The ability of *Sitophilus oryzae* (Coleoptera, Curculionidae) to transmit *Mycobacterium bovis*: Morphology, cultural biochemical properties of the bacteria

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The problem of tuberculosis has been relevant for many years due to active spread of the infection pathogen around the globe, in particular in Ukraine. In this article, we determined the epizootic role of the rice weevil (*Sitophilus oryzae* (Linnaeus, 1763); Coleoptera, Curculionidae) in the spread of *Mycobacterium bovis*. We identified the effect of the beetle on properties of the pathogen, particularly the changes in morphology, cultural, biochemical, and biological properties after the bacteria had travelled through the body of the rice weevil. To achieve our objectives, we used the museum 100th passage of the virulent strain of *M. bovis*. We employed microscopic, cultural, biochemical, biological (infecting the biological model), pathoanatomic, histological methods, and PCR studies. The rice weevil is able to retain mycobacteria and release them into their environment for 30 days after becoming infected, with gradual decrease in the number of microbial cells. According to morphology and cultural properties, the pathogen we isolated in the experiment was identical to the initial culture. Enzymatic activity of the bacteria varied. The bacteria that had passed through the intestines of *M. bovis* were observed to have changes in the biochemical parameters which helped them to adapt to the new environment. We measured the effect of the rice weevil on pathogenicity of *M. bovis*, isolated directly from the beetle and from grain contaminated with the insects during the experiment. The practical importance of the study consists in expansion of our understanding of the ways *M. bovis* spreads, identification of effect the rice weevil has on mycobacteria. It also might help in the search for ways to interrupt the chain of tuberculosis transmission – prevention of spread of the disease to favourable areas.

Keywords: mycobacteria; tuberculosis; histology; PCR; genotyping.

Introduction

Unfortunately, the world today is not free from tuberculosis. Outbreaks of the infection have been recorded in various regions. In Ukraine, the issue has been recently aggravated due to military engagements, which increase the movement of animals, both natural migrations and in the form of evacuation. This poses a threat, exacerbates the epizootic situation, and activates the spread of zoonoses, in particular tuberculosis infection. The main pathogens that are dangerous to agricultural animals are *Mycobacterium bovis* and *M. tuberculosis*, to which humans are also susceptible. Tuberculosis inflicts multiple economic and social losses (Pérez-Morote et al., 2020). To effectively combat this zoonosis, the priorities are identification of all possible ways by which the pathogen invades a favourable area, identification of potential sources of infection, and development of efficient antituberculosis drugs. Currently, special attention is paid to research of antimicrobial action of extracts from plants and derivatives of 1,2,4-triazole-3-thiols (Palchykov et al., 2019; Zazharskyi et al., 2019; Zazharskyi et al., 2020). Mycobacteria are able to adjust their microbial cell so as to adapt to altering ambient conditions, and the structure of the cellular wall (presence of lipids in it) provides additional protection for the bacteria. This flexibility and modification of morphological form are challenges to the diagnostics and elimination of the disease. Changes in the morphological specifics and biochemical and biological properties

make the mycobacteria resilient in a new living environment. Due to the variation of their properties, mycobacteria have acquired a broad range of potential biological and mechanical carriers. Numerous scientists have reported detecting mycobacteria in cockroaches, mites, lepidopterans, and a number of other invertebrates (Fischer et al., 2004; Wallace et al., 2010). Cano et al. (2018) noted that insects (Hemiptera) are potential reservoirs and carriers of *M. ulcerans*, and Marsollier et al. (2002) added that mycobacteria can be spread by water bugs of the Naucoridae family – carriers of *M. tuberculosis* complex. Crispell et al. (2019) found that macroorganisms are able to transmit mycobacteria both among one species and to other species.

Weevils of the *Sitophilus* genus are significant as a pest of barns (Martynov et al., 2019; Titov & Brygadyrenko, 2021). Our previous studies revealed that rice weevils *Sitophilus oryzae* (Linnaeus, 1763) are able to accumulate and spread dissociative forms of mycobacteria. We found that after the passage through the weevil body, dissociative bacteria maintained their morphology and cultural properties, though the number of viable bacteria was reduced. At the same time, we observed changes in enzymatic activity, oriented at survival of mycobacteria in a new environment. Passage through the organism of beetles did not restore pathogenic properties of avirulent strains (Tkachenko et al., 2021; Zazharskyi et al., 2022). However, there are no data about effect on biological properties of a virulent strain of mycobacteria, which poses the main threat to the wellbeing of populations of agricultural animals.

