Original Article

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Morphological Forms of *Mycobacterium bovis* Under Conditions of Long-Term Storage at Low Above-Zero Temperature (3°C)

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Abstract

Mycobacterium bovis cultures stored for a long time at a low above-zero temperature (3°C) are characterized by pronounced changes in their biological properties that occur during numerous passages in an artificial culture medium, as a result of which frequency of formation of small forms of bacteria and their adaptation to the conditions of their existence increase. Membrane filters with pore sizes of 0.1 and 0.05 μm let through filterable and L-forms of mycobacteria, which were cultivated at 3°C for a long time, and their cultures acquire the ability to show variability in tinctorial properties. It has been proved that dissociative elementary bodies are cultivated simultaneously with other morphological forms, preserving a possibility of reversion to acid fast-negative forms of the causative agent of tuberculosis, which is possibly determined by specificity of the life cycle of mycobacteria. Elementary bodies are found in subcultures of mycobacteria that were multiply passaged in a solid culture medium, generating rod-shaped acid fast-negative dissociative forms, which is due to the ability of small forms to adapt to a selective culture medium over time after multiple passages. Therefore, elementary bodies do not generate descendants of similar morphology but form acid fast-negative rod-shaped variants of mycobacteria instead. The study of archival strains of dissociative mycobacteria has shown that the frequency of in vitro isolation of filterable forms depends on the storage time of mycobacterial cultures and the frequency of isolation of cultures from filtrates does not depend on the number of passages in a solid culture medium. A low above-zero temperature of cultivation (3°C) results in a more frequent isolation of mycobacterial cultures from the filtrate, while the highest frequency of isolation of filterable forms of *M. bovis* was recorded after a single guinea pig passage. Electron microscopy has clearly demonstrated that L-forms of mycobacteria isolated from the control culture filtrate (F-0.05) are represented by thick rods that have semitransparent borders and uneven edges.

Keywords: Word Dissociants, filterable forms, L-forms, morphology, mycobacteria

Introduction

Variability of *Mycobacterium tuberculosis* determines the development and course of the epizootic process of tuberculosis (TB). An insight into the dynamics of bacterial excretion and specificity of the life cycle of *M. tuberculosis* complex is of paramount importance for the effective planning and organization of veterinary measures; however, failure to take into account the diverse origins of *Mycobacterium bovis* complicates the diagnosis and cannot provide a high economic effect in the struggle against the disease (Allen et al., 2010; Garbaccio et al., 2018; Ramos et al., 2015; Sales et al., 2001). A specific in-depth study of the morphological forms of this pathogen, in turn, will lead to significant changes in methodological approaches to the prevention and eradication of the disease in animal husbandry (Djachenko et al., 2008; Glebenjuk & Telizhenko, 2015; Lysenko et al., 2019; Tkachenko, 2004; Tkachenko et al., 2009).

A study of dissociation of *M. tuberculosis* complex is of particular relevance, since it determines methods for identifying various variants of the pathogen, which is of paramount importance for the study of its biology (Tkachenko et al., 2006, 2016, 2020). *M. bovis* exhibits variability in its ontogenetic development under different conditions of existence and goes through a number of stages and, accordingly, changes its morphology, biological properties, biochemical structure, and, as a result, virulence (Bentrup & Russell, 2001; Djachenko et al., 2009; Glickman & Jacobs, 2001; Kovaleva, 2005; Michailova et al., 2005; Niemann et al., 2000). Isolation of *M. tuberculosis* complex from pathological material is associated with certain

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difficulties due to specific genetic and phenotypic characteristics of this pathogen, such as slow growth on artificial culture media and its ability to L-transformation and long-term persistence in the altered (L-)form (Sekin, 2006; Slavchev & Markova, 2014). Polymorphism of mycobacteria significantly complicates the diagnosis and requires an in-depth study of their cultural properties depending on the pH level, temperature, and the number of passages in a culture medium (Jarovska & Sybirina, 2009; Lysenko et al., 2011; Prozorovski, 1981).

The research members of our department (Tkachenko et al., 2004, 2009, 2016, 2020b) experimentally proved that under conditions of multiple passages, mycobacteria retained their viability by rearranging their genetic apparatus and changing their morphological characteristics and tinctorial properties and acquired the ability to grow at low above-zero temperatures. Cultivation of *M. bovis* at 3°C significantly increases the efficiency of selection of microorganisms, especially their dissociative forms.

Today, formation of L-forms and the smallest filterable forms of mycobacteria, which, against the background of resistance to external factors of their existence, change their morphology, especially complicates the effective diagnosis of bovine tuberculosis (Huang & Lin , 1999; Mattman, 2000; Prozorovski, 1981; Shleeva et al., 2002, 2011). Moreover, changes in the morphology of mycobacteria and, as a consequence, a decrease in cell metabolism determines special requirements for the cultivation conditions of their L-forms (Markova et al., 2012; Michailova et al., 2005; Sekin, 2006). It was also found that both L-forms and filterable forms under certain conditions can revert to typical mycobacteria with restoration of their virulence, which increases the relevance of in vitro studies of *M. bovis*. It is assumed that such changes in the pathogen occur in response to the action of adverse agents or as a consequence of staging of their life cycle (Errington et al., 2016; Kochemasova, 1980). However, this issue requires further comprehensive research.

The question of pathogenicity of the bacterial L-forms has long remained controversial, because with the loss, or partial loss, of the cell wall, bacteria also lose most of their characteristics that deliver virulence factors (Clasener, 1972; Domingue, 2010; Guliang & Tefu, 1999; Onwuamaegbu et al., 2005). However, there are L-forms that are not inferior in their virulence to stock strains, and there is evidence that L-forms even acquire new properties that are absent in stock cultures (Errington, 2017; Galatova et al., 1990; Kaushal et al., 2002; Manganelli et al., 2002). One of the main biological features of L-forms of microorganisms is their ability to revert to the parental form of bacteria. The reversal process is of great theoretical and practical interest, since revertants fully or partially retain the properties of a microbial cell and can cause the disease (Howard et al., 2006; Prozorovski, 1981).

It is well known that typical forms of *M. tuberculosis* do not pass through bacterial filters; therefore, filterable small forms of mycobacteria, which belong to elementary bodies, make it extremely difficult to culture mycobacteria on conventional selective media (Kochemasova, 1980; Sekin, 2006). A similar phenomenon is observed during dissociation, where the released mycobacteria are avirulent and are not cultured or poorly cultured on conventional culture media. Reports of many authors indicate that the filterable forms and elementary bodies are similar in their biological structure. However, as our previous years research (Tkachenko et al., 2016, 2020a) shows, dissociative forms of mycobacteria, including filterable ones (elementary bodies), are cultivated at 3°C on conventional culture media having a pH value of 6.5–7.1. This research has indicated the possibility of studying the occurrence of the smallest forms (elementary bodies) in the population of *M. bovis* dissociants and investigating their role in the life cycle of this type of microorganisms.

Research members of our department (Tkachenko et al., 2016, 2020a, b) have repeatedly demonstrated that the frequency of isolation of filterable forms in the population of dissociative variants of *M. bovis* increases during passaging, and such filterable forms, when seeded on a culture medium, generate rod-shaped mycobacteria and, rarely, elementary bodies. The latter are an integral part of the mycobacterial life cycle, because it is the elementary bodies (filterable forms) that the typical rod-shaped forms of microorganisms are subsequently formed from.

However, to this date, the morphological forms of mycobacteria and their tinctorial properties remain understudied, especially at the ultrastructural level, and reports in the scientific literature are controversial and require further in-depth studies.

Methods

The study of morphology, tinctorial, and cultural properties of *M. bovis* was carried out on the basis of research laboratory of the Department of Epidemiology and Infectious Diseases of Animals of the Dnipro State Agrarian and Economic University. The study was approved by the Animal Researches Committee (ARC) of Dnipro State Agrarian and Economic University, Ukraine (approval no.: 2021/03/30).

Obtaining Test Samples of *M. bovis* Cultures

Archival virulent fast-growing strain of *M. bovis* passaged in a solid culture egg-based medium was used in the study. The original virulent mycobacteria were stored without subculturing at a temperature of 3°C for 3–20 months. After 20 months of storage, we observed an intensive growth of colonies and mycobacteria of different morphologies which were identified as dissociative variants and were subsequently passaged 124 times at 3°C. Dissociants of 60 and 110 generations (a, b, c and 118) were used in the experiments. At the same time, *M. bovis* (passages 54; 100; 115; 135, 143; 171; 180), which were stored under the same conditions (at 3°C and closed with rubber stoppers) for 9–12 years, were studied as well. Similar subcultures, before they were exposed to 3°C, were obtained by successive passages of the fast-growing strain of *M. bovis* at 37°C. To detect filterable forms of *M. bovis*, we also studied dissociative variants 117 a, b, c and 118 of subcultures passaged up to the 240th generation. The cultures were passaged 12–14 days after the appearance of the first signs of their growth (Tkachenko et al., 2020a, 2021). The experiments were carried out in five stages (Figure 1).

