Health, 2014, 6, 1866-1873
Published Online July 2014 in SciRes. http://www.scirp.org/journal/health
http://dx.doi.org/10.4236/health.2014.614219

Scientific Research

The Medium for Acceleration of *Mycobacterium tuberculosis* Growth

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Received 25 May 2014; revised 10 July 2014; accepted 22 July 2014

Abstract

This article has described the results of studies conducted on pre-culture samples in a modified environment Shkolnikova Dorozhkova at 37˚C during 24 - 48 hours. It was stated that this method helps grow *Mycobacterium tuberculosis*, which, when sown on the medium give rise visible colonies on the 5th - 7th day. These data confirm the possibility to accelerate the growth of mycobacteria and shorten the terms of diagnosing tuberculosis by 3 or 4 times thanks to pre-culturing of the diagnostic material in the experimental medium. The original conception of the *Mycobacterium tuberculosis* development step-by-step was elaborated which was based on changeability of mycobacteria and prominent character of their development from division of the bacterial cells to the development of the maturated cells and further biological transformation under conditions of the surrounding factors. This conception gave the explanation of the integrated picture of the biological changeability of *Mycobacterium tuberculosis*. It was stated that the cocci-like, flask-like, grain-like, virus-like, filtrating forms, L-forms and other agent forms are the stages of the biological development of tuberculosis mycobateria which are not always identified by the routine methods of microbiological diagnostics implemented in the veterinary practice. Thus, the use of the developed method of diagnostics of tuberculosis in animals together with agglutination test, ELISA and PCR allow shortening the terms of primary diagnosis of tuberculosis in animals by bacteriological method by 5 - 7 days.

Keywords

***Mycobacterium tuberculosis*, Experimental Infection of Animals, Histological Investigation**

How to cite this paper: Syrym, N.S., Turgenbaev, K.A., Yespembetov, B.A., Basybekov, S.Z., Bazarbayev, M.B., Borsynbaeva, A.M. and Bekembayeva, G.S. (2014) The Medium for Acceleration of *Mycobacterium tuberculosis* Growth. *Health*, **6**, 1866-1873. http://dx.doi.org/10.4236/health.2014.614219

1. Introduction

The application environment for pre-cultivation shortens the selection of *Mycobacterium tuberculosis* from the biomaterial. The growth of pathogenic mycobacteria in the experimental environment appears at 5 - 7 days, whereas in Lowenstein-Jensen medium first growth of pathogenic mycobacteria cultures observed at 30 days.

Tuberculosis usually runs chronic, often in a latent form in most cases by the lack of characteristic clinical signs of disease, which complicates its early detection. The disease is characterized by lesions of organs and tissues with the formation of tubercle in them.

The infection causes significant economic damage to the national economy because of forced slaughter of sick and reacting to tuberculin animals (possibly non-sick) as well as loss of the working population of the state.

Besides, it is a serious social problem of modern society: it is known that the incidence of disease is registered mainly in those countries where there is a sharp decline in living standards. Such living conditions remain in most countries of the so-called Third World, including, unfortunately, in the CIS countries, and in particular, in the Republic of Kazakhstan (in spite of the general increase in the living standards of our country, the situation is still very unstable). Risk of tuberculosis infection is expressed in the fact that there is a serious threat of mutual re-infection of humans and animals with other species of *Mycobacterium tuberculosis*, which complicates diagnosis and prevention of this infection.

Therefore, it is necessary to carry out comprehensive measures to control tuberculosis.

The primary effective stage of tuberculosis control is the timely and efficient diagnosis.

One of the main methods of the laboratory diagnostics of tuberculosis is the bacteriological method. This method includes the isolation of the culture from material to be investigated, determination of mycobacteria growing speed, founding-out of the particularities in colony formation, and bacterioscopy and laboratory biological sensibility testing *in vivo*.