The objectives of this study were to identify the epizootic significance of *S. oryzae* in the potential spread of tuberculosis and identify the effect the insects have on biochemical and biological properties of a pathogenic *M. bovis* strain. For this purpose, we identified how long the beetles took to accumulate and release the tuberculosis pathogen, analyzed changes in the enzymatic activity and pathogenicity of the causative agent after passage through the rice weevil.

Materials and methods

Ethical principles of the research. The studies were performed according to the positions of Article 26 of the Law of Ukraine No. 5456-VI as of 10/16/2012 On the Protection of Animals from Abuse, General Ethical Principals of Experiments on Animals, adopted on the First National Congress of Bioethics (Kyiv, 09/20/2001), requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 03/18/1986), the WMA Declaration of Helsinki (2000). The studies were carried out in the laboratory of the Department of Infectious Diseases of Animals of the Dnipro State Agrarian-Economic University and the Scientific-Research Laboratory of Novel Methods (ELISA and PCR) of the Bila Tserkva National Agrarian University in 2022–2023.

Collecting samples and creating experimental groups. Fast-growing virulent strain of *M. bovis* (passage No. 100: small colonies, R-shaped, ivory colour, acid-stable when performing a microscopy) was obtained by sequential inoculations of the culture onto a dense artificial Mordovskiy's egg medium (Nove), kept at low temperature (+3.0 ± 0.5 °C), which resulted in detachment of a part of the avirulent mycobacteria from the parental-strain bacteria. The insects were collected from forage wheat grain – fodder for rodents, and identified according to the morphological features. The necessary amount of weevils was collected during their six-month period of feeding on grain. Prior to infection, the rice weevils were examined for absence of natural bearing of acid-tolerant bacteria. Then, we created two groups: experimental and control (50 *S. oryzae* imagoes in each). The experimental group was placed on grain, which was previously autoclaved (20 min, 1.5 atm.) and then treated by a weighed amount of *M. bovis* – 10 mg of bacterial mass in 1 cm³ 0.9% solution of sodium chloride per each 10 g of wheat. The control group was placed on sterile grain.

The beetles were kept in grain, for 4 days, and afterward transferred to sterile grain for another 4 days, and then again transferred to sterile grain for 4 days. The next transfer was performed on the 20th day of the experiment and each 10 following days until day 90, inclusive.

Prior to each transfer onto sterile grain, we randomly chose two beetles, rinsed them in physiological solution, and macerated them on a microscope slide. Then, we stained the homogenate and rinses according to the Ziehl-Neelsen method and examined them under a microscope. At the same time, we gathered samples of the grain on which the weevils were kept. The grain was made into suspension, and then the prepared suspension was microscopically analyzed and inoculated on a Stonebrink growth medium. From the 30th to 90th day (each 10 days), onto the growth medium, we also inoculated the homogenate from previously rinsed rice weevils.

PCR studies. All the cultures (initial and isolated after each step of passage through the beetles) were subjected to PCR studies. From the medium's surface, we gathered a pure culture using a microbial loop and dispersed it in 1 mL of physiological solution. From the suspension, DNA was isolated using spin columns with commercial IndiSpin Pathogen Kit (Indical, Germany) according to the manufacturer's manual. Molecular-genetic identification was performed using classical PCR with detection in agarose gel. Amplification reaction was carried out in 25 μL of the reaction mixture, which included 12.5 μL of ready-to-use PCR mix OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs, USA), 7.5 μL of deionized water, 1 μL of each F and R primers, and 3 μL of matrix DNA, obtained at the previous stage. Amplification was carried out in a GeneAmp PCR System 2400 thermocycler (Applied Biosystems, USA) according to the following program: initial denaturation at 94 °C – 1 min., amplification – 30 cycles (denaturation at 94 °C – 30 sec., annealing (temperature depending on used primers (Table 1) – 30 sec., elongation at 68 °C – 60 sec.), and final elongation at 68 °C – 5 min.

For all the analyzed samples, we used two pairs of primers to confirm the identification to the *M. tuberculosis* complex and elucidate whether the bacteria were *M. bovis*. Using the VNTR method, we examined the culture prior to the passage and the isolate from homogenized beetles on the 30th day of the experiment. The cultures were genotyped using 8 pairs of primers flanking variable areas of genes of *M. bovis* (Table 1).

