ZAPOROZHYE STATE MEDICAL UNIVERSITY

THE CHAIR OF MICROBIOLOGY, VIROLOGY, IMMUNOLOGY

Microscopic (bacterioscopic) method of research in the microbiological practice

The methodical manual
on microbiology, virology and immunology
for medical the students of II - III courses

Zaporizhzhia

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

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

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

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

МІНІСТЕРСТВО ОХОРОНИ ЗДОРОВ'Я УКРАЇНИ
ЗАПОРІЗЬКИЙ ДЕРЖАВНИЙ МЕДИЧНИЙ УНІВЕРСИТЕТ

Кафедра мікробіології, вірусології та імунології

Мікроскопічний (бактеріоскопічний) метод дослідження у мікробіологічний практиці

Навчальний посібник
для іноземних студентів ІІ-ІІІ курсів медичних факультетів,
спеціальність «Лікувальна справа»

Запоріжжя
2017

Навчальний посібник затверджено на засіданні Центральної методичної Ради ЗДМУ (протокол № від 2017 р.) та рекомендовано для використання в навчальному процесі.

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Мікроскопічний (бактеріоскопічний) метод дослідження у мікробіологічний практиц: навчальний посібник для іноземних студентів ІІ-ІІІ курсів медичного факультету, спеціальність «Лікувальна справа» / Єрьоміна А.К. [та ін.]. – Запоріжжя, 2016. – 74 с.

Навчальний посібник з мікробіології, вірусології та імунології для іноземних студентів ІІ-ІІІ курсів медичного факультету спеціальністі «Лікувальна справа».

MICROSCOPIC (BACTERIOSCOPIC) METHOD FOR INVESTIGATING

MICROSCOPIC (BACTERIOSCOPIC) METHOD FOR INVESTIGATING A COMBINATION OF TECHNIQUES TO DETECT AND STUDY OF MORPHOLOGICAL AND TINCTORIAL (ABILITY TO BE PAINTED) MICROBIAL PROPERTIES IN THE MATERIAL (LABORATORY CULTURE, PATHOLOGICAL MATERIAL, SAMPLES FROM THE ENVIRONMENT) BY MICROSCOPY. THE MAIN PURPOSE IS TO ESTABLISH THE ETIOLOGY OF THE DISEASE, AS WELL AS DETERMINATION OF PURITY VYDE-UNIVERSE PURE CULTURE. IN LABORATORY PRACTICE, USE OF THE FOLLOWING TYPES OF MICROSCOPIC MICROPREPARATIONS:

A) BACTERIOLOGICAL SMEAR;

B) THE HANGING DROP;

C) PRESSED DOWN BY A DROP;

G) THIN SMEAR OF BLOOD, SPUTUM, ETC .;

D) THICK DROP;

G) DRUG-MARK.

THE STEPS OF THE METHOD

1. FENCE MATERIAL (PUS, SPUTUM, BLOOD, URINE, FECES, WASHINGS OF THE BRONCHI AND STOMACH, THE LIQUOR, THE CONTENTS OF THE NASAL CAVITY, VAGINA, CADAVEROUS MATERIAL).

2. TRANSPORTING THE MATERIAL.

3.PREPARATION MICROPREPARATIONS (IF NECESSARY FIXATION AND COLORATION).

4. MICROSCOPY WITH AN ESTIMATE OF THE SHAPE, SIZE, RELATIVE POSITION OF MICROBES, ETC.

5. CONCLUSION.

PRINCIPLES OF HEALTH PROTECTION AND SAFETY RULES IN THE MICROBIOLOGICAL LABORATORY. DESIGN, EQUIPMENT, AND WORKING REGIMEN OF A MICROBIOLOGICAL LABORATORY.

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

A microbiological laboratory usually comprises the following premises:

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

(2) washroom;

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

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

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

Laboratory animals employed for biological sampling are kept in separate isolated premises (an animal unit).

It is preferable that laboratory rooms should have only one entrance. To facilitate such procedures as washing and treatment with disinfectants, the walls are painted with light-coloured oil paint or lined with ceramic tiles, whereas the floors are covered with linoleum.

The infective material is examined in a separate room. The work requiring the observation of a microbiological regimen (inoculation of the material for sterility, contamination of tissue cultures, chicken embryos, etc.) requires special premises (box) whose floor space should be convenient for two workers.

Prior to and after work the entire box is treated with disinfectant solutions and irradiated with bactericidal lamps.

Equipment of the laboratory.

Laboratory furniture should be simple and convenient. Laboratory tables covered with a special enamel, linoleum, or other easily disinfecting materials are placed near windows. Safe-refrigerators are used for storing microorganism cultures.

The main pieces of equipment in the *bacteriological laboratory* include apparatuses for different types of microscopy, apparatuses for heating (gas and alcohol burners, electrical stoves, etc.), incubators, refrigerators, sterilizing apparatuses (sterilizer, Koch apparatus, Pasteur stove, coagulator, etc.), a centrifuge, distillator, etc.

The used material is rendered safe in the way which is employed in bacteriological laboratories.

Immunological laboratory is furnished with incubators, refrigerators, glassware, and apparatuses necessary to run serological reactions on a wide scale.

The design and furnishing of a virological laboratory somewhat differ from those of a bacteriological one.

The premises of a *virological laboratory* should include a box with pre-box inclosure separated by a glass partition where work with cell cultures and chicken embryos is conducted.

Apart from glassware and usual equipment, this type of laboratory should be furnished with chambers of deep and superdeep freezing (-30°-70 °C), refrigerator chambers (-20 °C), centrifuges with a rotation velocity of 1500-3000 Xg and over to ensure purification of the virus from ballast substances and its concentration. Other pieces of equipment include a homogenizer to comminute tissues, ovoscope, burners for ampule soldering, and a vacuum pump.

Before starting the work, the premises are disinfected in a way which is employed for disinfecting the box of microbiological laboratories.

The premises are treated, using disinfectant solutions and bactericidal lamps.

Rules of work in the laboratory.

1. The personnel working at laboratories is supplied with medical coats and kerchiefs or caps. While working in boxes, one should wear a sterile coat, cap, and gauze mash. To make an autopsy of animals, put on an oil cloth apron, oversleeves, and rubber gloves. Special clothes protect the worker and also prevent contamination of the material to be studied with foreign microflora.

2. Eating and smoking in the laboratory are strictly forbidden.

3. Unnecessary walking about the laboratory, sharp movements, and irrelevant conversations should be discouraged.

4. In the process of examination the working place should be kept clean and tidy. Bacteriological loops are rendered harmless by burning them in the burner's flame; used spatulas, glass slides, pipettes, and other instruments are placed into jars with disinfectant solution.

5. Upon the completion of work the nutrient media with inoculated cultures are placed into an incubator; museum cultures, into safe-refrigerators; devices and apparatuses are set up in places specially intended for them. Wipe tables with disinfectant solution and thoroughly wash the hands.

6. If the material to be analyzed or the culture of microorganisms is accidentally spilt onto the hands, table, coat, or shoes, they should be immediately treated with 1 per cent solution of chloramine.

Rules of work at a microbiological laboratory with a special regime.

Before entering the laboratory, all personnel take off the overcoats at the cloakroom. In the next room with individual closets they take off the remaining clothes and the underwear, put pyjamas, medical coats, kerchief (or cap), socks, and slippers (Set 1 of protective clothes of the fourth type). When working in the autopsy room, put on an anti-plague suit (Set 2), a second autopsy coat, helmet, cotton wool-gauze mask, rubber gloves, oil-cloth apron, and oversleeves. To protect the eyes, one should wear goggles. An anti-plague suit of the first type is put on in the following order: (1) overalls; (2) socks; (3) high boots; (4) helmet; (5) anti-plague coat; (6) cotton wool-gauze mask (place cotton wool tampons over the

wings of the nose); (7) goggles; (8) gloves; (9) oil cloth apron and oversleeves (these are put on while working in the autopsy room). A person working with infective material should have a towel soaked in 3 per cent solution of lysol. Upon completion of work immerse gloved hands into 5 per cent solution of lysol for 2 min and repeat this procedure after removing each item of the clothing. The anti-plague suit is taken off in the reverse order, with the exception of gloves which are the last to be taken off. Then. they are folded with the external surface inside and immersed into 5 per cent solution of lysol or I per cent solution of chloramine for 2 hrs. The goggles are put into 70 per cent alcohol.

Following autopsy, instruments and syringes are boiled in lysol for at least 40 min. All used material and corpses of animals are burnt or sterilized.

The Department of Microbiology is a place of work dangerous for health with the risk of professional infection.

Students are allowed to take off their clothes only in a cloakroom that must be locked and the key is placed in a reserved site to prevent any theft. If a student has any valuable things or larger sums of money with him, then he must announced it to his teacher who will secure its safe deposition.

However, taking any valuables in is not recommended.

The students come into the hall through the entrance from the waiting room under the teacher's surveillance. They are lend protective coats which must be taken off before entering other departments.

It is forbidden to damage these coats, to take away any infective material from the hall, as well as tools and coats. Students' own coats must not be worn.

Students must observe the principles of hygiene. They must disinfect and wash their hands always after contaminating them with a biological material and before leaving the hall.

For disinfecting hands, 0.5 % chloramine is used for 2 minutes. Then the hands are to be rinsed with warm water and washed with soap. It is forbidden to

eat, smoke and drink in the laboratory. It is also necessary to avoid rubbing one's eyes or nose, scratching one's head, biting nails, pencils, etc.

The space in front of the building and in the waiting room must be kept clear and quiet.

PRINCIPAL MICROBIOLOGICAL PROCEDURES

A complex of bacterioscopic, bacteriological, serological, allergological, and biological techniques is used in the microbiological diagnosis of bacterial infections. Depending on the nature of the given infectious disease, one of these methods is used as the main one, while the others are supplementary. Such biological substances as blood, faeces, urine, cerebrospinal fluid, bile, etc. serve as the material for microbiological diagnosis.

The main microbiological techniques pertaining to the laboratory diagnosis of bacterial infection are outlined below. Interpretation and specification of each technique with regard to specific infections are presented in the respective sections.

BACTERIOSCOPIC EXAMINATION

Modern methods of microscopic examination.

Contemporary microbiological laboratories employ not only conventional methods of optical microscopy in transmitted light **(Fig. 1)** but also such special ones as dark-field microscopy and phase-contrast, luminescent, and electron microscopy.

Light microscopy.

A light microscope 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 histological objects.

In examining microorganisms, the immersion objective with a small focal distance and a higher resolving power is predominantly employed.

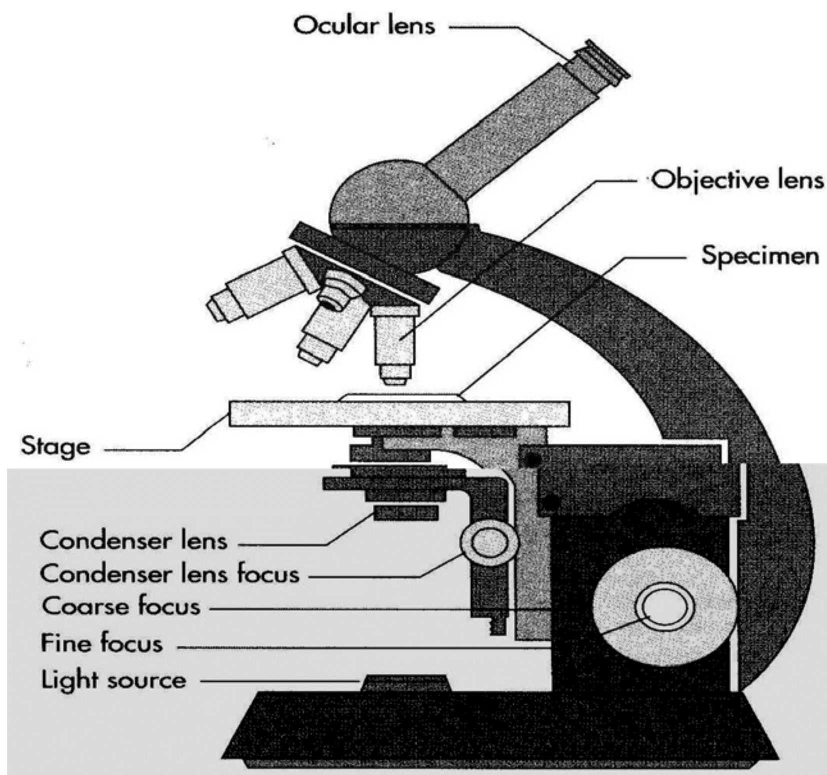


Figure 1. A light microscope

In microscopic examination with the help of an immersion objective the latter is immersed in oil (cedar, peachy, "immersiol", 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 **(Fig. 2).** The resolving power of the immersion objective is about 0.2 mcm. The maximum magnification of modern light microscopes is as high as 2000-3000.

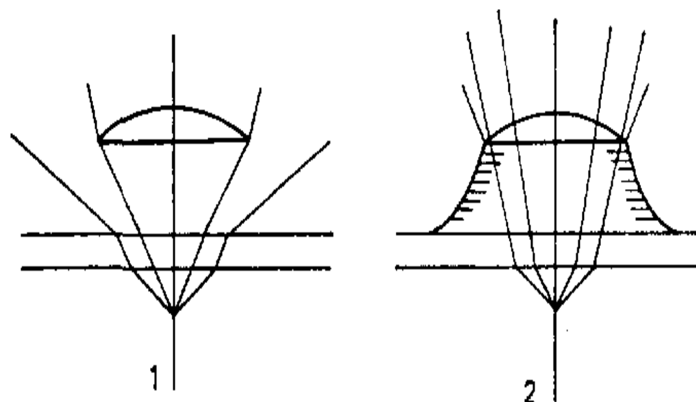


Figure 2. The course of rays in the dry (1) and oil-immersion (2) systems

Dark-field microscopy belongs to ultramicroscopic methods. Living objects 0.02-0.06 mcm in size are visualized in lateral illumination in a dark field of vision. In order to achieve bright lateral illumination, the usual condenser is replaced by a special parabolic condenser in which the central part of the lower lens is darkened, while the lateral surface is mirror **(Fig. 3).** This condenser intercepts the central portion of the parallel beam of rays forming a dark field of vision. The marginal rays pass through the circular slit, fall on the lateral mirror surface of the condenser, are reflected from it, and concentrate in the focus. On encountering in their path the cells of microorganisms or other optically non-homogeneous structures, the ray of light is reflected from them and gets into the objective. Cells of microorganisms and other objects are brightly illuminated in this case.

An electrical illuminator serves as a source of artificial light. To achieve lateral illumination, one needs a parallel beam of light which is created by means of a flat mirror of the microscope.

