AZERBAIJAN MEDICAL UNIVERSITY
DEPARTMENT OF MEDICAL MICROBIOLOGY and IMMUNOLOGY

Lesson 16.

Infection. Inoculation, diagnosis and examination of laboratory animals. Determination of pathogenicity and virulence

FACULTY: General Medicine
SUBJECT: Medical microbiology - 1

Discussed questions:

1. The concept of infection, infectious process, infectious disease.
2. Conditions of the infectious process
3. The role of microorganisms in infectious process.
4. Pathogenic factors of microorganisms (morphological structures, enzymes and toxins).
5. Definitions of pathogenicity and virulence (infectious dose ID).
6. Determination of virulence of microorganisms: lethal dose (Dlm, LD50, Dcl).
7. The role of macroorganisms in the infectious process (age, sex, hereditary factors, nervous system, endocrine system, immune system, normal microflora).
8. The role of environment in the infectious process (temperature, radiation, social factors, anthropogenic and environmental factors, iatrogenic factors).
9. The kind of infectious diseases (source and clinic expression)
10. The features of infection diseases (contagiousness, periodicity, the emergence of immunity).
11. The periods of infection and ways of infection.
12. Infection source and contamination ways
13. The essence of the biological method.
14. The methods of selection, preparation and contamination methods of laboratory animals.
15. Diagnosis and examination of infected animals.

Purpose of the lesson:

To inform students about the infectious process, the conditions of the infectious process, infectious disease, the characteristics, periods, forms and features of the infectious disease, to acquaint them with the source of infection and ways of infection. To explain to students the role of microorganisms in the infectious process, the concepts of pathogenicity and virulence, the essence of the biological method and its role in microbiological diagnosis.

Infection or infectious process

- ***Infection or infectious process*** cover pathological process occurring in macroorganism as a result of entry and reproduction of microorganism.
- The similar processes caused by protozoans, helminthes and insects are called invasion (lat, *invazio* – attack).
- The interaction of microorganisms with macroorganisms in the infectious process manifests itself pathogenetically and clinically as an infectious disease.

Infectious process conditions

- **Pathogenic microorganism**
- **Sensitive macroorganism**
- **Environmental conditions**

The role of microorganism in infectious process

- ***Saprophytic microorganisms*** **live in environment, human and animal organisms as commensals without causing disease** (greek, *sapros* – decay and *phyton* - plant).
- ***Pathogenic microorganisms*** (lat, *pathos* – suffering, *genos* - origin) enter sensitive macroorganism and cause infectious disease.
- Opportunistic microorganism can cause disease only under certain conditions. Their ability to cause disease is dependant on host macroorganism status.

Pathogenicity and virulence

- **Pathogenicity** is ability of microorganism to cause pathological process or disease. Pathogenicity is genetic feature of microorganims and specific for the majority of microorganisms in other words, each pathogenic microorganism causes specific disease.
- Pathogenicity may vary within the same species. The degree of pathogenicity is expressed in virulence (Latin, virulentus - toxic).
- For viruses, the term "infectivity" is used instead of "virulence".

Change of virulence

- Due to virulence a certain microorganism strains can be classified as strains with high, weak virulence and avirulent.
- Change of virulence – weakening or strengthening may be phenotypic or genotypic. Once the factor causing the change of virulence disappears, the virulence returns to its previous level.
- If the virulence change is due to genetic factor it is passed from one generation to another.

The factors influencing virulence

- Cultivation of microorganisms under unfavourable conditions, long-term cultivation on artificial media, passage in animal organism with weak sensitivity, impact of physical and chemical factors may cause weakening of virulence.
- Stabile weakening of virulence – attenuation is used in vaccine preparation.
- Passage of microorganism in organism of sensitive animal may strengthen the virulence. It may be due to selection of virulent population of microorganisms.

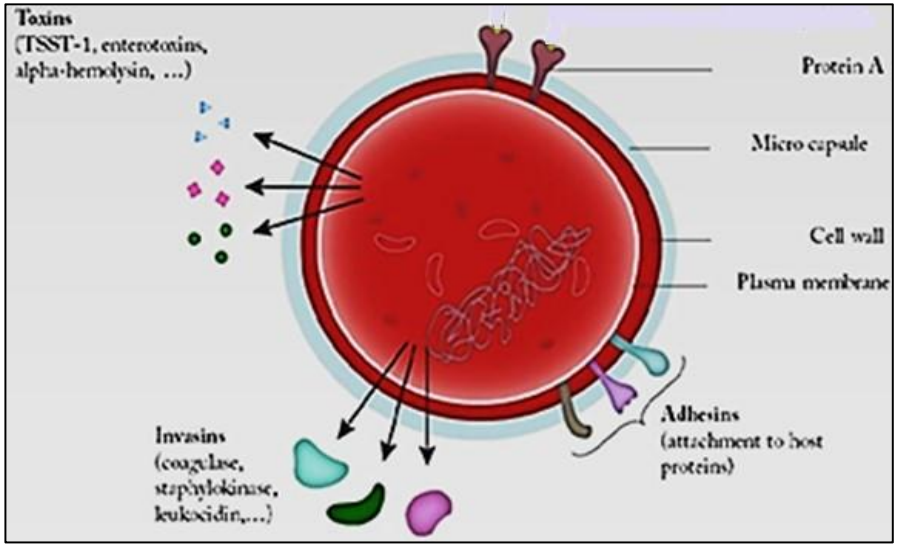
Virulence of microorganisms in the laboratory is usually assessed in laboratory animals, especially white mice. For this purpose, lethal and infectious doses are determined.

Lethal dose – the lowest number of microorganism or toxin causing death of certain number of animals over a period of time.

- ***Absolute lethal dose*** (DCL - *dosis certa letalis*) – the lowest number of microorganism or toxin causing death of 100% animals.
- ***Minimal lethal dose*** (DLM - *dosis letalis minima*) – the lowest number of microorganism or toxin causing death of the majority (approximately 90%).
- ***Median lethal dose*** (LD_{50}) – the number of microorganism or dose of toxin causing death of a half of experimental animals. This dose is commonly used for evaluation of virulence.
- **Infective doses are** ID_{100} and ID_{50}.

Pathogenicity factors of microorganisms

- Pathogenicity of microorganisms is determined by **pathogenicity factors**. The presence of these factors distinguishes pathogen microorganisms from saptophytes.
- Pathogenic factors include the **morphological structures, enzymes and toxins** of microorganism cells.
- These factors enable entry, adhesion on tissue and cells of organism and protection of microorganism from defense system of macroorganism.

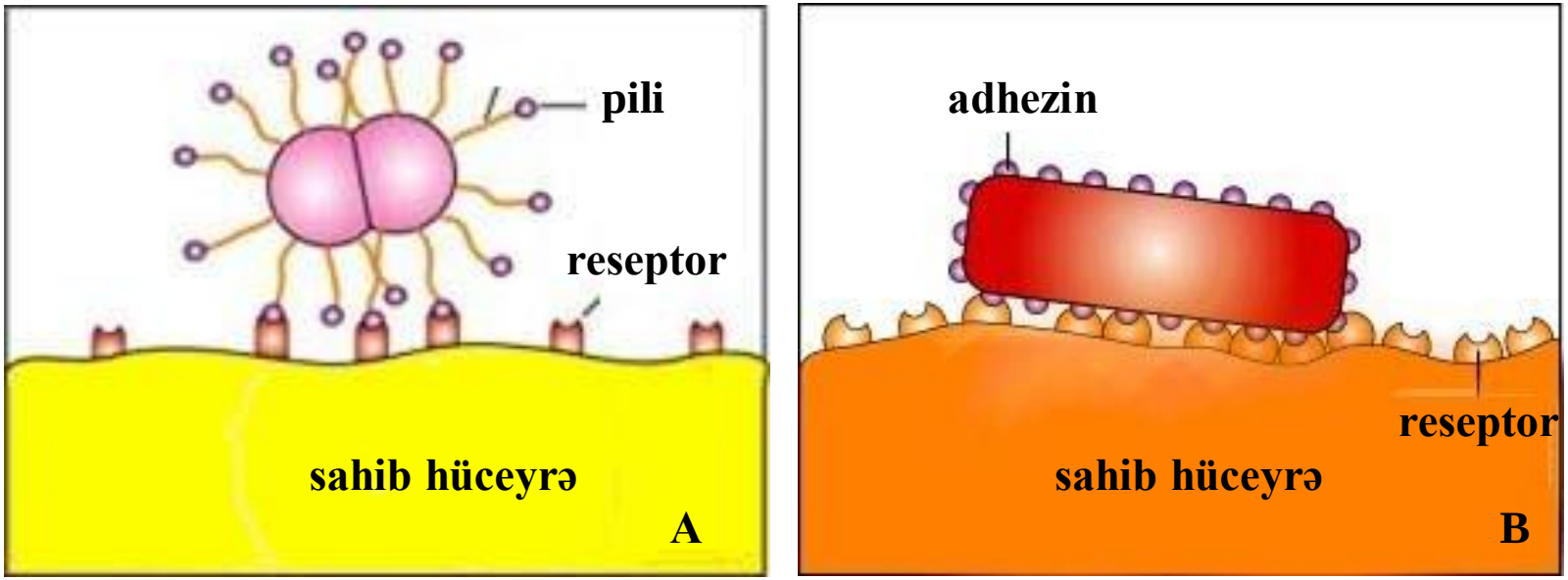


Pathogenicity factors of microorganisms

- ***Adhesion*** *– specific connection of microorganism to sensitive cell.*
- **Colonization -** multiplication of microbe on surface of sensitive cell.
- **Penetration** – ability of some pathogens to enter in cells (epithelial, leucocites, lymphocites etc.).
- **Invasion** – entry of microbe through mucous membrane and connective tissue into necessary tissues (neuraminidase, hyaluronidase)

