The Effect of Long-Term Storage on *Mycobacterium bovis*

OLEXIY TKACHENKO[1]* ⬤, NATALI KOZAK[1], MARYNA BILAN[1] ⬤, VOLODYMYR HLEBENIUK[1] ⬤,
NATALIA ALEKSEEVA[1] ⬤, LILIYA KOVALEVA[1], VITALII NEDOSEKOV[2] ⬤ and OLEXANDR GALATIUK[3] ⬤

[1] Dnipro State Agrarian and Economic University, Faculty of Veterinary Medicine, Dnipro, Ukraine
[2] National University of Life and Environmental Sciences of Ukraine, Faculty of Veterinary Medicine, Kyiv, Ukraine
[3] Polissya National University, Faculty of Veterinary Medicine, Zhytomyr, Ukraine

A b s t r a c t

It was established that when stored for many years (10–13 years) in low-temperature conditions (3°C), without sub-culture on a nutrient medium, *Mycobacterium bovis* grew as visible colonies along the line of inoculation. However, due to long-term storage in conditions of low temperature (3°C) morphology of mycobacteria differed significantly from initial cultures formed by rod-shaped bacteria. Some of them became pigment-forming and smooth on the surface. Unlike the initial strain of mycobacteria, a perennial bacteria stored under hard conditions did not cause the death of guinea pigs or their sensitization to a purified protein derivative for mammals. Morphological forms of the perennial mycobacteria had the following changes: pigment forming, L-forms of the vesicular type, non-acid-fast thread-like (filamentous) bacillary forms, and elementary bodies when compared to the initial strain. There were also some genetic changes in the target DNA due to the long-term storage of *M. bovis*. It may indicate a mutation in the pathogen's DNA. These mycobacteria had altered biochemical activity during storage. The number of passages on the solid nutrient medium did not affect their fermentative activity. However, the low cultivation temperature increases mycobacterial catalase activity and the ability to hydrolyze Tween-80.

Introduction

For many years, tuberculosis has remained relevant in veterinary and human medicine worldwide and in Ukraine. According to WHO, tuberculosis is one of the top ten causes of death in the world. In 2017, 10,000,000 people had a tuberculosis infection, and 1,600,000 died from the disease. In 2018, according to WHO, the overall incidence of tuberculosis in Ukraine was 36,000 (per 37,000,000 population).

Complexities of overcoming tuberculosis and mycobacteriosis can be interrelated to the variability of the bacterial cells that enable pathogens to survive both in hostile macrophages (Kumar and Sanyal 2012) and outside the organism. Mycobacteria have an extraordinary capacity to adapt to the changing environment (Velayati and Farni 2012). It has been shown that nutrient deficiency, hypoxia, temperature, pH, NaCl, and various exogenous stress can significantly influence the metabolism of mycobacteria and, at the same time, the microorganism's morphology (Vera and Rettger 1940; Smeulders et al. 1999; Bentrup and Russell 2001; Shleeva et al. 2002; Young et al. 2005; Anuchin et al. 2009; Velayati et al. 2009; 2011; Velayati and Farnia 2012).

It has been established that mycobacteria have a high degree of variability, and changes in the biological properties of *Mycobacterium bovis* (cultural, tinctorial, and morphology) may be accompanied by changes in their metabolism (Tkachenko et al. 2020b). The biological properties of mycobacteria and *M. bovis*, in particular, are far from being fully characterized. It leads to the tense situation of tuberculosis in individual countries, both among animals and humans.

Understanding the processes in a bacterial cell in response to the external factors could push the knowledge on the complex mechanism of mycobacterial adaptation and survival in macroorganisms and the environment.

There is no sufficient research on the impact of long-term storage of vaccine strains on the effectiveness

of preventive immunization for infectious diseases. The BCG strain is a good example. Long-term storage of a parent strain in various countries led to its variability and the formation of BCG sub-strains: Russia (Moscow), Japan (Tokyo), Moreau (Brazil, Rio), Sweden (Gothenburg), Birkhaug, Danish (Denmark, Copenhagen), Prague (Czech Republic), Glaxo (London), China, Phipps (New York, Park, Philadelphia), Tice (Chicago), Frappier (Montreal), Pasteur (Paris), Bulgaria (Sophia), Connaught (Toronto), etc. BCG sub-strains slightly differ in genetic traits and immunogenicity. Therefore, considering potential changes in bacteria's biological features after the long-term storage is essential for a comprehensive assessment of vaccines' safety and efficiency (Ritz et al. 2008; Zhang et al. 2016).

Therefore, there was necessary to investigate the morphology, growth rate, tinctorial properties, biochemical activity, pathogenic, and sensitizing properties of mycobacteria in long-term conditions of hypoxia, nutrient deficiency, and low temperature (3°C).

Experimental

Materials and Methods

Experiments were performed in a laboratory of the Department of Epizootiology and Infectious Diseases of Animals of the Dnipro State Agrarian and Economic University using the museum's fast-growing virulent strain *M. bovis*. The strain had been passaged many times (Tkachenko 2004) through solid Lövenstein-Jensen medium at pH 6.5 and 7.1. As a control (the initial mycobacterial strain), a pathogenic (parent) strain of *M. bovis* was used. The fast-growing strain of *M. bovis* was characterized by the formation of colonies after 2–3 days at 37°C, the absence of nitrate reduction reaction, hydrolysis of Tween-80, growth on the Lövenstein-Jensen medium containing sodium salicylate, and low catalase, dehydrogenase, and peroxidase activity (Tkachenko 2004; Kovaleva 2005; Glebenjuk and Telizhenko 2015; Tkachenko et al. 2020a).

Our study was approved according to the guidelines and roles of the Animal Researches Committee of the Dnipro State Agrarian and Economic University.

First, long-term storage of cultures (10–13 years at 3°C) in the test tubes was performed. The passages (variants of subculture, first-generation bacteria) No. 54, 115, 135, 135, 171, and 180 at pH 6.5, and passage No. 143 at pH 7.1 were grown in tubes covered with rubber stoppers. Each culture was stored in a nutrient medium in two tubes. It ensured the persistence of mycobacteria in an anoxic environment. Microscopy of smears was carried out, studying the tinctorial properties and morphology of mycobacteria. The *M. bovis* long-stored

strains were also inoculated onto Lövenstein-Jensen supportive nutrient medium (six test tubes), and subcultures were grown at 3°C and 37°C. The pigmentation, colonies, and their consistency were assessed. In the smears prepared from the first generations of mycobacteria (Manchenko et al. 1994), their tinctorial properties (acid resistance) and morphology were studied under the immersion system. The smears were stained by the Ziel-Nielsen method and viewed under a MicroMed XS-3330 microscope using an immersion lens (magnification 100-folds). The image fixation was performed using an eyepiece camera for the MDC-500 microscope and the CyberlinkYouCam program.

The culture purity was verified as follows: The bacterial suspension was divided into two parts, and one of them was heated at 130°C for 5 minutes. Then, both parts were inoculated on Lövenstein-Jensen medium at 37°C and Sabouraud agar at 30°C. Cultures were considered pure in the absence of growth of any foreign microorganisms on Lövenstein-Jensen nutrient medium after inoculation of heat-treated suspensions and the absence of colonies on Sabouraud agar.

At the second step, the pathogenic properties of mycobacteria were investigated. Seven guinea pigs were infected for each of six variants of subculture (a first passage). A control group of pigs was infected with the pathogenic parental strain of *M. bovis*. The suspension (2×10^5 CFU/ml) was injected parenterally (subcutaneous) from the inner side of the thigh. In total, 49 guinea pigs were infected. After euthanasia of the animals (on day 90th of the experiments), tissue specimens were examined bacteriologically after growth at 3°C and 37°C on the Lövenstein-Jensen nutrient medium. The pre-inoculation material was prepared separately from each guinea pig according to the method of Alikayeva. The inoculation of suspensions into ten tubes containing the Lövenstein-Jensen medium was performed (Manchenko et al. 1994).

