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МІНІСТЕРСТВО ОХОРОНИ ЗДОРОВ'Я УКРАЇНИ

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для англомовних студентів
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Special microbiology

LABORATORY DIAGNOSIS OF STAPHYLOCOCCAL INFECTIONS

Theme topicality. Staphylococcal infections are widely spread in many industries of medicine. Staphylococci belong to the potential pathogenic bacteria, representatives of normal microflora of human. They are pantropic bacteria and can cause the damage to all the organs and systems.

Staphylococci are most frequently isolated from clinical specimens in the microbiological laboratory. These bacteria are widespread in nature and can be obtained from the environment or as common inhabitants of the skin, mucous membranes and other body parts in humans and animals. *Staphylococcus aureus* causes pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, osteomyelitis, endocarditis and superficial skin lesions such as furunculosis. *S. aureus* is a major cause of hospital (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* also causes food poisoning by releasing enterotoxins into the food, and toxic shock syndrome. Coagulase negative staphylococci are normally abundant colonizers of humans and become pathogenic only under certain conditions. They are commonly isolated in clinical specimens and several species are recognized as important agents of nosocomial infections, especially in neonates. The most severe disease caused by *Staphylococcus spp*. is sepsis. The doctor of any specialization can run into this causative agent. It is therefore necessary to know the origin of staphylococcal infections and methods of diagnostics.

Primary objective: to be able to conduct and evaluate the microbiological diagnostics of staphylococcal infections.

QUESTIONS FOR DISCUSSION

1. Biological properties of *Staphylococcus spp*.
2. Staphylococcal infections (furuncles, carbuncles, sinusitis, ostitis, postinfluenza pneumonia, sepsis, etc.), microbiological diagnosis.
3. Epidemiology and pathogenesis of the diseases caused by *Staphylococcus spp*. Specific features of immunity in such cases.
4. Basic measures of prophylaxis and treatment of staphylococcal infections.

PROCEDURE OF PRACTICAL WORK

Task 1. Study the preparation of the pure cultures of *Staphylococcus areus* and *S. epidermidis* microscopically; draw them in the protocol.

All types of *Staphylococcus spp.* have identical staining and morphological properties. *S. aureus* are gram-positive cocci like “bunches of grapes”.

Task 2. Prepare and stain by Gram's method the preparation taken from the blood of the patient suspected of staphylococcal sepsis, draw it in the protocol.

Direct microscopy of the material taken from the patient with staphylococcal infection is not used. For example, microscopy of the mucus from the nose shows that staphylococci do not have classic morphology. However, in case of sepsis, microscopy of the blood makes it possible to reveal (1) *Staphylococcus spp.* in the shape of "bunches of grapes" (bacterium propagates itself in the blood) and (2) erythrocytes.

Task 3. Study the growth of *S. aureus* and *S. epidermidis* in milk nutrient agar. Fill in the protocol with their cultural properties.

Milk nutrient agar is a special medium capable to expose the ability of bacteria to form a pigment. Therefore, *S. aureus* forms yellow colonies in this medium. That is why they are called golden staphylococci. Other types of staphylococci do not have such a pigment.

Task 4. Study the growth of *S. aureus* and *S. epidermidis* in blood agar. Fill in the protocol with their cultural properties.

Blood agar is a special medium with the ability of bacteria to select haemolysin coming to light on.

In blood agar abundant growth of the most staphylococcal species occurs within 18–24 hours. Only individual colonies should be picked for preliminary identification testing at this time.

Since most species can not be distiguished from each other in the basis of the colony morphology within a 24-hour incubation period, colonies should be allowed to grow for at least additional 2–3 days before the primary isolation plate is confirmed for species or strain composition.

Task 5. Study the growth of *S. aureus* and *S. saprophyticus* in egg yolk salt agar. Fill in the protocol with their cultural properties.

For revealing lecitinase activity of *Staphylococcus spp*., cultures of bacteria grow on the special selective medium. This medium is yolk salt agar containing 8–10% of NaCl. *Staphylococcus spp*. tolerates to sodium chloride in concentrations of 5–10%. Salt-containing media are useful in isolating staphylococci from samples containing large numbers of other bacteria. The bacteria allocating lecitinase destroy yolk lecithin. Therefore round colonies with the turbidity zone (as enzyme diffusion on agar) are formed. Such bacterium in this case is *S. aureus*. Other kinds of staphylococci, such as *S. epidermidis, S. saprophyticus,* do not allocate such enzyme, therefore round colonies do not form lecitovitelase zone.

Task 6. Study the growth of other types of *Staphylococcus spp.* in citrate plasma. Draw the plasma in the test tubes before streaking of *Staphylococcus spp.* and after 24-hour growth.

It is known that plasmacoagulase concerns enzymes which promote invasive microbes on a macroorganism and their preservation in it. Plasmocoagulase leads to formation of the inflammatory centre round microbes. It leads to plasma coagulation that interferes those phagositosis and to action complement.

Coagulase is an enzyme of pathogenicity of bacteria, which rolls up plasma of blood, facilitates penetration of bacteria in tissues, use the reaction of plasmacoagulase. Plasmacoagulase is an enzyme that functions like thrombin to convert fibrinogen into fibrin. The ability to clot plasma continues to be the most widely used and generally accepted criterion for the identification of pathogenic staphylococci associated with acute infections.

Task 7. Study the sensitivity of the isolated staphylococcal culture to antibiotics; draw a conclusion. Write the results in the protocol.

Measure a zone of growth absence round a paper disk, study the sensitivity of isolated staphylococcal culture to antibiotic, and make the conclusion. Put down the results in the protocol.

Task 9. Study the main antimicrobial drugs used for treatment, prevention and diagnostics of suppurative diseases. Write them in your copybook.

Staphylococcal toxoid is a vaccine containing inactivated toxin. It is obtained by mix of the exotoxin *S. aureus* and formalin (0.4%) at temperature 40 °C with exposition 3–4 weeks. It is used specifically to prevent and treat staphylococcal infections.

Laboratory diagnosis of staphylococcal infections

Notion	Definition/explanation
Staphylococcus: classification and biological properties	The term *Staphylococcus* is derived from the Greek term *staphyl*, meaning "a bunch of grapes". This name refers to the fact that the cells of these gram-positive cocci grow in a pattern resembling a cluster of grapes; however, organisms in clinical material may also appear as single cells, pairs, or short chains. Most staphylococci are 0.5 to 1 μm in diameter and are nonmotile, aerobic or facultative anaerobic, and catalase-positive and grow in a medium containing 10% sodium chloride and at temperature ranging from 18 ºC to 40 ºC. The organisms are present on the skin and mucous membranes of humans and other mammals, and birds. *Staphylococcus* is an important pathogen in humans, causing a wide spectrum of life-threatening systemic diseases; infections of the skin, soft tissues, bones, and urinary tract; and opportunistic infections. The species most commonly associated with human diseases are *S. aureus* (the most virulent and best-known member of the genus), *S. epidermidis*, *S. saprophyticus*, and *S. haemolyticus*. *S. aureus* colonies are golden as the result of the carotenoid pigments that are formed during their growth, hence the species are called so. It is also the only species found in humans that produces enzyme coagulase; thus, all other species are commonly referred to as coagulase-negative staphylococci
Staphylococcus aureus	*S. aureus* is ubiquitous, existing everywhere in nature. It constitutes part of the normal flora of the skin, nose, throat, gastrointestinal tract, and genital tract of 25–50% of humans and animals. *S. aureus* is among the most resistant of the nonspore formers to adverse enviromental conditions and physical and chemical agents. The organism can survive for as long as 14 weeks in dried pus and is killed by 70% ethanol only after a 10-minutes contact period. Staphylococci produce diseases because of their ability to spread in tissues and form abscesses, produce extracellular enzymes or exotoxins and combat host defences
Cell wall virulence factor of *Staphylococcus aureus*	1. Capsule. A loose-fitting, polysaccharide layer (slime layer) protects bacteria by inhibiting the chemotaxix and phagocytosis. It also facilitates the adherence of bacteria to catheters and other synthetic material. 2. Teichoic acids are bound convalently to the peptidoglycan. It mediates the attachment of staphylococci to mucosal surfaces through their specific binding to fibronectin. 3. Protein A is a cell wall component also covalently linked to peptidoglycan and with the ability to bind to the Fc portion of Ig G and extracellular matrix glycoprotein. Protein A may contribute to adherence and possess antiphagocytic activity
Toxins	1. α, ß, δ, and γ toxins (produced by most strains of *S. aureus*) attack mammalian cell (including red blood cell) membranes, and are often referred to as haemolysins. α toxin exhibits dermonecrotic activity that contributes to tissue necrosis. 2. Toxic shock syndrome toxin-1 (TSST-1) is a protein produced by virtually all strains of *S. aureus* and responsible for the clinical manifestations of toxic shock syndrome. 3. Enterotoxin A, B, and D molecules are heat-stable proteins capable of withstanding boiling for 30 min and produced by 30% to 50% of all *S. aureus* strains. Synthesis is plasmid or chromosomally mediated. Enterotoxin A and D are responsible for staphylococcal food poisoning by inhibiting water

Table 3.8.1 continuation

Notion	Definition/explanation
	absorption from the intestinal lumen and inducing diarrhoea. Enterotoxin B damages the intestinal epithelium and produces colitis. 4. Exfolitative (epidermolytic) exotoxin is produced by some (5% to 10%) strains of *S. aureus*. Synthesis is plasmid or chromosomally mediated. By causing intraepidermal splitting of tissues and necrosis, it is responsible for the clinical manifestations seen in scalded skin syndrome (SSS)
Enzymes	1. Lipases are lipid-hydrolyzing enzymes, which allow the organisms to invade cutaneous and subcutaneous tissues by splitting fats and oils accumulating on the skin. All strains of *S. aureus* and more than 30% of the strains of coagulase-negative staphylococci produce several different lipases. 2. Leucocidin is an exotoxin that contributes to the survival of the organism, it destroys polymorphonuclear leukocytes. 3. Coagulase is an enzyme produced by the organism during its growth. The role of coagulase in the pathogenesis is speculative, but coagulase may cause the formation of fibrin layer around a staphylococcal abscess, thus localizing the infection and protecting the organisms from phagocytosis. 4. Hyaluronidase is produced by over 90% of *S. aureus* strains. It is an enzyme that hydrolyzes the hyaluronic acid constituent of connective tissue ground substances and thus facilitates the spread of the organism through the tissues. 5. Staphylokinase (fibrinolysin) is produced by virtually all *S. aureus* strains. It dissolves fibrin clots and thus contributes to the spread of the organism from local sites. 6. Nuclease is another enzyme for *S. aureus*. The role of this enzyme in the pathogenesis of infection is unknown. 7. Penicillinase. More than 90% of staphylococcal isolates were susceptible to penicillin in 1941, the year the antibiotic was first used clinically. Resistance to penicillin quickly developed, however, primarily because the organisms could produce penicillinase (ß-lactamase). 8. Catalase. All staphylococci produce catalase, which catalyzes the conversion of toxic hydrogen peroxide to water and oxygen. Hydrogen peroxide can accumulate during bacterial metabolism or after phagocytosis
Superantigens: enterotoxins and toxic shock syndrome toxin	*S. aureus* can express two different types of toxin with superantigen activity, enterotoxins (six serotypes A, B, C, D, E, G) and toxic shock syndrome toxin (TSST-1). Enterotoxins cause diarrhoea and vomiting when ingested and can causes staphylococcal food poisoning. When expressed systemically, enterotoxins can cause toxic shock syndrome (TSS) – indeed enterotoxins B and C cause 50% of non-menstrual TSS. TSST-1 is very weakly related to enterotoxins and does not have emetic activity. TSST-1 is responsible for 75% of TSS, including all menstrual cases. TSS can occur as a sequel to any staphylococcal infection if an enterotoxin or TSST-1 is released systemically and the host lacks appropriate neutralizing antibodies. Tampon-associated TSS is not a true infection, being caused by growth of *S. aureus* in a tampon and absorption of the toxin into the blood stream. TSS came to prominence with the introduction of superabsorbent tampons; and although the number of such cases has decreased dramatically, they still occur despite withdrawal of certain types of tampons from the market. Superantigens stimulate T cells nonspecifically without normal antigenic

	recognition. Up to one in five T cells may be activated, whereas only

Table 3.8.1 continuation

Notion	Definition/explanation
	1 in 10,000 are stimulated during antigen presentation. Cytokines are released in large amounts, causing the symptoms of TSS. Superantigens bind directly to class II major histocompatibility complexes of antigen-presenting cells outside the conventional antigen-binding grove. This complex recognizes only the Vb element of the T cell receptor. Thus, any T cell with the appropriate Vb element can be stimulated, whereas normally antigen specificity is also required in binding
Diseases of *S. aureus*	1. Localized skin infections include impetigo, folliculitis, furuncles, and carbuncles. Impetigo, a superficial infection affecting mostly young children, occurs primarily on the face and limbs. Initially, a small macule (flattened red spot) is seen, and then a pus-filled vesicle (pustule) on an erythematous base develops. Folliculitis is a pyogenic infection in the hair follicles. The base of the follicle is raised and reddened, and there is a small collection of pus beneath the epidermal surface. If this occurs at the base of the eyelid, it is called a stye. Furuncles (boils), an externsion of folliculitis, large, painful, raised nodules with an underlying collection of dead and necrotic tissue. Carbuncles occur when furuncles coalesce and extend to the deeper subcutaneous tissue. Multiple sinus tracts are usually present. 2. Scaled skin syndrome is a disease that occurs in infants and children 4 years of age or under. In this syndrome, the organisms release exfolitative toxin, which is resposible for the extensive intraepidermal splitting and bullous necrosis of the tissue. 3. Toxic shock syndrome is a disease that was initially evident in children, although it is now recognized as primarily a disease in menstruating women. At present, 80% to 90% of patients with TSS are menstruating women. Highly absorbent tampons contribute to the initiation of the disease by providing a favourable environment for the growth of resident *S. aureus*. The disease is initiated with the localized growth of toxin-producing strains of *S. aureus* in the vagina or a wound, followed by release of the toxin into the blood stream. Clinical manifestations start abruptly and include fever, hypotension, and diffuse macular erythematous rash 4. Food poisoning is one of the most common food poisoning in the world. The organisms are usually introduced into food, such as processed meat, pastries, potato, salad, and ice cream. The contaminated food is kept at room temperature, during that time the organisms multiply and release heat stable enterotoxin A or D. The following ingestion of the food, the onset of disease is abrupt and rapid with an incubation period of only 1–6 hours. Staphylococcal food poisoning is characterized by severe vomiting, diarrhoea, and abdominal pain. The absence of fever is an important observation in the differential diagnosis of staphylococcal food poisoning. 5. Colitis is a disease that is observed in patients whose normal bowel is altered by the oral administration of broad-spectrum antibiotics that selectively permit overgrowth by antibiotic resistant, enterotoxin B producing strains of *S. aureus*. Enterotoxin B damages the intestinal epithelium and produces fever, diarrhoea, and abdominal cramps. 6. Pneumonia is a disease that occurs among immunosuppressed patients, the aged, children under one year of age, and frequently in children with measles

	and influenza. 7. Other diseases like osteomyelitis, septicemia, and septic arthritis

Table 3.8.1 continuation

Notion	**Definition/explanation**
Principle of the laboratory diagnostics	Specimens obtained depend on the disease process and include lesion material, pus, sputum, blood, spinal fluid, and faeces. İsolation and identification of *S. aureus* requires initial cultivation on blood agar and/or specific medium and overnight incubation under aerobic conditions at 37 ºC. The organism may be identified as a gram-positive, catalase-positive coccus exhibiting coagulase
Resistance of staphylococci to antimicrobial drugs	Hospital strains of *S. aureus* are often resistant to many different antibiotics. Indeed, strains, resistant to all clinically useful drugs, apart from the glycopeptides vancomycin and teicoplanin, have been evident. The term MRSA refers to methicillin resistance and most methicillin-resistant strains are multiply resistant, too. Plasmid-associated vancomycin resistance has been detected in some enterococci and the resistance determinant has been transferred from enterococci to *S. aureus* in the laboratory and may occur naturally. *S. epidermidis* nosocomial isolates are also often resistant to several antibiotics including methicillin. In addition, *S. aureus* expresses resistance to antiseptics and disinfectants, such as quaternary ammonium compounds, which may aid its survival in the hospital environment. Since the beginning of the antibiotic therapy *S. aureus* has responded to the introduction of new drugs by rapidly acquiring resistance by a variety of genetic mechanisms including (1) acquisition of extrachromosomal plasmids or additional genetic information in the chromosome via transposons or other types of DNA insertion and (2) by mutations in chromosomal genes. Many plasmid-encoded determinants have recently become inserted into the chromosome at a site associated with the methicillin resistance determinant. There may be an advantage to the organism having resistance determinants in the chromosome because they will be more stable. There are essentially four mechanisms of resistance to antibiotics in bacteria: (1) enzymatic inactivation of the drug, (2) alterations to the drug target to prevent binding, (3) accelerated drug efflux to prevent toxic concentrations accumulating in the cell, and (4) a by-pass mechanism, whereby an alternative drug-resistant version of the target is expressed
Treatment	The antibiotics of choice are oxacillin (or other penicillinase-resistant penicillin) or vancomycin for oxacillin-resistant strains. The focus of infection (e.g., abscess) must be identified and drained. Treatment is symptomatic for patients with food poisoning
Coagulase-negative staphylococci	1. *S. epidermidis* is present in large numbers as a part of normal flora of the skin. It is frequently isolated from blood cultures. Despite its low virulence, it can cause infection of implants such as heart vulves and catheters. Cell envelope factors that facilitate attachment to plastic surfaces act as virulent factors. Acquired drug resistance by *S. epidermidis* is even more frequent than by *S. aureus*. 2. *S. saprophyticus* is a frequent cause of cystitis in women, probably related to it in occurrence as part of normal vaginal flora. It tends to be sensitive to most antibiotics, even penicillin G. *S. saprophyticus* can be distinguished from *S. epidermidis* and most other coagulase-negative staphylococci by its natural resistance to novobiocin

Laboratory test for identification the pure culture of *Staphylococcus spp.*

Species	Frequency of disease	Coagulase	Colour of colonies	Mannitol fermentation	Novobiocin resistanse
S. aureus	Common	+	Golden yellow	+	–
S. epidermidis	Common	–	White	–	–
S. saprophyticus	Occasional	–	Variable	–	+

Antimicrobial resistance

Antimicrobial	Resistance mechanism	Genetic basis
Penicillin	B-lactamase. Enzymatic inactivation of penicillin	Plasmid
Methicillin	Expression of new penicillin-resistant penicillin-binding protein. Bypass	Novel chromosomal locus acquired from unknown source
Tetracycline	Efflux from cell. Modification of ribosome	Plasmid. Novel chromosomal locus acquired from unknown source
Chloramphenocol	Enzymatic inactivation	Plasmid
Erythromycin	Enzymatic modification of ribosomal RNA. Prevents drug binding to ribosome	Plasmid. Transposon.
Streptomycin	Mutation is ribosomal protein. Prevents drug binding. Enzymatic inactivation	Mutation in chromosomal gene encoding drug target. Plasmid
Kanamycin	Enzymatic inactivation	Plasmid
Gentamicin	Enzymatic inactivation	Transposon is chromosome
Trimethoprim	Alternative dihydofolate reductase. Bypass	Plasmid
Mupirocin	Alternative isoleucine tRNA syntheses. Bypass	Plasmid
Fluoroquinolones	Altered DNA gyrase. Efflux	Mutation in chromosomal gene encoding drug target. Mutation increases expression of natural efflux mechanism
Antiseptics	Efflux	Plasmid

Scheme staphylococcal infections (furuncles, carbuncles, sinusitis, ostitis, postinfluenza pneumonia, sepsis) laboratory diagnosis

Mucus from pharynx, urine, vomit masses, etc.

