







Chronology of Morphological Forms of *Mycobacterium bovis* Rapid-Growing Strain

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Abstract

The study aimed to examine the changes observed in the morphology of rapid-growing tuberculosis pathogen strain, degree of pathogenicity, sensitized properties, and content of lipids, which depend on the pH of the medium, for 180 inoculations of 1–3 days culture. The reversion of non-acid-resistant forms of *Mycobacterium bovis* of the rapid-growing strain to bacterial acid-resistant forms occurred because of the following reasons: formation of non-acid-resistant grains in thread-like *M. bovis*, transformation and fragmentation of non-acid-resistant thread-like forms into acid-resistant rod-shaped bacteria, and output from non-acid-resistant thread-like *M. bovis* acid-resistant forms. It was found that increasing the

number of passages of *M. bovis* of the rapid-growing strain through the medium at pH 6.7 led to a probable decrease ($p < 0.05$) in the content of total lipids; fractions of phospholipids, triacylglycerols, and sterin ethers; and an increase in the number of diacylglycerols, sterins, and free fatty acids (FFAs). It is established that the decrease in saturated and long-chain fatty acids and the increase in unsaturated and short-chain fatty acids were accompanied by the loss of virulence of mycobacteria. Of the 19 FFAs, nine tended to disappear from the cell wall.

Keywords: *Mycobacterium bovis*, nutrient medium, passage, strain, virulence

Introduction

Tuberculosis is a zoonotic disease that has plagued humanity for centuries of its existence, notwithstanding more than 130 years of purposeful knowledge of the pathogen and the development of specific treatments, prophylaxis, and control. The Calmette and Guérin vaccine was developed and proposed in the 20th century, which resulted in the continuous gene loss of the vaccine stock (Behr, 2001). This prompted the scientists in the world to design a new preparation strategy against tuberculosis (Fine, 1995; Horwitz and Harth, 2003; Lisle et al., 2005; Majlessi et al., 2005; Pym et al., 2003; Rao et al., 2003; Sambandamurthy et al., 2005; Stead and Bates, 2000).

A problem question that remains is the lack of knowledge of the biological properties of mycobacteria. Over time, several

reports of their peculiarities appear, which largely determine the effect of mycobacteria on biological species of animal kingdom, creating a global problem.

With regard to this, studies have reported (Djachenko et al., 2008; Domingue and Woody, 1997; Kochemasova et al., 1980; Markova et al., 2012; Mattman et al., 1960; Michailova et al., 2005; Phelan et al., 2016; Tkachenko et al., 2020) that mycobacteria, which are pathogenic to humans and animals, under the influence of biotic and abiotic factors have altered morphology; tinctorial, cultural, sensitizing, biochemical (enzymatic), and other properties; and resistance to antituberculosis drugs. Importantly, the pathogenic potential of altered forms, and in particular cell wall-deficient bacteria, have partially or completely lost the cell wall, which may/may not reverse in vitro (Green et al., 1974; Gutman et al., 1965; Guze et al., 1976; Leon and Panos,

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1976; McDonald et al., 1981; Ponig et al., 1972; Schmitt-Slomska et al., 1973; Zykina et al., 1989).

At the same time, in long-term tuberculosis-disadvantageous farms, unique opportunities are created for changing the biological properties of mycobacteria, such as interaction in a closed environment of micro- and macroorganisms at numerous passages of different degrees of virulence of the pathogen over the years through an animal organism.

This, of course, leads to the emergence of new biovars of mycobacteria with different properties, which have not been reported in the literature. From the biological material of cows (with allergy to purified protein derivative tuberculin and absent pathological and anatomical changes), the highly virulent *Mycobacterium bovis* was isolated. The growth of the culture on the second day of cultivation at temperature 37°C was observed Tkachenko (2004). Therefore, the investigation of the morphogenesis and properties of mycobacteria in the dynamics of numerous passages through the dense nutrient medium with different pH values was observed.

With regard to this, it was necessary to investigate the lipids of rapid-growing isolates of mycobacteria and their pathogenicity. However, we found no such investigations of the rapid-growing field isolates of *M. bovis* in the available literature. This led us to perform this study.

Materials and Methods

This study was performed at the Department of Epidemiology and Infectious Diseases of Dnipro State Agrarian and Economic University according to the guidelines and roles of the Animal Research Committee of the Dnipro State Agrarian and Economic University (approval no.: 2014/09).

To determine the variability (conversion and reversion) in our study, we used a highly virulent strain of *M. bovis* (from the second-generation formed colonies on the second day at 37°C culture). The strain was passed through an artificial dense nutrient medium of Löwenstein-Jensen, which was made according to standard instructions in the conditions of Laboratory of Department with pH values 6.5, 6.7, and 7.1. To establish the desired acid-base balance, we used a pH meter. HCl or NH₄OH was added into the medium before the strain clotted.

Inoculation of the prepared suspension of mycobacteria (1 mg/cm³ of physiological salt solution) was performed with a bacteriological loop: two per medium (at different pH values) of each of the six test tubes of the three variants of the experiment according to the traditional technique in veterinary medicine (Manchenko et al., 1994).

For the next passage, one or three daily cultures of mycobacteria were used, and the suspension of mycobacteria was inoculated in the same medium of each subculture and cultivated under thermal conditions at 37°C.