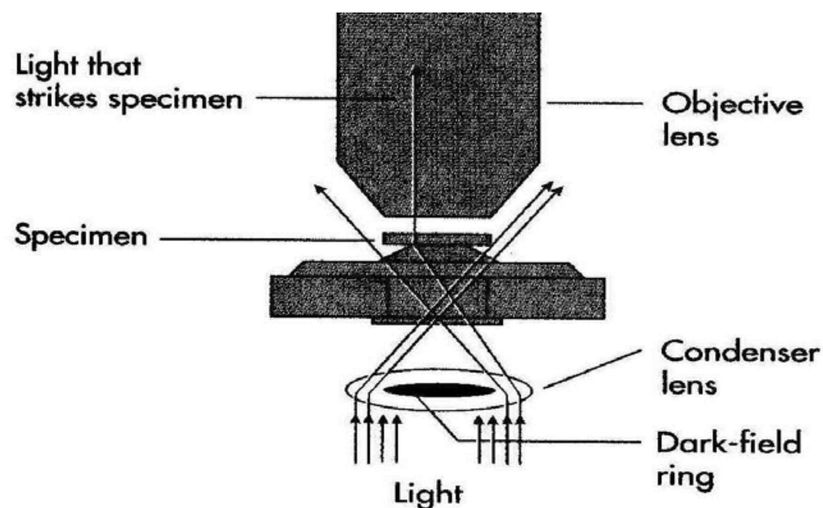


Figure 3. Diagram of a dark-field microscope showing the path of light. The dark-field ring in the condenser blocks the direct passage of light through the specimen and into the objective lens. Only light that is reflected off a specimen will enter the objective lens and be seen.

In dark-field microscopic examination a dry system is typically employed (objective 40). A small drop of the studied material is placed on the slide and covered with a cover-slip, taking care to prevent the formation of air bubbles. A drop of immersion oil is pipetted on the upper lens of the condenser.

This oil should fill the space between the condenser and the slide.

Microscopy with an oil-immersion system makes use of a special objective with a diaphragm trapping the rays which pass unobstructed through a homogeneous medium.

Dark-field microscopy is employed for detecting unstained causative agents of syphilis, recurrent typhoid fever, leptospirosis, and other illnesses, as well as for investigating the motility of microorganisms. Yet, dark-field microscopic examination does not allow a good study of their form, to say nothing about their internal structure.

Modified techniques of light microscopy are utilized for this purpose.

Phase-contrast microscopy is based on the fact that the optical length of the light travelling in any substance depends on its refractive index. Light waves transversing through optically denser sites of the object lag in their phase behind the light waves which do. not have to pass through these sites. The intensity of light in this case remains unaltered but the phase of fluctuation, detected by neither eye nor photoplate, is changed. To increase resolution of the-image, the objective is fitted with a special semi-transparent phase plate to create difference in the wave length between the rays of the background and the object. If this difference reaches one-fourth of the wave length, a visually tangible effect occurs when a dark object is clearly seen against a light background (positive contrast) or vice versa (negative contrast) depending on the structure of the phase plate.

Phase-contrast microscopy does not enhance the resolving power of the optical system but helps to elucidate new details of the structure of living microorganisms and to study different stages of their development, the effect on them of chemical agents, antibiotics, and other factors.

Luminescent microscopy.

Luminescence (or fluorescence) is the ability of some objects and dyes to fluoresce upon their exposure to. ultraviolet and other short-wave rays of light.

It is commonly accepted to distinguish between inherent (primary) and secondary fluorescence. In primary fluorescence the test object contains substances capable to fluoresce upon their exposure to ultraviolet rays.

Most of objects are not inherently fluorescent, so. prior to luminescent microscopy they have to be treated with dyes (fluorochromes) capable to fluoresce.

The following substances are usually used as fluorochromes: auramine (for tuberculosis mycobacteria), acridine yellow (for gonococci), coryphosphine (for Corynebacteria of diphtheria), fluoresceinisothiocyanate (FITC) (for making labelled antisera), etc.

A specimen to be examined by luminescent microscopy is prepared in the usual manner, fixed in acetone or ethanol for 5-10 min and exposed to a fluorochrome for 20-30 min.

Thereafter, the resultant preparation is washed with tap water for 15-20 min, covered by a cover-slip, and placed under a microscope.

Luminescent microscopes represent ordinary biological microscopes furnished with a bright source of illumination and a set of light filters which isolate a short-wave (ultraviolet or blue-violet) part of the spectrum inducing luminescence.

Fluorochromes, binding "with nucleic acids or proteins, form stable complexes that give away yellow-green, orange-red, and brown-red light under the lumimescent microscope.

Fluorescence microscopy has the following advantages as compared with the conventional microscopic methods: colour image; considerable contrasting; possibility to study both live and dead microorganisms, transparent and non-transparent objects; detection of individual bacteria, viruses, and their antigens and possibility of their localization; differentiation of individual components of the cell.

Electron microscopy.

In the electron microscope, a beam of electrons passing in vacuum and blocked by an anode is used instead of light. The source of electrons is an electron gun (a tungsten wire heated up to 2500-2900 °C).

The optic lenses are replaced by electromagnets. An electrical field of 30 000-50 000 V is generated between the tungsten wire and anode, which imparts high velocity to electrons which arrive at the first electromagnetic lens (a condenser) having passed through the anode opening. At their exit from the condenser the electron rays are accumulated in the plane of the studied object, deviate at different angles due to variable thickness and density of the preparation, and get into the electromagnetic lens of the objective equipped with a diaphragm.

Electrons showing only little deviation upon entering the object pass through the diaphragm, while those deviating at a greater angle are retained, which ensures contrasting of the image- The lens of the objective gives an. intermediate enlarged image which is viewed through the viewing window.

The projection lens may ensure a multiple magnification of the image; this image is perceived by a fluorescent screen and photographed. The most recent models of electron microscopes permit visualization of particles 1.4 nm in size.

Electron microscopy is extensively used in microbiology for detailed investigation into the structure of microorganisms. It is also employed in virology for diagnostic purposes.

To study preparations under the electron microscope, special films absorbing small numbers of electrons and fixed on supporting meshes are utilized instead of glass slides. Such films are made of collodium, aluminium oxide, and quartz. The material to be studied is thoroughly cleansed of various admixtures and placed on the film. A very thin layer remaining on the film after evaporation of the fluid is subjected to microscopic examination.

The electron microscope may also be used for studying sections of tissues, cells, and microorganisms which are obtained with the help of an ultramicrotome. The preparations are contrasted by means of electron-dense (electron-capturing)

substances, using such procedures as the spraying with heavy metals and treatment with phosphotungstic acid, uranyl acetate, salts of osmic acid, etc.

Scanning Electron Microscopy.

The scanning electron microscope uses a fine beam or spot of electrons that is focused rapidly back and forth over the specimen.

As the electrons strike the surface of particles in the sample, secondary electrons are emitted, which are collected by a detector to provide an image of the specimen's surface.

This instrument does not require that the sample be sectioned and provides some spectacular three dimensional images. In addition, because the energy of the secondary emitted electron is determined by the identity of the scattering atom, the energy spectrum of these electrons provides information about the location and content of the different elements.

Table 1

Comparrison of Various Types of Microscopes

Type of microscope	Maximum useful magnification	Resolution	Description
Bright-field	1,500x	100-200 nm	Extensively used for the visualization of micro organisms; usually necessary to stain specimens for viewing
Dark-field	1.500X	100-200 nm	Used for viewing live microorganisms, particularly those with characteristic morphology; staining not required; specimen appears bright on a dark background
Fluorescence	1,500X	100-200 nm	Uses fluorescent staining; useful in many diagnostic procedures for identifying microorganisms

Phase contrast	1.500X	100-200 nm	Used to examine structures of living microorganisms; does not require staining
TEM (trans-mission electron – microscope)	500,000-1,000,000X	0.1 nm	Used to view ultrastructure of microorganisms, including viruses; much greater resolving power and useful magni- fication than can be achieved with light microscopy
SEM (scanning electron microscope)	10,000-100,000X	1-10 nm	Used for showing detailed surface structures of microorganisms, produces a three-dimensional image

Preparation and Staining of Smears

Preparation and staining of smears, as well as other microbiological procedures, are performed in a prepared working place.

The working table should contain only those materials and objects which are necessary for the given examination, namely; the object to be studied (blood, pus, sputum, faeces, etc.), test tubes or dishes with a culture of microorganisms, sterile distilled water or isotonic sodium chloride solution, a stand for a bacteriological loop, a jar with clean glass slides, and felt tip pens.

Other necessary items include a gas or alcohol burner, staining solutions, a basin with a supporting stand (bridge) for slides, a washer with water, forceps, filtering paper, a jar with disinfectant solution used for sterilizing preparations and pipettes.

Methods of the treatment of cover-slips.

Now cover-slips are boiled in a 1 per cent solution of sodium hydrocarbonate. rinsed with water, immersed in a weak solution of hydrochloric acid, and then rinsed with water once again.

The used glass slips and slides are placed in a concentrated sulphuric acid (technical grade) for 2 hrs or in a mixture of sulphuric acid, potassium bichromate, and water (100:50:1000), thoroughly washed with water, boiled in sodium hydrocarhonate solution or ill sodium hydroxide, then washed with water once more, dried with clean linen cloth, and stored in alcohol or an alcohol-ether mixture in jars with ground stoppers.

Detergents are also utilized for washing slides. Prior to making smears take slides with a forceps from the solution whore they have been kept and blot them dry. Hold them by the edges with your fingers.

A drop placed on the properly prepared glass spreads uniformly and does not assume a spherical form.

Preparation of a smear.

Before making a preparation, glass slides are flamed to ensure their additional degreasing.

In preparing a *smear from bacterial culture grown on a solid medium,* a drop of isotonic saline or water is transferred onto the precooled glass.

A test tube with the culture is taken by the thumb and the index finger of the left hand. The loop is sterilized in the flame.

A cotton-wool plug is pinched by a small finger of the right hand, removed from the test tube, and left in this position. The edges of the test tube are flamed and then the loop is introduced into the test tube through the flame.

Having cooled the loop against the inner wall of the tube, the loop is touched to the nutrient medium where it meets with the glass wall (if the loop is not sufficiently cooled, it induces cracking and melts the medium).

Then the loop is touched to the culture of the microorganisms on the surface of the medium.

Then the loop is withdrawn, the edges of the test tube are quickly flamed, the tube is closed with a stopper passed through the flame, and then replaced into the test tube rack.

All the above described procedures are made above the flame. The culture sample is placed with the loop into a drop of water on the glass slide and spread uniformly with circular movements on an area of 1-1.5 cm in diameter, then the loop is flamed.

In preparing a *smear from bacterial culture grown in a fluid nutrient medium,* a drop of the culture is taken with a loop or a Pasteur pipette (the pipette is immersed in disinfectant solution), transferred onto the middle of the flamed glass slide and spread uniformly.

On the other side of the glass slide the preparation is delineated by a wax pencil since very thin smears are almost invisible.

The number of the analysis or culture is marked on the left side of the glass.
To prepare a *smear from pus or sputum,* two glass slides are used.

A small amount of the material is transferred with a sterile loop or needle onto the middle of the glass slide and covered with a second one so that one-third of the surface of both slides remains free.

Then, the glass slides are pulled gently aside **(Fig. 4),** which results in the formation of two large smears of the same thickness.

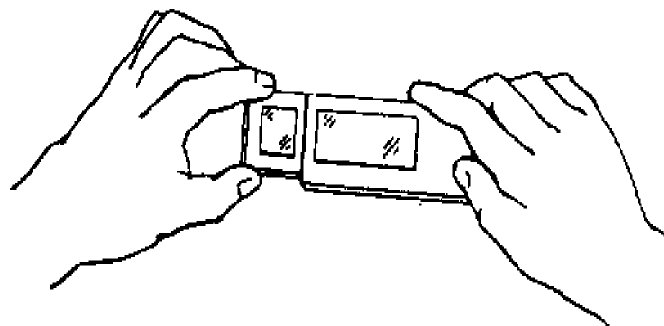


Figure 4. Preparation of a smear from sputum

Blood smear is prepared in the following way. Using a sterile needle, puncture a disinfected fourth finger of the left hand.

Wipe away the first drop of blood with a piece of dry cotton wool and touch the thoroughly-cleansed glass slide to the second drop of blood.

Quickly put the slide on the table, supporting it with the left hand.

Place the end of a second narrower cover slide in touch with the drop of the blood at the 45-degree angle **(Fig. 5).**

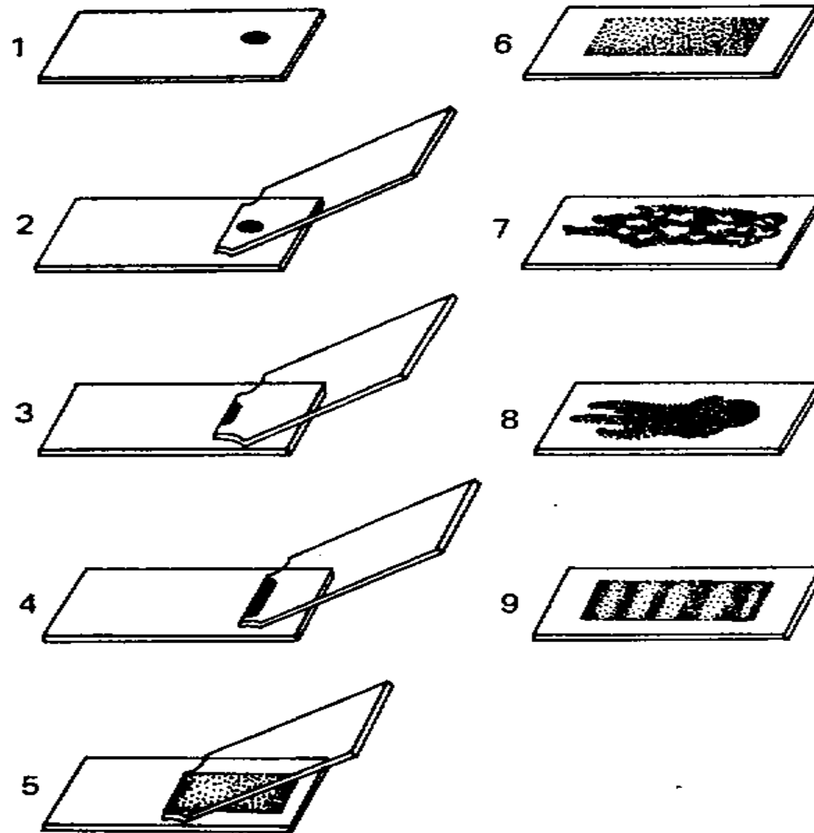


Figure 5. Preparation of a smear from blood:
1-6 — stages of thin smear preparation; *7-9* — inadequately prepared smears

Putting some pressure on the cover slide, smoothly and rapidly move it along the glass slide in the leftward direction stopping it at a distance of 1-1.5 cm from the edge. The correctly prepared smear is yellowish in colour and semitransparent.

Impression preparations are made of the internal organs of cadavers and solid foodstuffs (meat, sausage, ham, etc.).

The surface of the organs or of a foodstuff is burnt with a red-hot scalpel, and a piece of the material is cut off from this site.

The surface of the section is touched to the glass in two-three places.

Drying and fixation of the smear.

Thin smears usually dry rapidly in the air at room temperature; thicker ones are dried in an incubator or by holding them above the flame of a burner.

The slide is held by the edges with the thumb and forefinger, the smear upward, while the middle finger is placed under the glass to regulate the degree of its heating and to prevent coagulation of the bacterial protein and destruction of the cell structure.

The dried smears are flamed to kill and fix the bacteria on the glass slide, preventing thereby their washing off during staining.

The dead microorganisms are more receptive to dyes and present no danger for the personnel working with them.

The glass slide is grasped with a forceps or with the thumb and index finger of the right hand, the smear being in the upside position, and passed three times through the hottest part of the burner's flame.

Fixation with this technique takes about 5-6 s, with the exposure to the Game being about 2s.

