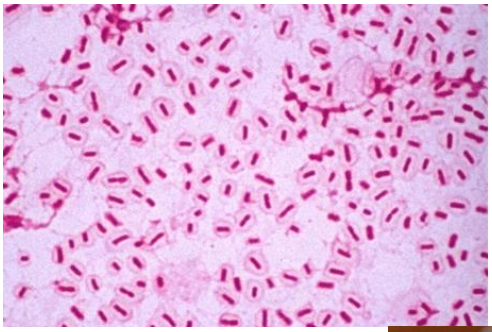

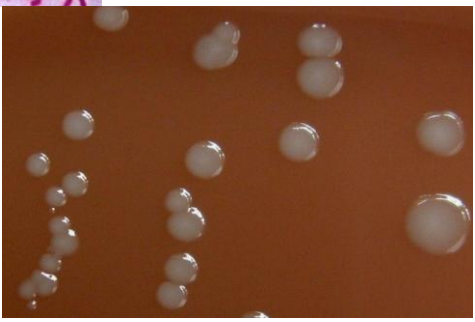

HEMOPHILIC BACTERIA

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

ГЕМОФІЛЬНІ БАКТЕРІЇ

Методичні вказівки з дисципліни "Мікробіологія, вірусологія та імунологія" для студентів II iIII курсів медичного та стоматологічного факультетів з англійською мовою викладання

МІНІСТЕРСТВО ОХОРОНИ ЗДОРОВ'Я УКРАЇНИ
Харківський національний медичний університет

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Compilers N. I. Kovalenko
T. M. Zamaziy

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

Learning guide includes sections of taxonomy, and biological characteristics of haemophilic bacteria. The most modern information on epidemiology, pathogenesis, immunity, immunoprophylaxis, and modern methods of laboratory diagnosis of H. influenzae and H. parainfluenzae is represented.

Гемофільні бактерії : метод. вказ. з дисципліни "Мікробіологія, вірусологія та імунологія" для студентів II і III курсів мед. та стомат. фак-тів з англ. мовою викладання / упоряд. Н. І. Коваленко, Т. М. Замазій. – Харків : ХНМУ, 2019. – 36 с.

Упорядники Н. І. Коваленко
Т. М. Замазій.

Theme: Laboratory diagnosis of hemophilic infection

Actuality of the theme

A group of hemophilic bacteria combines gram-negative bacteria that can grow only on enriched nutrient media that contain whole or lysed blood or its derivatives as growth factors. The name of the genus *Haemophilus* reflects the dependence of these bacteria on blood during growth on artificial nutrient media. Many microorganisms of this genus normally live on the mucous membranes of the human respiratory tract.

To date, there are 9 types of hemophilic bacteria that cause infection in humans (*table 1*).

Table 1

Species of the genus *Haemophilus*, isolated from humans and animals

Isolated species	
from human	from animals
H. influenzae	*H. paragallinarum* (poultry)
H. parainfluenzae	*H. haemoglobinophilus* (dogs)
H. haemolyticus	*H. felis* (cats)
H. parahaemolyticus	*H. parasuis* (pigs)
H. aphrophilus	*H. paracuniculus* (rabbits)
H. paraphrophilus	
H. paraphrohaemolyticus	
H. ducreyi	
H. segnis	

The most important pathogens for humans are H. influenzae, mainly type b, which causes infection with the respiratory mechanism of the lesion, as well as the causative agent of soft chancre *H. ducreyi*.

H. influenzae was first identified as a pathogen by R. Koch in 1883, which described gram-negative small rods in pus from patients with conjunctivitis. In 1892, R. Pfeiffer isolated *H. influenzae* in pure cultures from sputum in patients with influenza. Despite the fact that the viral etiology of the flu was later established, the original species name was left under the bacteria.

Annually in the world about 3 million cases of hemophilic infection are registered, of which about 400–500 thousand are fatal. Hemophilic infection is characterized by severe course and high mortality among young children (up to 60 %) in developing countries, the frequency of neurological complications to 25–35 %. The frequency of diseases of this etiology in children under 5 years in different countries reaches the rates from 25.5 to 130.0 per 100,000 children. Economic losses due to the cost of treatment for one patient reaches 22–56 thousand dollars.

Hemophilic infection (Hib infection) caused by *Haemophilus influenzae* type b (Hib) is one of the most important causes of morbidity and mortality among diseases that can be controlled by immune prophylaxis. The results of the study of Hib infection in many countries and on different continents have shown that this infection is a topical health problem almost everywhere. At

present, there is enough data to show that Hib infection is widespread and causes a high level of a number of topical diseases, among which the most frequent are meningitis, sepsis, epiglotitis and pneumonia.

A particular problem is the increasing frequency of *H. influenzae* antibiotic resistance. *Hemophilus* rod type b is one of the leaders among bacteria for resistance even to modern antibiotics. Etiotropic therapy of Hib-meningitis is often not effective and is associated with a decrease in the sensitivity of the hemophilic rods to 50–60 % of the known and new antibiotics. The diversity of clinical forms, on the one hand, and the growing resistance to antibiotics on the other, make it very difficult to diagnose and treat this infection.

The urgency of the problem is also determined by the widespread spread of bacterial carriers of *H. influenzae* type b to 11.3–20.8 % among children under the age of 5 years.

Under the prevailing conditions, a reliable method of protecting children from Hib infection is specific immune prophylaxis. The number of WHO member states using the hemophilic vaccine in their routine extended immunization programs is increasing, but there is still a problem of increasing the coverage of immunization in countries that use vaccine and introducing a vaccine in countries where there is still immunization program is not implemented.

Goal: Studying laboratory diagnosis of hemophilic infection.

Concrete goals:

1. Study of biological properties and classification of *Haemophilus* spp.
2. Study pathogenesis and clinical manifestations of hemophilic infection.
3. Study of the methods of laboratory diagnosis of hemophilic infection.

Students should be able to:

1. Isolate of pure cultures of *Haemophilus* spp. and examine growth on differential media.
2. Identify of pure culture of *Haemophilus* spp. on morphology, culture and biochemical properties, antigenic structure.
3. Interpret results of serological tests to diagnose hemophilic infection.

Equipment: slides, immersion microscope, biological preparations for laboratory diagnosis of hemophilic infection, tables, atlas.

Taxonomy.
Family: Pasteurellaceae
Genus: Haemophilus
Species: H. influenzae, H. parainfluenzae, H. ducreyi

Morphological and tinctorial properties. Hemophilic bacteria are small gram-negative spherical (1 × 0.3 μm), ovoid or rod-shaped bacteria, sometimes forming pairs, short chains or filaments (*figure 1*). This property of microorganisms is called pleomorphism. The morphology of these bacteria depends on the age of pure culture or on the type of nutrient medium. Hemophilic bacteria are immobile, do not form spores, have pili (fimbria).

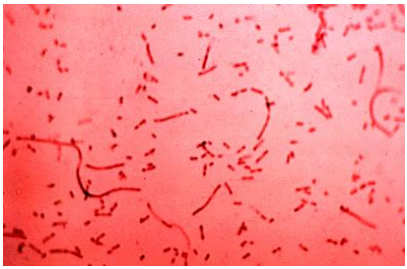


Fig. 1. Morphology of *H. influenzae*

Formation of the capsule is a non-persistent sign. The presence of a capsule is of great clinical importance, since it is a major virulence factor. Most invasive infections are caused by the strains of *H. influenzae* type b (Hib). The Hib capsule consists of polyribosylbutyol phosphate (PRP), which contains as monomer pentose (ribose), unlike other types containing hexose, which probably also determines a higher virulence. Non-encapsulated strains are denoted as non-typed.

Cultural properties. Hemophilic bacteria are facultative anaerobes, but they grow better in aerobic conditions. Almost all species require growth factors that are in the blood (hence the name of the genus *Haemophilus* – "loving blood"): a thermostable X-factor (protoporphyrin IX in hematite or hemin), as well as a thermolable V-factor (nicotinamide adenine dinucleotide – NAD). This is due to the fact that hemophilia are not able to synthesize heme, which is a part of the enzymes of the respiratory chain, and/or NAD, which is a cofactor of oxidative-reducing enzymes. However, in native sheep and human blood, there are enzymes (NADases) that destroy the V factor. Therefore, V-dependent types of hemophilia are poor or do not grow at all on blood agar, prepared on the basis of sheep or human blood. The need for bacteria in the factors X and V is an important criterion for the identification of *H. influenzae* (*figure 2*).

For cultivation of hemophilic bacteria, chocolate agar is used – a nutrient medium of brown color, which is obtained by heating the blood agar at 80 °C for 15 minutes. As a result of warming, hemolysis and release of hemin and NAD from red blood cells occurs. The optimum temperature of bacterial growth is 35–37 °C. Colonies appear after 36–48 hours. For *H. influenzae*, the ability to form R-, S-colonies is characteristic. Mucous, larger (3–4 mm in diameter), rainbow S-shaped colonies are characteristic of capsule strains (*figure 3*). Weakly virulent non-capsuleted variants of hemophilic bacteria form R-colonies – smaller (1 mm in diameter), fine-grained, with an uneven edge.

Hemophilic bacteria are characterized by a "feeder phenomenon" or "satellite phenomenon" (*figure 4*), which manifests itself in their ability to grow on a blood agar around colonies of staphylococci or other bacteria that produce NAD or cause α-hemolysis. The ability to cause hemolysis is not typical for hemophilic rods. Thus, small, rainbow colonies of hemophilic bacteria can be detected on a blood agar in the hemolysis zone formed by *S. aureus*.

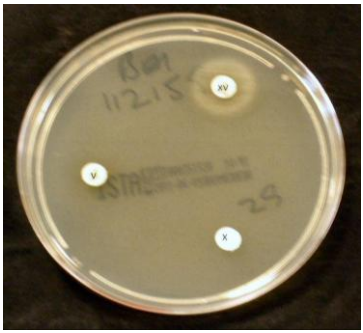


Fig. 2. Growth of *H. influenzae* in the presence of X and V factors

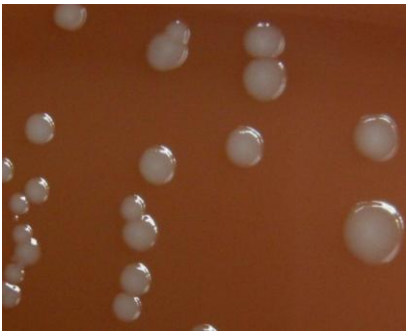


Fig. 3. Colonies of *H. influenzae* on chocolate agar

Identification of hemophilic rods is based on their need for growth factors and some biochemical tests.