The inoculations were inspected every day during the first week and once a week for 28 days from the time the colonies appeared. Colony form, time of appearance, and growth rate of the colonies were calculated and evaluated according to conventional methods (the beginning of growth was evaluated as the time of formation of macroscopically visible colonies formed in a dense egg medium). Growth character and morphological features of mycobacteria (cell form, presence of metachromatic granularity, and tinctorial properties (every 10th generation starting from the 60th generation)) were also evaluated. A total of 180 passages of mycobacteria were performed through the media at pH 6.7 and 6.5 and 132 passages at pH 7.1.

The pathogenicity of the 2nd, 100th, 130th, and 150th obtained subcultures of *M. bovis* was studied in guinea pigs weighing 250–300 g. The colonies were triturated in a mortar with 1 mg/cm³ physiologic solution. At this dose, the mycobacteria suspension was injected parenterally into the inner thigh area (experimental and control groups, n=6, in total 72 animals). The experimental animals were observed for 30 days to assess the nature of ulcer and the manifestation of allergy. After the death or euthanasia of guinea pigs, pathological and anatomical changes were identified (Manchenko et al., 1994).

To obtain a pure line (clone) of mycobacteria from the 100th subculture, the inoculation was sequentially performed from isolated initially formed colonies according to the time of their formation. In total, four inoculations were carried out for the same medium at pH 6.7.

To study the times of conversion of acid-resistant mycobacteria and reversion of non-acid-resistant mycobacteria of the 100th generation, 10 inoculations of three subcultures (variants) of the pathogen with smoke-colored growth were performed: (1) non-acid-resistant filamentous forms of mycobacteria, (2) non-acid-resistant thread-like and acid-resistant different shaped and sized rod-shaped bacteria, and (3) acid-resistant rod-shaped bacteria of traditional morphology.

The study of the timing of reversion of non-acid-resistant mycobacteria to acid-resistant or subsequent transformation of acid-resistant mycobacteria was carried out in 10 inoculations of all three clones of subcultures of the pathogen using the dense medium (pH 6.7). Direct inoculation was carried out with a bacteriological loop in a medium of two test glasses in the first or second day of the appearance of smoke-colored culture along the inoculation line. In the dynamics of inoculations, the time of cultivation of mycobacteria, under immersion, and the morphological aspects of the reversion of non-acid-resistant rod-shaped forms into acid-resistant bacterial cells were studied. For this purpose, the culture ("coating" and "haze") of non-acid-resistant mycobacteria and all 10 passages were kept in a thermostat for 5 months and were reviewed once a week. The smears prepared from the obtained cultures were examined. In these and other studies, rubber plugs were used to cork the test tubes (Rumachik, 1989).

The separation of total lipids from the samples was performed according to the method of Folche in Bligh-Dyer modification for microbiological samples (Keits, 1975). Biomass in the amount of 0.5 g of samples was diluted with distilled water to 1 cm³, and 3.5 cm³ of chloroform: ethanol (1:2) mixture was added to this and left for 2 h shaking occasionally. It was centrifuged for 5 min at a speed of 3500 rpm, the supernatant was poured, and 4.75 cm³ of chloroform: methanol: distilled water (1:2:0.8) mixture was added. The mixture was shaken and centrifuged (5 min at 3500 rpm). The supernatant was added to this mixture, and 2.5 cm³ of chloroform and distilled water mixture was added to this, shaken well, and left for separation. The bottom layer (chloroform with lipids) was collected and dried with benzene (30–35°C). The amount of total lipids was calculated as a percentage of the sample (by the conventional method).

The fractional composition of the lipids was studied by thin layer chromatography on silica gel plates (Silufol, Czech Republic) beforehand, which was ungreated with acetone and activated at 100°C for 1 h in a solvent system-hexane: diethyl ether: methanol: glacial acetic acid (9:2:0.2:0.3).

The determination of component composition of free fatty acid (FFA) fractions in the samples was performed by gas-liquid chromatography on a Chrom-5 gas chromatograph (Laboratori Pistroje, Czech Republic).

Methylation of FFA

To the samples of total lipids, 3 cm³ 5% dimethyl sulfate in methanol was added and heated for 15 min at 65°C. It was then cooled to 18–20°C, and 7 cm³ of distilled water and 1 cm³ of carbon tetrachloride were added. The mixture was shaken and left to settle to separate the layers. The bottom interlayer was taken using a syringe in a hydrolysis test tube and evaporated to dryness. To the evaporated samples were added 20 to 50 µL of hexane and 5 to 10 µL was introduced into the evaporator of the chromatograph.

Samples of fatty acid methyl esters were analyzed under the following conditions: column L=1 m×4 mm, on a Chromaton N-Super sorbent with 5% SP 2100 (0.16–0.20 mm). The temperature of the column was set as 180–270°C with a heating rate of 5°C/min. The evaporator temperature was 200°C, the detector temperature was 230°C, the carrier gas was nitrogen (*puriss. spec.*), and flame ionization detector was used (Ahrem and Kuznecova, 1964).

Qualitative analysis of fatty acid methyl esters was performed by comparing with the retention time of the standards, and quantitative analysis was performed by calculating the peak area and determining its percentage in the total peak area, which was taken as 100%.

