MINISTRY OF THE PUBLIC HEALTH OF UKRAINE
ZAPOROZHYE STATE MEDICAL UNIVERSITY
CHAIR OF MICROBIOLOGY, VIROLOGY AND IMMUNOLOGY

Module I

Collection of methodical recombinations

for practical classes

on microbiology, virology and immunology

for the students of 2nd year of the medical faculty.

Part I.

МІНІСТЕРСТВО ОХОРОНИ ЗДОРОВ'Я УКРАЇНИ

Запорізький державний медичний університет

Кафедра мікробіології, вірусології та імунології

Модуль I

Збірник методичних рекомендацій для підготовки практичних занять з мікробіології, вірусології та імунології для студентів II-III курсів міжнародного факультету, спеціальність «Лікувальна справа»

I частина

ЗАПОРІЖЖЯ- 2016

Збірник методичних рекомендацій для підготовки практичних занять з мікробіології, вірусології та імунології для студентів II-III курсів міжнародного факультету, спеціальність «Лікувальна справа».

Автори:

зав. кафедри, д.мед.н., професор **Камишний О.М.**

старший викладач кафедри , к.біол.н. **Єрьоміна А.К.**

доцент кафедри нормальної фізіології, к.мед.н. **Сухомлінова І.Є.**

доцент кафедри загальної гігієни та екології, к.мед.н. **Кірсанова Е.В.**

Рецензенти:

Затверджено ЦМР ЗДМУ: протокол № від .2016 р.

The independent practical work of students is an important part of the syllabus in the course of microbiology, virology and immunology. It helps students to study this fundamental subject.

The systematic independent work enables to reach the final goal in the students' education. It is also important while preparing the students for their future clinic work with patients.

These theoretical material, questions and tests help students to get ready for examination.

AUTHORS:

Kamyshny A.M., the heat of the chair of microbiology, virology, and immunology, professor of medicine.

Yeryomina A.K., senior lecturer of the chair of microbiology, virology and immunology, candidate of Biological Sciences.

Kirsanova E.V., assistant professor of the chair gygien and ecology, candidate of Medicine.

Sukhomlinova I. E., assistant professor of the chair of normal physiology, candidate of Medicine.

REVIEWERS:

The methodical manual for practical lessons on microbiology, virology, immunology for the medical students of II-III year of the study are approved by the Central Methods Board of ZSMU as a methodical manual on practical lessons for students of the medical faculty.

The order № from

Plan

of the lectures in microbiology for foreign students

of the medical faculty for spring semester

№	Theme	Amount of hours
1.	Subject and problems of microbiology in historical development. Structure and functions of the bacterial cells. Classification of microorganisms. Physiology of microorganisms. Nutrition, respiratory, growth and reproduction.	2
2.	Antibacterial chemotherapy of an infections diseases. Antibiotics. Genetics of bacteria. Genetic engineering. Biotechnology.	2
3.	The infection. The forms of infections. The infectious process. Pathogenicity and virulence of bacteria.	2
4.	Immunity. Immune system. Antibodies and antigens, their nature and properties. Immune diagnostics.	2
5.	Allergy. Reactions of immunity. Immunoprophylaxis and Immunotherapy of infectious diseases. Immunobiological preparations.	2
6.	History of virology. Morphology, structure, chemical composition, base of classification and reproduction of viruses. Antivirus immunity.	2
7.	Influensavirus and Parainfluenza viruses. Acute respiratory virus infections(respiratory-syncytial virus, reovirus, rhinovirus, adenovirus). Virus of measles. Mumps virus. Rubivirus.	2
8.	Herpesviruses. Virus of chickenpox. Smallpox.	2
9.	Virus of poliomyelitis, ECHO and Coxsackie. Hepatitis Viruses.	2
10.	Viruses of encephalitis, hemorrhagic fever. Viruses of immunodeficiency. AIDS. Principles and methods of the laboratory diagnostics. Oncogenic viruses.	2
	TOTAL	**20**

Plan

of practical classes in microbiology for foreign students

of the medical faculty for spring semester

№	Theme	Amount of hours
1.	Microbiological laboratory equipments and instructions for work. Structure of biological light to the microscope and rules of work with him. ***Bacterioscopical method of research.*** Microscopy of the prepared given. Morphology of bacteria. Preparations for a microscopy. Method of staining by Gram.	2,5
2.	Structure of the bacterial cell. Complex methods of staining by Anjesko, Neisser, Burri-Gins and Ziehl-Nilsen. Morphology of spirochetes, riskettsia, fungi and the protozoa.	2,5
3.	***Bacteriological method of research.*** Nutrition of bacteria. Nutrient media and methods of bacteria cultivation. Methods of sterilization. Asepsis and antiseptic. Disinfection. Chemotherapy. Chemotherapeutic preparations.	2,5
4.	Growth and reproduction of bacteria. Methods of isolation and cultivation of pure cultures of aerobes. Respiration of bacteria. Methods of isolation and cultivation of anaerobes. Biochemical properties of microorganisms.	2,5
5.	Genetics of microorganisms. Methods of biotechnology and gene engineering. ***Genetic method of diagnostics.*** Polymerase chain reaction (PCR). Polymerase chain reaction with reverse transcriprase (RT-PCR). Polymerase chain reaction in the real time.	2,5
6.	Ecological microbiology. Microflora of an environment and the human body. Methods of sanitary bacteriological research. Antibiotics. Bacteriophages.	2,5
7.	**Submodule 1. Morphology and physiology of microorganisms.**	**2,5**
8.	Infections, infectious and epidemiological process. Pathogenic	

	factors of microorganisms. Mechanisms of pathogenesis of an infectious diseases. Experimental infection of laboratory animals.	2,5
9.	Immunity. Kinds and forms of immunity. Types of immune answer. Innate immunity. Factors of non specific organism defence. Cells and receptors of innate immunity.	2,5
10.	Adaptive immunity.T- and B-lymphocytes. Description of antigens. Presentation of antigens. Activating of lymphocytes. Antiinfectious immunity.***Immunological method of research*** – serological reactions. Immunoglobulins. Serological reactions of agglutination and precipitation, immune lysis and compliment fixation test, their description and practical use. Coomb's test. Immunohematology. Application of monoclonal antibodies in immunocytochemical and immunohistochemical reactions.	2.5
11.	Immune serum and immunoglobulins. Reaction of flocullation (neutralization). Hypersensitivity. Allergic reactions for immunodiagnosis of infectious diseases. Autoimmune phenomena. Principles of the use of antibodies as medical, preventive and diagnostic preparations.	2,5
12.	Vaccines. Principles of making and application of vaccines. Immunobiological preparations. Immune status of man. Estimation of immune status. Immunomodulators. Immunocorrection. Transplantation immunology.	2,5
13.	Virologic laboratory. Morphology and ultrastructure of viruses. Principles of classification. Virologic methods of research. Cultivation of viruses in culture cells and in chick embryos. Indication of viral reproduction.	2,5
14.	Antiviral immunity. Immunoreactions in virology: hemagglutination reaction, hemagglutination inhibition assay, hemadsorption phenomenon, neutralization test. Radioimmunoassay, direct and indirect immunofluorescence, enzyme immunoassay. Enzyme linked immunosorbent assay (ELISA), immunoelectroblot technigues. Immunochromatography analisis. Immunology of tumours.	2,5
15.	**Submodule 2. Infection, immunity and general virology.**	**2,5**

16.	Laboratory diagnostics of flu and parainfluenza. Laboratory diagnostics of adenoviruses Laboratory diagnostics of mumps, measles and rubella.	2,5
17.	Laboratory diagnostics of chikenpox, smallpox, herpes, zoster, poliomyelitis, Coxsakie and ECHO.	2,5
18.	Laboratory diagnostics of viral hepatitis A, B, C, D, E, F, G, rabies and arboviruses infections.	2,5
19.	Laboratory diagnostics of AIDS. Oncogenic viruses. Viral genetic theory of tumours origin.	2,5
20.	**Final module control I.**	2,5
	TOTAL	**50**

INDEPENDENT WORK
on microbiology for foreign students

of II course of the medical faculty for spring semester

№	Theme	Hours quantity
1.	Morphology of microorganisms. Simple and complex methods of coloring the bacteria.	6
2.	Structure of bacterial cell. Complex methods of coloring the bacteria.	4
3.	Morphology of spirochetes and rickets.	4
4.	Morphology of fungi and elementary.	2
5.	Nutrition of microorganisms, nutrient mediums and methods of cultivation bacteria. Devices and methods of sterilization.	4
6.	Action of some physical and chemical factors on microorganisms, disinfection.	2
7.	Antibiotics and Chemotherapy.	6

8.	Genetics of microorganisms. Methods of biotechnology and gene engineering.	4
9.	An infection. Experimental infection of laboratory animals.	4
10.	Immunity, its kinds and forms. Nonspecific factors of protection of an organism.	6
11.	Immune reactoins for infectious diseases.	2
12.	Vaccines. Principles of manufacturing and application of vaccines. Immunobiological preparations.	2
13.	Prion diseases of humans and animals.	2
14.	Modern methods of the laboratory diagnostics of infectious diseases	2
	Total	**50**

Microbiology is the study of the organisms that are too small to be clearly perceived by the unaided human eye, called microorganisms.

Microbiology consists of general and special microbiology. The general microbiology studies structure, morphology, physiology, genetics and ecology of pathogenic and conditionally pathogenic microorganisms, is engages in their systematization.

The special microbiology studies etiology, pathogenesis, and methods of the laboratory diagnostics, preventive measures and treatment of infectious diseases.

Methods of laboratory diagnostic of the infectious diseases.

1. Microscopical (bacterioscopical) method is study of the microorganisms under the microscope.
2. Microbiological (bacteriological) method is study of the microorganism's physiology.
3. Biological method is the usage of the experimental animals.
4. Allergic method is the usage of skin allergic test for detection of dermal hypersensitivity to different infectious antigens.
5. Serological method is the research of the patient's blood serum for detection of antibodies to the given diseases.
6. Express-methods.

General laboratory safety rules for microbiology.

1. It is permited to attend the laboratory only in the gown and cap.
2. Every student have to work only at his working place.
3. It is necessary to use the proper equipments during the work with the infectious material, and after the work the equipments must be desinfected.
4. The working place and the microbiological laboratory must be kept clean.
5. All materials must be removed and the working place must be put in order.
6. If the dishes, containing infectious material, will be broken you will have to desinfect clothing and hands in the presence of the teacher.
7. It's not allowed to eat at the laboratory.
8. Hands should be washed after completing each task and always before leaving the laboratory.

Function of microbiological laboratory.

1. Helping to the doctors in exact diagnosis of the disease.
2. Helping in the choosing of the effective medicines for the treatment of patients.
3. Providing of the sanitary-bacteriological control of the conditions of environment (researching of water, air, etc).

Practical lesson # 1

Theme: **Microbiological laboratory equipments and instructions for work. Structure of biological light to the microscope and rules of work with him.** ***Bacterioscopical method of research.*** **Microscopy of the prepared given. Morphology of bacteria. Preparations for a microscopy. Method of staining by Gram.**

Questions for the learning.

1. Structure and functions of microbial laboratory.
2. General laboratory safety rules for microbiology.
3. Modern methods of microscopic examination: light, luminescent, phase-contrast, electron microscopy.
4. Methods of laboratory diagnostic of the diseases.
5. Classification of microorganisms.
6. Technique of smear preparation from specimen of microorganisms, fixation.
7. Tinctorial properties of microorganisms. Simple and complex methods of staining the bacteria, purpose of staining.
8. Gram stain procedure. The structure of gram-positive and gram-negative microorganisms and characteristic of their cell wall.

Topic relevance

B**acteriological laboratory –** important structure in the system of microbiology

diagnosis services of practical and diagnostic public health institutions. The corse of microbiology, virology and immunology students study in the bacteriological laboratory, which have the same equipment with practical bacteriological laboratories. Therefore on the first class very important to study structure, equipment and organization of working place of microbiologist. Besides, students have ability to master one of widespread microbiological method – smear preparation, stain with aniline dyes, and microscopy with immersion objective.

Students must know:

1. The principles of health protection, safety rules and structure of the microbiological laboratory.

2. The rules of microscopy with an immersion system.

3. The fundamental principles of dark-field, luminescent, phase-contrast, electron, scanning microscopy.

Students should be able to:

1. Prepare a smears from bacterial cultures.
2. Stain the smears by a simple method (fuchsin, methylene blue).
3. Examine it with immersion objective of light microscopy.

Topic content

Depending from their designation, microbiological laboratories may be bacteriological, parasitological, mycological, virological, immunological and special (for diagnosis of particularly virulent infections).

A microbiology laboratory usually consists of the following units:

(1) the prepreparatory room for preparing laboratory glassware, making nutrient media and performing other supplementary works;

2) washroom;

(3) autoclaving room where nutrient media and laboratory glassware are sterilized;

(4) room for obtaining material from patients and carriers;

(5) room for microscopic and microbiological studies comprising one or two boxes.

Laboratory animals are kept in separate isolated animal unit and employed for biological researches.

The role of the laboratory is assisting clinicians in the diagnosis of infection. Such biological substances as blood, faeces, urine, cerebrospinal fluid, bile, etc. are the samples (specimens) for microbiological diagnosis. The microbiology laboratory requests card accompanying the specimen to use the optimal method s necessary for identification of potential pathogens in particular infective syndromes. Samples for microbiological examination need to be carefully collected, if possible without contamination with commensals or from external sources.

GENERAL LABORATORY RULES

The microorganisms used for instruction in this course are pathogenic for humans or animals. The safety of every student depends upon the conscientious observation of rules that must be followed by all who work in the laboratory. Certain precautions must be followed to avoid endangering well being, that of neighbors and those who clean the laboratory. Any student who is in doubt about how to handle infectious material should consult an instructor.

The following rules <u>must be observed at all times</u>.

1. Always wear a laboratory coat when working in the laboratory classroom.
2. Put nothing in mouth which may have come in contact with infectious material.
3. Smoking, eating and drinking in the laboratory are not permitted at any time.
4. Mouth pipetting is not permitted under any circumstances. Use the safety pipetting devices which are provided. Dispose of used pipettes in the appropriate receptacle. Any infectious material which may accidentally fall from pipettes to the laboratory bench or floor should be covered with a disinfectant and reported to any instructor immediately.
5. Any spilled or broken containers of culture material should be thoroughly wet down with a disinfectant and then brought to the attention of an instructor
6. Report at once an accident which may lead to a laboratory infection.
7. The microscope issued to you is both an expensive and delicate instrument--treat it accordingly. Always, at the end of each laboratory period, carefully clean oil from the objective and condenser lenses, align the low power dry objective with the condenser and rack condenser up and body tube down. You will be held personally responsible for any defect found on microscope when it is recalled at the semester's end.
8. When finished for the day, dispose of all used glassware and cultures in the appropriate vessel. Wash hands thoroughly with soap and water before leaving the laboratory.
9. Do not throw refuse of any kind into the sink. Use the containers provided.
10. Be sure all burners are turned off at the end of the laboratory period. Double check to be sure that handles on <u>all</u> gas outlets are in the off position.

11. The inoculating needle should be heated until red hot before and after use. Always flame needle before you lay it down.

12. Always place culture tubes of broth or slants in an upright position in a rack. Do not lay them down on the table or lean them on other objects. They may roll onto the floor and break. All culture containers which are to be incubated should bear the following notations: 1) initials (or last name of the student), 2) specimen (name of organism or number of unknown) and 3) date. When using Petri plates, these notations should be entered on the bottom half, not the lid. Unless otherwise directed, all plates are to be inverted, all plugged tubes should have the plugs firmly set into the tubes.

13. Laboratory attendance is mandatory.

NOTES ON ASEPTIC TECHNIQUES

You will be working with many pathogenic species of bacteria in the laboratory. Therefore, you must learn to use careful aseptic technique at all times, both to protect self, and classmates, and to avoid contaminating cultures.

Remember that bacteria are in the air as well as on skin, the counter, and all objects and equipment that have not been sterilized.

The most important tool for transferring cultures is the wire inoculating needle or loop. It can be quickly sterilized by heating it to red hot in a bunsen burner flame.