The advantage of this method is that it allows making an accurate diagnosis with determining the species of mycobacterial cultures obtained. It is also possible to isolate defective in cell wall or lost it variants—L form of *Mycobacterium tuberculosis* that can reverse to the original bacterial species with recovery of virulence characteristic to pathogen. This allows the diagnosis of latent carrier of microbs in the test stage [1]-[10].

Difficulties in the isolation of *Mycobacterium tuberculosis* from pathological material related to the biological characteristics of the agent—one vegetative division of mycobacteria cell occurs in 14 - 18 hours as well as with a decrease in the viability of mycobacteria as a result of the use of antibacterial drugs with different methods of pre-processing of the material. Consequently, the time of primary diagnosis of tuberculosis by bacteriological method lengthened to 3 months [3] [6] [9] [11]-[13].

Currently used classical methods of allocation of the pathogen are often not sufficiently effective. The disadvantages that limit the possibility of culture-biological methods have led to the finding of accelerated and more efficient ways to display *Mycobacterium tuberculosis* in the materials.

The classical methods of pathological agent implemented nowadays are insufficiently in some cases. The attempts of elimination of the defects limiting the possibility of implementation of the cultural biological method led to finding out more accelerated and lesser expensive ways of indication of *Mycobacterium tuberculosis* in the material investigated.

Our task was to develop a method of cultivation of *Mycobacterium tuberculosis* and isolation of the pathogen from animal biomaterial repositories in both vegetative and in a modified form.

2. Material and Methods

In this work there were used the cultures of 3 species of pathogenic mycobacteria: *M. bovis*, *M. tuberculosis*, and *M. avium* and representatives of 2 groups of atypical mycobacteria by Runion' classification *M. scrofulaceum*, *M. phlei* obtained from the museum of tuberculosis laboratory.

The nutrient media by Gelberg, medium by Shkolnikova modified by Dorozhkova I.R. (DIR) and medium VCG were implemented to grow the mycobacterial cultures. The nutrient medium VCG (HANSA, Ukraine) was prepared by producer' protocol and poured out in the Petri' plate "Bion".

The samples of a biological material as a diagnostic material were obtained from 15 guinea pigs divided in 5 groups and infected with mycobacteria for modeling the experimental tuberculosis.

In this work there were implemented the serological, molecular biological, bacteriological and biological me-

thods of investigation.

3. Ethical Reasons

Research was considered by local ethical committee at the Research Institute for Biological Safety Problems and Kazakh Veterinary Research Instituteof the Republic of Kazakhstan. To each participant reported about the purposes, methods and potential dangers of research. Participants assured that all information collected for research, will be kept confidential, and that in any turning-out publication it wouldn't be possible to connect data with people and families in research. Written report that the consent was received from all participants and any didn't reduce participation in research. Participants subscribed/struck, print the written consent together with the witness to take part in research.

4. Results of Studies

The guinea pigs of the Groups I and II were inoculated sub-cutaneously with virulent culture *M. bovis* 8 and *M. tuberculosis* H37Rv in a dose of 0.001 mg, while the suspension of *M. avium* was induced to the guinea pigs of the Group III, suspension of *M. scrofulaceum* culture was induced to the animals of Group IV, suspension of *M. phlei* sub-cutaneously was induced to each guinea pig of the Group Y by 1 mg in 1 mil of saline solution.

30 days after infection blood samples were taken from the hearts of guinea pigs in a volume of 3 - 5 mil. Immediately after collection of blood in an equal volume was added growth stimulator, and the mixture was incubated in an incubator for 24 hours at a temperature of 37˚C - 38˚C.

Then all the animals were killed. After autopsy from each animal were taken to the lymph nodes, lungs, liver and kidneys for the microbiological study.

Results of pathological-anatomic photo are shown in the **Figures 1-3**.

The presented figures show that the highest contamination was found in the lungs, liver, spleen, and the lowest—in the retropharyngeal and bronchial lymph nodes.