Dissociants were obtained as a result of cleavage of avirulent cells from the virulent epizootic strain of *M. bovis*, which occurred after 116th passages of the virulent culture suspension on a solid culture medium and storage of cultures of the 117th passage for 20 months at a low above-zero temperature (Tkachenko et al., 2021). Highly virulent archival strain of *M. bovis* of the 100th and 124th passages served as a control.

Study of *M. bovis* Properties

Cultures were examined for the growth rate on a culture medium and appearance and pigment formation. Smears stained by the

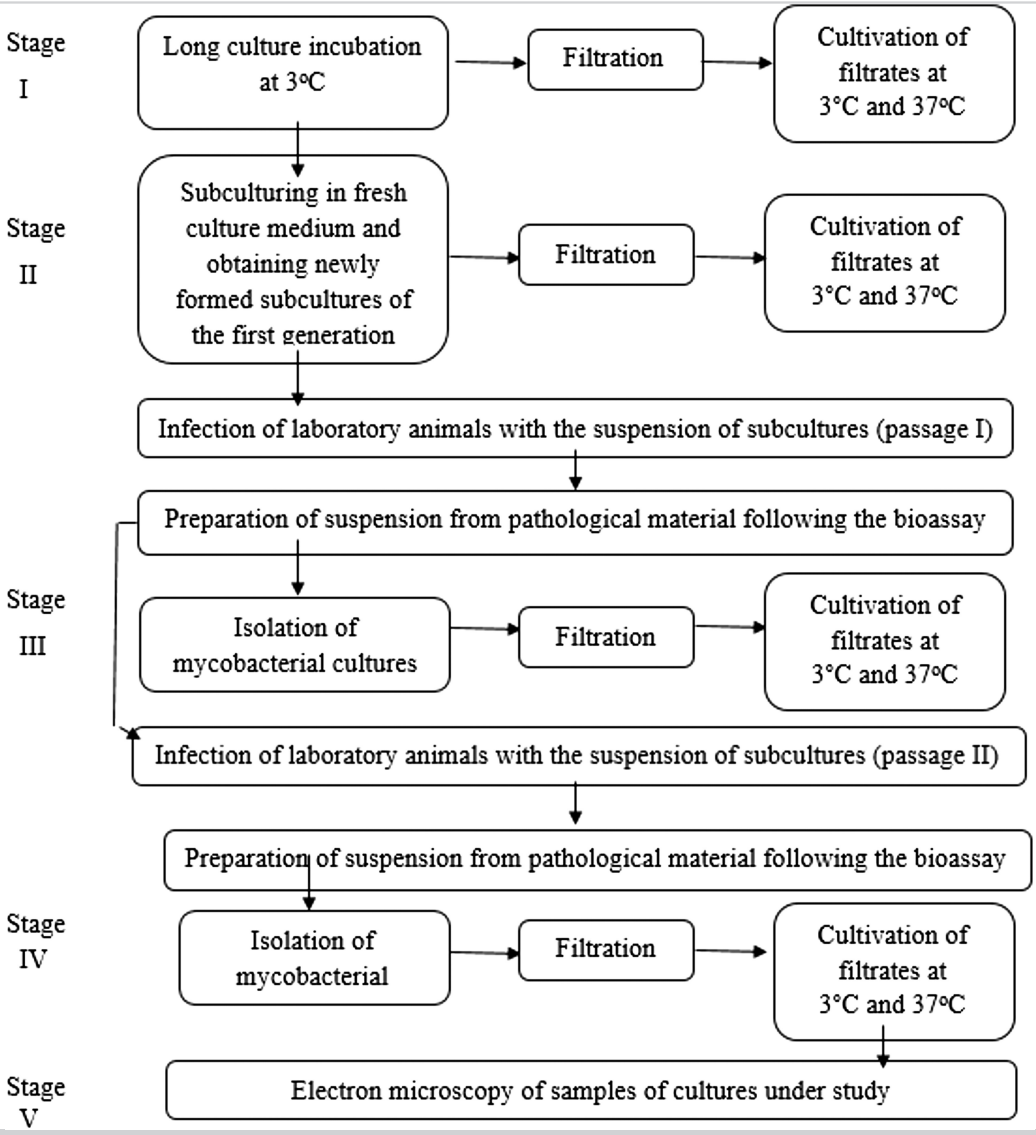

Figure 1
Experimental Design.

Ziehl–Nielsen technique were examined for the morphology and tinctorial properties of mycobacteria. Microscopy of smears from stock cultures and subcultures was performed after their passaging before and after filtration.

Virulent properties of the strains under study were assessed in vivo using guinea pigs. During the first passage, laboratory animals were infected with a suspension of each test culture (passages 54, 100, 115, 171, 143, 180) at a dose of 1 mg/cm^3 (8.0×10^5 CFU/cm^3) of isotonic solution. During the second and third passages, the animals were infected with a suspension from pathological material obtained after the first and second passages, respectively. Guinea pigs infected with the pathogenic maternal strain of *M. bovis* served as the control.

A total of 182 pigs weighing 250–300 g were used in the experiments, including the control. Conducting a biological test on guinea pigs was dictated by the need to determine the virulent properties of mycobacteria after many years of their exposure to stress (lack of oxygen and nutrients).

To infect laboratory animals with a suspension prepared from organs obtained from guinea pigs, the pathological material was pretreated using Alikaeva's method. The animals were infected by injecting 1.0 cm^3 of suspension into the inner side of the thigh. During the experiment (3 months), the animals were weighed and the formation of ulcers at the site of inoculation of mycobacteria and allergic reactions to bovine purified protein derivative (PPD) tuberculin injected at a dose of 25 IU in 0.1 cm^3 of isotonic solution were monitored. Tuberculinization was carried out on the 30th, 60th, and 90th days after inoculation, and the reaction was recorded 24–48 hours after administration of tuberculin.

After the animals were euthanized using ether anesthesia (on the 90th day of the experiment), pathologicoanatomic examinations

were carried out and bacteriological examination of biological material derived from their organs was performed following its cultivation at 3°C and 37°C.

Filtration of *M. bovis*

The cultures under study were filtered using MFAS-B1 and MFAS-B2 membrane disc filters. Membrane material used was microporous film made on the basis of a mixture of cellulose acetates with a pore size of 0.1 and 0.05 μm and a total porosity of 80–85%, manufactured by JSC STC Vladipor. A suspension of mycobacterial cultures under study with the concentration of microbial cells of 1 mg/cm^3 was used for filtration.

The mycobacterial suspension of the experimental samples was obtained by collecting a culture with a wire inoculating loop over the flame of an alcohol burner in a box and placed in a sterile mortar, followed by homogenization of the bacterial mass supplemented with sterile isotonic sodium chloride solution.

The suspension of test samples of mycobacteria of various morphological forms was filtered into sterile volumetric tubes using a luer cone syringe. To carry out the filtration process, the filter holder case was unscrewed, microfiltration membranes were placed on the support mesh (tribrach), the case screwed on, and then connected to the syringe. The suspension was filtered into sterile test tubes. Filtrates F-0.1 (MFAS-B1 filter, 0.1 μm pore size) and F-0.05 (MFAS-B2 filter, 0.05 μm pore size) of each test sample were plated on the fresh egg-based medium (pH 6.7) and cultivated at 3°C and 37°C. Tubes containing a culture medium seeded with a mycobacterial suspension which was not subject to filtration served as the control.

Electron Microscopy

Ultramicroscopic characteristics of mycobacteria were studied at the laboratory of electron microscopy of the Sumy National Agrarian University. The filterable forms of the control culture of the 115th passage of *M. bovis* and dissociative forms (cultures 117 a, b, c) were examined using a PEM-106-i scanning electron microscope. Sampling for scanning electron microscopy was carried out by preparing a suspension of microbial cells in distilled water. For this purpose, one inoculation loop of the bacterial mass was dissolved in 2 cm^3 of distilled water, previously poured into centrifuge tubes. To compact the cells of microorganisms, the tubes were centrifuged at 3000 rpm for 15 minutes. At the end of centrifugation, the excess supernatant was removed with a pipette.

The material was fixed with a glutaraldehyde-based fixative, which was introduced into test tubes containing 0.1 mL of the centrifuged sediment of bacteria samples. The tubes were shaken to obtain a uniform suspension of the sediment and left at the temperature of melting ice for 30 minutes. After 15 minutes, the tubes were shaken once again. At the end of the exposure, the samples were centrifuged at 1500 rpm for 15 minutes and the fixative was removed with a pipette, leaving a precipitate. The material was fixed twice. In order to remove excess water from bacterial cells, the test samples were dehydrated by treating them with increasing concentrations of ethyl alcohol (30–100%) added to the samples at the ratio of 1:10. With an increase in the concentration of ethyl alcohol used to treat each sample, the exposure time was also increased. After dehydration in absolute alcohol and centrifugation, the test samples were placed on a carbon tape and air dried. To ensure electrical conductivity, the test samples were sprayed with silver in a VUP-5 at a vacuum of about 10^{-5} mm of mercury. The prepared samples were placed in an electron microscope and subjected to scanning electron microscopy.