Adjust the air inlets of the burner so that there is a hotter inner cone and the outer, cooler flame.

A dry needle may be sterilized by holding it at a 30° angle in the outer part of the flame. A wet loop with bacteria on it should first be held in the inner part of the flame to avoid spattering, and then heated until red hot in the outer part of the flame.

Always flame the loop immediately before and after use! Allow it to cool before picking up an inoculum of bacteria. If the loop spatters in the agar or broth, it is too hot. Hold the loop or wire handle like a pencil.

A complex of bacterioscopic, bacteriological, serological, allergic and biologic techniques is used in the microbiological diagnosis of bacterial infections. Depending on the nature of the infectious disease, one of them is used as the main one, while the other supplementary.

Microscopy is one of important part of the examination of many specimens.

The Light Microscope

The resolving power of the light microscope under ideal conditions is about half the wavelength of the light being used. (Resolving power is the distance that must separate two point sources of light if they are to be seen as two distinct images.) With yellow light of a wavelength of 0.4 µm, the smallest separable diameters are thus about 0.2 µm, i.e., one-third the width of a typical prokaryotic cell.

The useful magnification of a microscope is the magnification that makes visible the smallest resolvable particles. Several types of light microscopes are commonly used in microbiology:

Bright-Field Microscope

The bright-field microscope is most commonly used in microbiology courses and consists of two series of lenses (objective and ocular lens), which function together to resolve the image. These microscopes generally employ a 100-power objective lens with a 10-power ocular lens, thus magnifying the specimen 1000 times. Particles 0.2 µm in diameter are therefore magnified to about 0.2 mm and so become clearly visible. Further magnification would give no greater resolution of detail and would reduce the visible area (field).

With this microscope, specimens are rendered visible because of the differences in contrast between them and the surrounding medium. Many bacteria are difficult to see well because of their lack of contrast with the surrounding medium. Dyes (stains) can be used to stain cells or their organelles and increase their contrast so that they can be more easily seen in the bright-field microscope.

Phase Contrast Microscope

The phase contrast microscope was developed to improve contrast differences between cells and the surrounding medium, making it possible to see living cells without staining them; with bright-field microscopes, killed and stained preparations must be used. The phase contrast microscope takes advantage of the fact that light waves passing through transparent objects, such as cells, emerge in different phases depending on the properties of the materials through which they pass.

This effect is amplified by a special ring in the objective lens of a phase contrast microscope, leading to the formation of a dark image on a light background.

Dark-Field Microscope

The dark-field microscope is a light microscope in which the lighting system has been modified to reach the specimen from the sides only. This is accomplished through the use of a special condenser that both blocks direct light rays and deflects light off a mirror on the side of the condenser at an oblique angle. This creates a "dark field" that contrasts against the highlighted edge of the specimens and results when the oblique rays are reflected from the edge of the specimen upward into the objective of the microscope.

Resolution by dark-field microscopy is quite high. Thus, this technique has been particularly useful for observing organisms such as Treponema pallidum, a spirochete which is less than 0.2 µm in diameter and therefore cannot be observed with a bright-field or phase contrast microscope.

Fluorescence Microscope

The fluorescence microscope is used to visualize specimens that fluoresce, which is the ability to absorb short wavelengths of light (ultraviolet) and give off light at a longer wavelength (visible). Some organisms fluoresce naturally because of

the presence within the cells of naturally fluorescent substances such as chlorophyll. Those that do not naturally fluoresce may be stained with a group of fluorescent dyes called fluorochromes. Fluorescense microscopy is widely used in clinical diagnostic microbiology. For example, the fluorochrome auramine O, which glows yellow when exposed to ultraviolet light, is strongly absorbed by Mycobacterium tuberculosis, the bacterium that causes tuberculosis.

When the dye is applied to a specimen suspected of containing M. tuberculosis and exposed to ultraviolet light, the bacterium can be detected by the appearance of bright yellow organisms against a dark background.

The principal use of fluorescence microscopy is a diagnostic technique called the fluorescent-antibody (FA) technique or immunofluorescence. In this technique, specific antibodies (e.g., antibodies to Legionella pneumophila) are chemically labeled with a fluorochrome such as fluorescein isothiocyanate (FITC). These fluorescent antibodies are then added to a microscope slide containing a clinical specimen.

If the specimen contains L. pneumophila, the fluorescent antibodies will bind to antigens on the surface of the bacterium, causing it to fluoresce when exposed to ultraviolet light.

The Electron Microscope

The high resolving power of the electron microscope has enabled scientists to observe the detailed structures of prokaryotic and eukaryotic cells. The superior resolution of the electron microscope is due to the fact that electrons have a much shorter wavelength than the photons of white light.

There are two types of electron microscopes in general use: the transmission electron microscope (TEM), which has many features in common with the light microscope, and the scanning electron microscope (SEM). The TEM was the first to be developed and employs a beam of electrons projected from an electron gun and

directed or focused by an electromagnetic condenser lens onto a thin specimen. As the electrons strike the specimen, they are differentially scattered by the number and mass of atoms in the specimen; some electrons pass through the specimen and are gathered and focused by an electromagnetic objective lens, which presents an image of the specimen to the projector lens system for further enlargement. The image is visualized by allowing it to impinge on a screen that fluoresces when struck with the electrons.

The image can be recorded on photographic film. TEM can resolve particles 0.001 µm apart. Viruses, with diameters of 0.01–0.2 µm, can be easily resolved.

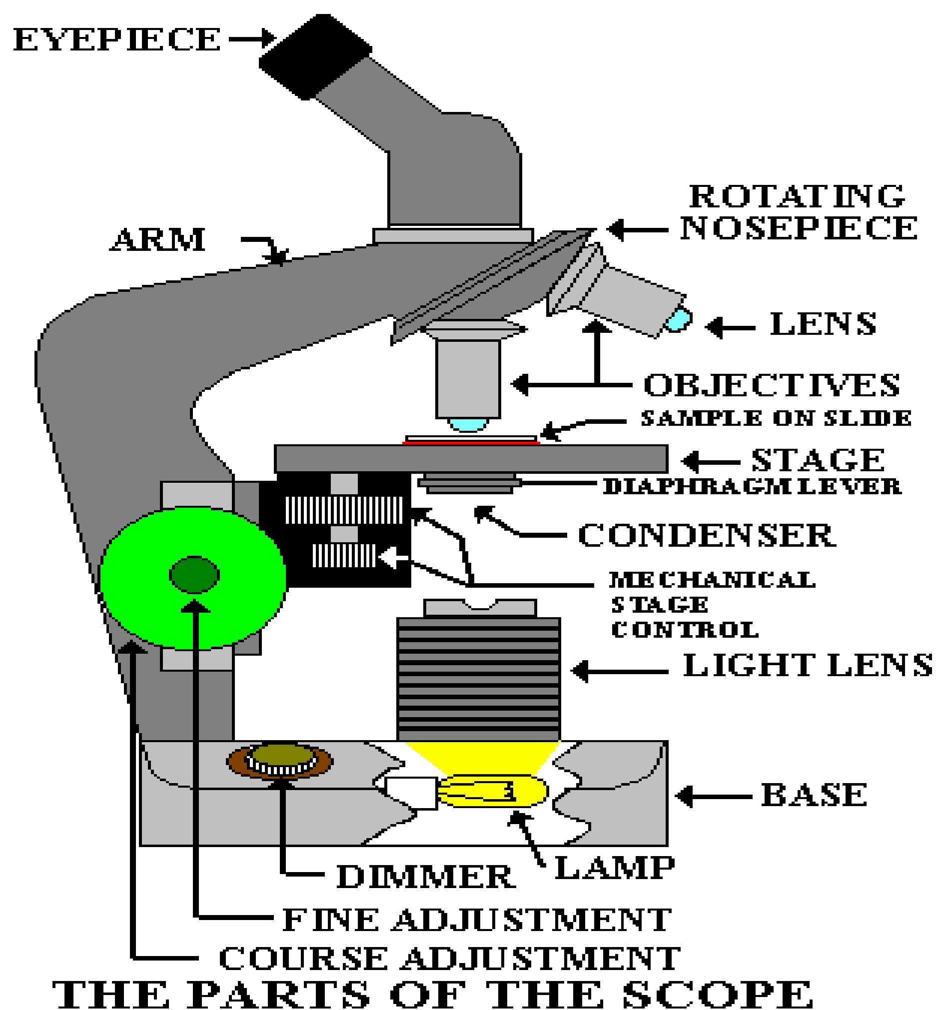
The SEM generally has a lower resolving power than the TEM; however, it is particularly useful for providing three-dimensional images of the surface of microscopic objects. Electrons are focused by means of lenses into a very fine point. The interaction of electrons with the specimen results in the release of different forms of radiation (e.g., secondary electrons) from the surface of the material, which can be captured by an appropriate detector, amplified, and then imaged on a television screen.

An important technique in electron microscopy is the use of "shadowing." This involves depositing a thin layer of heavy metal (such as platinum) on the specimen by placing it in the path of a beam of metal ions in a vacuum. The beam is directed at a low angle to the specimen, so that it acquires a "shadow" in the form of an uncoated area on the other side.

When an electron beam is then passed through the coated preparation in the electron microscope and a positive print is made from the "negative" image, a three-dimensional effect is achieved

Other important techniques in electron microscopy include the use of ultrathin sections of embedded material, a method of freeze-drying specimens that prevents the

distortion caused by conventional drying procedures, and the use of negative staining with an electron-dense material such as phosphotungstic acid or uranyl salts. Without these heavy metal salts, there would not be enough contrast to detect the details of the specimen.



THE PARTS OF THE SCOPE

Instructions for the use of microscope.

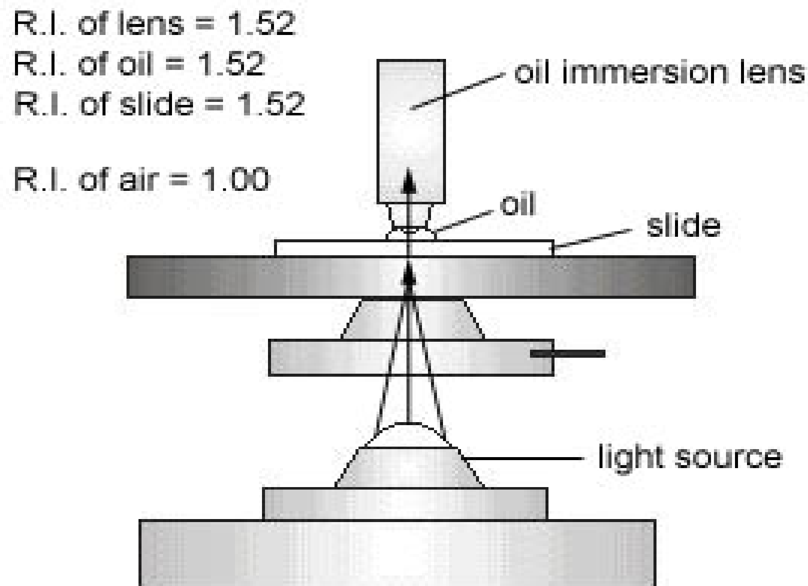
1. The objectives are clean and free from immersion oil
2. The eye pieces are free from dust
3. The plane side of the mirror is in position

4. The substage condenser is racked until its top surface is 1-2 mm below the object slide. For microbiological work it is recommended that artificial light should always be used.

Comparison of Various Types of Microscopes

Type of microscope	Maximum useful magnification	Resolution	Description
Bright-field	1,500X	100-200 nm	Extensively used for the visualization of microbes; usually necessary to stain specimens for viewing
Dark-field	1.500X	100-200 nm	Used for viewing live microorganisms, particularly those with characteristic morphology; staining not required; specimen appears bright on a dark background
Fluorescence	1,500X	100-200 nm	Uses fluorescent staining; useful in many diagnostic procedures for identifying microorganisms
Phase contrast	1.500X	100-200 nm	Used to examine structures of living microorganisms; does not require staining
TEM (trans-mission electron – microscope)	500,000-1,000,000X	0.1 nm	Used to view ultrastructure of microorganisms, including viruses; much greater resolving power and useful magnification than can be achieved with light microscopy
SEM (scanning electron microscope)	10,000-100,000X	1-10 nm	Used for showing detailed surface structures of microorganisms, produces a three-dimensional image

A light microscopy is fitted with dry and immersion objectives. A dry objective with a relatively large focal distance and weak magnification power is ordinarily utilized for studying large biological and histology objects. In examining microorganisms, the immersion objective with a small focal distance and higher resolving power is predominantly employed.



Using Immersion Oil to Create an Optically Homogeneous Light Path

The immersion oil has the same refractive index as the glass lens and the glass slide. This prevents distortion because the light waves follow a homogeneous path.

In microscopic examination with the help of immersion objective the latter is immersed in oil (cedar, peachy, “immersion”, etc.) whose refractive index is close to that of glass. When such a medium is used, a beam of light emerging from the slide is not diffused and the rays arrive at the objective without changing their direction.

The resolving power of the immersion objective is about 0,2 µm. Resolution is the capacity of optical system to distinguish or separate two adjacent object or point from one other. The maximum magnification of modern light microscopes is as high as 2000-3000.

Total power of magnification formed by the combined lenses (objective and ocular) is product of the separate powers of each lens:

Power of objective X Power of ocular = Total magnification.

Power of objective	Power of ocular	Total magnification.
40x high dry objective	10 x	400 x
100x oil immersion objective	10 x	1000 x
10x low power objective	20x	200 x

Preparation of smear for staining

The smear technique, developed by Robert Koch more 100 years ago, consists of spreading thin film made from a liquid suspension of cells on a slide and air drying it.

For preparation of satisfactory smear, the slide should be clean and grease free. Flame the slide to remove last traces of grease and moisture.

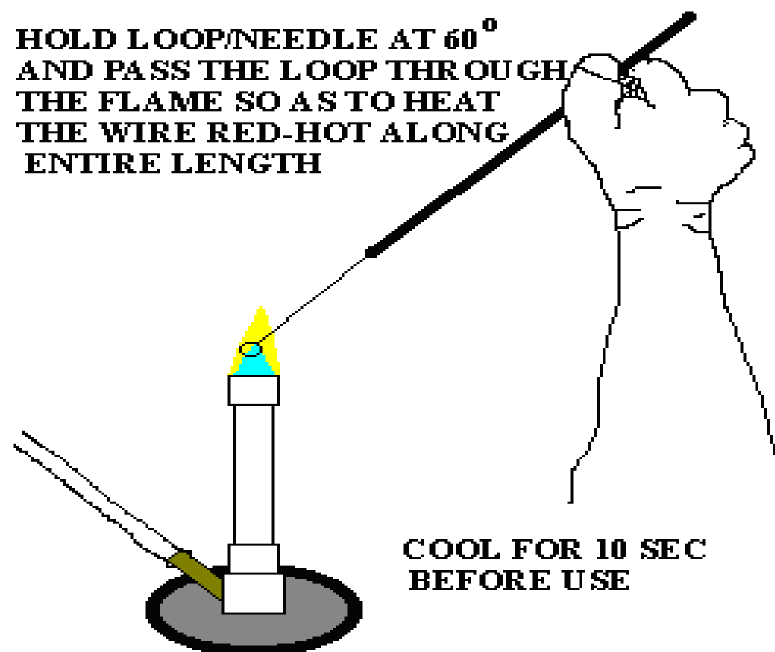
For examination of culture on a solid medium, place a drop of distilled water or normal saline on the clean slide. With the tip of inoculating loop (previously flamed

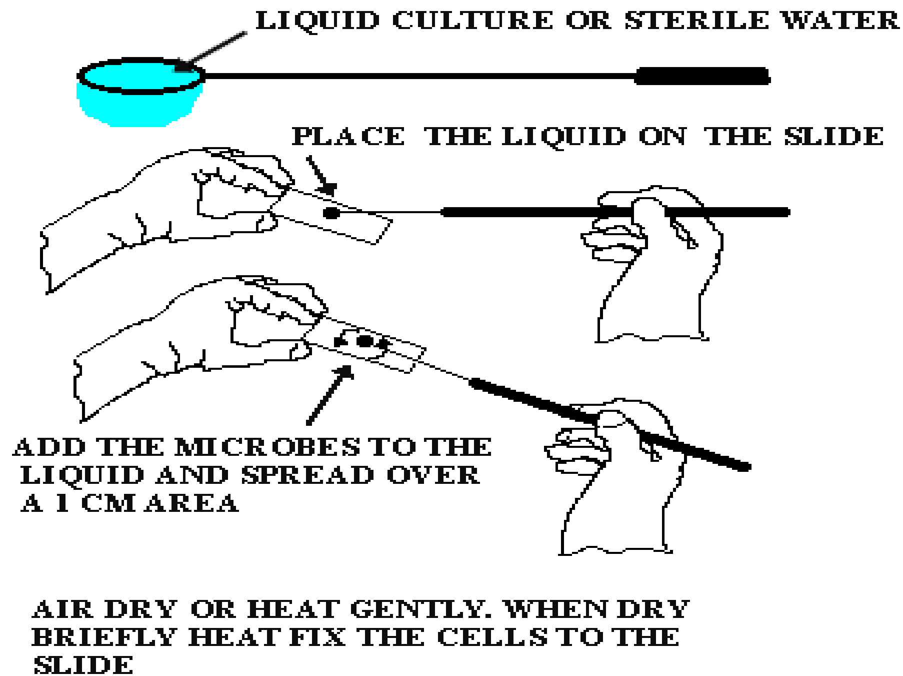
and cooled), the required colony is touched and the material mixed into the drop. Spread the emulsion into a thin film to occupy about 1 or 2 cm. In making smears from liquid medium, the

drop of culture is not spreaded but allowed to dry itself. When dry, fix smear on slide by slowly passing it three times through a flame. This process of heating is called fixing smear. Fixing smear kills and fixes the bacteria on glass slide, preventing thereby washing bacteria of during staining.

