Ministry of Public Health of Ukraine

Bogomolets National Medical University

Department of microbiology, virology and immunology

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STUDY GUIDE

OF THE PRACTICAL CLASSES COURSE

Part II

Specialties:

221 "Dentistry"

222 "Medicine"

225 "Medical Psychology"

226 "Pharmacy, industrial pharmacy"

228 "Pediatrics"

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Class №10

Topic: "Non-specific defense against microorganisms"

Relevance of the topic:

The complex processes and appearances in human body occur permanently. They are directed to support the functional integrity of the body, sustainability (permanence) of the chemical and cellular composition of the internal environment - that is, homeostasis. Protecting the body against foreign agents occurs at two levels: non-specific resistance and specific immunity. The first - an older phylogenetic level consists of nonspecific factors of host protection, which act against any foreign agent.

Nonspecific host defenses are the protective functions of the skin, mucous membranes, lymph nodes, gastric juice, hydrolytic enzymes; inhibitors production, lysozyme, interferon and others; antagonistic properties of the human microflora; bactericidal properties of blood: complement, β-lysine, leykins, normal antibodies and other substances with bactericidal action; phagocytosis.

Mechanisms of nonspecific host protection function in the body permanently and cause inflammatory reaction in cases of massive or microbial (or) other destabilizing actions, the same - various causative agents.

Various diseases are observed when components of nonspecific host protection are violated. Determination of quantitative changes, qualitative or functional state of various factors of nonspecific host defenses give possibility at value the state of the human immune system, help in the diagnosis, prevention and therapy of infectious and noninfectious diseases. All this provides for the relevance of topic and directs a positive motivation to study.

Educational purposes:

- To study theof technique of determination of lysozyme in human serum by titration method.
- The complement titration test in serum provides for 100% hemolysis, consider features of this test, make conclusions.
- To study the phenomenon of incomplete phagocytosis on the smears that are made from urethral discharge in a patient with gonorrhea.

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

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

Medical biology	Explain at the molecular and cellular level the patterns of biological processes.
Medical chemistry	Treat common physical and chemical principles that base the processes of cells develoupment.

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

Terms	Definitions
Immunity	Immunity – is a complex of processes and mechanisms that provide sustainability (permanence) of antigenic structure of an organism and its protection against infectious and other foreign agents for him.
Nonspecific host defenses	Nonspecific factors of host defenses, as evolutionarily emerged before specific immunity, are non-specific mechanisms, physic-chemical, cellular, humoral, and physiological protective reaction that ensure the constancy of internal environment and restoration of disturbed functions of macroorganism. Non-specific resistance factors act quickly and constantly against all are the foreign agents. The non-specific resistance factors are: barrier function of the skin, mucous membranes, lymph nodes, bactericidal components of organism liquid (saliva, blood serum, etc), secretion function, fever, antagonistic properties of normal microbiota etc.
Lysozyme	Lysozyme - is enzyme (acetylmuraminidase) that can destroy peptidopolysaccharide of grampositive bacteria cell wall, which consists from murein into the 90 %. Lysozyme is synthesized by macrophages and provides bactericidal properties of blood, saliva, and mucosa.
Complement	Complement – is a complex set of blood proteins, which consists of 9 factions, each of which has a certain property. Complement is synthesized by liver cells and performs some functions: 1) causes lysis of microbes and other cells, 2) taks part in specific immunological reactions and virus neutralization; 3) intensifies phagocytosis, chemotaxis, and inflammation.
Properdine	Properdine - is a high-(230 thousand Daltons) serum protein that participates in an alternative way of complement activation, eliminates some bacteria and viruses and stimulates phagocytosis.
Phagocytosis	Phagocytosis - is the oldest form of host defenses, is an active absorption and digestion of live or killed microorganisms or other foreign particles by the cells. Two types of cells carry out phagocytic function: 1) microphags (neutrophils, eosinophils);2)

	motile macrophages (monocytes, histiocytes, etc.) and motionless (cells of the spleen, lymphatic tissue, liver endotheliocytes, endothelium of blood vessels, etc.)..
Uncomplete phagocytosis	Uncomplete phagocytosis - is phagocytosis, in which microorganisms are absorbed by phagocytes, but not killed and digested, and sometimes multiply, causing the death of phagocytes.
Inflammatory reaction.	The inflammatory reaction - is a reaction in which the tissues release various substances (leukotoxins, leukophenic factor, histamine, serotonin, etc.), under the action of which leukocytes activate, that do not allow bacteria to spread in tissue, blood and organs. Inflammation causes fever, acidosis and hypoxia, that make detrimental effect on microorganisms.
Interferons	Interferons – make up a group of low-molecular-weight induced proteins that carry out control and regulatory functions aimed at preserving cellular homeostasis. The most important of these functions are antiviral, antitumor, immune-modulating, antibacterial and radioprotective. Interferon is synthesized by lymphocytes, leukocytes, fibroblasts, cells of the lymph nodes. Interferon is ubdivided into three types: interferon-α (from leukocytes), interferon-β (from fibroblasts) and interferon-γ (lymphocytic, or immune). Induction of interferon synthesis may be caused by viruses, bacteria, fungi, plant extracts, and synthetic compounds, various drugs, radiation, etc.
Acute phase proteins	Acute phase proteins – make up a large group of proteins that are produced in the body during inflammatory responses after infection or injury, during ontogenecity, pregnancy and have antimicrobial action, promote phagocytosis, complement activation, the formation and elimination of inflammation. The bulk of acute phase proteins consist from C-reactive protein, serum amyloid A and P. Other acute phase proteins - are blood coagulate factors, metalic-binding proteins, protease inhibitors and some components of complement.
β -lysine	Beta-lysine - is thermostable (destroyed at 65-70 ºC) bactericidal factor, that is active against anaerobes and aerobic spore-forming bacteria.
Inhibitors of viral activity	Inhibitors of viral activity, this is the first humoral barrier that prevents virus contact with the susceptible cells. Thermostable inhibitors can inactivate infectious, toxic, haemagglutinate properties sensitive to inhibition of strains of viruses.Thermostable inhibitors can block connections of virus

	with the host cell receptors. People with high levels of inhibitors in the blood have a greater resistance to viral infections.
Cytokines	Cytokines are the soluble mediators (like hormone) of host defense responses, both specific and nonspecific. The same cytokine can be produced by multiple cell types and can produce multiple effects on the same cell, and they can also act on many different cell types. Cytokines are the peptides or glucoproteins. As regulators of cytokines production there may be another cytokines, hormones, prostaglandins, antigens and many other agents, which can act to the cell.

Theoretical questions :

- Types of immunity that provide for protection of the organism from live organisms and substances that have geneticaly foreign information.
- Factors of nonspecific host defenses and their features in adults and children.
- The priority of Ukranian scientists in the discoveryof the factors (nonspecific host defenses).
- Phagocytosis. Cells that are capable of phagocytosis. Stages of this phenomenon.
- Complement and its components. Activation of complement. Methods of its determination.
- Properdine system. Its nature, properties and values.
- lysozyme, its nature. Lysozyme action on microorganisms and methods of its determination.
- Why does the normal microflora belong to nonspecific host defenses?
- Interferon’s types. Their role in antiviral, antitumor immunity, and in the regulation of immune functions of organism.
- The practical value of complement titer determination, of lysozyme, phagocytosis and other indicators of non-specific host defenses.

Practical tasks performed in class:

- Determination of lysozyme activity in human saliva with titration method.
- Reading the results of the complement titration test according to 100% hemolysis, examining the test and its features.
- Observing the phenomenon of uncomplete phagocytoses in the demonstrative smears.

Content topics:

In practice students should be able: to carry out determination of lysozyme activity in human saliva with titration method; to read the results f the complemen titration test according to 100% hemolysis, explin the test resalts; to observe phenomenon of uncomplete phagocytoses in the demonstrative smears.

The students write down in the protocol complete tasks and teacher sign it.

Recommendations for design of the protocol

Schematic representation of lysozyme titration

№ tube / Ingredients, ml.	Test tubes						Control
	1	2	3	4	5	6	7
0,5% Isotonic sodium chloride solution	1,8	1	1	1	1	1	1
Patient's saliva	0,2	1	1	1	1	1	
Dilution of patient's saliva	1:10	1:20	1:40	1:80	1:160	1:320	
2 mlrd cell/ml M.lysodeikticus	1	1	1	1	1	1	1
Final dilution	1:20	1:40	1:80	1:160	1:320	1:640	

Incubation in thermostate for 15 min at 45°C (previous registration).

The final registration of the results is made after incubation of tubes at 37°C for 24 hours.

Titer of lysozyme - is that most of amount of serum dilution, which is able to give complete lysis of bacteria (M.lysodeikticus)

Schematic representation of complement titration in human serum by 100% hemolysis.

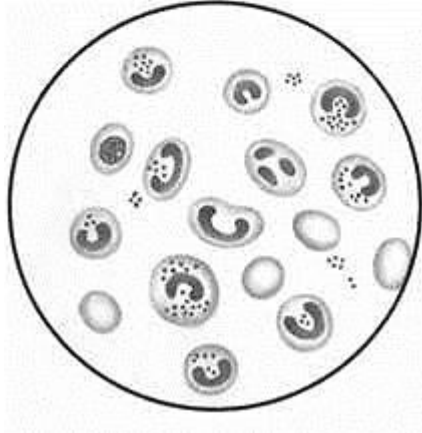
№ tube / Ingredients, ml	1	2	3	4	5	6	7	8	9	10	11
Tested serum in 1:10 dilution	0,1	0,2	0,3	0,4	0,5	0,6	0,7	0,8	0,9	1,0	1,2
Isotonic sodium chloride solution	1,4	1,3	1,2	1,1	1,0	0,9	0,8	0,7	0,6	0,5	0,3
Haemolytic system	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0
In thermostate on 40-50 min at 37°C											
The results*	-H	-H	-H	+H	+H	+H	+H	+H	+H	+H	+H

Note: ***-*H – no hemolysis; +H –hemolysis is present.***

Haemolytic system – a mixture of equal volumes of haemolytic serum (in 1:3 dilution) and 3 % suspension of erythrocytes of the sheep. The mixture is incubated for 30 minutes at 37 °C erythrocytes sensibilization.

Titer of complement – is the smallest number of tested serum that provides complete hemolysis of added volume of sensibilized erythrocytes.

To observe phenomenon of uncomplete phagocytoses in the demonstrative smears



Questions for self-control.

- What does immunology study?
- What are the factors of nonspecific host defenses? Advantages and disadvantages of nonspecific resistance mechanisms.
- What is the priority of Ukranian scientists in the discovery of the host defenses factors?
- Phagocytosis. Cells that are capable to provide phagocytosis. Types of phagocytosis. Describe the phagocytosis.
- Complement and its components. What are the similarities and differences of the two ways of complement activation? What are the methods of its determination?
- Properdine system. What is its nature, properties and values?
- What is a lysozyme, and its nature? How does lysozyme act on the microorganisms? What are the methods of its determination?
- Give the definition of "cytokines". Their immune-modulating and protective effect.
- What are interferons? What are the main interferons' types? What is their role in antiviral, antitumor protection and immune-modulating functions for ogranism?
- What is the practical value of determination of complement, lysozyme, phagocytosis and other indicators of nonspecific host defense?

Class №11.

"Serological tests (part 1)"

Relevance of the topic:

Methods of treatment of infectious disease are determined by biological characteristics of causative agent. That is, the doctor should not only put a clinical diagnosis, but also determine which organism caused the infection. Etiological diagnosis of many infectious diseases is based on an isolation of pure culture of causative agent and its identification. The identification of most bacteria and viruses is based on the determination of specific antigens, we use serological tests for this task. The purpose of this investigation is the serological identification. In addition, the diagnosis of infectious of diseases can be based on identification of specific microbial antigens directly in patient specimens (blood, spinal fluid, urine, etc..), especially, if we can not cultivate and make pathogen's identification by other methods.

On the other side, the immune response manifested, in particular, by development of specific antibodies to each species of causative agent. This allows us to put the etiological diagnosis by detection of these antibodies. For this purpose, we carry out serological tests for serological diagnosis.

Specific objectives:

- To familiarize with the purpose of using serological tests (ST).
- To examine the medicine, used for serological identification.
- To familiarize with the features of agglutination and precipitation tests.
- To master glass agglutination test and direct agglutination test (AT).
- To learn the technique of precipitation test (PT).

