



A Rapid Diagnostic Technique for Tuberculosis

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Received: April 19, 2016; Accepted: May 15, 2016; Published: May 25, 2016

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Abstract

This article presents the results of the study of a rapid bacteriological diagnosis of tuberculosis by stimulating the growth of *Mycobacterium* taken from the diagnostic material by precultivation in the medium of Shkolnikova within 24-48 h. Then there is the cultivation of bacilli in semi-liquid nutrient medium in a modification of Dorozhkova (DIR) within 5 days, and after that the kind of grown culture can be established by means of the immune diffusion reaction by adding a specific reagent directly to the culture medium.

Keywords: Tuberculosis; Immunology; Antigen; Antibody; Immune reaction; The colloidal gold conjugate

Introduction

Tuberculosis is a widespread infection among animals and humans [1]. The main reliable method of diagnosis is a bacteriological method [2]. However, the pathogenic mycobacterium cultivates very slowly in the artificial medium, and it is required at least for 3-4 weeks to obtain a preliminary response to the presence of the pathogen in the tested material [3]. In such cases, until we get the final results of bacteriological studies, serological and immunological diagnostic methods are used, but they are less specific [4].

Rapid identification of *Mycobacterium bovis* or its derivatives in the culture medium is a new and promising direction in the diagnosis of tuberculosis.

The aim of the research was to improve the methods for the bacteriological diagnosis of TB and to decrease the diagnosis period.

Materials and Methods

The work was carried out in the Department of Bacteriology of Limited Liability Partnership (LLP) "Kazakh Scientific Research Veterinary Institute." Materials used for the study were museum strains of *Mycobacterium* bovine species—*Mycobacterium bovis* no. 8, bird species *Mycobacterium avium*, *M. kansasii*, *M. scrofulaceum*, *M. phlei*, semi-liquid nutrient medium by method of Shkolnikova, rabbits weighing 3.5-4.0 kg, samples of biological material obtained from cattle that reacted positively to tuberculin.

All reagents including salts, acids, alkalis, and organic solvents were analytically and chemically pure. The gold-chlorine-hydrogen acid (manufactured by the company Sigma-Aldrich [USA]), solutions to obtain colloidal gold, and its conjugates were prepared with water that was deionized on the device Milli-Q.

Hyperimmunization of rabbits was performed according to the method of B. N. Kozmina-Sokolova [5].

The globulin fractions from a hyperimmune serum were extracted with a saturated solution of ammonium sulfate.

Colloidal gold was obtained by citrate method of Franse [6].

Results

The preparation of antigen A culture of *M. bovis* no. 8 that was grown in a liquid nutrient medium was decontaminated by autoclaving for 90 min at 1 atm. Then the bacterial mass was separated from the culture fluid by filtration through a cotton-gauze filter. Then a bacterial suspension was prepared and exposed to ultrasonic disintegration for 10 min in a mode of 22 kHz, with a power of 70-80 W/cm² on a device UZDN-A. The resulting suspension of mycobacterium was centrifuged for 30 min at 8,000 rpm, and the supernatant portion was decanted. After that 1 volume of incomplete adjuvant of Fraind was added to 1 volume of the supernatant fluid consisting of petrolatum and lanolin in 1:1 ratio. To prevent a separation of fractions, the mixing of adjuvant with antigens was carried out by means of ultrasound waves of low frequency (22 kHz) and high intensity (70 W) for 10 min.

Hyperimmunization of rabbits was performed with the obtained composition by subcutaneous injection of a volume of 0.1 cm³ in the finger pulp of the hind legs. One week after the first injection, the increased popliteal lymph node of the corresponding leg was reinjected with 0.2 cm³ of a mixture of an adjuvant with the antigen. On the 21st day after beginning of the cycle of immunization, if the titer of antibodies that was obtained from a serum in reaction of precipitation has not reached the required level, 1 mg of a suspension of *Mycobacterium* was intravenously administered to every rabbit through a marginal vein in the ear.

