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TUBERCULOSIS DIAGNOSTIC METHODS

STUDY GUIDE

for practical classes for students – foreign citizens of 4th course

on speciality «Phthisiology»

Zaporizhzhia 2016

UDC: 616-002.5-07(075.8)-111

Y31

Recommended of Zaporizhzhia state medical university Central Methodological Council as study guide for practical classes on speciality «Phthisiology»for students – foreign citizens of 4th course. The report №1 from 29.09.2016.

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Y31 Tuberculosis diagnostic methods : study guide for practical classes for students – foreign citizens of 4th course on speciality "Phthisiology"/ R. M. Yasinskyi, Yu.S. Solodovnik. – Zaporizhzhia : [ZSMU], 2016. – 81 p.

The study guide is made according to the work program of phthisiology for students – foreign citizens of 4 courses for practical classes. The manual described the tuberculosis screening methods, clinical and laboratory research, due to the correct interpretation of research methods, their advantages and disadvantages.

THE LIST OF ABBREVIATIONS

AFB – acid fast baccili

CFP10 – specific tuberculosis protein

CRI – colorimetric redox indicator assay

DST – drug susceptibility test

ESAT6 – specific tuberculosis protein

FG – fluorography

GERD – gastroesophageal reflux disease

IGRA – interferon gamma assay

IFN-γ – interferon gamma

INNO-LIPA Mycobacteria v2 – line probe assay for the identification of the genus 16 different mycobacterium species

LJ – Lowenstein-Jensen medium

MIC – minimal inhibitory concentration

MDR-TB – multi-drug resistant tuberculosis

MODS – microscopically observed drug susceptibility

NRA – nitrate reductase assay

NTB – non-tuberculosis mycobacteria

OADC – oleic acid, albumin, dextrose, catalase

PANTA – polymyxin, amphotericin B, nalidixic acid, trimethoprim, azlocillin

PBMCs – peripheral blood mononuclear cells

PCR – polymerase chain reaction

PPD – purified proteinum derivate

PZA – hydrolyse pyrazinamide

QFT-G – QuantiFERRON-TB gold test

RR – resistance ratio

RTA – recombinant tuberculosis allergen

TB – tuberculosis

TB7.7 – specific tuberculosis protein

UACS – upper airway cough syndrome

ZN – Zhiel Neelsen stain

CONTENT

1. THE INTRODUCTION

Study Guide is devoted to the actual problem of modern of phthisiology – methods of TB diagnostic. There are screening methods – scin and blood tests among kids and X-ray for adults. In tuberculosis suspicion cases clinical, bacteriological and radiological investigation take place. There are very old (ZN microscopy, X-ray, Lowenstein-Jencen methods and new, such as molecular epidemiology methods, tests for Mycobacteria tuberculosis genome identification, etc.

Study guide has a great practical importance because it allows students to evaluate the complexity of tuberculosis detection methods and to contribute finding ways to improve differential diagnostic results.

1. ANAMNESIS

With tuberculosis (TB) complains firstly patient appeals for help to family doctor. But mostly there are no any complains at the beginning of disease. So, sick people suffered from tuberculosis at home and disease course worsening.

Patient complains from a sub-febrile temperature up to $37,5^0$C,dry cough withoccasional presence of sputum, sometimes hemoptysis,dyspnoe, fatigue, night sweats, etc.

Medical care workers whatever their specialty must be aware of the TB prevalence.Here is a set of questions that are to be addressed in the case a doctor is faced with atuberculous patient:

1. Whether the given patient was prior infected by tuberculosis?

2. Whether his/her relatives were infected by tuberculosis?

3. Whether the patient had contact with tuberculous patients or animals (household,professional, industrial contact)?

4. Whether the patient is registered in a tuberculosis dispensary due to: tuberculin testing orhypersensitive reaction to the test, contact with tuberculous patients, and no clear diagnosisof tuberculosis.

5. When the patient had the X-ray examination?

6. Whether the patient was invited after the X-ray examination for additional research?

7. Whether he was in a prison or lived with someone who was in a prison.

8. Whether the patient is homeless, a refugee, migrant or being in unfavorable socialconditions?

It`s necessary to ask patient to the frequency of repeated respiratory infections. It may be TB manifestation in cases of long-term sub-febrile temperature combined with dry cough. If a patient had suffered from exudative or dry pleuritis, this might be an indication forthe presence of tuberculosis in past.

In cases of suspected primary tuberculosis among children and young adults it`s necessary to ask them about whether they had suffered from chronic

conjunctivitis;erythema nodosum; other poorly displayed signs of tubercular intoxication.

It`s necessary to find out social and medical TB risk factors: homeless, alcohol users, drug users, presence of diabetes mellitus, HIV, etc.

Upon gathering anamnesis, it is also necessary to find out, when the tuberculin testreacted positive for the first time.

After a well-collected anamnesis, it is easier for the doctor to confirm his assumptionof presence of tubercular process.

2. PATIENT`S CLINICAL INSPECTION

2.1. Complains.

If a patient has any of the following complains, consider him a "Tuberculosis Suspect":

1. Cough for over 3 weeks.

2. Haemoptysis.

3. Pain in the chest for over 3 weeks.

4. Fever for over 3 weeks.

All these can be due to some other diseases but sputum must be tested if any of thesymptoms are present.

Tuberculosis patients may complain of general and respiratory symptoms.

General Symptoms:

++ Loss of weight.

++ Fever and sweating.

+ Loss of appetite.

+ Dyspnea.

Respiratory Symptoms:

+++ Cough.

+++ Sputum.

++ Blood-spitting.

+ Tiredness.

+ Chest wall pain.

+ Localized wheeze in lungs.

+ Frequent colds.

(The number of plus (+) sign show which symptoms are most important for tuberculosis.)

It is important to remember that all the symptoms could be due to other illnesses.

One of the most important signs, that should raise suspicion of possible tuberculosis, is thatthe symptoms have ***developed gradually over weeks or months.***

Loss of appetite, weight loss,weakness, night sweats, and ***malaise*** are also common but aremore difficult to quantify and may relate to coexisting diseases.

2.1.1. Cough

According to cough duration there is such classification: acute, subacute and chronic cough. Acute cough lasts up to three weeks, subacute cough lasts three to eight weeks, and chronic cough lasts longer than eight weeks.

Mostly *acute cough`s*reasons are viral infection of the upper respiratory tract and an acute underlying cardiorespiratory disorder. It also may be in asthma or chronic obstructive pulmonary disease exacerbation cases, and occupational or environmental exposure to irritants.

The main reasons of s*ubacute cough*are post-infectious status, asthma and bacterial sinusitis (secondary reasons). It also may be Bordetella pertussis infection which can cause acute, subacute, orchronic cough. Cough can lust`s more than two weeks in this case and coughing paroxysms, or post-tussive emesis are typically for pertussis.

Chronic cough in adults mostly is caused by upper airway cough syndrome (UACS, also known as postnasal drip syndrome), asthma, or gastroesophageal reflux disease (GERD), alone or in combination. Chronic cough has two or more causes in 18 to 62 % of patients, and three causes in up to 42 % of patients (table 1).

Chronic cough differential diagnosis. Using anamnesis (smoking status, previously sickness, life conditions, medications using), clinical and X-ray investigation, sputum analisis data, instrumental diagnostic methods doctor can estimate cough reason.

Table 1

Etiology of chronic cough in adults and children

Freuqently	Common causes	Less common causes	Rare causes

Adults	Angiotensin-converting enzyme inhibitor use Asthma GERD UACS	Bronchiectasis Chronic bronchitis Irritants (cigarette smoke) Laryngopharyngeal reflux Nonasthmatic eosinophilic bronchitis Postinfectious cough	Arteriovenous malformation Aspiration Bronchiolitis Bronchogenic carcinoma Chronic interstitial lung disease Irritation of external auditory meatus Persistant pneumonia Psychogenic cough Sarcoidosis Tuberculosis
Children	Asthma GERD Upper or lower respiratory tract infection	Foreign body (young children) Pertussis Postinfectious cough	Aspiration Congenital abnormalities Cystic fibrosis Environmental exposure Immune deficiencies Primary ciliary dyskinesia Psychogenic cough Tourette syndrome Tuberculosis

2.1.2. Pains in the chest

Chest`s painoccur not often in tuberculosis. It may be just a dull ache or worst on breathing in (in pleurisy cases), or muscle strain from coughing.In case of pneumothorax it`s acute chest pain.

Outpatient and inpatient settings have different chest pain`s reasons.There are such chest pain reasons of inpatient (emergency) department: cardiovascular conditions such as myocardial infarction, angina, pulmonary embolism, and heart failure.In outpatient medical care musculoskeletal conditions, gastrointestinal disease, stable coronary artery disease, panic disorder or other psychiatric conditions, and pulmonary disease are typically for patients suffering from chest pain (Table 2).

Table 2

Epidemiology of chest pain

Diagnosis	Primary care in	Primary care in	Emergency

	United States	Europe	department
Musculo-skeletal condition	36	29	7
Gastro-intestinal disease	19	10	3
Serious cardiovascular disease	16	13	54
Stable coronary artery disease	10	8	13
Unstable coronary artery disease	1,5	-	13
Psychological or psychiatric disease	8	17	9
Pulmonary disease	5	20	12
Nonspecific chest pain	16	11	15

2.1.3. Dyspnea

Acute dyspnea appears suddenly or in a matter of minutes. The most common causes of acute shortness of breath include:respiratory tract infections, a severe allergic reaction (anaphylaxis), asthma, a blockage in the respiratory tract, a blood clot in an artery in the lungs,a collapsed lung (pneumothorax),interruptions in blood flow to the heart muscle, heart failure, pregnancy changes a woman's circulatory and respiratory systems, even before the woman is visibly pregnant.

Subacute dyspnea develops over hours to days. Common causes include acute asthma, exacerbation of chronic obstructive pulmonary disease, or pulmonary edema. Less common causes include myocarditis, superior vena cava syndrome, acute eosinophilic pneumonia, or cardiac tamponade.

Chronic dyspnea develops over weeks to months.The differential diagnosis of chronic dyspnea has been presented in table 3.

Dyspneain tuberculosis is due to lung`s infection, or to pleuraleffusion combinedwith the lung tuberculosis.Dyspnea may be inspiratory, expiratory and mixed, and the type (mechanism) of - restrictive, obstructive and mixed. The degree of dyspnea depends on the prevalence in the lungs, the development of cardiopulmonary failure and symptoms of intoxication. The descriptions of dyspnea note its nature, duration, intensity, relationship with physical activity.

Table 3

Differential diagnosis of chronic dyspnea

System	Type	Possible diagnosis
Pulmonary	Alveolar	Bronchoalveolar carcinoma, chronic pneumonia
	Interstitial	Drugs (e.g., methotrexate, amiodarone) or radiation therapy, lymphangitic spread of malignancy, passive congestion
	Obstructive	Asthma/bronchitis/bronchiectasis, bronchiolitis obliterans, chronic obstructive pulmonary disease, intrabronchial neoplasm, tracheomalacia
	Restrictive (extrinsic)	Kyphoscoliosis, obesity, pleural disease/effusion, pneumothorax
	Vascular	Chronic pulmonary emboli, idiopathic pulmonary hypertension
Cardiac	Arrhythmia	Atrial fibrillation, inappropriate sinus tachycardia, sick sinus syndrome/bradycardia
	Myocardial	Cardiomyopathies, coronary ischemia
	Restrictive	Constrictive pericarditis, pericardial effusion/tamponade
	Valvular	Aortic insufficiency/stenosis, congenital heart disease, mitral valve insufficiency/stenosis
Gastrointestinal	Dysmotility	Gastroesophageal reflux disease/aspiration, neoplasia
Neuromuscular	Metabolic	Acidosis
	Neurogenic	Amyotrophic lateral sclerosis, muscular dystrophies, phrenic nerve palsy, poliomyelitis
Other	Anemias	Iron deficiency, hemolysis
	Deconditioning/obesity	Sedentary lifestyle
	Pain/splinting	Pleural-based malignancy
	Psychological/functional	Anxiety/hyperventilation, depression

In the case of limited pulmonary tuberculosis lesions dyspnea is usually absent. If widespread tuberculosis developing (disseminated, caseous pneumonia or widespread infiltration of seeding), the shortness of breath will be mixed. Patients who have been suffering from tuberculosis dyspnea mixed nature becomes chronic and increases as the disease progresses.

2.1.4. Hemoptysis

Hemoptysis may be in the form of blood streaks in the sputum, or the presence of blood clots without the presence of sputum. Hemoptysismay be massive, if the expectorated blood volume is either more, than500 mlduring 24 hour period or if there are bleeding at a rate more, than100 ml/hour.Because of high mortality level (50 %) in massive hemoptysis, patients need emergency treatment. Butsuch severe lung`s bleedings are only in fewer than 5%. The most common causes of massive hemoptysis are: bronchogenic carcinoma, bronchiectasis and tuberculosisor other pneumonias.

It may be massive profuse hemorrhage in cases of untrited chronic tuberculosis: fibrous-cavernous, chronic disseminated, etc.

Nonmassive hemoptysis. The most common cases of hemoptysis in the primary care departments are acute and chronic bronchitis, pneumonia, tuberculosis, and lung cancer. The differential diagnosis is in table 4.

In 60-70 % of nonmassive hemoptysis the main reason is i*nfection*.The most common pathogenetic aspect of blood expectoration is tissues inflammation, that couse blood vessels rupture. According to literature bronchitis caused 26 % of cases, pneumonia - 10 %, and tuberculosis only 8 %. Staphylococcus aureus, Pseudomonas aeruginosa, Aspergillus species are the mostly causes of hemoptysis. Another infection, such as influenza also may cause severe hemoptysis.There are may be hemoptysis in Human immunodeficiency virus infected patients because of pulmonary Kaposi's sarcoma.

In the USA 23 % hemoptysis suffered patients have lung *cancers*. In 5-44 % of all cases bronchogenic carcinoma is a reason for hemoptysis. Pathogenesis of lung`s bleeding in cancer cases is blood vesselserosion. But metastatic carcinoma of lungs has significant less frequently of bleeding.

*Pulmonary venous hypertension.*Such cardiovascular diseases can cause hemoptysis: left ventricular systolic heart failure, severe mitral stenosis and pulmonary embolism.

In 7 up to 34 % of all hemoptysis cases are i*diopathic hemoptysis*.Their prognosis is good in most cases.

Table 4

Differential diagnosis of hemoptysis

Source	Diseases
Source other than the lower respiratory tract	Upper airway (nasopharyngeal) bleeding
	Gastrointestinal bleeding
Tracheobronchial source	Neoplasm (bronchogenic carcinoma, endobronchial metastatic tumor, Kaposi's sarcoma, bronchial carcinoid)
	Bronchitis (acute or chronic)
	Bronchiectasis
	Broncholithiasis
	Airway trauma
	Foreign body
Pulmonary parenchymal source	Lung abscess
	Pneumonia
	Tuberculosis
	Mycetoma ("fungus ball")
	Goodpasture's syndrome
	Idiopathic pulmonary hemosiderosis
	Wegener's granulomatosis
	Lupus pneumonitis
	Long contusion
Primary vascular source	Arteriovenous malformation
	Pulmonary embolism
	Elevated pulmonary venous pressure (especially mitral stenosis)
	Pulmonary artery rupture secondary to balloon-tip pulmonary artery catheter manipulation
Miscellaneous and rare causes	Pulmonary endometriosis
	Systemic coagulopathy or use of anticoagulants or thrombolytic agents

2.2. Physical investigation.

Objective examination begins with an external examination of the patient and estimating of the generalcondition, which in the TB beginning can be not changed. In case of disease dissemination it may be worsening of patient general condition, but he is still active. Only in miliary TB cases, or spontaneous pneumothorax when there is acute respiratory failure, the patient may take a forced position (orthopnea). Disorders of consciousness observed in tuberculous lesions of the meninges and brain cases.

