Ministry of Public Health of Ukraine

National O.O. Bohomolets Medical University

Microbiology, virology and immunology department

SHYROBOKOV V.P., PONIATOVSKYI V.A., SHYLOV M.V., MELNYK V.V.,
YEHOROV D.P., DOLINCHUK L.V., SVIZHAK V.K.

STUDY GUIDE

OF THE PRACTICAL CLASSES COURSE

Part I

Specialties:

221 "Dentistry"

222 "Medicine"

225 "Medical Psychology"

226 "Pharmacy, industrial pharmacy"

228 "Pediatrics"

Kyiv – 2020

Authors:

SHYROBOKOV V.P., PONIATOVSKYI V.A., SHYLOV M.V., MELNYK V.V., YEHOROV D.P., DOLINCHUK L.V., SVIZHAK V.K.

Edited by the Head of the Department of Microbiology, Virology and Immunology, Academician of the NAS and NAMS of Ukraine, Professor **V.P. Shyrobokov**.

Study guide of the practical classes was approved by cycle methodical commission on hygienic disciplines of the Bogomolets National Medical University on September 23, 2020.

CONTENTS:

Class №1

Topic "Structure of bacteriological laboratory. Dyes. Simple method of staining"

Topic relevance

Bacteriological 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. The sings and symptoms of some infectious diseases may be specific for the particular microorganisms, e.g. the specific spasm of mastication muscles of the tetanus and the characteristic rash of chickenpox. But many infections are unspecific, and any of several different pathogenic organisms may be the cause of an illness such as sore throat, bronchitis, pneumonia, meningitides, diarrhea, wound sepsis and fever. In these cases, microbiology laboratory help is required to elucidate the cause. The reliability of that help depends on the correct techniques being used in collection the appropriate specimen from the patient, and doctors must be properly instructed in these procedures. The precise identification of the patient's pathogenic organisms is generally necessary for the effective use of selective chemotherapeutic drugs. In other words, doctor has to identify and treat specific infections rather than clinical syndromes. Since, moreover, different strains of many bacterial species differ in their susceptibility to particular drug. It is usually desirable for the bacterium isolated from the patient to be tested for its drug sensitivity in the laboratory

Concrete objectives:

- To analyze the structure and functions of the bacteriological laboratory, its place in the system of practical medical and diagnostic healthcare institutions.
- To master the technique of making bacterial preparations from pure bacterial cultures using simple staining methods.
- To learn microscopy of bacterial preparations using immersion lenses.

Basic knowledge, skills, needed to study topics (interdisciplinary integration).

The names of the previous disciplines	Skills
Human anatomy	Analyze information about the structure of the human body, systems, of which it consists, organs and tissues.
Histology, cytology, embryology	Interpret the microscopic and submicroscopic structure of cells.
Medical and biological physics	Treat common physical and biophysical principles that base the biological processes.
Medical biology	Explain at the molecular and cellular level the patterns of biological processes.

Medical chemistry	Treat common physical and chemical principles that base the processes of cells develoupment.

List of terms, parameters, characteristics that a student should learn during class

Terms	Definition
Medical microbiology, virology and immunology	Medical microbiology, virology and immunology - a sciences of origin, evolution and properties of human pathogens, normal microflora of the human body, patterns of interaction between microorganisms and macroorganism and principles and methods of infectious diseases diagnosis.
Bacteriology laboratory	Bacteriology laboratory is the primary unit in the microbiological service system of practical health and diagnostic healthcare facilities.
Specimen (clinical material)	Specimen is substrates, tissues (pus, sputum, blood, urine, faeces, tissue biopsies, etc.) that are taken from a patient or environmental object for research to detect pathogens or antibodies to them (in serum), ie for the diagnosis of infectious pathology, or the detection of pathogens in the environment.
Smear (bacteriological praparation)	Smear is preparation for microscopic investigation. It is prepared from pure culture of microorganisms or specimen for detection or identification of microorganisms by morphological and tinctorial properties
Aniline dyes	Aniline dyes are aniline derivatives that are used to stain microorganisms to enhance their contrast and further microscopy.
Immersion system	The immersion system consists of an immersion lens (x 90) and an immersion oil that provides a concentration of rays passing through the microscopic object into the lens and thus provides the best conditions for the study of microorganisms.

Theoretical questions for class preparing:

- Structure of bacteriological laboratory, functions of different units.
- Organization and equipment of the workplace of a bacteriologist.
- Method of making a bacterial smear preparation.
- Aniline dyes and preparations for staining microorganisms.
- Simple and complex methods for staining microorganisms.
- Light microscope, structure and functions of individual parts.
- Definition of magnification and resolution of the microscope. Immersion system.

Tasks, performing during class:

- Familiarize yourself with the principles of organization of the bacteriology laboratory and its rules of operation.
- To study dyes and coloring solutions used in microbiology.

- To learn the methods of bacteriological preparation and staining with simple methods.
- Master the methods of microscopic examination of bacteriological preparations using an immersion lens.

Class content:

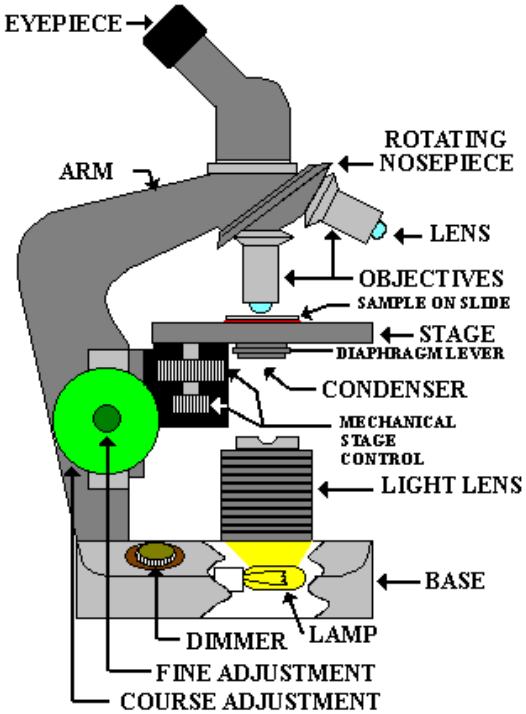
At the practical class students study the structure of the bacteriological laboratory, the rules of work in it, the organization of the workplace of a bacteriologist, prepare smears from pure cultures of staphylococcus and E. coli, stain smears by simple methods (methylene blue and fuchsin), study the methods of immersion microscopy. Students write complete tasks in the protocol and teacher sign it.

Recommendations for design of the protocol

Rules that must be followed by all who work in the laboratory

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 all 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.



THE PARTS OF THE SCOPE

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. 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.

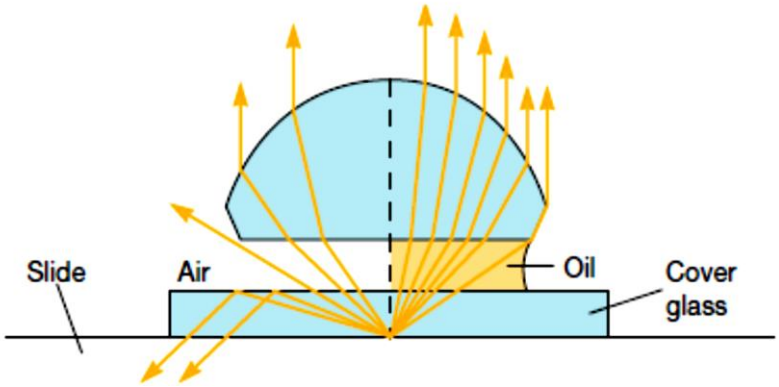
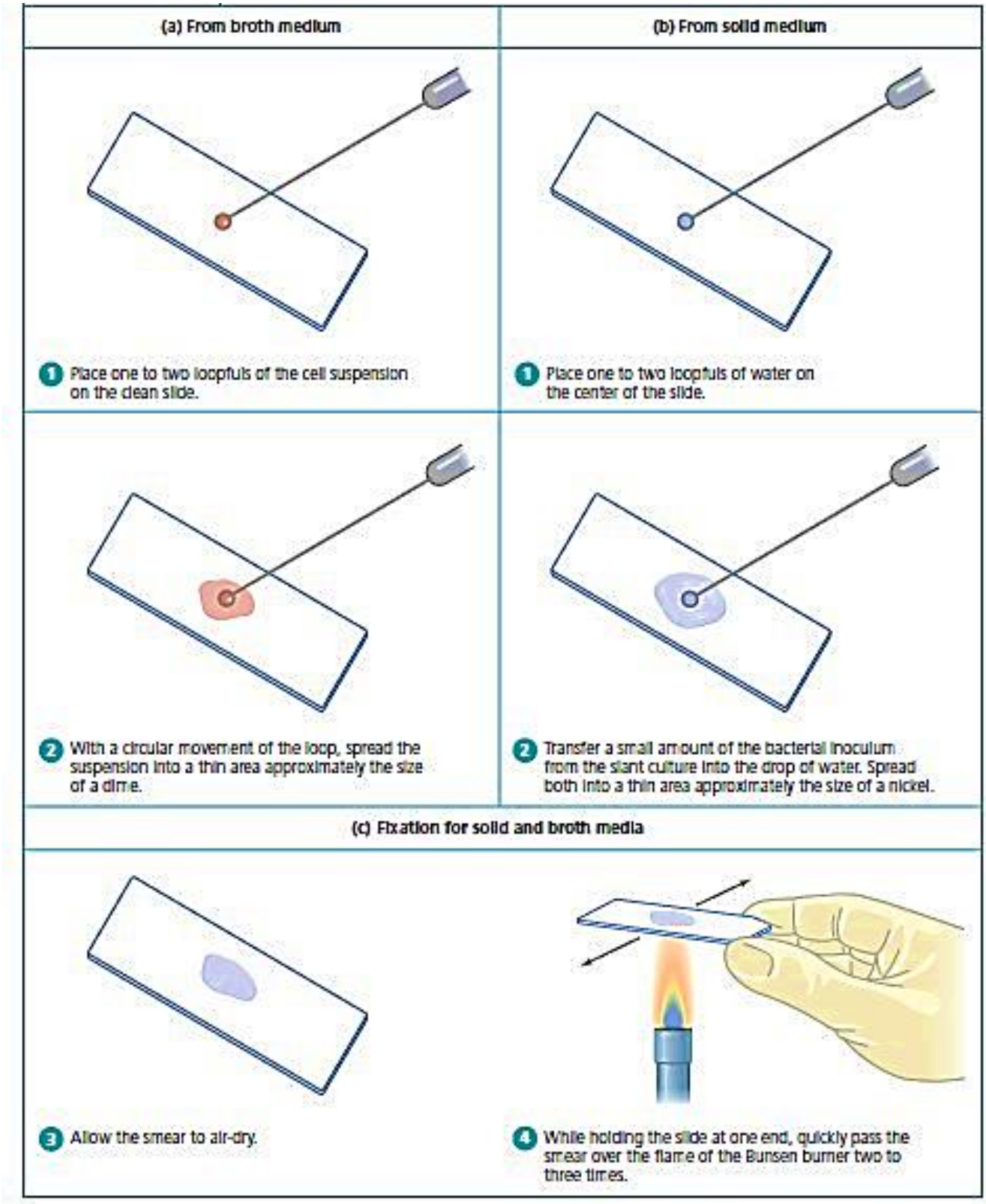


Fig 1. The oil immertion objective. An oil immertion objective lense opertion in air (left side) and with immertion oil (right side).

Bacterial smear preparation



Aniline dyes

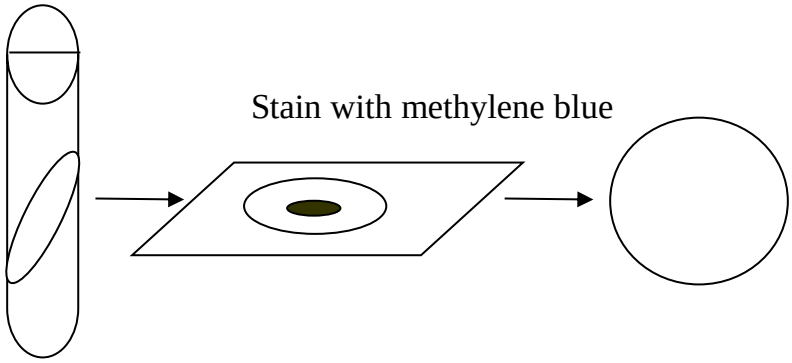
Aniline dyes are used to stain microorganisms. These are mainly derivatives of organic compounds (aniline and others). These are powders that do not dissolve in water but dissolve well in organic solvents (alcohol, acetone). Alcohol-aqueous solutions are used for staining of microorganisms.

Aniline dyes are basic, acidic and neutral. Basic dyes are used to stain bacteria. Methods for staining microorganisms are simple and complex. Simple - when using 1 dye. Complex - Use 2 or more dyes, or 1 dye and substances that do not stain germs but are involved in the staining process.

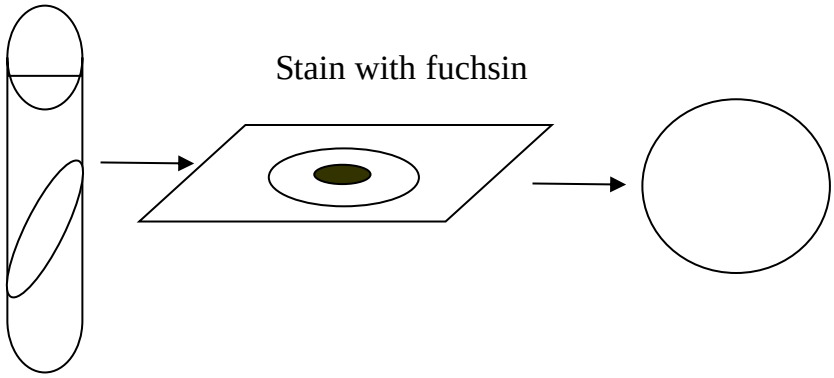
Aniline dyes

Color	Basic	Acidic
Red	Neutral red Safranin Basic fuchsin	Acid fuchsin Eosin
Violet	Crystal violet Gential violet	
Blue	Methylene blue	
Green	Malachite green Brilliant green	
Black	Inulin	Nigrosin
Yellow		Aurantin
Brown	Chryzoidin	

Practical activity N 1: Prepare the smears from agar culture of Staphylococcus epidermidis and Escherichia coli (the first smear to stain with methylene blue another one- with fuchsin) and examine smears with the oil objective.



Staphylococcus epidermidis



Escherichia coli

Conclusion:

Questions for self-control:

- What does medical microbiology study?
- What is the structure of the bacteriology laboratory and what is the purpose of its units?
- What is purpose of microorganisms staining?
- What are the properties of microorganisms that can be examined by microscopy of stained smear?
- How are aniline dye solutions prepared?
- For what purpose do smear fixation on a slide?
- What lenses are used in bacterial microscopy? What are the signs of such a lens?
- How to calculate the total magnification of a microscope?
- What is the resolving power ?

Class №2

Topic: “Differential Gram method”

Topic relevance:

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 colour 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.

Concrete objectives:

- To master the technique of colouring de Gram, using the cultures of gram-positive and gram-negative bacteria.
- To study properties of gram-positive and gram-negative bacteria.

Basic knowledge, skills, needed to study topic (interdisciplinary integration). See a class №1.

A list of key terms, parameters, characteristics that student should learn for the class:

Term	Definitions
Complex methods of staining of bacteria	Complex methods allow to differentiate one microorganism from other, and also to study the features of structure of microbial cells. Gram, Romanovsky-Geemse, Neisser methods are complex.
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 has an important differentially-diagnostic importance, helps to determine taxonomical position of bacteria
Tinctorial properties	The features of bacteria staining by different methods
Gram-positive bacteria	Gram-positive bacteria stain by Gram in dark-violet colour (able to make strong compound of crystal violet with iodine)
Gram-negative bacteria	Gram-negative bacteria stain by Gram in a red colour (compound of crystal violet with iodine is washed out by an alcohol; therefore they are stained by in a red colour).

Theoretical questions:

- Complex methods of bacteria staining
- Gram method
- Mechanism of staining by Gram.
- Gram staining technique
- Properties of gram-positive and gram-negative bacteria.
- Tinctorial properties of certain groups of microorganisms.

Practical activities performed in class:

- To study properties of gram-positive and gram-negative bacteria.
- To write down tinctorial properties of certain groups of microorganisms.
- To study factors which influence on Gram staining
- To study the features of chemical composition of cellular wall of gram-positive and gram-negative bacteria.
- To prepare smear from the pure cultures of bacteria, to stain by Gram investigate under the microscope, describe morphological and tinctorial properties.

Topic content:

In the practical class students study Gram method, prepare smears from the pure cultures of *S.epidermidis* and *E.coli*, stain by Gram, study the preparations with the use of oil immersion lens, familiarize yourself with the features of gram-positive and gram-negative microorganisms, tinctorial properties of certain groups of microorganisms. Students write down prepared tasks in the protocol and sign it at the teacher

Recommendations for the protocol design

Gram technique consists of a timed, sequential application of crystal violet (primary dye), Lugol's solution (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 decolourization. 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 colour. The second and key differentiating step is the addition of the mordant (intensifier) - Lugol's solution (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 colourless after decolourization, their presence is demonstration by applying the counter stain fuchsine in the final step.