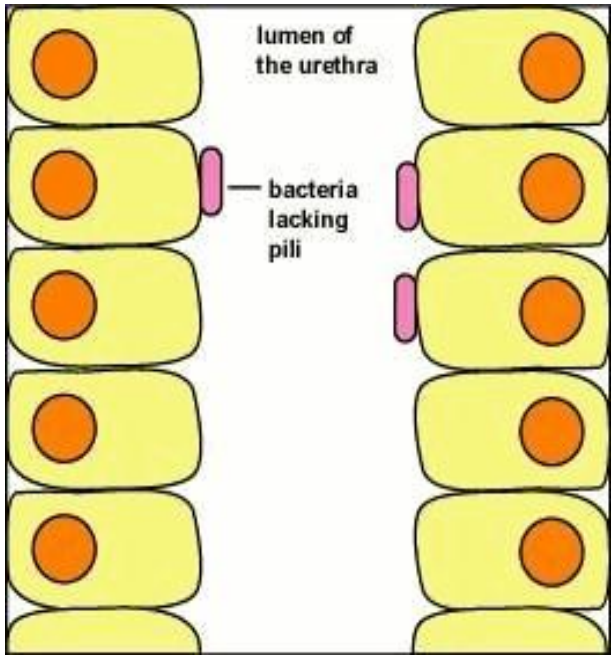
Adhesion

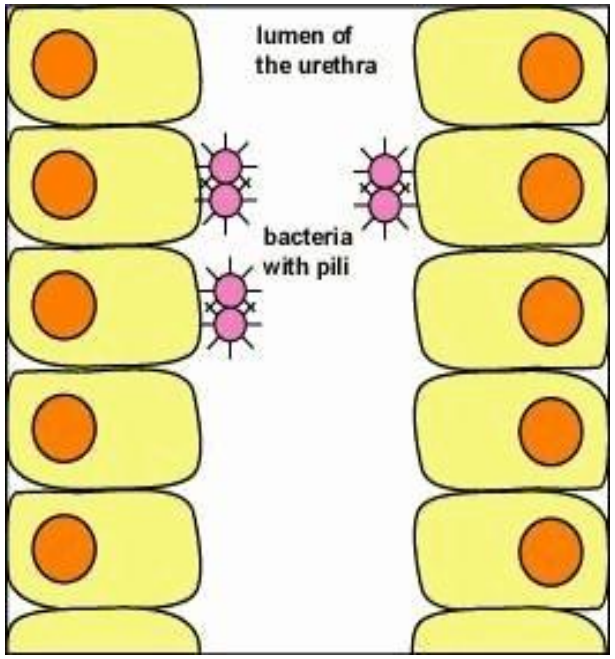
- **Adhesion** (lat, *adhesis* – stick) – ability of microorganism to stick cells and tissues.
- It is supported by pilis and other **structures (adhesins** and **ligands**).
- On the other hand there special structures of macroorganism cells called receptorswhich are able to interact with microbes.
- Adhesion of microorganisms is **ligand-receptror mediated** phenomenon.



The role of adhesion in pathogenicity: ligand-receptor mechanism. A – pili-mediated adhesion; B – adhesion-mediated adhesion

Adhesion as pathogenicity factor





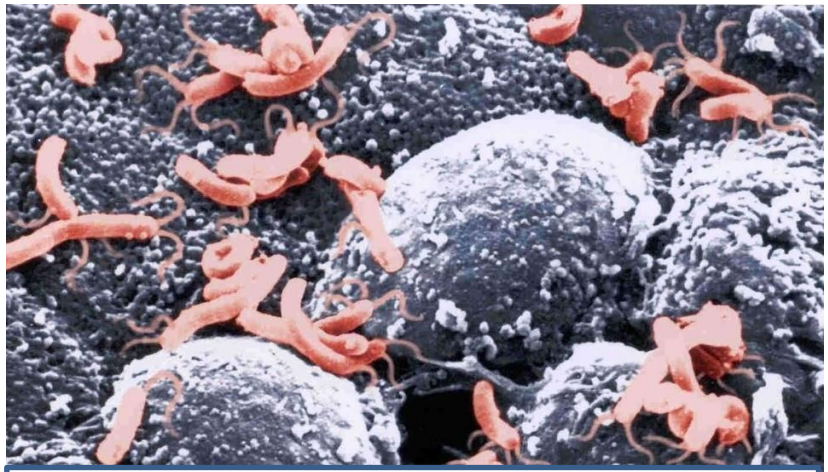
Colonization

- After adhesion microorganisms begin to multiply on certain areas – colonization.
- First, microorganism colonize skin and mucous membranes.
- Colonization may occur both inside and outside the cell.

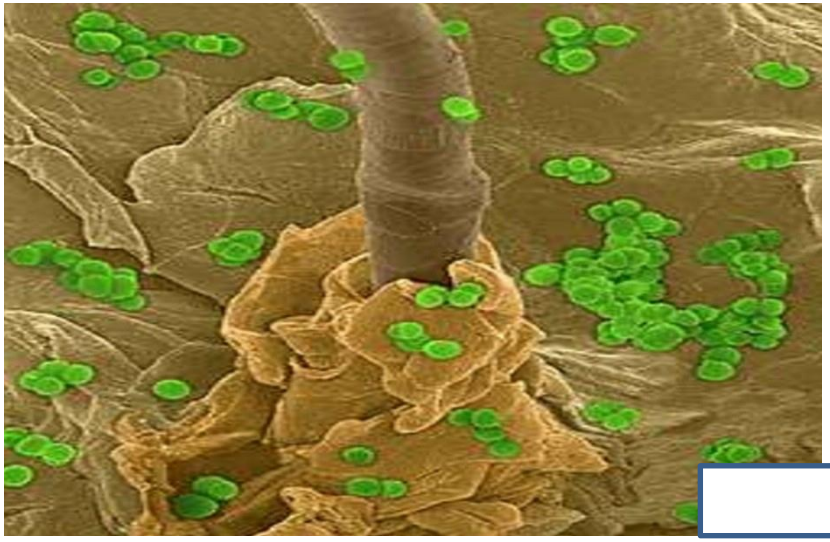
  For example, cholera causing microbe colonizes surface, while dysentery causing bacteria multiply inside the cell.