Pale skin, rosy cheeks, sparkling eyes were described as classics of tuberculosis and may be observed in long-sick and chronic forms of tuberculosis

and are manifestations of intoxication syndrome and sympathetic nervous system thoracic nodes irritation.

Examining the skin, pay attention to the presence of scarring in the areas of peripheral lymph nodes, especially in cervical group, that may testifies aboutprevious forms of tuberculosis with fistula.

Visible mucous membranes in patients with tuberculosis usually have no change, but the mucous membrane of the mouth can be affected by ulcer-necrotic tuberculosis, so you need to pay attention to it condition.

Subcutaneous tissue in patients long time sufferedof chronic forms of tuberculosis and may be poorly developed or absent (cachexia), and in the timely diagnosis of TB it can be kept normal. Edema is not characteristic of tuberculosis, but they can occur when complications such as blood circulation failure and amyloidosis.

Inspection and palpation of peripheral lymph nodes is carried on the front and back sides of the sternum clavicular-mastoid muscle, neck, around-ear, submandibular, subclavian over- and under-inguinal, elbow, inguinal and other groups.Estimatetheir number, size, shape, texture, pain sensitivity, cohesion with surrounding tissues mobility.

Tuberculosis changesof peripheral lymph nodes accompanied by increasing their size, amount (micro poly-adeniya in children), they have a rounded shape, tightly-elastic or soft, slightly painful, not fused with the surrounding tissue, fistula may form with the release of cheesy masses.Lesions in children of 5 groups of peripheral lymph nodes is a pathognomonic sign of tuberculosis.

The muscles in patients with tuberculosis are often poorly developed, they have reduced tone. But often they do not have deviation especially in patients with small form of tuberculosis.

Examining the bones and joints, pay attention to the curvature of the spine, vertebrae pain sensitivity, condition of the skin over the joints, their configuration, size, pain`s sensitivity, active and passive mobility. Manifestations of TB occur in cases of severe pathological changes in infected tissues.

Review of chest pulmonary tuberculosis patients has a great diagnostic value. Asymmetry, deformation, the volume reducing, retraction of some chest`s sections, tension of auxiliary respiratory muscles, superficial, shortness of breath, one half lag in breathing is evidence of pulmonary tuberculosis or meta-TB changes. Small forms of TB do not cause it severe changes.

Palpation of the chest conducted to identify pain areas, establish their connection with muscles, ribs, pleura, to determine reflex muscle tension, which significantly marked on the side of the affected lung. Determine the symmetry and voice tremor intensity,that is increased in patients with symptoms of cachexia.

Percussion in our time is a reliable method of clinical examination of patients. Comparative percussion can detect at the sites of abnormal thickening of the lung tissue (inflammation, tuberculosis, tumors, etc.) that appears shortening percussion sound. Small forms of pulmonary tuberculosis do not cause changes in percussion.

Auscultation is performed on the symmetric parts of the chest, comparing breathing in front, over-, under the clavicle and the sub-inguinal areas; on background: over-, inter and sub-scapule sections.

Lung tubercular process characterized by the weakening of vesicular breathing because of pleural lesions and sparing breathing in cases of lung`s atelectasis, bronchus obturation, pneumofibrosis and pneumosclerosis.

Bronchial breathing, which normally can listen at 7 cervical vertebrae in patients with tuberculosis listen to both phases of respiration in areas of cirrhosis due to loss of lung airiness and bronchoconstriction.

The large cavity with acute elastic wall and open the draining bronchus causing amphora breathing. Intermittent breath is caused by the emergence of barriers in the bronchi that prevents even the passage of air in them.Wheezing is not obligatory component of of pulmonary tuberculosis. For chronic tuberculosis is characterized mainly dry wheezing (whistling, squeaking, etc.), the feature of which is their own plase. Wet wheezing in patients with pulmonary tuberculosis often associated with infiltrative -form tuberculosis and lung destruction.

Crackling occurs in the alveoli and sometimes listen in patients with infiltrative pulmonary tuberculosis.

<u>However, often you will find nothing abnormal in the chest!</u>

2.3. Blood changes in tuberculosis patients.

Clinical evaluation of hemogram in TB patients aimed to determine the presence of intoxication, saturation, red blood cells and hemoglobin state of immunological reactivity. Usually limited forms of tuberculosis occur without changes in blood parameters.

Common forms of tuberculosis, with chronic course accompanied by a decrease in hematopoiesis and decrease the number of red blood cells in the peripheral blood of an increase in their hemoglobin (anemia hyperchromic).In cachexia the amount of hemoglobin in red blood cells decreases (hypochromic anemia), which makes gas exchange violations.

In patients with small form of tuberculosis, blood white acount does not change, while long-term cases and complicated forms of TB moderate or marked leukocytosis are typical, mainly due to the presence of intoxication.

Patients with tuberculosis in the early period of infection often have high number of eosinophil, but in chronic forms of tuberculosis their number can be reduced to extinction, which is evidence of anaergy. They also have increases in number of neutrophils because of caseous mass purulent transformation and nonspecific inflammation.Patients with TB disease have increase in number of lymphocytes in the early period of disease. In small forms of tuberculosis lymphocytes in the normal range.Severe or chronic tuberculosis accompanied by lymphopenia, a sign of immunodeficiency.Patients with tuberculosis have increase of monocytes that observed in hematogenous dissemination of pathogens cases.

ESR in patients with tuberculosis rarely changes.

There are no any pathognomonic blood changes for TB patients.

3. TUBERCULOSIS SCREENING METHODS

Tuberculosis screening methods should aim to detect Mycobacterium tuberculosis infected patients with.

3.1. Tuberculin skin test.

Tuberculin scin test is one of the most important screening methods of TB. A high importance of firstly reveald positive reactions is in early age children. It means an acute tuberculosis inflammation in child`s organism. For elder children

it`s important to find out not only “positive” reaction, but to identify it as “conversion” or “virage” of tuberculin test. Positive reactions may be as result of previously vaccination.

If patient has infected with mycobacterium tuberculosis – allergy to the tuberculin is development. In cases of tuberculin injections into the skin of infected human a delayed local reaction develops in 24-72 hours. Tuberculin includes purified protein derivative(PPD).

PPD consist of proteins with small molecular mass (10,000 Da), lipids and polysaccharides. Because of small size of PPD proteins it doesn’t react in persons, who weren’t infected mecobacterium tuberculosis with.

A batch of PPD (lot 49608) called PPD-S, which was producedby Seibert and Glenn in 1939, has continued to serve asthe international standard as well as the standard referencematerial in the United States.

In 1939 in Leningrad Research Institute of vaccines and serums dry tuberculin was produced under the direction Linnikova, it was called PPD-L.This drug is cleared (by ultrafiltration or ultracentrifugation), precipitated from chlorine-acetic acid, ether filled with alcohol and dried in a vacuum filtrate of killed by heating Mycobacterium tuberculosis cultures of human or bovine type.

In persons infected with Mycobacterium tuberculosis, or BCG vaccinated, in response to the tuberculin delayed type hypersensitivity allergic reaction occurs.In the place of injection tuberculin interacts with lymphocytes, monocytes, macrophages with antibodies to Mycobacterium tuberculosis. In reaction antigen - antibody mononuclear cells are destroyed with entering bioactive substances (kinins, skin allergy factor and so on.) and enzymes into the tissue.There is a different degree of inflammatory response at the site of tuberculin (local reaction). It can be in the form of redness, infiltration or pustules.Severity tuberculin reaction depends on the massiveness of tuberculosis infection, sensitivity to it body and its reactivity, dose of tuberculin. More severe reactions occur at the subcutaneous injection of tuberculin.

Mostly tuberculin reaction stars in 5-6 hours after injection and riches maximal expression in 48-72 hours. But in a few persons delayed type hypersensitivity may not rich maximum after 72 hours or may rich it less than 24 hours (immediate hypersensitivity).

Tuberculin test as a specific test to determine specific organism sensitization to Mycobacterium tuberculosis used for mass screening of the population (mass tuberculin tests) and individual examinations (individual tuberculin tests).

Mass tuberculinodiagnostics targets:

- identification of newly MBT-infected persons ("Virage" of tuberculin tests);
- identify persons with hyperergic and incremental reaction to the tuberculin;
- selection contingent of children to be revaccination against tuberculosis and vaccination if children that have not been vaccinated in the hospital aged 2 months or more;
- early diagnosis of tuberculosis in children and adolescents;
- identifying epidemiological indicators of tuberculosis (MBT infection of the population, the annual MBT infection risk).

To tuberculin preparations are related: PPD-L (purified protein derivative named after Linnikova), ATK-alttuberculin of Koch, tubercular diagnosticum erithrocyte dry and immune-enzyme analysis – system for definition of antibodies to the MBT. In Ukraine 2 kinds of PPD-L tuberculin are used in practice purified tuberculin:

- In the form of solutions, ready to the use, liquid form of tubercular allergen purified in standard solution for intradermal application (purified tuberculin in standard dilution, picture 1).
- Dry tubercular purified allergen (dry purified tuberculin).

Picture 1. **Tuberculin PPD-L**

For routine tuberculinodiagnostics as the only tuberculin reaction used Mantoux test with 2 TU (tuberculin units) of PPD-L ready for use. The drug is produced in ampoules as a solution in 0,1 ml which contained 2 TU and looks like a colorless transparent liquid.The use of a single tuberculin test eliminates errors and inaccuracies that occur when administered to tuberculin, simplify carrying out tuberculinodiagnostics and allows compare the results.

Tuberculin tests carried out annually regardless of the previous result. The use of tuberculinodiagnostics for the early detection of tuberculosis should allow the possibilityfor the comparison of sensitivity to tuberculin in dynamics, number and timing of BCG vaccinations, the presence and size of post-vaccinated scars, contact with TB patients, the appearance of clinical signs of disease.

In carrying out immunization schedule approved by the Health Ministry of Ukraine should take into account the time of tuberculin tests. Mantoux test is performed before preventive vaccinations against various infections. In cases where for various reasons Mantoux test is not performed before,but after immunization, then tuberculin test must be carried out not earlier than in 4 weeks (1 month) after an inoculation.

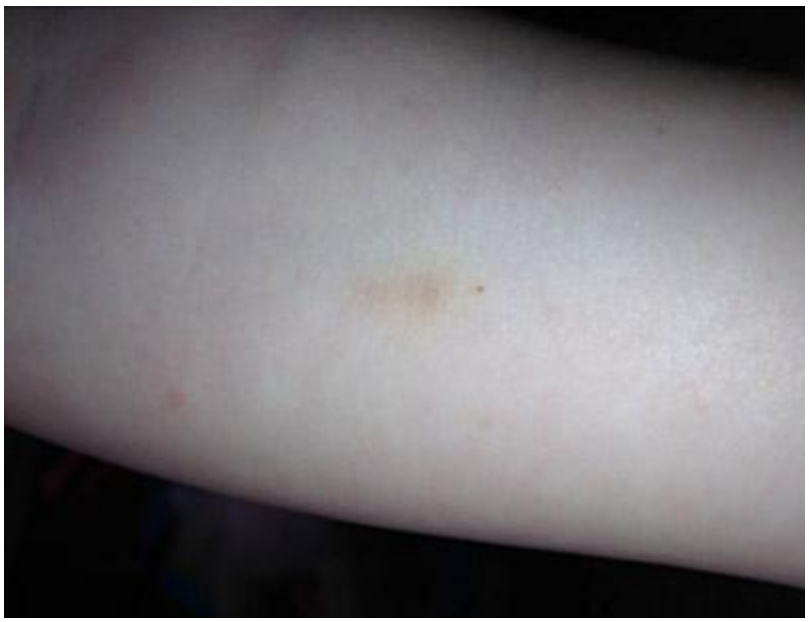
In order to early detection of TB Mantoux test with 2 TUcarried out in all vaccinated children from 4 yearto 14 yearof age and adolescents regularly annually once a year, regardless of the previous result.

Technique of Mantoux test.

For the Mantoux test one-gram disposablesyringes are used only. Ampoule of medication wiped with cheesecloth, soaked in 70 ° C of ethanol, open ampoule.Load 0,2 ml (i.e. two doses or 4 TU), poure 0,1 ml of solution. After opening the ampoule kept in aseptic conditions not more than 2 hours.The inner surface of the middle third of the forearm skin pretreated with 70 ° C ethanol and dried with cotton. A thin needle is introduced cut up into the upper layers of skin parallel to its surface - intradermally.With the right technology "a citric peel" papule in skin is formed with a size of 7-8 mm in diameter whitish color.

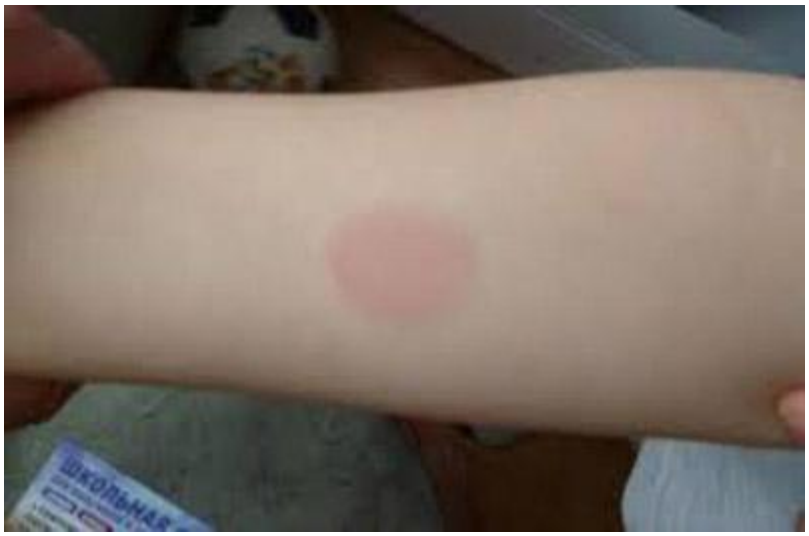
Mantoux scin test results:

- Negative if only signs of needle puncture is presented or 2 mm hyperemia (picture 2);



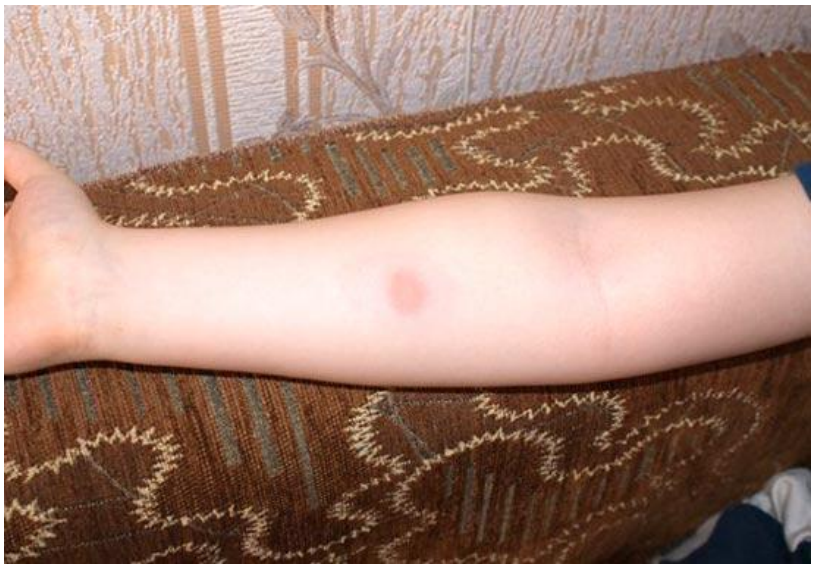
Picture 2. **Negative result of Mantoux test.**

- Doubtful – 2-4 mm of indurations or hyperemia of any size (picture 3).