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

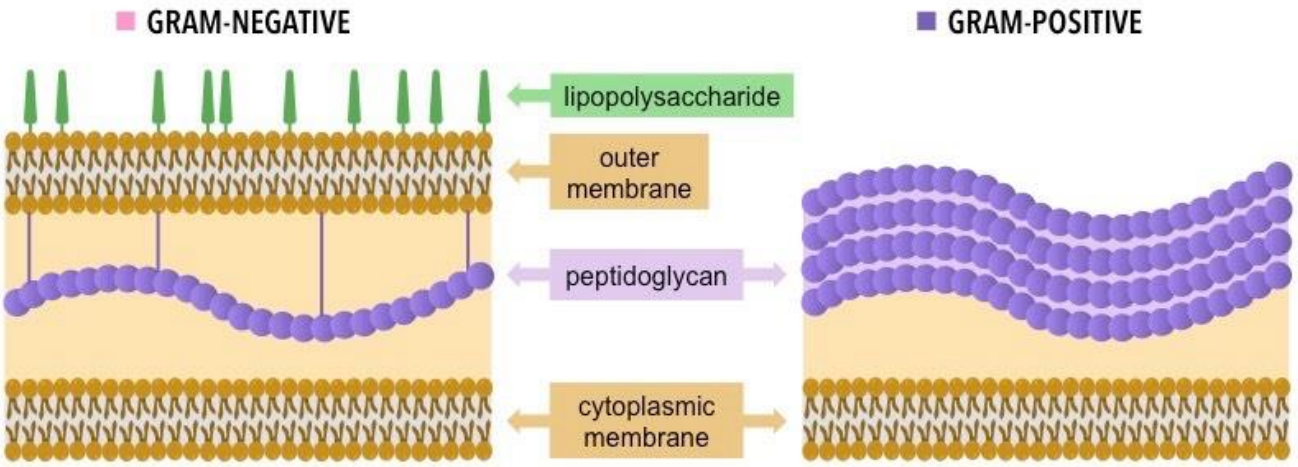
Property	**gram-positive**	**gram-negative**
Thickness of wall	20-80 nm	10 nm
Number of layers in wall	1	2
Peptidoglycan content	>50%	10-20%
Teichoic acid in wall	+	-
Lipid and lipoprotein content	0-3%	58%
Lipopolysaccharide	0%	13%
Outer membrane	No	Yes
Periplasmic space	Present in some	Present in all
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:**
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

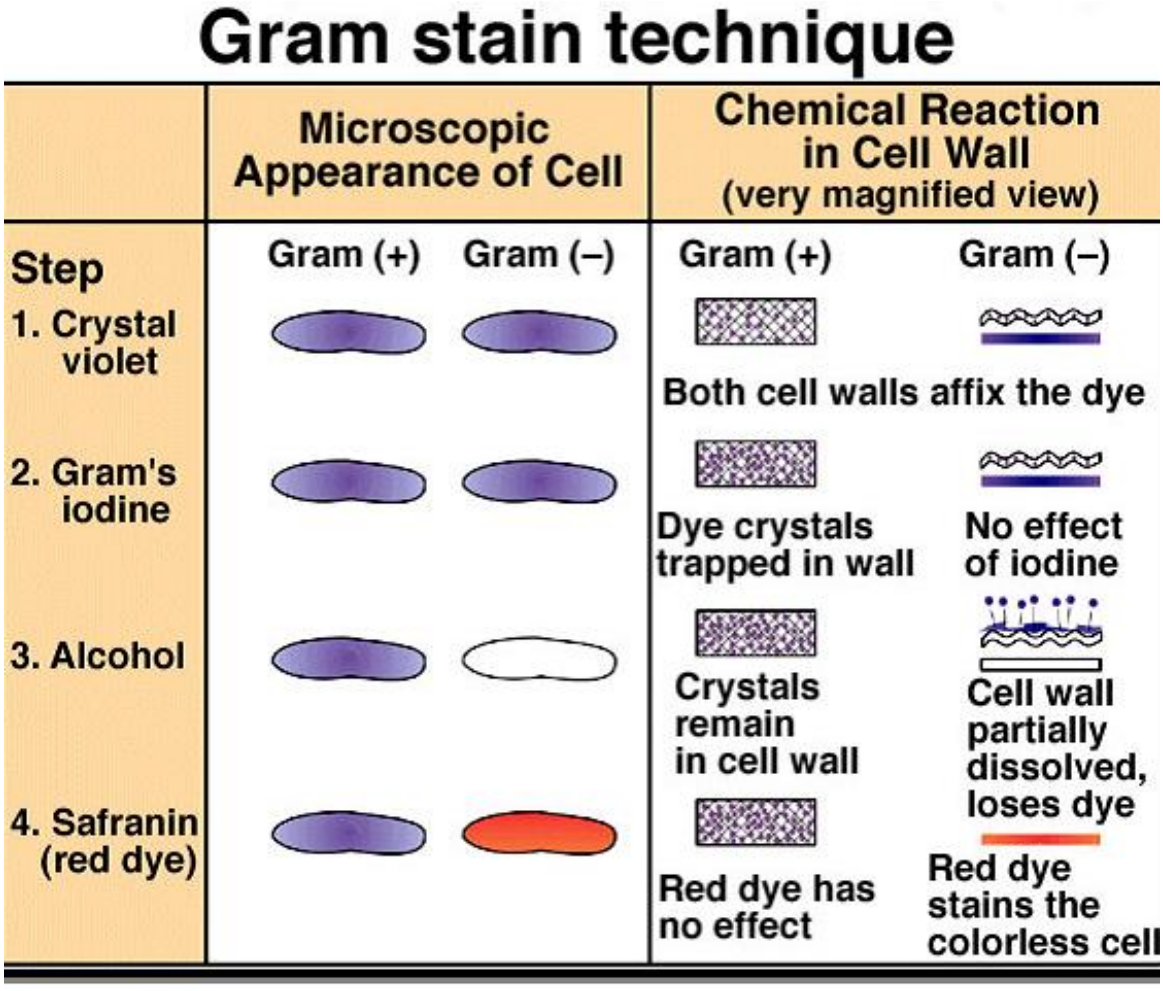
.
.

Scheme of cell wall Gram+ and Gram- bacteria



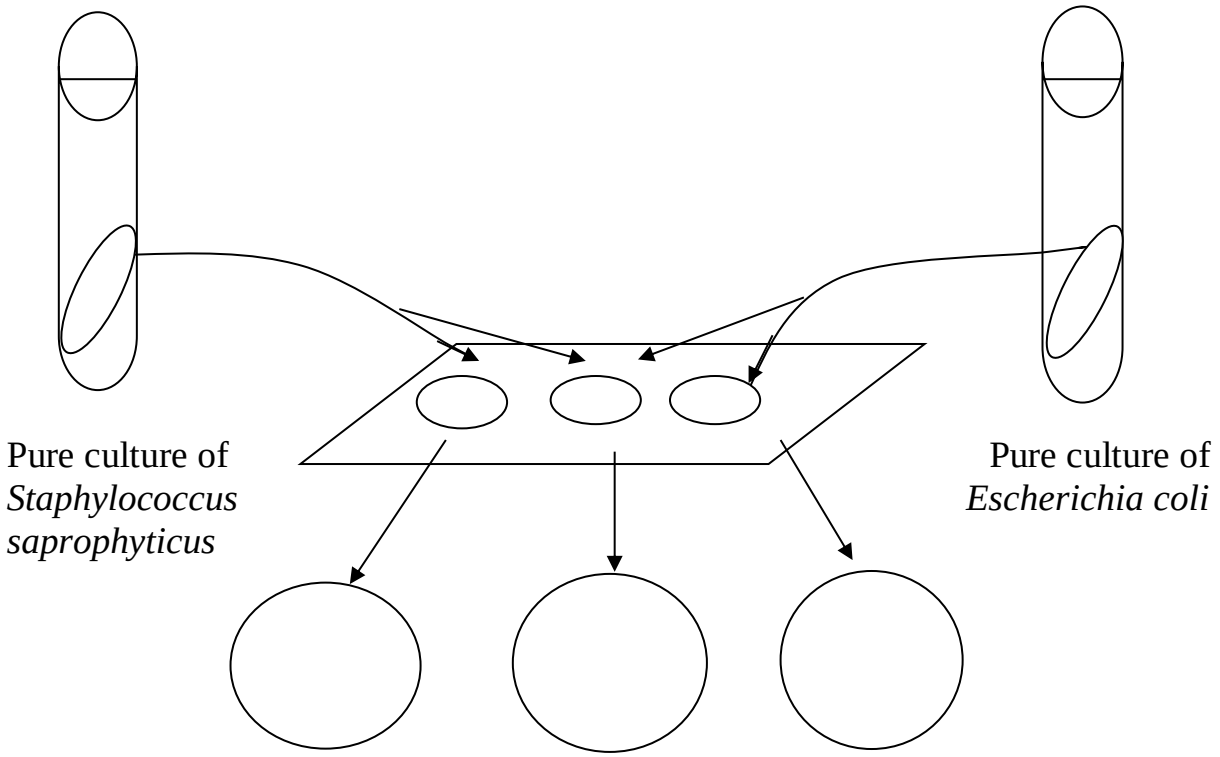
The Gram-positive cell wall appears as dense layer typically composed of numerous rows of peptidoglycan, and molecules of lipoteichoic acid, wall teichoic acid and surface proteins.

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 surface proteins. The lipopolysaccharide consists of lipid A and O polysaccharide.



Practical activity №1. Prepare smears from agar pure cultures and their mixture, stain by Gram, examine under microscope and make conclusion about staining of smears.

1. Prepare smear dry and fix by heat.
2. Cover the slide with crystal violet (or methyl violet) solution and allow to act for about 1 minute.
3. Pour out stain and replace with iodine. Allow iodine to act for 1 minute.
4. Wash off iodine with crystal violet with absolute alcohol, and treat with fresh alcohol tilting the slide from side to side until colour stops to come out of the preparation (caution: don't over or under decolorize)
5. Wash with water.
6. Apply the counter stain (0, 05% basic fuchsine) for 30 seconds.
7. Wash with water and dry between blotting paper.
8. Examine with oil immersion objective



Staining by Gram

Conclusion:

Control questions:

- Gram staining method
- Why Gram method is named differential?
- Name the features of chemical composition of cellular wall of gram-positive and gram-negative bacteria.
- Describe the features of structure of cellular wall of gram-positive and gram-negative bacteria.
- Name basic factors, influencing on staining by Gram.

Class №3

Topic: "Morphology and structure of bacteria"

Topic relevance:

Bacteria are microscopic unicellular prokaryotic non-chlorophyll organisms, which duplicate by non-sexual way.

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.

Concrete objectives:

- To familiarize with morphology of different bacteria.
- To study the essential structural elements of bacterial cells and their function.
- To analyse the role of nonessential structures in the vital functions of bacteria.
- To prepare microscopic preparations from the pure cultures of bacteria and describe their morphological and tinctorial properties

Basic knowledge, skills, needed to study topic (interdisciplinary integration). See a class №1.

A list of key terms, parameters, characteristics that student should learn for the class:

Term	Definitions
Prokaryotes	One-cellular organisms which have two strands circular DNA do not have the formed nucleus, typical cellular organells, mitosis.
Cocci	Bacteria of spherical form
Rod-form bacteria	Bacteria of cylindrical form
Curve bacteria	Bacteria of the bent form with one or a few curls
Essential structural elements of bacterial cells	
Nucleoid	Analogue of the nucleus for bacteria, double circular filament of DNA
Cytoplasm	Granular colloid system, which contains different organoids, organic and inorganic compounds. the Processes of metabolism occurs in it
Cytoplasm membrane	Separate a cytoplasm, has a difficult chemical structure, plays an active role in the processes of metabolism
Mesosoma	The analogue of mitochondria, derivative of a cytoplasmic membrane, plays role in power metabolism and division.
Cell wall	Bioheteropolymer with complex chemical composition; external shell of bacteria.
Ribosomes	Protein synthesis system

Nonconstant structural elements of bacterial cells	
Capsules	Protective mucous layer which covers a cellular wall
Spores	Form of existence of bacteria in the unfavourable condition for maintenance of genetic information
Flagella	Surface structures for some rod bacteria as thin filaments, provide active mobility.
Pili (fimbriae, cilia)	Thin hollow short filaments, covering the surface of bacterial cells, provide adhesion
Sex pili	Take part in conjugation of bacteria
Inclusions	Reserve nutrient substances (volutin, glycogen and other)

Theoretical questions:

- Distinctions in a structure, chemical composition, functions between prokaryotes and eukaryotes.
- Classification of bacteria on morphological signs.
- Description of essential structural elements of bacterial cells and their function.
- Nonessential structural elements of bacterial cells and their role is in the processes of vital functions of bacteria.
- Determination of different bacteria on morphological and tinctorial signs.
- Practical value of study of morphology of bacteria.

Practical activities performed in class:

- To study different shapes and arrangement of bacterial cells, stained by Gram
- To find out spores of rod bacteria, staining by the method of Ziehl-Neelsen
- To find out inclusions of bacteria on preparations, stained by the method of Loeffler.
- To determine capsules of bacteria, stained by Lugol (Iodine).
- To determine flagella of rod bacteria, stained by method of Loeffler.
- To prepare the smears from different agar cultures, stain by Gram's method and examine them, using an immersion objective of light microscope and describe morphological and tinctorial properties.

Topic content:

In the practical class students study differences in a structure and functions prokaryotes and eukaryotes, familiarize yourself with the morphological and structural features of bacteria cell, study the role of essential and nonessential structures in the vital functions of bacteria, prepare smears from the pure cultures of different bacteria, stain them by Gram method and characterize on the basis of morphological and tinctorial properties. Study the different methods of light and electronic microscopy. Students write down prepared tasks in the protocols and sign at teacher.

Recommendations for the protocol design

Students write down in the protocol the table of "Distinctions between prokaryotes and eukaryotes ", the methods of light and electronic microscopy, prepare smears from the pure cultures of microorganisms of different morphology, stain them by Gram. Study

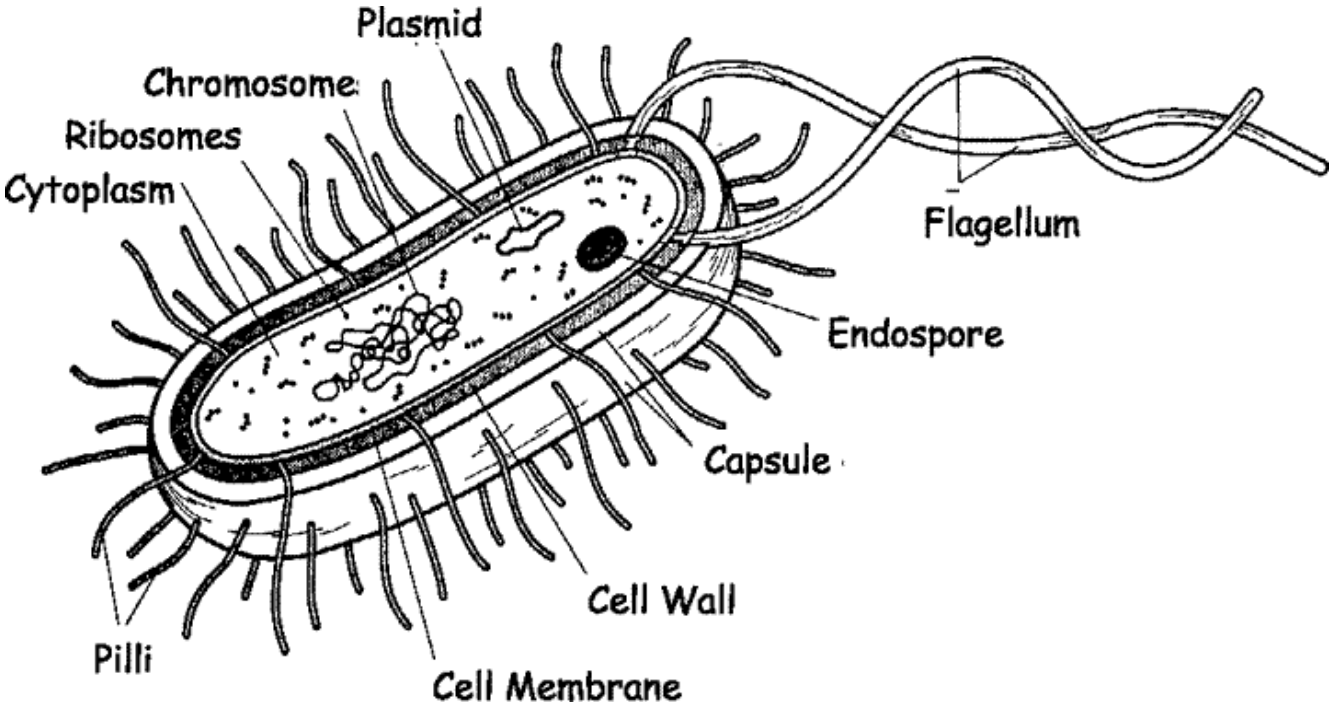
preparations under the microscope, sketch microorganisms in the protocols. Study demonstration preparations of microorganisms of different morphology and structure, sketch in protocols.

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 peptidoglycan	Yes	No
Membrane-bound organelles (mitochondria, lysosomes)	No	Yes
Endoplasmic reticulum	No	Yes
Golgi apparatus	No	Yes
Mitotic division	No	Yes
Peptidoglycan	Yes	No
Teichoic acids	In Gram(+) bacteria	No
Sterols in the cell membrane	No	Yes
Polyunsaturated fatty acids	No	Yes
Phagocytosis and pinocytosis	No	Yes
Intracellular digestion	No	Yes
Amoeboid movement	No	Yes

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 metabolism
Periplasm	Contains many hydrolytic enzymes



Bacterial shapes and arrangements

Coccus		Rod, or Bacillus		Curved forms: Spirillum/Spirochete
Diplococci (cocci in pairs)	Neisseriae (coffee-bean shape in pairs)	Coccobacilli		Vibrios (curved rods)
Tetrads (cocci in packets of 4)	Sarcinae (cocci in packets of 8,16,32 cells)	Mycobacteria	Corynebacteria (palisades arrangement)	Spirilla
Streptococci (cocci in chains)	Micrococci and staphylococci (large cocci in irregular clusters)	Spore-forming rods	Streptomycetes (moldlike, filamentous bacteria)	Spirochetes

Endospore staining

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

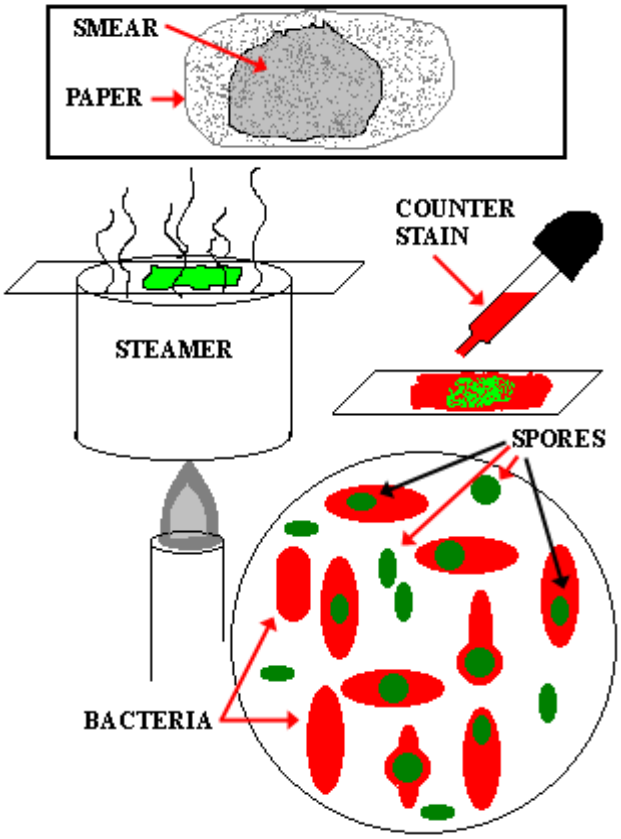


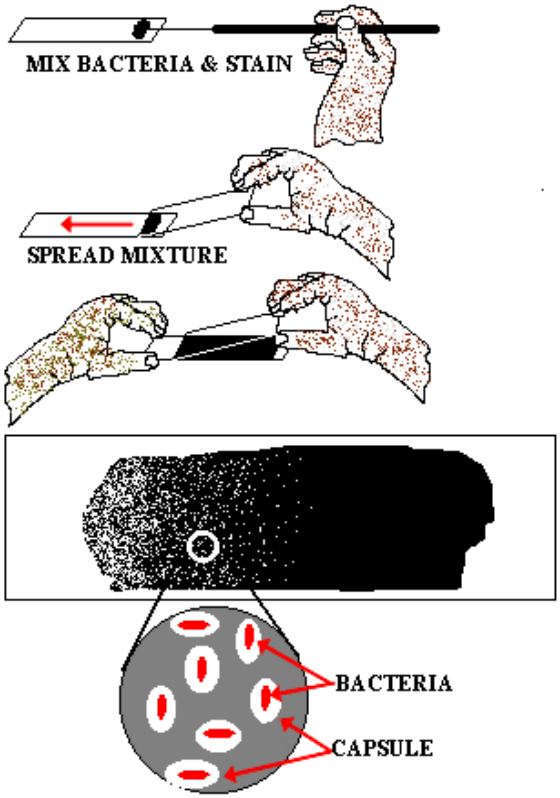
Illustration of spore staining protocol and example of various types of spore arrangements.