The dead microorganisms are more receptive to dyes and present no danger for the personnel working with them. Smears are sometimes fixed with methyl alcohol or other chemicals instead of heat. Allow the slide to cool before staining of smear.

Stages of smear preparing

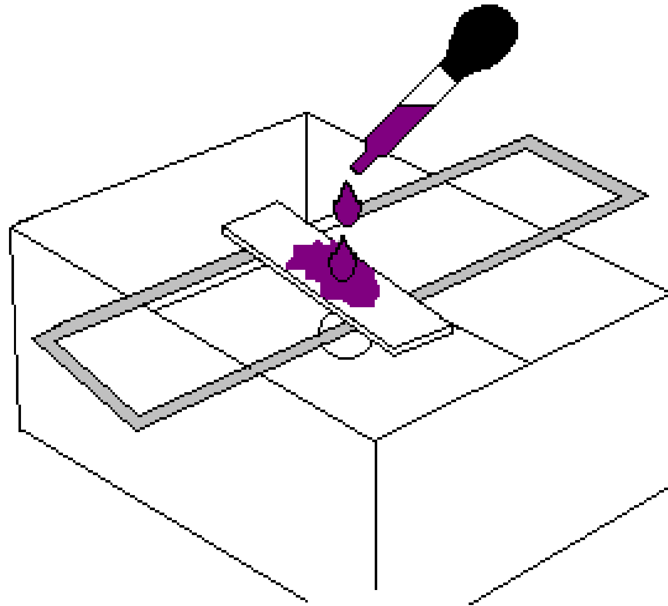




Staining is of primary importance for the recognition of bacteria since their clear protoplasm is so feebly refractive that it is difficult to see them with the ordinary microscope in the unstained condition, except when special method of illumination are used. Staining is any procedure in which colored chemicals (called dyes) are applied to specimens. Dyes impart a color to cells or cell parts by becoming affixed to them through a chemical reaction. In general, they are classified as basic (cationic) dyes, which have a positive charge, or acidic (anionic) dyes, which have a negative charge. Since chemicals of opposite charge are attracted to each other cell parts that are negatively charged will attract basic dyes and those that are positively charged will attract acidic dyes.

Many cells, especially those of bacteria, have numerous negatively charged acidic substances and thus stain more readily with basic dyes. Staining methods are classified as simple, differential, or special. Simple stains require only one a single dye and an uncomplicated procedure. Most simple staining techniques take advantage of the ready binding of bacterial cells to dyes like malachite green,

crystal violet, basic fuchsine, and safranin. Simple stains cause all cell in smear to appear more or less the same color, regardless of type, but they revealed such bacterial characteristic as shape, size and arrangement.



Adding stain to the fixed bacterial smear. After the bacterial smear has been heat-fixed to the slide, lay it over the sink on the slide-support. Carefully drop the appropriate staining solution onto the smear so as to cover it entirely. Allow it to sit for 30 to 60 seconds. Then tip the slide so the excess stain drops into the sink. Then gently run tap-water over the smear, washing off any remaining stain. Finally, dry the smear either in the air or by gently patting it with absorbent paper.

Examine under the microscope, first using the 10X to locate areas of stained material, then place a drop of oil on the dried, stained sample and rotate the oil-immersion lens into the oil drop.

4. Program of self-study training

1. The designs, equipment and working regime of bacteriological laboratory.
2. The structure of light microscope.

3. The principles of light microscopy (total magnification and resolving power).
4. The advantage and rules of work with immersion system of microscope.
5. Bright-field, dark-field, luminescent, phase- contrast, electron, scanning microscopy principles.The rules of work with immersion system of microscope.

The main stages of smear preparing.

1. The dyes used for bacteria staining.
2. Simple method of staining, its practical value.

Differential Gram method

Gram staining, a century old method named for its developer, Hans Christian Gram, remains the most universal diagnostic staining technique for bacteria. It permits ready differentiation of major significant categories based upon the color reaction of the cells: gram positive, which are stained purple, and gram negative, which are stained pink (red).

The Gram staining is the basis of several important bacteriological topics, including bacterial taxonomy, cell wall structure, identification of causative agent and diagnostic of infection; in some cases, it even guides the selection of the correct drug for an infection. For example, gram staining of fresh urine or throat specimen can help to determine the possible cause of infection, and in some cases it is possible to start drug therapy on the basis of this stain.

Even in these days of elaborate and expensive medical technology, the Gram stain remains an important and unbeatable first tool in diagnosis.

Students should be able

Prepare smears from different cultures and their mixture, staining by Gram Make conclusion about Gram staining of different smears.

Gram technique consists of a timed, sequential application of crystal violet (primary dye), Gram's iodine (IKI, the mordant), an alcohol rinse (decolorize), and fuchsine (the counter stain). In the finished product, bacteria that are stained purple were called gram positive and those are stained red were called gram negative. The hydrogen ion concentration of gram-positive bacteria (pH 2-3) is higher than of gram-negative bacteria (pH4-5).

The iodine treatment makes the cytoplasm further acidic and serves as a mordant, i.e. iodine combines with the dye and then fixes dye in bacterial cell.

Moreover, gram-positive organisms have more affinity for basic dyes than gram-negative bacteria. The dye-iodine complex formed within the cell is insoluble in water but soluble in alcohol or acetone. Gram-negative cell wall shows increased permeability to alcohol and acetone, so dye-iodine complex diffuses out through gram-negative cell wall.

Although these staining reactions involve an attraction of the cell to a charged dye, it is important to note that the terms gram positive and gram negative are not used to indicate the electrical charge of cells or dyes but whether or not a cell retains the primary dye-iodine complex after decolorization.

There is nothing specific in the reaction of the gram positive cells to the primary dye or reaction of gram negative cells to the counter stain.

The different results in the Gram staining are due to differences in the structure of the cell wall and how it affects the retention of the staining reagents.

In the first step, crystal violet is attracted to the cells in a smear and stains them all the same purple color. The second and key differentiating step is the addition of the mordant (intensifier)-Gram's iodine.

It causes the dye to form large crystals in the peptidoglycan meshwork of the cell wall. Because the peptidoglycan layer in gram positive cells is thicker, the entrapment of the dye is more extensive in them that in gram negative cells.

It does so by dissolving lipids in the outer membrane and removing the dye from the peptidoglycan layer and the cell itself. By contrast, the crystal of dye tightly

embedded in the peptidoglycan of the gram- positive bacteria are relatively inaccessible and resistant to removal.

Since gram –negative cells are colorless after decolorization, their presence is demonstration by applying the counter stain safranin in the final step.

Comparison of Gram-positive and Gram-negative bacterial cell wall

Property	Gram-positive	Gram-negative
Thickness of wall	20-80 nm	10 nm
Number of layers in wall	1	2
Peptidoglycan content	>50%	10-20%
Teichoic acid in wall	+	-
Lipid and lipoprotein content	0-3%	58%
Lipopolysaccharide	0%	13%
Outer membrane	No	Yes
Periplasmic space	Present in some	Present in all
Porin proteins	No	Yes
Sensitive to penicillin	Yes	Less sensitive
Digested by lysozyme	Yes	Weakly

Staining properties of disease causative agents

Gram-positive bacteria:	Gram-negative bacteria:
Staphylococci	Meningococci
Streptococci	Gonococci
Causative agent of diphtheria	E.coli
Causative agent of tuberculosis	Salmonella
Causative agent of tetanus.	Shigella
Causative agent of gas gangrene	Causative agent of plague
Causative agent of botulism	Causative agent of cholera

.

The Gram-positive cell wall appears as dense layer typically composed of numerous rows of peptidoglycan, and molecules of lipoteichoic acid, wall teichoic acid and surface proteins.

The Gram-negative cell wall is composed of a thin, inner layer of peptidoglycan and an outer membrane consisting of molecules of phospholipids, lipopolysaccharides (LPS), lipoproteins and sutface proteins. The lipopolysaccharide consists of lipid A and O polysaccharide.

Practical lesson # 2

***Theme*: Structure of the bacterial cell. Complex methods of staining by Anjesko, Neisser, Burri-Gins and Ziehl-Nilsen. Morphology of spirochetes, riskettsia, fungi and the protozoa.**

Questions for the learning.

1. Bacterial ultrastructure. Chemical structure of bacteria.
2. Spores of bacilli and clostridia, their role. Process of sporulation, spore localization in bacterial cell.
3. Spore-forming pathogenic bacteria. Methods of detection and staining of spores by Anjesky.
4. Capsule of bacteria, its biological role, constitution of encapsulated bacteria. Examples of bacteria making capsules in human organism.
5. A technique of staining by Burri-Gins.
6. Bacterial flagella. Methods of analysis of microorganisms in living condition ("Handing" and "crushed" drop).
7. Volutin granules (cytoplasm inclusions), its biological role. Staining of volutin granules by Neissers's method.
8. Staining of the acid-fast tuberculosis mycobacteria by Ziehl-Neelsen.
9. Spirochete, features of structure, function, classification (treponema, borrelia, leptospira), diseases, staining spirochetes by Romanowsky-Giemsa.
10. Position of rickketsiae in systematization of microorganisms. Morphology, structure, biological features, classification of rickketsiae, diseases. Staining of rickketsiae by Zdrodovsky.
11. Fungi: classification, structure, reproduction. The characteristic of penicillium, aspergillus and mucor. What are the methods of learning morphology and fungi structure?
12. Morphology and structure of yeast like fungus Candida-Method of staining.
13. Protozoa: morphology, structure, classification.
14. Method of staining by Romanowsky-Giemsa. Morphology of leichmania, trypanosome, entamoeba histolytica, toxoplasma, plasmodium malariae.

Morphology and structure of bacteria

1. Topic relevance

Bacteria are microscopic monocelled prokaryotic non-chlorophyll organisms, which duplicate by non-sexual way with cellular, but not organism properties.

The main groups of bacteria are distinguished by microscopic observation of their morphology and Gram staining reaction.

Knowledge of morphology and structure of bacteria helps to identify bacteria, understand influence of pathogenic bacteria on patient organism and find targets for antimicrobial therapy.

2. Educational objectives

Students must know:

1. Structure of microbial cell.

2. Classification of bacteria according morphological features

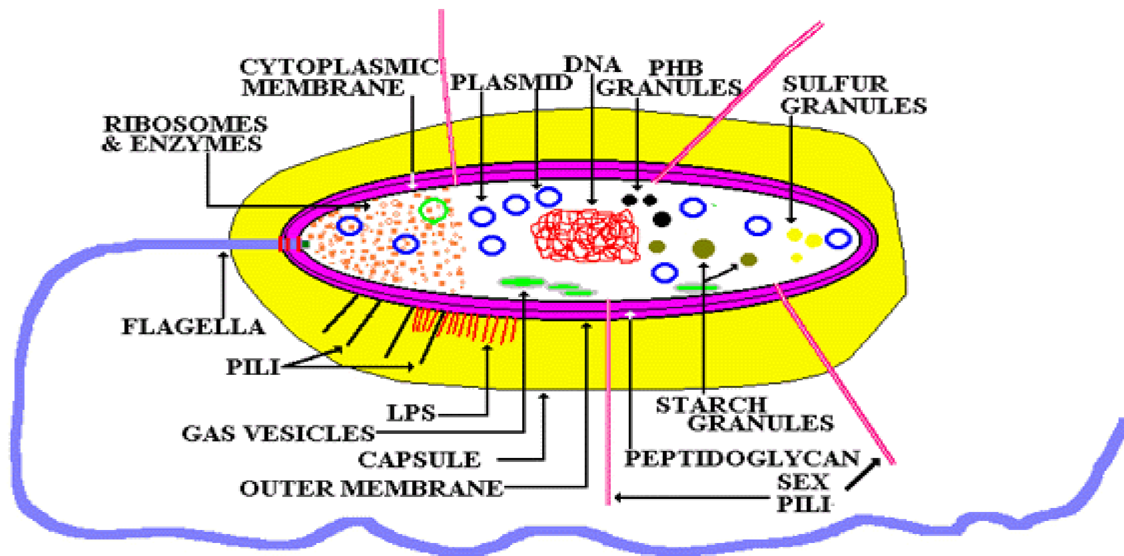
3. Composition and functions of essential structures of bacterial cell (nucleoid, cytoplasm, cytoplasm membrane, cell wall, ribosome, mesosomes) and non essential (capsules, spores, flagella, pili, inclusions).