Stage 1

Primary innoculation in the selective medium – egg yolk salt medium. Selective medium containing 8 to 10% NaCl. Staphylococci tolerate to sodium chloride in concentrations of 5–10%. Salt-containing media are useful in isolating staphylococci from samples containing large number of other bacteria

Result

END RESULT

LABORATORY DIAGNOSTICS OF STREPTOCOCCAL INFECTIONS

Theme topicality. Infection with group A streptococci (GAS) can result in a range of symptoms: no illness; mild illness (strep throat or skin infection, such as impetigo); severe illness (necrotizing fasciitis, streptococcal toxic shock syndrome).

Severe, sometimes life-threatening, GAS disease may occur when bacteria get into parts of the body where bacteria are usually not found, such as blood, muscles, or the lungs. These infections are termed “invasive GAS disease”. Two of the most severe, but least common, forms of invasive GAS disease are necrotizing fasciitis and streptococcal toxic shock syndrome. Necrotizing fasciitis destroys muscles, fat, and skin tissue. Streptococcal toxic shock syndrome (STSS), causes blood pressure to drop rapidly and organs (e.g., kidney, liver, lungs) to fail. STSS is not the same as the "toxic shock syndrome" frequently associated with tampon usage. About 20% of patients with necrotizing fasciitis and more than half with STSS die. About 10–15% of patients with other forms of invasive group A streptococcal disease die. Group B strep infection is fatal in about 20% of infected men and non-pregnant women and about 5 to 15% of infected newborns. Babies who survive can be left with speech, hearing, and vision problems as well as mental retardation.

Primary objective: to be able to carry out and evaluate microbiological diagnostics of streptococcal infections.

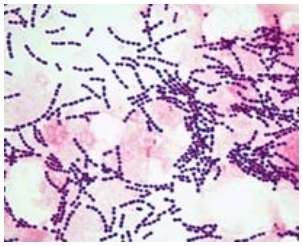
QUESTIONS FOR DISCUSSION

1. Classification of streptococci. Biological properties of *Streptococcus spp*.
2. Streptococcal infections (streptococcal pharyngitis or tonsillitis, scarlet fever, rheumatic fever, glomerulonephritis, sepsis and pneumoniae), microbiological diagnosis.
3. Epidemiology and pathogenesis of the diseases caused by streptococcus. Specific features of immunity in such cases.
4. Basic principles of streptococcal infections; prophylaxis and treatment.

PROCEDURE OF THE PRACTICAL WORK

Task 1. Study the preparation of pure cultures of *Streptococcus pyogenes, Streptococcus spp.* microscopically in the material taken from the patient and in pure culture, draw it in the copy book.

Streptococcus pyogenes in pleural puncture biopsy material is gram-positive cocci in twisted chains (in small number).



S. pyogenes is gram-positive single cocci or pairs rather than definite chains. Streptococci form different length chains.

Many bacteria (e.g., *S. pneumoniae*) contain a gelatinous covering called a capsule. The presence of a capsule indicates the organism's virulence. The capsule is examined by using Burri-Gins method of staining.

In a negative method of living bacteria stained by Burri's method, the bacteria remain unstained in a dark field. In a drop of Indian ink diluted with distilled water 1 to 10 the culture to be tested is inoculated and spread uniformly with a loop or the edge of the glass slide. The smear is air-dried. Nigrosin, Congo red, and other dyes may occasionally be used instead of Indian ink.

In this case, the bacteria are stained red, whereas unstained capsules are distinctly outlined against the dark background of the preparation. Sometimes, small colourless zones can be observed around those stained bacteria that do not form capsules. They are called false capsules and are the result of inadequate drying or fixation of the smear.

S. pneumoniae (pneumococcus) is the causative agent of many types of diseases (e.g., bacterial lobar pneumonia, conjunctivitis, otitis media, meningitis, peritonitis). Thirty to seventy percent of normal individuals harbour this bacterium in their pharynx. *S. pneumoniae* is a gram-positive coccus (the distal ends are lancet-shaped).

Task 2. Study the growth of *S. pyogenes* and *S. pneumoniae* in blood agar.

S. pyogenes and *S. pneumoniae* on blood agar forms the small, semitranslucent gray-white colonies of streptococci surrounded by zones of β-haemolysis. Streptolysins produced by *S. pyogenes* cause the lysis of red blood cells in vitro, producing β-haemolysis (a clear zone of haemolysis with no colour change) on blood agar. Two types of β lysins: streptolysin O and streptolysin S are produced. The former is oxygen-labile, while the latter is oxygen-stable. Streptolysin O is demonstrated only in deep colonies on the blood agar medium. Since most strains of *S. pyogenes* produce both types of lysins, surface of haemolysis is generally observed.

Different streptococci can produce haemolysins:

Colonies with **α-haemolysis** on blood agar are surrounded by green zone. This "greening" is caused by H_2O_2, which converts haemoglobin into methemoglobin.

Colonies with **β-haemolysis** on blood agar are surrounded by large, yellowish haemolytic zone in which no more intact erythrocytes are present and haemoglobin is decomposed.

Colonies with γ-haemolysis indicate the absence of macroscopically visible haemolytic zones.

Task 3. Study the main antimicrobial drugs used for treatment, prevention and diagnostics of suppurative diseases and write them down in the copy book.

O-streptolysin is an enzyme used for diagnosis. The streptolysin O (streptococcal haemolytic exotoxin) is oxygen-labile; the other antigen is oxygenstable streptolysin S. Both enzymes are involved in producing haemolysis, i.e., digestion of blood, particularly β-haemolysis. Antistreptolysin O antibody can be detected in an antistreptolysin O titre (antistreptolysin O titre AS(L) O titre is a measure of specific antistreptolysin O antibodies concentration). Titre testing employs serial dilution to obtain approximate quantitative information from an analytical procedure that inherently only evaluates as positive or negative of (serum) antistreptolysin O antibodies is a blood test used to assist in the diagnosis of streptococcal infection or indicate past exposure to streptococci.

Laboratory diagnosis of streptococcal infections

Notion	Definition/explanation
Streptococcus	The genus *Streptococcus* is a diverse collection of gram-positive cocci typically arranged in pairs or chains. Most species are facultative anaerobes, and some grow only in an atmosphere enhanced with carbon dioxide (capnophilic growth). Their nutritional requirements are complex, necessitating the use of blood or serum-enriched media for isolation. Carbohydrates are fermented, resulting in the production of lactic acid, and unlike *Staphylococcus species*, streptococci are catalase-negative
Group A, B haemolytic streptococci	*Streptococcus pyogenes* is the most virulent member of this group of gram-positive cocci. This bacterium is an important cause of a variety of suppurative and nonsuppurative diseases
Classification of streptococci	1. Haemolitic properties on blood agar (alfa-, betta-, and gamma-haemolitic streptococci). 2. Serological grouping (A and B). 3. Biochemical properties
Structure and physiology	Isolates of *S. pyogenes* are gram-positive, nonmotile cocci in short or long chains, and occasionally singly and in pairs. Freshly isolated strains of group A streotococci are encapsulated, but the capsules are lost rapidly during the stationary phase of in vitro cultivation. Group A, like most streptococci, are less resistant to environmental

	conditions than staphylococci, although they can survive on dry swabs for weeks. They are killed rapidly by physical and chemical agents. Structural features that are involved in the pathology or identification of the group A streptococci include: a) antigenic structure; b) extracellular products
Antigenic structure of group A, ß-streptococci	The outermost layer of the cell is the capsule (K-antigen), which is composed of hyaluronic acid, identical to that found in connective tissue. For this reason, the capsule is nonimmunogenic (in contrast with *S. pneumoniae*). C carbohydrates are cell wall polysaccharides whose antigenic diversity forms the basis for the classification of streptococci into 20 serogroups lettered from A to V. Lipoteichoic acid (LTA) exposed on the cell surface defines the ability of group A streptococci to bind to epithelial cells in the mouth and on the skin. M protein is a major antigen associated with virulent streptococci. In the abscence of M protein, the strains are not infectious. The M protein also prevents interaction with complement. Protein F (fibronectin-binding protein) mediates attachment to fibronectin in the pharyngeal epithelium
Extracellular products of	Streptolysin S is nonimmunogenic cell-bound haemolysin capable of lysing erythrocytes, as well as leukocytes and platelets, following direct cell

Table 3.9.1 continuation

Notion	**Definition/explanation**
group A, ß-streptococci	contact. Streptolysin O is inactivated reversibly by oxygen. Unlike streptolysin S, antibodies are readily formed against streptolysin O and are useful for documenting recent infection (ASO test). In addition to its ability to lyse human erythrocytes, streptolysin O is also capable of killing leukocytes by lyses of their cytoplasmic granules with release of hydrolytic enzymes. Pyrogenic (erythrogenic) exotoxins are proteins responsible for the rash of scarlet fever. There are three antigenically distinct types designated A, B, and C, which are produced by more than 95% of group A streptococcal strains. DNAse is used for identifying immunologically distinct deoxyribonucleases (A through D). These enzymes are not cytolytic but are capable of depolymerizing free DNA present in pus. This reduces the viscosity of the abscess material and facilitates spread of the organisms. Other enzymes such as hyaluronidase and streptokinase (fibrinolysin)
Diseases caused by streptococci group A (*Streptococcus pyogenes*)	Pharyngitis. Group A streptococcus is the major cause of bacterial pharyngitis, occasionally involved with group C and G. This is primarily a disease of children of 5 to 15 years old, but infants and adults are also susceptible. The pathogen is spread by person-to-person contact via respiratory droplets. Pharyngitis generally develops within 2 to 4 days after exposure to the pathogen, with an abrupt onset of sore throat, fever, malaise, and headache. The posterior pharynx can appear erythematous with an exudate, and cervical lymphadenopathy can be prominent. Scarlet fever is a complication of streptococcal pharyngitis seen when the infecting strain is lysogenized by a temperate bacteriophage that stimulates production of erythrogenic toxin. Within 1 to 2 days after initial clinical symptoms of pharyngitis, a diffuse erythematous rash will initially appear on the upper chest and then spread to the extremities. The area around the mouth is generally spared, as the palms and soles are. The tongue will initially be covered

	with a yellowish-white coating that will later be shed revealing a red, raw surface (“strawberry tongue”). Erysipelas. This disease can affect all age groups. İt is a disease of the skin and subcutaneous tissues usually occurring on the face or lower extremities and characterized by a fiery red, advancing erythema. Puerperal sepsis. This infection is initiated during or following soon after the delivery of a newborn. It can occur due to exogenous transmission (for example, by nasal droplets from an infected carrier, or from contaminated instruments), or endogenously, from the patient’s vaginal flora. It is a disease of the uterine endometrium in which patients suffer from a purulent vaginal discharge. Acute haemorrhagic glomerulonephritis occurs most commonly in children and can result when preceded by pharyngitis or skin disease caused by

Table 3.9.1 continuation

Notion	**Definition/explanation**
	goup A streptococci. This disease can occur in less than 1 to 15% of untreated patients from 1 to 5 weeks after pharyngeal or skin disease onset. Evidence supports the concept that renal damage is the result of immune complex diposition on the glomerular basement membrane. The major clinical manifestations are renal glomerular damage, hypertension, oedema, proteinuria, and haematuria. Other diseases are impetigo, cellulitis, lymphangitis
Antistreptolysin O titre	Immunoserologic test. Both the antistreptolysin O (ASO) and anti-DNAse B assays are useful in diagnosis. Antistreptolysin O titre (AS(L)O titre or AS(L)OT) – titre of (serum) antistreptolysin O antibodies is a blood test used to assist in the diagnosis of a streptococcal infection or indicate a past exposure to streptococci. The ASOT helps direct the antimicrobial treatment and is used to assist in the diagnosis of scarlet fever, rheumatic fever and post infectious glomerulonephritis
Treatment	Adequate drainage, debridement, and antibiotic therapy are essential for the treatment of localized, suppurative skin lesions. Penicillin is the drug of choice for acute diseases. Penicillin has no effect upon established rheumatic heart disease and acute haemorrhagic glomerulonephritis. Penicillin resistant strains have not been reported. Erythromycin is the drug of choice for penicillin allergic patients
Group B streptococci	Group B streptococci (*S. agalactiae*) are harboured in the female genital tract and male urethra of 15–25% of humans and animals, as well as in the pharynx and genital tract. The organism is transmitted from an infected mother to her infant in utero or at birth. Group B streptococci are encapsulated. These organisms far outnumber *E. coli* K1 as the leading cause of neonatal meningitis during the first 4 months of life. The antiphagocytic properties of the capsular polysaccharide allow the organisms to survive multiply, invade epithelial cells, and induce an acute inflammatory response. In adults, the organisms may produce pneumonia, septicemia, prosthetic joint disease, or puerperal sepsis originating from the female genital tract. Specimens for laboratory diagnosis depend on the disease process and include blood for culture, sputum, cervical swab, and spinal fluid. These specimens are cultured on blood agar and incubated aerobically at 37 ºC. Group B streptococci are beta-haemolytic, gram-positive, catalase negative cocci. They are only streptococci in which ability to hydrolyze hippurate and positive

	CAMP test. Early therapy with penicillin plus aminoglycoside is essential for the prevention of progressive, fatal disease. Heavily colonized mothers can be treated with penicillin intrapartum to prevent subsequent colonization of their newborns
Viridans group of streptococci	The viridans group often reffered to as “oral streptococci” do not contain C carbohydrate but have been grouped based on rRNA cataloging and nucleic acid hybridization investigations. These organisms are normal inhabitants of the oral, respiratory, and gastrointestinal mucosa of humans and animals. They are potential pathogens and have generally been thought to be of low virulence. Viridans streptococci are, however, the major aetiologic agents of bacterial endocarditis. Patients who develop streptococcal endocarditis usually possess a previously damaged heart valve (from previous rheumatic fever and other cause). Gingival disease or dental manipulations, including dental prophylaxis,

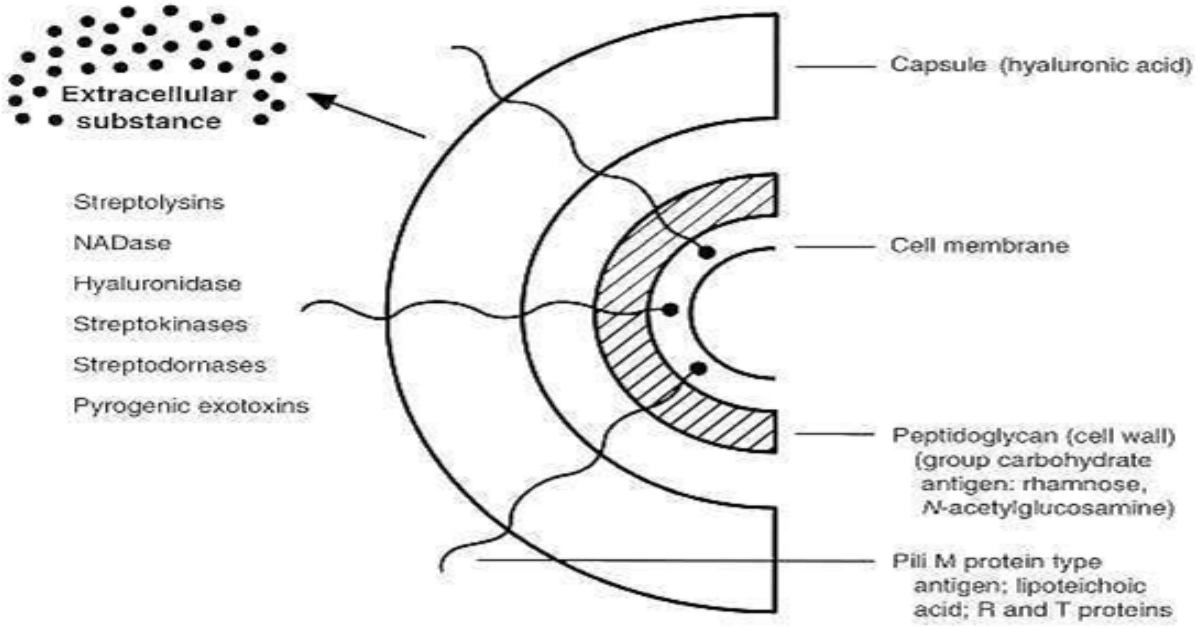
Table 3.9.1 continuation

Notion	Definition/explanation
	are often predisposing factors in the development of endocarditis. Viridans streptococci are able to adhere to epithelial and endothelial cells, and adherence is probably a key factor in their ability to cause disease. *Streptococcus mutans* has been definitively established as a major aetiologic agent of dental caries in addition to being a cause of endocarditis. Extracellular sugars, called dextrans, serve as attachment mediators for tooth surfaces as well as heart valves. Specimens for laboratory diagnosis depend on the disease process and include blood for culture and urine. These specimens are cultured on blood agar and incubated aerobically at 37 ºC. The species of the viridans group are alfa- or gamma-haemolytic, gram-positive, catalasenegative cocci that are not inhibited by optochin. Although treatment with penicillin is effective, the occurence of penicillin resistant strains necessitate the use of penicillin plus aminoglycoside
Streptococcus pneumoniae	*S. pneumoniae* are gram-positive, nonmotile, encapsulated cocci. They are lancet-shaped, and their tendency to occur in pairs accounts for their earlier designation as *Diplococcus pneumoniae*. *S. pneumoniae* is the most common cause of pneumonia and otitis media, and is an important cause of meningitis and bacteremia. The risk of disease is the highest among young children, older adults, smokers, and persons with certain chronic illnesses. Like other streptococci, *S. pneumoniae* is fastidious and is routinely cultured on blood agar and releases an α-haemolysin. This bacterium is an obligate parasite of humans and harboured in the nasopharynxs of 25–70% of the population. Pneumococci are very sensitive to environmental, physical, and chemical agents. Antiseptic agents kill them rapidly. The polysaccharide capsule is the sole basis for classification and the only known virulence factor. Distinct epitopes enable the recognition of more than 85 serotypes of pneumococci, 23 of which are responsible for greater than 85% of pneumococcal disease. The capsule inhibits phagocytosis and thus allows the organisms to establish themselves in host tissue, multiply, and produce disease
Disease of *S. pneumoniae*	*S. pneumoniae* is the most common cause of lobar and lobular (broncho) pneumonia. This organism also is the most common cause of meningitis among adults and a major cause of otitis media and sinusitis among children. Most infections are caused by endogenous spread from the colonized nasopharynx or oropharynx to distal site (e.g., lungs, sinuses, ears, blood, and meninges).