Results of the study

At the first stage, we studied the cultures of *M. bovis*, which were stored for 3 months at a low above-zero temperature (3°C) in the culture stock center of our department. It was found that the culture of the 60th generation, variant 117a of dissociative *M. bovis*, was characterized by continuous growth of yellow-orange color along the seeding line (Figure 2A).

Microscopy of smears of the culture under study revealed single small acid fast-negative (and sometimes acid-fast) grains and a significant number of short and longer filamentous rods (Figure 2B). No morphological forms of the examined mycobacteria were found in the filtrate (F-0.1 μm and F-0.05 μm) obtained from the stock culture of mycobacteria. However, the filtrate (F-0.05 μm) seeded on a culture medium stimulated the subculture growth (Figure 2C) on the 50th day of cultivation. Microscopy of smears prepared from the resulting subculture (F-0.05 μm) revealed acid fast-negative granular rods and single grains (Figure 2D). The other filtrate (F-0.1 μm) showed negative results.

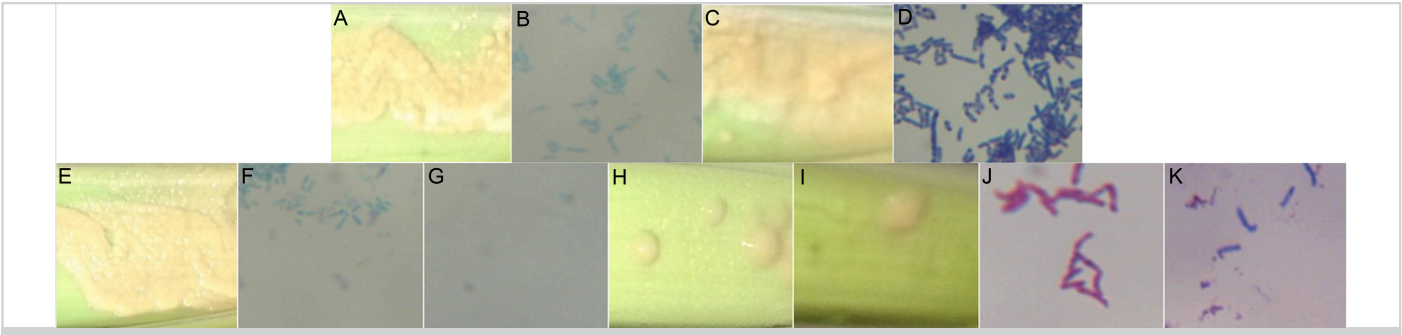

Figure 2
Cultural Properties and Morphology of Mycobacterium bovis *of Variant 117a. Culture: (A) the 60th Generation (C) from Filterable Forms of the 60th Generation (E) the 110th Generation, (H, I) from Small Forms of the 110th Generation; Morphology: (B) the 60th Generation (D) from Filterable Forms of the 60th Generation (F) the 110th Generation (G) small forms of the 110th Generation, (J, K)* M. bovis *Obtained from Small Forms of the 110th Generation (0.1 and 0.05 μm Pore Sizes). ×1600.*

After seeding the filtrate of the culture of the 110th generation, variant 117a, on a culture medium, the growth was observed along the seeding line on days 7–9 of cultivation (Figure 2E), and acid fast-negative coccoid, short and longer (sometimes filamentous) rods, were found in the field of view of the microscope under immersion (Figure 2F). The filtrates obtained from this subculture by 0.1 and 0.05 μm filtration contained single acid fast-negative microscopic grains (Figure 2G). Examination of the filtrates obtained by passing the culture through pores of filters of both sizes gave positive results. The culture growth in the form of scattered colonies was noted on days 19–22 of cultivation (Figure 2H and I). Microscopy of smears prepared from the obtained cultures revealed acid-fast short and longer straight and curved rods, as well as single microscopic grains (Φ–0.1 μm) (Figure 2J), as well as acid fast-negative granular rods and small acid fast-negative and acid-fast grains (Φ–0.05 μm) (Figure 2K).

When investigating the cultural properties of dissociative *M. bovis* of the 60th subculture of variant 117b (Figure 3), we observed continuous growth of an oily yellowish-orange culture on the culture medium (Figure 3A). A suspension of mycobacteria from this culture seeded on culture medium resulted in the growth of a similar subculture on days 7–8 of observation (Figure 3B). Microscopy of the resulting subculture revealed (Figure 3C) acid fast-negative coccoid, short and longer rods. Microscopic examination of the filtrate (F-0.1 and F-0.05 μm) obtained from the subculture did not detect any microorganisms.

During investigation of the properties of the 110th subculture, variant 117b, according to a similar scheme, the growth of a mucous gray-yellowish culture was observed (Figure 3D). Similar cultural properties were found after seeding a suspension prepared from the stock 110th culture (Figure 3E) on a culture medium. Smears prepared from a suspension of mycobacteria of variant 117b (subculture 110) were found to contain small acid fast-negative and acid fast-positive grains (30%) (Figure 3F). Microscopy of the filtrate (F-0.1 μm) obtained from this suspension revealed single microscopic acid fast-negative grains (Figure 3G). Similar grains were also found in the filtrate obtained by 0.05 μm filtration (Figure 3H). After seeding the first and second filtrates on the culture medium, an almost continuous growth of a yellow dryish culture, formed by scattered colonies, was recorded (Figure 3I and 3J). Microscopic acid fast-negative grains and rod-shaped forms of various lengths were found in smears prepared from cultures that stimulated the growth of small *M. bovis* (Figure 3K and L). Dissociative *M. bovis* of variant 117b in the 60th generation did not contain any small forms. They were found only in the 110th subculture.

Examination of the strain of dissociative *M. bovis* of variant 117b showed the following: the stock yellow-orange culture was formed by acid fast-negative single grains and rods and a large number of granular filamentous branched forms, some of which disintegrated into smaller particles. However, after they were filtered, microscopic examination of the prepared smears under immersion did not reveal any mycobacteria.

At the same time, examination of the 110th subculture of mycobacteria of variant 117b showed opposite results. The 110th yellowish stock subculture was formed by acid fast-negative grains and single (as an exception) granular rods. At the same time, rarely encountered acid fast-negative grains and sometimes thin short rods were found in the filtrate (F-0.1 μm). However, where the filtrate was seeded onto an artificial medium and incubated at 3°C, the culture grew in the form of separate colonies on days 23–26, which microscopically were formed by acid fast-negative granular rods.

It is known that L-forms of mycobacteria cultivated on a selective culture medium, despite their large size compared with ordinary rods, change their morphology due to the cell wall components and acquire properties that enable them to pass through bacterial filters. To clarify this issue, we investigated L-forms of the 118th generation (Figure 4).

The results of our study showed that within 20 months of storage of the 60th culture of the 118th generation at low above-zero temperature (3°C), virtually no changes occurred in its appearance; however, the morphological traits of mycobacteria and their tinctorial properties varied during the analyzed period (Figure 4A). The stock culture

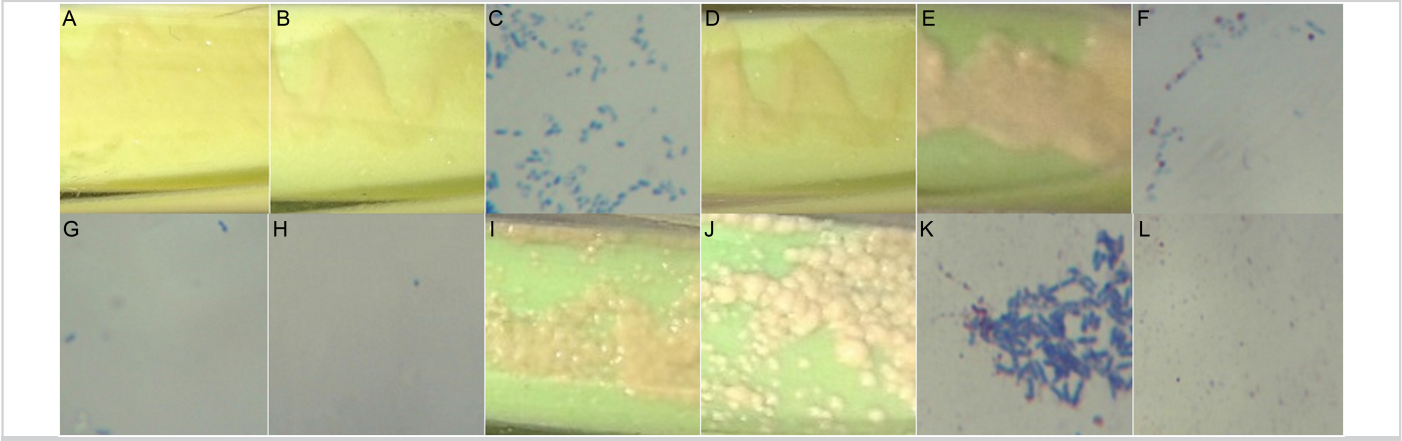

Figure 3
Cultural Properties and Morphology of Mycobacterium bovis *of Variant 117b. Culture: (A) the 60th Generation, (B) the 61st Generation, (D) the 11th Generation (Native), (E) the 111th Generation (Suspension), (I, J) from Small Forms of the 110th Generation (0.1 and 0.05* μm *Pore Sizes); Morphology: (C) the 61st Generation, (F) the 111th Subculture, (G, H) Small Forms of the 110th Generation (0.1 and 0.05* μm *Pore Sizes), (K, L)* M. bovis *Obtained from Small Forms of the 110th Generation (0.1 and 0.05* μm *Pore Sizes). ×1600.*