Capsule staining

It is method of observing the microbial capsule, an unstructured protective layer 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
4. Background is violet, cells are violet, and capsules are light violet, surrounded cells.



Flagella staining

Loeffler staining

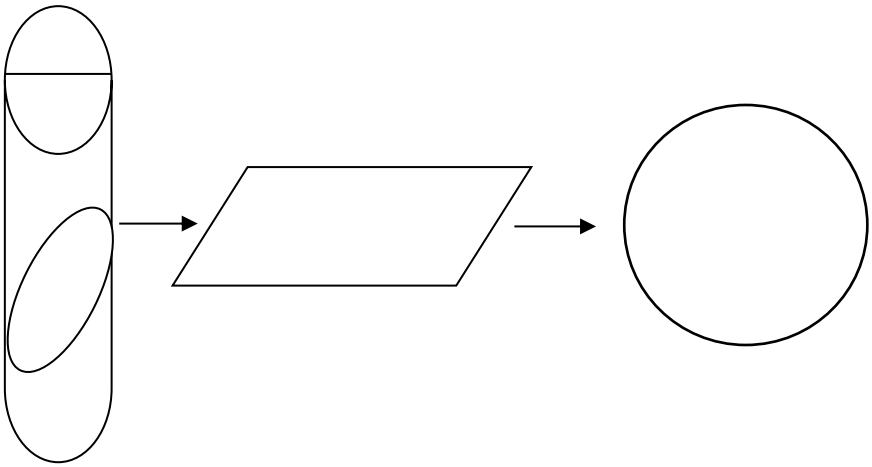
Because the width of bacterial flagella lies beyond the resolving power of the light microscope, in order to be seen, they must be enlarged by depositing a coating on the outside of the filament and then staining it. This stain works best with fresh, young cultures, since flagella are delicate and can be lost or damaged on older cells.

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

Volutin inclusion staining

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.

Practical activity №1. Prepare smears from agar pure cultures and their mixture, stain by Gram, examine under microscope and make conclusion about staining of smears.



1. Shape
2. Location of the cells
3. Tinctorial properties

Control questions:

- What does morphology of bacteria study?
- Define the bacteria.
- What are differences between prokaryotes and eukaryotes?
- How do bacteria classify on morphological signs?
- What structural elements of bacteria are essential, their functions?
- Name nonessential structural elements of bacteria and their functions
- What is the purpose of study the morphological and structural features of bacteria?

Class №4

Topic: "Morphology of fungi, actinomycetes, spirochetes and protozoa"

Topic relevance

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 ultra-structure, 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 cause spirochetoses (venereal syphilis (*T. pallidum*), endemic syphilis (*T. pallidum* subspecies endemicum), yaws (*T. pertenue*), pinta (*T. carateum*), relapsing fever (*B.recurrentis*), Lyme disease (*B. burgdorferi*), leptospiroses (*L. interorgans*)).

Concrete objectives:

- To study the morphological and structural features of fungi, actinomycetes, spirochetes and protozoa
- To define the morphological and tinctorial properties unknown culture of microorganisms.
- To study on morphological and tinctorial properties microflora of dental deposit.

Basic knowledge, skills, needed to study topic (interdisciplinary integration). See a class №1.

A list of key terms, parameters, and characteristics that student should learn for the class:

Term	Definitions
Eukaryotes	Eukaryotes are higher microorganisms (fungi, protozoa), which have the differentiated nucleus; typical cellular organells (mitochondria, endoplasmic reticulum, Golgi apparatus, and others like that).
Spirochetes	Prokaryotes. One-cellular mobile microorganisms, the thin helically coiled cells
Fibrils	Fibrils are long threadlike molecules of protein of flagellin, located under a cellular wall, which provide mobility of them.
Cysts	Cyst is a form of existence of microorganisms in the unfavourable condition

Actinomycetes	Prokaryotes. Threadlike branching cells, resembles hypha of fungi
Hypha	A basic structural element of actinomycetes and fungi is short rod and filamentous structure
Mycelium	Accumulation of interlacing hyphae.
Spores, spore formation	Form of existence of microorganisms in the unfavourable condition, type of reproduction of certain fungi and actinomycetes
Druse	Accumulations of hyphae of actinomycetes which appear in the pathological material
Moulds	Eukaryotes. Produce multicellular filamentous colonies
Yeasts, yeast-like fungi	Eukaryotes. Yeasts are single cells, usually spherical to ellipsoid in shape and varying in diameter from 3 mkm to 15 mkm
Budding	Type of reproduction of yeasts and yeast-like fungi
Pseudohyphae	Chain of elongated yeast cells as a result of reproduction by budding that characteristically fail to detach

Theoretical questions:

- Place of fungi, actinomycetes spirochetes and protozoa in the system of living creatures
- Morphological properties and structure of spirochetes, likeness to bacteria and protozoa.
- Morphology and tinctorial properties of actinomycetes. Likeness to bacteria and fungi.
- Basic morphological signs of fungi, criteria for their classification. Morphology of protozoa

Practical activities performed in class:

- To study morphological properties of yeasts on the demonstration preparations, yeast-like and moulds, actinomycetes, spirochetes, protozoa.
- To investigate morphology of mould (genus *Mucor, Aspergillus, Penicillium*) on solid nutrient media
- To prepare smears from the pure cultures of unknown microorganisms, to stain by Gram and define their morphological and tinctorial properties.
- To prepare a smear from own teeth scraping, stain, examine under microscope, describe morphological and tinctorial properties of found microflora, using demonstration pictures.

Topic content:

In the practical classes students study demonstration preparations of morphological and structural properties of actinomycetes, spirochetes, protozoa. Prepare preparations from the pure cultures of unknown microorganisms. Prepare a smear from the own dental sediment and determine morphological and tinctorial properties of found microflora,

according to the demonstrative picture. Students write down prepared tasks in the protocol and sign at the teacher.

Recommendations for the protocol design

It is necessary to write down in protocols information about fungi, the pictures of demonstrative preparations of fungi, actinomycetes, spirochetes, protozoa.

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 100,000 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 (e.g., penicillin) and immunosuppressive drugs (e.g., 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 defences. 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 cylindrical 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 that characteristically fail to detach 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.

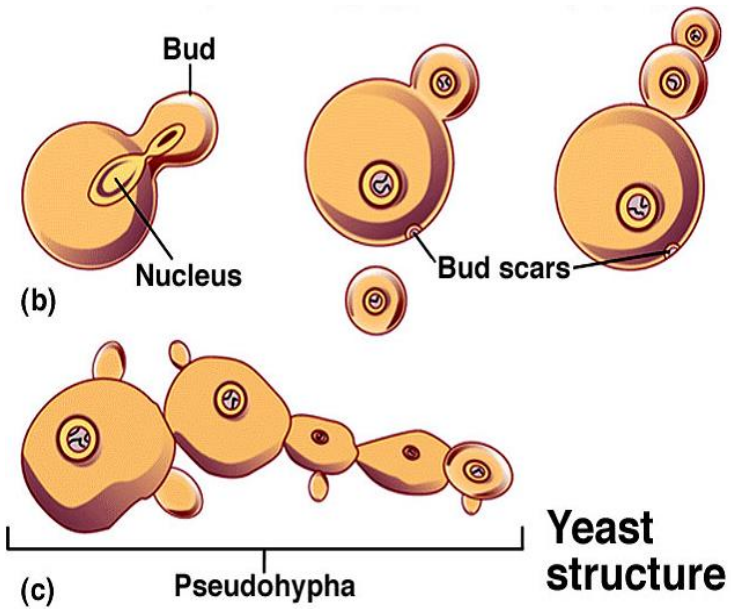
All fungi have an essential rigid cell wall that determines their shape. Cell walls are composed largely of carbohydrate layers—long chains of polysaccharides—as well as glycoproteins and lipids. During infection, fungal cell walls have important pathobiologic properties. The surface components of the cell wall mediate attachment of the fungus to host cells. Cell wall polysaccharides may activate the complement cascade and provoke

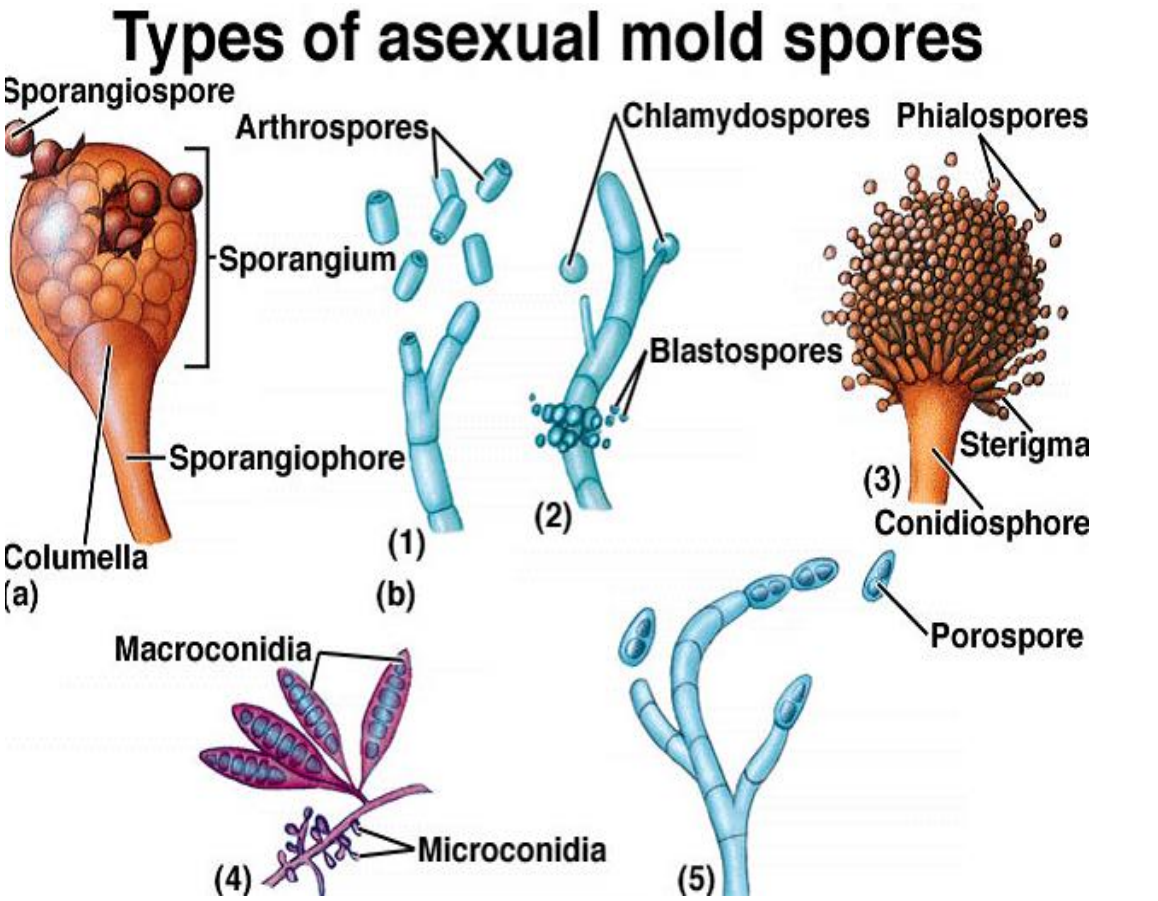
an inflammatory reaction; they are poorly degraded by the host and can be detected with special stains. Cell walls release immunodominant antigens that may elicit cellular immune responses and diagnostic antibodies. Some yeasts and molds have melanised cell walls, imparting a brown or black pigment. Such fungi are **dematiaceous.** In several studies, melanin has been associated with virulence.

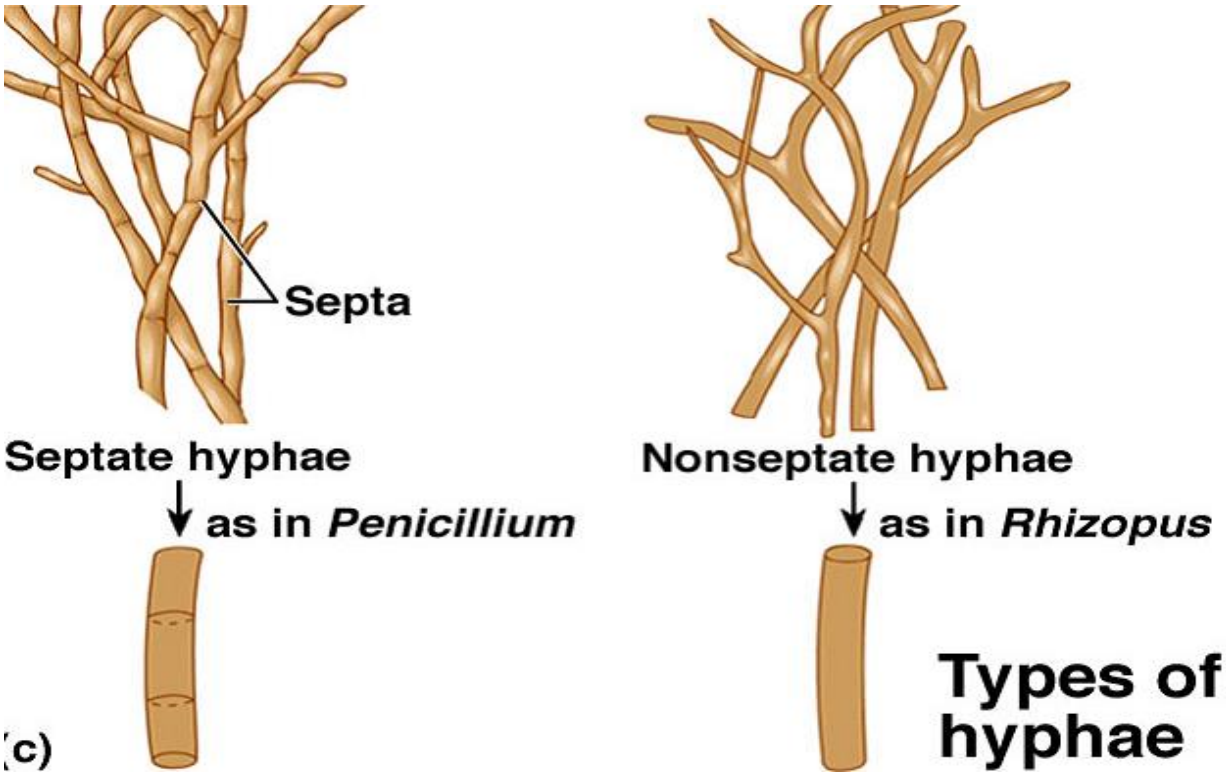
In addition to their vegetative growth as yeasts or molds, fungi can produce spores to enhance their survival. Spores can be readily dispersed, are more resistant to adverse conditions, and can germinate when conditions for growth are favourable. Spores can derive from asexual or sexual reproduction—the anamorphic and teleomorphic states, respectively. Asexual spores are mitotic progeny (i.e., 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, colour, and unicellularity or multicellularity). In some fungi, vegetative cells may transform into conidia (e.g., 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.