During the experiments (within 90 days), animals were weighed, an ulcer formation was studied in the injection site, and the manifestation of allergic reaction to PPD for mammals at a dose of 25 IU in 0.1 cm³ isotonic solution was monitored. The allergic tests were performed at 30, 60, and 90 days after infection of the animals, and the reaction was recorded 24–48 hours after tuberculin injection.

The guinea pigs were then infected for a second time (a second passage) with the mycobacteria recovered from the organs of guinea pigs infected with the bacteria of the first passage. The microorganisms recovered from the animal organs infected with the second passage were used to infect the next group of guinea pigs (a third passage). In the second and third rounds of infections, 84 (42 + 42) guinea pigs were infected (the control group's guinea pigs were not infected).

At the third step of our studies, *M. bovis* ability to grow on simple (basal) media (nutrient agar and nutrient broth) at 37°C, in the Lövenstein-Jensen medium with sodium salicylate at 3°C and 37°C was investigated. For this purpose, the suspensions (2×10^5 CFU/ml) were prepared from mycobacteria culture, and three tubes with the nutrient medium were inoculated (Manchenko et al. 1994).

To determine the biochemical activity of *M. bovis*, the first-generation bacteria were grown for 3–4 weeks. The activity of catalase, peroxidase (following modified Bogen method), and dehydrogenase, as well as nitrate reduction, and Tween-80 hydrolysis (following modified Wayne method) were assayed as described by Zhurylo et al. (2012).

The polymerase chain reaction was used to confirm or refute the mycobacterial changes in genetic material. DNA was isolated from cultures of the original highly virulent fast-growing strain (at the second passage), long-term variants of subculture (passages), as well as Vallee strain (KMIEV-9 modification, provided by the Suny National Agrarian University) and BCG strain (sub-strain of Russia BCG-1, used for vaccine production). These strains were inoculated at a volume of 0,2 cm³ and a concentration of 2×10^5 CFU/ml and grown in a nutrient medium for 30 days.

For RT-PCR, an amplifier iCycler iQ5 (Bio-Rad, USA) and reagent kits for DNA isolation and PCR («MIKO-GEN», producer NGO DNA-Technology, Russian Federation) were used.

Results

Initial cultures on a Lövenstein-Jensen nutrient medium were characterized by a predominantly moderate growth rate in the inoculation line (Fig. 1), the color of ivory. Some strains also grew as individually formed colonies. The colony surface was rough (R) in three subculture variants (passages No. 135, 171, and 180) or smooth (S) – also in three subculture variants (passages No. 54, 115, and 143).

The mycobacteria from passage 54 appeared as the acid-fast forms (thick rod with rounded ends and single grains) (Fig. 2a). All other subculture variants (passages No. 115, 135, 145, 171, and 180) displayed mixed morphological forms (Fig. 2b), i.e., acid-fast rods, non-acid-fast filamentous and rod-shaped forms, and grains.

After inoculation of the Lövenstein-Jensen nutrient medium, the mycobacteria were capable of growing at two temperatures (3°C and 37°C) (passages No. 115, 171), at one selected temperature 37°C (passages No. 54, 143, 180), or 3°C – one strain (passage No. 135). The subcultures obtained (Fig. 3) differed from the initial cultures, as shown in Fig. 1.

At 37°C, the bacterial growth became visible on the fourth day – passages No. 115, 143, on the fifth day – passages No. 171 and 180, and on the twentieth day – the passage No. 54. The growth rate in all cultures was considerable. In four subculture variants (passages No. 54, 115, 171, and 180), the growth was characterized by an oily coating (fur) of yellow or orange colors (Fig. 3), in one subculture variant (passage No. 143) – small translucent ivory-colored S-colonies were located in clusters.

At 3°C, the growth was noted on the sixth day – passages No. 115 and 171, and on the fiftieth day – passage No. 135. In two cultures (passages No. 115, 171), there was considerable growth in the form of blue-green mucous ropy fur, and in one culture (passage No. 135) – yellow fur.

Microscopy of smears prepared from the isolated cultures revealed the difference between the parental strain of *M. bovis* (as shown in Fig. 2) and the organisms that were stored for many years (Fig. 4). Thus, in passage No. 54 only blue thick thread-like forms

Fig. 1. Cultures of *Mycobacterium bovis*: a) passage No. 54, b) passage No. 180.
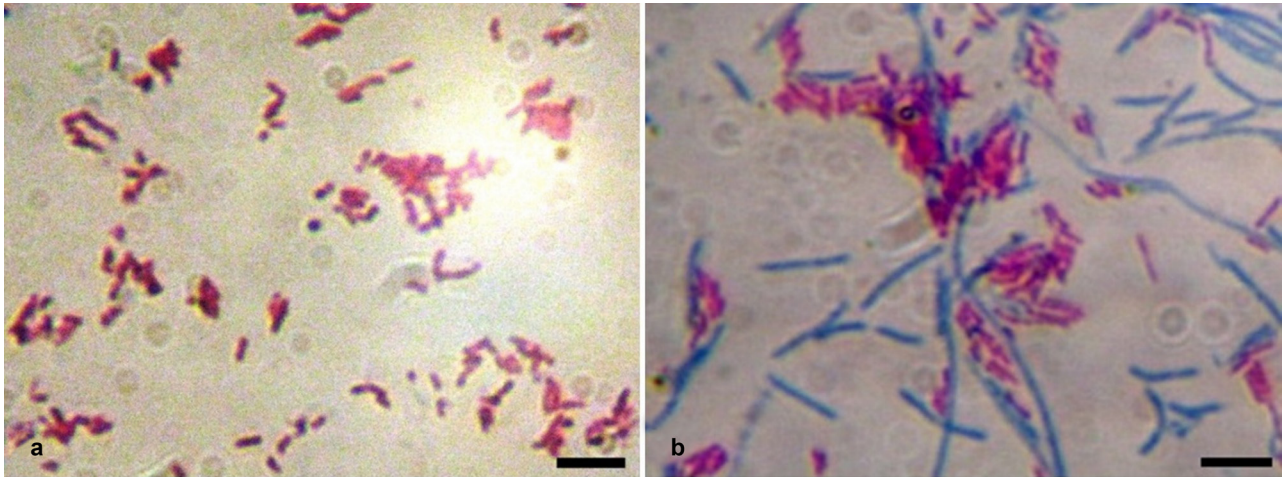
Fig. 2. Morphology of initial cultures of *Mycobacterium bovis*:
a) passage No. 54, b) passage No. 180 (stained by Ziehl-Neelsen). Bar = 10 μm.
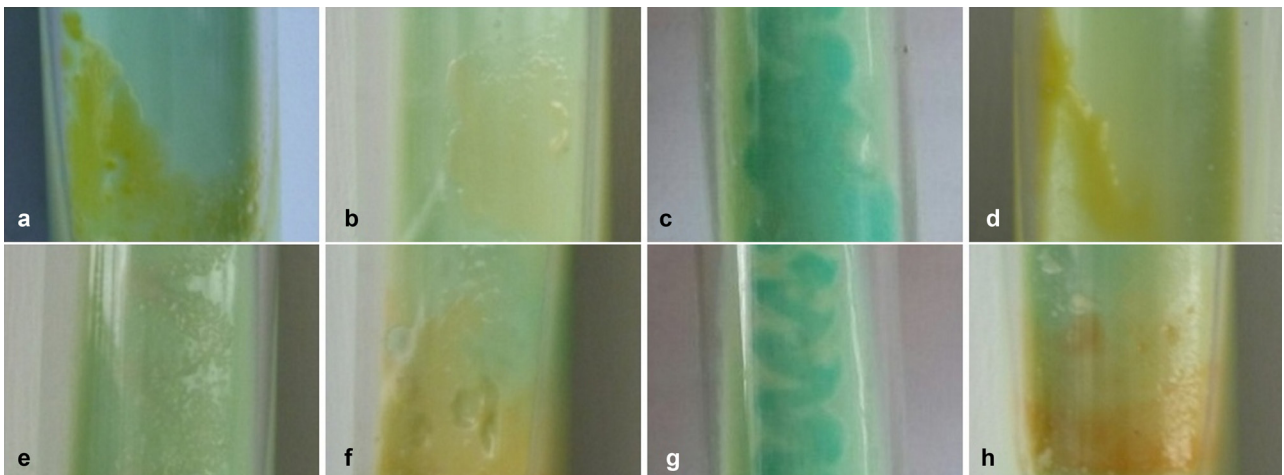