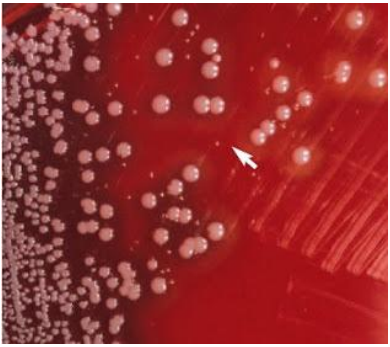


Fig. 4. "Satellite phenomenon"

Enzymatic properties. Hemophilic bacteria are chemoorganotrophs. They ferment glucose to acid, reduce nitrates to nitrites, and ferment other carbohydrates poorly. *H. influenzae* has 8 biovars, depending on their ability to produce indole, urease, ornithine decarboxylase. In addition, the species *H. influenzae* includes the aegyptius biovar. Catalase and oxidase activity in different types of hemophilic bacteria is a variable sign.

Antigenic properties. *H. influenzae* has a somatic O-antigen and a capsular polysaccharide K-antigen. Six serotypes of *H. influenzae* (a, b, c, d, e, f) are recognized depending on the structure of the capsular antigen. Serotyping of isolated capsule strains is carried out on the basis of sera to particular types. Non-encapsulated strains are denoted as non-typed, and biotyping suggested by M. Kilian is used for their characteristic.

Pathogenicity factors. The main factor of the virulence of *H. influenzae* is a capsule that protects bacteria from phagocytosis, provides survival of bacteria in the body and promotes the spread of infection.

The capsulated strains of *H. influenzae* are subdivided into 6 serotypes, depending on the antigenic properties of the capsule: a, b, c, d, e, f. The presence of a capsule is of great clinical importance, since it is a major virulence factor. Production of protective specific antibodies in the infectious process, carrier or vaccination is exactly against the antigens of the polysaccharide shell. Most invasive infections are caused by the strains of *H. influenzae* type b. The Hib capsule consists of polyribosylbutyol phosphate (PRP), which determines the higher virulence of the microorganism in comparison with other capsulated types, since it protects the microorganism from phagocytosis, opsonization and complement-mediated lysis.

Serotype "b" (Hib) is related to the development of severe forms of illness, which justifies the isolation of an independent variant (autonomous) of "Hib-infection" by some authors. The Hib serotype differs from other pathogens by 8 genes that control the formation of fimbria, which provide enhanced adhesion to the mucous membranes and simultaneously the implementation of the penetration properties of the microbe, which explains its ability to penetrate the bloodstream, causing a septic variant of the pathology.

Hemophilic rods can produce IgA protease that can inactivate secretory antibodies. Pili and IgA-protease of the pathogen play a leading role in attachment of microorganisms to the epithelium of the respiratory tract and its colonization.

LPSs of the outer membrane play the role of endotoxin, also taking part in the processes of adhesion and invasion of the hemophilic rods. Endotoxin can also cause paralysis of the ciliated epithelium of the human respiratory tract and promotes microbial colonization of the upper respiratory tract.

Resistance. Bacteria are not stable in the environment: they quickly die, being outside the human body. Hemophils are very sensitive to heating and normal disinfectants. *H. influenzae* has an ability to produce β-lactamase, which causes their high resistance to some β-lactam antibiotics.

Epidemiology. *H. influenzae* is pathogenic only to humans. The source of a hemophilic infection is a person, a sick person or a carrier. Non-encapsulated strains can normally colonize the mucous membranes of the upper respiratory tract of healthy children (about 60–90 %) and adults (about 35 %), strains of *H. influenzae* type b, having a capsule, are excreted from the nasopharynx in 2 % of asymptomatic carriers. The duration of the carrier is very variable, from several days to months, often characterized by resistance, despite the use of different antibiotics, and does not depend on the level of humoral immunity. The correlation between carrier frequency and crowding in children's collectives, from the period and the process of rearranging groups in children's collectives is noted.

Non-typed *H. influenzae* strains often colonize the lower respiratory tract in patients with chronic obstructive pulmonary disease and cystic fibrosis.

Colonization of mucous membranes with non-typed strains is a dynamic process in which "new" strains are periodically replaced by "old". Children who have non-typed strains of *H. Influenzae* in the first year of life have a higher

risk of developing acute otitis media. There is a direct relationship between colonization with such strains and the number of otitis media episodes.

The leading mechanism of transmission of a hemophilic infection is respiratory one, the rout of transmission is airborne (with cough, talking, sneezing). It is possible to be infected by contact with infected material, both from asymptomatic carriers and from patients. In children of the first year of life there is a contact mechanism. The most susceptible age is from several months to a year, although the highest level of lesion is from 6 months to one year. The emergence of infection is possible in older children (up to 5 years old), as well as in adults when the hemophilic infection develops on an unfavorable background (severe inborn pathology, oncopathology, immunodeficiencies of different genesis, non-European contingents, etc.).

Contagiousness of the pathogen is little therefore the disease is not epidemic in nature. The bacteria are localized on the mucous membrane of the nose, and most carriers do not have any clinical manifestations and can spread the infection by airborne droplets. Only a small number of persons who were in contact with the pathogen further developed clinical manifestations of the disease. The carriers are an important source of spread of the pathogen.

H. influenzae causes a large number of different infections, including those that endanger the lives of patients. In general, all infections caused by a *Haemophilus* can be divided into 2 types: invasive and non-invasive (*table 2*).

Table 2

Infectious diseases caused by *H. influenzae*

Infections	Age group	Strains
Invasive: meningitis epiglotitis pneumonia septic arthritis osteomyelitis cellulitis bacteremia	90 % – children under 4 years old; 10 % – older children and adults	90 % – type b. 10 % – non-typed types 1 % – types e and f
Sepsis	Newborns, pregnant women	> 90 % – non-typed types
Non-invasive: otitis media sinusitis conjunctivitis exacerbation of chronic bronchitis	Children and adults	> 90 % – non-typed types

In "invasive" forms of infection caused by Hib, the pathogen is detected in the fluids and tissues of the body, sterile under normal conditions (blood, cerebrospinal fluid, peritoneal and pleural fluid, etc.). Non-invasive forms include non-bacterial pneumonia (in the absence of a pathogen in the blood), acute otitis media, conjunctivitis, and others.

Non-invasive infections occur in the spread of microorganisms through the mucous membrane of the respiratory tract. Acute sinusitis, acute otitis media and exacerbation of chronic bronchitis are usually complications of viral infections that reduce local immunity and disturb the mucociliary clearance.

Most non-invasive infections are caused by non-typed strains, for which the presence of P2 protein in the outer membrane is a major virulence factor. In the pathogenesis of pneumonia, an important role is played by protease that destroys IgA1, and celleotoxin.

Invasive infections, especially meningitis and epiglotitis, are mainly caused by Hib strains and are haematogenic in origin. The b-type capsule, consisting of PRP, is the most important factor of virulence, since it protects the microorganism from phagocytosis, opsonization and complement-mediated lysis. The low incidence of invasive infections in children during the first two months of life is due to the presence of maternal antibodies to the PRP. With the growing population of people with antibodies to PRP, the frequency of invasive infections also decreases.

The most common forms of hemophilic infection are acute respiratory infections, including pneumonia, bronchitis, and meningitis. Other forms – purulent cellulitis (inflammation of fatty tissue) of the face, epiglottitis (inflammation of the epiglottis), arthritis (inflammation of the joints) and sepsis are less common.

Hemophilic infection is the cause of 35 % to 50 % of all purulent bacterial meningitis in children under the age of 5 years. The infection is poorly treated because the hemophilic rod is resistant to antibiotics.

The children with the age from 2 months to 6 years are most susceptible to hemophilic infection. However, meningitis and septicemia caused by *H. influenzae* type b are more common in children between the ages of 6 months and 2 years. Pneumonia, sinusitis and other respiratory tract infections may be among the elderly, patients with chronic pulmonary pathology, with reduced immunity, and also those who smoke.

Pathogenesis and clinical manifestation. *H. influenzae* passes into the body through the nasal mucous membrane. This is facilitated by repeated contact with a source of infection, which is especially characteristic for children of the first two years of life. As a rule, such penetration into the body leads to the persistence of the pathogen, which must multiply on the mucous membrane to a complete "infective doses". Then the process goes into the manifest form. Concomitant infection (often viral) which provides this process and immunodeficiency states of different genesis play particularly aggravating role.

There are also values of invasive properties of the pathogen, which is especially characteristic of "Hib". All this provides the causative agents the opportunity to overcome the protective mechanisms and entry of the microorganism into the blood. If less invasive serovars mainly cause localized manifestations (sinusitis, otitis, bronchitis, pneumonia, cellulitis, etc.), then "Hib", penetrating into the bloodstream, leads to the generalization of the infection with the development of sepsis, meningitis, arthritis. Overcoming the blood-brain barrier, the agent penetrates into the subarachnoid space and multiplies in the brain meninges and brain

vessels, causing an increase in intracranial pressure, circulation disorders, hypoxia, and, eventually, the formation of inflammatory foci in the central nervous system.

In the pathogenesis of infection, certain features of the formation of specific immunity are noted. In connection with the expressed ability of Hib to suppress phagocytosis, the inhibition of the formation of humoral immunity is due to the weak response of T-helper cells. The formation of local immunity, due to the small production of IgA, is also inhibited. Such a weak immune response to the infection persists, which is reflected in the low expressiveness of the booster effect in the rise of antibodies to re-infection of the pathogen. Quite a complex mechanism of activating complement in hemophilic infection occurs both through alternative pathway and through classic variant. In addition, it is known that capsular hemophilic pathogens are capable to produce protease-like enzymes that destroy antibodies, which further exacerbates the inferiority of humoral immunity.

Non-encapsulated variants of the hemophilic bacteria often remain in the entrance gate of the infection, without causing symptoms of the disease (asymptomatic carriers). In people with reduced immunity, they are able to penetrate the submucosal layer and with the help of endotoxin, cause local purulent-inflammatory diseases – secondary otitis (middle ear lesion), sinusitis (inflammation of the sinuses), laryngotracheitis, bronchitis, and pneumonia.