The lowest	**The highest**	**Perfect**	**Imperfect**
Chitridiomycetes	*Ascomycetes*	*Ascomycetes*	*Deuteromycetes*
Hyphochitridiomycetes	*Basidiomycetes*	*Basidiomycetes*	
Oomycetes	*Deuteromycetes*		
Zygomycetes			

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




Types of asexual mold spores
Sporangiospore
Arthrospores
Chlamydospores
Phialospores
Sporangium
Blastospores
Sterigma
Sporangiophore
(3)
Conidiosphore
Columella
(a)
(1)
(b)
(2)
Porospore
Macroconidia
Microconidia
(4)
(5)


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

Basic Morphological Elements of Fungi

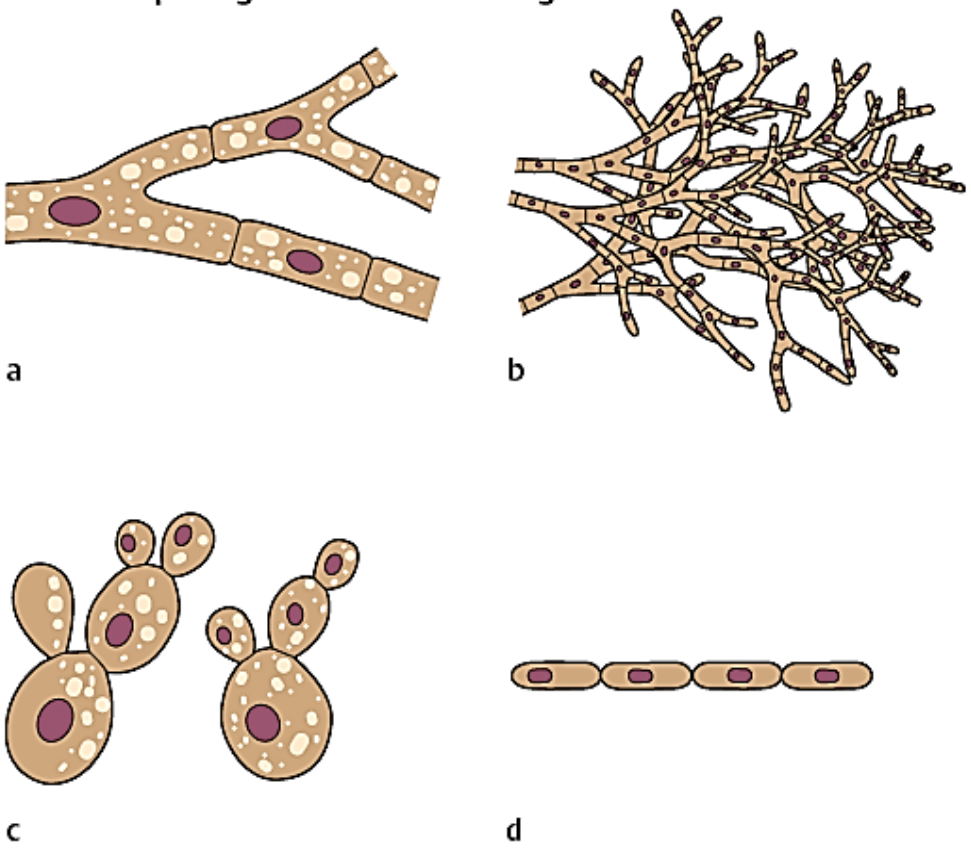
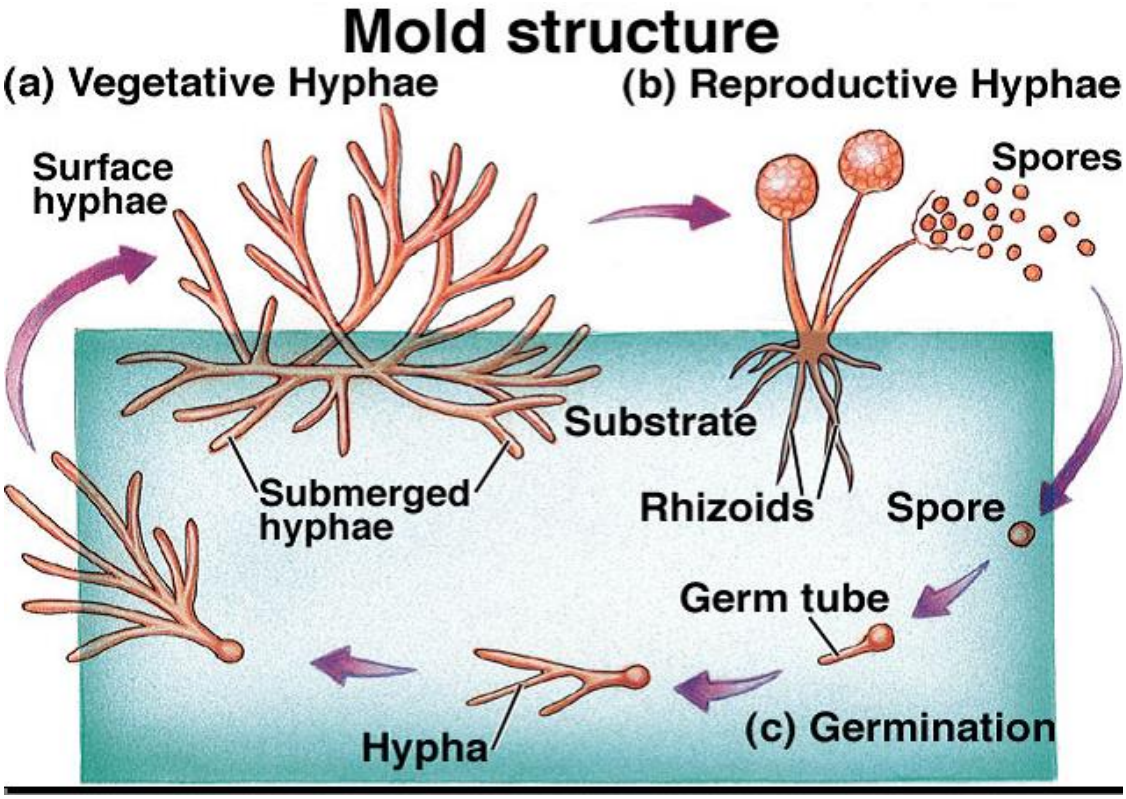
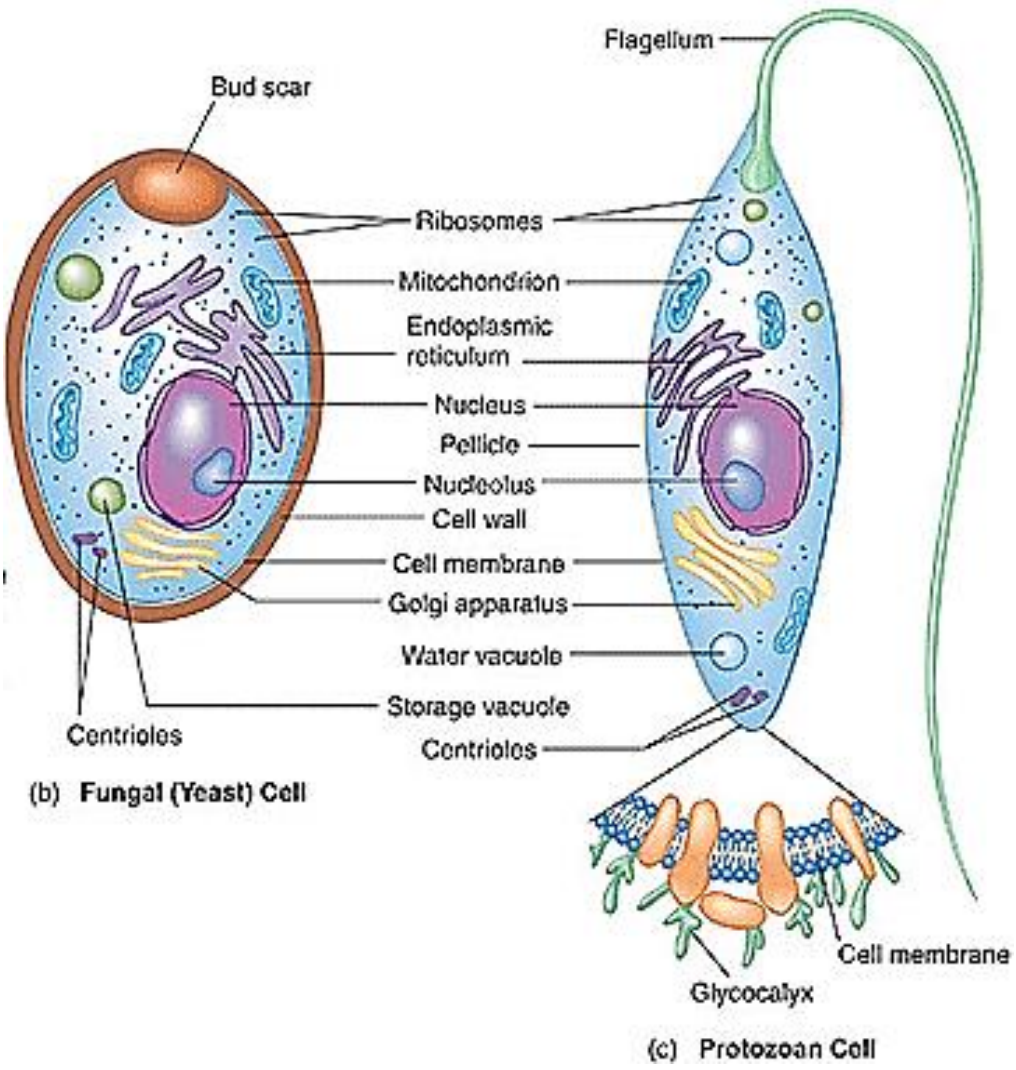


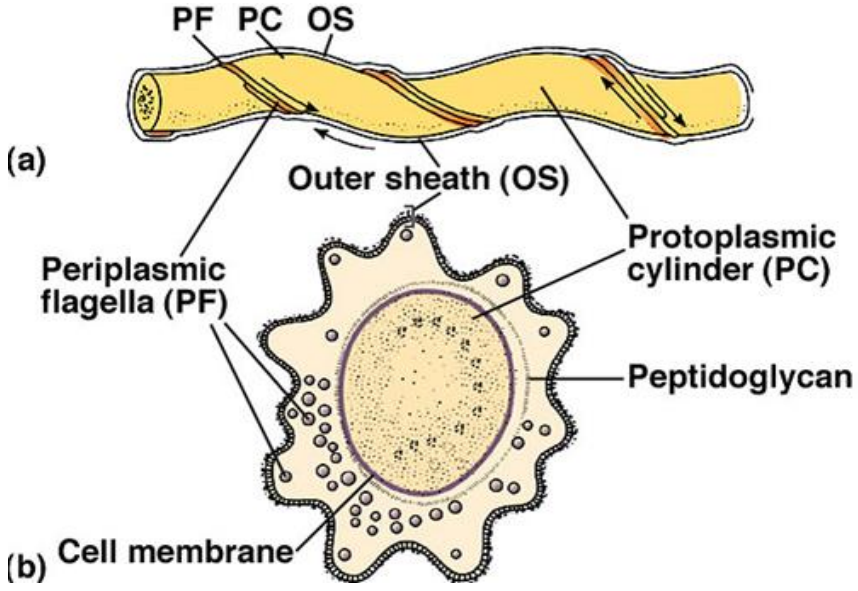
Fig. 5.1 There are two basic morphological forms: hypha and yeast.
a Hypha, septate, or nonseptate.
b Mycelium: web of branched hyphae.
c Yeast form, budding (diameter of individual cell 3–5 µm).
d Pseudomycelium.



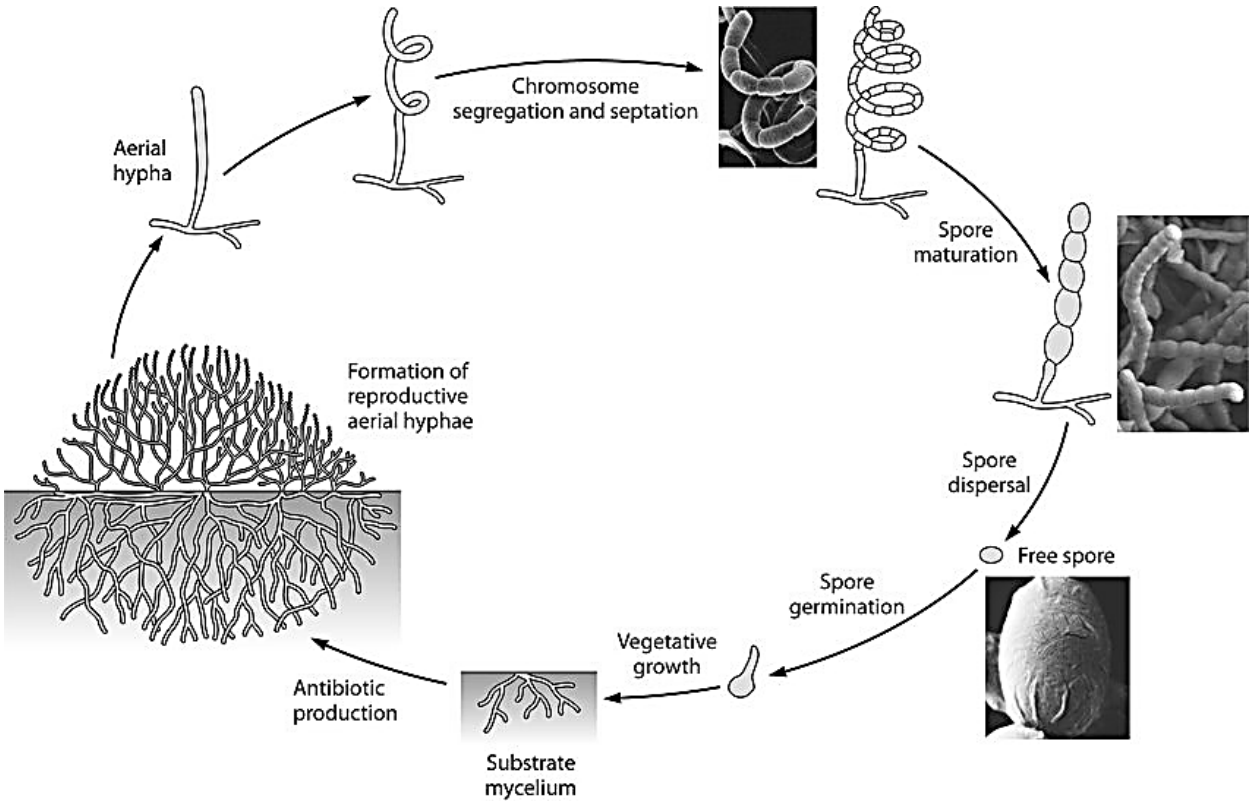
Comparative morphology of fungi and protozoa



Endoflagella of Spirochetes

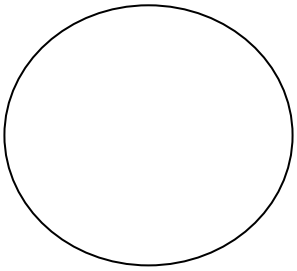


Growth and development of *Actinomycetes*



Practical activity №1. Prepare a smear from own teeth scraping, stain, examine.

Prepare smear (see practical N1), stain by Gram (see practical N2), examine it microscopically (see practical N1). Make conclusion about microorganisms of teeth scraping.



Microflora of oral cavity

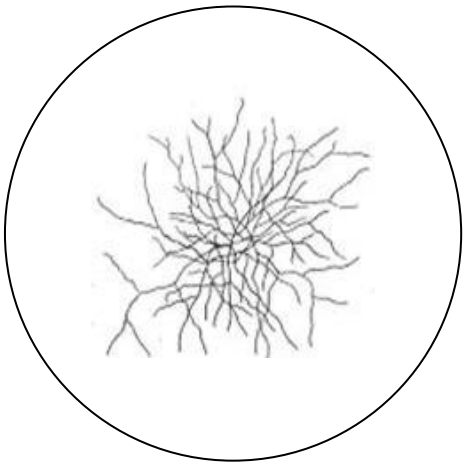
Gram (+) microorganisms

1. *Candida*
2. *Micrococci*
3. *Streptococci*
4. *Staphylococci*
5. *Lactobacilli*
6. *Veilonella*
7. *Fusobacteria*

Gram(-) microorganisms

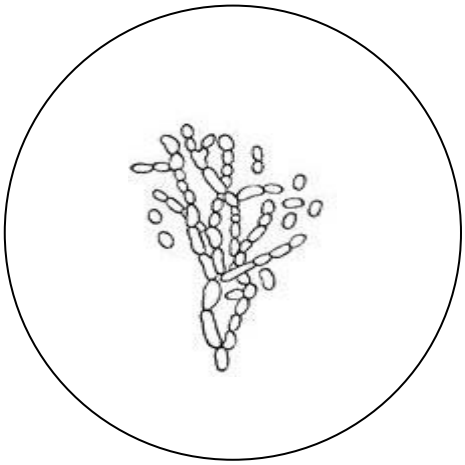
8. *Vibrio*
9. *Spirillas*
10. *Spirochetes*
11. *Bacteroides*
12. *Leptotrichia*
13. *Endothelial cell*

Practical activity №2. Examine smears of microorganisms different groups. Draw them.

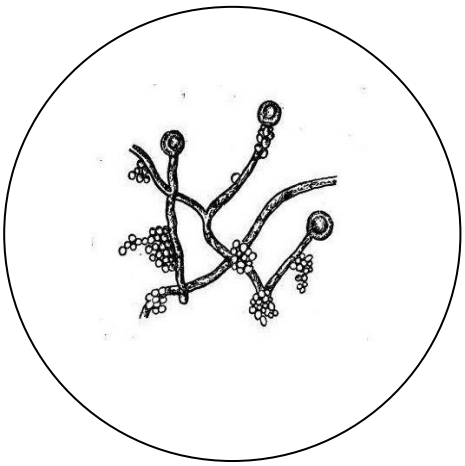


Yeasts

Spirochetes

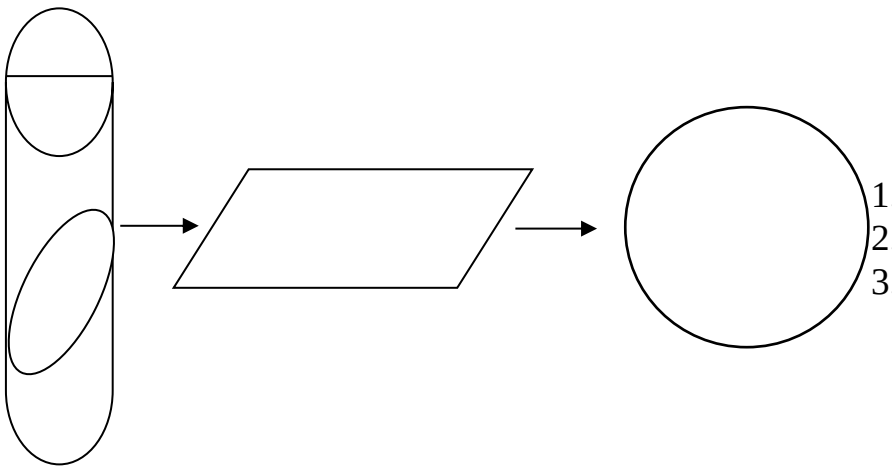


Actinomycetes



Candida

Practical activity №3. Prepare smears from different (agar and liquid) cultures, stain by Gram, examine them.



1. Shape
2. Location of the cells
3. Tinctorial properties

Control questions:

- Taxonomic position of fungi, spirochaetes, actinomycetes and fungi.
- Morphological features of spirochaetes. What are their likeness to bacteria and protozoa?
- Morphological properties of actinomycetes. Name their similar properties with bacteria and fungi.
- Morphological properties of moulds. Classification. Septated and nonseptated moulds. Perfect and imperfect moulds.
- What are morphological differences and similarities between yeasts and yeast-like fungi
- Protozoa morphology and structure.

Class №5

Topic “Nutrient media for cultivation of bacteria. Sterilization”

Topic relevance:

Microbiological work, and first of all practical tasks, is related to preparation of nutrient media for raising of microorganisms. Media are needed for an accumulation, selection and storage of microorganisms. Nutrient media are used for the bacterial diagnosis of infectious diseases. Except it, they are used for raising of cultures with the aim of research of their metabolism, for a production by means of microorganisms of valuable products of metabolism: enzymes, antibiotics, vitamins, etc. Quality of diagnostics and quality of bacterial preparations often depend on the full valu e of nutrient media.

To master this topic, students should study the chemical composition of the microbial cell, the metabolism of microorganisms, the mechanism of nutrition, the classification of microorganisms by type of breathing and other. Students are given an opportunity to get acquainted with the classification of nutrient media, the principles of their preparation and requirements for them; to learn methods for determining the enzymatic activity of bacteria.

No less relevant is the topic "Sterilization". Studying this topic gives students an opportunity to become acquainted with the influence of physical, chemical and biological factors on the life of microorganisms. To get acquainted with the methods of sterilization and sterilization equipment, to master the methods of control of sterilization in an autoclave.

All this determines the relevance of the topic of the lesson and aims at forming a positive motivation for its study.