	Colonization is the highest in young children. Person-to-person transmission through infectious droplets is rare
Laboratory diagnosis of *Streptococcal pneumoniae*	Specimens obtained depend on the disease process and include a nasopharyngeal swab, sputum (which may be rusty), blood for culture, spinal fluid, and pus. Gram staining of pus, sputum, and spinal fluid often shows gram-positive, lancet shaped, diplococci and numerous polymorphonuclear leukocytes. Primary isolation and identification require initial cultivation on blood agar or in blood culture broth. Overnight incubation under aerobic conditions at 37 ºC is optimal for isolation of the organism. The organism may be identified as α-haemolytic, gram-positive, catalase-negative coccus that is bile soluble and inhibited by optochin. Rapid identification of pneumococcal serotypes in spinal fluid can be accomplished by latex agglutination using serotype-specific anticapsular antibody for the detection of capsular polysaccharide
Treatment of	Although penicillin is still the drug of choice, multiple-resistant strains are

Table 3.9.1 continuation

Notion	Definition/explanation
Streptococcal pneumoniae	now appearing. Cephalosporins, erythromycin, chloramphenicol, vancomycin are used for patients allergic to penicillin or for treatment of penicillin-resistant strains



Cell surface structure of *Streptococcus pyogenes* and secreted products involved in virulence

Scheme streptococcal infections laboratory diagnosis

Mucus from pharynx, urine, vomit masses, etc.

Stage 1

Primary growth in the blood agar and sugar peptone water

RESULT

Stage 2

Stage 3

END RESULT

Scheme streptococcal pneumonia laboratory diagnosis

LABORATORY DIAGNOSIS OF MENINGOCOCCAL AND GONOCOCCAL INFECTIONS

Theme topicality. Gonorrhoea (also called the clap and the drip) caused by *Neisseria gonorrhoeae* is an important public health problem and the most common reportable infectious disease. Gonorrhoea is most frequently spread during sexual contact. However, it can also be transmitted from the mother's genital tract to the newborn during birth, causing ophthalmia neonatorum and systemic neonatal infection.

Meningococcal (*Neisseria meningitidis*) bloodstream infections (known as meningococcemia) can range in severity from a transient bacteremia that is relatively benign to an overwhelming infection that is rapidly fatal. Meningitis commonly occurs during the course of meningococcemia. In rare cases, *N. meningitidis* organisms can spread haematogenously to other sites, such as pericardium, joints, and eyes.

Primary objective: to be able to conduct and evaluate the microbiological diagnosis of meningococcal and gonococcal infections.

QUESTIONS FOR DISCUSSION

1. Biological properties of *Neisseria spp*.
2. Gonococcal infections (gonorrhoea, conjunctivitis of the newborn, pelvic inflammatory disease (PID); microbiological diagnosis. Principles of Bordet-Gengou test (CFT) for diagnosis of gonorrhoea.
3. Epidemiology and pathogenesis of the diseases caused by gonococci. Specific features of immunity in such cases.
4. Principles of gonococcal infections; prophylaxis and treatment (features of chronic gonorrhoea treatment).
5. Principles of meningitis microbiological diagnosis.
6. Epidemiology and pathogenesis of the diseases caused by meningococci. Specific features of immunity in such cases.

PROCEDURE OF PRACTICAL WORK

Task 1. Examine microscopically the preparation (Gram staining) isolated from the urethra of the women suspected of gonorrhoea. Draw a conclusion in relation to the results of microscopic investigation. Complete the protocol.

There are gram-negative diplococci and leucocytes in it (incomplete phagocytosis) in the smear from urethra of women that are suspected of gonorrhoea. Such microscopic picture is characteristic at acute gonorrhoea. At the chronic form of gonorrhoea gonococci can not come to light microscopically.

Task 2. Examine microscopically the preparation (Gram staining) from the spinal fluid of the child suspected of meningitis. Draw a conclusion in relation to the results of microscopic investigation. Complete the protocol.

In spinal fluid of a child suspected of meningitis, you will observe the presence of intra- and extracellular gram-negative diplococci (meningococci).

Finding out the causative agents in the spinal fluid (by microscopy or bacteriological methods) is absolute proof of the proper infectious disease of the CNS.

Task 3. Examine the growth of *Neisseria meningitides* on blood agar. Write the description of cultural properties in the protocol.

Meningococci are strict aerobes, very demanding to the nutrient media and terms of cultivation. Therefore, grow only media with addition of albumens natively (serum, blood and other). In this case, meningococci are cultivated on the blood agar at the temperature of 37 °C. On a blood agar, meningococci form the shallow, tender colonies of oily consistency.

Task 4. Study CFT with gonococcal antigen. Fill in the result of reaction in the protocol, explaining the mechanism of its formation.

CFT is more frequently used for the serodiagnosis of chronic gonorrhoea. CFT is carried out on Bordet-Gengou on a standard chart. The reaction is positive in 3–4 weeks of illness.

Task 5. Examine the sensitivity of *N. gonorrhoeae* to antibiotics, make the conclusion. Write down the results of the experiment in the protocol.

Certainly, specific preparation for treatment of gonorrhoea gets out depending on the form of this infection.

Task 6. Study the main antimicrobial drugs used for diagnosis, treatment, and prevention of meningococcal and gonococcal diseases, write them down in the copy book.

Gonococcal inactivated liquid vaccine (gonovaccine) is a suspension for intramuscular introduction, represents a suspension inactivated cultures of *N. gonorrhoeae* in 0.9% solution of sodium chloride. General biological properties of this vaccine are increase of specific reactance of an organism. It is used for immunotherapy (auxiliary method of treatment of gonorrheal infection from 3-years-old age) the chronic form of gonorrhea; and for provocation of chronic gonorrhoea. This vaccine can be used for diagnosis (to establish whether a patient was cured completely of gonorrhoea).

Meningococcal chemical vaccine contains purified polysaccharide of *Neisseria meningitidis* of group A and C. It is used for prophylaxis of cerebrospinal meningitis caused by meningococcus of serogroup A and C. Vaccination is recommended in endemic regions, and also in case of epidemic caused by meningococcus of serogorup A or C.

Gonococcal antigen is inactivated by *N. gonorrhoeae*. Preparation is used for serological diagnosis of gonorrhoea in CFT.

Laboratory diagnosis of gonococcal and meningococcal infections

Notion	Definition/explanation
	Neisseria gonorrhoeae
N. gonorrhoeae	*N. gonorrhoeae* is a fastidious organism, requiring complex media for growth and adversely affected by drying and fatty acids. Soluble starch is added to the media to neutralize the toxic effect of the fatty acids. It is highly susceptible to environmental, physical, and chemical agents. The optimum growth temperature is 35 ºC to 37 ºC, with poor survival of the organism at lower temperature. A humid atmosphere supplemented with CO_2 is required or enhances growth of *N. gonorrhoeae*
Antigenic structure relation to virulence	The outer surface is not covered with a true carbohydrate capsule, as is found in *Neisseria meningitidis*. Pili mediate attachment of the organism to epithelial and mucosal cell surfaces and are antiphagocytic. Three outer membrane proteins (OMPs) have been studied extensively: 1. **Por** proteins are porin proteins that form pores channels in the outer membrane. 2. **Opa** (opacity) proteins are a family of membrane proteins that mediate binding to epithelial cells. 3. **RMP** (reduction-modifiable) proteins prevent complement-mediated bactericidal antibody function and thus may contribute to dissemination of the disease. Another major antigen in the cell wall is lipooligosaccharide (LOS). This antigen is composed of lipid A and a core oligosaccharide, similar to gram-negative lipopolysaccharide (LPS), and possesses endotoxin activity. Other important gonococcal proteins are an immunoglobulin (Ig) A1 protease; the enzyme inactivates local secretory Ig A and thus may play a role in facilitating the adherence of gonococci to mucosal surfaces; ß-lactamase, which degrades penicillin
Diseases caused by *N. gonorrhoeae*	Gonorrhoea is one of the most commonly reported sexually transmitted diseases. A higher proportion of females than males are generally asymptomatic; these individuals act as the reservoir for maintaining and transmitting gonococcal infections. More than one sexually transmitted disease may be acquired at the same time, for example gonorrhoea in

	combination with syphilis, chlamydia, human immunodeficiency virus (HIV), and hepatitis B virus. Patients with gonorrhoea may therefore have to be treated for more than one pathogen. Genital infection in men is primarily restricted to the urethra. Purulent urethral discharge and dysuria are developing after the 2–7 day incubation period. Although complications are rare, epididymitis, prostatitis, and periurethral abscesses can occur. The primary site of infection in women is the cervix, although gonococci can be isolated in the vagina, urethra and rectum. Vaginal discharge, dysuria, and abdominal pain are commonly reported in symptomatic patients. Other diseases associated with *N. gonorrhoeae* include purulent conjunctivitis particularly in newborns infected during vaginal delivery (ophthalmia), anorectal gonorrhoea in homosexual males, and pharyngitis

Table 3.10.1 continuation

Notion	**Definition/explanation**
Laboratory diagnosis	Specimens obtained depend on the disease process and include urethral, cervical, rectal, pharyngeal, and/or conjunctival exudates. The direct demonstration of gram-negative intracellular diplococci within PMNs is diagnostic only when observed in the urethral exudates of males with characteristic clinical manifestations. Gram stains of smears from female urethral and cervical exudates, from rectal, pharyngeal, and conjunctival exudates of male and female, are unreliable due to the potential presence of nonpathogens resembling gonococcal morphology, and presence of meningococci. All such specimens must be cultured and the isolated organism identified. These techniques require initial cultivation on Thayer-Martin medium. Incubation for 48 hours at 35–37 ºC under aerobic conditions in the presence of 3–10% carbon dioxide is optimal for isolation of the organism, which may be identified as gram-negative, oxidase positive, diplococcus that ferments glucose, but not maltose, sucrose, or lactose
Treatment	Ceftriaxone, cefixime, ciprofloxacin, or ofloxacin can be administered in uncomplicated cases. In vitro susceptibility should be determined in cases unresponsive to therapy, because antibiotic resistance is increasing. Penicillin should be avoided, because resistance is common. Doxycycline or azithromycin should be added for infections complicated by chlamydia
Neisseria meningitidis	
N. meningitidis	*N. meningitidis* causes endemic or epidemic disease of worldwide prevalence. The most commonly recognized form of this disease is meningitis. *N. meningitidis* is an obligate parasite of humans, harboured in the nasopharynx, and transmitted by droplet nuclei or direct intimate contact with a sick people or bacteria carrier. Factors contributing to sesceptibility include fatigue and exposure to inclement weather. Meningococci are destroyed rapidly in the environment and are highly susceptible to physical and chemical agents. This organism is a gram-negative, nonmotile, encapsulated, piliated diplococcus flattened on one site to give the appearance of a “kidney bean” or “coffee bean”. The antigenic diversity of the capsule forms the basis for classification of the meningococci into 13 serogroups. The great majority of meningicoccal disease is caused by serogroups A, B, C, W, and Y. The

	capsules are antiphagocytic and may facilitate meningeal invasion. Outer membrane proteins (OMPs) of the organism are also antigenically diverse, which enables serotyping meningococci within each serogroup. In addition, OMPs acts as porins. The organisms are strict aerobes that grow best on chocolate agar at 35–37 ºC in the presence of 3–10% carbon dioxide
Pathogenesis and clinical manifestations	The basic pathogenesis process of primary meningococcal disease is initiated in the nasopharynx from a sick people or carrier. Adherence to the mucosal surface with resultant colonization is mediated by pili and possibly facilitated by secretory Ig A cleavage by Ig A1 protease. Phagocytosis is inhibited by the capsule, and the organisms continue to multiply, producing nasopharyngitis. Most patients produce complement-dependent bactericidal antibody, predominantly of the Ig M type, and opsonic antibody, both of which restrict the organisms to the mucosal surface of the nasopharynx and eventually cause their riddance. Disseminated meningococcal disease

Table 3.10.1 continuation

Notion	Definition/explanation
	manifests most often as septicemia and meningitis. Onset may be correlated with the absence of complement-dependent bactericidal antibody and opsonic antibody, the presence of serum Ig A antibody that blocks the initiation of immune lysis, or complement component deficiencies. The clinical manifestations of septicemia are the result of lipooligosaccharide (LOS), which is an abundant component of the organism, is released upon multiplication and autolysis. LOS differs from LPS in that the former has shorter, nonrepeated, O antigenic side chains and thus has a lower molecular weight. Meningitis is the most common complication of meningococcal septicemia. Clinical manifestations are fever, stiff neck, vomiting, severe headache, convulsions, bulging of the fontanels, and progression to coma within a few hours
Laboratory diagnosis	Specimens obtained depend on the disease process and mainly include nasopharynx swabs, blood culture, and cerebrospinal fluid. Gram stains of specimens may show gram-negative, intracellular and extracellular diplococci in association with PMNs. Primary isolations require initial blood cultures and culture on chocolate agar plates or, if a mixed flora is anticipated, Thyer-Martin medium, which is an enriched chocolate agar medium, containing antibiotics to inhibit gram-positive organisms and gram-negative rods. Incubation for 48 hours at 35–37 ºC under aerobic conditions in the presence of 3–10% carbon dioxide is optimal for isolation of the organisms, which may be identified as gram-negative, oxidase-positive, diplococcus that ferments glucose and maltose, and agglutinates in the presence of serogroup-specific anticapsular antibody
Treatment	Early treatment with penicillin reduces the case fatality rate in disseminated disease from 40–90% to 10–15%, but the antibiotic is ineffective in eradicating the carrier state. Chloramphenicol and ceftriaxone are effective in penicillin-allergic individuals. Polysaccharide vaccines conjugated with protein carriers offer protection for infants younger than 2 years

Scheme meningococcal infections and bacteria carriers laboratory diagnosis

Mucus from pharynx and nose, blood, pus, spinal fluid, exudates

RESULT

END RESUL

Scheme gonococcal infection laboratory diagnosis

LABORATORY DIAGNOSIS OF ANAEROBIC INFECTIONS

Theme topicality. Causative agents of tetanus and gas gangrene to cause wound infections. The diseases caused by them are taking hard course and without the professional medical intervention can result in the death of the patient. The preparations that can form the antitoxic immunity (active and passive) play an important role in the prevention of these diseases. The method of approach can be explained by the biological properties of the causative agents of these diseases. Non-clostridia anaerobes cause the diseases at first in the debilitated people (for example, in postoperative period). Microbiological diagnostics of infections caused by non-clostridia anaerobes is not used widely in the medical practice because isolation of anaerobic pure cultures is specific. This influences the effectiveness of the treatment.

Primary objective: to be able to conduct and evaluate the microbiological diagnosis of the anaerobic infection.

QESTIONS FOR DISCUSSION

1. General characteristics of the pathogenic spore-forming anaerobes (clostridia) – *C. tetani, C. perfringens, C. septicum, C. histoliticum, C. novyi.*
2. Epidemiology, pathogenesis, microbiological diagnostics, treatment and prevention of tetanus and gas gangrene.
3. General characteristics of non-clostridia anaerobes – bacteroides, fusobacteria, propionibacterium, veillonella, eubacterium, peptococcus, peptostreptococcus, bifidobacterium.

PROCEDURE OF PRACTICAL WORK

Task 1. Examine microscopically the preparation of the patient's material. Draw the preparation in the protocol.

C. tetani is gram-positive rod with length from 1 to 6 mm. The bacteria are placed isolated and form round terminal spore. The size of the spore is thicker than bacteria, therefore the cell is drumstick – shaped. When the smear is stained by Gram's method the spore becomes colourless with violet contour. When the smear is stained by Ziehl–Neelsen method the spore is red because it is acid-stable structure, the vegetative body of the bacteria stains blue.

B. bifidum is nonspore-forming anaerobes, members of the normal flora of the large intestine, gram-positive, like branchy rods.

Peptostreptococcus is gram-positive streptococcus, nonspore-forming anaerobe.

Task 2. Examine the growth of anaerobes on the media. Enter the description of cultural properties in the protocol.

C. perfringens on the blood-sugar Zeissler's agar forms a small round grey colony. The medium contains glucose, blood and agar. This medium is placed on the Petri dish and is used for isolation of the colony.

Fortner dish is a dish with Zeissler's agar and it is a biological mechanical method of making anaerobic conditions. The medium is divided into two parts (the furrow is made in the dish centre by scalpel). The aerobic and anaerobic bacteria are growing on the different parts of the medium.

Sugar nutrient agar consists of the nutrient medium and glucose as a reduced agent. The reduced agents promote to lowering the oxidizing possibilities of the medium.

Kitt-Tarozzi medium is a nutrient broth with 0.5% glucose and piece of a liver and minced meat for oxygen absorption. Before inoculation the medium is warmed thoroughly on the water bath for 10–15 minutes to remove oxygen (regeneration of the medium). After inoculation the medium is covered with vaseline oil to prevent interaction of gases with medium. *C. perfringens* grows on it with formation of the gas and dimness of the liquid.

Litmus milk is fatless and is regenerated for oxygen absorption like Kitt-Tarozzi medium. *C. perfringens* is formed in it after 3–4 hours of cultivation like sponge clot rice. It contains blebs of gas and transparent liquid.

Wilson-Blerr medium is an iron-sulfite agar. It consists of nutrient medium with glucose, Na_2SO_3, $FeCl_2$. The tube with medium is allowed to harden in an upright position, the tube is designated an agar deep tube. The inoculation is made by prick in the nutrient medium. Clostridia reduce Na_2SO_3 in Na_2S that binds with $FeCl_2$ and makes black sediment in place of the inoculation. After cultivation, *C. perfringens* forms black culture in the deep of the Wilson-Blerr medium.

C. perfringens on lactose egg-yolk medium forms the dimness of the media around the colonies.

Task 3. Examine the methods of the anaerobic state creation.

Anaerobic jar is an apparatus that can maintain the constant temperature and it allows produce anaerobic conditions for cultivation. It is closed hermetically. Vacuum pump pumps air outside the camera of the anaerobic jar.

Veyon-Vinyal method is used for isolation of anaerobes colonies. It consists of the preparing serial dilutions of the investigated materials in the fusion nutrient agar. The nutrient agar is taken in the Paster pipettes with solder end. Thus, in that way, in the thickness of the medium anaerobic conditions are made. The pipette is placed in the thermostat with optimal temperature. The grown colony is obtained when the pipette is cut up or the end of the pipette is broken.

Exsiccator can be used for anaerobes cultivation. The culture is placed inside the exsiccator. The chemical method is used for the evolution of oxygen (the steam of the chemical matter that interacts with oxygen is placed on the bottom of the exsiccator).