Colonization

Oral cavity



Stomach

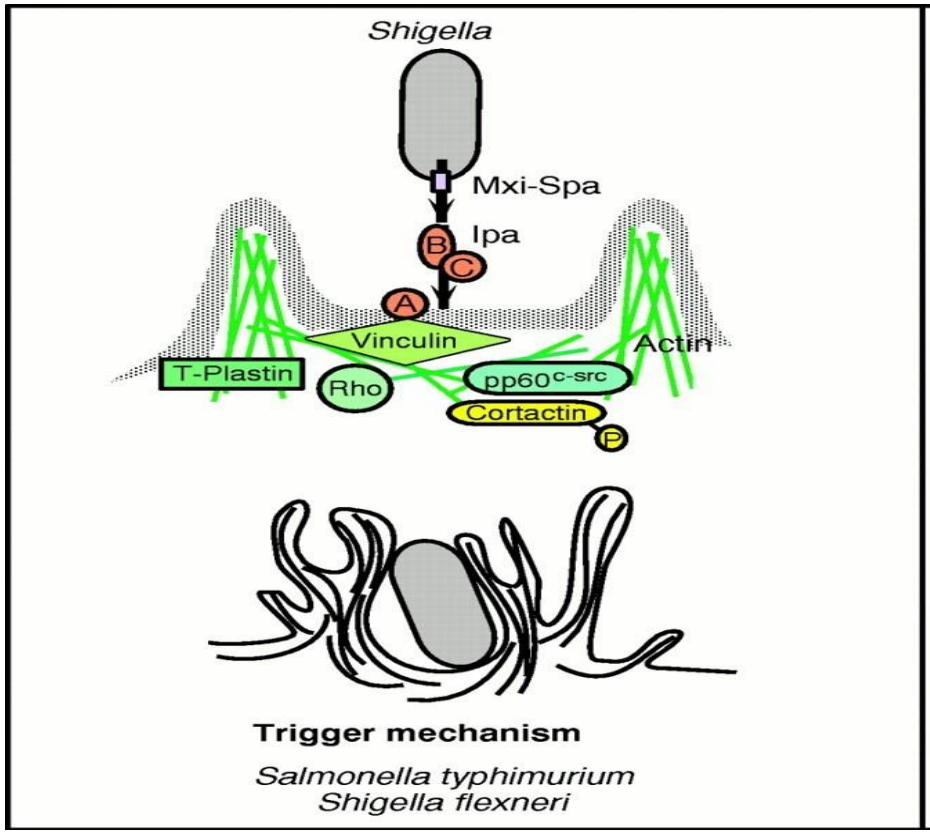


Skin

Penetration and invasion

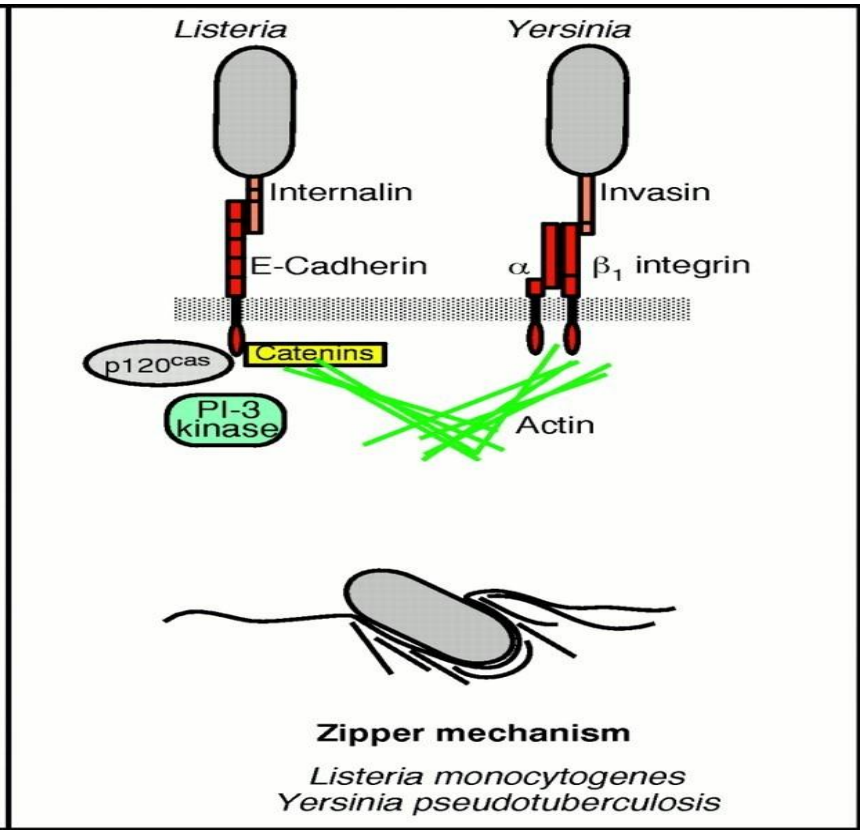
- Ability to penetrate is related to invasiveness of microorganism.
- **İnvasiveness** - is ability to enter cells and tissues.
- Colonization of skin and mucose membranes is not always limited to surface layers. Pathogenicity of some bacteria (Shigellae, iersinia etc.) is related to their ability for penetration.
- Penetration is mediated by special factors among which **invasins** – special proteins of outer layer are well studied. Interaction of invasins with cell surface receptors – integrins results with endocytosis(“swallowing”).

Invasion in various microorganisms



Trigger mechanism

Salmonella typhimurium
Shigella flexneri



Zipper mechanism

Listeria monocytogenes
Yersinia pseudotuberculosis

Agression enzymes

Invasiveness is closely linked with ability to produce enzymes – aggression ferments. They commonly break down membrane of cells, extracellular substance enabling spread of microorganism in tissues.

Hyaluronidase

Lesitinase (phospholypase)

Neuraminidase

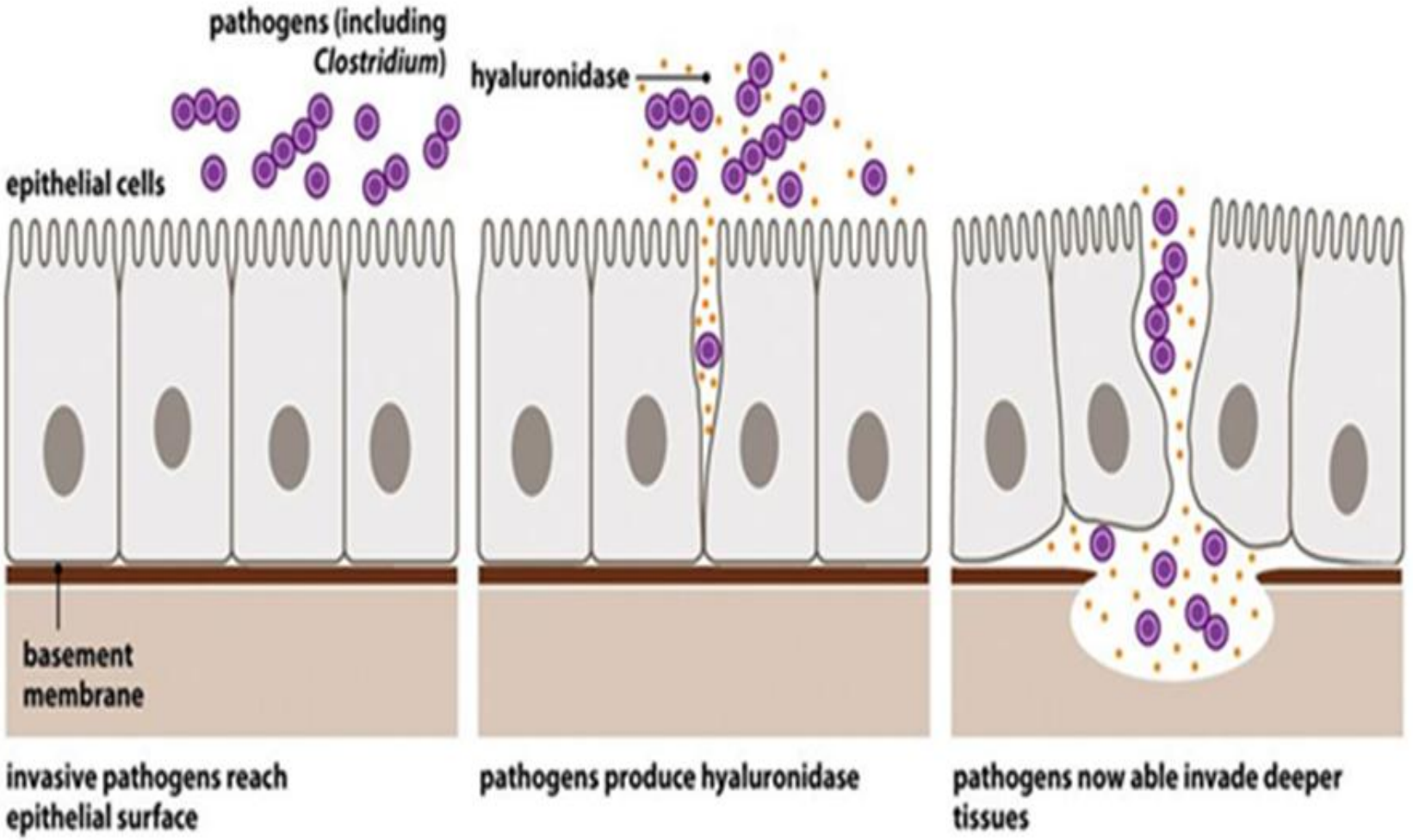
Collagenase

Plasmacoagulase

Fibrinolysin

Citolysins (***hemolysins***), ***leucosydins***, ***IgA1-proteases***

Agression enzymes

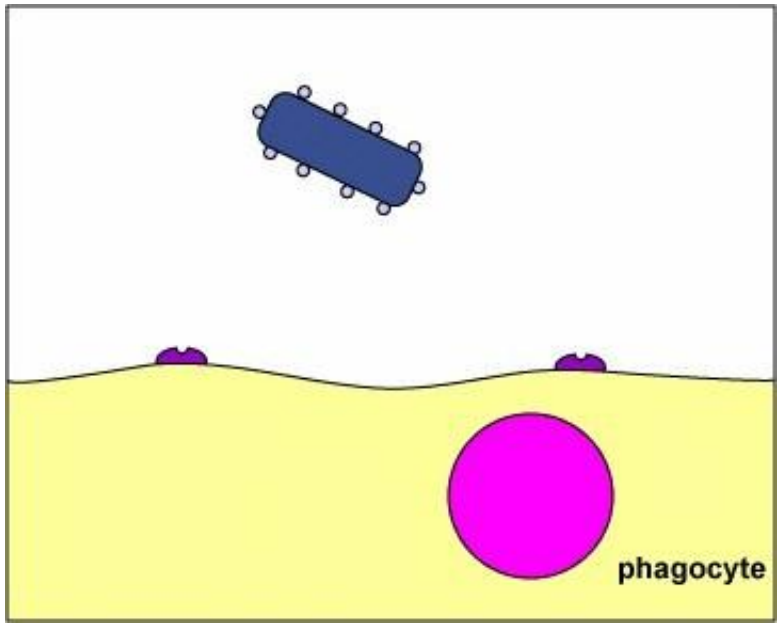


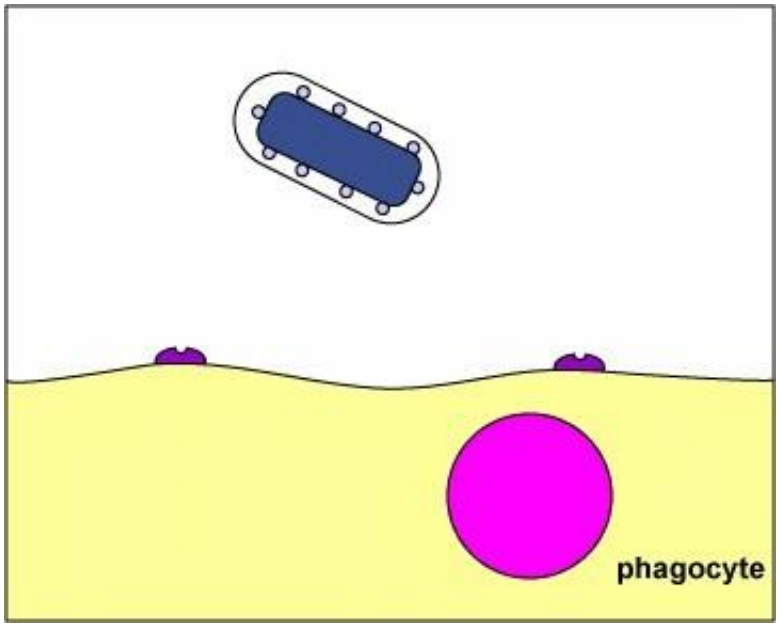
Factors preventing phagocytosis

- Many pathogenic microorganisms especially bacteria have pathogenic factors preventing phagocytosis – **microcapsule, capsule, slime layer.** Some microorganisms synthesise **s**ubstances **weakening phagocytosis or** breaking down **chemoattractants.**
- There are also factors preventing intracellular killing of bacteria:

- ❑ Substances inhibiting fusion of phagosome with lysosome
- ❑ Protection from oxydasing factors of phagososmes
- ❑ Resistance to lysosomal enzymes
- ❑ Factors causing lysis of phagosome(exp. listeriolysin);
- ❑ Some microorganisms (trypanosomes) can leave phagolysosome thus preventing themselves from phagocytosis

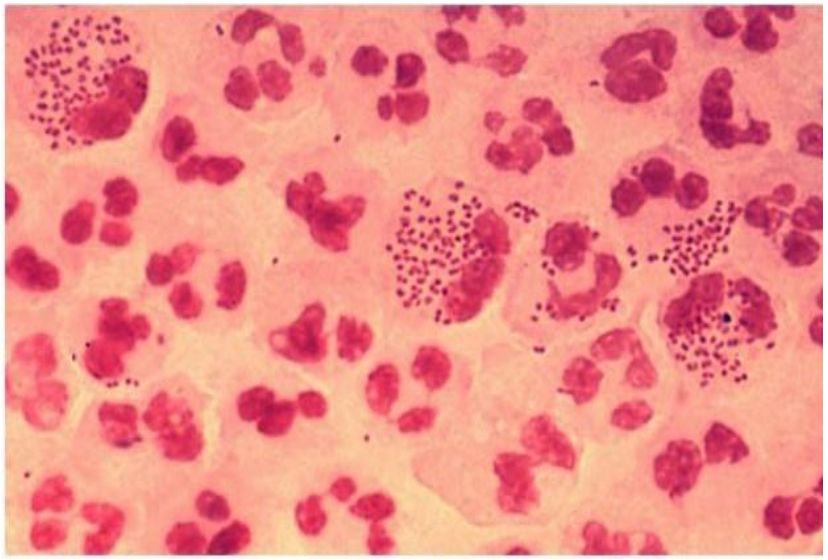
Capsule protects from phagocytosis





Incomplete phagocytosis

- These factors support survival of microorganisms inside the pahgocytes.
- This phenomenon enables spread (dissemination) of microbe in organism through blood and lympha.



Bacterial toxins

- One of the most important pathogenic factors of bacteria are their toxins.
- Two main groups of toxins exist: **exotoxins** and **endotoxins**.

Exotoxins

Exotoxins *are proteins (enzymes) which in small concentrations have lethal effect on macroorganisms cells*.

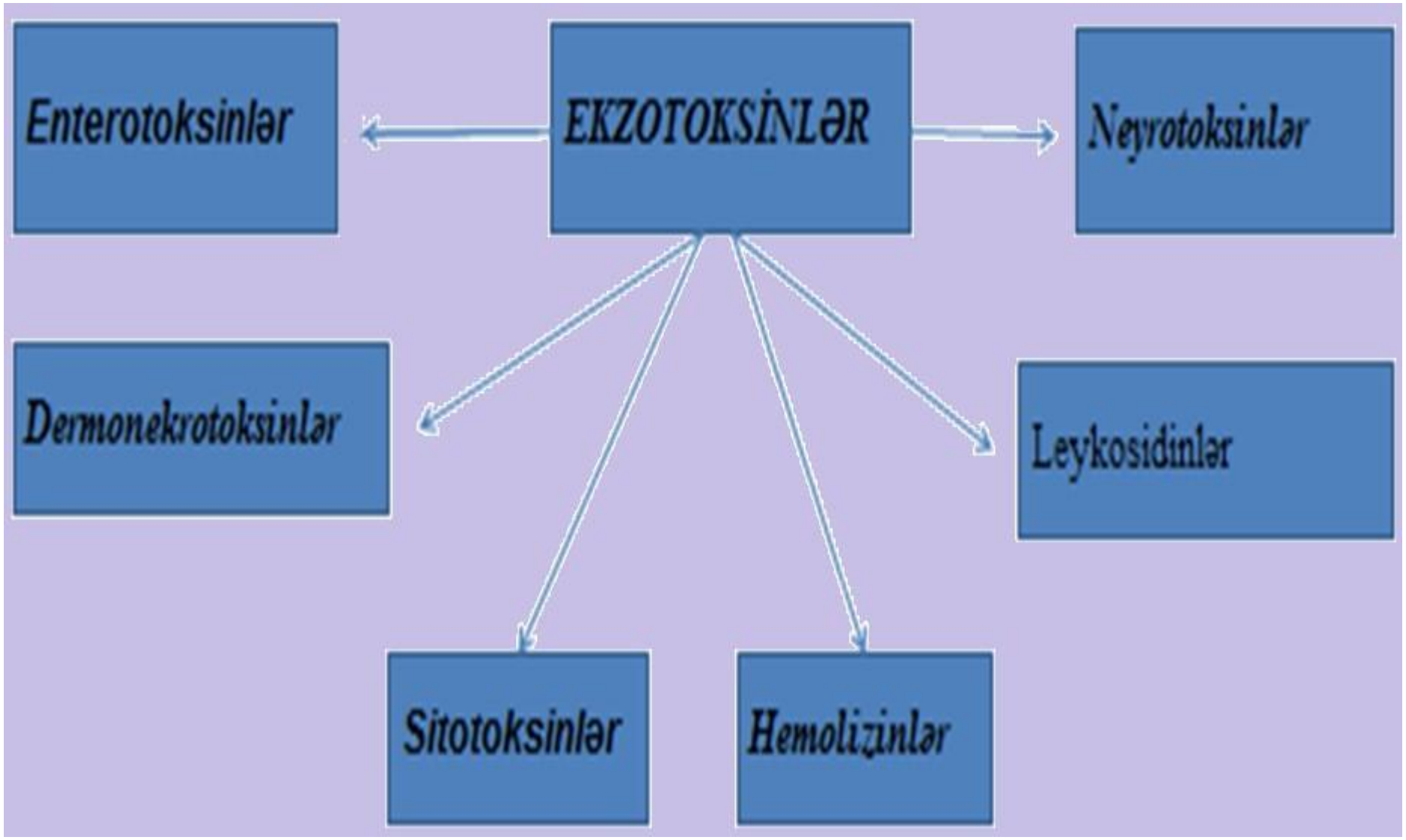
They can be secreted by the cell or exist inside the cell and released after death of cell.

Thus, extracellular secretion of toxin is not essential. Thus, recently a term protein toxin is used instead of exotoxin.

Exotoxin features

- Proteins (enzymes)
- They are not structural part of the cell
- Have high toxicity
- Relatively termolabile
- Have selective effect on organ and tissues.
- formaline, acids, heat causes their inactivation – conversion to anatoxins (toxoids)
- Synthesized by both Gram negative and Gram positive microorganisms.

Due to ability to bind with specific receptors of target cells exotoxins are divided to different groups:



Endotoxins

- **Endotoxins** differ sharply from exotoxins in many aspects
- Endotoxins are lipopolysaccharides(LPS) of Gram negative outer layer

Endotoxin features

- Lipopolysacharides
- They are a structural part of cell
- Relatively low toxic
- Termostabile
- Cause general intoxication
- Can not be converted to anatoxin
- Commonly exist in gram negative bacteria

Lipopolysacharide

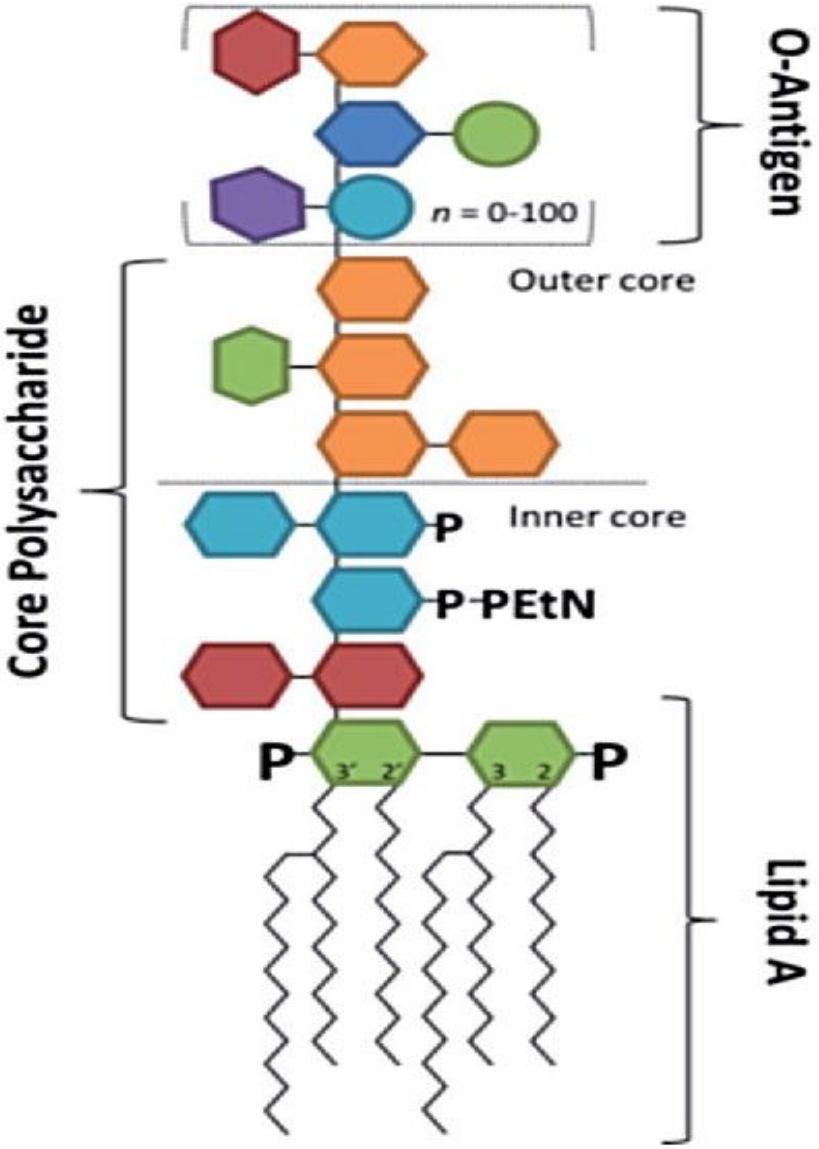
LPS consists of ***polysachariide and lipid***

- **Polisacharide complex consists of O-antigen and core part and determines antigenic feature of LPS. O-antigen is variable and may be different even among same species.**
- Thus, there different serovars within the same species which have diffrenet antigenic structure.
- The core part is stabile and the same within the species or genera. It is the cause of cross-reaction phenomenon in microorganisms.