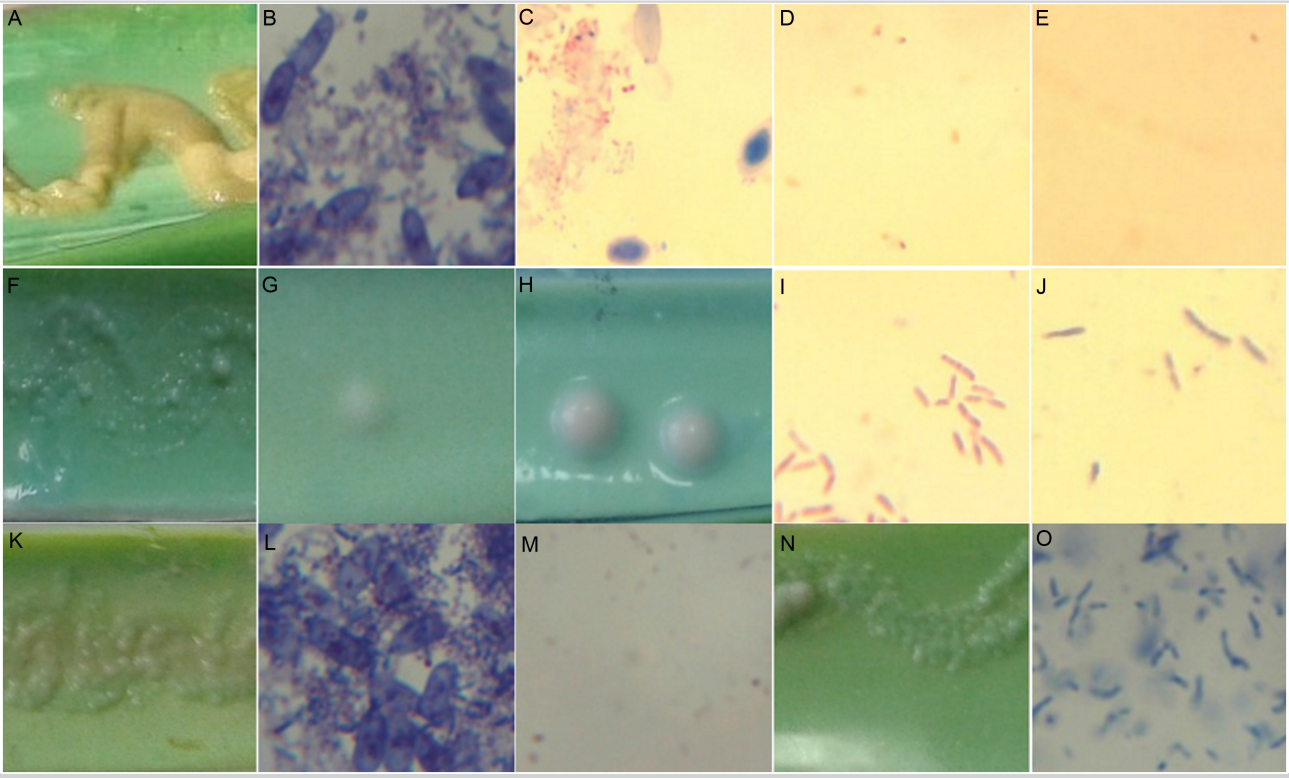

Figure 4
Cultural Properties and Morphology of Mycobacterium bovis *of Variant 118. Culture: (4A) the 60th Generation, (4F) the 61st Generation (Control), (4G, 4H) from Filterable Forms of the 60th Generation (0.1 and 0.05* μm *Pore Sizes), (4K) the 110th Generation, (4N) from Filterable Forms of the 110th Generation; Morphology: (4B) the 60th Generation (Native), (4C) the 60th Generation (Suspension), (4D, 4E) Small Forms of the 60th Generation (0.1 and 0.05* μm *Pore Sizes), (4I,4J)* M. bovis *Obtained from Filterable Forms of the 60th Generation, (4L) the 110th Generation, (4M) Small Forms of the 110th Generation (0.1* μm *Pore Size), (4O)* M. bovis *Obtained from Small Forms of the 110th Generation. ×1600.*

was formed (Figure 4B) by single acid fast-negative small grains, coccoid, granular short and longer rods, filaments (which were formed by grains), and L-forms (ovals) that released granular formations. Microscopy of this subculture after 20 months of its incubation at low above-zero temperature revealed a different morphology and tinctorial properties of mycobacteria. Reddish bacteria with predominantly blue grains in the middle prevailed in the smears. Such grains, mostly reddish in color, were pushed out (released) through the shell. In the field of view of the microscope, grains dominated and only single acid fast-negative L-forms with different optical densities were seen.

Microscopy of the suspension of mycobacteria stored for 20 months at 3°C, which was obtained by 0.1 and 0.05 μm filtration, revealed only a few small acid fast-negative grains (Figure 4D and E). Seeding of the filtered material (mycobacterial suspension (control)) (Figure 4F) resulted in the growth of a significant number of colonies as early as on days 8–10. Seeding of the culture filtrate obtained from mycobacteria, regardless of the filter pore size, onto the culture medium followed by its cultivation at 3°C, resulted in the growth of single colonies (1–3 per test tube) on the 23rd day (Figure 4G and 4H). Microscopy of the culture revealed acid fast-negative (partially) short and long granular rods and isolated single grains (Figure 4I). Another culture (Figure 4J), obtained by 0.05 μm filtration, examined using the microscope immersion system, was found to contain acid fast-negative rods, long with rounded ends and grains in the middle. At the same time, microscopic single acid fast-negative grains (elementary bodies) were found in smears. Obviously, filters of different pore sizes let through small forms (elementary bodies) of various sizes having a certain potential ability to generate acid-fast and acid fast-negative forms of mycobacteria, which is possibly related to the diversity of their biological significance.

When examining the culture of the 110th passage (Figure 4K) of the same strain of dissociative mycobacteria using the immersion objective, we found (Figure 4L) acid fast-negative forms similar to those of the 60th generation: short coccoid rods and elongated L-forms with dark grains in the middle, which in some cases were released through the shell. Microscopy of the filtrate prepared from mycobacterial suspension (F-0.1 μm) revealed small grains (Figure 4M). After seeding the filtrate onto a culture medium, a continuous growth culture was obtained (Figure 4N) on day 20, and a smear prepared from the resulting culture was found to contain only acid fast-negative grains and coccoid short and long granular rods (Figure 4O) in the absence of L-forms.

When examining *M. bovis* of pathogenic maternal strain of the 124th generation (Figure 5) cultivated at 37°C, we found numerous, small and medium-sized, regular-shaped opaque colonies yellow-white in color (Figure 5A), while smears (Figure 5B) demonstrated acid fast rods, short, thin and straight with rounded ends, both singly scattered and gathered in clusters.

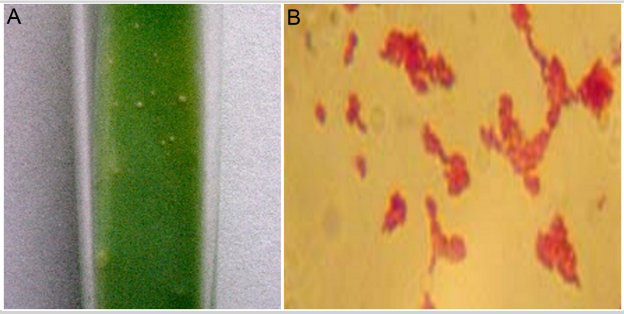

Figure 5
Subculture (A) and Morphology (B) of Pathogenic Mycobacterium bovis *of Passage 124. ×1600.*

Having filtered the investigated *M. bovis* of pathogenic strain of the 124th generation and examined smears prepared from the filtrate after 3-month incubation using an oil immersion technique, we did not find any mycobacteria and did not observe any culture growth.

At the second stage of our study, we examined filterable forms of bacteria in the *M. bovis* cultures stored in the culture stock center of our department for 9–12 years (Table 1).

During long-term storage, no passaging onto a fresh culture medium was carried out, which, in turn, led to the depletion of nutrient qualities of the medium, as well as to a strong decrease in the oxygen level inside the test tubes. In parallel, cultures of dissociants, 117a, b, c, 118, and a virulent fast-growing strain of *M. bovis* of the 100th passage were examined for the presence of filterable forms (Table 2). After passing the mycobacterial suspension through the 0.05 μm filter and cultivating the resulting filtrate at 3°C, new cultures were obtained from 49.8% of the filtrate vs. only from 16.6% of cultures when cultivated at 37°C.