Task 4. Examine the biochemical properties of bacteria and make the conclusion. Write down the results of the experiment.

Biochemical properties of clostridia

Causative agent	Fermentation of carbohydrates	Curdle of milk	Dilution of gelatin
C. perfringens	+	+	+
C. tetani	–	–	+

Task 5. Study and write in the copybook the main antimicrobial drugs used for treatment, prevention and diagnostic of anaerobic infections.

Tetanus toxoid (TT) is a monovaccine, which can be used both for planned vaccination of children and for epidemiological indications. There it is defused at 37 °C by formalin 0.4% exotoxin of *Clostridium tetani*.

Sextatoxoid consists of toxoids *C. botulinum* (serovar A, B, F), *C. perfringens, C. septicum, C. tetani*. Preparation is used for prophylaxis of tetanus.

DTaP (diphtheria, tetanus, acelular pertussis) is a vaccine adsorbed on the aluminium hydroxide, that contains killed pertussis bacteria and diphtherial and tetanic toxoids. It is used for planned prophylaxis.

DT is adsorbed toxoid of diphtheria and tetanus causative agents; used for planned revaccination.

Tetanus antitoxic serum is the preparation that is obtained by hyperimmunization of the horse by tetanus toxoid. The use of it requires specific rules of introduction of heterogenic serum – to prevent the development of anaphylactic shock and serum illness.

Task 6. **Prepare the smear from soil and stain it by Zeill-Nelson method.**

For the preparing of the smear, some quantity of soil is placed in the Kitt-Tarozzi medium for cultivation of the anaerobes. Anaerobic bacteria and their spores are placed in the bottom of the

medium; therefore, taking the material you must pass one's loop over one's bottom of the media. The oil layer is on the surface of the medium and prevents from preparing qualitative preparation. When you take the culture you must incline the tube and pass the loop through that place where the oil is absent. After that carefully place the loop in the flame, avoid burns. When you examine the smear, choose the places where there are more artefacts.

Laboratory diagnosis of anaerobic infections

Notion	Definition/explanation
Morphology of anaerobic infection causative agents	They are large gram-positive spore-forming mobile rods (*Clostridium),* gram-positive bacilli (*Actinomyces, Lactobacillus, Propionobacterium, Eubacterium, Bifidobacterium*), gram-positive cocci (*Peptostreptococcus*), gram-negative bacilli (*Bacteroides, Prevotella, Porphyromonas, Fusobacteria*), gram-negative cocci (*Veillonella*)
Diseases caused by *Clostridium spp.*	*C. tetani* causes tetanus. *C. botulinum* causes botulism. *C. perfringens, C. septicum, C. histoliticum, C. novii* cause gas gangrene. *C. difficile* causes pseudomembranous colitis
The natural habitat of the clostridium	Soil
Condition of the clostridium cultivation	Clostridia grow only in anaerobic conditions, on the special media
Causative agent of the tetanus	The causative agent is *Clostridium tetani*, an anaerobic, sporeforming, gram-positive, rod-shaped bacterium, shows two striking features: (1) a spherical endospore that forms at the end of the bacillus, in contrast to the oval endospore that develops near the center of the cell in other pathogenic species of Clostridium, (2) swarming growth that quickly spreads over the surface of solid media, making it easy to obtain pure cultures
Pathogenesis of tetanus	Vegetative cells of the *C. tetani* produce tetanospasmin and release it mainly when they lyse. Tetanospasmin acts in several ways upon the central nervous system. It inhibits the release of acetylcholine, thus interfering with neuromuscular transmission. The most important action is the inhibition of postsynaptic spinal neurons by blocking the release of the inhibitory mediator resulting in hyperreflexia and muscle spasms that may be generalized
Typical clinical manifestations of tetanus	The incubation period may range from 4–5 days to as many weeks. The disease is characterized by tonic contraction of voluntary muscles. Muscular spasms often involve first the area of injury and infection and then the muscles of the jaw, which contract so that the mouth cannot be opened. Any external stimulus may precipitate a titanic generalized muscle spasm. Death usually results from intervention with the respiratory mechanism

Causative agent of gas gangrene	Several species of *Clostridium* can produce life-threatening gas gangrene when they invade injured muscle, but by far the most common offender is *C. perfringens*. These encapsulated gram-positive rods are shorter and fatter than *C. tetani* and usually do not exhibit spores in material from wounds or cultures
Pathogenesis of gas gangrene	The clostridia produce a large variety of toxins and enzymes that results in spreading infection. These toxins have lethal, necrotizing, and haemolytic properties. When the spores contaminate the tissue they transform in the vegetative cells. These cells produce the ferment of carbohydrates and gas. The distention of tissue and intervention with blood supply, together with the secretion of the necrotizing toxin and hyaluronidase, favour the spread of infection. Tissue necrosis extends,

Scheme tetanus laboratory diagnosis

Scheme gas gangrene laboratory diagnosis

LABORATORY DIAGNOSIS OF DIPHTHERIA AND WHOOPING COUGH (PERTUSSIS)

Theme topicality. *Corynebacterium diphtheriae* is the most important member of the group, it can produce exotoxin that causes diphtheria in humans. *Bordetella pertussis*, a highly communicable and important pathogen of humans, causes whooping cough (pertussis).

Primary objective: to be able to conduct and evaluate the microbiological diagnosis of the diphtheria and pertussis (whooping cough).

QESTIONS FOR DISCUSSION

1. Biological properties of diphteria and pertussis causative agent.
2. Pathogenesis of diphtheria and pertussis in humans.
3. Features of antidiphtherial and antipertussis immunity.
4. Laboratory diagnostics of diphtheria and pertussis.
5. Specific prophylaxis and treatment of the disease.

PROCEDURE OF PRACTICAL WORK

Task 1. Prepare the smears of pure culture of *C. diphtheria* by Gram's method, Neisser's method, and Loeffler's method.

Diphtheria bacteria are gram-positive, pleomorphic, often club-shaped rods. The individual cells tend to group in V, Y, or palisade arrangements.

At Loeffler staining *Corynebacterium spp*. is blue. In both cases of valutins grain, stained more intensively than the central part of the cells (phenomenon of metachromasia). Neisser staining reveals the polar bodies (polyphosphates stored at one end of the rod).

Task 2. Study the growth of *Corynebacterium diphtheriae* cultures on Loeffler nutrient medium and Buchin's medium.

The usual media employed for cultivation of the diphtheria bacillus are Loeffler's serum slope and tellurite blood agar. Diphtheria bacilli grow on Loeffler's serum slope very rapidly and colonies can be seen in 6–8 hours, long before other bacteria grow. Colonies are at first small, circular white opaque discs but enlarge on continued incubation and may acquire a distinct yellow tint.

Tellurite (0.4%) inhibits the growth of most other bacteria, acting as a selective agent. Diphtheria bacilli reduce tellurite to metallic tellurium, on the tellurite medium and colonies become grey or black. The growth of diphtheria bacilli may be delayed. It may take 2 days to appear.

Buchin's medium consists of agar, fish hydrolysat, sodium chloride, glucose, indicators. It is prepared of powder, according to the label instructions. It is boiled for 2–3 minutes and cooled to 50 °C, after that 50 ml of defibrinated blood (rabbit or human) is added. The prepared medium is dark blue. On this medium *C. diphtheriae* forms dark blue colonies, diphtheroids – light blue ones.

Task 3. Study the diphtheria bacillus cultures toxigenicity in gel-precipitation assay (Elek test).

The gel precipitation test is based on the interaction of homologous antibodies and antigens in gel and the formation of visible bands of precipitation. Because of counter-diffusion into gel, the antibodies and antigen form immune complexes (aggregates) visualized in the form of opalescent white bands.

When several antigens diffusing irrespective of each other are present, the number of bands corresponds to the number of antigens. Serologically homogeneous antigens form precipitation bands which merge with each other, whereas bands of heterogeneous antigens cross each other. This property permits determination of the homogenecity of the antigenic structure of various objects tested.

Components of the gel precipitation test are gel, antigen, and antibodies. For quality control of the gel precipitation test, the test system comprised of known homologous antibodies and antigens is used. The antigen used in a precipitation test should be concentrated, while the sera (of patients or immunized animals) should be of a high titre.

Procedure. In a solidified agar cut the wells and remove the agar from them with a pasteur pipette. Into 1 series of walls place serum, into the other – antigens and put the slides into a humid chamber for several days. In reading the results of the reaction, compare the localization and nature of precipitation lines in the test and control wells. To measure the levels of the antigen and antibodies, study their multiple dilutions. The precipitation test in gel is widely used in the diagnosis of diseases caused by viruses, rickettsiae, and bacteria producing exotoxins. It has become of great practical significance with regard to determining the toxigenicity of *Corynebacteria diphtheriae.*

Task 4. Study the differentiative properties of corynebacteria.

Type of diphtheria bacilli differentiation

Properties	**Gravis**	**Intermedius**	**Mitis**
Morphology	Usually short rods, with uniform staining, few or no granules. Some degree of pleomorphism, of irregularly barred, snowshoe and tear-drop forms	Long-barred rods with clubbed ends; poor granulation, very pleomorphic	Long, curved, pleomorphic rods with prominent granules
Colony on tellurite blood agar	In 18 hours, the colony is 1–2 mm in size, with greyish black centre, paler, semitranslucent periphery. In 2–3 days, 3–5 mm in size, flat colony with raised dark centre and crenated edge with radial striation	In 18 hour, the colony is small, 1 mm in size, misty. Does not enlarge in 48 hours, dull granular centre with smoother, more glistening periphery and lighter ring near the edge – “frog’s egg” colony	Variable in size, black. In 2–3 days, colonies become flat, with central elevation, “poached egg” colony
Consistency of colonies	Like “cold margarine”, not easily picked out or emulsifiable	Intermediate between gravis and mitis	Soft buttery, easily emulsifiable
Haemolysis	Variable	Nonhaemolytic	Usually haemolytic
Growth in broth	Surface pellicle, granular deposit, little or no turbidity	Turbidity in 24 hours, clearing in 48 hours, with fine granular sediment	Diffuse turbidity with soft pellicle later
Glycogen fermentation	Positive	Negative	Negative

Task 5. Study the cistinase test.

Investigated culture is inoculated by a prick in the column of agar with cysteine and test tube is incubated in the thermostat. In 1 day, a medium becomes blak along a prick, and brown “cloud” appears on the depth of 1 cm from the surface. Diphtheroids do not form a “cloud”.

Task 6. Study the urease test.

Urease activity (the urease test) is detected by growing bacteria in the medium containing urea and using a pH indicator such as phenol red. When urea is hydrolyzed, ammonia accumulates in the medium and makes it alkaline. This increase in pH causes the indicator to change from orange-red to deep pink or purplish red (cerise) and is a positive test for urea hydrolysis. Failure of a deep pink colour develops in case of a negative test.

Task 7. Read the indirect haemagglutination test, make the conclusion about antidiphtherical immunity.

Indirect haemagglutination test determines the antitoxin antibodies concentration in blood serum. It is made according to the formula: X=10xA/B, where X is a content of diphtheria antitoxin in assayed serum (IU), 10 – titre of control serum (IU/ml), A – maximal dilution of assayed serum with positive result, B – maximal dilution of control serum with positive result.

Task 8. Prepare the smears of pure culture of *Bordetella pertussis,* stain it by Gram's method.

B. pertussis is small, coccoid, nonmotile, gram-negative rods.

Task 9. Study the differentiating features of bordetella.

Differentiating features of bordetella

Feature	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. avium*
Motiliti	–	–	+	+
Appearance on MacConkey agar	–	–	–	+
Appearance on Bordet-Gengou medium (days)	3–6	1–2	1	1
Urease	–	+	+	–
Nitrate to nitrite	–	–	+	–
Citrate use	–	Variable	+	Variable
Oxidase	+	–	+	+
Heat labile toxin and tracheal cytotoxin	+	+	+	+
Adenylate cyclase toxin	+	+	+	–
Pertussis toxin	+	–	–	–

Task 10. Examine the agglutination test with patient's paired sera and whooping cough diagnosticum. Make the conclusion.

The agglutination test with patient's paired sera and whooping cough diagnosticum

Component	The number of the test tube				
	1	2	3	4	5
0.9% NaCl solution, ml	1.0	1.0	1.0	1.0	1.0
Serum dilution	1:40	1:80	1:160	1:320	1:640
Diagnosticum, ml	0.1	0.1	0.1	0.1	0.1

Incubation at 37 °C for 1 hour, then at room temperature for 18 hours						
Reading	1st serum					
	2nd serum					

Note. Reaction is performed simultaneously with patient's paired sera.

Task 11. **Describe the immunological preparations for treatment and prophylaxis of diphtheria and pertussis.**

DTaP (diphtheria tetanus acelular pertussis) is a vaccine adsorbed on the aluminium hydroxide, that contains killed pertussis bacteria and diphtherial and tetanic toxoids. It is used for planned prophylaxis.

DT is an adsorbed toxoid of diphtheria and tetanus causative agents; used for planned revaccination.

ADT-M is a preparation with the diminished amount of antigens, which is used for persons with violation of immune status.

Diphtheria toxoid (DT) is a monovaccine, which can be used both for the planned vaccination of the chidren and on epidemiological indications. It is kept at temperature 37 °C and by formalin 0.4% exotoxin of *Corynebacterium diphtheriae*.

Antidiphtheria antitoxic serum is got by hyperimmunization of horse, diphtherial toxoid. Effective remedy is for specific therapy of diphtheria. The rules of heterogenic serum introduction should be followed to prevent the development of anaphylactic shock and serum diseases.

Antidiphtherial gamma globulin contains antitoxins which are able to neutralize diphtherial exotoxin. It is gamma globulin fraction of blood serum of the hyperimmunized animals. It contains diminished amount of ballast substances.

Schick test. This procedure has been used during outbreaks of diphtheria to determine which case contacts are susceptible to the disease, and thus in need of immunization. A small amount of diphtheria toxin is injected intracutaneously into one forearm and a heated toxin into the other for control. Redness and induration appear within 1 to 2 days at the test place only, or their appearance at the both places with persistence at the test place but disappearance from the control place in 4–7 days, signifies susceptibility due to lack of sufficient antitoxin. In contrast, no reaction at both the test and control places, or redness at both places within 1 day, reaching a maximum in 2–3 days, and fading rapidly from both places, signifies immunity due to the presence of sufficient neutralizing antitoxin.

Laboratory diagnosis of the diphtheria and pertussis

Notion	Definition/explanation
Diphtheria	*Corynebacterium diphtheriae* causes diphtheria. Usually the bacteria multiply on the surface of the mucous membranes of the throat where they

Table 3.12.3 continuation

Notion	Definition/explanation
	cause inflammation. The inflammation may spread to the voice box (larynx) and may cause throat swelling, and airway narrowing. Disease-causing strains of *C. diphtheriae* release a damaging substance (toxin), which can also involve the heart, brain and nerves. The bacteria may cause a thick, gray covering in the nose, throat or airway a marker of diphtheria that distinguishes it among other respiratory diseases. This covering is usually fuzzy gray or black and causes respiratory difficulties and painful swallowing
Risk factors for diphtheria	People who are at increased risk of diphtheria infection: children and adults who don't have up-to-date immunizations; people living in crowded or unsanitary conditions; undernourished people; immunocompromised patients
Morphology, staining, and culturing of *Corynebacterium diphtheriae*	Diphtheria bacteria are gram-positive, pleomorphic, often club-shaped rods. The individual cells tend to group in V, Y, or palisade arrangements. Neisser staining reveals the polar bodies (polyphosphates stored at one end of the rod). Loeffler's nutrient medium, which consists of coagulated serum and nutrient broth, is used for the primary cultures. Selective indicator media containing tellurite are used in selective culturing. K tellurite is used to inhibit the accompanying flora. The K tellurite is also reduced to tellurium, staining the colonies brownish black
Extracellular toxin of *Corynebacterium diphtheriae*	Diphtheria toxin consists of two functionally distinct fragments, A and B, whereby B stands for binding to receptors of target cells and A stands for toxic activity. Fragment A irreversibly blocks protein synthesis translation in the target cells, which then die. The toxin gene is always a prophage genome component
Epidemiology of diphtheria	Infection sources include infected persons and carriers (rare)
Transmition of diphtheria	The disease is usually transmitted by droplet infection, or less frequently indirectly via contaminated objects
Laboratory diagnosis of diphtheria	Specimens obtained depend on the disease process and include a nose, throat, nasopharyngeal, and wound swab. Specimens are cultivated on cysteine-tellurite agar and Loeffler's coagulated serum. In addition, differential diagnosis necessitates primary cultivation on blood and chocolate agar. Identification of gray-black colonies on cysteine-tellurite agar, the typical Chinese characters, beaded, barred, or palisade arrangment of pleomorphic rods with accentuated metachromatic granules by methylene blue staining of colonies on Loeffler's coagulated serum, constitute presumptive evidence for *C. diphtheriae*. Definitive identification of the organism is based upon the demonstration of exotoxin production by a virulence test in guinea pigs or by a modified in vitro "Elek" test, with using specific antitoxic serum
Treatment of diphtheria	Specific antitoxic serum must be administered immediately. Inasmuch as the antitoxin is generated in horses, a skin test to ensure the absence of hypersensitivity to horse protein is essential prior to its use. Although antibiotics have no effect upon the toxemic disease process, penicillin or erythromycin is effective in killing organism and thus preventing further toxin production

Table 3.12.3 continuation

Notion	Definition/explanation
Prevention and control of diphtheria	1. Active immunization with formalin-inactivated toxoid. Vaccination is highly effective. It is first administered early in infancy along with tetanus toxoid and killed *Bordetella pertusis* (DTaP) and must be followed by periodic toxoid boosters throughout childhood and adulthood. 2. The schick test
Bordetella pertussis	*B. pertussis* organisms do not grow on universal laboratory media. Bordet-Gengou medium contains potatoes, glycerol, and sheep blood. It is traditionally the medium of choice. These organisms are nonmotile and oxidize amino acids but do not ferment carbohydrates. The other bordetella species are less fastidious and can grow on blood and MacConkey agars
Pertussis toxin	Pertussis toxin is a classic A–B toxin consisting of toxic subunit (S1) and five binding subunits (S2 to S5). The S2 subunit binds to ciliated respiratory cells. The S3 subunit binds to receptors on phagocytic cells. Two S4 subunits are present in each toxin molecule
Clinical syndromes of pertussis of whooping couph	*B. pertussis* is a human disease with no other recognized animal or environmental reservoir. The disease is still endemic worldwide and affects more than 60 million people annually. The infection is initiated by inhalation of infectious aerosol droplets and attachment and proliferation of the bacteria on ciliated epithelial cells. After a 7–10 day incubation period, the patient will experience the first of three stages. The catarrhal stage resembles a common cold, with serous rhinorrhea, sneezing, malaise, anorexia, and a low-grade fever. Patients in the catarrhal stage pose the highest risk to their contacts. After 1–2 weeks the paroxysmal stage begins, with classic whooping cough paroxysms. The paroxysms are characterized by a series of repetitive coughs followed by inspiratory whoop. The paroxysms are frequently terminated with vomiting and exhaustion. After 2–4 weeks the disease enters the convalescent stage when the paroxysms diminish in number and severity, but secondary complications can occur
Laboratory diagnosis of pertussis	Secretions collected on nasopharyngeal smears consisting of calcium alginate or dacron on wire handles are the best specimen. The smears must be inoculated immediately onto media at the patient's bedside or transported in a moist, protective medium to the laboratory for cultivation. Direct fluorescent antibody staining of the organism is the fastest diagnostic tool. Although method is only 60% sensitive, it is very specific. Specimens are inoculated onto special media, such as Regan-Lowe agar and freshly made Bordet-Gengou agar. Cultures are incubated in a humidified atmosphere without added carbon dioxide for at least 10 days. Once the characteristic colonies (colonies are shiny and have a characteristic metallic or pearly sheen) appear, they can be identified using fluorescent antibody stain. *B. pertussis* is oxidase–positive and microscopic examination should reveal very tiny gram-negative coccobacilli
Treatment of pertussis	Treatment with macrolide (erythromycin, azithromycin) is effective in eradicating organisms and reduces the duration of infectious stage. Treatment does not alleviate symptoms
Prevention and control of pertussis	Vaccination is the key to control; susceptible population should be immunized. Immunization of unvaccinated children older than 7 years of age or adults is not recommended at this time because of the decreased severity

Table 3.12.3 continuation

Notion	Definition/explanation
	of disease among the age groups. Immunization with the current vaccine of completely killed cells of *B. pertussis* in a trivalent vaccin containing diphtheria and tetanus toxoids confers a high degree of protection

LABORATORY DIAGNOSIS OF TUBERCULOSIS AND LEPROSY

Theme topicality. According to the data of the World Health Organization, one third of the globe population are infected with tuberculosis mycobacteria. In some countries, infectiousness of

the population with tuberculosis reaches 80–90%. Every year each tuberculosis patient can infect 10–15 and more persons, 5–10% of which will catch the disease.