Blood smears, impression smears, and smears from bacterial culture deforming at high temperature are treated with one of the following fixatives:

(1) methyl alcohol (for 5 min);

(2) ethyl alcohol (10-15 min);

(3) Nikiforov's mixture: equal volumes of ethyl alcohol and ether (10-15 min);

(4) acetone (5 min);

(5) fumes of osmic acid and formalin (several seconds).

Staining of a smear.

Smears are stained with aniline dyes. Chemically, acid, alkaline, and neutral dyes can be distinguished. Alkaline dyes, whose staining portion of the molecule is charged positively, more actively conjugate with a negatively charged bacterial cell.

Staining of bacteria is a complex physicochemical process. Interaction of the dye with the cell substances results in the formation of salts ensuring stability of staining. Relationship between various types of microorganisms and dyes is called a tinctorial property.

The following dyes are employed most extensively:

(1) red (basic fuchsine, acid fuchsine, safranine, neutral red, Congo red);

(2) blue (methylene blue, toluidine blue, trypan blue, etc.);

(3) violet (gentian, methyl or crystal); and

(4) yellow-brown (vesuvin, chrysoidine).

All the employed dyes are powder-like or crystalline. Such dyes as basic fuchsine, gentian violet, and methylene blue are usually used to prepare in advance saturated alcoholic solutions (1 g of the dye per 10 ml of 96 per cent alcohol).

Saturated alcohol and phenol dye solutions are utilized to prepare water-phenol or water-alcohol solutions to be employed in staining by simple and complex techniques.

Simple techniques of staining make use of only one dye and demonstrate the form of bacteria.

Preparation of dye solutions for simple staining.

Basic fuchsine is used for preparing Ziehl's phenol fuchsine which is stable upon storing. Ziehl's fuchsine is employed to stain in red colour the acid-fast microorganisms and spores.

Ziehl's phenol fuchsine

Basic fuchsine	1 g
95 per cent alcohol	10 mg
Crystal phenol	5 g
Glycerol	several drops
Distilled water	100 ml

Fuchsine, together with phenol crystals and some drops of glycerol, is homogenized by grinding in a mortar, adding simultaneously small amounts of alcohol.

Then, with the obtained mass being continuously stirred, distilled water is gradually added.

The resultant dye is allowed to stand at room temperature for 48 hrs and is then filtered. Shelf life is prolonged.

Pfeiffer's fuchsine

Ziehl's fuchsine	1 ml
Distilled water	9 ml

In using Pfeiffer's fuchsine, the solution should be freshly prepared.

Saturated alcoholic solution of methylene blue

Methylene blue	10 g
95 per cent alcohol	100 ml

Alkaline solution of methylene blue as proposed by Loeffler

Saturated alcoholic solution of methylene blue	30 ml
Sodium hydroxide or potassium hydroxide (1 per cent solution)	1 ml
Distilled water	100 ml

Water-alcoholic solution of methylene blue

Saturated alcoholic solution of methylene blue	10 ml
Distilled water	100 ml

Old solutions of this dye have a better staining ability.

The fixed preparation is placed, the smear upward, on the support. A dye solution is pipetted onto the entire surface of the smear.

With Pfeiffer's fuchsine the staining lasts 1-2 min, with alkaline solution of Loeffler's methylene blue or water-alcoholic solution of methylene blue, 3-5 min. Following the staining procedure the dye is dispensed, the preparation is washed with water, dried between sheets of filter paper, and then examined under the oil-immersion objective.

Live staining of microorganisms is made with methylene blue, neutral red, and other weakly poisonous dyes in a 1:10 000 dilution.

For this purpose, a drop of the test material is mixed with the dye solution on a glass slide and covered with a cover-slip. Microscopic examination is carried out with a 40X objective.

In a negative method of living bacteria staining by Bum's technique, the bacteria remain unstained against a dark background.

In a drop of Indian ink diluted with distilled water 1 to 10 the culture to be tested is introduced and spread uniformly with a loop or the edge of a glass slide.

The smear is air dried. Nigrosin, Congo red, and other dyes may occasionally be utilized instead of Indian ink.

Students practical activities

1. To study the rules of work with immersion system of a microscope.

Positioning the slide

Place the slide specimen-side-up on the stage so that the specimen lies over the opening for the light in the middle of the stage. Secure the slide **between** (not under) the arms of the mechanical stage. The slide can now be moved from place to place using the 2 control knobs located on the right of the stage.

Adjusting the illumination

Adjust the **total light available** by turning the flat mirror. Adjust the **amount of light coming through the condenser** using the **iris diaphragm lever** located below and to the front of the stage.

Light adjustment using the iris diaphragm lever is critical to obtaining proper contrast.

For oil immersion microscopy (900X), the iris diaphragm lever should be set almost all the way open (to your left for maximum light).

Obtaining different magnifications

The final magnification is a product of the 2 lenses being used.

The **eyepiece or ocular lens** magnifies **7X, 10X, 15X**.

The **objective lenses** are mounted on a turret near the stage. They make magnifications: **10X**; **40X**, and **90X** (**black-striped oil immersion lens**).

Final magnifications are as follows:

Ocular lens	X	Objective lens	=	Total magnification

10X	X	10X	=	100X
10X	X	40X	=	400X
10X	X	100X (black)	=	900X

Focusing from lower power to higher power:

a. Rotate the **10X objective** until it locks into place (total magnification of 100X).

b. Turn the **coarse focus control** (larger knob) all the way **away from you** until it stops.

c. Look through the eyepiece and turn the **coarse focus control** (larger knob) **towards you** slowly until the specimen comes into view.

d. Get the specimen into sharp focus using the **fine focus control** (smaller knob) and adjust the light for optimum contrast using the iris diaphragm lever.

e. If higher magnification is desired, simply rotate the **40X objective** into place (total magnification of 400X) and the specimen should still be in focus. (Minor adjustments in fine focus and light contrast may be needed.)

f. For maximum magnification (**900X or oil immersion**), rotate the 40X objective slightly out of position and place a **drop of immersion oil** on the slide. Now rotate the **black-striped 90X oil immersion objective** into place. Again, the specimen should remain in focus, although minor adjustments in fine focus and light contrast may be needed.

Cleaning the microscope

Clean the exterior lenses of the eyepiece and objective before and after each lab using lens paper only. (Paper towel or kim-wipes may scratch the lens.) Remove any immersion oil from the oil immersion lens before putting the microscope away.

Reason for using immersion oil

Normally, when light waves travel from one medium into another, they bend. Therefore, as the light travels from the glass slide to the air, the light waves bend and are scattered (the "bent pencil" effect when a pencil is placed in a glass of water). The microscope magnifies this distortion effect. Also, if high magnification is to be used, more light is needed.

Immersion oil has the same refractive index as glass and, therefore, provides an optically homogeneous path between the slide and the lens of the objective. Light waves thus travel from the glass slide, into glass-like oil, into the glass lens without being scattered or distorting the image. In other words, **the immersion oil "traps" the light and prevents the distortion effect that is seen as a result of the bending of the light waves.**

2. To prepare the smears from agar cultures of Staphylococci or Escherichia coli (the first smear to stain with methylene blue, another one – with fuchsin).

Before making a preparation, glass slides are flamed to ensure their additional degreasing.

In preparing a smear from bacterial culture grown on a solid medium, a drop of isotonic saline or water is transferred onto the precooled glass.

A tost tube with the culture is taken by the thumb and the index finger of the left hand. The loop is sterilized in the flame.

A cotton-wool plug is pinched by a small finger of the right hand, removed from the test tube, and left in this position. The edges of the test fube are flamed and then the loop is introduced into the test tube through the flame.

Having cooled the loop against the inner wall of the tube, the loop is touched to the nutrient medium where it meets with the glass wall (if the loop is not sufficiently cooled, it induces cracking and melts the medium). Then the loop is touched to the culture of the microorganisms on the surface of the medium.

Then the loop is withdrawn, the edges of the test tube are quickly flamed, the tube is closed with a stopper passed through the flame, and then replaced into the test tube rack.

All the above described procedures are made above the flame. The culture sample is placed with the loop into a drop of water on the glass slide and spread uniformly with circular movements on an area of 1– 1.5 cm in diameter, then the loop is flamed.

Then you should do drying an fixation of the smear.

The fixed preparation is placed, the smear upward, on the support. A dye solution is pipetted onto the entire surface of the smear. With Pfeiffer's fuchsine the staining lasts 1-2 min, with alkaline solution of Loeffler's methylene blue or water-alcoholic solution of methylene blue, 3-5 min.

Following the staining procedure the dye is dispensed, the preparation is washed with water, dried between sheets of filter paper, and then examined under the oil-immersion objective.

To examine live microorgansms with a help of a dark-field (Leptospirae) and fhase-contrast (Proteus vulgaris) a microscopies.

III. Tests and Assignments for self–assessment

1. Choose the correct statement:

a – the scanning microscopy gives the possibility to spectacular three-dimensional image of bacteria; b – luminescence (or fluorescence) is the ability of some objects and dyes to fluoresce upon their exposure to ultraviolet light; c – the dark-field microscopy is useful for detecting unstained bacteria; living objects are visualized in lateral illumination in a dark field of vision; d – in microscopic examination with the help of immersion objective the latter is immersed in oil (cedar, peachy, "immersiol", 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; e – the wave length of an electron beam is in 10000 times less, than a visible light.

2. Are these statements correct?

A. Luminescent microscopy is the ability of some objects to fluoresce upon their exposure to a beam of electrons; b – ordinary light microscopy allows to achieve the greater magnification then immersion microscopy; c – electron microscopy provides the flat image of object, and scanning electron microscopy – three dimensional- image; d –a dark-field microscopy gives the possibility to examine the staining preparations; e – bacteria in the smear stain in different colors using simple technique of staining; f – Pfeiffer's fuchsin stains bacteria in red colour, and methylene blue – in blue.

3. Answers the questions:

A. What is the resolving power of the immersion objective? B. What is the principle of phase-contrast microscopy? C. What is the purpose of a condenser at a dark field method? D. What is the resolving power of an electron microscope? E. Can we increase the resolving power of light microscope by use of ultra– violet beams? F. What beams are used for luminescent microscopy?

4. Give the answers to questions:

A. Main stages of preparation of a staining smear. B. What mirror are used use for illumination at immersion microscopy? C. How can we differentiate immersion objective from dry one? D. Why is forbidden to laboratory personnel to bring own things into microbiological laboratory? E. Why the ring of the bacterial loop should be closed?

IV. The answers to the self– assessments;.

1. A, b, c, d, e. 2. C, f. 3. A. 0.2 mcm.

B. Phase-contrast microscopy is based on the fact that the optical length of the light travelling in any substance depends on its refractive index. Light waves transversing through optically denser sites of the object lag in their phase behind

the light waves which do. not have to pass through these sites. The intensity of light in this case remains unaltered but the phase of fluctuation, detected by neither eye nor photoplate, is changed. To increase resolution of the image, the objective is fitted with a special semi-transparent phase plate to create difference in the wave length between the rays of the background and the object.

If this difference reaches one – fourth of the wave length, a visually tangible effect occurs when a dark object is clearly seen against a light background (positive contrast) or vice versa (negative contrast) depending on the structure of the phase plate.

C. This condenser intercepts the central portion of the parallel beam of rays forming a dark field of vision (illumination in lateral beams);

D. It is approximately in 10000 times less than at a light microscopy.

E. Yes, we can.

F. Ultraviolet beams.

4. A. Smear preparation, drying, fixation, staining, washing and drying. B. Flat mirror. C. It has black ring (strip), label "MI" (oil immersion), total magnification of 90x, numerous aperture is more then 1. D. For prevention of removal biological material from microbiological laboratory. E. For keeping a drop of water or saline solution in it.

STUDENTS MUST KNOW:

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

2. The rules of microscopy with an immersion system.

3. Fundamental principles of a dark-field, luminescent, phase-contrast, electron, scanning microscopies.

STUDENTS SHOULD BE ABLE TO:

– prepare a smears from bacterial cultures;

– stain the smears by a simple method (fuchsin, methylene blue);

– examine it using an immersion objective of light microscope.

MAIN RESEARCHING METHODS OF BACTERIA MORPHOLOGY. STAINING PREPARATION FROM DIFFERENT CULTURES OF MICROORGANISMS. COMPLETE METHODS OF STAINING. GRAM'S METHOD

I. STUDENTS' INDEPENDENT STUDY PROGRAM

1. Prokaryote and eukaryote:

a - common properties and differences;

b - features of bacterial cells structure.

1. Classification of Prokaryotes (Manual Determinative of Bacteriology Bergey's):

a – common principles of classification;

b - to make definition of such terms: species, subspecies, serovar, biovar, pathovar, chemovar, strain, clone, population.

2. Chemical composition of prokaryotes:

a - chemical composition of bacteria;

b - features of chemical composition rickettsiae, spirochaetes, chlamydiae.

4. Morphology of bacteria:

a – classification of bacteria by the form on cocci, rods, spiral-shaped, thread-shaped;

b - morphology of cocci and division then in dependence segmentation, to give examples of pathogenic ones;

c – rod-shaped bacteria (bacteria, bacillus, clostridia) and their locating in staining, to give examples of pathogenic ones;

d –spiral-shaped forms of bacteria (vibriones, spirilla, spirochaetes) and give examples of pathogenic representatives.

5. Complete staining methods, Gram's method:

a - to give definition of complete staining methods;

b - procedure and mechanism of Gram's staining;

c - practical value of Gram's staining;

d - Gram's staining by Sinev's.

A. Morphology of Bacteria

Bacteria (Gk bakterion small staff) are, for the most part, unicellular organisms lacking chlorophyll.

Their biological properties and predominant reproduction by binary fission relates them to prokaryotes

The size of bacteria is measured in micrometres (mem) and varies from 0.1 mem (Spiroplasma, Acholeplasma) to 16-18 mem (Spirillum volutans). Most pathogenic bacteria measure 0.2 to 10 mm

The shape of spherical bacteria represents a certain ratio of surface area ($As = 4\pi r^2$) to volume ($Vs = 4/3\pi r^3$).

For those cells having a proper cylindrical shape the formulae will be $At = 2\pi b(b + 2a\}$; $Vi = 2\pi ab^2$, where a is equal to one-half the maximum length, b is equal to one-half the maximum width, and r is equal to the radius of the spherical cell.

The shape as well as the dimensions of microbes is not absolutely constant Morphological differences are found in many bacterial species.

The organisms are subject to change with the surrounding environmental conditions.

However, in relatively stable conditions, the microbes are capable of retaining their specific properties (size, shape) inherited during the process of evolution.

Morphologically, bacteria possess three main forms **(Fig. 6).**

They are either spherical (cocci), rod-shaped (bacteria, bacilli, and clostridia) or spiral-shaped (vibrios and spirilla).