Passing the test material through a 0.1 μm pore size filter and cultivation of the filtrate at 3°C resulted in obtaining new cultures from 64.4% of the filtrates, and the same result was obtained after cultivation of cultures at 37°C. No dependency of the frequency of culture isolation on the number of passages was observed.

The culture of passage 135 in the control (a suspension that was not subject to filtration) was found to be able to produce the colony growth only at a low temperature of cultivation (3°C). Cultures of the 115th and 171st passages in the control produced the growth at both cultivation temperatures while all other cultures were productive only at 37°C.

In the control, the dissociative variants were found to be able to grow at both cultivation temperatures, whereas the virulent fast-growing strain of *M. bovis* grew only at 37°C. When using a filtrate from the 0.1 μm pore size filter, new cultures were obtained from 80.0% of the filtrate at both cultivation temperatures. After filtration using a 0.05 μm filter and cultivation at 3°C, new cultures were obtained from 40.0% of the filtrate, while cultivation at 37°C caused no new culture growth. It is significant that among the dissociants, mycobacteria of variant 118 were unable to pass through bacterial filters at all, since there was no culture growth from any of the filtrates. It can be concluded that the low temperature of cultivation had a positive effect on the frequency of isolation of mycobacterial cultures from the filtrate (F-0.05 μm).

At the second stage of our study, we investigated the presence of filterable forms of bacteria in early cultures of the first generation (Table 3) obtained after passaging the long-stored stock cultures of *M. bovis* onto a fresh culture medium and cultivating them at 3°C and 37°C. It should be noted that mycobacteria of passages 115 and 171 were able to form colonies at both cultivation temperatures, passage 135—only at 3°C—and passages 54, 143, 193—only at 37°C. Therefore, for filtration, we afterwards used both variants

Table 1
Growth of Cultures from Filtrates of Long-Stored M. bovis *Cultures*

	37°C			3°C		
Passage	**F-0.05**	**F-0.1**	**Control**	**F-0.05**	**F-0.1**	**Control**
54	–	–	+	–	–	–
115	+	+	+	+	–	+
135	–	–	–	–	–	+
143	–	+	+	–	+	–
171	–	–	+	+	+	+
180	–	–	+	+	–	–

Note: culture growth recorded +; no culture growth –.

Table 2
Culture Growth from Filtrates of Dissociants and Virulent Mycobacterium bovis

	37°C			3°C		
Passage	**F-0.05**	**F-0.1**	**Control**	**F-0.05**	**F-0.1**	**Control**
100	–	+	+	+	+	–
117a	–	+	+	–	+	+
117b	–	+	+	+	+	+
117c	–	+	+	–	+	+
118	–	–	+	–	–	+

Note: culture growth recorded +; no culture growth –.

Table 3
Culture Growth from Filtrates of Early Mycobacterium bovis *Cultures*

	37°C			3°C		
Passage	**F-0.05**	**F-0.1**	**Control**	**F-0.05**	**F-0.1**	**Control**
54	–	–	+	+	+	–
115 (37°C)	–	–	+	+	+	+
115 (3°C)	–	–	+	–	–	+
135	–	–	–	–	–	+
143	–	–	+	–	–	–
171 (37°C)	–	+	+	+	–	+
171 (3°C)	–	–	+	–	–	+
180	–	–	+	+	+	+

Note: culture growth recorded +; no culture growth –.

of cultures of the 115th and 171st passages grown at 3°C and at 37°C. As a result, after cultivation at 37°C, not a single culture was obtained from the F-0.05 filtrates, and after cultivation at 3°C, we obtained new cultures from only 50.0% of the filtrate. Cultivation of the F-0.1 filtrates at 37°C resulted in the growth of new cultures from 10.0% and at 3°C—from 37.5% of the filtrates.

At the third stage of our study, we infected guinea pigs with a suspension of mycobacteria from early cultures. For 90 days of the bioassay, where experimental animals were sensitized with bovine PPD tuberculin, none of them were found to develop an ulcer at the mycobacterial suspension injection site or lose weight. Following the bioassay, after euthanasia and autopsy, none of the experimental animals showed any pathological changes characteristic of tuberculosis.

Guinea pigs infected with the control maternal pathogenic strain of *M. bovis* reacted to tuberculin on days 30 and 60 of the experiment. The animals were observed to lose weight and develop ulcers at the injection site of the mycobacterial suspension on the 27th day. Guinea pigs of the control group showed pathological changes characteristic of tuberculosis: hyperplasia of the spleen and inguinal lymph nodes and specific lesions (granulomas) in the lungs.

Eight cultures of mycobacteria were isolated as a result of bacteriological examination of biological material: three cultures at 3°C (passages 115, 135 and 171) and five cultures at 37°C (passages 54, 115, 143, 171, 180). Morphologically, mycobacteria of the isolated cultures examined using the microscope immersion system were characterized by acid fast-negative rods and grains. The obtained cultures were also examined for the presence of filterable forms (Table 4).

After a single passage of early mycobacterial cultures through guinea pigs, the maximum number of cultures isolated from the filtrates was obtained under their cultivation at 3°C: 100% from the filtrates obtained after passing the suspension through the 0.05 μm filter and 87.5% from the filtrates obtained from the 0.1 μm filter. As a result of cultivation at 37°C, only 12.5% of cultures were obtained from each of 0.05 μm and 0.1 μm filtrates.

Table 4

Culture Growth from Filtrates Obtained After One Passage of Mycobacterium bovis *Through Laboratory Animals*

	37°C			3°C		
Passage	**F-0.05**	**F-0.1**	**Control**	**F-0.05**	**F-0.1**	**Control**
54	–	–	+	+	+	–
115 (37°C)	+	–	+	+	+	+
115 (3°C)	–	–	+	+	+	+
135	–	+	–	+	–	+
143	–	–	+	+	+	–
171 (37°C)	–	–	+	+	+	+
171 (3°C)	–	–	+	+	+	+
180	–	–	+	+	+	–

Note: culture growth recorded +; no culture growth –.

After completion of the first bioassay, the next group of laboratory animals (second passage) was infected with the suspension obtained from the organs of guinea pigs. Experimental animals did not show any allergic reactions to bovine PPD tuberculin, such as ulceration at the injection site or changes characteristic of tuberculosis. However, as a result of bacteriological examination of biological material, four cultures were isolated at 37°C (passages 54, 171, 143, 180). Smears from these cultures examined under immersion were found to contain acid fast-negative rods and grains. The cultures obtained in the second passage were examined for the presence of filterable forms (Table 5).

After the second passage of the modified forms of mycobacteria cultivated at 37°C through guinea pigs, no cultures were isolated from the F-0.05 and F-0.1 filtrates, while after cultivation at 3°C, an equal percentage of cultures (75.0%) were obtained from each filtrate. The following group of guinea pigs (third passage) was infected with a suspension from organs obtained from guinea pigs from the second passage: the animals did not exhibit any allergic reactions to the bovine PPD tuberculin, ulceration at the site of injection, weight loss, or changes characteristic of tuberculosis. No culture was isolated as a result of bacteriological examination of the biological material from experimental animals. The frequency of in vitro isolation of filterable forms depends on the storage time of mycobacterial cultures. The frequency of isolation of cultures from the filtrate does not depend on the number of passages through a solid culture medium. The low above-zero temperature of cultivation causes a more frequent isolation of mycobacterial cultures from filtrates. The frequency of isolation of filterable forms is the highest after a single passage through guinea pigs.

Using an example of the 115th passage culture, which was stored at the culture stock center of our department at a low above-zero temperature of cultivation (3°C) for 12 years, we examined its features in comparison with the control culture obtained from a suspension that was not subject to filtration (Figure 6).

The growth of culture of the control sample was recorded on the 73rd day and of the culture from the filtrate as soon as on the 66th day of cultivation. The control culture was represented by greenish flat mucous colonies of irregular shape. Properties of the obtained cultures are described in Table 6.

At the fourth stage, tinctorial properties and morphology were investigated by light microscopy of smears from cultures stained using the Ziehl–Neelsen method (Figure 7). Examination of cultures obtained by seeding the suspension and F-0.1 filtrate from

Table 5

Culture Growth from Filtrates Obtained After Two Passages of Mycobacterium bovis *Through Laboratory Animals*

	37°C			3°C		
Passage	**F-0.05**	**F-0.1**	**Control**	**F-0.05**	**F-0.1**	**Control**
54	–	–	+	+	+	–
143	–	–	+	+	+	–
171 (37°C)	–	–	+	–	–	+
180	–	–	+	+	+	–

Note: culture growth recorded +; no culture growth –.