Monitoring of the results was conducted using amplicon electrophoresis in 2% agarose gel with addition of 0.5% of ethidium bromide in 1x TBE buffer. Electrophoresis was carried out for 80 min and after the procedure the reaction was read and the results of electrophoresis were photographed in a transilluminator in UV light. The study results were analyzed according to the length of the specific fragment in agarose gel, compared with molecular-weight marker 100 bp DNA Ladder # N3231S (New England Biolabs, USA). Comparison of genotypes by VNTR analysis was conducted by evaluating sizes of fragments for each pair of primers.

Table 1

Oligonucleotide primers for PCR identification of *M. bovis* strain and its VNTR genotyping

Names of primers, loci (VNTR)	PCR primer (5′–3′)	Size of fragment + size of repetition	Annealing temperature	Source
M. tuberculosis complex	CTGGCGGGTCGCTTCCACGA TTCGACCGGCGGGACGTCGC	375	63	Vázquez-Marrufo et al. (2008)
M. bovis	TCGTCCGCTGATGCAAGTGC CGTCCGCTGACCTCAAGAAC	500	55	
ETR-A / 2165	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGCGATTT	195 + 75	52	
ETR-B / 2461	GCGAACACCAGGACAGCATCATG GGCATGCCGGTGATCGAGTGG	121 + 57	58	
ETR-C / 577	GTGAGTCGCTGCAGAACCTGCAG GGCGTCTTGACCTCCACGAGTG	44 + 58	60	
ETR-D / 580	GTCAAACAGGTCACAACGAGAGGAA CCTCCACAATCAACACACTGGTCAT	114 + 77	55	Kam et al. (2006)
ETR-E / 3192	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTCAT	492 + 53	52	
QUB-11a / 2163a	CCCATCCCGCTTAGCACATTCGTA TTCAGGGGGGGATCCGGGA	167 + 69	58	
QUB-11b / 2163b	CGTAAGGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT	67 + 69	49	
QUB-3232 / 3232	CAGACCCGGCCGTCATCAAC CCAAGGGCGGCATTGTGTT	423 + 56	56	

Biochemical assays. During the study of biochemical activity, we identified the catalase, peroxidase, dehydrogenase activities, presence of reduction of nitrates, and ability to hydrolize Tween 80. Catalase and peroxidase activities were identified using the modified Bogen's technique (Tkachenko et al., 2019). Into test tubes with the culture, we added 2% solution of hydrogen peroxide and 0.5% solution of pyrogallol A. After 15 and 30 min., we measured the catalase activity ("+++" – generation of a large number of oxygen bubbles, "++" – moderate amount, "+" – single gas bubbles, "–" – absence of the bubbles). After 1.5–2.0 h, we identified peroxydase activity by detecting changes in colour of the colonies ("+++" – dark-brown colour of colonies, "++" – brown, "+" – pale-brown, "–" – colour did not change).

The studies of dehydrogenase activity were carried out using the Bloch technique (1950), modified by Tkachenko et al. (2019). We gathered 4.0 cm³ of weighed amount of the culture of microbial cells with the concentration of 10.0 mg/cm³ in phosphate buffer (pH 7.4–7.6), mixed with 1.0 cm³ of 1.0% of glucose solution and 0.1 cm³ of 0.02% solution of methylene blue. The content developed in the test tube was overlaid by sterile liquid paraffin. The prepared test tubes were put in a thermostat (at +38.0 ± 0.5 °C) and we monitored how long it took for methylene blue to lose colour, making records about the reaction after 15–30 min and 24 h.

To assess the activity of nitrate reductase, we collected 10.0 mg of microbial mass and transferred it into a test tube with 1.0 cm³ of 0.067 M phosphate buffer with 0.1% solution of sodium nitrate. The content was accurately mixed and incubated in thermostat (at +37.0 ± 0.5 °C) for 20–22 h. After this, to the test tube, we added 2 drops of 2.0% solution of p-dimethylaminobenzaldehyde in 1.0% solution of hydrochloric acid (and noted whether a yellow coulr emerged).

The ability to hydrolyze Tween 80 was identified according to the Wein's method (1962). Reagents (1/15 M phosphate buffer (pH 7.0) – 100.0 cm³, Tween 80 – 0.5 cm³, and the main red 0.1% – 2.0 см³) were mixed and poured into the test tubes, 4.0 cm³ to each, and autoclaved (15 min., 1.5 atm.), Then, the test tubes were incubated at +37.0 ± 0.5 °C for 24 h, after which we introduced 3 bacterial loops of mycobacterial mass, made suspension of them and incubated them for 10 days at +37.0 ± 0.5 °C. We evaluated the reaction after 4 h, 5 days, and on the 10th day. When the reaction was positive, we observed a pink colour.

