, 12, 17].

Slide agglutination reaction on the glass for serological identification of a microorganism

Principle of reaction: a drop of agglutinating diagnostic antiserum is applied to a glass slide and next to it is a drop of physiological solution (for con-

trol). The studied microbial culture is looped into a drop of physiological solution and a drop of antiserum and carefully emulsified until a homogeneous suspension is obtained, then the glass is slightly shaken. The reaction occurs at room temperature. The results of the reaction are taken into account with the naked eye after 2–4 minutes; sometimes a magnifier is used for this. With a positive reaction in a drop with known serum, clustering of bacteria in the form of agglutinate flakes (large or small) against a background of a clear liquid is observed, and the control drop of physiological solution remains uniformly cloudy (figures 26, 27) [6, 14].
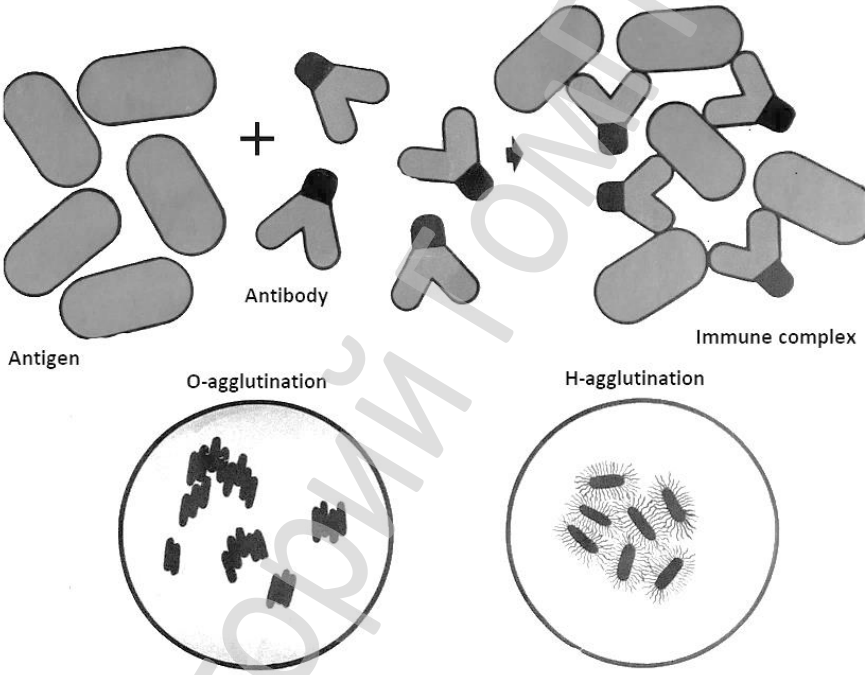
Figure 26 — Scheme of agglutination reaction
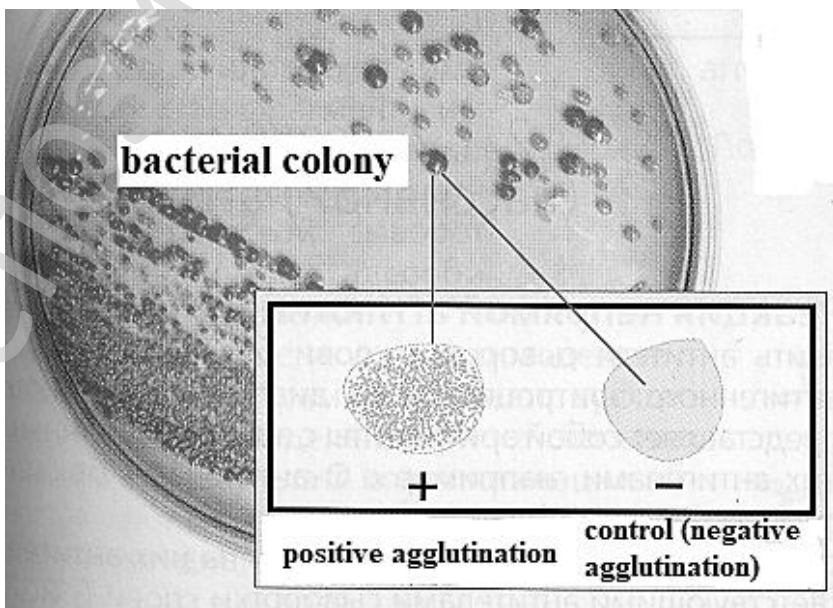
Figure 27 — Slide agglutination reaction

The flakes are clearly visible on a dark background when the glass slide is slightly shaken. In case of a negative reaction, the liquid remains uniformly turbid, as in the control [17, 18].

Tube agglutination reaction

Tube RA is carried out in test tubes. It is used mainly for serodiagnostics of infectious diseases. The use of RA for the serological diagnosis of infectious diseases — typhoid and paratyphoid fever (Widal reaction), brucellosis (Wright reaction), tularemia and other diseases — based on the determination of antibodies (agglutinins) in the serum of patients [17, 18].

Principle of reaction: the patient's blood serum is diluted with an isotonic sodium chloride solution from 1:5 or 1:10 to 1:80 or 1:160 and more and mixed with diagnosticum (figure 28). **Diagnosticum** is used as an antigen in this reaction — suspension of known killed microorganisms.
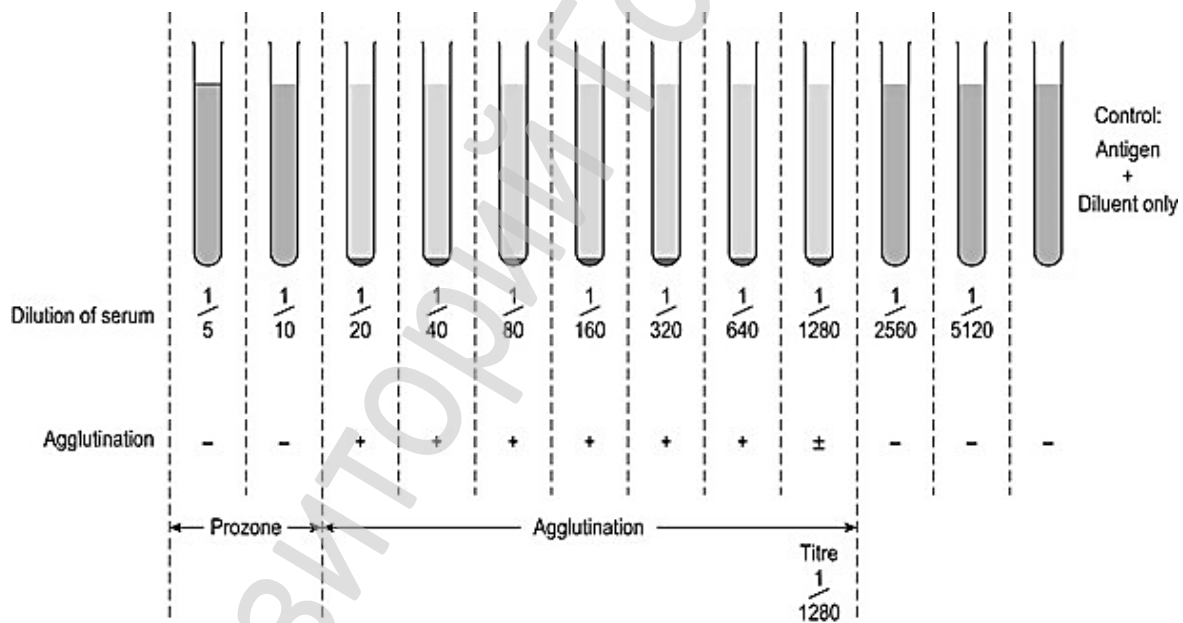
Figure 28 — Tube agglutination reaction

If *O-diagnosticum* is used (bacteria killed by heat or alcohol retain the O-antigen), the agglutination is fine-grained (the bodies of the bacteria combine). If *H-diagnosticum* is used (formalin-killed bacteria retain the flagellar H-antigen), agglutination in the form of large flakes (bacterial flagella combine). The last serum dilution is taken, in which a positive reaction result is still observed (agglutination in the form of sediment) will be considered as the *serum titer* [1, 6, 14].

The reaction of indirect (passive) hemagglutination (RIHA)

The serum antibodies of the patient for serodiagnostics are detected using an erythrocyte antigen diagnosticum, which is red blood cells with antigens adsorbed on them (figure 29) [6, 17].

Diagnostic RBCs   **Antigens**   **Erythrocyte antigenic diagnosticum**
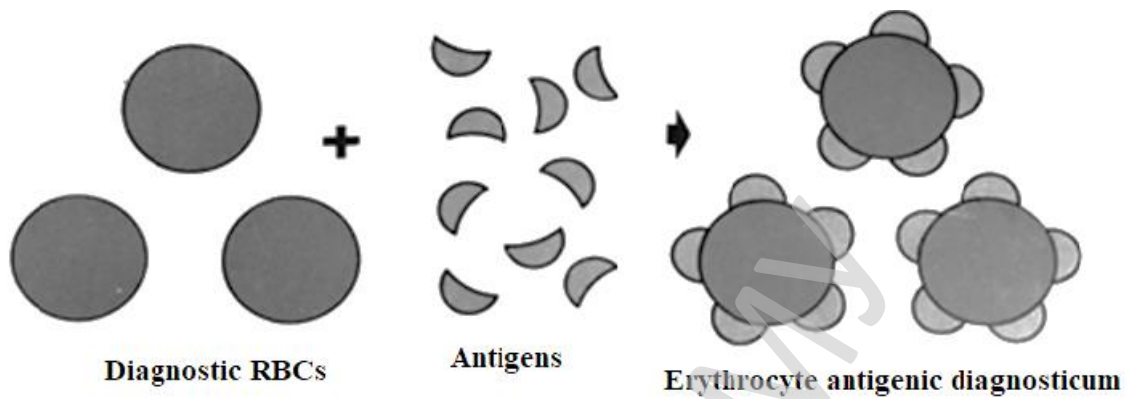
Figure 29 — Formation of antigenic erythrocyte diagnosticum

Principle of reaction: serial two-fold dilutions of the patient's serum are prepared in the wells of polystyrene plates. Then, 0.1 ml of diluted erythrocyte antigenic diagnosticum is added to all wells, and polystyrene plate is placed in a thermostat for 2 hours. In the positive case, red blood cells with antigens adsorbed on them interact with the patient's blood serum antibodies; as a result, red blood cells stick together and fall to the bottom of the tablet cell in the form of scalloped sediment — an «umbrella». In case of a negative reaction, red blood cells settle in the form of a «button» (figure 30) [1, 2].
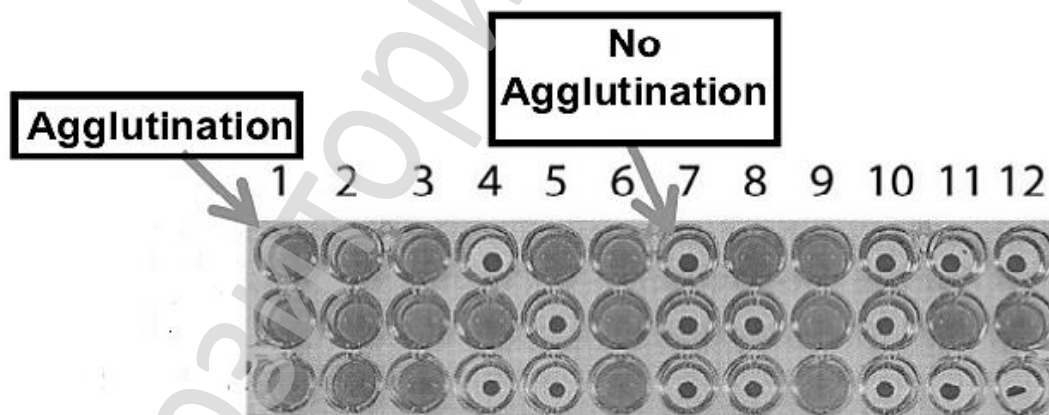
Figure 30 — Registration of RIHA results

Latex agglutination reaction

In addition to red blood cells, latex particles can be used as carriers of antigens or antibodies, which serve as carriers of antigen and play the role of «bridges» between antibody molecules in the formation of immune aggregates.

Principle of reaction: a drop of a suspension of latex diagnosticum is added to a drop of the test serum, mixed thoroughly. With a positive reaction, small but well visible agglutinates are visible to the naked eye. The latex agglutination reaction is more often carried out to detect antigens during express-diagnostics (figure 31) [7, 14].
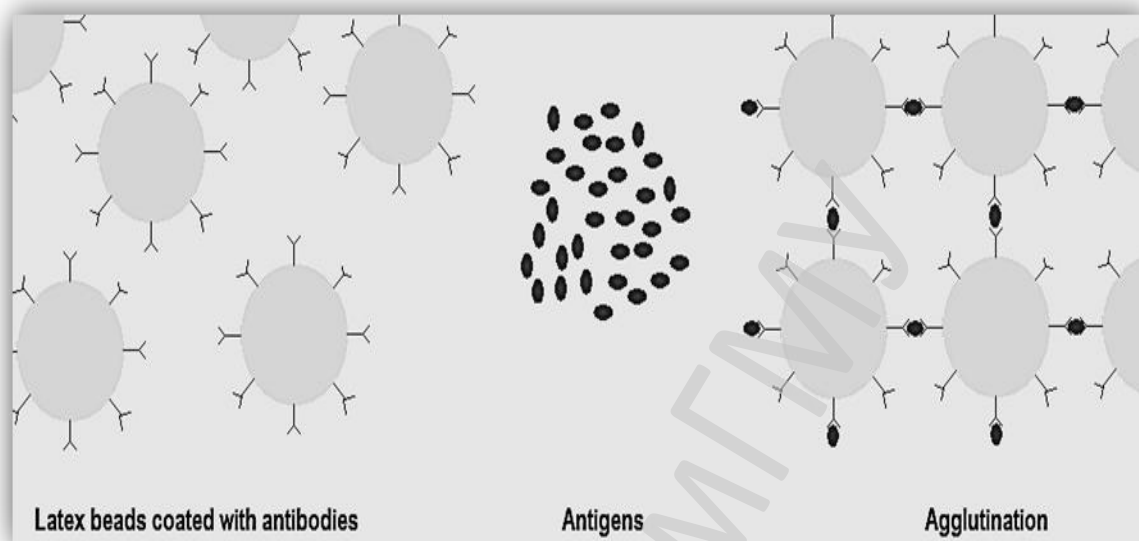
Figure 31 — Scheme of latex-agglutination for express diagnostics

Coagglutination reaction

Protein A of some strains of *Staphylococcus aureus* is capable of nonspecifically adsorbing Fc-fragments of IgG on its surface. The resulting molecule is able to agglutinate homologous antigens [14].

Coombs reaction (direct, indirect)

Using direct and indirect agglutination reactions, complete (divalent) antibodies are determined. Incomplete (monovalent, blocking) antibodies are not detected by these methods, because incomplete Ab have only one active center, and the 2nd active center for an unknown reason does not work (blocked) [2].

To identify incomplete antibodies, a special Coombs reaction is used. Using the Coombs reaction, incomplete anti-erythrocyte (Rh+) antibodies are detected that are fixed on the surface of red blood cells or are in a free state in plasma. They are found in Rh-negative mothers with Rh conflict, with hemolytic disease of newborns, hemolytic anemia, with some infectious diseases (for example, brucellosis), in patients with systemic lupus, with infectious polyarthritis and with a number of collagen diseases and autoimmune disorders [2, 13].

Incomplete antibodies with a specific interaction with red blood cells (Ag) do not cause their agglutination.

Direct Coombs reaction: allows identifying incomplete antibodies fixed on red blood cells. Erythrocyte agglutination is carried out after adding antiglobulin serum to incomplete antibodies located on red blood cells (table 16) [2].

Table 16 — Components of the direct Coombs reaction and general scheme

Direct reaction components	1 — Red blood cells of the patient (with incomplete antibodies); 2 — Rabbit antiglobulin serum (contains antibodies against human immunoglobulins (divalent), obtained by hyperimmunization of a rabbit with human immunoglobulins)
Reaction scheme	
Reaction result	3 — Agglutination of red blood cells and the appearance of a visible precipitate as a result of binding of two incomplete (monovalent) antibodies (fixed on red blood cells) using complete (divalent) antiglobulin antibodies

Indirect Coombs reaction: allows identifying incomplete antibodies that are in a free state in blood serum. It got its name — indirect — due to the fact that the reaction proceeds in two stages. Initially, the patient's blood serum containing incomplete antibodies interacts with the added normal blood group 0 red blood cells without visible manifestations. At the second stage, the introduced antiglobulin serum interacts with incomplete antibodies adsorbed on red blood cells with the appearance of a visible hemagglutination (table 17) [2, 13].

Table 17 — Components of the indirect Coombs reaction and general scheme

Indirect reaction components	1 — Patient's serum in which incomplete antibodies are determined; 2 — Red blood cells of a healthy person; 3 — Rabbit antiglobulin serum
Reaction scheme	
Reaction result	4 — Erythrocyte agglutination and the appearance of a visible precipitate as a result of binding of two incomplete (monovalent) antibodies using complete (divalent) antiglobulin antibodies

Precipitation reactions

The **precipitation reaction** (RP) is based on the formation and precipitation of «antigen-antibody complexes». The precipitation reaction involves solu-

ble antigens — *precipitinogens* (products of microorganisms, chemicals and drugs). Antibodies (*precipitins*), when combined with soluble antigens, cause their aggregation, which is manifested in turbidity of clear liquids or precipitate formation in the agar gel. An immune serum containing precipitins is called precipitating serum. Diagnostic precipitating sera are obtained by hyperimmunization of laboratory animals (e.g. rabbits) with the appropriate antigen. In the precipitation reaction, the antigen is not diluted. The titer of precipitating serum is the maximum dilution of the antigen that precipitates with this serum [2, 12, 14].

Precipitation reactions can determine both an unknown antigen and unknown antibodies, but in practice it is used only for the first purpose.

The precipitation reaction is used in laboratory practice to diagnose infectious diseases of bacterial etiology, for example, anthrax (Ascoli precipitation reaction). In forensic science, the precipitation reaction is used to determine the species of the protein (blood stains, sperm, etc.). The reaction is especially widely used in experimental biology; with its help, for example, the relation degree of various animal species was determined. The use of RP for sanitary-hygienic control of food products reveals the falsification of meat, fish, flour products, impurities in milk, etc [16].

The precipitation reaction is carried out:
• In the liquid phase (Ascoli precipitation reaction);
• In agar gel (different types of immunodiffusion).

Ascoli precipitation reaction

Principle of reaction: the reaction is placed in narrow precipitation tubes where precipitating serum is added, and the dissolved unknown antigen is carefully layered on top. The tube should be kept in an inclined position. After adding the soluble antigen, the tube is carefully transferred to the upright position. If the reaction is positive, after a few minutes, a cloudy precipitation ring will appear at the interface between the two fluids (figure 32). Ascoli precipitation reaction is used to diagnose anthrax antigen in raw animal materials [17, 18].
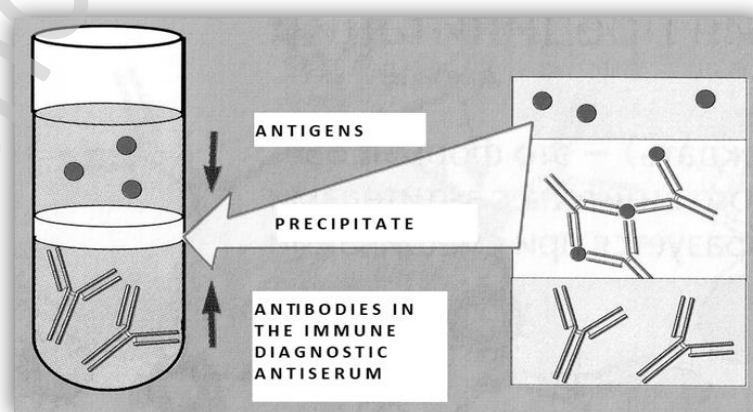
Figure 32 — Reaction scheme of Ascoli ring precipitation

Agar gel precipitation reaction (immunodiffusion)

The essence of the immunodiffusion lies in the fact that antigens and antibodies, placed in different wells in an agar gel, diffuse towards each other and when they interact, the immune complex is formed, which manifests itself in the form of a precipitation line [6, 14].

Distinguish between simple and double immunodiffusion. In case of simple immunodiffusion, one component of the reaction diffuses, in case of double immunodiffusion, both components diffuse. Depending on how immunodiffusion occurs, it is called linear, radial, or angular.
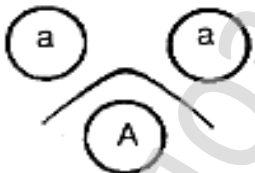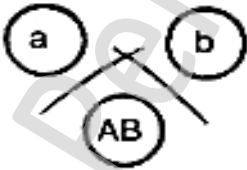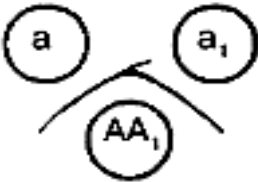
Ouchterloni double radial immunodiffusion

The reaction is carried out on plates or in Petri dishes with agar gel. Antigen and antiserum solutions are placed in wells cut at a certain distance from each other. Immunoreagents (antigen and antibody) diffuse in the gel, when they meet; they form immune complexes that appear as precipitation lines. This method allows studying several samples of immunoreagents at once. For example, several wells with different antisera can be placed around a well with antigen or vice versa [1].