Fig. 3. Subcultures of *Mycobacterium bovis*:
a) passage No. 54, b) passage No. 115 (at 37°C), c) passage No. 115 (at 3°C), d) passage No. 135,
e) passage No. 143, f) passage No. 171 (at 37°C), g) passage No. 171 (at 3°C), h) passage No. 180.

with a barely noticeable red granularity inside could be found. The morphology of mycobacteria was quite diverse: short and long, straight and curved, thin and thick rod-shaped bacteria with rounded ends, coccal and rod-shaped forms, and grains. In passages No. 171 and 180, the L-forms and other rod-shaped mycobacteria, thread-like forms, and grains were noticed. In passage No. 143 only L-forms with a different optical density were recorded. As proof of the affiliation of L-forms to mycobacteria is the destruction of vesicular L-forms and the release of acid fast-negative grains and acid fast-positive shaped bacteria (rods).

At the end of the animal experiments and euthanasia, an autopsy was performed to reveal any pathological-anatomical changes characteristic for tuberculosis in the experimental animals. The infection of guinea pigs by all studied mycobacterial subcultures (passages) was not accompanied by sensitization of the macroorganism to PPD for mammals, the formation of ulcers at the site of mycobacteria suspension, or bodyweight decrease.

Guinea pigs infected with the parental pathogenic strain of *M. bovis* responded to tuberculin after 30 and 60 days of the experiment, and their body weight decreased. At the point of inoculation of the mycobacterial suspension, the ulcers were formed after 27 days. The death of one guinea pig was reported on day 69. The other guinea pigs were euthanized after 90 days. The pathological changes characteristic of tuberculosis were noted in infected all guinea pigs (Fig. 5).

Through bacteriological investigations of specimens from each guinea pig, eight cultures of mycobacteria were isolated: after four days of culture, there were three cultures at 3°C (passages No. 115, 135, and 171) and five cultures at 37°C (passages No. 54, 115, 143, 171, and 180). Non-acid-fast rod-shaped mycobacteria and grains were therefore visible under the immersion in the microscope.
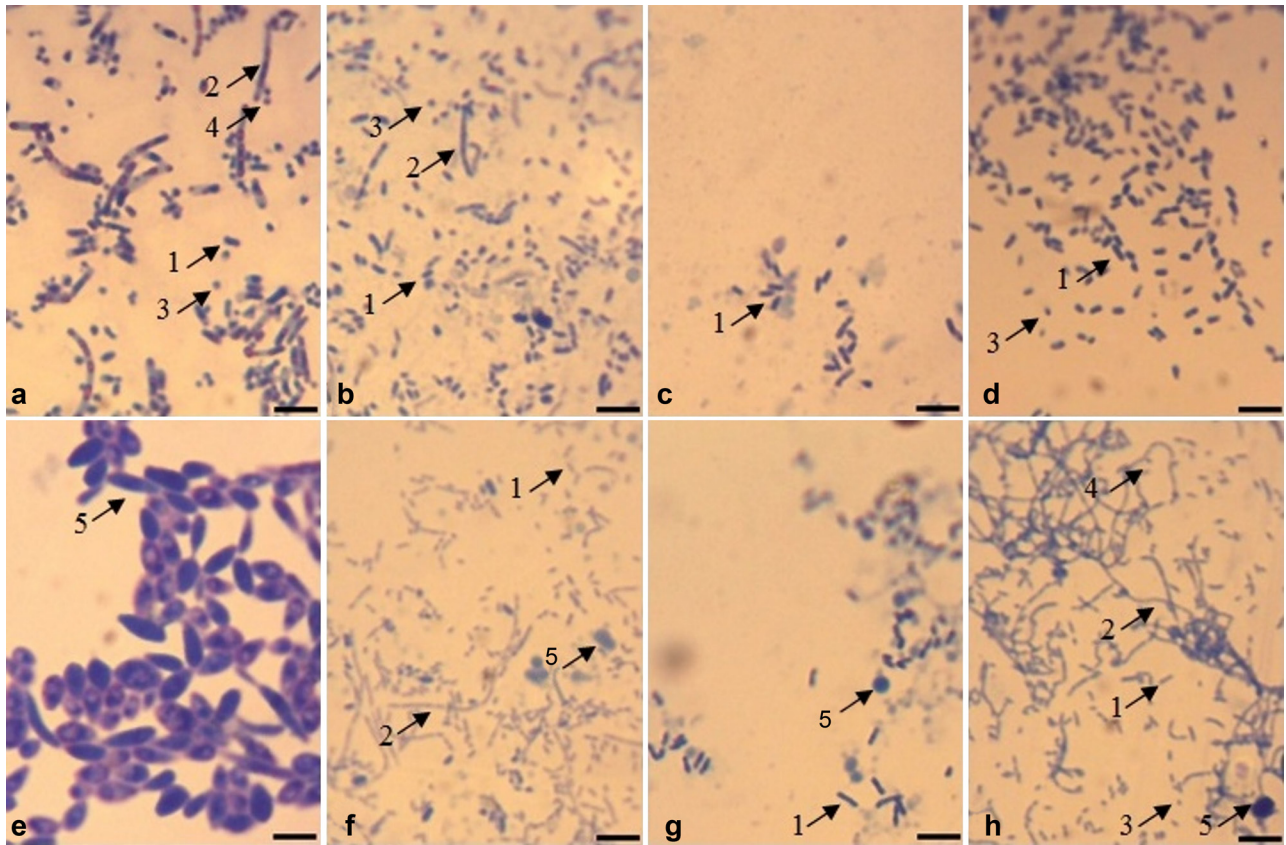
Fig. 4. Morphology of *Mycobacterium bovis* subcultures:
a) passage No. 54, b) passage No. 115 (at 37°C), c) passage No. 115 (at 3°C), d) passage No. 135, e) passage No. 143,
f) passage No. 171 (at 37°C), g) passage No. 171 (at 3°C), h) passage No. 180;
1 – rod-shaped bacteria, 2 – thread-like forms, 3 – grains (elementary bodies), 4 – output of grains from thread-like forms,
5 – L-forms. Bar = 10 μm.

Fig. 5. The spleen of the guinea pig:
1) normal, 2) with tuberculous foci.

In animals infected with a second passage, no allergic reactions to PPD for mammals, formation of ulcers in the injection site, or tuberculosis characteristic features were observed. However, the bacteria growth from tissue specimens at 37°C was observed on the 6th day (passages No. 54 and 180) and on the 10th day (passages No. 171 and 143). Under microscopy, the smears from cultures revealed acid fast-negative rod-shaped bacteria and grains.

The animals infected with a third passage did not suffer allergic reactions to the PPD for mammals, the formation of ulcers in the site of inoculation of material, decrease in body weight, or and symptoms of tuberculosis. No bacteria were grown from the animal tissues.

These mycobacteria could grow on nutrient media. In the nutrient broth, colony growth was observed for all passages except for passage No. 54. In the Lövenstein-Jensen medium with sodium salicylate at 3°C, colony growth was observed for all passages except for 54, 135, and 171 (Fig. 6).