The most severe of the "invasive" forms of hemophilic infection and the most investigated form of infection is purulent bacterial meningitis. Detection and diagnosis of other forms of the hemophilic infection is extremely complicated. An ecological niche for Hib in macroorganism serves is the mucous membrane of the nasopharynx. It was established that the pathogen is able to spread from the entrance gate of the infection through the lympho- and hematogenous pathways to other organs and tissues. It is precisely because of the high ability to invade the extremely diverse localization and character of the complications that arise even in acute respiratory infections of hemophilic etiology, which are registered in 54,9 % of patients, can be explained.

According to world statistics, hemophilic infection is one of the first among the causes of infant mortality.

The incubation period during this infection is difficult to establish due to the peculiarities of infection and the transition from the phase of persistence to the clinical (invasive) form. Adhesion of the microbe to the mucous membrane, overcoming local immunity and phagocytosis lead to local forms of the disease (sinusitis, otitis, bronchitis, pneumonia, cellulitis). With the penetration into the blood variants of the disease are formed, which reflect the generalization of infection: sepsis, meningitis, arthritis. Among all forms in the order of their severity and frequency of registration in the first place purulent meningitis should be attributed, then pneumonia, septicemia, cellulitis, epiglottis, arthritis, pericarditis and the local lesions: sinusitis, otitis media.

Meningitis. According to WHO, meningitis is about 50 % (from 12 % to 73 %) among all cases of invasive forms of *H. influenzae* type b. The incidence of meningitis is sporadic, does not differ in seasonal or cyclic elevations and does

not have outbreaks. Prior to the introduction of mass vaccination, the incidence of meningitis of hemophilic etiology was, on average, 18 cases (from 2 to 60) per 100,000 children under the age of 5 years per year. Even with timely diagnosis and proper treatment, mortality from hemophilic meningitis is about 5 %. Children in the age group of 4 to 18 months of life, as well as elderly people over 65 years of age suffer from meningitis caused by Hib most often. Children under the age of 3 months and over 6 years old suffer from this disease rarely. In patients over the age of 10 years, the most frequent are the aggravating factors: anatomical (skull traumas, nasal liquorice, spina bifida, etc.) or immunological (deficiencies of complement components and other types of immunodeficiencies). Residual phenomena in the form of neurosensory hearing loss, speech disorders, mental retardation, developmental delay persist in 15–30 % of patients.

The disease is characterized by both acute and gradual development, and begins with moderate events of nasopharyngeal lesions, accompanied by headache, fever and the appearance of meningeal signs with frequent convulsive syndrome, and further by focal manifestations (III, VI, VII pairs of cranial nerves). Meningitis is often combined with pneumonia, arthritis, disorder of the gastrointestinal tract in the form of moderate diarrhea.

Intoxication is most pronounced against hyperthermia, often accompanied by vomiting. In part of cases when meningitis develops against the background of pneumonia, bronchitis, otitis, meningeal symptoms develop intensively, but intoxication grows slowly. Disease with late diagnosis and the presence of background pathology (congenital diseases, oncopathology, immunodeficiency) gives the highest rates of fatality – up to 10–15 %.

In cases where meningitis develops as a manifestation of common septicemia, an infectious and toxic shock often occurs with the presence of a hemorrhagic rash, which is extremely difficult to verify the pathology.

When lumbar puncture fluid is cloudy, sometimes with a greenish tinge at relatively low pressure and often drops indicating the prevalence of severe inflammation against the background of weak liquor production. Among the severe consequences of meningitis it should be noted: hearing loss, sometimes deafness, vision loss, the formation of a hypertensive syndrome with a result in hydrocephalus, tetra-and hemeneresis, possible decortication.

The peculiarity of hemophilic meningitis is a lethargic, torpid or wavy progression with a slow rehabilitation of liquor, culminating in disabling effects.

Less common is *hemophilic sepsis*. The most vulnerable contingent is the children of 4 to 18 months of life. The disease develops against the background of severe congenital pathology, with primary and secondary immunodeficiencies. The result is unfavorable due to background pathology.

Hemophilic pneumonia is the second most frequent clinical form. It may be focal or partial, with very often (2/3 of cases) combined with pleurisy, pericarditis, and sometimes with meningitis. The course of pneumonia is prolonged with the continued preservation of physical data.

In the WHO documents, it is noted that on average 1 case of meningitis caused by Hib in children under 5 years accounts for 5–10 cases of acute pneumonia of this etiology. In Europe, the incidence of hemophilic pneumonia before the start of vaccination was an average of 150–300 cases per 100,000 children up to 5 years of age per year, that is 10–20 times more than the incidence of meningitis: the share of this pathogen accounted for almost a third bacterial pneumonia. It can be noted the severity of the course and a significant number of complications in pneumonia of hemophilic etiology (in 58,3 % of patients) in the form of pericarditis, meningitis and pleural empyema, requiring pleuroectomy, with the largest number of patients was observed at the age of 2–8 years.

Epiglotitis. Acute inflammation of the epiglottis and larynx with obstruction of the respiratory tract is one of the most severe infections caused by Hib. Sometimes it can occur with septicemia. As a rule, the temperature rise is acute, marked by intoxication and rapidly progressing croup with asphyxiation.

Epiglottiitis often affects children 2–7 years of life. Mortality is 5–10 %, the cause of death is always not eliminated airway obstruction in time.

Arthritis and osteomyelitis. Prior to the vaccination, a Hib was the leading causative agent of purulent arthritis in children younger than 2 years old. In total, purulent arthritis accounts for about 8 % of invasive infections caused by this microorganism. Purulent arthritis often combines with meningitis.

Phlegmon. The most frequent localization of phlegmon due to Hib infection is head and neck. Most cases occur in the first 2 years of life.

Latent bacteremia. In most children with bacteremia caused by Hib, the foci of infection can not be detected and the only initial manifestation of the disease is fever. This situation is common in children younger than 2 years of age. Prior to the introduction of specific vaccination, the hemophilic rod of type b was the second most frequent cause of latent bacteremia, second only to *S. pneumoniae*. Bacteremia caused by Hib in 30–80 % of cases is complicated by secondary foci of infection, including meningitis.

Pericarditis. The classic manifestations of Hib pericarditis are intoxication, fever and respiratory failure in the child in the absence of changes in the lungs. Pericarditis often accompanies pneumonia and meningitis. Sometimes the pericardial lesions develop on the background of antibacterial therapy.

Newborn infections. In recent years cases of bacteremia and meningitis of hemophilic etiology become more frequent in newborns. Infection is manifested by early sepsis; more than 80 % of children develop it in the first week of life. It is not excluded intrauterine infection of the future baby, because this form of infectious pathology is often accompanied by prematurity, low birth weight, and complications in the mother (premature withdrawal of amniotic fluid, chorioamnionitis).

Other invasive infections. In rare cases, bacteremia results in the appearance of secondary foci of infection such as endophthalmitis, glossitis, uvulitis, thyroiditis, endocarditis, abscess, epididymitis, peritonitis, abscess of abdominal cavity, liver and biliary tract defeat, brain abscess.

Diagnosis and differential diagnosis. Hemophilic pathology is characterized by sporadicity, although some researchers consider it possible to be activated it in winter. The absence of sharply expressed epidemic elevations complicates the diagnosis. When verifying particularly difficult forms it is necessary to remember that in severe background pathology in children (rarely in adults), the hemophilic infection essentially manifests the impairment of the immune status of patients.

Prior to the etiological detection of the hemophilic infection, the most important for verifying the diagnosis is the fact of contact with the patient with the diagnosis of this disease, the development of the most frequent form meningitis against the background of otitis, sinusitis, bronchitis, progressive, often wave-like course of infection, prolonged preservation of meningeal signs with signs of lesion of cranial nerves, slow sanitation of the liquor. These features are nonspecific, however, the more often they are detected, the more they should be alarmed by the clinician, especially if, in addition to the above "small" forms, pneumonia and septicemia develop. Of great importance is the "tendency" of the hemophilic infection to the background pathology and immunodeficiencies of different genesis.

This infection is characterized by leukocytosis, neutrophilosis and a shift to the left in the formula in blood. However, there is a form of indifferent picture of the blood. Pleocytosis (elevated levels of cellular elements in the cerebrospinal fluid) ranges from 300 to 900 per 1 μL, it has neutrophil character, sometimes mixed composition or even with predominance of lymphocytes, due to early initiated antibacterial therapy prior to the study.

Immunity. During the first 3–6 months of life, children are protected from infection by maternal IgG, so at this age, the disease is very rare. Most children are ill from 6 months to 2 years old. It is known that up to 5–6 years in the serum of blood of many children, even non-immunized and non-infected, there are natural acquired protective antibodies against the capsular antigen of *H. influenzae* type b.

Microbiological diagnostics. Microbiological study is mandatory for confirmation of the etiology of the disease and determination of the sensitivity of the isolated pathogen to antimicrobial agents.

Due to the fact that the *Haemophilus* rod causes a wide range of infections, a different clinical material may be sent for microbiological examination. The greatest diagnostic value is the study of sterile in normal biologic liquids: blood, pleural, pericardial, synovial and cerebrospinal fluid.

The main condition for the proof of the etiological role of *H. influenzae* in the development of lower respiratory tract infection is prevention of the contamination of clinical material with the microflora of upper respiratory tract. To do this, it is desirable to use techniques that help to avoid contact with the microflora of upper respiratory tract (bronchoalveolar lavage, bronchoscopy with "protected" brushes).

The collection of material in patients with epiglotitis (epiglottis smear) has limited diagnostic significance and may pose a great threat to the life of the patient (the risk of developing laryngospasm). Therefore, this study should be conducted only with the availability of conditions for emergency assistance to maintain the patency of the respiratory tract.

It is inappropriate microbiological study of nasopharyngeal smears. Even positive cultures have dubious diagnostic value due to the high frequency of carriage of hemophilic rods by healthy children and adults.

Due to the fact that the hemophilic rod has a low viability in the environment, it is recommended to use transport media and immediately (no later than 2 hours) to deliver the material to the clinical laboratory.

The microscopic study is not informative, but it is used for purulent meningitis (swabs from cerebrospinal fluid, stained after Gram).

For express diagnosis and differentiation of the hemophilic rod from other pathogens of meningitis, serological tests are used to detect capsular antigen. At high concentrations of the causative agent in the material under investigation, the "swelling capsule test" may also be used.