Ouchterloni double counter immunodiffusion

Wells are formed on the agar gel into which antigens and serum are added. Antibodies and antigens diffuse towards each other in agar and form precipitation lines. There are three possible locations of the precipitation lines (table 18) [2, 17, 18].

Table 18 — Options for the location of the lines of precipitation

Scheme	Explanations
	Both precipitation lines are completely fused, which indicates the identity of both antigens
	Precipitation lines intersect, indicating the dissimilarity of antigens
	If the antigens are partially identical, a single precipitation line is formed that is divorced from the general one

Mancini simple radial immunodiffusion

Principle of reaction: the agar gel is mixed with antiserum and poured in an even layer on a glass surface, for example, in a Petri dish. After solidification in the gel, the wells are cut out and the solutions of the tested and standard antigens are poured into them. The reaction is carried out in a wet chamber.

In the positive case, a ring-shaped precipitate forms around the antigen well. The diameter of the precipitation ring is proportional to the concentration of antigen (the higher the concentration of introduced antigen, the larger the diameter of the ring). If several standards with a known antigen concentration are used in the reaction, then by comparing the diameters of the precipitate zone and constructing a calibration curve, the antigen in the samples is quantified [12, 14].

The reaction is used to determine the concentration of immunoglobulins of various classes in blood serum. The Mancini simple radial immunodiffusion scheme is shown in figure 33 [12].
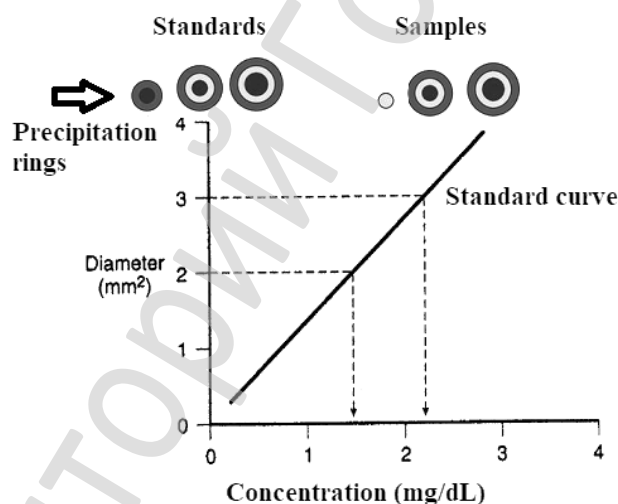
Figure 33 — Reaction scheme of Mancini simple radial immunodiffusion

Immunoelectrophoresis

Immunoelectrophoretic analysis is a combination of agar gel electrophoresis with immunodiffusion.

Principle of reaction: first, electrophoretic separation of proteins (antigens) is carried out in a buffered agar gel; after voltage in the electric field is removed, a groove is cut out in the gel into which precipitating immune serum is introduced. The antigen and antiserum diffuse into the gel towards each other, and in the place of their interaction precipitate lines appear, the number, position and shape of which give an idea of the qualitative composition of the initial mixture of antigens. Using this method, in clinical immunology, the concentration of immunoglobulins of various classes is determined quantitatively, and myeloma proteins are identified. This method is also used in the diagnosis of immunodeficiency states [6, 14, 17].

Neutralization reactions

Neutralization reactions (RN) are mainly used to identify bacterial exotoxins, as well as to determine the concentration of antitoxin in the test serum. Bacterial exotoxin in a mixture with homologous antitoxic serum is not destroyed, but is neutralized (bound).

Antitoxic serum (contains antibodies — antitoxins) is obtained by hyperimmunization of the horse with the corresponding toxoid (Ag). Anatoxin (toxoid) is an exotoxin, devoid of its toxic properties, but retaining immunogenic properties. To obtain the toxoid, 0.3–0.4 % formalin is added to exotoxin and placed in a thermostat at a temperature of 39–40 °C for 4 weeks (according to Ramon). The resulting toxoid is controlled for safety by introducing it to guinea pigs or white mice, as well as for sterility and immunogenicity [17, 18].

The neutralization of exotoxin with an antitoxin is obtained in strictly defined proportions and with the simultaneous addition of the entire amount of toxin to the antitoxin [1].

The neutralization reaction can be carried out:
- *in vitro*;
- *in vivo* (in guinea pigs or white mice, and skin immunological tests).

Detection of the toxigenicity of the diphtheria pathogen in the gel precipitation reaction according to Ouchterloni (Elek test)

Principle of reaction: the studied material is isolated cultures of the diphtheria pathogen *(Corynebacterium diphtheriae).* Diagnostic preparation is antitoxic serum (in a paper strip), containing antitoxin antibodies, obtained by hyperimmunization of a horse with diphtheria toxoid. Immunoreagents (antigen and antibody) diffuse in the gel, upon meeting they form immune complexes precipitating in the form of precipitation lines (figure 34) [15, 17, 18].
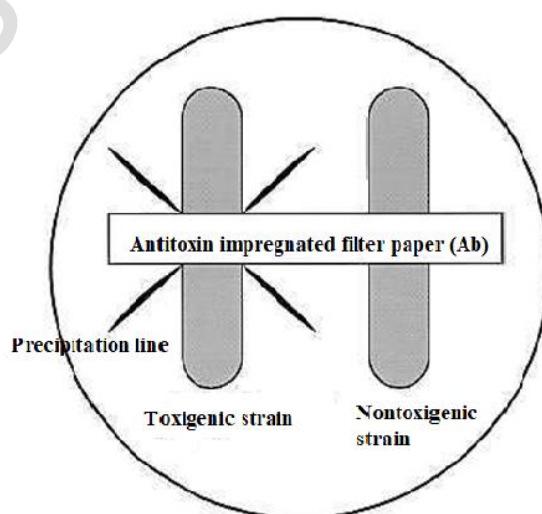
Figure 34 — Detection of toxigenicity of *Corynebacterium diphtheriae* strains

And if there are precipitation lines, the microbial culture is considered as toxigenic, i.e. produces exotoxin. If there are no precipitation lines, there is a non-toxigenic culture of *Corynebacterium diphtheriae*.

Animal neutralization reaction

Animal RN is used to identify bacterial exotoxin. To set up a neutralization reaction in vivo, the studied material, in which the presence of exotoxin is assumed, is mixed with diagnostic antitoxic serum, incubated in a thermostat and administered to laboratory animals (white mice, guinea pigs). When exotoxin is neutralized with antitoxins (Ab), which are contained in antitoxic serum, experimental animals survive. Control animals, which injected with filtrate of the test material, containing only exotoxin, die [1, 6].

Skin immunological tests

Skin immunological tests are based on the intradermal injection of small doses of exotoxin into the palmar surface of the patient's left forearm. In the absence of antitoxic immunity, redness is observed at the site of exotoxin injection, and in the presence of antitoxic immunity, the introduced toxin is neutralized by serum antitoxins and only an injection mark remains at the injection site [1, 6].

Reactions with complement participation (lysis reactions)

Three components take part in lysis reactions: antigen, antibody and complement. The essence of these reactions is that when specific antibodies interact with cellular antigens (red blood cells, bacteria), an immune complex forms on their surface that activates complement along the classical pathway, and a membrane-attacking complex forms on the cell membrane, resulting in the lysis of these cells. Complement is an integral part of any warm-blooded serum. It is thermolabile, completely inactivated at a temperature of 56 ºC for 30 minutes. In laboratories, guinea pig serum taken immediately before the experiment is used as a complement; dry complement can also be used [1].

Bacteriolysis reaction

An **immune bacteriolysis reaction** is a reaction in which bacteria are lysed by the action of immune serum (contains specific bacteriolysin antibodies) in the presence of complement. In a sterile tube, a mixture is made from a suspension of bacterial culture, inactivated immune serum and complement. Instead of immune serum, the same volume of normal serum is added to the control tube. The tubes are placed for 2 hours in a thermostat at a temperature of 37°C, after which a small amount of material from the tested and control tubes is inoculated onto nutrient media. The next day, abundant growth is observed for inoculation from

a control tube. In the inoculation from a test tube with immune serum, the growth is poor and absent [2, 12, 16, 17].

Hemolysis reaction

An **immune hemolysis reaction** is a reaction in which red blood cells (Ag) are lysed by the action of hemolytic immune serum (contains specific hemolysins antibodies) in the presence of complement.

Hemolysis reaction components:

1. Sheep red blood cells.

2. Hemolytic rabbit serum obtained by hyperimmunization of a rabbit with sheep erythrocytes; contains antibodies — hemolysins.

3. Complement (guinea pig serum).

The hemolysis reaction is used to determine the titer of complement and titer of hemolytic serum [17, 18].

Complement fixation test (CFT)

It is a reaction in which complement and two antigen-antibody systems are involved:

• The first is specific;

• The second is an indicator (hemolytic) system.

Thus, 5 components are required for CFT (table 19) [1]:

Table 19 — Components of CFT

Components of CFT	Systems «Ag-Ab»
1. Antigen	Specific system
2. Antibody	
3. Complement (use guinea pig serum)	
4. 3 % suspension of sheep erythrocytes (Ag)	Indicator (hemolytic) system
5. Hemolytic rabbit serum (obtained by hyperimmunization of a rabbit with sheep erythrocytes; contains Ab — hemolysins)	

Principle of reaction: the formation of antigen-antibody complex (specific system) and fixation of complement by this complex is not accompanied by visible changes. To detect complement binding, an additional indicator hemolytic system is used. This system consists of sheep erythrocytes and rabbit hemolytic serum. In the presence of complement, red blood cell lysis (hemolysis) occurs in the hemolytic system (figure 35) [6, 17, 18].

If an antigen-antibody complex has formed in a specific system that binds complement, then there will be no red blood cell lysis in the indicator hemolytic system (CFT is positive, there is no hemolysis, an antigen or antibody is detected).
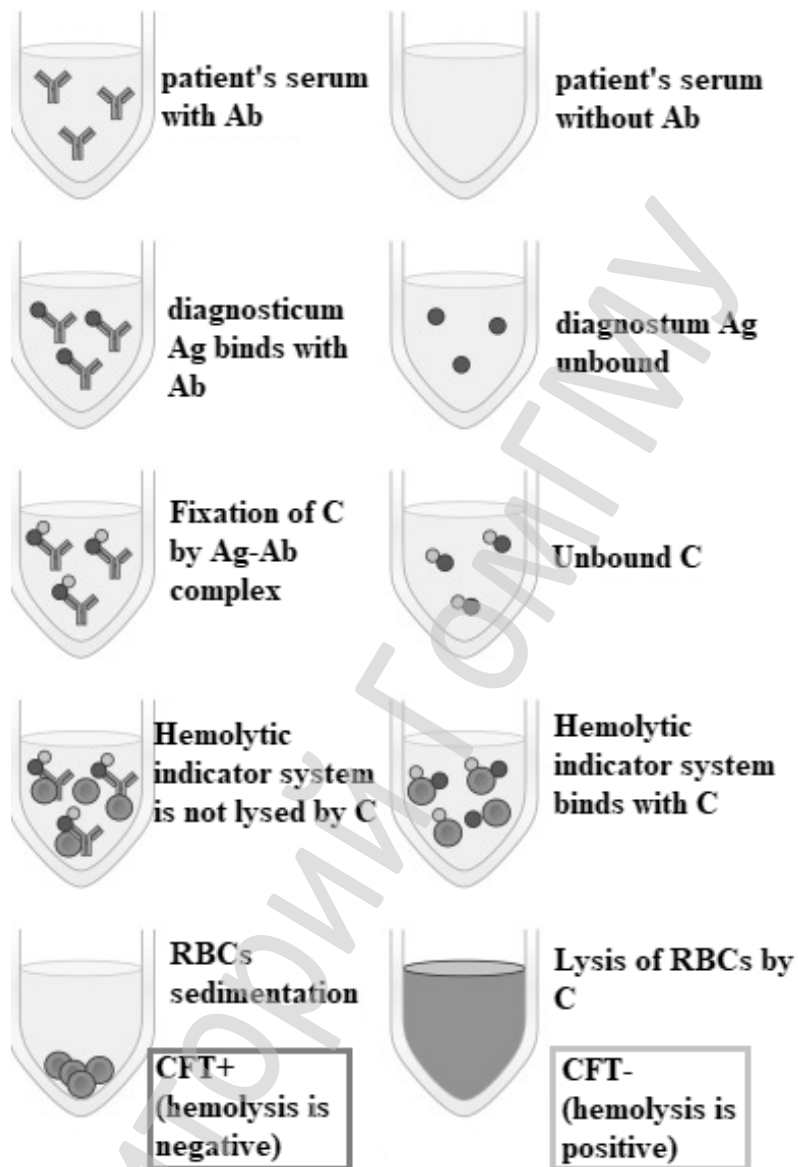
Figure 35 — Scheme of CFT (C is complement system)

If the antigen-antibody complex does not form in the specific system (the antigen and antibody do not match each other, or the desired antigen or antibody is not found in the test sample), then the complement remains free, interacts with the hemolytic system and causes sheep erythrocyte hemolysis (CFT is negative, there is hemolysis, no antigen or antibody detected) [1].

To obtain a reliable result, it is necessary that all components are taken in optimal quantities (working doses). For this purpose, both complement and hemolytic serum are preliminarily titrated (before CFT) for subsequent calculation of their working doses. For titration of complement and hemolytic serum, an immune hemolysis reaction is used.

CFT is used both for serological identification of an unknown antigen and for serodiagnostics of infectious diseases (determination of an unknown antibody in the patient's serum) [1].

Immunological reactions using labeled antibodies and antigens

To immunological reactions using labeled antibodies and antigens include (table 20):
1. Immunofluorescence assay (IFA);
2. Enzyme linked immunosorbent assay (ELISA);
3. Radioimmune assay (RIA) [1, 10].

Table 20 — General characteristics of reactions using labeled antigens or antibodies

Name of the method	Labels	Label identification
Immunofluorescence assay	Fluorochromes	Observe the emission of light by a microbe in a luminescent microscope
Enzyme linked immunosorbent assay	Enzymes	The color change is determined spectrophotometrically using the optical density of the colored solution in the spectrophotometer
Radioimmune assay	Radioisotopes	Determine the radioactivity of the formed immune complex in the appropriate radiation counter

Immunofluorescence assay (IFA)

The direct immunofluorescence method is based on the interaction of an antigen with antibodies labeled with fluorochrome. Fluorescein has been one of the most popular fluorophores. It gives a green luminescence in ultraviolet rays. Other historically common fluorophores are derivatives of rhodamine, coumarin, and cyanine. Two IFA methods are proposed: direct (primary) and indirect (secondary) [14, 17, 18].

Advantages of IFA: simplicity, sufficient sensitivity and specificity; it is often used as a method of rapid diagnosis of infectious diseases (express diagnostics). IFA disadvantages: the method is not quantitative [1].

The use of IFA to determine the unknown microbial antigen in an investigated material (express diagnostics)

The *direct IFA method* is "one-stage method": diagnostic antimicrobial rabbit luminescent antiserum is applied to a fixed smear of cells with an unknown antigen. This luminescent antiserum contains known antibodies labeled with fluorochrome (e.g. fluorescein). Next, the smear is incubated in a moist chamber and washed. With a positive result (the presence of microbial antigen), the luminescence of microbial antigens is observed in a luminescent microscope. The disadvantage of the direct method is that in order to detect various microbial antigens, it is necessary to label with fluorochrome a specific diagnostic immune antiserum against every microbial antigen studied [16, 17, 18].

The use of the IFA to determine the unknown microbial antigen in an investigated material (express diagnostics) is presented in figure 36.
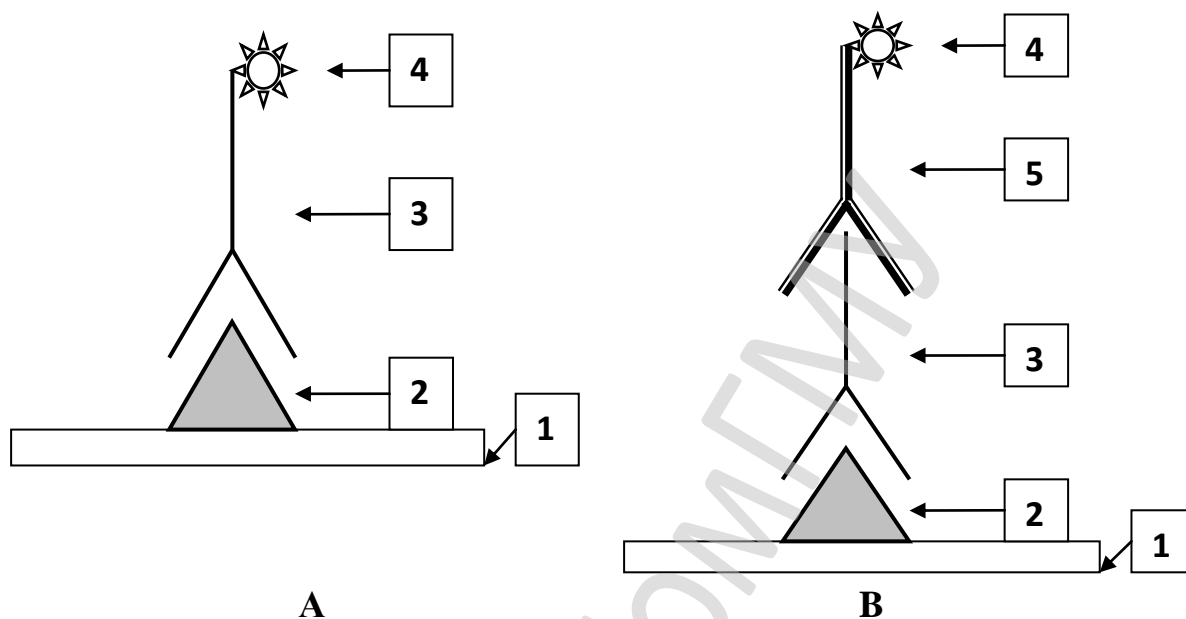
**Figure 36 — Schemes of IFA for determination of unknown microbial antigen:
A — direct method, B — indirect method. Note. IFA components: 1 — glass;
2 — the unknown antigen (investigated material from the patient);
3 — diagnostic antimicrobial rabbit antiserum; 4 — label — fluorochrome
(e.g. fluorescein); 5 — AGS — antiglobulin (anti-rabbit) serum**

An *indirect IFA method* is that first the unknown antigen is treated with the usual diagnostic antiserum (rabbit antimicrobial antiserum), which is obtained by hyperimmunization of the rabbit with the corresponding antigen. Then antibodies that are not bound by microbial antigens are washed. Further, *antiglobulin serum* (AGS) against rabbit immunoglobulins (anti-rabbit serum) fluorochrome-labeled is used to detect the resulting antigen-antibody complex. Such serum is obtained by hyperimmunizing a sheep (or other animal) with rabbit immunoglobulins. As a result, an immune complex is formed: a microbe (Ag) + antimicrobial rabbit antibodies (Ab) + anti-rabbit antibodies (AGS) labeled with fluorochrome. This complex is observed in a luminescent microscope, as in the direct IFA method. One of the advantages of the indirect method compared to the direct one is that the indirect method makes it possible to detect various antigen-antibody complexes using one labeled antiglobulin serum; specific antisera (containing antibodies against antigen) will not be labeled [1, 11].

The use of IFA to detect unknown antibodies in the serum of the patient's blood (serodiagnostics)

To detect antibodies in the patient's blood serum, an indirect IFA method is used. To do this, a suspension of a reference microorganism is applied to the glass, after fixing it on the glass and washing, drops of the patient's blood serum are applied. After washing, luminescent (labeled with fluorochrome, e.g. fluo-

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rescein) diagnostic «antihuman» antiserum against the globulin fraction of human serum proteins is layered on the smear. The resulting antigen-antibody complex is detected by luminescence microscopy: when it is illuminated with ultraviolet rays, it gives a bright green glow, which indicates the presence of antibodies in the patient's blood serum [1].

