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Харківський національний медичний університет

ZOONOSIS

Learning guide for the 2nd and 3rd year English media students of the Faculty of Medicine and the Faculty of Dentistry (Microbiology, virology and immunology)

ЗООНОЗИ

Методичні вказівки з дисципліни «Мікробіологія, вірусологія та імунологія» для студентів II і III курсів медичного та стоматологічного факультетів з англійською мовою викладання

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Learning guide is related to the program of Ministry of Health of Ukraine and is recommended to students of medical and dentistry faculties of high medical schools of III-IV level accreditation.

Learning guide includes sections of taxonomy, and biological characteristics of causative agents of plaque, tularemia, anthrax, and brucellosis. The most modern information on epidemiology, pathogenesis, immunity, immunotherapy, immunoprophylaxis, and modern methods of laboratory diagnosis of zoonotic diseases is represented.

Theme: LABORATORY DIAGNOSIS OF PLAGUE AND TULAREMIA

Actuality of the theme.

Goal: Studying of laboratory diagnostics of plague and tularemia.

Concrete goals:

1. Study of biological properties and classification of Y.pestis and F.tularensis.
2. Study pathogenesis and clinical manifestations of plague and tularemia.
3. Study of the methods of laboratory diagnosis of plague and tularemia.
4. Study of specific prophylaxis and therapy of plague and tularemia.

Students should be able to:

1. Isolate of pure cultures of Y. pestis and examine growth on blood agar.
2. Identify of pure culture of Y. pestis and F. tularensis on morphology, culture and biochemical properties, antigenic structure.
3. Perform ring test to diagnose of plague and tularemia.
4. Interprete results of serological tests and skin allergic test to diagnose tularemia.

Equipment: slides, immersion microscope, biological preparations for laboratory diagnosis plague and tularemia, specific prophylaxis and therapy of plaque and tularemia, tables, atlas.

Plague

Plague (pestis) is acute, natural focal, especially dangerous zoonotic transmissible infection, prone to epidemic spread. The causative agent of the plague Yersinia pestis (formerly called Pasteurella pestis) was discovered in 1894 by A. Yersen in Hong Kong. The name comes from the Arabic "jumma" – bean, because inflamed lymph nodes resemble beans in appearance.

Morphology. Yersinia pestis – gram-negative pleomorphic small nonmotile non-spoeforming rods, with rounded ends (ovoid form), with a size of 1–2 × 0.3–0.7 microns (*Fig. 1*). Y.pestis forms a capsule in the patient's body and during reproduction on nutrient media and can produce a filtering form (L-form) in the patient's body. Y.pestis is often located in chains in smears from pathological material (sputum, bubonic pus, blood, contents of ulcers, smears-prints of organs); when stained with methylene blue, bipolarity is revealed. In smears from agar cultures bipolarity is less pronounced, chains are not formed. When reproducing on solid media, elongated forms of Yersinia predominate.

Physiology. Y.pestis are facultative anaerobes. They multiply on simple nutrient media, but grow better with the addition of hemolyzed blood and sodium sulphite. The optimal cultivation temperature is 28 °C, but they can multiply at a temperature of 2 to 42 °C. On solid media, after seeding a large number bacteria (10,000 or more bacteria), the initial growth of colonies in the form of "broken glass" or "melting snow" is detected by microscopy in 8–12 hours, which becomes light with uneven edges in 18–20 h. The scalloped edge of the colonies gives them the appearance of a "crumpled lace handkerchief" in

48 hours. Large colonies with a grainy center of yellowish or brown color, with a scalloped (lacy) periphery (R-form) are characteristic of avirulent bacteria. Smooth colonies with even edges (S-form) contain avirulent bacteria.

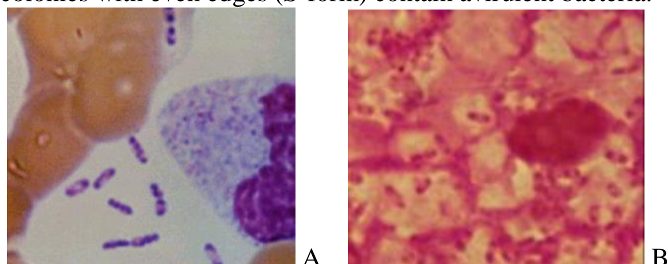

Fig. 1. Morphology of Y. pestis: A – Giemsa stain, B – Gram stain

In liquid nutrient media, Y. pestis form flakes, a crumbly sediment on the bottom; with prolonged cultivation, a film on the surface, and filaments descending down to the flaky deposit, similar to stalactites.

Biochemical activity is characterized by the ability to split a number of carbohydrates (fructose, galactose, etc.) with the formation of acid, Y.pestis do not decompose lactose and sucrose. According to the ability to ferment glycerol species Y.pestis is divided into chemovares (capable and not able to ferment glycerol). Proteolytic activity is poorly expressed - gelatin is not liquefied, milk is not coagulated. When proteins are decomposed Y.pestis produces H_2S, and does not produce indole. There release a number of exoenzymes.

Antigens. Y.pestis contains a complex of antigens (16–18), the main ones of which are somatic (thermostable, specific for genus) and capsular (polysaccharide, thermolabilite, variable in different serovars, antigenic specificity of Yersinia is associated with it). Among antigens, there are common with representatives of the Enterobacteriaceae family and O-group people erythrocytes. F_1 surface thermolabile protein antigen is used in the HAT as a diagnosticum in serological reactions for plague. V- and W-antigens possess antiphagocytic activity.

There are three antigenic fractions:

I. Capsular antigen (synthesized at 37 °C) has pronounced immunogenic properties, is represented by two varieties: Ia (polysaccharide) and Ib (protein), water soluble.

II. Complex protein and polysaccharide is water soluble, has a toxic effect on mice rodents.

III. Protein antigen has immunogenic properties, insoluble in water. Ten antigens of Yersinia pestis are identical to Yersinia pseudotuberculosis so in serological analysis differential diagnosis should be performed.

Resistance. In conditions of growth on artificial media, Y.pestis has a pronounced resistance to the products of its vital activity. Getting from the body of sick animals and humans into the environment, Y.pestis can remain

viable for a long time. They tolerate low temperatures well, they persist up to 6 months at 0 °C, in frozen corpses, fleas – 1 year or more, in water, soil, clothes – from 1 to 5 months; in bread, on vegetables and fruits – for 4–6 days.

Y.pestis are sensitive to sunlight (they are killed by direct sunlight for 2–3 hours), UV irradiation, drying and exposure to high temperatures. They die by boiling in 1 minute, when heated to 80 °C in 5 minutes, and when heated to 60 °C in 1 hour. Sensitive to disinfectants. The harmful effect of disinfectants is manifested when exposed to a 3 % phenol solution in 5–10 minutes, 5 % lysol solution in 2–10 minutes. Sensitive to many antibiotics (especially tetracycline).

Pathogenicity. Yersinia pestis is pathogenic for many animal species (for 164 rodent species, for some domestic animals (cats), for some farm animals (camels), for some predators (foxes, jackals, ferrets, weasels), from laboratory animals for guinea pigs, white mice and rats, rabbits). For the occurrence of the disease in humans, it is sufficient to penetrate one bacterial cell. In all species of sensitive animals and in humans, the causative agent of the plague suppresses the protective function of the phagocytosis system, this is realized by the action of certain virulence factors.

Virulent strains of plague bacillus are characterized by the following properties:

● they produce a toxin that combines the properties of exo- and endotoxin. It is contained in the microbial cell, partially bound to the shell, partially secreted, most toxic to mouse-like rodents ("mouse poison"). Mechanism of action: it blocks the transfer of electrons in the mitochondria of the heart muscle and liver, affects platelets and blood vessels, causes hemorrhagic syndrome. Toxin is an antagonist of adrenergic receptors, it inhibits cardiac activity, reduces vascular tone;

● during the breakdown, endotoxin (lipopolysaccharide) is released, which in addition to the toxic effect has allergic properties;

● Y.pestis produce enzymes of aggression: fibrinolysin, plasmocoagulase, neuraminidase (responsible for adhesive properties), aminopeptidases (destroy important regulatory proteins, enzymes, individual complement fractions);

● have pili that provide adhesion;

● have a capsule that inhibits the activity of phagocytes;

● have F-antigen;

● have V- and W-antigens (formerly called Vi-antigen);

● have pH6 antigens (show activity when the pH is lowered);

● form thermoinduced proteins of the outer membrane.

These factors inhibit phagocytosis, promote reproduction in macrophages, exhibit a cytotoxic effect against macrophages.

● Develop adenylate cyclase, which blocks oxidative enzymes of macrophages;

● Form pesticides (a kind of bacteriocins) that suppress the normal microflora of the macroorganism.

The formation of most of the aggression factors is controlled by plasmid genes (a common feature with other enterobacteria).

Virulent strain of plague bacilli produces bacteriocin and it is coagulase positive also showing fibrinolytic activity. Colonies may be pigmented (hemin media).