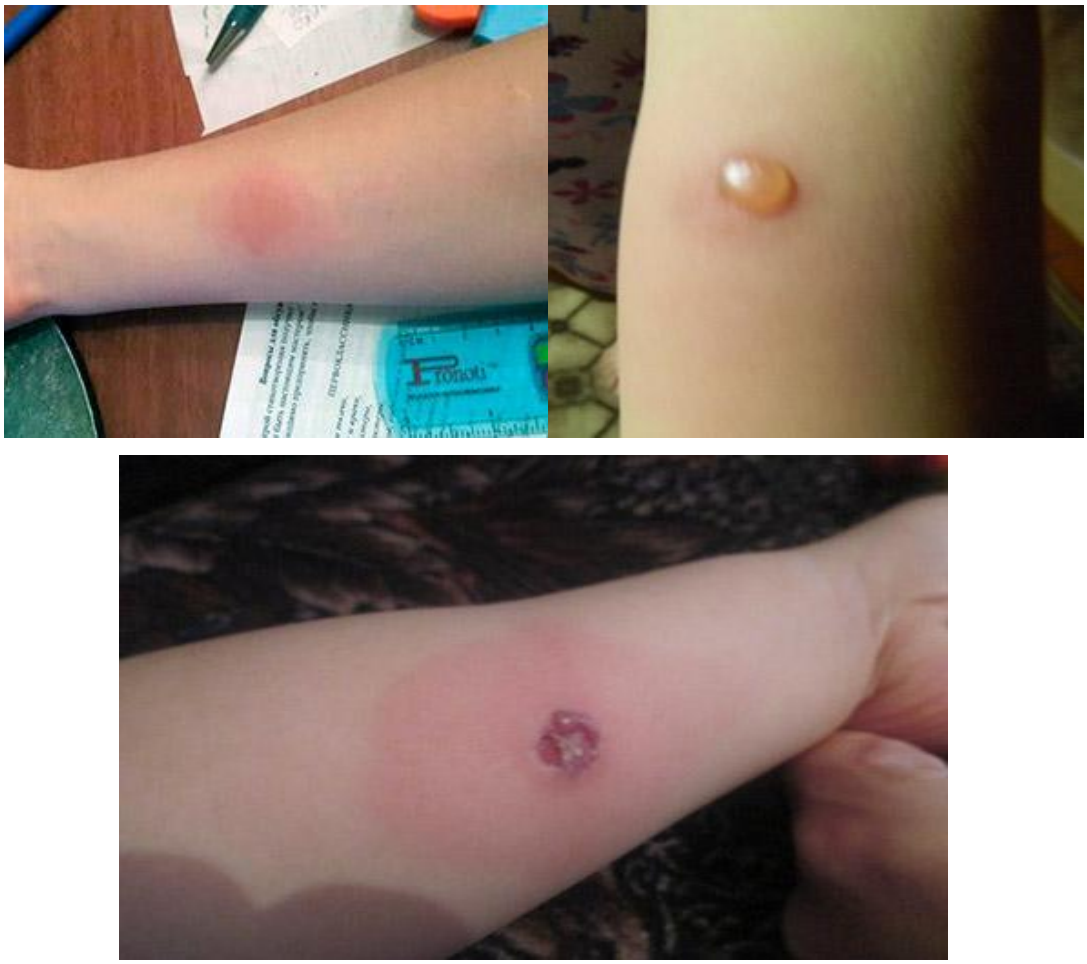


Picture 3. **Doubtful result of Mantoux test (hyperemia only).**

- Positive – 5-17 mm of indurations among children and adolescents and indurations 5-21 among adults (picture 4);
- Hyperergic – indurations more than 17 mm among children and adolescents and more than 22 mm among adults (picture 5).



Picture 4. **Positive result of Mantoux test (papula size more, than 5 mm).**



Picture 5. **Hyperergic reactions: papula more than 17 mm, vesicula, papula with vesicula.**

Mantoux skin test “virage” determined in cases:

- the first positive reaction to the tuberculin after negative or doubtful;
- an increase doubtful or positive reaction to the tuberculin 6 mm or more but not linked to post-vaccination allergy compared to a preliminary investigation;
- increased positive reaction less than 6 mm, but with the development of infiltration size 12 mm or more;
- a stable conservation of the infiltration reaction 12 mm or more, not linked to post-vaccination allergy.

Differences between postvaccinal and infectious allergies

In deciding whether this is related positive Mantoux test in children (teenagers) with infections (Mycobacterium infection, Mycobacterium

tuberculosis), or it reflects postvaccinal allergy (associated with immunity to the vaccine BCG) should be considered:

- The intensity of positive tuberculin reaction;
- The number of BCG vaccinations carried out;
- The availability and size of postvaccinal scars;
- Time elapsed after vaccination;
- Duration of residual reaction to the injection of tuberculin;
- The presence or absence of contact with TB patients;
- The presence of clinical signs of disease.

Postvaccinal allergy has less intensity and it tends to weaken when compared to the infectious allergy dynamic observation.The average size of infiltration at postvaccinal allergy is 7-9 mm, at infectious – 11-12 mm.If there arelarge scars (6-9 mm or more in diameter) tuberculin reactions with infiltration of 12 mm or more can simulate infectious allergy but actually are detection of postvaccinal allergies.Dynamic monitoring by intensity reactions that tend to weaken a 1,5 years or more after BCG vaccination helps the differentiation.

When inspection papule associated with Mycobacterium tuberculosis infection is clearly delineated, bright red color, rises above the surface of the skin.Residual reaction (pigmentation) in infectious allergy persists for more than 2 weeks. Hyperergic reaction (17 mm or more) is not characteristic for postvaccinal allergy.

Contraindications to Mantoux scin test:

- Skin diseases, acute and chronic infectious disease in acute phase, including convalescence,
- Allergic condition in acute and subacute stages,
- Rheumatism in acute and subacute stages,
- Worsening of chronic somatic diseases,
- Epilepsy,
- Quarantined because childhood diseases in children's groups.

According to American Thoracic Society Tuberculin PPD-S is use. The result depends on size of papula and risk factors presense (table 5).

Table 5

Classification of the tuberculin skin test reaction

An induration of **5 or more millimeters** is considered positive in	An induration of **10 or more millimeters** is considered positive in	An induration of **15 or more millimeters** is considered positive in
– HIV-infected persons – A recent contact of a person withTB disease – Persons with fibrotic changes onchest radiograph consistent withprior TB – Patients with organ transplants – Persons who are immunosuppressed for other reasons (e.g.,taking the equivalent of >15 mg/day of prednisone for 1 month orlonger, taking TNF-alpha antagonists)	– Recent immigrants (< 5 years) from high-prevalence countries – Injection drug users – Residents and employees of highriskcongregate settings – Mycobacteriology laboratory personnel – Persons with clinical conditions thatplace them at high risk – Children < 4 years of age – Infants, children, and adolescents exposed to adults in high-risk categories	positive inany person, including persons with noknown risk factors for TB. However,targeted skin testing programs shouldonly be conducted among high-riskgroups.

False-Positive Reactions

It may be positive TST in patients, who are not infected with mycobacterium tuberculosis. The main reasons of such false-positive reactions are:

- Infection with nontuberculosis mycobacteria,
- Previous BCG vaccination,
- Incorrect method of TST administration,
- Incorrect interpretation of reaction,
- Incorrect bottle of antigen used.

False-Negative Reactions

It may be negative TST in infected patients. The main reasons of these are:

- Cutaneous anergy (*anergy* is the inability toreact to skin tests because of a weakenedimmune system),
- Recent TB infection (within 8-10 weeks ofexposure),
- Very old TB infection (many years),

- Very young age (less than 6 months old),
- Recent live-virus vaccination (e.g., measles andsmallpox),
- Overwhelming TB disease,
- Some viral illnesses (e.g., measles and chickenpox),
- Incorrect method of TST administration,
- Incorrect interpretation of reaction.

Boosted Reactions and Serial Tuberculin Testing

In most individuals, PPD skin test sensitivity persists throughoutlife. However, over time, the size of the skin test may decreaseand may disappear. When tuberculin ijected and result is negative – repeated administration of TST can lead to increase of reaction. It`s called the“booster effect”.

Boosted reactions may be in patients, infected with other mycobacteria or in previously vaccinated persons. To identify correct result there is two-steps way. Firstly medical care workers prescribe TST and if there is negative result it repeated in 2-3 weeks. If next result becomes positive – it would be correct answer, so patient was infected previously.If second result is still negative – patient isn’t infected.

3.2. Diascintest.

Diaskintest – it`s a new screening skin test, it was founded by Russian scientists. It has higher specificity and sensitivity, than TST. The procedure of doing and estimating is the same as for TST. Diaskin test – is intradermal test.

Sample result is estimated as well as in the Mantoux test:

backlash – in the absence of papules,

doubtful reaction – if redness without papules,

positive reaction – if the papules of any size,

hyperergic reaction – if more than 15 mm papules and vesicular changes.

When doubtful, positive or hyperergic reactionstest is repeated no earlier than a month. Children are sent to the TB dispensary for more detailed diagnostics.

Recombinant tuberculosis allergen (RTA) uses for diaskin test It contains two antigens – CFP10 and ESAT6, present in strains of virulent mycobacteria. These antigens are absent in strains of mycobacteria, of which always prepared BCG and BCG-M tuberculosis.

Picture 6. **Recombinant tuberculosis allergen**

Diaskin test administred to differentiate post-vaccinated reactions and TST "virage".It`s positive only in cases of TB infection, because there are no ESAT-6 and CFP-10 proteins in BCG mycobacteria. But it may be negative in TB-infected persons (false negative reactions).

Advantages

More specific and sensitive test, there are no false positive reactions.

Disadvantages

It is believed that diaskin test may eventually replace the Mantoux test – but still can not use it to identify the indications for BCG revaccination. Children, adolescents, 7 and 14 years, it is still necessary to put the Mantoux test. There are the same contraindications to diascintest as to Mantoux test.

3.3. Interferon-γ release assays (IGRAs)

The QuantiFERON-TB Gold and the T-SPOT are two in-vitro tests, that measured cell immune response to mycobacterial antigens (proteins).Such antigens

(ESAT-6, CFP-10 and TB7.7)presented only in M. tuberculosis complex. They are absent from all BCG strains.

Sensitized T-lymphocytes of patients, infected from M. tuberculosis produce interferon-γ (IFN-γ) against specific proteins. The principle of IGRAs is in quantification estimation of IFN-γ, measured by enzymelinkedimmunoassay (QuantiFERON) or enzyme-linked immunospot (T-SPOT).

Advantages of IGRAs over TST include: greater sensitivity; higher specificity; there are significantly less problems with result`s interpretation, there are no contraindications.

Disadvantages.This test, as TST can`t differentiate latent TB infection and active TB, distinguish reactivation from reinfection. These tests needed for expensive equipment and are not cheep for patients.

3.3.1. QuantiFERON-TB Gold and QuantiFERON-TB GoldPLUS

General principles

The QuantiFERON-TB Gold IT system uses blood collection tubes that contain antigens representing specific M.tuberculosis proteins or controls. After blood collection (nil control, TB antigen and a mitogen tube for QFT-G andnil control, two antigen tubes, and a mitogen tube for QFT-GP, picture 7), tube incubation at 37°C ± 1°C for 16 to 24 hoursfollows. When incubation is complete, the tubes are centrifuged, plasma is harvested and the amount of IFN-γproduced is measured by ELISA. Results for test samples are reported in International Units relative to astandard curve prepared by testing dilutions of the secondary standard supplied by the manufacturer. The effect ofheterophile antibodies is minimised by adding normal mouse serum to the green diluent and using F(ab')2monoclonal antibody fragments as the IFN-γ capture antibody coated to the microplate wells.

Picture 7. **QuantiFERON-TB Gold In-Tube**

Baseline epidemiological data

Before performing the QuantiFERON-TB Gold IT test, baseline epidemiological data should be recorded: name, fulladdress, contact information, gender, occupation, place of birth, time since immigration (if applicable), travelhistory, history of BCG vaccination and tuberculin scin test, clinical data (medication uptake, immunosuppression, weight loss,night sweats, fever, cough, abnormal chest X-ray, previous TB treatment/chemoprophylaxis, etc.). Baselinedata should be recorded on the patient data sheet that accompanies the specimen.

Report interpretation

The predictive value of QFT-G results depends on the prevalence of M. tuberculosis infection in the testedpopulation. Each QFT-G result and its interpretation should be considered in conjunction with otherepidemiological, historical, physical, and diagnostic findings. The magnitude of the measured IFN-γ level cannot becorrelated to stage or degree of infection, level of immune responsiveness, or likelihood for progression to activedisease. Actual test data should not be reported. QuantiFERON-TB Gold IT results are interpreted using thefollowing criteria (Tables 6 and 7).

Limitations

- Diagnosis of LTBI means tuberculosis disease must be excluded by medical evaluation.

Table 6

QuantiFERON-TB Gold results interpretation

TB antigen minus Nil (IU/ml)	**Nil (IU/ml)**	**Mitogenminus Nil(IU/ml)**	**QuantiFERON-TBGold IT Result**	**Report/interpretation**
<0,35OR≥0,35 and <25% of Nil value	≤8,0	≥0,5	**Negative**	MTB infection NOT likely
≥0,35 and ≥ 25% of Nil value	≤8,0	Any	**Positive**	MTB infection likely
<0,35OR ≥0.35 and <25% of Nil value	≤8,0	<0,5	**Indeterminate**	Results cannot beinterpreted as a result oflow mitogen response
Any	>8,0	Any	**Indeterminate**	Results cannot beinterpreted as a result ofhighbackground response

Table 7

QuantiFERON-TB Gold PLUS results interpretation

Nil (IU/ml)	**TB1 minus Nil orTB2 minus Nil (IU/ml)**	**Mitogenminus Nil(IU/ml)**	**QFT-Plus Result**	**Report/interpretation**
≤8,0	≥0,35 and ≥ 25% of Nil	Any	**Positive**	M. tuberculosis infection likely
≤8,0	<0,35	≥0,5	**Negative**	M. tuberculosisinfection NOTlikely
≤8,0	≥0,35 and <25% of Nil	≥0,5	**Negative**	M. tuberculosisinfection NOTlikely
≤8,0	<0,35	<0,5	**Indeterminate**	Results are indeterminate for TBantigenresponsiveness
≤8,0	≥0,35 and <25% of Nil	<0,5	**Indeterminate**	Results are indeterminate for TBantigenresponsiveness
>8,0	Any	Any	**Indeterminate**	Results are indeterminate for TBantigenresponsiveness

- A negative result must be considered in conjunction with the individual's medical and historical data,particularly for individuals with impaired immune function.

- There are technical factors related to indeterminate results:

* Longer than 16 hours from blood drawing to incubation at 37°C ± 1°C;

* Storage of filled blood collection tubes outside the recommended temperature range (22°C ± 5°C)prior to 37°C ± 1°C incubation;

* Insufficient mixing of blood collection tubes;

* Incomplete washing of the ELISA plate.

If technical issues are suspected with the collection or handling of blood samples, the entire QuantiFERON-TB GoldIT test should be repeated. Please note that responses to the mitogen positive control (and occasionally TBantigen) can be outside the range of the microplate reader. This has no impact on test results.