4. Methods of staining of them (by Gram, Loeffler, Jone, Ziehl-Neelsen)

Students should be able to:

1. Prepare fix smear, wet-mount preparation.
2. Stain by Gram
3. Make conclusions about shape and arrangement of bacteria

Schematic structure of bacteria



Essential structure	Function
Cell wall with peptidoglycan	Gives rigid support, protect against osmotic pressure
Cytoplasmic membrane	Site of oxidative and transport enzymes
Cytoplasm	Motionless colloid system bounding all structures
Ribosome	Protein synthesis
Nucleoid	Contains genetic material
Mesosome	Participates in cell division and secretion
Periplasm	Contains many hydrolytic enzymes

Comparison of prokaryotic and eukaryotic cells

FEATURES OF CELLS	PROKARYOTIC	EUKARYOTIC
Cytoplasmic membrane	Yes	Yes
Nucleus containing a nuclear membrane surrounding DNA	No	Yes
DNA associated with	Polyamines	Histone proteins
Chromosome number	1	More then 1
Ribosomes	70S	80S
Cell wall containing of peptidoglican	Yes	No
Membrane-bound organelles (mitochondria, lysosomes)	No	Yes
Endoplasmic reticulum	No	Yes
Golgi apparatus	No	Yes
Mitotic division	No	Yes

Endospore stain

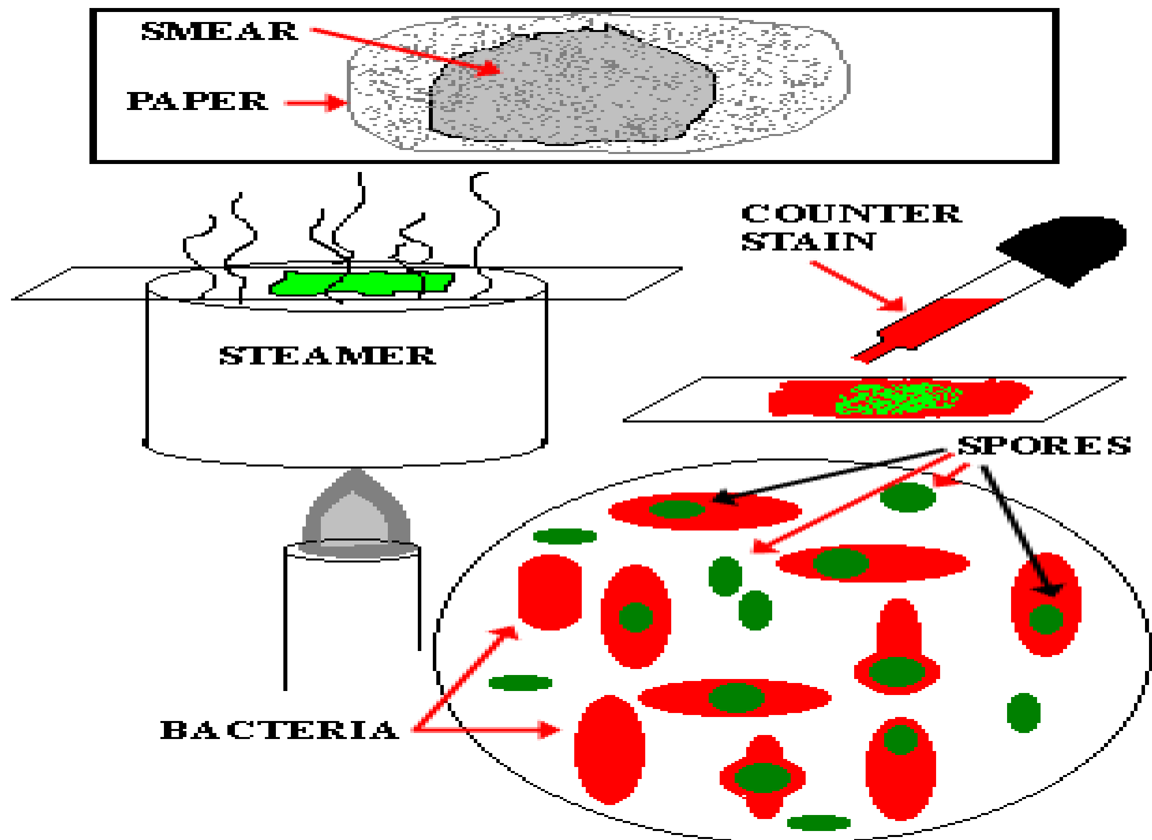
Ziehl-Neelsen staining is intended for detecting acid-fast bacteria and spores. Dye is forced by heat into resistant bodies called spores or endospores.

This stain is designed to distinguish between spores and the cells that they come from (so-called vegetative cells) Procedure includes the following stages***:***

1. *Put a slip of filter paper on a fixed smear and pour Ziehl's phenol fushsin on it. Heat the smear over the flame until the steam rises, then draw it aside for cooling and add a new portion of the dye. Repeat heating 2-3 times.*
2. *Allow the smear to cool, take off the filter paper, and wash the preparation with water.*
3. *Decolorize the preparation with 5% solution of sulfuric acid and wash several times with water.*

4. Stain the preparation with aqueous-alcoholic solution of methylene blue for 3-5 minutes, wash with water and dried.

Upon staining by Ziehl-Neelsen spores acquired bright red color, while the vegetative parts are stained light- blue.



Capsule staining

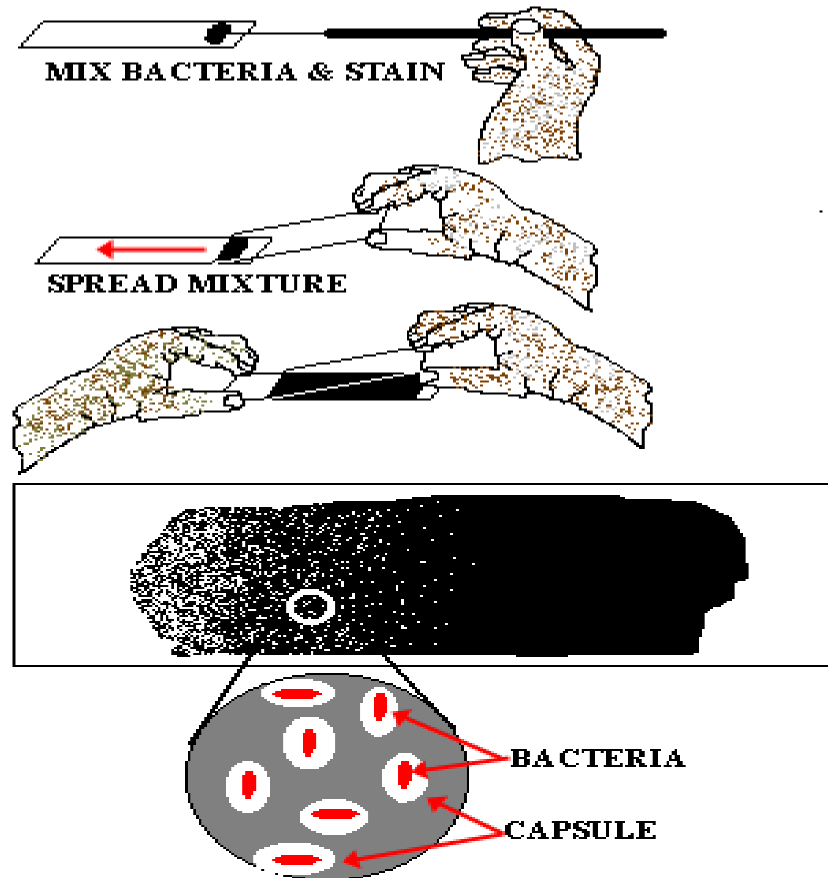
It is method of observing the microbial capsule, an unstructured protective laye surrounding the cells of some bacteria and fungi.

Ione staining

1. Cover the dry but not heat- fixed smear from pure culture of capsule-produced bacteria with the 2% aqueous solution of crystal violet (or methyl violet) to act for about 2 minutes.
2. Apply the 2% acetic acid for 10 seconds.

3. Wash with water, dry, and examine with oil immersion objective

Background is violet, cells are violet, and capsules are light violet, surrounded cells.



Flagella staining

Loeffler staining

Because the width of bacterial flagella lies beyond the resolving power of the light microscope, in order to be seen, they must be enlarged by depositing a coating on the outside of the filament and then staining it.

This stain works best with fresh, young cultures, since flagella are delicate and can be lost or damaged on older cells.

Loeffler staining uses special treatment with mordant (solution tannin and basic fuchsine) and next treatment with carbol solution fuchsine.

Volutin inclusion stain

On fixed smear pour alkaline methylene blue to act for 3-5 minutes, wash with water dry with filter paper, and examine under the microscope.

The cytoplasm of diphtheria corynebacteria is stained light- blue, while volutin granules are dark-blue.

Morphology of fungi, actinomycetes, spirochetes

Actinomycetes are unicellular microorganisms, which belong to class Bacteria, the order Actinomycetales.

They persist in the soil freely and another objects of environment, have typical bacterial ultrastructure, tendency to branching, some of special produce some antibiotics. They are the causative agents of actinomycoses.

The Kingdom ***Myceteaes (Fungi)*** is composed of non-photosynthetic haploid species with cell walls of chitin. The fungi are either saprobes (free living) or parasites. Economically beneficial as sources of antibiotics; several fungi cause infections or mycoses.

Spirochaetes are prokaryotic microorganisms, which belong to class Bacteria, the order Spirochaetales.

They are flexible, spiral - shaped microorganisms. They move by unique motility structures (axial filaments).

Medical important spirochaetes are found in genera Treponema, Borrelia and Leptospira. They causes spirochetoses (veneral syphilis (T.pallidum), endemic syphilis (T.pallidum subspecies endemicum), yaws (T. pertenue), pinta (T. carateum), relapsing fever (B.recurrentis), Lyme disease (B. hurgdorferi), leptospiroses (L. interorgans)).

Properties	Fungi	Bacteria
Nucleus	Eukaryotic; nuclear membrane; more than one chromosome; mitosis	Prokaryotic; no membrane; nucleoid; only one "chromosome"
Cytoplasm	Mitochondria; endoplasmic reticulum; 80S ribosomes	No mitochondria; no endoplasmic reticulum; 70S ribosomes
Cytoplasmic membrane	Sterols (ergosterol)	No sterols
Cell wall	Glucans, mannans, chitin, chitosan	Murein, teichoic acids (Gram-positive), proteins
Metabolism	Heterotrophic; mostly aerobes; no photosynthesis	Heterotrophic; obligate aerobes and anaerobes, facultative anaerobes
Size, mean diameter	Yeast cells: 3–5–10 µm. Molds: indefinable	1–5 µm
Dimorphism	In some species	None

Mycology is the study of fungi. Approximately 80,000 species of fungi have been described, but fewer than 400 are medically important, and less than 50 species cause more than 90% of the fungal infections of humans and other animals.

Rather, most species of fungi are beneficial to humankind. They reside in nature and are essential in breaking down and recycling organic matter. Some fungi greatly enhance our quality of life by contributing to the production of food and spirits, including cheese, bread, and beer.

Other fungi have served medicine by providing useful bioactive secondary metabolites such as antibiotics (eg, penicillin) and immunosuppressive drugs (eg, cyclosporine).

Fungi have been exploited by geneticists and molecular biologists as model systems for the investigation of a variety of eukaryotic processes. Fungi exert their

greatest economic impact as phytopathogens; the agricultural industry sustains huge crop losses every year as a result of fungal diseases of rice, corn, grains, and other plants.

All fungi are eukaryotic organisms, and each fungal cell has at least one nucleus and nuclear membrane, endoplasmic reticulum, mitochondria, and secretory apparatus. Most fungi are obligate or facultative aerobes. They are chemotrophic, secreting enzymes that degrade a wide variety of organic substrates into soluble nutrients which are then passively absorbed or taken into the cell by active transport.

Fungal infections are **mycoses.** Most pathogenic fungi are exogenous, their natural habitats being water, soil, and organic debris. The mycoses with the highest incidence—candidiasis and dermatophytosis—are caused by fungi that are part of the normal microbial flora or highly adapted to survival on the human host. For convenience, mycoses may be classified as superficial, cutaneous, subcutaneous, systemic, and opportunistic Grouping mycoses in these categories reflects their usual portal of entry and initial site of involvement.

However, there is considerable overlap, since systemic mycoses can have subcutaneous manifestations and vice versa.

Most patients who develop opportunistic infections have serious underlying diseases and compromised host defenses. But primary systemic mycoses also occur in such patients, and the opportunists may also infect immunocompetent individuals.

During infection, most patients develop significant cellular and humoral immune responses to the fungal antigens.

Fungi grow in two basic forms, as **yeasts** and **molds** (or **moulds).** Growth in the mold form occurs by production of multicellular filamentous colonies. These colonies consist of branching cylindric tubules called **hyphae,** varying in diameter from 2 mkm to 10 mkm. The mass of intertwined hyphae that accumulates during active growth is a **mycelium.** Some hyphae are divided into cells by cross-walls or **septa,** typically forming at regular intervals during hyphal growth. One group of

medically important molds, the zygomycetes, produces hyphae that are rarely septated.

Hyphae that penetrate the supporting medium and absorb nutrients are the vegetative or substrate hyphae. In contrast, aerial hyphae project above the surface of the mycelium and usually bear the reproductive structures of the mold. Under standardized growth conditions in the laboratory, molds produce colonies with characteristic features such as rates of growth, texture, and pigmentation.

The genus—if not the species—of most clinical molds isolated can be determined by microscopic examination of the ontogeny and morphology of their asexual reproductive spores, or conidia

Yeasts are single cells, usually spherical to ellipsoid in shape and varying in diameter from 3 mkm to 15 mkm. Most yeasts reproduce by budding. Some species produce buds that characteristically fail to detach and become elongated; continuation of the budding process then produces a chain of elongated yeast cells called **pseudohyphae.** Yeast colonies are usually soft, opaque, 1–3 mm in size, and cream-colored. Because the colonies and microscopic morphology of many yeasts are quite similar, yeast species are identified on the basis of physiologic tests and a few key morphologic differences. Some species of fungi are dimorphic and capable of growth as a yeast or mold depending on environmental conditions.

All fungi have an essential rigid cell wall that determines their shape. Cell walls are composed largely of carbohydrate layers—long chains of polysaccharides—as well as glycoproteins and lipids.

During infection, fungal cell walls have important pathobiologic properties. The surface components of the cell wall mediate attachment of the fungus to host cells. Cell wall polysaccharides may activate the complement cascade and provoke an inflammatory reaction; they are poorly degraded by the host and can be detected with special stains. Cell walls release immunodominant antigens that may elicit cellular

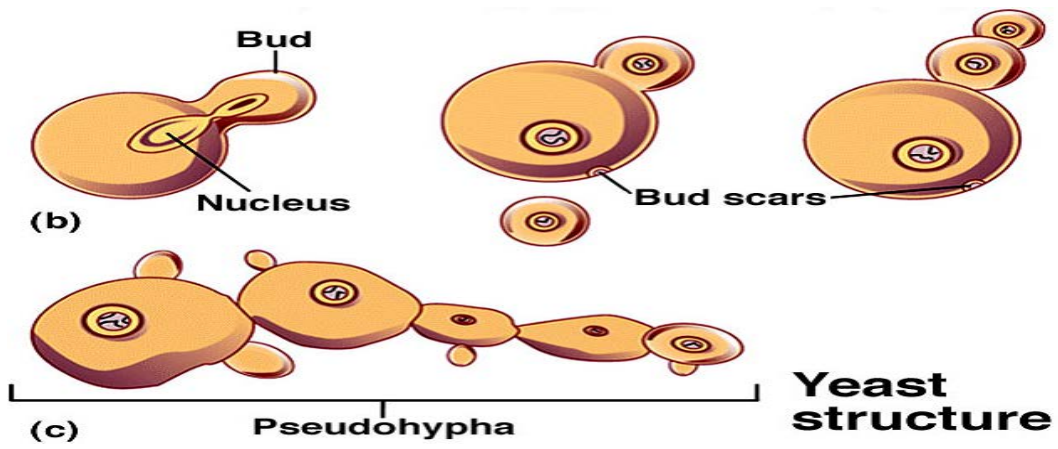
immune responses and diagnostic antibodies. Some yeasts and molds have melanized cell walls, imparting a brown or black pigment. Such fungi are **dematiaceous.** In several studies, melanin has been associated with virulence.

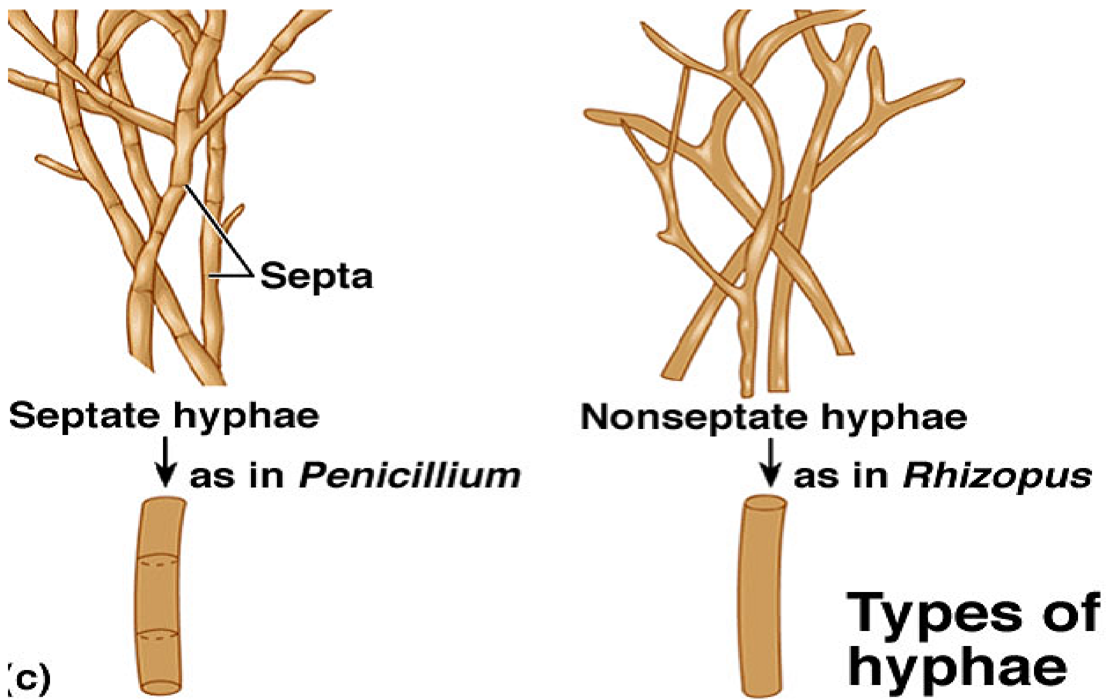
In addition to their vegetative growth as yeasts or molds, fungi can produce spores to enhance their survival. Spores can be readily dispersed, are more resistant to adverse conditions, and can germinate when conditions for growth are favorable. Spores can derive from asexual or sexual reproduction—the anamorphic and teleomorphic states, respectively. Asexual spores are mitotic progeny (ie, mitospores) and genetically identical.