Concerning the biochemical activity of the cultures studied, variations in the activity of mycobacterial enzymes were ascertained depending on the number of passages and the cultivation temperature. The tendency to change the biochemical activity of the microorganisms studied was established: catalase activity increased along with the decrease of the cultivation temperature in passages 115 and 171 (Fig. 7).
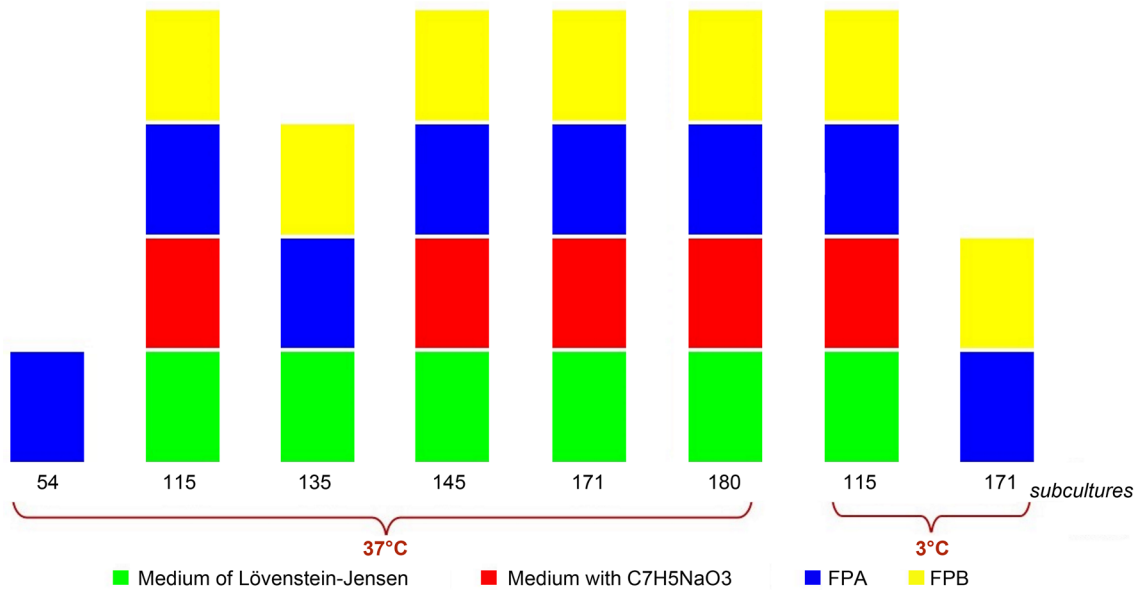
Fig. 6. Growth of pathogenic *Mycobacterium bovis* strain and its subcultures (variants) on nutrient media.
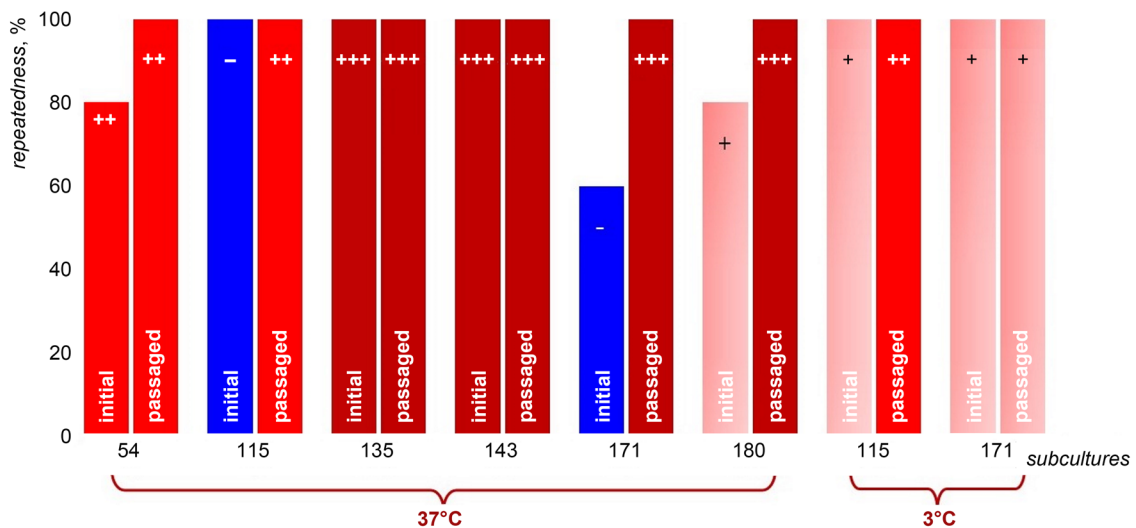FPA – nutrient agar, FPB – nutrient broth.

Fig. 7. Catalase activity of *Mycobacterium bovis* initial subcultures and the subcultures passaged through the guinea pigs (repeatedness, %):
"–" – negative reaction, "±" – doubtful reaction, "+", "++", "+++" – the magnitude of the positive reaction.

Peroxidase activity was observed only in the control samples of the highly virulent strain. Dehydrogenase activity in the first 15–30 minutes was not detected in any culture, but after 24 hours was observed in almost all cultures, except those on the 54 passage (Fig. 8).

Nitrate reduction was recorded in all cultures except for passages No. 143 and 171 grown at 37°C (Fig. 9).

Hydrolysis of Tween-80 in the first 4 hours was not found in all subcultures (passages) of *M. bovis*. Passages No. 115 and 171, which were grown at a low temperature, had a higher ability to hydrolyze Tween-80 on the fifth day.

When the biochemical activity of mycobacteria at subsequent passages in guinea pig was compared with the initial strain, the following phenomena become apparent: an increase in the activity of the enzyme dehydrogenase – passages No. 54, 135 (grown at 37°C) and 171 (grown at 3°C); loss of ability to reduce nitrates, especially in cultures grown at low temperature (3°C) – passages No. 115, 171, and at 37°C – for the passage No 195; increase of catalase activity – passages No. 115 and 171 at both cultivation temperatures (3°C and 37°C); the ability to hydrolyze Tween-80 in the vast majority of cultures was decreased for all passages except passage No. 54 (Fig. 10).