Many factors play direct and indirect roles in *Y. pestis* pathogenesis.

Low calcium response (*lcr*): This is a plasmid-coded gene that enables the organism to grow in a low Ca^{++} (intracellular) environment. It also coordinates the production of several other virulence factors, such as V, W and *yop*s (*Yersinia* outer proteins).

V and W proteins: These plasmid-coded proteins are associated with rapid proliferation and septicemia.

***Yop*s:** A group of 11 proteins, which are coded by plasmids, are essential for rodent pathogenesis and are responsible for cytotoxicity, inhibition of phagocyte migration and engulfment and platelet aggregation.

Envelope (F-1) antigen: This is a protein-polysaccharide complex which is highly expressed at 37 ° in the mammalian host but not in the flea and is anti-phagocytic.

Coagulase and Plasminogen activator: Both of these are plasmid-coded proteins. Coagulase is responsible for micro thrombi formation and plasminogen activator promotes the dissemination of the organism. It also destroys C3b on the bacterial surface, thus attenuating phagocytosis.

Ecology, distribution and epidemiology. Plague is a zoonotic, natural focal disease. Foci are found on all continents (except Australia). The foci differ in the carriers of the pathogen and the biological properties of Yersinia, which are common in a certain zone. According to the biochemical characteristic and the correspondence with the place of distribution, there are 3 biovars (*Table 1*).

Table 1. Biochemical characteristic and distribution of biovars of Y.pestis

Y. pestis biovars	Fermentation with acid production		Reduction of nitrate to nitrite	Site of distribution
	glycerol	melibiase		
v. antiqua	+	-	+	Central Asia, Central Africa
v. medievalis	+	+	-	Iran, Russia
v. orientalis	-	-	+	Everywhere

Notation: + – presence of a characteristic, - – no indication.

The main hosts (reservoir) of Y.pestis in nature are rodents, as wild-growing (gophers, tarbagans, etc.), and synanthropic (adapted to inhabiting near human habitation). The most epidemiologically dangerous are black, gray and Egyptian rats. Rodents suffer mainly in acute form. Fleas living on infected rats are 100% infected, because the plague in rats proceeds according to the type of sepsis. After the death of rats, fleas attack random feeders – people. In fleas stomach plague block forms, consisting of pathogens of plague. Flea regurgitates it when bitten and it can get into

the wound. With a bite, itching also occurs, which leads to the combing of the bite site and rubbing the plague block into the skin.

Consequently, in natural foci of plague, the infection is transmitted to humans by fleas, by contact (can penetrate through intact skin), and alimentary ways. People sick with pulmonary plague, infect others with airborne route (ie, the transmission of the pathogen from person to person, which is excluded in the case of other zoonotic infectious diseases).

Pathogenesis of plague. The clinical manifestations of the plague largely depend on the portal of entry of the infection. At the site of penetration of the pathogen on the skin and mucous membranes, no changes are observed (only in 3–4 % of cases primary affect can arise on the skin: bright red spot → ulcer, ulcerous carbuncle). The causative agent enters the regional lymph nodes by lymphogenous pathway, intensively multiplies there, which leads to hemorrhagic inflammation with the formation of bubo – an enlarged, inflamed lymph nodes package. From the primary nodes the pathogen spreads to new lymph nodes lymphogenically or hematogenously, this is accompanied by bacteremia and toxicomia, while the cardiovascular system, the nervous system and other organs are affected. Necrosis, infiltration, serous impregnation of the vascular wall occur with vascular lesions. Insufficient barrier function of regional lymph nodes, usually accompanied by incomplete phagocytosis of the Y.pestis and their increased reproduction, leads to the development of a primary septic form of the plague. This form is characterized by the development of severe infectious-toxic shock, the formation of septicopyemic foci in various organs with destruction of the tissues, blood coagulability disorder. The secondary-septic form developes on the basis of bubonic or pulmonary forms.

Clinical manifestations. The incubation period lasts from several hours (primary-pulmonary and septic form) to 6 days. In vaccinated people incubation period is up to 10 days. The disease begins suddenly without a prodromal period, with severe chills, fever, severe headache, which can be accompanied by nausea, vomiting. The patient experiences a feeling of fear. In the severe course of the disease cyanosis, pointed features, expression of suffering and horror on the face (facies pestica) develope.

According to clinical manifestations, the following forms are distinguished:

– local: cutaneous, bubonic, cutaneous-bubonic;

– generalized: primary and secondary-septic; primary and secondary pulmonary,

– disseminated: intestinal.

Secondary pulmonary form can begin as cutaneous or bubonic (getting the pathogen into the lungs by hematogenous or lymphogenous pathway). More common is the bubonic form (lethality 60 %), in primary and secondary-pulmonary lethality is even higher.

Bubonic plague: Patients develop sudden onset of fever, headache, chills, and weakness and one or more swollen, tender and painful lymph nodes (called buboes). This form usually results from the bite of an infected flea. The bacteria multiply in the lymph node closest to where the bacteria entered the human

body. If the patient is not treated with the appropriate antibiotics, the bacteria can spread to other parts of the body.

Septicemic plague: Patients develop fever, chills, extreme weakness, abdominal pain, shock, and possibly bleeding into the skin and other organs. Skin and other tissues may turn black and die, especially on fingers, toes, and the nose. Septicemic plague can occur as the first symptom of plague, or may develop from untreated bubonic plague. This form results from bites of infected fleas or from handling an infected animal.

Pneumonic plague: Patients develop fever, headache, weakness, and a rapidly developing pneumonia with shortness of breath, chest pain, cough, and sometimes bloody or watery mucous. Pneumonic plague may develop from inhaling infectious droplets or may develop from untreated bubonic or septicemic plague after the bacteria spread to the lungs. The pneumonia may cause respiratory failure and shock. Pneumonic plague is the most serious form of the disease and is the only form of plague that can be spread from person to person (by infectious droplets).

Immunity. There is no inherent immunity. After the disease, a long-term immunity remains. Immunity is based on the T-effector arm, which realizes its effect through the system of macrophages (the main role in protecting from the plague belongs to the completed phagocytosis). In persons who have recovered from the plague or have been vaccinated, phagocytosis is complete, unvaccinated –- incomplete.

Laboratory diagnostics. Plague is a especially dangerous infection. Work with materials containing the causative agent of the disease is carried out in special laboratories. The material for investigation can be pus from bubos, peptic ulcer, carbuncle, sputum, throat material, blood, liquor, sectional material, rodents (live and dead), rodents fleas, water, air, food products, etc. There are bacterioscopic , bacteriological, biological and serological researches.

Microscopy. The smears are stained by Gram and by methylene blue according to Leffler. For the purpose of rapid diagnosis, smears are also treated with a labeled luminescent antiserum against Y. pestis (direct IF). If a gram-negative ovoid form of bacteria is detected, surrounded by a gentle capsule and stained bipolar according to Leffler, giving a specific luminescence, a preliminary response is given based on the results of express diagnostics.

For the indication of antigens of the pathogen PHA is applied with immunoglobulin plague diagnosticum, ELISA, as well as PCR. Based on the results of rapid diagnostics, a preliminary response can be given in 4 hours from the beginning of the research, the final one in 18–20 hours.

Bacteriological method. The patient's material is seeded in the appropriate nutrient media: the blood – in MPB; the contents of bubo, ulcer's exudate, sputum and other material – in Petri dishes with MPA (pH 7.2–7.3). To stimulate the growth

of bacteria, 0.1 % of whole or 1–2 % of hemolyzed blood of a rabbit or a horse is added to the MPA. To inactivate the plague phage, 0.1 ml of antiphage serum is applied to the surface of the medium and uniformly distributed. To suppress the growth of extraneous microflora in the test material, 1 ml of a 0.1 % solution of gentian violet and 2.5 % of sodium sulfite is added to 100 ml of MPA. To this end, a selective medium with antibiotics is also used. The cultures are incubated at 25–28 °C. After 16–20 h on the Petri dishes at low magnification, colonies resembling a cluster of broken glass fragments are found which, by 48 h, acquire the form of an R-shape with a compact raised center and a delicate translucent periphery ("lace handkerchiefs"). In the broth after 24 hours a crumbly film is formed, from which the filaments in the form of "stalactites" descend. The pure culture of bacteria is identified by morphological, cultural, enzymatic, antigenic properties and sensitivity to the plague bacteriophage, as well as its sensitivity to antibiotics (*Table 2*).

Table 2. Differentiation of Yersinia species

Species	Motility at 25 °C	Fermentation with acid production				Production	
		ram-nose	sor-bose	raffi-nose	inosi-tol	ure-ase	ornithine decarbo-xylase
Y. pestis	-	-	-	-	-	-	-
Y. pseudotuberculosis	+	+	-	±	-	+	-
Y. enteroco-litica	+	-	+	-	+	+	+

Notation: + – presence of the characteristic, - – no indication, ± – the sign is unstable.