Biological study. In order to evaluate the effect of the rice weevil on virulent properties of mycobacteria after their passage through the beetle, we performed biological studies on guinea pigs (*Cavia porcellus* (Linnaeus, 1758), Rodentia, Caviidae) in two variants. In the first variant, subcutaneous infection, we used cultures harvested during the experiment and a culture that did not travel through the body of the weevil (initial culture of mycobacteria). In the second variant, the guinea pigs had alimentary infection, entailed by consumption of grain on which the weevils had been kept until their next transfer.

The experimental groups of guinea pigs in the first variant were divided as follows: group I – subcutaneously infected with a weighed amount of the initial culture; II – subcutaneously infected with a culture harvested from grain on the 4th day of the experiment, III – on the 8th, IV – on the 12th, V – on the 20th, VI – subcutaneously infected with a culture of mycobacteria isolated from homogenate of beetles on the 30th day; VII – control (intact) group. In the second variant (alimentary infection of the guinea pigs), we formed 5 groups: I – the guinea pigs were fed wheat contaminated with a weighed amount of the initial culture, II – grain on which the beetles were kept during days 4–8; III – for 8–12 days, IV – for 12–20 days of the experiment, V – control group (fed with only sterile grain). Before the experiment, the guinea pigs were kept on quarantine for two weeks, during which the animals were subjected to allergic test (intradermal administration of solution of PPD-tuberculin for mammals (0.1 cm³ 25 IU) to rule out natural carrying of tuberculosis.

To induce subcutaneous infection of the animals we administered a weighed amount of the experimental cultures in the amount of 1.0 mg of mycobacterial mass per 1.0 cm³ of 0.9% solution of sodium chloride. During alimentary infection, animals of the experimental groups were each given 20 g of the wheat on which the beetles had been feeding prior to each placing. For this purpose, the guinea pigs were put in a sterile box with experimental grain and kept until the wheat grain was eaten completely. The control group consumed only sterile grain.

The study lasted for 90 days, during which the guinea pigs were clinically examined daily, weighed weekly, and subjected to tuberculin skin test on the 30th, 60th, and 90th days of the experiment to identify sensitizing properties of the experimental forms. Evaluation of the biological study was based on the results of pathoanatomic, histological, cultural, and microscopic examinations.

During the necropsy, we determined distinctive changes in the lungs, liver, spleen, intestines, and lymph nodes. Pieces of the affected organs were gathered for histological studies. The degree of pathogenicity was evaluated according to the damage to the internal organs, expressed in points (scheme of M. V. Trius). According to this scheme, the specific damages were expressed as follows: "+" – single, "++" – several, "+++" –

numerous nodes; and after this the pluses were converted into numerical parameters (each "+"equaled 1 in the lymph nodes, 2 in the spleen, 3 in the liver, and 4 in the lungs), and thus the maximal index of damage to the organism could equal 30.

Biological material for the histological study was obtained during necrosis and fixated in 10% neutral solution of formalin followed by rinsing in tap water, dehydration by ethyl alcohol of ascending concentrations, introduction to a densifying environment (histoplast), preparation of histological sections with their following staining with hematoxylin and eosin and further microscopy according to the generally approved histological methods (Horalskyi et al., 2005). At the same time, we made impression smears from the internal organs, fixated them and stained according to the Ziehl-Neelsen method. Also, we prepared a suspension from damaged organs according to the method of Alikaieva (1950): the suspension was inoculated onto the Stonebrink medium (Tkachenko et al., 2019). The inoculations were incubated at +37.0 ± 0.5 °C.

Results

Microbiological study. During a microscopic study of the non-infected weevils, no acid tolerance of microflora was found. We also observed no growth after inoculating the homogenate of beetles on the growth medium, which rules out natural carrying of *M. bovis* by the experimental insects. During microscopy of smears of rinses from the experimental weevils, we detected rods up until the 20th day after they had been infected. Analyses of smears from homogenized beetles revealed mycobacteria up until the 30th day after infection (Fig. 1). During each study, we observed gradual decrease in the number of rods in the field of view, which was especially noticeable starting from day 12 after being infected: 88 ± 7 on the 4th day, 62 ± 8 on the 8th, 56 ± 4 on the 12th, 31 ± 6 on the 20th, and 5 ± 2 microbial cells on the 30th day since the beetles were infected.