It should also be noted that the biochemical activity of one subculture strain No. 143 after passage through the body of guinea pigs remains unchanged.
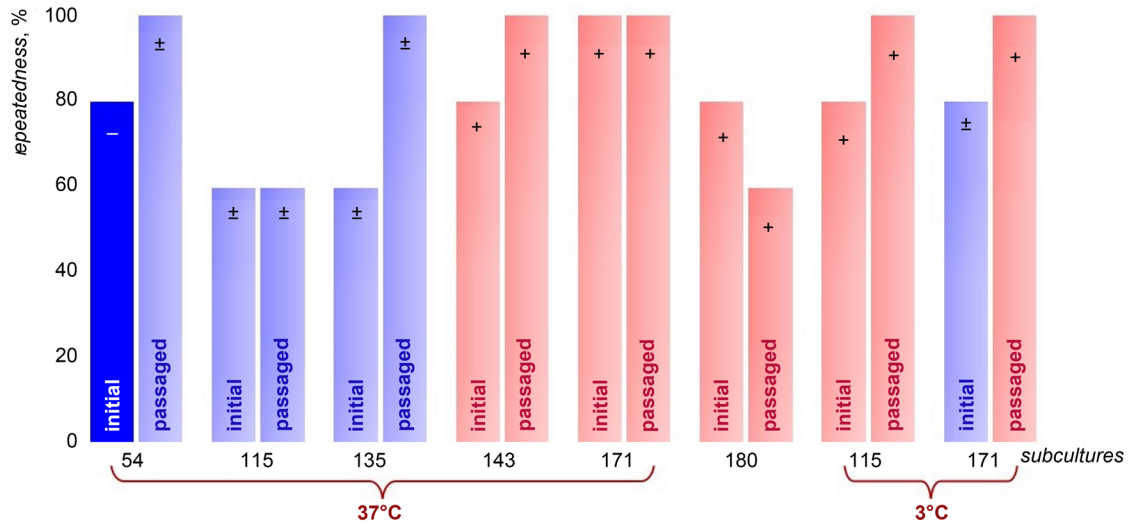
Fig. 8. Dehydrogenase activity of *Mycobacterium bovis* initial subcultures and the subcultures passaged through
the guinea pigs (repeatedness, %):
"–" – negative reaction, "±" – doubtful reaction, "+", "++", "+++" – the magnitude of the positive reaction after 24 hours.
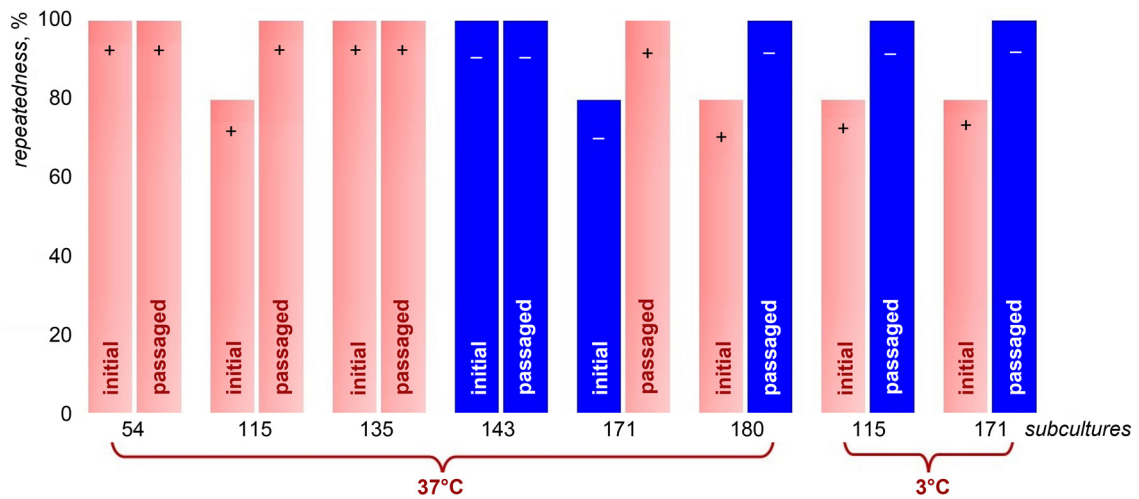

Fig. 9. Reduction of nitrates by *Mycobacterium bovis* initial subcultures and the subcultures passaged through
the guinea pigs (repeatedness, %):
"–" – negative reaction, "±" – doubtful reaction, "+", "++", "+++" – the magnitude of the positive reaction.

Using PCR, the amplicons were detected in the initial pathogenic *M. bovis* fast-growing strain and both Vallee and BCG strains (Table I). At the same time, the amplicons were not observed after amplification of DNA derived from the stored for long-time *M. bovis* fast-growing strain.

Table I
The results of DNA amplification of mycobacteria

Type of mycobacteria	Strain of mycobacteria	Result of detection of amplification products
Mycobacterium bovis	fast-growing (original)	+
	after the long-term storage and reinoculation of cultures on the nutrient medium), passages 54, 115, 135, 135, 171, and 180	–
	Vallee strain	+
	BCG strain	+

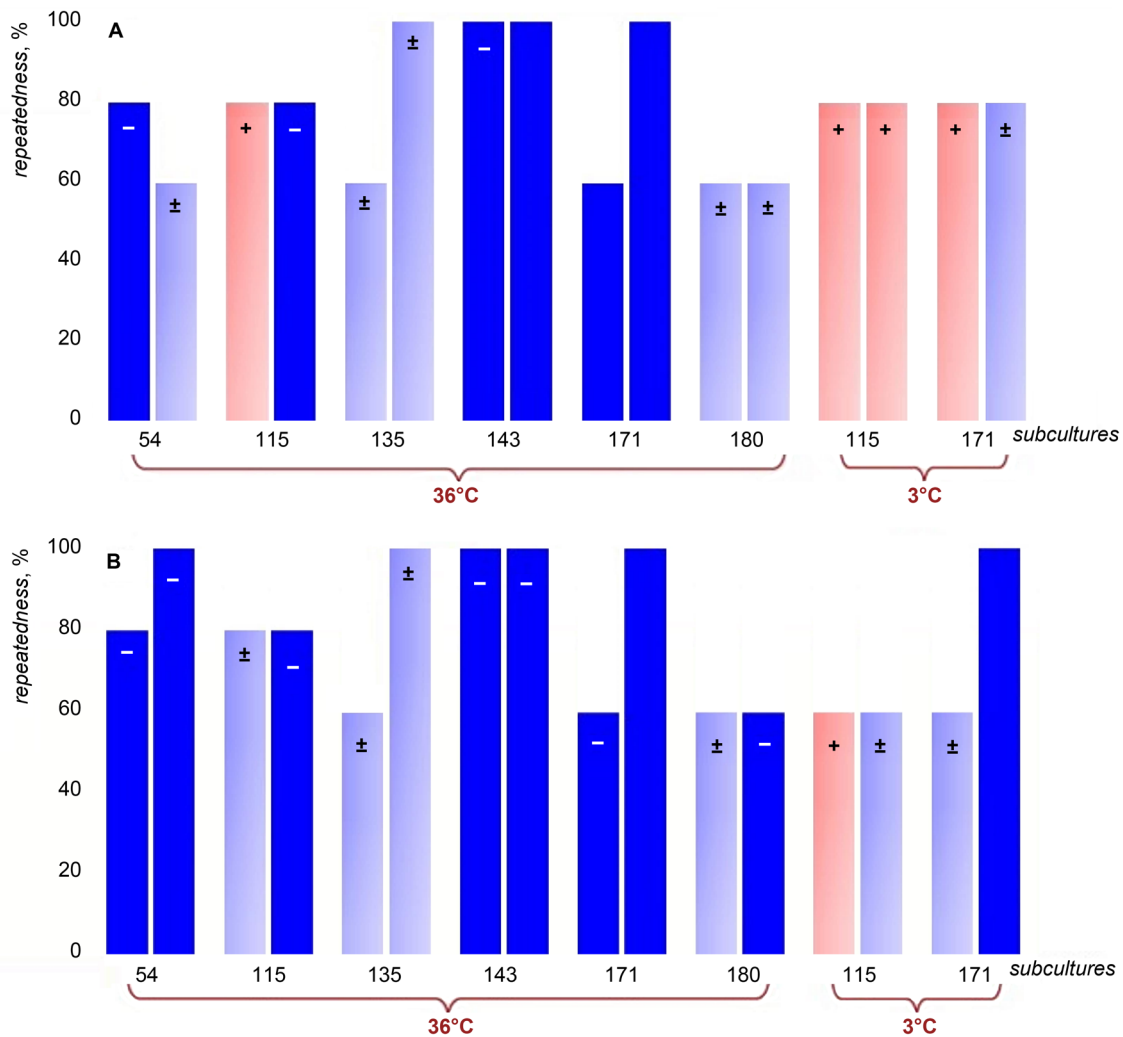+ – positive result;   – – negative result

Fig. 10. Tween-80 hydrolysis by *Mycobacterium bovis* initial subcultures and the subcultures passaged through the guinea pigs: A) after five days, B) after ten days.

Discussion

According to the experimental findings of this study, mycobacteria stored in the nutrient medium for 9–12 years manifested viability on the Lövenstein-Jensen medium. After long-term storage, it was possible to trace the non-acid-fast elements and L-forms of vesicular type in the subsequent cultures.

