

МІНІСТЕРСТВО ОХОРОНИ ЗДОРОВ'Я УКРАЇНИ
Харківський національний медичний університет

CAUSATIVE AGENTS OF WHOOPING COUGH

***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)***

ЗБУДНИКИ КОКЛЮШУ

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

Затверджено
вченою радою ХНМУ.
Протокол № 2 від 21.02.2019.

**Харків
ХНМУ
2019**

Causative agents of whooping cough: 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) / comp. N. I. Kovalenko, T. M. Zamaziy. – Kharkiv : KhNMU, 2019. – 40 p.

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

Learning guide includes sections of taxonomy, and biological characteristics of causative agents of whooping cough. The most modern information on epidemiology, pathogenesis, immunity, immunoprophylaxis, and modern methods of laboratory diagnosis of whooping cough is represented.

Збудники коклюшу : метод. вказівки з дисципліни «Мікробіологія, вірусологія та імунологія» для студентів II і III курсів мед. та стомат. факультів з англ. мовою викладання / упоряд. Н. І. Коваленко, Т. М. Замазій. – Харків : ХНМУ, 2019. – 40 с.

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Theme: Microbiological diagnosis of whooping cough.

Actuality of the theme.

Whooping cough is an acute infectious disease of the respiratory tract, the main symptom of which is convulsive coughing attacks. The causative agent of whooping cough *Bordetella pertussis* was isolated in 1906 by J. Bordet and O. Gengou. In 1937, *Bordetella parapertussis* was discovered.

Susceptibility to whooping cough is high: the contagiousness index is up to 70–100 % in unvaccinated children of the first year of life, especially newborns and premature babies. In the age structure, the majority of cases are schoolchildren of 7–14 years old – up to 50 %, children 3–6 years old – up to 25 %, the smallest share is children aged 1–2 years old – 11 % and children under 1 year old – 14 %. Frequent diseases occur among adults. According to the observations made in the outbreaks, the frequency of adult diseases is up to 24 %.

After suffering whooping cough under conditions of high immunization of children and low circulation of pathogens, persistent immunity lasts for 20–30 years, after which repeated cases of the disease are possible.

Mortality is currently low however its risk persists in newborns and premature babies, as well as in patients with congenital infections.

Goal: Studying laboratory diagnosis of whooping cough.

Concrete goals:

1. Study of biological properties and classification of *Bordetella* spp.
2. Study pathogenesis and clinical manifestations of whooping cough.
3. Study of the methods of laboratory diagnosis of whooping cough.

Students should be able to:

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

Equipment: slides, immersion microscope, biological preparations for laboratory diagnosis of whooping cough, tables, atlas.

Taxonomy.

Family: *Brucellaceae*

Genus: *Bordetella*

Species: *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*.

Morphological and tinctorial properties. *Bordetella* spp. are ovoid (coccobacilli), $0.2\text{--}0.5 \times 1\text{--}1.2 \mu\text{m}$ in size (*B. parapertussis* – $0.6 \times 2 \mu\text{m}$), placed in smears singly, sometimes in pairs (Fig. 1). They do not form spore, produce a capsule, have pili, are non-motile, gram-negative bacteria.

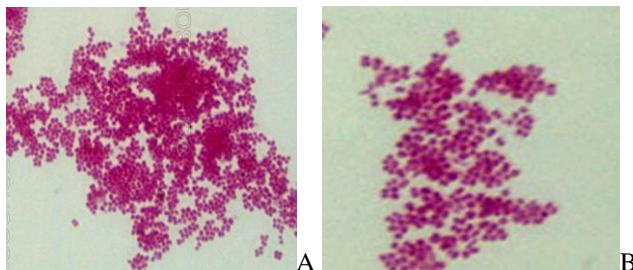


Fig. 1. Morphology of *B. pertussis* (A) and *B. parapertussis* (B)

Cultural properties. *Bordetella* are obligate aerobic hemophilic bacteria. Optimum growth temperature is 35–36 °C, pH 6,8–7,4; they require high humidity and the content of CO₂.

B. pertussis is fastidious and does not grow on the MPA and MPB. It requires three amino acids: proline, cysteine, glutamic acid for its development. In addition, during the life of *Bordetella* metabolites (sulfides, unsaturated fatty acids, peroxide compounds) accumulate in the nutrient medium that capable of suppressing growth. Therefore, the nutrient media for the cultivation of *Bordetella* contain substances of high adsorption capacity (blood, albumin, activated carbon). The Bordet-Gengou (potato-glycerol agar with blood) and casein-charcoal agars are used for the primary selection of the culture. Antibiotics penicillin or cefalexin are added to the media to suppress the foreign microflora.

Colonies appear after 3–4 days, small (up to 1–4 mm in diameter), smooth, shiny, transparent, dome-shaped, with metallic shine, similar to droplets of mercury or half pearls, viscous consistency (fig. 2, 3). *Bordetella* spp. form a zone of weak hemolysis around colonies.

B. parapertussis and *B. bronchiseptica* are not fastidious to nutrient media, grow on MPB, MPA, casein-charcoal and Bordet-Gengou agars. The colonies of *B. parapertussis* are almost the same in appearance as the colonies of *B. pertussis*, but larger and appear after 2–3 days (fig. 2). In addition, on casein-charcoal agar *B. parapertussis* causes brown coloration and darkening of the medium because of production of tyrosinase, which splits the amino acid tyrosine (this amino acid is always present in the proteins) with the formation of products of such color. Colonies of *B. bronchiseptica* appear after 1–2 days, they are similar to colonies of other *Bordetella*, but more flat with raised center. During a stereoscopic microscopy, a narrow ray of light ("tail", "span") is seen that extends from the center of the colony (reflected by the surface of the colony).

Enzymatic properties. *Bordetella* spp. have low enzymatic activity (table 1). They do not ferment carbohydrates, do not dilute gelatin, do not form indole and H₂S. *B. pertussis* is less active in enzymes (positive oxidase test).

B. parapertussis produces tyrosinase and urease and does not form oxidases. Tyrosinase catalyzes the production of pigment from tyrosine contained in nutrient media, which causes their darkening. *B. bronchiseptica* is the most active: it produces urease, oxidase, utilizes citrates, reduces nitrates to nitrites.

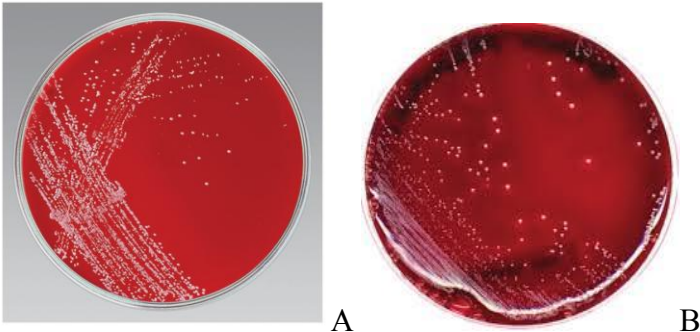


Fig. 2. Colonies of *B. pertussis* (A) and *B. paraptertusis* (B) on Bordet-Gengou agar



Fig. 3. Colonies of *B. pertussis* on casein-charcoal agar

Table 1. Differentiating characteristics of *Bordetella* spp.

	Oxidase	Urease	Tirosinase	Nitrate to nitrite	Citrate use
<i>B. pertussis</i>	+	–	–	–	–
<i>B. paraptertussis</i>	–	+	+	–	–
<i>B. bronchiseptica</i>	+	+	–	+	+

Antigenic properties. *Bordetella* spp. are antigenically heterogeneous. There are genus, species and type-specific antigen. The antigen, which causes the formation of agglutinins (agglutininogen), consists of several components. They are called factors and are designated by numbers from 1 to 16 (*table 2*). Factor 7 is generic, *B. pertussis* contains factor 1, *B. paraptertussis* – 14, *B. bronchiseptica* – factor 12, the rest factors are found in different combinations; for *B. pertussis* these are factors 2, 3, 4, 5, 6, for *B. paraptertussis* – 8, 9, 10. Agglutination reaction with adsorbed factor sera allows differentiation of *Bordetella* species and their antigenic variants.

Table 2. Agglutinogens of *Bordetella* spp.

	Generic	Species	Strain
<i>B. pertussis</i>	7	1	1, 2, 3, 4, 5, 6, 13, 15, 16
<i>B. parapertussis</i>		14	8, 9, 10
<i>B. bronchiseptica</i>		12	8, 9, 10, 11

Depending on the presence of agglutinogens 2 and 3 in the bacterial cell, four serotypes of *B. pertussis* are distinguished: 1.2.0; 1.0.3; 1.2.3; 1.0.0. The concept of agglutinogens is closely related to fimbria (fim). In the genome of all *B. pertussis*, the fim2 and fim3 genes are present, that is, theoretically, any strain can produce agglutinogens 2 and/or 3. Fimbria are included in some acellular pertussis vaccines. Serotyping *B. pertussis* is based on the agglutination of bacterial cells with monofactor sera, i.e. antibodies to agglutinogens, or monoclonal antibodies to fimbrial antigens in an agglutination reaction on glass and in a microplate.

Factors of pathogenicity:

- surface antigens (filamentous hemagglutinin, pertactin and protective agglutinogens) – adhesion factors; tropism to ciliary (in novolat. cilia – villus) epithelium of respiratory tract is a characteristic for *Bordetella* spp..

Filamentous hemagglutinin is a surface protein that participates in adhesion and has protective properties. It is included in acellular pertussis vaccines. Unlike toxin, hemagglutinin is not strictly specific for *B. pertussis*, it is also present in *B. parapertussis*, and can cross-react with *H. influenzae*, *C. pneumoniae*, and a number of other bacteria.

Pertactin is a protein of the outer membrane, belongs to the system of adhesins produced by bacteria when entered into the human body. It has protective properties, and is part of a number of acellular pertussis vaccines.

- Thermolabile exotoxin (pertussis toxin, lymphocytosis stimulating or histaminesensitizing factor) is the main factor of the pathogenicity of *B. pertussis*, affects the nervous and vascular systems, similar to diphtheria exotoxin, has a systemic effect (hematologic and immunosuppressive). It is exotoxin, a protein with a molecular weight of 117,000 Da, consisting of two functional parts (A and B) and five structural subunits (S1-S5): fragment A (corresponding to subunit S1) has enzymatic activity, inhibits cell adenylate cyclase. Fragment B (consisting of S2-S5 subunits) is responsible for attaching toxin to receptors of target cells. Toxin has high immunogenicity, its inactivated form is included in all non-cellular pertussis vaccines. The detection of antibodies against pertussis toxin by the ELISA is used to diagnosis whooping cough and monitoring vaccine efficacy.

- endotoxin has sensitizing and general toxic effects;
- the thermolabile dermo-necrotic toxin causes tissue necrosis, stimulates the formation of hypersensitivity to histamine, serotonin, which can lead to anaphylactic shock;

– tracheal cytotoxin leads to local lesions, destroys the ciliated epithelium. Tracheal cytotoxin is a fragment of a peptidoglycan of the cell wall. It has various biological properties: pyrogenicity, adjuvance, arthritogenicity, stimulation of IL-1 production. In vitro, the toxin infects tracheal epithelial cells and causes ciliostasis. At the same time, mucociliary clearance is violated - the first line of defense and conditions are created for the persistence of infection;

– capsule suppresses phagocytosis;
– enzymes of aggression: hyaluronidase, plasmocoagulase, lecithinase.
– adenylate cyclase-hemolysin (adenylate cyclase exoenzyme complex, which catalyzes the formation of cAMP, with toxin hemolysin; along with pertussis toxin causes the development of characteristic convulsive (spasmodic) cough). Toxin is the main pathogenicity factor acting at the initial stage of the infection, besides it is associated with the protective properties of the complex.