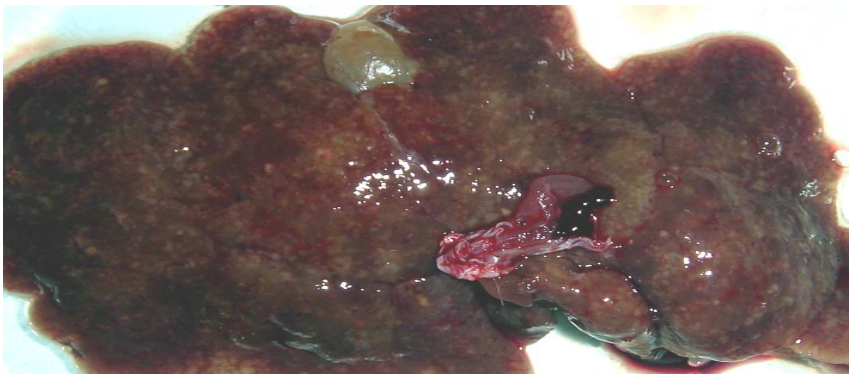

Figure 1. Liver of guinea pig infected with *M. bovis*.

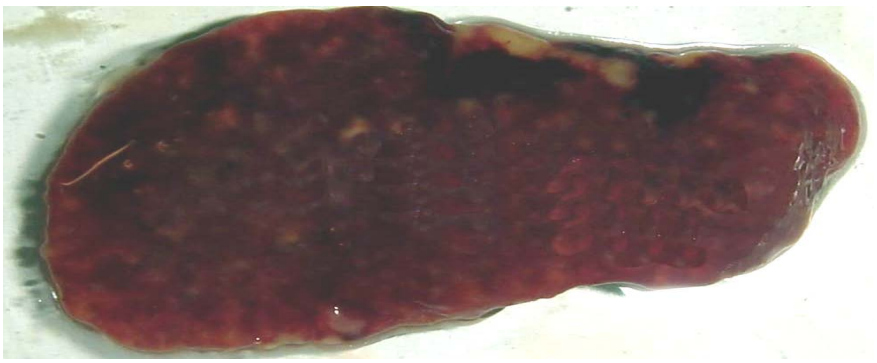

Figure 2. Spleen of guinea pig infected with *M. bovis*.

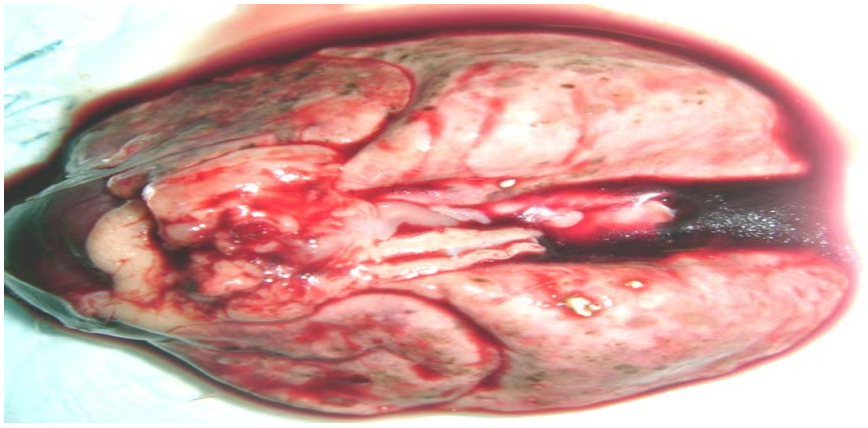

Figure 3. Lungs of guinea pig infected with *M. bovis*.

Also smears were prepared from these bodies and painted by Ziehl-Nielsen.

Material for diagnosing has been undergoing to the pre-inoculation treatment by Alikayeva method, *i.e*. it was crumbled carefully, added 5% sulfuric acid during 20 min, washed by saline solution, homogenized, and one part of it was inoculated to the Lewenstein-Jensen medium (control 1).