Educational purposes:

- To get acquainted with a variety of nutrient media for the cultivation of microorganisms and their classification.
- To study samples of prepared nutrient media, their components. To get acquainted with the techniques of preparation, bottling and preparation for sterilization of various nutrient media (MPA, MPB, Marten’s broth, Hottinger’s broth, meat-peptone gelatin, peptone water, blood agar, Endo’s medium, Levin’s medium, Hiss’s medium, etc).
- To learn the method of determination of sucrolytic, lipolytic, proteolytic, peptolytic, hemolytic properties of bacteria.
- To study the principles and methods of sterilization, sterilization equipment, modes of sterilization in different devices and control of sterilization efficiency in autoclave and dry-heat sterilizer.
- To explain the effect of physical, biological and chemical factors on microorganisms.

Basic knowledge, skills, needed to study topics (interdisciplinary integration). See a class №1.

List of terms, parameters, characteristics that a student should learn during class:

Terms	Definition
Physiology of microorganisms	Part of general microbiology, which studies the metabolism of microorganisms, their nutrition, growth, reproduction, chemical composition, cultivation. The physiological properties of microorganisms are used in their systematics, they are important for the study of pathogenesis of infectious diseases, microbiological diagnostics, as well as in the development of biotechnological processes for the production of diagnostic, therapeutic and prophylactic drugs and biologically active substances of microbial origin.
Groups of nutrient media	
Main nutrient media	Media that by the composition and presence of nutritives suitable for cultivation of many types of bacteria (MPB, MPA).
Special nutrient media	Used in cases where microorganisms do not grow on simple nutrient media. These include blood agar, serum agar, serum broth, ascitic broth.
Elective media	Media where microorganisms of a particular species grow faster, more intensively, pass ahead other types of bacteria in their development. For example, 1% alkaline peptone water is an elective medium for cholera vibrios, Ru and Leffler medium – for diphtheria causative agents.
Selective media	Media which by the addition of certain components (bile, aniline dyes, antibiotics, etc.), are able to inhibit the development of certain types of microorganisms but do not affect other species. For example, Müller's medium is selective for typho-paratyphoid bacteria, furazolidone-twin agar – for corinebacteria and micrococci. Adding antibiotics to the media makes them selective for fungi (Saburo's medium, etc.).
Differential media	Media that allow to determine certain enzymatic properties of microorganisms and to differentiate them. They are divided into media to determine the proteolytic, peptolytic, sucrolytic, lipolytic, hemolytic, reducing properties of bacteria (Endo's, Levin's, Ploskirev's, Hiss's media).
Transport media	Used for short-term storage and transportation of microorganisms. Usually, such media are placed material that must be delivered to the laboratory for research. The cultivation of microorganisms in these media is not carried out. Media of this type contain components that provide optimal pH and consistency.
Enrichment media	Used in cases where there is a need to increase the concentration of bacteria of a particular species (10% bile MPB for the causative agent of typhoid fever).

Sterilization	Complete destruction of vegetative and spore forms of microorganisms on objects, materials, in nutrient media.
Desinfection	A set of measures for the complete, partial or selective destruction of pathogens potentially pathogenic to humans at various environmental sites in order to prevent the transmission of the pathogen from the source of infection to the susceptible organism.

Theoretical questions for class preparing:

- What is studied in the «Microbial Physiology»?
- Features of metabolic processes in bacteria, enzymes of bacteria, their classification.
- Bacterial feeding mechanisms. Classification of microorganisms by type of nutrition.
- Breathing methods in bacteria. Classification of microorganisms by breathing.
- Nutrient media for cultivation of bacteria, classification. Requirements for nutrient media.
- Characteristics and principles of production of individual groups of media.
- Methods for determining the enzymatic activity of bacteria.
- The sensitivity of microorganisms to the action of physical, chemical and biological agents. Mechanisms of their action on a bacterial cell.
- Sterilization, disinfection, aseptics, antiseptics. Their principles and methods.
- Sterilization equipment, the mode of operation of these devices.
- Monitoring the efficiency of sterilization in an autoclave and dry-heat sterilizer.

Tasks, performing during class:

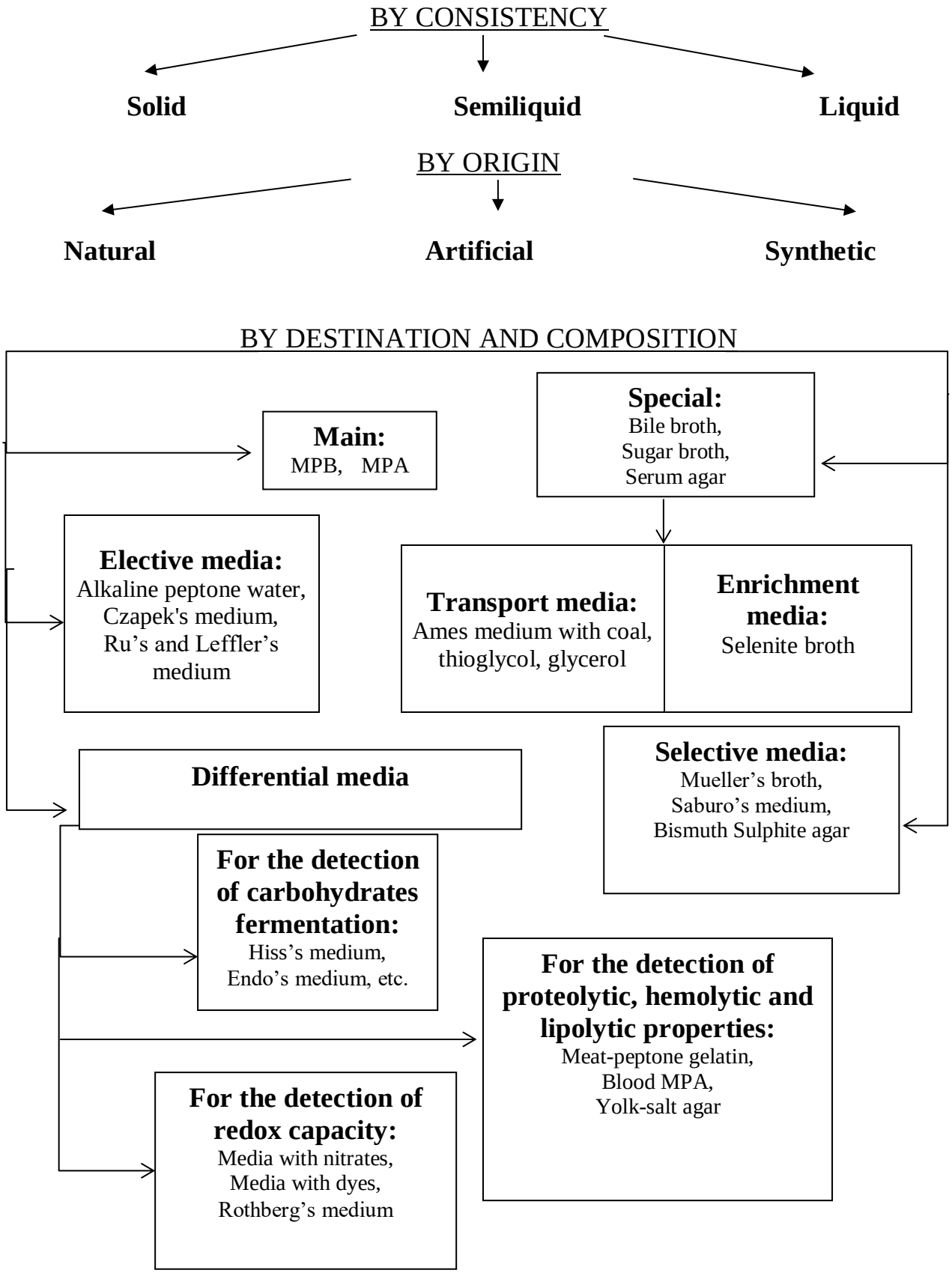
- To get acquainted with the classification and principles of production of different groups of nutrient media: main, special and differential-diagnostic.
- To learn the samples of nutrient media and constituent components.
- To learn the method of determination of sucrolytic, proteolytic, peptolytic, reducing, hemolytic properties of bacteria.
- Familiarize yourself with sterilization methods, sterilization equipment and principles of autoclave sterilization control.
- To get acquainted with the sterilization laboratory of the department and the equipment in it (autoclave, dry-heat and fluid-steam sterilizer, apparatus for inactivation of serum).

Class content:

At the practical class students study the nutrient media for the cultivation of microorganisms, their purpose and principles of production. They study the method of determining the enzymatic properties of bacteria. Familiarize themselves with sterilization equipment, study methods of sterilization, which are used in microbiological practice and principles of efficiency control. Students write complete tasks in the protocol and teacher sign it.

Recommendations for design of the protocol

Classification of nutrient media

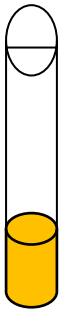


REQUIREMENTS FOR NUTRITIONAL ENVIRONMENTS

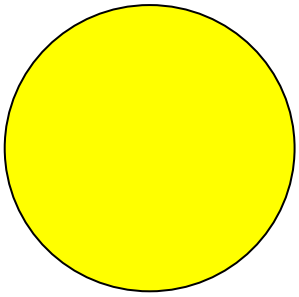
- Completeness in chemical composition.
- Presence of growth factors
- Stabilization of the pH optimum
- Buffering
- Appropriate redox potential
- Isotonicity
- Viscosity
- Humidity
- Transparency
- Sterility

Practical activity №1. Draw in the protocol samples of various nutrient media.

Main nutrient media

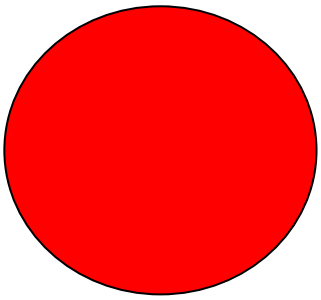


Meat-peptone broth
(MPB)

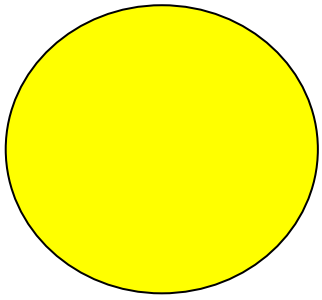


Meat-peptone agar
(MPA)

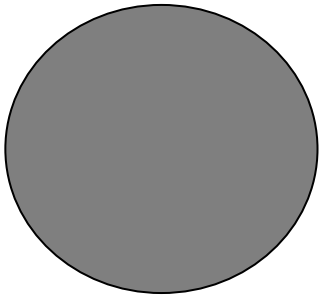
Special media



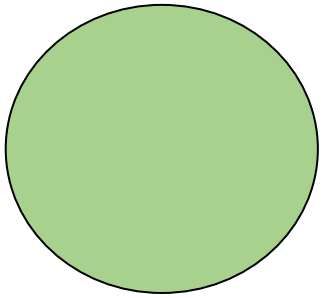
Blood MPA



Yolk-salt agat
(YSA)

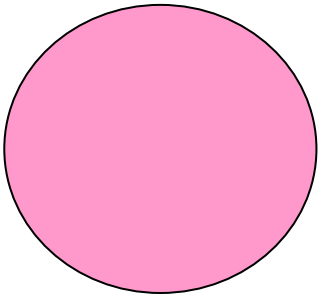


Mansuro's medium

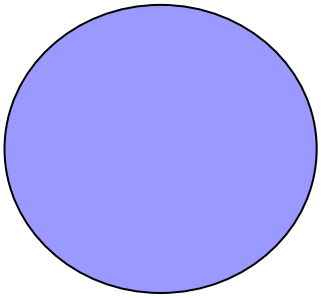


Bismuth Sulphite agar

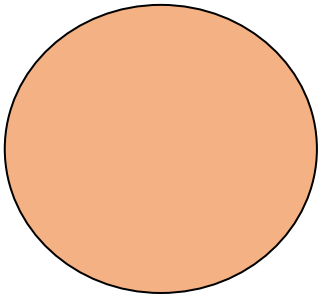
Differential media



Endo's



Levin's

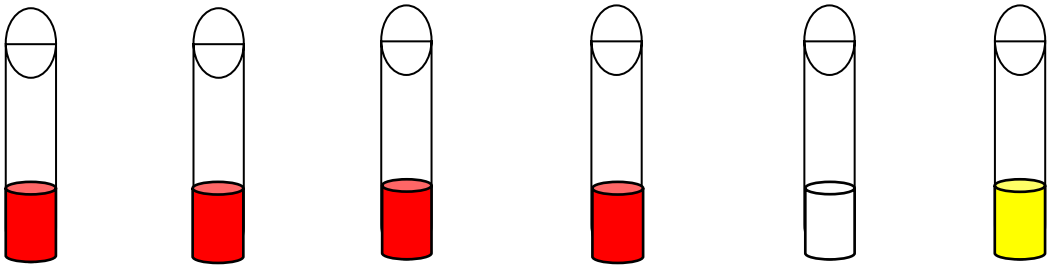


Ploskirev's

Composition of the Endo's medium:

Meat-peptone agar, 1% lactose, pH indicator - basic fuchsin (discolored with sodium sulfite solution).

Hiss's color series

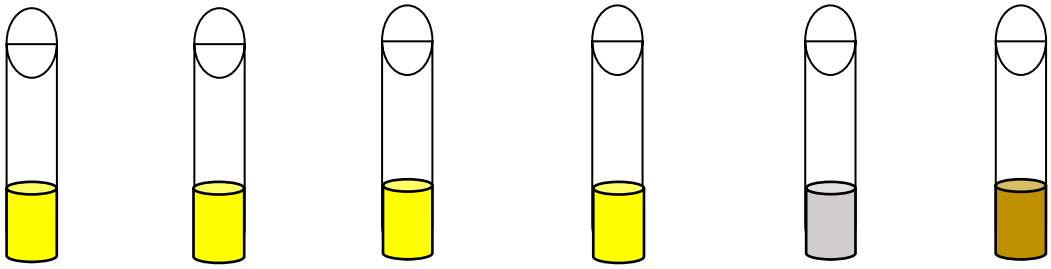


Glucose Lactose Mannitol Sucrose Milk MPB

Hiss's color series:

MPA (MPB)+1% carbohydrate + pH indicator

Practical activity №2. Determine the enzymatic activity of the tested bacterial culture on the Hiss's color series.



Hiss's color series a day after inoculation of pure bacterial culture (glucose and lactose fermentation took place).

To determine the enzymatic properties of bacteria, carry out the culture of *E. coli* on a Hiss's series (detection of sucrose). Students should become familiar with the changes that are taking place in the Hiss's medium.

The principles and methods of sterilization.

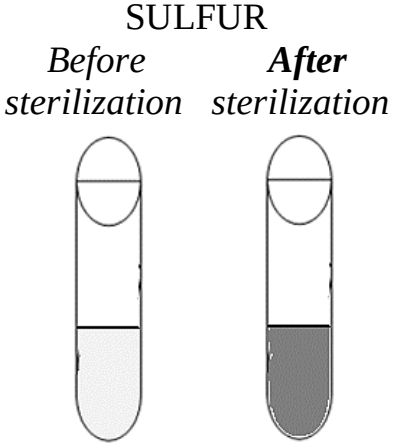
PHYSICAL	PHYSICAL AND CHEMICAL	CHEMICAL	BIOLOGICAL
Thermal Ultrasound Radiant Electric current and ultra-high frequency current	Adsorption-filtration, filters (disks, Chamberlain's and Berkefeld's candles: asbestos, nitrocellulose, porcelain, diatomite)	Processing: halides, acids, aldehydes, alkalis, esters, alcohols, etc.	Processing with antibiotics and other chemotherapeutic drugs

Thermal sterilization

Methods of sterelization	**Appliances**	**Sterilization conditions**		**Sterilizing objects**
		Temperature	Time	
Annealing and firing	Burner	> 200 ºC	2-3s	Loops, spreaders, pin tweezers, slide
Boiling	Sterilizer	100ºC	30-60 min	Needles, syringes, surgical instruments, rubber tubes
Liquid steam (with unscrewed autoclave cover). Steam sterilization under pressure	Liquid steam sterilizers (Koch's apparatus) Autoclaves	100ºC 120ºC 134ºC 1,5-2atm	3 days in a row for 30 min (fractional sterilization) 20 min 40 min	Gelatin, milk, carbohydrate nutrient medium, MPB, MPA. Spent bacterial cultures
Tyndalization (multiple steam)	In the water bath	60-65ºC	1 hour 5 days	Protein fluids, vitamins, some medicine
Pasteurization	--- // ---	70ºC 80ºC	30 min 10 min	Milk, wine, juices
Dry heat in a dry-heat sterilizer	Dry-heat sterilizers	160º 180º	120-150 min 45-60 min	Glassware, oil, tools, etc.

Autoclave sterilization efficiency control.

Chemical control – use substances with a certain melting point (sulfur - 119ºC, benzoic acid - 120-122ºC, benzonaphthol - 110ºC, mannose and urea - 132-133ºC, temperature indicator paper).



Biological control – use biotests made from the test culture of microorganisms (from the group of anthracoids). At least 5 tests are placed in the sterilizer and 1 (control) is left at room temperature. After sterilization, biotest washes are seeded into nutrient media. The result is read as follows: there should be no growth in the washes that were in the sterilizer; in rinses from the control test - abundant culture growth.

Conclusion:

Questions for self-control:

- What is the physiology of microorganisms studying?
- What are the characteristics of metabolic processes in a bacteria?
- What are the enzymes in the bacteria, their classification?
- What is the mechanism of nutrition in bacteria and how microorganisms are divided by type of nutrition?
- How microorganisms are divided by breathing type?
- Classification of nutrient media for the cultivation of microorganisms. How nutrient media are divided by destination, by origin?
- What are the principles of preparation of different groups of nutrient media, requirements for them?
- In what environments the enzymatic activity of the bacteria is determined?
- How sensitive the spore and vegetative forms of microorganisms are to the action of physical, chemical and biological agents?
- What are the principles and methods of sterilization?
- What sterilization devices and sterilization regimens are used in microbiological practice?
- What are the methods of controlling the efficiency of sterilization in an autoclave and dry-heat sterilizer?

Class №6

Topic: "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).

Concrete objectives:

- To study principles, methods, stages of isolation of bacteria pure cultures.
- To master the technique of specimen inoculation for purpose of isolated bacterial colonies obtaining.
- To master the methods of cultivation of anaerobic bacteria.

Basic knowledge, skills, needed to study topic (interdisciplinary integration). See a class №1.

A list of key terms, parameters, characteristics that student should learn for the class:

Term	Definition
Pure culture	A pure culture is population of one species microorganisms that grow on a sterile nutrient media
Population of microorganisms	Aggregate of microorganisms of one species, which exists on certain territory (in the biotope) relatively long time
Colony-forming unit	A measure of viable cells in which a colony represents an aggregate of cells derived from a single progenitor cell.