Basic knowledge, skills, skills needed to study topics (interdisciplinary integration). See a practice № 10.

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

Terms	Definitions
Serological tests	The reaction of a specific interaction between antigens (Ag) and antibodies (Ab).
Agglutination test	Agglutination – is clumping of corpuscular Ag (bacteria, erythocytes) under the action of specific antibodies in the presence of electrolyte. Agglutination is a method of founding and quantitate detection Ag or Ab, based on their ability to form visible conglomerates.
Precipitation test	Precipitation is sedimentation of dispersed or soluble molecular Ag under the action of specific immune serum.
Serological identification	Serological identification - serological tests for detection (identification) unknown Ag using familiar Ab

The immune-diagnostic serum	Immune diagnostic serum (IDS) - a standard preparation, which contains antibody to a definite group of microbes. It is used for serological tests.

Theoretical questions to studies:

- Phylogenetic peculiarities of the immune system of animals.
- Central and peripheral organs of the immune system, their functions.
- Immunocompetent cells and their functions.
- To give the definition: “antigens”, “antigenecity”.
- Antigenic structure of bacterial cells on the example of E.coli
- Structure and function of immunoglobulins.
- Features of antigens and antibodies interaction.
- Serological tests, differences between simple and complex ST.
- Aims of the serological tests.
- Components of serological test for serological identification.
- The agglutination test – definition.
- The precipitation test – definition.
- Immune diagnostic serum, use and stages of their obtaining.

Practical tasks are performed in class:

- To carry out glass-agglutination test with adsorbed serum, to read its results.
- To carry out precipitation test in liquid phase, to readits results.
- To carry out direct agglutination test for serological identification of bacterial culture
- To examine samples of immune diagnostic serum.

Contents subject.

In practice, students should familiarize with the serological tests (ST) definitions, to determine components of ST, to familiarize with agglutination and precipitation tests peculiarities. Students carry out glass-agglutination test with adsorbed serum, carry out precipitation test in the liquid phase, read their results. Students examine the results of direct agglutination test (AT), carry out direct AT for serological identification.

The students write down complete tasks in the protocol and teacher sign it.

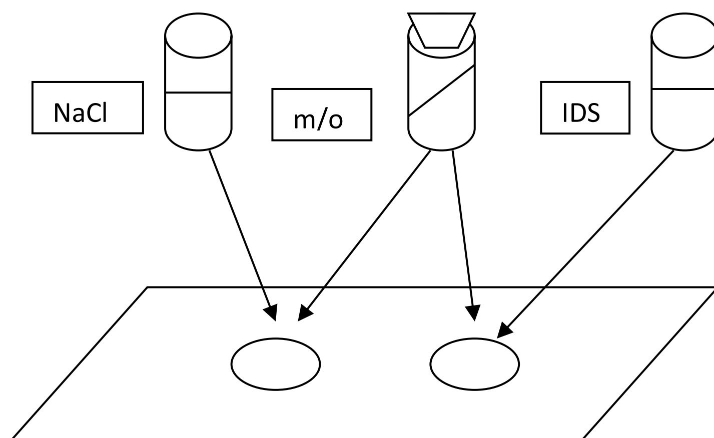
Recommendations for the design of the protocol.

The glass-agglutination test

Materials:

• Culture of bacteria
• Adsorbed immune diagnostic serum (IDS) for glass-agglutination test
• 0,5% solution of NaCl
• Glass for smears, bacteriological loop

The procedur:



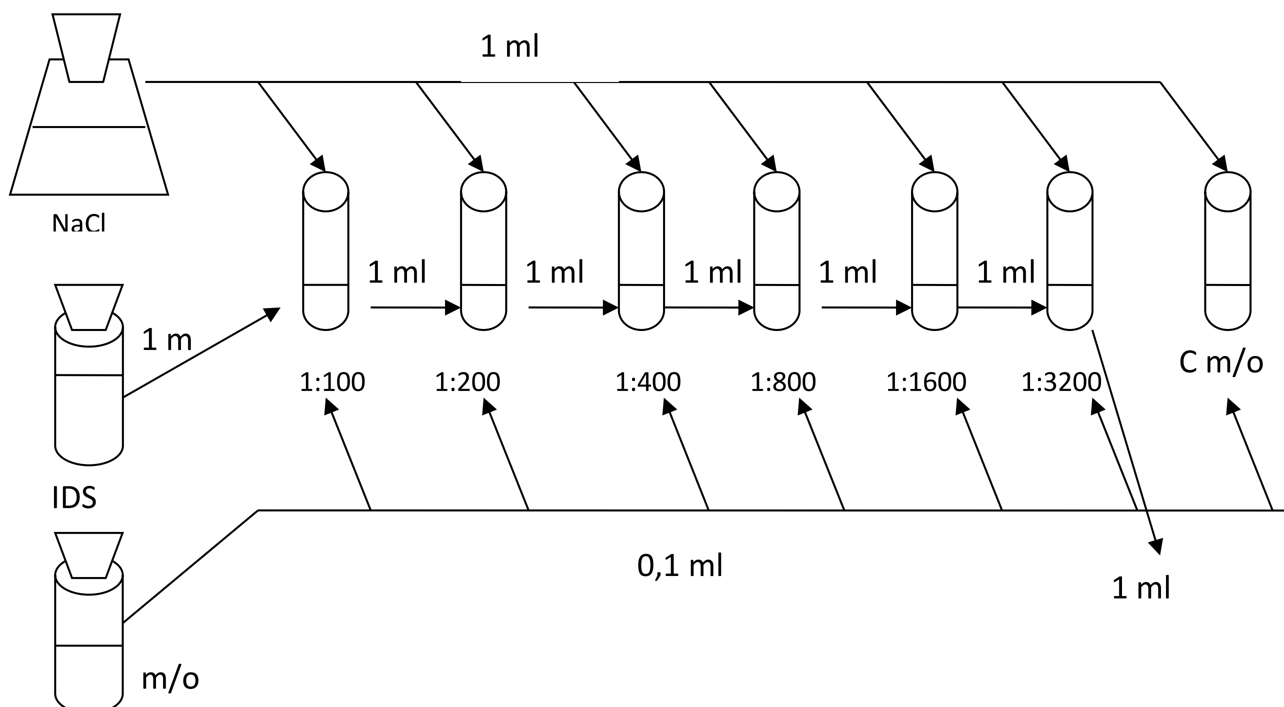
The results of test: Positive test: is manifested by clumping formation.

The direct agglutination test for serological identification of bacteria culture

Materials:

- Bacteria culture (suspension)
- Immune-diagnostic serum in the working dilution (1:50), titer - 1:3200
- 0,5% solution of NaCl
- Test tubes, pipettes

The procedure:



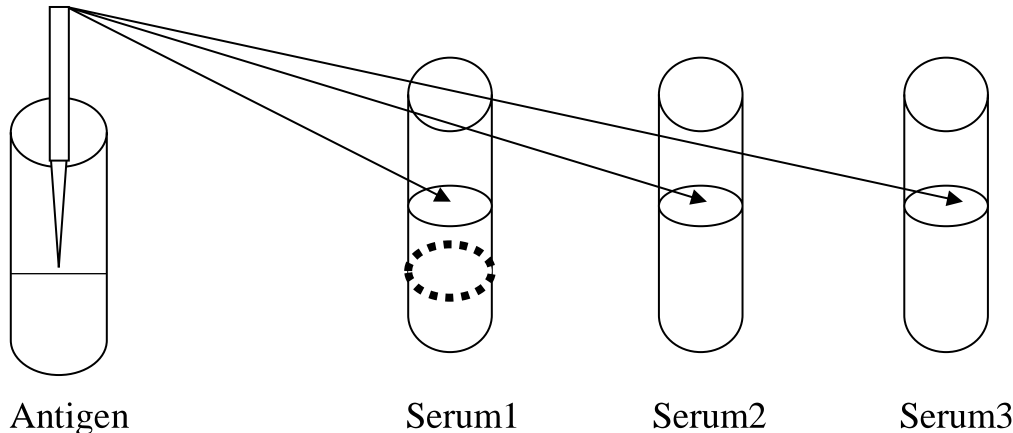
The results of test: Positive test – is manifested by agglutinate formation; Previous registration - after 2 hours, final regisration – after 18-24 hours.

Precipitation test in liquid phase.

Materials:

- Antigen (precipitinogen);
- Serum (precipitin) - № 1, № 2, control serum (№ 3);
- Pipettes

The procedure:



The results of test: Positive test is manifested by formation of precipitation ring (turbidity).

Questions for self-control.

- What are the immune system organs that belong to the central and peripheral system, their functions?
- What are the immuno competent cells, their function?
- What are the cells involved in the synthesis of antibodies, the nature of their cooperation?
- To give definition to such terms: “antigen”, “antigenecity”.
- What are the bacterium antigens (at E.coli example)?
- Structure and function of immunoglobulins.
- What are the serological tests (ST),difference between simple and complex ST?
- For what purpose may serological tests be used?
- The agglutination test - definition.
- The precipitation test - definition.
- What are the components of simple ST?
- What are the stages of immune diagnosis serum obtaining?

Practical activity №12

Topic: “Serological tests (part 2)”

Relevance of the topic:

The immunological mechanisms play a main role in defence of the body against foreign agents. The immunological mechanisms in the body are realized by antibodies (immunoglobulins) and immune cells (lymphocytes). The basis of immunological mechanisms is the specific interaction of antigen and antibody, which was formed in response to an antigen penetration. All diagnostic immunological reactions are based on the principle of interaction between antigens and antibodies. They called serological reactions (serological tests), because antibodies are in the blood serum. Serological tests are used for serological diagnostic of infectious disease and for serological identification pathogens isolated from patients. It is necessary to establish the diagnosis of infectious diseases. The students have possibility to master the basic serological tests (agglutination test, passive hemagglutination reaction, the reaction of hemolysis). To study the role of antibodies in forming of the immune response is necessary for understanding the complex mechanism of functioning of the immune system and its role in the development of immune-depending pathologies. The ability to select the correct serological tests, carry out them and interpret its results are important for: diagnosis of infectious diseases; formation clinical thinking of the students; understanding of the pathological process in infectious diseases.

Objective purpose:

- To analyze the mechanism of interaction of antibodies with antigens
- To know the patterns of serological reactions
- To explain the immune system cells involved in immune response and phase of the immune response
- To be able to read the results agglutination test for serological identification
- To master the agglutination test for serological diagnosis (Widal’s reaction)
- To master the immune hemolysis reaction
- To learn the technique of indirect hemagglutination reaction

Basic knowledge, skills, skills needed to study topics (interdisciplinary integration). See a practice № 10.

The list of principal terms, parameters, characteristics, which student should be study for lesson:

Term	Definition
Lyses reaction	Lyses reaction is antigen dissolving under the action of antibody in the presence of complement. Fresh human immune serum can do lyses, because contain antibodies and complement. If serum was heated or stored some time, lyses can be only with adding of complement.

Immune haemolysis reaction	The hamolysins (specific protective antibodies) are formed in the animal's blood serum after animal's immunization by erythrocytes. The haemolysins can destroy connection haemoglobin with erythrocytes' stroma in the presence of complement, and provide haemolysis reaction. The immune serum that contains haemolysins has the name of haemolytic serum. Such serums are produced by special laboratories.
The power of haemolytic serum	The power of haemolytic serum measured in titers – this is the maximum haemolytic serum dilution in volume 0,5 ml, that lead to fool haemolysis of 0,5 ml 3% sheep erythrocytes in the presence of 0,5 ml complement (in 1:10 dilution), and tubes incubation for 1 hour at 37^0 C.
Indirect haemagglutination (IHA) reaction	The essence of indirect haemagglutination (IHA) reaction is the ability sheep erythrocytes (or other spacies of animal) to absorbe Ag on its surface, and becom "sensitive" (sensibilized) to the corresponding immune serum. Sensibilized red blood cells stick together under the influence of specific antibody and form sediment (haeamagglutinate) at the bottom of the tube. We can detecte a minimum number of antibodies with IHA reaction because of high specifity and sensitivity of this reaction.
Complement system	Complement system – are the group of serum proteins, which after their activation are converted to effector molecules that lead to the development of inflammation (C3a,C, C4a), phagocytosis (C3b) and destruction of cells (C6-9).The complement proteins are involved in the development of inflammatory reactions, reactions opsonizations and lysis of cell membranes.
Diagnosticums	Diagnosticums – are the standard antigens. They can be suspensions of live bacteria (or inactivated), viruses or their antigens in isotonic solution. Diagnosticums are used for serological diagnosis of infectious diseases.