The titer of antibodies in the serum of rabbits was determined by the reaction of precipitation (RP) in the agar gel. Agar gel was prepared according to the recipe; it was melted in a water bath at 60°C and was poured into 25 cm³ in a Petri dish. After the solidification of agar gel, special holes were made in it with a special punch on a stencil at a distance of 4-10 mm from each other. The bottom of the hole was filled with a drop of molten agar to prevent leakage under the agar ingredients of the reaction. The antigen in the working dilution was added through the central hole and hyperimmune serum was added at different dilutions through the peripheral holes. Petri dishes with the ingredients were placed into a desiccator, and a wet cotton gauze was placed at the bottom of it. A review and an evaluation of the results of the reaction were carried out in 72-96 h. Its activity was dependent

upon the formation of precipitation lines in agar gel. The precipitation reaction is presented in Figure 1.

After the accumulation of antibodies in serum in titer up to 1:64, rabbits were totally exsanguinated by the resection of the jugular vein; the serum was sludged by blood sedimentation for 2-3 h, and then it was used for further work.

The separation of the globulin fraction of hyperimmune sera was performed by the ammonium sulphate solution at a temperature of 2-4°C. An equal volume of 36.6% solution of ammonium sulfate (7.2 pH) was added to the 20 cm³ of the hyperimmune rabbit serum. The mixture was left for 1 day in the refrigerator under the constant stirring of a magnetic stirrer. The next day the mixture was centrifuged for 20 min at 5,000 rpm. The supernatant fluid was decanted, and the precipitate was dissolved in a bicarbonate buffer (8.5 pH) and was passed through a column with Sephadex (pD-10 desalting columns contain Sephadex G-25). The result is a purified (enlightened) specific immunoglobulin (IgG) in a volume of 7.5 cm³ (Figure 2).

The process of purification of the sera of rabbits is presented in Figure 2.

The activity of the obtained antibodies (IgG) was tested in the reaction of precipitation according to the method described above. In this way, 300 µl of antigen was added to the central dimple, that was made from cultures of *Mycobacterium bovis* at a concentration of 1 mg of protein in 1 cm³, and a purified immunoglobulin was added at the external edge of a circle in a breeding of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, and then it was left for 1 day in a thermostat at a temperature of 37°C.

As a result, the line of precipitation in agar gel was detected in all dimples; the activity of IgG was not less than 1:32. (Figure 3). The resulting immunoglobulin (IgG) was used for making of the conjugate.

The obtaining of a solution of colloidal gold for staining of specific tuberculosis antibodies In this way, 1.0 cm³ of a 1% solution of gold-chlorine-hydrogen acid was added to 97.5 cm³ of deionized water,



Figure 1: Reaction of precipitation (RP) in the agar gel



Figure 2: Purification through the column (pD-10 desalting columns contain Sephadex G-25)



Figure 3: Activity of the separated specific immunoglobulin IgG in the RP

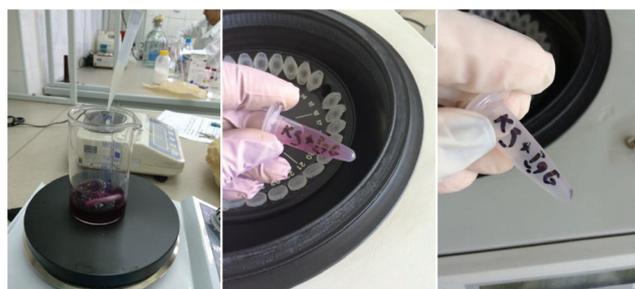


Figure 4: Obtaining of a conjugate of colloidal gold with proteins

brought to boiling point and 1.5 cm³ of 1% solution of sodium citrate was added by stirring. The mixture was boiled for 25 min until a deep purple color appeared, and the particle size of a colloidal gold consisted of 10 nm. Then it was cooled and stored at 4-60°C.