– lipopolysaccharide: it contains two lipids: A and X. The biological activity of the lipopolysaccharide is related to the X-fraction. It has multiple functions, including immunogenicity; reactogenicity of the cellular vaccine is associated with it.

All of the above factors are present in freshly isolated strains of the *B. pertussis*. However, when stored on artificial nutrient media, the causative agent is variable. It has been established that in the process of saprophytization, *B. pertussis* goes through four phases: a freshly isolated microbe (a smooth strain) with high virulent and immunogenic properties belongs to the first phase. With the transition to the fourth phase, immunogenicity and virulence are gradually lost, and cultural and biological properties change.

Resistance. *Bordetella* spp. are not stable in the environment, they are sensitive to ultraviolet rays, disinfectants in normal concentrations. At a temperature of 56–60 °C they die after 10–15 minutes, but recently strains are isolated that withstand this temperature within 30–40 minutes. In the dried sputum, they die after several hours, are sensitive to low temperature, less susceptible to antibiotics, resistant to penicillin.

Epidemiology. Whooping cough is anthropogenic infection. The reservoir and source of infection is only human (patients with typical and atypical forms of whooping cough, as well as healthy bacteria carriers). The most dangerous source of infection is children in the first 2 weeks of the disease. *Bordetella* can also be transmitted at the last stage of incubation period, when signs are not yet expressed. Carrier state of pathogens in young children is noted very rarely, in children 3–10 years old – in 1–2 % and it does not exceed for 14 days.

The source of the disease caused by *B. bronchiseptica* may be domestic and wild animals, among which there are epizootics (pigs, rabbits, dogs, cats, rats, etc.). It is believed that *B. bronchiseptica* may be part of the normal human respiratory microflora.

Transmission of the pathogen occurs in the catarrhal stage and the stage of convulsive coughing by respiratory route (with coughing, sneezing, talking).

People of any age are susceptible to the infection, but most of all – children aged from 1 to 10 years. Children under 1 year are sick too, which is associated with a lack of maternal immunity and a possible lack of transplacental transmission of specific antibodies. Perhaps, whooping cough is more common among adults, but it is not detected because its course is not accompanied by convulsions. Morbidity after contact reaches 90 % among non-immunized persons.

Pathogenesis. The portal of entry is the mucosa of the upper respiratory tract. *B. pertussis* spreads by the bronchogenic route, reaching the bronchioles and alveoli. Bacteremia is not typical for patients with whooping cough.

Bacteria attach to cells of the ciliated epithelium, where they multiply on the surface of the mucous membrane, without penetrating the bloodstream. An inflammatory process develops at the site of pathogen introduction, the activity of the ciliary apparatus of epithelium cells is inhibited, and secretion of mucus increases. Subsequently, ulceration of the airway epithelium and focal necrosis occur. The pathological process is most manifested in the bronchi and bronchioles, less manifested changes develop in the trachea, larynx and nasopharynx. The destroyed epithelium of the respiratory tract creates big risk of secondary infection, which leads to serious complications. In addition, mucosal-purulent corks close small bronchi, resulting in local respiratory failure and circulatory changes in the lungs. Peribronchial infiltration is observed. Pulmonary ventilation is impaired, vascular permeability increases → hypoxemia and acidosis. The reaction of delayed hypersensitivity is formed (strong bacterial allergen). Sensitization of an organism to pertussis toxins is important in the genesis of convulsive seizures. Constant irritation of the respiratory tract receptors causes coughing and leads to the formation of a dominant-type excitation focus in the respiratory center. As a result, typical attacks of spasmodic cough can also be caused by non-specific irritants. From the dominant focus, irritation may radiate to other parts of the nervous system, such as the vasomotor (increase in blood pressure, vasospasm). Irradiation of irritation also explains the appearance of convulsive contractions of the muscles of the face and body, vomiting, and other symptoms of whooping cough.

In the development of whooping cough, there are three stages, in which the leading role is played by various pathogenicity factors:

- adhesion, in which pertactin, filamentous hemagglutinin, agglutinogens are involved;
- local damage, the main factors of which are the tracheal cytotoxin, adenylate cyclase-hemolysin and pertussis toxin;
- systemic lesions caused by pertussis toxin.

Pertussis toxin, having adenosine-diphosphate-ribosyltransferase activity, influences the intracellular exchange of ionized calcium (the work of the “calcium pump”). It causes the development of a convulsive component of

cough, convulsions in severe whooping cough, and hematological and immunological changes (including the development of leukocytosis and lymphocytosis, increased body sensitivity to histamine and other biologically active substances with the possibility of hypersensitivity with an IgE-mediated mechanism of allergic reactions).

The structure of systemic lesions in whooping cough is dominated by:

1. Disorder of the central regulation of respiration.
2. Impaired respiratory function with the development of a spastic condition of the respiratory tract in combination with productive inflammation in the peribronchial, perivascular and interstitial tissue.
3. Violation of the capillary blood flow due to damage to the vascular wall with an acute disorder of blood and lymph circulation (plethora, hemorrhage, edema, lymphostasis), mainly at the site of inflammation (respiratory organs).
4. Dyscirculatory disorders in the brain and impaired intracellular metabolism of brain tissue, mainly due to hypoxia with the possibility of necrobiotic changes in nerve cells (their lysis followed by glial reaction in severe forms of the disease).
5. Inhibition of vascular centers and the blockade of β -adrenoreceptors under the action of pertussis toxin, along with impaired capillary blood flow and exposure to hypoxia, are the cause of disorders of the cardiovascular system.
6. Reduction of nonspecific resistance (phagocytosis) and violation of the mechanisms of cytokine regulation of T-cell immunity with the development of a secondary immunodeficiency state.

B. pertussis and its metabolic products cause long-term stimulation of the receptors of the afferent fibers of the vagus nerve, the impulses from which are sent to the central nervous system, in particular the respiratory center, which leads to the formation of a stagnant focus of irritation characterized by signs of dominant according to A. Ukhtomskyi.

The main features of the dominant focus for whooping cough are:

- ✓ increased excitability of the respiratory center and the ability to summarize irritations (sometimes a slight irritant is enough to cause an attack of convulsive cough);
- ✓ the ability for a specific response to a non-specific irritant: any stimuli (pain, tactile, etc.) can lead to a convulsive cough;
- ✓ the possibility of irradiation of excitation to neighboring centers:
 - a) vomiting (often results from convulsive coughing);
 - b) vascular (the response is an increase in blood pressure, vasospasm with the development of cerebral circulation disorder and swelling of the brain);
 - c) skeletal muscle center (with tonic-clonic seizure response);
- 1. persistence (activity is long kept);
- 2. inertness (having formed, the focus periodically weakens and increases);

3. possibility of transition of the dominant focus to the state of parabiosis (parabiosis of the respiratory center explains the delay and stop of breathing in patients with whooping cough).

The formation of a dominant focus occurs already at the beginning of the disease (in the pre-convulsive period), but its symptoms are most clearly manifested in the paroxysmal period, especially at the 2nd or 3rd week.

The response is a cough (like an unconditioned reflex), which at the stage of local injuries (initial catarrhal period) has the character of the usual tracheobronchial, subsequently (at the stage of systemic lesions during the period of convulsive cough) acquires paroxysmal convulsive nature.

Clinical picture (table 3). The incubation period lasts for 3–14 days, most often 5–8 days. Symptoms consist of 3 periods: catarrhal, paroxysmal and convalescence.

Table 3. Clinical criteria for the diagnosis of whooping cough

Sign	Characteristic
Intoxication syndrome	It is absent throughout the entire illness (both in the catarrhal period and in the period of convulsive cough) in the absence of complications or co-infections
Fever	It is absent throughout the entire illness (both in the catarrhal period and in the period of convulsive cough) in the absence of complications or co-infections
Catarrhal syndrome	In the first 1–2 weeks of the disease: the presence of dry, unproductive, obsessive cough, increasing in dynamics, despite the symptomatic therapy, on the background of a satisfactory condition and well-being of the child, the absence or weak expression of other catarrhal phenomena and objective changes from the respiratory tract. From 2–3 weeks of the disease, a change in the nature of cough on paroxysmal convulsiveness with hyperemia or facial cyanosis, reprises, lacrimation, viscous sputum or vomiting after an attack of cough. Characterized by increased cough at night, when waking up, during meals, after physical and emotional stress.
Edema syndrome	Decrease in diuresis at 1–2 weeks of the period of a convulsive cough; swelling of the face
Hemorrhagic syndrome	Nosebleeding during a coughing attack, subconjunctival hemorrhages in the corners of the eyes, elements of petechial rash on the face.
Characteristic symptom	Tear or sore of tongue bridle

The catarrhal period. The first manifestations of the whooping cough are similar with the acute respiratory viral or bacterial infection of the upper respiratory tract:

- ✓ an increase in body temperature to +38 °C, accompanied by chills, although the course of the disease is possible without hyperthermia;
- ✓ weakness, muscle aches, headache;
- ✓ catarrhal phenomena: clear discharge from the nasal passages, nasal congestion, dry cough, mucosal swelling.

There are also hyperemia of the pharynx, rapid heartbeat, accelerated breathing rate. The duration of the catarrhal stage of whooping cough is 7–10 days. If a newborn is ill, then a rapid development of the disease may occur, when the convulsive stage occurs in 2–3 days after the first signs of pathology appear.

During this period, the causative agent is most intensely secreted from the body.

The paroxysmal period (from the Greek paroximos - irritation, excitement) lasts from 2–3 weeks to 4–8 weeks. The characteristic symptom of whooping cough paroxysmal convulsive cough develops due to tonic spasm of the respiratory muscles.

The paroxysmal period is characterized by an unstable cough attack of up to 20–30 times per day, which can be provoked up with nonspecific stimuli (light, odor, smoke, sound, medical examination, manipulation). During an attack, the face of the patient turns red, then becomes a bluish tint, tears appear in the eyes, sometimes hemorrhages appear in the eyes, the viscous sputum is secreted, the cervical veins swell, the muscles of the neck are tense, and the involuntary discharge of urine and feces is possible. The tongue protrudes from the mouth to the limit, its tip rises up. As a result of friction of the bridle of the tongue on the teeth and its mechanical overstretching, tear or formation of ulcer occurs. Tear or ulcers of the tongue is a characteristic symptom of whooping cough.

An attack of cough begins with the characteristic sensations of lack of air, the presence of a foreign object in the throat. Young children can not single out the approach of attack, but a child from 5–6 years old is already able to recognize the onset of characteristic coughing attacks.

Then follow:

- little cough impulses on the exhale with a characteristic "barking" sound;
- long convulsive breath-reprise, which occurs when air passes through a constricted glottis (due to laryngospasm) and is accompanied by a hoarse, whistle;
- the next series of cough on the exhale.

After each attack caused by spasm, the sputum begins to move away from the respiratory tract. Inclusions of blood may be present in the dense secretion with significant damage to the epithelium.

Abrupt spasms of the airways, first of all tracheas, can lead to vomiting caused by overstressed muscles. Stress during cough is the cause of the characteristic appearance of patients with whooping cough: puffiness of the face, hemorrhages on the sclera of the eyes, in the corners of the mouth (*fig. 4*). White dense sores may develop on the surface of the tongue, on the bridle – trauma due to friction on the teeth during coughing attacks.

Coughing may be accompanied by convulsions (from Latin convulsivus – convulsive), cyanosis, respiratory depression. After an attack in children, especially early age, there is a painful inspiratory stridor (from Latin stridor – whistle, creak), caused by spasm of the larynx.