	CFU is used to determine the number of viable bacterial cells in a sample per mL. Hence, it tells the degree of contamination in samples of water, vegetables, soil or fruits, or the magnitude of the infection in humans and animals.
Aerobic microorganisms	Microorganisms, metabolism of which takes place at presence of free oxygen in the environment. Oxygen is final electron acceptor.
Anaerobic microorganisms	Microorganisms get energy without access of free oxygen by breaking up of nutrients, for a synthesis and structure of microbial cell, her structural components and for the processes of vital functions
Ethology of microbes	Ethology of microbes studies their "biosocial" conduct, "language" and mechanisms of intercourse ("Quorum sensing"). Microbes live as biosocial creatures are in colonies, cultures, biofilms.

Theoretical questions:

- Concept of pure culture of microorganisms.
- Purpose of isolation of pure culture of microorganisms.
- Principles and methods of isolation of pure culture of bacteria.
- Stages of isolation of pure culture of bacteria.
- Concept of colony of microorganisms.
- Methods of isolatation colonies of microorganisms obtaining
- Methods of creation of anaerobic condition for cultivation of anaerobic microorganisms.

Practical activities performed in the class:

- To study principles, methods, stages of receipt of Isolation of pure culture of bacteria.
- To master work with a bacteriological loop, Petri dish, test tubes to carry out subinoculation of specimen on solid media for isolated colonies obtaining
- To familiarize yourself with the methods of cultivation of anaerobes.

Topic content:

Students study principles, methods and stages of pure cultures isolation. Master the technique of specimen inoculation on solid nutrient media by the method of the streak method. Carry out the first stage of pure cultures of aerobic and anaerobic bacteria isolation, subinoculating corresponding specimen on solid media and creating corresponding conditions, that necessary for microorganisms with different biological properties. A purpose is obtaining of the isolated colonies. Students write down ready tasks in the protocol and sign it at teacher.

Recommendations for the protocol design

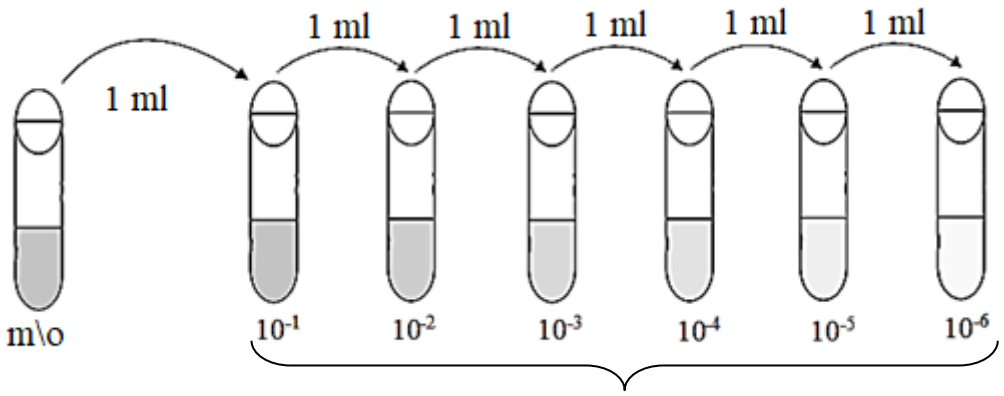
Main stages of pure culture isolation

Stages	Objective
1.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
1. Investigation of isolated colonies **a.** Examine cultural properties **b.** 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
3. Examine cultural properties and Microscopic examination (staining by Gram)	To be sure that culture is not contaminated and conduct morphological, tinctorial and cultural identification.

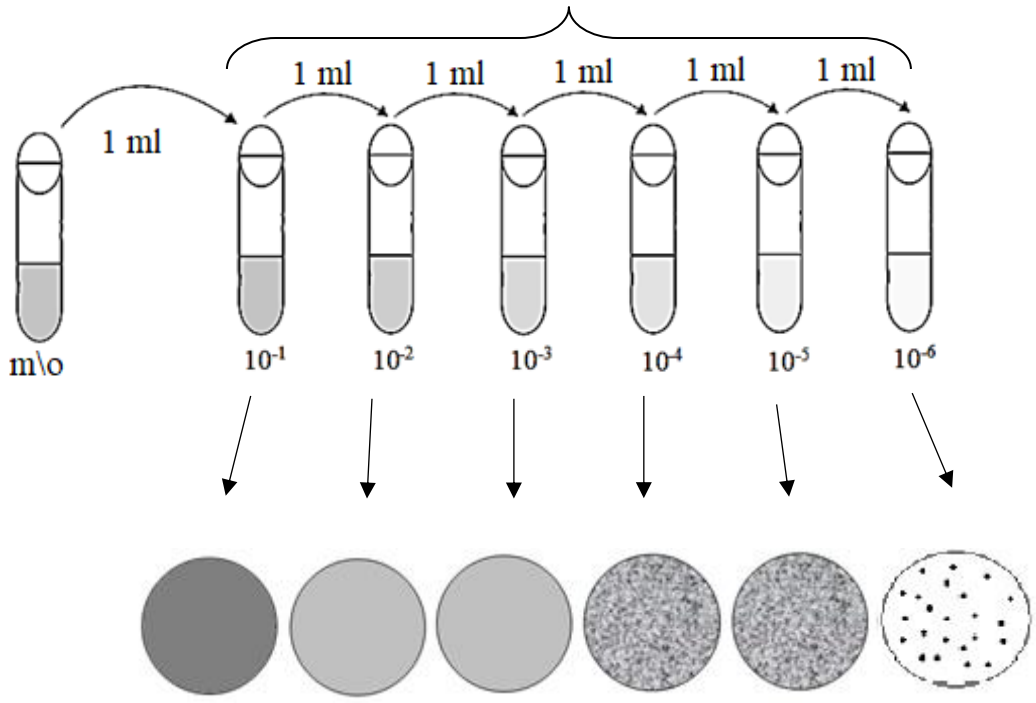
Principles and methods of isolation of pure culture

Methods of isolation of pure culture, based on:	
mechanical separation principle	***biological properties of bacterial***
1. Pasteurs' method of serial dilution; 2. Method of "plate" dilutions by Koch; 3. Drigalsky method; 4. Streak method: 5. Clone isolation	1. Method of isolation of moving bacteria (Shukhevich method) 2. Method of isolation of heat resistant bacteria 3. Method of isolation of strict anaerobes 4. Method of isolation of acid resistance 5. Method of isolation of sporeforming bacteria 6. Method of isolation of antibiotic resistant bacteria 7. Method of isolation of bacteria on selective (elective) media 8. Method of isolation of bacteria in sensitive laboratory animal

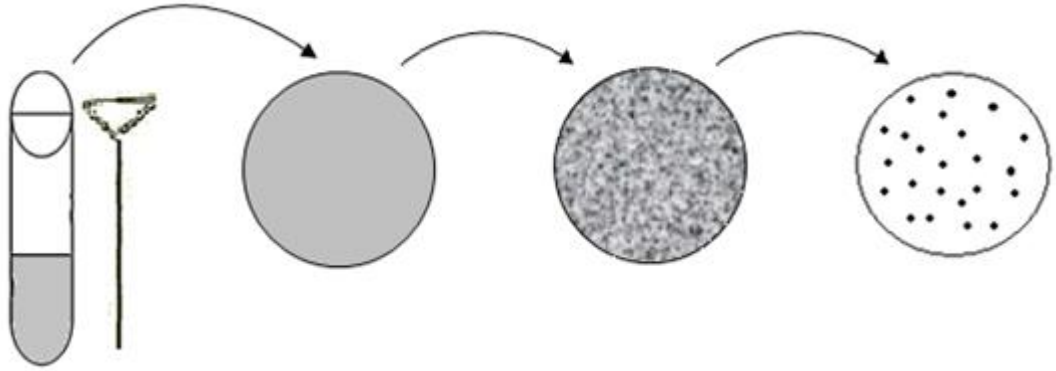
Pasteur developed the concepts of pure culture. This was an important intellectual step forward because the ability to work with pure cultures allowed Pasteur (and subsequent microbiologists) to study individual bacterial species. Pasteur's method for developing pure culture- repeatedly diluting broth 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.

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 thelid) 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. Sterilize the transfer loop before obtaining a specimen.

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.

a. Open the culture and collect a sample of specimen using the sterile loop.

b. Insert the loop into the culture tube and remove a loop full of broth.

c. Replace the cap of the test tube and put it back into the test tube rack.

Streak the first sector. Hold the inoculation loop handle in one hand as you would hold a pencil.

d. 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.

2. 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.

a. 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.

3. Flame and cool the inoculation loop. 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.

4. Flame and cool the inoculation loop. Turn the plate 90 degrees counter-clockwise. Streak the fourth quadrant in a same manner. Hold the agar plate in the opposite hand

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.

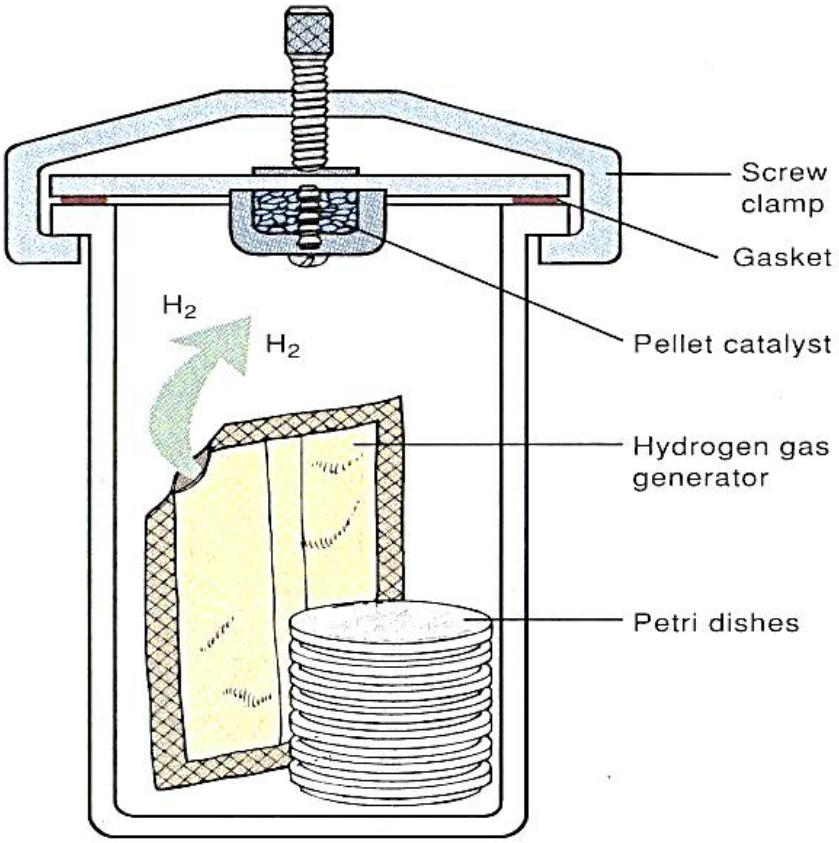
Micromanipulators have been built, which permit one to pick out a single cell from a mixed culture. This instrument is used in conjunction with a microscope to pick a single cell (particularly bacterial cell) from a hanging drop preparation.

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.

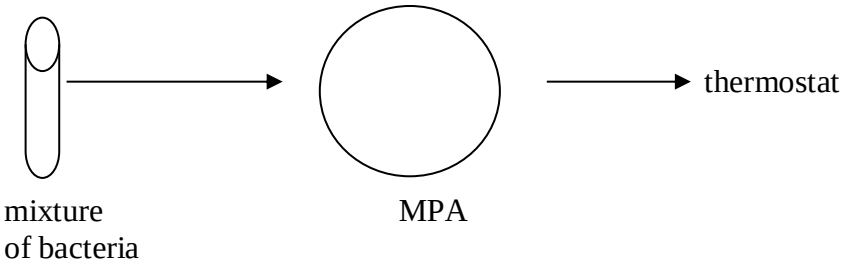
General methods of cultivation of anaerobe microorganisms:

- Anaerobe jars
- Special medium for anaerobic growth



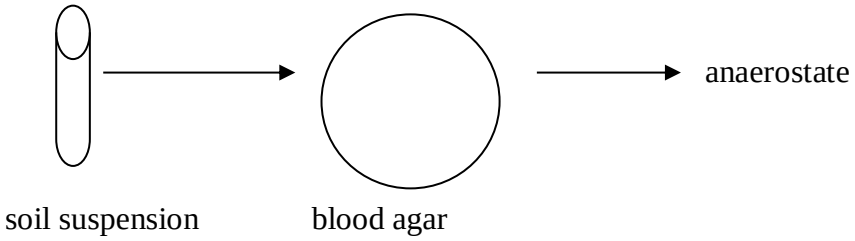
Practical activity №1. To start aerobic bacteria pure culture isolation from bacterial mixture by streak method.

You will need: tube with bacterial mixture, Petri dish with MPA (meat peptone agar), bacterial loop, incubator.



Practical activity № 2. To start anaerobic bacteria pure culture isolation from soil suspension by streak method.

You will need: tube with soil suspension, Petri dish with blood MPA, anaerostate.



Control questions

1. Describe bacteriological method of diagnoses of infectious diseases.
2. Define pure culture of microorganisms.
3. What are signs of bacteria culture purity?
4. Name purpose and importance of pure bacteria culture isolation.
5. Describe methods of inoculation of pure culture of bacteria.
6. Name principles of pure culture isolation.
7. What biological properties of microorganisms can be used for pure culture isolation?
8. Describe main stages of pure culture isolation.
9. Name methods of creation of anaerobic condition for cultivation of microorganisms.

Class №7

Topic: "Growth and reproduction of microorganisms. 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).

Concrete objectives

- To master the methods of colony investigation (external signs, microscopy, mobility of microorganisms).
- To master the technique of subculturing of bacteria colonies on nutrient media (aerobic bacteria on slant MPA, anaerobic bacteria on the special nutrient media).

Basic knowledge, skills, needed to study topic (interdisciplinary integration). See a class №1.

A list of key terms, parameters, characteristics that student should learn for the class:

Term	Definition
Cultural properties	Cultural properties are types of growth bacteria on liquid and solid nutrient media (properties of colonies).
Colony	A population of bacterial cells of same species which have grown from one bacterial cell on solid medium in isolated accumulation.
Dissociation of microbes	One of forms changeability, when in population appear individuals and clones which differ from an initial type in the form of colonies (S - R dissociation) and other signs (for example, by the loss of capsule, mobility; decline of enzyme activity, virulence, antigenicity; sensitiveness to the phages, physical and chemical factors of and other).
Growth of microorganisms (microorganism level)	Concerted increasing of all components of bacterial cell
Reproduction of Microorganisms (growth on population level)	An increase of number of cells in the population

Forms of colonies	S -colonies - round, protuberant, have an even edge and smooth and shiny surface. At subculturing in a liquid nutrient media form diffuse turbidity. R - colonies - irregular shape, uneven edge and wrinkled, rough surface. At subculturing in a liquid nutrient media form grainy sediment.
Methods of reproduction of bacteria	Binary fission (most bacteria), fragmentation of cell (actinomycetes); by spores (streptomycetes); by fragmentation and budding (mycoplasm); development cycle chlamidia (they have 2 forms: extracellular infectious elementary bodies (not capable of dividing) and intracellular, capable of dividing resulting in elementary bodies
Batch culture	Batch culture is a cultivation of bacteria culture in the closed system (without adding new nutrients and removing of metabolite waste products). Batch culture has a few phases of development: 1) Lag phase of growth involves no increase in cell numbers) Exponential phase of growth is the time during which cell number increases exponentially. 3) Stationary phase is reached when the numbers of viable cells stops increasing. In the stationary phase the total number of viable microorganisms remains constant. This may result from a balance between cell division and cell death, 4)Death phase is characterized by an exponential decrease in the numberin the number of viable cells

Theoretical questions

- Methods of colonies research.
- Signs of colonies.
- Concept of morphological, tinctorial, cultural properties of microorganisms.
- Pigments of bacteria, their physiology value.
- Type of microorganisms growth in liquid nutrient media
- Concepts of "growth" and "reproduction" of microorganisms.
- Methods of reproduction of bacteria.
- Factors, inhibiting reproduction of microbes.
- Concept of batch culture, phases of development.
- Methods of determination of mobility of bacteria.

Practical activities performed in class:

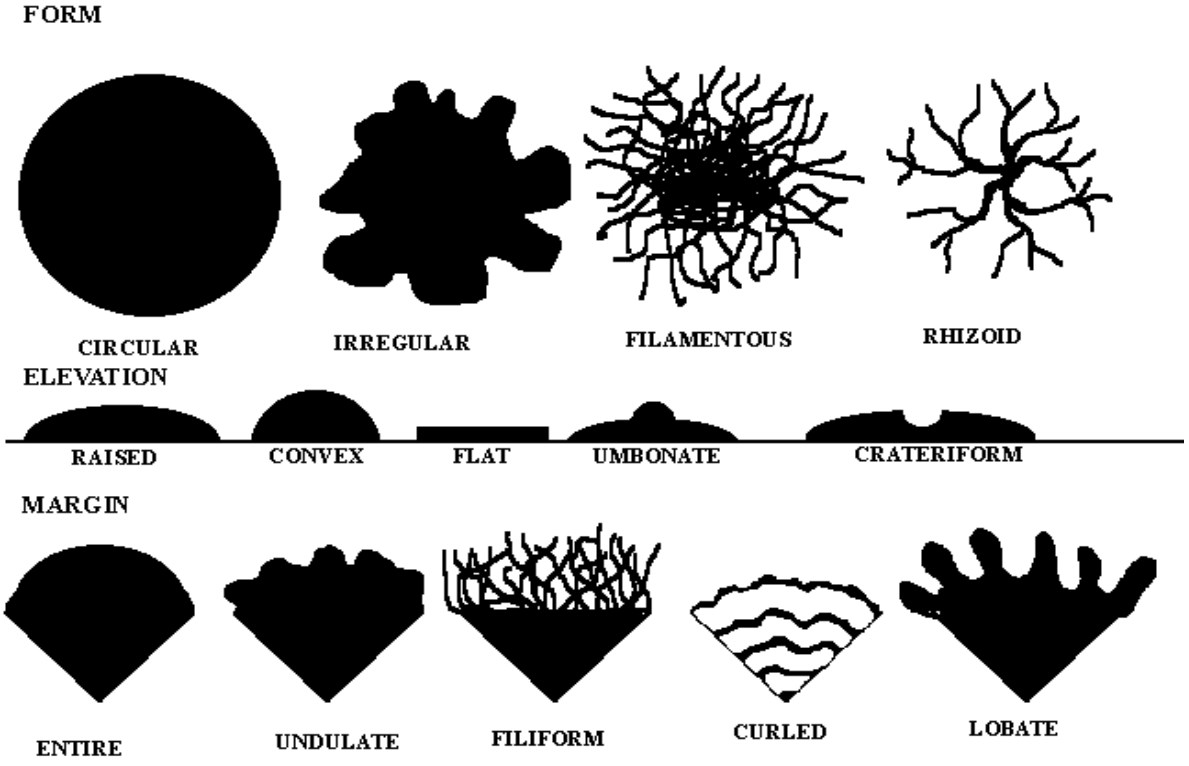
- To study signs of colonies.
- To master methods of macro- and microscopic methods of colony research.
- To define mobility of aerobic bacteria by wet mount slide with cover slip
- To carry out subinoculation of microorganisms on solid and liquid nutrient media with the purpose of pure culture obtaining.