Primary objective: to be able to conduct and evaluate the microbiological diagnosis of the tuberculosis and leprosy.

QUESTIONS FOR DISCUSSION

1. Biological properties of *Mycobacterium tuberculosis*, *M. bovis*, *M. leprae.*
2. Epidemiology and pathogenesis of tuberculosis and leprosy.
3. Features of antituberculosis immunity.
4. Laboratory diagnosis of tuberculosis and leprosy.
5. The tuberculin skin test, the interpretation of the results.
6. Specific prophylaxis and treatment of the disease.

PROCEDURE OF PRACTICAL WORK

Task 1. Prepare the smear from vaccine BCG strain, stain it by Ziehl-Neelsen method, perform the microscopy and draw it, make the conclusion.

Causative agents of the tuberculosis and leprosy are the distinctive property called acid-fastness, due to the presence of lipids (mycolic acid) in the cell wall. These organisms quickly absorb red carbolic fuchsin in the presence of a detergent or when warmed and retain dye; per washing with an acidified alcohol solution. All non-acid-fast bacteria, pus, cells, and so forth lose the carbolic fuchsin when treated with adding alcohol and take a contrasting counter staining (e.g., methylene blue, brilliant green).

Tubercle bacilli are more strongly acid-fast than other members of the acid-fast group. Both Gram staining and acid-fast staining depend on the integrity of the cell wall. Broken or disintegrated bacilli or their parts are neither gram-positive nor acid-fast.

Ziehl-Neelsen staining procedure

1. Fix the smear.
2. Put carbolfuchsin on the slide and steam it gently for 5 minutes over low flame, and do not allow drying, add more stain if necessary. Cool. Alternatively, carbolfuchsin containing phenol and alcohol (cool) may be used without heating.
3. Apply 90% alcohol containing 3% to 5% HC1 until all but the thickest parts of the smear cease to give off color (approximately 1 to 3 minutes). Wash.
4. Stain for 1 minute with methylene blue. Wash.
5. Examine smear using the oil-immersion lens of the light microscope.

Task 2. Perform the microscopy and draw ready preparation from patient's sputum (stained by Ziehl-Neelsen method), make the conclusion.

An acid-fast staining is used to diagnose the presence of mycobacteria in tissue and cytologic preparations. It is thin red rod-like organism.

Task 3. Learn the chemical structure of media for cultivation of tubercle bacilli. Examine the growth of mycobacteria (*M. tuberculosis*) on Löwenstein-Jensen medium, make the conclusion.

The solid media contain egg (Löwenstein -Jensen, Petragnini’s or Dorset’s), blood (Tarshis’ medium), serum (Loefller's serum slope) or potato (Pawlowsky's). The solid medium most widely employed for routine culturing is the Löwenstein -Jensen medium without starch, as recommended by the International Union against tuberculosis. It consists of coagulated hen's egg, mineral salt

solution, asparagine and malachite green, the last acting as a selective agent inhibiting other bacteria.

A simple medium containing only eggs, malachite green and coconut water has been reported to be a useful and cheap alternative to the Lowenstein-Jensen medium.

Among the several liquid media described, Dubos', Middlebrook's, Proskauer and Beck's, Sula's and Sauton's media are the most common. On solid media *M. tuberculosis* forms dry, rough, raised, irregular colonies with a wrinkled surface. They are creamy white initially, becoming yellowish or buff coloured later. They are tenacious and not easily emulsified. The colonies of *M. bovis* are in comparison, flat, smooth, moist and white, breaking up easily when touched. Liquid media are not generally employed for routine cultivation but are used for sensitivity tests, chemical tests and preparation of antigens and vaccines. In liquid media without dispersing agents, the growth begins at the bottom, creeps up the sides and forms a prominent surface pellicle that may extend along the sides above the medium. Diffuse growth is obtained in Dubos' medium containing a detergent Tween-80 (sorbitan monooleate). Virulent strains tend to form long serpentine cords in liquid media, while avirulent strains grow in a more dispersed manner. The cord factor by itself is not responsible for virulence. It is present in some nonpathogenic species of mycobacteria as well. Colonial morphology may be modified by the presence of bacteriophage in the strain. Tubercule bacilli may also be grown in chick embryos and in tissue culture.

Task 4. Study the differential properties of mycobacteria.

For identification of the selected cultures of tuberculosis causative agents and their differentiation from other types of mycobacteria lot of signs are used.

The differential properties of mycobacteria for identification

	Time of growth, days	Catalase, 68 °C	Urease	Nicotin-amidase	Niaci-nase	Nitratere-ductase	Pigment
M. tuberculosis	12–25	–	±	+	+	+	–
M. bovis	24–40	–	+	–	–	–	–
M. africanum	31–42	–	+	+	–	–	–
M. kansasii	10–20	+	+	+	–	+	+
M. avium	10–12	+	–	+	–	+	–
M. smegmatis	3–5	±	+	+	–	+	–

Niacine test and ability to synthesize plenty of nicotin acid (niacinum) help distinguish *M. tuberculosis* from other mycobacteria. Catalase activity is relatively weak and is lost at 68 °C.

Task 5. Read the results of sensitivity tests to antibiotics of *M. tuberculosis*, make the conclusion. Draw the slant and the growth of mycobacteria in the test tubes. Make the conclusion.

The determination of MBT sensitivity to antimycobacterial preparations is of paramount importance for the treatment tactics, correction of antimycobacterial therapy and the illness prognosis. MBT sensitivity to antituberculous preparations is defined by the preparation minimum concentration, which inhibits MBT growth on the nutrient medium. MBT are considered to be sensitive to either preparation if less than 20 colonies have grown in a test tube, with abundant growth in the control. The culture is supposed to be stable if more than 20 colonies have grown. MBT are considered to be stable if they grow at the concentrations of the preparation in 1 ml of the nutrient medium: for isoniazidum – 1 μg, rifampicinum – 20 μg, streptomycini – 5 μg, ethambutolum – 2 μg, all other preparations – 30 μg.

Medicinal resistance of tuberculosis causative agents is determined by the method of the serial delutions before the beginning of treatment, in 3 months and farther at continuation of tubercular rods isolation of through each 6 months. It is done by growing of cultures on medium with the

different concentration of tuberculostatics. The most widespread methods of mycobacteria medicinal stability determination are cultivation on the solid Lowenstein-Jensen medium; slide microcultivation by Price's method; deep inoculation in semisynthetic agar medium. Pure culture suspension is inoculated in test tubes that contain different concentration of preparations and control (without tuberculostatics). A culture is sensible, if in the test tube there are less than 20 colonies. Culture is steady if more than 20 colonies grew. Resistance of this culture expresses the maximal concentration of antibacterial preparation, at which yet there is growth, close growth in a control test tube.

Scale of estimation of mycobacteria resistance to medicinal preparations

Preparation	Resistance at growth on media, which contain preparation, µg/ml	
	solid	liquid
Streptomycini sulfats	5	
Isoniazid	1	
Rifampicin	5	0
Ethambutol	50	0
Kanamycin sulfats	25	0
Ethionamide	30	
Biomycin	30	0

Task 6. Study the antimicrobial agents for treatment of tuberculosis. Write the main data to the protocol.

Isoniazid is the principal representative of hydrazide isonicotine acid group (HINA), the most effective among all antimycobacterial drugs, strictly specific only against MBT, penetrates through cell and tissue membranes and through haematoencephalic barrier well.

Rifampicin is a semisynthetic antibiotic with a wide action spectrum. The drug possesses an expressed bacteriostatic activity to MBT, which are distributed extracellularly and in the cells (intracellularly), as well as to the ones that multiply quickly and slowly.

Task 7. Study and write into the protocol information about the main diagnostic preparation.

Tuberculin is an allergen that is used for tuberculin skin test or Mantoux test. It is used for determination of population infection, mass inspection, tuberculosis of children and teenagers, detection of persons, which need to undergo revaccination, check up its efficiency. In addition, this test is used with the purpose of diagnostics of tuberculosis and determination of activity of infectious process. Tuberculin is prepared of mixture of human and bovine *Mycobacteria* types, inoculation is held in glycerin pure broth. A culture is sterilized in steam for 30 minutes, evaporated, filtered through a bacterial filter and poured out in ampoules. Preparation is introduced subcuteneously.

Lepromin (allergen) is prepared of sterilized in the autoclave of the staggered fabrics that contain plenty of mycobacteria. Lepromin introduced intracutaneously in the middle third of forearm in the volume 0.1 ml. In 48 hours in positive cases spot of eritema or papula is formed (Fernandes's reaction), later (1–2 months) tubercle can appear, often with necrosis (Mitsuda's reaction). In patients with lepromatous form and healthy people an allergic reaction is negative, in patients with tuberculoid form the test is positive. The test does not have a diagnostic value, it is used only for determination of clinical form of disease and prognosis.

Erythrocytic tuberculosis diagnosticum is used in serum diagnosis of tuberculosis for PHAT carrying out, which is put with the purpose of exposure of specific antibodies in the patient's blood serum. On the surface of sheep red blood cells, the phosphatidic antigen of *M. tuberculosis* is adsorbed. Serum method belongs to the additional methods of tuberculosis diagnosis. A positive reaction is marked at active tubercular process, at infection of tuberculosis mycobacteria and after vaccination.

BCG (bacillus Calmette-Guerin) is live or attenuated, liophilically dried up culture of unpathogenic *M. tuberculosis* strain. It was found by the French scientists Calmette and Guerin. It is used for the active specific prophylaxis of tuberculosis. It is put down into the calendar of inoculations. It is contraindicated for people with violation of cellular immunity.

BCG-M differs from previous preparation only by an amount of mycobacteria (less than 2 times). It is intended for an active specific prophylaxis for hyposthenic children.

Table 3.13. 3 – Laboratory diagnostics of the tuberculosis and leprosy

Notion	Definition/explanation
Etiopathogenesis of tuberculosis	Tuberculosis is an infectious disease and its causative agent is *Mycobacterium tuberculosis* (MBT) of the genus *Mycobacterium* of *Actinomycetacae* family
Morphology of *Mycobacterium tuberculosis*	MBT looks like a bacillus 0.8 to 5 μg long, 0.2 to 0.3 μg thick. MBT can exist in various forms: typical rods, chips, L-forms and filtrating forms
Groups of mycobacteria	3 groups of mycobacteria are distinguished: true (pathogenic for a human being), atypical and acidic-stable saprophytes. There are three types (species) of pathogenic MBT: human (*M. tuberculosis*), bovine (*M. bovis*) and African (*M. africanum*)
Cord factor of MBT	Lipoid cell wall of mycobacteria forms its virulence and capacity for formation of bacteria as scythes in culture accumulations (cord factor). Cord-factor is related to the unusual biological matter of trehalose 6, 6-dimycolate, which possesses high virulence
L-forms of MBT	One of the important types of MBT changeability is L-forms forming. L-forms are characterized by reduced metabolism and loss of virulence. Remaining viabile, they can be in an organism and induce antituberculosis immunity for a long time
Resistance of *Mycobacterium tuberculosis* in the environment	All of them are very stable in the environment. Particularly, they are preserved in the soil for 1–2 years, in basins – up to 5 months, in the road dust – up to 10 days, in premises at the dissipated sunlight – up to a month and a half, in excrements and on pasture-grounds up to a year, in butter, cheese kept in a fridge – 8–10 months, on books' pages – 3 months. At the temperature of 20 °C MBT preserve their vital activity for 7 years. Boiling liquid sputum kills MBT within 5 minutes. Under the action of sun rays MBT die in an hour and a half, and that of ultraviolet radiation – in 2–3 minutes
Sourse of	Sick people and animals, secreting MBT, are the source of human

tuberculosis infection	tuberculosis infestation. A pathogene, depending on the affected organ, is secreted into the environment with sputum, excrements, urine, milk, sperm, etc. Infectation occurs most often by aero-genic (90%), contact (5–6%), rarely alimentary (1–2 %) and extremely rarely by intrauterine route
Matherial for microbiological investigation of tuberculosis	The materials for MBT revealing are sputum, bronchial lavage waters, faeces, urine, fistula pus (matter), pleural cavity exudate, spinal fluid, punctates and biopsy matherial of various organs and tissues

Table 3.13.3 continuation

Notion	Definition/explanation
Methods of mycobacteria investigation	Bacterioscopy (flotation method, luminescent microscopy). Bacteriological method. Biological method
Principle microscopic methods for tuberculosis diagnosis	Bacterioscopy is one of the main methods of MBT revealing; it includes ordinary bacterioscopy, flotation and luminescent microscopy. An ordinary bacterioscopy is accessible to everybody; it is simple and quick to do. In smears, stained by Zeihl-Neelsen method, MBT are revealed when not less than 50000 microbic bodies are present in 1 ml of pathologic material. Under the microscope MBT look like bacilli of the red colour on the blue background. Flotation method (enrichment or concentration of MBT in a small volume, caused by droplets of benzine, benzole, xylol or toluene on the surface of a retort ring) is applied in cases when there is a small number of MBT in the pathologic material and at negative results of ordinary bacterioscopy. Flotation method provides for 10–15% more often revealing MBT in comparison to direct bacterioscopy. Luminescent microscopy is based on the ability of MBT, stained with fluorochroms, to illuminate under the influence of ultraviolet rays and performing microscopy at small magnification, increasing by 15–30% the sensitivity of the method in comparison to direct bacterioscopy and by 10% in comparison to the flotation method
Principle of bacteriological method for tuberculosis diagnostics	Bacteriological method consists in the following: sputum or another material, after preliminary special treatment, is inoculated on nutrient media (hard, blood, semisynthetic). More often hard egg Löwenstein-Jensen medium is used. 20–100 microbial bodies in 1 ml of sputum is enough for revealing MBT culture. The first colonies appear on the 18–30th day of cultivation. The negative result is given only in 3 months of inoculaion. This method of revealing MBT allows to define their vitality, virulence, group (differentiate from acid resistant saprophytes and atypical MBT) and species origin, as well as their resistance to antimycobacterial preparations. In addition to this, according to the data of bacteriological examination quantitative assessment of bacterial secretion is made: up to 10 colonies on a nutrient medium, moderate – from 10 to 50 and massive – more than 50 colonies
Principle of biological method for tuberculosis diagnostics	It is infection with sputum or another pathologic material of guinea pigs, which are highly sensitive to MBT. A biological testing is the most sensitive method of revealing MBT, as far as in laboratory animals tuberculosis develops after the introduction of the material in which there may be less than 5 microbial bodies in 1 ml. However, it should be noted that MBT are stable to chemical preparations, particularly to isoniazidum,

	avirulent for guinea pigs. That is why various methods of microbiological examination should be used for revealing MBT in pathologic material. Generally, before starting to treat a patient, he should undergo complex bacteriological examination. It is three times direct bacterioscopy of sputum or, when it is absent, three times examination of the material after provoking inhalations or bronchial lavage fluid; with negative results – three times examination by flotation method; three times sputum inoculation on nutrient medium, irrespective of the results of the previous examinations, with a view to define MBT sensitivity to antimycobacterial

Table 3.13.3 continuation

Notion	Definition/explanation
	preparations. Further on, in the process of the treatment, the examination is repeated every month until the bacterial secretion ceases, and then once for 2 months till the end of the main course of chemotherapy
Tuberculin	Robert Koch obtained tuberculin for the first time (1890) which was later named old Koch's tuberculin (alt Tuberculin Koch – ATK). It is manufactured in ampules as 100% solution and is a liquid of dark-brown colour, which contains, in addition to specific active substances (tuberculo- proteins), products of MBT vitality, elements of their cells and the medium on which they grow. In 1934 F. Seibert obtained a more specific (dried) tuberculin preparation – purified protein derivative (PPD-S) – purified tuberculin protein derivate, for which bacteria were grown on a synthetic protein-free medium. In 1939 in the USSR M. A. Linnykova obtained an analogical tuberculin preparation, named PPD-L. One ampule contains 50000 TU of dry rectified tuberculin. The solvent is isotonic solution of sodium chloride with the addition of 0.25% carbolic acid. Preservation time is 5 years, in a dark place at the temperature of +4 °C. In Ukraine PPD is manufactured as a solution ready for use, the sterility of which is guaranteed by the presence of 0.01% chinozol in it. The solution is packed in ampules of 3 ml (30 doses) or in bottles of 5 ml (50 doses). Each dose (0.1 ml) contains 2 TU. 0.005 g of twin-80 is added to stabilize biological activity of the solution. In accordance to the WHO international standard 1 TU PPD-L contains 0.00006 mg of dry preparation. Tuberculin is an incomplete antigen and therefore it does not cause the formation of antibodies, but it calls forth a reaction in a sensibilized organism with a complete antigen (MBT, vaccine strain of BCG). Depending on the mode of tuberculin introduction, cutaneous Pierquet test (Pierquet, 1907), intracutaneous Mantoux (Mantoux, 1909) and subcutaneous Koch test (Koch, 1890)
Tuberculin skin test	Reactivity to intradermal injection of mycobacterial antigens can differentiate between infected and noninfected individuals. The tuberculin test is a measure of DTH as determined by the intradermal injection of 0.1 ml of intermediate strength purified protein derivative (PPD), which is a tuberculoprotein derived by fractionation of a broth culture filtrate of *M. tuberculosis.* A positive PPD reaction usually develops within 3 to 4 weeks after exposure. Some infected patients may have less than 10 mm (5–9 mm) induration, but this level of reactivity generally represents exposure to other mycobacteria. Less than 5 mm induration represents

	negative reaction
The aim of tuberculin skin test using	1. Early tuberculosis revealing. 2. Revealing persons with an increased risk of tuberculosis illness. 3. Contingent selection for BCG revaccination. 4. Determination of infestation index of MBT population. 5. Differential diagnosis between infectious and postvaccinal allergy
Posible results of Mantoux testing, which are estimated in 72 hours	1. Negative – absence of a papule or a papule after an injection to 1 mm. 2. Doubtful – a 2–4 mm papule or hyperaemia only. 3. Positive is a papule of 5 mm or more. 4. Hyperergy – in children and teenagers a papule of 17 mm and more, in