Fig. 4. The appearance of the patient with whooping cough

The attack may be preceded by aura (feeling of fear, anxiety, sneezing, sore throat, etc.). Coughing attacks may be short-term or last for 2–4 minutes. Paroxysms are possible – the concentration of coughing attacks for a short period of time.

Cough attacks, frequent vomiting lead to a violation of food intake, dehydration of the body. As a result, depletion and depression of consciousness develop. In the intervals between cough attacks children feel satisfactorily.

Outside of a coughing attack, puffiness and pastyness of the patient's face, eyelid edema, pale skin, perioral cyanosis persist; subconjunctival hemorrhages, petechial rash on the face and neck are possible.

Characteristic is the gradual development of symptoms with maximum participation and worsening of attacks of convulsive cough on the 2nd week of the convulsive period; at week 3, specific complications are identified; on the 4th week – non-specific complications on the background of secondary immunodeficiency.

In the convulsive period there are marked changes in the lungs. Coughing attacks gradually increase and reach their maximum on the second week of the spasmodic period.

Damage to the respiratory system is the main symptom of whooping cough. There are 4 variants of pathological changes: 1) pneumonic form or "pertussis lung"; 2) bronchitis; 3) pneumonia and 4) atelectasis.

Pneumonia with whooping cough most often occurs due to the addition of a secondary respiratory infection – more often, SARS and mycoplasmal infection.

The symptoms get worse by the evening due to general agitation and fatigue, and subside with fresh air. Coughing can be triggered by pain, physical exertion, food intake or medication in solid form.

Tachycardia, shortness of breath, pronounced attacks of an exhausting cough are the reason for hospital treatment of a child to assist if there is a need to connect to an artificial respiration apparatus.

Severe seizures of convulsive cough, accompanied by spasms of the bronchial tree and trachea, leading to myocardial hypoxia, symptoms of oxygen

insufficiency of the brain and muscle tissue are dangerous. Severe spastic syndrome is more often observed in children under the age of 3 years.

The lack of timely treatment can lead to changes in the heart muscle, the occurrence of necrotic lesions in the lungs.

The final period (*the period of reconvalescence*) lasts for 2–4 weeks, sometimes to 2–6 months. Cough attacks become less prolonged, occur less often. During the cough, pieces of necrotized areas of the mucous membrane of the trachea and bronchi are separated and exuded.

The period of reverse development (early convalescence) lasts from 2 to 8 weeks. Cough loses its typical character, occurs less frequently and becomes milder. The health and condition of the child improves, vomiting disappears, sleeping and appetite are normalized.

The period of late convalescence lasts from 2 to 6 months. At this time, high excitability of a child remains, and possible trace reactions (return of paroxysmal convulsive cough in case of other diseases).

It is marked with the beginning of a reduction in the number of coughing episodes and a general improvement in the child's well-being. On average, for 2 weeks, there is still a residual cough that does not cause an oversteering of the body. Manifestations of the disease persist within next 2 weeks, after which the attacks end.

During the period of reverse development, it is important to protect the child from viral and bacterial infections, severe stress, emotional experiences, including joyful ones: they can often provoke coughing attacks.

On average, cough and other signs of disease disappear independently within 1–2 months, but coughing seizures may recur on the background of illness, hypothermia, or stress in the six-month period after the end of the illness.

The temperature during the whole period is normal.

There may be subclinical course (mainly in the vaccinated) – no coughing episodes, it is an epidemiological hazard.

Classification of whooping cough.

By type:

1. Typical.
2. Atypical:
 - abortive;
 - subclinical;
 - asymptomatic;
 - transient bacteriocarrier.

By severity:

1. Mild form.
2. Moderate form.
3. Severe form.

The severity of the disease is determined by the frequency and nature of coughing attacks, the presence of complications and the severity of signs of oxygen deficiency between coughing attacks (*table 4*).

Criteria for severity:

- severity of symptoms of oxygen deficiency;
- frequency and nature of attacks of convulsive cough;
- condition of the child between cough attacks;
- severity of edema syndrome;
- presence of specific and nonspecific complications;
- severity of hematological changes.

Table 4. Criteria for assessing the severity of whooping cough by clinical signs

Sings	Form of infection		
	Mild (70–80 %)	Moderate (20–29,5 %)	Severe (0,5 %)
Hypoxia	absent	cyanosis of the nasolabial triangle	facial cyanosis when coughing
Duration of catarrhal period	7–14 days	7–10 days	3–5 days
Coughing frequency	up to 10 per day; reprise rarely	10–20 per day; reprises often	more than 20 per day; paroxysms
Vomiting after coughing	absent	characteristic	possible
State between cough attacks	active, appetite saved	active, appetite reduced	inactive, appetite absent
Terms of complications	absent	on the 3-4 weeks	from the 1 st week
Apnea	absent	absent	characteristic
Dysfunction of the cardiovascular system	absent	mild	meaningful
Convulsive syndrome	absent	absent	characteristic
Leukocytosis	$10-15 \times 10^9$ c/l	up to $20-30 \times 10^9$ c/l	more than 40×10^9 c/l
Lymphocytosis	up to 70 %	70–80 %	more than 80 %

Atypical forms of whooping cough. *Abortive form:* the period of convulsive cough begins typically, but ends very quickly (within a week). *Subclinical form:* a child has a dry, obtrusive cough during the entire period of the disease, attack-like convulsive cough is absent. *Asymptomatic form:* there are no clinical manifestations of the disease, but the pathogen is isolated, there is re-isolation of its DNA from a smear from the posterior pharyngeal/nasopharyngeal wall and (or) increase in titers of specific antibodies in the blood. *Transient bacteriocarrier:* isolation of bacteria or identification of bacterial DNA in the absence of clinical manifestations of the disease and without an increase in specific antibody titers in the dynamics. Bacterial carrier in children is rarely observed (in 1.0–2.0 % of cases), as a rule, in vaccinated children. The carrier of infection is the source of the pathogen, infecting others. Atypical forms of whooping cough are more common in adults and vaccinated children.

Features of whooping cough in unvaccinated young children. The incubation and pre-convulsive periods are shortened to 1–2 days, the period of convulsive cough is extended to 6–8 weeks. Severe and moderate forms of the disease predominate. Coughing attacks may be typical, but reprises and protrusions of the tongue are less common and not clearly expressed. Cyanosis of the nasolabial triangle and face is often noted. In newborns, especially premature ones, the cough is weak, not very sonorous, without reprise and abrupt face hyperemia, but with cyanosis. When coughing there released less sputum as children swallow it. As a result of the discoordination of various parts of the respiratory tract, including the soft palate, mucus can be released from the nose.

In children of the first months of life, instead of typical coughing attacks, their equivalents are noted (sneezing, unmotivated wailing, crying). Hemorrhagic syndrome is characteristic: hemorrhages in the central nervous system, less often in the sclera and skin. The general condition of patients in the period without cough attacks is violated: children are lethargic, skills acquired by the time of the disease are lost. Specific, life-threatening complications (apnea, encephalopathy) with the development of emergency conditions (respiratory rhythm disturbances, convulsions, depression of consciousness, hemorrhage and bleeding) often develop.

Disturbances of the respiratory rhythm (delay and stop breathing) can occur both during an attack of cough and outside the attack (in a dream, after eating). Apnea with whooping cough in children of the first months of life is divided into spasmodic and syncopal. Spasmodic apnea occurs during a coughing attack, lasts from 30 seconds to 1 minute. Syncopal apnea, otherwise known as paralytic, is not associated with coughing. The child becomes lethargic, hypotensive. Paleness appears first, and then cyanosis of the skin. There comes a cessation of breathing while maintaining cardiac activity. Such apneas last for 1–2 minutes.

In premature babies, in the presence of morphofunctional immaturity, perinatal lesions of the central nervous system or concomitant whooping cough cytomegalovirus infection, apnea occurs more often and can be long lasting. Apnea is observed mainly in children of the first months of life. At present, there are no severe respiratory rhythm disturbances in children over the age of one year.

Encephalopathy is consequence of dyscirculatory abnormalities in the brain on the background of hypoxia and develops after frequent and prolonged respiratory arrest in unvaccinated young children, as well as due to intracranial hemorrhage.

The first signs of incipient neurological disorders are general anxiety or, on the contrary, hypodynamia, increased daytime sleepiness and sleep disturbance at night, limb tremor, increased tendon reflexes, and light convulsive twitching of individual muscle groups. With more severe course of encephalopathy, convulsive syndrome is observed with a brief loss of consciousness.

Of the non-specific complications, pneumonia most often occurs. Lethal outcomes are possible.

Secondary immunodeficiency develops early (from 2–3 weeks of spasmodic cough) and manifests significantly.

Features of whooping cough in vaccinated children. Children vaccinated against whooping cough can become ill due to insufficient development of immunity or reduction of its intensity. Mild and moderate forms of the disease are more common, severe course is not characteristic. Often there are atypical forms. Specific complications are rare and not life threatening. Fatal outcomes are not noted. More often, atypical forms of whooping cough are recorded. The incubation and pre-convulsive periods are extended to 14–20 days, the period of spasmodic cough is shortened to 2 weeks. Reprises and vomiting are less common. Hemorrhagic and edematous syndromes are not characteristic; the course of the disease is often mild. Hematological changes are weakly expressed, there is insignificant lymphocytosis. While bacteriological examination serotypes 1.0.3 and 1.0.0 of *B. pertussis* more often are isolated. The increase in the titer of specific antibodies is more intense and noted at the beginning of the 2nd week of the period of convulsive cough.

Features of whooping cough in adolescents and adults

- More often women are ill.
- Atypical or mild forms are predominantly noted.
- Specific complications are rare, life-threatening complications and deaths do not occur.

Complications.

Severe form of whooping cough can lead to prolonged hypoxia, impaired blood supply to brain tissue and myocardium.

Prolonged hypoxia is the cause of structural changes in the organs, expansion of the ventricles and atria, dangerous pathologies associated with impaired brain activity.

Often there is the development of a secondary asthmatic complex with regular attacks of suffocation on the background of viral diseases of the respiratory tract.

Specific:

- 1) atelectasis (complete bronchial collapse) → impaired ventilation → reproduction of anaerobic microflora → lung gangrene,
- 2) valve obturation → emphysema → bronchiectasis (reproduction of pyogenic cocci (*Staphylococcus*, *Streptococcus*)).
- 3) severe emphysema, mediastinal emphysema,
- 4) respiratory rhythm disturbances (holding the breath – up to 30 seconds and respiratory arrest – apnea – more than 30 seconds),
- 5) pertussis encephalopathy,
- 6) bleeding (from the nose, the posterior pharyngeal space, the bronchi, the external auditory canal), hemorrhages (into the skin and mucous membranes, the sclera and retina, the brain and spinal cord),

7) hernia (umbilical, inguinal), prolapse of the rectal mucosa,

8) ruptures of the eardrum and diaphragm.

Non-specific complications (pneumonia, bronchitis, sore throat, lymphadenitis, otitis, etc.). Most complications are related to secondary bacterial infections. On the background of weakened immunity and a reduction in the intensity of movement of the lymph in the lung tissue, stagnations begin, which leads to the formation of a favorable conditions for the addition of staphylococcal, streptococcal, pneumococcal, and *P. aeruginosa* infections.

Residual changes: chronic bronchopulmonary diseases (chronic bronchitis, bronchiectasis); psychomotor retardation, neurosis, convulsive syndrome, various speech disorders; enuresis; rarely in the unvaccinated in the absence of etiopathogenetic therapy – blindness, deafness, paresis, paralysis.