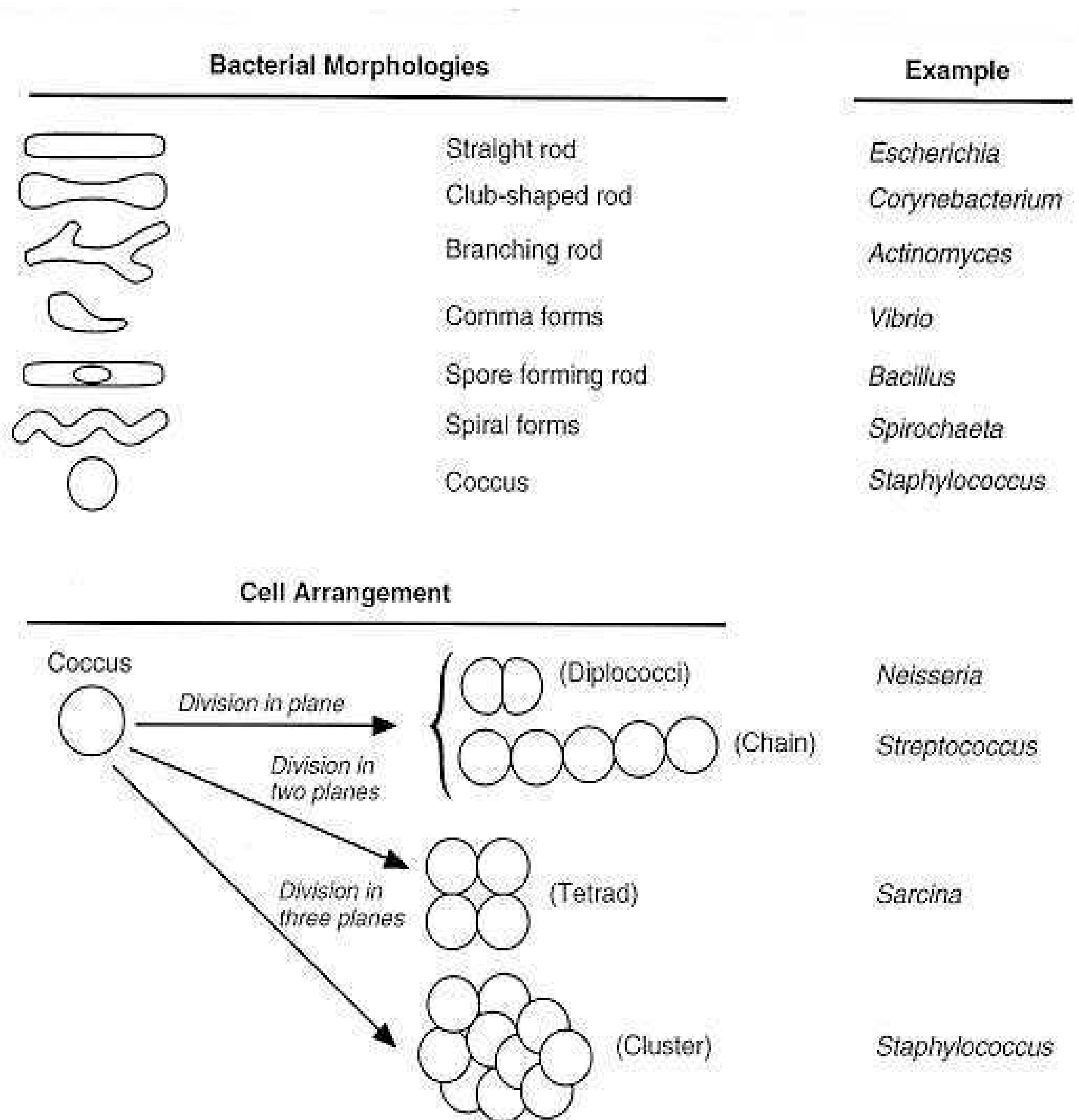


Figure 6. Typical shapes and arrangements of bacterial cells

Cocci (Gk. chokes berry) These forms of bacteria **(Fig.7)** are spherical, ellipsoidal, bean-shaped, and lancelet Cocci are subdivided into six groups according to cell arrangement, cell division and biological properties

1. Micrococci (Micrococcus) The cells are arranged singly or irregularly They are saprophytes, and live in water and in air (M agilis, M.roseus, M luteus, etc)

2. Diplococci (Gk. diplos double) divide in one plane and remain attached in pairs These include, menmgococcus, causative agent of epidemic cerebrospinal meningitis, and gonococcus, causative agent of gonorrhoea and blennorrhoea.

3. Streptococci (Gk. streptos curved, kokkos berry) divide in one plane and are arranged in chains of different length. Some streptococci are pathogenic for humans and are responsible for various diseases.

4. Tetracocci (Gk. tetra four) divide in two planes at right angles to one another and form groups of ours. They very rarely produce diseases in humans

5. Sarcinae (L sarcio to tie) divide in three planes at right angles to one another and resemble packets of 8, 16 or more cells.

They are frequently found in the air Virulent species have not been encountered.

6. Staphylococci (Gk. staphyle cluster of grapes) divide in several planes resulting in irregular bunches of cells, sometimes resembling clusters of grapes. Some species of Staphylococci cause diseases in man and animals.

Rods. Rod-shaped or cylindrical forms **(Fig. 8)** are subdivided into bacteria, bacilli, and clostridia. Bacteria include those microorganisms which, as a rule, do not produce spores (colibacillus, and organisms responsible for enteric fever, paratyphoids, dysentery, diphtheria, tuberculosis, etc).

Bacilli and clostridia include organisms the majority of which produce spores (hay bacillus, bacilli responsible for anthrax, tetanus, anaerobic infections, etc).

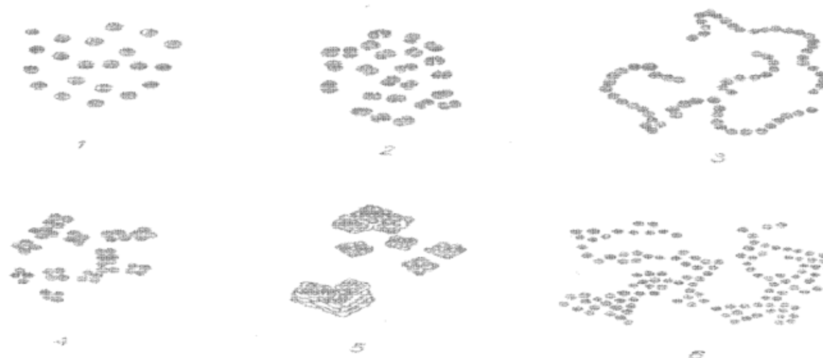


Figure 7. Spherical forms of bacteria
1 - micrococci, 2- diplococci, 3-streptococci, 4-teracocci; 5-sarcina; 6 -Staphylococci

Rod-shaped bacteria exhibit differences in form. Some are short (tularaemia bacillus), others are long (anthrax bacillus), the majority have blunted ends, and others have tapered ends (fusobacteria).

According to their arrangement, cylindrical forms can be subdivided into three groups (1) diplobacteria and diplobacilli occurring in pairs(bacteria of pneumonia); (2) streptobacteria or streptobacilli occurringin chains of different length (causative agents of chancroid, anthrax), (3) bacteria and bacilli which are not arranged in a regular pattern (these comprise the majority of the rod-shaped forms).

Figure 8a

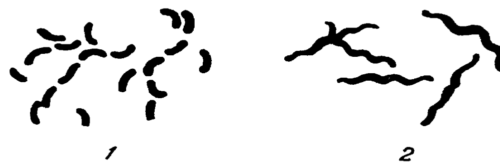


Figure 8b

Figure 8. Rod-shaped bacteria and some spiral-shaped bacteria

Fig. 8a: 1 – diplobacteria; 2- rods with rounded, sharpened and\or thickened ends; 3- different rod-shaped forms and streptobacteria

Fig. 8b: 1-vibriones 2—spirilla

Some rod-shaped bacteria have pin-head thickenings at the ends(causative agents of diphtheria); others form lateral branchings (bacilli of tuberculosis and leprosy).

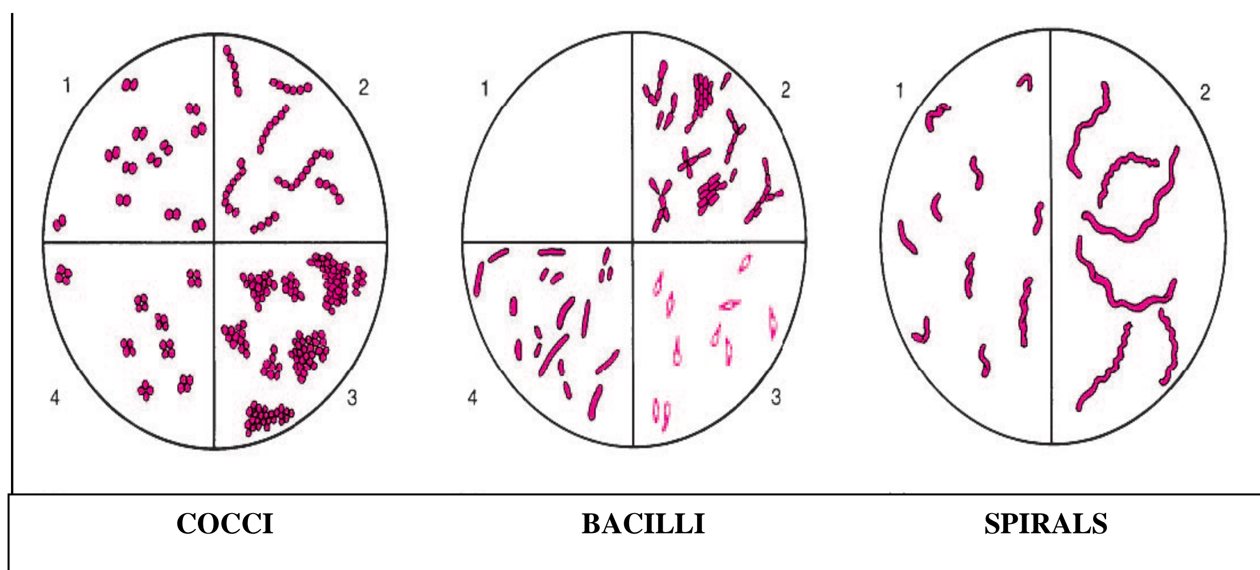
There is a significantly greater number of rod-shaped bacteria than coccal-shaped organisms. This is explained by the fact that in rod-shaped bacteria the ratio of surface area to volume is higher.

Thus, a larger surface area is in direct contact with nutrient substances in the surrounding medium.

Spiral-shaped bacteria. Vibriones and spirilla belong to this group of bacteria.

1. *Vibriones* (L. vibrio to vibrate) are cells which resemble a comma in appearance- Typical representatives of this group are Vibrio comma, the causative agent of cholera, and aquatic vibriones which are widely distributed in fresh water reservoirs.

2. *Spirilla* (L. spira coil) are coiled forms of bacteria exhibiting twists with one or more turns. Only one pathogenic species is known (Spirillum minus) which is responsible for a disease in humans transmitted through the bite of rats and other rodents (rat-bite fever, sodoku).



Microbes exhibit pleomorphism, they are subject to individual variations, unassociated with age or stage of development, causing the existence of different forms of cells in the same species. They are extremelylabile, and susceptible to changes which are associated with such factors as temperature, nutrients, salt concentration, acidity, metabolites, disinfectants, drugs, and body resistance.

PROKARYOTIC CELL STRUCTURE

The cytoplasm is enclosed within a lipoprotein cell membrane, similar to the prokaryotic cell membrane.

Most animal cells have no other surface layers; many eukaryotic microorganisms, however, have an outer cell wall, which may be composed of a polysaccharide such as cellulose or chitin or may be inorganic, as in the silica wall of diatoms

The prokaryotic cell is simpler than the eukaryotic cell at every level, with one exception: the cell wall may be more complex.

The prokaryotic nucleus can be seen with the light microscope in stained material. It is Feulgen-positive, indicating the presence of DNA.

The negatively charged DNA is at least partially neutralized by small polyamines and magnesium ion, but histonelike proteins have recently been discovered in Cytoplasmic Structures.

Prokaryotic cells lack autonomous plastids, such as mitochondria and chloroplusts. The electron transport enzymes are localized instead in the cell membrane; in photosynthetic organisms, the photosynthetic pigments are localized in lamellae underlying the cell membrane.

In some photosynthetic bacteria, the lamellae may become convoluted and pinch off into discrete particles called chromatophores.

Bacteria often store reserve materials in the form of insoluble cytoplasmic granules, which are deposited as osmotically inert, neutral polymers. In the absence of a nitrogen source, carbon source material is converted by some bacteria to the polymer poly-β-hydroxybutyric acid and by other bacteria to various polymers of glucose such as starch and glycogen.

The granules are used as carbon sources when protein and nucleic acid synthesis is resumed.

Similarly, certain sulfur-oxidizing bacteria convert excess H_2s from the environment into intracellular granules of elemental sulfur.

Finally, many bacteria accumulate reserves of inorganic phosphate as granules of polymerized metaphosphate, called volutin.

Volutin granules are also called metachromatic granules because they stain red with a blue dye. They are characteristic features of corynebacteria.

Microtubular structures, characteristic of eukaryotic cells, are generally absent in prokaryotes. In a few instances, however, the electron microscope has revealed bacterial structures that resemble microtubules.

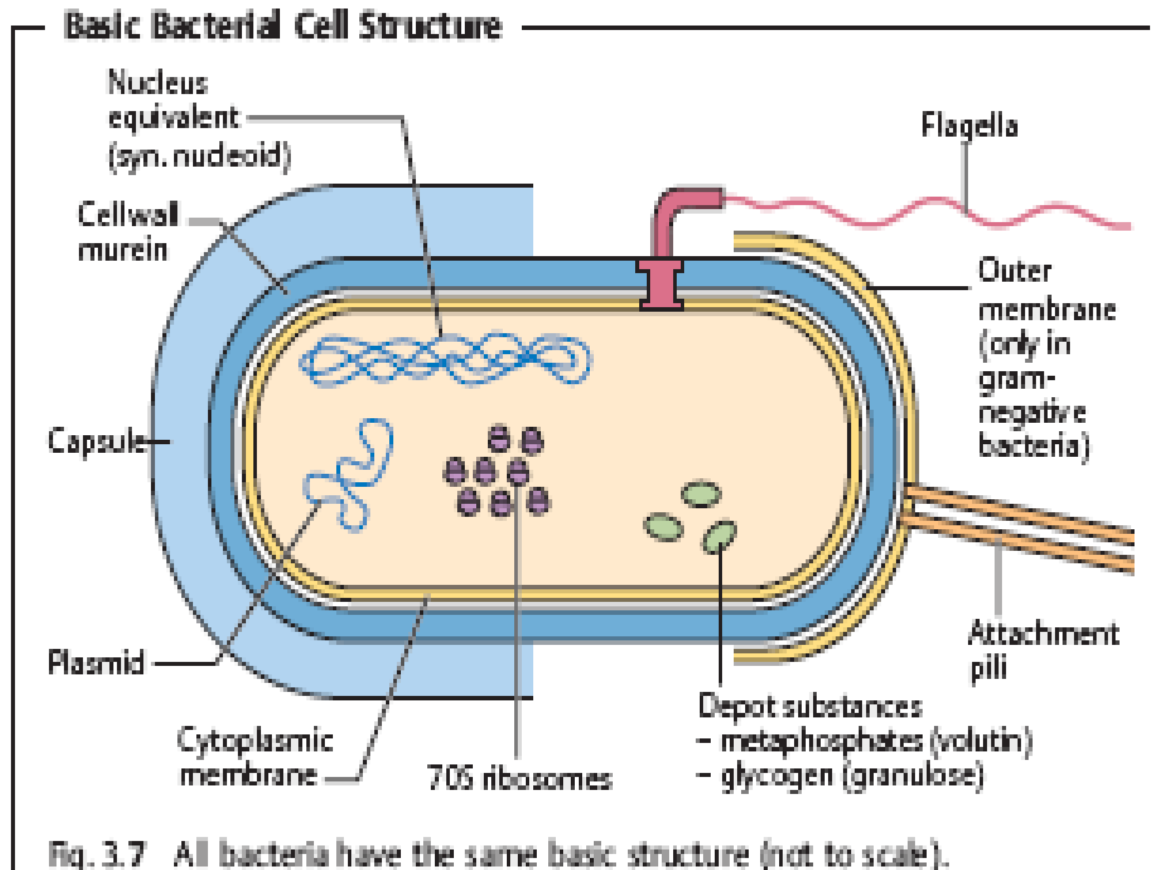


Fig. 3.7 All bacteria have the same basic structure (not to scale).