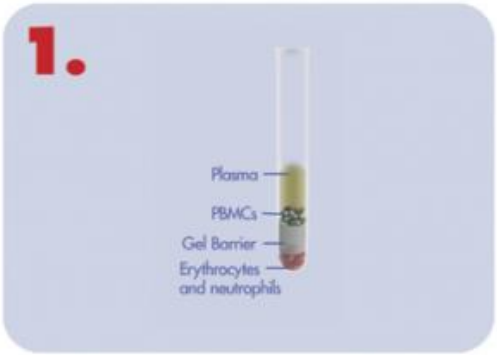
3.3.2. T-SPOT procedure

General principles

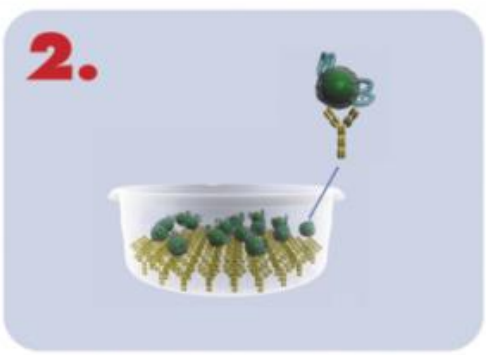
T-SPOT (Oxford Immunotec, Abingdon, UK, picture 8), unlike QuantiFERON-TB Gold, uses an enzyme-linked immunospot(ELISPOT) technique based on enumeration of activated specific T-cells responding to stimulation by specificantigens (ESAT-6 and CFP10) and resulting in IFN-γ secretion. Stimulation by ESAT-6 and CFP10 antigens takesplace in separate microtitre plate wells.

Picture 8. **T-SPOT test**

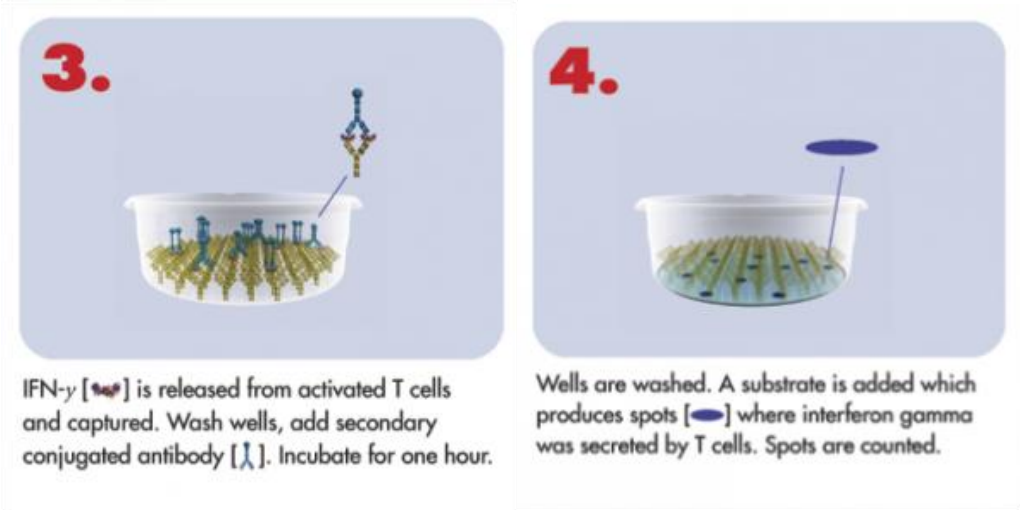
During the course of the procedure, peripheral blood mononuclear cells (PBMCs) are separated from a whole bloodsample and counted so that a standardised cell number is used in the assay (picture 9.1). The PBMCs are incubated with theantigens to allow stimulation of any sensitised T-cells present(picture 9.2); secreted IFN-γ is captured by specific antibodies onthe membrane at the base of the well. A second antibody, conjugated to alkaline phosphatase and directed to adifferent epitope on the (cytokine) IFN-γ molecule, is then added and binds to the cytokine captured on themembrane surface (picture 9.3). Finally, a soluble substrate is added to each well; this is cleaved by bound enzyme to form aspot of insoluble precipitate at the site of the reaction (picture 9.4). Each spot therefore represents the footprint of an individualcytokine-secreting T-cell, and evaluating the number of spots obtained provides a measurement of the abundanceof M. tuberculosis-sensitive effector T-cells in the peripheral blood.



Collect the blood sample. At the lab, PBMCs are separated from whole blood, washed, counted and inoculated into 4 separate microtiter wells.



PBMCs [●] and specific TB antigens [ ] are added to wells pre-coated with antibodies to IFN-γ [Y] and incubated 16 to 20 hours (37o C, CO2).



Picture 9. **Stages of T-SPOT procedure**

Baseline epidemiological data

As for the QuantiFERON-TB Gold assay, baseline epidemiological data are necessary for the correct clinicalinterpretation of the test results. Data should include name and surname, full address, contact information, gender,occupation, place of birth, time since immigration (if applicable), travel history, history of BCG vaccination and tuberculin scin test,relevant clinical data (medication uptake, immunosuppression, weight loss, night sweats, fever, cough, abnormalCXR, previous TB treatment/chemoprophylaxis, etc.). Baseline data should be recorded on the patient data sheetthat accompanies the specimen.

Reading and results interpretation

T-SPOT.TB results are interpreted by subtracting the spot count in the nil control well from the spot count in eachof the panels, according to the following algorithm:

- The test result is ‘positive’ if (Panel A minus nil control) and/or (Panel B minus nil control) ≥ 6 spots, AND anil control count <10 spots;

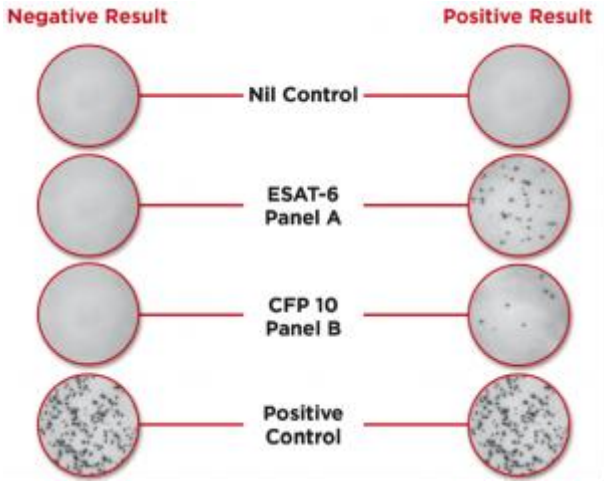
- The test result is ‘negative’ if both (Panel A minus nil control) and (Panel B minus nil control) ≤ 5 spots (thisincludes values less than zero), AND a nil control count <10 spots AND a positive control count >20 spots(or show saturation);

- The test result is “indeterminate” if:

* a nil control count >10 spots regardless of spot counts in Panel A AND Panel B; or

* a positive control count <20 spots if both (Panel A minus nil control) AND (Panel B minus nil control)≤ 5 spots (picture 10).

Due to potential biological and systematic variations, where the highest of Panel A minus nil control and Panel Bminus nil control is 5, 6 or 7 spots, the result may be considered as borderline (equivocal).Borderline (equivocal)results, although valid, are less reliable than results where the spot count is further from the cut-off. Retesting ofthe patient, using a new sample, is therefore recommended.



Picture 10. **T-SPOT test interpretation**

If the result is still borderline (equivocal) on retesting,then other diagnostic tests and/or epidemiological information should be used to help determine TB infection statusof the patient.

Reporting

The manufacturer recommends using the following wording in the laboratory reports:

- A “positive” result indicates that the sample contains effector T-cells reactive to M. tuberculosis.

- A “negative” result indicates that the sample probably does not contain effector T-cells reactive to M.Tuberculosis.

4. RADIOLOGICAL INVESTIGATION

Methods of radiological (X-ray) TB diagnosis based on the fact that the X-rays passage through the body organs and tissues resulting in their shadow image on photosensitive screen or recorded on film. There are following X-rays methods:

1. rontgenscopy;
2. radiography;
3. tomography;
4. fluorography.

*Rontgenoscopy*the principle is in imaging of organ on the screen during X-raying.This method is cheep, useful for fluid in the pleural cavity detection, but examination documentation absence, bad revealing of small changes are the bad sides of this method.

Radiography (rontgenography). Standard radiograph is a projection of «shadows» of humanorgan on X-ray film. It`s the main X-ray method for tuberculosis reveiling. It allows to see pathological process in dynamic.

Tomography Radio-tomography of the chest cavity enables to get X-ray filmswithout summation effect. This method allows detect dicay in lung tissue, to identify localization of pathological process and to reveal pathology counturs.

Fluorography – is photographing of the x-ray image from fluorescent screen. Fluorogramscould be with size of 34*34 mm, 70*70 mm and 100*100 mm and electronic. The electronicfluorograms carry out with the help of special fluorography installations, equipped with acomputer. Fluorography applies for massive preventive X-ray examinations of the population,with the purpose of revealing of latent forms of lung diseases, mainly lung tuberculosis andtumors.

Screening fluorography

In Ukraine screening fluorographyexamination (FG) is conducted every two years from 15years.According to the organization of mass preventive screening all population is divided into groups:

1. "The organized population"– employees of large companies, institutions and students in higher education. Planning preventive FG-examination and the number of contingents reported bycompanies medical and sanitary units institutions personnel department, district education departments and others. Their examination conducted by mobile x-ray stations.
2. "Employees of small businesses"– employees of agencies, enterprises conducting examination in district city clinics.
3. "Disorderly population" – housekeepers, don't working pensioners, self-employed persons. They inspection conducted in clinics in the city of residence.

Additionally "obligatory contingents" divided – employees of institutions and professionals who need to perform prior (when applying for a job) and periodic medical examination by use of fluorography inspection **annually**.

"Obligatory contingents" include:

- Students of higher and specialized secondary educational institutions;
- Persons living in the hostel;
- Employees of kindergartens and school children's institutions;
- Employees of medical and pharmaceutical institutions;

- Food industry workers who work in all phases of preparation and sale of food;
- Domestic service workers;
- Trade workers;
- Employees of public transport;
- Water utility workers;
- Workers, working in hazardous occupational conditions with high air pollution. In rural areas these contingents also includes machine operators and cattle farms employees;
- Mothers to their discharge from the hospital.

Annual x-ray, except "obligatory contingents" should be conductedto individuals with risk factors **("high risk" group):**

by medical and biological factors:

- Persons who were or are in contact with TB patients, including employees of tuberculosis institutions;
- Persons who have changes on radiographs;
- Patients, who had pleural effusion of unknown etiology (during lust year);
- Patients with pneumonia, that repeated many times;
- Persons, working on adverse for tuberculosis farms and those with TB patients animals;
- HIV-positive and AIDS patients;
- Persons with immunodeficiency any origin (prolonged use of corticosteroids, cytotoxic drugs, radiation therapy, hemosorbtion, organ transplantation, the consequences of the Chernobyl accident);
- Persons with chronic pesticide poisoning;
- Persons suffering from gastric 12-duodenal ulcer ulcer, diabetes, chronic nonspecific and occupational respiratory diseases;
- Persons suffering from mental illness;
- Those suffering from alcoholism and drug addiction.

by social factors:

- Persons without permanent residence (refugees, migrants to getting the status citizens and etc.);
- Persons, held in penitentiary system;
- Persons, who have returned from the prison (for 3 years);
- Persons, who got in remand centers and are there for a week or more;
- Unemployed;
- Persons, who are registered in the state employment as job seekers and the unemployed and those registered more than a year;
- Members of low-income families who are registered in the Department of Labor and Social Protection;
- Novices, monks;
- Pilgrims, pilgrims upon arrival at place of pilgrimage;
- Persons, who provide paid sex services.

Besides the above persons, obligatory screening should be administerto patients, who addressed for medical helpwith symptoms of respiratory diseases (cough for three weeks or more, discharge sputum, coughing up blood, chest pain, etc.).

5. BACTERIOLOGICAL INVESTIGATION

5.1. Sputum samples collection

In order to collect diagnostic material shall be used special containers:

- made from impact-resistant and transparent material that prevents leakage of fluid and allows to estimate the quantity and quality of samples collected without opening the cover;
- can be easily marked and keeps it throughout the period of storage, transportation and carrying out research;
- with the compaction screw tops (do not use bottles with closely corked cover, because at the opening of the container there is rarefied space that leads to the formation of aerosol, creating potential danger intra-laboratory contamination);
- have the volume 30,0-50,0 ml;
- have a wide hole for sputum collection (at least 30 mm in diameter) for the patient can easily separate the mucus inside the container without pollution exposing its outer surface.

The best option is to use to collect diagnostic material transparent disposable plastic containers of about 50,0-100 ml in volume. The material from which the container is made, should melt (warp) during autoclaving (picture 11).

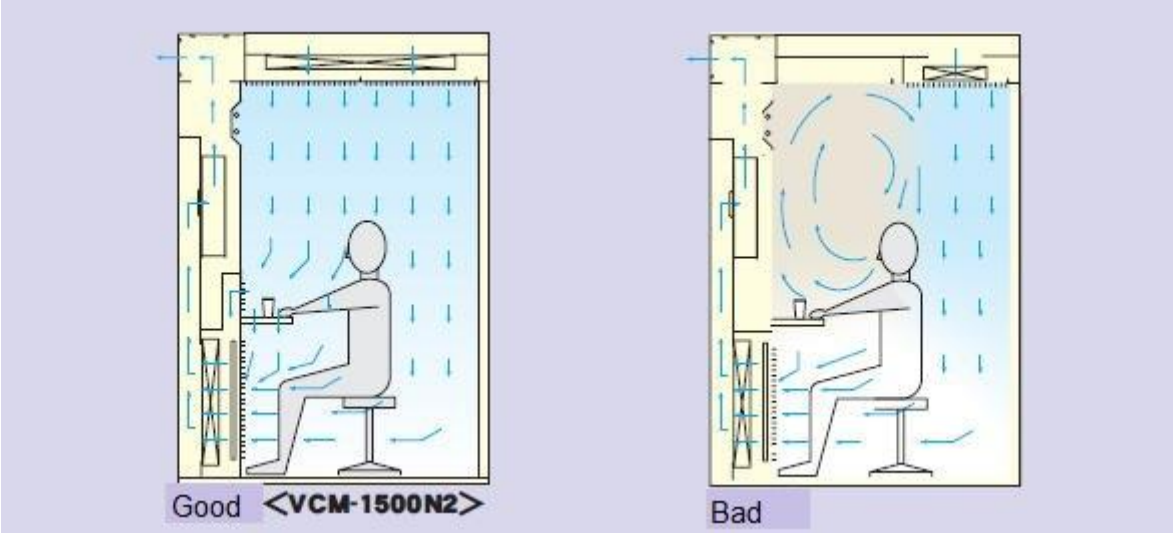
Picture 11. **Sputum containers.**

Using of these containers makes it easy to assess the quality and volume of the collected material and during preparation of native sputum smears - to make a choice of purulent lumps smear preparation directly from the container, that preventing mucus pouring phase in a petri dish, it is extremely dangerous due to the formation of aerosol.

Sputum collection for Mycobacterium tuberculosis detection - a very important stage of diagnostic procedure, investigation result depends on the clarity of which.

It`s well known that at the cough moment patients creates a very high risk of airborne infection. Therefore, it is advisable to collect sputum in specially dedicated for this purpose a separate well-ventilated room (sputum collection point), and equipped with a bactericidal lamps disinfectant or outdoors.

The ideal situation is to establish special booths in the room for sputum collection with intensive ventilation or separate sputum collection places with glass wall in the room to isolate and protect of health worker (pictures 12-13).