Differential diagnosis of whooping cough. In the period of convulsive cough, it is necessary to carry out differential diagnostics of whooping cough with *B. parapertussis* infection and diseases occurring with whooping cough syndrome (mycoplasma, chlamydial and respiratory syncytial (RS) infections, cystic fibrosis), as well as with aspiration of a foreign body.

In rare cases, it is necessary to exclude diseases, accompanied by increase intrathoracic lymph nodes (lymphogranulomatosis, leukemia, tuberculosis of the intrathoracic lymph nodes).

Infection caused by *B. parapertussis* can be diagnosed only with laboratory confirmation (bacteriological, PCR, serological). According to clinical signs and epidemiological data (contact with a long-term coughing patient), *B. parapertussis* infection is indistinguishable from whooping cough, but there are no characteristic changes in the hemogram (leukocytosis, lymphocytosis). A child who has had *B. parapertussis* infection needs preventive vaccinations. In one focus of infection it is possible to identify diseases caused by both *B. pertussis* and *B. parapertussis*.

RS-infection often begins gradually, but its initial period is shorter: 2–3 days. The syndrome of intoxication is mild or moderate, temperature is subfebrile. Catarrhal phenomena of the upper respiratory tract are mild: a slight swelling of the mucous membrane, a small serous discharge from the nose, an obtrusive cough, unproductive, often paroxysmal. The clinical picture is dominated by the phenomena of respiratory failure. In the lungs, dry whistling and fine-bubble crepitus wheezes are heard. There identified the radiological signs of emphysema. Blood tests include leukopenia, lymphocytosis, normal ESR. The diagnosis is confirmed by detection of respiratory syncytial virus RNA by PCR, detection of specific IgM in ELISA, and detection of virus antigens in smears imprinted from the mucous membrane of the posterior pharyngeal wall by immunofluorescence or immunocytochemistry.

Respiratory chlamydiosis begins gradually, often on the background of normal or subfebrile temperature. Severe catarrhal syndrome: rhinopharyngitis, conjunctivitis. The cough is dry at the beginning, but gradually acquires the character of paroxysmal with perioral cyanosis, tachypnea, and vomiting. It is possible expiratory dyspnea. In the lungs there are crepitating wheezes at the top of the inhale, a tympanic shade of the pulmonary sound, and its shortening is possible. Cervical lymphadenitis and hepatosplenomegaly are also characteristic. In hemogram – leukocytosis, neutrophilia with a shift of the formula, increased ESR. The diagnosis is confirmed by the detection of specific IgM in serum by ELISA, an increase in IgG titer over time; detection of pathogen antigens using IF or DNA fragments of *C. pneumoniae* by PCR in smears from the nasopharynx.

Respiratory mycoplasmosis can begin abrupt or gradually. It is characterized by febrile fever or long subfebrile condition, as well as a mismatch between high fever and moderately severe intoxication syndrome. From the first days of the disease, hyperemia of the face, rhinopharyngitis, scleritis are detected. The patient is worried about paroxysmal cough, often with abdominal pain, discharge of viscous sputum or vomiting. With the gradual onset of the disease, the nature of the cough can vary from dry obsessive to paroxysmal. Radiographic signs of interstitial, focal, lobar pneumonia, and atelectasis are characteristic. Along with the indicated clinical features, patients often have lymphadenopathy, hepatomegaly; exanthema, dyspeptic syndrome, serous meningitis are also possible. In the hemogram – leukocytosis, neutrophilia with a shift of the formula, increased ESR. The diagnosis is confirmed by the detection of specific IgM in serum by ELISA, an increase in IgG titer over time; identification of pathogen antigens using IF or DNA fragments of *M. pneumoniae* by PCR in smears from the nasopharynx, seeding of sputum on the selective medium is possible.

Cystic fibrosis is characterized by the presence of family cases of the disease, a gradual onset from the first days of life. There is no intoxication syndrome, body temperature is normal, there are no catarrhal phenomena. Gradual increase in cough to paroxysmal, with cyanosis, shortness of breath and a discharge of viscous sputum are characteristic. There are peribronchial, infiltrative, sclerotic changes in lung tissue, emphysema. The hemogram is related to the age norm. Laboratory confirmation of the diagnosis includes: "sweat test" (increasing the concentration of chlorine and sodium in the sweat); "Meconial test" in newborns (an increase in the amount of albumin in the faeces); reduction of pancreatic enzymes in the duodenal contents, scatological signs of enzymatic deficiency.

Foreign bodies of the larynx, trachea and bronchi are characterized by an acute onset on the background of normal body temperature, the absence of signs of intoxication syndrome and catarrhal phenomena. In the history, as a rule, a child's play with small objects or gagging and coughing when eating or

playing is noted. A cough is immediately paroxysmal in convulsive nature, with a foreign body of the larynx it may be accompanied by asphyxia, with a foreign body of the trachea and bronchi - vomiting or asphyxia attacks. The X-ray picture depends on the X-ray contrast of the foreign body and the level of the lesion. Hemogram is without features. To confirm the diagnosis a laryngo- or bronchoscopy is necessary.

Immunity. The survived whooping cough (as well as the vaccination) does not provide intense lifelong immunity therefore repeated diseases of whooping cough are possible (about 5 % cases occur in adults). Cross-immunity against other bordetella is not induced.

Despite the presence of transplacental transmission of some pertussis antibodies, newborns are not protected from the disease in the first months of life. Congenital immunity due to maternal antibodies does not develop. The probability of infection by contact is 90 %. Disease is very dangerous for children under 2 years old.

Passively obtained antibodies disappear after a few months. At six months of age, only 4 % of children have antibodies against whooping cough. The period for which the content of anti-pertussis toxin (anti-PT), anti-hemagglutinin (anti-HA) and agglutinins is reduced by half, presumably equal to 36, 40 and 55 days, respectively.

The frequency of detection of pertussis antibodies in different age groups depends on the coverage of vaccination and the intensity of the circulation of the pathogen in the general population.

Serological studies show that the proportion of individuals with identified IgG-antibodies against pertussis toxin increases with age, reflecting the frequency of contacts with *B. pertussis*.

Microbiological diagnostics. Microbiological diagnostics is carried out by three methods: bacteriological, serological and molecular genetic (*table 5*).

Table 5. Methods of laboratory diagnosis of whooping cough

Method	Indications
Bacteriological method	Patients with clinical symptoms of whooping cough, children and adults who cough more than 7, but not more than 21 days; contact children and adults working in children's educational and medical institutions
Molecular genetic method (PCR)	Patients with clinical symptoms of whooping cough, children and adults who cough for more than 7 but not more than 28 days; contact children and adults working in children's educational and medical institutions
Serological methods (ELISA, AT)	Patients with clinical symptoms of whooping cough, children and adults who cough for more than 14 days for vaccinated children and adults and more than 21 days for unvaccinated, contact children and adults working in children's educational and treatment-and-prophylactic institutions, regardless of vaccination status
Express-method (IF)	Patients with clinical symptoms of whooping cough, children and adults coughing for more than 7 days

Laboratory researches are carried out for diagnostic purposes and for epidemic indications:

1) for diagnostic purposes:

- children who cough for 7 days or more, independently on indications of contact with patient with whooping cough;
- children with suspected whooping cough according to clinical data;
- adults with suspected whooping cough who are working in maternity homes, children's hospitals, sanatoriums, children's educational institutions and schools;

2) according to epidemic indications (to persons who have been in contact with the patient):

- children attending children's educational institutions or resided in children's hospitals, sanatoriums in which patients with whooping cough were detected, as well as all children under 14 years old who communicated with a sick person at home;
- adults working in children's institutions, in case of revealing in them patients with whooping cough, as well as when communicating with a patient with whooping cough at home.

Laboratory diagnosis is determined by the stage of the disease. In the catarrhal period and the initial stage of the convulsive period, the diagnostic method of choice is the direct detection of the pathogen using the polymerase chain reaction (PCR). This method allows you to quickly and efficiently examine nasal-pharyngeal swabs for the presence of *B. pertussis* and *B. paraptussis*.

Molecular genetic methods (PCR): detection of the genetic material of *B. pertussis* in a smear from the pharynx or the nose, or in the wash waters of the nose.

Material for research for PCR:

- deep nasal-pharyngeal smear without transport medium: introduce a applicator gently through the nasal passage to the posterior pharynx, wrap once and remove from the nasal passage. Remove the applicator and insert it into the tube without medium.

- Liquid after washing the nasal and pharyngeal cavity, nasal-pharyngeal aspirate.

The PCR allows detecting the pathogen's DNA within 6 hours at a later stage of the disease than the bacteriological method, and on the background of antibiotic therapy, but the maximum efficiency of the method falls on the earliest terms (1–3 weeks from the onset of the disease). Vaccination does not affect PCR results. However, in PCR, DNA is found not only of living, but also of dead microbes, which are stored in biological material from 1 week to 1 month. In this regard, DNA can be detected on the background of clinical recovery and after successful treatment with antibiotics, therefore, PCR is not recommended to be used to confirm the effectiveness of treatment.

The bacteriological method is the main method of laboratory confirmation of the diagnosis of whooping cough. The frequency of isolation depends on the timing of taking the material; on the 1st week of the disease, positive results can be obtained in 95 % of patients, on the 4th – only in 50 %, and starting from the 5th week, the microbe cannot be isolated.

Isolation of *B. pertussis* from the mucus of the posterior wall of the pharynx or nasopharynx is performed on casein-charcoal agar or Bordet-Gengou medium (potato-glycerol agar with the addition of blood and penicillin or cephalosporins of the first generation to suppress coccal microflora). The material is taken before the start of antibiotic therapy no earlier than two hours after eating. Bacteriological examination for diagnostic purposes should be carried out twice daily or every other day in the early stages of the disease (no later than the 3rd week of illness). At a later date, *Bordetella* seeding rate decreases sharply.

The selection of the material for research (mucus from the posterior pharynx) is carried out by two methods: the method of "cough plates" and a posterior pharyngeal swab (Fig. 5). The material is taken in a specially designated room, excluding contact with other children, in order to completely avoid the possible mutual contamination of those who are examined.



Fig. 5. Selection of the material by the posterior-pharyngeal swab (A) and the method of "cough plates" (B)

The material is taken up to the use of antibiotics, on an empty stomach or not earlier than in 2–3 h. after eating. If antibiotic therapy is started, then the material should be taken only 2–3 days after the cessation of treatment.

The material, selected with a dry swab, is immediately sown on heated nutrient media (cotton wool suppresses the growth of *B. pertussis*). The material selected with a wet swab is delivered to the laboratory. Material is transported within the temperature range of 4–37 °C.

When removing the material using the "cough plates" method, an open Petri dish with heated agar to 37 °C is kept in an upright position at the time of coughing attack at a distance of 8–10 cm from the child's mouth for 6–8 cough

shocks. In the case of short cough attacks, this cup can be raised again (the cup in the break between the attacks should be kept in the thermostat at 37 °C). It is necessary to prevent contamination of the medium by sputum, vomiting, mucus.

During transportation, material should be protected them from cooling (it is wrapped in paper, cotton wool, and placed in a heated container).

It is inappropriate to use the method of "cough plates" in the early stages of the disease, in the mild and atypical course of the disease, and during the examination of young children. To increase the positive result both methods of selection of material are used. The isolated pure culture is identified by the cultural, morphological, biochemical and antigenic properties (*table 6*).

Table 6. Differentiation of *Bordetella* spp.