Y. pestis restores nitrates to nitrites, ferments glucose with the formation of acid (without gas), xylose, mannitol, but unlike many other representatives of the family has a lower temperature optimum of growth and physiological activity at 28 °C. During identification, the causative agent of the plague must be differentiated with other Yersinia, especially the causative agent of pseudotuberculosis. Identification is also carried out in AT using diagnostic sera against capsular antigen.

Biological method. Guinea pigs or mice are infected in various ways – subcutaneously, intraperitoneally or dermally (a material abundantly contaminated with foreign microflora). Inoculation of the material from the corpses is carried out in the following way: the animal is shaved off the skin area and scarified it, 2 to 3 drops of the test liquid are applied to the skin and rub it by the flat part of the scalpel. The blood of the patient and the suspension of the isolated pure culture are inoculated intraperitoneally; sputum, punctate of bubo – subcutaneously in the inner surface of the thigh. Animals infected cutaneously, die on the 5th–7th day, in other ways – in 2–3 days (from a progressive septic process). From the blood and internal organs of the corpse, a culture is isolated, which is identified in the manner described above. In smears from internal organs, blood, exudate, a large number of gram-negative, bipolar-stained bacteria are detected.

When examining decomposed corpses of rodents, a precipitation reaction is used, since it is difficult to isolate a pure bacterial culture in this case. Smears-prints are prepared from internal organs and pure culture is isolated on nutrient media. Biological method is mandatory.

Express diagnostics:

- Detection of the pathogen with a bacteriophage (including the reaction of increasing the titer of a bacteriophage);
- immunofluorescent method (response in 2–3 hours);
- PHAT with an antiviral erythrocyte diagnosticum;
- antibody neutralization reaction (RNAT);
- immuno-enzyme analysis (ELISA);
- the pathogen in the organs of the animal is detected by the precipitation reaction (response in 6–8 hours); in materials from rotten corpses by the thermal precipitation reaction.

Serological examination. Serodiagnostics is carried out for the purpose of retrospective diagnosis, as well as for epizootic examinations in natural foci of plague. PHAT is carried out with red blood cells, on which the capsular antigen of Y.pestis is adsorbed. The antigen neutralization reaction, ELISA and indirect IF are also used. In the study of patient's sera or dead rodents, the result is considered positive at a titer of 1 : 40 and higher.

Prevention. *Nonspecific prevention:* immediate hospitalization and isolation of patients, contact persons (isolation for 6 days, emergency prophylaxis – streptomycin, tetracycline).

Specific prophylaxis: immunization of people by living (strain EV) or chemical anti-plague vaccine. After a single subcutaneous or dermal administration, relatively stable immunity is created for 6 months. Vaccination for epidemiological indications, primarily groups at increased risk: geologists, agricultural workers, hunters, workers of anti-plague establishments.

Treatment: streptomycin, antiplague immunoglobulin.

Yersinia pseudotuberculosis

The causative agent of the pseudotuberculosis Yersinia pseudotuberculosis was detected and described by Malassa and Vinyal in 1883. Diseases etiologically related to Y. pseudotuberculous are defined as gastroenteritis, mesenteric lymphadenitis, polyarthritis, regional ileitis, and others.

Morphology, physiology. Y. pseudotuberculosis – coccobacteria that do not form spores, Gram-negative, have flagella and capsule. Phacultative anaerobes. They multiply well on simple nutrient media at the optimum temperature of 20–28 °C, but at 0–4 °C there is intensive reproduction.

Antigens. Y. pseudotuberculous have H-flagellar and O-somatic antigens. According to the O-antigen, 10 serovars are distinguished.

Ecology and distribution. Bacteria Y.pseudotuberculous are common in many countries. The diseases they cause are registered in the countries of Asia, Africa, and Europe. The source of the pathogen are rodents (voles, house mice, rats, hares). Infection of people occurs by an alimentary route, at the use of foodstuff (mainly vegetables, fruit), polluted by feces and urine of animals.

The ability of Y. pseudotuberculosis to multiply at a temperature of 4–6 °C leads to a massive accumulation of them in foods that are stored in the refrigerator for a long time.

Pathogenesis of human diseases. After entering the gastrointestinal tract Y. pseudotuberculosis penetrates into the intestinal mucosa, where (mainly in the submucosa layer) multiple granulomas and hemorrhages form and symptoms of gastroenteritis occur. The penetration of microorganisms into the lymph nodes leads to the development of mesenteric lymphadenitis, in the epigastric region pains intensify, symptoms of irritation of the peritoneum appear, in the ileocecal angle infiltrate is determined. Bacteremia is possible, which is accompanied by a rash, arthralgia and other manifestations of the generalization of the pathological process.

Immunity. Antibodies to the pathogen appear during the course of the disease, but they do not possess protective properties. Cases of repeated pseudotuberculosis are described.

Laboratory diagnosis is carried out by bacteriological and serological methods. Cultures of the pathogen are isolated from feces, sputum and other materials, and differentiated from other Yersinia and similar bacteria of other genera. ELISA is used to identify the pathogen in materials from patients and indications in the environment. In serodiagnosis, the agglutination reaction and PHAT are used.

Prevention and treatment. Specific prophylaxis is not developed. For treatment tetracyclines, levomycetin, streptomycin are used.

The causative agent of intestinal yersiniosis

Yersinia enterocolitica was isolated from animals (rabbits, hares, monkeys, pigs). The study of yersiniosis began in 1964.

Morphology, physiology. Y. enterocolitica are Gram-negative mobile rods that do not form spores and capsules.

They are cultivated on simple nutrient media at a temperature of 20–26 °C. There are 5 biovars, of which the 3rd and 4th are pathogenic for humans.

Antigens. According to the specificity of the O-antigen Yersinia enterocolitica is divided into 30 serovars. In humans, O3 and O9 serovars are most common.

Pathogenicity. Y. enterocolitica are facultative intracellular parasites. Their pathogenicity is associated with the invasive properties and effects of cytotoxins, virulent strains are resistant to phagocytosis and bactericidal action of the serum. These properties are encoded by the plasmid genes. The virulence markers are calcium dependence and autoagglutination.

Ecology and distribution. Y. enteocolitica are widely distributed: they are isolated from sick people and animals, they are found in the environment. In open reservoirs, they not only survive, but also reproduce. They are resistant to low temperatures. In milk, they can multiply both at room temperature and in a refrigerator. Vegetables, fruits, ice cream, contaminated with Yersinia, in the conditions of their storage in the refrigerator, are massively seeded with these microorganisms. The main source of Y. enterocolitica are animals and people (patients and carriers). The rout of infection is alimentary. Diseases are recorded in the form of outbreaks or sporadic cases.

Pathogenesis of human diseases and immunity. Infection of the human Y. enterocolitica is realized in different ways: from asymptomatic carrier and mild forms to severe and generalized, septic, which are more common in elderly people suffering from chronic diseases (cirrhosis, diabetes). Yersinia of this species can be the cause of the occurrence of polyarthritis, nodosa erythema.

During the course of the disease, antibodies to the pathogen accumulate. Their titer in the agglutination reaction is no more than 1 : 800.

Laboratory diagnostics of yersiniosis is carried out by bacteriological and serological methods. From the patient's material (feces, nasopharyngeal swab, blood, urine), the culture is isolated, the serovar is identified.

Serodiagnostics is performed in agglutination reactions, indirect haemagglutination, immunofluorescence analysis.

Prevention and treatment. Specific prophylaxis is not developed. Treatment of patients with intestinal yersiniosis is carried out with antibiotics (streptomycin, gentamicin, tetracycline, ampicillin are usually prescribed) and sulfonamides.

Tularemia

Tularemia is an acute zoonotic infection that affects the lymphatic system. The causative agent of tularemia, Francisella tularensis, was discovered in 1912 by G. McCoy and S. Chepin. The name of the microorganism was given by the name of the Tulare region in California, where the researchers isolated the pathogen. Modern classification of tularemia bacteria is attributed to the genus Francisella.

Morphology. F.tularensis are very small, 0.2–0.7×0.2 μm in size, polymorphic, coccoid and rod-shaped (in liquid media) gram-negative bacteria (*Fig. 2*). They do not form spores, do not have flagella, form a capsular like mucus. In smears from liquid cultures they are stained bipolar. In smears from the organs they are located intracellularly and in the form of clusters, forming a gentle capsule.

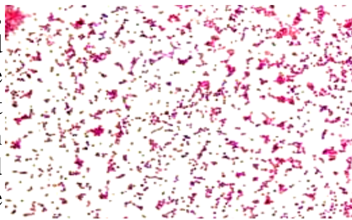

Fig. 2. Morphology of F. tularensis Gram stain

Physiology. They are facultative anaerobes and facultative intracellular parasites. F.tularensis are extremely fastidious, and do not grow on simple nutrient media. For reproduction, the introduction of cysteine as a growth stimulant is required. Cultivation is possible on nutrient media containing egg yolk, liver, spleen, brain, on blood agar with the addition of glucose and cysteine. The optimum temperature is 36–37 °C. On a egg yolk medium, a colorless, delicate deposit is formed. Colonies are from 0.5 to 2 mm, smooth, shiny, whitish in color. On liquid media they reproduce worse than on solid ones.