Picture 12. **Air circulation in sputum collection booth.**

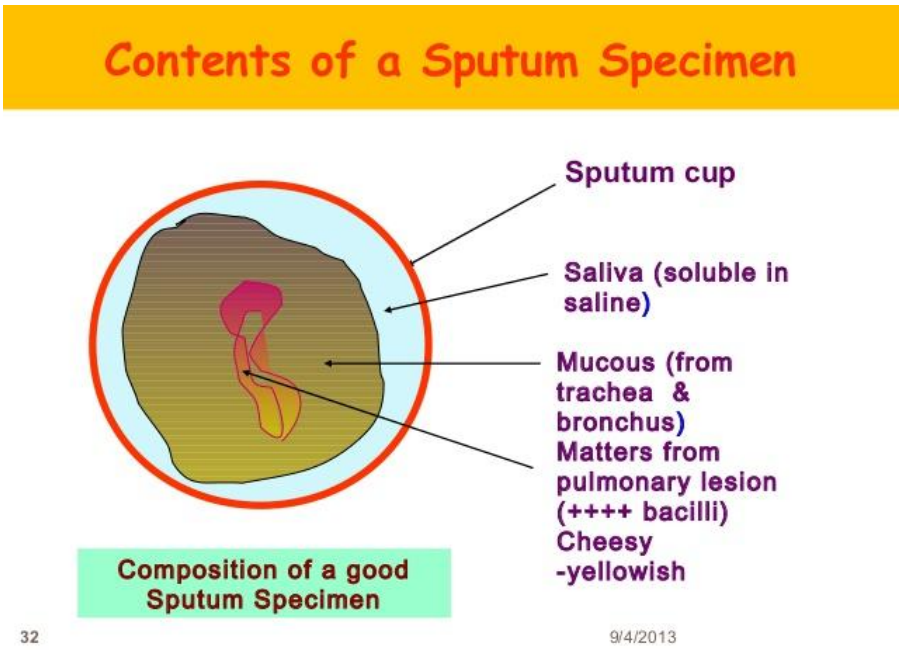
In this case, the a medical care worker can transmit to the patient sputum container for collection without opening it, and observe the process of collecting sputum through the glass.

Standing behind the patient or in an isolated part of the room, a medical worker oversees on the collection of sputum. Health workers should be advised to the patient keep container closest to the lips and immediately spit sputum into it as far as his cough.

Picture 13. **Types of sputum collection booths.**

After the sputum collection procedure a medical worker cover the container (or test how tightly it was closedby patient) and assess the quantity and quality of collected sputum.

Satisfactory quality of material assumes presence in the material mucous or muco-purulent sputum. The volume of collected material should be in the range 3,0-5,0 ml, although satisfactory quality is acceptable less(picture 14).



Picture 14. **Composition of a good sputum specimen.**

It should be noted the quality of the material in journal of registration and researches, in blank of issuing the investigation. In case of material rejection to invite new portion of the sputum.

5.2. Smear preparation

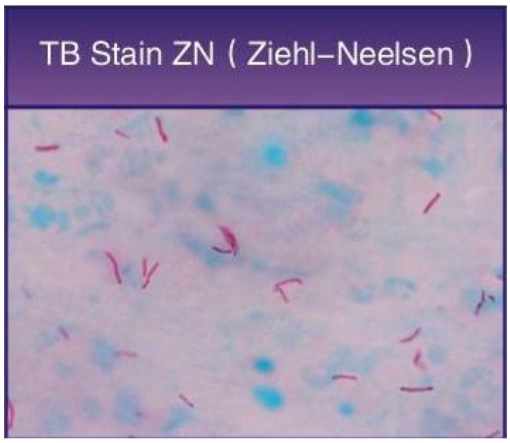
Pathology identification starts with sputum smear preparation. It allows identify acid fast baccili (AFB).

5.2.1.*Ziehl-Neelsen staining method*

This method is based on the MBT resistance to acids, alkalis and alcohol.For the detection of Mycobacterium tuberculosis in biomaterial, prepare smear on the glass, cover the entire surface of each heat-fixed slide with carbol-fuchsin,dried by air, fixed over the alcohol lamp flame upto the appearance of vapor, poured colorant and remove the filter paper, rinse smear in water, washed with 3% solution of muriatic alcohol and dried it.Stained with methylene blue or pikryn

solution that forms the background. Conduct light microscopy in immersion. Browsing the entire smear. MBT looks like as bright red rod.

Its sensitivity is comparatively low: you should have 50-100 thousand of MBT in 1 mL of sputum to detect AFB.Acid fast bacilli stain pink, straight or slightly curved rods, at times having beaded appearance. The background appears blue due to methylene blue (picture 15).



Picture 15. **Ziehl-Neelsen stain.**

Microscopy smears results estimating are in table 8.

Table 8

Reporting of microscopy smears

Results	**Explaining** **Bright field(1000x magnification:1 length = 2 cm = 100 fields)**
Negative	Zero AFB/1 length
Scanty	1–9 AFB/1 length or 100 HPF
1+	10–99 AFB/1 length or 100HPF
2+	1–10 AFB/1 HPF in at least50 fields
3+	>10 AFB/1 HPF in at least 20fields

Examine one length of the smear (2 cm) or 100 fields with light microscope, using 1000x magnification. If lessthan 10 AFB are found in 100 fields, the number of AFB should be counted. For 2+ and 3+ results only20 to 50 fields examinated.

5.3. Culture tests for Mycobacteriumtuberculosis complex

5.3.1. Sample collection

Sputum samples

Most often in order to diagnose TB in patient sputum investigated. There are no problems with sputum obtaining in patient with a productive cough. If the patient can not expectorate sputum, you should use other methods (expectorant drugs, the use of irritating aerosol inhalation, washing trachea, bronchi, etc.).

Other specimens

Biological fluids (cerebrospinal fluid, pericardial, synovial, ascitic fluid, blood, bone marrow) should be collected aseptically by physician in a sterile container using appropriate methods.To liquids have a tendency to clotting, add sterile heparin (0,2 mg in 1,0 ml) or potassium oxalate (0,01-0,02 ml of 10,0% neutral oxalate in 1,0 ml of material). Material must be delivered to the laboratory immediately.

Aseptically collected tissues are placed in a sterile container without adding any conservants. In the case of long transportation tissue must be placed in sterile isotonic, imposed of dry ice to cool at 4-8° C. Material must be delivered to the laboratory immediately.

Urine (middle portion of the morning or the whole morning portion) is collected in a sterile container thoroughly after toilet vulva. Analysis of urine for mycobacteria should provide for the compulsory three-fold investigation. The laboratory centrifuged urine using a method of accumulating sediment.The feature existence of M. tuberculosis in liquids is their ability to stay a long time in a suspension. Therefore recommended centrifugation at 3000 g of whole material, not its bottom fraction derived after sedimentation in vivo.

Daily urine collection for bacteriological research is not practiced. This is because the accumulation of urine during the day can not save it without accumulating of organisms. Keeping containers in the urine in a cool place can lead to falling of salt, which adversely affects the further processing of sludge.

In addition, urine contains antibacterial products that can not only inhibit the viability of mycobacteria, but during the day even destroy microbial cells. At the same time, long-term storage of urine can not avoid purulent microflora multiplication.

Storage of specimens

In order to increase the cultural method results period between collecting the material and its processing should be minimal. The material should be sent to the laboratory immediately after collection (within 24 hours). In the case of laboratories distance from the taking material place it`s sending to the laboratory may be twice a week.In this case, the containers of collected material should be stored in a refrigerator at 4-8° C up to 72 hours. If it`s necessary, store of material over 72 hours may be if conservantis added to the diagnostic, in this case storage time increases up to 5 days.

Aseptic material should be sent to the laboratory immediately!

For other materials if their transportation is expected at high environment temperature or delivery to the laboratory is more than 24-72 hours after collection (registration), it is recommended to use these chemical conservantes:10,0% solution of tri-sodium phosphate, 1,0% tsetylpirydyn chloride solutionin 2,0% sodium chloride, 2,0-3,0% boric acid solution.

Listed solutions recommended, primarily, to preserve samples of sputum. If their application, material can be kept at room temperature.But conservants are toxic for mycobacteria, and their use can reduce the seeding of mycobacteria. To reduce the toxicity of conservants is recommended to keep samples in the refrigerator at a temperature of (+4 to +8) °C.

Diagnostic material can be frozen and in case it will not be subjected to repeated unfreezing and freeze viability of Mycobacterium will be kept.

Transportation of specimens

For safe transportation of bacteriological material it should be packed in a waterproof, not beating container that as well protected from concussions, shock and other possible damages.The majority of material that is sent to the laboratory, sent to it in the same container where the sputum is removed, so it is advisable to have laboratory several special metal or plastic transport boxes (picture 16).

They constructed so, that can fix of 20-30 containers with diagnostic material upright. The boxes cover should be securely closed to preclude spontaneous containers cover opening with samples rash. For transportation you can use metal boxes. When transporting the material must, if possible be cooled and not be in the sun.

Material acceptance and registration

Depending on the purpose of the investigation direction form number 200 -1 "Destination on bacterioscopic research TB 05" filled to the samples, or the form number 200 - 2 "Destination on bacteriological examination TB 06".

Picture 16. **Transportation of M. tuberculosis cultures.**

In addition, to each container (box) filling form number 240 - 1 "Description of sputum samples, which is sent to the laboratory 05a TB".The listed documentation separately placed from the material (in the file, envelope, plastic bag, etc.) out of the transport container (box) with samples.

Before sending the collected material a medical worker should check:1) whether the number of sputum containers with their numbers indicated in the list of TB 05a; 2) whether the number of each container with number on the TB 05a; 3) whether a list of all the necessary information about each patient.

Material admission should be conducted in the special space. Transport boxes with containers should be opened in a fume cupboard, or on a specially desk with the following requirements.

- Wear disposable gloves. If a transport container opened in biosafety cupboard, you need to wear anti-aerosol respirator.
- Carry out external container processing with appropriate disinfectant.
- Carefully open container and check the integrity of containers. Broken containers decontaminated by immersion in disinfectants, boiling or autoclaving. From these containers samples are not investigated. In this case, you must request a new sample for analysis.
- Extract containers with material from transport box. To carry out processing with disinfectants of exterior surfaces of the containers, which are in box.
- Disinfect the inside of box. If there is contamination of transport box, to getit an autoclave or immerse in disinfectant solution.
- To verify compliance numbers in accompanying documentation with numbers marked on containers of material.
- Assign laboratory serial number according to laboratory journal.

5.3.2. Homogenisation and decontamination of specimens

Most clinical samples of material, supplied to the microbiological laboratory for bacteriological research for diagnosis and control of tuberculosis chemotherapy, , are contaminated with bacteria that are growing rapidly in varying degrees, their lush growth on rich nutrient media hinder mycobacteria development and complicates their selection.

Therefore, diagnostic material before seeding on nutrient media subjected to special treatment, providing decontamination, i.e. the utilization of putrefactive microflora.

Mycobacterium tuberculosis isolated from the respiratory tract of the patient, usually surrounded by a large number of mucous substances that complicate their selection. So sputum and other similar materials before seeding simultaneously with decontamination subjected to dilution and homogenization.

All drugs currently used for diagnostic material dilution and decontamination have expressed toxicity regarding to mycobacteria.To ensure the survival of sufficient mycobacterial population we should use processing techniques that allow, on the one hand, to suppress fast-putrefactive bacteria, on the other - to preserve the viability of mycobacteria present in the material.

The frequency of contamination of cultures (number of not-growing cultures) in laboratories usually riches 2,0-5,0%.If clinical material before entering the laboratory was kept for several days in unregulated conditions, the frequency of contamination can reach more than 5,0%, which is unacceptable.If the number of not-growingcultures less than 2,0%, it indicates too hard mode of decontamination, which can lead to the death of a large part of MBT contained in the diagnostic material.

The following solutions used: 10 % tri-substituted sodium phosphate (Na_3PO_4); N-acetyl-L-cysteine and sodium hydroxide(NACL-NaOH); 4,0% sodium hydroxide; 3,0% sulfuric acid; 5,0 % oxalic acid or 4,0% sulfuric acid.

5.3.3. Culture media

There are 4 main groups of various culture media for diagnostic material sowing:

- egg-based media: Löwenstein-Jensen (LJ) medium, Finn II and Ogawa medium;
- agar-based media: Middlebrook 7H10 and Middlebrook 7H11;
- liquid media: Middlebrook 7H9 broth;
- liquid synthetic and semi-synthetic nutrient media.

Each of these media has its advantages and disadvantages. The optimum environment for the MBT cultivation should be inexpensive, easy to prepare, contain available components.In addition, the media should inhibit the growth of accompanying microorganisms, provide good growth during the sowing of small number of mycobacteria and the possibility of grown colonies pre-differentiating, based on morphological characteristics.That is the suitable medium should have good inhibiting, growth and differential properties. Egg media most of all respond the above requirements during the sowing of sputum.

Solid media

Egg-based media

The *advantages* of egg-based media:

- cost (the cheapest of all the media, used for the Mycobacterium selection) and ease of preparation;
- can be kept in the refrigerator for up to 4 weeks;
- well support the growth of most strains of Mycobacterium tuberculosis;
- allow preliminary identification of mycobacteria colonies on morphology;
- malachitegreen, which is part of media, inhibits the growth of accompanying flora that grows quickly, reducing the probability of contamination.

*Disadvantages*of egg-based media:

- the appearance of mycobacteria growing within 2 to 12 weeks and more.

- if in cultivation process accompanying microflora growth appears, it observed on the entire surface of the culture medium, so that these tubes must be culled.

To improve bacteriological examination performance it`s recommended to seed diagnostic material simultaneously on 2- 3 nutrient media of different composition.

Results of numerous comparative tests established, that the bacteriological diagnosis of tuberculosis should use at least two different composition of the culture medium. The most widely used in Ukraine 2 egg-based media - LJ and Finn-II.

LJmedium is used worldwide as the standard medium for the initial selection of Mycobacterium tuberculosis and determine its sensitivity to antimycobacterial drugs.This medium recommended for use in all TB service microbiological laboratories of Ukraine in order to obtain comparable results.

LJ medium - an egg-based medium, where good growth of Mycobacterium tuberculosis receive approximately in 18-25 days after clinical material sowing with positive microscopy for AFB.

The composition of the culture medium is glycerol, which promotes the growth of M. tuberculosis. To isolate M. bovis it`s recommended to use version of LJ medium, to which instead of glycerol is 0,5% solution of sodium pyruvate.

Finn-II medium is recommended in Ukraine as the second standard medium for mycobacteria selection.It differs from the LJ medium that instead of L-asparagine it uses glutamic acid-sodium (monosodium glutamate) and salt composition is designed so that the final acidity of the medium has lower values (pH 6,3-6,5) than LJ medium (pH 7,2-7,4) and more stability.These properties lead to higher medium efficiency during the treated with alkaline detergents material sowing. The growth of mycobacteria appears in this environment for a few days before on LJ medium, and the selection cultures percentage is 6,0 – 8,0% higher.

Agar-based media

These media are prepared in slant tubes or plates and are less likely than egg-based media to becomecontaminated. Middlebrook 7H10 and 7H11 media are usually prepared in the laboratory from commerciallyavailable agar-powdered bases, with the addition of Middlebrook oleic acid-albumin-dextrose-catalase (OADC)enrichment. Because of the transparency of 7H10 and 7H11 plates, M. tuberculosis micro colonies with typical cordformation can be detected and counted using a microscope as early as one week after incubation.