Characteristic	<i>B. pertussis</i>	<i>B. parapertussis</i>	<i>B. bronchiseptica</i>
Growth on Bordet-Gengou agar (days)	3–6	1–2	1
Motility	–	–	+
Nitrate to nitrite	–	–	+
Urease	–	+	+
Oxidase	+	–	+
Citrate use	–	+/-	+
Pertussis toxin	+	–	–

Serological method

The timing of the research. During primary infection, antibodies of the IgM and IgA classes are formed not earlier than the second week after the onset of clinical symptoms, after another 1 week IgG antibodies begin to be detected, reaching their maximum by 6–8 weeks, after which their level decreases. After vaccination, IgG antibodies are formed. IgG antibodies can be detected before reaching adulthood in low concentrations. In this regard, the serological diagnosis of whooping cough and *B. parapertussis* infection should be used no earlier than the third week of the disease; the optimal time for serological diagnosis is from 3 to 6 weeks of illness. Within 1 year after vaccination against *B. pertussis* it is not recommended to conduct serological research for diagnostic purposes. It should be considered that in children under the age of 3 months, their own antibodies are not produced, but maternal antibodies may be present, which, as a rule, are defined in low titers.

The serological method is a method of late diagnosis. It is used to confirm the disease, in patients with negative results of bacteriological studies, for a retrospective diagnosis and identification of risk groups. The risk groups include those in whose blood the level of anti-pertussis antibodies is lower than the protective, that is, below 1:80. Serological reactions – AT, CFT, PHAT, ELISA – are put with paired sera taken at intervals of 1–2 weeks. Diagnostic titer for single examination of unvaccinated patients – 1 : 80; the greatest significance is the fourfold increase in the titre of specific antibodies in paired

sera taken with an interval of 7–14 days, depending on the vaccine status. The vaccinated children should be tested only in a dynamic interval of at least 7 days, regardless of the initial titre of specific antibodies.

For rapid diagnosis of whooping cough, the reaction of microlatex-agglutination is used to determine anti-pertussis antibodies in saliva.

Using express-methods (IF), *B. pertussis* antigens are detected in the brush-biopsy specimen from the posterior pharyngeal wall.

Recently, *an enzyme-linked immunosorbent assay* (ELISA) has been successfully used to detect IgM antibodies in the serum (in the early stages) and IgG (in the late stages of the disease) and in the nasopharyngeal mucus (immunoglobulins IgA). These antibodies appear from the 2–3rd week of the disease and persist for 3 months. Whooping cough is differentiated in the catarrhal period of the disease from acute respiratory infections, in the period of spasmodic cough from other diseases, accompanied by persistent cough at normal body temperature and the absence of signs of general intoxication. The method is more sensitive compared to AT and allows detection of antibodies to individual pathogen antigens (pertussis toxin, filamentous agglutinin), which is important for monitoring the effectiveness of immunization with cell-free vaccines. Enzyme-linked immunosorbent assay uses purified *B. pertussis* protein antigens (such as HA, PT, or AG).

ELISA is used to detect specific antibodies to the pertussis toxin in the serum (limited probability given the difficulty in interpreting the results). IgG in older children and adults is the result of an infection or vaccination; if the patient has not been vaccinated against whooping cough during the last 12–24 months, then an elevated IgG titre to PT in a single sample indicates a fresh infection. The confirmation of the disease is also an increase of $\geq 100\%$ or a decrease of $\geq 50\%$ of the antibodies found in the second serum sample taken in 2–4 weeks after the first specimen was taken. Antibodies IgA confirm the fresh infection (also synthesized in the vaccinated children), circulate for several months. They are detected only in case of a doubtful or unlikely result for IgG.

Agglutination test (AT) is the first method for detection of the level of anti-pertussis antibodies, still remains the most commonly used reaction. In AT, agglutinating antibodies to *B. pertussis* and *B. parapertussis*, which are presented in blood, are detected. When performing AT, antibodies induced by agglutinogens of the antigenic form of pertussis pathogens in the 1st phase are determined. Freshly isolated encapsulated *B. pertussis* belong mainly to phase I. Passage of the pathogen in culture can lead to the appearance of other forms lacking immunogenic properties and are defined as phase II, III, or IV *B. pertussis*. Strains of phase I cause disease in humans.

The disadvantages of AT are low sensitivity and non-standard. Agglutinin titers are highly dependent on the bacterial strain used as an antigen. The results of measurements of the antibodies level in AT correlate with the results of the detection of IgG and IgA by ELISA.

A 1 : 80 dilution is considered as a diagnostic titer in AT in unvaccinated and children who were not sick with whooping cough previously. In immunized children and adults, positive results of AT are taken into account only in the study of paired sera with an increase in titer of at least 4 times. It should be taken in mind that in children under the age of 3 months, their own antibodies are not produced, but maternal antibodies may be present, which, as a rule, are defined in low titers.

In order to determine the protective antibody titer, a serological examination is carried out in 1.5 months after the third vaccination or revaccination using AT when immunizing with a vaccine with whole-cell pertussis component (DTP), ELISA with detection of IgG to PT (or PT and FHA) when immunizing with a cell-free vaccine.

The Schick test is a test for determining a person's susceptibility to diphtheria. A small amount of diphtheria toxin is injected intracutaneously. The toxin is injected into the skin of the left hand. The same amount of toxin heated at 100 °C for 10 minutes is injected into the skin of the right hand for control. Results are noted after 24 and 96 hours. Redness and swelling at the site of toxin injection indicate that a person does not have immunity to diphtheria, so if there is a risk of infection with this disease, he should be immunized. If there is a sufficient amount of antitoxin in the blood of the test subject, the latter neutralizes the injected toxin, and a “negative” reaction is observed.

Laboratory Confirmation Criteria

The diagnosis of whooping cough caused by *B. pertussis* is made when confirming the clinical diagnosis of whooping cough with at least one of the following methods:

- isolation of *B. pertussis* culture;
- detection of a specific fragment of the *B. pertussis* genome by PCR;
- in vaccinated children and adults: manifested seroconversion, i.e. an increase or decrease of 4 or more times the level of specific IgG and / or IgA (ELISA) or the level of agglutinating antibodies in the research of paired sera taken at least 2 weeks apart;
- in adults: single detection of specific IgM (ELISA) is permissible;
- in unvaccinated children: single detection of specific IgM and / or IgA and / or IgG (ELISA) or antibodies in a titer of 1/80 or more (AT).

The diagnosis of whooping cough caused by *B. parapertussis* is made in the case of:

- isolation of culture *B. parapertussis*;
- or if a fragment of the *B. parapertussis* genome is detected by PCR;
- or when antibodies to *B. parapertussis* are detected by AT in a titer of at least 1/80.

Criteria for special diagnostics using laboratory methods:

- bacteriological method is the “gold standard”, which is an absolute confirmation of whooping cough in case of positive seeding, however, in

recently vaccinated children, with or after antibiotic therapy, after 21 days from the onset of the disease, the method becomes poorly effective. The method is more informative in the early stages of the disease (up to the 2nd week of the period of spasmodic cough).

- molecular genetic method is a modern high-tech method of etiological diagnosis, which allows DNA of *B. pertussis*, *B. parapertussis*, and *B. bronchi-septica* to be detected in real-time by polymerase chain reaction in smears from the posterior pharynx in patients with suspected "whooping cough", contact children and adults, regardless of vaccination status, on the background or after antibiotic therapy and up to 4 weeks from the onset of the disease;

- serological methods (ELISA, AT) are methods of retrospective diagnosis, effectively confirming whooping cough in vaccinated children and adults. In newborns and the first months of life children, as well as in patients with immunodeficiency states, they have no diagnostic significance;

AT with the determination of the titer of specific antibodies against the pertussis and parapertussis antigen in the dynamics is used for the diagnosis of disease in late periods or epidemiological analysis (examination of foci of infection). Diagnostic titer with a single examination is 1:80. In vaccinated patients, a fourfold increase in the titer of specific antibodies in paired sera has diagnostic value.

ELISA is used to detect anti-pertussis IgM antibodies in the blood in the early stages and a fourfold increase in the titer of specific IgG during the examination in dynamics with an interval of 10–14 days.

The reaction of indirect immunofluorescence is a method of rapid diagnosis, allowing identify the antigens of *B. pertussis* in the material from the oropharynx, nasopharynx within a few hours.

Therapy of adolescents and adults should begin within 3 weeks from the appearance of coughing. Antibioticotherapy, administered in the early phase of the catarrhal period, facilitates the course of the disease, whereas, after the onset of spasmodic cough, it does not affect the symptoms, but reduces the period of contagiousness. Drugs of choice: macrolides (azithromycin, clarithromycin, erythromycin), penicillin group (ampicillin, amoxicillin, ceftriaxone, cefepime and others). In case of hypersensitivity or intolerance to macrolides and penicillins: cotrimoxazole, tetracyclines, aminoglycosides.

Specific prophylaxis. Currently, there are 2 types of pertussis vaccine in the world. Vaccines with killed whole *B. pertussis*: DTP, which contains bacterial component (killed *B. pertussis* at a concentration of 20 billion/cm³) and purified from the ballast proteins diphtheria and tetanus toxoid adsorbed on aluminum hydroxide. Cell-free or acellular vaccine in which bacterial component is presented by three high purified antigens.

The vaccine is administered in accordance with the schedule of vaccinations three times at the 3rd, 4th and 5th months after the birth of the baby, and revaccination is carried out in 1.5–2 years.

For emergency prevention of whooping cough in unvaccinated children, a normal human immunoglobulin can be used: twice with an interval of 24 hours in a single dose of 3,0 ml at the earliest possible time after contact with the patient.

Laboratory algorithms:

Algorithm: *"The course of microbiological study of material for suspicion of whooping cough."*

Material for research: Mucus from the nasopharynx.

With diagnostic purpose, children are examined for clinical signs or those who cough for 5–7 days without signs of inflammation in the oropharynx, regardless of the presence of contact with the patient with whooping cough; adults with suspicion of whooping cough, who work in maternity homes, children's hospitals, kindergartens, etc., as well as adults who work with children and cough for 5–7 days or more.

According to epidemiological indicators, persons who communicated with patients with whooping cough are examined. The material is collected on an empty stomach, or 2–3 hours after breakfast. With diagnostic purpose, a three-time analysis is performed every day or every other day; according to epidemic indicators, it is two-fold. Later on the third week of the disease the possibility of the pathogen selection is sharply reduced.

There are two ways to select a material: 1) by posterior pharyngeal swab; 2) by the method of "cough plates".

Material is taken with the posterior pharyngeal swab both for diagnostic purposes and for epidemiological indications, and in any case for infants. For this purpose, two tampons are used: dry and moisturized. Taking a material with a dry swab stimulates the cough and increases the possibility of secretion of the pathogen when the material is taken by another a moisturized swab. A dry swab is used to seed bacteria (necessarily), and moisturized swab is sent to the laboratory no later than 2–4 hours after taking the material.

To diagnosis, mucus is removed from the posterior pharynx. It should be remembered that there are avirulent strains in the upper parts of the nasopharynx, that prone to autolysis, and virulent strains colonize the lower part of the upper respiratory tract.

The method of taking the material with dry and wet tampons is the same. The medical worker fixes with the left hand with the spatula the tongue's root, and inserts with the right hand the tampon into the oral cavity, pushing it over the root of the tongue (*fig 6*). The tampon should not touch the mucous membranes of the cheeks, tongue and tonsils. One touches the posterior pharynx with the tip of the swab and its convex part, moving it from right to left 2–3 times. Then also carefully, pull out a tampon from the oral cavity above the spatula. In both cases it is desirable to seed on 2 Petri dishes. If the child shows signs of anxiety, then the assistant fixes his head behind his back.