Enzymatic activity is poorly expressed (splitting glucose and maltose to an acid), biochemical properties are unstable. F.tularensis are oxidase-negative, produce hydrogen sulphide.

Antigens. F.tularensis contains two antigenic complexes: superficial Vi-antigen and somatic O-antigen. Virulent and immunogenic properties of the pathogen are associated with the Vi-antigen. Virulent strains have S-form colonies, R-forms lose their virulence. Antigens of F. tularemia induce the synthesis of agglutinins, precipitinins. An antigenic commonality with another species of the genus, Francisella novicida, non-pathogenic to humans, and a cross-reacting in the agglutination reaction with Brucella and Yersinia have been noted.

Resistance. In the environment, the causative agent of tularemia remains viable for a long time: in the corpses of rodents, in water at 1 °C – up to 9 months, at 4 °C– 4 months. With the rise in temperature, the survival time is shortened - microorganisms survive 1–2 months in water at 20 °C, but do not reproduce. In frozen meat, it remains viable up to 90 days. In the skin of a sick rodent at room temperature it remains up to 1.5 months. To the action of high temperature, the causative agent of tularemia is unstable, dies at 60 °C in 20 minutes, and when boiled – instantly. The disinfecting substances (solutions of carbolic acid, lysol and other disinfectants) are disastrous for micro-organisms in normal concentrations. F. tularemia is sensitive to many antibiotics: streptomycin, gentamycin, kanamycin, neomycin, tetracyclines, chloramphenicol but resistant to penicillin and its derivatives.

Ecology and distribution. Tularemia is a zoonotic disease with natural foci. Natural hosts of the pathogen are 145 species of animals, maximum sensitivity in rodents (water rats, voles, house mice, hamsters, hares). An important role in the preservation and spread of the pathogen belong to ixodex tixes (in the body of the tick, the pathogen is present throughout its life (530 days)), the ticks have transfacial transmission of the pathogen.

The infection of a human with the causative agent of tularemia occurs with direct contact with a sick animal or corpses of dead, airborne dust, as well as through infected water and food products. It is possible to transmit the agent with ticks (not through the bite, but through the intact skin whith the tick's escreta), by other blood-sucking arthropods (77 species) when bitten (by mosquitoes, flies) which are carriers.

From a person with tularemia, healthy people practically do not become infected.

Pathogenesis of human disease.

Factors of virulence:

● presence of a capsule;

● endotoxin;

● neuraminidase;

● antiphagocytic activity, anticomplementary activity, and a receptor to the Fc fragment of IgG, which upon attachment provides loss of antibidy activity.

In the human body, the causative agent of tularemia penetrates through the skin and mucous membranes of the eyes, mouth, nose, respiratory tract and digestive tract. Possessing high invasive ability, microorganisms can penetrate through intact skin.

Clinical manifestations. The disease in people is febrile, toxic-allergic, less often septic. After 2–7 days of the incubation period, the pathogen appears in regional (close to the site of penetration) lymph nodes, where it intensively multiplies (lymph nodes become inflamed, buboes appear) and appears in the blood. Depending on the routes of penetration, various clinical forms of tularemia develop: glandular (lymphadenopathy without ulcer), ulceroglandular, oculoglandular, intestinal (typhoidal), pneumonic, and rarely oropharyngeal (pharyngotonsillitis with lymphadenopathy), primary septic with an lesion of the lymph nodes is posible. Tularemia is accompanied by the development of a specific allergy that occurs already on the 3–5th days of the disease and persists after recovery for many years, and sometimes for life.

The signs and symptoms of tularemia vary depending on how the bacteria enter the body. Illness ranges from mild to life-threatening. All forms are accompanied by fever, which can be as high as 40 °C. Main forms of this disease are listed below:

Ulceroglandular. This is the most common form of tularemia and usually occurs following a tick or deer fly bite or after handing of an infected animal. A skin ulcer appears at the site where the bacteria entered the body. The ulcer is accompanied by swelling of regional lymph glands, usually in the armpit or groin.

Glandular. Similar to ulceroglandular tularemia but without an ulcer. Also generally acquired through the bite of an infected tick or deer fly or from handling sick or dead animals.

Oculoglandular. This form occurs when the bacteria enter through the eye. This can occur when a person is butchering an infected animal and touches his or her eyes. Symptoms include irritation and inflammation of the eye and swelling of lymph glands in front of the ear.

Oropharyngeal. This form results from eating or drinking contaminated food or water. Patients with orophyangeal tularemia may have sore throat, mouth ulcers, tonsillitis, and swelling of lymph glands in the neck.

Pneumonic. This is the most serious form of tularemia. Symptoms include cough, chest pain, and difficulty breathing. This form results from breathing dusts or aerosols containing the organism. It can also occur when other forms of tularemia (e.g. ulceroglandular) are left untreated and the bacteria spread through the bloodstream to the lungs.

Typhoidal. This form is characterized by any combination of the general symptoms (without the localizing symptoms of other syndromes)

Immunity. After disease there remains a persistent, long-lasting immunity.

Laboratory diagnosis. Serological, biological, bacteriological methods and allergic tests of investigation are used. Allergic tests and serological diagnostics are performed in usual hospital conditions and microbiological laboratories. Detection and isolation of bacteria are carried out in special laboratories.

Serological examination. In the serum of patients, specific agglutinins are determined, which appear from the 10th to the 12th day of the disease. Antibodies are determined in the agglutination test and PHAT with a tularemia corpuscular diagnosticum (formalin-killed bacteria). In case of mass examinations, a rapid microAT is carried out on slides (blood-drop sample). At repeated researches increase of an antibody titer is observed.

Tube AT. For this test, 2–3 ml of blood from the ulnar vein is taken from the patient on the 10th-15th day of the disease. The serum is serially diluted from 1 : 25 to 1 : 400. 0.5 ml of tularemia diagnosticum is added to each tube containing 0.5 ml of diluted serum, resulting in final serum dilutions (1 : 50 to 1 : 800). The reaction is followed by monitoring the serum and diagnosticum. The tubes are placed for 2 hours in a thermostat. Preliminary results of the reaction are taken into account after removing the tubes from the thermostat, and finally after their stay for 18 to 20 hours at room temperature. The diagnostic value is the detection of agglutinins in a titer of 1 : 100 and above. It is recommended to examine paired sera (the second time in 7–10 days after the initial examination). Increasing the antibody titer in 4 times or more has a high diagnostic value.

Blood-droplet AT. Blood from the finger in the form of a thick drop is applied to a slide and an equal volume of distilled water is added to it to cause hemolysis. Next to the blood, a drop of tularemia diagnosticum is put, both drops are mixed with a glass rod. In patients with tularemia in the seropositive period (at an antibody titer of 1 : 100 and above) agglutination occurs immediately. In persons who have suffered tularemia, specific agglutinins are found in the blood for a long time.

Passive hemagglutination test (PHAT) can be positive at the end of the first and the beginning of the second week of the disease. Tularemia erythrocytic diagnosticum (sheep erythrocytes with absorbed tularemia antigen) is used as an antigen. In patients with tularemia, antibody titers in this reaction may be 1 : 1280–1 : 2560 and higher. For the detection of antibodies to Francisella tularensis, ELISA is also used.

Allergological research. The earliest method of diagnosing tularemia is allergic test with tularin. The test becomes positive from the 3rd–5th day of the disease. Intradermal tularin is administered in a dose of 0.1 ml inside the skin of the palmar part of the forearm (pretreated with alcohol). The results of the sample are taken into account in 24–48 h by examining and palpating the injection site. The presence of severe infiltration and hyperemia with a diameter of 1.0 cm and more is regarded as a positive reaction. Individual patients may have pustules and even necrosis of the skin at the injection site, lymphangitis, regional lymphadenitis with an increase in body temperature to 37.5–38.0 °C for 24–48 hours.

Biological method. Isolation of the pathogen is carried out in special laboratories. It is not usually possible to isolate the culture on nutrient media. Therefore, the investigated material (punctate from bubo, scraping from the ulcer, conjunctival discharge, pharyngeal swab, sputum, blood) is inoculated in white mice or guinea pigs. The material is administered subcutaneously, ocularly, intraperitoneally and / or through the mouth. If the experimental animals do not die within 7–15 days, they are killed and the corpses are opened. Blood, bone marrow, parts of internal organs and lymph nodes of the animal's corpse are rubbed into the surface of one of the media: yolk, McCoy, glucosocysteine agar with blood and antibiotics.