Enzyme linked immunosorbent assay (ELISA)

ELISA is used to diagnose viral, bacterial and parasitic diseases, in particular to diagnose HIV infection, viral hepatitis and other infections, as well as to determine hormones, enzymes, drugs and other biologically active substances [19].
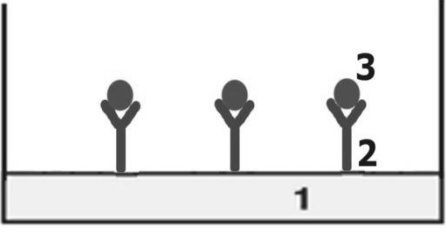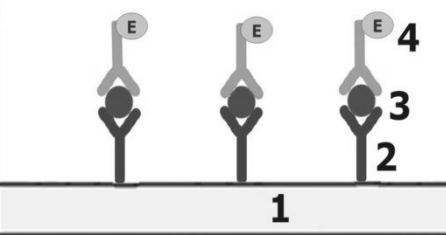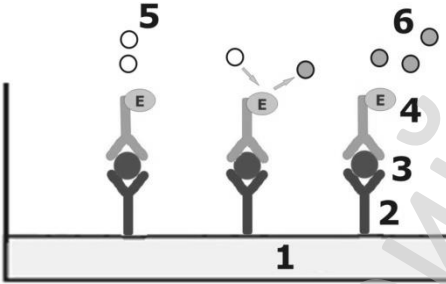
ELISA is a method for the quantification of antibodies or antigens, which uses antibodies labeled with an enzyme. Horseradish peroxidase, β-galactosidase or alkaline phosphatase are often used as an enzyme. When a substrate (for example, hydrogen peroxide if peroxidase is used in the reaction) and chromogen are added to the enzyme, the enzyme changes color. Chromogens are substances containing chromophores, i.e. groups of atoms responsible for the color of the compounds. Depending on the form (oxidized or reduced), the chromogen may be colorless or colored. In ELISA, orthophenylenediamine, tetramethylbenzidine are mainly used as a chromogen. The color intensity is directly proportional to the number of bound antigen and antibody molecules. Intensity of color change is determined spectrophotometrically — by the optical density of the colored solution [1, 17, 18, 19].

There are many formulations of ELISA, and *solid phase* enzyme-linked immunosorbent assay is the most widely used. As the solid phases polystyrene or polyvinyl microplate wells on which antigens or antibodies are adsorbed are most often used. Depending on the antigen-antibody combination, the assay is called a direct ELISA, indirect ELISA, sandwich ELISA, competitive ELISA etc.

The use of ELISA to determine the unknown microbial antigen in an investigated material (express diagnostics)

To detect unknown investigated Ag, a modification of the *direct* ELISA is used, which is shown in figure 37. On the surface of the microplate well (solid phase), specific Ab to the investigated Ag is adsorbed. If the investigated Ag is present in the analyzed material, an immune complex is formed. The enzyme is able to cleave the substrate introduced there, atomic oxygen is released, which oxidizes the chromogen to form a colored product. If the investigated Ag is absent in the analyzed material, then an immune complex is not formed and, as a result, the chromogen does not form a colored product [19].

	The analyzed material is added to the microplate well containing specific antibodies, and if test material contains antigens (3) corresponding to these antibodies (2), then during the incubation, mutual recognition takes place, as a result of which the antigen/antibody complex is formed (2/3)
	After washing the wells, antibodies specific for the investigated antigen are added to them (4), labeled with an enzyme (e.g. peroxidase), i.e. conjugate. During incubation, the conjugate is fixed on the antigens bound to the fixed antibodies.
	After washing the unbound conjugate, a substrate is added to the well - hydrogen peroxide (H_2O_2) with a colorless chromogen (5); atomic oxygen is released during enzymatic decomposition of the substrate, which oxidizes the chromogen and turns it into a colored product (6)
	The amount of colored product is measured on a photometer at a specific wavelength. Thus, the amount of colored product is directly proportional to the amount of enzyme in the well, and hence the amount of conjugate in the sandwich. The amount of conjugate is directly proportional to the amount of antigen/antibody complex that is obtained in the first stage of ELISA. Therefore, the intensity of the color reaction correlates exactly with the presence of specific antigens in the analyzed material.

Figure 37 — ELISA for the determination of antigens

The use of ELISA to detect unknown antibodies in the serum of the patient's blood (serodiagnostics)

The modification of the *indirect* ELISA for the determination of antibodies is presented in figure 38. The figure schematically shows the polystyrene microplate well in which all stages of the ELISA are performed sequentially [17, 18, 19].
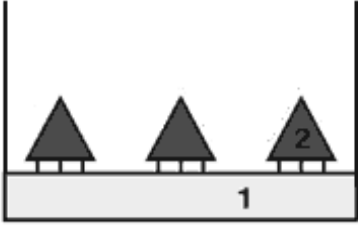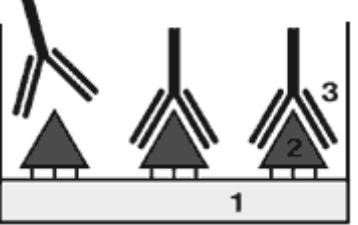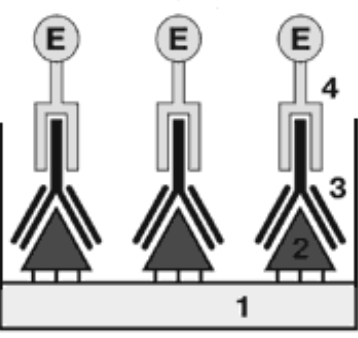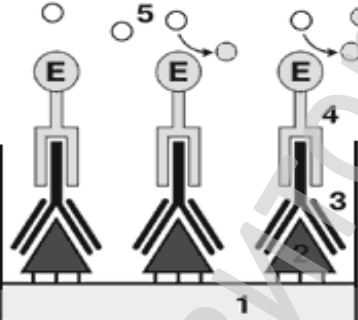
	Specific antigens (2) are adsorbed on the surface of the microplate wells (1)
	The analyzed serum of the patient is added to the well, and if it contains antibodies (3) to these antigens (2), then during the incubation, mutual recognition occurs, as a result of which an antigen/antibody complex is formed (2/3)
	After washing the wells from unbound substances, specific antiglobulin antibodies (i.e., antibodies against human immunoglobulins) (4) labeled with the enzyme (E) are added to them. Such a compound is called a conjugate. During incubation, the conjugate is fixed on human antibodies bound to the antigen, as a result of which a sandwich-like structure is formed in the well (2/3/4)
	After washing the unbound conjugate, a substrate is added to the well - hydrogen peroxide (H_2O_2) and a colorless chromogen (5); during enzymatic decomposition of the substrate, atomic oxygen is released, which oxidizes the chromogen and turns it into a colored product (6)
	The amount of colored product is measured on a photometer at a specific wavelength. Thus, the amount of colored product is directly proportional to the amount of enzyme in the well, and hence the amount of conjugate in the sandwich. The amount of conjugate is directly proportional to the amount of antigen / antibody complex that is obtained in the first stage of ELISA. Therefore, the intensity of the color reaction correlates exactly with the presence of specific antibodies in the analyzed serum.

Figure 38 — ELISA for the determination of antibodies

Immunoblotting

Immunoblotting allows to identify individual antibodies to various proteins of microorganisms. The pathogen antigens are separated by polyacrylamide gel electrophoresis, and then transferred to a strip of nitrocellulose. In this case, different antigens are recorded separately in different parts of the strip. Then the patient's serum is applied to this strip and further studies are carried out according to the usual ELISA scheme. Positive reactions look like dark spots («blots»), each of which corresponds to a discrete pair of «antigen-antibody» (figure 39) [17].

Reading of results:
1 — Positive control (strong)
2 — Positive control (weak)
3 — Negative control
4 — Indeterminate profile
5 — Indeterminate profile (highly suggestive)
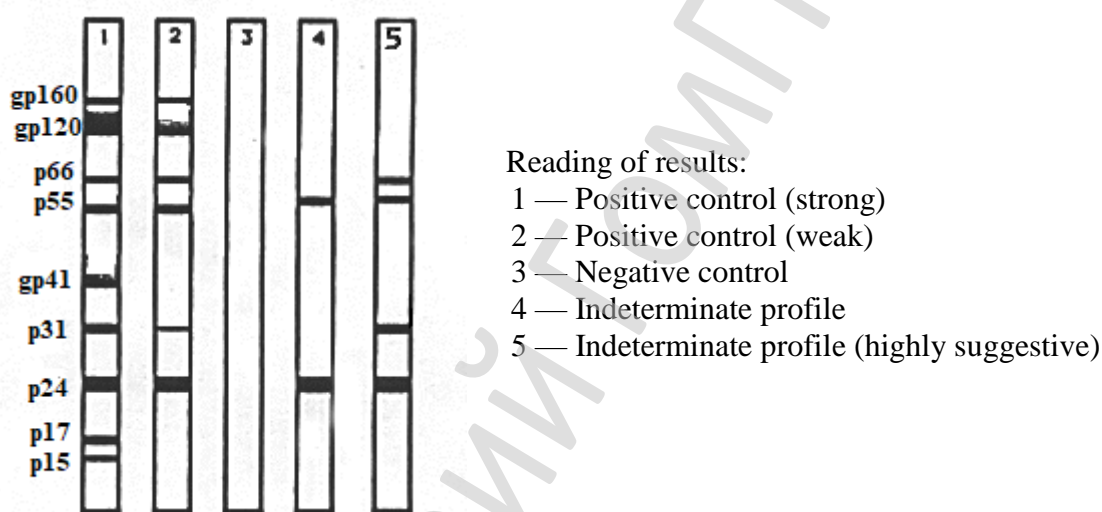
Figure 39 — ELISA for the determination of antigens

Radioimmune analysis (RIA)

Radioimmune analysis – a method for determining antibodies or antigens using antigens or antibodies labeled with a radioactive isotope (^{125}J, ^{14}C, ^{3}H, ^{51}Cr, etc.). After binding of the antibody and antigen, the resulting radioactive immune complex is separated and its radioactivity is determined in the appropriate counter (beta or gamma radiation): the radiation intensity is directly proportional to the number of bound antigen and antibody molecules [14, 17, 18].

Immunohistochemistry, immunocytochemistry

Immunohistochemistry is a complex of methods that allow to identify (visualize) certain antigens in the composition of the natural cellular or tissue microenvironment in normal and pathological conditions. Immunohistochemistry is based on solid-phase ELISA, which is carried out *in situ*, i.e. on histological sections (immunohistochemistry) or smears, cytological preparations (immunocytochemistry). The resulting insoluble stained product is localized at the site of antigen expression and counted using light microscopy.

Setting up the immunohistochemistry involves the following steps:

1. Material sampling;

2. Preparation of histological (cytological) preparations;

3. Fixation, preparation of antigens (depends on the nature of the antigen and antibodies);

4. Staining itself (solid-phase ELISA: direct, indirect, indirect with signal amplification);

5. Accounting: microscopy, photographing, conclusion.

Immunohistochemistry can significantly improve the specificity and sensitivity of the pathomorphological research method, which is very important for the diagnosis of tumors (metastases), their tissue origin, degree of differentiation, etc. In addition to oncology, immunohistochemistry is used for the diagnosis of autoimmune pathology, some infectious diseases, phenotyping of blood cells and bone marrow, for scientific purposes [16, 19].

Immunochromatographic analysis

Immunochromatographic analysis (ICA) is one of the modern and popular methods of laboratory diagnostics, which determine the presence of certain concentrations of substances in biological materials (urine, whole blood, serum or plasma, saliva, feces, etc.). This analysis method is based on the principle of thin layer chromatography and includes the reaction between an antigen and its corresponding antibody [1, 16, 19].

ICA can be attributed to the group of reactions with labeled antibodies. As a label, dyes (dyed latex particles or colloidal gold nanoparticles) or enzyme labels are used. Immunochromatographic analysis is carried out using special indicator strips, panels, microplates or test cassettes [1].

The principle of the immunochromatographic test (for example, using strips) is that when the test is immersed in a physiological fluid, it begins to migrate along the strip according to the principle of thin-layer chromatography. Together with the liquid, specific antibodies labeled with a dye are also deposited on the lower part of the test strip. If the studied antigen is present in this fluid (infectious or oncological marker, hormone), then it binds to antibodies. In this case, antibodies with a dye accumulate around antibodies rigidly immobilized in the test zone of the ICA strip, which appears as a bright dark band. Unbound dye antibodies migrate further along the strip and inevitably interact with secondary antibodies in the control zone, where the second dark strip is observed. The interaction (and the dark band) in the control zone should always occur (if the analysis is carried out correctly), regardless of the presence of antigen in physiological fluid (figure 40). The results are determined visually or by computer processing of the scanned image [1].
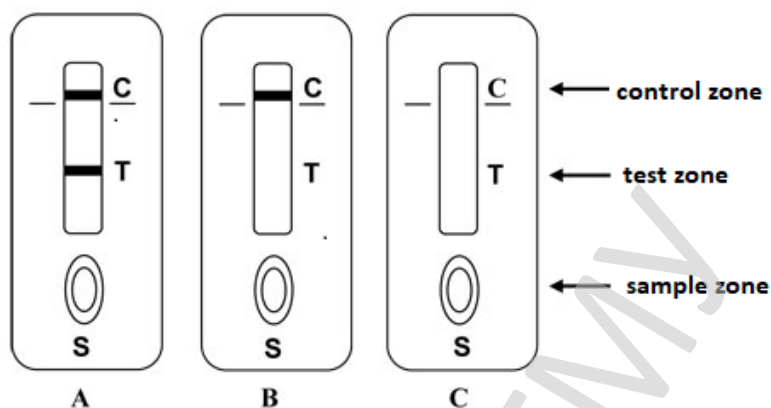
Figure 40 — Scheme of performing the ICA test using plastic plate and record keeping (A — test is positive, B — test is negative, C — test is invalid)

An immunochromatographic test can be a plastic microplate, examples of which are shown in figure 41.
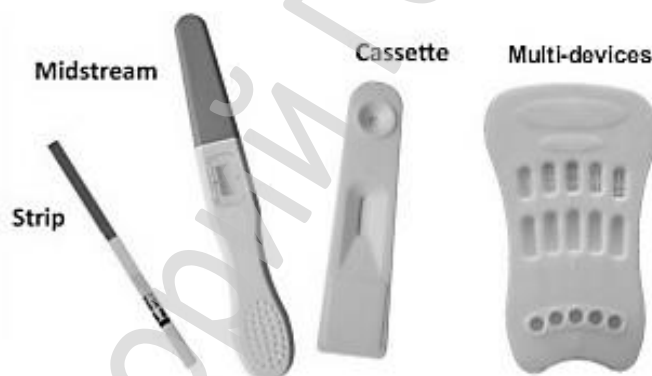
Figure 41 — Immunochromatographic test examples

The following antibodies are used in rapid tests:

1. Movable monoclonal antibodies to the test antigen or antibody conjugated («crosslinked») with colloidal gold — a dye that can be easily identified even in the smallest concentrations. These antibodies are applied near the immersion site of the test strip in physiological fluid.

2. Polyclonal antibodies to the test antigen or antibody, rigidly immobilized in the test zone of the strip.

3. Secondary antibodies to monoclonal antibodies, rigidly immobilized in the control zone of the test strip.

The plastic microplate contains a hole for introducing sample material (sample zone), a hole for recording the result (test zone) and one or more holes for internal control/monitoring. A small amount of prepared investigated material (5–7 drops) is introduced into the start hole of the investigated system (sample zone). Here, the antigen interacts with antibodies adsorbed on the particles, and the formation of the immune complexes begins due to the capillarity of the car-

rier. When they reach the antibodies located on the carrier in the hole for recording the result, these complexes bind, and the particles of latex or colloidal gold appear as a line in blue (latex) or brown. Since particles loaded with antibodies are taken in excess, part of them moves on and binds in the hole of the internal reaction control. The band in this hole indicates the correct operation of the test system. Thus, in the case of a positive result, two bands are formed: in the test and control zones. If the result is negative (lack of the investigated antigen), labeled antibodies, not remaining in the test zone, immediately migrate to the control zone, where they bind to immobilized antibodies, forming only one band in control zone (figure 42) [1, 15].
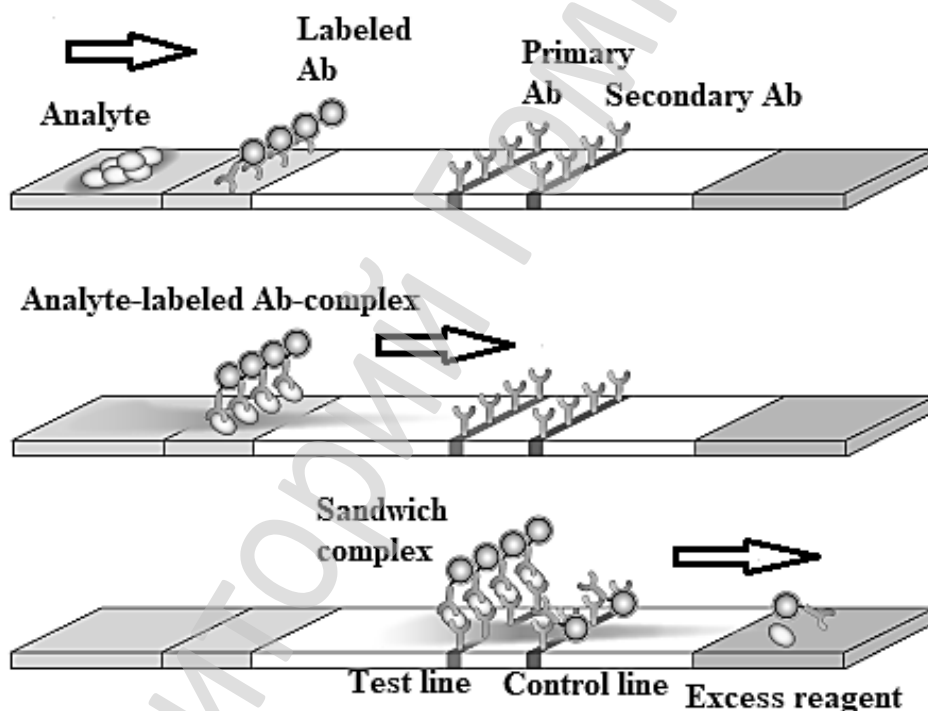
Figure 42 — Immunochromatographic test: principle of operation

The reaction of ICA uses:
• The *first antibodies* to the investigated antigen immobilized in the form of a strip on a chromatographic carrier in the hole for recording the result (above the hole for sample zone);
• *Second antibodies* to the same antigen adsorbed on microparticles of gold or latex (placed in the hole for sample zone)
Internal controls include:
• Detectable antigen applied after the hole to account for the result (positive control, control of all stages of the main reaction);
• Anti-species antibodies against the second (labeled) antibodies, fixed in the form of a strip on the carrier (negative control, control of the transfer of ingredients on the carrier, correct material);

• Nonspecific first antibodies of the same origin (the control of the specificity of the binding for the second antibodies) [1].

ICA test systems have been developed for the detection of group A streptococci in throat swabs, tuberculosis pathogens in sputum, chlamydia in urethral and cervical scrapings, *Clostridium difficile* toxin in feces, Epstein-Barr virus in the blood, and influenza viruses A and B and PC virus in scrapings and swabs from the nasopharynx, for the diagnosis of infectious mononucleosis, determination of markers of myocardial damage (myocardial infarction), calcium metabolism disorders (osteoporosis), for the establishment of ovulation and pregnancy, and others.

Immunochromatographic analysis can be used both for rapid indication of antigens in a sample and for the identification of isolated pure cultures of microorganisms.

Thus, the ICA test systems have undoubted advantages:
• High specificity;
• Speed of obtaining the result;
• Accessible, easy to interpret;
• Do not require medical or laboratory qualifications;
• Can be used by patients independently in any conditions.

However, the sensitivity of the ICA test systems is inferior to other methods of immunoassay, which allows them to be used only as an indicative test [1].

Allergological method of research

Almost every doctor is faced with allergic diseases in patients, with cases of intolerance to drugs and food products, unusual reactions to chemicals in a domestic or professional environment. An allergological research method is used to detect antibodies or sensitized T-lymphocytes to the allergen in the body [6, 13].

Method objectives:

1) The diagnosis of allergic diseases (immediate hypersensitivity);

2) Diagnosis of chronic infectious diseases, in the pathogenesis of which there is sensitization to microbial antigens (delayed-type hypersensitivity);

3) Development of specific desensitizing therapy of immediate hypersensitivity [1].

Stages of the allergological method:

I stage. Collection of an allergic history to:

1. Establishing a hereditary predisposition to the onset of an allergic disease;

2. Identifying the relationship between environmental factors and the development of the disease; establishing a relationship with the seasonality of the manifestation of an allergic disease;

3. Identifying factors aggravating a personal allergic history (acute infectious diseases; presence of a number of chronic inflammatory diseases; detection of food and inhalation allergies, contact dermatitis, insect allergy);

4. Obtaining information about reactions to the introduction of any antibacterial drugs, information about intolerance to other drugs;

5. Obtaining information about preventive vaccinations, the introduction of heterologous serums, and reactions to them; the presumptive determination of a group of allergens or single allergens that could cause the occurrence of this disease;

7. Establishment of the effect of elimination of the allergen: the relationship between contact with the allergen and a change in the patient's condition [1].

II stage. Depending on the type of allergic reaction, various tests, tests and laboratory methods are used (table 21) [1, 16].