The studies were performed using *M. bovis* 2nd, 130th, and 150th passages through an artificial nutrient medium with pH 6.7 in three replications of test. For the study of lipids, mycobacteria

were inoculated in media at different pH values. Four-week culture was collected using a spatula without touching the medium. Calculations and statistical analyses of results of research were performed using a personal computer in the Excel spreadsheets of the Office XP Professional software package.

Results

The results of inoculation of the rapid-growing pathogenic strain (Figure 1) showed dynamic significant differences: in medium at pH 7.1, the time of colony formation from the 19th passage decreased. The decrease in time of colony formation is defined as slow growing. In media at pH 6.7 and 6.5, this phenomenon was detected only from the 109th to the 120th inoculation (Figure 2). Therefore, 180 generations of the rapid-growing strain were obtained in the latter media, and only 122 generations in the former medium.

In the rapid-growing strain of three subcultures of *M. bovis*, dynamic significant changes were observed depending on the content of acid-alkaline gram equivalents in the medium. First, it depends on the form of colonies and the intensity of adaptation of mycobacteria in an artificial nutrient medium.

During the observation period, the forms of colonies changed from small, dry, and solitary, to large and moist ones with

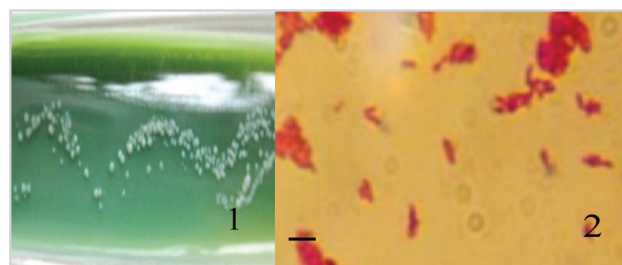


Figure 1. Subculture (1) and morphology (2) of pathogenic *M. bovis* at passage 2; bar=5 µm

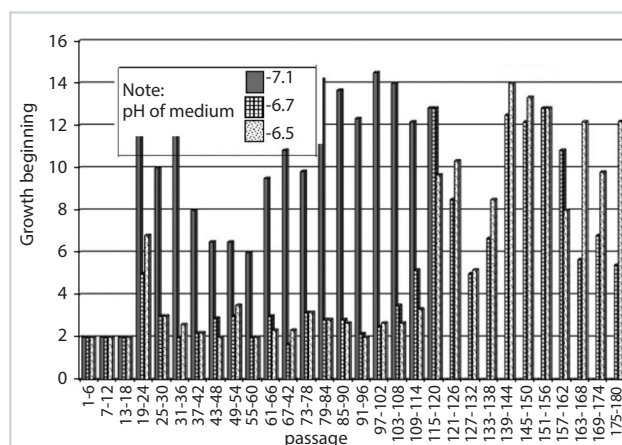


Figure 2. Growth of colonies of *M. bovis* at different passages

Table 1. Conversion of *M. bovis* into non-acid-resistant forms

pH medium	Passage												
	60	70	80	90	100	110	120	130	140	150	160	170	180
6.5	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+
6.7	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+
7.1	-/-	-/-	-/-	-/-	-/-	+/-	+/+	+/+	Not investigated				

–: barely visible; +: clearly formed non-acid-resistant mycobacteria

Table 2. Growth of *M. bovis* culture at different passages

pH of the medium	Passage												
	60	70	80	90	100	110	120	130	140	150	160	170	180
6.5	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+
6.7	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+
7.1	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	Not investigated				

Numerator: colonies; denominator: a haze, a fur/coating

continuous growth during prolonged cultivation to a slight smoke-colored (coating) continuous growth in the last 20 generations along the lines of inoculation of mycobacterium suspension.

However, on the 14th day since the beginning of the formation of colonies in media at pH 6.5 and 6.7, there was a continuous growth up to the 114th passage, and in medium at pH 7.1, continuous growth was observed only on 21–28 days. This indicates a more negative effect of the acid–base gram equivalents (pH 7.1) on the adaptive capacity of *M. bovis*, relative to the nutrient medium.

The morphological features and tinctorial properties of mycobacteria, depending on the medium, varied with the increase in the number of inoculations (Table 1).

Beginning from the 90th generation, thick and thin, granular, red colored, short- and long-segmented rod-shaped bacteria were observed under immersion, and barely visible single thread-like, non-acid-resistant forms of rod-shaped bacteria, with a fuzzy granularity (smear from a colony formed in medium with pH 6.7), were observed.

From the 145th passage, long (6–10 times longer) forms of mycobacteria appeared, in contrast to the short traditional acid-resistant ones.

In the last generations, starting at the 160th generation, single thread-like, acid-resistant, segmented mycobacteria with a large number of grains, but with a less intensely colored mantle layer, appeared on medium at pH 6.7 compared with those in the first inoculations.

The number and color intensity of non-acid-resistant mycobacteria remained the same as in the inoculation until the 90th generation.

From the 170th and subsequent generations, both acid-resistant rod-shaped bacteria and non-acid-resistant rod-shaped and thread-like forms of mycobacteria were detected by microscopy (Figure 3).

The appearance of non-acid-resistant forms in the population of mycobacteria was accompanied by changes in the external appearance, shape of colonies, and timing of their formation (Table 2).