Theoretical questions to studies:

- The diagnosticums, its characteristic, principl of obtaining and practical using.
- Three- and many-component serological tests (immune lysis, complement fixation test, opsonophagocyting, indirect agglutination), its main point, practical using.
- The complement system, way of compliment activation, biological value.
- Immune haemolysis test, mechanism, practical using.
- Valuation of results of serological tests.
- The bacteriolysis tests, citolysis, its mechanism and practical using.

Practical tasks are performed in class:

- To read the resut agglutination test for serological identification of bacterial culture.
- To carry out Widal test for serological diagnostic of typhoid fever.

- To read the result of indirect haemagglutination test for serological diagnostic typhoid fever, to make conclusion.
- To read the result of immune haemolysis test, to make conclusion
- To study sampls of diagnosticums.

Topic content:

At the practical activity the students familiarize with compound of serological tests, considerate peculiarity and components of agglutination test and indirect haemagglutination test (IHAT) for serological diagnostic infection diseases. Students read the resut of agglutination test for serological identification bacterial culture; carry out Widal test for serological diagnostic typhoid fever; read the result of IHAT for serological diagnostic typhoid fever; carry out immune haemolysis test; study sampls of diagnosticums.

The students write down in the protocol complete tasks and a teacher sign it.

Recommendations for the design of the protocol.

Red the result agglutination test for serological identification of bacteria cultures.

The students red the results agglutination test for identification the bacteria cultures. The positive agglutination test denotes with pluses:

++++ - Complete agglutination, the liquid is completely transparent, while on the bottom of the test tube there is a white colour sediment.

+++ - the liquid is not completely transparent, the lesse number of sediment;

++ - the liquid is not transparent, the more lesse number of sediment ;

+ - the liquid is turbid, the very little number of sediment;

- -suspension remains uniformly turbid, as the antigen control, there is no sediment. There is a negative test.

Correspondence between the type of microbe and diagnostic agglutination serum is reliable at the condition of clear expressed agglutination reactions in test tubes with dilution of serum in the within of titer, but not less than 2 / 3 of its titer in the control serum and culture tubes with absence of sediment in there.

Carry out agglutination test for detecting antibodies in serum of patients with suspected typhoid and paratyphoid A and B.

For the agglutination test we shoul obtaine serum from patient's blood and solve it with isotonic sodium chloride solution from 1:50 to 1:800 according to the scheme. We use as the antigen – diagnosticums (suspension of the known, killed typhoid and paratyphoid A and B microorganisms) in this test.

Scheme of agglutination test for finding antibodies in patient serum.

Ingredient (in ml)	Number of the test tube						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Isotonic sodium chloride solution	1,0	1,0	1,0	1,0	1,0	1,0	---
The patient's serum in a 1: 25 dilution (0,1 ml serum + 2,4 ml Isotonic sodium chloride solution)	1,0	1,0	1,0	1,0	1,0	in disinfect solution	1,0
diagnosticum	0,1	0,1	0,1	0,1	0,1	0,1	---
The obtained dilution of the serum	1:50	1:100	1:200	1:400	1:800	antigen control	serum control

The previous results of reaction are registered after 2 hours, and final – after 18-20 hours of thermostat incubation at 37^{0}C.

The principle of of indirect hemagglutination test (IHAT) for serological diagnosis.

The essence of the indirect haemagglutination test is that erythrocytes with adsorbed on its surface any soluble antigens can be agglutinate by antibodies, which are specific to the adsorbed antigen. Erythrocytes that are sensibilized by antigens we call as erythrocytic diagnosticums. Sheep's erythrocytes are used more often because of its high adsorptive activity. The test carry out according to the scheme: tested serum, heated for 30 min at 58°C, we dilute in tubes from 1:10 to 1:320 in volume 0.25 ml and then, we add 2 drops of erythrocytic diagnosticum in the each tubes. Controls for test are: on the spontaneous agglutination of sensibilized erythrocytes; with normal erythrocytes; controls of diagnosticum with positive and negative sera. Test tubes place in a thermostat for 30-45 minutes at 37ºC after light shaking. Registeration of results carries out after appeirence of sediment from erythrocytes in control tubs. Reliable results should be: a lack of hemagglutination in the control tubes with normal sensibilized erythrocytes and with a negative serum; and the presence of hemagglutination in tube with positive serum. Hemagglutination in test tubes (with corresponding controls) indicates the presence of specific antibodies in test serum. The results we read according the appearance of erythrocytes sediment in the tubes. In the case of positive result (+) - erythrocytes are in the bottom of the tube as "umbrella" form. When a negative result (-) is - erythrocytes looks like "buttons" (compact sediment in the bottom of the tube).

Immune haemolysis test.

Immune lysis – is a disolve the **immune haemolysis test** cells (antigens) under the action of specific antibodies (lysins) in the presence of a complement. We called it as bacteriolis, spirochaetolysis, haemolysis, etc., it is depende from the nature of antigens in the lysis reaction. Haemolysis reaction is used as indicator system for the complement-fixation test. For carry out of the lysis reaction we need: 1) antigens – 3% suspension of erythrocytes; 2) antibodies – haemolysis serum ageinst sheep erythrocytes; 3)

complement – the serum of guinea-pig in 1:10 dilution; 4) isotonic sodium chloride solution.

Scheme of the basic immune haemolysis test

№ tubes / Ingredient (in ml)	test	controls		
	1	2	3	4
antibodies – haemolysis serum	0,5	---	0,5	---
antigens – 3% suspension of sheep erythrocytes	0,5	0,5	0,5	0,5
comlement (1:10)	0,5	0,5	---	---
isotonic sodium chloride solution	---	0,5	0,5	1,0

The results of the reaction are readed after test tubes incubation at 37 °C for 45 min. Haemolysis should be present in the test tube (a result of specific interaction between haemolytic serum and erythrocytes in the presence of complement). Haemolysis should be absent in the control tubes: in the first tube (haemolytic serum control) comlement is absent, in the second tube (complement control) haemolytic serum is absent, in the third tube (erythrocytes control) complement and haemolytic serum are absent.

Complement fixation (CF) test for detection of antibodies in the test serum.

Complement fixation test is based on the ability of a specific complex antigen + antibody adsorb (bind) a complement. As the process of complement fixation is not visuale, hemolytic system (sheep erythrocyte +hemolytic serum) is used as an indicator, which shows the effects of the reaction between antigen and antibody. If the antigen and antibody are homologous with each other, then this complex binds complement and hemolysis does not occur, and if the complex does not bind with complement, the hemolysis follows.

CF test belongs to the complex serological tests and for its implementation there should be not less than 5 ingredients: antigen, antibody and complement (first system), sheep red blood cells and their homologous hemolytic serum.

Scheme of complement fixation (CF) test for detection of antibodies in the test serum

Number of tube / ingredients (in ml)	Test tube	Control tubes				
	1	2	3	4	5	6
Serum assayed in dilutions 1:10	0,5	---	0,5	0,5	---	---
Antigen (working dose)	0,5	0,5	---	0,5	0,5	0,5
Complement (working dose)	0,5	0,5	0,5	---	0,5	0,5
Positive serum (1:10)	---	---	---	---	0,5	---
Negative serum (1:10)	---	---	---	---	---	0,5
Isotonic sodium chloride solution	---	0,5	0,5	0,5	---	---
Incubation at 37 °C for 2 h						
hemolytic system	1,0	1,0	1,0	1,0	1,0	1,0
Incubation at 37 ºC for 45 min						

Questions for self-control.

- What does it mean such defenitions: “serological identification of causative agent of disease” and “serological diagnosis of infection disease”?
- How serological tests does classify?
- What is standard diagnisticums? How standart diagnisticums does make?
- What serological reactions are many-components?
- What is complement system and its way activation?
- What is the lysis test?
- What is the immune haemolysis test? What is the aim of its using?
- What is indirect haemagglutination test? What is the aim of its using?

Practice №13

Topic: "Serological tests with labels"

Relevance of the topic:

Among in the immunological methods for diagnosis of infectious diseases serological tests with labeled antigens or antibodies (immune fluorescence (IF) test, enzyme immunoassay (ELISA) and radioimmune analysis (RIA)) are widecy. This is because of high sensitivity and specificity of these reactions, and because of the possibility to use it for express-diagnoties of infectious diseases. In practice, the students continue to study two-component and many-component serological tests (agglutination test, complement fixation test), to structurize the knowledge. In addition, the students have possibility familiarize with method of serological test with label, and they have possibility to evaluate the results of RIF and ELISA. All this factors provide for the topic relevance and students'activization of studying.

Specific objectives:

- To learn the principles, methods, and read the result of complement fixation test (CFT) for detection of antibodies in the test serum.
- To interpret of agglutination test for serological diagnosis, make conclusions.
- Carry out identification of microbial culture in smears with immunofluorescence test (demonstration).
- To learn the principles of enzyme-linked immunosorbent assay (ELISA) and radioimniun assay (RIA). To read the results of ELISA for serological diagnosis.

Basic knowledge, skills, skills needed to study topics (interdisciplinary integration). See a practice № 10.

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

Term	Definition
Monoclonal antibodies	Monoclonal antibodies (MCA) – are antibodies obtained by using hybridome technologies. They belong to one class of immunoglobulins and react with specific antigen epitopes against which they are produced.It has many advantages in serological reactions with labeled antibodies compared to polyclonal antibodies, namely: high homogeneity, ease of recognition, the absence of nonspecific antibodies, and lack of variability in various samples.
Serological reactions with labels	Serological reactions with labels based on the detection of interaction of antigen and antibody with immune complex (antigen-antibody) formation, where one of the reaction

	participants has labels. Labels can be detected visually or by special highly sensitive equipment. Serological reactions with labels allow to detect labeled substrate quantitatively and, accordingly, unknown antigen or antibody.
Label	As the labels are used: fluorescent dye in ultraviolet light (isothiocyanate fluorescein) in the immunofluorescence (IF) test; enzyme (peroxidase, alkaline phosphatase) that is detected by a color change of the corresponding substrate in enzyme immunoassay (ELISA) test; isotope, that is detected by radiometry in radioimmune assay (RIA).
Immunofluorescence (IF) test	The immunofluorescence (IF) test is based on the properties of fluorcscence antibody to connect specifically with homologous antigen and cause them to fluoresce in violet and ultraviolet parts of spectrum of luminescent microscope. IF test is specific and sensitive. It used mainly to identify the antigen and can be performed in several ways. 1.Direct IF test is based on the use of immunofluorescence sera against each investigated antigen. 2. Indirect IF test is based on the us of two different sera. Initially, on the first stage we use the unlabeled antibodies against the studied antigens and antigen-antibody complex is formed. On the second stage of reaction we treate this antigen-antibody complex by immunofluorescence serum (this serum contains antibodies against gamma-globulins the same animal species, that is used for obtained unlabeled specific antiserum).
Enzyme-linked immunosorbent assay (ELISA)	Enzyme immunoassay (ELISA) is based on the using enzyme as a label antibodies. Enzyme can decompose substrates with formation of colored products. They are direct and indirect variants of ELISA. Its essence is that antigens (or antibodies) adsorbed on some solid material (solid-phase) and then add other ingredients of this serological tests. Plastic plates, balloons, foil, tubes of various synthetic materials are used as solid-phase widely. Antigens or antibodies that adsorbed on the surface of such materials can keep their immunological specificity and ability to react in serological tests even in the dried state for a long time. The presence and activity of antibody or antigen-antibody complex bound with the enzyme can detect and evaluate by the intensity of staining visually

	after its incubation with the appropriate substrate. can be done with special equipment. ELISA results is recorded by photometer (reader).
Radio immune assay (RIA)	Radioimmune assay (RIA) is based on the use of radioactive isotopes as a label of one of the serological reaction components. The method is the most sensitive and can detect small amounts of reagents. RIA is used in both direct and indirect ways. The special radiometric equipment is required for the RIA assay and recording its results.
Western blot	The western blot (sometimes called the protein immunoblot), or western blotting, is a widely used analytical technique in molecular biology and immunogenetics to detect specific proteins in a sample of tissue homogenate or extract. In brief, the sample undergoes protein denaturation, followed by gel electrophoresis. A synthetic or animal-derived antibody (known as the primary antibody) is created that recognises and binds to a specific target protein. The electrophoresis membrane is washed in a solution containing the primary antibody, before excess antibody is washed off. A secondary antibody is added which recognises and binds to the primary antibody. The secondary antibody is visualised through various methods such as staining, immunofluorescence, and radioactivity, allowing indirect detection of the specific target protein.