Table 21 — Principles for the diagnosis of allergic diseases, depending on the type of allergic reaction

Type of allergic reaction	Main methods
I type Anaphylactic, IgE-mediated, atopic (actually immediate type of hypersensitivity — ITH)	1. Skin allergic tests with allergens: a) application (cutaneous); b) scarification (test by injection or prick-test); c) intradermal. Evaluation of the results is carried out during the first hour (20 minutes) after application of the allergen to the skin 2. Provocation tests: (conjunctival, nasal, inhalation and others) 3. Elimination tests (for example, diet). 4. Laboratory methods: a) ELISA to determine the amount of total IgE; b) ELISA, radioallergosorbent test (RAST) for determining allergen-specific IgE to various types of allergens; c) mast cell degranulation reaction (Shelley test)
II type Cytotoxic hypersensitivity (ITH)	Laboratory methods: IFA, RIA, Coombs test for autoimmune hemolytic anemia
III type Immune complex hypersensitivity (ITH)	Laboratory methods: a) the detection of circulating immune complexes (CIC) in blood serum by the method of their deposition by polyethylene glycol; b) determination of fixed immune complexes by indirect IFA method
IV type Cell-mediated type of allergic reactions or delayed type of hypersensitivity (DTH)	1. Skin allergic tests (differ in time and evaluation criteria when taking into account the reaction: the maximum of their development is observed after 48–72 hours). For example, an intracutaneous Mantoux skin tests (tuberculin test). 2. Laboratory methods: a) macrophage migration inhibition reaction; b) the reaction of blast transformation of lymphocytes, and others

Allergic skin tests

Skin allergic tests are methods for identifying specific sensitization of the body by introducing an allergen through the skin and assessing the magnitude and nature of the resulting edema or inflammatory reaction.

Variants of skin tests:
a) Application (cutaneous);
b) Scarification (test by injection or prick-test);
c) Intradermal tests [6, 11, 17].

Skin allergic tests for the diagnosis of ITH

The following solutions of allergens are used for skin tests: herbs, pollen, animal epidermis, insect venom, food, drugs.

Application tests (patch tests) — a gauze swab is moistened with an allergen solution and applied to an intact skin area (inner surface of the forearm or back). Then this section is fixed with a plaster (figure 44).

Scarification tests — drops of allergens are applied to the skin of the forearm, small scratches are made through a disposable, separate for each allergen scarifier (without damaging the blood vessels). **Prick tests** (an injection test) is a variant of a scarification test: allergens are applied to the skin of the forearm, and only epidermis is injected with disposable needles (one millimeter deep). The test is considered as positive if hyperemia occurs at the injection site and a blister (blister) forms. The test is considered as negative if only the injection point remains (figure 43) [1, 6, 17].
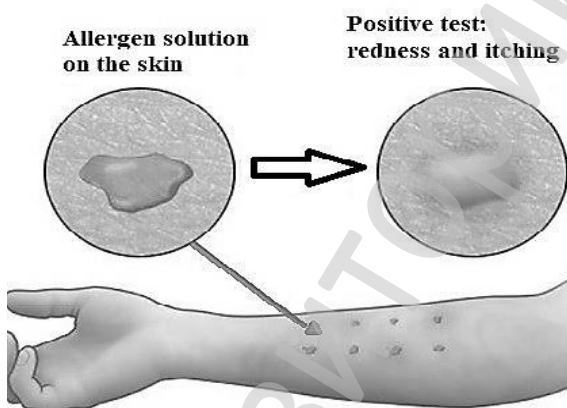
A — Prick test

C — scarifier
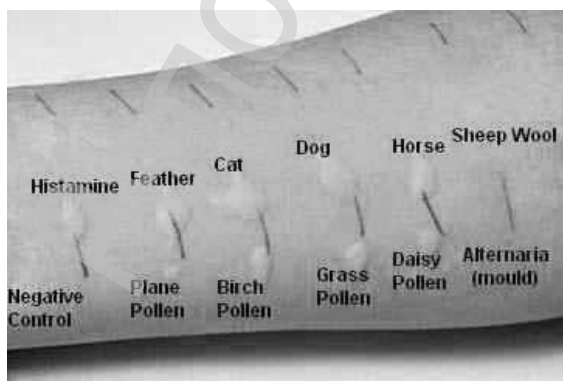
B — Visualization of prick test

D — Patch test

**Figure 43 — Examples of staging and visualization
of skin test results for the diagnosis of ITH**

Intradermal tests — a diluted allergen is injected intradermally into the skin of the forearm in a volume of 0.1 ml. The test is considered as positive if, 20 minutes after contact with the allergen, hyperemia occurs at the injection site and a vesicle (blister) forms; signs of a general reaction may appear (dizziness, nausea). The test is considered as negative if only the injection point remains.

The positive results of skin testing indicate only the presence of sensitization to the allergen and are not an evidence of its causative significance in the occurrence of the disease. An allergen, for which a skin test is positive, can be considered the cause of the disease only if it coincides with the clinical manifestations and history data. At the same time, the timely detection of sensitization to allergens plays a decisive role in the appointment of preventive measures [1, 6].

Skin allergic tests for the diagnosis of DTH involve intradermal administration of the allergen. The expected allergen is administered intradermally in a volume of 0.1 ml. The result is taken into account 24–72 hours after the introduction of the allergen according to the diameter of the papule (infiltrate). Skin tests have found application in the diagnosis of diseases such as tuberculosis (Mantoux test with tuberculin), brucellosis (Burnet test with brucellin) and others. The staging and recording of the Mantoux intracutaneous test (tuberculin test) is shown in figure 44 [16, 17].
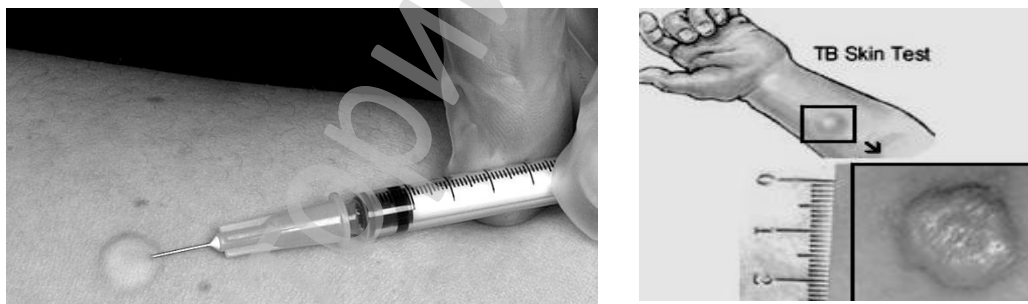
Figure 44 — Staging and recording of the intracutaneous Mantoux test

Provocative tests

A provocation test is a method of etiological diagnosis of allergic reactions, based on the reproduction of this reaction by introducing an allergen into a shock organ. By the type of shock organ (i.e., the organ whose defeat is leading in the picture of the disease), the following types of provocative tests are distinguished: conjunctival (for allergic conjunctivitis or hay fever), nasal (for allergic rhinitis), inhalation (for bronchial asthma), cold (with cold urticaria) and others. If an allergic reaction develops in response to the administration of a suspected allergen, then the allergen may be considered as significant cause of allergy [17, 18].

Elimination tests

The elimination test is a method for the etiological diagnosis of an allergic reaction, based on the disappearance or attenuation of this reaction after the pa-

tient stops contact with the allergen. It is usually used in the diagnosis of food and (rarely) drug allergies and is combined after the onset of remission using a provocative test with this allergen. Elimination tests in the diagnosis of the causes of food allergies are very different — from diets with the exception of a single product to complete starvation [17, 18].

Laboratory methods

In vitro laboratory diagnostic methods are preferred for safety and potential use at any time during the illness. They are of primary importance in cases where skin allergy tests and provocative tests cannot be used.

When using laboratory methods, some methodological points should be kept in mind. Firstly, all immunological methods reveal only the state of sensitization. They cannot serve as evidence that an allergic reaction will develop specifically for this allergen, because the sensitization and allergen are not enough to realize an allergic reaction, a number of additional conditions are needed. Secondly, given the differences in the immunological mechanisms of different types of allergic reactions and the fact that many methods give information only about a particular immune mechanism, for diagnostic purposes, it is necessary to apply several methods to assess the possible participation of all 4 types of hypersensitivity (see table 20). When interpreting the results of laboratory methods, it should be remembered that in the case of negative tests, the possibility of developing an allergic reaction is not excluded [1, 17, 18].

Determination of general and specific IgE (for the diagnosis of type I hypersensitivity)

The study of *total IgE* is carried out using ELISA and paper radioimmunosorbent assay (PRIST method).

Determination of *allergen-specific* IgE is carried out using ELISA and a radioallergosorbent test (RAST method). To determine the specific IgE, commercial test systems for a large list of pollen, household, food, drug and occupational allergens are offered. An immunologist can determine the amount of a laboratory examination and make a list of possible causally significant allergens based on data from a carefully collected medical history [17].

Radioallergosorbent test (RAST) principle: the allergen is adsorbed on a porous carrier (paper, polymer membranes, granules, sponges). Next, patient serum (with IgE) is added, incubated for 3 hours, washed and anti-globulin serum (against IgE) labeled with a radioactive isotope is added. After washing unbound reagents, radioactivity is counted on the counter.

III stage of allergological method

The conclusion about the type of process and its cause is given on the basis of a generalization of the results of an allergological history and the indicated research methods [17, 18].

Methods for studying the human immune status. Immunogram

The development and application of methods for assessing the immune status of the human body is engaged in clinical immunology.

Immune status is a structural and functional state of an individual's immune system, determined by a complex of clinical and laboratory immunological parameters. Immune status characterizes the ability of the body of a given individual to immune response to a specific antigen at a given time [9, 10, 12].

The study of the parameters of the immune status is important for identifying violations of the immune response, resolving the pathogenesis of the disease, setting or clarifying the immunological diagnosis, as well as conducting immunotherapy, immunoprophylaxis and immunorehabilitation.

Assessment of immune status includes [1, 9]:

1) Clinical examination data:

• patient complaints;

• anamnesis (the frequency of infectious diseases, the nature of their course, the presence of chronic infections, reactions to vaccination or the administration of drugs, the presence of allergic, autoimmune, tumor diseases, etc.);

• clinical condition;

• general blood test (total number of lymphocytes and phagocytes).

2) The state of non-specific resistance factors:

• functional activity of phagocytes;

• assessment of the complement system;

• the level of interferon and lysozyme.

3) Indicators of humoral immunity:

• Ig level of various classes in blood serum (Mancini radial immunodiffusion);

• titer of specific antibodies (serological reactions);

• Ig catabolism (radionuclide method);

• the number of B-lymphocytes in the peripheral blood (cluster analysis, i.e. the detection of specific CD molecules using monoclonal antibodies);

• blast transformation of B-lymphocytes (response of B-cells to stimulation by mitogens).

4) Indicators of cellular immunity:

• the number of T-lymphocytes; the number of subpopulations of T-lymphocytes;

• blast transformation of T-lymphocytes;

• level of hormones of the thymus and cytokines secreted by T-lymphocytes (ELISA with monoclonal antibodies)

5) Results of additional tests:

• bactericidal activity of blood serum;

• the number of C3 and C4 components of complement system;

• C-reactive protein level;
• level of rheumatoid factor and other autoantibodies;
• other indicators [1, 15].

Immune status assessment levels

Evaluation of the immune status involves a «two-level» examination. Tests of the first level (approximate) are simple, economical, do not require sophisticated laboratory equipment, reflect the nonspecific patterns of the immune system reactions, and allow to identify severe defects in the immune system and reveal typical immunopathological processes.

The list of tests of the first level with the main indicators is presented in table 22 [1, 12].

Table 22 — Tests of the first level for assessment of the immune status

Test	Indicator	Level
White blood cell total count; differential white blood cell count	Total white blood cell count	4.5–9.5 thousand/μl
	Neutrophils	50–77 %
	Lymphocytes	18–38 %
	Monocytes	2–10 %
	Basophils	0.5–1/0 %
	Eosinophils	1–5 %
T- and B-lymphocytes	T-lymphocytes	55–75%
	T-lymphocytes total	1000–2000 in 1 μl
	B-lymphocytes	10–15 %
	B-lymphocytes total	100–300 in 1 μl
Serum Ig level	Ig M	0.5–2.0 g/l
	Ig G	10.0–20.0 g/l
	Ig A	1.25–3.0 g/l
Phagocytic activity of blood leukocytes (indicators of phagocytosis)	Phagocytic index	For Candida 1–2.5 For Staphylococcus 4–9
	Phagocytic number	For Candida 40–90 For Staphylococcus 40–80

Tests of the second level (analytical) are a more detailed and in-depth study of the parameters of the immune system, and allow to clarify the nature of the defect identified by tests of the first level. This level of immunological research requires the deployment of a special laboratory, involves the high qualification of a laboratory doctor and the availability of material resources. Analytical tests of the second level are prescribed selectively depending on the clinical symptoms of a particular patient.

The tests of the second level include:

1. Definition of subpopulations of T-lymphocytes (T-helpers, T-suppressors, cytotoxic T-lymphocytes/T-killers).

85

2. Evaluation of the proliferative activity of T- and B-lymphocytes (lymphocyte blast transformation reaction — RBTL).

3. Determination of spontaneous leukocyte migration and a test of inhibition of leukocyte migration.

4. Determination of lymphocytes carrying surface immunoglobulins of various classes (B-lymphocytes).

5. Determination of mediators of the immune system (cytokines).

6. Assessment of Ig subclasses.

7. Skin delayed hypersensitivity tests [1, 6].

Methods for determining indicators of immune status

Quantification of lymphocytes

The total (absolute) and relative number of lymphocytes is determined according to a clinical blood test. The level of T- and B-lymphocytes is counted in a luminescent microscope in an immunofluorescence reaction using labeled monoclonal fluorescent serums to specific surface antigenic markers denoted by CD (cluster of differentiation) symbols. There are several dozen such antigenic markers, but some of them are characteristic of one or another type of cell [17, 18]:

• receptor CD3 — all T-lymphocytes;
• receptors CD19, 21, 22, 72 — B-lymphocytes;
• CD4 receptors — T-helpers;
• CD8 receptors — cytotoxic T-lymphocytes;
• CD16 receptors — NK cells (natural killers).

Modern methods for assessing the level and differentiation of T-lymphocytes, their subpopulations, and other immunocompetent cells are methods also based on the use of monoclonal antibodies to CD markers — **flow cytometry method** [1, 17, 18].

The principle of the method: the investigated cells, or rather their markers — CD antigens, are stained with fluorescent antibodies. For staining of cells (for example, CD3 — T-lymphocytes, CD4 — T-helper lymphocytes, CD8 — cytotoxic T-lymphocytes, CD19, 21, 22, 72 — B-lymphocytes), monoclonal antibodies against their CD antigens labeled with fluorescein isothiocyanate, giving green fluorescence, or labeled with phycoerythrin, giving a red glow. A blood sample after treatment with labeled monoclonal antibodies is passed through a thin tube. A laser beam is passed through the test sample, which excites the fluorochrome illumination. A photomultiplier picks up light scattering, which analyzes the size and granularity of the cell, and records fluorescence, indicating the number of labeled antibodies associated with the cell. The fluorescence intensity correlates with the density of antigens on the cell surface and can be quantified using a photocell. Flow cytometry is used to determine the level of the main populations of lymphocytes, intracellular and extracellular cytokines, the functional activity of NK-cells, the activity of phagocytosis, and features of apoptosis [1, 8].

To determine T- and B-lymphocytes, the rossete method is more accessible and simple, but less accurate and outdated. It is based on the fact that B-lymphocytes can adsorb mouse erythrocytes on their surface, and T-lymphocytes – sheep erythrocytes. A lymphocyte with red blood cells adhering to it – this is a socket, or a rosette-forming cell; they are counted in Romanovsky-Giemsa-stained smears from a mixture of lymphocytes and corresponding red blood cells. What is the number of rossetes, therefore, the total number of lymphocytes [1].

Study of the functional state of lymphocytes

The ability of lymphocytes to multiply (assessment of their ability to proliferate) is determined in response to the introduction of mitogens (substances that stimulate reproduction) in the reaction of blast transformation of lymphocytes (RBTL) with phytohemagglutinin (PHA), a mitogen for T-cells. A decrease in the proliferative response to PHA indicates the presence of immunodeficiency, but its causes may be different. An increase in the proliferative response to PHA may indicate the presence of infection or some kind of autoimmune pathology.

Assessment of the functional state of phagocytes

To assess the phagocytic activity of blood neutrophils, the phagocytic number (percentage of active phagocytes) and the phagocytic index (average number of microbial cells absorbed by one leukocyte) are determined.

To assess the oxygen-dependent microbicidal ability of neutrophils, the **NBT test** is used (NBT is a dye nitrosine blue tetrazolium). The absorption of microbes by phagocytes is accompanied by an increase in oxygen consumption by these cells and the formation of hydrogen peroxide and free oxygen radicals (both can be combined under the name — active oxygen forms). The essence of the reaction is that the NBT in the presence of reactive oxygen species is colored blue, and in the absence thereof remains colorless [6, 16, 17, 18].

The concentration (level) of immunoglobulins of different classes G, M, A and E in blood serum is determined in the gel precipitation reaction (Mancini radial immunodiffusion) with antiglobulin antisera to IgG, IgM, IgA, and IgE [6].

Determination of the concentration of individual cytokines, the main regulatory molecules that determine the type of immune response, is carried out using enzyme-linked immunosorbent and radioimmune methods.

Assessment of the complement system. The hemolytic activity of complement in the hemolysis reaction is determined using the hemolytic system (consists of sheep erythrocytes and hemolytic serum). The quantity of complement components is also evaluated and activation products are detected [1, 6].

Immunogram

Immunogram — the results of an analysis of the immune status of the particular patient, which contains data on the normal parameters of the immune status

of persons of the corresponding age. Based on a comparison of the immunogram of the individual (patient) with the norm data, taking into account the rules of interpretation of the obtained indicators, the specialist in clinical immunology (immunologist) concludes that there are deviations and the need for immunotherapy [1].

The basic rules for interpreting an immunogram:

1. A comprehensive analysis of the immunogram is more informative than the evaluation of each indicator separately.

2. It is necessary to take into account both relative and absolute values of each indicator of the immunogram.

3. Analysis of indicators can only be carried out taking into account the clinical picture of the disease.

4. The real information in the immunogram is carried by strong shifts of indicators; weak shifts can increase confidence in the correctness of the conclusion.

5. The analysis of the immunogram in dynamics is always more informative both in diagnostic and prognostic terms.

6. The absence of shifts in the immunogram in the presence of clinical symptoms indicates the inclusion of adaptive mechanisms, stress reactions.

7. The revealed shifts in the immunogram without clinical symptoms should be taken into account and included in the patient's risk group for the development of immunodeficiency.

8. For diagnostic and prognostic evaluation of the immunogram, the age, gender and individual norms of the patient should be taken into account.

9. Of great importance in the immunogram are the ratios of cell subpopulations and spontaneous and stimulated tests, rather than the absolute values of each indicator [1, 6, 12].

Methods for monitoring the post-vaccination immunity efficiency

Collective immunity is understood as the immunity of society to one or another infectious disease. It is created with the help of specific preventive measures (for example, vaccination) and other measures used by health authorities, as well as through measures to improve the material and cultural standard of living of the population (improving living conditions, nutrition, etc.) [8, 13].

Immune layer of the population is the percentage of the population that is immune to a specific infectious disease (including vaccinated and ill). It is determined using various immunological reactions. If the number of susceptible individuals is small and they find themselves surrounded by unresponsive people, then the disease does not spread [8, 11].

Post-vaccination immunity is immunity that develops after the introduction of the vaccine. The following methods are used to assess the status of artificial post-vaccination immunity:

• Formulation of serological reactions with sera of vaccinated persons;
• Skin immunological tests;
• Skin allergic tests.

The assessment of the state of immunity in the population is carried out mainly against infections controlled by specific, such as diphtheria, tetanus, pertussis, measles, rubella, mumps, etc. There are effective vaccines against these infections. In addition, the effectiveness of immunoprophylaxis and the state of collective immunity to influenza, polio, tuberculosis, tularemia, brucellosis and other infections are selectively monitored [1].

Serological monitoring of the state and duration of post-vaccination immunity should be carried out systematically by the method of selective serological examination of various population groups. For example, vaccinated children and adolescents from 3 to 17 years old and adults over 20 years old are subject to examination. Serological monitoring should be carried out starting with groups of 3-year-old children not earlier than 6 months after the last vaccination. Four teams of the same age group, at least 25 people in each team, should be chosen for examination.

Serological monitoring is carried out by serological testing of blood serum of vaccinated people. Most often, serological reactions are used: RIHA (reaction of indirect hemagglutination) with erythrocyte antigenic diagnostics, ELISA (enzyme-linked immunosorbent assay) to detect specific antibodies and determine their concentration. In addition to these reactions, other methods are also used. To detect immunity to whooping cough, RA is placed (agglutination reaction); the state of immunity to influenza viruses with the help of RIHA (reaction of inhibition of hemagglutination) is constantly monitored. A selective control of immunity to polio in children is also carried out using the neutralization reaction of the virus with antibodies in the serum on cell culture [1].