Table 3.13.3 continuation

Notion	Definition/explanation
	adults – 21 mm and more, and also for various age groups, reactions with the availability of vesicules, necrosis or lymphangitis, irrespective of the papule size
Individual tuberculin diagnosis	Depending on the indications at individual tuberculin diagnostics Mantoux test with 2 TU is applied, as well as with various tuberculin doses. Generally, Mantoux test with 2 TU is of importance for children and teenagers; for adults, in some cases, hyperergic results of Mantoux test testify to the active tuberculosis, while the negative ones – of tuberculosis absence, therefore sometimes the necessity arises to apply Koch's test (10-100 TU). A negative reaction to 100 TU of tuberculin with the probability of 97–98% allows to exclude tuberculosis infection. Koch's testing is done with a view of diagnostics and differential diagnostics of tuberculosis, the definition of the activity of tuberculosis process. Before the Koch's testing, Mantoux test with 2 TU is done for ascertaining the tuberculin titre. After this near the lower angle of a shoulder blade or in the upper third of the outer surface of the shoulder, after rubbing the skin with 70% ethylic alcohol, tuberculin is injected subcutaneously in the dose from 20 to 100 TU. Two or three days before the procedure the clinical blood analysis is done every day, the intake of bronchial lavage fluid for MBT, measuring the body temperature every 4 hours; a day prior to Koch's test the protein fractions of the blood serum are defined. In 24–48–72 hours after subcutaneous tuberculin injection the examinations analogous to those before the tuberculin injection are done. Roentgenological examination before and after Koch's testing (in 48 hours and on the 7th day) is performed depending on the process localization
Koch's test results	Koch's test results are evaluated in 24, 48 and 72 hours, based on the results of local, nidal and general reaction. The local reaction is supposed to be positive at the formation of subcutaneous infiltrate of the 15 mm size and more. The nidal is positive at the availability of the intensification of inflammatory reaction in the site of specific wound. The general (overall) is characterized by the worsening of the general state of the person under examination. It is rise of the body temperature (not less than 0.5 °C), joint pain, headache, increased disposition to perspire, as well as changes of the formula and protein fractions of the blood serum (each index, which has deviated not less than 20% from the initial index is taken into account). Simultaneous changes of not less than 3–4 indices are of diagnostic importance
Disposition	An active vaccination reduces the risk of contracting the disease by

prophylaxis of tuberculosis	about one-half. It contains the live vaccine BCG (lyophilized bovine TB of the Calmette-Guerin type). Vaccination of tuberculin-negative persons induces allergy and (incomplete) immunity that persists for about 5 to 10 years. In countries with low level of tuberculosis prevalence, the advisory committees on immunization practices no longer recommend BCG vaccination, either in tuberculin-negative children at high risk or in adults who have been exposed to TB. Preventive chemotherapy of clinically inapparent infections (latent tuberculosis bacterial infection, LTBI) with INH (300 mg/d) over a period of six months has proved effectivity in high-risk persons, e.g., contact persons who therefore became tuberculin-positive, in tuberculin-positive persons with increased susceptibility

Table 3.13.3 continuation

Notion	**Definition/explanation**
	(immunosuppressive therapy, therapy with corticosteroids, diabetes, alcoholism) and in persons with radiologically confirmed residual tuberculosis. Compliance with the therapeutic regimen is a problem in preventive chemotherapy
Contraindications for BCG vaccination	1. Prematurely born child, when the body mass at birth is less than 2000 g. 2. Intrauterine infection. 3. Purulent septic diseases. 4. Haemolytic disease of newborns (moderate and severe forms). 5. Severe puerperal traumas with neurologic symptomatic. 6. Generalized skin wounds. 7. Any acute illness. 8. Generalized BCG infection of other children in the family. Children (babies), not immunized at a maternity home, in connection with contraindications are vaccinated after recovery at a children's polyclinic or hospital-assistant's health station with BCG-M vaccine during 1–6 months. However, if a baby has reached a 2-months age and more, the Mantoux test with 2 TU should be done before inoculation. Children with negative tuberculine reaction are vaccinated. The interval between Mantoux test and vaccination must be not less than 3 days and not more than 2 weeks.
Morphology and culture properties of *Mycobacterium leprae*	In morphological terms, these acid-fast rods are identical to tuberculosis bacteria. They differ, however, in that they cannot be grown on nutrient media or in cell cultures
Epidemiology of leprosy	Person-to-person spread by direct contact or inhalation of infectious aerosoles. People in close contact with patients who have lepromatous disease are at greatest risk
Pathogenesis and immunity of leprosy	Leprosy manifests as tuberculoid leprosy or lepromatous leprosy forms. Patients with tuberculoid leprosy have a strong cellular immune reaction but a weak humoral antibody response. Infected tissues typically have many lymphocytes and granulomas but relatively few bacilli. Patients with lepromatous leprosy, however, have a strong antibody response and specific defect in the cellular response to *M. leprae* antigens. Thus, an abundance of bacilli are typically observed in dermal macrophages and Schwann cells of the peripheral nerves. As would be expected, this is the most infectious form of leprosy

Table 3.13.3 continuation

Notion	Definition/explanation
Treatment of leprosy	Although at one time the sulfone drug dapsone was the preparation of choice in the treatment of leprosy, the development of a high degree resistance has prompted the recommendation of combined dapsone, rifampin, and clofazimine for patients with lepromatous leprosy and dapsone and rifampin for those with the tuberculoid type

Mantoux test is performed to evaluate whether a person has been exposed to tuberculosis. If there has been a prior exposure, sensibilized lymphocytes are formed and remain in the body. During the skin test, the tuberculosis antigen is injected under the skin and if sensebilized lymphocytes are present, the body will have an immune response. There will be an area of inflammation at the site of the injection.

Scheme leprosy laboratory diagnosis

LABORATORY DIAGNOSIS OF PLAGUE AND TULAREMIA

Theme topicality. Causative agents, which you can learn in this topic, are related to highly pathogenic and particularly dangerous diseases for the humans and animals. Their biological properties are such that in the absence of the professional intervention when these diseases occur the epidemics of these diseases may occur. The course of the disease is usually severe. Plague in the absence of medical intervention can lead to the death of the patient. Causative agents of these diseases are the basis of biological weapons developed earlier in some countries. Natural and geographical characteristics of Ukraine are such that there are conditions for human infection with any of these pathogens and epidemiological outbreaks of these diseases are missing only through the efforts of health services.

Primary objective: to be able to conduct and evaluate the microbiological diagnosis of plague and tularemia.

QESTIONS FOR DISCUSSION

1. Special danger infections.
2. Biological properties of *F. tularensis* causative agent of tularemia.
3. Microbiological diagnosis of tularemia.
4. Biological properties of *Y. pestis* causative agent of plague.
5. Microbiological diagnosis of plague.
6. Specific prevention of special danger infections.

PROCEDURE OF PRACTICAL WORK

Task 1. Examine microscopically the smears of the patient's materials, fill in the protocol.

Yersinia pestis is oval in shape, length to 2 mm, width – 0.3–0.7 microns, gram-negative. The bacteria are isolated. In preparations that are prepared of cultures growing on the solid media may be of bacillary form, similar to *E. coli*. The bipolar staining (poles are coated more intensely than the central part of cells) is seen better if the smears are stained by methylene blue by Lefler’s method than Gram’s method.

Francisella tularensis is very small gram-negative coccobacteria (the forms are intermediate between rhabdoid and orbed) with pronounced polymorphism. Certainly, their size is 0.2–0.7 microns.

Task 2. Study the growth of the *Francisella tularensis* on the solid media.

Francisella tularensis forms small white colonies on the solid media.

Task 3. Study and write to the protocol information about immunobiological preparates for diagnostics, treatment and prophylaxis of the plague and tularemia.

Tularemia diagnosticum is a frozen culture of the tularemia causative agent in the isotonic sodium chloride solution with formalin. Diagnosticum contains 25 billion microbial bodies in 1 ml. It can be used for blood-drip reaction (used in undiluted form); tube agglutination test to determine the type of Widal's reaction (in this case the diagnosticum is diluted by isotonic sodium chloride solution at 5 times). The tube agglutination test is often made with paired sera for detection the growth of antibody titre. Serum of the patient is diluted as 1:12.5 to 1:400. Reaction is considered positive if the titre is 1:100 and more.

Pestilential erythrocytic antibody diagnosticum is erythrocytes with adsorbed on them antibodies against the causative agent of plague. It is used to identify the causative agent in PHAT in rapid diagnosis.

Pestilential bacteriophage contains a live bacteriophage. It is used for the phagolysis reaction to identify isolated pure cultures in bacteriological method of diagnosis.

Tularin is allergen. There are two types of tularin and two administration methods: tularin for dermal administration and tularin for intradermal use. The first preparation is more appropriate for mass examination for detection of the postvaccinal immunity or immune layer among the population. Intradermal tularin is used for diagnosis of acute tularemia. Various tularins contain various concentrations of allergens, so it is not recommended to use dermal tularin for intradermal tests and vice versa.

Plague live EV vaccine is dried culture of the *Y. pestis* of the live vaccine strain EV. It is used for prevention of plague by epidemiological indications.

Tularemia live vaccine contains suspension of live microorganisms of tularemia causative agent vaccine strain. People living in the epizootic tularemia territory and arriving to such places are liable to vaccination. Vaccination is supracutaneous and one-time. In 1–2 weeks, the efficiency of the vaccination is verified by making the allergic skin test with tularin or serological reaction (AT and PHAT). In case of the negative result, make revaccination. The vaccine provides immunity, which is kept to 5–15 years.

Immunoglobulin antipestilential is gamma globulin fraction from the blood serum of the hyper-immunization vaccine EV horses. It is used with preventive and curative purposes.

Plague serum labeled FITS is used for direct IFT when rapid diagnosis of plague is necessary.

Tularemia serum labeled FITS is obtained in a classical way. It is used to identify the causative agent in blood smears and impressive smears of the animal organs that are contaminated by the patient's material (bubo bioptate, sputum, etc.).

Task 4. Read the PHAT with the patient's paired sera and make the conclusion.

The serological method refers to additional methods of plague diagnosis. PHAT is combined with the patient's serum.

The purpose of the test is antibodies detection to the plague antigens (it is adsorbed on the surface of sheep erythrocytes), which may indicate the causative agents of the disease (plague). Positive reaction appears as turned umbrellas.

Scheme plague laboratory diagnosis

Scheme tularemia laboratory diagnosis

LABORATORY DIAGNOSIS OF ANTHRAX AND BRUCELLOSIS

Theme topicality. Anthrax, a prototype disease in the history of microbiology, is caused by *Bacillus anthracis*. Anthrax remains an important disease of animals and occasionally of humans. *B. anthracis* is a major agent of bioterrorism and biological weapons.

The disease caused by brucella is called brucellosis (undulant fever, Malta fever). It is characterized by an acute bacteremic phase followed by a chronic stage that may extend over many years and may involve many tissues.

Primary objective: to be able to conduct microbiological investigation of anthrax and brucellosis.

QUESTIONS FOR DISCUSSION

1. Biological properties of the *B. anthracis* and brucellae, antigenic structure.
2. Microbiological diagnosis of athrax and brucellosis.
3. Epidemiology and pathogenesis of athrax and brucellosis. Features of immunity at such diseases.
4. Principles of prophylaxis and medical treatment of athrax and brucellosis.

PROCEDURE OF PRACTICAL WORK

Task 1. Carry out precipitation test in tube acorrding to the table 15.1.

Table 3.15.1 – Precipitation test with investigated material

Ingredient	Test	Control of Ag	Control of serum
0.9% NaCl solution, ml		0.5	0.5
Diagnostic serum , ml	0.5		0.5
Diagnostic material extract, ml	0.5	0.5	
Result	+	–	–

Task 2. Study the smears of patient's materials.

B. anthracis is the typical cells, arranged in long chains, have square edns; spores are located in the centre of the nonmotile bacilli.

B. anthracis in tissue is gram-positive rods, which form capsule.

B.melitensis is gram-negative, nonmotile, nonspore-forming coccobacilli.

Task 3. Study and write to the protocol information about immunobiological preparations for diagnostics, treatment and prophylaxis of athrax and brucellosis.

Brucellosis diagnosticum is common for Hedelson's and Wright's test. It consists of killed brucrella, 12% NaCL and aniline due.

Anthraxin is an allergen. The allergogenic diagnosis is based on the ability of the patient's organism to react to the injection of the anthraxin by local reddish skin. The skin-allergic test is made into the forearm. The result will be estimated in 24–48 hours. The reaction will be positive if oedema and redness of the skin are more than 8 mm within 24 hours. The result of the test must be estimated in complex with clinical manifestation.

Brucelin is used for making the skin allergic Burne's test. It is 0.1% solution of the polysaccharide-protein complex that is received from vaccine strain by vinegar hydrolysis. The result of the reaction is estimated in 24 hours. Presense of the intensive oedema of the skin is a positive reaction. Absence of the local pain and redness do not except the positive test.

Live anthrax vaccine contains the spore of the avirulent noncapsular strain. It is used for planned vaccinal prevention of people with high risk of anthrax infection and also by epidemiological indications.

Live brucellosis vaccine contains live cow-type brucella. It is used for prevention of brucellosis by epidemic and epizootic indications. However, the vaccination is considered as a temporary prophylactic means in brucellosis control. The main thing is strict observance of sanitary and veterinary rules and making the complex of the nonspecific measures.

Killed brucellosis vaccine contains the killed by heat the cow-type and sheep-type species of the brucella. It is used for immunotherapy at chronic brucellosis. It has high sensibilization and allergic ability and is used only for patients with normegric reaction of the organism to the intracutaneous injection of the brucelin. For the patients with hyperergic and allergic reaction and with generalized infection the vaccination is dangerous. The intracutaneous therapy is performed more often than intravenous. The injection of the brucelin is useful for the same patients.

Antianthrax gamma globulin is a preparation containing antitoxins. It is gamma globulin fraction of serum of the hyperimmunized animals. There is the diminished amount of ballast substance in such preparation that diminishes probability of by-reactions development.

Antianthrax precipitating serum is received by hyperimmunisation of animals by killed bacteria. It is used for thermoprecipitation that allows to detect thermostable antigens of *B. antracis* in very small quantity. The antigen is extracted by boiling the investigated materials (skin, wool, fur).

Anthrax luminescent serum is used for detection of the antigens in patient's materials in IFT. It consists of specific antibody with luminescent label.

Scheme antrax laboratory diagnosis

Scheme brucelosis laboratory diagnosis

LABORATORY DIAGNOSIS OF SPIROCHAETOSIS (SYPHILIS, BORRELIOSIS, AND LEPTOSPIROSIS)

Theme topicality. Syphilis is a social and venereal disease. There was an increase in number of the patients with syphilis in comparison with 1980–90ies. Borellia may cause epidemic relapsing fever. It does not occur today, but was widely spread in wartime. Newcomers get endemic relapsing fever in the endemic regions. The water rats play significant role in spread of leptospirosis. People having contact with infected water (fishers, swimmers in the lake and river) suffer from leptospirosis mainly.

Primary objective: to be able to conduct microbiological diagnosis of syphilis, borreliosis and leptospirosis and estimate its results.

QUESTIONS FOR DISCUSSION

1. General description of bacteria of the family *Spirochaetes*.
2. Microbiological diagnosis of leptospirosis.
3. Microbiological diagnosis of boreliosis.
4. Biological properties of *T. pallidum*.
5. Microbiological diagnosis of *T. pallidum*.
6. Principles of prophylaxis and medical treatment of syphilis.

PROCEDURE OF PRACTICAL WORK

Task 1. Carry out venereal disease research laboratory (VDRL) or rapid plasma reagin (RPR).

When you carry out venereal disease research laboratory (VDRL) you must put 0.1–0.2 ml of the patient's serum in the tubes, then add carefully the dilution of the antigen. If you do everything correct, the measure between serum and antigen will be clear. The tubes are incubated at 37 °C for 30 minutes. In work time, you must be careful because the patient's serum may contain spirochaetes. If the result is positive, white ring will be at the measure between serum and antigen.

Task 2. Carry out Wassermann's test according to the table 16.1.

Table 3.16.1 – The Wassermann's test with patient's sera

Tube number Content	1 (test)	2 (serum control)	3 (antigen control)
Patient's serum	0.5	0.5	–
Antigen	0.5	–	0.5

Complement	0.5	0.5	0.5
0.9 % NaCl	–	0.5	0.5
37 °C, 30 minutes			
Haemolytic mixed	1.0	1.0	1.0
37 °C, 60 minutes			
Result			

Task 3. Study the smears of patient's materials and draw them in the protocol.

***T. pallidum* in IFT**. Tissue fluid or exudate is spread on a glass slide, air dried, and sent to the laboratory. It is fixed, stained with a fluorescein-labeled antitreponeme serum, and examined by means of immunofluorescence microscopy for typical fluorescent spirochaetes.

T. pallidum is a slender spiral measuring about 0.2 μm in width and 5–15 μm in length; it has 10–12 wave curls and actively moves. It will have green luminescence in IFT test.

T. pallidum in the the dark field examination. 1–2 drops of tissue fluid or exudates are placed on the slide. Make a thin layer. The preparation is examined under oil immersion with dark field illumination for typical motile spirochaetes.

Borelia persica stained by Giemsa's method are purple irregular spirals 10–30 μm long and 0.3 μm wide. The distance between turns varies from 2 to 4 μm.

Leptospira interrogans does not stain with aniline. Leptospirae have 18–20 prime coils and two secondary (one of the each ends).

Task 4. Describe the immunobiological preparations for diagnostics, treatment and prophylaxis of spirochetosis.

Cardiolipin antigen for Wassermann's test is an extract of bull's cardiac muscle lipids with addition of 0.25–0.3% cholesterin and lecithin. It has permanent composition and does the standard results.