The main method of diagnosis of diseases caused by Hib remains the culture – the seeding blood, cerebrospinal fluid and other material, isolated from the foci of infection (articular, pericardial fluid, pus). *The bacteriological method (culture)* is based on seeding on chocolate, blood agar or on agar with heart-brain extraction and incubation for 24–48 hours. Since the pathogen is very demanding for the conditions of cultivation, seeding is conducted immediately upon receipt of the material on adequate media that provide the growth of the pathogen. In biological fluids (serum, urine, synovial fluid, spinal fluid), capsid Hib polysaccharide can be detected. For this purpose, the following methods are most commonly used: IF, counter immunoelectrophoresis, latex agglutination and coagglutination reaction with staphylococcal protein A. Biochemical properties are investigated. Antigens of bacteria are detected by precipitation test in agar. *H. influenzae* is differentiated from other closely related gram-negative rods for their need for X- and V-factors of growth, lack of hemolysis in blood agar and other tests.

Since classical bacteriological method, even in the ideal application, can provide etiological identification of no more than 40–50 % of cases of bacterial meningitis, it is necessary to supplement them with non-culture methods, with which it is possible to provide identification to 60–80 % (with the application of the reaction of latex-agglutination) and up to 80–90 % (in the case of a polymerase chain reaction).

Prevention. The only reliable means of specific prophylaxis of diseases caused by Hib is active immunization. To create artificial acquired active immunity against *H. influenzae* type b, a sub-corpuscular vaccine with purified capsular antigen is used. However, given the low immunogenicity of this drug, it is prescribed to children older than 1.5 years. The use of the vaccine does not protect against the carrying of hemophilic rods.

At present, the following vaccines against hemophilic infection are used:

1. Act-HIB (conjugated monovalent vaccine containing capsular Hib polysaccharide, conjugated to the tetanus anatoxin protein).

2. Hyberix (a monovalent vaccine containing purified capsular polysaccharide isolated from *H. influenzae* strain type b and conjugated to tetanus toxoid).

3. Combined vaccines:

a) Pentaxim (diphtheria, tetanus, pertussis, polio and hemophilic vaccine containing diphtheria anatoxin, tetanus anatoxin, pertussis anatoxin, phylamentous *B. perrussis* hemagglutinin, poliomyelitis virus 1–2–3 type inactivated, capsular Hib polysaccharide).

b) Infanrix Hexa (diphtheria, tetanus, pertussis, poliomyelitis, hemophilic vaccine and hepatitis B vaccine containing diphtheria anatoxin, tetanus anatoxin, pertussis anatoxin, filamentous hemagglutinin, the surface antigen of the hepatitis B virus (HBsAg), poliomyelitis virus inactivated type 1, 2, 3, capsular Hib polysaccharide).

All vaccines are administered intramuscularly in the thigh or upper shoulder. Vaccines Act-HIB or Hyberix can be administered concurrently with vaccines of DTP, hepatitis B and poliomyelitis vaccine in different parts of the body. There are three schemes for the use of the Hib vaccine, depending on the age at which the vaccination schedule begins. Given the high level of age-related illness for type-B haemophilus infection in the early years of life, it is recommended to vaccinate all children, starting from 2–3 months of life.

Passive immunization with donor serum preparations that have high IgM concentrations can be given to children with a weak immune response to the vaccine and immunodeficiency.

Non-specific prophylaxis. Hospitalization of the patient is based on clinical indications. Patients with meningitis or suspicion of meningitis are immediately hospitalized in an infectious disease hospital or specialized divisions and boxes. Patients with pneumonia and other clinical forms of the disease of hemophilic etiology are hospitalized depending on the severity of the disease. They can be treated at home if there are no other children under the age of 5 in the family or apartment under regular medical supervision.

Healthy children and adults who have been in contact are not isolated or delimited. Patients with mild forms of disease are treated and only then allowed in the team. In children's institutions (children under 5 years of age), where cases of hemophilic infection were registered, contact healthy children are not transferred to other groups for 10 days, as well as those who are not present at the time of contact during this period, are not accepted into the team.

Treatment. Antibacterial therapy. Preparations of choice: cephalosporins of the third generation (ceftriaxone, cefotaxime), β-lactam antibiotics with β-lactamase inhibitors.

Ampicillin maintains its position in antibacterial therapy in a daily dose of 200–400 mg/kg/day in children and 6 g/day in adults. In the torpid course, clinical experience suggests the effectiveness of its combination with levomycetin at 100 ml/kg/day and 4 g/day adult intravenously in 6 hours.

There is evidence of the feasibility of using fluoroquinolones (cyprofloxacin), amoxiclav. In recent years, the use of co-trimoxazole (biseptol) has decreased due to the relatively high susceptibility to the pathogen (up to 40 %). In the treatment of "local" forms, erythromycin, asytromycin, and others are used.

Practical skills on the topic.

1. Detection of morphotincitorial and cultural properties of hemophilic bacteria.

Algorithms of laboratory work

Algorithm *"Scheme of microbiological research".*

First day

- Primary seeding of clinical material on chocolate agar, blood agar.
- Direct microscopy of a stained smear.
- If necessary (in CSF) detection of Hib-antigen in latex agglutination.

Second day

- Selection of suspicious colonies, their study and transfer to a Petri dish with chocolate agar. Seeding is carried out by the sector method.
- Microscopy of a stained smear.

Third day

- Detection of catalase activity.
- Detection of cytochromoxidase activity.
- Seeding to a Petri dish to determine the requirements for the X- and V-factors.
- Detection of β-galactosidase activity (preliminary analysis).
- Seedings to determine the sensitivity to ampicillin and β-lactamase (with nitrocephin).

Fourth day

- Analysis of needs in X and V factors.
- Analysis of β-galactosidase activity.
- Response to the presence of *H. influenzae*.
- Detection of ampicillin sensitivity and the presence of β-lactamase.
- Indol production test.
- Detection of the presence of urease.
- Detection of the presence of ornithine decarboxylase.

Fifth day

- Detection of the *H. influenzae* biotype.

Algorithm *"The course of microbiological research of a material for suspected hemophilic infection".*

H. influenzae is highly susceptible to cultivation on artificial nutrient media. A prerequisite for their growth is the presence of factors X and V in the medium.

Hemophilus rods can grow in small colonies on media based on horse or rabbit blood. The exception is blood agar containing native sheep or human red blood cells, due to the presence of enzymes that inactivate the V factor. Therefore, blood agar is not suitable for the isolation of *H. influenzae*.

To improve the isolation of *H. influenzae* from clinical material, it is recommended to use chocolate agar or selective agar for hemophilic bacteria.

Chocolate agar (*figure 5*) is prepared by adding blood to an enriched agar having a temperature of about 80 °C in order to destroy red blood cells and

release X and V factors. In this case, excessive and/or prolonged heating should be avoided to prevent inactivation of the thermolabile V factor. In order to improve the growth properties of the nutrient medium, it is recommended to add NAD to chocolate agar cooled to a temperature of 45–50 °C to obtain a final concentration of 15 μg/ml.

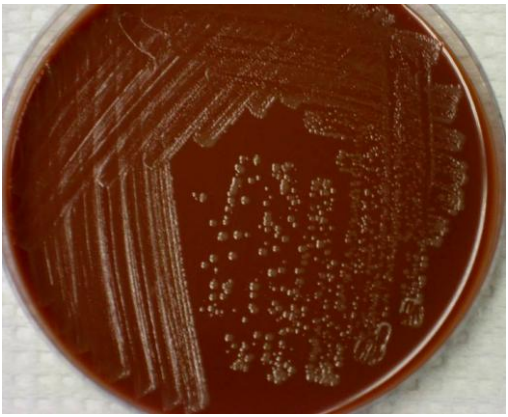


Fig. 5. Growth of *H. influenzae* on chocolate agar

Commercial Petry dishes with chocolate agar (bioMerieux, BBL) usually contain a mixture of hemin (X factor) and "cocktail" of growth factors added to the base – gonococcal agar.

Gonococcal agar contains peptones, corn starch, mono- and dibasic phosphate buffers, sodium chloride and agar. Growth factors include NAD (V factor), vitamins (B_1, B_{12}), cysteine, glutamine and glucose.

A number of companies offer ready-made supplements that include the listed growth factors: PolyVitex (bioMerieux), IsoVitalex (BBL) and Supplement B (Difco Laboratories).

The disadvantage of chocolate agar is the inability to observe hemolytic properties of hemophilic bacteria, which allow differentiation of *H. haemolyticus* and *H. parahaemolyticus* from *H. influenzae* and *H. parainfluenzae*.

Selective agar for the isolation of bacteria of the genus Haemophilus. Nutrient media containing bacitracin may be used to selectively isolate hemophilia from the clinical material of the upper respiratory tract. The high concentration of this antibiotic suppresses the growth of most other microorganisms that are representatives of the respiratory tract microflora (staphylococci, micrococci and streptococci), which allows the growth of the hemophilic rods from a highly contaminated clinical material. In addition to commercially available antibiotic-containing media, it is possible to prepare and use chocolate agar with bacitracin at a concentration of 300 μg/ml in clinical laboratories.

Instead of antibiotic-containing media, commercial disks with bacitracin (10 U) may be used when releasing a hemophilic rods from contaminated material (eg, sputum). Naturally resistant to bacitracin hemophilia will grow around the disc.

Selective medium for selection and differentiation of H. influenzae and H. parainfluenzae (Taylor D. C. et al.). It consists of hemin- and NAD-enriched heart-brain agar, sucrose (10 mg/ml), an indicator of phenol red (100 μg/ml) and bacitracin (300 μg/ml). On this medium, the colonies of *H. parainfluenzae* are yellow in color due to their ability to produce sucrose acid, and the colonies of *H. influenzae* are colorless because they do not ferment sucrose. The disadvantage of this medium is the inability to observe the hemolytic properties of hemophilia.

Conditions for the incubation of H. influenzae. The optimal conditions for *H. influenzae* incubation are a moist atmosphere with high content of CO_2 (5–10 %) and a temperature of 35–37 °C. Similar conditions can be created in a CO_2-thermostat or during incubation of Petry dishes in a desiccator with an inflamed candle. As a result of the burning of the candle, the concentration of oxygen decreases and the level of CO_2 increases, reaching 3 %.

Isolation of H. influenzae from clinical material.

Staining of clinical material after Gram and with methylene blue. A preliminary diagnosis of the infection caused by *H. influenzae* may be established on the basis of a study of a smear of clinical material stained after Gram and/or with methylene blue.

When staining after Gram bacteria of the genus *Haemophilus* look like small, pale painted gram-negative rods, sometimes forming thin filaments. Small sizes, cellular polymorphism and insufficient staining with safranin can greatly complicate the detection of the hemophilic rods. Therefore, some authors suggest staining with methylene blue along with Gram stain. In this case, microorganisms have a blue color on a gray-and-blue background.