To control the immunity to diphtheria in children's groups, a Schick skin immunological test was previously used — an intradermal injection of the minimum dose of diluted diphtheria toxin (if there is a sufficient titer of antibodies (antitoxins) in the blood, the introduced toxin is neutralized and the skin reaction is absent). Currently, in addition to the use of, for example, RIHA, antibodies to diphtheria toxin in human serum can be determined in the neutralization reaction in cell culture [16].

Skin allergic test with tuberculin (Mantoux test) allows to detect the presence of non-sterile immunity to tuberculosis. The effectiveness of vaccination of tularemia is also monitored by setting a skin allergic test with tularin. With a negative test, there is no immunity.

Immunological control of the effectiveness of vaccine prophylaxis allows to carry out an epidemiological assessment of the level of protective immunity (i.e. to assess the actual protection against this infection) and the quality of vaccination work. Data on seronegative individuals who do not have a protective ti-

ter of antibodies are transmitted to the clinic to develop individual immunization regimens. The level of protective immunity is not lower than the minimum in each age group in the population of children as a whole should be 90 %. With a decrease in this indicator, it is necessary to carry out correction of vaccine prophylaxis. The level of protective immunity not lower than the minimum in adults should be 75 %. If a significant decrease in the incidence disease rate is achieved as a result of vaccination, but the immunological efficiency coefficient is not high (less than 90 % in children and 75 % in adults), this indicates the instability of the achieved epidemiological well-being and a possible increase in the incidence of disease, first of all, in weakly protected groups [1].

Biological (experimental) method

Biological research method is a set of methods for artificial reproduction of the clinical picture of infectious diseases or their syndromes in laboratory animals [8, 10].

This method also has a number of other goals:

1. Diagnosis of infectious diseases.
2. Isolation and identification of the pure culture of the pathogen.
3. Determination of pathogenicity and virulence of the microorganism.
4. Isolation and identification of microbial exotoxins.
5. Obtaining immunological preparations (immune sera).
6. Verification of the safety and efficacy of medical drugs (including chemotherapeutic drugs, drugs for immunotherapy).
7. Cultivation of viruses (because of viruses are obligate intracellular parasites) [1].

Stages of the biological method:

1. The collection of the pathological material. Material for research is taken under aseptic conditions, into sterile dishes, homogenized and used for infection as quickly as possible [2].

2. The choice of a *laboratory animal*. The choice of the species, line, age and sex of animals is dictated by the objectives of the research. The most widely used laboratory animals are white mice, guinea pigs, white rats, and rabbits. Special studies are carried out on monkeys, dogs, cattle and small cattle, horses, and some species of wild animals. Healthy animals of the same species, sex, age, weight and kept in the same conditions are taken into experience. Animals are marked with paint, either by fastening the rings on their feet or by other means [2, 6].

3. Fixation of animals during research. During infection, the animal must be motionless, it is fixed using special devices (machines, tables) or with your hands. For example, a white mouse is captured on the back skin on the nape of the neck with the thumb and forefinger of the left hand, while the remaining fingers of the same hand hold the hind limbs and tail (figure 45). The same manipulations can be performed separately with two hands. A fixed animal is placed in the desired position [2, 6].
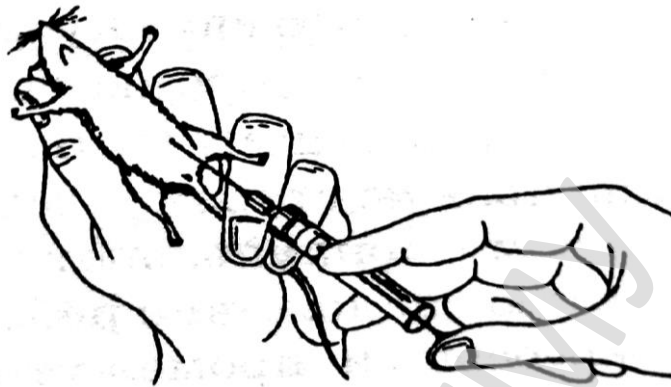
Figure 45 — Fixing a white mouse when conducting research

4. Infection of laboratory animals

Methods of infection in laboratory animals. Infection of animals is carried out in one of the ways (cutaneous, subcutaneous, intradermal, intramuscular, intravenous, intraperitoneal, intracerebral, oral, intranasal, etc.), depending on the tropism of the microbe. Before infection, the wool at the injection site is removed (shaved). The material is administered in compliance with aseptic rules (the skin is subjected to antiseptic treatment (iodine tincture); use only sterile instruments). With painful interventions, anesthesia is performed [2, 14].

Cutaneous infection method. It is a rubbing of a material with a sterile glass rod into a shaved area of skin or into scarified skin.

Subcutaneous infection. The skin of the animal is captured in a fold and a syringe needle is inserted into the base of the skin fold, then the right amount of material (0.5–2 ml) is slowly injected, depending on the type of animal, then the fold is released.

Intradermal infection. Material in a volume of 0.1–0.2 ml is injected into the skin with a thin sharp needle (tuberculin syringe) beveled upward at an acute angle. A correctly inserted needle is visible through the epidermis and a bloating similar to a «lemon peel» forms at the injection site.

Intramuscular infection. The material is introduced into the muscle tissue of the upper part of the hind limb of the animal. The needle tip is directed almost perpendicular to the site.

Intravenous infection. The injection is made into the tail vein of mice or rats, the marginal vein of the rabbit ear, the jugular vein of guinea pigs. The skin is treated over a vein. For better filling of the vein, it is not controlled below the future introduction or the skin is heated with warm (55ºC) water. Material is introduced slowly in the direction of blood flow.

Intraperitoneal infection. The animal is placed upside down or in an inclined position to avoid damage to the intestines by the needle (internal organs move closer to the diaphragm). Also, to avoid damage to internal organs, use a needle with a blunt end. Injection is done in the lower third of the abdomen, on the side of the midline, holding the needle at a right angle, pierce the abdominal wall.

Intracerebral (intracranial) infection. Mice and rats are infected both under general anesthesia and without it. The material is injected with a syringe with a thin needle at a distance of 1–2 mm from the point of intersection of the midline of the skull with the line connecting the outer corners of the eyes. Rabbits and guinea pigs are infected by piercing a thin bone in the area of the infraorbital sulcus. Craniotomy is used for large animals.

Oral infection. The infectious material is introduced into the animal in the mouth or directly into the stomach through an elastic probe. The volume of fluid administered orally depends on the type and age of the animal.

Intranasal infection (through the nose). The material is instilled into the nose with a sterile pipette or a needle on a syringe. With this method of infection, mild anesthesia is used [2, 6, 14].

5. Observation of animals. Registration of signs of disease of an infected animal or its death.

6. Lifetime sampling of material from an animal and conducting bacteriological and serological studies, staging an allergic test.

7. Autopsy of an animal, examining the pathological, anatomical and pathomorphological picture, bacteriological inoculation of organs of dead or killed animals (to detect contamination and isolation of pure culture), preparation of smear-prints from internal organs.

At autopsy of a laboratory animal, the following conditions must be observed [2, 14]:

1. Between death and autopsy of the animal, as little time as possible should pass, since the intestinal flora, even at the temperature of the refrigerator, enters tissues, blood, organs after 20–27 hours.

2. The autopsy, the taking of the material and its investigation are carried out under conditions that protect against contamination by extraneous microorganisms.

3. The recovered material must not come into contact with disinfectants prior to bacteriological inoculation.

4. All observations during the autopsy, as well as the results of the study, are recorded in a special register.

5. At autopsy and its investigation, it is necessary to prevent any possibility of infection of the laboratory staff.

Preparing the dead animal for dissection is as follows: the animal is captured with tweezers, placed abdomen up on a special board placed in an enameled tray, and attached to the board for four legs. Inspect the corpse and note external pathological changes, if any. All animal wool is moistened with a disinfectant (alcohol, 5 % carbolic acid, 5 % chloramine). The skin is cut along the midline of the abdomen from the symphysis to the lower jaw, and then lateral cuts are made to the front and hind legs. Separate the skin, turn it to the sides and pin it to the board (figure 46). Mark changes in the subcutaneous tissue: vasodilation, edema, hemorrhage, etc. [2].
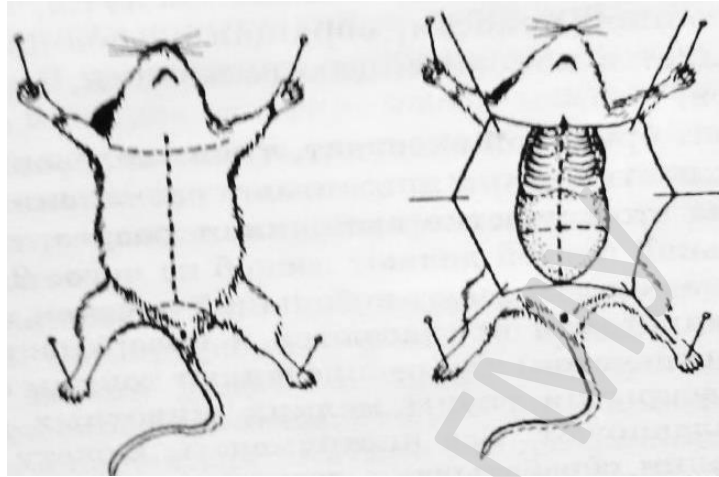
Figure 46 — Fixation of a corpse animal for dissection

An autopsy begins from the chest cavity, cutting and tilting the sternum up. Inspect the chest cavity; note the presence or absence of exudate in it, from which smears and culture are made into a nutrient medium. The appearance of the lungs and heart is noted. Blood is taken from the heart by a puncture with a Pasteur pipette; followed by blood culture. Make smears imprints from the tissues of the lungs and pleura. If necessary, pieces of tissue are taken from the organs of the chest cavity. Next, an autopsy and examination of the abdominal cavity are performed. Having lifted the abdominal wall with tweezers, they cut it with scissors from the diaphragm to the pubis and the formed muscle flaps are removed to the sides. In the presence of exudate, it is sown in a nutrient medium. Then carefully examine the abdominal organs (intestine, liver, kidneys, spleen, etc.). The material is taken from the parenchymal organs as follows: the surface of the organ is cauterized with a scalpel heated in the flame of an alcohol lamp; the cauterized portion of the organ is pierced with a sterile Pasteur pipette with a drawn end or with a sterile loop; the material taken is inoculated in liquid and solid nutrient media. For the preparation of smears, a piece of organ tissue is cut out and smear-prints are prepared with tweezers [2, 13].

After opening the animal's corpse and cadaveric material are disinfected by autoclaving or burning. Crops are signed and placed in a thermostat. All tools and board are disinfected and sterilized. Corpses of animals that have died from especially dangerous infections are opened in special rooms with special precautions.

8. Identification of the selected culture of microorganisms.

9. The conclusion of the study.

Evaluation of the biological method

The method is highly sensitive, can be used at the early stages of the disease, but is not always available, expensive, long, and unsafe.

Currently, as a diagnosis of infectious diseases, the biological method has been replaced by more modern, cost-effective methods of laboratory diagnostics [1].

Molecular genetic method

The modern period of the development of microbiology is characterized by the study of microorganisms at the genetic level. In this regard, in modern medicine, molecular genetic methods of microbiological diagnostics are becoming more widespread, which are express diagnostic methods (detection and identification of microorganisms directly in the biological material of a patient, without isolation of a pure culture). The molecular genetic methods are based on the use of complementary nucleotide binding [10, 18].

Currently known reactions can be divided into two large groups.

The first group includes methods for the direct detection of specific nucleotide sequence (DNA or RNA) using short oligonucleotide single-stranded fragments with a label. These reactions are named as follows: a **nucleic acid hybridization reaction** or a **DNA probe method** or a **molecular hybridization method** [10, 20].

The second group of methods is based on *amplification* – cyclically repeated replication *in vitro* of the investigated DNA fragment (or RNA). The amplified fragment is then detected by gel electrophoresis or using a hybridization assay. This group of methods includes the **polymerase chain reaction (PCR)**. Currently, many modifications of this reaction have been developed, as well as amplification tests, which are fundamentally different from PCR; however, it is the polymerase chain reaction that is most widely used for the diagnosis of various forms of infectious pathology [10].

Molecular hybridization method (DNA probe method)

The method is based on the ability of nucleic acids to hybridize — the formation of double-stranded structures due to the interaction of complementary nucleotides, in particular, the hybridization reaction of specific oligonucleotide probes labeled, for example, with a radioactive isotope (or fluorochrome) with a sample of the extracted DNA of the studied microorganism. A **DNA probe** is a single-stranded labeled (radioactive isotope (^{32}P), fluorochrome, etc.) DNA fragment that carries a gene specific for this type of bacteria. These nucleic acid fragments are obtained: a) upon restriction (splitting of a molecule into two chains) of a DNA site of known microorganisms; b) by chemical synthesis [1, 6, 9].

Variants of molecular hybridization statement:

A. *Solid phase hybridization method*. The principle of the method is based on hybridization of the probe on a solid surface, which is most often used as a polymer membrane filter, for example, a nylon membrane [9].

The hybridization reaction consists of the following steps:

1. Isolation of DNA from the studied microbes, denaturation and restriction (cutting with restriction enzymes) of a DNA molecule (single-stranded sections are needed).

2. Fixation of a nucleic acid sample on a polymer membrane. When applying fragments of the studied DNA to the membrane in the form of dots (round spot shape) — *dot blot hybridization*, in the form of strips — *slot blot hybridization*. *Southern blot hybridization or Southern blot*: small fragments of the studied DNA are previously separated by relative molecular weight by polyacrylamide gel electrophoresis, and then the DNA is transferred from the gel to the surface of the membranes (figure 47). An adaptation of Southern blotting is *Northern blotting*, in which RNA molecules are electrophoresed through the gel instead of DNA.

3. The introduction of a DNA probe. If the probe is complementary to the DNA of the studied microbes, then binding will occur, i.e. hybridization; unbound probes are removed by washing.

4. Detection and accounting of reaction results, for example, using autoradiography (when using DNA probes labeled with a radioactive isotope) or in a luminescent microscope (in the case of using fluorescent DNA probes), etc. [1, 9].

B. In situ hybridization method. At the same time, DNA manipulations are carried out in intact tissue samples (tissue sections) fixed on a glass slide. After incubation with a DNA probe, a photographic emulsion is applied to the glass. In areas of radioactive emission (i.e. where hybridization has occurred) dark microscopic granules appear on it. In situ hybridization is one of the most effective methods for mapping DNA sequences on chromosomes complementary to a DNA probe. This technique is effective in studying the distribution of repetitive DNA sequences across the genome; determining not only the chromosomal affiliation, but also the intrachromosomal localization of unique genes in those cases when there are corresponding DNA probes [9, 13, 20].

C. The hybridization method in solution. When hybridizing in solution, the investigated nucleic acid and probe freely interact in the aqueous reaction mixture, which increases the speed of the hybridization process. Detection of the results of hybridization is carried out by nuclease hydrolysis of single-stranded DNA and isolation of the remaining double-stranded hybrids containing a labeled probe. The method is good because it requires minimal volumes and quantities of biological and clinical samples, therefore, it can be used for diagnostic purposes. At the same time, this method has one significant disadvantage — on its basis it is possible to create diagnostic test systems for detecting specific fragments of small sections of DNA subject to a sufficiently high concentration of these fragments in the test sample. This reduces the method sensitivity to the level of ELISA and even lower [20].

D. The hybridization method on biochips (microarray hybridization) is the most advanced method. It allows to apply and fix up to several hundred thousand DNA probes on the surface of a glass biochip, which makes it possible to study simultaneously many genes present in the DNA of a microorganism. The method is used in the diagnosis of diseases, the identification of genes and the study of their expression, a comparative analysis of genomes and proteomes [4, 9, 20].
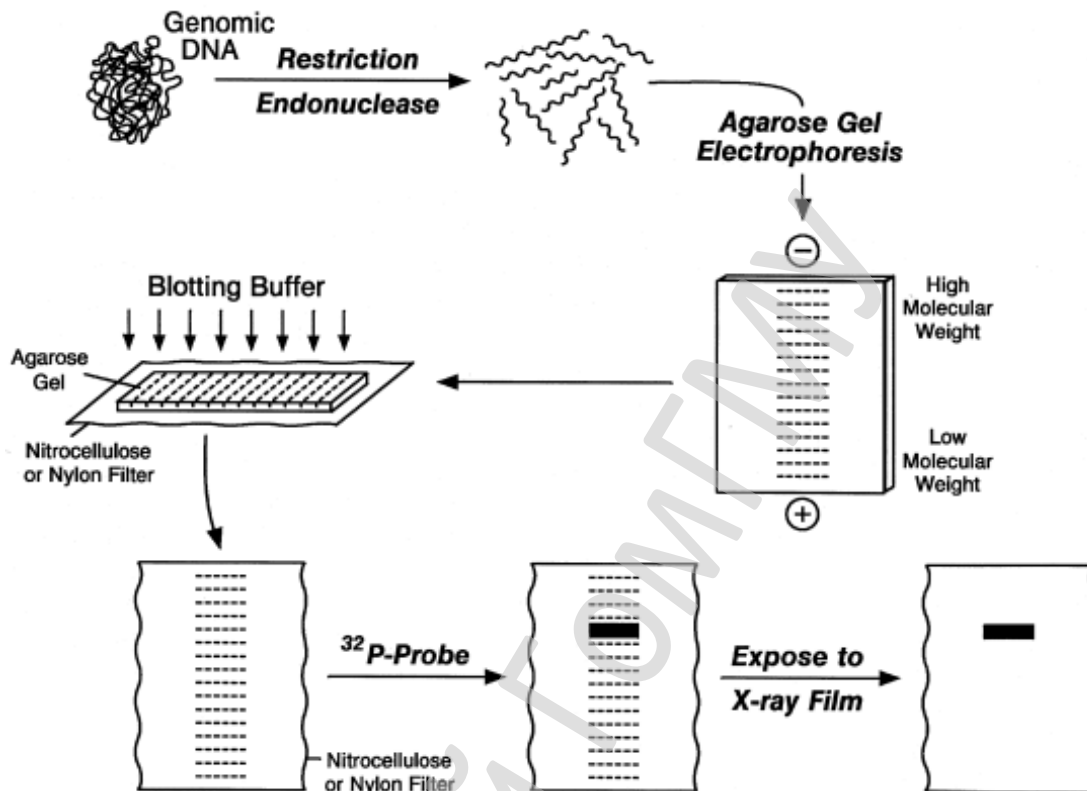
Figure 47 — Principle of Southern blotting

Polymerase chain reaction

Polymerase chain reaction (PCR) is the process of multiple copying (amplification) of genome fragments under *in vitro* conditions, which leads to the rapid accumulation of a specific DNA sequence of interest to a researcher in a sufficient amount necessary for subsequent detection [10].

The initial components of PCR:

1. DNA matrix (sample to be analyzed) — DNA or its part containing the investigated specific fragment; is a target for subsequent multiple copying.

2. Primers are synthetic oligonucleotides (sequences of 20–30 nucleotides) that are complementary to DNA sequences at the boundaries of a specific fragment. The choice of a specific fragment and the selection of primers plays a crucial role in the specificity of amplification and determines the quality of the analysis.

3. A **mixture of deoxynucleotide triphosphates** (dNTPs) is a mixture of four types of nucleotides: deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dTCP), deoxythymidine triphosphate (dTTP); are the material for the synthesis of new complementary DNA strands.

4. Taq polymerase enzyme is a thermostable DNA polymerase that catalyzes the extension of primer chains by sequential addition of nucleotide bases to the growing chain of synthesized DNA. Taq polymerase is obtained from thermophilic microorganisms *Termophilus aquaticus*.

5. Buffer solution — a reaction medium containing Mg^{2+} ions, necessary to maintain the activity of Taq polymerase enzyme.

6. A positive control is a DNA sample reliably containing the specific DNA sequence of interest to a researcher.

The entire research process using PCR includes:

1) Sample preparation — DNA extraction from the sample and its purification;

2) Carrying out the polymerase reaction itself, aimed at the multiplication (amplification) of DNA fragments of the pathogen;

3) Detection of amplified fragments and evaluation of results.

To carry out PCR, a special device is used – a **thermal cycler (amplifier)**, which is an apparatus in the nests of which special tubes from the thermally conductive plastic with a reaction mixture are placed (figure 48). Reaction mixture contains all initial components of PCR in certain volumes [9, 13, 20].

Figure 48 — Examples of PCR amplifiers

The amplifier allows to automatically, according to a specific program, change the temperature regime of the reaction mixture. The temperature parameters play an extremely important role for the successful reaction [1].

Each PCR cycle includes three steps (figure 49):

Stage 1. **DNA denaturation** (weaving a double helix of DNA into two separate chains). It proceeds at a temperature of 93–95 °C for 30–40 seconds [18, 20].