Before the emergence of polymorphic forms, acid-resistant mycobacteria were formed in the environment of separate colonies with subsequent continuous growth along the inoculation line. Mixed forms (acid and non-acid-resistant) stimulated smoke-colored growth of the culture along the sowing line and after growth retardation (from 109–120 to 157–168 passages), showing almost the previous rate of reproduction (5–7 days) from almost 169–180 passages.

Identical changes were recorded in the rapid-growing strain of *M. bovis*, which was transplanted through an artificial nutrient medium at pH 6.5. However, a clear appearance of non-acid-resistant polymorphic mycobacteria was observed at the 176th generation, that is, nine passages later than that observed in medium at pH 6.7.

On medium at pH 7.1, the rapid-growing *M. bovis* strain, as well as the two previous ones, stimulated the smoke-colored culture (coating) along the inoculation line of suspension with the appearance of non-acid-resistant forms of the pathogen.

Table 3. Time of death of guinea pigs infected with *M. bovis*, cultivated in media at different pH values

Subculture	pH of the medium								
	7.1			6.7			6.5		
	MurRAIN day	Allergy	Ulcer	MurRAIN day	Allergy	Ulcer	MurRAIN day	Allergy	Ulcer
2	35–45	+	+	35–47	+	+	39–41	+	+
100	45–47	+	+	–	–	–	–	±	–
130	49–53	+	+	–	–	–	–	–	–
150	Not investigated	–	–	–	–	–	–	–	–
Control	–	–	–	–	–	–	–	–	–

+: positive; ±: doubtful; –: negative result

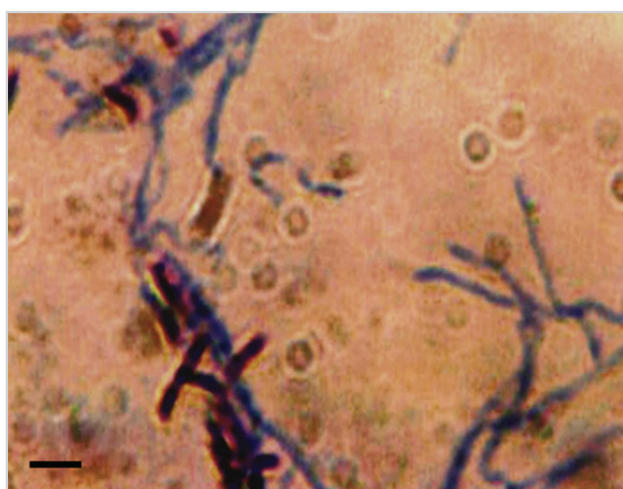


Figure 3. *M. bovis*: acid-resistant and non-acid-resistant forms of first generation; bar=10 µm

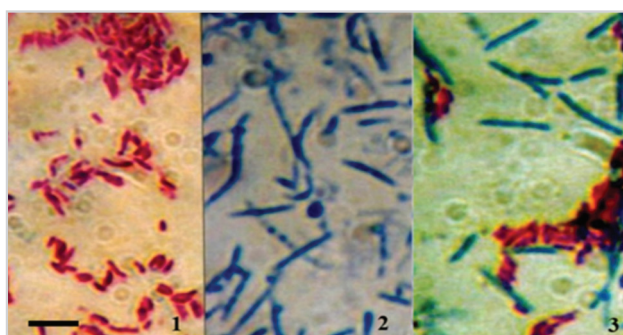


Figure 4. Clones of *M. bovis*. (1) Acid-resistant, (2) non-acid-resistant, and (3) acid-resistant and non-acid-resistant forms; bar=10 µm

However, a clear transformation of mycobacteria into non-acid-resistant forms was noted at the 120th inoculation, that is, much earlier than that in the two previous cases, with a slightly accelerated growing tendency of the coating culture. At the same time, in this medium, single indistinctly formed non-ac-

id-resistant forms of mycobacteria, as well as the other two, were observed under the microscope much earlier before the appearance of clearly formed conversion (transformed) forms (60th inoculation).

Furthermore, over time (from 3 to 8 weeks after inoculation of suspension of acid-resistant and non-acid-resistant mycobacteria) in the medium of all three subcultures, against the background of "coating," "haze" appeared as singly formed yellow-gray colored colonies, which were the same as the initially formed acid-resistant and non-acid-resistant forms.

In addition to the changes in the form of the colonies, morphological features, and tinctorial properties of passaged mycobacteria in medium at different pH values, the pathogenicity (virulence) of the pathogen also changed (Table 3). More pronounced changes occurred in mycobacteria cultivated on media with pH 6.5 and 6.7 because they did not cause guinea pig deaths (except for the original subculture). While others were infected with *M. bovis* and passaged through the medium with pH 7.1 died from tuberculosis within 35–53 days.

Inoculating mycobacteria of mixed culture from sequentially isolated ones initially formed colonies according to the time of their formation (in the same medium at pH 6.7). On the fourth inoculation of individual colonies, the clones were obtained as follows: (1) acid-resistant rod-shaped bacteria, (2) non-acid-resistant thread-like and rod-like and acid-resistant rod-shaped bacteria with different forms and sizes, and (3) acid-resistant and non-acid-resistant thread-like and rod-shaped mycobacteria (Figure 4), which formed smoke-colored cultures.