Theoretical questions:

- Serological tests with many components, their characteristic.
- Serological tests with labels, requirements for them.
- Monoclonal antibodies, principles of obtaining, their application in serological tests with labels.
- Complement fixation (CF) test, mechanism, features, practical use.
- Coons' reaction, mechanism, the practical use.
- Immunofluorescence (IF) test, the essence, practical use.
- Radio immune assay (RIA), the essence, methods, recording the results.

Practical activities for the esson:

- To read the results of Vidal test for detection of antibodies for the causative agent of typhoid and paratyphoid A and B in the patient’s blood serum. Make conclusions.
- To read the results of complement fixation test for detection of antibodies in the patient’s blood serum (demonstration work). Make conclusions.

- To make identification of fungi genus Candida in the smears, which are stained with antiserum labeled by fluorehrome, using the immunofluorescence method (demonstration work). Make conclusion.
- To read the results of ELISA for identification of HBsAg in serum of patients with suspected viral hepatitis B (demonstration work). Make conclusions.
- Examine samples of test-systems fo ELISA for detection of HBs Ag.

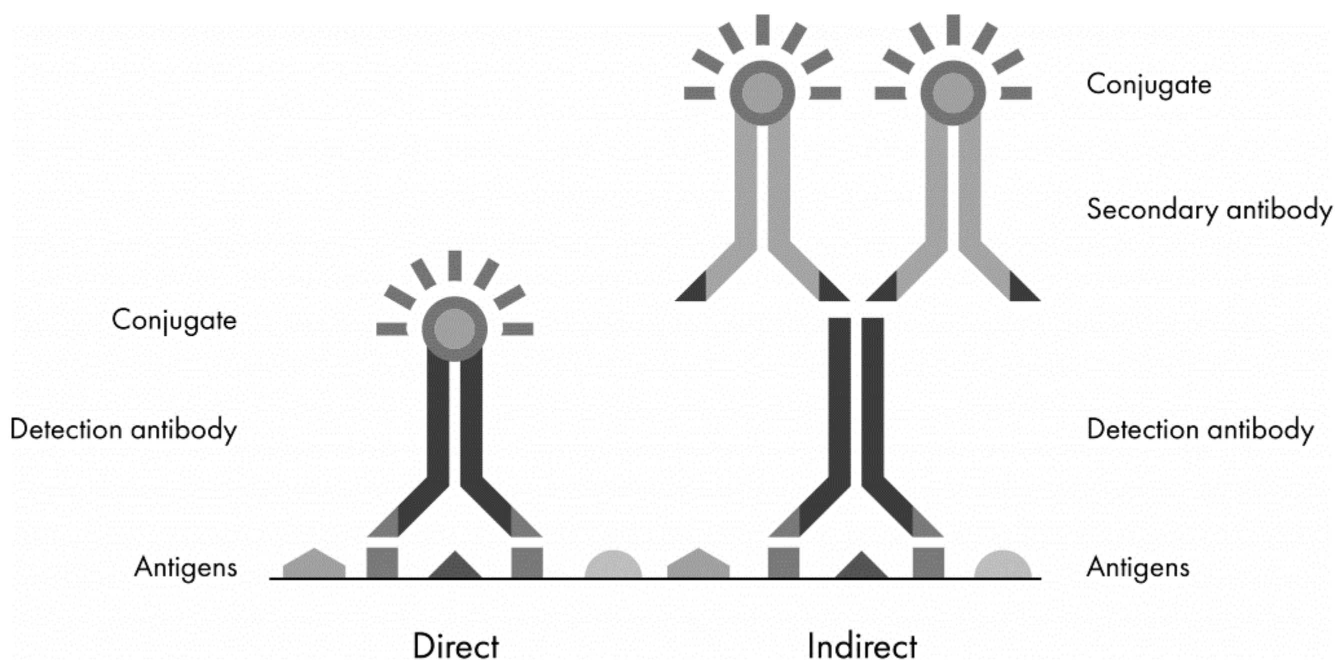
Content topics:

The students learn with the peculiarities of serological reactions' methods using labeled antibodies and antigens (ELISA, RIA, IF- test) in practice; read the results of Vidal's test for determining antibody (Ab) against the causative agent of typhoid fever and paratyphoid A and B in the patient's blood serum; read the results of complement fixation test, for detection of Ab in the patient's serum; carry out the identification of fungal culture of the genus Candida, which are stained with fluorehrome labeled with antiserum in smears by immunofluorescence method; read the results of ELISA, for detecting of HBsAg in the serum of the patients with suspected viral hepatitis B; study samples of ELISA test systems for detection of HBsAg.

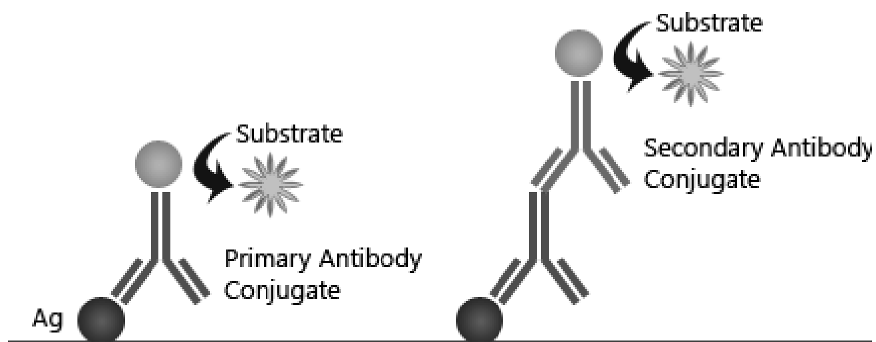
The students write down complete tasks in the protocol and teacher sign it.

Recommendations for design of the protocol.