Stage 2. **Attaching primers (annealing).** The primers attach complementary to the corresponding sequences on opposite DNA strands at the boundaries of a specific site. Each pair of primers (F-primer ― forward, R-primer ― reverse) has its own annealing temperature, the values of which are in the range of 50–65°C. Annealing time is 20–60 sec.

Stage 3. **Elongation** (completion of DNA chains). The process is catalyzed by the enzyme with thermostable DNA polymerase (Taq polymerase), which attaches to the 3′-end of the primer; complementary completion of DNA chains in opposite directions occurs, starting from the primer attachment sites. The material for the synthesis of new DNA strands are deoxyribonucleotide triphosphates (dNTPs) added to the solution. It proceeds at a temperature of 72 °C for 20–40 seconds [20].
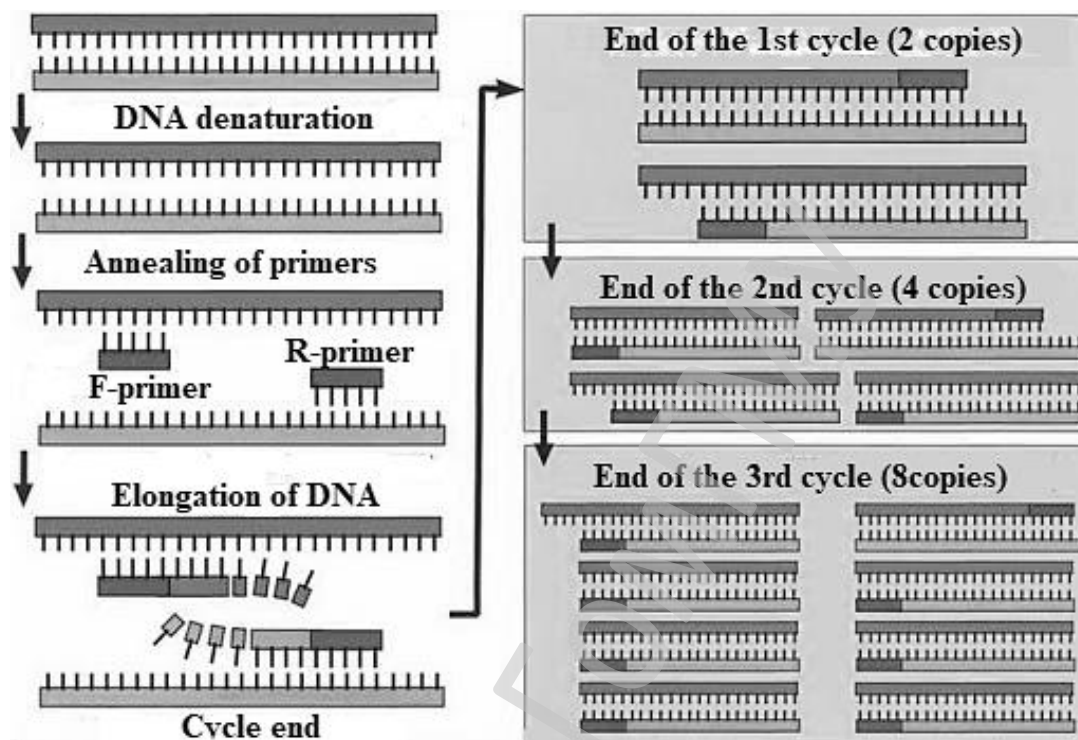
Figure 49 — Scheme of polymerase chain reaction

Two new DNA strands formed in the first amplification cycle serve as templates for the second amplification cycle. The accumulation of **amplicons** (specific amplification products limited in length by primers) in solution occurs according to the formula 2^n, where n is the number of amplification cycles. *To obtain a sufficient number of copies of the desired DNA fragment, amplification involves 20–40 cycles*. Amplicons are the direct subject of detection [20].

Detection of amplification products, i.e. identification of amplified fragments is carried out using *agarose gel electrophoresis* or a *hybridization-enzymatic method*, followed by evaluation of the results.

The polymerase chain reaction described above is a **«classic» PCR**.

When interpreting the results of PCR, it should be remembered that both false positive and false negative results can be obtained.

• *False positive results* can be observed as a result of contamination with amplification products (amplicons), since amplicons accumulate in large quantities during operation and are very easily transferred with aerosols and through instruments. Therefore, in order to avoid cross-contamination of the studied samples, nucleic acids should be separated under a number of conditions (for example, separation of the PCR laboratory into zones (separate rooms); mandatory use of clean gloves, disposable tubes and tips for automatic pipettes, preliminary UV treatment of the room and work surfaces of tables and devices, etc.); continuous internal laboratory monitoring of the quality of the results is also required.

• *False negative results* can be observed as a result of a decrease in the sensitivity of PCR during inhibition of the reaction by components of biological

samples (for example, in the study of whole blood samples, inhibition of PCR can occur due to hemoglobin, lactoferrin, plasma) [20].

The main advantages of the polymerase chain reaction are its versatility, high sensitivity and specificity. PCR can be used to detect any infectious agent if there is an appropriate primer for it. The use of PCR is especially shown in cases where it is difficult to isolate a pure culture of the pathogen due to the complexity of the cultivation method, or uncultivated microbes, persistent forms of microorganisms in latent and chronic infections, or in the test sample the concentration of the pathogen is very low (legionellosis, chlamydia, mycoplasmosis, the initial period of HIV infection, etc.). Test systems with primers for the use of PCR in order to detect various pathogens have been developed and are being put into practice [1].

Modifications of PCR

Classical PCR is an amplification of DNA sections up to 5000 bp in size using one pair of primers. It is described in the above mentioned material [20].

Multiplex (multiprimer) PCR. The technique allows for PCR with two to four or more non-overlapping primers of several pathogens. Multiplex PCR makes it possible to determine in one sample nucleic acids of several pathogens at once, which is especially important because often during diagnosis, mixed forms of infection are detected. This approach is already used in diagnostic practice [11, 20].

Nested PCR has greater sensitivity and specificity; allows the determination of nucleic acids present in very low concentrations. It is carried out sequentially with two different pairs of primers (first with a pair of external primers, then with a pair of internal primers). DNA products formed in the first PCR after using the first pair of primers are transferred to another tube, where they interact with the second (internal) pair of primers [13, 20].

Reverse transcription PCR (RT-PCR). The technique allows PCR to be carried out with RNA-containing pathogens, reading information from RNA and creating DNA complementary to it. This process can be performed using special enzymes with reverse transcriptional properties (RT) and deoxynucleotide triphosphates. Using the reverse transcriptase or revertase (RT) enzyme and one amplification cycle, RNA reverse transcription is performed according to a special program: one RNA strand is converted into double complementary DNA, and then the DNA can be amplified by «classical» PCR [4, 11, 20].

Real-time PCR has the following features:

1) determining the formation of the reaction product after each amplification cycle;

2) construction of the kinetic curve of PCR from these data;

3) determination of the relative concentration of the substrate based on the analysis of this curve.

To detect the PCR product, fluorescent dyes are used that provide fluorescence directly proportional to the amount of the PCR product. Real-time PCR is

used to determine point mutations, the quantitative content of DNA in a sample, as well as to determine the level of gene expression. This type of PCR is becoming more widespread; since amplicons are detected by fluorescence of probes or SybrGreen dye and does not require electrophoresis (analysis time and labor are reduced) [4, 20].

Long-PCR is a modification of PCR designed for amplification and subsequent study of extended sections of DNA. The principle of the method is to increase the time of polymerization and selection of new varieties of Taq-DNA polymerase with endonuclease (editing) activity, i.e. able to cut out erroneous (non-complementary) nucleotides.

RAPD analysis (random amplified polymorphic DNA analysis). It uses short primers that can bind to different sections of DNA, resulting in the formation of numerous fragments of different lengths, the number and size of which judge the group or species of the microorganism [13, 20].

PCR in situ. The method is relatively recently proposed for practice; allows PCR to be performed on a tissue section or smear. The method has recommended itself well and is used in oncology, although the spectrum of its application is not limited to oncological diseases. There is no sample preparation in this method. The same PCR mixture is used as in the «classical» method; however, the reaction itself takes place on the surface of the tissue section or smear. To conduct this technique, a special amplifier is required. Detection of the results is carried out using an enzymatic-hybridization method with a visual assessment of the results in a conventional light microscope [15, 20].

Broad-range PCR. It used to determine the presence of microorganisms, including unknown and uncultivated, in the clinical material from the patient. For this, universal primers are used that interact with highly conserved DNA regions found in many microorganisms (16S rRNA genes) [20].

Sequencing

Sequencing is determination of the nucleotide sequence of DNA. The principle of sequencing is to obtain a series of complementary DNA molecules that vary in length by one base [9, 10].

Analysis of the information contained in the sequenced DNA fragment allows us to solve the following problems:

• decoding the genome with determining the number of genes, their length, localization, functions;

• determination of mutations in genes to identify resistant microorganisms and study the molecular mechanisms of resistance;

• identification of the presence of transposons, IS-elements;

• identification of unknown microorganisms by comparing the nucleotide structure of some of their genes with the corresponding sequences of known species;

• identification of evolution directions of microorganisms (construction of dendrograms that allow to assess the similarity of microorganisms, the degree of kinship and their origin) [20].

Basic sequencing methods:

• **chemical sequencing** — the Maxam-Gilbert method, which uses the chemical cleavage of DNA on one base;

• **enzymatic sequencing** (dideoxy sequencing) — the Sanger method, where the desired DNA strand is synthesized, specifically stopping the synthesis on a given basis.

More often during sequencing they use the Sanger method, since it is more reliable and simple to execute.

Stages of sequencing:

1. DNA is denatured to produce single-stranded molecules.

2. A *sequencing primer* is added — an artificially synthesized oligonucleotide sequence complementary to a specific region of the original DNA molecule.

3. A complementary strand is synthesized using DNA polymerase on the DNA being studied as a template, in which, at any time from the beginning of the synthesis, the corresponding terminator can be inserted instead of the usual deoxynucleotide triphosphate (dATP, dTTP, dGTP, dCTP) — specific dideoxynucleotide triphosphate (ddATP, ddTTP, ddGTP, ddCTP), labeled with fluorochrome. After embedding the terminator, DNA polymerase is unable to attach the next nucleotide and DNA synthesis stops, the DNA chain breaks.

4. Further electrophoresis in a denaturing polyacrylamide gel allows the separation of fragments differing in size of one nucleotide.

5. Since the terminators are labeled with a fluorochrome, the fragments fluoresce, which is recorded by the device — automated DNA-sequencing instruments (**DNA sequencers**), which give the results in graphical and text format [13, 20].

Directions of research in infectious pathology using molecular genetic methods

Diagnostics of infectious diseases. The main marker of the pathogen is its gene, namely species-specific genetic sites, which determine the presence of a certain species of microorganism in the clinical material (that is, microorganisms are identified). PCR plays a leading role in the diagnostics of chronic infections caused by the persistence of microorganisms (for example, urogenital chlamydia). PCR is an indispensable tool in the identification of intracellular and membrane parasites, such as viruses, rickettsia, chlamydia, mycoplasma; also widely used to identify long-cultivated bacteria (e.g., *Mycobacterium tuberculosis*) [1].

Determination of the microbial resistance to antibiotics, and genes that determine antibiotic resistance. PCR allows the determination of antibiotic resistance in slowly growing and difficult to cultivate bacteria. Recently, molecu-

lar genetic methods have been widely used to identify resistance markers — resistance genes or mutations in genes. For example, the determination of *Enterobacteriaceae* representatives resistant to β-lactam antibiotics is carried out by the presence of blaSHV, blaCTX-M or blaTEM genes in their genome that determine the synthesis of extended-spectrum β-lactamases (ESBL) of the corresponding classes [1].

Epidemiology and infection control. Using molecular genetic methods, differences are determined between microorganisms of the same species (microorganisms are typed), isolated from different patients, in different hospitals, in different geographical regions. This allows to determine the sources of the pathogen, the ways of its distribution in the hospital, country, world and develop measures to control the spread of epidemic clones [1].

The use of molecular genetic methods is also possible in other areas: to decipher the structure and function of genomes, to identify directions of the evolution of microorganisms, in biotechnology and vaccinology, to determine genetically modified foods, and to detect infectious agents in the environment [1].

SECTION 4
MICROBIOLOGICAL METHODS OF RESEARCH APPLICABLE FOR DIAGNOSTICS OF VIRAL INFECTIONS

For the purpose of laboratory diagnostics of viral infections express diagnostics, virological and serological methods are used (table 23) [1].

Table 23 — Methods of laboratory diagnostics of viral infections

Methods	Principle
1. Express diagnostics	The express diagnostic method is based on the direct and rapid detection of the pathogen or its components (viral antigens, inclusions, and genome) directly in the clinical material taken from the patient
2. Virological method	Virological method is based on the cultivation of viruses on sensitive test systems (chicken embryo, cell culture, laboratory animals) with subsequent identification of the isolated virus
3. Serological method (serodiagnostics)	Serological diagnostics is based on the detection of antibodies in the patient's blood serum that are specific for viral antigens.

The choice of method depends on the biological properties of the virus, the period of the disease, as well as the technical equipment of the laboratory.

Express diagnostics

For express diagnostics, the following methods are used:
1. Electron microscopy and immune electron microscopy;
2. Light microscopy (virusoscopy);
3. Immunological reaction for detection of viral antigens;
4. Molecular genetic methods [1, 10].

Electron microscopy and immune electron microscopy

Electron microscopy (EM) is used to detect virions in pathological material. However, EM does not allow the identification of viruses, since many of them have no morphological differences within the family. One of the options for EM used for diagnostic purposes is **immune electron microscopy** (IEM). IEM — electron microscopy of viruses treated with the corresponding specific antibodies. As a result of the interaction of antibodies with viruses, complexes are formed which, after negative contrasting, are more easily detected. IEM is somewhat more sensitive than EM; it allows to identify various types of viruses detected by electron microscopy (for example, various types of herpes viruses), which is impossible to do based on morphological features [17].

Electron microscopy involves the use of an electron microscope (figure 50), in which electron rays are used instead of a light beam to illuminate objects, which makes it possible to increase the sensitivity of the method by several orders of magnitude.
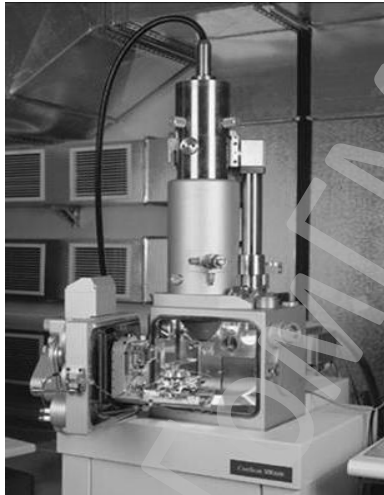
Figure 50 — Electron microscope

Principle of electron microscopy

An electron gun is used as a source of electron beams, the basis of which is a tungsten filament heated by electric current. The passage of electron beams in vacuum through electromagnetic fields created by electromagnetic lenses concentrates and directs the electron beam. This provides a sharp increase in the resolution of the electron microscope to 0.2 nm and an increase to 10^9.

Electronoscopic preparations (smears). They are prepared from purified and concentrated virus-containing suspensions or ultrathin sections of tissue infected with viruses. Ultrathin sections are obtained from the test material (pieces of virus-infected tissue), which are fixed in a special fixative, dehydrated, poured into epoxy resins, cut with glass or diamond knives on ultratomes. Viral objects are applied to special films-substrates placed on supporting grids. Substrate films must be very thin (no more than 30 nm thick), transparent and strong. Colloidal coal films have proven themselves well. They are applied to supporting grids of copper with numerous holes. Further, the smears are processed in various ways, which are listed below [11, 17].

Metal spraying methods (shadow processing). They are used to obtain contrast smears. Vapors of heavy metals (gold, platinum, uranium, etc.) formed in a special device under vacuum and high temperature are directed at an acute angle to the virus-containing preparation (smear). Viruses are covered with a thin layer of metal, with the exception of the side covered by the object, which creates the effect of shadow. The spraying method creates a three-dimensional image, allows well studying the shape and size of the virions, the structure of their surface. But the internal structure of the virus is not available for observation [9, 15].

The method of negative contrast. The most commonly used method for producing contrast agents, which allows revealing both the surface structure of virions and their internal structure. The principle of this method is based on the fact that when the smear is treated with salts of heavy metals, a denser layer is created that does not pass electrons, on which more electronically transparent objects under study are clearly visible. The method of positive contrasting, in which salts of heavy metals, combining with the substances that make up the virions, «stain» them, as a result of which darker viral structures are visible on a light background.

The method of ultrathin sections in combination with negative contrasting. It is the best method for studying the detail structure of virions and studying the stages of the interaction of viruses with the cell, but at the same time it is the most complicated. The studied pieces of infected tissue or other virus-containing material are fixed in a special fixative (for example, osmium) and dehydrated by successive placement in alcohols of increasing strength. The samples are poured with special plastic, after the polymerization of which solid transparent blocks form. Ultrathin sections 10–20 nm thick are prepared from the blocks using a special microtome. The obtained sections are contrasted by, for example, placing in a solution of phosphoric-tungsten acid. The prepared preparations are studied in a transmission electron microscope, whose resolution reaches 0.2–0.3 nm. The image of the preparation is observed on the fluorescence screen of an electron microscope and special photographic plates are photographed from which fingerprints are obtained [9, 13, 14].

Scanning electron microscopy. It is carried out using a scanning (scanning) electron microscope, in which a thin electron beam moves rapidly over the object under study, that is, scans its surface. As a result, radiation of secondary electrons occurs, which, passing through the cathode ray tube, is converted into a three-dimensional image of the object on a fluorescent screen (the process is similar to the formation of a television image). Scanning microscopy allows to obtain three-dimensional images of virions (the smear should be pre-sprayed with metals), to distinguish the details of the structure of their surface, but does not reveal their internal structure. The resolution of the scanning microscope is 7–20 nm. Figure 51 shows an example of influenza virus in a scanning electron microscope [9, 14, 21].
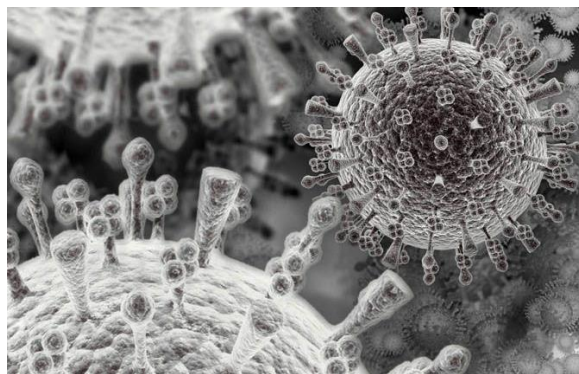
Figure 51 — Scanning electron microscopy

Light microscopy (virusoscopy)

Large viruses can be seen in the light microscope, the sizes of which are within the resolution of the microscope — at least 0.2 microns, as well as intracellular inclusions in the tissues affected by the virus. Large viruses, such as poxviruses, and inclusions are detected using special staining methods and *phase contrast, dark field* and luminescent microscopy. As a rule, these studies give only preliminary results, not eliminating the use of other methods of laboratory diagnostics of viral infections. Large viruses are detected by staining according to *Morozov* (impregnation by silver salts). To identify intracellular inclusions, histological sections from infected tissues, usual smear or smear-prints are prepared. Typically, smears are stained according to *Romanovsky-Giemsa*, sometimes by other methods. The detection of Negri inclusions (Negri bodies) in the nerve cells of the brain during rabies is of the greatest practical importance (figure 52). For this purpose, the preparations are stained according to *Turevich, Muromtsev* and others [6, 22].
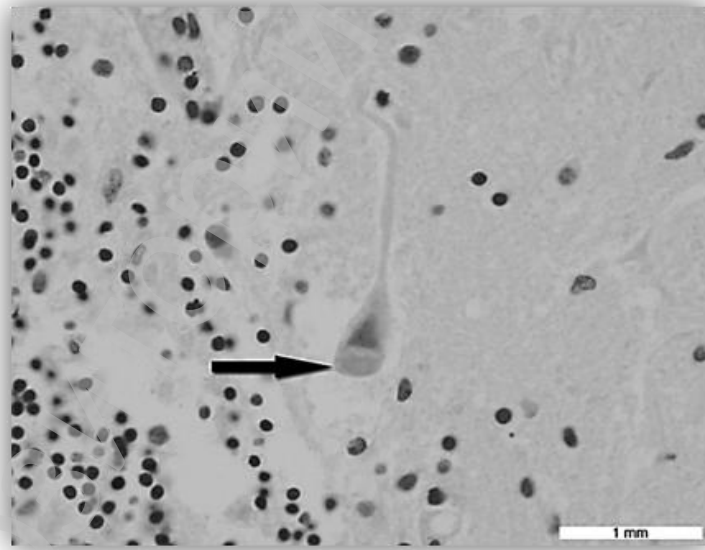
Figure 52 — Negri body (arrow) is identified within a Purkinje cell. Photomicrograph of the cerebellum (hematoxylin-eosin stain; original magnification ×400)

Luminescent microscopy is one of the highly sensitive methods of light microscopy, is quite widely used in virology. Smears are made from materials containing large viruses, intracellular inclusions, stained with fluorochrome dyes. Fluorescein, auramine, acridine orange and others fluorochromes are used. Under luminescent microscopy in UV light, acridine-orange-stained clusters of RNA viruses and the inclusions formed by them are visible as luminous red granules against a pale green cell cytoplasm; DNA viruses give an emerald green glow [1, 14].