After inoculating selected clones of mycobacteria 10 times on the nutrient medium, 10 subcultures of acid-resistant mycobacteria grew into non-acid-resistant and acid-resistant forms of the pathogen (Table 4). The latter were morphologically identical to the original clone of mycobacteria.

The passages of the clone of acid-resistant and non-acid-resistant mycobacteria were accompanied by the complete disappearance of acid-resistant forms on the fifth inoculation, and

Table 4. Mycobacteria conversion time for 1–3 days' culture

Clone no. of <i>M. bovis</i>	Form of mycobacteria	Passage (subculture)									
		1	2	3	4	5	6	7	8	9	10
1	Acid-resistant and non-acid-resistant	k-nk	k-nk	k-nk	k-nk	nk	nk	nk	nk	nk	nk
2	Acid-resistant	k	k	k	k	k	k	k	k	k	k-nk
3	Non-acid-resistant	nk	nk	nk	nk	nk	nk	nk	nk	nk	nk

k: acid-resistant; nk: non-acid-resistant; k-nk: acid-resistant and non-acid-resistant

Table 5. Time of reversion of non-acid-resistant mycobacteria to acid-resistant forms and colony formation

Subculture no.	Formation of acid-resistant forms and colony formation (week)																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
2	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
3	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+
4	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+
5	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+
6	-/-	-/-	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+
7	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+
8	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+
9	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+
10	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-

Numerator: acid-resistant mycobacteria; denominator: colonies

Table 6. Content of total lipids in *M. bovis* (% by weighted portion)

Index	Passage		
	2	130	150
Total lipid content	8.05±0.20	3.90±0.55**	3.66±0.42***

p≤0.01; *p≤0.001

the clone of non-acid-resistant ones generated thread-like forms in all 10 subcultures.

The investigated clones of mycobacteria formed the culture on the 3rd–10th day in the form of coating (haze), which disappeared after 2–4 weeks of cultivation (except individual subcultures), whereas the smoke-colored culture (coating) remained for 5 months.

At the same time, cultivation in a thermostat (37°C) of non-acid-resistant mycobacteria of the third clone of all 10 subcultures for 5 months showed (Table 5) that the reversion of the pathogen to acid-resistant forms and the time of colony formation, though only solitary, are interconnected.

Thus, the first generations of non-acid-resistant mycobacteria, reversed significantly earlier, but only partially, to acid-resistant

forms (from the third week) than the other (8th to 10th) generations—which reversed only from the seventh week. Consistent with this, only in long term, single colonies are formed.

Mycobacteria of subculture no. 10 did not form macroscopically visible colonies until 5 months. However, microscopy of mycobacteria that formed colonies of nine subcultures revealed the presence of both non-acid-resistant polymorphic and acid-resistant forms. In the latter form, we observed extremely long rod-shaped bacteria with a large number of grains (small bodies), up to nine or 12.

Furthermore, it was determined that the reversion of non-acid-resistant mycobacteria into acid-resistant mycobacteria occurs stage by stage (Figure 5).

Results of the studies of total mycobacterium lipids showed that their tendency decreased in the dynamics of passages. Table 6 shows that the total content of lipids was significantly higher in the second passage than that in passages 130 and 150 (2.0 and 2.2 times, respectively).

The distribution of total lipids into fractions in *the M. bovis* of the second subculture passaged 130 and 150 times revealed dynamic changes (Table 7). Displacements were ambiguous:

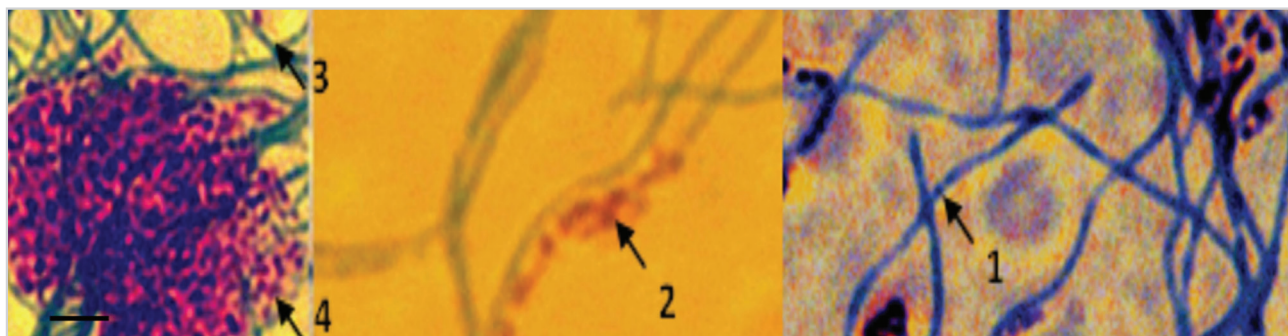


Figure 5. Stages of reversion of acid-resistant filamentous *M. bovis* to acid-resistant bacterial forms. (1) Acid-resistant grains in thread-like *M. bovis*, (2) transformation and fragmentation of non-acid-resistant thread-like forms into acid-resistant rod-shaped bacteria, (3) outlet of acid-resistant thread-like *M. bovis* acid-resistant forms, and (4) initial stage of formation of the microcolony of mycobacteria