Lipopolysacharide

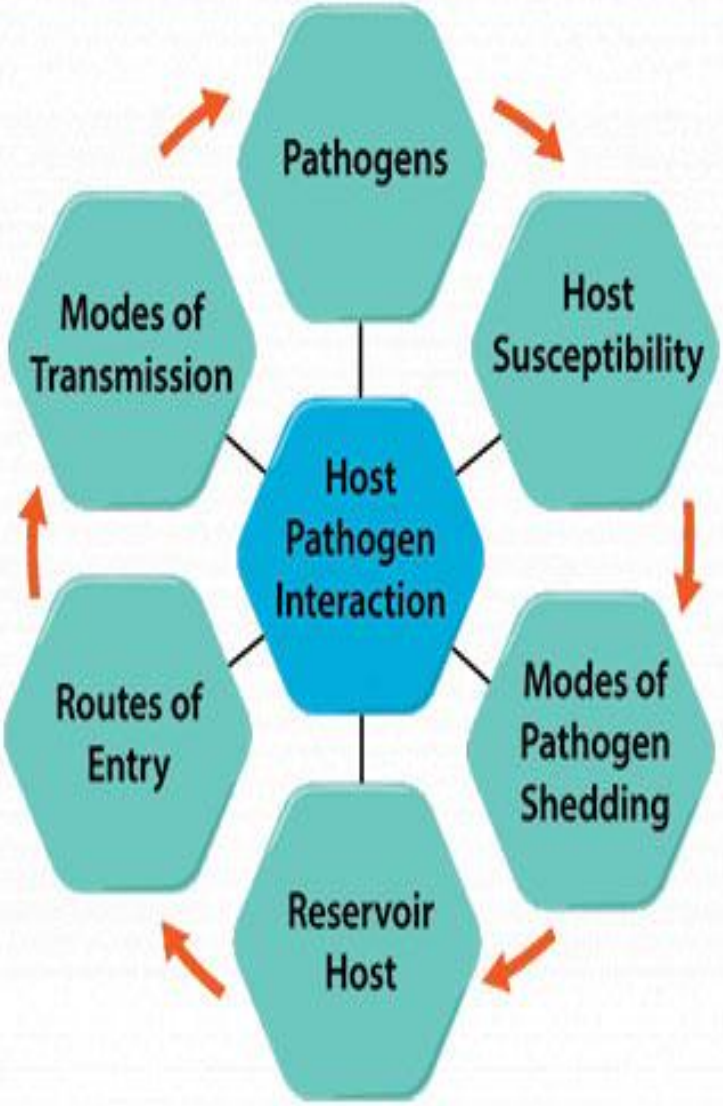
- **Lipid complex consists of** lipid A and responsible for toxicity of LPS.
- As a core part of LPS polysaccharide, lipid A is also conservative in all Gram negative bacteria (some bacteria - Bacteroides Fragilis, Borderacelis, Borderus, Borderus, Borderus are exceptions)

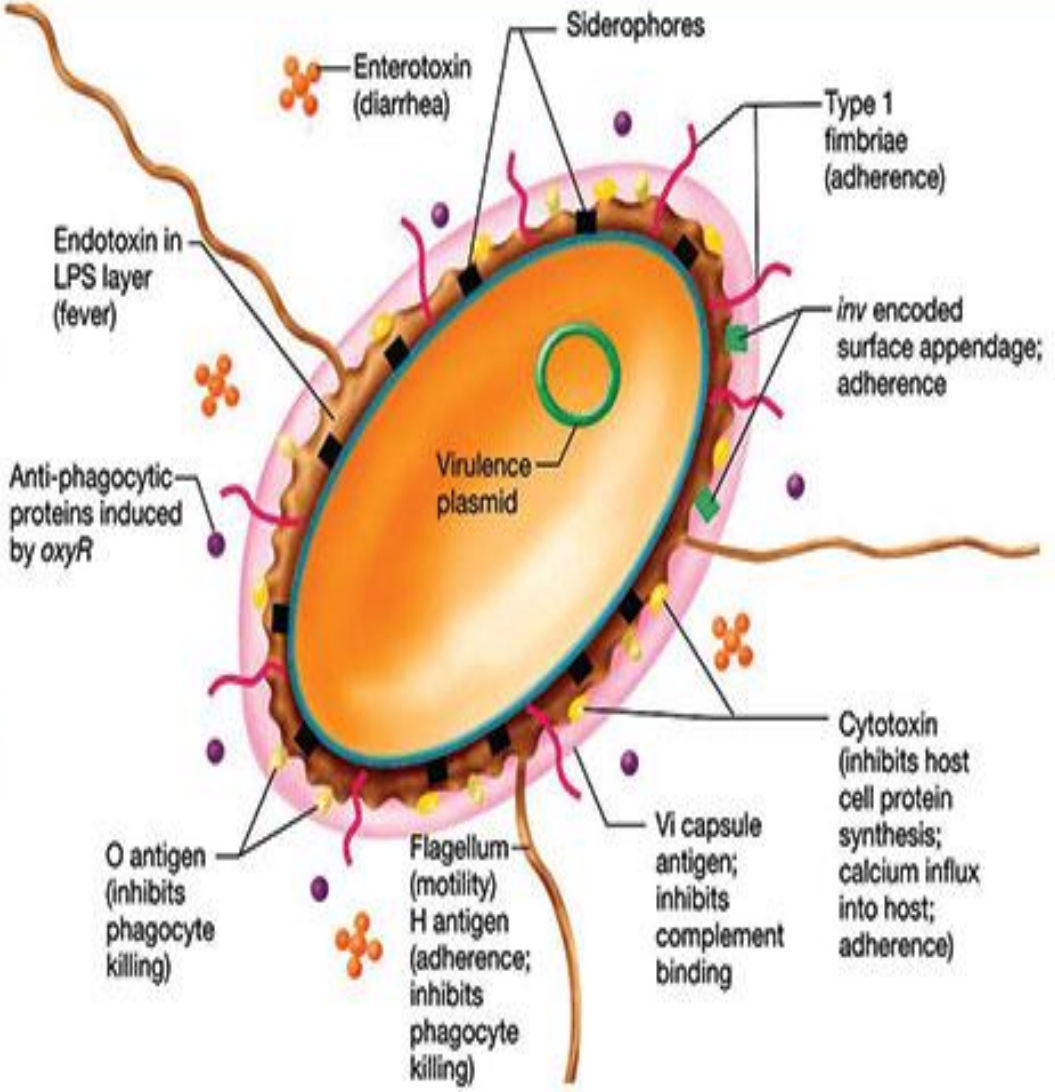
Structure of lipopolysaccharide



Exotoxins	Endotoxins
Synthesized by living microbial cells and accumulates in high concentrations in a liquid culture medium.	*Gram-negative bacteria are part of the cell wall and are removed after the bacterial cell is destroyed.*
Produced by both gram negative and gram positive bacteria.	*Exist only in gram negative bacteria*
Proteins with molecular weight 10000-900000 D.	*Lipopolisacharide complex. Toxicity is related to lipid A.*
Relatively thermolabile – rapidly destroyed by 60 C and higher.	*Relatively thermostable and do not lose their toxicity for one hour at a temperature higher than 60 C.*
High antigenic properties	*Weak antigenic properties*
Some factors cause their conversion to anatoxins.	*Do not convert to anatoxins.*
High toxicity.	*Low toxicity.*
Do not cause fever.	*Cause fever by mediating interleukin-1 production.*
Production may be coded by extrachromosomal genes.	*Production is coded by chromosomal genes.*
Selective effect on organs and tissues.	*Have no selective action.*

Factors affecting bacterial pathogenicity





The role of macroorganism in infectious process

- **Age** («*child infections*»)
- **Nervous system condition**
- **Endocrine system condition**
- **Nutrition**
- **Sex**
- **Genetic factors**
- **Immune system condition**
- **Normal microbiota role** (*colonization resistance*)

The role of environment in infectious process

- **Temperature** («cold» diseases)
- **Radiation**
- **Social factors**(«social diseases»)
- **Antropogenic and ecological factors** (natural disasters)
- **Iatrogenic factors**

Features of infectioyus process

- Each infectious disease has its **own pathogen** (**etiological factor**), in other words, each pathogenic microorganism causes only a certain disease (or diseases).