Immunological reactions for detection of viral antigens

Immunological reactions are used to determine the viral antigens in the investigated material using known *diagnostic antiviral antisera*.

Immunofluorescence assay (IFA)

Direct IFA. Its essence consists in treatment of smear-prints made from pathological material with an *immune luminescent diagnostic antiserum* containing specific antibodies to a specific virus and labeled with fluorochrome. If the viral antigen and labeled serum containing antibodies specific for this virus correspond to each other, then the formation of the antigen-antibody complex occurs, and it is detected using a luminescent microscope by specific glow.

Indirect IFA. Specific diagnostic antiserum (for example, rabbit antiserum) is applied to the investigated material, the antibodies of which bind to the viral antigen in the material. Then *fluorochrome-labeled antiglobulin serum* (anti-rabbit serum) containing antibodies against rabbit gamma-globulins is used. An advantage of the indirect IFA is the need for only one type of labeled antibody.

The successful use of IFA methods for direct detection of the virus in clinical material is possible only if it contains a sufficiently large number of virus-infected cells and insignificant contamination by other microorganisms, which can give a non-specific glow [17, 18].

Enzyme linked immunosorbent assay (ELISA)

The principle of the method is the detection of antigen by specific antibodies labeled with enzymes (peroxidase or phosphatase). The enzyme is manifested with various substrates, giving it a characteristic staining, which is determined by the photometric method. ELISA can be used both in direct and indirect form. Since soluble antigens can be measured by ELISA, the presence of intact cells in the sample is not required and thus various types of clinical material can be used. Another important advantage of the ELISA is the ability to quantify antigens, which allows it to be used to assess the clinical course of the disease and the effectiveness of chemotherapy [17, 18, 19].

Radioimmune analysis (RIA)

The method is based on the use of radioisotope-labeled antibodies. When a viral antigen interacts with a specific antibody, a labeled immune complex antigen-antibody is formed. The result is determined using gamma counters [1, 17].

Reaction of indirect (passive) hemagglutination (RIHA/RPHA)

The viral antigen is detected using an *erythrocyte antibody diagnosticum*, which is red blood cells with antibodies adsorbed on them. In the positive case, red blood cells with antibodies adsorbed on them interact with the viral antigen;

as a result, red blood cells stick together and form a scalloped sediment — «umbrella» — at the bottom of the plastic plate well. In case of a negative reaction, red blood cells settle in the form of a «button» [17].

Reaction of inhibition of hemagglutination (RIHA)

Its principle is that specific antiviral antibodies (in the diagnostic immune antiserum or patient's serum), interacting with a viral antigen, neutralize it, as a result of which the virus loses its ability to agglutinate (stick together) red blood cells, i.e. hemagglutination is inhibited (a positive reaction result is visible at the bottom of the plastic plate well as a «button» sediment), figure 53 [2, 18, 21].
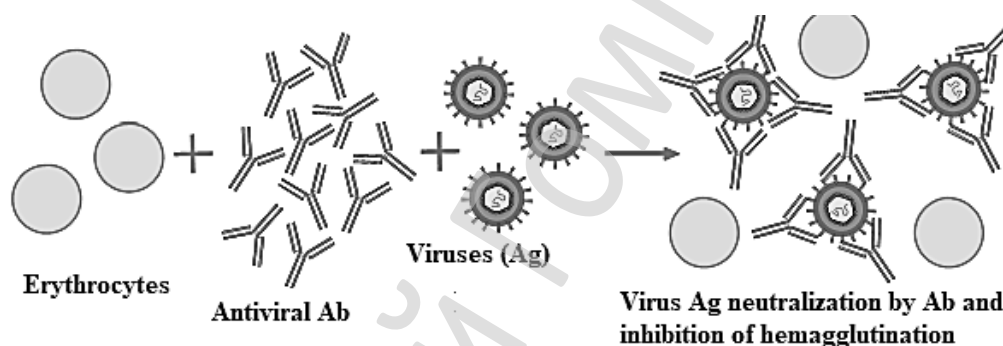
Figure 53 ― Scheme of RIHA

With a negative reaction, hemagglutination is not inhibited; red blood cells stick together and drop to the bottom of the plastic plate well in the form of a scalloped sediment ― «umbrella».

The high specificity of RIHA allows to determine the species and serotype (or subtype) of viruses with the help of known immune antisera. RIHA is used to diagnose many viral diseases, the causative agents of which (influenza, measles, rubella, tick-borne encephalitis, etc.) can agglutinate red blood cells of various animals and birds (due to the presence of hemagglutinin antigen in the virus envelope) [2, 21].

Molecular genetic methods

They are used to detect the genome of the virus in the patient's pathological material. Such methods include molecular hybridization and polymerase chain reaction, which, due to their specificity and sensitivity, have become widespread in recent years [1].

Molecular hybridization of nucleic acids

The method is based on the hybridization of complementary DNA or RNA strands with the formation of double-stranded structures and their detection us-

ing special DNA or RNA probes, labeled, for example, with a radioactive isotope or fluorescent label. This method is used primarily to determine persistent viruses in pathological material; for latent viral infection; to detect viruses that cannot be cultured in cell cultures [20].

Polymerase chain reaction (PCR)

The essence of the method is the repeated copying (amplification) of fragments of the pathogen genome, which leads to the rapid accumulation of a certain virus-specific DNA sequence in sufficient quantity necessary for subsequent detection. The method is highly specific and very sensitive. It allows to detect multiple copies of viral DNA in the test material. Using PCR, it is possible to determine not only the nucleotide sequence of DNA, but also RNA and, therefore, the identification of RNA viruses (for this, PCR with a preliminary stage of reverse transcription (RT-PCR) is used). In recent years, PCR has been increasingly used for the diagnostics and monitoring of viral infections (hepatitis viruses, herpes viruses, papillomas, etc.). The method is especially valuable for the diagnostics of latent viral infections and HIV infection.

In many cases, the concentration of the virus in the pathological material is insufficient for direct detection of the virus, its genome or antigen (express diagnostics of infection), and then it is necessary to use the virological method [9, 11, 20].

Virological method

The **virological method** is based on the isolation and cultivation of viruses on **sensitive test systems** such as **chicken embryo, cell culture, and laboratory animals** with subsequent identification of the isolated virus [10, 21, 22].

Viruses are cultivated only on sensitive living cells, as viruses are *obligate intracellular parasites* that are not able to multiply in any of the cell-free environments.

The virological method should be carried out in a specialized virological laboratory. This is a time consuming process.

It should be noted that the isolation of the virus is not always evidence of the etiology of the infection, since the persistence of viruses in the human body or the appearance of a mixed infection caused by two viruses is possible [1].
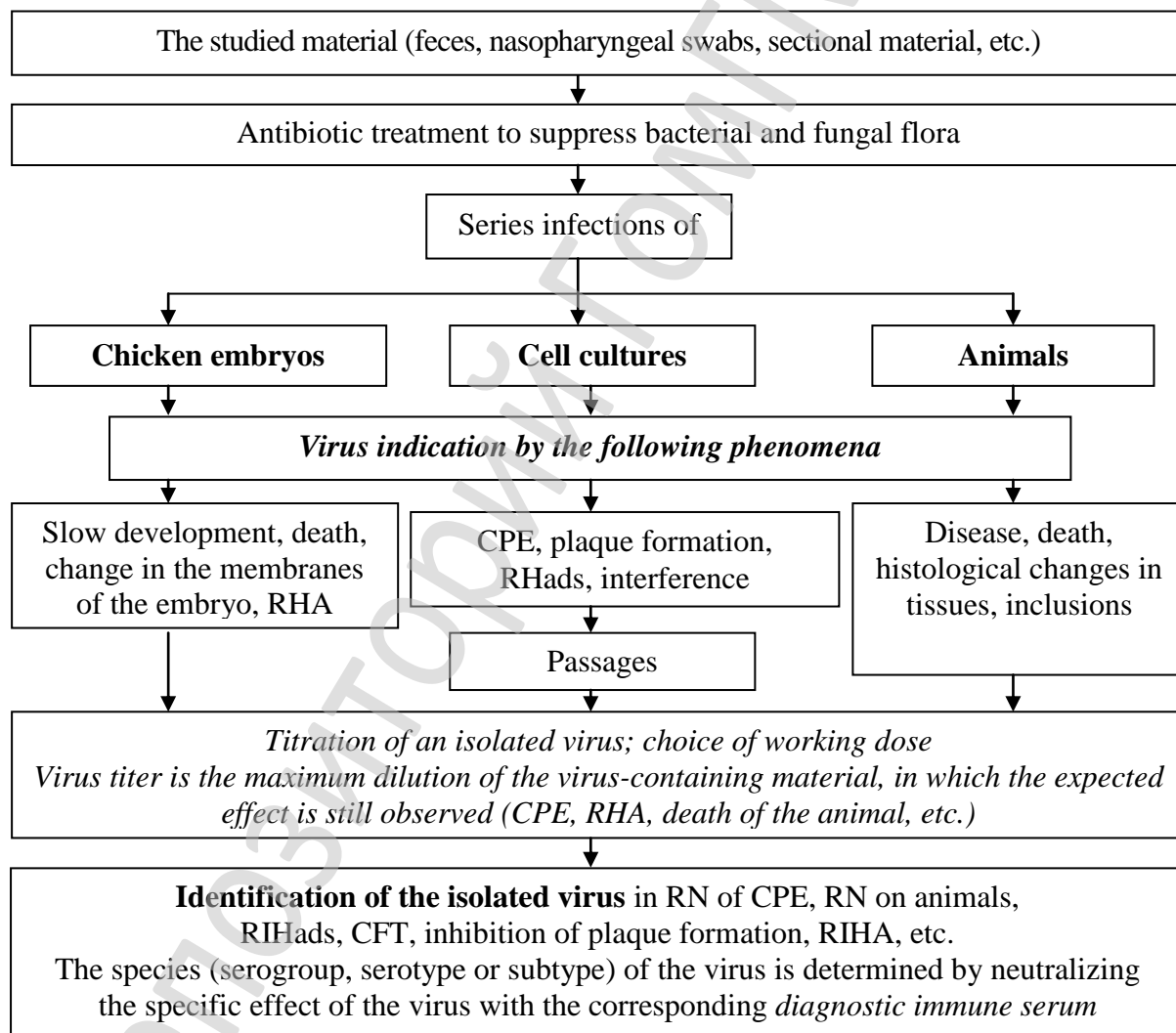
Basic stages of the virological method

1. Collection of the investigated material.
2. The choice of a sensitive test system by the principle of cytotropism (or tissue tropism), the determination of test system viability.
3. Infection of the selected test system.

4. *Indication* of the virus based on the detection of the corresponding phenomena in sensitive test systems, the nucleic acid of the virus, Ag.

5. *Identification* and titration of the virus is carried out on the basis of:

• determination of virus antigens with help of known diagnostic antisera using immunological reactions (RN, RIHA, IFA, ELISA, CSC, etc.);

• various variants of the cytopathic effect of the virus;

• histopathological examination of organs and tissues [1, 10].

The virological method is shown in figure 54.

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┌─────────────────────────────────────────────────────────────────────────┐
│     The studied material (feces, nasopharyngeal swabs, sectional material, etc.)     │
└─────────────────────────────────────────────────────────────────────────┘
                                    │
                                    ▼
┌─────────────────────────────────────────────────────────────────────────┐
│          Antibiotic treatment to suppress bacterial and fungal flora          │
└─────────────────────────────────────────────────────────────────────────┘
                                    │
                                    ▼
                       ┌────────────────────────┐
                       │    Series infections of    │
                       └────────────────────────┘
              ┌─────────────────────┼─────────────────────┐
              ▼                     ▼                     ▼
    ┌──────────────────┐  ┌──────────────────┐  ┌──────────────────┐
    │  Chicken embryos │  │   Cell cultures  │  │     Animals      │
    └──────────────────┘  └──────────────────┘  └──────────────────┘
              │                     │                     │
    ┌─────────────────────────────────────────────────────────────────┐
    │          *Virus indication by the following phenomena*           │
    └─────────────────────────────────────────────────────────────────┘
              │                     │                     │
              ▼                     ▼                     ▼
    ┌──────────────────┐  ┌──────────────────┐  ┌──────────────────┐
    │ Slow development,│  │ CPE, plaque      │  │ Disease, death,  │
    │ death, change in │  │ formation,       │  │ histological     │
    │ the membranes of │  │ RHads,           │  │ changes in       │
    │ the embryo, RHA  │  │ interference     │  │ tissues,         │
    │                  │  │                  │  │ inclusions       │
    └──────────────────┘  └──────────────────┘  └──────────────────┘
              │            ┌──────────────────┐           │
              │            │     Passages     │           │
              │            └──────────────────┘           │
              ▼                     ▼                     ▼
    ┌─────────────────────────────────────────────────────────────────┐
    │     *Titration of an isolated virus; choice of working dose*     │
    │ *Virus titer is the maximum dilution of the virus-containing material, in which the expected* │
    │      *effect is still observed (CPE, RHA, death of the animal, etc.)*      │
    └─────────────────────────────────────────────────────────────────┘
                                    │
                                    ▼
    ┌─────────────────────────────────────────────────────────────────┐
    │       **Identification of the isolated virus** in RN of CPE, RN on animals,       │
    │          RIHads, CFT, inhibition of plaque formation, RIHA, etc.          │
    │  The species (serogroup, serotype or subtype) of the virus is determined by neutralizing  │
    │       the specific effect of the virus with the corresponding *diagnostic immune serum*       │
    └─────────────────────────────────────────────────────────────────┘
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Figure 54 — Diagram of the virological diagnostic method

Notes. Indication, titration and identification of the virus are carried out, if it is possible, using the same phenomenon. Passage (subculture) is transfer of cell from one culture vessel to another.

Indications for virus cultivation and isolation may be as follows:

1. Determination of the type and variant of the virus circulating among the population when examining sporadic cases of infection, outbreaks and epidem-

ics (for example, to identify the «wild» and vaccine strain of polio virus, influenza virus serotype/subtype, etc.) [21, 22].

2. Cases where the diagnostics is important for urgent epidemiological measures, for example, laboratory confirmation of the diagnoses of influenza, poliomyelitis, arbovirus encephalitis, is a signal for immunization of the population or to vector control measures.

3. Infections caused by new types of viruses, for example, causative agents of previously unknown viral diseases or new serotype or subtype of the influenza virus.

4. The need to confirm preliminary microbiological diagnostics, established, for example, according to express diagnostic methods.

5. Cases when, using the immunological method, it is not possible to distinguish one virus from another or when the clinical manifestations of the disease are similar.

6. Indication of viruses in environmental objects [1, 21, 22].

Isolation of viruses in chicken embryos and methods of their indication

For infection, 7–12-day chicken embryos are selected. Before infection, the viability of the embryo is determined — with ovoscopy, live embryos are mobile (a mobile shadow is visible), with pronounced blood vessels [2, 21, 22].

Chicken embryos are infected under aseptic conditions with sterile instruments, having previously treated the shell over the airspace with iodine and alcohol. Methods of infection of chicken embryos can be *open* (the test material is introduced by opening the shell) or *closed* (without opening the shell). Methods of infection of chicken embryos can be different: applying the virus to the chorionallantoic membrane, to the amniotic and allantoic cavities, to the yolk sac, etc. The choice of the infection method depends on the biological properties of the studied virus [1].

The structure of the chicken embryo and methods of its infection are shown in figure 55.
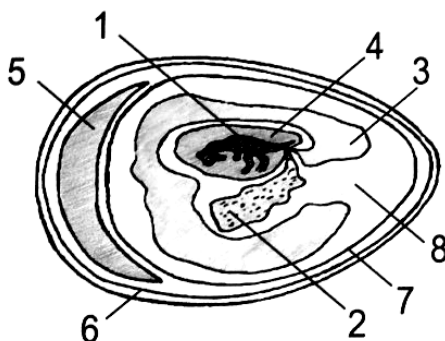
Figure 55 — Structure of the chicken embryo and methods of its infection:
1 — the embryo; 2 — the yolk sac; 3 — the allantoic cavity; 4 — the amniotic cavity;
5 — the air sac; 6 — the inner lining of the shell; 7 — the chorionallantoic
membrane (CAM); 8 — the extraembryonic cavity

Indication of the virus in the chicken embryo is carried out according to the following phenomena:
• slow development, death of the embryo;
• change in the membranes of the embryo (for example, "plaques" on the chorioallantoic membrane);
• positive hemagglutination reaction with an allantoic or amniotic fluid [1].

Reaction of hemagglutination (RHA)

RHA is based on the ability of certain viruses to agglutinate red blood cells of certain animal or bird species due to hemagglutinins (figure 56).
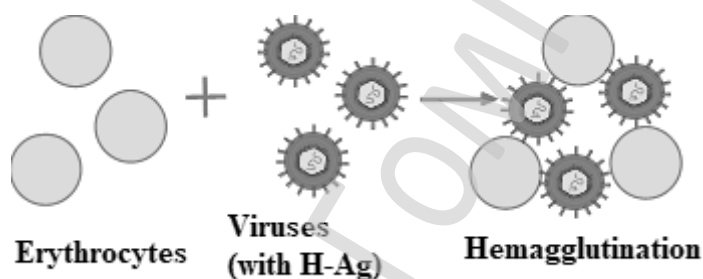

Figure 56 — Scheme of hemagglutination reaction

Using RHA the presence of a *hemagglutinating virus* in the test material (indication of the virus) is determined, as well as its titer. The *titer of the virus* is called its greatest dilution, in which erythrocyte agglutination is still observed. With a positive reaction (the presence of a virus), hemagglutination occurs, i.e. erythrocytes stick together and fall to the bottom of the plastic plate well in the form of scalloped sediment — «umbrella». In case of a negative reaction (no virus), the red blood cells do not stick together — the sediment is in the form of a «button» [17, 22].

Isolation of viruses in cell cultures and methods of their indication

Cell cultures are of great importance in virology. They are widely used in the diagnostics of viral infections, in the production of vaccines, are indispensable for research in the field of virology. Cell cultures are the most universal way to isolate and cultivate viruses, since the vast majority of viruses manage to select highly sensitive cultures on which this virus can multiply. A great convenience in the work is the presence of the visible cytopathic effect of viruses on cells, due to which it is easy to detect the presence of the virus directly in the virus-infected cell culture [1].

Cell cultures create the most standard conditions for the cultivation of viruses in comparison with other methods, as they consist of homogeneous cells under similar conditions and do not contain antibodies and non-specific inhibitors. Among the disadvantages, the laboriousness of preparing cell cultures, as

well as the frequent infection of the tissues from which the primary cell cultures are prepared with latent viruses, as well as the contamination of these cells with mycoplasmas, bacteria, and fungi, are noteworthy [21, 22].

By origin, cell cultures are divided into embryonic, tumor, and adult organisms; on morphogenesis — on fibroblast, epithelial, etc.

The most widespread in virology are single-layer cell cultures that can be divided into primary (primary trypsinized), continuous, and semi-continuous (diploid), the characteristics of which are presented in table 24 [21, 22].

Table 24 — Cell cultures characteristics

Cell cultures	Characteristics
Primary cell cultures	**Primary cell cultures** are cells of various types (monkey kidneys, human embryo kidneys, human amnion, and chicken embryos). They are obtained from tissue after mechanical milling, treatment with proteolytic enzymes (trypsin) and standardization of the number of cells. The lifespan of such cell cultures is limited to ≤10 passages. Only certain groups of cells of primary cultures can retain the ability to grow and multiply, and with repeated passages they give rise to continuous cell cultures
Continuous and diploid cell cultures	The **continuous cell lines (cell cultures)** are characterized by potential immortality and a heteroploid set of chromosomes. The most commonly used lines of continuous cell lines, such as Vero — from the kidneys of the green monkey, RH — from the human kidney; BHK-21 — from the kidneys of baby Syrian hamsters; GPK — from a kidney of a guinea pig; from tumor cells of malignant neoplasms (HeLa — from cervical carcinoma, Hep-2 — from laryngeal carcinoma), etc. **Semi-continuous cell cultures (diploid) cell cultures** are cells of the same type, capable of withstanding a maximum of 50 passages *in vitro*, while maintaining their original morphology and diploid set of chromosomes. They are used for the isolation and cultivation of viruses, but they were truly indispensable in the production of viral vaccines (cultivation of vaccine strains of viruses)

In order to successfully obtain cell cultures and the subsequent reproduction of viruses in them, cultured cells must constantly be in a balanced physiological environment containing all the necessary components for their life and reproduction. For this purpose, *balanced salt solutions (BSS)* and *virological culture media (growth media)* are used [1].