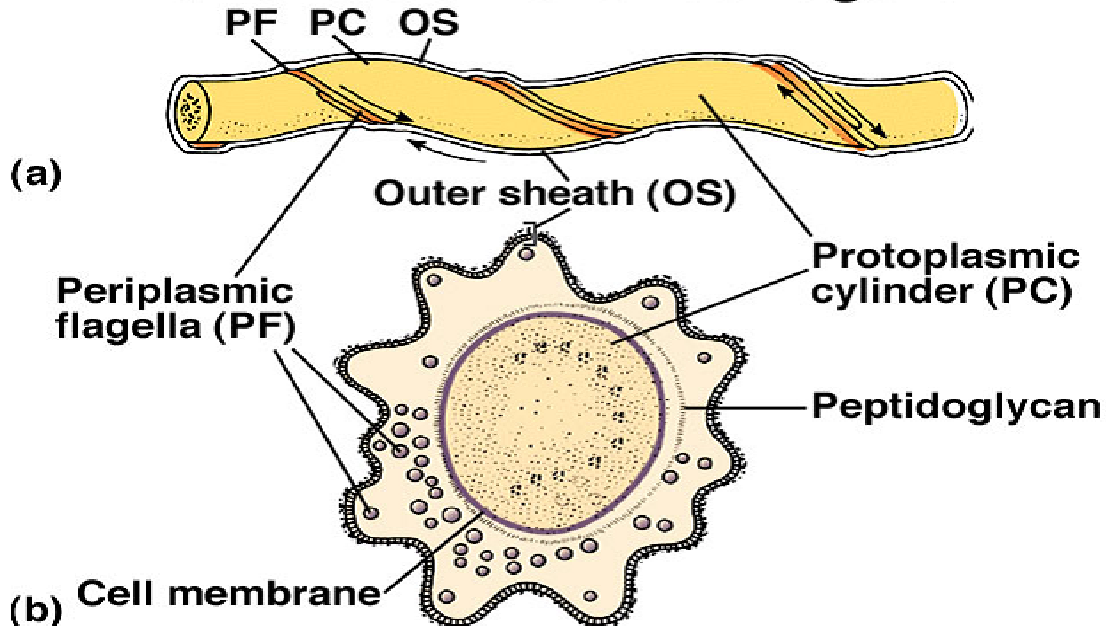
The medical fungi produce two major types of asexual spores, **conidia,** and, in the zygomycetes, **sporangiospores.** Informative features of spores include their ontogeny (some molds produce complex conidiogenic structures) as well as their morphology (size, shape, texture, color, and unicellularity or multicellularity). In some fungi, vegetative cells may transform into conidia (eg, arthroconidia, chlamydospores).

In others, conidia are produced by a conidiogenous cell, such as a phialide, which itself may be attached to a specialized hypha called a conidiophore. In the zygomycetes, sporangiospores result from mitotic replication and spore production within a sac-like structure called a sporangium, which is supported by a sporangiophore.

Major groups: The four important subgroups among the true fungi that given by sexuel spores type, are: Zygomycota(zygospores), Ascomycota(ascospores), Basidomycota (basidiospores), and Deuteromycota(no sexual spores).


Bud
Nucleus
(b)
Bud scars
(c)
Pseudohypha
Yeast
structure


Septa
Septate hyphae
as in Penicillium
Nonseptate hyphae
as in Rhizopus
(c)
Types of
hyphae



Practical lesson # 3

Theme: *Bacteriological method of research.* **Nutrition of bacteria. Nutrient media and methods of bacteria cultivation. Methods of sterilization. Asepsis and antiseptic. Disinfection. Chemotherapy. Chemotherapeutic preparations.**

Questions for the learning.

1. Chemical structure of bacteria.
2. Classification of bacteria according to the type of nutrition. Features and mechanism of nutrition.
3. The basic principles of the bacteria cultivation.
4. Nutrient media for bacteria, classification, requirements. Differential-diagnostic nutrient media.
5. Methods of sterilization. The apparatus for sterilization. Principles of structure and work of autoclave, thermostat (incubator). Dry-heat sterilizer, hot-air oven, e.t.c.
6. The influence of the chemical matter on the microorganisms.

7. Disinfection.The main disinfectants and antiseptics used in the medical practice. Asepsis and antiseptic.

8. The definition of the terms “Chemotherapy”, “Chemotherapeutics drugs”.

9. The effect of chemotherapeutics drugs on microorganisms. The classification of chemotherapeutics drugs.

Bacterial physiology.
Nutrient media for cultivation of bacteria.
Sterilization and disinfection.

Topic relevance

Bacterial physiology is important part of general microbiology, which study metabolisms, nutrition, growth, multiply, respiration, cultivation of bacteria and other. The choice of the method of cultivation and the composition of the nutrient medium largely depends on the type of nutrition and respiration of microorganism to be investigated.

Students should be able to:

Make conclusion about hemolytic activity of bacteria on blood agar.
Make conclusion about ability of bacteria to ferment carbohydrates on Giss,
Endo, Ploskirev media.
Choose proper media for different bacteria.
Prepare simple media
Prepare the material and laboratory equipments for sterilization;
Carry out of a sterilization control
Choose proper method of sterilization of different materials

Chart of topic content

Media can be classified on three primary levels:
(1) physical form, (2) chemical characteristics, and (3) functional type.

Liquid media are defined as water-based solutions that do not solidify at temperatures above freezing and that tend to flow freely when the container is tilted. These media, termed broths, milks, or infusions, are made by dissolving various solutes

in distilled water. Growth occurs throughout the container and can then present a dispersed cloudy or particulate appearance. A common laboratory medium, nutrient broth contains beef extract and peptone dissolved in water. Methylene blue milk and litmus milk are opaque liquids containing whole milk and dyes.

Fluid thioglycollate is a slightly viscous broth used for determining oxygen utilization of bacteria. At ordinary room temperature, **semisolid media** exhibit a clotlike consistency. This is because they contain an amount of solidifying agent (agar or gelatin) that thickens them but does not produce a firm substrate.

Semisolid media are used to determine the motility of bacteria and to localize a reaction at a specific site. Both motility test medium and SIM contain a small amount (0.3-0.5%) of agar. The medium is stabbed carefully in the center and later observed for the pattern of growth around the stab line. In addition to motility, SIM is used to test for physiological characteristics used in identification (hydrogen sulfide production and indole reaction).

Solid media provide a firm surface on which cells can form discrete colonies and are advantageous for isolating and subculturing bacteria and fungi. They contain a solidifying agent that is thermoplastic—its physical properties change in response to temperature. By far the most widely used and effective of these agents is **agar,** a complex polysaccharide isolated from the red alga *Gelidium.* The benefits of agar are numerous. It is solid at room temperature and most incubation temperatures, and it melts (liquefies) at the boiling temperature of water (100°C).

Once liquefied, agar does not resolidify until it cools to 42°C, allowing it to be inoculated and poured in liquid form at temperatures (45°-50°C) that will not harm the microbes or the handler. Agar is flexible and moldable, and it provides a basic framework to hold moisture and nutrients, though it is not itself a digestible nutrient for the vast majority of microorganisms.

Any medium containing 1% to 5% agar usually has the word agar in its name. Nutrient agar is a common one. Like the broth, it contains beef extract and peptone, as well as 1.5% agar by weight. Many of the examples covered in the section on functional

categories of media contain agar. Although gelatin is not nearly as satisfactory as agar, it will create a reasonably solid surface in concentrations of 10% to 15% (but it probably will not remain solid).

Media whose compositions are chemically defined are termed **synthetic.** Such media contain highly pure organic and inorganic compounds that vary little from one source to another and have a molecular content specified by means of an exact formula. Synthetic media come in many forms. Some media such as minimal media for fungi contain nothing more than a few essential compounds such as salts and amino acids dissolved in water.

Others contain a variety of defined organic and inorganic chemicals. Such standardized and reproducible media are most useful in research and cell culture, where the exact nutritional needs of the test organisms are known. If even one component of a given medium is not chemically definable, the medium belongs in the next category.

Complex, or **nonsynthetic, media** contain at least one ingredient that is *not* chemically definable — not a simple, pure compound and not representable by an exact chemical formula. Most of these substances are extracts of animals, plants, or yeasts, including such materials as ground-up cells, tissues, and secretions. Examples are blood, serum, and meat extracts or infusions. Infusions are high in vitamins, minerals, proteins, and other organic nutrients. Other nonsynthetic ingredients are milk, yeast extract, soybean digests, and peptone. Peptone is a partially digested protein, rich in amino acids, that is often used as a carbon and nitrogen source. Nutrient broth, blood agar though different in function and appearance, are all nonsynthetic media. They present a rich mixture of nutrients for microbes with complex nutritional needs.
Microbiologists have many types of media at their disposal, with new ones being devised all the time.

Depending upon what is added, a microbiologist can fine tune a medium for nearly any purpose. As a result, only a few species of bacteria or fungi cannot yet be cultivated artificially. Media are used for primary isolation, to maintain cultures in the

lab, to determine biochemical and growth characteristics, and for numerous other functions. Note that some media can serve more than one function.

For example, a medium such as brain-heart infusion is general purpose and enriched; yolk salt agar is both selective and differential; and blood agar is both enriched and differential.

General purpose media are designed to grow as broad a spectrum of microbes as possible. As a rule, they are nonsynthetic and contain a mixture of nutrients that could support the growth of pathogens and nonpathogens alike. Examples include nutrient agar and broth, brain-heart infusion contains partially digested milk protein (casein), soybean digest, NaCl, and agar.

An **enriched medium** contains complex organic substances such as blood, serum, hemoglobin, or special **growth factors** (vitamins, amino acids) that certain species must have in order to grow. Bacteria that require growth factors and complex nutrients are termed fastidious**.** For example, the fastidious species ***Streptococcus pneumonia*** (a cause of pneumonia) is cultured on blood agar, which is made by adding sterile sheep, horse, or rabbit blood to a sterile agar base. Pathogenic ***Neisseria*** (one species causes gonorrhea) are grown on chocolate agar, which is essentially cooked blood agar.

Selective and Differential Media

Some of the most clever and inventive media belong to the categories of selective and differential media. These media are designed for special microbial groups and they have extensive applications in isolation and identification. They can permit, in a single step, the preliminary identification of a genus or even a species.

A selective medium contains one or more agents that inhibits the growth of a certain microbe or microbes (A, B, C) but not others (D), and thereby encourages or *selects* microbe D and allows it to grow. Selective media are very important in primary isolation of a specific type of microorganism from samples containing a highly mixed population—for example, feces, saliva, skin, water, and soil. They hasten isolation by suppressing the background organisms and favoring growth of the desired ones.

Yolk salt agar (YSA) contains a concentration of NaCl (7.5%) that is quite inhibitory to most human pathogens.

One exception is the genus ***Staphylococcus,*** which grows well in this medium and consequently can be amplified in very mixed samples. Bile salts, a component of feces, inhibit most gram-positive bacteria while permitting many gram-negative rods to grow. Media for isolating intestinal pathogens (Ploskirev's medium, MacConkey agar, eosin-methylene blue [EMBJ agar) contain these salts as a selective agent. Dyes such as methylene blue and crystal violet also inhibit certain gram-positive bacteria.

Other agents that have selective properties are antimicrobic drugs and acid. Some selective media contain strongly inhibitory agents to favor the growth of a pathogen that would otherwise be overlooked because of its low numbers in a specimen. Selenite and brilliant green dye are used in media to isolate ***Salmonella*** from feces, and tellurite is used to isolate oral streptococci from saliva.

A differential medium can grow several types of microorganisms, but it is designed to highlight differences among these microorganisms. Differentiation shows up as variations in colony size and color, in media color changes, and in the formation of gas bubbles and precipitates. The disparities in appearance are due to the type of agents added and the way the cells react to them. For example, when microbe X metabolizes a certain substance not used by organism Y, then X will cause a visible change in the medium and Y will not.

The simplest differential media demarcate two reaction types— showing the use or nonuse of a particular nutrient, or one colony reacting with a dye and the other one not Dyes serve as differential agents by acting as pH indicators, changing color in response to the production of an acid or base. For example, Endo medium contains lactose and fucshin , a dye that is light red (pink) when neutral and dark red(cherry) when acidic. A common intestinal bacterium like *Escherichia coli* that gives off acid when it metabolizes lactose develops dark red colonies, and one like *Salmonella* that does not give off acid remains its natural color (off-white)

A reducing medium contains a substance (thioglycollie acid or cystine) that absorbs oxygen or slows the penetration of oxygen in a medium, thus reducing its availability. Reducing media are important for growing anaerobic bacteria or determining oxygen requirements

Transport media are used to maintain and preserve specimens that have to be held for a period of time prior to clinical analysis, or to sustain delicate species that die rapidly if not held under stable conditions. Stuart's transport medium contains salts, buffers, and absorbants to prevent cell destruction by enzymes, pH changes, and toxic substances, but will not support growth.

Main demands to media. Nutrient media should be easily assimilable, and they should contain a known amount of nitrogen and carbohydrate substances, vitamins, a. required salt concentration In addition they should be isotonic, and sterile, and they should have buffer properties, an optimal viscosity, and a certain oxidation-reduction potential

Sterilization means the destruction of all kinds and forms of microorganisms present in a material. The methods of sterilization are used in a microbiology laboratory, surgical practice and hospital service, pharmacies, etc. Nutrient media, glassware, containers and instruments used for the collection of specimens and isolation of organisms have to be sterile before using.

Disinfection is the process of reducing or eliminating pathogenic microorganisms or viruses in or on material so that it no longer presents a hazard. Disinfection refers to the use of a physical process or a chemical agent (a disinfectant) to destroy vegetative pathogens but not bacterial endospores. It is important to note that disinfectants are normally used only on inanimate objects because, in the concentrations required to be effective, they can be toxic to human and other animal tissue. Disinfection processes also remove the harmful product of microorganisms (toxins) from materials.

Antisepsis is the term used to describe disinfection applied to living tissues such as a wound.

Pasteurization is the process of heating food or other substances under controlled conditions of time and temperature to kill pathogens and reduce the total number of microorganisms without damaging the substance.

The most complete way to dispose of infectious materials is incineration, although precautions must be taken to prevent spilling and to assure that everything is fully burned. Things that cannot be incinerated are sterilized to free them not only of pathogens but of all living organisms. For dry materials, glassware, syringes, dressings, filters, and pipettes, this may be done in a sterilizer oven at 165 °C for 2 hours This treatment destroys fungi, bacteria, spores, and viruses.

Program for self- study training

1. Constructive and power metabolism of bacteria, their interrelation.

2. To name types of a bacteria nutrition and to give examples:

a - on a source of Carbon;

b - on a source of nitrogen;

c - on a source of energy and donors of electrons;

3. To describe the mechanism of nutrition:

a -passive diffusion, facilitated diffusion, active transport;

b -the role of permeases in metabolism of bacteria;

c - the role of cytoplasm membrane in metabolism of bacteria.