After inoculation of the homogenate from the experimental weevils on the Stonebrink medium, the growth was seen only in the test tubes in which we inoculated the material on the 30th day of the experiment. The initial growth was manifest on the 18th day after inoculation. The colonies were identical to the initial culture, during microscopy – acid-tolerant rods.

When microscopically studying the suspension from grain on which the weevils were kept, we determined the presence of rods of M. bovis on the 4th, 8th and 12th days, i.e. after 1–3 transfers of the beetles. Morphologically, the microorganisms were identical to the initial culture. Density of the rods in the smear gradually decreased. At the same time, we inoculated the suspensions on the growth medium. Growth was seen after the first-fourth transfers. After the first transfer (on day 4), the growth of numerous colonies was observed along the inoculation line, and after the second-fourth transfers (on the 8th, 12th, and 20th days) the colonies were single (Fig. 2), their number being 67 ± 4 on the day 4, 29 ± 6 on day 8, 17 ± 3 on day 12, and 15 ± 4 on day 20. When studying smears from the grown colonies, we saw red rods after the Ziehl-Neelsen staining.

By generalizing the obtained data, we can state the ability of rice weevil to accumulate, transmit, and release pathogenic mycobacteria. On the body surface and in the grain on which the insects had been kept, we found experimental microorganisms until day 20 after being infected, and inside the insects until day 30 after the initial contact between the beetles and tuberculosis pathogen.

PCR study. Molecular-genetic identification and genotyping. The initial culture was identified using PCR with primers for *M. tuberculosis* complex, which identify *M. tuberculosis* and *M. bovis*. Then, we determined that the culture contained specific DNA of *M. bovis*. The following experimental cultures at each stage of the study also went through molecular-genetic identification to confirm the species (Fig. 3). The culture of the initial strain and culture from homogenized beetles on the 30th day of the experiment were genotyped using VNTR method (Fig. 4).

Analysis of length of amplicon fragments, obtained as a result of PCR with primers flanking VNTR loci revealed that the both strains were identical to the strains of *M. bovis*. While mycobacteria passed through the organism of insects, the microorganisms' genome underwent no changes that could be identified by VNTR-genotyping method (no changes occurred in the number of tandem repeats in variable loci which were researched).
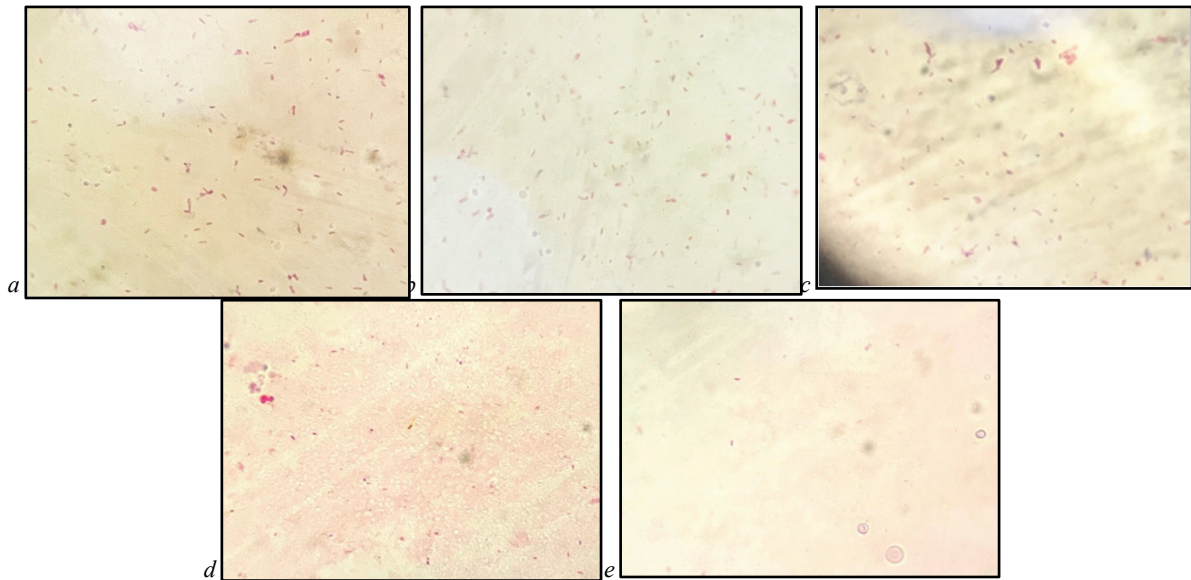
Fig. 1. Microscopy of smears from homogenate of the beetles: *a* – on day 4, *b* – day 8, *c* – day 12, *d* – day 20, *e* – day 30 since the *Sitophilus oryzae* weevils were infected by *Mycobacterium bovis*; the Ziehl-Neelsen staining
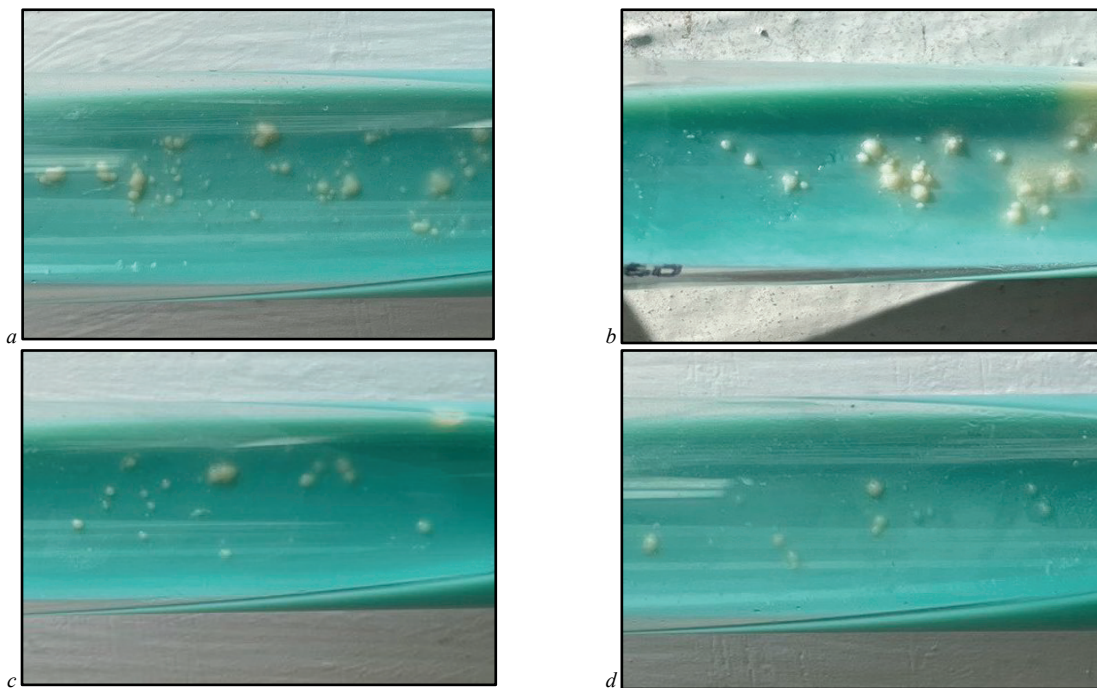