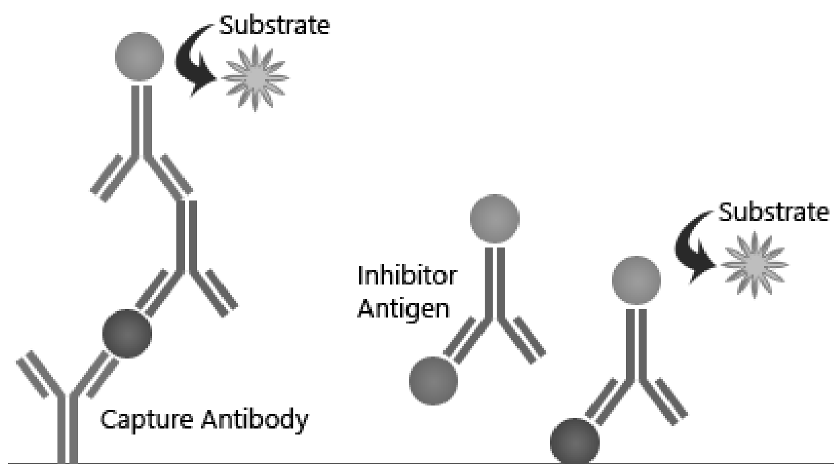
Immunofluorescence assay



Types of ELISA

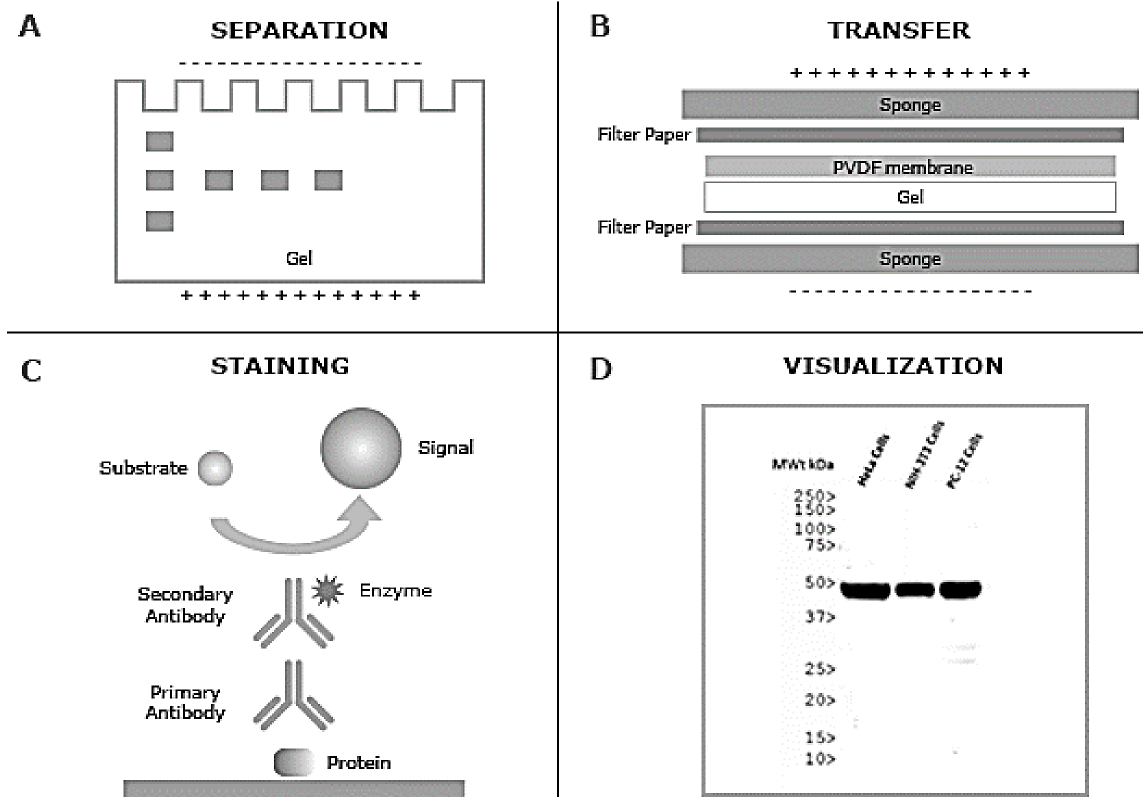


DIRECT ELISA **INDIRECT ELISA**

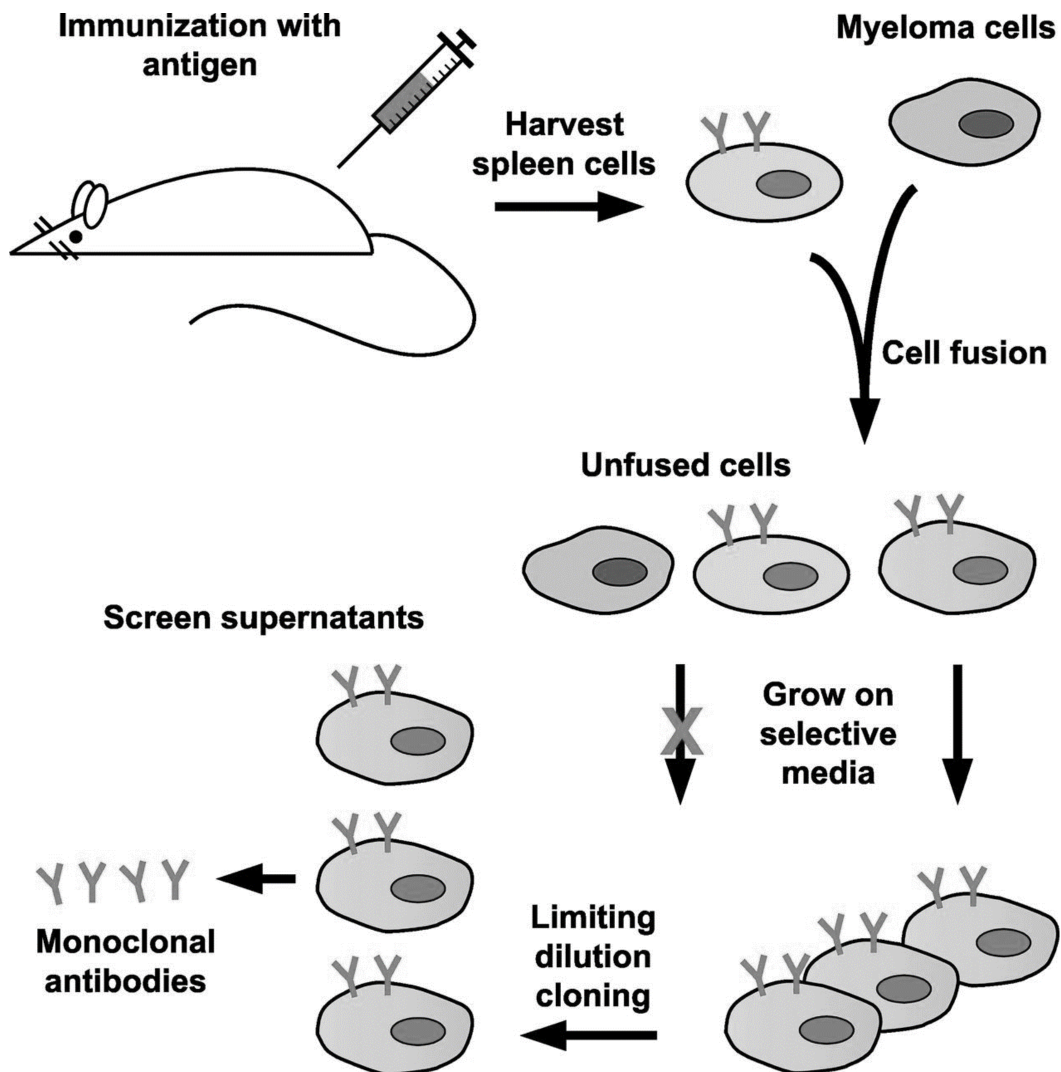


SANDWICH ELISA **COMPETITIVE ELISA**

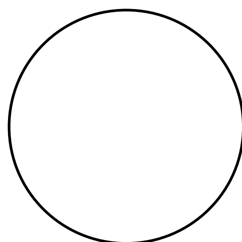
Western blot scheme



Scheme for producing monoclonal antibodies



Task 1. The identification of fungal culture of the genus Candida, which is stained with fluorehrome-labeled antiserum in smears by immunofluorescence method (IF test) (demonstration work). Conclusions.

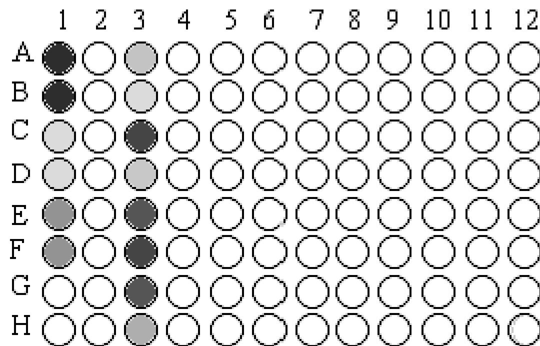


Fungi of the genus *Candida*, stained immune serum labeled with fluorochrome

For the direct IF test studied material (culture of the genus *Candida*) is applied to skim a glass slide with bacteriological loop, we obtain a thin smear, dry it in air and fix. We put 1-2 drops of fluorehrome-labeled antiserum in fixed smear serum and we stain it in a moist chamber for 20-30 min. at 25^0 C. After incubation, the smear is washed 2-4 times with isotonic solution for 10-15 min., and then we wash it with distilled water and dry. After that, a drop of fluorehrome oil is put on the smear and we examin it with fluorescent microscope through immersion lens. This fungusa of the genus Candida give a glisten brightly on a dark background.

Task 2. Read the results of ELISA, for detecting HBsAg in serum of patients with suspected viral hepatitis B (demonstration work).

The presence and activity of the enzyme bound to an antibody or antigen-antibody complex can be detected and evaluated visually by the intensity of staining after incubation with the appropriate substrate.



Control:

1A-B – positive
1C-D – negative
1E-F – *«cut-off»**

Determine ELISA results according to control wells 1A-F:

3A –
3B –
3C –
3D –
3E –
3F –
3G –
3H –

Questions for self-control.

- What principle is complement fixation test based on, what are its advantages over other serological reactions?
- What serological tests can be applied for rapid diagnosis of infectious diseases?
- What are the perspectives and aspects of the use of monoclonal antibodies? Why are serological reactions with labels preferred for monoclonal antibody detection?
- What are the principles of direct and indirect IF test, ELISA, RIA, what are the advantages and disadvantages of these methods?

Class №14

Topic: "Vaccines and immune serum"

Relevance of the topic:

Specific prevention and therapy of infection disease are of important significance in general complex preventive measures. Vaccines, immune serum and immunoglobulins have particular importance in these preventive measures, - trhey saved the lives of millions of people at different times. Vaccines and toxoids led to formation of active antiinfection immunity, mobilizing mechanisms of immunological memory. Inoculation of immune sera and immunoglobulins creates immediate passive humoral immunity, which can protect the body from infection or intoxication. Diagnostic immune sera are used for determination the antigenic structure of causative agent, its serological identification to establish the etiology of infectious diseases.

Students are given the possibility: to study the curatives and preventive medicines, diagnostic medicines, which are used in medical practice; to learn the principles of their production, methods of standardization and control.

All this provides for the relevance of the topic and formation of positive motivation for learning.

Educational purpose:

- To analyse the principle of obtaining vaccines, to give comparative characteristics to each of them, to study their standartization and control methods, practical use.
- To study the vaccines, which are used in medical practice, to study principles of vaccine classification.
- To study the principles of immune sera obtaining, methods of their standartization and control. Practical value.

Basic knowledge, skills, needed to study topics (interdisciplinary integration). See practical №10.

The list of principal terms, parameters, characteristics, which a student should study for the lesson:

Term	Definition
Immunological prophylaxis	Prevention of infectious diseases by creating immunity to them with immunological methods - active and passive immunization.
Immunotherapy	Treatment of patients by influencing the immune system.
Vaccines	Biological substances obtained from microorganisms, their metabolic products, synthetic, genetic engineering analogy, or anti-idiotype antibodies, that are used for active immunization of people for the prevention and treatment of infectious diseases.

Attenuation	Sustained irreversible weakening of the virulence of pathogenic microorganisms, it is used for obtaining vaccine strains.
Live vaccine	Consists of viable strains of pathogenic microorganisms with the most reduced virulence, but with safe antigenic properties. Live vaccine creates stress immunity, similar to postinfectious.
Inactivated vaccine	Inactivated vaccine (killed) consists of microorganisms that have expressed immunogenic properties, obtained under the action of physical and chemical factors.
Chemical vaccine	Consists of specific antigens that were extracted from bacteria and purified from ballast substances.
Toxoid	Qualitatively new medicine that is obtained from exotoxin by treatment with 0.3% solution of formalin at 37 ^{o}C for 30 days.
Genetic-engineering vaccine	It's obtained on the basis of microbe genomes sequence detecting: genes that control the required antigenic determinants, we transfer gene into other micro-organisms and clone them, promoting the expression of these genes in the new environment.
Anti-idiotype antibodies	Vaccines, obtained from anti-idiotype antibodies that are characterized by similar structure between the antigen epitope and active center of anti-idiotype antibodies.
Antiserum	Serum, obtained from human or animals that were immunized with an antigen and contains some antibodies to this antigen, used for therapy or diagnostics.
Flocculation	A variety of precipitation reactions in the liquid in which the antigen-antibody complexes (often toxin-toxoid) form a visible precipitate (flocculants), which can be characterized quantitatively.
Toxoid activity	The smallest amount of toxoid which reacts in the flocculation reaction in the initial test-tube with one unit of antitoxic serum at 45 ^{o}C.
Initial tube	Initial tube – a tube, which contain equivalent amount of toxoid and antitoxin. Sediment appear for the first time in initial tube.

Theoretical questions:

- The history of immunization and immunotherapy of infectious diseases.
- Principles of obtaining, methods of standardization and characteristics of the first generation vaccines - live and inactivated.

- Principles of obtaining, methods of standardization and characteristic of second generation vaccines - chemical and toxoid.
- Genetic-engineering vaccine, anti-idiotype antibodies, the theoretical basis for their creation, properties, prospects use.
- Immune sera and immunoglobulins, the principles of obtaining, practical value.
- Methods of vaccine and sera preparations control.

Practical tasks are performed in class:

- To study the vaccines that are used in medicine, to determine their properties, advantages and disadvantages, and practical use.
- Examine the principles of obtaining treatment-and-prophylactic sera, using in medicine.
- To learn the method of determination the toxoid activity in flocculation units.
- To analyze methods of control of vaccine and serum medicine.

Content topics:

In practice, the students learn the principles of classification, methods of obtaining different immune prophylactic, therapeutic and diagnostic drugs, that are used in medicine. Students learn their properties, advantages and disadvantages, the principles of standardization and control. To learn the method of determination the toxoid activity with flocculation test. The students write down in the protocol completed tasks and a teacher sign it.

Recommendations for design of the protocol.

Practical work №1. To determine activity of diphtheria toxoid with flocculation test.

Antitoxic serum of known activity (200 IU/ ml) is poured on the tubes in such quantities: 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml. Then, we add 2 ml toxoid in each tube and we placed all tubes in a water bath at 45^{0}C. After this procedure we observe the appearance of opalescence in the initial tube. If we know the number of antitoxic serum units in the initial tube, we can account number of international unit (IU) of toxoid. We make the calculation of toxoid activity and conclusion.

Immune sera and immunoglobulins

Immune sera and immunoglobulins for treatment and prophylactic	Immune sera for diagnostic
Antitoxic and antibacterial • Diphtheria sera; • Tetanus sera and tetanus • Immune globulin; • Gas gangrene mono-and polyvalent serum;	***For identification of bacterial infections*** • agglutination • precipitation • lyzise

• Staphylococcal immunoglobulin; • Antianthrax globulin ***Antiviral*** • Measles immunoglobulin • Rabies gamma -globulin • Influenza immunoglobulin • Smallpox immunoglobulin • Immunoglobulin for tick-borne encephalitis virus	***For virus identification*** • Virus neutralisation • Complement fixation • Antihaemagglutination

BASIC PRINCIPLES CONTROL OF VACCINE DRUGS AND PREPARATIONS WITH SERA

1. We make control vaccine drugs and preparation with sera on the sterility (the live vaccines should not contain other microorganisms).
2. We make control vaccine drugs and preparation with sera on the harmfool absence. This control we performe on the sensitive laboratory animals (according to the death or survival of the animals, clinical manifestations of infection or the presence of intoxication, bacteriological parameters and change the weight of animals).
3. We make control vaccine drugs and preparation with sera on the reactivity (on laboratory animals or, sometimes, on a limited number of people - volunteers). The assessment is carried out by temperature reaction of the organism, the development of inflammation at the injection site and other indicators.
4. We check specific activity of **vaccine preparations**:
   - according to concentration of microbes;
   - according to antibody productions after the injection of animals;
   - according to ability induce relevant to infection in animals after its immunization by virulent microorganisms.
5. We check specific activity of **preparations with sera** according the antibodies concentration.
6. We make control vaccine drugs and preparation with sera on the oncogenicity. Corpuscular vaccine should be verificate into experimental animals.

Questions for self control

- What is the immunoprophylaxis and immunotherapy?
- What medicines are used to create artificial active antimicrobial immunity?
- What are the principles of classification of vaccines?
- Who made the first live vaccine, what is the basis of their production?
- What medicines are used to create artificial active antitoxic immunity, how is it made?
- What medicines are used for artificial passive antimicrobial and antitoxic immunity?
- How to use diagnostic immune serum?

Class №15

Topic: "Methods of laboratory diagnosis of infectious diseases"

Topic relevance

The clinical presentation of an infectious disease reflects the interaction between the host and the microorganism. This interaction is affected by the host immune status and microbial virulence factors. Signs and symptoms vary according to the site and severity of infection. Diagnosis requires a composite of information, including history, physical examination, radiographic findings, and laboratory data.