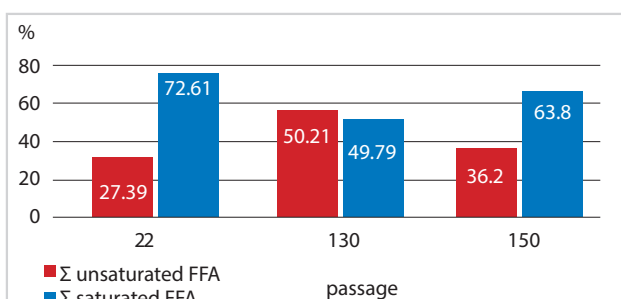


Figure 6. The ratio of unsaturated and saturated free fatty acids in the dynamics of passages of *M. bovis*

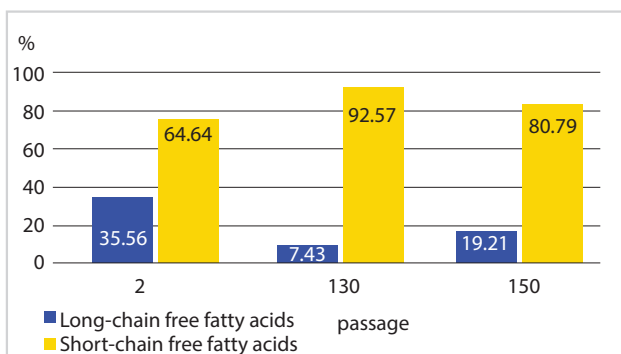


Figure 7. The ratio of short- and long-chain free fatty acids in the dynamics of *M. bovis* passages

the content of some fractions increased, while that of others decreased.

Thus, the levels of phospholipids, triacylglycerols, and sterol esters decreased significantly compared with the original data by 1.2, 1.3, and 1.2 times, respectively, and the levels of diacylglycerols, sterols, and FFAs probably increased by 1.5, 1.4, and 1.1 times, respectively.

Table 7. Fractional composition of lipids in passaged *M. bovis* (% by weighted portion)

Total lipid fraction	Passage		
	2	130	150
Phospholipids	27.97±0.26	20.72±0.85**	23.41±0.45***
Diacylglycerols	12.54±0.27	20.36±0.86***	16.71±0.43**
Sterols	12.23±0.20	18.93±0.74***	16.00±0.70**
Free fatty acids	14.47±0.23	16.07±0.36*	16.00±0.14**
Triacylglycerols	18.32±0.16	12.50±0.49***	15.61±0.32**
Esters of sterols	14.47±0.24	11.42±0.80*	12.27±0.50*
Σ lipids	100	100	100

*p≤0.05; **p≤0.01; ***p≤0.001

The qualitative and quantitative compositions of FFAs in the dynamics of *M. bovis* passages changed significantly (Table 8).

Thus, if *M. bovis* of the original subculture was placed at the diagnostic level 19 FFAs, then in 130th passages they were reduced to 14, and by 150th - to ten.

It should be noted that at the 150th passage, acids such as tri-decanoic, hexacosanoic, and heptacosanoic acids completely disappeared from the cell membrane. The last two acids were not detected at the 130th passage. The contents of palmitic, oleic, and stearic acids increased significantly by 1.4, 1.5, and 1.6 times, respectively, by passages 130–150 compared with those in the original second subculture.

Figure 6 shows that the number of passages had a significant effect on increasing the amount of unsaturated and reducing the amount of saturated acids. If the original culture contained 27.39% unsaturated acids, then at passages 130–150, it was 43.2%. This is 1.6 times more than in the original subculture of mycobacteria.

Table 8. Content of free fatty acids in the samples, % of the amount

Free fatty acids	Code	Passage		
		2	130	150
Lauric	C ₁₂₀	0.04±0.02	0.10±0.06	Traces
Tridecanoic	C ₁₃₀	0.10±0.02	0.15±0.08	–
Myristic	C ₁₄₀	0.27±0.04	0.51±0.30	Traces
Pentadecanoic	C ₁₅₀	0.22±0.02	0.33±0.23	Traces
Palmitoleic	C _{16:1}	0.55±0.07	1.63±0.21*	0.08±0.05**
Palmitic	C ₁₆₀	19.62±0.53	26.43±1.01**	28.97±0.91***
Margaric	C ₁₇₀	0.81±0.07	3.05±0.4**	0.91±0.07
Oleic	C _{18:1}	23.87±0.60	44.72±1.55***	27.16±0.55*
Stearic	C ₁₈₀	7.42±0.11	10.57±0.64**	13.58±0.52***
Linoleic-linolenic	C _{18:2} +C _{18:3}	2.97±0.16	3.86±0.02**	8.96±0.57*
Nonadecanoic	C ₁₉₀	3.34±0.06	1.22±0.07***	1.13±0.36**
Arachic	C ₂₀₀	5.23±0.14	Traces	Traces
Heneicosanoic	C ₂₁₀	6.74±0.23	2.14±0.03***	3.39±0.38**
Behenic	C ₂₂₀	5.78±0.18	3.17±0.01***	10.17±0.54**
Tricosanoic	C ₂₃₀	5.89±0.35	–	Traces
Tetracosanoic	C ₂₄₀	5.57±0.19	–	Traces
Pentacosanoic	C ₂₅₀	7.43±0.26	2.12±0.02***	5.65±0.65
Heptacosanoic	C ₂₆₀	2.53±0.30	–	–
Heptacosanoic	C ₂₇₀	1.62±0.19	–	–
Σ unsaturated		27.39±0.44	50.21±1.80***	36.20±0.70***
Σ saturated		72.61±0.50	49.79±1.70***	63.80±0.75***
Σ short-chain		64.44±0.61	92.57±0.53	80.79±0.50
Σ long-chain		35.56±0.47	7.43±0.40	19.21±0.32