Liquid media

Liquid media offer a considerable time advantage over solid media: 7–14 days in Middlebrook 7H9 liquid medium,compared with 18–28 days in Middlebrook 7H11 agar, or 21–42 days in LJ medium.

5.3.4. Culture tube inoculation

Solid media

Before the seeding procedure to prepare the tubes with culture medium, numbered them, according to the registration numbers of samples, and consistently place in a vertical tripod. Similarly, prepare and numbered glasses.

The precipitate, obtained after diagnostic material preprocessing by one of the above methods should be subjected to microscopic and bacteriological parallel research in the following order.

Loadin sterile dimensional pipette (preferably disposable plastic Pasteur) 1,0-1,2 ml of prepared sediment, leaving about 0,1-0,2 ml for further preparation for smear microscopy.

Compliance with sterility, apply equal volumes of of the dialed material (approximately 0,5-0,6 mL) in 2 tubes with different media.

Tubes with medium during the sowing must be in an inclined position (at an angle of 40-45 °).

Put in diagnostic material on the upper third of culture medium.

Sown tubes close with cotton-gauze corks and place in an upright position in the a tripod so that the sowing material evenly distributed across the surface of the culture medium; it`s better to use tubes with screw tops.

Remaining precipitate pick up with the same pipette and put on a prepared numbered glass slide 2-3 drops of sediment to obtain smear for microscopic examination, distributing the material evenly in the center of the glass in the area of about 1,0 x 2,0 cm.

Used for sowing and preparation smear pipette to drop a container of disinfectant solution.

After seeding completing all samples in the tubes move in horizontal tripods and placed in an incubator at 37° C. The culture medium surface should be in the horizontal plane and inclination tripod should eliminate seeding material corc wetting when cotton-gauze plugs using.

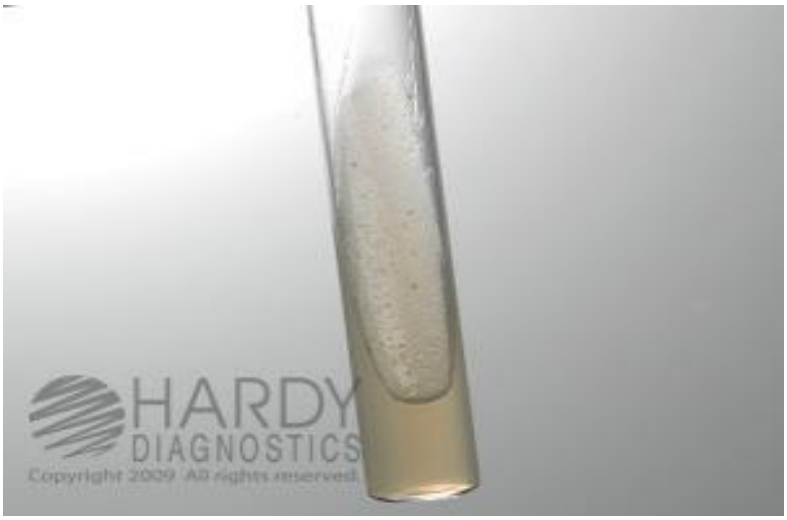
Liquid media

Detection of Mycobacterium of bouillon culturing using automated systems necessarily involves samples seeding parallel to the egg-basedcultural media.

Diagnostic material inoculation in the liquid medium is carried out simultaneously with sowing in egg-basedmedia that is needed for better results, which can give rise to only one of the media. This principle can also avoid some of the errors associated with technical errors, incorrect interpretation of positive growth in vitro.

In order to confirmation of the culture that has grown in a liquid medium of any analyzer it should be carried out ZN microscopy and sub-culturing on egg-based media.

The growing of mycobacterium TB on Middlebrook medium showed on picture 17.



Picture 17. **Mycobacterium TB growing on Middlebrook medium.**

5.3.5. Culture incubation

Incubation of cultures in order to detect MBT requires for long term growing of visible colonies. Long incubation necessitates adherence to a set of rules to preserve the mycobacterial cell viability and growth properties of the nutrient medium.

The optimal incubation temperature is 37° C.

During the first seeding of microscopically negative material average duration of mycobacteria growing on solid nutrient media can be 20-46 days. The growth of some strains appears after 60 or more days. This necessitates, in the absence of growing of mycobacteria colonies in the thermostat to 10 weeks for the issuance of a negative result.

Incubation is carried out for 10 weeks with obligatory weekly viewing of tubes with cultures.

5.3.6. Culture examination

In evaluating culture results of diagnostic material is necessary to adhere the following rules:

- Observation and tubes viewing should be performed weekly.

- In the absence of growing tubes should be left in an incubator for 10 weeks. The negative result of bacteriological research can only be issued after this period of incubation.
- During the regular review all the tubes with growth of colonies should be taken away, put in numerical order of registration material.

Estimating results register the following parameters:

"The appearance of growth" - the date of the appearance of growth in test tubes (in the case of growthappears simultaneously in both tubes). If the culture is grown only in onewith tubes (with a good growth culture in relevan terms), and the secondgrowth is not recommended to register the date of growth appearance and use firstube for further work without waiting for the appearance of growth of colonies in another tube.The second tube is left inincubator for further incubation and if it continues to register growthresults;

"Intensity of growth" - the number of colonies, that grew in each tube.If simultaneous growth in all tubes is recommended to evaluatethe number of colony forming units in each tube, which was sown from this material;

"Sprouted up"when foreign microorganisms or fungi are present;

"Absence of growth" (specified parameter is recorded after 10 weekscultivation).

The appearance of MBT growing in 7-10 days of cultivation on solid nutrient media may indicate the selection of non-tuberculous mycobacteria that rapidly grow and not in the M. tuberculosis complex, so before answers such culture should undergo initial identification.

The appearance of colonies growing after 3-4 weeks of culturing shows the selection M. tuberculosis, and other slow growing mycobacteria, which may be of potentially pathogenic non-tuberculous mycobacteria or saprophytes.

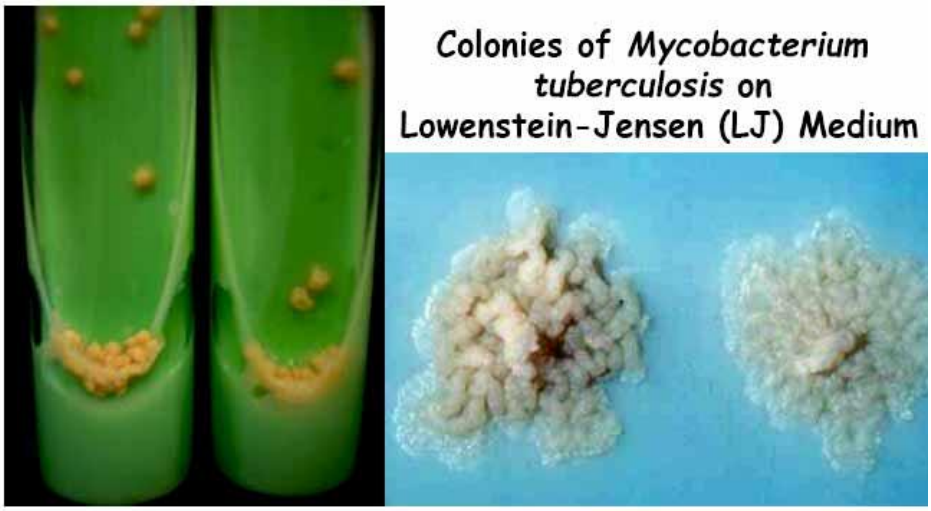
In assessing the results, should remember that used for sowing enriched with substrate nutrient medium is easily utilized by other microorganisms. This leads to a high risk of contamination by various bacteria and fungi, colonies are visually difficult to distinguish from mycobacteria.

During the cultivations weekly views in contamination of extraneous microflora suspected it is necessary, first of all, remove and neutralize those tubesin with marked contamination of the entire surface or changing the culture medium (vacuum or discoloration).

Seedings with partial contamination is desirable to sustain the expiration or the incubation to appear of at least several colonies of Mycobacterium because late appearance contamination does not exclude growth of M. tuberculosis. In such cases it is necessary to make ZN smear and in case of MBT presence try to process the culture that has grown with 3,0-4,0% of sulfuric acid, and then launder it with isotonic sodium chloride to sow precipitate again into nutrient media.

Characterization of M. tuberculosis colonies

Cultures should be read within 5to 7 days after inoculation and once a week thereafter for up to 8 weeks.Typical non pigmented, rough, dry colonies are seen on LJmedium. The green color of the medium is due to the presence of malachite green which is one of the selective agents to prevent growth of most other contaminants (picture 18).



Picture 18. **M. tuberculosis growing on LJ medium.**

Virulent Mycobacterium tuberculosis cultures typically grow on solid media in the form of R-colonies of various sizes andhave a yellowish or slightly creamy

shade (ivory color), a rough surface that resembles semolina or cauliflower.Colonies are usually dry, wrinkled, but in the case of dissociation they may be moist, lightly pigmented colonies, pink and yellow pigment whichvery different from the orange or yellow pigment or saprophytic some nontuberculous mycobacteria.

It should be noted that the Finn-II media colony often lookwetter than the Lowenstein-Jensen medium.After the chemotherapy of tuberculosis patients sleek colonies with moist growth (S-shaped) can be released.

When preparing smears for microscopic examination of coloniesMycobacterium tuberculosis they show their physical-chemical characteristics: they are notemulsifiable in isotonic solution, and form a granular crumb-likesuspension.

Colony growth quantitative estimation.

Depending on the number of colonies grown MBT estimated result: negative, positive 1-20 colonies 1+, 2+, 3+ and 4+ (table 9).

Table 9

Reporting of colony growth

Results	**Explaining**
Negative	Zero
Scanty	1–20colonies
1+	20-100colonies
2+	100-200colonies
3+	200-500 colonies
4+	More, than 500 colonies

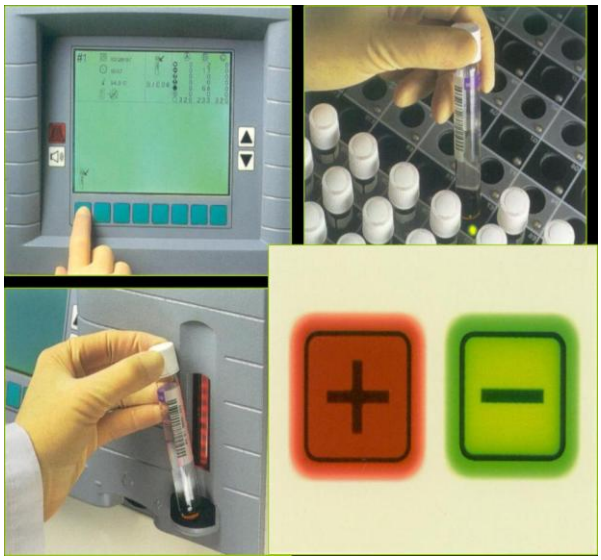
5.3.7. BACTEC MGIT 960 system

BACTEC MGIT 960 is a fully automated system for simultaneous incubation and monitoring of 960 tubes. Cultivation of Mycobacterium carried outthe indicator tube MGIT, containing 7,0 ml of modified environmentMiddlebrook 7H9.This system can detect clinical specimens of most strains of Mycobacterium tuberculosis within 10-20 days and determine the sensitivity of culturethe causative agent of drugs in a period not exceeding two weeks.

It should be emphasized that the BACTEC MGIT 960 is the only fullyautomated system for determining mycobacteria susceptibility todrugs that provides rapid culture testto almost all drugs, including pyrazinamide.

Advantages of the method: the receiving culture twice reduces and determinationthe sensitivity of mycobacteria to medicinal drugs, increases the frequencydetection of the pathogen in oligo-bacillary material from patients with tuberculosis andalso improves the accuracy and repeatability of the results of microbiologicalresearch.

The device weighs 351 kg, its size is small (92h135h85sm), not required specialconditions for its placement in a laboratory. It consists of threesections that accommodate over 320 tubes each, so the maximalsimultaneous loading device - 960 tubes. Control over included in theindicator tube material, carries a built-in a device computer.Liquid crystal display and custom indicators on each section giveinformation about the presence of positive and negative results (picture 19).

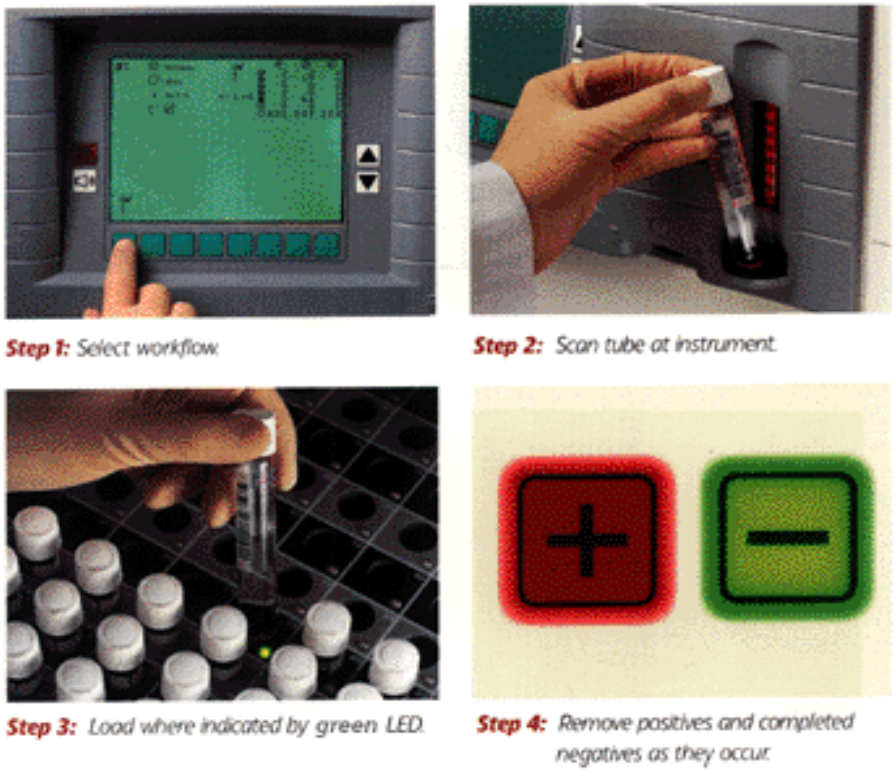


Picture 19. **BACTECMGIT 960 system.**

An important component of the system is Mycobacteria growth indicator tube with luminescence fluorescent indicator, which extinguished by oxygen. Microbial actively multiplayingpopulationabsorbs oxygen, releasing fluorescent component that startsilluminate in the rays of ultraviolet light.

To accelerate growth and reduce contamination of Mycobacterium providedaddition liquid nutritional supplements OADC and five lyophilized antibiotic PANTA added to 7H9, which contribute to the indicator tubebefore sowing.The device evaluates tube as positive if the number of living organisms in it reached10^5-10^6per 1,0 ml of medium.

The BACTEC MGIT 960 System was designed with simplicity in mind, ensuring maximum productivity with minimal staffing and training. Bar code scanning guides the simple 4-step operating procedure, eliminating potential errors (picture 20).



Picture 20. **BACTEC MGIT 9604-step operating procedure.**

Firstly you should press «Tube enter» on BACTEC MGIT 960 display regimen "Loading tubes". Thus scanner lamp switches on to read the bar code on the tube.Scan a tube barcode and install it into the slot that recommends.“Positive results” (growth of mycobacteria) introduced as redpositive indicator signals on the relevant box and on icon display.When the information about the positive result you should open set, press the «positive», which appeared on the screen, pull out tube from the slotand scan the barcode.