Topic content

Students study signs of the colonies. Master macro- and microscopic methods of colonies research. Master the technique of specimen inoculation by a loop on solid nutrient media (in test tubes with slope MPA) and in the liquid nutrient media (Vrublesky media) for the obtaining of pure cultures of aerobic and anaerobic bacteria.

Recommendations for protocol design

Various types of colonies:



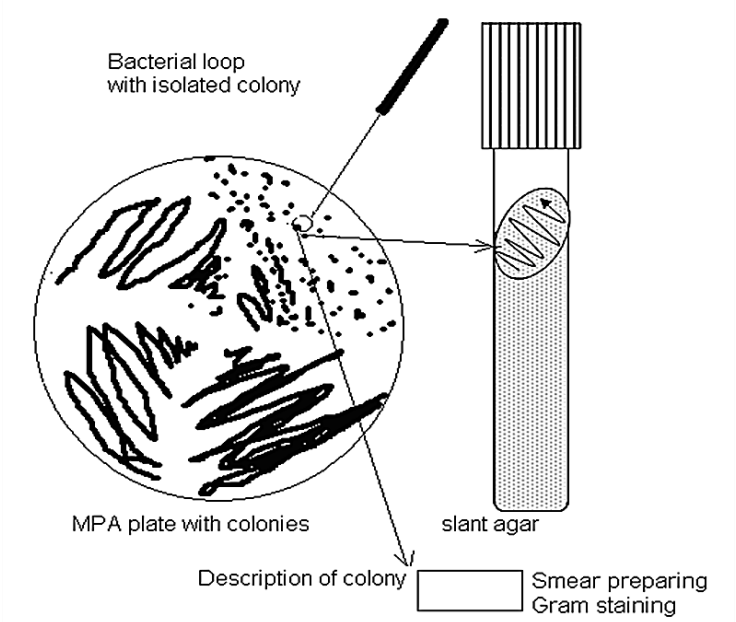
1. **Size** of colonies (large, 4-5 mm in diameter or more; medium, 2-4 mm; small, 1-2 mm; minute, less than 1 mm);
2. **Configuration** of colonies {regularly or irregularly rounded, rosette-shaped, rhizoid, etc.);
3. **Colour**: of the colonies: colourless, pigmented, the colour of the pigment (white, yellow, red ets.); colorless. It is noted whether pigment secretion on medium.
4. **Profile** *of the colonies:* flat, convex, raised, pulvinate, umbonate, etc.
5. **Margin:** entire, serrated, erose, curled, lobate, filamentous, jagged, wavy, fringy, etc.
6. **Transparenc** (non-transparent, semitransparent, transparent).
7. **Structure** (homogeneous or amorphous, granular, fibriliar, etc.)
8. **Nature of the surface** (smooth, glassy, moist, wrinkled, lustreless, dry)
9. **Position of the colonies on the nutrient medium** (protruding above the medium, submerged into the medium; flat, at the level of the medium; flattened, slightly above the medium).

Practical activity № 1. Continue the isolation of pure culture of aerobic bacteria

Materials*: Petri plate cultures of various bacteria*

Procedure

1. Obtain an test culture. Note that the bacteria grow in clearly defined groups, known as colonies. In most cases, each colony is the outgrowth from an individual cell, although they may overlap if excessive numbers of cells were plated.
2. Visually examine the individual colonies of bacteria and describe them according to the main characteristics (see table)
3. Determine and record the identity of your colonies.
4. Prepare the smear from studied colony, check up of the purity and describe morphological and staining properties of the culture.
5. Sub- inoculate tested colony on slope agar.
   a) Place the slope agar tube to be inoculated between the thumb and forefinger of the left hand with the sloped surface of the medium towards the worker.
   b) Take the holder of the inoculation loop between the thumb and first two fingers of the right hand (like a pen). Sterilize the wire by holding it vertically in the flame.
   c) Remove the lid of Petri dish and pick a portion of single colony by touching it with the sterile cool loop.
   d) Remove the stopped and flame the mouth of the tube.
   e) Insert the loop charged with the growth and make zigzag streaks on the surface of the agar. Take care not to dig into the agar.
   f) Withdraw the loop and sterilize it, flame the mouths of the tubes and replace the stoppers.
   g) Write the nature of the inoculated material, date and student's name on tube by means of labeling pencil.
   h) Place the test tubes with the inoculated medium into a 37 °C incubator for 18-24 hrs.

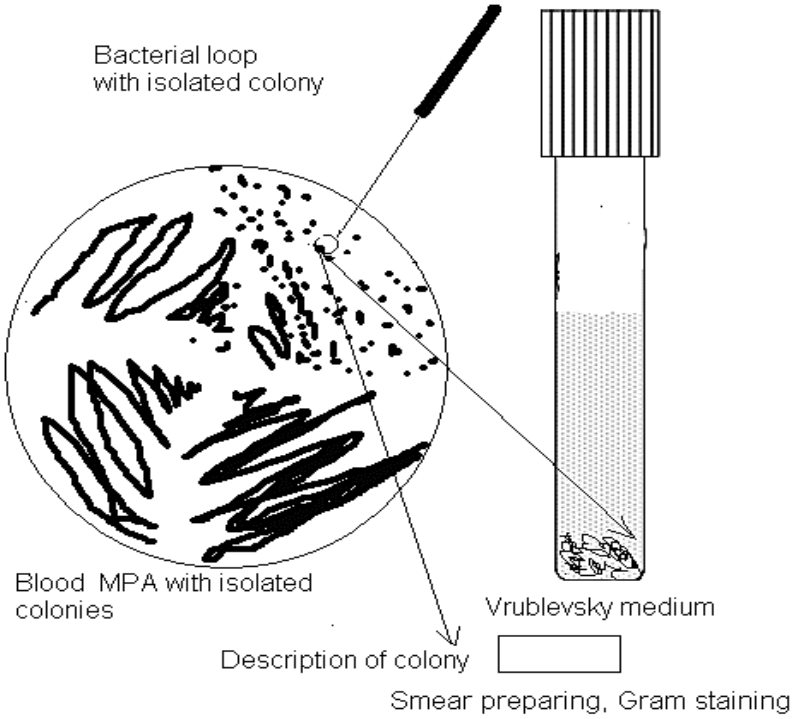


External characteristic bacterial colony:
size –
configuration –
coloure –
profile –
marigin –
transparence –
surface–
structure –

Characteristic morphology of cells:
1. Shape
2. Location of the cells
3. Tinctorial properties

Conclusion:

Practical activity № 2. Continue the isolation of pure culture of anaerobic bacteria.



External characteristic bacterial colony:
coloure –
profile –
marigin –
transparence –
surface–
structure –

Characteristic morphology of cells:
1. Shape
2. Location of the cells
3. Tinctorial properties

Conclusion:

Control questions

- What are methods of bacterial colonies research?
- Name sings of colony purity?
- Describe biological importance of S - R dissociation.
- Name physiological importance of bacteria pigments.
- Can appearance of colony and ability bacteria to form pigments be consistent signs of bacteria species and used for it identification?
- What does growth of microorganisms on solid media differs from growth in liquid media?
- What factors can influence microbial growth patterns in liquid media?
- How are descendants of one microbial cell, which grew on (or in) solid nutrient media named?
- How exponential phase of batch culture differ from stationary phases?
- What biological property of microorganisms is determined by the method of routine wet mount slide with cover slip?

Class №8

Topic:" Isolation of pure culture (part 3)"

Topic relevance

In clinical laboratory, the bacterial culture is indicated in isolation of organisms in pure culture from clinical specimens and their final identification. Identification means the correct naming of isolates according to agreed systems of taxonomy and nomenclature.

Since a species is the "basic unit" of taxonomy, representing a specific, recognized type of organism, bacteria identification means determining a species of unknown bacteria.

Precise identification of bacteria is time consuming and contention and is best carried at in specialized reference centers. For the most clinical purposes what is required is clear guidance on the likely case of an infection, not a rigorously accurate, but belated description of what the patient has already recovered (or possibly died) from. In clinical laboratories, identifying the genus or species of an organism is more important than understanding evolutionary what species of bacteria they have- they are just interested in getting well. Their recovery often depends on the proper identification of the infectious agent.

Concrete objectives

- To give definitions of main taxonomical categories in classification of microorganisms.
- To define morphological, tinctorial properties and check up the purity of culture.
- To define a purpose and master the basic stages of identification of pure culture.
- To master the microscopic method of research of mobility of bacteria - method of wet mount slide with cover slip
- To define enzyme properties of pure culture of aerobic bacteria.

Basic knowledge, skills, needed to study topic (interdisciplinary integration).

See a class №1.

A list of key terms, parameters, characteristics that student should learn for the class:

Term	Definition
Species	Collection of microorganisms that has high level of DNA similarity (70%), shares phenotype properties and derived from common ancestor
Strain	Pure culture of microorganisms, isolated from a certain source (organism, environment), or from the same source in different times.
Clones	Descendants of one microbial cell.

Identification	Determination of species of microorganisms on the basis of study of their morphological, tinctorial, cultural, biochemical, antigenic and other properties.

Theoretical questions :

- Taxonomical categories in microbial classification
- Signs of culture purity?
- Name properties of microorganisms using for identification.
- Classification of enzymes.
- How to determine biochemical activity of microorganisms?
- Methods of determination of active movement of microorganisms.

Practical activities performed in class:

- To investigate the culture of aerobic bacteria - to define cultural, morphological, tinctorial signs, active mobility by wet mount slide preparing. To make up conclusion about purity of the culture.
- To define enzyme properties of pure culture of aerobic bacteria.
- To investigate the culture of anaerobic bacteria - to define tinctorial, cultural, biochemical, morphological properties. To make up conclusion about purity of the culture.

Topic content

Students study the taxonomical categories of microorganisms; determine the morphological, tinctoria properties. Investigate the culture of anaerobic bacteria, draw conclusion about purity of the culture. Determine the purpose of identification of pure culture and master the basic stages of identification of pure culture of bacteria. Determine enzyme properties of pure culture of aerobes and investigate the capacity of bacteria for active motion by means of microscopic method – method (wet mount slide preparing). Students write down prepared task in the protocol and sign protocols

Methods of identification of bacteria

Examination of morphological properties and staining affinity (Gram staining, or other methods)

Morphology and staining reactions of individual organisms generally serve as preliminary criteria to place an unknown species in its appropriate biological group. A Gram stain smear suffices to show the Gram reaction, size, shape and grouping of the bacteria, and the arrangement of any endospores.

An unstaining wet film may be examined with the dark- ground microscope to observe the morphology and delicate spirochaetes; an unstaining wet film or “hanging grope” preparation is examined with the ordinary microscope for observation of motility.

To identify of mycobacteria, or other acid- fast organisms, a preparation is stained by the Ziehl- Neelsen method or one of its modifications.

The microscopic characters of certain organisms in pathological specimens may sufficient for final identification, e.g. tubercle bacilli in sputum, or *Treponema pallidum* in exudate from a chancre. However, many bacteria share similar morphological features and further tests must be applied to differentiate them.

Cultural characteristic (see previous class)

The appearance of colonial growth on the surface of a solid medium, such as nutrient agar, is often very characteristic. Attention is paid to the diameter of the colonies, their outline, their elevation, their translucency and colour. Changes brought about in the medium (e. g. hemolysis in a blood agar medium) may also be significant.

The rage of conditions that support growth is characteristic of particular organisms. The ability or inability of the organism to growth on media containing selective inhibitory factors. (e. g. bile salt, specific antimicrobial agent, low or high pH) may be of diagnostic significance.

Biochemical reactions

Species that be distinguished by morphology and cultural characters may exhibit metabolic differences that can exploited. In general, these methods fall into the realm of biochemical test because they can determine fundamental chemical characteristics such as nutrient requirement, products given off during growth, temperature and gas requirements, and mechanisms for deriving energy. It is usual to test the ability of organism to produce acid and gaseous end-products, when presented with individual carbohydrates (glucose, lactose, sucrose, mannitol, etc.) as the sole carbon source. Other tests determine whether the bacterium produces particular end-products (e. g indol, hydrogen sulphide) when growth in suitable culture media, and whether it possesses certain enzyme activities such as oxidase, catalase, urease, gelatinase or lecithinase.

Sometimes more elaborate procedures may be used the analysis of metabolic products. For example, gas- liquid chromatography is widely used to recognize characteristic volatile fatty acids and alcohols produced by anaerobic bacteria.

Antigenic properties

Isolated cultures are investigated by the agglutination test with specific sera and other serological tests.

Animal pathogenicity and toxigenicity

Virulence is determined by infecting experimental animal. Biological tests may be utilized for causative agents of tuberculosis, plaque, tularemia and demonstration of bacterial toxins.

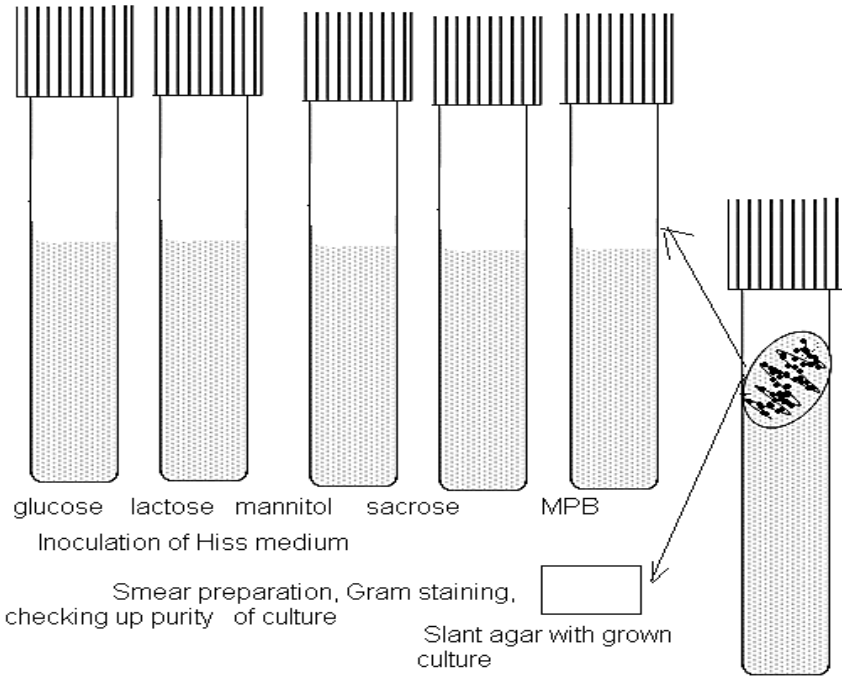
Determining of phagotyping, bacteriocinetyping, antibiotic sensetivity tests

DNA-analysis

Several modern analytical and diagnostic tools that focus on the genetic and molecular characteristics can detect the exact nature of microbial DNA.

Recommendations for protocol design

Practical activity №1. Checking up purity of the isolated culture, which has grown on slant agar, making smear, staining by Gram method. Sketching in album.



Biochemical propertis:
Glucose
Lactose
Mannitol
Sacrose

Characteristic morphology of cells:
1. Shape
2. Location of the cells
3. Tinctorial properties

Conclusion:

Developed slope agar culture should be cleared for purity by observation the colonies by naked eye and examination of smears under microscope. Try to determine whether or not the culture is pure. Pure culture shows one type of colonies and in Gram staining smears you observe only one type of bacteria as well in appearance as staining affinity.

Take long, thick, straight wire in the right hand. Open tube with slope agar culture. Charge sterilized wire with culture material. Close tubes. Open the first Hiss' tube (with glucose) deep inside the medium. Close the tube. Heat the loop. Repeat with other Hiss' tubes. This culture is named stab culture.

MPB is inoculated by touching with a charged loop. Place the strip of filter paper soaked in lead acetate solution at the top along the cotton plug in a tube of meat peptone broth.

Inoculation onto Giss medium allows to:

1. Detect sugar fermentation to acid and gas.

2. Detect reduction properties and how intensively indicator changed it own color. (if bacteria ferment any one sugar medium-pH will lower due to the production acids).

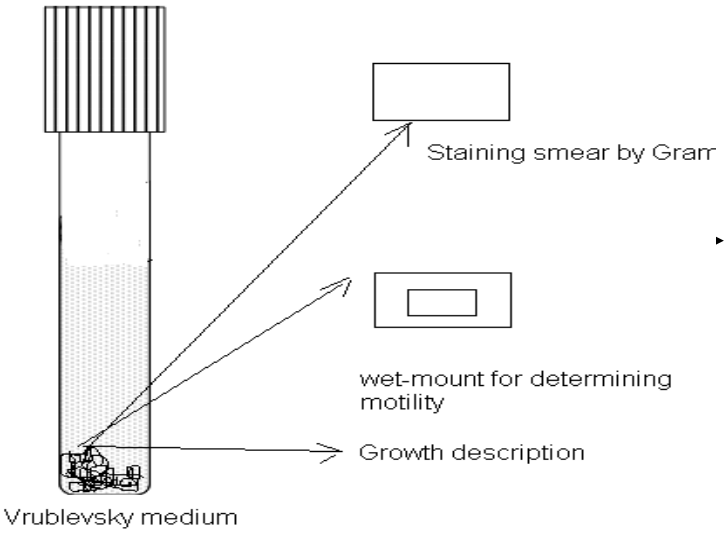
3. detect proteolytic properties (on MPB). We make conclusion about peptone decomposition from final compounds:

- H_2S (indicator paper became black);
- indole (indicator paper became pink).

4. Determine growth character on the broth medium.

Practical activity№3. Prepare the smears from Vrublevsky medium and stain by the Gram method. Prepare wet- mount and investigate under microscope

Make conclusion about purity of the isolated culture after observation of stained smears.



Characteristic morphology of cells:

4. Shape
1. Location of the cells
2. Tinctorial properties

Conclusion:……..

Control questions

- Name basic taxonomical ranks of microorganisms.
- Describe signs of culture purity.
- What properties of microorganisms do we study to identify them?
- Why microorganisms are so biochemically active?
- Name types of enzyme classification.
- Describe methods of determination of microorganism enzyme activity.
- Name microorganism species that can produce pigment, using for identification of them.
- Define terms: stain, species, clone.

Class N9:

Topic: “Chemotherapy. Antibiotics”

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.