STAINING

Stains combine chemically with the bacterial protoplasm; if the cell is not already dead, the staining process itself will kill it. The process is thus a drastic one and may produce artifacts.

The commonly used stains are salts. Basic stains consist of a colored cation with a colorless anion (eg, methylene blue chloride"); acidic stains are the reverse (eg, sodium + eosinate"). Bacterial cells are rich in nucleic acid, bearing negative charges as phosphate groups. These combine with the positively charged basic dyes. Acidic dyes do not stain bacterial cells and hence can be used to stain background material a contrasting color (see Negative Staining, below).

The basic dyes stain bacterial cells uniformly unless the cytoplasmic RNA is destroyed first. Special staining techniques can be used, however, to differentiate flagella, capsules, cell walls, cell membranes, granules, nuclei, and spores.

Gram Stain

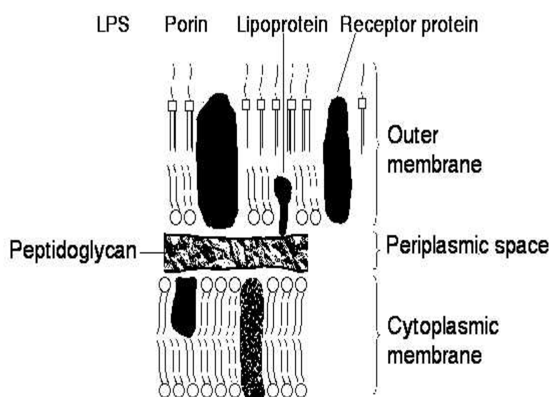
An important taxonomic characteristic of bacteria is their response to Gram's stain. The gram-staining property appears to be a fundamental one, since the Gram reaction is correlated with many other morphologic properties in phylogenetically related forms. An organism that is potentially gram-positive may appear so only under a particular set of environmental conditions and in a young culture.

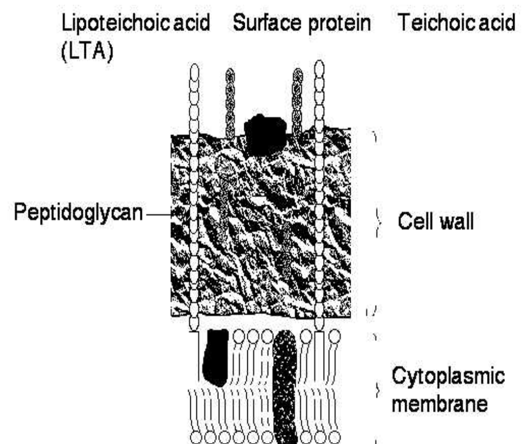
The gram-staining procedure begins with the application of a basic dye, crystal violet.

A solution of iodine is then applied; all bacteria will be stained blue at this point in the procedure. The cells are then treated with alcohol.

Gram-positive cells retain the crystal violet-iodine complex, remaining blue; gram-negative cells are completely decolorized by alcohol.

As a last step, a counter stain such as the red dye safranin) is applied so that the decolorized gram-negative (cells will take on a contrasting color; the gram-positive cells now appear purple.





The Gram-Positive Cell

As previously mentioned, Gram-positive bacteria are characterized by their blue-violet color reaction in the Gram-staining procedure.

The violet color reaction is caused by crystal-violet, the primary Gram-stain dye, complexing with the iodine mordant.

When the decolorizer is applied, a slow dehydration of the crystal-violet/iodine complex is observed due to the closing of pores running through the cell wall. Because the crystal-violet is still present in the cell, the counter stain is not incorporated, thus maintaining the cell's blue-violet color. If you recall, most cell walls contain peptidoglycan, a molecule made of amino acids and sugar.

A distinguishing factor among Gram-positive bacteria is that roughly 90% of their cell wall is comprised of peptidoglycan and a Gram-positve bacteria can have more than 20 layers of peptidoglycan stacked together to form the cell wall. Examples of common Gram-positive cells include Staphylococcus aureus and Streptococcus cremoris, a bacterium used in dairy production.

The Bacterial Cell Wall

The bacterial cell wall is a unique structure which surrounds the cell membrane. Although not present in every bacterial species, the cell wall is very important as a cellular component.

Structuraly, the wall is necessary for:

- Maintaining the cell's characteristic shape- the rigid wall compensates for the flexibility of the phospholipid membrane and keeps the cell from assuming a spherical shape
- Countering the effects of osmotic pressure- the strength of the wall is responsible for keeping the cell from bursting when the intracellular osmolarity is much greater than the extracellular osmolarity
- Providing attachment sites for bacteriophages- teichoic acids attached to the outer surface of the wall are like landing pads for viruses that infect bacteria

- Providing a rigid platform for surface appendages- flagella, fimbriae, and pili all emanate from the wall and extend beyond it

The cell walls of all bacteria are not identical. In fact, cell wall composition is one of the most important factors in bacterial species analysis and differentiation. There are two major types of walls: Gram-positive and Gram-negative.

The cell wall of Gram-positive bacteria consists of many polymer layers of peptidoglycan connected by amino acid bridges. A schematic diagram provides the best explanation of the structure. The peptidoglycan polymer is composed of an alternating sequence of N-acetylglucosamine and N-acetyl-muraminic acid. It's a lot easier to just remember NAG and NAMA. Each peptidoglycan layer is connected, or crosslinked, to the other by a bridge made of amino acids and amino acid derivatives.

The particular amino acids vary among different species, however. The crosslinked peptidoglycan molecules form a network which covers the cell like a grid. Also, 90% of the Gram-positive cell wall is comprised of peptidoglycan.

The cell wall of Gram-negative bacteria is much thinner, being comprised of only 20% peptidoglycan. Gram-negative bacteria also have two unique regions which surround the outer plasma membrane: the periplasmic space and the lipopolysaccharide layer. The periplasmic space separates the outer plasma membrane from the peptidoglycan layer. It contains proteins which destroy potentially dangerous foreign matter present in this space.

The lipopolysaccharide layer is located adjacent to the exterior peptidoglycan layer. It is a phospholipid bilayer construction similar to that in the cell membrane and is attached to the peptidoglycan by lipoproteins. The lipid portion of the LPS contains a toxic substance, called Lipid A, which is responsible for most of the pathogenic affects associated with harmful Gram-negative bacteria.

Polysaccharides which extend out from the bilayer also contribute to the toxicity of the LPS. The LPS, lipoproteins, and the associated polysaccharides together form what is known as the outer membrane.

Keep in mind that the cell wall is not a regulatory structure like the cell membrane. Although it is porous, it is not selectively permeable and will let anything pass that can fit through its gaps.

The Gram-Negative Cell

Unlike Gram-positive bacteria, which assume a violet color in Gram staining, Gram negative bacteria incorporate the counter stain rather than the primary stain. Because the cell wall of Gram(-) bacteria is high in lipid content and low in peptidiglycan content, the primary crystal-violet escapes from the cell when the decolorizer is added.

This is because primary stains like to bind with peptidoglycan- something the G(-) cell lacks. The pathogenic nature of Gram(-) bacteria is usually associated with certain components of their cell walls, particularly the lipopolysaccharide (endotoxin) layer.

The Black Plague, which wiped out a third of the population of Europe, was caused by the tiny G(-) rod, Yersinia pestis. Most enteric (bowel related) illnesses can also be attributed to this group of bacteria.

I. Gram-staining Procedure

Gram-staining is a four part procedure which uses certain dyes to make a bacterial cell stand out against against its background. The specimen should be mounted and heat fixed on a slide before you precede to stain it.

The reagents you will need to successfully perform this operation are:

- Crystal Violet (the Primary Stain)
- Iodine Solution (the Mordant)
- Decolorizer (ethanol is a good choice)
- Safranin (the Counterstain)
- Water (preferably in a squirt bottle)

Before starting, make sure that all reagents, as well as the squirt-bottle of water, are easily accessible because you won't have time to go get them during the staining procedure.

Also, make sure you are doing this near a sink because it can get really messy. Wear the appropriate lab attire.

STEP 1: Place your slide on a slide holder or a rack. Flood (cover completely) the entire slide with crystal violet. Let the crystal violet stand for about 60 seconds. When the time has elapsed, wash your slide for 5 seconds with the water bottle. The specimen should appear blue-violet when observed with the naked eye.

STEP 2: Now, flood your slide with the iodine solution. Let it stand about a minute as well. When time has expired, rinse the slide with water for 5 seconds and immediately precede to step three. At this point, the specimen should still be blue-violet.

STEP 3: This step involves addition of the decolorizer, ethanol. Step 3 is somewhat subjective because using too much decolorizer could result in a false Gram (-) result. Likewise, not using enough decolorizer may yield a false Gram (+) results.

To be safe, add the ethanol dropwise until the blue-violet color is no longer emitted from your specimen. As in the previous steps, rinse with the water for 5 seconds.

STEP 4: The final step involves applying the counterstain, saffranin. Flood the slide with the dye as you did in steps 1 and 2. Let this stand for about a minute to allow the bacteria to incorporate the saffranin.

Gram positive cells will incorporate little or no counterstain and will remain blue-violet in appearance.

Gram negative bacteria, however, take on a pink color and are easily distinguishable from the Gram positives.

Again, rinse with water for 5 seconds to remove any excess of dye.

After you have completed steps 1 through 4, you should dry the slide with bibulous paper or allow it to air dry before viewing it under the microscope

II. Students practical activities

1. To teach upon microscope and to sketch in an album stains of the main forms of bacteria:

- spherical (micrococci, diplococci, streptococci, teracocci, staphylococci, sarcina),
- rod-shaped (monobacteria, monobacilla, diplobacteria, streptobacteria, Streptobacilla),
- spiaral-shaped (vibriones, spirilla, spirochaetes).

2. Preparation of stains from different cultures and staining by a simple method.

3. Preparation of stains from an mixture of bacteria of Escherichia coli and Staphylococci and staining by Gram's method of .

III. Tests and Assignments for Self-assessment

Chose the correct answers:

1. Select the correct statements:

a – prokaryote are haploid microorganisms;

b – prokaryote contain one gene;

c – prokaryote have no mitochondria, chloroplusts and Golgi apparatus;

d – prokaryote have no ameba-like motility;

e – prokaryote have nucleoid, haven't nuclear membrane;

f – prokaryote have mesosoms.

3. To indicate, where are indicated properties of prokaryote and where are eukaryote:

The nucleoid has no membrane separating it from the cytoplasm	Karyoplasm is separated from the cytoplasm by membrane
Chromosome is a one ball of double twisted DNA threads Mitosis is absent	Chromosome is more than one, There is a mitosis
DNA of cytoplasm are represented in plasmids	DNA of cytoplasm are represented in organelles
There aren't cytoplasmic organelle which is surrounded by membrane	There are cytoplasmic organelle which is surrounded by membrane
The respiratory system is localized in cytoplasmic membrane	The respiratory system is localized mitochondrion

There are ribosome 70S in cytoplasm	There are ribosome 80S in cytoplasm
Peptidoglycan are included in cell's wall (Murein)	Peptidoglycan aren't included in cell's wall
There aren't phagocytosis, pinocytosis	There are phagocytosis, pinocytosis

5. You should get in (paste) percentages of a contents in a bacterial cell:
a - water...; b- dry matter...; c- nucleic acids...; d - polysaccharides...;
e - lipids...; f- mineral substances...

6. Select the correct statements: a - the bacteria are subdivided on cocci, rod-shaped, threat-shaped, filiform by their morphology; b - the forms of cocci are bean-shaped, ellipsoidal, lancelet; c – rod-shaped bacteria, which frame spores, are named as the bacilli; d - Spirilla are the threat-shaped form; e - Vibriones have some convolutions; f – the Bacillus and Clostridia is rod-shaped microbes, which frame spores.

IV. The answers to the self-assessments

1. A, b, c, d, e, f. 2. At the left - prokaryote, on the right - eukaryote. 3. Water - 75-85 %, dry matter–25-15 %, proteins - 50-80 % of dry matter, nucleic acid - 10-30 % of dry matter, polysaccharides - 12-18%, Lipids - 10 % of dry matter, mineral substance - 2-14 % of dry mass. 4. A, b, c, d, e, f.

Students must know:

1.The main forms of bacteria (depends on shape).

2.The definitions: species, subspecies, serovar, biovar, pathovar, chemovar, strain, clone, population.

3. The mechanism of bacteria Gram's staining.

4.Pathogenic representatives of different shaped microorganisms.

Students should be able to:

1. Stained preparation;
2. Gram's staining;
3. Staining of acid-fast microbes.

MAIN METHODS OF BACTERIA EXAMINATION. COMPLETE METHODS OF STAINING. ANJESKY'S STAINING. METHODS OF EXAMINATION FLAGELLA, HANGING DROP AND WET-MOUNT TECHNIQUES. SPORES AND FLAGELLA OF BACTERIA.

I. STUDENTS' INDEPENDENT STUDY PROGRAM

1. Sporulation for bacteria:

a – to identify a difference between bacteria, bacilli and clostridia;
b – function of a sporulation process for bacteria
c – chemical composition of spores;
d – spores locating in pathogenic bacteria;
e – stages and condition of a sporulation;
f – influence of environmental factors on spores;
g – methods of spores staining.

2. Locomotor organoids of bacteria:

a – creeping and swimming bacteria;
b –structure of flagella, chemical composition;
c – division of bacteria by location of flagella;
d – function of flagella, mechanism of bacteria motility;
e – methods of flagella examination.

3. Fimbriae (cilia, filaments, pili) of bacteria. Their types and value.

Endospores.

Endospores are highly heat-resistant, dehydrated resting cells formed intracellularly in members of the genera Bacillus and Clostridium **(fig. 9).** Endospores are small spherical or oval bodies formed within the cell. A spore is formed at a certain stage in the development of some microorganisms and this property was inherited in the process of evolution in the struggle for keeping the species intact.

Some microorganisms, principally rod-shaped (bacilli and clostridia), are capable of sporulation. These include the causative agents of anthrax, tetanus, anaerobic infections, botulism and also saprophytic species living in the soil, water and bodies of animals.

Spore formation only rarely occurs in cocci *{Sarcina lutea, Sarcina ureae)* and in spiral forms *(Desulfovibrio desulfuricans}*. Sporulation occurs in the environment (in soil and on nutrient media), and is not observed in human or animal tissues.