Negative results of microscopy do not exclude the possibility of a hemophilic infection, as in the clinical material there may be insufficient amount of microorganisms (resolution of light microscopy is 10^4–10^5 bacterial cells in 1 ml). Therefore, cultural bacteriological study is mandatory.

Detection of capsular Hib antigen. For the rapid diagnosis of infections caused by *H. influenzae* type b, immunological methods for detecting capsular antigen in the cerebrospinal fluid, blood, pleural fluid and urine are used: latex agglutination (LA) (*figure 6*), coagulation with staphylococcal protein A (CoA), counter immunoelectrophoresis (ZIEF) and enzymelinked immunoassay (ELISA).

Latex agglutination and coagglutination with samples of CSF are the most widely used. Antibodies (IgG) against the capsular Hib antigen are applied to latex particles or staphylococcal cells as "carriers." When the antigen contained in the clinical material interacts with specific antibodies in less than 10 minutes visible flakes are formed.

Tests can be false positive if the baby has recently been vaccinated with an Hib vaccine (within 21 days of immunization). In addition, nonspecific false-positive results may be observed in some diseases not related to the hemophilic rods.

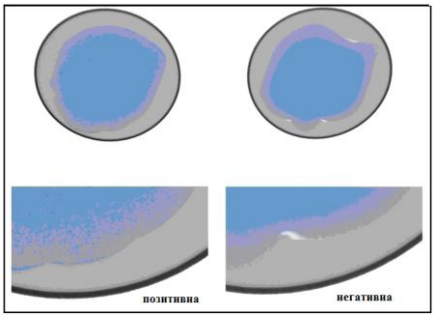
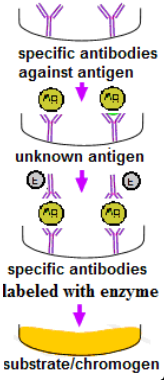


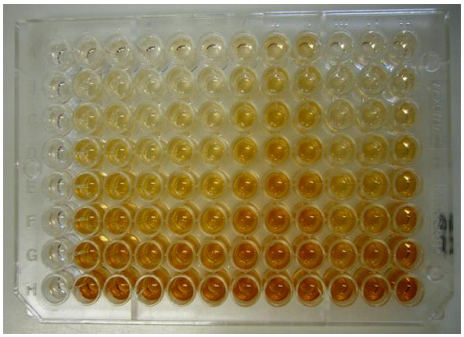
Fig. 6. Latex agglutination

However, none of these studies can replace the cultural research, which remains the "gold standard" of microbiological diagnosis.

Solid-phase ELISA is used to detect the capsular antigen. The reaction is carried out on plastic plates with wells in which there are specific antibodies. The material being studied is introduced into the well. To determine the antigen, a specific serum is added with antibodies labeled with the enzyme and then the substrate/chromogen for the enzyme. Each time, when adding the component unbound components are removed from the wells by washing. When a reaction is positive the color of the chromogen changes (*figure 7*).



A



B

Fig. 7. ELISA: A – scheme of reaction, B – results

Study of the cerebrospinal fluid. When a sample of CSF is received it is necessary to begin the research immediately or store the material in the thermostat at a temperature of 35–37 °C or, at least, at room temperature not more than 30 minutes. Samples of CSF should not be stored in the refrigerator!

Prolonged storage of samples can lead to bacterial death and false negative results (with the exception of the antigen test!).

To increase the concentration of microorganisms it is recommended to centrifuge the cerebrospinal fluid (at least 1 ml of the sample of clinical

material for 5–15 minutes at 1 500–3 000 r/min). The supernatant fluid should be aseptically transferred to a sterile test tube, after which carefully mix the resulting precipitate by repeated pipetting.

The study of the cerebrospinal fluid includes the following diagnostic tests:

1) staining after Gram and/or with methylene blue. To do this, a drop of sediment is applied to the surface of a sterile glass, after drying the second drop is added, which allows increase the amount of bacterial cells in the smear. It is not necessary to distribute sediment over a large surface, as this reduces the probability of detecting microorganisms in the material in a small amount;

2) detection of Hib antigen in supernatant fluid. Some authors believe that the detection of antigens is a more sensitive test than a culture study in patients who received antibiotics prior to the capture of CSF;

3) bacteriological study of sediment. Several drops of a cerebrospinal fluid are placed on the surface of Petry dish with chocolate and blood agar, as well as in a two-phase or liquid medium. The Petry dishes are incubated at a temperature of 35–37 °C in an atmosphere of 5–10 % CO_2.

Other biological fluids (synovial, pericardial, pleural) are stained after Gram and are examined by culture method.

Blood tests. Blood samples placed in vials with a biphasic or liquid medium are incubated for 18–24 hours at a temperature of 35–37 °C. Due to the weak growth of the hemophilic rods in the liquid medium, the expressed cloudiness of the medium may be absent. Therefore, it is recommended to conduct subcultivation or staining of the smear 6–12 hours after the start of incubation without visible growth. Preference is given to subculture, since the resolution of light microscopy (10^4–10^5 CFU/ml) slightly exceeds the density at which visible growth (10^6–10^7 CFU/ml).

For subcultivation under aseptic conditions a few drops of pre-carefully mixed medium are collected and distributed over the surface of chocolate agar. The Petry dishes are incubated at a temperature of 35–37 °C and 5–10 % CO_2 for 24–48 hours.

After intermediate subcultivation, the incubation of the vials should be continued. A smear should be prepared after 24 hours incubation, regardless of the absence or presence of turbidity in the vial.

If there is visible clouding of the mediumt, even with a negative smear or if there is a positive smear in the absence of turbidity, then you should subcultivate the material on chocolate agar and blood agar.

Research of materials containing microbial associations (sputum, material from the middle ear and sinuses of the nose, etc.). Before seeding the received clinical material it is recommended to conduct microscopic examination of stained smears. This allows you to get preliminary data on possible pathogens.

It is recommended to use media containing bacitracin (300 μg/ml) or bacitraz discs (10 OD) or saponinabacitracin disks to improve the selection of the hemophilic rods.

Identification of Haemophilus influenzae.

The colonies of *H. influenzae* on chocolate agar may have the following forms after incubation for 24 hours:

– Capsule strains form mucous, round, grayish, and radiating (give rainbow color) in the light colonies of up to 2 mm in diameter. Strains with a less pronounced capsule form translucent, round, smooth, non-radiaiting colonies;

– non-capsulated strains form small, opaque, non-radiating colonies with rough edges.

The presence of a specific "mouse" odor is characteristic of pure culture of hemophilic rods.

H. influenzae has cytochrome oxidase and catalase activity and needs X and V factors, which is one of the main qualities that distinguishe them from other representatives of the genus of hemophilia.

The requirement for *H. influenzae* in the indicated factors is determined by means of stripes or disks with X and V factors. In their absence, you can use a test with saponin or determine the ability to satellite growth (the method of "feeding").

Saponin test is based on the ability of saponin to lyse red blood cells. Saponin leads to the release of X and V factors that are present in erythrocytes, which provides growth of the hemophilic rods.

Disc with saponin is placed on the surface of 5 % blood agar, inoculated with the test culture (suspension in physiological saline sodium chloride). Test results are taken into account in 24–48 hours of incubation at a temperature of 35–37 °C and 5–10 % CO_2.

Growth of colonies around discs with saponin and its absence outside the hemolysis area is a differential feature of the genus *Haemophilus*.

Satellite growth test (method "feeders"). The principle of the method of "feeders" is similar to the above method discs with saponin. A surface of 5 % blood agar is inoculated with a suspension of the test culture in a physiological solution of sodium chloride, after which two parallel lines of the hemolytic strain of *S. aureus* are applied (the distance between the lines is 5–6 mm). After incubation at a temperature of 35–37 °C in an atmosphere with an elevated (5-10 %) content of CO_2 during 18-24 hours the growth of colonies (in the form of a roller) in the zone of hemolysis caused by *S. aureus* indicates the identity of the investigated microorganism to *Haemophilus* spp. (*figure 8*).

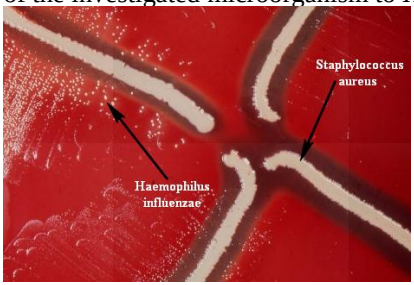


Fig. 8. "Satellite phenomenon

Detection of b-galactosidase. An important diagnostic test for identification of *H. influenzae* is the presence of b-galactosidase activity. *H. influenzae* does not possess this enzyme. Thus, on the basis of this test, it can be differentiated from other types of hemophilia that require X and V factors.

The dependence of hemophilia on growth factors, as well as other properties are given in *table 3.*

Table 3

Differential-diagnostic properties of the genus *Haemophilus*

Species	Need for		catalase	oxydase	*ONPG*	Hemolysis*	Acid production from			
	X and V factors	CO_2					glu	suc	lac	man
H.influenzae	*X,V*	+	+	+	-	-	+	-	-	-
H.influenzae біовар aegypticus	*X,V*	-	+	+	-	-	+	-	-	-
H.haemolyticus	*X,V*	-	+	+	+	+	+	-	-	-
H.parainfluenzae	*V*	-	v	+	+	-	+	+	-	+
H.parahaemolyticus	*V*	-	+	+	v	+	+	+	-	-
H.aphrophilus	h	+	-	-	+	-	+	+	+	+
H.paraphrophilus	*V*	+	-	+	+	-	+	+	+	+
H.segnis	*V*	-	v	-	в	-	wr	wr	-	-
H.ducreyi	X	-	-	-	-	-	-	-	-	-

Note**:** glu – glucose; suc – sucrose; lac – lactose; man – mannose; ONPG – *b-galactosidase* test; h – hemin is required for primary isolation; wr – weak reaction; w – variable sign. * – with horse's blood.

Biotyping H. influenzae. M. Kilian, who studied the taxonomy of the genus *Haemophilus*, proposed the use of a series of biochemical tests for the biotyping H. influenzae. Based on tests for indole, urease and ornithine decarboxylase activity 8 biotypes of *H. influenzae* are isolated (*table 4*).