The yolk agar. The medium containes egg yolks (pH 7.0–7.4). On the 3rd–5th day (at 37 °C), and sometimes on the 20th and later, there appear delicate small colonies, the growth of which resembles shagreen skin.

Glucosocystin agar with blood. To the melted MPA, cystine and glucose are added (pH 7.2 to 7.4), boiled for several minutes, cooled to 45–50 °C, and 5–10 % defibrinated rabbit blood is added. The colonies of bacteria are moist, milky white in color. The causative agent of tularemia reproduces well in the yolk sac of the 12-day-old chick embryo. It grows poorly in liquid nutrient media. Simultaneously smear-prints are prepared and stained by Romanovsky-Giemsa.

As an express diagnostics, the pathogen is detected in the material by the methods of IF and ELISA. The isolated pure culture is identified by morphological, antigenic and biological properties, its sensitivity to antibiotics is determined. Freshly isolated pathogens of tularemia are biochemically little active. They can ferment glucose, release hydrogen sulphide, with further cultivation they acquire the ability to ferment maltose, mannose and other carbohydrates. To identify the isolated culture, AT with tularemia agglutinating serum and IF with luminescent serum are used. The virulence of culture is established by infecting sensitive animals.

PCR is used.

The antigen in a boiled suspension of the spleen and liver of the deceased animal is determined by ***the thermoprecipitation reaction***.

Prevention and treatment. Prevention of tularemia is carried out in the foci of the pathogen. Immunization of people is carried out by the live

attenuated vaccine of Gaisky-Elbert on epidemiological indications. The effectiveness of this vaccine is high, a single cutaneous (or subcutaneous) vaccination creates immunity for 5–6 years.

To treat tularemia, streptomycin, tetracyclines and chloramphenicol are used.

Practical tasks, being carried out during practical classes:

1. Studying morphology of Y.pestis and F.tularensis (in atlas and microslides).
2. Studying biological preparations for serological methods (antigens and diagnostic sera).
3. Studying the scheme of laboratory diagnosis of plague and tularemia.
4. Studying vaccines for specific prophylaxis of plague and tularemia.

Therminology: Y.pestis, F.tularensis, blood agar, "lace handkerchiefs", "stalactites" descend, glucosocystin agar with blood, yolk agar, tularin.

Theoretical questions for control:

1. Genus Yersinia, major characteristics, antigenic structure.
2. Genus Francisella, major characteristics, antigenic structure
3. Culture properties of Y. pestis and F. tularensis.
4. Routes of transmission and pathogenesis of plague and tularemia.
5. Laboratory diagnosis of plague and tularemia.
6. Treatment and control of plague and tularemia.

Test tasks for control:

1. Small, Gram-negative coccobacilli showing bipolar staining were revealed in sputum of the patients with pneumoniae. Which of the following bacterium has such morphological characteristic?

A. B.melitensis
B. C.diphteriae
C. C. S.typhi
D. M.tuberculosis
E. Y.pestis

2. In the patients with high fever and severe toxemia (septicemia) doctor revealed increased lymph node (bubon). Which of the following reaction may confirm diagnosis “tularemia”?

A. Agglutination test with tularin
B. Widal test
C. Agglutination test with tularemia diagnosticum
D. Askoly test
E. Wasserman test

3. The selective medium for isolation of Y.pestis is:

A. Blood agar
B. Milk agar
C. Hiss medium
D. Endo medium
E. MPB

4. Which of the following infections quickly spreads to the draining lymph nodes, which become hot, swollen, tender, and hemorrhagic, giving rise to the characteristic black buboes responsible for the name of this disease:

A. Diphtheria
B. Plaque
C. Enteric fever
D. Tuberculosis
E. Anthrax

5. Each of the following statements about Francisella tularensis is true, except:

A. Obligate aerobe *C. Nonmotile* *E. Pleomorphic*
B. Gram positive *D. Non sporeforming*

6. Laboratory diagnosis of tularemia is based on, except:

A. Agglutination test *C. Animal inoculation* *E. Culture*
B. ELISA *D. Skin test*

7. Which of the following is not factor of pathogenicity of Yersinia pestis:

A. Coagulase *C. Shiga-like toxin* *E. F-1 antigen*
B. Plasminogen activator *D. Yops*

8. Which of the following is not true about Yersinia pestis:

A. Pleomorphic *C. Facultative aerobic* *E. Bipolar staining*
B. Gram-negative *D. Motile bacillus*

9. Stalactite growth in ghee broth is seen in case of:

A. C.diphthteria *C. Y. pestis* *E. C. E.coli*
B. S.pyogenes *D. S.aureus*

10. Laboratory diagnosis of plaque is based on, except:

A. Microscopy *C. Cuture* *E. IF*
B. Askoly test *D. Animal inoculation*

LABORATORY DIAGNOSIS OF ANTHRAX AND BRUCELLOSIS

Goal: Studying of laboratory diagnostics of anthrax and brucellosis.

Concrete goals:

1. Study of biological properties and classification of B.anthracis and Brusella.
2. Study of pathogenesis and clinical manifestations of anthrax and brucellosis.
3. Study of the methods of laboratory diagnosis of anthrax and brucellosis.
4. Study of specific prophylaxis and therapy of anthrax and brucellosis.

Students should be able to:

1. Isolate pure cultures of B.anthracis and examine growth on blood agar.
2. Identify pure culture of B.anthracis and Brusella on morphology, culture and biochemical properties, antigenic structure.
3. Perform ring test to diagnose of anthrax.
4. Interprete results of serological tests and skin allergic test.

Equipment: slides, immersion microscope, biological preparations for laboratory diagnosis anthrax and brucellosis, specific prophylaxis and therapy of anthrax and brucellosis, tables, atlas.

Anthrax

Anthrax is an acute infectious disease that proceeds mainly in the form of a dermal form, and less frequent pulmonary and intestinal forms. It is refered to zoonoses. According to current classification, this microorganism is related to the genus Bacillus of the family Bacillaceae.

Morphology. The causative agent Bacillus anthracis is a gram-positive, large (3-8 x 1-1.5 μm) nonmotile rod. In the body of animals and humans, on nutrient media containing blood or serum, bacilli form a capsule (protein nature). In stained preparations, the bacilli are arranged in chains, look chopped off at the ends, so that their chains look like a bamboo cane (Fig. 3).

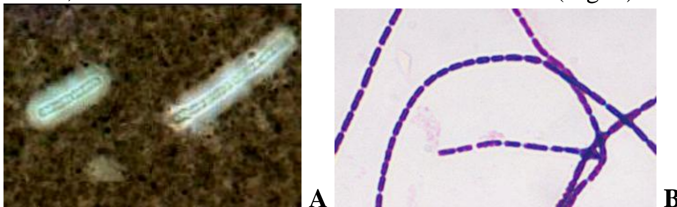

Fig. 3. Morphology of B. anthracis: A – Burry stain, B – Gram stain

Spore formation. In the environment, mainly in the soil, where the pathogen enters with excreta, urine of sick animals or corpses of animals, in the presence of oxygen and at a temperature of 12 to 40 °C bacteria form oval spores. Spores do not exceed the diameter of the microbial cells and are located centrally. In living organisms, spores are not formed. Spores are characterized by extreme

resistance to the action of environmental factors, which allows them to persist for a long time. They are of primary importance in the infection of animals in natural conditions (grass contaminated by spores, water reservoirs).

Cultural properties. The causative agent of anthrax is aerobic or facultative anaerobic. They reproduce well on simple nutrient media. On the surface of the agar crimped, arched, rough colonies with uneven edges are formed in a day, resembling curls, lion's mane or the head of Medusa of the Gorgon. Growth in the broth is characterized by the appearance of white flakes (like cotton wool), settling to the bottom of the test tube, and the broth itself remains transparent.

Seeding a stab in the gelatin column reveals a characteristic feature of growth in the form of an overturned "fir-tree" (a white cord with outgoing processes decreasing downwards).

Biochemical activity of anthrax bacillus is high: besides gelatin liquefaction, they hydrolyze starch, casein, decompose a number of carbohydrates (glucose, maltose, etc.), and restore nitrates.

Resistance. Spores are very resistant: in soil and water they can sustain life for decades. In the soil they can germinate, multiply and again form spores. Disinfectants (5 % solution of carbolic acid, 5–10 % chloramine solution) kill the spores only in several hours of action. Spores are heat-resistant and can withstand even boiling for 15–20 minutes. Tanning and pickling of hides and flesh does not destroy spores. The vegetative forms possess the usual resistance for bacteria – they perish at 55 °C in 40 minutes, at 60 °C in 15 minutes, at boiling – instantly.

Antigens. Anthrax bacilli have a species antigen of protein nature, located in a capsule, and a group, somatic antigen of polysaccharide nature, localized in the cell wall of the microorganism. The somatic antigen is heat-stable, does not break down when boiled.