Nutrient media and saline solutions for cell cultures

Balanced salt solutions have a salt composition that qualitatively and quantitatively corresponds to the composition of the fluids of an animal organism. The main purpose of these salts is to create a buffering and isotonic environment and provide the most important inorganic ions. Salt solutions provide the survival of cells outside the body; they serve to wash tissues and cells during the preparation of cell cultures; are the basis for the preparation of virological culture media. The most common salt solutions are *Hank's, Earle's solutions* and *Dulbecco's phosphate-buffered saline*.

Cell culture media

Cell culture media are nutrient media are necessary for the vital functions of cultured cells. As intended, they are divided into *cell growth media* and *maintenance media*. Growth nutrient media should contain more nutrients that ensure active cell multiplication and monolayer formation. They are used before infection of the cell culture with viruses. Maintenance media are used after infection of the cell culture with viruses; they contain less nutrient components (distinctive feature of maintenance media is absence of serum in their content) and ensure the survival of cells in the already formed monolayer during the period of virus reproduction in them.

Nutrient media for cell cultures in most cases are prepared on the basis of a *balanced salt solution* (for example, Hank's solution). An integral component of most growth media is the *blood serum of animals* (calf, bovine, equine), without the presence of 5–20 % of which the reproduction of cells and the formation of a monolayer does not occur. Example of growth nutrient media is *lactalbumin hydrolysate-yeast extract medium* (LAH-YE medium). It consists of following components: lactalbumin hydrolysate, yeast extract, Earle's solution, calf serum and antibiotics. Antibiotics are added to the nutrient media in order to prevent the possible growth of microorganisms. Other components of cell culture media are *glucose/glutamine, amino acids, vitamins, phenol red dye,* etc. [21, 22].

The following nutrient media are distinguished:

1. Natural nutrient media (rarely used) — prepared on the basis of Hank's and Earle's salt solutions, to which serum, amniotic fluid, and embryonic extract are added.

2. Enzymatic hydrolysates of protein substances (more commonly used is the enzymatic lactalbumin hydrolyzate, which is obtained from milk).

3. Synthetic culture media (widespread use). They are prepared from a specific set of chemicals (amino acids, vitamins, minerals). They have a constant and well-known composition (**chemically defined media**). Most often used *medium 199* (Parker's medium), *Eagle's minimum essential medium, RPMI-1640, Ham's F 12*, etc. [13, 15].

Virus isolation and infection of the cell culture

For infection, cell cultures sensitive to the virus with a well-developed continuous monolayer located in test tubes, flat bottles (special cell culture vessels), or well cell culture plates are used (figure 57).

Before infection of the cells, the growth medium is removed and 0.1–0.2 ml of suspension of the investigated material pretreated with antibiotics is added to the cell culture. After 30–60 minutes of contact of the virus with the cell culture, excess material is removed, a maintenance medium is introduced into the vessel and left in the thermostat until signs of virus reproduction are detected. When cell culture is infected by viruses, various visible manifestations of the virus action are observed (**phenomena of virus indication in cell culture**).

Figure 57 — Vessels, plates for cell cultures

Phenomena that can detect a virus in cell culture (indication of the virus reproduction in the cell culture):

1. *Cytopathic effect (CPE)* — changes in cells under the influence of the virus multiplying in them. It is assumed that the main cause of CPE is a disturbance of cell metabolism [21, 22].

The CPE can be of the following main types (figure 58):

a) *Round cell degeneration or small cell degeneration*; complete or partial degeneration of monolayer cells. With a rapid degeneration, the cell monolayer dies: the cells exfoliate from the glass and float freely in the culture fluid in the form of structureless grains, and then they are lysed [1].

| 1 | 2 | 3 | 4 |
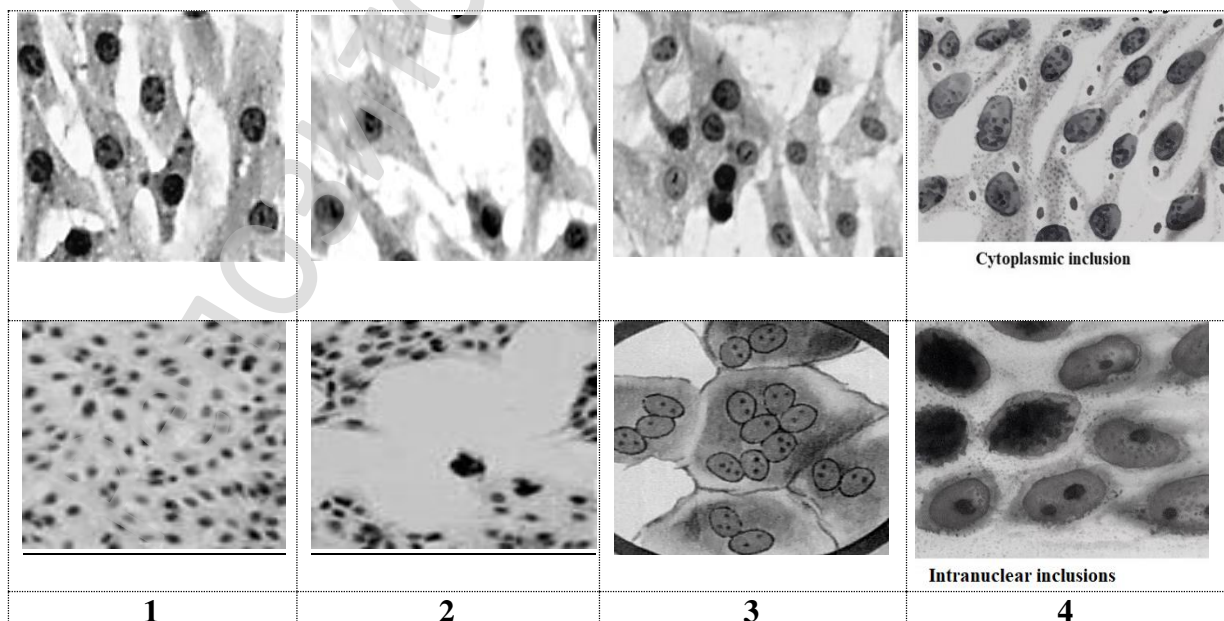
Figure 58 — Monolayer cell culture before infection and the variants of the virus CPE:
1 — continuous monolayer of cells before infection with the virus (normal cells);
2 — degeneration of cell monolayer; 3 — symplast formation; 4 — inclusions

b) *Symplast (syncytium) formation* — under the influence of some viruses, cells fuse with each other with the formation of enormous giant multinucleated cells — symplasts and syncytia (set of symplasts).

c) Formation of *intranuclear or cytoplasmic inclusions*;

d) *Proliferative type of changes* — arises as a result of cell transformation upon infection of the cell culture with oncogenic viruses, under the influence of which the cells begin to divide uncontrollably and are not located in a monolayer, but grow randomly in several layers.

The cytopathic effect of various viruses on different cell cultures has certain specificity. Related viruses usually give a cytopathic reaction of a similar type, the effect of viruses distant in their properties is often different, and therefore, by the type of CPE, one can often identify the family or genus to which the studied virus belongs. So, enteroviruses (poliomyelitis, Coxsackie, ECHO viruses) are characterized by CPE in the form of homogeneous cell degeneration; the cells acquire a rounded shape, are arranged quite evenly. For the cytopathic effect of adenoviruses, the transformation of the cell layer into clusters of small, rounded cells arranged in the form of clusters of grapes is typical. Parainfluenza viruses, respiratory syncytial, measles and mumps viruses give a cytopathic effect in the form of the formation of symplasts. During the reproduction of herpes viruses, round-shaped cells are diffusely located throughout the monolayer and inclusions are formed [1, 22].

2. *The phenomenon of haemadsorption* (reaction of haemadsorption — RHads)

Principle of the method: the contact of cell culture with red blood cells leads to the adsorption of red blood cells on the surface of cells affected by the virus with hemagglutinating properties. The effect can be seen with a small magnification of the microscope (figure 59) [21].
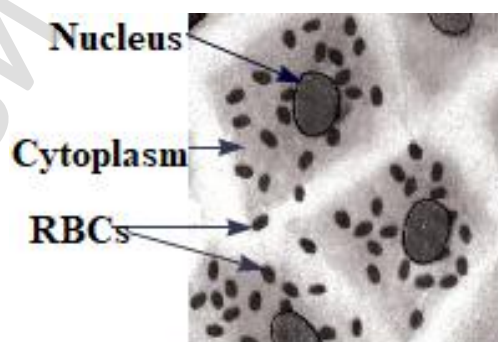
Figure 59 — Positive RHads result

In case of positive hemadsorption, the accumulation of red blood cells on the cells can be very characteristic. Influenza virus gives islet type haemadsorption; parainfluenza virus — diffuse haemadsorption; for poxviruses, the location of red blood cells around the cells in the form of a corolla or necklace is characteristic [12, 21].

3. *The phenomenon of interference*

Principle of the method: the phenomenon of interference is used to detect viruses that do not give a clear CPE in cell culture. The test culture is re-infected with a virus that regularly causes CPE. Indicator virus is the VVS is a virus of vesicular stomatitis. If there is a non-cytopathic virus in the investigated material, the CPE of the indicator virus will also be absent (the cell is "occupied" by the investigated virus) [21, 22].

4. Plaque formation is a Dulbecco phenomenon

Principle of the method: a monolayer of cells after infection with the virus is poured with a layer of semi-solid agar, which includes a nutrient medium and a neutral red dye. In those areas of the monolayer where the cytopathic virus multiplies, the groups of destroyed cells do not acidify the medium and look like *transparent spots on a pink background* called plaques (figure 60).

One of the first poliomyelitis virus plaques in a single-layer cell culture was obtained. The ability to form plaques is currently found, in addition to polio viruses, and in many other viruses: ECHO, encephalitis, influenza, measles and several others [21, 22].
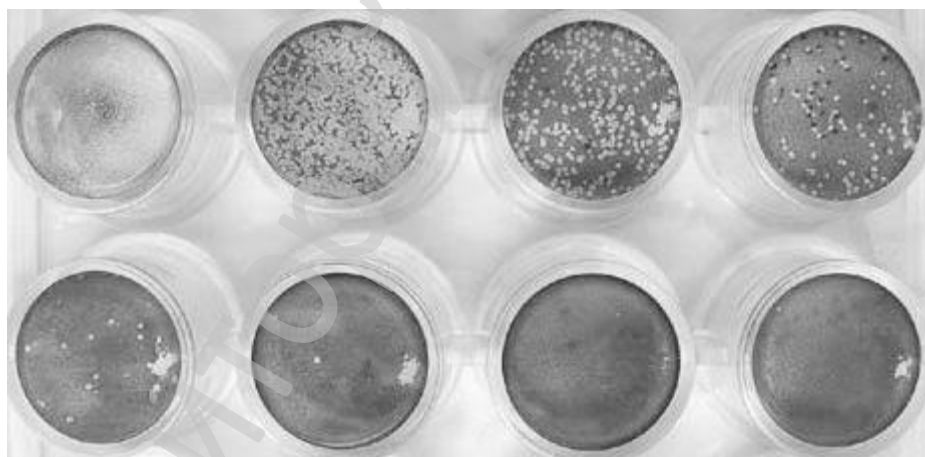
Figure 60 — Plaque formation in the cell cultures

When studying the morphology of plaques, it was found that different viruses, as a rule, form plaques that differ in size, shape, nature of the edges, in terms of appearance and other properties, which can be used for preliminary identification of the virus [1].

5. Salk color test

The «color test» method is also a method for assessing the multiplication of viruses in tissue culture. In the absence of the virus and its reproduction, cells in a cell culture multiply in a nutrient medium with an indicator and, due to the formation of acidic metabolic products, the medium changes color (the initial red color of the medium changes to yellow). During the reproduction of the virus, normal metabolism of culture cells is disturbed, acidic products are not formed, and the medium retains its original (red) color [13, 21, 22].

Isolation of viruses in laboratory animals and methods of their indication

Mainly newborn white mice, hamsters, guinea pigs, white rats are used. Animals susceptible to the viruses studied are called the *experimental model*. As a model, animals with species sensitivity and high susceptibility to certain viruses are selected. Experienced animals must be of the same species, of a certain age and kept in the same conditions [1].

A prerequisite for the successful isolation of viruses is the use of animals free of latent infections of a viral, bacterial or protozoal etiology, which are often found in many types of laboratory animals. Such diseases include viral pneumonia, viral encephalomyelitis in white mice, protozoal encephalitis in many mammals and birds, etc.

For virological work, the so-called *«pure (inbred) lines»* of experimental animals are most often used. They have the same type of heredity, have a minimal number of latent infections, and are highly susceptible to viruses [1].

Infection of animals is carried out according to the principle of *virus cytotropism*: pneumotropic viruses are administered intranasally, neurotropic — intracerebrally, dermatotropic — on the skin.

Indication of the virus in laboratory animals is based on the manifestation of signs of disease in animals, their death, pathomorphological changes in tissues and organs, as well as on a positive hemagglutination reaction with extracts from organs.

Virus titration is performed on laboratory animals, and a neutralization reaction is performed; animals are used to obtain viral vaccines, diagnosticums, and diagnostic antiviral antisera [21, 22].

General scheme for identification of the virus identification

Isolation, titration and identification of the virus are carried out using the same phenomenon.

I stage. Titration of an isolated virus; working dose selection

Virus titer is maximum dilution of virus-containing material, in which the expected effect is still observed (CPE, plaque formation, RHA, RHads, death of the animal, etc.) [1].

II stage. **Identification of isolated virus**

Essential for the identification of viruses (by genus, species, and serotype) is the study of their antigenic structure, which is carried out in the *reactions of virus neutralization* (for example, RN of CPE or RN on laboratory animals) or *inhibition reactions* (RIHA, RIHads) with the corresponding diagnostic antisera [22].

The *reaction of virus neutralization* is carried out by introducing a mixture of antigen (isolated virus in a working dose) and antibody (diagnostic antiviral

serum) in sensitive test system (cell cultures, chicken embryos, in the body of laboratory animals). The essence of this reaction is that after treatment with homologous antibodies, the virus loses its biological activity ("neutralized") and the test system cells develop in the same way as noninfected virus [22].

III stage. Registration of results

If the immune serum is able to bind the virus in the test system (i.e., neutralize virus effect or inhibit it), then, therefore, the antibodies in this serum are specific for viral antigens in the experiment. As a result, the CPE will be absent in the RN of CPE, hemadsorption will be inhibited in RIHads, hemagglutination also in RIHA, animals will survive in the RN, etc. (figure 61) [17, 18, 22].
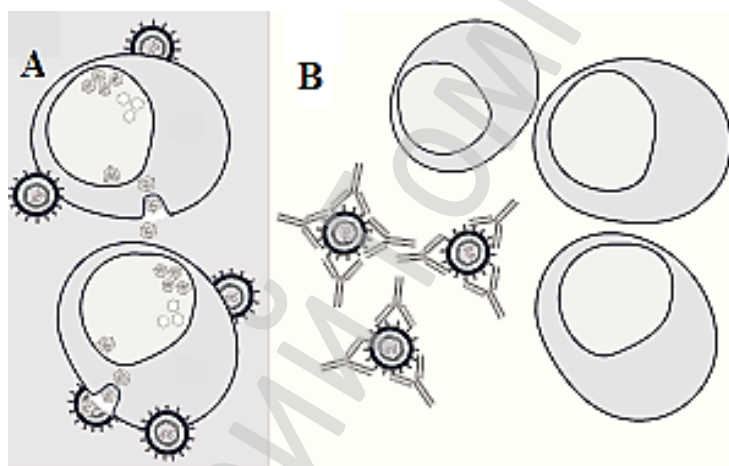
Figure 61 — Scheme of the neutralization of viruses in cell culture:
A — CPE as a result of the multiplication of viruses (binding the virus
with a cell leading to cell destruction); B — CPE is absent due to neutralization
of viruses by specific antibodies

The species (serogroup, subtype or serotype) of the virus is determined by neutralizing the specific effect of the virus with the corresponding immune serum (for example, if the antipoliomyelitis diagnostic serum for the first type of poliovirus is able to neutralize the CPE of the virus, then this virus is poliomyelitis virus of the first type) [1].

Serological method

Serological diagnostics is based on the detection of *specific antiviral antibodies* in the patient's blood serum to certain viral antigens [1].

Criteria of serodiagnostics

In the serodiagnostics of viral infections, the «paired sera» approach is used, which allows to determine the *Ab titer increase* over a certain period of the disease using a set of viral diagnosticums. Paired sera — two sera collected

from one patient at the beginning of the disease and after 1–3 weeks. *Diagnostically significant increase in antibody titer is considered an increase of 4 or more times during the development of the acute phase of the disease*.

A ***diagnostic titer*** is an antibody titer indicating a disease, i.e. in this titer; the reaction is positive only in patients and negative in healthy ones. If the obtained serum titer of the patient is the same as the diagnostic titer or higher, therefore, the patient is given a serological diagnosis of the corresponding disease; if the obtained serum titer is lower than the diagnostic titer, then at this time stage the serological diagnosis of the disease is not confirmed [1].

It is known that most specific antibodies belong to the ***IgG*** and ***IgM classes***, which are synthesized at different times of the infectious process. At the same time, IgM antibodies are early, and the reactions used to determine them (for example, ELISA or indirect IFA) are used for early diagnosis (it is enough to examine one serum). Detection of antibodies of the IgM class will indicate an acute infection. IgG antibodies appear in the late stages of the disease, persist for a long time, and indicate a past illness or latent (persistent) infection. ***IgG avidity test*** also helps differentiate between past and recently acquired infection because IgG produced initially bind weakly to the antigen (low avidity of IgG indicates recent infection); and IgG with high avidity is marker of past or persistent infection [1].

***Basic immunological reactions applicable
in serodiagnostics of viral infections***

Neutralization reaction (RN)

The neutralization reaction is performed with different dilutions of the patient's serum and a constant dose of the known virus (diagnosticum) to detect unknown antiviral antibodies. Antibodies of the immune serum are able to neutralize the damaging effect of viruses on cell cultures (RN of CPE) or laboratory animals, which is associated with the blockade of viruses by antibodies, i.e. their neutralization [17, 18].

Reaction of inhibition of hemagglutination (RIHA)

Its principle is that when the virus comes into contact with homologous antibodies, the antibodies bind to the hemagglutinins of the virus, as a result of which the virus loses its ability to agglutinate (stick together) red blood cells. To do this, the patient's serum is mixed in different dilutions with the same doses of the known virus and, after contact; a suspension of red blood cells is added to all the mixtures. At the maximum dilution of the investigated serum, when hemagglutination is still inhibited, the titer of antibodies in this serum will be determined. A positive result of the reaction is sediment in the form of a «button»; in case of a negative reaction, hemagglutination does not slow down, red blood

cells stick together and fall to the bottom of the plate well in the form of scalloped sediment — an «umbrella». RIHA detects antibodies in the patient's serum that are synthesized against hemagglutinating viruses (e.g., influenza virus) [17, 18].

Immunofluorescence assay (IFA)

To detect antibodies in the patient's blood serum, an indirect IFA method is used. To do this, a suspension of the reference microorganism (diagnosticum) is applied to the glass, after fixing it on the glass and washing, drops of blood serum are applied in certain dilutions. After washing, luminescent antiglobulin serum (serum labeled with fluorochrome, for example, FITC, and containing antibodies against human serum proteins) is layered. The resulting complex «antigen + antibody + anti-human Ig antibody» is detected by luminescent microscopy: when illuminated with ultraviolet rays, it gives a bright green glow [17, 18].

Enzyme-linked immunosorbent assay (ELISA)

It allows to identify IgM and IgG separately, which can explain the dynamics of the infectious process or possibility of recovery. To detect antibodies, a known antigen is adsorbed onto a solid substrate (for example, plastic microplates) and various dilutions of the patient's serum are introduced. After appropriate incubation, unbound antibodies are removed, an anti-human Ig antiserum (antiglobulin serum) labeled with an enzyme (e.g., peroxidase) is added. Then, the procedure of incubation and washing of unbound antibodies is repeated and any chromogenic substrate (sensitive to the action of the enzyme) is introduced. The substrate is cleaved by the enzyme and the color of the reaction product changes. The amount of colored product is measured spectrophotometrically [17, 18].

Immunoblotting

Immunoblotting not only detects antibodies but allows differentiating individual antibodies to various proteins of microorganisms. The microbial antigens are separated by polyacrylamide gel electrophoresis, and then transferred from the gel to the nitrocellulose membrane. In this case, various antigens are fixed separately in different parts of the strip. Then the patient's serum is applied to this strip and further studies are carried out according to ELISA scheme: they are washed from unbound antibodies of the patient's serum and antiglobulin serum labeled with the enzyme is applied. The complex «antigen + patient's antibody + anti-human Ig antibody» formed on the strip is detected by the addition of a chromogenic substrate that changes color under the action of the enzyme. Positive reactions look like dark blots on the strip [17, 18].

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