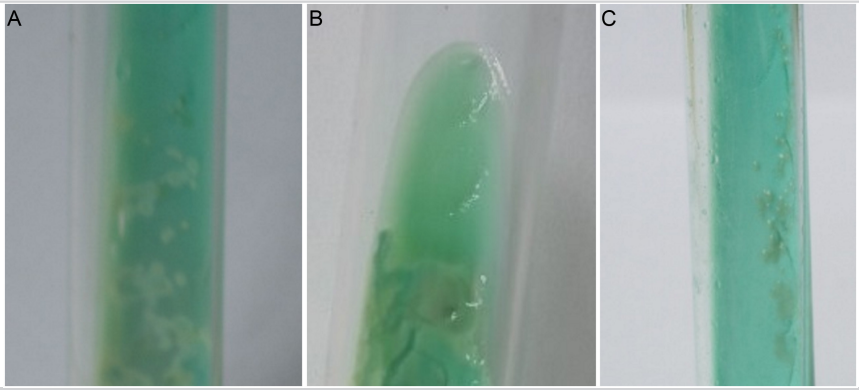

Figure 6
Culture Growth on a Solid Egg-Based Medium. A-Control; B-0.1 μm Filtrate (F-0.1); C-0.1 μm Filtrate (F-0.05).

the bacterial culture under study onto a culture medium showed that the morphology of microorganisms significantly differed from their parent culture. Both obtained cultures were morphologically similar to each other and were represented by acid fast-negative rods with rounded ends (Table 7); the culture from the filtrate, in addition to rods, also contained single grains (elementary bodies).

A microscopic picture of the culture from the 0.05 μm filtrate is represented only by giant elongated oval-shaped L-forms which contain a large number of grains. Some mycobacterial cells are able to pass through a bacterial filter with a very small pore size (0.05 μm) and subsequently give rise to a new generation of bacteria, not typical rod-shaped but cell wall-deficient ones (L-forms). Such result of the L-form formation can be explained by the effect of a stress factor on bacteria, and filtration is such a factor, and the very fact of the cell wall loss can be considered as a mechanism of bacterial adaptation to unfavorable conditions.

When investigating cultural properties of 117a, 117b, 117c dissociative forms, we found that the cultures obtained from the filtrates and control cultures had certain differences (Figure 8).

Table 6
Properties of Cultures Obtained from Filtrates

Characteristic	Control Group	Culture from 0.1 μm Filtrate	Culture from 0.05 μm Filtrate
Growth rate	Moderate	Slow	Moderate
Growth pattern	Clustered colonies	Scattered colonies	Clustered colonies
Quantity	Numerous	Singly scattered	Numerous
Size	Small	Big	Small
Form	Irregular	Irregular	Regular
Surface	Smooth	Smooth	Smooth
Consistency	Mucous	Mucous	Mucous
Pigmentation (color)	Greenish	Greenish	Yellowish
Transparency	Semitransparent	Semitransparent	Semitransparent
Emulsification	Satisfactory	Satisfactory	Satisfactory

Control cultures are represented by small and medium-sized opaque yellow-orange S-colonies. In the samples obtained from the filtrates, the culture growth rate on the culture medium is lower. In culture 117a from filtrate F-0.1, there were clusters of S-colonies, which significantly rose above the medium surface, larger than those in the control culture. Single large S-colonies rising in the center were found in culture 117b obtained from the filtrate. In culture 117c, the most pronounced differences were found between the control culture and the culture obtained from the filtrate—the colonies of the latter had the form of flat green spots of irregular shape with a smooth surface.

It was found that passing a mycobacterial culture suspension through a bacterial filter led to changes in the cultural properties of dissociative forms.

According to the results of light microscopy, mycobacteria in the control samples of dissociative variants 117a, 117b, 117c were represented by acid fast-negative short and long rods and single grains (Figure 9).

Mycobacterial cultures obtained from filtrates in variants 117a and 117b are morphologically similar to the control culture, while their morphology in variant 117c has changed dramatically—the culture is completely represented by acid fast-negative grains. The cultural properties of the control mycobacteria and mycobacteria of the culture obtained from filtrate 117c also differ.

At the fifth stage of our study, electron microscopy analysis of the samples of cultures under study was carried out (Figure 10). The control culture of the 115th passage was represented by thick somewhat convex rods with rounded ends, 2–3 μm in size, and grains of various sizes. The culture obtained from filtrate F-0.1, morphologically, was mainly represented by more convex thick rods similar to ovals, 2–3 μm in size (Table 8). The culture from filtrate F-0.05 was represented by somewhat arched thick rods of larger size (4.5–5 μm) with rounded ends and a semitransparent border with uneven edges, which is typical for L-forms.

According to the results of electron microscopy, cultures 117a and 117b, both control and obtained from the filtrates, have a similar morphology and are represented by short and long straight and slightly bent rods. Samples of variant 117b also contain long

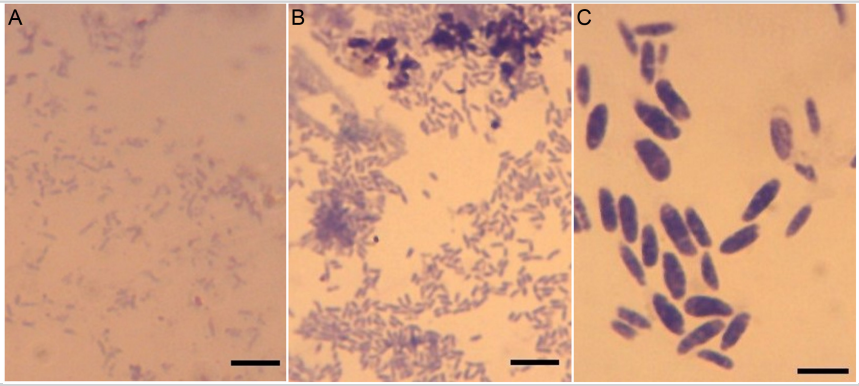

Figure 7
Light Microscopy of Mycobacterial Cultures of the 115th Passage. (Ziehl–Neelsen Staining, ×1600): A-Control, B-Culture from F-0.1 Filtrate, C-Culture from F-0.05 Filtrate. Bar = 10 μm.

filamentous rods (Figure 11). Morphology of the 117c cultures is different. The control cultures and the culture obtained from the filtrates are similar to each other and are represented by short convex rods with rounded ends, oval-shaped variants, and grains of various sizes.

Discussion, Conclusion and Recommendations

Tuberculosis is an extremely urgent problem for mankind (World Health Organization [WHO], 2020). The studies of phenotypic and genotypic properties of mycobacteria have actually been carried out since the discovery of these microorganisms by R. Koch. Bacteria, discovered for the first time, were of relatively stable forms that had a cell wall, were able to grow, and exhibited their metabolic activity in vitro.

As known, typical forms of mycobacteria cannot pass through bacterial filters. However, it is the filterable forms of the causative agent of TB that prove the ability of bacteria to exist as populations in the forms with a deficient cell wall, which is an adaptive strategy for bacteria to survive and reproduce in unfavorable conditions of existence (Allan, 2009; Domingue, 1982; Mattman, 2000). Obviously, filters with different pore sizes let through small forms (elementary bodies) of various sizes, which have a certain potential ability to generate acid-fast and acid fast-negative forms of mycobacteria, in our opinion, is associated with the diversity of their biological significance (Tkachenko et al., 2016).

Table 7
Characterization of Morphological Traits and Tinctorial Properties of Mycobacterial Cultures Under Study

Characteristic	Control Culture	Culture from 0.1 μm Filtrate	Culture from 0.05 μm Filtrate
Tinctorial properties	Acid fast-negative	Acid fast-negative	Acid fast-negative
Morphological traits	Short rods	Short rods	ElongatedL-forms
Thickness	Thin	Thick	Thick
Rod shape	Straight	Straight	Straight
Ends	Rounded	Rounded	Rounded
Granulation	Absent	Absent	Pronounced
Arrangement	Singly scattered, clusters	Singly scattered, clusters	Singly scattered, clusters
Elementary bodies	–	Acid fast-negative	Acid fast-negative
Ovals (*L*-forms)	–	–	Acid fast-negative

The authors (Domingue, 1982; Klieneberger-Nobel, 1951; Markova et al., 2012; Prozorovski, 1981; Tulasne, 1958), who investigated filterable forms of bacteria suggested that they were small reproductive elements and played an important role in the life cycle of L-forms. Ductility of L-form elements is precisely the factor that determines their filterable ability. Bacterial L-form conversion is considered a universal phenomenon well described by many authors (Allan et al., 2009).

Cell wall-deficient bacteria were first discovered in 1935 and were later named "L-form" after the Lister Institute in London. Today, most researchers of the L-forms of mycobacteria believe that this is their form of survival, that is, reproduction of microorganisms that have completely or partially lost their rigid cell wall due to natural or artificially induced reasons and have the ability to revert to the parental cell-walled form (Prozorovski, 1981). However, there is also an opinion that L-transformation is a pathological process that occurs under the influence of a resistant acting factor or is the result of spontaneous mutation (Errington, 2017; Kaushal et al., 2002; Manganelli et al., 2002), and L-forms are a product of lethal degeneration of microbial cells. According to other data, L-transformation is a manifestation of adaptive variability of microorganisms under the influence of a resistant acting environmental factor, and, accordingly, L-forms are forms of microorganisms highly resistant to the action of corresponding agent (Domingue, 1982; Mattman, 2000; Prozorovski, 1981; Slavchev & Markova, 2014).