Table 4

Biotyping *H. influenzae*

Biotype	Indol	Urease	ODC
H. influenzae I	+	+	+
H. influenzae II	+	+	–
H. influenzae III	–	+	–
H. influenzae IV	–	+	+
H. influenzae V	+	–	+
H. influenzae VI	–	–	+
H. influenzae VII	+	–	–
H. influenzae VIII	–	–	–

Note**:** ODC – Ornithine decarboxylase.

The biotyping the hemophilic rods has only an epidemiological significance. Different biotypes are associated with certain types of infections. For example, according to a number of studies, the vast majority of meningitis are caused by

biotype I (93.1 %), while the biotypes II and IV have respectively 4.6 and 2.3 %. Most of the Hib strains belong to the biotypes I.

A number of studies also noted the association of biotypes II and III with conjunctivitis. Biotype IV causes infections more often in obstetric and gynecological practice, perinatal and neonatal infectious diseases.

Urease test. The ex tempore mixture of reagents A and B are prepared in 0.1 ml of narrow tubes and several drops of a dense suspension of the investigated culture of *H. influenzae* in a nutritional broth are added. The test tubes are placed in the thermostat at a temperature of 35–37 C. Pre-registration is possible after 30 minutes.

In the case of a positive reaction, the medium acquires a crimson-red color, while negative – the color does not change (*figure 9*). The account of a negative reaction is possible only after 24 hours of incubation.

Indole production test. To determine the ability of bacteria to form an indole, it is necessary to use media containing tryptophan. Culture is incubated at 35–37 °C for 18–24 hours. With a positive result, a pink color appears (*figure 10*).

Fig. 9. Urease test

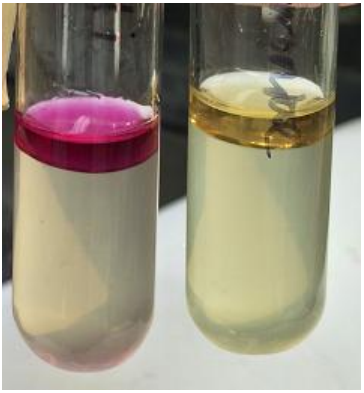


Fig. 10. Indole production test

Ornithine dicarboxylase test. When determining ornithine decarboxylase, disks with ornithine (SIB, bioMerieux, BBL) are used. The disk is placed in a 0.3–0.5 ml sample of physiological saline and several drops of a daily broth culture of *H. influenzae* are added, then poured into sterile vaseline oil and incubated for 18–24 hours at a temperature of 35–37 °C.

With a positive result, a blue or intense green color appears (*figure 11*).

Algorithm *"Detection of H. influenzae sensitivity to antibiotics".*

The choice of antibiotics to be used in testing *H. influenzae* depends on the spectrum of activity, the frequency of acquired antibiotic resistance in the region, the localization and degree of severity of the course of the infectious disease.

Fig. 11. Ornithine dicarboxylase test

The following antibiotics has potential activity against *H. influenzae*: aminopenicillins (ampicillin, amoxicillin), ureiodopenicillins (piperacillin), inhibitoryprotected penicillins (amoxicillin / clavulanate, ampicillin / sulbactam, piperacillin / tazobactam, ticarcinin / clavulanate), cephalosporin II (cefuroxime, cefaclor), III (ceftriaxone, cefotaxime, cefoperazone) and IV (cefepim, cefpirom) generations, carbapenems, macrolides (azithromycin, clarithromycin), tetracyclines (tetracycline, doxycycline), fluoroquinolones (ciprofloxacin, ofloxacin, efloxacin, levofloxacin) rifampicin, chloramphenicol, co-trimoxazole.

The most significant problem is the resistance of the hemophilic rods to aminopenicillin due to b-lactamase production. Such microorganisms are usually sensitive to inhibitory-protected penicillins and cephalosporins, and for the rapid detection of b-lactamase products it is enough to conduct a nitrocefin test.

In recent years, strains of *H. influenzae* have been described, whose resistance to ampicillin is associated with a change in the target of the action of b-lactam antibiotics (penicillin-binding proteins) or decreased permeability of the outer cell wall. These strains have been called b-lactamase-negative ampicillin-resistant (BLNAR) and are considered to be resistant to inhibitory-protected penicillins and cephalosporins such as cefaclor, cefuroxime, cefixime, ceftibutene.

BLNAR strains of *H. influenzae* are found very rarely (on average 0.2 % of cases) and do not have a significant clinical significance.

In accordance with international recommendations, in the routine laboratory practice, to determine the ampicillin resistance of the hemophilic rods, it is sufficient to determine the sensitivity to ampicillin by the disc diffusion method and the test for the production of b-lactamases with nitrocephin.

These two tests allow the strains to be divided into ampicillin-susceptible, beta-lactamase-producing ampicillin-resistant (sensitive to inhibitory-protected penicillins and cephalosporins II–IV generations) and BLNARs, which should be considered as resistant to inhibitory-resistant penicillins and some cephalosporins. Moreover, testing with a disk containing inhibitory-protected penicillins, such as amoxicillin/clavulanate, does not allow distinguish BLNAR from ampicillin-susceptible strains of *H. influenzae*.

Detection of the sensitivity of *Haemophilus* rods to macrolides (azithromycin, clarithromycin) is an unresolved problem all over the world, due to the wide range of values and the monomodal distribution of strains in the population. Therefore, the subdivision of strains in the sensitivity category according to in vitro studies is always subjective and is significantly exposed to minimal differences in the methodology and testing conditions.

Currently, most researchers are guided by the standards of the National Committee on Clinical Laboratory Standards (NCCLS) in determining the sensitivity of microorganisms to antibiotics. As a result, the main studies of the sensitivity of the hemophilic rods to antibiotics are carried out in accordance with these recommendations.

According to NCCLS guidelines, to determine the sensitivity of the hemophilic rods the *Haemophilus* Test Medium (HTM), which contains all the factors necessary for bacteria growth, to be used. HTM is the Muller-Hinton agar with the addition of yeast extract and the factors X and V.

Detection of sensitivity of bacteria to antibiotics by disc diffusion method. The procedure for detecting the sensitivity of the hemophilic rods to antibiotics is similar to the testing of "non-urgent" microorganisms and has only a few peculiarities.

1. To prepare the inoculum, the colonies of the daily culture of *Haemophilus* spp. grown on chocolate agar, in a nutrition broth (for example, a Muller-Hinton broth) or a sterile saline solution are suspended.

The density of the inoculum should correspond to the standard of turbidity 0,5 for McFarland ($1{,}5 \times 10^8$ CFU/ml).

2. The inoculation of Petry dishes with HTM agar should be carried out for 15 minutes after preparing inoculum. Sterile cotton swabs are used for inoculation. The tampon is immersed in a tube with suspension, squeezes an excess of inoculum onto the wall of the test tube, and applied to the surface of the agar by strokes in 3 directions at an angle of 60 degrees, without adding an additional amount of suspension.

3. The Petry dishes are incubated at 35 °C in an atmosphere with high content of CO_2 (5–10 %) in a desiccator or CO_2-incubator for 16–18 hours, after which the received zones of growth inhibition are measured (*fig. 12*).

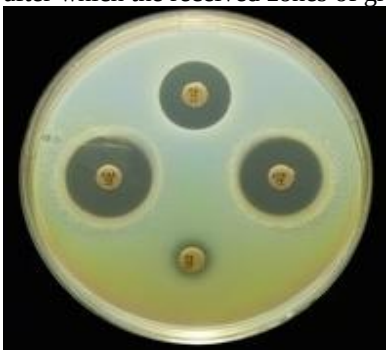


Fig. 12. Detection of sensitivity of bacteria to antibiotics by disc diffusion test

The growth inhibition zone is measured using a ruler or caliper. And it is necessary to measure its diameter (not radius!). The end point is the distance in the zone where there is no growth of microorganisms. The results are interpreted after the size of the growth inhibition zone around the disc.

4. To interpret the results of detection of the sensitivity of the hemophilic rods to antibiotics, specific criteria are used that differ from the criteria for interpreting the results of the detection of the sensitivity of "non-urgent" microorganisms (*table 5*).

Table 5

Criteria for interpreting the sensitivity of the hemophilic rods by disc-diffusion method on HTM agar (NCCLS, 2016)

Antibiotics	Diameter of the zone of inhibiuion of growth, mm		
	resistant	moderately resistant	sensitive
Ampicillin (10 μg)	< 18	19-21	> 22
Amoxicillin / clavulanate (20/10 μg)	< 19	–	> 20
Meropenem (10 μg)	–*	–*	> 20
Imipenem (10 μg)	–*	–*	> 16
Trimetoprim/sulfamethoxazole (1.25/23.75 μg)	< 10	11–15	> 16
Cefotaxime (30 μg)	–	–	> 26
Ceftriaxone (30 μg)	–*	–*	> 26
Ceftazidime (30 μg)	–*	–*	> 26
Chloramphenicol (30 μg)	< 25	26–28	> 29
Azithromycin (15 μg)	–*	–*	> 12
Clarithromycin (15 μg)	< 10	11–12	> 13
Tetracycline (30 μg)	< 25	26–28	> 29
Ciprofloxacin (5 μg)	–*	–*	> 21

* Resistant strains are not isolated.

Quality control of the detection of sensitivity of microorganisms to antibiotics is carried out by testing standard strains. As a control, strains of the American Collection of Typical Cultures (ATCC) are used that are distinguished by genetic stability and well-studied phenotypic characteristics (*table 6*).

For quality control in detection of the sensitivity of hemophilia on HTM agar strains of *H. influenzae* ATCC 49247 and *E. coli* ATCC 35218 (in the testing of inhibitory protected penicillins) are used. The method and accounting corresponds to the method of work with the tested strain. The results are evaluated according to the criteria set out in the *table 6*.

Each series of Petry dishes with nutrient media should be checked for their fitness for growth. For this, a control strain of *H. influenzae* ATCC 10211 is used, from which a daily culture of microbial suspension corresponding to a turbidity of 0.5 for McFarland is prepared. Series of sequential dilutions of 1:10 are prepared from the obtained microbial suspension. Then, on Petry dishes with the medium of HTM, 0.1 ml of suspension of -5, -6, -7 dilutions are seeded. With good nutritional properties agar should be marked by the growth of microorganisms from -6 and -7 dilutions.