Obtaining of a conjugate of colloidal gold with antibodies

The 98 cm³ of colloidal gold (pH 8.5) was added dropwise to 200 µl of purified antibody (IgG) at a concentration of 1 mg/cm³ until the concentration of 20 µl/cm³ was obtained. The mixture was left for 30 min for complete dissolution at room temperature and centrifuged at 12,000 rpm for 30 min; the supernatant fluid was decanted, and the precipitate was dissolved in 10 mm of TRIS (oxymethyl) aminomethane buffer, pH 9.0.

During the interaction of colloidal gold with the antibodies against the *Mycobacterium bovis*, electrical charges and the non-covalent binding of molecules of proteins with granules of gold occur. The obtained conjugates of colloidal gold with proteins are stable; the molecules of proteins retain the biological properties (Figure 4).

A culture method allows to identify a pure culture of *Mycobacterium* and to identify the species that is of great importance in the epidemiology of tuberculosis. In addition, it allows to make an accurate diagnosis without delay, allows to establish biochemical and biological characteristics and to determine drug sensitivity of the pathogen, and to determine with great precision one of the important properties of bacteria—its virulence.

The diagnostic materials that were taken from the animals with tuberculosis (lymph nodes, liver, lungs, and kidneys) were subjected to pretreatment method according to Alikieva [7]. The biomaterial was cut with scissors into small pieces in a mortar, soaked in a 5% solution of sulfuric acid and was left for 10-20 min.

Then the acid was decanted; the material was washed 2-3 times with the isotonic physiological solution; the pieces were carefully ground by a pestle (pounder) in a small volume of NaCl 0.9%; the resulting suspension was used for seeding.

The treated suspension was gathered in the Pasteur pipette, and 0.25 cm³ of it was added into 2 glass tubes with the medium of Shkolnikova. Then they were incubated at 37°C for 1-2 days for the cultivation of *Mycobacterium*. Then, using a Pasteur pipette, the 0.25-0.5 cm³ of the cultivated material along with the liquid medium of Shkolnikova were recultivated into 3-4 glass tubes with semisynthetic medium of Shkolnikova in the modification of Dorozhkova (DIR) [8].

For control, another portion of the biomaterial from the mortar was sown according to the standard technique on a dense nutrient medium of Lowenstein-Jensen.

The cultivated bacilli were placed in a thermostat, incubated at a temperature of 37°C and viewed in transmitted light daily.

As a result, a growth of mycobacterium in the form of a cloud or haze in the nutrient medium was detected on the fifth day in test tubes with the DIR medium (Figure 5).

At this stage of bacteriological diagnosis, a culture of *Mycobacterium* grown inside the liquid nutrient medium in the form of diffused clouds cannot be differentiated into species by cultural-morphological features, and reseeded on a dense nutrient medium of Lowenstein-Jensen lengthens study periods up to 20 days, just like the direct seeding of the biomaterial by conventional method of TB diagnosis (control).

To establish the type of cultures of *Mycobacterium* (differentiation), a prepared conjugate of colloidal gold with IgG against *Mycobacterium* bovine in a volume of 0.25 cm³ was added in the test tubes with growth of *Mycobacterium*. For control, a conjugate was added dropwise to the test glass tubes with the sterile DIR medium.

For the second control, the glass tubes with cultures of *M. bovis* no. 8 in the DIR medium without the conjugate were used.

All tubes were placed in a thermostat at 37°C, the reactions were examined after 30-60 min.

As a result, after 30 min in a culture with *Mycobacterium bovis* inside the semiliquid DIR nutrient medium a diffused staining of culture in purple color was detected (Figure 6).

Thus the conjugate had penetrated into the interior of the nutrient medium, and the specific antibodies (labeled with colloidal gold) have associated with the antigen—*Mycobacterium bovis*, having painted them in purple color.

In the first control in a sterile DIR nutrient medium, the conjugate remained on the surface of the nutrient medium, and after 1 h, it began to lose its color. The thickness of the medium remained unchanged, and the medium retained its transparency.

In the second control, the color of colonies of *Mycobacterium* with a form of a white cloud remained unchanged.

The specificity of the resulting conjugate was studied by adding it dropwise to the test glass tube with a culture in DIR medium with *Mycobacterium* of four groups according to the classification of Runyon: 1—photochromogens, *M. kansasii*; 2—cattle chromogens, *M. scrofulaceum*; 3—nonphotochromogens, *M. avium*; 4—fast growing, *M. phlei*.