The method of "cough plate" is used only for diagnostic purposes in the presence of cough. The material is collected on 2 Petri dishes with a nutrient

medium. During the onset of coughing, remove the lid of the Petry dish with the left hand, and bring the opened Petry dish to mouth with the right hand at a distance of 10–12 cm so that droplets of mucus from the respiratory tract hit the surface of the medium (*fig. 6*). The Petry dish is held in this position for some time (within 6-8 cough pushes). With a short coughing, you can bring the Petry dish again. The nutrient medium should not be contaminated with saliva, vomit mass, sputum. Then the Petry dish with the nutrient medium is covered with a lid and delivered to the laboratory.

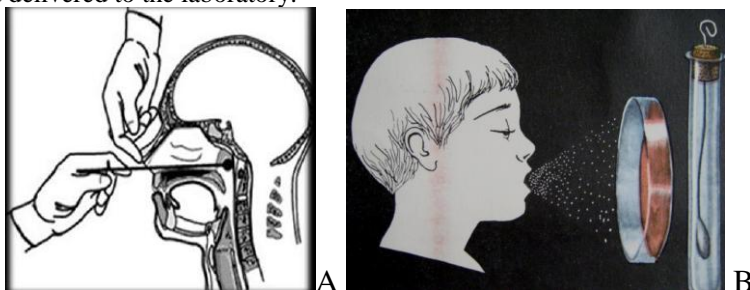


Fig. 6. Selection of material from patient with whooping cough:

A – by posterior pharyngeal swab; B – by the method of "cough plates".

When transporting the material for research it should be protected from direct sunlight, keeping it at a temperature of 35–37 °C, for which it is recommended to place the material in special boxes, boxes with a protective lining and a heater.

Bacterioscopic method: small gram-negative rods of ovoid form.

Bacteriological method: The bacteriological method involves the selection of the pathogen by seeding on a solid nutrient medium, the isolation of pure culture and the identification of pathogens of the genus *Bordetella* to the species. The examination lasts for 5 to 7 days.

Seeding the material for diagnostic purposes is performed on two Petry dishes, according to epidemiological indications – on one. Seeding is carried out on media treated with antibiotics (penicillin) to suppress the foreign microflora. When taking the material using the method of "cough plates", a medium without antibiotics is used, because the concomitant microflora will be insignificant in this case. For seeding it is necessarily to use a warm nutrient medium. In the case of taking the material with a dry swab, seeding is carried out immediately because of the low resistance of the pathogen.

The material taken with a swab can be seeded in the following ways:

a) seeding the material is made by carefully rubbing it with a tampon first on the periphery of the medium on Petry dishes in the form of 4–5 plaques, and then like Z-shaped strokes in the center of Petry dish, then rub in the central part with steril spatula, without touching the plaques;

b) The sectoral method (Gould method) can be used: the material is rubbed with a tampon in sector A, after which 4 lines are seeded with a sterile spatula or a loop from sector A into sector I, then the loop/spatula is cooled and similarly seeding from sector I in sector II and from II to III is carried out (fig. 7).

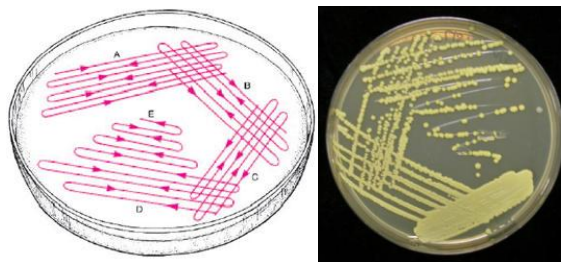


Fig. 7. Sectoral method (Gould method) of isolation of pure culture

If seeding is carried out at the site of taking the patient's material, that it is rubbed with a swab in one half of the medium, and then in the laboratory it is straightened with bacteriological loop for 2 more sectors.

First day of the examination:

Petry dishes with casein-charcoal agar are placed in a thermostat at 35–37 °C. For moistening the air into the thermostat put an open Petri dish with water.

Fourth day of the examination (72 hours):

1. Examine seeding on Petry dishes with a nutrient medium in order to select suspicious colonies of bacteria of the genus *Bordetella* (table 7). Examination of colonies is performed using a binocular stereoscopic microscope or binocular magnifier with a large focal length. After 24–72 hours, the colonies of *B. pertussis* are small, with a diameter of 1–2 mm on casein-charcoal agar, colonies of *B. parapertussis* are larger, shiny, grayish-cream color (droplets of mercury). Colonies can be green, cream, pink, chocolate and milky. The ray of light falling from the side of the colony is reflected by its surface, which results in the formation of a light cone (a little ray) falling from the center of the colony to the surface of the nutrient medium. This can be seen in a stereoscopic microscope. The seeding is remained up to 7 days (3–4 days in the thermostat, last days at room temperature (cellophane package)). Colonies of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* have a soft (butyric) consistency and are easily removed. Colonies of *B. bronchiseptica* in the subculture can be of two types: similar to the *B. pertussis* and more flat, with the raised center.

Terms of appearance of colonies are different: colonies of *B. bronchiseptica* appear after 18–24 h, *B. parapertussis* – after 24–48 h. and *B. pertussis* – after 48–72 hours. Different growth rate influence on the size of the colony when viewed through 72 hours. A zone of weak hemolysis around the colonies is almost always formed on blood agar.

The growth of *B. pertussis* and *B. bronchieptica* on the nutrient medium is not accompanied by a change in its color. *B. parapertussis* with abundant growth on the medium causes a diffused coloration of the medium in brown color, and also darkening of the blood agar.

Table 7. Growth characteristics of *Bordetella* spp.

Characteristics	<i>B. pertussis</i>	<i>B. parapertussis</i>	<i>B. bronchiseptica</i>
Time required for the growth of colonies (day):			
– on the casein-charcoal agar	2–3	1–2	18–24
– on the Bordet-Gengou agar	3–6	2–3	1–2
The size of the colonies on the casein-charcoal agar	1–2 mm	2–3 mm	4 mm
Growth on MPA	–	+	+

2. In the presence of suspected colonies on a medium isolation of pure culture is performed on Petri dishes with one of the nutrient medium. In this case, the surface of the medium is divided into several sectors and each colony is seeded on a separate sector, carefully rubbing it in the medim with circular motions.

3. In the presence of a significant number of similar colonies, in addition to the isolation of pure culture, smears from the remaining colonies are prepared in the physiological solution to determine the morphology of culture after Gram staining. At the same time, the absence of spontaneous agglutination is also determined. Microbes of the genus *Bortedetella* have the appearance of monomorphic gram-negative small ovoid rods (cocobacteria), arranged in a smear regularly. Sometimes *B. parapertussis*, especially from the Bordet-Gangou medium, have the form of elongated polymorphic rods.

The culture from the remaining colonies is checked in the agglutination reaction on a glass with specific non-adsorbed sera diluted 1 : 10, and with adsorbed monoreceptor sera to agglutinogens (factors) 1 and 14.

If the number of suspected colonies is significant, then it is possible to perform Zacks test to determine the presence of the urease enzyme.

4) A preliminary response can be given based on the examination of the colonies and morphology of cells in smears stained after Gram, a positive agglutination reaction with the characteristic non-adsorbed and adsorbed sera to factors 1 and 14 on the third day.

5) In the absence of growth of colonies on the culture medium, Petri dishes are placed again in a thermostat for 24–48 hours and re-reviewed at appropriate times.

Fifth to sixth day of examination (96–120 hours):

1. Growth of cultures is observed and the change in the color of the medium is noted: *B. parapertussis* colors casein-charcoal medium in brown color.

2. On a slide in a drop of a saline solution smears are prepared and they are stained after Gram; the morphology of the selected culture its purity, and also the absence of spontaneous agglutination are determined.

3. The serological properties are checked in the slide agglutination reaction with specific non-adsorbed serum diluted 1 : 10, as well as with adsorbed monoreceptor sera to factors 1 and 14. Serotypes of *B. pertussis* are determined in agglutination with monoreceptor sera to factors 1, 2, 3 (fig. 8).



Fig. 8. Slide agglutination test

4. To test biochemical properties, pure culture is seeded on agar with tyrosine (determination of tyrosinase), the presence of urease in the Zacks test or seeding on Hootinger's broth with urea are determined. If pure culture of *B. bronchiseptica* is suspected the utilization of citrate on the Simmons medium and mobility by seeding on semi-solid agar (0.5 % agar-agar) are determined.

B. pertussis is biochemically inert: it does not grow on MPA, does not change the color of the medium with tyrosine, does not produce enzyme urease (Zacks test is negative), does not utilize citrate.

B. parapertussis can grow on simple nutrient media, changes the medium with tyrosine in brown color, produces urease enzyme (Zacks test is positive).

B. bronchiseptica is characterized by rapid growth on simple nutrient media, mobility, does not change the color of the medium with tyrosine, produces urease enzyme (Zacks test is positive after 4 hours), is capable of utilizing citrate on the Simons agar.

5. A final positive response may be given based on the examination of pure culture: the morphology of colonies and cells, the agglutination reaction with non-adsorbed sera and adsorbed monoreceptor sera to factors 1 and 14, the results of the test for urease, changes in the color of the medium with tyrosine, the utilization of citrates.

The result is usually given in 4-6 days. The presumptive positive result can be given on 4th day with the formulation: "Microorganisms are found to be suspicious of bacteria of the *Bordetella* genus; research continues." The final positive response can be given on 5-6 days and is worded as follows: "*B. pertussis* (*B. parapertussis*, *B. bronchiseptica*)" was detected. Negative result is given on 6th day in the absence of suspected colonies of bacteria of the genus *Bordetella*, and it is formulated: "*B. pertussis* (*B. parapertussis*, *B. bronchiseptica*) have

not been detected." In the case of slow growth of microbes or the isolation of an atypical culture, preliminary and final responses can be given later (7–8 days).

Algorithm "*Study of enzymatic activity and its accounting*":

Determination of biochemical properties (*table 8*):

- the presence of tyrosinase;
- determination of urease;
- mobility;
- the ability to utilize citrates (the Simons medium);
- study of saccharolytic properties on the Olkenitsky medium.

Table 8. Differentiation of *Bordetella* spp. based on biochemical properties

Species of microorganism	Growth on MPA	Production of tyrosinase	Production of urease	Utilization of citrates
<i>B. pertussis</i>	-	-	-	-
<i>B. parapertussis</i>	+	+	+	+
<i>B. bronchiseptica</i>	+	-	+ slowly	+

1. To determine urease activity of microbes, a mixture of reagents A (1 part) and B (19 parts) is poured into 0.1–0.2 ml in sterile agglutination test tubes (with cork) and 1–2 loops of the test culture are added. The test tubes are placed in the thermostat at 35–37 °C for 30 minutes. The results are recorded after incubation, and in the absence of a change in the color of the mixture, the test tubes are left at room temperature and the result is taken into account the next day. At the same time control of the reagent is performed without introducing a culture. When urease is produced, the cleavage of urea to ammonia occurs, which leads to the alkalization of the medium, changing its color from yellow to crimson (*fig. 9*).

2. In order to determine the need for citrates, seeding of the tested culture on the slanted Simon's agar is carried out. The test tubes are incubated at 37 °C for one day. Bacteria that utilize citrate grow well, make the environment alkalify and cause its coloration in blue. Microbes that do not utilize citrates on this medium do not grow or change its color (*fig. 10*).