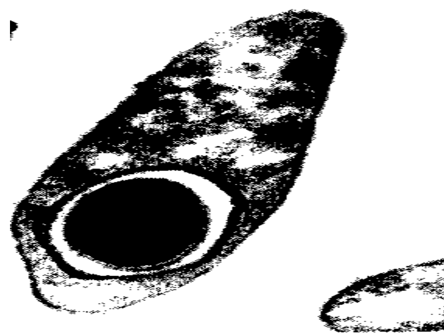


Figure 9. Thin section through a sporulating cell of bacilli

The series of biochemical and morphologic changes that occur during sporulation represent true differentiation within the cycle of the bacterial cell. The process, which usually begins in the stationary phase of the vegetative cell cycle, is initiated by depletion of nutrients (usually readily utilizable sources of carbon or nitrogen, or both).

The cell then undergoes a highly complex, well-defined sequence of morphologic and biochemical events that ultimately lead to the formation of mature endospores.

As many as seven distinct stages have been recognized by morphologic and biochemical studies of sporulating Bacillus species:

stage 0, vegetative cells with two chromosomes at the end of exponential growth;

stage I, formation of axial chromatin filament and excretion of exoenzymes, including proteases;

stage II, forespore septum formation and segregation of nuclear material into two compartments;

stage III, spore protoplast formation and elevation of tricarboxylic acid and glyoxylate cycle enzyme levels;

stage IV, cortex formation and refractile appearance of spore;

stage V, spore coat protein formation;

stage VI, spore maturation, modification of cortical peptidoglycan, uptake of dipicolinic acid (a unique endospore product) and calcium, and development of resistance to heat and organic solvents;

stage VII, final maturation and liberation of endospores from mother cells (in some species).

When newly formed, endospores appear as round, highly refractile cells within the vegetative cell wall, or sporangium. Some strains produce autolysins that digest the walls and liberate free endospores.

The spore protoplast, or core, contains a complete nucleus, ribosomes, and energy generating components that are enclosed within a modified cytoplasmic membrane. The peptidoglycan spore wall surrounds the spore membrane; on germination, this wall becomes the vegetative cell wall. Surrounding the spore wall is a thick cortex that contains an unusual type of peptidoglycan, which is rapidly released on germination.

A spore coat of keratinlike protein encases the spore contained within a membrane (the exosporium). During maturation, the spore protoplast dehydrates and the spore becomes refractile and resistant to heat, radiation, pressure, desiccation, and chemicals; these properties correlate with the cortical peptidoglycan and the presence of large amounts of calcium dipicolinate.

Recent evidence indicated that the spores of Bacillus spharicus were revived which had been preserved in amber for more than 25 million years. Their claims need to be reevaluated. The thin section of the spore shows the ruptured, thick spore coat and the cortex surrounding the spore protoplast with the germinal cell wall that becomes the vegetative wall on outgrowth.

The spores of certain bacilli are capable of withstanding boiling and high concentrations of disinfectants. They are killed in an autoclave exposed to saturated steam, at a temperature of 115-125 °C, and also at a temperature of 150-170 °C in a Pasteur hot-air oven.

Sporulation: The sporulation process begins when nutritional conditions become unfavorable, depletion of the nitrogen or carbon source (or both) being the most significant factor Sporulation occurs massively in cultures that have terminated exponential growth as a result of such depletion. Sporulation involves the production of many new structures, enzymes, and metabolites along with the disappearance of many vegetative cell components. These changes represent a true process of differentiation: A series of genes whose products determine the formation and final composition of the spore is activated, while another series of genes involved in vegetative cell function is inactivated. These changes involve an alteration in the specificity of RNA polymerase.

The sequence of events in sporulation is highly complex asporogenous mutants reveal at least 12 morphologically or biochemically distinguishable stages, and at least 30 operons (including an estimated 200 structural genes) are involved During the process, some bacteria release peptide antibiotics, which may play a role in regulating sporogenesis.

Morphologically, sporulation begins with the isolation of a terminal nucleus by the inward growth of the cell membrane. The growth process involves an infolding of the membrane so as to produce a double membrane structure whose facing surfaces correspond to the cell wall-synthesizing surface of the cell envelope. The growing points move progressively toward the pole of the cell so as to engulf the developing spore.

The 2 spore membranes now engage in the active synthesis of special layers that will form the cell envelope: the spore wall and cortex, lying between the facing membranes; and the coat and exosporium, lying outside of the facing membranes.

In the newly isolated cytoplasm, or core, many vegetative cell enzymes are degraded and are replaced by a set of unique spore constituents.

Properties of Endospores:

1. Core - The core is the spore protoplast. It contains a complete nucleus (chromosome), all of the components of the protein-synthesizing apparatus, and an energy-generating system based on glycolysis. Cytochromes are lacking even in aerobic species, the spores of which rely on a shortened electron transport pathway involving flavoproteins. A number of vegetative cell enzymes are increased in amount (eg, alanine racemase), and a number of unique enzymes are formed (eg, dipicolinic acid synthetase). The energy for germination is stored as 3-phosphoglycerate rather than as ATP.

The heat resistance of spores is due in pan to their dehydrated state and in part to the presence of large amounts (5-15% of the spore dry weight) of calcium dipicolinate, which is formed from an intermediate of the lysine biosynthetic pathway. In some way not yet understood, these properties result in the stabilization of the spore enzymes, most of which exhibit normal heat lability when isolated in soluble form.

2. Spore wall -The innermost layer surrounding the inner spore membrane is called the spore wall. It contains normal peptidoglycan and becomes the cell wall of the germinating vegetative cell.

3. Cortex -The cortex is the thickest layer of the spore envelope. It contains an unusual type of peptidoglycan, with many fewer cross-links than are found in cell wall peptidoglycan. Cortex peptidoglycan is extremely sensitive to lysozyme, and its autolysis plays a key role in spore germination.

4. Coat -The coat is composed of a keratinlike protein containing many intramolecular disulfide bonds. The impermeability of this layer confers on spores their relative resistance to antibacterial chemical agents.

5. Exosporium -The exosporium is a lipoprotein membrane containing some carbohydrate.

Germination: The germination process occurs in 3 stages: activation, initiation, and outgrowth.

1. Activation-Even when placed in an environment that favors germination (eg, a nutritionally rich medium), bacterial spores will not germinate unless first activated by one or another agent that damages the spore coat.

Among the agents that can overcome spore dormancy are heat, abrasion, acidity, and compounds containing free sulfhydryl groups.

2. Initiation - Once activated, a spore will initiate germination if the environmental conditions are favorable. Different species have evolved receptors that recognize different effectors as signalling a rich medium: thus, initiation is triggered by L-alanine in one species and by adenosine in another.

Binding of the effector activates an autolysin that rapidly degrades the cortex peptidoglycan. Water is taken up, calcium dipicolinate is released, and a variety of spore constituents are degraded by hydrolytic enzymes.

3. Outgrowth - Degradation of the cortex and outer layers results in the emergence of a new vegetative cell consisting of the spore protoplast with its surrounding wall.

A period of active biosynthesis follows; this period, which terminates in cell division, is called outgrowth. Outgrowth requires a supply of all nutrients essential for cell growth.

In bacilli and clostridia, spores are located (1) *centrally,* in the centre of the cell (causative agent of anthrax); (2) *terminally,* at the ends of the rod (causative agent of tetanus); (3) *subterminally,* towards the ends (causative agents of botulism, anaerobic infections, etc.) **(Fig. 10).**

In some species of sporulating microorganisms, the spore diameter is greater than the width of the bacterial cell.

If the spore is located subterminally, the microbes take on the form of a spindle (closter).

In tetanus clostridia the spore diameter is also greater than the width of the vegetative cell, but the spore is located terminally, and hence the drum-stick appearance.

This property of sporulation is important in characterizing and identifying spore-forming microbes, and also when selecting methods of decontaminating objects, housings, foodstuff's, and other substances.

The microbe may lose its ability to sporulate by frequent cultivation on fresh media or by subjecting it to high temperatures.

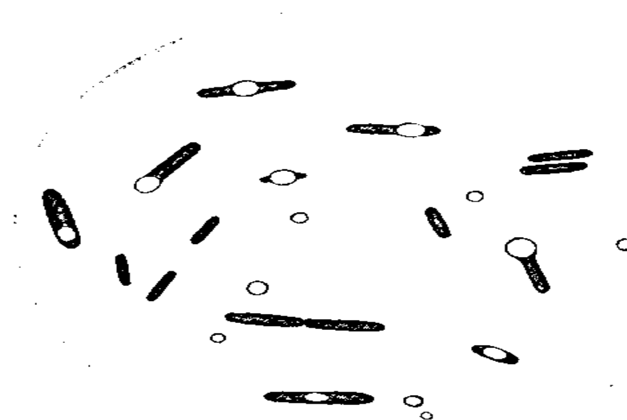


Figure 10. Shapes and arrangement of spores in bacilli and clostridia

Flagella. Motile bacteria are subdivided into creeping and swimming bacteria. Creeping bacteria move slowly (creep) on a supporting surface as a result of wave-like contractions of their bodies, which cause periodic alterations in the shape of the cell.

These bacteria include Myxobacterium, Beggiatoa, Thiothrix. Swimming bacteria move freely in a liquid medium. They possess flagella, thin hair-like cytoplasmic appendages measuring 0.02 to 0.05 mcm in thickness and from 6 to 9 mcm in length. In some spirilla they reach a length of 80 to 90 mcm.

Investigations have confirmed that the flagella are made up of proteins the composition of which differs considerably from that of the bacterial cell proteins (keratin, myosin, fibrinogen).

Figure 11. The flagella of Proteus vulgaris demonstrated by electron microscopy.

With the aid of paper chromatography, it has been discovered that the flagellate material contains several ammo acids: lysine, aspartic and glutamic acids, alanine, etc. It has been suggested that the flagella are attached to basal granules which are found in the outlying zones of the cytoplasm

The flagella can be observed by dark-field illumination, by special methods involving treatment with mordants, adsorption of various substances and dyes on their surfaces, and by electron microscopy The latter has made it possible to detect the spiral and screw-shaped structure of the flagella. The axial filament of the flagellum consists of two entwined hair-like processes enclosed in a sheath.

According to a pattern in the attachment of flagella motile microbes can be divided into 4 groups:

(1) monotrichates, bacteria having a single flagellum at one pole of the cell (cholera vibrio, blue pus bacillus),

(2) amphitrichates, bacteria with two polar flagella or with a tuft of flagella at both poles (Spirillum volutans),

(3) lophotrichales, bacteria with a tuft of flagella at one pole (blue-green milk bacillus, Alcaligenes faecalis),

(4) peritrichales, bacteria having flagella distributed over the whole surface of their bodies (colibacillum, salmonellae of enteric fever and paratyphoids A and B). **(Fig. 12).**

The above mentioned classification is provisional. While studying the flagella under an electron microscope, it was revealed that the flagellum in some monotrichates is not located at the end of the cell, but at the point of transition of the lateral surface to the pole.

It has been established that bacteria which once were considered to be monotrichous possess a number of flagella As to amphitrichates, their independent existence is a subject of controversy

It has been suggested that the amphitrichate cell is actually comprised of two cells which have been separated incompletely, having flagella at their distal ends.

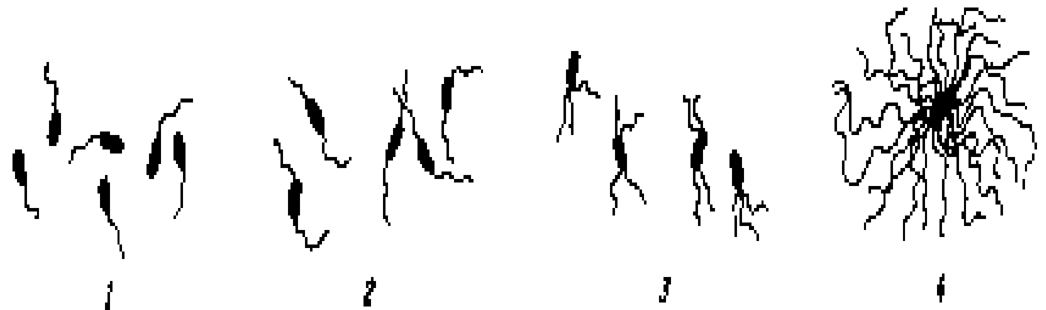


Figure 12. Bacterial flagella
1 – monotrichates, 2 – amphitrichates, 3 – lophotrichates 4 – peritrichates

The flagella are main locomotor organoid of bacteria. As the result of their vigorous movements, resembling the twiddling of a corkscrew, the fluid moves along them and the micro-organism moves at a rate of about 50 mcm/sec.

The mechanism of the contraction is not quite clear. It has been suggested that the protein of the sheath surrounding the flagellum forms with flagella a bicomponent system which contracts like actomyosin.

The contraction of the flagella is due to the existence of two configurations of flagellin molecules differing in ammo acid composition.

The type of motility in bacteria depends on the number of flagella, age and properties of the culture, temperature, amount of chemical substances and on other factors. Monotrichates move with the greatest speed (60 mcm per second).

Peritrichates move at rates ranging from 25 to 30 mcm per second. Certain species of motile microbes move at a rate of up to 200 mcm per second.

Motile bacteria also possess the power of directed movements, or taxis. According to the factors under the effect of which motion occurs, chemotaxis, aerotaxis, and phototaxis are distinguished.

Motility in bacteria can be observed by the hanging drop in wet conditions. The determination of motility in microbes is employed in laboratory practice as a means to identify cholera vibrio, dysentery, enteric fever, paratyphoid and other bacteria. However, although the presence of flagella is a species characteristic, they are not always essential to life, since a flagellate forms of motile bacteria exist.

Various types of microbes have pili (cilia, filaments, fimbriae), structures which are much shorter and thinner than the flagella **(Fig. 13).** They cover the body of the cell and there may be 100 to 400 of them on one cell. Pili are 0.3-1.0 mcm long and 0.01 mcm wide.

It is supposed that cilia are not related to the organs of locomotion and that they serve to attach the microbial cells to the surface of some substrates.

Nine different types of pili have been studied. They consist of protein. Just like in the case of flagella, it is not necessary that all bacterial cells have pili.

Of most interest are the F-pili within which there is a canal through which the genetic material from the donor to the recipient is transferred durin conjugation (see section on conjugation).

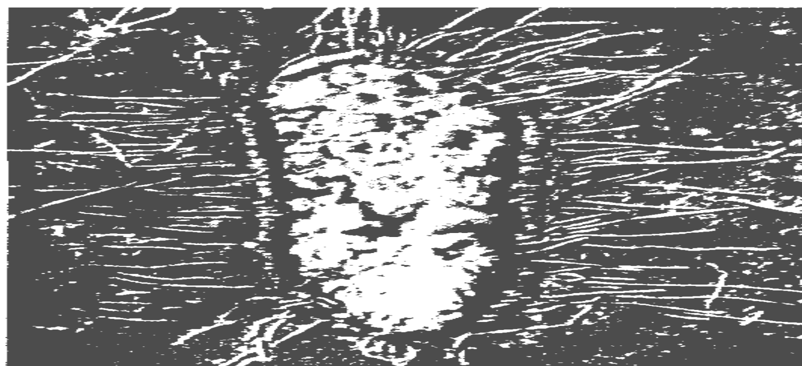


Figure 13. Cilia (pili) of Shigella flexneri demonstrated by electron microscopy

It is possible that the pili contribute to the nutrition of bacteria since they greatly increase the surface area of the bacterial cell.

Besides actively moving by means of flagella or by cell contraction, microbes are capable of molecular, passive or brownian movement, due to the thermal molecular motion of the surrounding medium.

Two types of surface appendage can be recognized on certain bacterial species: the flagella, which are organs of locomotion, and pili (Latin hairs), which are also known as fimbriae (Latin fringes).