Researchers have studied filterable forms of mycobacteria quite extensively using different experimental methods. The studies conducted by authors (dos Santos et al., 2019; Kochemasova, 1980) indicate that filterable forms are not cultivated or poorly cultivated on conventional artificial media. However, we have discovered (Tkachenko et al., 2016, 2020b) that dissociative forms of mycobacteria, including filterable ones, are cultivated at 3°C on solid egg-based

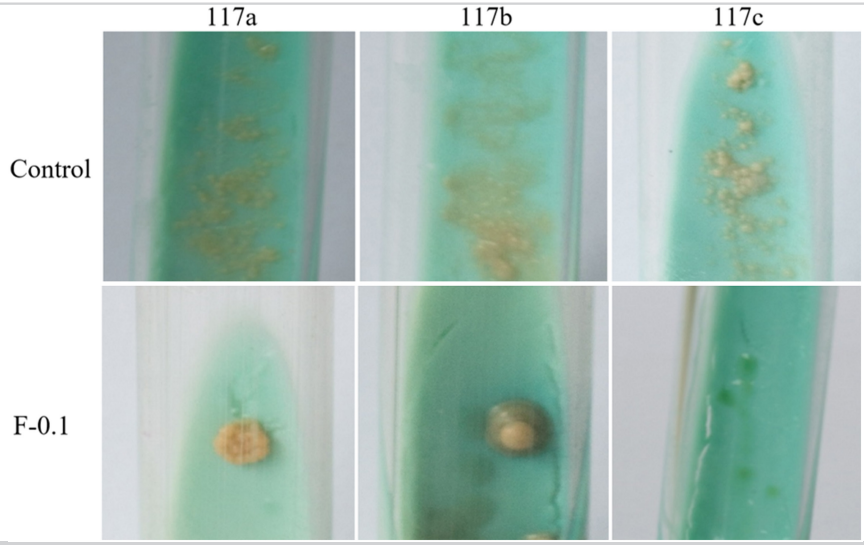

Figure 8
Growth of Dissociant Cultures on a Solid Egg-Based Medium.

media with a constant pH value of 6.5–7.1. We have not encountered any information in the scientific literature about the studies that would use similar methodological approaches.

We have found that long-term storage of the *M. bovis* culture in conditions of low above-zero temperatures, which are not typical for the existence of mycobacteria without their passaging onto a supporting medium, influenced the microbial cell metabolism and, accordingly, determined the mechanism of bacterial survival in adverse conditions. In our study, it was hypothesized that multiple passages and, especially, storage of mycobacterial cultures under conditions unsuitable for optimal metabolism of mycobacteria caused dissociation of certain microbial cells, which began to proliferate at 2–3°C during 20 months of incubation. Such variants of mycobacteria in their biological properties are closer to atypical mycobacteria. They did not cause death of guinea pigs in an in vivo experiment; however, they caused an infection and supposedly stimulated a latent infectious process in the animals, as evidenced by the long-term

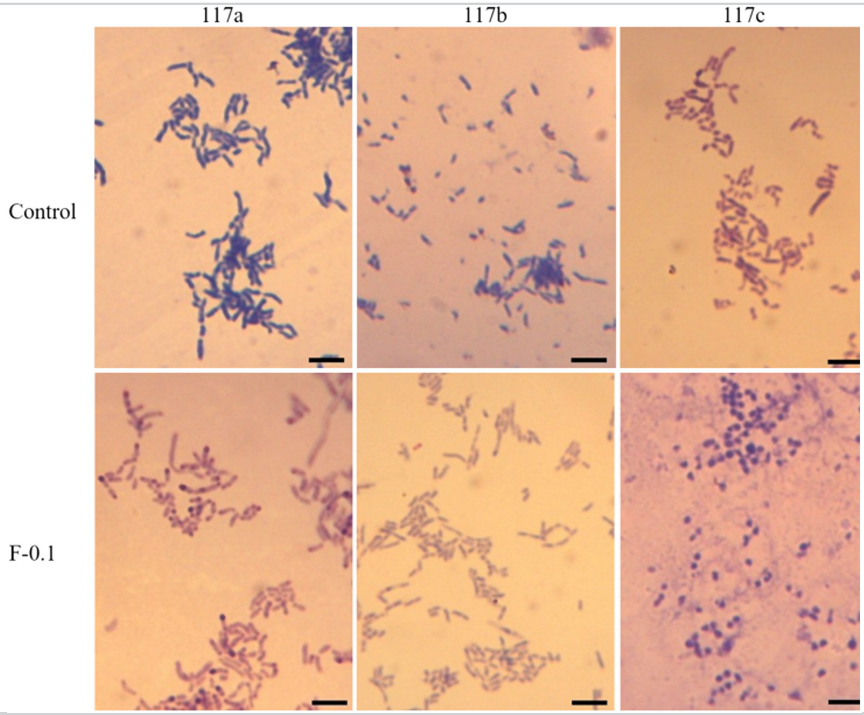

Figure 9
Light Microscopy of Mycobacteria of Dissociative Forms. (Ziehl–Neelsen Staining, ×1600): Bar = 10 μm

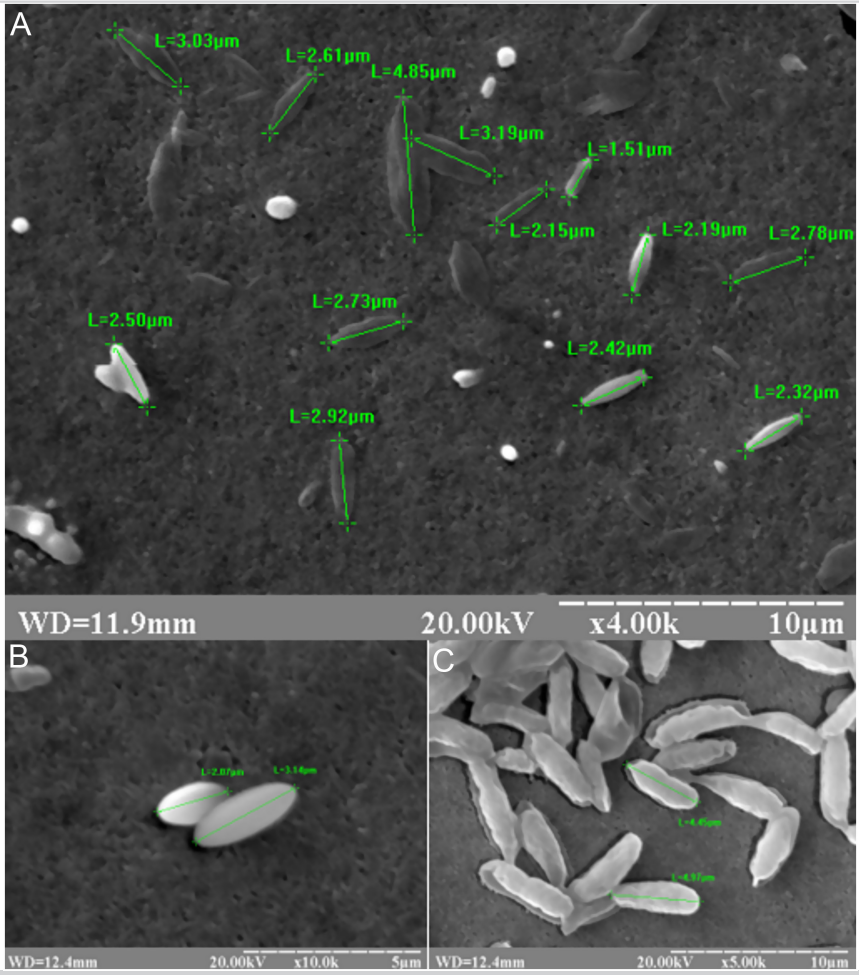

Figure 10
Electron Microscopy of Mycobacteria of Cultures Under study. A-Control; B-Culture from F-0.1 Filtrate; C-Culture from F-0.05 Filtrate.

persistence of inoculated mycobacteria in the tissues of experimental guinea pigs, which were isolated bacteriologically.

During our experiments, we have found that long-term storage of L- and other forms of the 60th generation of mycobacteria at low above-zero temperature was mainly accompanied by transformation of acid fast-negative rods and single grains into acid-fast ones, and L-forms demonstrated a clear tendency toward destruction. Our investigation of dissociative *M. bovis* of variant 117a showed that small forms of mycobacteria were constantly generated in the populations of microorganisms. Small grains in the first subculture generated polymorphic rods and grains, including microscopic ones. This obviously indicates that small forms of various sizes are one of the stages of the biological cycle of growth of the causative agent of TB. However, Slavchev G. et al. (2013) recorded the presence of small cellular elements, mainly granules, which were released from giant cells due to stressful conditions of existence. Some authors (Domingue, 2010; Mattman, 2000), on the contrary, believe that small granule-like forms have a protective role against unfavorable conditions of existence and take part in the L-form proliferation. It is believed that small forms contain a specific bacterial genome and are characterized by a minimal metabolic capacity sufficient for subsequent microbial cell proliferation. Other researchers (Ghosh et al., 2009; Lamont et al., 2012) confirm the ability of mycobacteria to form spore-like forms, short "empty" or ovoid forms containing granules.