The part of isolates was investigated through PCR (the set "Biokom") by producer' protocol. The purification of mycobacterial DNA with sorbent method using the set AmpliSens to isolate the DNA from cultures. The classical PCR conduction was performed by set AmpliSens.

Another part of homogenate was used to stimulate the mycobacteria growth through preliminary culturing. For this 1 - 2 ml of homogenate, after preliminary treatment with acid were placed in the tubes with 10 ml of medium by Shkolnikova modified by Dorozhkova and were incubated 1 or 2 days in the thermostat at 37˚C. Then inoculation material together with liquid medium by Shkolnikova was re-inoculated with Paster' pipette by 0.25 ml into bacteriological tubes with meet-peptonic agar (MPA) and incubated at 37˚C during 5 - 7 days. For the second control samples of biomaterial was inoculated to the ВКГ medium containing the growth factor by recommended protocol (**Table 1**).

As it is seen in **Table 1**, growth of mycobacteria cultures of *M. bovis*-8 and *M. tuberculosis* in Lewenstein-Jensen medium was observed in the conventional term by 30th days after treatment of biomaterial with well-known method, while the growth of colonies of cultures of *M. avium* did by 17th day, *M. scrofulaceum* did by 14th day and *M. phley* did by 7th day.

ELISA positive result was obtained in I group of guinea pigs infected with *Mycobacterium bovis*, while PCR positive result groups of animals I and II.

Growth of colonies and cultures *M. bovis*, *M. tuberculosis* on Lowenstein-Jensen medium, made respectively from biomass grown on MPA was marked on day 21.

In MPA medium examination of tubes transparently revealed the growth of small, whitened, transparent and wax-like colonies of cultures 3 - 5 days later (**Figure 4**, **Figure 5**). In the same terms analogous growth of mycobacterial colonies was found-out in the VCG medium.

After appearance of growth in MPA medium scrapings with bacteriological loop from surface of medium were done, they were transported on the slides, grinded in the small quantity of distillated water, fixed over the flame of spirituous burner and stained by Zhil-Nilsen method. There were identified as no acid fast cocci-like forms stained into blue color at the smear microscopy (**Figure 6**).

The smears were prepared (paint by Zhil-Nilsen) from mycobacterial colonies. Bacterial mass was investigated by reaction of agglutination (RA) in slide with antiserum. The fast-free slides for RA conduction were used. The investigation was conducted on or two colonies of different morphology from population grown in one tube. The antiserum and negative control serum were put on the slide by 20 - 40 mcL. The bacterial mass by 0.5 - 1 mg was suspended in each serum using the bacteriological loop. The reading of reaction was conducted during 5 minutes using the light at the bottom (**Figure 7**).

As a result, it has been stated that the positive reaction has been marked when the bacterial mass had been obtained from animals infected with *M. tuberculosis* and *M. bovis*. Agglutinate was formed with antiserum to *M.*

Table 1. Results of biomaterial samples investigation of guinea-pigs for tuberculosis.

Number of group and mycobacteria species	Methods of investigation						
	Treatment by Alikayeva (control 1)	Pre-culturing	Growth in VCG medium (control 2)	Microscopy	RA*	ELISA**	PCR***
Group I *M. tuberculosis*	+	+	+	+	+	–	+
	+	+	+	+	+	–	+
	+	+	+	+	+	–	+
Group II *M. bovis*-8	+	+	+	+	+	+	+
	+	+	+	+	+	+	+
	+	+	+	+	+	+	+
Group III *M. avium*	+	+	+	+	–	–	–
	+	+	+	+	–	–	–
	+	+	+	+	–	–	
Group IV *M. scrofulaceum*	+	+	+	+	–	–	–
	+	+	+	+	–	–	–
	+	+	+	+	–	–	–
GroupV *M. phley*	+	+	+	+	–	–	–
	+	+	+	+	–	–	–
	+	+	+	+	–	–	–

*RA—reaction of agglutination; **ELISA—Enzyme immunological assay; ***PCR—Polymerase-chain reaction.