Flagella occur on both Gram-positive and Gram-negative bacteria, and their presence can be useful in identification. For example, they are found on many species of bacilli but rarely on cocci.

In contrast, pili occur almost exclusively on Gram-negative bacteria and are found on only a few Gram-positive organisms (e.g., Corynebacterium renale).

Some bacteria have both flagella and pili.

The electron micrograph in **Fig. 14** shows the characteristic wavy appearance of flagella and two types of pili on the surface of Escherichia coli.

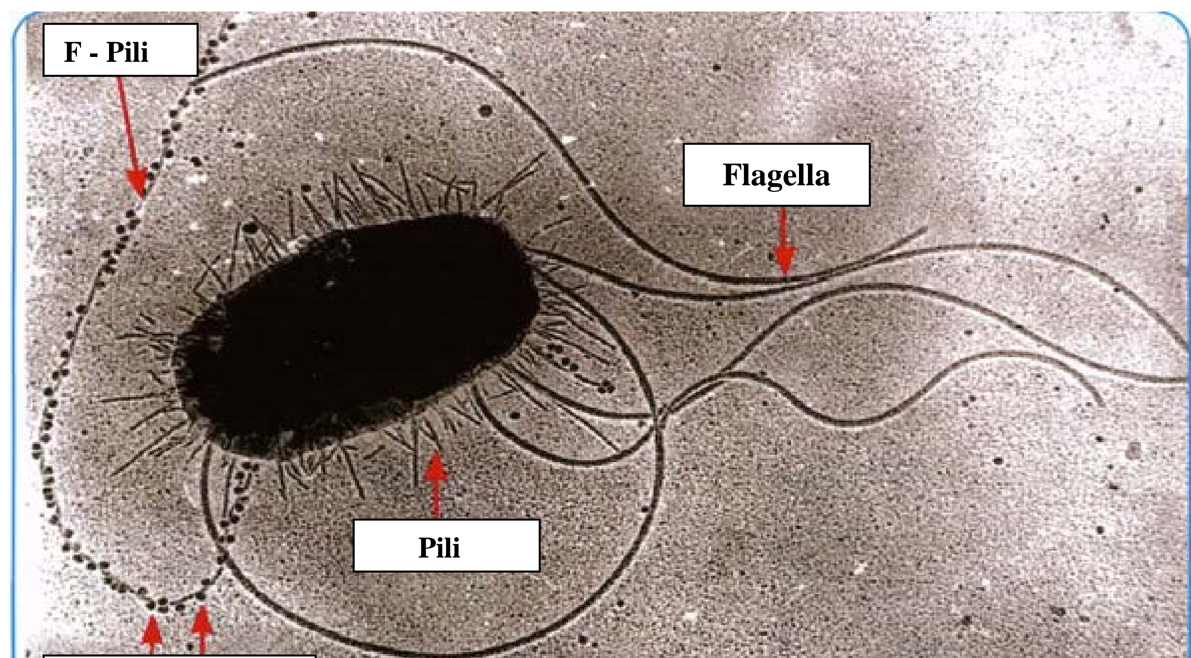


Figure 14. Flagella and pili of E.coli.

II. Students practical activities:

1. To study and to sketch ready smears of bacteria with spores and flagella.
2. To stain bacteria with spores by Anjesky technique.

Spores are most simply observed as intracellular refractile bodies in unstained cell suspensions or as colorless areas in cells stained by conventional methods. The spore wall is relatively impermeable, but dyes can be made to penetrate it by heating the preparation. The same impermeability then serves to prevent decolorization of the spore by a period of alcohol treatment sufficient to decolorize vegetative cells. The latter can finally be counterstained. Spores are commonly stained with malachite green or carbolfuchsin.

To demonstrate bacterial spores, special staining methods proposed by Anjesky, Peshkov, Bitter, Schaeffer-FuIton, and others are used.

Anjesky's staining. A thick smear is dried in the air, treated with 0.5 per cent sulphuric acid, and heated until it steams.

Then, the preparation is washed with water, dried, fixed above the flame, and stained by the Ziehl-Neelsen’s technique. Spores stain pink-red, the cell appears blue.

3. To study of microorganisms with flagella (Proteus vulgaris) by wet-mount and hanging drop technique.

Study of living microorganisms using the wet-mount and hanging-drop techniques. Using living microorganisms, one can study the processes of their propagation and spore formation, as well as the effect on them of various chemical and physical factors.

In clinical laboratories living microorganisms are investigated to determine their motility, i.e., indirect confirmation of the presence of flagella.

Preparations in this case are made using wet-mount or hanging-drop techniques and then subjected to dry or immersion microscopy. Results are better when dark-field or phase-contrast microscopy is employed.

Wet-mount technique. A drop of the test material, usually 24-hour broth culture of microorganisms, is placed into the centre of a glass slide. The drop is covered with a cover slip in a manner preventing the trapping of air bubbles; the fluid should fill the entire space without overflowing.

An inherent drawback of the wet-mount technique is its rapid drying. In prolonged microscopy it is recommended that the edges of a cover slip be sealed with petrolatum.

Hanging drop technique. To prepare this kind of preparation, special glass slides with an impression (well) in the centre are utilized. A small drop of the test material is put in the middle of the cover slip. The edges of the well are ringed with petrolatum. The glass slide is placed onto the cover slip so that the drop is in the centre of the well. Then. it is carefully inverted and the drop hangs in the centre of the sealed well, which prevents it from drying.

The prepared specimens are examined microscopically, slightly darkening the microscopic field by lowering the condenser and regulating the entrance of light with a concave mirror. At first low power magnification is used (objective 8 X) to detect the edge of the drop, after which a 40 x or an oil-immersion objective is mounted.

Occasionally, molecular (Brownian) motility is mistaken for the motility of microorganisms. To avoid this error, it should be borne in mind that microorganisms propelled by flagella may traverse the entire microscopic field and make circular and rotatory movements.

After the examination the wet-mount and hanging-drop preparations should be immersed in a separate bath with disinfectant solution to kill the microorganisms studied.

4. To study morphology of bacteria fimbriae by electronic photo.

III. Tests and Assignments for Self-assessment.

1. Select the correct statements:

a – the creeping bacteria move by waved-liked contractions;

b – the swimming bacteria have flagella;

c – the spores are formed in tissues of men and animals;

d – the spores would grow up in favourable conditions and turn into vegative forms;

e – all motile bacteria have the fimbriae;

f – bacteria, which are able to an adhesion have pili of the first (common) type;

g – pili (fimbriae) are examined by a hanging drop or wet-mount methods.

2. Give the correct answer statements:

a – what is a main organoid of bacteria motility?

b – call four groups of bacteria by locating of flagella;

c - is the sporogenesis the function of bacilli multiplication?

d – to give an example of fimbriae (pili) second type and to define either they are obligatory structure of a cell.

3. Insert missed word or words in the next statements:

a – a falgella is connected with bacterial body by two disks: external one is included in ………., internal one is included in …………...;

b – there are four stages of sporogenesis process: ………., …………….., ……………., ...;

c – the sporogeneses are function was delivered as saved the species in unfavorable conditions as …………………… for Actinomyces

in Actinomyces spore formation fulfils a function not only saving species in unfavorable conditions but also …………….. .

IV. The answers to the selfassessments.

1. A, b, d, f. 2. A – flagella; b – monotrichates, amphitrichates, lophotrichates, Peritrichates; c – no; d – sex-pili, no. 3. A – cell wall, cytoplasmic membrane; b – preparatory stage, forespore stage, stage of cell wall formation, maturation stage; 3 – reproduction.

Students must know:

1. Locomotory organoids of bacteria.

2. Kinds, location and function of spores.
3. Chemical composition of flagella.
4. Mechanism of motility.

Students should be able to:

1 – to stain of spores by Anjesky's method.

2 – to prepare of hanging drop peparation.

3 – to prepare of wet-mount preparation.

Protocol # 1

***Theme*: Microbiological laboratory, its equipment and rules of work. Biological light microscope. Microscopy of ready preparations.**

Questions for the learning (homework).

1. Structure and functions of microbial laboratory.
2. General laboratory safety rules for microbiology.
3. Modern methods of microscopic examination: light, luminescent, phase-contrast, electron microscopy.
4. Methods of laboratory diagnostics of the diseases.

Inderpendent work.

1. Learn microscopy, using the immersion system of a light microscope.
2. Master the microscopic methods of microorganism's study for of infectious diseases (a microscopic method).
3. Master the technology of immersion microscopy system by examining in microscope the preparations from bacterial culture.
4. Study the morphology of microorganisms in ready glass – sliders used in immersion objective.
5. Draw the studied microorganisms in protocol.

1. Study the immersion microscopy and the rules of work.

Mechanical part

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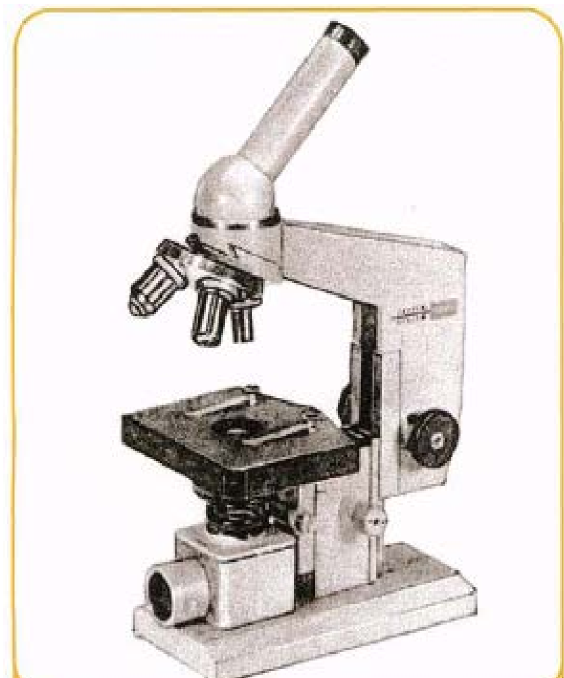

Light microscope

Optical part

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2. Name general increase in immersion microscopy.

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3. Mark max permit able capacity of the immersion microscopy.

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4. Name the types of microscopes and methods of microscopy used in the microbiological investigations.

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5. Name methods of diagnostics used in the microbiological (bacteriological) laboratory.

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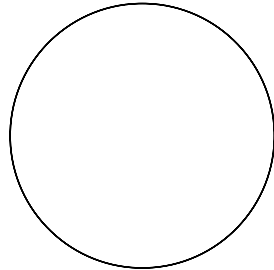
6. For the recognition of the microbes under microscope it is necessary to determine:

1.____

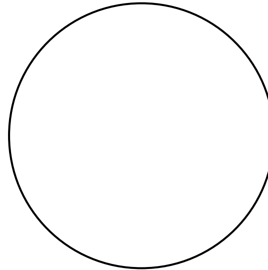
2.____

3.____

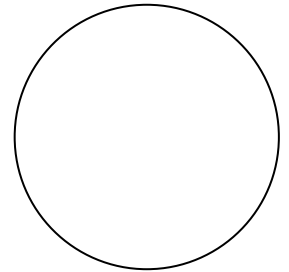
7. Microscopy of ready preparations and draw the studied microorganisms in protocol.



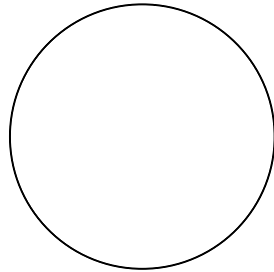
Staphylococci
(Gram's stain)



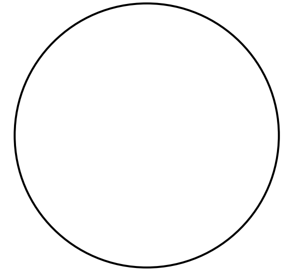
Streptococci
(Gram's stain)



Escherichia coli
(Gram's stain)



Yeast
(simple method of staining with methylene blue)



Bacillus antracoides
(Gram's stain)

Protocol # 2

***Theme:* Morphology of microorganisms. Simple and complex methods of the bacteria staining.**

Questions for the learning (homework).

1. Classification of microorganisms.
2. Technique of smear preparation from specimen of microorganisms, fixation.
3. Tinctorial properties of microorganisms. Simple and complex methods of staining the bacteria, purpose of staining.
4. Gram stain procedure. The structure of gram-positive and gram-negative microorganisms and characteristic of their cell wall.
5. Ziehl –Nielsen stain procedure for acid-fast bacteria (Mycobacterium tuberculosis).

Inderpendent work.

1. Study the main forms of bacteria and draw in protocol.
2. Master the technique of preparing the glass – slides from bacterial culture of Staphylococcus aureus and Escherichia coli.
3. Master the technology of staining ready preparations according to Gram.
4. Master the technology of staining ready preparations according to Ziehl - Nielsen.
5. Draw the studied microorganisms in protocol.

Stages of making a smear preparation.

1.____
2.____
3.____
4.____

Characteristic of the cell wall

Criteria	Gram-positive bacteria	Gram-negative bacteria

Name Gram-positive bacteria

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Name Gram-negative bacteria

____
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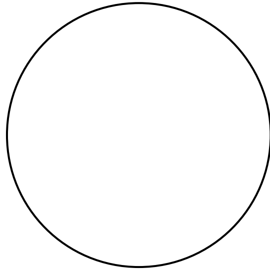
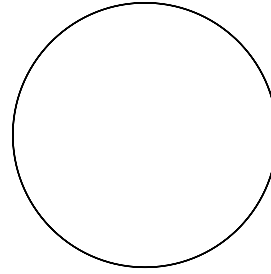
Differential-diagnostics methods of staining.

Gram's Stain			**Ziehl – Nielsen Stain**		
Stage	Ingredients	Exposition	Stage	Ingredients	Exposition
1			1		
2			2		
3			3		
4			4		
5			5		
6			6		
7			7		
8			8		



Staphylococci and Escherichia coli
(Gram's Stain)



Mycobacterium tuberculosis in sputum
(Ziehl – Nielsen Stain)

Protocol # 3

Theme: Structure of the bacterial cell. Complex methods of bacteria staining.

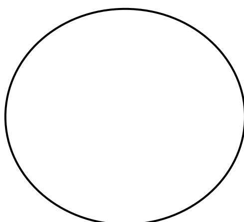
Questions for the learning.

1. Bacterial ultrastructure. Chemical structure of bacteria.
2. Spores of bacilli and clostridia, their role. Process of sporulation, spore localization in bacterial cell. Spore-forming pathogenic bacteria. Methods of detection and staining of spores by Anjesky.
3. Capsule of bacteria, its biological role, constitution of encapsulated bacteria. Examples of bacteria making capsules in human organism. A technique of staining by Burri-Gins.
4. Bacterial flagella. Methods of analysis of microorganisms in living condition ("Handing" and "crushed" drop).
5. Volutin granules (cytoplasm inclusions), its biological role. Staining of volutin granules by Neissers's method.

Inderpendent work.

1. Prepare smear from culture of Bacillus anthracoides and stain according to Anjesko method for reviling spores. Microscopy and draw in protocol.

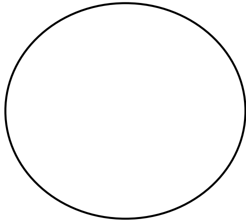
Structural element _____________________



Bacillus antracoides stained according to ________________ method

2. Prepare smear from culture of Asotobacter and stain according to Burri - Gins method for reviling capsules. Microscopy and draw in protocol.