Diagnosis of infectious disease sometimes involves identifying an infectious agent either directly or indirectly. In practice most minor infectious diseases such as warts, cutaneous abscesses, respiratory system infections and diarrheal diseases are diagnosed by their clinical presentation. Conclusions about the cause of the disease are based upon the likelihood that a patient came in contact with a particular agent, the presence of a microbe in a community, and other epidemiological considerations. Given sufficient effort, all known infectious agents can be specifically identified. The benefits of identification, however, are often greatly outweighed by the cost, as often there is no specific treatment, the cause is obvious, or the outcome of an infection is benign.]

Specific identification of an infectious agent is usually only determined when such identification can aid in the treatment or prevention of the disease, or to advance knowledge of the course of an illness prior to the development of effective therapeutic or preventative measures. For example, in the early 1980s, prior to the appearance of AZT for the treatment of AIDS, the course of the disease was closely followed by monitoring the composition of patient blood samples, even though the outcome would not offer the patient any further treatment options. In part, these studies on the appearance of HIV in specific communities permitted the advancement of hypotheses as to the route of transmission of the virus. By understanding how the disease was transmitted, resources could be targeted to the communities at greatest risk in campaigns aimed at reducing the number of new infections. The specific serological diagnostic identification, and later genotypic or molecular identification, of HIV also enabled the development of hypotheses as to the temporal and geographical origins of the virus, as well as a myriad of other hypothesis. The development of molecular diagnostic tools have enabled physicians and researchers to monitor the efficacy of treatment with anti-retroviral drugs. Molecular diagnostics are now commonly used to identify HIV in healthy people long before the onset of illness and have been used to demonstrate the existence of people who are genetically resistant to HIV infection. Thus, while there still is no cure for AIDS, there is great therapeutic and predictive benefit to identifying the virus and monitoring the virus levels within the blood of infected individuals, both for the patient and for the community at large.

Educational objectives

- Determine the main goals and tasks of laboratory diagnosis of infectious diseases.
- Familiarize oneself with modern methods of laboratory diagnosis of infectious diseases.

- Determine the advantages and disadvantages of main methods of infectious diseases laboratory diagnosis
- Choose appropriate method of diagnosis for different infectious diseases
- Create scheme of microscopic, cultural, serological and express method of diagnosis
- Describe the main principle of gen diagnosis (by example of PCR)

Basic knowledge, skills, skills needed to study topics (interdisciplinary integration). See a practice № 10.

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

Term	Definition
Microbial Causes of Infection	Infections may be caused by bacteria, viruses, fungi, and parasites. The pathogen may be exogenous (acquired from environmental or animal sources or from other persons) or endogenous (from the normal flora).
Specimen Selection, Collection, and Processing	Specimens are selected on the basis of signs and symptoms, should be representative of the disease process, and should be collected before administration of antimicrobial agents. The specimen amount and the rapidity of transport to the laboratory influence the test results.
Microbiologic Examination	Direct Examination and Techniques: Direct examination of specimens reveals gross pathology. Microscopy may identify microorganisms. Immunofluorescence, immuno-peroxidase staining, and other immunoassays may detect specific microbial antigens. Genetic probes identify genus- or species-specific DNA or RNA sequences.
Culture	In many instances, the cause of an infection is confirmed by isolating and culturing microorganism either in artificial media or in a living host. Bacteria (including mycobacteria and mycoplasmas) and fungi are cultured in either liquid (broth) or on solid (agar) artificial media. Liquid media provide greater sensitivity for the isolation of small numbers of microorganisms; however, identification of mixed cultures growing in liquid media requires subculture onto solid media so that isolated colonies can be processed separately for identification. Growth in liquid media also cannot ordinarily be quantitated. Solid media, although somewhat less sensitive than liquid media, provide isolated colonies that can be quantified if necessary and identified. Some genera and species can be recognized on the basis of their colony morphologies. Chlamydiae and viruses are cultured in cell culture systems, but virus isolation occasionally requires inoculation into animals, such as suckling mice, rabbits, guinea pigs, hamsters, or

	primates. Rickettsiae may be isolated with some difficulty and at some hazard to laboratory workers in animals or embryonated eggs. For this reason, rickettsial infection is usually diagnosed serologically. Some viruses, such as the hepatitis viruses, cannot be isolated in cell culture systems, so that diagnosis of hepatitis virus infection is based on the detection of hepatitis virus antigens or antibodies.
Differential media	In some instances one can take advantage of differential carbohydrate fermentation capabilities of microorganisms by incorporating one or more carbohydrates in the medium along with a suitable pH indicator. Such media are called differential media (e.g., eosin methylene blue or MacConkey agar) and are commonly used to isolate enteric bacilli. Different genera of the Enterobacteriaceae can then be presumptively identified by the color as well as the morphology of colonies.
Selective media	Isolation of infectious agents frequently requires specialized media. Nonselective (noninhibitory) media permit the growth of many microorganisms. Selective media contain inhibitory substances that permit the isolation of specific types of microorganisms Culture media can be made selective by incorporating compounds such as antimicrobial agents that inhibit the indigenous flora while permitting growth of specific microorganism's resistant to these inhibitors. One such example is Thayer-Martin medium, which is used to isolate Neisseria gonorrhoeae. This medium contains vancomycin to inhibit Gram-positive bacteria, colistin to inhibit most Gram-negative bacilli, trimethoprim-sulfamethoxazole to inhibit Proteus species and other species that are not inhibited by colistin and anisomycin to inhibit fungi. The pathogenic Neisseria species, N gonorrhoeae and N meningitidis, are ordinarily resistant to the concentrations of these antimicrobial agents in the medium.
Microbial identification	Microbial growth in cultures is demonstrated by the appearance of turbidity, gas formation, or discrete colonies in broth; colonies on agar; cytopathic effects or inclusions in cell cultures; or detection of genus- or species-specific antigens or nucleotide sequences in the specimen, culture medium, or cell culture system. Identification of bacteria (including mycobacteria) is based on growth characteristics (such as the time required for growth to appear or the atmosphere in which growth occurs), colony and microscopic morphology, and biochemical, physiologic, and, in some instances, antigenic or nucleotide sequence characteristics. The selection and number of tests for bacterial identification depend upon the category of bacteria present (aerobic versus

	anaerobic, Gram-positive versus Gram-negative, cocci versus bacilli) and the expertise of the microbiologist examining the culture. Gram-positive cocci that grow in air with or without added CO_2 may be identified by a relatively small number of tests. The identification of most Gram-negative bacilli is far more complex and often requires panels of 20 tests for determining biochemical and physiologic characteristics. The identification of filamentous fungi is based almost entirely on growth characteristics and colony and microscopic morphology. Identification of viruses is usually based on characteristic cytopathic effects in different cell cultures or on the detection of virus- or species-specific antigens or nucleotide sequences.
Interpretation of culture results	Some microorganisms, such as *Shigella dysenteriae*, *Mycobacterium tuberculosis, Coccidioides immitis*, and influenza virus, are always considered clinically significant. Others that ordinarily are harmless components of the indigenous flora of the skin and mucous membranes or that are common in the environment may or may not be clinically significant, depending on the specimen source from which they are isolated. For example, coagulase-negative staphylococci are normal inhabitants of the skin, gastrointestinal tract, vagina, urethra, and the upper respiratory tract (i.e., of the nares, oral cavity, and pharynx). Therefore, their isolation from superficial ulcers, wounds, and sputum cannot usually be interpreted as clinically significant. They do, however, commonly cause infections associated with intravascular devices and implanted prosthetic materials. However, because intravascular devices penetrate the skin and since cultures of an implanted prosthetic device can be made only after incision, the role of coagulase-negative staphylococci in causing infection can usually be surmised only when the microorganism is isolated in large numbers from the surface of an intravascular device, from each of several sites surrounding an implanted prosthetic device, or, in the case of prosthetic valve endocarditis, from several separately collected blood samples. Another example, *Aspergillus fumigatus,* is widely distributed in nature, the hospital environment, and upper respiratory tract of healthy people but may cause fatal pulmonary infections in leukemia patients or in those who have undergone bone marrow transplantation. The isolation of *A fumigatus* from respiratory secretions is a nonspecific finding, and a definitive diagnosis of invasive aspergillosis requires histologic evidence of tissue invasion. Physicians must also consider that the composition of microbial species on the skin and mucous membranes may be altered by

	disease, administration of antibiotics, endotracheal or gastric intubation, and the hospital environment. For example, potentially pathogenic bacteria can often be cultured from the pharynx of seriously ill, debilitated patients in the intensive care unit, but may not cause infection.
Serodiagnosis	Infection may be diagnosed by an antibody response to the infecting microorganism. This approach is especially useful when the suspected microbial agent either cannot be isolated in culture by any known method or can be isolated in culture only with great difficulty. The diagnosis of hepatitis virus and Epstein-Barr virus infections can be made only serologically, since neither can be isolated in any known cell culture system. Although human immunodeficiency virus type 1 (HIV-1) can be isolated in cell cultures, the technique demanding and requires special containment facilities. HIV-1 infection is usually diagnosed by detection of antibodies to the virus. The disadvantage of serology as a diagnostic tool is that there is usually a lag between the onset of infection and the development of antibodies to the infecting microorganism. Although IgM antibodies may appear relatively rapidly, it is usually necessary to obtain acute- and convalescent-phase serum samples to look for a rising titer of IgG antibodies to the suspected pathogen. In some instances the presence of a high antibody titer when the patient is initially seen is diagnostic; often, however, the high titer may reflect a past infection, and the current infection may have an entirely different cause. Another limitation on the use of serology as a diagnostic tool is that immunosuppressed patients may be unable to mount an antibody response.
Molecular (gen) diagnostics	Like the evidence left at the scene of a crime, The DNA, RNA, or proteins of the infectious agent in clinical sample can be used to help identify the agent. In many cases the agent can be detected and identified in this way even if can not be isolated or detected by immunological means. The advantages of molecular techniques are their sensitivity, specificity, and safety. From the standpoint of the safety, these techniques do not require isolation of the infectious agent and can be performed on chemically fixed (inactivated) samples or extracts. Because of their sensitivity, very dilute samples of DNA (e.g., viral DNA) can be detected in a tissue even if the agent is not replicating or producing other evidence of infection. The potential specificity of these techniques allows strains to be distinguished on the basis of differences in their genotype (i.e., mutants). This is especially useful for distinguishing antiviral drug- resistant strains, which may by a single nucleotide.

	DNA probes can be used like antibodies as sensitive and specific tool to detect, locate and quantify specific nucleic acid sequences in clinical specimens.
The polymerase chain reaction (PCR)	The polymerase chain reaction (PCR) can detect single copies of microbial DNA an is one of the newest techniques of genetic analysis. In this technique, a sample is incubated with two short DNA oligomers that are complementary to the ends of a known genetic sequence of microbial DNA, a heat stable DNA polymerase (Taq polymerase obtained from hemophilic bacteria), nucleotides and buffers. The oligomers hybridize to the appropriate sequence of DNA and act as primers for polymerase, which copies the segment of the DNA. The sample is then heated to denature the DNA (separated strand of the double helix) and cooled to allow hybridization of the primer to the new DNA. Each copy of DNA becomes a new template. The process is repeated many (20-40) times to amplify the original DNA sequence in an exponential manner. A target sequence can be amplified a million fold in a few hours using this method. This technique is especially useful for detecting latent and integrated virus sequences, such as is the case for retroviruses, herpesviruses, papillomaviruses and other papovaviruses. The RT- PCR technique is a variation of PCR, and it involves the use of the reverse transcriptase of retroviruses to convent viral RNA or messenger RNA to DNA before PCR amplification. In 1993, Hantavirus sequences were used as primers for RT- PCR to identify the agent causing the outbreak of hemorrhagic pulmonary disease in the Four Corners area of New Mexico. It showed the infectious agent to be a Hantavirus. Technologies based upon the polymerase chain reaction (PCR) method will become nearly ubiquitous gold standards of diagnostics of the near future, for several reasons. First, the catalog of infectious agents has grown to the point that virtually all of the significant infectious agents of the human population have been identified. Second, an infectious agent must grow within the human body to cause disease; essentially it must amplify its own nucleic acids in order to cause a disease. This amplification of nucleic acid in infected tissue offers an opportunity to detect the infectious agent by using PCR. Third, the essential tools for directing PCR, primers, are derived from the genomes of infectious agents, and with time those genomes will be known, if they are not already. Thus, the technological ability to detect any infectious agent rapidly and specifically are currently available. The only remaining blockades to the use of PCR as a standard tool of diagnosis are in its cost and application, neither of which is

	insurmountable. The diagnosis of a few diseases will not benefit from the development of PCR methods, such as some of the clostridial diseases (tetanus and botulism). These diseases are fundamentally biological poisonings by relatively small numbers of infectious bacteria that produce extremely potent neurotoxins. A significant proliferation of the infectious agent does not occur, this limits the ability of PCR to detect the presence of any bacteria.