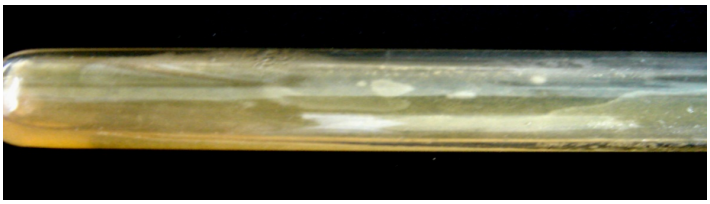

Figure 4. Growth of mycobacteria in MPA medium after culturing of organs of guinea-pigs infected with *M. bovis*.

Figure 5. Growth of mycobacteria in MPA medium after pre-culturing of organs of guinea-pigs infected with *M. tuberculosis*.

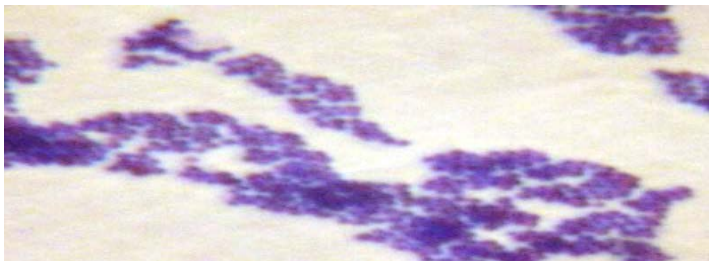

Figure 6. Shows the presence of ruby-red mycobacteria in the smear prepared from guinea pig lung infected with *M. bovis*.

Figure 7. Agglutination reaction in the glass.

tuberculosis-M. bovis only, while no agglutination was observed in any sample, and the suspension remained homogenous if the control negative serum used.

5. Conclusions

By results of investigations conducted, it had been stated that the preliminary cultivation of the samples to be studied in the medium by Shkolnikova modified by Dorozhkova incubated at 37˚C during 24 - 48 hours led to the growth of the changeable forms of Mycobacteria which being incubated in MPA gave the visible growth of colonies by 3rd - 5th days of incubation. These data confirm the possibility to accelerate the terms of diagnostics by 3 - 4 times through preliminary culturing of diagnostic material in the experimental medium.

Question about the viability of *Mycobacterium tuberculosis*, lost the ability to grow on nutrient media was discussed many times, and most recently in connection with the use of chemotherapy relevant observations became frequent. Griffith [14] at cultivation of pus bacteria from 176 patients with tuberculosis lymphadenitis in which acid-fast bacteria were detected microscopically, in 97 cases, growth is not observed.

In 1941 year Pryce proposed a method for obtaining microcultures of mycobacteria—smear from pathological material is prepared on a object-plate, after it should be processed with sulfuric acid and placed in a nutrient medium (citrate, defibrinated or lysed blood). This method of cultivation allows the causative agent of tuberculosis to multiply rapidly, forming a microculture, visible under the microscope after staining the smear according to the method of Ziehl-Nielsen. According to Pryce, the visible growth can be seen within 24 - 48 hours, and clearly visible microcolonies are detected in 3 - 4 days.

A. S. Holtzman, E. Shkolnikova [15] applied the method of Pryce in their modifications, using as a nutrient medium citrated blood mixed with distilled water. From microscopically positive sputum in 93% and from microscopically negative—in 21.9% of cases, the authors obtained microculture on the fourth day.