Ecology and distribution. In natural conditions such animals are sick with anthrax: large and small cattle, horses, pigs, deer, camels. They are infected by alimentary tract, engulfing spores of the pathogen along with the fodder. Pathological process develops in the intestine → generalization – severe septic form by the end of 2 days → bleeding from all natural openings. Isolated vegetative forms in the external environment are transformed into spores → contamination of surrounding objects. Animals have infected wool, skin, meat, bones.

Epidemiology. A human becomes infected from sick animals by direct contact, as well as by contact with livestock products (skins, leather, fur products, wool, bristles), contaminated by spores. Infection can occur through the soil in which the spores of the anthrax pathogen persist for many years. Spores enter the skin through micro-trauma. At alimentary infection (the use of the infected products) there is an intestinal form.

Transmission of the pathogen can be carried out aerogenic (inhalation of infected dust, bone meal). In these cases, pulmonary and generalized forms of

anthrax arise. Transmission of infection through the bites of bloodsucking insects is possible. Human-to-human transmission is usually not observed.

Pathogenicity of the causative agent of anthrax. Bacilli form a toxin, secreted into the environment, where microorganisms multiply. It consists of three components:

– "edematous factor", causing dermonecrotic reaction in guinea pigs;

– lethal toxin ("mouse" toxin) causes edema of the lungs and severe hypoxia;

– protective antigen.

The pathogenicity of the anthrax bacilli also is determined by the capsule, which is a factor of virulence. It has antiphagocytic activity. Bacteria covered with a capsule are fixed on the cells of a macroorganism, the encapsulated cultures are non-virulent.

Pathogenesis. The incubation period ranges from a few hours to 8 days (usually 2–3 days).

Depending on the place of penetration of the pathogen and the primary lesion caused by it, anthrax in man manifests itself in three clinical forms: skin, intestinal and pulmonary.

Portal of entry of infection is often the skin. Usually the pathogen is introduced into the skin of the upper extremities (about half of all cases) and the head (20–30 %), less often the trunk (3–8 %) and legs (1–2 %). A few hours after infection the multiplication of the pathogen begins at the site of the infection. In this case, pathogens form capsules and release exotoxin, which causes dense edema and necrosis. From sites of primary reproduction, pathogens along lymphatic vessels reach regional lymph nodes, and in the future, hematogenous distribution of microbes to various organs is possible.

In case of aerogenic contamination, spores are phagocytosed by alveolar macrophages, then they enter the mediastenal lymph nodes where the pathogen multiplies and accumulates, and the lymph nodes of the mediastinum are necrotic, which leads to hemorrhagic mediastenitis and bacteremia. As a result of bacteremia, secondary hemorrhagic anthrax pneumonia occurs.

When using infected meat, spores penetrate into the submucosa and regional lymph nodes. An intestinal form of anthrax develops, in which pathogens also penetrate into the blood and the disease developes into the septic form. The last two forms are severe and usually end lethal. In any clinical form, weakened people may develop anthrax septicemia with fatal outcome.

Immunity. The anthrax induces a strong immunity, the main role is played by the phagocytic reaction and antibodies. During the course of the disease, specific sensitization develops, which is detected by an intradermal test with an allergen-anthraxin.

Clinical manifestations. The cutaneous form is characterized by local changes in the area of the infection. In the beginning, a red spot appears on the

lesion site, which rises above the skin level, forming a papule, then the vesicle develops in place of the papule, after a while the vesicle turns into a pustule, and then into an ulcer. The process proceeds quickly, several hours pass from the moment of the appearance of the spot to the formation of a pustule. Locally, the patients notice itching and burning. The contents of the pustule often have a dark color due to the admixture of blood.

When the integrity of the pustule is violated (more often with combs) an ulcer is formed, which is covered with a dark crust. Around the central scab, there are secondary pustules in the form of necklaces, the destruction of which increases the size of the ulcer. Around the ulcer there is swelling and hyperemia of the skin. Characteristic is the decrease or total absence of sensitivity in the area of the ulcer.

In the place of localization of the pathogen, a carbuncle is formed. First a bright red spot → papula of copper-red color → in several hours vesicule, first serous, then bloody (maybe with a purulent strip), sometimes it has a purple color → a blackish scab forms in the center quickly, around it swelling tissue (crimson color) is located. Carbuncle is painless. In the treatment and favorable course the carbuncle disappears, with unfavorable course it can go into the septic form.

Signs of general intoxication (fever up to 40 °C, general weakness, headache, adynamia, tachycardia) appear at the end of the first day or on the 2nd day of the disease. Fever lasts for 5–7 days, body temperature decreases critically. Local changes in the area of the ulcer gradually heal, and by the end of the 2–3rd week the scab is rejected. Before the introduction of antibiotics in patients older than 50 years, lethality was 5 times higher (54%) than in younger patients (8–11 %). In vaccinated against anthrax, skin changes can be very slight, resembling an ordinary boil, and general signs of intoxication may be absent.

The pulmonary form of the anthrax begins sharply, proceeds severe and even under modern methods of treatment can end up lethal. The condition of patients from the first hours of the disease becomes severe. The body temperature quickly reaches high figures (40 °C and above). There are strong stitching pains in the chest, dyspnea, cyanosis, tachycardia (up to 120–140 beats/min), blood pressure decreases. Catarrhal phenomena of the upper respiratory tract (sneezing, runny nose, hoarse voice, cough) are observed. Death comes in 2–3 days.

The intestinal form is manifested by severe intoxication, nausea, vomiting with bile, diarrhea with blood.The patient's condition progressively worsens and patients die when infectious-toxic shock occurs. With any of the described forms, anthrax sepsis may develop with bacteremia and the appearance of secondary foci (meningitis, liver, kidney, spleen, and others).

Laboratory diagnosis is carried out by bacterioscopic, bacteriological, biological methods.

The laboratory confirmation of the diagnosis is the isolation of the culture of the anthrax bacillus and its identification. For the study, the contents of pustules, vesicles,

tissue effusions from the scab are taken. When suspected of a pulmonary form, blood, sputum, and bowel movements are taken.

Bacterioscopic method. A smear prepared from the test material is stained by Gram and other methods that reveal the capsule. It is effective the use of capsular luminescent serum. The pathogen is detected also according to the characteristic shape and location of the chains, but it is necessary to make differential diagnosis with Bacillus anthracoides.

Bacteriological method. Isolation of the pure culture of the pathogen is performed by seeding the material into liquid and on solid nutrient media (meat-peptone agar). Identification is carried out according to morphological, tinctorial, cultural (specific form of colonies on solid media and on gelatin) signs, and also on lysability by a specific phage and "pearl necklace" test.

In the Petry dishes with MPA, characteristic rough colonies ("head of the jellyfish"), whose edges with microscopy with a small increase, appear as curly locks ("lion's mane"). In the broth, bottom growth appears, resembling a wad of cotton wool, the medium remains transparent. In the smear, uncapsular Gram-positive bacilli are found, which are located in long chains.

On the nutrient agar with penicillin, the transformation of bacteria into protoplasts in the form of separate balls located in a chain is observed - the phenomenon of a "pearl necklace" (since they lose the cell wall). Soil bacilli retain the shape of rods arranged in chains.

Bacilli of anthrax, unlike non-pathogenic rods similar to them, are lysed by a specific anthrax phage.

Identification of the isolated culture is carried out by seeding it in gelatin, other differential media, lysis by phage and infection of animals, as well as determining the susceptibility of the culture to antibiotics.

The anthrax bacilli give characteristic growth in the column of gelatin in the form of a rod, from which the shoots looking like an inverted fir-tree, later gelatin is liquefied. The study of the enzymatic and other biological properties of the isolated culture makes it possible to differentiate the anthrax bacilli and soil bacilli.

To investigate the material (corpses, skins, wool), a thermoprecipitation reaction whith a specific serum is used (Ascoli reaction). The desired antigen is extracted by boiling for 10 to 45 minutes. Then, the liquid is filtered and the resulting extract is carefully layered onto the precipitating anthrax serum in a narrow tube. Serum is obtained from the blood of animals, hyperimmunized with the STI vaccine or by the killed culture of the pathogen. At the same time, controls are placed: extracts and precipitating serum with a known positive and negative test, with normal horse serum, etc. A white ring (precipitate) appears on the interface between the two liquids within 1 to 5 minutes, which is regarded as a positive reaction. When the precipitate appears after 10 minutes, the reaction is considered non-specific.

Serological method: IF, reverse indirect hemagglutination reaction (with erythrocytes loaded with antibodies), immunoassay analysis (ELISA).

Serological examination is carried out in those cases when it is not possible to detect the pathogen in the material. To determine antibodies in the patient's blood serum, highly sensitive reactions of latex agglutination and passive agglutination with a protective anthrax antigen are used.

Allergological research. An allergic test is used to determine the immunological changes after a disease and vaccination. Allergen anthraxin is injected intradermally into the palmar surface of the forearm in a volume of 0.1 ml. The results are taken into account in 24 hours. The sample is considered positive in the presence of hyperemia and infiltration with a diameter of more than 15 mm. A positive reaction becomes in the first days of the disease and persists for many years after recovery.