Theoretical questions to the class:

- The historical stages of microbiological diagnosis
- The main features and development trends of modern microbiology.
- Bacterioscopic research method. Stage.
- Bacteriological research method. The principles of isolation of pure bacterial cultures and their identification.
- Stages of bacteriological diagnosis of infectious diseases.
- The main advantages and disadvantages of immunological research methods.
- Laboratory animals. The characteristic of the biological method.
- Genetic methods for the laboratory diagnosis of infectious diseases, their characteristics.

Students' practical activities:

- Create scheme of microscopic, cultural, serological and express method of diagnosis
- Familiarize oneself with PCR laboratory.

Content of the topic:

Cultures are generally incubated at 35 to 37°C in an atmosphere consisting of air, air supplemented with carbon dioxide (3 to 10 percent), reduced oxygen (microaerophilic conditions), or no oxygen (anaerobic conditions), depending upon requirements of the microorganism. Since clinical specimens from bacterial infections often contain aerobic, facultative anaerobic, and anaerobic bacteria, such specimens are usually inoculated into a variety of general purpose, differential, and selective media, which are then incubated under aerobic and anaerobic conditions. The duration of incubation of cultures also varies with the growth characteristics of the microorganism. Most aerobic and anaerobic bacteria will grow overnight, whereas some mycobacteria require as many as 6 to 8 weeks

Microbial Causes of Infection

Infections may be caused by bacteria, viruses, fungi, and parasites. The pathogen may be exogenous (acquired from environmental or animal sources or from other persons) or endogenous (from the normal flora).

Specimen Selection, Collection, and Processing

Specimens are selected on the basis of signs and symptoms, should be representative of the disease process, and should be collected before administration of antimicrobial agents. The specimen amount and the rapidity of transport to the laboratory influence the test results.

Microbiologic Examination

Direct Examination and Techniques: Direct examination of specimens reveals gross pathology. Microscopy may identify microorganisms. Immunofluorescence, immuno-peroxidase staining, and other immunoassays may detect specific microbial antigens. Genetic probes identify genus- or species-specific DNA or RNA sequences.

Culture

In many instances, the cause of an infection is confirmed by isolating and culturing microorganism either in artificial media or in a living host. Bacteria (including mycobacteria and mycoplasmas) and fungi are cultured in either liquid (broth) or on solid (agar) artificial media. Liquid media provide greater sensitivity for the isolation of small numbers of microorganisms; however, identification of mixed cultures growing in liquid media requires subculture onto solid media so that isolated colonies can be processed separately for identification. Growth in liquid media also cannot ordinarily be quantitated. Solid media, although somewhat less sensitive than liquid media, provide isolated colonies that can be quantified if necessary and identified. Some genera and species can be recognized on the basis of their colony morphologies.

In some instances, one can take advantage of differential carbohydrate fermentation capabilities of microorganisms by incorporating one or more carbohydrates in the medium along with a suitable pH indicator. Such media are called differential media (e.g., eosin methylene blue or MacConkey agar) and are commonly used to isolate enteric bacilli. Different genera of the Enterobacteriaceae can then be presumptively identified by the color as well as the morphology of colonies.

Culture media can also be made selective by incorporating compounds such as antimicrobial agents that inhibit the indigenous flora while permitting growth of specific microorganisms resistant to these inhibitors. One such example is Thayer-Martin medium, which is used to isolate Neisseria gonorrhoeae. This medium contains vancomycin to inhibit Gram-positive bacteria, colistin to inhibit most Gram-negative bacilli, trimethoprim-sulfamethoxazole to inhibit Proteus species and other species that are not inhibited by colistin and anisomycin to inhibit fungi. The pathogenic Neisseria species, N gonorrhoeae and N meningitidis, are ordinarily resistant to the concentrations of these antimicrobial agents in the medium.

Chlamydiae and viruses are cultured in cell culture systems, but virus isolation occasionally requires inoculation into animals, such as suckling mice, rabbits, guinea pigs, hamsters, or primates. Rickettsiae may be isolated with some difficulty and at some hazard to laboratory workers in animals or embryonated eggs. For this reason, rickettsial infection is usually diagnosed serologically. Some viruses, such as the hepatitis viruses, cannot be isolated in cell culture systems, so that diagnosis of hepatitis virus infection is based on the detection of hepatitis virus antigens or antibodies.

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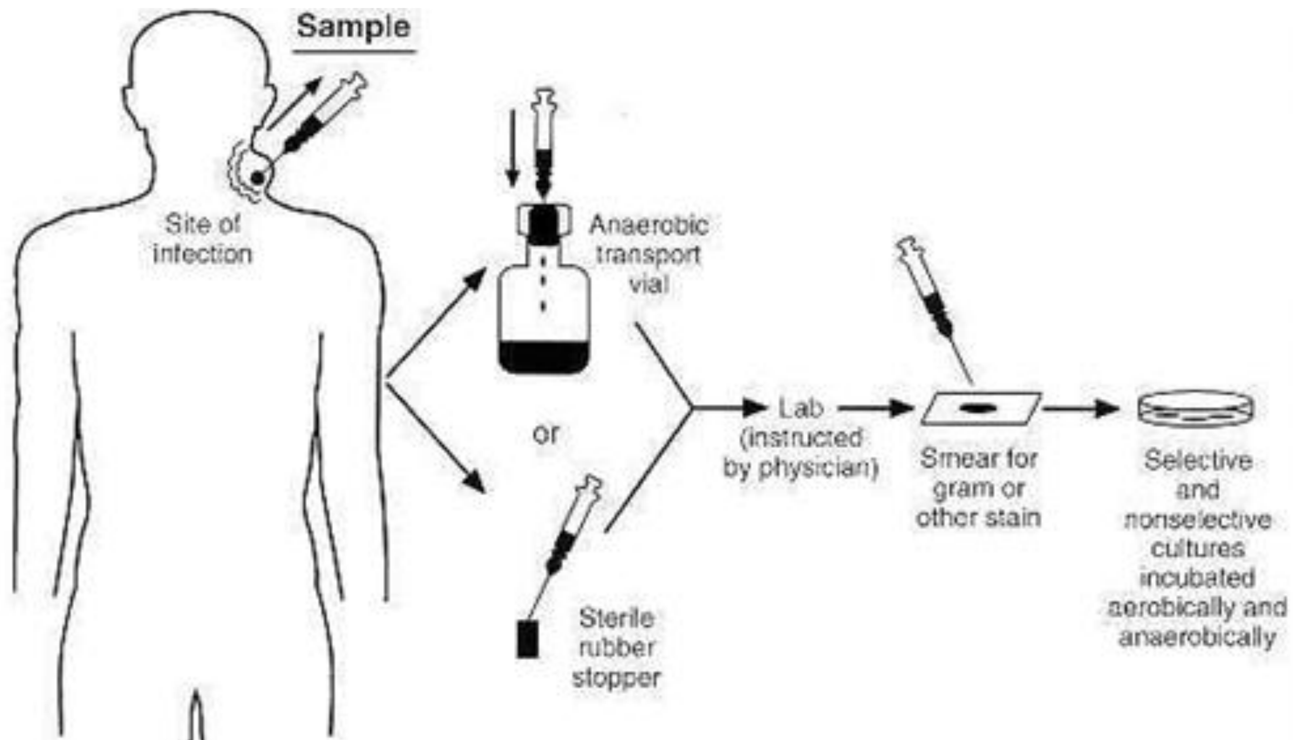

FIGURE 1 General procedure for collecting and processing specimens for aerobic and/or anaerobic bacterial culture.

The duration of incubation of cultures also varies with the growth characteristics of the microorganism. Most aerobic and anaerobic bacteria will grow overnight, whereas some mycobacteria require as many as 6 to 8 weeks

Microbial Identification

Microbial growth in cultures is demonstrated by the appearance of turbidity, gas formation, or discrete colonies in broth; colonies on agar; cytopathic effects or inclusions in cell cultures; or detection of genus- or species-specific antigens or nucleotide sequences in the specimen, culture medium, or cell culture system.

Identification of bacteria (including mycobacteria) is based on growth characteristics (such as the time required for growth to appear or the atmosphere in which growth occurs), colony and microscopic morphology, and biochemical, physiologic, and, in some instances, antigenic or nucleotide sequence characteristics. The selection and number of tests for bacterial identification depend upon the category of bacteria present (aerobic versus anaerobic, Gram-positive versus Gram-negative, cocci versus bacilli) and the expertise of the microbiologist examining the culture. Gram-positive cocci that grow in

air with or without added CO_2 may be identified by a relatively small number of tests. The identification of most Gram-negative bacilli is far more complex and often requires panels of 20 tests for determining biochemical and physiologic characteristics. The identification of filamentous fungi is based almost entirely on growth characteristics and colony and microscopic morphology. Identification of viruses is usually based on characteristic cytopathic effects in different cell cultures or on the detection of virus- or species-specific antigens or nucleotide sequences.

Interpretation of Culture Results

Some microorganisms, such as *Shigella dysenteriae*, *Mycobacterium tuberculosis, Coccidioides immitis*, and influenza virus, are always considered clinically significant. Others that ordinarily are harmless components of the indigenous flora of the skin and mucous membranes or that are common in the environment may or may not be clinically significant, depending on the specimen source from which they are isolated. For example, coagulase-negative staphylococci are normal inhabitants of the skin, gastrointestinal tract, vagina, urethra, and the upper respiratory tract (i.e., of the nares, oral cavity, and pharynx). Therefore, their isolation from superficial ulcers, wounds, and sputum cannot usually be interpreted as clinically significant. They do, however, commonly cause infections associated with intravascular devices and implanted prosthetic materials. However, because intravascular devices penetrate the skin and since cultures of an implanted prosthetic device can be made only after incision, the role of coagulase-negative staphylococci in causing infection can usually be surmised only when the microorganism is isolated in large numbers from the surface of an intravascular device, from each of several sites surrounding an implanted prosthetic device, or, in the case of prosthetic valve endocarditis, from several separately collected blood samples. Another example, *Aspergillus fumigatus,* is widely distributed in nature, the hospital environment, and upper respiratory tract of healthy people but may cause fatal pulmonary infections in leukemia patients or in those who have undergone bone marrow transplantation. The isolation of *A fumigatus* from respiratory secretions is a nonspecific finding, and a definitive diagnosis of invasive aspergillosis requires histologic evidence of tissue invasion.

Physicians must also consider that the composition of microbial species on the skin and mucous membranes may be altered by disease, administration of antibiotics, endotracheal or gastric intubation, and the hospital environment. For example, potentially pathogenic bacteria can often be cultured from the pharynx of seriously ill, debilitated patients in the intensive care unit, but may not cause infection.

Serodiagnosis

Infection may be diagnosed by an antibody response to the infecting microorganism. This approach is especially useful when the suspected microbial agent either cannot be isolated in culture by any known method or can be isolated in culture only with great difficulty. The diagnosis of hepatitis virus and Epstein-Barr virus infections can be made only serologically, since neither can be isolated in any known cell culture system. Although human immunodeficiency virus type 1 (HIV-1) can be isolated in cell cultures,

the technique demanding and requires special containment facilities. HIV-1 infection is usually diagnosed by detection of antibodies to the virus.

The disadvantage of serology as a diagnostic tool is that there is usually a lag between the onset of infection and the development of antibodies to the infecting microorganism. Although IgM antibodies may appear relatively rapidly, it is usually necessary to obtain acute- and convalescent-phase serum samples to look for a rising titer of IgG antibodies to the suspected pathogen. In some instances the presence of a high antibody titer when the patient is initially seen is diagnostic; often, however, the high titer may reflect a past infection, and the current infection may have an entirely different cause. Another limitation on the use of serology as a diagnostic tool is that immunosuppressed patients may be unable to mount an antibody response.

Molecular (gen) diagnostics

Like the evidence left at the scene of a crime, The DNA, RNA, or proteins of the infectious agent in clinical sample can be used to help identify the agent. In many cases the agent can be detected and identified in this way even if can not be isolated or detected by immunological means.