*p≤0.05; **p≤0.01; ***p≤0.001

C_{Ad}: saturated fatty acid (A is the number of carbon atoms); C_{AdH}: unsaturated fatty acid (H is the number of double bonds); Traces: have an acid content of less than 0.01%

With increase in the number of inoculations in mycobacteria, the content of long-chain FFAs significantly reduced (Figure 7) and the content of short-chain FFAs increased. Thus, if the ratio of these acids in the original sample of mycobacteria was 1:0.55, then at passages 130 and 150, it was 1:0.15.

Discussion

The occurrence of tuberculosis disease in humans and animals, even after the discovery of the pathogen and its purposeful, versatile knowledge, is conditioned probably in consequence of variety of biological properties that are changing easily depending on the environment and inducing factors. Our investigations established the existence in the nature of previously unrecognized strains of *M. bovis* with rapid metabolism, which allows *M. bovis* to form colonies in the medium on the second day after inoculation of suspension. However, the main peculiarities of this type of mycobacterium are pre-

served: pathogenicity and sensitizing ability (Glebenyuk and Telijenko, 2015).

Furthermore, these traditional properties of *M. bovis*, especially pathogenicity, are changing quite rapidly depending on the pH of the medium in which they are cultivated (are multiplied). The investigations have shown that media at pH 6.5 and 6.7 promote long-term increased metabolism and transformation into adaptive, transient forms of mycobacteria (non-acid-resistant rod-shaped bacteria, thread-like mycobacteria). Similarly, the virulence of the pathogen decreases until it is completely lost by 100th inoculation. In medium at pH 7.1, on the contrary, metabolism in the microbial cell (by 13–24th inoculation) slowed down to the normal level. This determined the formation of colonies at a longer duration of time (6.5 and more days), which is characteristic of this type of mycobacteria. This is also accompanied by the preservation of virulence: Guinea pigs died because of tuberculosis after being infected with 130 subcultures for 49–53 days, which almost corresponds to the time of death of guinea pigs infected with the original subculture (35–45 days).

These data are consistent with the reports of other authors (Tkachenko et al., 2012; Veisfeiler, 1975), who noted death of guinea pigs in similar time after infection with mycobacteria cultivated in medium at pH 7.1. However, we did not investigate the virulence of rapid-growing *M. bovis* cultivated in media at pH 6.5 and 6.7.

Changing the metabolic processes of the microbial cell could not affect the form of colonies: small, solitary, dry at the beginning of inoculations to the slight smoke-colored (coating) continuous growth in the last subcultures with the stability of color of culture. Such changes in colony morphology have been reported by a number of researchers (Djachenko et al., 2008; Michailova et al., 2005; Tkachenko et al., 2016), who argue that this may be caused by the adaptive mechanisms of mycobacteria, in particular, the activation of genes with adaptive character.

However, we found that in media at pH 6.5 and 6.7, the culture growth rate exceeded by almost twice compared with that in medium at pH 7.1. This indicates that a more acidic medium contributes to a more intense metabolism and biomass accumulation. As reported previously by researchers (Prozorovskiy et al., 1981; Timakov and Kagan, 1973), the medium pH also affects the conversion of *M. bovis* since the occurrence of the first adaptive forms of non-acid-resistant rod-shaped bacteria, thread-like forms, which was at 90th inoculation at pH 6.5 and 6.7, on medium with pH 7.1 at 122nd inoculation.

The formation of non-acid-resistant mycobacteria in the acid-resistant population led to a change in the growth pattern of the culture: acid-resistant mycobacteria formed single colonies at the beginning of inoculations, and mixed forms (acid-resistant and non-acid-resistant) stimulated the smoke-colored growth of the culture in the line of inoculation. This was reported by oth-

er authors (Kalina, 1962) who found that transformed mycobacteria alter the nature and form of colonies, and this was evident because such altered microorganisms have, if not fundamentally, different metabolism, at least substantially altered.

Changing the cultural, tinctorial properties and morphology of mycobacteria was accompanied by the decrease in their virulence, except mycobacteria cultivated in medium at pH 7.1.

Mycobacteria cultivated in media at pH 6.5 and 6.7 significantly reduced virulence already by the 100th inoculation (guinea pigs remained alive for 3 months, and no pathological or anatomical changes were detected at dissection). Reduced virulence in altered mycobacteria was reported by other researchers as well (Domingue and Woody, 1997).

At the same time, mixed cultures of mycobacteria formed separate colonies at different times. Studying the separated initially formed colonies, three clones of mycobacteria were selected and examined for 10 inoculations. The clone of acid-resistant and non-acid-resistant mycobacteria transformed into a non-acid-resistant form at the 5th inoculation, acid-resistant form on the 10th inoculation - in acid-resistant and non-acid-resistant; non-acid-resistant - remained non-acid-resistant for ten times inoculations.