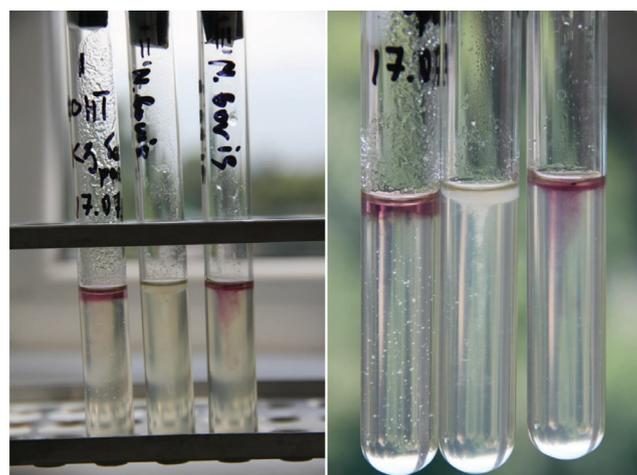


Figure 6: Staining of culture of *Mycobacterium bovis* with a conjugate of colloidal gold with IgG in the DIR medium

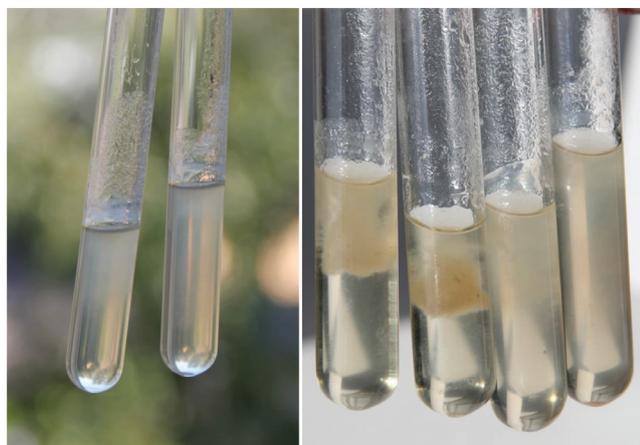


Figure 5: The growth of cultures of *Mycobacterium* in a DIR medium on the fifth day

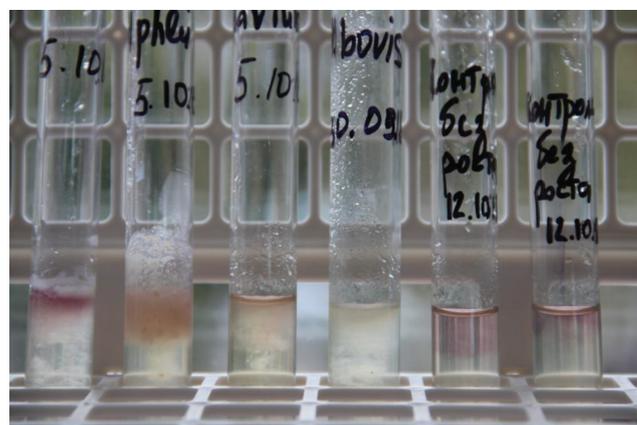


Figure 7: The specificity of the conjugate in the cultivation of different species of *Mycobacterium* in the DIR medium

As a result of the formation of immune complex, a binding of antibody conjugated with colloidal gold to a specific antigen has occurred in the staining medium in magenta color only in the tube with cultivated *Mycobacterium bovine* species *M. bovis* no. 8.

In other cultures of atypical *Mycobacterium*—*M. kansasii*, *M. scrofulaceum*, *M. avium*, *M. phlei*—staining of the colonies did not occur within 30-60 min. That indicates a specificity of the developed diagnosis (Figure 7).

As a result of the conducted researches, a possibility of accelerated bacteriological diagnosis of tuberculosis was established by promoting the growth of arthropod of *Mycobacterium* in the diagnostic material, i.e., in the medium of Shkolnikova with the precultivation for 24-48 h, and then with the cultivation of bacilli in a semiliquid DIR nutrient medium for 5 days.