4. Main growth factors of bacteria.

5. Enzymes of bacteria, their feature:

a - classification;

b - function;

c - practical usage.

6. Physical and environmental requirements of bacterial growth:

a. effect of oxygen
b. effect of pH
c. effect of temperature

7. To describe media:

a - classification of media;

b - main demands to media.

8. Methods of a biological objects decontamination. Sterilization, pasteurization, disinfection, asepsis, antiseptic.

9. Methods of sterilization. Sterilization by moist heat (steam under pressure;live, non pressirized steam; boiling water; pasterization). Sterilization by dry heat(incineration, hot air oven). Ionizing and UV radiation. Sterilization by filtrating ("cool sterilization").

10. Controls of sterilization.

Practical lesson # 4

***Theme*: Growth and reproduction of bacteria. Methods of isolation and cultivation of pure cultures of aerobes. Respiration of bacteria. Methods of isolation and cultivation of anaerobes. Biochemical properties of microorganisms.**

Questions for the learning.

1. Growth and reproduction of bacteria, phases of reproduction. Methods of isolating pure cultures of aerobes.
2. What are the necessary conditions for the cultivation of the bacteria?
3. Classification of the bacteria according to the types of respiration.
4. Mechanism of respiration. Cultivation methods of strict anaerobes.
5. Enzymes involving in respiration of the bacteria.
6. Methods of isolating pure cultures of anaerobes.
7. Classification of enzymes. Enzymes, which are important for the identification and species differentiation of the microorganisms. Methods of learning of the bacterial enzymatic activity. Enzymes of pathogenicity.
8. Methods of definition of saccharolytic and proteolytic enzymes.
9. Role of the enzymes in live activity of bacteria. Practical usage of the enzymes.

Isolation of pure culture (part 1)

Topic relevance

In nature, bacteria exist as a mixed population of various genera and species that have became adapted to particular environment. But in laboratory the various species may be separated one from other and cultivated separately. A culture, which contains just one species of microorganisms, is called a pure culture.

The process of obtaining a pure culture by separating one species of microorganism is spoken of as isolation of organism. Isolation of causative organism in pure culture (bacteriological method) is the most available method of laboratory diagnosis.

Isolation and identification of bacteria from patients aids treatment since infectious diseases caused by different bacteria have a variety of clinical courses and consequences. Susceptibility testing of isolates (i.e. establishing the minimal inhibitory concentration or MIC) can help in selection of antibiotics for therapy. Recognizing that certain species (or strains) are being isolated atypically may suggest that a disease outbreak has occurred e.g. from contaminated hospital supplies or poor aseptic technique on the part of hospital personnel.

This method is used in medical, pharmaceutical (antibiotic production, preparation of antigens for serological tests, vaccines, serums and other), and food industry (bread, wine, cheese, yoghurt production and other).

Pure culture – a population of microorganisms belonged to one species and cultivated on (in) nutrient media.

Pure culture – a population of microorganisms isolated on nutrient medium (predominately solid) that shares morphological, tinctorial, cultural, biochemical and antigenic properties.

Species – a group of microorganisms that has high level of DNA similarity (70%), shares phenotype properties and derived from common ancestor.

Colony – a population of bacterial cells of same species which have grown from one bacterial cell on solid medium isolated accumulation.

Clone – a group of cells derived from a single cell.

Pasteur developed the concepts of pure culture. This was an important intellectual step forward because the ability to work with pure cultures allowed Pasteur (and subsequent microbiologists) to study individual bacterial species.

Pasteur's method for developing pure culture- repeatedly diluting broth cultures until he thought he had individual species was very inefficient.

Robert Koch, in 1880s, developed the procedure of diluting in solid media (it is unknown in gelatin or agar). The purpose of this procedure was to obtain isolated colonies. Remember that an isolated colony is populations of millions of cells that are identical and are descendent from single founder cell.

Koch could pick cells from the single colony to inoculate into new nutrient medium. This process presented a powerful new way to develop pure culture.

Drigalsky method based on repeatedly mechanical spreading of specimen on surface of agar by **Drigalsky** spatula (glass spreader) using set of agar plates.

Streak method used only one Petri dish making streaks in separated sectors (see below) This method consists of spreading source material over an agar surface until one microorganism at a time falls off of the loop.

The medium is then incubated until colonies arise. Theoretically, each colony represents a single type of microorganism that originated from a single cell.

Single Cell Isolation method

An individual cell of the required kind is picked out by this method from the mixed culture and is permitted to grow.

Micromanipulator method.

Micromanipulators have been built, which permit one to pick out a single cell from a mixed culture. This instrument is used in conjunction with a microscope to pick a single cell (particularly bacterial cell) from a hanging drop preparation.

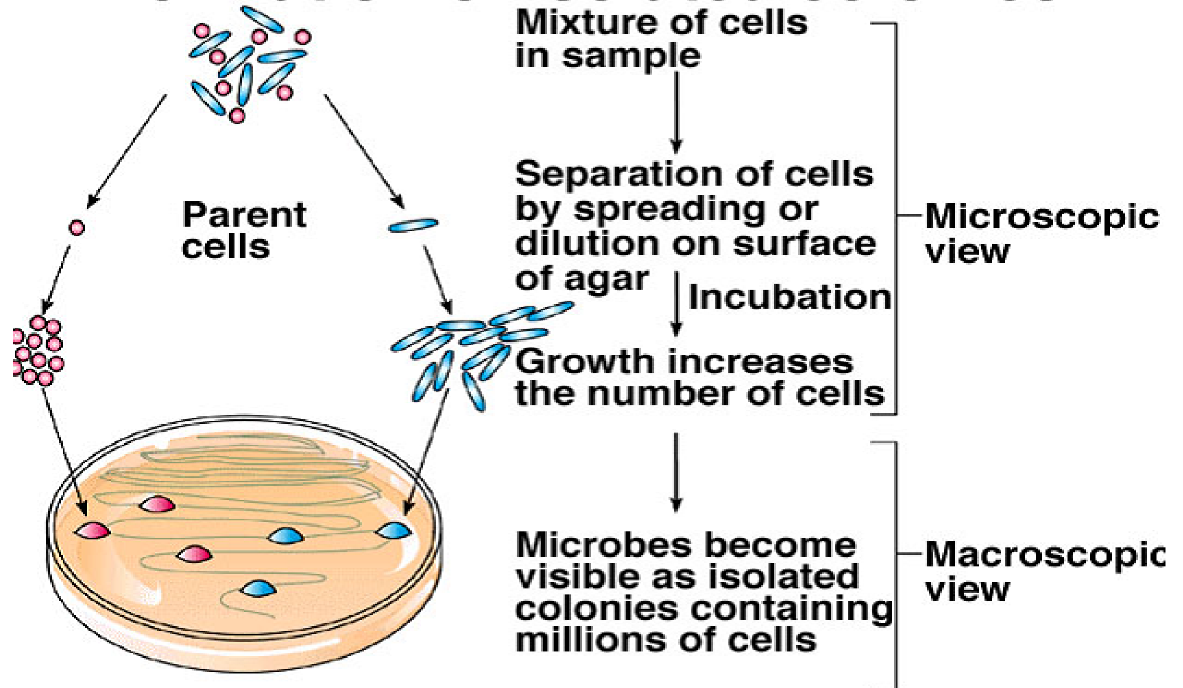
The advantages of this method are that one can be reasonably sure that the cultures come from a single cell and one can obtain strains with in the species.

The disadvantages are that the equipment is expensive, its manipulation is very tedious, and it requires a skilled operator.

This is the reason why this method is reserved for use in highly specialized studies.

Main stages of pure culture isolation

Stages	Objective
Taking of specimen from patient Inoculation of solid media	To take a specimen, in which there is causative agent of this disease, to save it and not to infect people To have isolated colonies
Investigation of isolated colonies Examine cultural properties Microscopic examination (staining by Gram) Subinoculation of isolated colony onto solid media (or liquid) media	Select isolated colony(suspected) Checking up purity of the isolated culture and study morphological and tinctorial properties Accumulate pure culture
Examine cultural properties and Microscopic examination (staining by Gram)	To be sure that culture not contaminated and conduct morphological, tinctorial and cultural identification



Streak agar procedure

Necessary equipment

Test tube with mixture of bacteria. It is used a source of cells from which to inoculate new cultures.

CAUTION: Always hold the glass test tube (not the lid) when carrying them.
Bunsen Burner
Inoculating Loop
Test Tube Rack
Sterile Agar Plate

1. Draw lines on the bottom of the agar plate to visually separate the plate into four sectors.

2. Sterilize the transfer loop before obtaining a specimen.

3. Remove the test tube cap. It is recommended that the cap be kept in your right hand (the hand holding the sterile loop). Curl the little finger of your right hand

around the cap to hold it or hold it between the little finger and third finger from the back. Open the culture and collect a sample of specimen using the sterile loop.
Insert the loop into the culture tube and remove a loopful of broth.
Replace the cap of the test tube and put it back into the test tube rack.

4. Streak the first sector. Hold the inoculation loop handle in one hand as you would hold a pencil.
Open the lid of the agar plate enough to insert the loop and spread the inoculum over the surface of one quadrant of the agar plate.
Do not streak over previously streaked lines. The loop should glide over the surface of the agar; take care not to dig into the agar. Done properly, this process will result in a series of parallel lines at the top of the plate.

5. Flame and cool the inoculation loop. This will kill all the cells on the loop. This is important because we want to spread out the cells from sector 1. We don't want to add more cells to the plate.

6. Turn the plate 90 degrees counter-clockwise. Streak the second quadrant of the plate by touching the loop into the first quadrant and streaking all the way across the second quadrant.

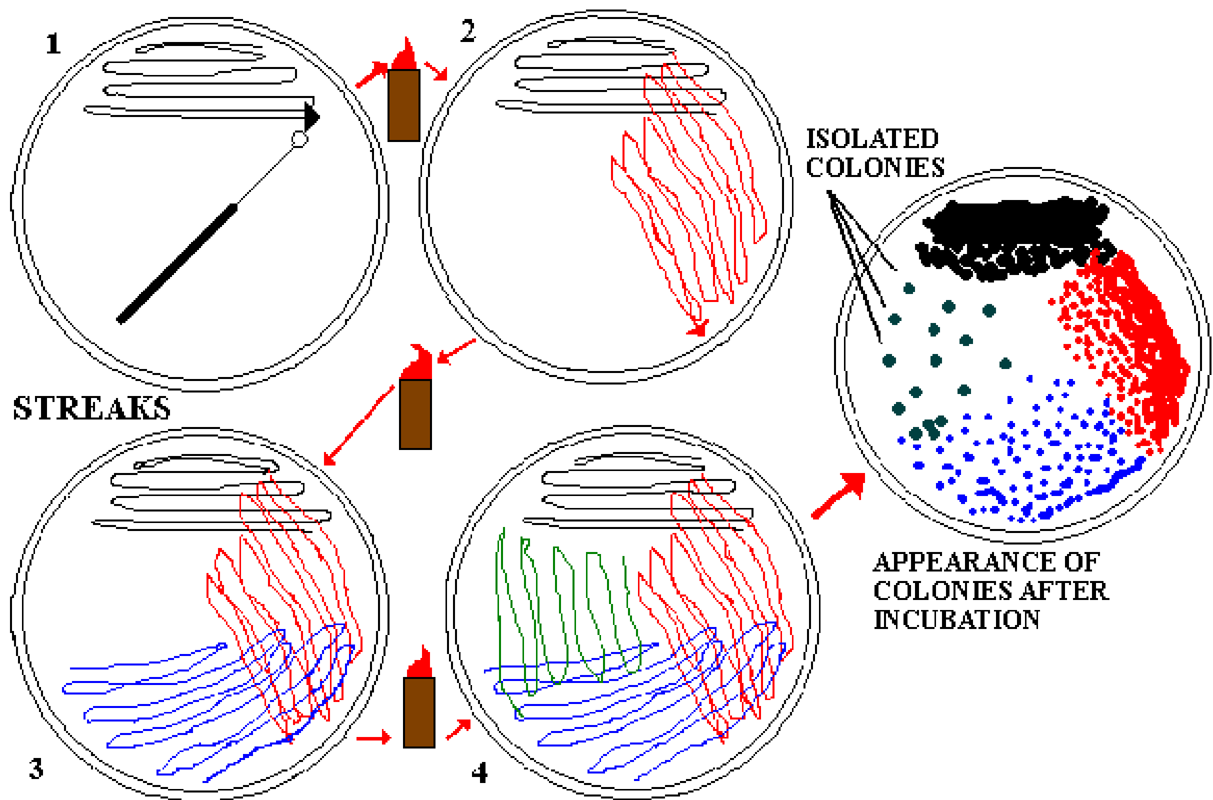
7. Flame and cool the inoculation loop.

8. Turn the plate 90 degrees counter-clockwise. Streak the third quadrant touching the loop into the second quadrant and streaking all the way across the third quadrant.

9. Flame and cool the inoculation loop.

10. Turn the plate 90 degrees counter-clockwise.

11. Streak the fourth quadrant in a same manner. Hold the agar plate in the opposite hand.



Isolation of pure culture (part 2)

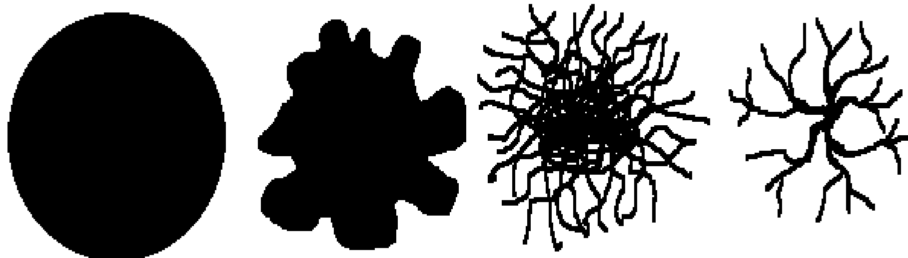
Topic relevance

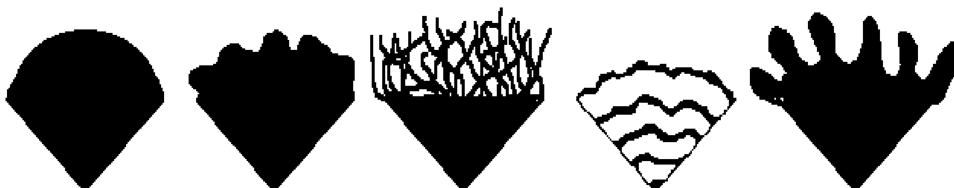
Colony isolation is essential technique for bacteriological method ("golden" standard of infectious diseases diagnosis).

Every species has specific features of growth on solid and liquid media. Colony investigation (macroscopic and microscopic) helps to determine a species (make preliminary diagnosis) and isolate pure culture (make final diagnosis).

Topic content

Colony characteristics





Dynamics of Bacterial Population Growth—Four Well-recognized Phases

A. Lag phase of growth involves no increase in cell numbers—the "tooling-up" phase.
B. Exponential phase of growth is the time during which cell number increases exponentially.
C. Stationary phase is reached when the numbers of viable cells stops increasing.
D. Death phase is characterized by an exponential decrease in the number of viable cells.

Practical lesson # 5

Theme*: Genetics of microorganisms. Methods of biotechnology and gene engineering.** ***Genetic method of diagnostics. **Polymerase chain reaction (PCR). Polymerase chain reaction with reverse transcriprase (RT-PCR). Polymerase chain reaction in the real time.**

Questions for the learning.

1. Stage of development of science about the variability and hereditary of microorganisms.
2. Modificational, non-hereditant forms of microbes variability. Hereditant forms of variability. Properties of the hereditant information. Mutations, their classification.
3. Organization of the genetic material in the bacterial cell. Genotype and phenotype.
4. Types of the genetic recombinations (transformation, transduction and conjugation), their role in variability of microorganisms. Plasmids.
5. Importance of genetics for the development of the medical microbiology.
6. Practical values of bacterial genetics for production of antibiotics, obtaining of live vaccines.
7. Polymerase chain reaction (PCR). Polymerase chain reaction with reverse transcriprase (RT-PCR).