The tubes, which are not fixed growth of Mycobacterium during 42days, the system evaluated as negative. Negative result(no growth of mycobacteria) introduced as green signal of negativeindicator on the relevant box and icon on the display.

5.3.8. Identification of Mycobacterium tuberculosis

After morphological identification for differential diagnosis of Mycobacterium tuberculosis and non-tuberculosis mycobacteria (NTB) a number of tests carry out. Mycobacterium tuberculosis grow no less, than 10 days.

Growth in different media

In LJ medium MBT colonies are ivory have dry form with irregular edges.Growth possible only at 35-37° C.Growth on solid nutrient media appears no earlier than 3 weeks.

The absence of MBT growth in the medium with 500 mg / ml of salicylic acid-sodium or 500 mg / ml paranitro-benzoic acid (PNBK), and with 1000 mg / ml tioatsetazon (tibon).

Growth on the medium with 5,0% NaCl

The method is based on the ability of nontuberculous mycobacteria of IV group grow on the medium with 5,0% NaCl. Besides this group, in this environment grow only M. terrae complex (including M. triviale, M. terrae, M.

nonchromogenicum (III group)) and M. flavescens (group II), as well as some mycobacteria from I groups (M. marinum). All other mycobacteria, including M. tuberculosis and M. bovis, do not grow on this environment.

Detection of cord factor

Nontuberculous mycobacteria grow diffusely in the form of humps, unlike true tuberculous mycobacteria, growing looks like film or bottom, with cord-factor and grow as a "braid", "strands", "mustaches" - with close intertwining of individual sticks with one another.

Sensitivity to cycloserine

All strains of M. bovis-BCG observed resistance to 30,0-50,0 mg / ml of cycloserine. This biological feature of the BCG vaccine strain is an important diagnostic test to identify it.

Biochemical test of identification

Niacin test

Niacin producedby all mycobacteria, but M. tuberculosis as a result of blocking a number of metabolic pathways nicotinic acid accumulates in large quantities. Therefore, this test is a major, which allowsdistinguishing M. tuberculosis from other mycobacteria.

The principle of the method is determining of nicotinic acid by chemical methods in the culture medium, but not in the mycobacteria using cyanide compounds, nicotinic acid gives a bright yellow color.

Nitrate reduction test

To identify M. tuberculosis reaction of reduction of nitrate to nitrite also used. The reaction of nitrate reduction makes it possible to differentiate M. tuberculosis, which have nitrate reductase from M. bovis, M. avium and some non-tuberculous mycobacteria in which this enzyme is absent. The exceptions are photo-chromogenic MBT (M. kansasii) and some of the groups III and IV.

The activity of nitrate reductase is determined by the amount of reduced nitrate from nitrite, which gives the color reaction with para-dimethylamino-benzaldehyde.

Determining ability to growth in the medium with nicotinamide

M. tuberculosis is susceptible to nicotinamide. M. bovis vaccine strain has a natural resistance to nicotinamide. Differentiation is based on this features of tuberculosis complex.

Determination of catalase and peroxidase activity simultaneously

Principle of catalase reaction consists in disjoined of hydrogen peroxide by enzyme catalase to water and atomic oxygen, which is accompanied by of bubbles of oxygen and transition pyrogallol in purple-galin in the presence of hydrogen peroxide under the influence of peroxidase.

Thermostability catalase

Catalase in MBT is different. In virulent MBT it quickly and easily destroyed when heated to 65-68° C. In y nontuberculous MBT and saprophytes it is thermostable.

The reaction of hydrolysis of Tween-80

An important reaction for MBT identification in the second and third groups is the tvin-80 hydrolysis reaction. Tween-80 binding neutral red and the mixture reaction has straw-yellow color.Principle of reactions is inenzyme hydrolysisof tween-80. This releases a neutral color red from pink to red. The positive reaction observed in M. aquae (unlike M. scrofulaceum, in which the reaction is negative), on the group III it is positive only for M. terrae.

5.4. First and second-line drug susceptibilitytesting for Mycobacterium tuberculosiscomplex

5.4.1. General method – DST by culture

Currently there is no a single universal method for determining the sensitivity of the MBT. There are cultural methods using solid and liquid culture media with automatic detection of mycobacteria growing and express molecular genetic methods.

The choice of a method is determined according to methodical approaches traditionally used and developed in this country.Effective monitoring management, providing epidemiological surveillance of resistance of mycobacteria to medicinal drugs and the spread of resistant strains of the pathogen and for comparing the results of research and treatment effectiveness within the international community on the scale of each country is recommended to use only one of the available standardized methods regulated by internal regulations countries.

All available methods for determining the sensitivity of MBT can be divided into 2 categories:

- Direct methods of determine of the MBT sensitivity;
- indirect methods of determining the MBT sensitivity.

When using direct methods of the sensitivity of MBT determining, sputum or other clinical materials previously disinfected and homogenized, sown directly in medium containing the appropriate drug. Number of inoculum is determined by the amount of AFB defined in the smear.

Methods of direct determination of sensitivity have a number of drawbacks:

- for research can not be used diagnostic material samples with negative microscopy;
- during this research increases the risk of contamination;
- there may be a an insufficient culture growth that does not allow reliable conclusions;
- the main drawback is the inability to standardize the methodology.

When using indirect methods of determining the sensitivity of MBT selection of microorganismsperformed from clinical samples by culturing and then

on medium containing drug homogeneous culture suspension, grown in broth is sown.

There are three main classical microbiological methods of indirect determination of MBT sensitivity:

- the method of proportions, proposed in 1963 by Canetti, Rist and Grosset and detailed in 1985 by Middlebrook and Cohn.
- absolute concentration method on solid and liquid media, modified in 1970 by Meissener.
- resistance coefficient method, developed in 1961 by Mitchison and others.

The best known from the classical culture methods for determining susceptibility to Mycobacterium TB drugs is method of proportions, At present time it`s generally accepted in Ukraine, which allows you to determine what part of mycobacterial population in percentage terms is resistant to the drug.

5.4.2. The proportion method on Löwenstein-Jensen medium

The principle of the method is to determine the ratio (proportion) between resistant and susceptible individuals in the M. tuberculosis population strain, which is selected from a patient with TB to TB drugs in "critical" concentration.

"Critical" concentration it`s one of the criteria of resistance. This is a strictly defined quantity of each drug preparation, which should contain the medium for DST setting.

"Critical" proportion it`s another criteria of resistance - a percentage of resistant individuals in the bacterial population in which or above which the strain is considered resistant to this drug.

If the number of resistant individuals to some antibacterial agent in the population will be less than 1,0%, a strain considered susceptible to the drug. If the resistance individuals in a population is more than 1,0% - the strain is considered resistant to the drug (picture 21).

Picture 21. **TB susceptibility agar proportion test.**

Indirect absolute concentrations drug susceptibility testing

The method is performing by dosed seeding carefully prepared suspension of mycobacterial culture from tubes with nutrient LJ containing certain concentrations of antituberculosis drugs and test tubes without drugs.

Usually "critical" concentration of drugs used, which is the criterion of resistance, inhibits the growth of all or almost all mycobacteria, defined as the presence of 20 or fewer colonies of the pathogen and allows to define the culture of MBT as sensitive or resistant to TB medication.

The evaluation results to determination resistance of mycobacteria to antituberculosis drugs conduct in 3 weeks of incubation in an incubator. If MBT do not grow on the control nutrient medium it should wait 1-2 weeks to get a pronounced growing in control, and then give the final answer.

When using the method of absolute concentrations MBT culture is considered resistant if on the culture medium with a certain drug grows more than 20 colonies with abundant microbial growth in vitro control (no drug).

5.4.3. Drug susceptibility testing in liquid media(MGIT 960)

The system BACTEC MGIT 960 AST allows determining the sensitivity of mycobacteria to low and high concentrations of drugs, similar investigation methods in solid media.

A set of indicator tubes growing control (no drug) and containing TB drug is placed in a special medium with a bar code by which the device provides continuous monitoring of introduced to the culture tube.

Results are interpreted automatically, based on accounting multiplication of mycobacteria in vitro without drug at the time of control growth in 4-13 day after inoculation culture.

Set MGIT 960 SIRE Kit includes 4 bottles of major anti-TB drugs and 8 bottles of enriching liquid. Critical (low) drug concentrations achieved in nutrient broth after dilution.To determine the sensitivity of mycobacteria to pyrazinamide PZA) as control using MGIT tube with special pH = 5.9. Kit BACTEC MGIT 960 PZA includes 2 bottles with pyrazinamide lyophilized and six bottles with nutritional supplements.

Definition DSTof isolated M. tuberculosis from cultures in newly diagnosed patients and relapsed patients with tuberculosis must be necessarily to spend DST to first-line drugs: isoniazid, rifampicin, ethambutol, pyrazinamide, streptomycin. In the case of drug resistance to these drugs or multidrug resistance is recommended to conduct DST to Ethionamidum, amikacin, capreomycin and fluoroquinolones (ofloxacin).

In previously treated patients (treatment failure and treatment after an interruption), and patients with chronic tuberculosis is necessary to determination DSTof M. tuberculosis drugs to all immediately with the results of previous studies.

DST inMGIT system should be carried out as follows:

- a positive result in MGIT system + positive resultin LJ medium culture →MGIT culture andDST MGIT;
- a positive result in MGIT system + negativeresultin LJ medium culture →MGIT culture andDST MGIT;
- a negative result in MGIT system + positiveresultin LJ medium culture → LJ culture andDST MGIT;

5.4.4.Investigation of the sensitivity of mycobacteria to medicinal preparations by the coefficient of resistance

The principle of method consists in determining the minimum inhibitory concentration of anti-TB drugs for clinical strains of Mycobacterium and minimum inhibitory concentration ratio of these drugs for deliberately sensitive laboratory strain of mycobacteria (typically, H37Rv).This is the most time consuming and expensive method because it requires the use of a large number of tubes with nutrient because it is used mainly for scientific research.

5.4.5. Absolute concentration method

DST results counted in 3- 4 weeks of incubation in a thermostat, so the necessary correction of chemotherapy can be made in the best case only in 2-2,5 month from the moment of receipt the laboratory diagnostic material.

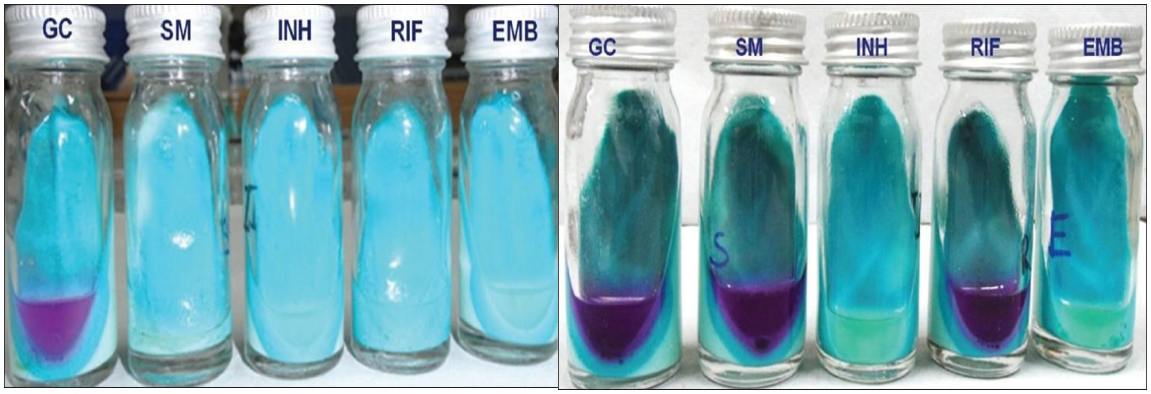
To accelerate research direct method of absolute concentrations can be used. When setting this method performed direct seeding precipitate processed detergents diagnostic material simultaneously to control culture medium and environment with appropriate anti-tuberculosis drugs.Seeding of material to standard culture media is performed simultaneously (in order to obtain culture).

Culture of Mycobacterium considered as resistance, if in the indirect method of absolute concentrations grows more than 20 colonies.

However, the direct method of absolute concentrations can be used only for research material if bacterioscopic result is positive with massive bacterial excretion at least 2+.In this case, increases the risk of contamination. Also, necessary to consider that this method is not performed dosed seeding, which may complicate the interpretation of results. Therefore, in some cases, the results may be unreliable.

5.4.6. Nitrate reductase assay

The nitrate reductase assay (NRA) is a technique based on the capacity of M. tuberculosis to reduce nitrate tonitrite, which is detected by adding the Griess reagent to the medium. By incorporating 1 mg/ml potassium nitrate(KNO_3) in the LJ medium, the reduction of nitrate can be detected using the Griess reagent, which produces acoloured reaction. In the presence of rifampicin or isoniazid at the critical concentration, the appearance of a red–pink colour indicates strain growth, which is interpreted as resistance to the drug (picture 22). Results can be obtained fasterthan by macroscopic detection of colonies, as the NRA uses the detection of nitrate reduction as an indicator ofgrowth.

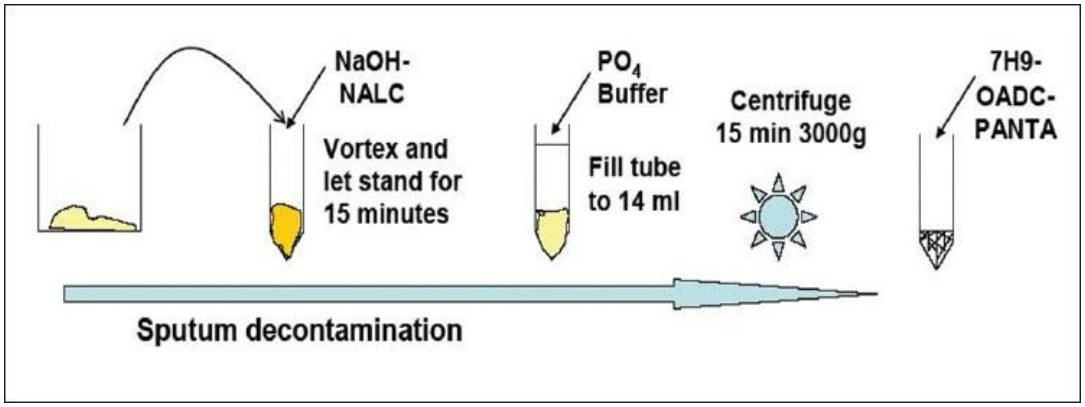


Picture 22. **NRA method: 1) sensitive to all tested drugs, 2) resistant to two drugs.**

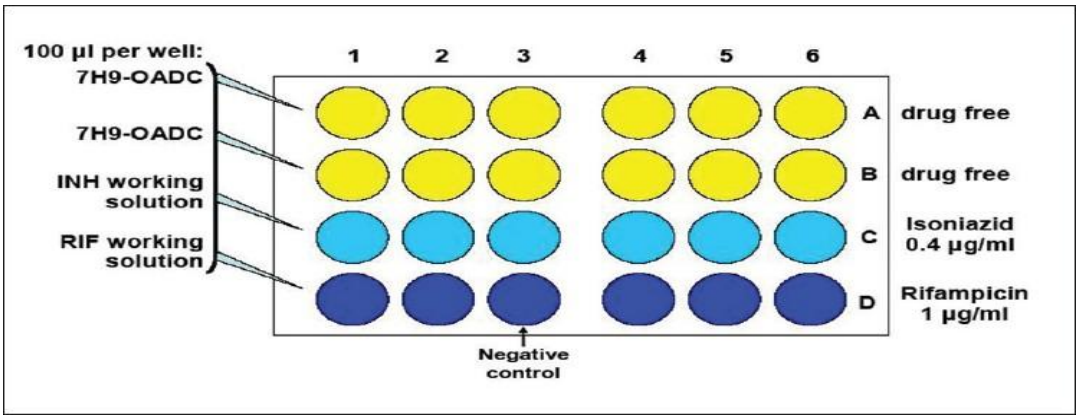
The results are classified as negative (no colour change) or ± (pale pink) to 5+ (deep red to violet). An isolate isconsidered resistant to a certain drug if there is a colour change in the antibiotic tube in question greater than thatin the 1:10-diluted growth control on the same day.