D. Esopo [16] in 113 of 139 cases failed to get growth of mycobacteria from sputum of TB patients treated with streptomycin, although microscopically he detected acid-fast bacteria. Meissner [17] [18], Martinek [19], Boszormenyi *et al.* [20] recently observed also increase the number of cases in which the sputum seeding prevented growth despite the fact that acid-fast bacteria were detected microscopically. In such cases, attempts were made to improve methods for the cultivation of mycobacteria. Hobby *et al*. [21] in some cases when the conventional method of sowing gave negative results, used the seeding material on solid medium followed by inoculation on dense medium and after 9 - 12 months was observed substantial growth of *Mycobacterium tuberculosis.* Bernsten, Steenken [22] Medlar [23] and Wayne [24] at sowing of resected lung tissue, where *Mycobacterium tuberculosis* were detected microscopically in outbreaks using the Hobby method, could not get the results that would have been better than the conventional method of sowing. Coletos [25] [26] developed a medium that contained monkey liver extract, gelatin solution and oligodynamic metals. The author has received “reanimation” and the growth of *Mycobacterium tuberculosis* from pathological material, seeding of which gave a negative result when using conventional methods. McCune *et al*. [27] used combined chemotherapy GINK with pyrazinamide on white mice infected with tuberculosis and reached the sterile state of *Mycobacterium tuberculosis*, in which the last are not bred on artificial media. After 6 months at the end of treatment, especially after handling animals with cortisone, from their bodies in some cases it was possible to isolate mycobacteria culture with new features, which were considered by the authors as newly viable modified *Mycobacterium tuberculosis.* I. R. Dorozhkova modified the medium of Shkolnikova adding to the composition 1% of agar and 200% of sucrose, which create high osmotic pressure, suitable not only for vegetative but for L-forms of mycobacteria having a cell wall defects. *Mycobacterium tuberculosis* grows at 14 - 21 days and more and views up to the light in the test tube as a cloudy translucent cloud in the background of culture medium [28]. Now becoming famous common methods of targeted detection and evidence of specific belonging of biologically changed, including granu-

lar, ultrafine L-forms of mycobacteria. Ukrainian scientists proposed commercial growing medium VCG (HANSA, Ukraine) with a growth factor for the rapid detection of *Mycobacterium tuberculosis* [29]-[31], which has the properties to neutralize the shortcomings of traditional culture media. According to the authors data, growth stimulator contains components that can break the peptidoglycan backbone of tuberculosis pathogen and intensify "potassium-sodium pump" cells that accelerates the process of reproduction and growth of mycobacteria on medium VCG located both in conventional and in a transformed form. This allows selecting *Mycobacterium tuberculosis* from pathological material and biological fluids of the animal body: blood, sputum, urine, etc. for 2 - 4 days. But for worldwide application of VCG medium exists patented limit of the content of mycobacteria growth stimulant.

Researchers repeated attempts to improve the method of cultivation of mycobacteria which lost the ability to grow on nutrient media [32]-[38]. So, Hobby *et al*. [21] in some cases, when conventional culture methods did not give positive results, used the seeding material on solid medium followed by inoculation on dense medium and after 9 - 12 months were observed substantial growth of *Mycobacterium tuberculosis*.

The scientists of Kazakhstan elaborated the original conception of staged development of *M. tuberculosis*, the base of which is the changeability of mycobacteria and stage' nature of their development from division to development of matured cells and their further biological transformation under impact of different environmental factors. This conception gave the explanation for the integral presentation on biological mutability of *M. tuberculosis*. It had been stated that the cocci-like, bulb-like, granular, virus-like, filterable, L-forms and other forms of tuberculosis agent are the stages of the biological development of tuberculosis mycobacteria which can be not always identified through routine methods of microbiological diagnostics implemented in veterinary practice [39] [40].

Thus, the use of this method of animal tuberculosis diagnosis in conjunction with RA, ELISA and PCR reduces setting time of initial diagnosis of TB animals' bacteriological method to 5 -7 days.

Competing Interest

We declare that we have no competing interest in writing this article.

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