  - Bacterial infections, viral infections, mycoses

  - Protozoosis, helminthosis, infestations

- Infectious disease is **contagious**.

  - *Contagious index* – a ratio of infected people number to number of people which were in contact with infection source.

- Infectious**Acquired immunity** disease has **periodical course**

- is formed after infectious disease

Infection source

Antroponoses- the source of infection are people

Zoonotic infections- the source of infection are animals

Sapronoses - the source of infection is the environment

Infection mechanisms

- ***Air-droplet mechanism -*** *the causative agent is mainly localized in the upper respiratory tract spreads to environment when talking, sneezing, coughing and infects through air-droplet, air-dust mechanism. Respiratory tract pathogens are transmitted through this mechanism. Sneezing*
- ***Fecal-oral mechanism*** - the causative agent is mainly localized in the intestines, excreted in the environment with feces and transmitted by an alimentary route (food, water). Intestinal infections are transmitted by this mechanism.
- ***Contact mechanism***– pathogens are localized in different places and spread through different ways.
- *- Direct and indirect infections possible*.
- ***Transmissive mechanism***. The causative agent is in the blood of a person or an animal and is transmitted by blood-sucking insects (malaria, smallpox, etc.).
- *- Parentheral infection can also be attributed to the transmissive mechanism*

Infectious process stages

- **The incubation period, or latent period,** covers the period from the entry of a pathogenic microbe into an organism until the first signs of the disease are observed. In most diseases, the latent period lasts 1-2 weeks**.**
- **Prodromal (Greek, prodromos - evangelist),** or the period of awareness is a period after the latent period, with non-specific symptoms (fever, headache, weakness, malaise).
- **The period of clinical manifestations,** beginning after the prodromal period, is accompanied by the symptoms characteristic of each infectious disease.
- - General signs, characteristic symptoms, pathognomonic symptoms.
- **Reconvalescence period -** decresase of symptoms and recovery of organism functions.
- ***- healing, microbe carriage, chronic form, lethal***

Infectious disease forms

- **Depending on the origin**
- - *exogenous infection, endogenous infection, or autoinfection*
- **Depending on the location of the causative agent in the body**
- - *Focal infection, generalized infection*
- **Distribution of the causative agent and its toxin in the body**
- - *Bacteremia (sepsis), virusemia, toxemia*
- **Depending on number of he pathogen**

  - *monoinfection*, *mix-infection*
- **Superinfection-** infection with the same agent before the disease is cured
- **Reinfection -** infection with the same agent after complete recovery of the infectious disease
- **Recidive -** recurrence of syptoms without new infection

Infectious process forms

- **Depending on how long the pathogen stays in the body**
- **-** Acute infections are relatively short, lasting from 1 week to 1 month (flu, measles, plague, etc.).
- - Chronic infections, as a rule, have a long course (6 months and more) (tuberculosis, leprosy, brucellosis, syphilis, etc.).Chronic infections are accompanied by long-term stay of microorganism in body –*persistence*.
- ***Microbial carriage*** *(*bacterial, parasitic, viral, mycobacterial, etc.) - the pathogen can remain in the body for a certain period of time, sometimes for life. Microbial carriage sometimes manifests as a latent, hidden, or dormant infection**.-**
- ***Depending on clinical manifestations***

  - Typical, atypical, inapparant (latent, hidden, subclinical, asymptomatic), fulminant), abortive.

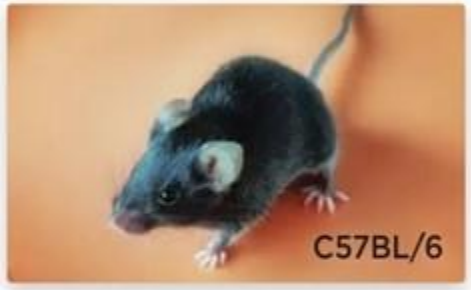
Spread of infectious diseases

- ***An epidemic*** is a mass spread of an infectious disease in a certain area and for a certain period of time.
- If a disease spreads to countries or even continents, it is called a pandemic.
- Sometimes the infection occurs in the form of a single disease ***- sporadic disease.***
- Infectious diseases are called endemic if they are found only in a certain area. Endemics are *natural-focal* disease with source and vectors localized in certain areas.

Biological method

- Laboratory animal inoculation is performed to:
- Evaluate of pathogenicity and virulence of microbes,
- Obtain of pure culture from pathological material,
- Create of experimental infections

Laboratory animals



Preparation of laporatory animal for experiment

- Selection of animals by weight, sex and age
- - When choosing laboratory animals, their sensitivity to the studied pathogen is taken into account (for example, guinea pigs - susceptible to tuberculosis, diphtheria, plague, black sores, white mice - tularemia, botulism, tetanus, etc.).

Preparation of tools and material

- All instruments used during operation must be sterile.
- The material to be injected into the animal is dissolved in a sterile saline. The prepared solution is collected in a syringe. Air bubbles and excess of material should be discarded on sterile cotton soaked in 5% chloramine, 5% carbolic acid, or alcohol.
- After the animals have been infected, all used tools must be sterilized in an autoclave.

Methods of laboratory animal inoculation

Laboratory animals (rabbits, guinea pigs, white mice, rats, etc.) can be inoculated by

- scarification,
- intracutaneously,
- subcutaneously,
- intramuscularly,
- intravenously,
- intra-abdominally,
- intranasally,
- orally,
- intratracheally,
- intracerebrally, etc.