Table 6

Allowable values ranges of the diameters of the growth inhibition zones for control strains *H. influenzae* ATCC 49247 and *E. coli* ATCC 35218 (NCCLS, 2016)

Antibiotic	*H. influenzae* ATCC 49247	*E. coli* ATCC 35218
Ampicillin (10 μg)	13–21	–
Amoxicillin / clavulanate (20/10 μg)	15–23	18–22
Trimetoprim / sulfamethoxazole (1.25/23.75 μg)	24–32	–
Meropenem (10 μg)	20–28	–
Imipenem (10 μg)	21–29	–
Cefotaxime (30 μg)	31–39	–
Ceftriaxone (30 μg)	31–39	–
Chloramphenicol (30 μg)	31–40	–
Azithromycin (15 μg)	13–21	–
Clarithromycin (15 μg)	11–17	–
Tetracycline (30 μg)	14–22	–
Ciprofloxacin (5 μg)	34–42	–

Detection of the minimum inhibitory concentration. Method of serial dilutions in broth.

Macromethod. The method of serial dilutions in the broth (macromethod) allows determine the minimal inhibitory concentration (MIC) without significant material costs. Testing a small number of strains in routine practice should be performed by a macromethod.

Materials:

1) sterile HTM broth; you can use a ready-made commercial HTM broth or cooked in a laboratory based on a sterile Muller-Hinton broth with a stabilized cationic composition (for Ca ++, Mg ++ ions) with the addition of the same components as in HTM agar;

2) substances of known antibiotics;

3) sterile tubes measuring 13 × 100 mm or 14 × 140 mm;

4) sterile pipettes;

5) dosing pipettes with sterile tips;

6) standard of turbidity 0,5 for McFarland.

Procedure. Testing is carried out in a volume of 1 ml of each dilution of the antibiotic with a final concentration of *H. influenzae* approximately 5×10^5 CFU/ml.

Preparation of serial dilutions of antibiotics. The serial dilutions of the antibiotic are prepared from the "starting" solution on the HTM broth (*table 7*), which then is diluted in 2 times in each test tube. Then *H. influenzae* broth culture is added. The number of test tubes is determined by the required range of antibiotic dilutions and increased by two for "negative control" and "growth control".

Table 7

Test antibiotics and dilution range

Antibiotic	Dilution range, mg /l	Starting concentration, mg/l
Ampicillin	0,016–16	32
Amoxicillin/Clavulanate	0,032/0,016–32/16	64/32
Trimetoprim/sulfamethoxazole	0,032/0,608–32/608	64/1216
Meropenem	0,004–4	8
Imipenem	0,016–16	32
Ceftriaxone or cefotaxime, or ceftazidime	0,008–8	16
Chloramphenicol	0,064–64	128
Azithromycin	0,032–32	64
Clarithromycin	0,064–64	128
Tetracycline	0,064–64	128
Ciprofloxacin	0,004–4	8

The base antibiotic solution is prepared from the chemically pure substance of the preparation by dissolving it in the calculated amount of solvent to obtain a concentration that exceeds the initial concentration of the antibiotic 100 times (for example, 3 200 mg/l for ampicillin).

• It is inappropriate to use medicines instead of substances!

The starting antibiotic solution is prepared by adding 0.1 ml of the antibiotic base solution to 9.9 ml of HTM broth, 0.5 ml of which is transferred to a sterile tube containing 0.5 ml of broth. It is mixed thoroughly and 0.5 ml of antibiotic solution in a broth is transferred into a second tube containing 0.5 ml of broth.

This procedure is repeated until all the necessary dilutions are prepared. From the last test tube, 0.5 ml of broth is removed. Thus, a number of tubes with solutions of antibiotics in the amount of 0.5 ml are obtained, the concentration of which differs in adjacent tubes 2 times. Simultaneously, the series of serial dilutions of the antibiotic are prepared for the testing of control strains of the hemophilic rods and *E. coli*.

Preparation of inoculum. To prepare the inoculum, a daily culture of a hemophilic rod on chocolate agar is used. Colonies of *H. influenzae* are suspended in a sterile 0.9 % solution of sodium chloride to a turbidity equivalent to 0.5 on the McFarland standard.

Further dilution of this suspension 100 times is prepared on the HTM broth, after which the concentration of the hemophilic rod will be approximately 10^6 CFU/ml. 0.5 ml of the inoculum are placed in each test tube containing 0.5 ml of the appropriate antibiotic dilution, and in 2 tubes containing 0.5 ml of HTM without an antibiotic ("negative control" and "growth control").

The final concentration of *H. influenzae* in each test tube will reach the required level of about 5×10^5 CFU/ml. The inoculum must be introduced into the test tubes with antibiotic dilutions no later than 30 minutes since its preparation.

Incubation. Test tubes with tested strains, except the "negative control" tube, are incubated in the usual atmosphere at 35 °C for 20–24 hours. The

"negative control" test tube is placed in the refrigerator (temperature 4 °C), where it is stored to obtaining the results.

Accounting for results. To determine the presence of growth of the hemophilic rod, the test tubes with growth of bacteria are examined in passing light. The growth of culture in the presence of the antibiotic is compared to the reference test tube ("negative control") containing the initial inoculum and stored in the refrigerator. The MIC is determined by the lowest concentration of antibiotic that suppresses the apparent growth of *H. influenzae* (*figure 13*).

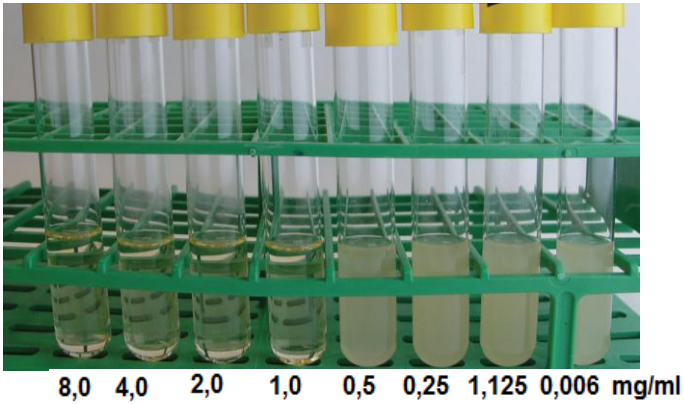


Fig. 13. Tube dilution test

Micromethod. If it is necessary to determine the MIC in 8 or more strains it is expedient to use a micromethod. It allows you to simultaneously test a large number of strains to several antibiotics.

Testing is carried out in a volume of 0.1 ml (0.05 ml of HTM broth and 0.05 ml of inoculum), which allows to significantly reduce the amount of materials. The method has no differences from the macromethod, except for the volume, but requires additional laboratory equipment with multichannel pipettes, microtiter plates (with a round or conical bottom) with sterile lids, a special device with indirect illumination to account for the results (*figure 14*).

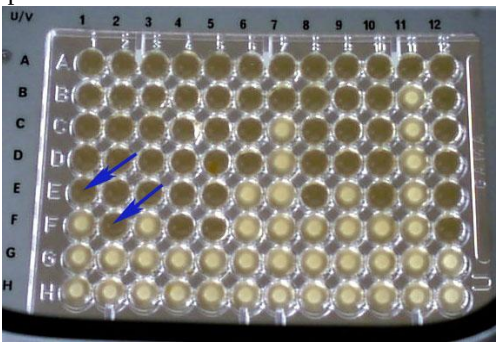


Fig. 14. Micromethod for determining the sensitivity of *H. influenzae* to antibiotics

Growth of the microflora in the presence of the antibiotic is compared with the growth of culture in the medium without antibiotic.

Each testing of strains is accompanied by an internal control using control strains of *H. influenzae* ATCC 49247 and *E. coli* ATCC 35218.

Method of E-tests. The E-test is a 5 × 50 mm plastic strip with a gradient of antibiotic concentration (0.002–32, 0.016–256 or 0.063–1024 mg/l depending on the preparation). The method is based on the diffusion of antibiotics into agar, which creates a concentration gradient of the antibiotic in the agar. The growth inhibition zone has the form of an ellipse, the size of which is increased from the smaller concentration of antibiotic in the strip to the greater.

Materials:

1) HTM agar, prepared in the laboratory, or commercial ready-made agar;
2) strips of E-tests with antibiotics;
3) Muller-Hinton sterile broth;
4) standard sterile tampons;
5) standard of turbidity 0,5 for McFarland.

The procedure for the preparation of HTM agar and the application of culture is similar to the one under the disc diffusion method. The surface of the microbial lawn must be dry, for which the E-tests are applied no earlier than after 15 minutes from the moment the inoculum is applied. The strips of E-tests are placed on the agar surface with a plastic surface with marks of the concentration gradient upwards. No more than 2 strips are applied to a 90 mm diameter Petry dish. The cultures are incubated for 20–24 hours at a temperature of 35 °C in an atmosphere with high content of CO_2.

Accounting for results. The results should be taken into account only if there is a solid dense lawn of culture. If the growth is weak, it is necessary to continue the incubation. In the case of "sparse" lawn growth, testing should be repeated by checking the quality of the agar and inoculum. The results are taken into account in reflected light and/or under the magnifying glass to look good at the edge of the growth. The zone of complete growth inhibition is taken into account. The size of the MIC is determined by the concentration value at which the ellipse intersects with the scale of the strip (*figure 15*).

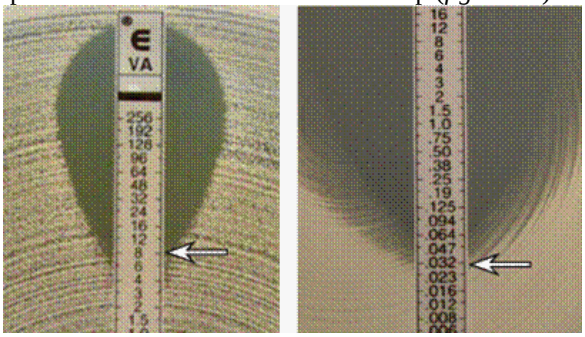


Fig. 15. E-test

Interpretation. The E-test method defines the MIC, based on the continuous concentration gradient, including the values between two-time dilutions. To determine the sensitivity category, the obtained values should be rounded to the nearest values of double dilutions. Example:

1) for sensitive strains the value of MIC of ampicillin is 10 mg/l; the method of E-tests obtained MIC 0.064 mg/ml, the result is interpreted as sensitive;

2) MIC of moderately resistant strains is 2 mg/l; by the method of E-tests, MIC is defined as 1.5 mg/l, the result is interpreted as moderately resistant.