Biological method. Laboratory animals are infected. In smears from internal organs and blood, typical bacilli are found surrounded by a capsule.

Prevention and treatment. For specific prevention, the STI vaccine (Sanitary Technical Institute) - a suspension of live spores of avirulent, non-capsular (S-form) anthrax bacteria, can be used. The vaccine is administered once dermally or subcutaneously, the immunity is created for 1 year, if necessary, revaccination is used.

For the treatment of anthrax, both immunoglobulin and antibiotics (penicillin, chlorotetracycline, streptomycin, ciprofloxacin, doxycycline) are used.

Brucellosis

The causative agents of brucellosis, a chronic zoonotic infection, belong to the genus Brucella. For a human pathogenic species: Brucella melitensis, Brucella abortus, and Brucella suis (they are referred to bacteria of the second pathogenicity group).

Each pathogen causes disease in a certain species of animals, but all three species of brucella (Brucella melitensis, Brucella abortus, Brucella suis) cause disease in humans. They are morphologically indistinguishable, biochemical methods and AT with specific sera are used for differential diagnostics.

Morphology, physiology. Brucellae are small, gram-negative coccobacteria in the size of 0.6–1.5×0.5–0.7 microns (*Fig. 4*). They do not have flagellum and do not form spores. Freshly isolated strains can form a tender capsule.

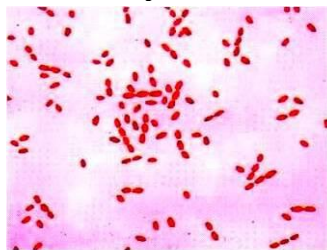

Fig. 4. Morphology of Brucella

Brucella are fascidious for nutrient media. For their cultivation special media with the addition of serum, blood, glucose, thiamine, biotin are used. Isolated from the body of

patients, they reproduce very slowly, growth can be detected only in 1–3 weeks after seeding the material. Multiple seeding in the laboratory makes cultures capable of growing within 1–2 days. On solid nutrient media brucella form small, convex, colorless with pearl gloss colony of S-form, which easily dissociates into mucoid and rough. In liquid media, there is uniform turbidity. Under the influence of antibiotics they produce L-forms.

Brucella are strict aerobes. Brucella abortus in the first generations needs an increased concentration (5–10 %) of CO_2.

Biochemical activity of brucella is characterized by the ability to break down glucose and some other carbohydrates, decompose urea and asparagine, hydrolyse protein, peptones, amino acids, release catalase, hyaluronidase, peroxidase, lipase, phosphatase and other enzymes. In Brucella species, biovars are distinguished. Their differentiation is based both on biochemical differences and on the ability to grow on media with fuchsin and thionine, lysis of phage T6, agglutination by monospecific sera.

Antigens. Brucella contain a superficially located Vi-antigen and somatic species-specific antigens A and M, the quantitative ratio of which is different for different species. M-antigens dominates in Brucella melitensis, in Brucella abortus and Brucella suis – A-antigen. To identify brucella by antigenic properties, the adsorption of agglutinins by Castellani or mono-receptor sera are used.

Ecology, distribution and epidemiology. Brucellosis is a zoonotic infection. The causative agents of various species circulate among a certain range of animals, from which people are infected. Brucella melitensis causes diseases of small cattle, Brucella abortus – cattle, Brucella suis – pigs. Other brucella species affect rams and sheep – Brucella ovis, dogs – Brucella canis but do not cause disease in humans,

Sick animals excrete the pathogen with urine, feces. In animals, the pathogen is contained in the placenta, in amniotic waters, because in the placenta there is a protein erythritol, which is a good substrate for the growth of brucella.

The main symptoms of the disease in animals:

- fever,
- disturbance of generative organs and gestation,
- lesion of joints → claudicatio intermittens.

Animals are infected from animals with a common watering place (waterway), on pasture (alimentary route), and sexually. In cattle, the incidence of brucellosis is the second most frequent after tuberculosis.

Man is not the natural reservoir of the pathogen. Penetration of the causative agent of brucellosis into the human body is possible by alimentary, contact and airborne pathways. Veterinarians, zootechnicians, meat-packing plant workers are more often infected by contact with the care of sick animals, processing of raw materials. Infection is possible when working with infected wool, rags, when spraying takes place, and penetration of brucella into the air.

The alimentary route of infection is associated with the consumption of animal products (cheese, butter, milk, meat), obtained from sick animals. There are also contact-household and water ways of infection.

Laboratory workers working with the causative agent of brucellosis are infected frequently. Breathing in the bacteria that causes brucellosis may also lead to infection. This risk is generally greater for people in laboratories that work with the bacteria.

For humans, the most pathogenic are B. melitensis, the causative agent of small-horned cattle disease (sheep, goats). Infection of man from man is impossible.

Resistance. Brucella are characterized by great resistance to the action of environmental factors. They persist at low temperatures for a long time. Pathogens survive in soil, urine, feces of animals, sick with brucellosis, in manure for 4–5 months, in sheep wool – 3–4 months, in dust – 1 month. In milk and dairy products (in brynza, butter), cooked without additional heat treatment they remain viable for 4 months, in frozen meat – up to 5 months. To high temperature and disinfectants Brucella are highly sensitive: they die at 60 °C for 30 minutes, at boiling – instantly. All disinfectants destroy brucella for several minutes.

Pathogenicity of brucella. The portal of entry of infection in humans is mucous membranes and skin. Invasive and aggressive properties of brucella determine the ability of the pathogen to penetrate the body through intact mucous membranes.

Factors of virulence:

● intracellular localization → prolonged existence in the body. In the pathogenesis of brucellosis, the ability of the pathogen to reproduce in the cells of the lymphoid macrophage system is important.

● presence of a capsule → blockage of phagocytosis;

● endotoxin production, which is released when microorganisms are destroyed → endotoxinemia → fever.

● enzymes (hyaluronidase, etc.) that promote the spread of microbes in tissues.

Pathogenesis of brucellosis:

● First of all, the pathogen enters the lymph nodes and multiplies there, localizing intracellularly. In the lymph nodes, an reservoir is created, resistant to the action of protective factors (phagocytosis) → long-term retention.

● Generalization: the spread by lymphogenous and hematogenous pathways through the body → damage to other lymph nodes and other organs and tissues (spleen, bone marrow).

● Later the disease turns into chroniocepsis - the periodic inflow of microorganisms into the blood from the lymph nodes containing their reservoir, → the destruction of microorganisms → the release of endotoxin → a rise in temperature.

● Since the first days of the disease, the reaction of deleyed cytotoxicity occurs (because the lipopolysaccharide of the cell wall and proteins have a

sensitizing effect), which persists throughout the disease and for a long time after recovery. In the affected tissues granulomas are formed (the first – to 20 days from the onset of the disease). Granulomas occur according to a mechanism similar to that of granulomas of tuberculosis (but there is no specific necrosis). Brucellosis is a chronic granulomatous infection inclined to the formation of chroniocepsis.

The main signs of the disease in humans. The incubation period lasts from 1 to 3 or more weeks. The beginning is gradual (the person feels well, although the temperature is above 40 °C), then the main symptom appears - a prolonged fever (wavy, intermittent), can last for months. It is possible to defeat of large joints (arthritis, in some cases – with destruction of tissue), in severe forms the heart, lungs, liver, nervous system are affected, the spleen may increase. The normal course of pregnancy can be disturbed.

Immunity. The base of immunity in brucellosis is the activity of the T-lymphocyte system. An important role is played by phagocytosis and the state of allergy. Brucella decontamination occurs with the participation of antibodies – opsonins, agglutinins. Immunity is non-sterile, i.e. its protective function is expressed weakly and the causative agent persists in the body.

Laboratory diagnosis is carried out by bacteriological and serological methods.

Bacteriological method. The material for bacteriological research at the height of fever is blood, feces and urine of patients, outside of fever – punctate from lymph nodes. The causative agent can be isolated from the bone marrow. Sometimes the cerebrospinal fluid is examined. In addition, aborted animal fetuses, amniotic fluid and placenta areas, milk, air and other objects of the environment are examined.

● *I stage.* Seeding the material in two flasks with a liver broth, one of which has a high concentration of CO_2 (for Brucella abortus). Antiphage serum is added to the medium (as most cultures are lysogenic and the bacteriophage can be activated when the conditions change). Observations for the growth are conducted for 3–4 weeks. Seeding and bacterioscopy are conducted every 4–5 days. On liquid media brucella grow with the formation of light turbidity and a small mucous sediment.

● *II stage.* Seeding on the accumulation medium: liver agar + antiphage serum.

On solid media colonies appear no earlier than 48 h: rounded, up to 5 mm in diameter, grayish-white, shiny, transparent with an amber tint in transmitted light. When forming isolated colonies on a solid medium, a pure culture is obtained and the brucella species is determined.