The advantages of molecular techniques are their sensitivity, specificity, and safety. From the standpoint of the safety, these techniques do not require isolation of the infectious agent and can be performed on chemically fixed (inactivated) samples or extracts. Because of their sensitivity, very dilute samples of DNA (e.g., viral DNA) can be detected in a tissue even if the agent is not replicating or producing other evidence of infection. The potential specificity of these techniques allows strains to be distinguished on the basis of differences in their genotype (i.e., mutants). This is especially useful for distinguishing antiviral drug- resistant strains, which may by a single nucleotide.

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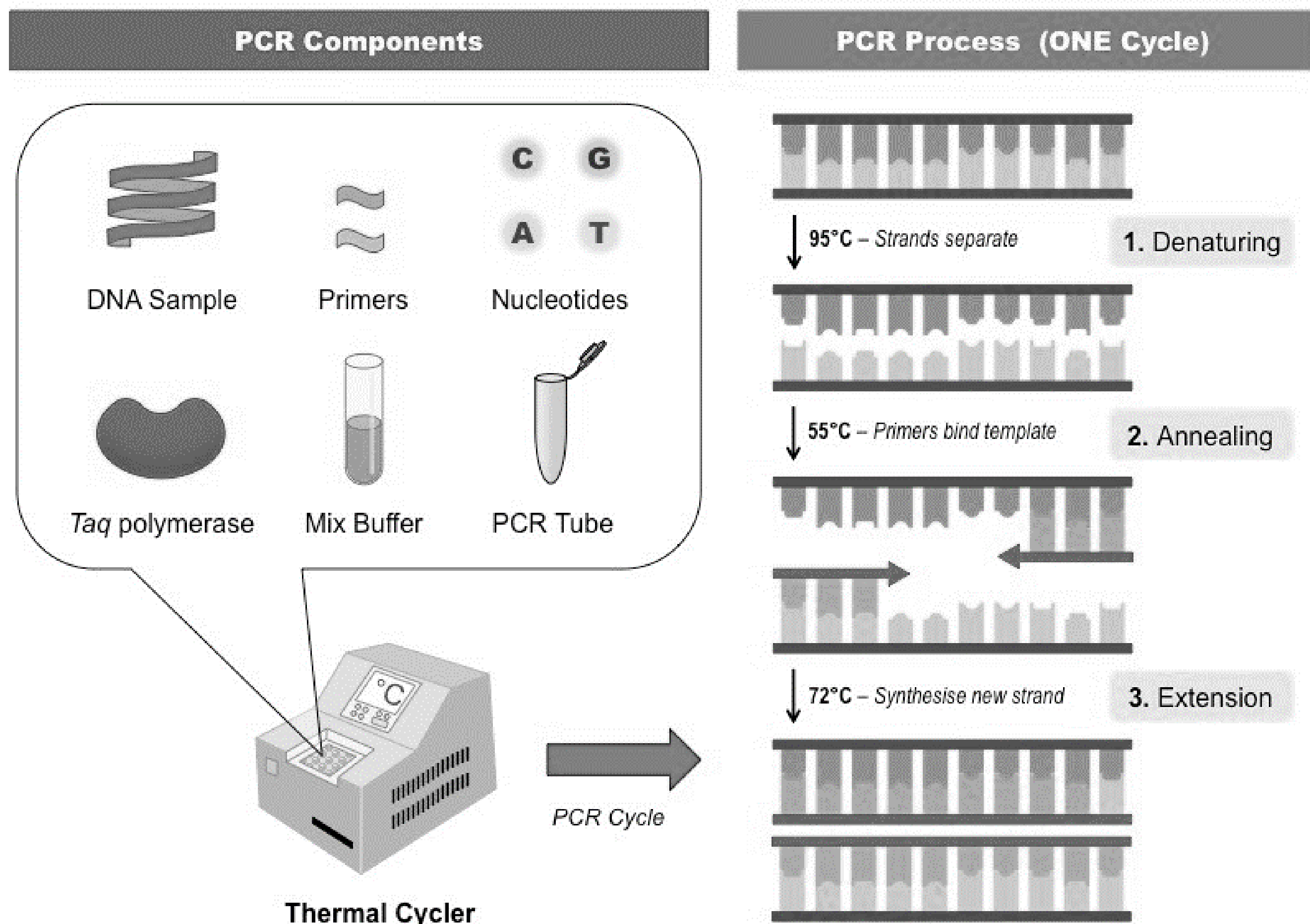
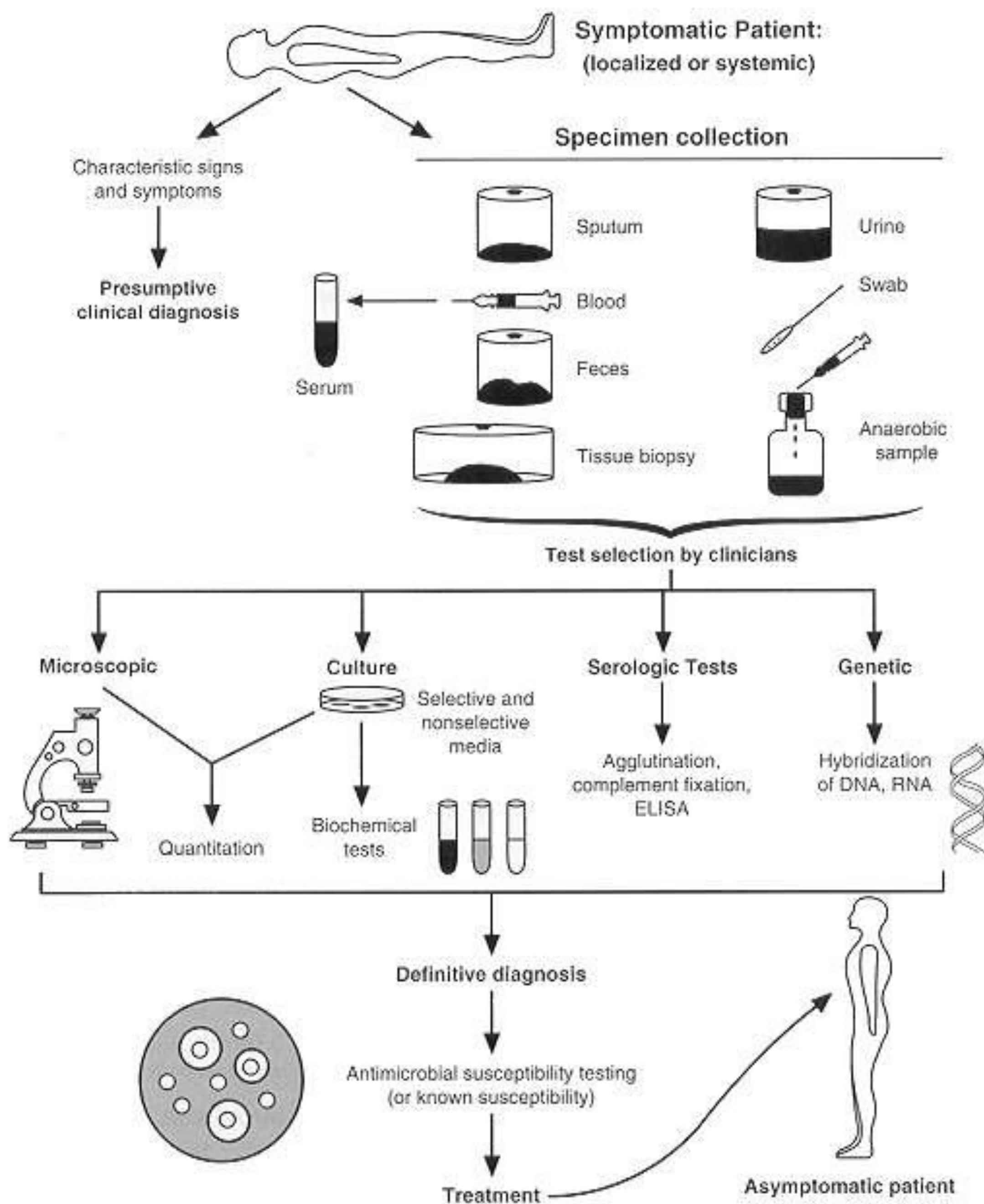
Recommendations for design of the protocol

Scheme of PCR



Laboratory procedures used in confirming a clinical diagnosis of infectious disease with a bacterial etiology.



Culture: Isolation of infectious agents frequently requires specialized media. Nonselective (noninhibitory) media permit the growth of many microorganisms. Selective media contain inhibitory substances that permit the isolation of specific types of microorganisms.

Microbial Identification: Colony and cellular morphology may permit preliminary identification. Growth characteristics under various conditions, utilization of carbohydrates and other substrates, enzymatic activity, immunoassays, and genetic probes are also used.

Serodiagnosis: A high or rising titer of specific IgG antibodies or the presence of specific IgM antibodies may suggest or confirm a diagnosis.

Antimicrobial Susceptibility: Microorganisms, particularly bacteria, are tested in vitro to determine whether they are susceptible to antimicrobial agents

The staff of a clinical microbiology laboratory should be qualified to advise the physician as well as process specimens. The physician should supply salient information about the patient, such as age and sex, tentative diagnosis or details of the clinical syndrome, date of onset, significant exposures, prior antibiotic therapy, immunologic status, and underlying conditions. The clinical microbiologist participates in decisions regarding the microbiologic diagnostic studies to be performed, the type and timing of specimens to be collected, and the conditions for their transportation and storage. Above all, the clinical microbiology laboratory, whenever appropriate, should provide an interpretation of laboratory results.

Laboratory diagnosis.

Goals:

- ❖ Diagnostics of disease – definition of infectious disease etiology
- ❖ Control of disease development
- ❖ Choosing of adequate individual therapy
- ❖ Estimation of treatment effectiveness
- ❖ Detection of donor blood or organ contamination
- ❖ Control of epidemic development

Materials for laboratory diagnosis

- ➢ Blood – influenza, AIDS
- ➢ Spinal liquid – viral and bacterial meningitis
- ➢ Urine – genitourinary infections
- ➢ Feces – enteroviral infection, cholera
- ➢ Material from wound, pus
- ➢ Sputum – respiratory infections (rhinoviral infection)

Methods of laboratory diagnostics

- ***Direct*** – to reveal presence of agent (microorganism) or its components in patient organism:
  - ❖ Microscopy
  - ❖ Cultivating method
  - ❖ Biological method
  - ❖ Indication of microbial antigens
  - ❖ Gen diagnosis
- ***Indirect*** – to detect response of human organism to agent (microorganism):
  - ➢ Serological diagnosis
  - ➢ Histological assay
  - ➢ Allergic assay

Microscopy

Based on microscopic identification the morphological and structural features of microorganisms directly in the specimen from a patient

- ***Positive:***
  - ❖ Rapid
  - ❖ In early stage of a disease
  - ❖ Simple
- ***Negative***
  - ➢ Only presumptive diagnosis
  - ➢ Non universal
  - ➢ Low sensibility

Some disease that can be diagnosed by microscopy:

- ❖ Acute gonorrhea
- ❖ Meningococcal meningitis
- ❖ Primary syphilis
- ❖ Rotaviral infection
- ❖ Relapsing fever

Microbiologic diagnosis (cultural method)

Method is based on obtaining a pure culture of microorganisms from the patient material and next identification the culture to species

- ***Positive*:**
  - ❖ The most exact, permits to make a final diagnosis
  - ❖ In early stage of a disease
  - ❖ Give possibility to choose the antimicrobial treatment
  - ❖ May be used different systems for cultivation of microorganisms (for instance, nutrient medium for bacteria and cell culture for viruses)
- ***Negative***
  - ➢ Some microorganisms can not be cultivated and isolated in pure culture
  - ➢ Long time for examination and making the diagnosis

Questions for self- assessment:

- What are goals and tasks of infectious diseases laboratory diagnosis?
- What is the basic difference between direct and indirect methods?
- Which diseases can be diagnosed by microscopic method only?
- What are advantages and disadvantages of main methods of infectious diseases laboratory diagnosis?
- What are stages of basic methods of infectious diseases laboratory diagnosis (microscopic, cultural, serological, express)
- What is sensitivity and specificity of a test?
- Gen diagnosis. Principle, types of tests, stages

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