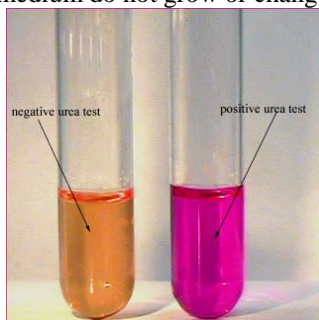


Fig. 9. Detection of urease activity

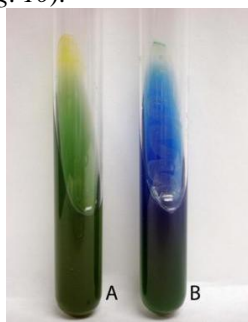


Fig.10. Detection of the need for citrates: A – negative, B –positive

3. To determine pigmentation, cultures are seeded on a simple nutrient agar containing 0.1% tyrosine with incubation at 37 °C for 24 hours. When tyrosine is cleaved, the medium is stained with a yellowish-brown color due to formation of melanin from tyrosin (fig. 11).

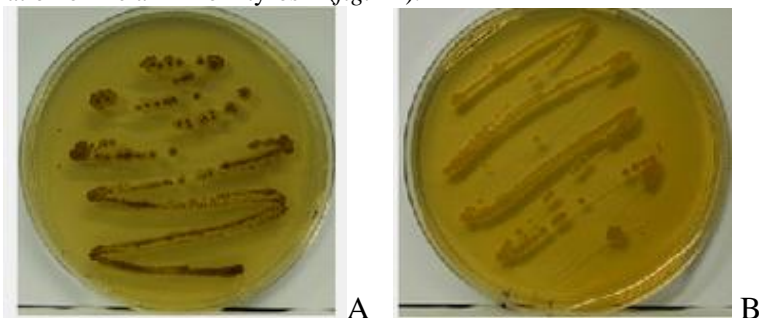


Fig. 11. Detection of tyrosinase activity: A – positive, B – negative

Algorithm "Sampling a pathological material with a posterior pharyngeal swab":

- 1) take a sterile test tube with a swab in the left hand, mark it the analysis number;
- 2) bend the tampon with your right hand about 2 cm from the end at an angle of 120° when removing the swab from the tube; hold the tampon in the right hand;
- 3) take a spatula to your left hand;
- 4) offer the patient to open the mouth wide;
- 5) hold the root of the tongue with a spatula, insert the tampon by the curved end down into the pharynx to "feeling of falling";

Warning! When taking the material, you can not touch by the tampon to the mucous membrane of the cheeks, tongue, tonsils, palate!

- 6) move the end of the tampon and its convex side on the posterior pharynx wall from right to left 2–3 times;

•remove the tampon from the mouth and, straightening it, lower it in the test tube.

Algorithm "Polymerase Chain Reaction":

The detection of DNA of *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* is carried out by polymerase chain reaction (PCR). The essence of the method is the multiple replication (amplification) of specific DNA regions of the bacteria under study, in the process of repeating from 35 to 45 times programmed cycles, consisting of three (usually) temperature regimes maintained for a certain time. The amplification of DNA sites takes place on a special equipment (amplifier) using a thermostable enzyme Taq polymerase, which complements the nucleotide sequence after short oligonucleotides (primers), complementarily bind to the desired DNA region.

At the first stage of the analysis, the treatment of the biological material is carried out in order to extract (isolate) the DNA of the pathogen and to remove or neutralize the inhibitors of the amplification reaction. In order to ensure the

quality of the research procedure for each sample, it is advisable to use an internal control sample that is added to the biological sample at the extraction stage of the DNA. At the second stage, the amplification of the DNA fragment of the microorganism is carried out and then the visualization (detection) of the result. The last stage is detection of fragments of amplification that can be carried out by different methods. The cheaper method is an agarose gel electrophoresis, but this method is associated with an increased risk of laboratory contamination with PCR fragments, which may lead to false-positive results of the study, especially in the absence of strict rules for organizing the laboratory.

Fluorescently labeled oligonucleotide probes are used in the PCR. They hybridize to the complementary regions of the amplified DNA target, which results in an increase in the fluorescence intensity during the reaction, and allows recording the accumulation of a specific amplification product by measuring the intensity of the fluorescence signal both during amplification (in real mode time), and at its end. This method greatly reduces the risk of laboratory contamination with PCR products and reduces the duration of the analysis.

In the case of the high coverage of children with vaccines against whooping cough, the effectiveness of bacteriological research is reduced and the role of PCR as a rapid (within 4 to 6 hours) method is increasing. PCR allows detect the DNA of the agent at a later time of the disease than the bacteriological method, and on the background of antibiotic therapy. In this case, the presence in the history of vaccination against *B. pertussis* does not affect the results of PCR.

It should be taken in mind that PCR also detects DNA of dead microbes, which is stored in biological material longer than viable microorganisms (from 1 week to 1 month). In this regard, DNA can be detected on the background of clinical recovery and after successful antibiotic treatment, so PCR is not recommended for confirmation of treatment efficacy, as in the case with a bacteriological study or using the NASBA method, in which bacterial RNA can be found (less a stable component of the genome than DNA).

The maximum level of specificity and sensitivity of the research is provided by tests based on PCR with hybridization-fluorescence detection of amplification products. Optimal terms of use of PCR for diagnostic purposes - from the first days of the catarrhal period of the disease and up to three weeks of illness at the time of the examination.

The material for research by PCR is smears from the mucous of the nasopharynx and the posterior wall of the oropharynx. It is recommended to examine both types of swabs from each patient. For this purpose, it is advisable to combine and investigate both a single swab as a single sample, collected successively with different probes in one test tube. At the same time, first swab from the nasal mucous membrane is taken, then swabs from the wall of the posterior pharynx wall. If the cavity of the nose is filled with mucus, it is recommended to remove it before the procedure. Two hours before taking swabs from the nasopharynx you can not drink and take food. Within 6 hours

before the procedure, you can not use medicines that irrigate the nasopharynx or oropharyngeal, and drugs for resorption in the mouth.

Smears from the mucous of nasopharynx are taken with a dry sterile nasopharyngeal tampon on a plastic applicator. The probe is introduced by light motion on the outer wall of the nose to a depth of 2–3 cm to the bottom of the turbinate, slightly lowered downward, inserted in the lower nasal passage underneath the turbinate to the nasopharynx. Probe is removed along the outer wall of the nose by rotational motion. The total depth of introduction of the probe should be about half the distance from the nostril to the eardrum (3 to 4 cm for children and 5 to 6 cm for adults). After taking the material, the end of the probe with a swab is dropped into a sterile tube with 0.5 ml of transport medium to the place of the fracture, wherein the flexible part of the probe is curtailing with a spiral, then, covering the top of the test tube with the lid, the probe handle is lowered, achieving complete break off of the upper part of the probe. The tube is sealed hermetically.

The swabs from the oropharynx are taken by dry sterile polystyrene probes with viscose applicator by rotational movements from the posterior wall of the oropharynx, gently pressing the tongue of the patient with a spatula, without touching the cheeks, tonsils and tongue. After taking the material, the working part of the probe with a swab is placed at a depth of 1.5 cm into a sterile tube containing 0.5 ml of transport medium. The handle of the probe with a swab is lowered down and broken off by holding the lid of the test tube, so that it allows tight close of the test tube. The test tube is sealed hermetically and marked.

Preparation of biological material for PCR research. Immediately before the examination, the contents of the closed test tube with swabs from the nasopharynx are mixed in a vortex and centrifuged for 5 seconds at 5,000 g on a microcentrifuge to remove droplets from the inner surface of the test tube. Open the test tube and, without extracting the probes, remove the required amount of sample for examination.

The order of conducting of PCR. The analysis is carried out in two separate rooms (zones: nucleic acid extraction zone and nucleic acid amplification zone) during PCR with real-time detection and in three separate rooms (zones: nucleic acid extraction zone, nucleic acid amplification zone and nucleic acid detection zone) during PCR with electrophoresis detection. Work should begin in the zone of extraction of nucleic acids, continue in the zones of amplification and detection. You can not return samples, equipment and reagents to the area in which the previous stage of the process was carried out. All laboratory equipment, including dispensers, tripods, laboratory utensils, as well as all working solutions, should be strictly stationary. It is prohibited to transfer them from one room to another. Unused reagents, reagents with expired term, as well as used reagents (including buffer and gels) should be removed. When working with PCR with real-time detection, it is unacceptable to open the samples and spray the contents, as this may lead to contamination of the laboratory area,

equipment and reagents with PCR products. Strict requirements should be presented to the organization of work in the zone of electrophoresis: the transfer of personnel from the laboratory for electrophoresis to other laboratories is forbidden. Changing working overalls, hats, shoes and gloves is mandatory when leaving the room for electrophoresis.

Algorithm "Serological methods":

In the conditions of mass vaccine prophylaxis, the number of severe forms of the disease has decreased, cases of late appeals for medical assistance are more frequent, often on the background of antibiotic therapy. At the same time, the significance of serological methods as means of late (retrospective) diagnostics has increased.

Detection of specific antibodies to *B. pertussis* and *B. parapertussis* is carried out using agglutination (AT) and enzyme-linked immunosorbent assay (ELISA). In AT, agglutinating antibodies to *B. pertussis* and *B. parapertussis* are detected in the blood. ELISA allows detect IgM, IgA, and IgG antibodies to various *B. pertussis* antigens (most commonly to pertussis toxin (PT) and filamentous hemagglutinin (FHA)); antibodies to PT have the highest specificity.

At primary infection, IgM and IgA antibodies are formed not earlier than the second week from the appearance of clinical symptoms, after one week, IgG antibodies begin to be detected, reaching their maximum for 6–8 weeks, after which their level is reduced. IgG antibodies can be detected in young in low concentration. In connection with this, serological diagnosis of the whooping cough and disease caused by *B. parapertussis* should be used not earlier than the second week of the disease; optimal terms for serological diagnosis - from 3 to 6 weeks of the disease. After vaccination, IgG antibodies are produced. Therefore, within 1 year after vaccination, serological testing for diagnostic purposes is not recommended, and in children vaccinated against pertussis, and in adults for serological diagnosis, only paired sera of blood obtained at intervals of at least 2 weeks should be used. In the study of a single serum, the result of detection of IgG antibodies can be regarded as positive only in the case of high titre. The indicators considered as high titers may vary in sets of reagents from different manufacturers and should be specified in the instructions for the use of the diagnostic kit.

Detection of significant levels of IgM (with different combinations of IgG or IgA) in nonimmunized children and adults should be considered as acute infection. However, in some patients with acute infection, IgM antibodies are determined at a low level. Children younger than 6 months may not respond to the infection by producing IgA antibodies, so the negative IgA result in children of this age does not mean the absence of the disease.

As a confirmatory test for samples with borderline or positive ELISA results, obtained by ELISA an immunoblot method can be used to detect IgG and IgA antibodies using *B. pertussis* antigens (PT in two concentrations and FHA).

Diagnostic titres in AT in non-vaccinated and non-diseased children were considered to be 1 : 80. In immunized children and adults, the positive results of AT are taken into account only when examining pairs of sera with an increase in the titre not less than 4 times. It should be taken into account that in children under 3 months their own antibodies are not produced, but there may be maternal antibodies, which are usually determined in low titres.

In order to determine the protective titre of antibodies, a serological study is carried out in 1.5 months after the third vaccination or revaccination using AT in case of immunization with a whole-cell pertussis component vaccine (DTP), or ELISA with the detection of IgG antibodies to PT (or PT and FHA) – in case of immunization with a non-cellular vaccine (DTaP).