The bone marrow for the isolation of myeloculture is seeded on the same media. It has been established that myelocultures in brucellosis can be obtained 1.5–2 times more often than hemocultures.

● III stage. The isolated culture is identified according to biochemical tests, a phage test, bioassays (virulence determination), a Heddleson bacteriostatic

method (based on the different ability of microorganisms to grow on media with dyes), serological reactions; biovars are detected.

Kozlovsky stain. The smear is fixed in a flame, stained with a 3 % aqueous solution of safranin with heating over the flame until the appearance of fumes, washed with water and dyeed with a 1 % aqueous solution of methylene blue for 1.0–1.5 minutes, washed again with water and dried. Brucella are stained pink after Kozlovsky method.

Allergic method. Intradermal allergic Burne test is used to identify the hypersensitivity to brucella allergens in patients and recovered from brucellosis. Positive results become in 2–3 weeks after infection and persists so throughout the course of the disease. The Burne test is used with brucellin (most often performed in animals). Intracutaneously, 0.1 ml of brucellin is injected into the region of the middle third of the forearm. A positive reaction is considered if in the day after the introduction painfulness, hyperemia and infiltration of the skin appear on the average size of 3–3 cm (slightly positive –- 1×1 cm, sharply positive – 6×6 cm). The allergic reaction is positive for many years after the disease and after vaccination.

Serological method. Serodiagnostics is carried out from the 10th to the 12th day, when antibodies appear.

● Haddleson reaction – slide agglutination reaction with undiluted serum – only the presence or absence of antibodies can be determined. The Haddleson agglutination reaction, due to the rapid accounting and simple formulation technique, is most often used in foci of brucellosis (in the field). The glass plate is divided by a wax pencil into 6 squares and undiluted test serum in amounts of 0.08; 0.04; 0.02; 0.01 and 0.02 ml is applied by a micropipette, the same diagnosticum as for Wright's reaction (0.03 ml to each dose of serum) is added (except for the 5th square in which 0.03 ml of saline is added (serum control)). In the 6th square there is 0.03 ml of antigen and 0.03 ml of saline (diagnosticum control).

Serum and antigen drops are mixed with a glass rod. The reaction develops already in the first minutes in the form of the appearance of fine-grained colored agglutinate. Absence of agglutination in all doses of serum is assessed as a negative reaction, the presence of agglutination in the first dose (0.08 ml of serum) is doubtful, in the second (0.04 ml) is weakly positive, in the 3rd or 4th doses (0,02–0,01 ml) - positive, in all doses – a sharply positive reaction.

The disadvantage of the Haddleson agglutination reaction is often the observed positive result in healthy people due to normal antibodies. Therefore, it is necessary to put tube AT with sera, which gave a positive Haddleson reaction. In acute, subacute and chronic form of brucellosis, antibodies in the serum of patients can be detected with the help of IF, which is more sensitive than the agglutination reaction.

● Wright's reaction – tube agglutination reaction – antibody titer can be detected. Wright's reaction is put at the beginning of the second week of the disease. As a diagnosticum killed by heating and phenol brucella, stained with gentian violet or methyl violet are used. For the Wright and Haddleson reactions, a single color brucellosis diagnosticum containing antigens of three pathogenic human brucella is produced.

The reaction is considered positive at a titer of 1 : 200 and doubtful at a titer of 1 : 50. Wright's reaction can be positive in vaccinated and sick people, therefore, as the disease progresses, it is repeated, taking into account the growth of antibody titer.

● PHAT, immunofluorescence, immunoassay, CFT.

With the help of PHAT in patients with acute and subacute brucellosis, agglutinins can be detected in 90–100 % of cases. The high diagnostic value in the acute phase of brucellosis ELISA and IF have which allow detecting antibodies of IgM class in patient's serum. They unlike IgG appear in the early period after infection. CFT is very specific for brucellosis. A positive result is recorded from the 20th–25th day of the disease and persists for a long time.

● Incomplete antibodies are detected by the Coombs and Wiener reaction.

After infection IgM are found, IgG appear in a week. In the treatment of IgG titer decreases, recovery can last for weeks and months. In case of relapse (if symptoms appear again) IgG is immediately detected, when disease occurs de novo, IgM first appear.

Serological methods are used more often, because brucellosis is a particularly dangerous infection, and when using serological methods, direct contact with the pathogen is excluded.

Biological method. This research is carried out only in special laboratories. The white mice and guinea pigs are infected. It is used to determine virulence, excretion of the pathogen from the organs. The blood of the patients is administered to the animals intraperitoneally, the urine sediment is administered subcutaneously. Blood is taken from the guinea pig (or mouse) in 20–30 days after infection and AT is performed. Then the animals are slaughtered, and bacteria are isolated from blood, internal organs and lymph nodes. Identification of the pathogen is carried out as described above.

PCR. Given the complexity of the cultivation of brucella, the use of PCR allows increase the sensitivity of detection of the pathogen, reduce the analysis time significantly. The sensitivity of the method is 100 cells in the sample.

Prevention and treatment. Vaccination is carried out by a live attenuated brucellosis vaccine, which is administered to animals on a scheduled basis, as well as to people who are at risk of infection. For treatment, streptomycin, levomycetin, tetracycline, erythromycin as etiotropic agents are used.

Practical tasks, being carried out during practical classes:

1. Studying morphology of B. anthracis and Brucells (in atlas and microslides).
2. Studying biological preparations for serological methods (antigens and diagnostic sera).
3. Studying the scheme of laboratory diagnosis of anthrax and brucellosis.
4. Studying vaccines for specific prophylaxis of anthrax and brucellosis.

Therminology: *Bacillus anthracis, Brucella melitensis, Brucella bovis, Brucella suis*, "pearl necklace" test, "head of the jellyfish", "lion's mane".

Theoretical questions for control:

1. Genus Bacillus, major characteristics, antigenic structure, culture properties.
2. Genus Brucella, major characteristics, antigenic structure, culture properties.
3. Routes of transmission and pathogenesis of anthrax and brucellosis.
4. Laboratory diagnosis of anthrax and brucellosis.
5. Treatment and control of anthrax and brucellosis.

Test tasks for control:

1. Bacteriological method is the most definite one for the diagnosis of brucellosis. Which of the following specimens is used to obtain the pure culture:

A. CSF
B. Lymph node
C. Urine
D. Blood
E. Al of the above

2. The intracellular persistence of bacteria results in granuloma formation in the reticuloendothelial system organs and tissue damage due to hypersensitivity reactions, mostly:

A. Type 1
B. Type 2
C. Type 3
D. Type IV
E. All of the above

3. Which of the following methods is used to detect a capsule in the smear prepared from tissue of infected animals?

A. Staining with methylene blue
B. Silver impregnation
C. Ziehl-Neelsen's stain
D. Burri-Gins' stain
E. Gansen's stain

4. Laboratory diagnosis of anthrax is based on, except:

A. Microscopy
B. Askoli test
C. Animal inoculation
D.
E. Bacteriological method

5. Medusa head colonies are produced by:

A. Brucella melitensis
B. Staphylococcus aureus
C. Mycobacterium tuberculosis
D. Corynebacterium diphtheriae
E. Clostridium tetani

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Навчальне видання

ЗООНОЗИ

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та стоматологічного факультетів
з англійською мовою викладання

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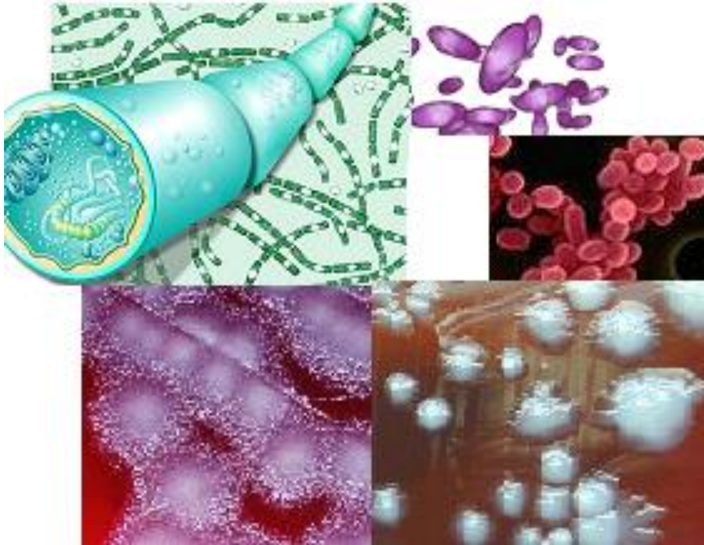

ZOONOSIS

Learning guide for the 2nd and 3rd year English media students of the Faculty of Medicine and the Faculty of Dentistry (Microbiology, virology and immunology)

ЗООНОЗИ

Методичні вказівки з дисципліни «Мікробіологія, вірусологія та імунологія» для студентів II і III курсів медичного та стоматологічного факультетів з англійською мовою викладання