Other authors have also reported that the causative agent of tuberculosis of the horned presented highly adaptive properties (Djachenko et al. 2008). The transformation of mycobacteria may occur when environmental hostility leads to the change in their lifestyle (Gomez and McKinney 2004; Lewis 2007). To minimize the need for nutrients, bacteria may undergo hibernation and increase their resistance.

The phenotypic change that occurred in mycobacteria in our laboratory may stem from the decrease in the oxygen level in tubes due to its gradual use for bacterial metabolism during storage under strictly closed stoppers.

According to Kumar and Sanyal (2012), such a process of increasing resistance by reducing metabolism may be caused by the decrease in oxygen concentration in the habitat, which leads to anaerobiosis. It has appeared that the slight decrease in oxygen content in the tubes allows mycobacteria to survive. In contrast, the rapid decrease of oxygen concentration leads to the sharp decrease in the number of colony-forming units (Wayne and Lin 1982; Dick et al. 1998; Usha et al. 2002; Lewis and Falkinhan 2015).

The oxygen limitation causes some specific changes in mycobacteria. The microorganisms become thermotolerant, reduce overall protein synthesis, lose acid resistance, and their cell wall display a thickening process (Gillespie et al. 1986, Cunningham and Spreadbury 1998, Hu et al. 1998, Primm et al. 2000).

Adaptation to nutrient deficiency is also crucial for the long-term survival of mycobacterial cells. *Mycobacterium tuberculosis* can survive on starvation for up to two years *in vitro* while maintaining the ability to resuscitation on the nutrient media (Nyka 1974; Primm

et al. 2000). *Mycobacterium smegmatis* retain the viability for more than 650 days and long-term maintenance of stable numbers of colony-forming units (Smeulders et al. 1999). After the long-term storage (10–13 years), *M. bovis* retained its viability in this study. Moreover, the mycobacteria could grow on a nutrient medium and Levenstein-Jensen medium with sodium salicylic acid. They could also reduce nitrates, hydrolyze Tween-80, and display an increase in catalase, peroxidase, and dehydrogenase activity.

The results of guinea pig infection in this study demonstrated that mycobacteria underwent a strong transformation during the years of storage because they became avirulent and lost their sensitizing properties. Even multiple direct passages through the laboratory animals failed to restore their pathogenicity. On the contrary, after the second passage through guinea pigs, only half of the mycobacterial subcultures were isolated from animals, and no cultures were grown after the third passage. No allergic reactions to tuberculin in any of the infected guinea pigs were observed for three passages. In our opinion, the multiple passages of mycobacterial subcultures through the nutrient medium and subsequent long-term preservation at 3°C affected their vital properties. Due to the influence of the above-named factors, mycobacteria adapted so much to the conditions of existence in vitro that the immune system overcame them when they enter the macroorganism.

Already described by many authors (Tkachenko 2004; Djachenko et al. 2009; Yavorska and Sybirna 2009; Glebenjuk and Telizhenko 2015; Lysenko et al. 2019), the high variability of mycobacteria is confirmed by our investigations of the biochemical activity. *M. bovis* (unlike *M. tuberculosis*) is traditionally considered to have no pronounced nitroreductase activity (Bönicke et al. 1970; Fritz et al. 2002). Some researchers have suggested that *M. bovis* BCG (vaccine strain) uses nitrates as a critical source of nutrition, supporting bacterial metabolism in the lungs, liver, and kidneys by restoring nitrate to nitrite. They argue that nitrate could provide energy for bacterial metabolism even in anaerobic environments (Philippot and Højberg 1999; Fritz et al. 2002). In addition, nitrate reductase enzyme analysis is widely used in laboratory practice as an alternative method for detecting the resistance of nitrate reductase-positive strains of mycobacteria to anti-tuberculosis medicines such as isoniazid, rifampicin, ethambutol, ofloxacin, and streptomycin. A mycobacterial strain is considered to be resistant if it has nitrate reductase activity (Angeby et al. 2002; Martin et al. 2005; Montoro et al. 2005; Lemus et al. 2006; Fonseca et al. 2012).

On this basis, we can assume that the changes that have occurred in *M. bovis* enzymatic activity reflect the rearrangement of metabolic reactions within the bacterial cell. Such processes took place throughout long-term storage in the same medium and aimed at providing nutrition to the microorganisms at the expense of the energy obtained by reducing nitrates.

After the passage of *M. bovis* through the living organism, the bacterial activity of dehydrogenase and catalase increased. These are multifunctional heme-dependent enzymes (Bertrand et al. 2004) actively involved in the antioxidant protection of the microbial cell (Kondratjuk and Sybirna 2008).

It has been reported (Li et al. 1998) that catalase promotes *M. tuberculosis* ability to survive in host tissues. In the work of Manca et al. (1999), the catalase and peroxidase activity of the laboratory, clinical, and recombinant strains of *M. tuberculosis* were investigated. These strains had minimal catalase activity both intracellularly (in human monocytes) and in the nutrient medium. Significantly more bacteria (85 %) survived when they were exposed to the influence of exogenous H_2O_2.

According to some assertions, catalase and peroxidase activity acts as a virulence factor of mycobacteria (Manca et al. 1999; Kondratjuk and Sybirna 2008).

However, our results indicate that mycobacteria's final typing and species identification based only on biochemical tests are impossible. These characteristics may differ between members of the same species depending on the environmental conditions of the specific microorganism. (Torkko et al. 1998; Yavorska and Sybirna 2009; Lysenko et al. 2011). Thus, mycobacteria can rapidly alter metabolic processes and adapt to environmental conditions. To identify mycobacteria, researchers (Wayne and Sramek 1992; Torkko et al. 1998; Tkachenko 2004) recommend other tests, for instance, fatty acid analysis, detection of bacterial growth rate, sequence analysis, or PCR.

In the microbiological diagnosis of tuberculosis, PCR is recognized as a method of high specificity and sensitivity (Rodriguez et al. 1995; Portillo-Gómez et al. 2000). However, sequencing of the complete genome of *M. tuberculosis* revealed a similar frequency of mutations both during latency and during active disease or in a logarithmically growing culture. Such a mutational load on mycobacteria *in vivo* is assumed to be due to oxidative DNA damage (Ford et al. 2011). After long-term storage of the BCG strain, numerous genetic differences were detected in the form of large-sequence polymorphisms, including deletions and duplications, as well as single nucleotide polymorphisms among different groups of the BCG strain (Zhang et al. 2016).

Repeatedly passaged *M. bovis* (acid fast-positive rod-shaped bacteria, acid fast-negative thread-like, and rod-shaped forms) under long-term persistence in the unfavorable conditions, underwent permanent changes leading to growth as yellow, orange, or blue-green pigment colonies. Also, the generation of L-forms of vesicular type, acid fast-negative thread-like, rod-shaped

variants, and elementary bodies pushed out of the latter were observed. Secondly, the mycobacterial metabolism changed because they were able to grow on nutrient medium and Lövenstein-Jensen medium with sodium salicylate. Their capability to reduce nitrate and hydrolyze Tween-80 was diminished. The catalase, peroxidase, and dehydrogenase activities of subcultured mycobacteria increased and were not dependent on the number of passages. After passages through animal tissues, the activity of the dehydrogenase and catalase enzymes increased while virulence and sensitizing activity were lost. We conclude that *M. bovis* was considerably changed during long-term storage in tubes under hypoxia conditions.