Specific antigen for Wassermann's test is made of spirochaetes, which are growing in the rabbit testicles and are sonicated.

Cardiolipin antigen for venereal disease research laboratory on the slide is a solution of the cardiolipin, cholesterin, and lecithin.

Wassermann's test is based on the fact that the particles of the lipid antigen react with specific antibody and fix complement. If the test is positive, we will see the sediment of the erythrocytes at the bottom of the tube.

Killed leptospirosis vaccine contains killed by heat and conserved by phenol leptospires. It is used by epidemiological indications.

Algoritm of action during conduction of syphilis microbiological diagnosis

***T. pallidum* immobilization tests (TPT)** – dilutions of the serum are mixed with complement and with live, actively motile *T. pallidum* from the testicular chancre of the rabbit, and the mixture is observed microscopically. If specific antibodies are present, spirochaetes are immobilized; in normal serum active motion continues.

Scheme syphilis laboratory diagnosis

Scheme epidemic relapsing fever laboratory diagnosis

Scheme leptospirosis laboratory diagnosis

LABORATORY DIAGNOSIS OF RICKETTSIAL DISEASES

Theme topicality. Rickettsia diseases are caused by small bacteria that are obligate intracellular parasites and – except for Q fever – are transmitted in humans by arthropods.

Primary objective: to be able to conduct and evaluate the microbiological diagnosis of rickettsial diseases.

QUESTIONS FOR DISCUSSION

1. General description of bacteria of the family *Rickettsia*.
2. Microbiological diagnosis of rickettsial diseases.

PROCEDURE OF PRACTICAL WORK

Task 1. Carry out and estimate haemagglutination test.

The main method of rickettsial diseases diagnosis is serological. This test is carried out with patient's paired sera. There is four times increase of antibody titre in the second serum (patient's serum taken after 14 days from the disease onset). It has diagnostic value. When you will make the passive haemagglutination test (PHAT) gut 5 drops of the different solvent of the serum in the hole of the plane-table, then add 1 drop of diagnosticum in each hole.

Table 3.17.1 – Haemagglutination test with patient's paired sera

Ingredient, ml	**Number of the test wells**							**Control**	
	1	2	3	4	5	6	7	8	9
0.9% solution of NaCl	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Assayed serum 1:62.5	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	–
Diagnosticum	0.1	0.1	0.1	0.1	0.1	0.1	0.1	–	0.1
Serum dilution	1:125	1:250	1:500	1:1000	1:2000	–	–	1:125	–
Control erythrocytes	–	–	–	–	–	–	–	0.1	–
Standard immune serum, diluted to the titre	–	–	–	–	–	–	–	–	–

Task 2. Study the smears of patient's materials and draw them in the protocol.

R. prowazekii is ovoid cells with green luminescence around them in the picture.

R. prowazekii are red rods in the blue cytoplasm of the epithelial cell.

R. prowazekii are small black rods in cytoplasm of the epithelial cells and in the intracellular spaces.

Task 3. Describe the immunobiological preparations for diagnosis, treatment, and prophylaxis of spirochaetosis.

Type-specific Muzer's serum for CFT is blood serum of the hyperimmunized animals. The preparation is used for diagnostic in complement fixation test for detection of *Rickettsia prowazekii* antigen.

Ricketsia epidemic typhus prowazekii erythrocyte diagnosticum for PHAT is erythrocytes bound with specific antigens. The preparation is used for diagnosis in PHAT for determination of specific antibody.

Coxiella burnetti dry antigen for CFT, Muzer's dry antigens for CFT are the preparations that are used for diagnostic, in complement fixation test for specific antibody detection.

Table 3.17.2 – General characteristic of ricketsial diseases

Group	Organism	Disease	Vector	Mammalian reservoir	Clinical features	Diagnostic test
Typhus group	*Rickettsia prowazekii*	Epidemic typhus, Brill-Zinsser disease	Louse	Humans	Fever, chills, myalgia, headache, rash	Serology
	Rickettsia typhi	Endemic typhus, murine typhus	Flea	Rodents	Fever, myalgia, headache, rash; mider typhus then epidemic typhus	Serology
Other	*Coxiella burnettii*	Q fever	None (airborne transmission)	Sheep, cattle, goats, etc.	Fever, fatigue, headache, pneumonia, can have major complications	Positive CFT to phase I, II antigens

Table 3.17.3 – Laboratory diagnosis of ricketsial diseases

Notion	Definition/explanation
Morphological, physiological and tinctorial features of *Rickettsiae*	Pleomorphic, asporogenic, immobile short rods, 600x300nm size, or cocci, and they occur singly, in pairs, in short chains, or in filaments. At Giemsa staining, they are blue; at Macchiavello's staining they are red, in contrast with the blue-staining cytoplasm around them. They have cell wall gram-negative bacteria and microcapsules
Distribution in nature: the bacteria of the family *Rickettsiae*	1. Isolation of rickettsia should be carried out only in reference laboratories for biosafety. 2. Rickettsiae lose their activity when they are stored at 0 °C, or incubated for a few hours at 36 °C. 3. Rickettsiae grow on different parts of the cell. 4. Rickettsiae are quickly destroyed by heat, drying, bacteriacidal chemicals

Table 3.17.3 continuation

Notion	Definition/explanation
Pathology	Rickettsiae multiply in endothelial cells of small blood vessels and produce vasculitis. The cells become swollen and necrotic; there is thrombosis of the vessel, leading to rupture and necrosis. Vascular lesions are in the skin and other organs

Pathogenic factors	Pili, endotoxin, some surface proteins, phospholipasae A2
Antigens of rickettsiae	Lipopolysaccharides, glycoproteins
Epidemiology of epidemic typhus, Brill-Zinsser disease	The louse obtains the organism by stinging infected human beings and transmits the agent by faecal excretion on the surface of the skin of another person. The scratching of the area of the bite allows rickettsiae excreted in the faeces to penetrate the skin. Brill-Zinsser disease is a recrudescence of an old typhus infection. The rickettsiae can persist for many years in the lymph nodes of the individual without any manifested symptom
Epidemiology of endemic typhus	*Rickettsia typhi* has reservation on the rat. The fleas can carry rickettsiae from fleas to human
Epidemiology of Q fever	*Coxiella burnettii* is found in ticks, which transmit the agent to sheep, cattle, and goats. Worker in slaughterhouses and in the plants that process wool and cattle hides have contracted the disease because of handling infected animal tissues. *C. burnetti* is transmitted by the respiratory route rather than through the skin, sometimes through milk
Methods of endemic and endemic typhus microbiological diagnosis	Serological, biological, and microscopic examination and cultivation in cell cultures and chick embryo
Serological tests for rickettsiosis diagnosis	CFT, tube agglutination test, PHAT, IFT, ELISA
Methods of Q fever microbiological diagnosis	Serological, biological, microscopic examination, cultivation in cell cultures and chick embryo and skin allergic test
The way *C. burnetii* is differentiated from other rickettsiae	*C. burnetii* have structure lipopolysaccharide (phase 1) or have none (phase 2) in cell wall
Treatment of rickettsial diseases	Antibiotics
Prevention of epidemic typhus	Live epidemic typhus vaccines, killed epidemic typhus vaccines
Immunity formed after epidemic typhus	Cellular and humoral
Prevention of Q fever	Live *Coxiella burnetii* vaccines

Scheme rickettsial diseases laboratory diagnosis

LABORATORY DIAGNOSIS OF MYCOPLASMAL AND CHLAMYDIAL DISEASES

Theme topicality. There are over 150 species in the class cell wall-free bacteria known as Mollicutes. In humans, four species are of primary importance: *Mycoplasma pneumonia, Mycoplasma hominis, Mycoplasma genitalium,* and *Ureaplasma urealiticum.* It causes pneumonia, urethritis, postpartum fever, and other infections.

Primary objective: to be able to conduct and evaluate the microbiological diagnosis of mycoplasmal and chlamydial infections.

QUESTIONS FOR DISCUSSION

1. General description of bacteria of the family *Mycoplasma* and *Chlamydia*.
2. Microbiological diagnosis of the diseases caused by *Mycoplasma* and *Chlamydia*.
3. Epidemiology and pathogenesis of the diseases caused by *Mycoplasma* and *Chlamydia*. Features of immunity in such diseases.
4. Principles of prophylaxis and treatment of the diseases caused by *Mycoplasma* and *Chlamydia*.

PROCEDURE OF PRACTICAL WORK

Task 1. Inoculate the patients' sputum with candidiasis pneumonia on the medium.

Inoculate 0.1 ml of the diluted (1:100) sputum on Petri dish with potatoes medium. Spread the material on the surface of the media in order to obtain growth in the form of lawn. After that, put Petri dish for incubation.

Task 2. Examine microscopically the smears of the patient's materials, fill in the protocol.

The arrow shows the inclusion of chlamydia in cytoplasm of the epithelial cells.

Chlamydiae have distinctive staining properties (similar to those of rickettsiae). Elementary bodies stain purple with Giemsa staining in contrast to the blue of host cell cytoplasm. The larger, noninfective reticulate bodies stain blue with Giemsa staining.

Task 3. Study the growth of the *Mycoplasma hominis* on the solid medium.

Mycoplasma hominis has been sealed to prevent evaporation, isolated colonies measuring 20–500 μm can be detected with the hand lens. These colonies are round, with a granular surface and a dark centre typically buried in the agar. They can be subcultured by cutting out a small square of agar containing one or more colonies and streaking this material on a fresh plate or dropping it into liquid medium.

Task 4. Study and write to the protocol information about immunobiological preparations for diagnosis of chlamydial infection.

Dry chlamydia diagnosticum for CFT is used for diagnosis in complement fixation test for specific antibody detection. It contains killed chlamydia.

Scheme chlamydial diseases laboratory diagnosis

Scheme mycoplasmal diseases laboratory diagnosis

MEDICAL MYCOLOGY

Theme topicality. Fungal infection is caused by the type of fungus called dermophyte that infects the top layer of the skin, hair or nails. Fungal infections of the skin are known as ringworm (tinea). There are many types of ringworms, including body ringworm (tinea corporis), jock itch (tinea cruris), athlete's foot (tinea pedis), scalp ringworm (tinea capitis), nail ringworm (tinea unguium), and beard ringworm (tinea barbae), which is rare. In most cases, these infections are not life threatening. However, they may lead to more serious bacterial infections in the elderly and those who have conditions that affect the immune system, such as AIDS.

Fungal infections such as ringworm are caused by types of fungi that are like worm, moist areas of the skin, such as between the toes or fingers, in the groin, and on other parts of the body where there are folds of skin. A worm does not cause ringworm. The fungus can be carried by kittens and puppies, combs, brushes, pillows, hats, and towels, and is found in areas that are warm and moist, such as locker rooms and showers.

There are variables, which can incrise the susceptibility to fungal infection. If patients have recently taken a course of antibiotics or taken oral steroids, they are more likely to contract an infection. In addition, if patients have diabetes or cancer, or if HIV has weakened their immune system, fungal infection is not common.

Primary objective: to be able to conduct and evaluate the microbiological diagnostics of fungal diseases.

QUESTIONS FOR DISCUSSION

1. General characteristics of fungi (definition and taxonomy, morphology).
2. General aspects of fungal disease (primary mycoses, opportunistic mycoses, subcutaneous mycoses, cutaneous mycoses, fungal allergies, and fungal toxicoses).
3. Primary mycoses (coccidioidomycosis, histoplasmosis, blastomycoses): characteristics of pathogen, pathogenesis, principle diagnosis, treatment and prophylaxis.
4. Opportunistic mycoses (surface and deep yeast mycoses, aspergillosis, mucormycoses, phaeohyphomycoses, hyalohyphomycoses, cryptococcoses; penicilliosis, pneumocystosis): characteristics of pathogen, pathogenesis, principle diagnosis, treatment and prophylaxis. Features of candidiasis diagnosis.
5. Subcutaneous mycoses (sporotrichosis, chromoblastomycosis, Madura foot (mycetoma): characteristics of pathogen, pathogenesis, principle diagnosis, treatment and prophylaxis.
6. Cutaneous mycoses (pityriasis versicolor, dermatomycoses): characteristics of pathogen, pathogenesis, principle diagnosis, treatment and prophylaxis.
7. Principle of antifungal immune response.

PROCEDURE OF PRACTICAL WORK

Task 1. Study microscopically material from the vagina.

The material for investigation was stained by Gram's method. The cells of *Candida spp*. are gram-positive. This micrograph illustrates the dimorphic nature of this fungus with yeast cells and mycelia both being present. Mycelial cells are more commonly found in cases of clinically apparent candidosis. *Candida spp.* will be yeast-like with asymptomatic infection.

Task 2. Examine microscopically the smears from the patient's materials, fill in the protocol.

Preparation is preliminary processed by 10 % KOH solution. Potassium hydroxide preparations are used to examine a variety of clinical samples including hair, nails, skin scrapings, fluids, or exudates. The potassium hydroxide solution serves to clear away tissue cells and debris, making the fungi more prominent. Slides must be examined with reduced illumination to allow fungal structures to be seen. A diagnostic value has arrangment of spores, presence of gas and drops of fat in the hair. Spores of fungus (*Microsporum spp*.) can be inside (endotrix) and outside of hair. Fill the results in the protocol.

Candida spp. is large gram-positive cells arranged like butterfly wings.

Task 3. Study the growth of the fungi on the solid media.

Trichophyton rubrum and *Trichophyton violaceum* can be cultivated on the Sabouraud dextrose agar. Material before inoculation is processed by antibiotics and incubating by temperature 30 °C. In positive cases, growth is observed on 3–5 days as various colonies. *Trichophyton rubrum* form colonies with a red pigment. This feature is characteristic only for this type of fungus.

Trichophyton violaceum forms colonies with a violet pigment. The colonies of this fungus at a senescence form white coverage (because they are covered with spores). The study of cultural properties of fungus needs identification.

Task 4. Study the growth of *Candida* from the patient's sputum (stage 2):

1. Count colonies of *Candida spp.*, determining the amount of fungus cells in 1 ml of the diluted sputum (1:100).
2. Prepare a smear, stain the preparation by Gram's method, microscopic examination, draw it in protocol.
3. Make a conclusion.

At determining the amount of fungi it is necessary to take into account in 1 ml of sputum, that a sputum was preliminary diluted 100 times, and 0.1 ml of the diluted sputum was inoculated on Petri dish. It is needed to count up the amount of colonies of fungus which grow on Petri dish and to increase the obtained number by 1000 (taking into account aforesaid). If amount of fungus $\geq$ 100 000/ml material, it gives ground to diagnose candidiasis. The amount of fungi from 50 000 to 100 000/ml material is an index higher than norm and for clarification of diagnosis, in this case, it is needed to perform the investigation in a few days. The isolation of the fungi colonies from patient without clinical manifestation are estimated as carrier.

Task 5. Study and write to the copybook the main diagnosis of suppurative diseases.

Polysaccharide antigen of Candida spp. for CFT is an extract of polysaccharide antigens of *Candida spp.*, used for diagnosis of candidiasis in serological method (for determination of specific antibodies in the patient's blood serum). The reaction (CFT) is conducted with paired sera.

Erythrocytes Candida diagnosticum contains the antigens of *Candida spp.* that are adsorbed on the erythrocytes. This diagnosticum is used for serological diagnosis of candidiasis in PHAT with paired sera.

Task 6. Study PHAT (demonstration) for serological diagnosis of candidiasis. Write the conclusion in the protocol.

The concentration of antibodies in candidiasis patient's serum is not of high titre, that is why it is important to trace the changes of their concentration during disease. Diagnostic value has a fact of 4 times and anymore increase of anticandidiasis antibodies titre. Positive reaction is characterized by formation of grainy sediment of erythrocytes with an unequal edge as an "umbrella", while in small holes, where the reaction is negative; there is compact sediment with an even edge as a "button".

The components of PHAT are: NaCl-solution, patient's paired sera (unknown antibody), erythrocytes candida diagnosticum (known antigen). Because *Candida spp.* is a normal microflora a serum reaction needs to be carried out in dynamics. Candidiasis is diagnosed on increase of antibody titre in the paired sera. For example:

Table 3.19. 1 – Laboratory diagnosis of mycosis

Notion	Definition/explanation
General characteristics of fungi	Microbiologists use the term fungus (*pl*., fungi; *Latin* fungus) to determine eukaryotic, spore-bearing, nonchlorophyllic organisms with absorptive nutrition that reproduce sexually and asexually. Scientists who study fungi are mycologists (*Greek* mykes, and logos, discourse). Scientific discipline dealing with fungi is called mycology. The study of fungal toxins and their effects is called mycotoxicology. The diseases caused by fungi in animals are known as mycoses. The five-kingdom system places the fungi in the kingdom Fungi. Fungi are eukaryotic microorganisms (domain eucarya) that occur ubiquitously in nature. Only about 200 of the thousands of species have been identified as human pathogens, and among these known pathogenic species fewer than a dozen are responsible for more than 90% of all human

Table 3.19.1 continuation

Notion	Definition/explanation
	fungal infections. The basic morphological element of filamentous fungi is hypha and web of intertwined hyphae is called a mycelium. The basic form of unicellular fungus is the yeast cell. Dimorphic fungi usually assume the form of yeasts in the parasitic stage and the form of mycelia in the saprophytic stage. The cell walls of fungi consist of nearly 90 % carbohydrate (chitin, glucans, and mannans) and fungal membranes are rich in sterol types not found in other biological membranes (e.g., ergosterol). Filamentous fungi reproduce either asexually (mitosis), by hyphal growth and tip extension, or with the help of asexual spores. Yeasts reproduce by a process of budding. Sexual reproduction (meiosis) on the other hand, produces sexual spores. Fungi imperfecti or deuteromycetes are the designation for a type of fungi in which the fructification forms are either unknown or missing entirely
Mycosis	A fungal infection in or on the part of the body

Morphology of fungi	Two morphological forms of fungi are observed: 1. Hypha – the basic element of filamentous fungi with a branched, tubular structure, 2–10 μm in width. Mycelium – the web- or mat-like structure of hyphae. Substrate mycelia (specialized for nutrition) penetrate into the nutrient substrate, whereas aerial mycelia (for asexual propagation) develop above the nutrient medium. Fungal thallus is an entirety of the mycelia, it is also called the fungal body or colony. 2. Yeast is the basic element of the unicellular fungi. It is round to oval and 3–10 μm in diameter. Several elongated yeast cells chained together and resembling true hyphae are called pseudohyphae. Some fungal species can develop either the yeast or the mycelium form depending on the environmental conditions. This property is called dimorphism. Dimorphic pathogenic fungi take the form of yeast cells in the parasitic stage and appear as mycelia in the saprophytic stage
Structure of fungi	The body or vegetative structure of a fungus is called a thallus (*pl*. thalli). It varies in complexity and size, ranging from the single-cell microscopic yeasts to multicellular molds, macroscopic puffballs, and mushrooms. The fungal cell usually is encased in a cell wall of chitin. Chitin is a strong but flexible nitrogen-containing polysaccharide consisting of N-acetylglucosamine residues. Yeast is a unicellular fungus that has a single nucleus and reproduces either asexually by budding and transverse division or sexually through spore formation. Each bud that separates can grow into new yeast, and some group together to form colonies. Generally, yeast cells are larger than bacteria, vary considerably in size, and are commonly spherical to egg shaped. They have no flagella but do possess most of the other eukaryotic organelles
Fungi reproduction	Asexual reproduction includes the vegetative propagation of hyphae and yeasts as well as vegetative fructification, i.e., formation of asexual spores. Asexual reproduction is accomplished in several ways: 1. A parent cell can divide into two daughter cells by central constriction and formation of a new cell

Table 3.19.1 continuation

Notion	**Definition/explanation**
	2. Somatic vegetative cells may bud to produce new organisms. This is very common in the yeasts 3. The most common method of asexual reproduction is spore production. Asexual spore formation occurs in an individual fungus through mitosis and subsequent cell division. There are several types of asexual spores: a) a hyphae can fragment (by the separation of hyphae through splitting of the cell wall or septum) to form cells that behave as spores. These cells are called arthroconidia or arthrospores. b) if the cells are surrounded by a thick wall before separation, they are called chlamydospores. c) if the spores develop within a sac (sporangium; *pl*., sporangia) at a hyphal tip, they are called sporangiospores. d) if the spores are not enclosed in a sac but produced at the tips or sides of the hypha, they are called conidiospores. e) spores produced from a vegetative mother cell by budding are called blastospores.