Table 8
Morphological Forms of Mycobacterium bovis

Cultures	Rods (%)	Spherical (%)	Oval (%)	Destroyed (%)
Control	52	7	4	6
F-0.1 filtrate	11	14	67	8
F-0.05 filtrate	77	13	5	5
Dissociative forms				
117-a	73	13	8	6
117-б	87	4	4	5
117-в	15	20	55	10

The regularity we revealed (Tkachenko et al., 2020b) made it possible to conduct a comprehensive study of elementary bodies in the population of *M. bovis* dissociants and to determine their role in the life cycle of this type of microorganisms, as well as to confirm a certain

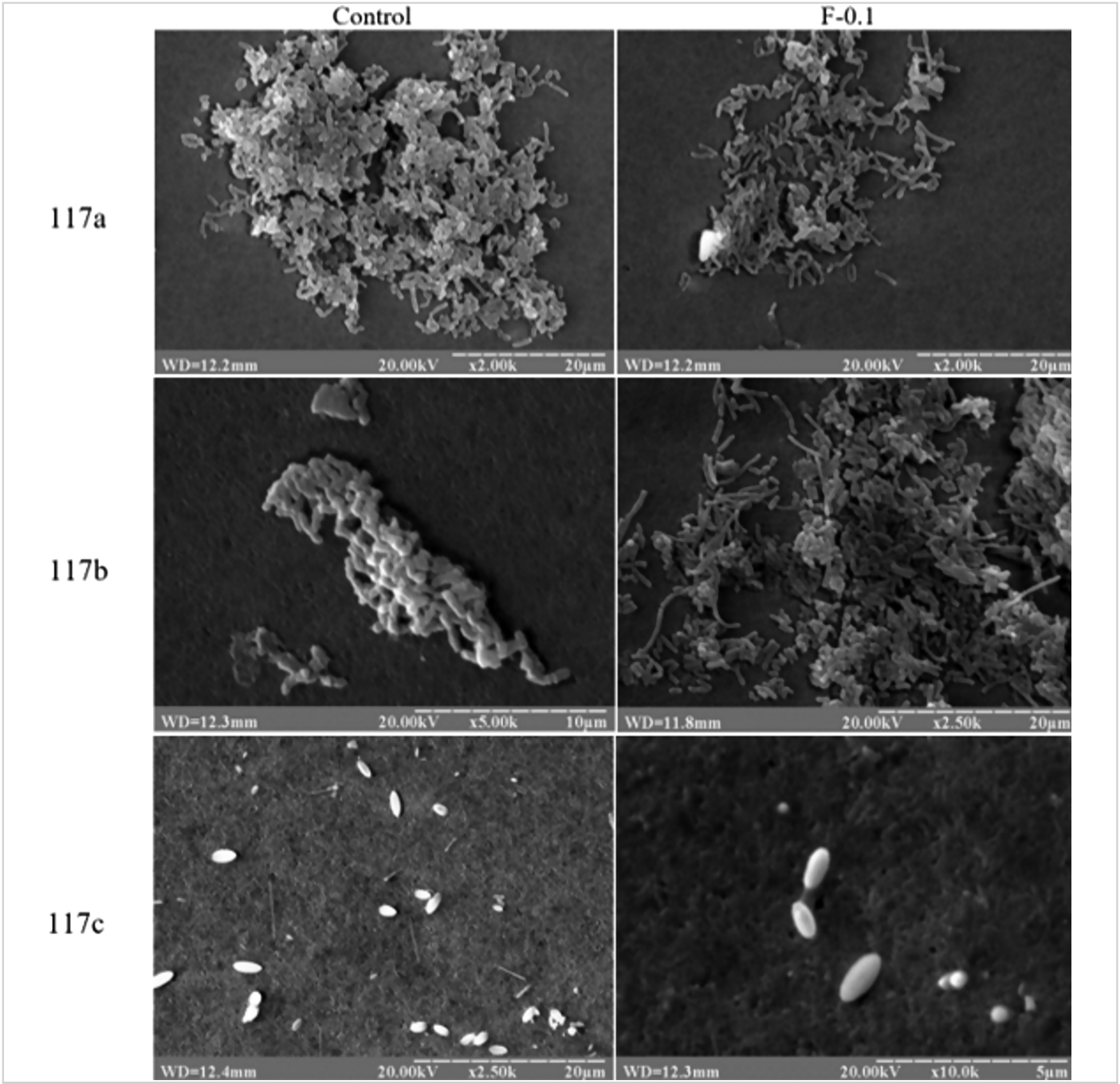

Figure 11
Electron Microscopy of Mycobacteria of Cultures of Dissociative Forms.

reorganization of the genetic code in individual mycobacterial cells. Small forms (elementary bodies) were more often found in subcultures of mycobacteria that were multiply passaged in a solid culture medium, generating rod-shaped acid fast-negative dissociative forms. This is probably due to the ability of small forms (elementary bodies) to adapt to a selective culture medium over time after multiple passages. However, on an artificial medium, elementary bodies do not generate descendants of similar morphology but form acid fast-negative rod-shaped variants of mycobacteria.

In a study by Markova et al. (2012), *M. bovis* BCG L-forms were obtained by starvation stress in vitro. Electron microscopic examinations performed by the authors demonstrated a typical ultrastructural morphology of L-bodies of various sizes and cell wall-deficient forms. Large spherical bodies, giant "mother" cells, small elementary bodies, and granules observed by the authors in the cultures of BCG L-forms allowed them to suggest that these bodies undergo division processes. It is significant that L-form cultures obtained from filterable bacille Calmette-Guérin (BCG) strains are similar in morphology,

ultrastructure, and reproductive processes. Elementary bodies, according to Markova et al. (2012), are the final stage of the life cycle of mycobacteria, which can give rise to their rod-shaped variants.

Domingue (1982) suggests that small, electron dense L-bodies are capable of developing along several different routes depending on the stimulus received and they have the potential for unlimited growth and division. On the other hand, the author considers the smallest filterable forms as minimal reproductive cells, which can be formed from large L-bodies in all possible ways.

Of considerable interest is the work of Markova et al. (2012) where the authors argue that *M. bovis* can transform into cell wall-deficient forms inside macrophages in guinea pigs and that the phenomenon of L-conversion significantly increases their ability to survive and persist in vivo.

According to the authors (Markova et al., 2012; Udou et al., 1982), in the absence of inducing factors in the population of mycobacteria, a gradual appearance of L-shaped cells is observed: transitional acid fast-negative resistant rod-shaped bacteria, filamentous variants, L-forms of protoplast (spheroplast) type, and grains (elementary bodies). In our study, the phenomena of dissociation were accompanied by a one-time appearance in individual cells of the ability to transform into non-pathogenic mycobacteria, while the maternal population of cells continued generating pathogenic, virulent cultures.

Lysenko et al. (2019) have found that *M. tuberculosis* transforms into a kind of "protective" forms that can survive heating even up to 121°C and can pass through filters with pore sizes in the nano range (3–10 nm), restoring their viability in the cell wall-deficient forms. The nature of the established phenomena, according to the authors, is not entirely understood, but they must be taken into account when assessing the risks of using biological products, food products, and studying pathological conditions of unclear etiology.

In our study, we, on the contrary, have found that it is the low temperature of cultivation (3°C) that has a positive effect on the frequency of isolation of mycobacterial cultures from the filtrates of early *M. bovis* cultures. However, we did not record any dependence of the frequency of culture isolation on the number of passages.

The intensity of in vitro isolation of filterable forms of *M. bovis* correlates with the culture storage duration. A low above-zero temperature of cultivation (3°C) on a solid egg-based medium stimulates isolation of mycobacterial cultures from the filtrate.

Subcultures of dissociative *M. bovis* (117 a, b, c and 118) are characterized by polymorphism and contain small forms, the frequency of isolation of which increases depending on the number of generations (passages), and the frequency of their isolation from filtrates obtained from cultures stored for 9–12 years (without passaging) does not depend on their number. Granular, mainly acid fast-negative small forms in the population of dissociative *M. bovis* are capable of generating morphologically altered microorganisms, which confirms their importance in the life cycle of the causative agent of tuberculosis.

Elementary bodies are an integral part of mycobacterial ontogenesis and are found in subcultures of mycobacteria that were multiply passaged in a solid culture medium generating rod-shaped acid fast-negative dissociative forms, which is due to the ability of small forms to adapt to a selective culture medium over time after multiple passages elementary bodies do not generate descendants of similar morphology but form acid fast-negative rod-shaped variants of mycobacteria.

Electron microscopy of L-forms of mycobacteria isolated from the control culture filtrate (F-0.05) is represented by thick rods having a semitransparent border and uneven edges. Rods of different lengths prevail in cultures of filterable and dissociative forms of mycobacteria, while samples of the 0.1 μm filtrate and 117a culture mostly contain oval forms.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Animal Researches Committee (ARC) of Dnipro State Agrarian and Economic University (Date: March 30, 2021, Approval No: 2021/03).

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