ORCID

Olexiy Tkachenko https://orcid.org/0000-0003-0978-6575
Maryna Bilan https://orcid.org/0000-0003-3178-201X
Volodymyr Hlebeniuk https://orcid.org/0000-0001-5599-651X
Natalia Alekseeva https://orcid.org/0000-0003-1984-5209
Vitalii Nedosekov https://orcid.org/0000-0001-7581-7478
Olexandr Galatiuk https://orcid.org/0000-0002-9720-0660

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Angeby KA, Klintz L, Hoffner SE. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. J Clin Microbiol. 2002 Feb;40(2):553–555. https://doi.org/10.1128/jcm.40.2.553-555.2002

Anuchin AM, Mulyukin AL, Suzina NE, Duda VI, El-Registan GI, Kaprelyants AS. Dormant forms of *Mycobacterium smegmatis* with distinct morphology. Microbiology. 2009 Apr;155(4):1071–1079. https://doi.org/10.1099/mic.0.023028-0

Bentrup KH, Russell DG. Mycobacterial persistence: adaptation to a changing environment. Trends Microbiol. 2001 Dec;9(12):597–605. https://doi.org/10.1016/S0966-842X(01)02238-7

Bertrand T, Eady NA, Jones JN, Jesmin, Nagy JM, Jamart-Grégoire B, Raven EL, Brown KA. Crystal structure of *Mycobacterium tuberculosis* catalase-peroxidase. J Biol Chem. 2004 Sep 10; 279(37):38991–38999. https://doi.org/10.1074/jbc.M402382200

Bönicke R, Juhasz SE, Diemer U. Studies on the nitrate reductase activity of mycobacteria in the presence of fatty acids and related compounds. Am Rev Respir Dis. 1970 Oct;102(4):507–515. https://doi.org/10.1164/arrd.1970.102.4.507

Cunningham AF, Spreadbury CL. Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton α-crystallin homolog. J Bacteriol. 1998 Feb;180(4):801–808. https://doi.org/10.1128/JB.180.4.801-808.1998

Dick T, Lee BH, Murugasu-Oei B. Oxygen depletion induced dormancy in *Mycobacterium smegmatis*. FEMS Microbiol Lett. 1998 Jun 15;163(2):159–164. https://doi.org/10.1111/j.1574-6968.1998.tb13040.x

Djachenko GM, Kravchenko NO, Golovach OV, Dmytruk OM, Il'i'nyh VV. [Variability of phenotypical markings of mycobacteries of different kinds with acquired medicamentose stability] (in

Ukrainian). Scientific Messenger of Lviv National University of Veterinary Medicine and Biotechnologies named after S.Z. Gzhytskyj. 2008;10(3):72–77.

Djachenko GM, Kravchenko NO, Il'i'nyh VV, Dmytruk OM, Golovach OV. [Adaptation and variability of the properties of mycobacteria of different species for the influence of antibacterial drugs] (in Ukrainan). Sil's'kogospodars'ka mikrobiologija: Mizhvid. temat. nauk. zb. 2009;9:158–165.

Fonseca Lde S, Vieira GB, Sobral LF, Ribeiro EO, Marsico AG. Comparative evaluation under routine conditions of the nitrate reduction assay, the proportion assay and the MGIT 960 assay for drug susceptibility testing of clinical isolates of *Mycobacterium tuberculosis*. Mem Inst Oswaldo Cruz. 2012 Feb;107(1):142–144. https://doi.org/10.1590/s0074-02762012000100021

Ford CB, Lin PL, Chase MR, Shah RR, Iartchouk O, Galagan J, Mohaideen N, Ioerger TR, Sacchettini JC, Lipsitch M, et al. Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. Nat Genet. 2011 May; 43(5):482–486. https://doi.org/10.1038/ng.811

Fritz C, Maass S, Kreft A, Bange FC. Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific. Infect Immun. 2002 Jan;70(1):286–291. https://doi.org/10.1128/iai.70.1.286-291.2002

Gillespie J, Barton LL, Rypka EW. Phenotypic changes in mycobacteria grown in oxygen-limited conditions. J Med Microbiol. 1986 May;21(3):251–255. https://doi.org/10.1099/00222615-21-3-251

Glebenjuk VV, Telizhenko KV. [Species affiliation of mycobacterium isolated from animals in the Dnipropetrovsk region] (in Ukrainian). Scientific and Technical Bulletin of the National State Center of Biosafety and Environmental Control of Resources of Agroindustrial Complex. 2015;3(1):61–64. Available from http://biosafety-center.com/wp-content/uploads/2015/06/2.pdf

Gomez JE, McKinney JD. *M. tuberculosis* persistence, latency, and drug tolerance. Tuberculosis. 2004;84(1–2):29–44. https://doi.org/10.1016/j.tube.2003.08.003

Hu YM, Butcher PD, Sole K, Mitchison DA, Coates ARM. Protein synthesis is shutdown in dormant *Mycobacterium tuberculosis* and is reversed by oxygen or heat shock. FEMS Microbiol Lett. 1998 Jan 1;158(1):139–145. https://doi.org/10.1111/j.1574-6968.1998.tb12813.x

Kondratjuk N, Sybirna R. [Usage and modification of biochemical tests confirming the belonging of isolated cultures to tuberculosis mycobacteria] (in Ukrainian). Visnyk L'viv Univ. 2008;47:70–73. Available from http://old.franko.lviv.ua/faculty/biologh/wis/47/2/8/8.pdf

Kovaleva LO. [Special adaptive capabilities of fast-growing strains of *M. bovis* on artificial hard places for numerous passages] (in Ukrainian). Bulletin of the Poltava Service of the Agrarian Academy. 2005;1:166–168.

Kumar R, Sanyal S. *Mycobacterium tuberculosis*: dormancy, persistence and survival in the light of protein synthesis. In: Cardona PJ, editor. Understanding tuberculosis – deciphering the secret life of the bacilli. Rijeka (Croatia): IntechOpen; 2012. p. 217–238. https://doi.org/10.5772/31098

Lemus D, Montoro E, Echemendía M, Martin A, Portaels F, Palomino JC. Nitrate reductase assay for detection of drug resistance in *Mycobacterium tuberculosis*: simple and inexpensive method for low-resource laboratories. J Med Microbiol. 2006 Jul;55(7):861–863. https://doi.org/10.1099/jmm.0.46540-0

Lewis AH, Falkinham JO 3rd. Microaerobic growth and anaerobic survival of *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*. Int J Mycobacteriol. 2015 Mar; 4(1):25–30. https://doi.org/10.1016/j.ijmyco.2014.11.066

Lewis K. Persister cells, dormancy and infectious disease. Nat Rev Microbiol. 2007 Jan;5(1):48–56. https://doi.org/10.1038/nrmicro1557

Li Z, Kelley C, Collins F, Rouse D, Morris S. Expression of katG in *Mycobacterium tuberculosis* is associated with its growth and persistence in mice and guinea pigs. J Infect Dis. 1998 Apr;177(4):1030–1035. https://doi.org/10.1086/515254

Lysenko AP, Vlasenko IG, Vlasenko VV, Babijchuk JV. [Biochemical properties of bacillary and modified forms of mycobacteria grown on nutrient media] (in Russian). Scientific Messenger of Lviv National University of Veterinary Medicine and Biotechnologies named after S.Z. Gzhytskyj. 2011;13(4):249–252.

Lysenko OP, Vlasenko VV, Palii HK, Vlasenko IH, Nazarchuk OA. [Mycobacterium of tuberculosis with defective cell wall, determined in the brain of the biological model with spongional changes] (in Ukrainian). Reports of Vinnytsia National Medical University. 2019;23(1):12–19. https://doi.org/10.31393/reports-vnmedical-2019-23(1)-02

Manca C, Paul S, Barry CE, Freedman VH, Kaplan G. *Mycobacterium tuberculosis* catalase and peroxidase activities and resistance to oxidative killing in human monocytes *in vitro*. Infect Immun. 1999 Jan;67(1):74–79. https://doi.org/10.1128/IAI.67.1.74-79.1999

Manchenko VM, Trocenko ZR, Pavlenko MS. [Guidelines for diagnosing tuberculosis] (in Ukrainian). Kyiv. 1994:39.