Concrete objectives:

- To analyze the phenomenon of microbial antagonism.
- To explain the mechanism of action of antibiotics on a microbial cell.
- To evaluate the methods of determination of sensitiveness of microorganisms to the antibiotics.
- To make conclusion about the sensitiveness of microorganisms to the antibiotics.
- To interpret the mechanisms of resistance of microorganisms to the antibiotics.
- To explain the mechanisms of complications of chemotherapy.

Basic knowledge, skills, needed to study topic (interdisciplinary integration) See a class №1**.**

A list of key terms, parameters, characteristics that student should learn for the class:

Term	Definition
Chemotherapeutic drugs	Chemicals are capable even in high dilutions of destroying or inhibiting microorganisms while harming the host nothing or as little as possible.
Chemotherapy	Antimicrobial chemotherapy is the treatment and prevention of infectious diseases by means of chemicals called chemotherapeutic drugs.
Therapeutic index	The therapeutic index is the ratio of maximal tolerated dose i to ihe minimal drug
Maximal tolerated dose	Maximal dose of chemotherapcutic preparation, which does not cause side actions for a patient.
Minimum curative dose	Minimum dose of chemotherapeutic preparation, able to inhibit a growth and reproduction of microorganisms.

Antimetabolite	A substance such as a drug that competes with, substitutes for, or interferes with a normal metabolite
Antagonism	Type of relations between organisms, at which one microorganism represses development other
Antibiotics	Chemotherapeutic preparations of biological origin or their semisynthetic derivatives and synthetic analogues, which are capable in low concentrations preferentially to damage or kill microbes or tumor cell, repress in an organism the patient of causative agents of diseases or to delay the growth of malignant formations.
Antibiotic of narrow spectrum of action	Able to influence on the separate groups of microorganisms (for example, only on gram-positive or only on gram- negative)
Antibiotic of broad spectrum of action	Able to influence on the different groups of microorganisms (both on gram-positive and on gram-negative)
Bactericidal antibiotic	Destroys microorganisms.
Bacteriostatic antibiotic	Agent that inhibits bacterial growth.
Oxford unit	The minimum amount of penicillin that will prevent the growth of Staphylococcus aureus in the 50 ml of meat peptone broth 1 unit equals 0.6 mkg of crystalline sodium salt of penicillin.
Unit of action	Minimum concentration of antibiotic, which stops the growth of standard sensible microorganism in the certain volume of nutrient media .
International unit	As a rule, 1 microgram of antibiotic
Minimum inhibitory concentration	Is the lowest concentration of a drug that prevents growth of a particular pathogen
Drug resistance	Ability of microorganism to grow in the presence of minimum inhibitory concentration of antibiotic.

Theoretical questions:

- History of development of antimicrobial therapy. Periods of development of chemotherapy.
- Papers of D.L. Romanovsky, P. Erlich, G. Domagk. Discovering of sulfanilamide.
- Basic principles of rational chemotherapy.
- Concept of antimicrobial preparation, chemotherapeutic index.
- Microbial antagonism, mechanisms. Microbes-antagonists are producers of antibiotics. Studies of I.I. Metchnikoff about the physiological role of lactic- acid bacteria in the large bowel.
- History of the first antibiotics discovery. A. Fleming, Z. Waksman. Antibiotics, definition, biological role in the nature. Principles of antibiotics obtaining.

- Classification of antibiotics originally, to chemical composition, on a mechanism and spectrum of antimicrobial action. Natural, semisynthetic and synthetic antibiotics.
- Mechanism of antibiotic action in the microbial cell. Antibiotics are inhibitors of synthesis of peptidoglycan of cell wall, synthesis of protein, nucleic acids, and alteration of cytoplasm membrane function. Bactericidal and bacteriostatic actions of antibiotics.
- Units of antimicrobial activity of antibiotics. Methods of determination of sensitiveness of bacteria to the antibiotics. Concept of a minimum inhibitive concentration. Antibiogram.
- Complications of chemotherapy. Dysbacteriosis. Antibiotic resistance, antibiotic tolerant and antibiotic depending strains of bacteria.
- Natural and acquired resistance to the antibiotics. Genetic and biochemical mechanisms of resistance to the antibiotics. A role of plasmids and transposons in the antibiotic resistance formation. Ways of prevention of antibiotic resistance formation Principles of rational chemotherapy.
- Intercellular communication for bacteria («sense of quorum») and prospect of creation on it basis of antimicrobial preparations of new generation.

Practical activities performed in class:

- To familiarize oneself with the methods of determination of microbal antagonism.
- To study demonstration of determination to the minimum inhibitory concentration of antibiotics, by the method of the serial dilutions.
- To master the methods of determination of sensitiveness of microorganism to the antibiotics by the method of disks.

Topic content

Students study the phenomenon of microbial antagonism, methods of determination of antagonism and methods of it practical use, basic classifications of antibiotics and examples of preparations, which illustrate these classifications, meet with the methods of determination of minimum inhibitory concentration of antibiotics by serial dilutions and disks methods. Students write down prepared tasks in the protocol and sign it at the teacher.

Recommendations for protocol design

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:

$$\frac{\textbf{Maximal tolerated dose (DT—Dosis tolerata)}}{\textbf{Minimal curative dose (DC—Dosis curativa)}} \Rightarrow \textbf{3}$$

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 produsedby the fungi *Penicillium notatum, Penicillium chrysogenum*
2. *Cephalosporin* was isolated from *Cephalosporium acremonium*

Antibiotics produced by actinomycetes

- *Streptomycin* is obtained from *Streptomyces griseus*
- *Chloramphenicol* is obtained from the cultural fluid of a strain of *Streptomyces venezuelae,* isolated from the soil in tropical South America.
- *Chlortetracycline* (biomycin, aureomycin) is produced by *Streptomyces aureofaciens.*.
- *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.

- *Erythromycin* is obtained from *Streptomyces erythraeus.*
- *Neomycin* has been isolated from *Streptomyces fradiae.*
- *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.
- *Kanamycin* is an antibiotic produced by *Streptomycin kanamycetius.*
- *Cycloserine* obtained from *Streptomyces lavendula*
- *Oleandomycin* obtained from *Streptomyces antibioticus*
- *Amphotericin (A and B)* are antimycotic antibiotics obtained from *Streptomyces nodosum.*
- *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.

The activity of antibiotics is expressed in **unit of activity (AU).** Thus, for example, 1 IU of penicillin (**Oxford unit**) is the smallest amount of preparation inhibiting the growth of standard Staphylococcus aureus strain. Recently the method of determining the activity of antibiotics according to the weight of the preparation has received wide application.

One unit of activity (AU) corresponds to the activity of 0.6 micrograms (μg) of the chemically pure crystalline sodium salt of benzylpenicillin. Consequently, in 1 mg of sodium salt of benzylpenicillin there may be 1667 AU, and in 1 mg of potassium salt —

1600 AU. For practical purposes both preparations are manufactured with an activity not less than 1550 AU.

The concentration of dry preparations as well as of solutions is expressed as the number of micrograms of active substance in 1 g of preparation or in 1 mg of solution.

Specific mechanisms of drug resistance

- Synthesis of enzymes that inactivate the drug
- Decrease in cell permeability and uptake of the drug
- Change in the number or affinity of the drug receptor sites
- Modification of an essential metabolic pathway

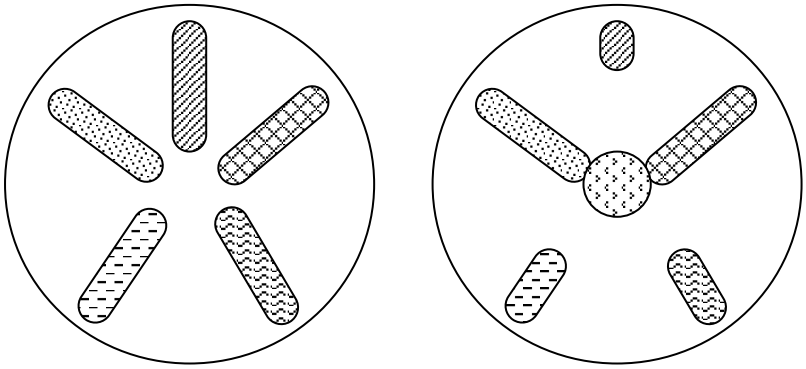
Types of drug resistance

- Natural
- Acquired
  - ❖ Mutation
  - ❖ Recombination
  - ❖ Transferred with plasmids and transposons

Antibiotic requirement

- Great activity against microbes
- Selectively toxic to the microbe but nontoxic to host cells
- Relatively soluble and functions even when highly diluted in body fluids
- Remains potent long enough to act and is not broken down or excreted prematurely
- Not subject to the development of antimicrobial resistance
- Complements or assists the activities of the host's defenses
- Does not disrupt the host's health by causing allergies or predisposing the host to other infections

Practical activity №1. To study the phenomenon of microbial antagonism (special Petri dish).



Control Test

Microorganism:

Shigella disenteriae

Staphilococcus aureus

Escherichia coli

Salmonella typhi

Candida albicans

Practical activity №2. To examine the sensitivity of staphylococci to benzylpenicillin by serial dilutions method in a liquid media.

Method of serial dilutions in a liquid medium. Hottinger's broth (or another medium suitable for the growth of the given micro-organism) is poured by 2-ml portions into test tubes mounted in a tube rack by ten in each row. Prepare antibiotic solution containing 100 U per ml and add 2 ml of this solution into the first test tube. Following thorough mixing, transfer with a new sterile measuring pipette 2 ml of the culture from this tube into the next one, and so on until the ninth tube is reached, from which 2 ml is poured off. The tenth tube containing no antibiotic serves as a control of culture growth.

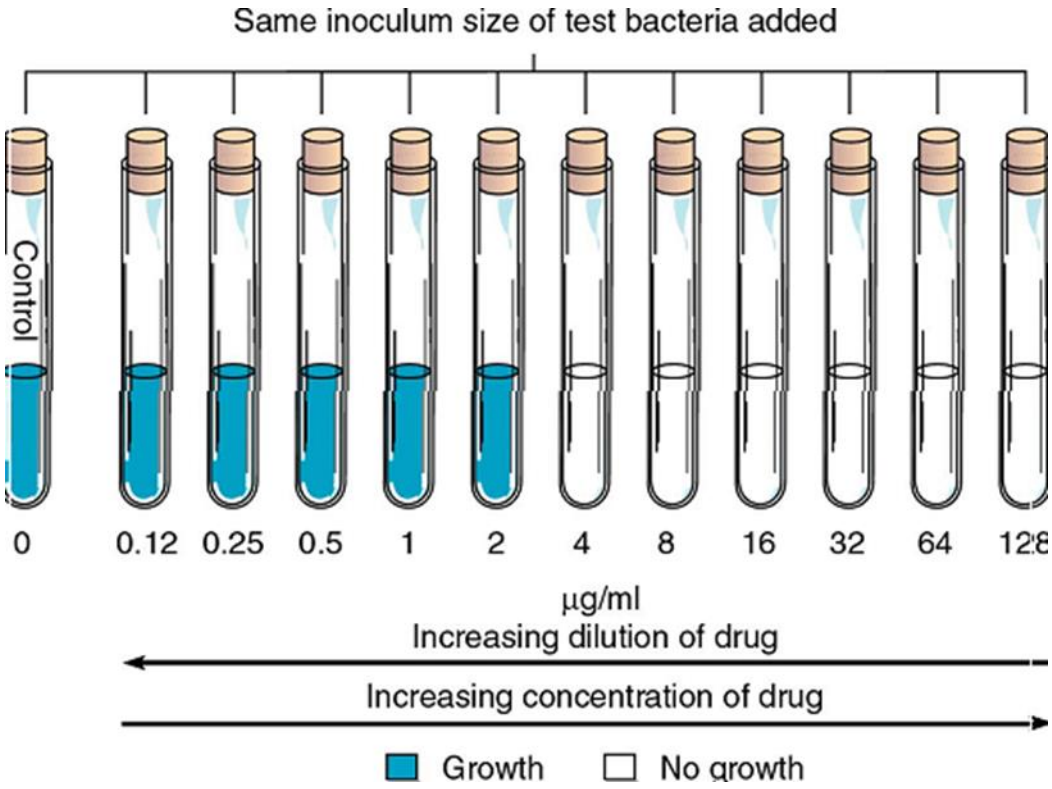
Wash the 24-hour agar culture of the studied microorganism with isotonic sodium chloride solution, determine the density of the suspension by the turbidity standard, and dilute to a concentration of 10000 microorganisms per ml. A sample of 0,2 ml of the obtained suspension is inoculated into all tubes of the row beginning from the control one. Thus, all tubes contain 1000 microorganisms per 1 ml. The results of the experiment are read following incubation of the tube at 37 °C for 18-20 hrs. The minimal concentration of the antibiotic suppressing the growth of the given microorganism is determined by the last test tube with a transparent broth in the presence of an intensive growth in the control one.

One may also prepare antibiotic solution in molten nutrient agar to subsequently streak the tested culture onto the surface of this medium.

Another approach to antimicrobial susceptibility testing is the determination of the **minimum inhibitory concentration (MIC)** that will prevent microbial growth. **The MIC is the lowest concentration of antimicrobic that prevents the growth of a microorganism in vitro.**

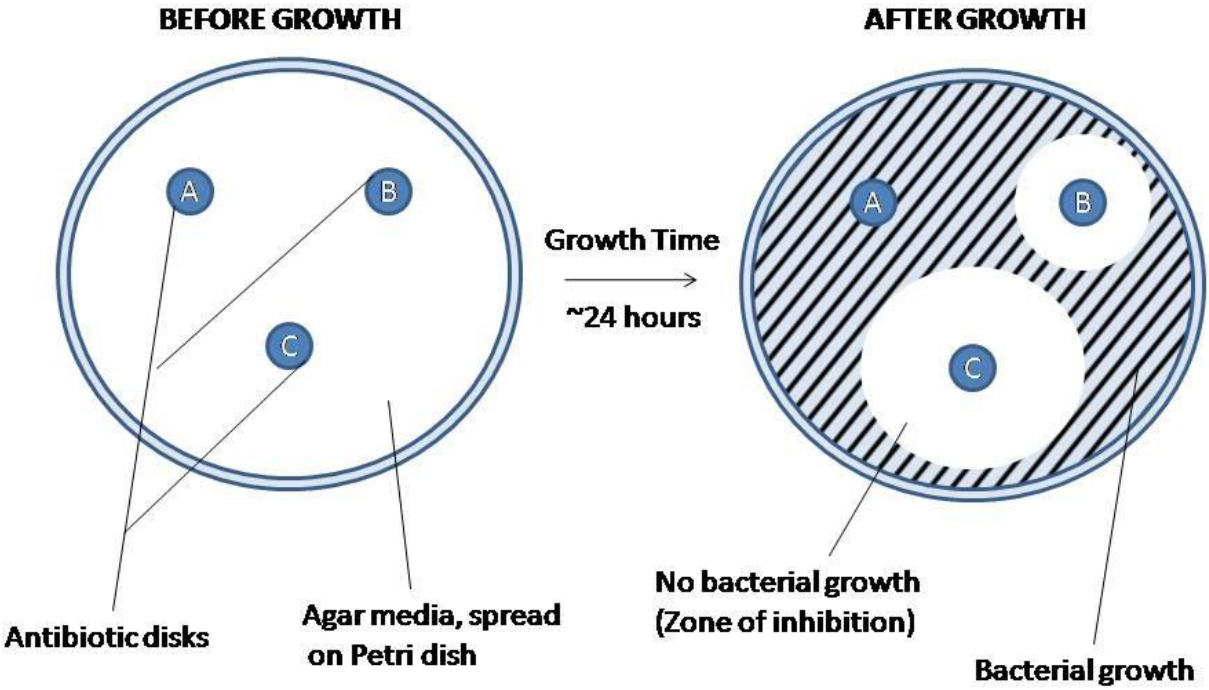
The minimum inhibitory concentration indicates the minimal concentration of the antibiotic that must be achieved at the site of infection to inhibit the growth of the microorganism being tested. By knowing the MIC and the theoretical levels of the antibiotic that may be achieved in body fluids, such as blood and urine, the physician can select the appropriate antibiotic, the dosage schedule, and the route of administration. Generally, a margin of safety of 10 times the MIC is desirable to ensure successful treatment of the disease.

Determination of the MIC is adequate for establishing the appropriate concentration of an antibiotic that should be administered for controlling the infection in patients with normal immune response levels.



Practical activity №3. To determine sensitivity of staphylococci to antibiotics by standard disks method.

Disk method. Into sterile Petri dishes placed on a horizontal surface, pour 15 ml of solid nutrient medium (most often 2 per cent agar on Hottinger's broth containing 0.11-0.13 per cent of amine nitrogen). On the surface of solidified and slightly dried agar, pour 1 ml of suspension of 24-hour culture of the causative agent or, if no pure culture has been isolated, of the pathological material (pus, exudate) obtained for the study and diluted with isotonic saline. Spread uniformly over the agar surface the bacterial suspension, removing its remainder with a Pasteur pipette. Disks with antibiotics (5-6 disks per plate) are placed onto the surface of the inoculated plate at a distance of 25 mm from its centre. The plates are incubated at 37 °C for 16-18 hrs, after which the results of the test are read by measuring the zones of growth retardation of microorganisms around the disks, including the diameter of the disk itself. The size of the zones depends on the degree of sensitivity of the causative agent to a given antibiotic. This method cannot be considered strictly quantitative.



Control questions

- What are differences between pharmacotherapy and chemotherapy?
- What contributions to chemotherapy were made by Ehrlich, Domagk, Fleming, and Waksman?
- What are antibiotics?
- How is chemotherapeutic index calculated?
- Name main sources of antibiotics?
- What are antibiotics requirements?
- Describe main mechanisms of action of antibiotics.
- How is activity of antibiotics measured?
- How the microorganism sensitiveness to the antibiotics is determined?
- Why is it necessary to do?
- Describe complications of antibiotic therapy.

RECOMMENDED LITERATURE

1. K. Talaro, A. Talaro. Faundations in microbiology. Basic principles. Pasadena, 1999,by TMHE group. - P. 72-88.
2. E.Alcamo. Fundamentales of microbiology, third edition. 1991. - P. 19-20.
3. I.S. Gaudash, V.V. Flegontova. Microbiology, virology and immunology. Lugansk, 2004. - P. 34-54.
4. W.Levinson, E.Jawetz. Microbiology and immunology Medical: examination and board review, 6th ed. Companies McGraw-Hill The, 2000, 582.
5. Medical microbiology and immunology: examination and board review, 6th ed. The McGraw-Hill Companies, 2000, 582 p.
6. Медична мікробіологія, вірусологія та імунологія: підручник для студентів вищ. мед. навч. закладів / За ред. В.П. Широбокова / Видання 2-е. – Вінниця: Нова Книга, 2011. – 952 с.