The obtained data indicate certain regularities of biological properties of the pathogen development, because in all experiments, the conversion of acid-resistant mycobacteria to non-acid-resistant ones was observed. However, the clone of non-acid-resistant mycobacteria of all 10 passages within 20 months of cultivation at 37°C reversed to acid-resistant forms with some regularity: the first passages from the third week of cultivation, and the last (9–10 inoculations) from the ninth week. Similarly, the times of formation of single colonies were from 13 and 18 weeks, respectively.

This indicates that the converted non-acid-resistant forms of mycobacteria at an early stage can reverse in some time to the original acid-resistant variants of the pathogen. This was achieved through mechanisms such as the conversion of non-acid-resistant grains to acid-resistant with subsequent exit from the mother cell and the acquisition of acid-resistant end of thread-like mycobacteria. The conversion and reversion of transient forms of bacterial cells are apparently related to nucleic acid stability.

The formation of acid-resistant forms of mycobacteria from non-acid-resistant, thread-like forms occurs from small bodies (grains) that fill (placed one after another) the thread-like forms of the pathogen.

However, only some of them are reversed to acid-resistant small bodies. The vast majority of small bodies, as well as their mother's non-acid-resistant, thread-like forms, over time (4–5 months), probably lose their reproductive capacity and viability.

In addition, with increase in the number of mycobacteria inoculations of conversion forms, the reversing ability of the non-acid-resistant to acid-resistant mycobacteria decreases. However, to conclude on this point, studies of a longer duration are needed.

At over 180 inoculations, we could not observe L-forms of protoplasmic (spheroplasmic) type, which were reported by several researchers (Downing et al., 2005; Markova et al., 2012; Udou et al., 1982), under the influence of inducing factors (e.g., lysozyme, antibiotics, starvation, radiation, temperature, factors of the internal environment of the macroorganism). This determines to some extent the peculiarities of the genetic code of the rapid-growing strain of *M. bovis*.

Generally, the processes of transformation of acid-resistant virulent mycobacteria are closely related to the chemical constituents of the cell wall. In parallel with the modification of the phenotype of the microorganism, the number of total lipids decreases from $8.05 \pm 0.20\%$ in the original to $3.66 \pm 0.42\%$ at passage 150. The fractional composition of total lipids did not reveal changes in their quality, but the content of phospholipids, triacylglycerols, and esters of sterols decreased quantitatively, while others tended to increase.

The qualitative and quantitative composition of the fraction of FFAs in the dynamics of *M. bovis* passages has significantly changed against the background of the significant increase in their level.

The data obtained indicate that passages of *M. bovis* through an artificial nutrient medium with pH 6.7 significantly affect their quantitative and qualitative composition, which can lead to changes in the biological activity of the microorganism (Bilan et al., 2007; Tkachenko et al., 2007; Tkachenko and Kovaleva, 2008).

Adaptive physiological processes of the microbial cell cause changes in the ratio of the amount of saturated and unsaturated FFA. The number of passages significantly influenced the increase in the amount of unsaturated and the decrease in the amount of saturated acids, which is consistent with the reports of Suutari and Laakso (1993).

If the initial culture contained 27.39% unsaturated acids, then at 130–150 passages, it was 43.2%, which was 1.6 times more than in the mycobacteria of the original variant. An increase in the amount of unsaturated FFAs may indicate an increase in the adaptive physiological processes of the microbial cell under adverse conditions, in particular constant long-term inoculations.

Marked and analyzed indicators of lipid composition of mycobacteria in the dynamics of numerous inoculations are interrelated and aimed at adaptation to different conditions, that is, to survival in the biological flora as a species.

Therefore, prolonged inoculations of mycobacteria were accompanied by the appearance in the population of microorganisms that differed from the original variant of the pathogen, in particular, changes in terms of morphological characteristics, and tinctorial, cultural, and virulent properties. However, the changes did not affect the entire population of the pathogen and its variants, but separate species that give onset to the changed clones in the subculture of them.

Furthermore, it can be argued that the appearance of non-acid-resistant forms of mycobacteria, whose epidemiological and etiological significance are completely unclear, is a natural process of the biological cycle of the species development of the studied microorganism, although they arise under the influence of certain factors. Not all biovars of microorganisms of the same strain are generated against the background of the influence of different inducing factors over time, as they lose acid resistance.

Obviously, the variability of mycobacteria has great importance in the epidemiology of tuberculosis, and in particular with regard to the loss of mycobacteria by acid resistance. It is the non-acid-resistant form of mycobacteria, as well as others, that determine the development and course of the epidemic and infectious processes of tuberculosis.

The chronology of the morphological forms of *M. bovis* of the rapid-growing strain depends on the acid-base gram equivalents of the medium. At pH 6.5–6.7, a rapid propagation with more intensive transformation into non-acid-resistant rod-shaped and thread-like variants of mycobacteria is observed than that at pH 7.1.

The change in morphological characteristics of the pathogen of tuberculosis is accompanied by the formation of the culture in the form of coating (haze), the decrease in virulence with increase in the cell wall and short-chain FFAs, and simultaneous reduction and disappearance of individual long-chain FFAs.

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