Methods of laboratory animal inoculation

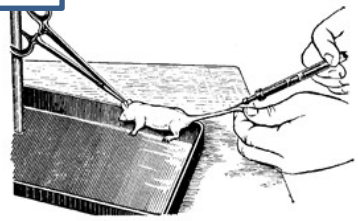
Intradermal

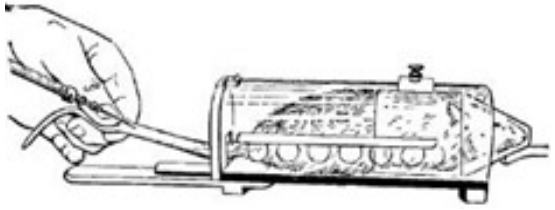
Intracranial

Intramuscular

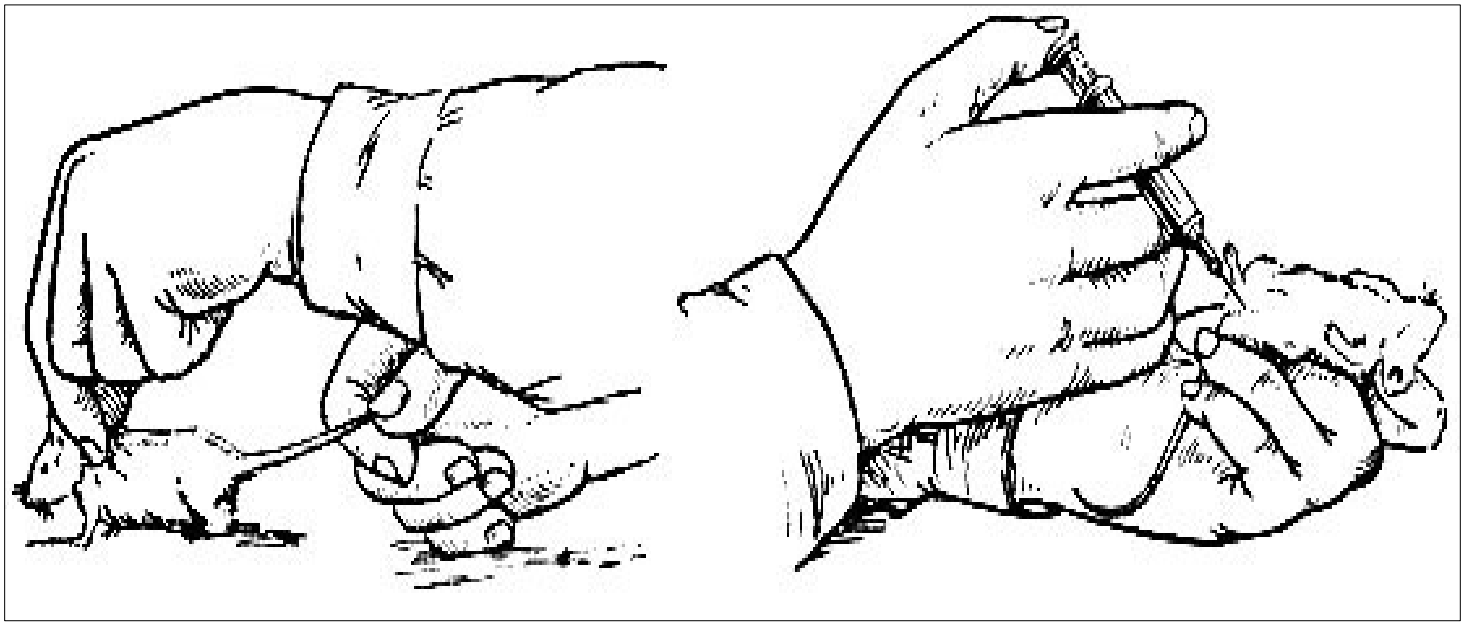
Intestinal tract

Abdominal cavity





Intraperitoneal inoculation of white mouse

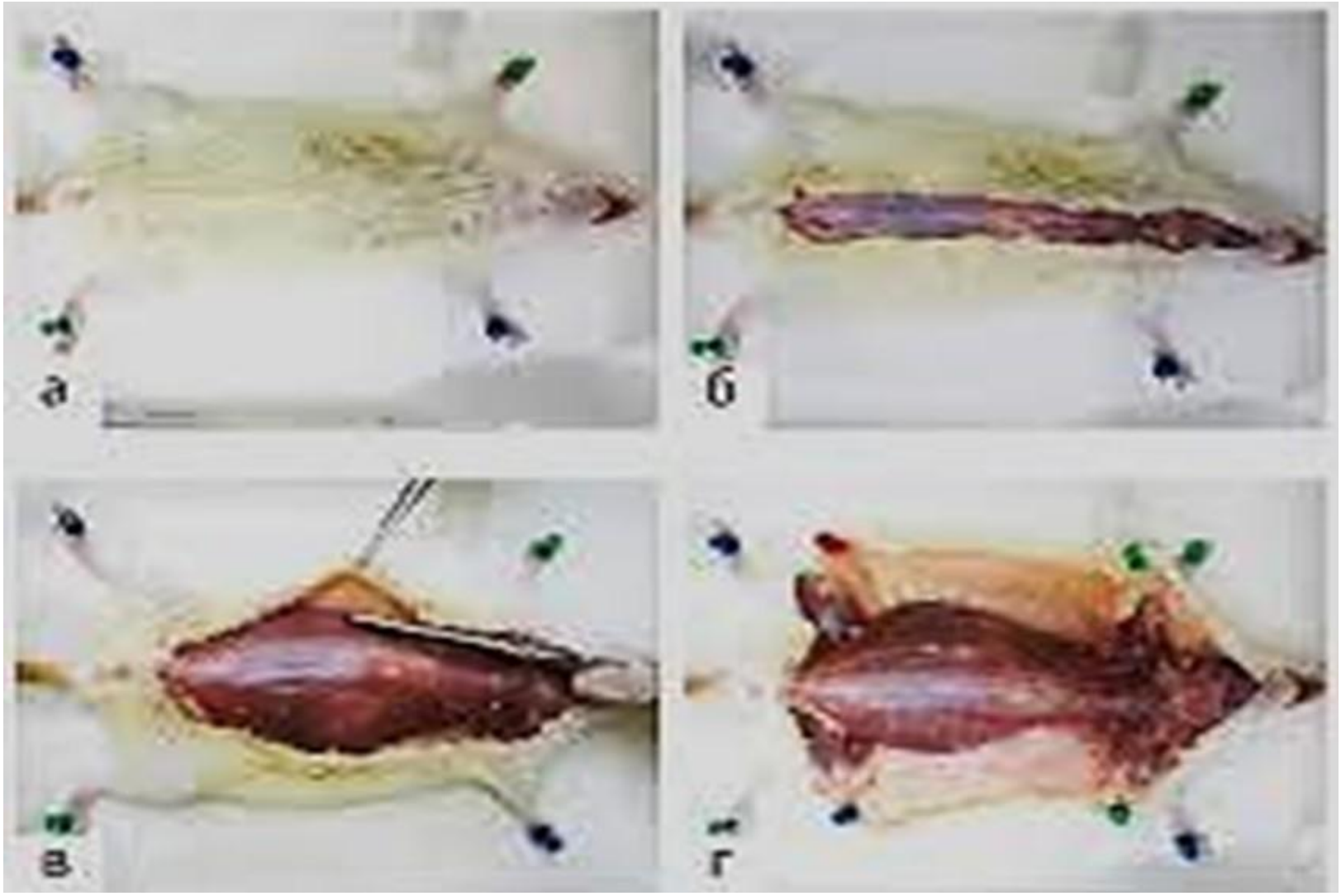


Keeping of infected animals in vivarium

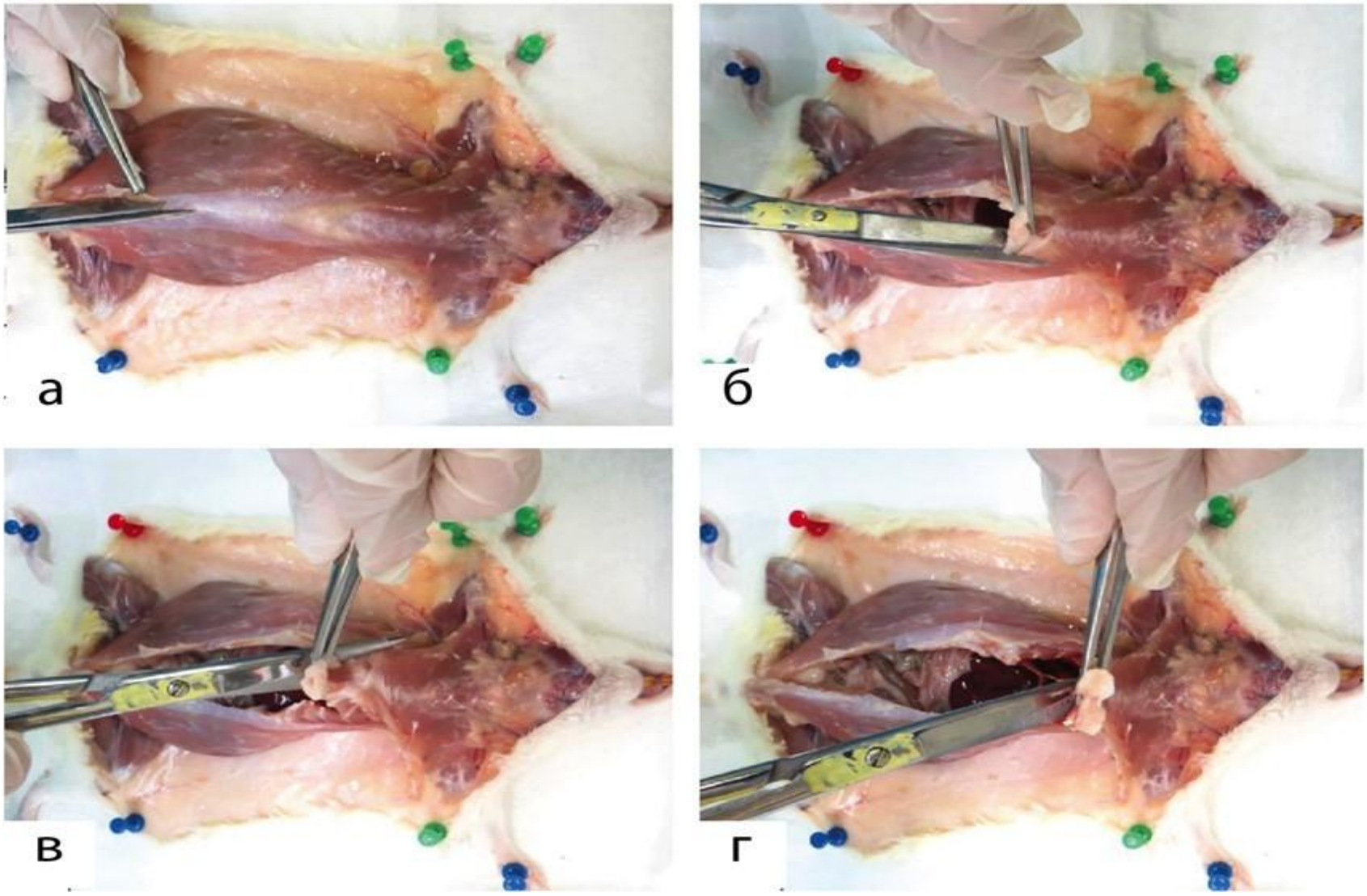
Examination of laboratory animal

- The purpose of bacteriological examination of an animal's body is to detect the microbe that caused the animal's death or illness, to find its location in the body, and to obtain a culture of the pathogen.
- In order to protect the specimen from contamination with microbes, the autopsy and culture materials are taken immediately after the death of the animal and in accordance with aseptic rules.
- If animals does not die, they are killed in accordance with the principles of ***bioethics***. These principles are based on the fact that manipulations on laboratory animals are performed under complete anesthesia.

Examination of laboratory animal (white mouse) corpse



Examination of laboratory animal (white mouse) corpse



Bacteriological examination of laboratory animal (white mouse) corpse

If animal alive:

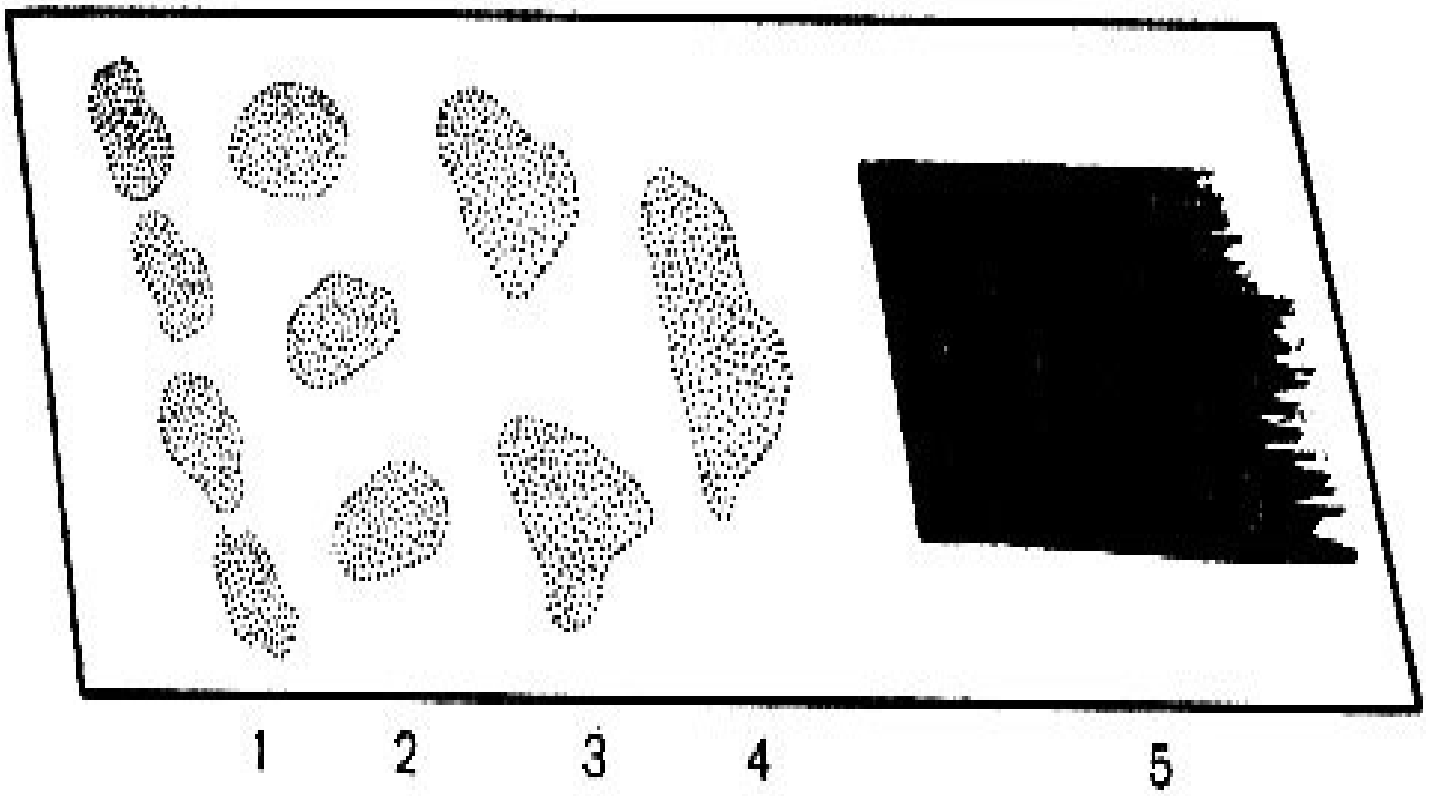
- Blood
- Abdominal cavity exudate.

If animal dead:

- Blood
- Spinal fluid
- Fluids from different cavities.

Bacteriological examination of laboratory animal (white mouse) corpse

- After dissection, the internal organs are examined, swabs prepared from the organs are inoculated onto blood (the surface of the organ section is touched to the surface of the nutrient medium).
- Parallely, smears from liver, spleen, kidneys are prepared and fixed in Nikiforov solution (a mixture of equal volumes of alcohol and ether), stained with methylene blue or Gimza and examined under microscope.
- Inoculated nutrient media are incubated for 24-48 hours at 37°C.
- Microorganism isolated after cultivation are identified based on their morphological, cultural, biochemical etc. features.



Preparation of smears (1-4) and thin blood smear (5) on the same slide.

Neutralization of animal corpse

- After dissection, the animal's corpse is burned, sterilized in autoclave or boiled in phenol solution for 1-2 hours.
- All tools, tubs and plates for fixation are treated with disinfectant solution or sterilized in an autoclave.

Determination of pathogenicity and virulence (lethal doses)

For this purpose, average lethal dose is determined(LD_{50}).

- When evaluating LD_{50} species, sex, weight, are taken into account.
- Several dilutions (10^{-1}, 10^{-2}, 10^{-3} etc.) of microorganism culture are injected in different groups of animals(at least 5-6 animals in one group).
- After a certain period of time LD_{50} evaluated in each group based on number of death animals.

LD_{50} *calculation by Kerber method*

There are many methods for calculation. Kerber method is one of the most widely used methods. LD_{50} is calculated by applying the number of dead and surviving animals in the Kerber formula.

$$IgLD_{50} = IgD_N - S\,(\sum Li - 0{,}5)$$

- Ig –logarithm;
- ∑ - sum;
- S - the decimal logarithm of the ratio subsequent dose to the previous dose;
- Li – the ratio of the number of animals that died from the same dose to the number of animals in that group;
- N – total number of doses (dilutions) applied;
- IgD_N – the maximum dose among the applieddoses.

Determination of pathogenicity and virulence

- At present, due to the **principles of bioethics** the use of laboratory animals is limited.
- For this purpose, other methods are used - infection of cell cultures, chicken embryos, primitive cultures.
- Individual pathogenic factors of microorganisms or their genetic determinants are alsoevaluated.

Evaluation of pathogenicity and virulence (adhesion, invasion, cytotoxicity)

- To determine the ability of microbes for adhesion, invasion and cytotoxicity, standard one layer tissue cultures (HeLa, Hep-2, etc.) is infected. After certain periods of time, the culture fluid is discarded, the tissue layer is washed to remove unbound microorganisms, and microscopy is performed after fixatio.
- Under the microscope, 200-300 tissue cells are counted taking into account cytopathic effect, intracellular and extracellular microorganisms.
- The number of intracellular and extracellular microorganisms per cell (**adhesion and invasion indexes**), the percentage of cytopathic cells (**cytotoxicity index**) is determined.

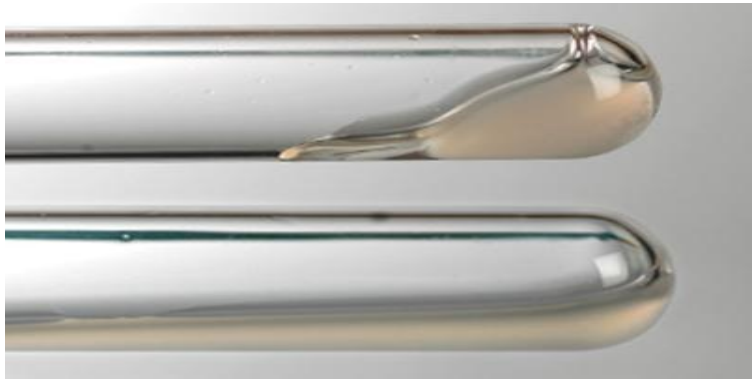
Evaluation of pathogenicity and virulence (pathogenicity enzymes)

- Pathogenicity enzymes are direct indicators of microorganism pathogenicity.
- In practice, it is used to differentiate pathogenic microorganisms from saprophytes.

Plasmacoagulase detection

- The examined microbial culture is inoculated into sterile blood plasma with citrate and incubated at 37^0C for 2-5 hours.

Plasmacoagulase positive microbes clot the plasma, while in the control tube the plasma remains liquid.



Positive and negative tests

Lecithinase detection

Determination of the ***lecithinase*** is based on the breakdown of a substrate containing lecithin.

The examined microbial culture is inoculated into Petri dishes containing egg-wrapped agar and incubated for one day at 37^0C.

Lecithinase activity is manifested by the formation of a blurred border around the colonies.

Detection of hyaluronidase

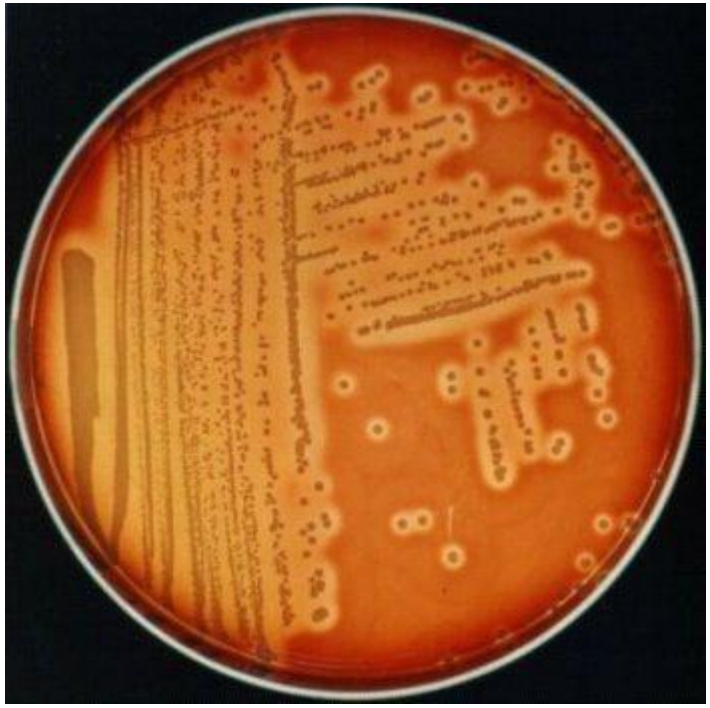
Hyaluronidase detection is based on the hydrolysis of hyaluronic acid by the action of this enzyme.

The examined microbial culture is inoculated on a substrate containing hyaluronic acid. After incubation for 15 minutes at 37^0C, 2-3 drops of acetic acid are added.

In the presence of hyaluronic acid, solid mucus clots are formed in the test tubes.

Detection of hemolytic activity

- In order to determine the ***hemolytic activity,*** the microbial culture is inoculated into Petri dishes with bloody agar.
- Incubated for one day at 37^0C.
- a hemolysis zone formed around the colonies indicates hemolytic activity



Detection of exotoxins

- The synthesis of exotoxins by microorganisms is one of the main indicators of its pathogenicity. In classical experiments, this feature was usually studied on laboratory animals.
- At present, the ability to synthesize exotoxins can be determined in cell cultures, chicken embryos, and primitive cultures, as described above.
- Genetic determinants of toxins of microorganisms, such as toxigenicity genes, are also identified.
- The serological method - the precipitation reaction (Sieve method) is used to determine the exotoxin of diphtheria.

In vivo neutralization of toxin by antitoxin