Blood collection (for ELISA necessarily on an empty stomach) is carried out from a vein in volume from 3 to 4 ml or from a pad of a third phalanx of the middle finger in the volume of 0.5–1.0 ml (in young children) in a single-use plastic tube without anticoagulant.

Blood collection from the elbow vein is carried out with a single needle (diameter 0.8–1.1 mm) in a Vacuette (R) tube without anticoagulant or with 5 ml syringe. When collected into a syringe, blood from it is carefully (without the formation of foam) transferred to a one-used glass flask. Capillary blood is taken from the finger in aseptic conditions in a test tube without anticoagulant. Before taking blood, the patient's hand skin is warmed with hot water, then wiped dry. The finger pad is rubbed with 70° alcohol and pierced with sterile scarifier for single use. Blood is collected directly through the edge of a sterile disposable centrifuge tube. After taking the blood, place of the injection is treated with 5% iodine solution. Blood samples taken without anticoagulant are stayed at room temperature for 30 minute or placed in a thermostat at 37 °C for 15 minute. Then they are centrifuged for 10 minutes at 3 000 rpm/min. At the end of centrifugation, the serum is transferred to sterile tubes, using for each sample a separate tip with an aerosol barrier.

The term of storage of blood is not more than 6 hours. The blood serum is stored at room temperature for 6 hours, at a temperature of 4–8 °C for 5 days, longer – at a temperature not higher than –16 °C. Multiple freezing/defrosting of serum is unacceptable.

Each test tube is marked in accordance with the list in the accompanying document and placed in a polyethylene package of a suitable size with cotton wool (or other hygroscopic material) in an amount sufficient to adsorb the entire sample in case of its leakage and seal hermetically. It is possible to transport samples from different patients in one package.

Polyethylene bags are placed in an insulating container (refrigerator bag) adapted for the transport of biological materials, and transported at temperatures from 4 to 8 °C. When transporting and storing blood in the winter season, it is necessary to create conditions under which it does not freeze.

Packages containing material for PCR and serological examination may be transported in one thermo-insulating container.

Agglutination reaction. When using AT, serological examination of blood serum should be carried out at the same time for *B. pertussis* and *B. paraper-tussis* using diagnostic kits, allowed in the prescribed manner, in accordance with the manufacturer's instructions.

From the patient serum, 9–10 dilutions are prepared: 1 : 5, 1 : 10 and so on up to 1 : 1280 or 1 : 2560. In the 10th (or 11th) test tube, instead of the serum, 0.25 ml of physiological saline (control) is added. The reaction of agglutination is performed in a volume of 0.5 ml: to 0.25 ml of the related dilution of serum add 0.5 ml of diagnosticum. The test tubes are placed in a thermostat at 37 °C for 2 hours and then left at room temperature. The results are estimated the next day with agglutinoscope. The reaction is considered to be positive if there is clear agglutination in the test tube for four or three crosses (4+ or 3+).

The diagnostic titre of the agglutination reaction in the non-immunized and non-diseased children is considered to be 1 : 80 dilution. In immunized children and adults, positive results of the reaction are taken into account only in the study of pairs of blood sera taken at intervals of at least 2 weeks, with an increase in titre not less than 4 times.

Immunoenzyme analysis. Serological research with ELISA is carried out using kits of reagents allowed for use in the prescribed manner in qualitative, semi-quantitative and quantitative formats. Blood serum is used for research, all manipulations and interpretation of results are carried out according to the manufacturer's instructions for diagnostic kit. The results are taken into account on a spectrophotometer at a wavelength of 450/620 nm. Calculations are carried out by the graph-analytical method. The presence of a suspension or serum precipitate may be the cause of getting the wrong results. Such samples should be centrifuged or filtered before the examination.

Immunoblot that detects IgG and IgA antibodies in serum using *B. pertussis* antigens (PT in two concentrations and FHA) may be used as a confirmatory test for specimens with border or positive ELISA results or for self-dependent diagnosis.

The principle of the method consists in carrying out a reaction of binding of specific antibodies with highly purified recombinant antigens applied in the form of streaks on strips of a nitrocellulose membrane, pre-treated with a solution of protein, in order to block free sites of non-specific binding immunoglobulins. During the analysis, the strips are incubated with diluted samples of human serum. If specific antibodies are present in the sample, then during incubation they bind to antigens fixed on the strip. After washing, the strips are incubated with antibodies to human IgG or IgA, conjugated to horseradish peroxidase, and repeat the washing. Specifically bound antibodies are detected by color reaction, adding a substrate that interacts with the horseradish peroxidase. The dark strips at the appropriate site of the strip indicate the presence of the antigen-antibody complex. Pertussis toxin is

applied to the strip at two concentrations – PT and PT-100. Concentration of PT is standardized: a positive IgG response (banding) in the interaction of the test serum with PT-100 indicates that serum IgG levels exceed 100 IU/ml according to the WHO standard.

The control of the reaction is carried out using four bands arranged parallel to each other on the upper edge of the strip:

1) a band of positive control of the reaction to be present in the analysis of each serum;

2) two bands of positive control of the conjugate (IgG/IgA), which serve as controls for the detection of each class of antibodies;

3) "Cut-off control" (to control the coloration reaction): the intensity of this band is fundamental for the evaluation of antibody reactivity and the interpretation of the result of the analysis of a specific strip.

The analysis results are taken into account only if adequate control results are obtained. The intensity of the bands depends on the concentration of antibodies specific to *B. pertussis* present in the serum under study, and the result is evaluated in relation to the intensity of the Cut-off band.

The positive reaction to detect IgG to PT in the titre of more than 100 IU/ml of the WHO standard can be considered as a sign of acute infection in unvaccinated children or vaccinated more than three years ago. In other cases, a re-examination of serum or blood plasma taken 2 weeks after the first is recommended.

Scheme of bacteriological research

1st day

Seeding the test material to the nutrient media and incubation in a thermostat at 35–37 °C.

4th day (after 72 hours)

1. Study of morphology of colonies on nutrient media using a stereoscopic microscope.

2. Reseeding the colonies to obtain a pure culture of the bacteria.

3. In the presence of a large number of colonies in the nutrient medium:

a) preparation of smears, Gram stain, microscopy;

b) performance of slide agglutination reaction with specific non-adsorbed pertussis and parapertussis sera in dilution 1 : 10 and specific monoreceptor sera to agglutininogen (factors) 1 and 14;

c) performance of urease test.

4. Issuing preliminary positive result of the conducted research.

5th day (after 96 hours)

Study of isolated pure culture in case of presence of *B. parapertussis* and *B. bronchiseptica* in the investigated material:

a) studying the nature of the growth of pure culture and changing the color of the nutrient medium;

b) preparation of smears, Gram stain, microscopy;

- c) performance of the slide agglutination test with specific non-adsorbed and monoreceptor sera to agglutininogen (factors) 1, 14 and 12;
- d) performance of the urease test and evaluation of the results;
- e) seeding on MPA with tyrosine;
- g) if necessary, determine the mobility by seeding in semi-solid agar;
- e) seeding on Simon's agar.

6th day (after 120 hours)

1. Study of the isolated pure culture of *B. pertussis* or, in the case of late growth, *B. parapertussis* according to the scheme of the fifth day of the research.
2. Accounting for the results of the fifth day of the research.
3. Detection of serovariate of *B. pertussis*.
4. Issuing the final result: positive or negative.

Note: In the absence of growth of suspicious colonies on the fourth day of the research, Petry dishes with the culture medium are revised on the fifth and sixth days. The further course of research proceeds according to the scheme.

Practical tasks, being carried out during practical classes:

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

Therminology: *Bordetella pertussis*, *Bordetella parapertussis*, Bordet-Gengou agar.

Theoretical questions for control:

1. Genus *Bordetella*, major characteristics, antigenic structure.
2. Morphology and biological properties of *Bordetella* spp. Classification.
3. Routes of transmission and pathogenesis of whooping cough. Immunity.
4. Laboratory diagnosis of whooping cough.
5. Treatment and control of whooping cough.

Test tasks for control:

1. For serological diagnostics of the whooping cough it was made large-scale reaction with parapertussis and pertussis diagnosticums. At the bottom of the test-tubes with diagnosticum of *Bordetella parapertussis* grain-like sediment was formed. What antibodies have this reaction revealed?

- | | | |
|-------------------|----------------|----------------|
| A. Bacteriolysins | C. Antitoxins | E. Precipitins |
| B. Opsonins | D. Agglutinins | |

2. A patient has been suffering from elevated temperature and attacks of typical cough for 10 days. Doctor administered inoculation of mucus from the patient's nasopharynx on the agar. What microorganism is presumed?

- | | | |
|-----------------------|------------------------------|--------------------------|
| A. <i>Menigococci</i> | C. <i>Klebsiella</i> | E. <i>Staphylococcus</i> |
| B. <i>Listeria</i> | D. <i>Pertussis bacillus</i> | |

3. A patient has severe catarrhal symptoms. Material growth on Bordet-Gengou agar showed mercury-drop like colonies. Examination of the blood smears revealed some small ovoid gram-negative bacilli. What microorganisms were isolated?

A. Bordetella

C. Mycobacteria

E. Brucella

B. Corynebacteria

D. Meningococcus

4. During bacteriological examination of sputum of a child with choking cough and fever there were revealed glossy smooth colonies growing on casein-charcoal agar and reminding of mercury drops. Microscopic examination revealed short gram-negative bacteria. What microorganism was secured from the sputum?

A. K. pneumoniae

C. S. pyogenes

E. C. diphtheriae

B. B. pertussis

D. H. influenzae

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Навчальне видання

ЗБУДНИКИ КОКЛЮШУ

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для студентів II і III курсів медичного
та стоматологічного факультетів
з англійською мовою викладання**

Упорядники Коваленко Наталія Іллівна,
 Замазій Тетяна Миколаївна

Відповідальний за випуск

Н. І. Коваленко

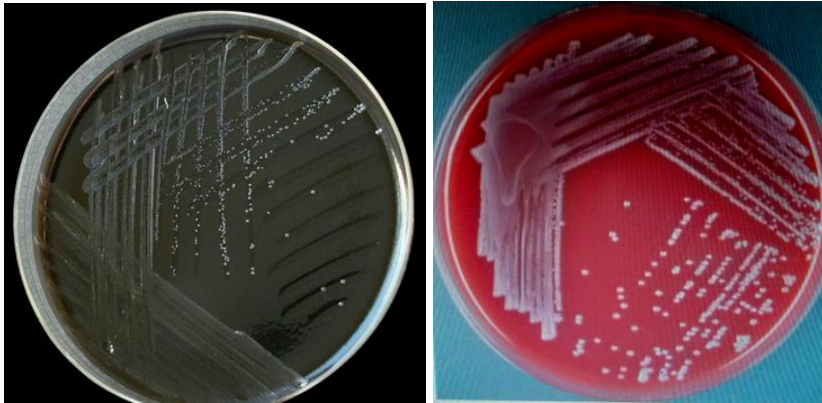


Комп'ютерний набір Н. І. Коваленко
Комп'ютерна верстка О. Ю. Лавриненко

Формат А5. Ум. друк. арк. 2,5. Зам. № 19-33702.

**Редакційно-видавничий відділ
ХНМУ, пр. Науки, 4, м. Харків, 61022
izdatknmurio@gmail.com**

Свідчення про внесення суб'єкта видавничої справи до Державного реєстру видавництв, виготівників і розповсюджувачів видавничої продукції серії ДК № 3242 від 18.07.2008 р.



CAUSATIVE AGENTS OF WHOOPING COUGH

*Learning guide for the 2nd and 3rd year English
media students of the Faculty of Medicine
and the Faculty of Dentistry
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