Practical lesson # 6

***Theme*: Ecological microbiology. Microflora of an environment and the human body. Methods of sanitary bacteriological research. Antibiotics. Bacteriophages.**

Questions for the learning.

1. Characteristic of the antibiotics, the methods of their production, the principles of their classification,mechanism of their influence on the bacteria, demands to them.Methods of definition of microbe’s sensitivity to antibiotics.
2. Resistance of microbes to antibiotics. Factors determining the formation of the drug resistance of microbes.

3. Morphology, structure and other biological properties of the bacteriophage. Interaction between phages and bacterial cell.
4. Virulent and moderate phages. Lysogenic, phage conversion.
5. Usage of bacteriophages for microbial indication and identification, for therapy and prophylaxis.
6. Characteristic of the air, water and soil microflora contents.
7. What are the main microorganisms of the skin, mucous membranes and internal cavities microflora?
8. Methods of the environmental microflora examination.
9. The detection of the general microbial number, coli-titer, coli-index and the meaning of them for the sanitary-microbiological research.
10. Sanitary-indicative microorganisms of the air, water, soil.
11. Value of normal microflora of the human body.

Antibiotics

1. Topic relevance

Treatment of infectious diseases has obviously been a primary goal of medicine for centuries. Paul Ehrlich's search for the ''magic bullet'' that would kill an infectious agent without harming the patient was a systematic approach to the problem of antimicrobial therapy.

Antibiotics occupy a special place in the modern medicine; they are irreplaceable components of complex therapy of bacterial, fungal and tumor diseases.

At the same time, uncontrolled use of antibiotics and antimicrobial drugs can lead to develop multiple complications.

Doctor who uses chemotherapy should understand mechanisms of antibiotic actions, possible benefit and harm and prescribe them according balance of first and last.

2. Educational objectives

Students must know:

- Phenomenon of microbial antagonism, methods of microbial antagonism studying.
- Main groups of chemotherapeutic drugs and demands to them.
- Antibiotics, classification of antibiotics according to their origin, spectrum, mechanism of action, chemical structure, and mechanisms of their action
- Antimicrobial susceptibility testing (serial dilutions, disks agar diffusion)
- Main principles of a rational chemotherapy;
- Side effects of antibiotics, complications of chemotherapy.
- Mechanisms, which cause drug resistance.

Students should be able to:

- determine antibiotic sensitivity of bacteria by agar diffusion susceptibility testing;
- determine antibiotic sensitivity of bacteria by serial dilution method in liquid.

3. Topic content

Chemopreparations should have a specific action, a maximal therapeutic effectiveness, and a minimal toxicity for the body.

As a characteristic of the quality of a medicinal preparation, P. Ehrlich introduced the *chemotherapeutic index* which is the ratio of the maximal tolerated dose to the minimal curative dose:

Maximal tolerated dose (DT—Dosis tolerata)

-- > 3

Minimal curative dose (DC—Dosis curativa)

The chemotherapeutic index should not be less than 3.

Antibiotic - the chemotherapeutic preparation produced by living organisms or their synthetic analogs that can selectively inhibit disease agents or inhibit growth of tumor cells in patient organism

Antagonism (ammensalism). Mechanisms of antagonism:

- Competition for nutrient substrate (different spread of growth)
- Excretion of acids, alcohols, ammonia by microorganisms-antagonist
- Excretion antibiotics, bacteriocines by microorganisms-antagonist
- Predation

Antibiotics are classified according to the chemical structure, the molecular mechanism, and the spectrum of activity.

Mechanisms of action

Inhibition of cell wall synthesis: penicillin, cephalosporin, vancomycin

Inhibition of protein synthesis:

drugs that act on the 30S subunit: streptomycin, tetracycline

drugs that act on the 50S subunit: chloramphenicol, erythromycin, clindamycin

Inhibition of nucleic acid synthesis:

inhibition of DNA synthesis: norfloxacin

inhibition of mRNA synthesis: rifampin

Alteration of cell membrane function:

Bacterial: *polymyxin*

Fungal: *amphotericin*

Uncertain mecanisms: izoniazid, metroninazole

According to origin, antibiotics are subdivided into the following groups.

Antibiotics produced by fungi.

1. *Penicillin* is prodused by the fungi *Penicillium notatum, Penicillium chrysogenum*

2. *Cephalosporin* was isolated from *Cephalosporium acremonium*

Antibiotics produced by actinomycetes. 1. *Streptomycin* is obtained from *Streptomyces griseus.*

2. *Chloramphenicol* is obtained from the cultural fluid of a strain of *Streptomyces venezuelae,* isolated from the soil in tropical South America.

3. *Chlortetracycline* (biomycin, aureomycin) is produced by *Streptomyces aureofaciens..*

4. *Tetracycline* is a derivative of chlortetracycline. It is obtained by reductive dechlorination of chlortetracycline5. *Oxytetracycline* (terramycin) is obtained from *Streptomyces rimosus.* In spectrum and mode of action it is close to chlortetracycline. Rondomycin (6-methyl-5-hydroxytetracycline) is a homologue of oxytetracycline. It is absorbed rapidly. Rondomycin possesses a broad spectrum of action (suppresses Gram-positive and Gram-negative bacteria, i. e. cocci, *Salmonella* organisms, *Shigella* organisms, pathogenic *E. coli* serotypes) and is administered per os.

6. *Erythromycin* is obtained from *Streptomyces erythraeus.*

7. *Neomycin* has been isolated from *Streptomyces fradiae.* 8. *Nystatin* has been extracted from the cultural fluid of *Streptomyces noursei.* It inhibits many

pathogenic fungi and some pathogenic protozoa. It is non-toxic when used per os. It has received wide application in treatment of candidiasis.

9. *Kanamycin* is an antibiotic produced by *Streptomycin kanamycetius.*

10.Cycloserine obtained from *Streptomyces lavendula*

11. Oleandomycin obtained from *Streptomyces antibioticus*

12. Amphotericin (A and B) are antimycotic antibiotics obtained from *Streptomyces nodosum.*

13. Levorin produced by *Actinomyces levoris* is employed for treating superficial and deep candidiases.

Antibiotics produced by bacteria.

1. *Gramicidin* isolated from a culture of *B. Brevis*.

2. *Soviet gramicidin* (gramicidin C) is produced by a special subspecies of *B. brevis.*

3. *Polymyxins A, B, C, D, E* and *M* are produced by *Bac. polymyxa.*.

Semisynthetic antibiotics. This group includes some penicillins obtained on the basis of6-aminopenicillmic acid, the nucleus of penicillin (methicillin, oxacillin, dioxacillm, ampicillin, etc.) and on the basis of 7-amino-cephalosporanic acid, the nucleus of cephalosporin (cephalothin, cephaloridine, etc.). Semisynthetic penicillins and cephalosporins are used in the treatment of diseases induced by penicillin-resistant staphylococci and other causative agents. The antibiotic levomycetin (an analogue of natural chloramphemcol) is obtained by synthesis.

Combined preparations have also been produced on a mass scale, e. g. vitacycline (tetracycline with vitamins C, B(and B, and some others).

New medicinal forms of tetracyclines having weaker side effects have been devised.

<u>Classification of antibiotics on chemical structure.</u>

<u>1st group –β-lactams</u>. Penicillins and cephalosporins are members of a broad group of antimicrobial agents called "β-lactams".

Several types of penicillin-binding proteins are present in all bacteria that are susceptible to the penicillins and cephalosporins.

Once the cell wall structure is no longer, bacteria are killed by lysis. Penicillins are considered to be bactericidal.

Vancomycin is a glycopeptide antimicrobial agent and important cell wall of microbe active agent. Vancomycin is used against methicillin-resistant Gram-positive cocci, particularly Staphyloccus as well Streptococcus and Enterococcus (it's active only against gram-positive bacteria).

<u>2nd grop-aminoglycosides.</u> Aminoglycosides include gentamicin, amikacin, netilmicin, kanamycin, streptomycin, neomycin, tobramycin. Due to their consistent activity against gram-negative bacilli, they are among the most useful antimicrobial agents, despite some well-known toxicities.

Aminoglycosides also to cause disruption of ribosomal activity by breaking up polysomes.

<u>3rd group-macrolides.</u> Agents such as clindamycin prevent peptide chain initiation. Chloramphenicol inhibits elongation of peptide chain on the ribosome. Erythromycin and other macrolides inhibit the translocation of the growing peptide chain on the ribosome.

4th group – tetracyclines. Tetracyclines inhibit protein chain elongation. Tetracyclines serves as a broad-spectrum antibiotic; tetracyclines have the widest spectrum of activity of any antibiotics.

5th group – polymyxins. The mode of action of polymyxin involves disruption of the gram-negative outer cell membrane (it additionally serves to inhibit the toxic effects of endotoxins though this comes at costs associated with its low chemotherapeutic index). Because of the toxicity of polymyxin, it's used rarely except in topical ointments.

6th gropus-rifampin. Rifampin, a bactericidal agent is never used alone for therapy because resistance develops rapidly, but it's an effective therapeutic agent for Neisseria meningitides and has synergistic or additive activity with other antimicrobial agents against staphylocci and mycobacteria.

7th group – antiblastomic antibiotics. The mechanism of action is connected with DNA-synthesis inhibition or with inhibition of DNA activity in system of DNA-dependent RNA-polymerase.

Drug resistance can be nongenetic and genetic by the origin.

Nongenetic origin. Active microbial reproduction is needed for the manifestations of the activity of most of the antimicrobial substances.

Genetic origin. Most of the microbes acquire drug resistance as the result of the genetic changes with the following selection. Genetic changes can be chromosome and nonchromosome by the origin and transfer from one bacterial species to another by various mechanisms.

Antibiotics influence not only on pathogenic microorganisms, but also on the sensitive normal microflora of the organism. Balance violation of normal microflora in itself can result in illness.

The demands to the antibiotics are:

1. High targeted action (to the certain microorganism only).
2. The favorable form of introduction.
3. Absence of the microorganism resistance.
4. The ability to dissolve in liquors of the human organism.
5. Absence of the allergic reactions or toxicity.

The rational choice of antibiotic preparations depends on the number of factors. Should be put up special aetiological diagnosis which is often set on the clinical data.

For the right choice of the preparation the laboratory definition of the antibiotic sensitivity should take place. Laboratory tests on the sensitivity to the antibiotics are hold in the following cases:

1) When the microorganism concerns to the species which is frequently resistant to antimicrobial agents (e.g. Gram-negative intestinal bacteria);

2) When the infection turns out to be mortal (e.g. meningitis, septicemia):

3) In the case of some infections when for the destruction of the infectious agent the usage of remedies with fast bactericidal effect is needed (e.g. infectious endocarditis, acute osteomyelitis).

Intensive circulation of drug-resistant microbes among the people and their spread with things in domestic use produce a big problem for health services. Some drag-resistant microbes probably have elevated viralence or ability for a dissimilation.

Principles of the rational antibiotic therapy

Microbiological principle.

Antibiotics must be used only according to the indications. For the selection of the medicines it's necessary to take patient's material for the investigation and isolation of the pure culture of infections agent.

Then we have to determine the sensitivity to the antibiotics. The method (disks with antibiotics and serial dilutions in liquid medium method) establish not only the effectiveness on the antibiotic against certain the microorganism But also the minimal inhibitory concentration (MIC).

Pharmacologic principle.

It means the definition of the right dosage, the intervals between the introductions of the antibiotics, the terms of the antibiotic therapy and of course the methods of introduction.

Clinical principle.

It's necessary to pay attention to common status of patient, age, sex, status of the immune system, pregnancy, presence of the various side-diseases.

Epidemiologic principle.

It's necessary to know for what antibiotics the microbes from environment of the patient (department, hospital, geographical region) are resistible.

Pharmaceutical principle.

It's necessary to consider the term and the conditions of storage, so as wrong and long term storage of the antibiotics causes the appearance of the toxic medicine degradation products.

Genetics of bacteria.

Bacterial genetics is the science studing laws of heredity and variability of prokaryotic microorganisms. Value of bacterial genetics in the theory and practice of medicine is great. On bacterial genomes models it has been established, that DNA is

material basis of heredity; its structure has been deciphered, the genetic code was determined, laws of heredity and variability were established.

The data of bacterial genetics have allowed to establish mechanisms of transfer of pathogenic properties and resistance to medical products; they have made theoretical base of gene engineering and biotechnology.

Success in genetic of bacteria and virus were the basis of solving lots of medical problems: fight against infectious and hereditary diseases, production of antibiotics and others. With the help of the method of oriented mutation alive vaccines are produced which are successfully used in infectious diseases prophylaxis.

In formulating a general concept of the mechanism of inheritance, two basic biologic phenomena must be accounted for: heredity, or stability of type (e.g., the progeny formed by the division of a unicellular organism are identical with the parent cell); and the rare occurence of heritable variations.

Genetic and cytologic analyses of plant and animal cells have established that the unit of heredity is the gene. *Gene* is a segment of chromosomal DNA that carries in its nucleotide sequence information for a specific biochemical or physiologic property. The genes are located along the thread – like chromosomes in the cell nucleus. The chromosomes undergo duplication (replication) prior to cell division; when the cell divides, each cell receives an identical set of chromosomes and therefore an identical set of genes.

In prokaryotic cells the chemical substance of the chromosome which is responsible for both gene replication and gene function is deoxyribonucleic acid (DNA). In viruses, it can be either DNA or RNA.

The traditional approach to genetics has been to identify genes on the basis of their contribution to phenotype, or the collective structural and physiologic properties of a cell or an organism.

A phenotypic property, be it eye color in a human or resistance to an antibiotic in a bacterium, is generally observed ad the lever of the organism. The

chemical basis for variation in phenotype is change in genotype, or alteration in the sequence of DNA within a gene or in the organization of genes.

Genetic material of a bacteria contains the genome (bacterial chromosome) and nonchromosome genetic elements: plasmids and transposans.

Plasmids are in cytoplasm of bacteria. Plasmid is additional DNA molecule which is extrachromosome bacterial genetic factor. Plasmids can be independent (not connected with nucleoid) or integrated in nucleoid.

Independent plasmids represent the ring DNA molecules containing insignificant amount of genes, frequently determining bacterial virulence.

In addition to chromosomes, many bacteria harbor one or more plasmids, which can be considered minichromosomes (e.g., circular, supercoiled ds DNA molecules) that contain their own genes for DNA replication. Also, some of the larger plasmids encode their own sex pilus, which permits transfer of one DNA strand to an attached sensitive bacteria through the mechanism of conjugation. Plasmids can exist outside a host chromosome, but not outside the host bacterium. If the plasmid is integrated into the host DNA, then the entire host bacterial chromosome can also be transferred during conjugation. This can generate new bacterial strains.

Many toxins as well as enzymes involved in antibiotic resistance (R-factors) are encoded by plasmids.

Nowadays plasmids are discovered: factors of medicine resistance (R-factor) sexual factor (F-factor) and others. The F-factor (fertility factor, also known as the sex-factor) codes for a series of genes necessary for conjugal transfer.

Plasmids have been discovered in more than 50 species of bacteria.

Mobile genetic elements. The 2 major groups are ***insertion sequences*** (IS) and ***transposons.*** IS elements are unable to replicate autonomously like plasmids. They are small (approximately 10^3 base pairs) DNA molecules that are inserted into another DNA molecule.

IS elements contain one gene, which encodes the enzyme transposase. The transposase permits the IS to move among DNA molecules in the host. Some IS elements also contain regulatory sequences near or into which they have been inserted, which the resident host gene can turn on or off.

Transposons are mobile genetic elements that usually contain two IS elements flanking a segment of DNA and can move the segment of DNA from one to another chromosomal site.

Single or multiple antibiotic-resistant genes can be moved this way, even among different bacterial strains.

Genetic studies not only hereditary but variability of microorganisms as well. The basis of variability are in changing of genotype reaction to the factors of environment and changes within of a genotype itself as a result of genes mutation and their recombination.

A bacteria has nonhereditary (modification, phenotype) variability which is caused by the influence of environment and results changes in genotype.

The range of this variability is caused by genotype.