Criteria for interpreting the results of the determination of sensitivity to some antibiotics by dilution and E-tests are given in the *table 8.*

Table 8

Criteria for the interpretation of *H. influenzae* sensitivity by dilution and E-tests (NCCLS, 2016)

Antibiotic	Diameter of the zone of suppression of growth, mm		
	resistant	moderately resistant	sensitive
Ampicillin	> 4	2	< 1
Amoxicillin/Clavulanate	> 4/2	–	< 2/1
Trimetoprim/sulfamethoxazole	> 4	1	< 0,5
Ceftriaxone or cefotaxime, or ceftazidime	–*	–*	< 2
Meropenem	–*	–*	< 0,5
Imipenem	–*	–*	< 4
Chloramphenicol	> 8	4	< 2
Azithromycin	–	–*	< 4
Clarithromycin	> 32	16	< 8
Tetracycline	> 8	4	< 2
Ciprofloxacin	–*	–*	< 1

- Resistant strains are not highlighted.

Quality control. Strains of *H. influenzae* ATCC 49247, *H. influenzae* ATCC 49766 (for carbapenem test) and *E. coli* ATCC 35218 (for testing inhibitor-protected penicillins) are used. The method and accounting corresponds to the method of work with the tested strain. The results are interpreted according to the following criteria (*table 9*).

Algorithm *"Test for the ability to produce catalase"*.

Catalase is an enzyme that converts hydrogen peroxide (H_2O_2) into water and oxygen. Chemically, catalase is a hemoprotein similar in structure to hemoglobin, except that it contains trivalent iron, not divalent.

Table 9

Allowable ranges of MIC values for control strains (NCCLS, 2016)

Antibiotic	*H.influenzae* ATCC 49247	*H.influenzae* ATCC 49766	*E. coli* ATCC 35218
Ampicillin	2–8	–	–
Amoxicillin / Clavulanate	2/1–16/8	–	4/2–16/8
Trimetoprim / sulfamethoxazole	0,03–0,25	–	–
Meropenem	–	0,03–0,12	–
Imipenem	–	0,25–1	–
Chloramphenicol	0,25–1	–	–
Cefotaxime	0,12–0,5	–	–
Ceftriaxone	0,06–0,25	–	–
Azithromycin	1–4	–	–
Clarithromycin	4–16	–	–
Tetracycline	4–32	–	–
Ciprofloxacin	0,004–0,03	–	–

Reagents and materials:

1. A 3 % solution of hydrogen peroxide (stored in a vial of dark glass in a refrigerator).

2. Daily culture of the investigated microorganism.

Quality control: positive control – agar cultures of *S. aureus* (ATCC 25923), negative control – *S. pyogenes* (ATCC 12344).

Method. Daily agar culture of the microorganism is suspended in a drop of 3% solution of hydrogen peroxide on the glass. Bubbles of varying intensity appear immediately or after 3–5 c (*figure 16*).

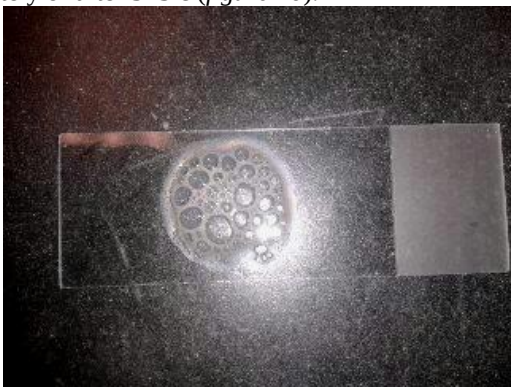


Fig. 16. Catalase test

It should not be used to test a culture older than 24 hours, since the enzyme is present only in living microorganisms, and false negative results can be obtained. Some microorganisms have other enzymes that destroy hydrogen peroxide. Therefore, small bubbles in small quantities that appear after 20–30 seconds are not considered as a positive test.

In addition, catalase is present in erythrocytes, therefore, a false-positive result may be observed in the capture of colonies from the blood-containing medium.

Algorithm *"Identification of H. influenzae using X and V factors"*.

The need for hemophilia in the X and / or V factors can be determined by using stripes or disks impregnated with X, V and XV factors. When placed on the surface of the medium, the discs with the appropriate factors, they are easily diffused into the nutrient medium around the discs. After incubation, the need is evaluated depending on the nature of the growth of the testing microorganism around the disks.

Materials:

1) strips or disks of filter paper, saturated with the factors X and V (BBL, Oxoid);

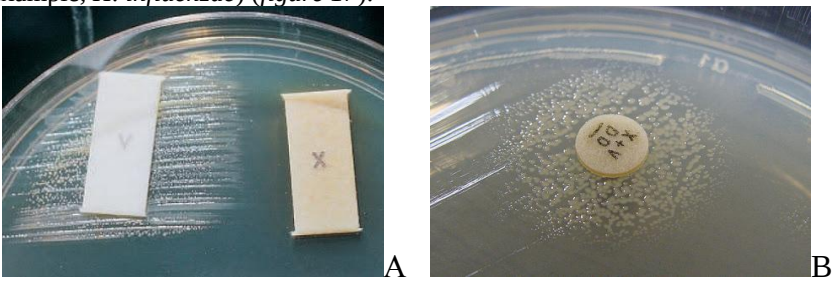
2) nutrient medium that does not contain the X and V factors (triptycase-soy agar, heart-brain agar);

3) heart-brain nutrition broth.

Quality control: *H. parainfluenzae* –- requires factor V, *H. influenzae* – requires X and V factors.

Method. Prepare a light suspension of pure daily culture of the test microorganism in the nutritional broth. It is necessary to avoid transferring together with the colonies of the hemi-containing medium, which may lead to false-positive results. Using a sterile swab, inoculate the surface of the nutrient medium with the prepared suspension. Place on the surface of the agar discs or strips containing X, V and XV factors, at a distance of 2 cm apart. Incubate 18–24 hours at a temperature of 35–37 °C in an atmosphere with 5–10 % CO_2.

Accounting for the nature of growth is made visually. The presence of microorganism growth only around disks with X and XV or V and XV factors indicates the need for the X or V factor respectively. Growth only around a disk with factor XV is characteristic of hemophilia that requires both factors (for example, *H. influenzae*) (*figure 17*).



A B

Fig. 17. Growth of hemophilic bacteria (A)
and *H. influenzae* (B) in the presence of X and V factors

Algorithm *"Test for the detection of b-lactamase production"*.

Beta-lactamases are enzymes that destroy b-lactam antibiotics. The production of b-lactamase is the main mechanism of resistance to aminopenicillins and some cephalosporins in the strains of *H. influenzae*. Several methods for the

detection of b-lactamase production have been developed. The most common is chromogenic method using disks with nitrocephin.

The principle of the method is based on the fact that b-lactamases hydrolyze the amine bond in the b-lactam ring of nitrocephin, resulting in a change in color that is visible to the naked eye.

Materials:

1) discs with Cephinaz (BBL);

2) sterile distilled water;

3) glass bottles or Petri dishes;

4) sterile pipettes;

5) sterile wooden sticks-applicators or loops.

Procedure:

1) place the required number of disks on a clean slide or on a Petri dish;

2) moisten each disk with 1 drop of sterile distilled water;

3) using a sterile loop or a stick-applicator to apply several isolated, morphologically similar colonies to the surface of the disk; use a pure daily culture on chocolate agar;

4) observe the change of color; positive results appear within 15 seconds – 5 min; in the absence of color change for 5 minutes the test is considered negative.

Interpretation of the test: positive is the formation of a red disc in the place where the culture of the investigated microorganism is applied, negative - no color change (*fig. 18*).

Fig. 18. Test for the detection of b-lactamase production

Quality control: positive control – *S. aureus* ATCC 29213, negative control – *H. influenzae* ATCC 10211.

The control strains should be stored on the slant trypticasesoy (*S. aureus*) and chocolate agar (*H. influenzae*) at a temperature of 2–8 °C for 1 month. Before testing, you should subculture the strains on a Petry dish with blood agar and chocolate agar. Quality control should be performed daily when testing each new lot of disks.

Therminology.

Family: Pasteurellaceae

Genus: Haemophilus

Species: H. influenzae, H. parainfluenzae, H. ducreyi.

Practical tasks, being carried out during practical classes:

1. Staining and microscopy of smears of *Haemophillus* spp.
2. Primary seeding of "pathogenic material" on blood agar.
3. Determination of cultural and enzymatic properties of *Haemophillus* spp.
4. Slide agglutination reaction for differentiation of *Haemophillus* spp.
5. Studying biological preparations for serological methods.
6. Studying the scheme of laboratory diagnosis of hemophilic infection.

Theoretical questions for control:

7. Genus *Haemophillus*, major characteristics, antigenic structure.
8. Morphology and biological properties of *Haemophillus* spp. Classification.
9. Routes of transmission and pathogenesis of hemophilic infection. Immunity.
10. Laboratory diagnosis of hemophilic infection.
11. Treatment and control of hemophilic infection.

Test tasks for control:

1. It is known that for the hemophilic bacteria are characterized by the phenomenon of feeders. The phenomenon is related to:

A. The need for hemophilic bacteria in high concentrations of CO_2.
B. Growth of hemophilic bacteria around colonies of other bacteria that produce NAD or cause α-hemolysis.
C. Adding lincomycin to the medium.
D. Adding vitamin B to the medium.
E. Growth of hemophilic bacteria in the presence of small concentrations of CO_2.

2. Name the leading factor of the pathogenicity of hemophilic bacteria:

A. Exotoxin.
B. Endotoxin.
C. Capsule.
D. Enzymes.
E. Vi-antigen.

3. Which medium is more appropriate to use for the isolation of hemophilic bacteria?

A. MPA, MPB.
B. Levenshtein-Jensen agar.
C. Chocolate or blood agar.
D. Casein-charcoal agar.
E. Serum agar.

4. In the bacteriological laboratory pathological material from a patient suspected of hemophilic etiology pneumonia was received. What method of microbiological research should be used?

A. Microscopic.
B. Bacteriological.
C. Microscopic and bacteriological.
D. Bacteriological and serological.
E. Biological and serological.

5. In the bacteriological laboratory pathological material from a patient suspected of purulent meningitis was received. Microscopy revealed microorganisms similar to hemophilic bacteria. What morphological properties are characteristic for them?

A. Gram-positive rods with thickening at the ends, located in smears in the form of Roman numerals V and X.

B. Gram-positive rods, arranged in the form of a chain.

C. Gram-negative rods, peritrichous.

D. Gram-negative rods, arranged in short chains.

E. Gram-negative spherical, ovoid or rod-shaped bacteria.

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Навчальне видання

ГЕМОФІЛЬНІ БАКТЕРІЇ

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

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