Martin A, Palomino JC, Portaels F. Rapid detection of ofloxacin resistance in *Mycobacterium tuberculosis* by two low-cost colorimetric methods: resazurin and nitrate reductase assays. J Clin Microbiol. 2005 Apr;43(4):1612–1616. https://doi.org/10.1128/JCM.43.4.1612-1616.2005

Montoro E, Lemus D, Echemendia M, Martin A, Portaels F, Palomino JC. Comparative evaluation of the nitrate reduction assay, the MTT test, and the resazurin microtitre assay for drug susceptibility testing of clinical isolates of *Mycobacterium tuberculosis*. J Antimicrob Chemother. 2005 Apr;55(4):500–505. https://doi.org/10.1093/jac/dki023

Nyka W. Studies on the effect of starvation on mycobacteria. Infect Immun. 1974 May;9(5):843–50. https://doi.org/10.1128/iai.9.5.843-850.1974

Philippot L, Højberg O. Dissimilatory nitrate reductases in bacteria. Biochim Biophys Acta. 1999 Jul 7;1446(1–2):1–23. https://doi.org/10.1016/s0167-4781(99)00072-x

Portillo-Gómez L, Morris SL, Panduro A. Rapid and efficient detection of extra-pulmonary *Mycobacterium tuberculosis* by PCR analysis. Int J Tuberc Lung Dis. 2000 Apr;4(4):361–370.

Primm TP, Andersen SJ, Mizrahi V, Avarbock D, Rubin H, Barry CE. The stringent response of *Mycobacterium tuberculosis* is required for long-term survival. Bacteriol. 2000 Sep;182(17): 4889–4898. https://doi.org/10.1128/JB.182.17.4889-4898.2000

Ritz N, Hanekom WA, Robins-Browne R, Britton WJ, Curtis N. Influence of BCG vaccine strain on the immune response and protection against tuberculosis. FEMS Microbiol Rev. 2008 Aug;32(5):821–841. https://doi.org/10.1111/j.1574-6976.2008.00118.x

Rodriguez JG, Mejia GA, Del Portillo P, Patarroyo ME, Murillo LA. Species-specific identification of *Mycobacterium bovis* by PCR. Microbiology. 1995 Sep;141(9):2131–2138. https://doi.org/10.1099/13500872-141-9-2131

Shleeva MO, Bagramyan K, Telkov MV, Mukamolova GV, Young M, Kell DB, Kaprelyants AS. Formation and resuscitation of "non-culturable" cells of *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* in prolonged stationary phase. Microbiology. 2002 May;148(5):1581–1591. https://doi.org/10.1099/00221287-148-5-1581

Shleeva MO, Kudykina YK, Vostroknutova GN, Suzina NE, Mulyukin AL, Kaprelyants AS. Dormant ovoid cells of *Mycobacterium tuberculosis* are formed in response to gradual external acidification. Tuberculosis. 2011 Mar;91(2):146–154. https://doi.org/10.1016/j.tube.2010.12.006

Smeulders MJ, Keer J, Speight RA, Williams HD. Adaptation of *Mycobacterium smegmatis* to stationary phase. J Bacteriol. 1999 Jan;181(1):270–283. https://doi.org/10.1128/JB.181.1.270-283.1999

Tkachenko O, Bilan M, Hlebeniuk V, Alekseeva N, Nedosekov V, Galatiuk O. Chronology of morphological forms of *Mycobacterium bovis* rapid-growing strain. Acta Vet Eurasia. 2020a;46(3):104–114. https://doi.org/10.5152/actavet.2020.20007

Tkachenko O, Bilan M, Hlebeniuk V, Kozak N, Nedosekov V, Galatiuk O. Dissociation of *Mycobacterium bovis*: morphology, biological properties and lipids. Adv Anim Vet Sci. 2020b;8(3):312–326. https://doi.org/10.17582/journal.aavs/2020/8.3.317.326

Tkachenko OA. [Rapid-growing *M. bovis* in the problem of tuberculosis] (in Ukrainian). Veterinary Medicine of Ukraine. 2004; 7: 14–17. Available from http://dspace.dsau.dp.ua/jspui/handle/123456789/745

Torkko P, Suutari M, Suomalainen S, Paulin L, Larsson L, Katila ML. Separation among Species of *Mycobacterium terrae* Complex by Lipid Analyses: Comparison with Biochemical Tests and 16S rRNA Sequencing. J Clin Microbiol. 1998 Feb;36(2):499–505. https://doi.org/10.1128/JCM.36.2.499-505.1998

Usha V, Jayaraman R, Toro JC, Hoffner SE, Das KS. Glycine and alanine dehydrogenase activities are catalyzed by the same protein in *Mycobacterium smegmatis*: upregulation of both activities under microaerophilic adaptation. Can J Microbiol. 2002 Jan;48(1):7–13. https://doi.org/10.1139/w01-126

Velayati AA, Farnia P, Ibrahim TA, Haroun RZ, Kuan HO, Ghanavi J, Farnia P, Kabarei AN, Tabarsi P, Omar AR, et al. Differences in cell wall thickness between resistant and nonresistant strains of *Mycobacterium tuberculosis*: using transmission electron microscopy. Chemotherapy. 2009;55(5):303–307. https://doi.org/10.1159/000226425

Velayati AA, Farnia P. Division-cycle in *Mycobacterium tuberculosis*. Int J Mycobacteriol. 2012 Sep;1(3):111–117. https://doi.org/10.1016/j.ijmyco.2012.08.003

Vera HD, Rettger LF. Morphological variations of the tubercle bacillus and certain recently isolated soil acid fasts with emphasis on filterability. J Bacteriol. 1940 Jun;39(6):659–687. https://doi.org/10.1128/JB.39.6.659-687.1940

Wayne LG, Lin KY. Glyoxylate metabolism and adaptation of *Mycobacterium tuberculosis* to survival under anaerobic conditions. Infect Immun. 1982 Sep;37(3):1042–1049. https://doi.org/10.1128/iai.37.3.1042-1049.1982

Wayne LG, Sramek HA. Agents of newly recognized or infrequently encountered mycobacterial diseases. Clin Microbiol Rev. 1992 Jan; 5(1):1–25. https://doi.org/10.1128/CMR.5.1.1

Yavorska GV, Sybirna RI. [Morphologic-cultural and physiologic-biochemical properties of atypical mycobacteria] (in Ukrainian). Mikrobiolohichnyi zhurnal. 2009;71(4):27–34. Available from http://dspace.nbuv.gov.ua/bitstream/handle/123456789/7790/05-Yavorivska.pdf

Young M, Mukamolova GV, Kaprelyants AS. Mycobacterial dormancy and its relation to persistence. In: Parish T, editor. Mycobacterial molecular biology. Norfolk (UK): Horizon Bioscience; 2005. p. 265–320.

Zhang L, Ru HW, Chen FZ, Jin CY, Sun RF, Fan XY, Guo M, Mai JT, Xu WX, Lin QX, et al. Variable virulence and efficacy of BCG vaccine strains in mice and correlation with genome polymorphisms. Mol Ther. 2016 Feb;24(2):398–405. https://doi.org/10.1038/mt.2015.216

Zhurylo OA, Barbova AI, Glushkevych TG, Tretjakova LV. [Standards of bacteriological diagnosis of tuberculosis in laboratories of tuberculosis establishments of Ukraine] (in Ukrainian). Ministerstvo ohorony zdorov'ja Ukrai'ny i Nacional'na akademija medychnyh nauk Ukrai'ny Derzhavna ustanova "Nacional'nyj instytut ftyziatrii' i pul'monologii' im. F.G. Janovs'kogo NAMN Ukrai'ny". Navchal'nyj posibnyk dlja fahivciv bakteriologichnyh laboratorij zakladiv protytuberkul'oznoi' sluzhby Ukrai'ny. 2012;188. Available from http://www.ifp.kiev.ua/ftp1/metoddoc/posibnyk_1_2012.pdf