	Sexual reproduction in fungi perfecti (eumycetes) follows essentially the same patterns as in the higher eukaryotes. The nuclei of two haploid partners fuse to form a diploid zygote. The diploid nucleus then undergoes meiosis to form the haploid nuclei, finally resulting in the haploid sexual spores: zygospores, ascospores, and basidiospores. Sexual spores are only rarely produced in the types of fungi that parasitize human tissues. Sexual reproduction structures are either unknown or not present in many species of pathogenic fungi, known as fungi imperfecti (deuteromycetes)
General aspects of fungal disease	Fungal diseases fall into four clinical patterns: superficial infections on surface epithelial structures (skin, hair, nails), systemic infections of deep tissues, and subcutaneous and opportunistic infections, mycotoxicoses (aflatoxicosis)
Aspect of laboratory diagnosis	Laboratory diagnosis of fungal infection depends on the direct microscopic detection of fungal structures in clinical samples and/or the recovery in culture and subsequent identification of the fungus. Fungi may be isolated from a variety of clinical specimens representing the focus of infection (sputum, spinal fluid, tissue, pus aspirated from lymph nodes or other lesion, bone marrow aspirates, skin scrapings). All specimens of sufficient quantity submitted for fungal culture should be examined microscopically for fungi. When there is not sufficient specimen to allow both a culture and direct microscopic examination, the culture has priority over the smear because culture is more sensitive than microscopic examination. However, observing a fungus in a clinical specimen is often valuable in establishing the significance of the fungus (i.e., ruling out contamination) and in providing early information that may be crucial for determining appropriate patient therapy. In general, serological tests have limited application for the diagnosis of most fungal infections. Exceptions to this rule include certain dimorphic fungal diseases, such as histoplasmosis and coccidioidomycosis. The purpose of this laboratory exercise is to acquaint the student with some

Table 3.19.1 continuation

Notion	**Definition/explanation**
	direct microscopic and cultural methods that are available for establishing the laboratory diagnosis of a human mycosis Histopathology. The visualization of fungal structures (hyphae, conidia, etc.) in tissue obtained by biopsy or at autopsy. Specialized tissue stains such as Giemsa, methenamine silver or mucicarmine may be used to facilitate the detection of the fungus in tissue. The particular fungal structures that are seen in tissue can sometimes confirm the identity of the fungus (e.g., spherules (the yeast form) of *Coccidioides immitis* or cysts of neumocystis carinii) or suggest the presence of a particular fungal group. In this latter case, culture is used to confirm the presence and identity of the fungal pathogen. Direct smears of patient material other than tissue are often made to detect the resence of fungal elements microscopically. Several types of stains or reagents are used to facilitate the detection of certain fungi: 1. 10% KOH are used to examine a variety of clinical samples including

	hair, nails, skin scrapings, fluids, or exudates. The potassium hydroxide solution serves to clear away tissue cells and debris, making the fungi more prominent. Slides must be examined with reduced illumination to allow fungal structures to be seen. 2. Calcofluor white is used with most specimen types to detect the presence of fungi by fluorescence microscopy. The cell walls of the fungi bind the stain and fluoresce blue-white or apple green, depending on the filter combination used with the microscope. This stain is useful for examining skin scrapings for the presence of dermatophytes and tissues and body fluids for yeast and filamentous fungi 3. India ink is usually ordered to screen for the presence of *Cryptococcus neoformans* in spinal fluid samples. This yeast is encapsulated, and the capsule can be visualized readily against the black background of the India ink as a clear halo surrounding the yeast cell. The India ink test is very insensitive (detecting only 40% of cases of cryptococcal meningitis) and therefore has been superseded by other tests, such as the cryptococcal antigen latex agglutination test, which detects more than 90% of cases of cryptococcal meningitis. The India ink test is rarely performed in clinical microbiology laboratories. 4. Wright, Giemsa, or Diff-Quik stains. These specialized stains are often used on blood and bone marrow smears to look for intracellular yeast forms of *Histoplasma capsulatum*. 5. Most fungi are not stained well by the Gram staining procedure, and therefore, it is of limited use when examining specimens for fungal forms. It is generally reliable only for detecting the presence of *Candida species, Sporothrix schenkii*, and perhaps a few other fungi in clinical material. In Gram-stained spinal fluid specimens, *Cryptococcus neoformans* may appear as irregularly staining gram-positive yeast cells surrounded by an orange capsule. Culturing is possible on universal and selective mediums. Sabouraud dextrose agar can contain selective agents (e.g., chloramphenicol and cycloheximide), this medium has an acid pH of 5.6. The main identifying structures are morphological, in particular the asexual and, if present, sexual reproductive structures. Biochemical tests are used mainly to

Table 3.19.1 continuation

Notion	**Definition/explanation**
	identify yeasts and are generally not as important in mycology as they are in bacteriology Serology is used for identification of antibodies to special fungal antigens in patient's serum. The interpretation of serological findings is quite difficult in fungal infections. Antigen detection is used for finding of specific antigens in the diagnostic material by direct means using known antibodies, possible in some fungal infections (e.g., cryptococcosis). Cutaneous (allergy) tests with specific fungal antigens can be useful in diagnosing a number of fungal infections. Nucleic acid detection. Combined with amplification, such tests are useful for rapid detection of mycotic diseases in immunocompromised patients
Histoplasmosis	Histoplasmosis is caused by *Histoplasma capsulatum var.*

	capsulatum, a facultative parasitic fungus that grows intracellularly. It appears as a small budding yeast in humans and on culture media at 37 °C. At 25 °C it grows as a mold, producing small microconidia (1 to 5 µm in diameter) that are borne singly at the tips of short conidiophores. Large macroconidia or chlamydospores (8 to 16 µm in diameter) are also formed on conidiophores. In humans the yeast-like form grows within phagocytic cells. *H. capsulatum var. capsulatum* is found as the mycelial form in soils throughout the world and is localized in areas that have been contaminated with bird or bat excrement
Pathogenesis and clinical picture of histoplasmosis	The natural habitat of *H. capsulatum* is the soil. Spores (conidia) are inhaled into the respiratory tract, are taken up by alveolar macrophages, and become yeast cells that reproduce by budding. Small granulomatous inflammatory foci develop. The pathogens can disseminate haematogenously from these primary infection foci. The reticuloendothelial system (RES) is hit particularly hard. Lymphadenopathies develop and the spleen and liver are affected. Over 90% of infections remain clinically silent. The clinical picture depends heavily on any predisposing host factors and the infective dose. A histoplasmosis can also run its course as a respiratory infection only. Disseminated histoplasmoses are also observed in AIDS patients. Histoplasmosis is a disease of the monocyte-macrophage system; thus, many organs of the body can be infected. More than 95% of “histo” cases have either no symptoms or mild symptoms such as coughing, fever, and joint pain. Lesions may appear in the lungs and show calcification; most infections resolve on their own. Only rarely does the disease disseminate
Diagnosis of histoplasmosis	Suitable material for diagnostic analysis is bronchial secretion, urine, or scrapings from infection foci. For microscopic examination, Giemsa or Wright staining is applied and yeast cells are looked for inside the macrophages and polymorphonuclear leukocytes. Cultures on blood or Sabouraud agar must be incubated for severalweeks. Laboratory diagnosis is accomplished by complement fixation tests and and agar gel precipitation. Most individuals with this disease exhibit a hypersensitive state that can be demonstrated by the histoplasmin skin test
Epidemiology, therapy and	Histoplasmosis is endemic to the midwestern USA, Central and South America, Indonesia, and Africa. Prevention and control involve wearing

Table 3.19.1 continuation

Notion	**Definition/explanation**
prevention histoplasmosis	protective clothing and masks before entering or working in infested habitats. Soil decontamination with 3 to 5% formalin is effective where economically and physically feasible. The most effective treatment is with amphotericin B, ketoconazole, or itraconazole
Coccidioidomycosis	Coccidioidomycosis, also known as valley fever, San Joaquin fever, or desert rheumatism because of the geographical distribution of the fungus, is caused by *Coccidioides immitis. C. immitis* exists in the dry, highly alkaline soils of North, Central, and South America
Morphology and culture *C.immitis*	*C. immitis* is an atypical dimorphic fungus. In cultures, this fungus always grows in the mycelial form; in body tissues, however, it neither buds nor producesmycelia. What is found in vivo are spherical structures (spherules) with thick walls and a diameter of 15–60 µm, each filled with

	up to 100 spherical-to-oval endospores. *C. immitis* is readily cultivated on the usual fungus nutrient mediums. After five days of incubation, a white, wooly (fuzzy) mycelial colony is observed. One of the morphological characteristics of the mycelium is the asexual arthrospores seen as separate entities among the hyphae
Pathogenesis and clinical picture of coccidioidomycosis	The infection results from inhalation of dust containing arthrospores. Primary coccidioidomycosis is always localized in the lungs, whereby the level of manifestation varies from silent infections to severe pneumonia. Five percent of those infected develop a chronic cavernous lung condition. In fever than 1%, haematogenous dissemination produces granulomatous lesions in skin, bones, joints, and meninges. Most cases of coccidioidomycosis are asymptomatic or indistinguishable from ordinary upper respiratory infections. Almost all cases resolve themselves in a few weeks, and a lasting immunity results. A few infections result in a progressive chronic pulmonary disease. The fungus also can spread throughout the body, involving almost any organ or site
Diagnosis of coccidioidomycosis	The available tools are pathogen detection in sputum, pus, cerebrospinal fluid or biopsies, aspirates and antibody identification. The spherules can be seen under the microscope in fresh material. Culturing clinical samples in the presence of penicillin and streptomycin on Sabouraud agar also is diagnostic. The fungus can be readily cultured on Sabouraud agar at 25 °C. The resulting arthrospores are highly infectious and must be handled very carefully. Newer methods of rapid confirmation include the testing of supernatants of liquid media cultures for antigens, serology (the complement fixation test, gel precipitation or latex agglutination), and skin testing
Therapy and prevention of coccidioidomycosis	Amphotericin B can be used to treat the disseminated forms. An oral azole derivative will serve as an alternative, or for use, in clinically less severe forms: miconazole (lotrimin), itraconazole, ketoconazole, and amphotericin B are the drugs of choice for treatment. Prevention involves reducing exposure to dust (soil) in endemic areas
Cryptococcosis	Cryptococcosis is a systemic mycosis caused by *Cryptococcus neoformans*. This fungus always grows as large budding yeast. In the environment *C. neoformans* is a aprophyte with a worldwide distribution. Aged, dried pigeon droppings are an apparent source of infection.

Table 3.19.1 continuation

Notion	**Definition/explanation**
	Cryptococcosis is found in approximately 15% of AIDS patients. The fungus enters the body by the respiratory tract, causing a minor pulmonary infection that is usually transitory. Some pulmonary infections spread to the skin, bones, viscera, and central nervous system. Once the nervous system is involved, cryptococcal meningitis usually results. Diagnosis is accomplished by detection of the thick-walled spherical yeast cells in pus, sputum, or exudate smears using India ink to define the organism. The fungus can be easily cultured on Sabouraud dextrose agar. Identification of the fungus in body fluids is made by immunologic procedures. Treatment includes amphotericin B or itraconazole. There are no preventive or control measures
Opportunistic	Candidiasis is the mycosis caused by the dimorphic fungus *Candida*

mycoses - candidiasis	*albicans, C. parapsilosis, C. tropicalis, C. guillermondii, C. kruzei*, and a few other rare Candida species. In contrast to the other pathogenic fungi, *C. albicans* is a member of the normal microbiota within the gastrointestinal tract, respiratory tract, vaginal area, and mouth
Morphology and culture of *Candida spp.*	Gram staining of primary preparations reveals *Candida spp*. to be a gram-positive, budding, oval yeast with a diameter of approximately 5 µm. Gram-positive pseudohyphae are observed frequently and septate mycelia occasionally. *Candida spp.* can be grown on the usual culture mediums. After 48 hours of incubation on agar mediums, round, whitish, somewhat rough-surfaced colonies form. They are differentiated from other yeasts based on morphological and biochemical characteristics
Pathogenesis and clinical pictures of candidiasis	Candida is a normal inhabitant of human and animal mucosa (commensal). Candida infections must therefore be considered endogenous. Candidiasis usually develops in persons whose immunity is compromised, most frequently in the presence of disturbed cellular immunity. The mucosa is affected most often, less frequently the outer skin and inner organs (deep candidiasis). Oral candidiasis or thrush is a common disease in newborns. It is seen as many small white flecks that cover the tongue and mouth. At birth, newbornsdo not have a normal microbiota in the oropharyngeal area. If the mother vaginal area is heavily infected with *C. albicans,* the upper respiratory tract of the newborn becomes colonized during passage through the birth canal. Thrush occurs because growth of *C. albicans* can not be inhibited by the other microbiota. Once the newborn has developed its own normal oropharyngeal microbiota, thrush becomes uncommon Paronychia and onychomycosis are associated with Candida infections of the subcutaneous tissues of the digits and nails, respectively. These infections usually result from continued immersion of the appendages in water. Intertriginous candidiasis involves those areas of the body, usually opposed skin surfaces that are warm and moist: axillae, groin, and skin folds. Napkin (diaper) candidiasis is typically found in infants whose diapers are not changed frequently and therefore are not kept dry. Candidal vaginitis can result from diabetes, antibiotic therapy, oral contraceptives, pregnancy, or any other factor that compromises the female host. Normally the omnipresent lactobacilli (Döderlein's bacilli) can control Candida in this area by the low pH they create. However, if their numbers are

Table 3.19.1 continuation

Notion	**Definition/explanation**
	decreased by any of the aforementioned factors, Candida may proliferate, causing a curdlike, yellow-white discharge from the vaginal area. Candida can be transmitted to males during intercourse and lead to balanitis; thus it also can be considered a sexually transmitted disease. Balanitis is a Candida infection of the male glans penis and occurs primarily in uncircumcised males. The disease begins as vesicles on the penis that develop into patches and are accompanied by severe itching and burning
Criteria of candidiasis diagnostics	1. Causative agents in sterile liquids (blood, spinal fluid), punctuate of the closed cavities (pleura cavity). 2. The mycelium or pseudomicelium in pathological material. 3. Repeated excretions of the same type of fungus in great numbers from

	the mucous membranes, skin and its appendages; abscesses. 4. High concentrations of *Candida spp.* in urine ($\geq 10^4$ cell in 1 ml). 5. Fungi antigens in the serum
Therapy of candidiasis	There is no satisfactory treatment for candidiasis. Cutaneous lesions can be treated with topical agents such as sodium caprylate, sodium propionate, gentian violet, nystatin, miconazole, and trichomycin. Ketoconazole, amphotericin B, fluconazole, itraconazole, and flucytosine also can be used for systemic candidiasis
Pneumocystosis	The disease that this protist causes, pneumocystis pneumonia or *Pneumocystis carinii pneumonia* (PCP), occurs almost exclusively in immunocompromised hosts. Extensive use of immunosuppressive drugs and irradiation for the treatment of cancers and following organ transplants accounts for the formidable prevalence rates noted recently. This pneumonia also occurs in more than 80% of AIDS patients. Both the organism and the disease remain localized in the lung–even in fatal cases. Within the lungs, pneumocystis causes the alveoli to fill with a frothy exudate
Morphology and developmental cycle of the *P. carinii*	Three developmental stages are known for *P. carinii*. The trophozoites are elliptical cells with a diameter of 1.5–5 μm. Presumably, the trophic form reproduces by means of binary transverse fission, i.e., asexually. Sexual reproduction does not begin until two haploid trophozoites fuse to make one diploid sporozoite (or precyst), which are considered to be an intermediate stage in sexual reproduction. After further nuclear divisions, the sporozoites possess eight nuclei at the end of their development. The nuclei then compartmentalize to form eight spores with a diameter of 1–2 lm each, resulting in the third stage of development, the cyst. The cysts then release the spores, which in turn develop into trophozoites
Diagnosis of pneumocystis pneumonia	Laboratory diagnosis of pneumocystis pneumonia can be made definitively only by microscopically demonstrating the presence of the microorganisms in infected lung material or by a PCR analysis

Scheme candidiasis laboratory diagnosis

Scheme coccidioidomycosis, histoplasmosis, blastomycoses laboratory diagnosis

Abreviation

ABS – antigen-binding site
BGEC – Bacteria Group of *Escherichia coli*
CFT – complement fixation test
CNS – central nervous system
DLM – doses letalis minima
DTH – delayed type of the hypersensitivity
ELISA – enzyme-linked immunosorbent assay
EMB – Eosin methylen blue
EPEC – enteropathogenic *E. coli*
EIEC – enteroinvasive *E. coli*
ETEC – enterotoxigenic *E. coli*
EHEC – enterohaemorrhagic *E. coli*
EYA – egg yolk agar
FA – fagocytic activity
FI – fagocytic index
FACS – fluorescence-activated cell sorter
GAS – group A streptococci
IFT – immunofluorescent test
IU – international units
MBT – *Mycobacterium tuberculosis*
MIC – minimal inhibition concentration
MHC – major histocompatibility complex
MPA – meat pepton agar
MRSA – methicillin-resistant Staphylococcus aureus
NBT – nitrat blue tetrasolium
NT – neutralization test

PHAT – indirect (passive) hemagglutination test
PMNL – polimorphonuclear leucocyte
RPR – rapid plasma reagin
RIA – radioimmunoassay
STSS – Streptococcal toxic shock syndrome
TCBS – thiosulfate-citrate-bile salts-sucrose
VDRL – venereal disease research laboratory

Recommended reading list

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Informational resourses:

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