Hereditary (genotype) variability is connected with mutations changes in an initial structure of DNA when influenced by mutation of genes factors. It means loss or changing of any hereditary indification (e.g. of virulence). Such variability is called mutable and it plays an important part in micro- organism evolution.

Mutations can be spontaneous and induction. Spontaneous appears independently, and induction under the influence of mutagenes (physical, chemical and biological factors) change the microorganisms properties directly.

Variation of the Main Characters of Microorganisms

Changes in morphological characters. Under the influence of physical and chemical effects some cells assume the form of large spheres, thickened filaments, flask-shaped formations, and branchings resembling fungal mycelia. Acetic acid bacteria under the effect of a temperature of 41 °C easily form very long, strongly

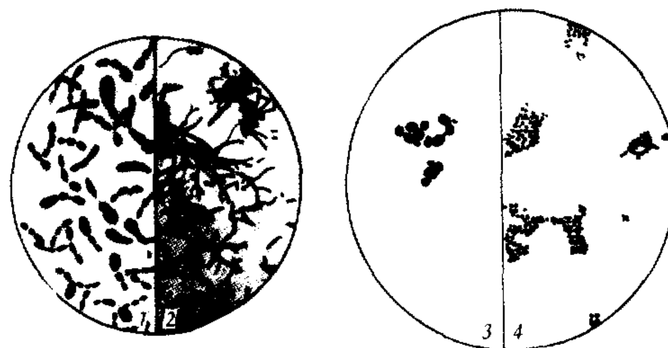
swollen filaments. The cultivation of these bacteria at ordinary temperatures is accompanied by the appearance of rod-shaped forms.

Gamaleia observed morphological changes in a number of microbes, e. g. the formation of giant spheres, amoeboid forms, thickened filaments, etc. He named this phenomenon heteromorphism which arises due to the adaptation of bacteria to unusual environmental conditions.

Heteromorphism easily occurs under the influence of lithium salts, phage, caffeine, sulphonamides, antibiotics, different types of irradiation, and also many other factors.

The phenomenon of heteromorphism is relatively often observed when the culture ages. Figure 1 shows the flask-shaped, filamentous, yeast-like, and coccus-like forms of diphtheria bacilli. The variation of morphological forms is most distinctly expressed in mycoplasmas and L-forms of bacteria.

N. Vavilov called intraspecies polymorphism (a phenomenon common among the vegetable kingdom) the law of homologous series in hereditary variation. Its essence consists in similar traits being manifested now and again in some varieties or races which had originated from one and the same species and, less frequently, in the progeny of remote species. It was established that genetically related species are characterized by similar and parallel series of hereditary forms. Not only genetically related species but genera too display resemblance in the series of hereditary variation.



Diphtheria bacilli. Corynebacterium diphtheriae: *1– flask-shaped form; 2 – filamentous form; 3 – yeast-like form; 4— coccus-like form*

The law of homologous series may also be applied in relation to micro-organisms. It was described in Ascomycetes and Basidomycetes organisms, algae, bacteria, and protozoa.

The parallel variation is proof of the repeated character of variation cycles in different families and genera. Mimicry (the imitation of one species by another in shape and colour) and convergence (similarity in signs) are considered common phenomena of the repetition of forms characteristic of the whole organic world and particularly in micro-organisms which were found to possess structures (antigens) in common with animal and human cells**.**

Other traits (the affinity for dyes, the formation of flagella, cilia, spores and capsules, the structure of the hereditary apparatus) are also subject to variation. It should be noted that any change in the morphological features is attended with a change in the physiological properties too.

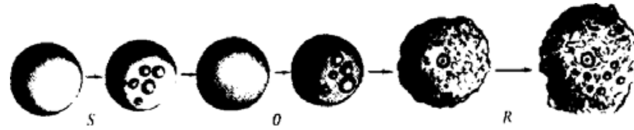
Therefore the subdivision of the types of bacterial variation into morphological, cultural, enzymatic, biological, etc. is conventional and serves merely for more illustrative discussion of the multiform material on the subject.

Changes in cultural properties. Besides morphological deviations in microbes, changes are often observed in the cultural properties.

In 1920-1924 P. De Kruif, G. Arkwnaht, and many other scientists established that the cultures of one and the same species of bacteria may differ among themselves. When a pure culture is seeded onto a solid nutrient medium, different forms of colonies of two main types are produced: (1) smooth, S-forms, and (2) rough, R-forms.

Between these two types of colonies there are transitional, unstable forms, and more often O-forms (Fig. 2). The difference between S- and R-forms is not only

limited to the forms of the colonies, but includes other characters. This kind of variation is known as dissociation (Table 1). In some cases the formation of daughter colonies is observed. There are other forms of colonies: D-colonies (dwarf) and G-colonies (gonidial) which originate on the surface or margin of normal colonies and L-colonies.



Changes in the forms of colonies during bacterial dissociation

S – smooth; O – opaque; R – rough

Properties of Cells from S- and R-colonies

S-type	R-type
Colonies are smooth, regular, and convex	Colonies are rough, irregular, and flattened
Colonies form daughter buds	Daughter buds are rarely formed
Motile species possess flagella	Motile species may have no flagella
Capsulated species have well developed capsules	Capsules are absent
More active biochemically	Less active biochemically
Most pathogenic species have a virulent stage	Virulent stage is weak or absent
Usually isolated in the acute stage of the disease	Associated predominantly with the chronic forms of the disease and the carrier state
Sensitive to phage	Less sensitive to phage
Poorly phagocytable	Easily phagocytable

Variation in requirement metabolites. Under the influence of antibiotics and chemotherapeutic substances. X-rays, ultraviolet irradiation, and other effects, in some microbes the need of certain amino acids and growth factors appears which the original cultures did not require. These varieties which, for their development require special conditions are known as auxotrophic in contrast to original strains—prototrophic.

Thus, auxotrophs differ from prototrophs in that part of their metabolic processes is blocked, and they lack the ability of synthesising the necessary metabolites. Thus, for example, after the effect of X-rays on E. coli, it began to require for its growth many factors (casein hydrolysate or yeast extract) while the original strain could develop in a synthetic medium in which amino acids and vitamins (minimal medium) were absent.

Auxotrophic variants lost the ability to synthesise leucine or vitamin B_1, and their growth depended on the presence of a certain substrate containing the amino acid leucine and vitamin B_1.

One of the means of revealing auxotrophic variants is the use of penicillin which has a bactericidal effect only on cells in the state of division. Blocking can be reproduced in relation to tryptophan, atranil acid, and other factors.

Physical and chemical factors can induce different changes in the ability to synthesize important metabolites in bacterial cultures. These changes take place under the effect of the mechanisms concerned with genetic information.

Variation in enzymatic functions. Variation in microbes is not limited to the morphology, size or cultural characters, but includes other properties.

Of special theoretical and practical interest is the variation of enzymatic ability in bacteria and their adaptation to the changed internal and external environmental conditions.

The addition of a definite substance to the medium may cause activation of the enzyme which had been in a latent state. For example, the induction of the biosynthesis of the ferment β-galactozydaze in E. coli may be reproduced by cultivating this bacteria in the presence of lactose.

The ability for induction is determined by the nucleoid genes and the presence of the inducing factor in the external environment.

The catalytic activity of bacteria can be increased many times by adding substrates inducing the synthesis of enzymes in the corresponding conditions of cultivation (certain amount of vitamins, definite pH level and degree of aeration).

By the action of certain toxic substances on bacteria it is possible to deprive them of their ability to produce various enzymes.

The cultivation of Clostridium perfringens on a medium with a low content of iron brings about a decrease in the enzymatic ability of the microbe. Thus, the enzymatic activity is variable, and can be lost or gained. The loss of enzymatic activity may be constant or temporary.

Under the effect of an unusual metabolite (inducer or represser) induction or repression of the synthesis of a definite enzyme takes place.

The production of enzymes (specific proteins) takes place according to genetic processes in the cell. They are governed by the general laws of protein synthesis.

Of great interest is the problem concerning the possibility of the production of adaptive enzymes not only in intact cells, but in their protoplasts.

The production and disappearance of adaptive enzymes, as numerous investigations have shown, is a widespread phenomenon among microorganisms. Changes of the biological properties of microorganisms take place as a result of the genetic recombinations of a genotype, the exchange of genotype material between species of microorganisms.

There are three traditional mechanisms for the transfer of bacterial DNA from cell – conjugation, transformation and transduction – which have been used to analyze bacterial genes important for many pathogenic processes.

Transformation is the changing of a bacterial cell property as a result of the process carrying the information from place to place within which a fragment DNA cell-donor penetrates into a kindred (related) bacterium. By means of transformation different indifications can be replaced: synthesis of a capsule polysaccharyd, resistance to antibiotics, synthesis of ferments and so on.

Ability of transformation was demonstrated by lots of species of microorganisms: pneumococci, staphylococci, gonococci, meningococci, E. coli and others.

Transduction is carryng genetic material from a cell-donor to a cell- recipient with the help of phag.

Transductive phag carries DNA fragment from the former owner and brings in this DNA the same way as its own DNA molecule, it brings it in a sensitive bacterial cell.

Phenomenon of transduction is one of the sources of formation species of bacteria new properties, which are more adapted to environment.

Conjugation os the transmission of genetic material from a cell to cell, direct contact of bacteria is available within the process. Research work with E. coli were carried out.

Transmission of genetic material is taking place only in one direction; one cell was a donor, and the other one was a recipient.

The process of conjugation is connected with the presence of sexual factor (F) in a bacterial cell (an outside chromosome separate determinant).

Sexual factor is a DNA molecule (it's much less than a chromosome), which has a circular structure and ability of self replication.

All three processes of genetic recombination in bacterium (transformation, transduction and conjugation) are different in their forms but similar in their essence;

as a result of each process the carrying of the DNA fragment from one cell to another takes place.

Fundamentals of Biotechnology.
Genetic Engineering.

Biotechnology is preparing products from biological objects or by means of using biological objects. One can use the organisms of man and animals, unicellular microorganisms, animal and vegetable cells as biological objects.

Biotechnology uses the production of these cells as raw material and as a result of technological processing we can get products used in medicine. Biotechnology produces: antibiotics, vitamins, enzymes, aminoacids, hormones, vaccines, antibodies, blood components, diagnostic preparations, immunomodulators, alkaloids, nutritious proteins, nucleic acids, lipids, antioxidants, antihelminthic and antitumoral preparations, etc.

The main branch of biotechnology is the production of diagnostic, preventive and medical preparations.

Biotechnology is applied in veterinary medicine, agriculture, food and chemical industries, energetics, ecology.

Modern biotechnology appeared as a result of the inventions of molecular biology, microbiology, genetics and genetic engineering, immunology and chemical technology. But it is based on genetic engineering, which study the production of recombinant DNA.

Recombinant DNA code the biological properties of microorganisms. It became possible after discovering the enzyme of reverse transcriptase, ligase, DNA- polymerase, restrictase, transferase.

The method of genetic engineering helped produce recombinant strains of yeast, pyocyanic bacillus, intestinal bacillus.

Recombinant strains of intestinal bacillus produce interferon, insulin, the hormone of growth; strains of yeast – interlakin –2, antigens of viral hepatitis B. The are produced recombinant strains of pox virus, rabies, tick encephalitis.

There were introduced into practice the following preparations produced according to the method of genetic engineering: interferons, interlakins, factor VIII , insulin, the hormone of growth, the tissue activator of plasminogen, vaccine against hepatitis B, monoclonal antibodies for the prevention of rejection at transmitting kidneys; diagnostic preparations for the revelation of AIDS, etc.

Practical lesson # 7

***Theme*: *Submodule 1.* Morphology and physiology of the microorganisms.**

1. Definition of microbiology as a science. Fields of microbiology. Subject and tasks of medical microbiology. The main features and trends of modern microbiology.
2. Discovering of the microorganisms by Antonie van Leeuwenhoek. Stages of the microbiology development. The contribution of Louis Pasteur and Robert Koch to microbiology.
3. Formation of the major directions of microbiological science. Role of D. Samoilovych, E. Jenner, I.I. Mechnikov, D.Y. Ivanovskiy, P. Ehrlich, S.M Vinogradskiy, E. Behring, G. Ramon, F.A. Losch, G. Domagk, A. Fleming, D.K. Zabolotniy, L.A. Zilber, V.M. Zhdanov, M.P. Chumakov, F. Burnett and other scientists. The development of microbiology in the Ukraine.
4. The main differences between prokaryotes and eukaryotes. Forms of bacteria with the cell wall synthesis defect, protoplasts, spheroplasts. L-form of bacteria.
5. Morphology and structure of bacteria. The role of separate structures for the life of bacteria in the pathogenesis of infectious diseases. Vegetative forms and spores.
6. Morphology and classification of protozoa. Classification and morphology of fungi.The methods of microscopy. Production of bacteriological slides. Dyes and staining solution, simple and complex methods of painting.

7. The principles of organization, equipment and operating mode in bacteriological, serological and virological laboratories. Bacterioscopic method. Stages.
8. Types and mechanisms of microorganisms nutrition. Mechanisms of penetration of nutrients into bacterial cells. The chemical composition of microorganisms. The value of the components. Growth media and requirements for them. Classification of growth media, which are used in microbiology.
9. Respiration of microorganisms. Aerobic and anaerobic types of respiration. Enzymes and structures of the cells involved in the process of respiration. Methods of cultivation of anaerobic bacteria.
10. Enzymes of microorganisms and their role in metabolism. Role of enzymes in differentiation of bacteria. Enzymes pathogenicity.
11. Growth and ways reproduction of bacteria. The mechanism of cell division, phases of bacteria culture multiplication in stationary conditions.
12. Bacteriological method. Principles of isolation of pure cultures of bacteria and their identification.
13. Influence of physical, chemical and biological factors on microorganisms. Methods of sterilization. Control of the effectiveness of sterilization. Asepsis. Antiseptics.
14. Origin and evolution of microorganisms. Modern classification of prokaryotes. Main taxonomic ranks. Taxonomy and nomenclature of bacteria. Species as basic taxonomic rank.
15. Taxonomy and nomenclature of bacteria. Basic principles of systematics. Classification of bacteria. Characteristics of the species.
16. The material basis of heredity of microorganisms. Genotype and phenotype. Types of variability. Non-hereditary variability.
17. Hereditary variability. Mutations and their variations. Physical, chemical, biological mutagens. Genetic recombination: transformation, transduction, conjugation. Dissociation of bacteria.

18. Nonchromosomal heredity factors of bacteria. Plasmids and their basic genetic functions. Migratory elements. The role of mutation, recombinations and selection in the evolution of microbes. The main factors of evolution.
19. The value of genetics in the development of general and medical microbiology, virology and molecular biology. Microbiological basis of genetic engineering. The scheme of gene structures and genetically modified organisms production. Achievement of genetic engineering, the use genetically engineered drugs in medicine.
20. Chemotherapy and chemotherapeutic preparations. Chemotherapeutic index. The mechanism of antibacterial action of sulfonamides. The role of P. Ehrlich and G. Domagk in the development of the chemotherapy doctrine.
21. The phenomenon of microbes antagonism. The role of national microbiologists in the development of the microbes antagonism doctrine. Antibiotics, characteristics, principles of producing, units of measurement. Classification by mechanism of action on microorganisms.
22. Drug-resistant microbes, the mechanism of resistant forms formation. Methods of determining the sensitivity of microbes to antibiotics. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentration. Practical value. How to combat with drug-resistant microorganisms.

Literature:

1. Jawetz, Melnik, E. Adelberg's. Medical Microbiology, 1995.
2. Gaidash I.S., Flegontova V.V.,: Microbiology, Virology and Immunology.
3. K. Talaro, A. Talaro, Faundations in microbiology. Basic principles. Pasadena, 2005, by TMHE group.
4. M.T. Nester, E.V. Nester,C.E.Roberts, Microbiology. A human perspective, 1995.
5. W. E. Levenson, E. Javetz , Medical microbiology and immunology, 1994, Norwalk.
6. Yu.S. Krivoshein, Handbook on microbiology, 1989, Mir Publishers, Moscow.
7. D.Greenwood, R. Slack, J. Peutherer, Medical microbiology. A guide to microbial infections pathogenesis, immunity, laboratory diagnosis and control, 1995.