The type of culture of grown *Mycobacterium* was performed by immune diffusion reaction in agar gel by adding a specific reagent to the culture medium directly. The formation of the complex of antigen with antibodies labeled with colloidal gold and painted in purple color allows to differentiate the selected cultures of bacteria and to visualize the result.

Discussion

One of the main methods of TB diagnosis is a bacteriological method, involving a cultivation of the studied material in a nutrient medium in order to separate colonies of *M. bovis*, the determination of nature and speed of its growth, and a biological method—a reproduction of the manifestations of the disease on biological models and laboratory animals.

By means of a bacteriological method during a research, a culture of *Mycobacterium* can be evaluated if the content is ranges from 20 to 100 bacilli in 1.0 cm³ of the tested material. When the nutrient medium favors the growth of different *Mycobacterium* species, they form colonies that are visible to the naked eye.

A traditional microbiological diagnosis of tuberculosis with the cultivation of bacilli on thick egg media (such as Lowenstein-Jensen) gives a visual result after 6-12 weeks. In addition, microbiological tests for tuberculosis have a low sensitivity. These methods currently cannot satisfy practitioners in terms of detection.

The developed nutrient media, methods of cultivation, ELISA, and PCR for detection of *Mycobacterium* enable us to obtain the results of the studies, ranging from 2 h to 5 days after admission of the diagnostic material, but they have some significant disadvantages—they require expensive equipment, and the methods of estimation are indirect.

The well-known, ultrasmall, L-transformed, branched, coccoid, non-acid resistant and other forms are not only the result of adaptation of the pathogen to the immune system, or the action of chemotherapeutic agents but also a natural stage, when the classical “Koch’s Bacillus” is just one of the stages of the life cycle of the causative agent of tuberculosis [9]. It is believed that *Mycobacterium* growth activates the sodium-potassium pumps and some enzymes of *Mycobacterium* [10], which activates adaptive structures and promotes fast growth of bacteria in the form of polymorphic non-acid resistant cells that have common antigens with bacillary forms of *Mycobacterium* tuberculosis and the identical stretches of DNA.

In rapid diagnosis of TB, the average time for detection of *Mycobacterium* on the semiliquid nutrient DIR medium is 5-7 days.

The developed method of diagnosis of tuberculosis includes the precultivation treatment, the cultivation of a diagnostic material in a liquid nutrient medium, and the addition of the conjugate in the form of specific antibodies labeled with gold colloid in a test glass tube with a culture of *Mycobacterium bovis*.

Under the surface of the immunological complex of antigen-antibody is the invisible cloud that changes into purple and diffuses in a liquid medium if *Mycobacterium bovis* is detected.

The results of the research showed the possibility of a rapid bacteriological diagnosis of tuberculosis by promoting the growth of arthropods of *Mycobacterium* from the diagnostic material with the precultivation in the medium of Shkolnikova within 24-48 h. Then the bacilli are cultivated in a semiliquid nutrient DIR medium within 5 days and the kind of grown culture in the reaction of immune diffusion by adding a specific reagent directly to culture medium is established.

The proposed method of bacteriological diagnosis of tuberculosis allows a decrease in the period of diagnosis of TB up to 7 days, allows to visualize the growth of *Mycobacterium* in a semiliquid nutritional medium and to differentiate species of *Mycobacterium bovis*. This is a specific, rapid, and reliable method for diagnosing and determining the safety of resulting animal foods.

Conclusions

1. The developed method of conjugation of antibodies by colloidal gold is specific—the staining of a medium in purple color occurred only in the test glass tube with a culture of *Mycobacterium bovine* species *M. bovis* no. 8.
2. The proposed method of bacteriological diagnosis of tuberculosis allows a decrease in the period of diagnosis of TB up to 7 days.
3. The introduction of the conjugate into a test glass tube with a culture of *Mycobacterium bovis* makes it possible to visualize the growth of *Mycobacterium*, staining it in purple color.

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