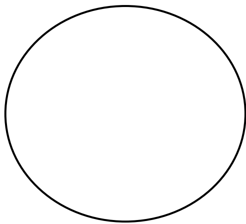
Structural element ______________________



Asotobacter stained according to ________________ method)

3. Microscopy a ready smear from culture of Corynebacterium diphtheriae (stained according to Neisser's method) for reviling volutine granules and draw in protocol.

Structural element ______________________



Corynebacterium diphtheriae stained according to ________________ method)

4. Name the basic structural elements of bacterial cell.

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5. Name the special structural elements of bacterial cell.

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6. Name the main methods of studying motility in microbes.

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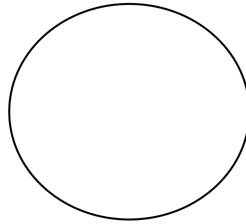
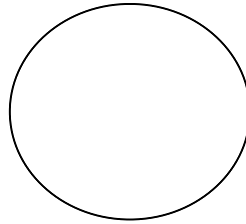
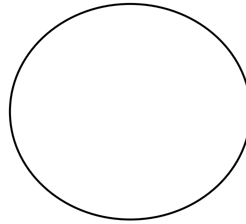
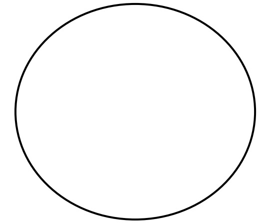
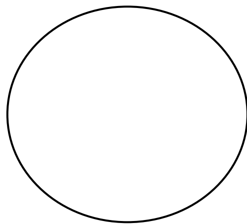
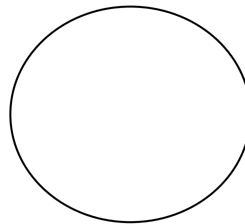
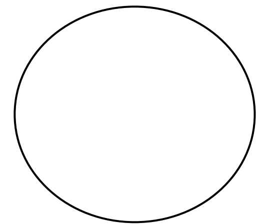
7. Motile microbes are divided into 4 groups according position of flagella. Draw:

Monotrichous Lophotrichous Amphitrichous Peritrichous

8. Draw the types of positions of spores in body of bacilli.

Central position Subterminal position Terminal position

Protocol # 4

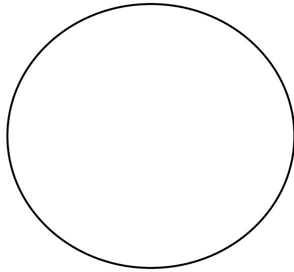
***Theme*: Morphology of Spirochetes, Rickettsia, Fungi and Protozoa.**

***Questions for the learning*.**

1. Spirochete, features of structure, function, classification (treponema, borrelia, leptospira), diseases, staining spirochetes by Romanowsky-Giemsa.
2. Position of rickketsiae in systematization of microorganisms. Morphology, structure, biological features, classification of rickketsiae, diseases. Staining of rickketsiae by Zdrodovsky.
3. Fungi: classification, structure, reproduction. The characteristic of penicillium, aspergillus and mucor. What are the methods of learning morphology and fungi structure?
4. Morphology and structure of yeast like fungus Candida-Method of staining.
5. Protozoa: morphology, structure, classification. Method of staining by Romanowsky-Giemsa.
6. Morphology of leichmania, trypanosome, entamoeba histolytica, toxoplasma, plasmodium malariae.

Inderpendent work.

1. Stain a ready smear from rickettsia according to Zdrodovsky method. Microscopy and draw in protocol.



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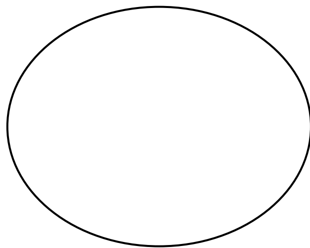
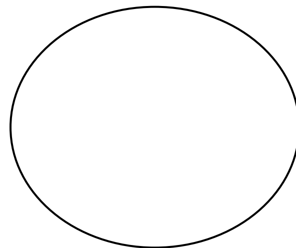
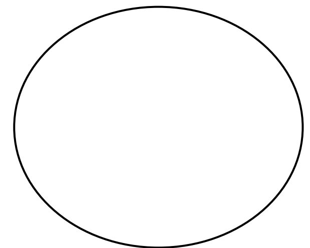
2. There are 3 types of pathogenic spirochetes. Name and draw them.







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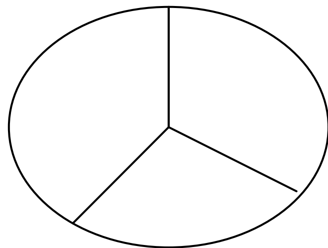
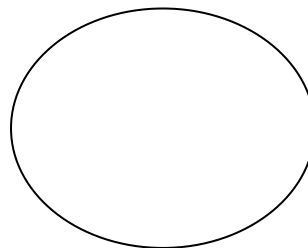
3. Name the methods of studying spirochetes in clinical material.

Treponema pallidum



1. ____________________
2. ____________________
3. ____________________

Leptospira



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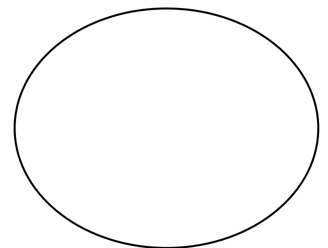
Borrelia



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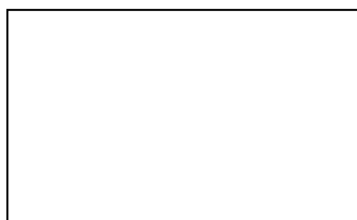
4. Draw rickettsia in the cell. Name the method of staining



Method of staining ____________________

5. Draw the filamentous fungi.

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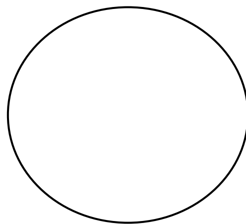
6. Prepare a smear of a yeast-like fungi and stain with simple method of staining, perform its microscopy. Draw the stained smear in protocol.

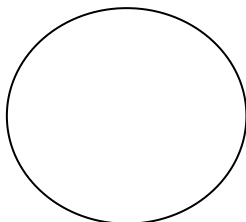


Method of staining ____________________

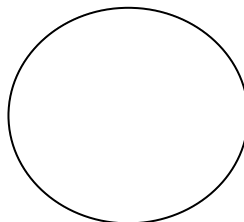
Yeast-like fungi Candida

6. Draw protozoa in protocol.

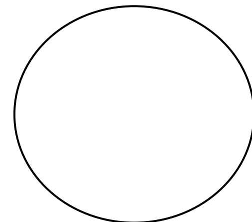
Method of staining ____________________________



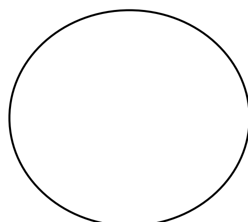
Entamoeba histolytica



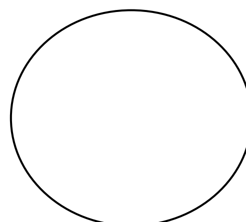
Toxoplasma gondii



Trypanosoma cruzi



Leishmania donovani



Plasmodium malariae.

QUIZZES

1. A bacteriologist has revealed the big bacilli of blue colour with terminally situated spore of red coloring in a slider prepared from wound discharge. What method of colorings did the bacteriologist use?

A.Gram

B.Neisser

C.Burry

*D.Anjesko

E.Ziel - Neelsen

2. The slider from the patient's sputum is the coloured according to Gram. On microscopy the red bacilli and violet cocci were revealed. Why is the coloring of microorganisms different?

A.The proportion of DNA and RNA

B.The peculiarities of nucleus substance

C.Isoelectric point

*D.The presents of magnasian salt of RNA

E.Flagellae

3. The patient sputum was presented for confirming the diagnosis of tuberculosis to bacteriological laboratory. The coloring of slider was carried out by complex differential - diagnostic method to reveal the Mycobacteria of tuberculosis. What method of coloring was used?

A.Gram

B.Neisser

C.Simple

D.Anjehko

*E.Ziehl – Neelsen

4. During microscopy of slider prepared from the discharge of tonsils of a patient with lacunal quinsy and coloured according to Neisser the yellow bacilli situated in V form and with blue bipolar - situated including were revealed. What element of the bacteriological cell did a doctor reveal?

A.Flagella

B.Capsule

C.Cytoplasm

D.Mesosomes

*E. Grains of volutine

5. Gram-positive cocci situated as grape's bunch were revealed in the slider prepared from pus. How can such situation of microorganisms be explained?

A. The localization of purulent process
B. The technique of a slider preparation
C. The technique of coloring
D. The peculiarities of a bacterial cell's division
*E. The effect on bacteria dye

6. The white spots were revealed on the mucous membrane of cheeks of the mouth cavity of eight - month-old child. The big blue cells of oval form were revealed in the slider from discharge. What are these microorganisms?

*A. Candida
B. Staphylococci
C. Streptococci
D. Spirochetes
E. Actinomycetes

7. A patient with fever lasting two weeks was admitted to infectious clinic. The spiral microorganisms with acute ends of blue - violet colour were revealed in the blood slider coloured according to Romanovskiy-Gimza. What microorganisms caused this infection?

A. Candida
B. Staphylococci
C. Streptococci
*D. Spirochetes
E. Actinomycetes

8. A bacteriologist used the standard set of ingredients in coloring according to Gram. Which of the dyes is used on the first stage of staining to this method?

*A.Crystal violet
B.Iodine solution
C.Alcohol
D.Fuchsin
E.Methylen blue

9. The intracellular parasites of cocci form were revealed in the sliders coloured according to Zdradovsky. What group of microorganisms is an agent referred?

A.Cocci
B. Mycoplasma
C.Spirochetes
D.Bacilli
*E. Rickettsiae

10. For the identification of the microorganisms it is necessary to determine their mobility. What method can be used for it?

A. Gram
B. Neisser
*C. Occurring during their lifetime color
D. The method of luminescent microscopy
E. The isolation of pure culture

11. Many bacteria on the surface of cell wall form a protective layer badly coloured with dyes. What is the reason of bad coloring of this layer?

A. Stability to an acid
B. Sensitivity to enzymic action
C. Sensitivity to alcohol
*D. High content of lipids and waxes
E. Chemical composition of cell wall

12. The coloured smears are studied under the microscope with the help of objective which is called:

A. Dark field
B. Phase contrast
*C. Immersion
D. Electronic
E. Dry

13. Colorless formations of right form with red coloured body of microbic cell were revealed in the sputum of the patient with pneumonia in dark field. It may be a colorless capsule. What method was the preparation coloured with?

A.Burry
B.Neisser
*C.Burry-Ginse
D.Anjesko
E.Ziehl - Neelsen

14. Under unfavorable conditions of environment, the microorganisms, for the preserving their type form the special structures. To reveal them, a bacteriologist used the method of coloring according to Anjesko. For revealing of what structural elements is it used?

A. Capsules
*B.Spores
C.Flagellae
D.Grains of valutin

E.Nucleus

15. The spirochetes with 8-14 equal eddies performing different forms of movements were revealed in the smears while studying the ulcer content. In what way was the agent discovered?

A.Colouring according to Gram
B.Silvering according to Morosov
C.Colouring according to Neisser
D.Colouring according to Romanowsky-Giemsa
*E.Hanging drop technique

16. Bacteria whose flagella are located on the whole surface of a cell are called:

A.Monotricheal
B.Lophotricheal
C.Amphitricheal
D.Polytricheal
*E.Peritricheal

17. The physical method of fixation of a glass slider is included in?

*A. Moving the smear in the gas
B.Immersion of the smear into formalin
C.Drying the smear in the air
D.Immersion of the smear into alcohol
E.Treatment of the smear with 5 % sulfuric acid

18. There is a method of coloring which gives the possibility to paint in different colour the spirochetes of the genus Borrelia, Leptospira, Treponema. How do we call it?

A.Colouring according to Gram
B.Silvering according to Morosov
C.Colouring according to Neisser
*D.Colouring according to Romanowsky-Giemsa
E.Colouring according to Burry

19. One of the complex methods is used for coloring the acid-fast bacteria. When colored they become red under the influence of the main due. How do we call this method?

A.Colouring according to Gram
B.Silvering according to Morosov
C.Coloring according to Neisser
D.Coloring according to Romanowsky-Giemsa’s

*E.Colouring according to Ziehl-Neelsen

20. The microorganisms which are painted according to Gram in violet color are called:

*A. Gram-positive
B.Gramnegative
C.Acid-fast
D.Cocci
E.Fungi

21. Acid fast microbes are resistant to acid because they contain in cell wall:

A. Lipopolysaccharides
*B. Fatty waxes, fatty acid
C. Acetylglucosamine
D. Diaminopimelic acid
E. Polyphosphates

22. Cell wall of gram-negative bacteria contain all, except:

A. Lain monolayer peptidoglycan
B. Lipoproteins
*C. Polilayer peptidoglycan
D. Lipopolysaccharide
E. Outer membrane

Recommended reading list

Main literature

1. Ananthanarayan R. Textbook of Microbiology [Текст] / R. Ananthanarayana, Jayaram CK. Paniker ; ed. by.: A. Kapil. - 9th ed. - India : Universities Press (Verlag), 2015. - 710 p.
2. Gaidash I. Microbiology, Virology and Immunology. Vol. 1 / I. Gaidash, V. Flegontova; Ed. N. K. Kasimirko. - Lugansk : S. N., 2004. - 213 p.
3. Gaidash I. Microbiology, Virology and Immunology. Vol. 2 / I. Gaidash, V. Flegontova; Ed. N. K. Kasimirko. - Lugansk : S.N., 2004. - 226 p.
4. Jawetz, Melnik & Adelberg's Medical Microbiology [Текст] : учебное пособие. - 22 Edition. - New York : Lange Medical Books/McGraw-Hill, 2001. - 695 p.
5. Medical Microbiology : textbook / D. Greenwood [et al.]. - 17th ed. - Toronto : Churchill Livingstone, 2007. - 738 p.

Further Reading

1. Talaro K. Faundations in microbiology. Basic principles. - Talaro K., Talaro A. - Pasadena, 2005, by TMHE group.
2. Microbiology. A human perspective / M. T. Nester, E. V. Nester, C. E.Roberts. - 1995.
3. Levenson W. E. Medical microbiology and immunology / W. E. Levenson, E. Javetz. – Norwalk, 1994,
4. Krivoshein Yu.S.Handbook on microbiology / Yu. S. Krivoshein– Moscow : Mir Publishers,.1989

Informational resourses:

1. http://commons.wikimedia.org/wiki/Category:Medical_illustrations_by_Patrick_Lynch
2. http://www.yteach.co.uk/index.php/search/results/AQA_GCSE_Science_A_(4461)_Biology,3,0,7033;7230,0,25,1,wa,1.html

3. American Society for Microbiology — http:// asm.org.;
4. http://journals.asm.org; (American Society for Microbiology) — http:// asm.org.;
5. http://www.news-medical.net/health/Virus-Microbiology-(Russian).aspx;
6. http://www.rusmedserv.com/microbiology; http://www. rusmedserv.com/
7. http://rji.ru/immweb.htm; http://www.rji.ru/ruimmr;
8. http://www. infections.ru/rus/all/mvb journals.shtml;
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