5.4.7. Microscopically Observed Drug Susceptibility Assay (MODS)

The microscopically observed drug susceptibility (MODS) assay is a method for direct testing of resistance to antituberculousdrugs. The sputum pellet is inoculated in microplate wells containing liquid media afterdecontamination. Cultures are examined on a daily basis using an inverted microscope(picture 23).



a)



b)

Picture 23. **MODS: a)Sputum decontamination by the NaLC-NaOH method; b) inoculation into the MODS culture wells**

Wells are subsequently examined using an inverted light microscope at 400 magnification every day from Day 4through to Day 21. Positive MODS cultures are identified by presence of characteristic cord formations in the drugfreecontrol wells.

Growth, as observation of cord formations, in drug-free control wells but not in drug-containing wells indicatessusceptibility. Growth observed in both the drug-

free wells and the drug containing wells on the same day isinterpreted as resistance.

5.5. Identification of Mycobacteriumtuberculosis and drug resistance in culturesand sputum using molecular assays andimmunoassays

Molecular genetic tests offer considerable time advantages in the identification of mycobacteria, enabling a morerapid initiation of resistance tests and specific treatment. They are useful tools for the detection and differentiationof mycobacteria from cultures and can have a high specificity and sensitivity. It should be noted however, that theycannot/should not replace the currently endorsed standard methods of detecting mycobacteria and determiningdrug-susceptibility patterns. Instead they should be used to support the diagnostic work-up. Test results shouldalways be confirmed using the standard methods.

5.5.5. GeneXpertMTB/RIF test

Test system GeneXpertMTB/RIF is recommended by WHO for use in the diagnosis of tuberculosis only from 2010. It allows the following:

- isolation and amplification is carried in the cartridge, pretreatment diagnostic material is reduced to a minimum of manipulation;
- the possibility of contamination is greatly reduced;
- it`s only determines the MBT resistance to rifampicin.

Test system GeneXpertMTB/RIF is a semi-nested PCR in real time in the cartridge that is conducted to identify:

- M. tuberculosis DNA in sputum samples or concentrated sputum precipitates;
- mutations in rpoB gene (resistance to rifampicin) in samples received from patients with a risk of resistance to this drug.

The principle of the PCR method is amplification - repeated increase in specific sections of mycobacteria DNA sequence in the tubes microvolumes at cyclic repetition of three reaction steps, each of which takes place under different temperature conditions:

The first stage - the change in the structure of DNA (denaturation) when heated with separation of it`s circuits;

Second stage - denatured DNA binding with synthetic nucleotide sequences (primers) complementary to the end sections of DNA fragment specific for Mycobacterium tuberculosis;

Third stage - the completion,or synthesis of the limited on the flanks chain of DNA fragment using thermostable DNA polymerase.

Test system GeneXpert Dx includes automated processing of samples, nucleic acid amplification and determine the samples sequences the that we are interested in using the real-time PCR methods with reverse transcriptase.The test system requires the use of disposable cartridges GeneXpert, containing reagents for PCR. Because the cartridges are autonomous, cross-contamination of samples is not possible.

Indications for use of the test system GeneXpert MTB/RIF

Group A

This group includes individuals (both adults and children)suspected of having TB who are considered to be at risk of harbouring drug-resistant TB bacilli (these risk groups should be defined according to national policies or as defined in WHO's Guidelines for the Programmatic Management of Drug-resistant TB).

It also includes both adults and children who have been treated with anti-TB drugs and in whom TB has again been diagnosed, that is, all retreatment categories (failure, return after loss to follow-up, return after relapse).

Xpert MTB/RIF should be used as the initial diagnostic test in these individuals rather than conventional microscopy, culture and DST.

Group B

Individuals (adults and children) suspected of having HIV-associated TB should ideally be offeredHIV testing routinely, preferably before investigation with Xpert MTB/RIF. HIV testing should beperformed according to national guidelines.Among adults and adolescents living with HIV, a person suspected of having TB is defined asanyone who reports any one of the following symptoms:

current cough, fever, weight loss or night sweats. Among children living with HIV, TB should be suspected in any child who has any oneof the following symptoms: poor weight gain, fever, current cough or a history of contact with someone who has TB.

Xpert MTB/RIF should be used as the initial diagnostic test rather than conventional microscopy, culture and DST in all persons living with HIV who have signs or symptoms of TB, in persons who are seriously ill and suspected of having TB regardless of their HIV status, and in those whose HIV status is unknown but who present with strong clinical evidence of HIV infection in settings where there is a high prevalence of HIV or among members of a risk group for HIV.

Group C

This group includes adults suspected of having TB but who are not at risk of MDR-TB or HIVassociatedTB (that is, adults who are HIV-negative or whose HIV status is unknown and who arenot a member of a risk group for HIV or who live in a setting with a low prevalence of HIV).

These individuals may receive an Xpert MTB/RIF test as an initial diagnostic test for TB. When resource limitations do not allow Xpert MTB/RIF to be used for all individuals, sputum-smear examination may be conducted first; using Xpert MTB/RIF for smear-negative individuals will identify TB cases missed by smear microscopy.

Group D

This group includes all individuals suspected of having TB (adults and children).

Xpert MTB/RIF may be used as an initial diagnostic test for TB. This can result in more bacteriologically confirmed patients and shortened time to treatment. Resource limitations may affect the ability of national programmes to undertake Xpert MTB/RIF testing in all individuals in this group.

The test requires a specific hardware platform, as well as the test kits, disposable pipettes, and gloves.The platform integrates sample processing, PCR, and the analysis of the PCR fragment.

The test is based on a disposable plastic cartridge containing all reagents required for bacterial lysis, nucleic acidextraction, amplification, and amplicon detection. The only manual step is the addition of a bactericidal lysis bufferto sputum or decontaminated sputum.The closed sputum cup or centrifuge tube isincubated at room temperature for 15 minutes, during which time the samples must be manually agitated twice. The inactivated sample mixture is transferred to the cartridge. Cartridges are then inserted into the platform and theprogramme is started. The extraction, PCR and detection process takes 90 minutes and the results areautomatically generated by the GeneXpert system unit (picture 24).

Results in the detection of TB bacteria can be positive, negative or indeterminate.

1. M. tuberculosis identified. Resistance to rifampicin is established. It`s the case of the risk of multi-resistant tuberculosis.

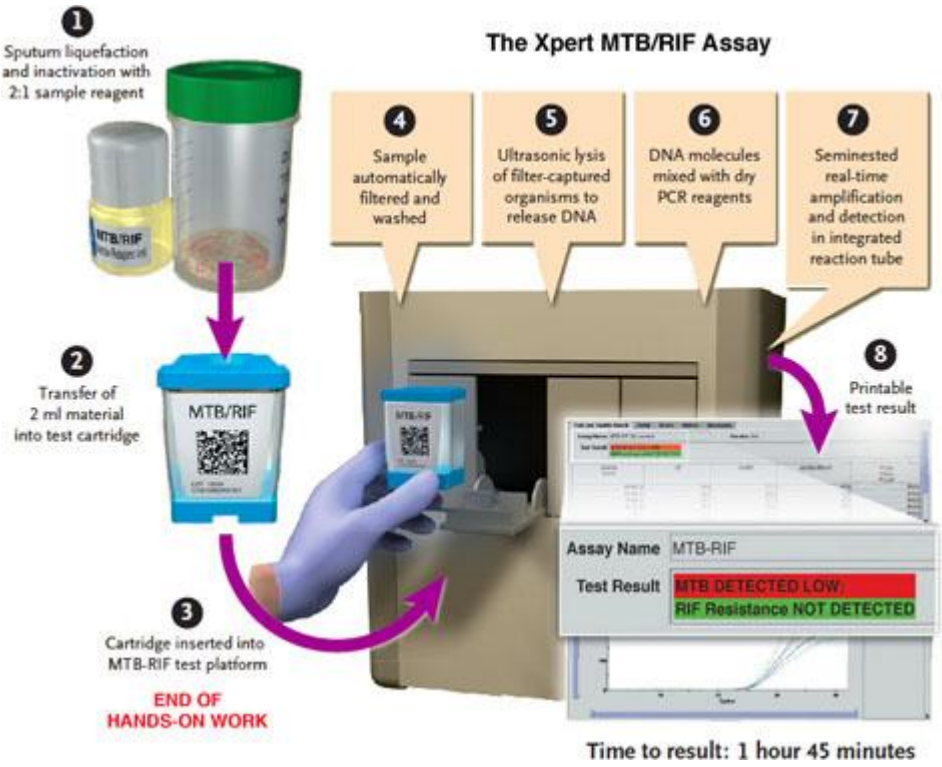
The patient starts treatment in 4 category as Rif TB case.It is necessary determine resistance to first and second drugsin liquid and / or solid nutrient media.

2. M. tuberculosis identified. Resistance to rifampicin is not installed. A case of tuberculosis, sensitivity to rifampicin.

The patient starts treatment according categories 1 or 2.

3. Test system GeneXpert MTB/RIF not revealed M. tuberculosis.

It is necessary to conduct a survey of the patient’s sputum by bacterioscopic and culture methods using liquid and / or solid culture media. When positive culture results it`s necessary to do DST to anti-tuberculosis drugs of the first line.



Picture 24. **Xpert MTB/RIF: The first automated diagnostic test for TB.**

5.5.6. GenoType test system

The GenoType test is based on the DNA-streap technology. The whole procedure is divided into three steps: 1) DNA extraction from clinical specimens or cultured material; 2) a multiplex amplification with biotinylated primers and 3) a reverse hybridization.

Length of research itself is low and is only 4 - 5 hours.

Test system GenoType® only used in III level laboratories for microbiological diagnosis of tuberculosis and is for diagnosis of tuberculosis mycobacteria identification and sensitivity to rifampicin, isoniazid, fluoroquinolones, aminoglycosides / cyclic peptides and ethambutol.

Indications to GenoType test:

- HIV-infected patients with suspected TB.

- Patients with suspected pulmonary tuberculosis with the presence of MDR-TB risk (risk according to the national guidelines):

• patients from MDR-TB contacts;

• patients, previously treated for tuberculosis;

•patients, who were born in a foreign country with a high TB incidence;

-Children or teenagers (0-17 yearsage group) with suspected TB.

Indications for patients with AFB in the sputum:

-TB patients with negative clinical and radiological dynamics and / or continuation or resumption of bacterial-excretion;

- Patients from social risk groups;

- Patients with newly diagnosed tuberculosis.

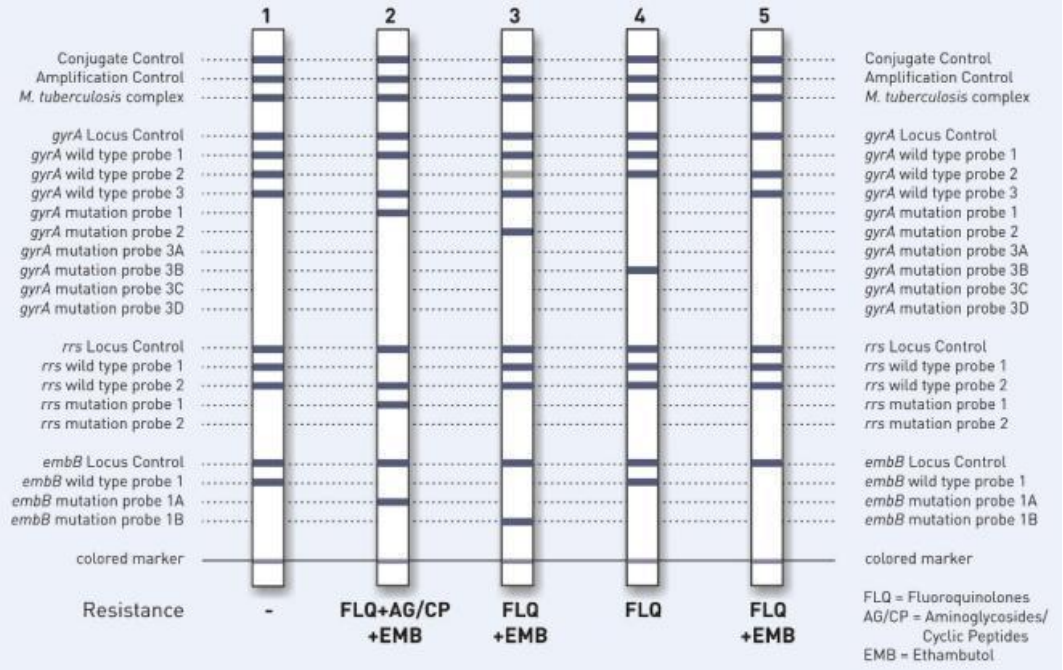
The GenoType MTBDRplus test allows for the detection of M. tuberculosis complex and simultaneously its resistanceto rifampicin and/or isoniazid by mutations in the rpoB and katG/inhA (high/low isoniazid resistance) genes, respectively. The GenoType MTBDRsl simultaneously detects M. tuberculosis complex and its resistance tofluoroquinolones (e.g. ofloxacin and moxifloxacin) and/or aminoglycosides/cyclic peptides (injectable antibiotics ascapreomycin, viomycin/kanamycin, amikacin) and/or ethambutol. The MTBDRplus and MTBDRsl are validated for DNAextracted from both positive cultures and smear-positive pulmonary specimens. These tests should not be usedto detect mycobacteria directly from smear-negative materials unless the laboratory independently validates their use.

All procedures are identical and are divided into three steps: DNA extraction, a multiplex amplification usingbiotinylated primers, and reverse hybridisation.The GenoType series all use the same reagents except for the primer/nucleotide/dye mix, which is specificto each test kit (picture 25).Interpretation rules are available in the manufacturer's inserts.

Results interpretation.

1. M. tuberculosis identified. Resistance to rifampicin and isoniazid is established. It`s the case of the risk of multi-resistant tuberculosis.

The patient starts treatment in 4 category. It is necessary using the test system GenoType® determine resistance to fluoroquinolones, aminoglycosides / cyclic peptides and ethambutol and determine DST simultaneously to first and second drugsin liquid and / or solid nutrient media.



Picture 25. **Examples of different GenoType® MTBDR*sl* strip readouts.**

2. M. tuberculosis identified. Resistance to rifampicin and susceptibility to isoniazid established.

The patient starts treatment in 4 category as Rif TB case.It is necessary using the test system GenoType® determine resistance to fluoroquinolones, aminoglycosides / cyclic peptides and ethambutol and determine DST simultaneously to first and second drugsin liquid and / or solid nutrient media.

3. M. tuberculosis identified. Resistance to isoniazid and susceptibility to rifampicin established.

The patient starts treatment in 1-2 categories.It is necessary using the test system GenoType® determine resistance to fluoroquinolones, aminoglycosides / cyclic peptides and ethambutol and determine DST simultaneously to first and second drugsin liquid and / or solid nutrient media.

4. M. tuberculosis identified. Resistance to rifampicin and izoniasid is not installed.

The patient starts treatment according categories 1 or 2.

5. Test system GenoType not revealed M. tuberculosis.

The patient starts treatment according categories 1 or 2.

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