Fig. 2. Cultural properties of *Mycobacterium bovis* isolated on days 4–20 after infecting the *Sitophilus oryzae* insects: *a* – on day 4, *b* – on day 8, *c* – on day 12, *d* – on day 20 (the Ziehl-Neelsen staining)
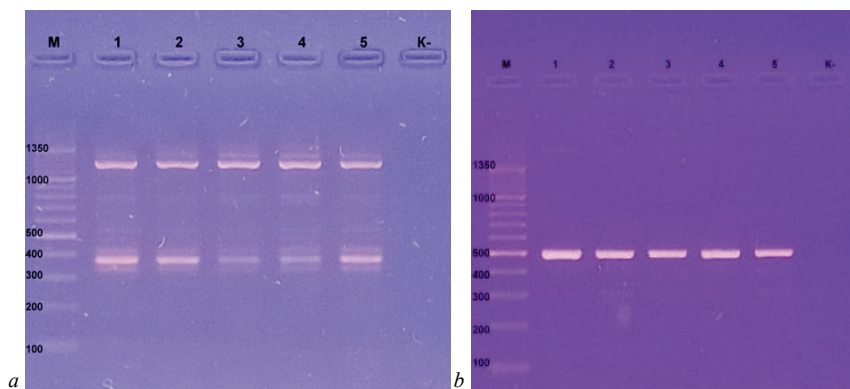

Fig. 3. Results of PCR studies of the cultures on days 4, 8, 12, 20, and 30 of the experiment (probes 1, 2, 3, 4, 5, respectively): *M* – marker of molecular weight, *K* – negative control (deionized water); *a* – identification of target DNA fragment for *M. tuberculosis* complex, measuring 375 base pairs in length, *b* – identification of target DNA fragment for *M. bovis*, measuring 500 base pairs in length
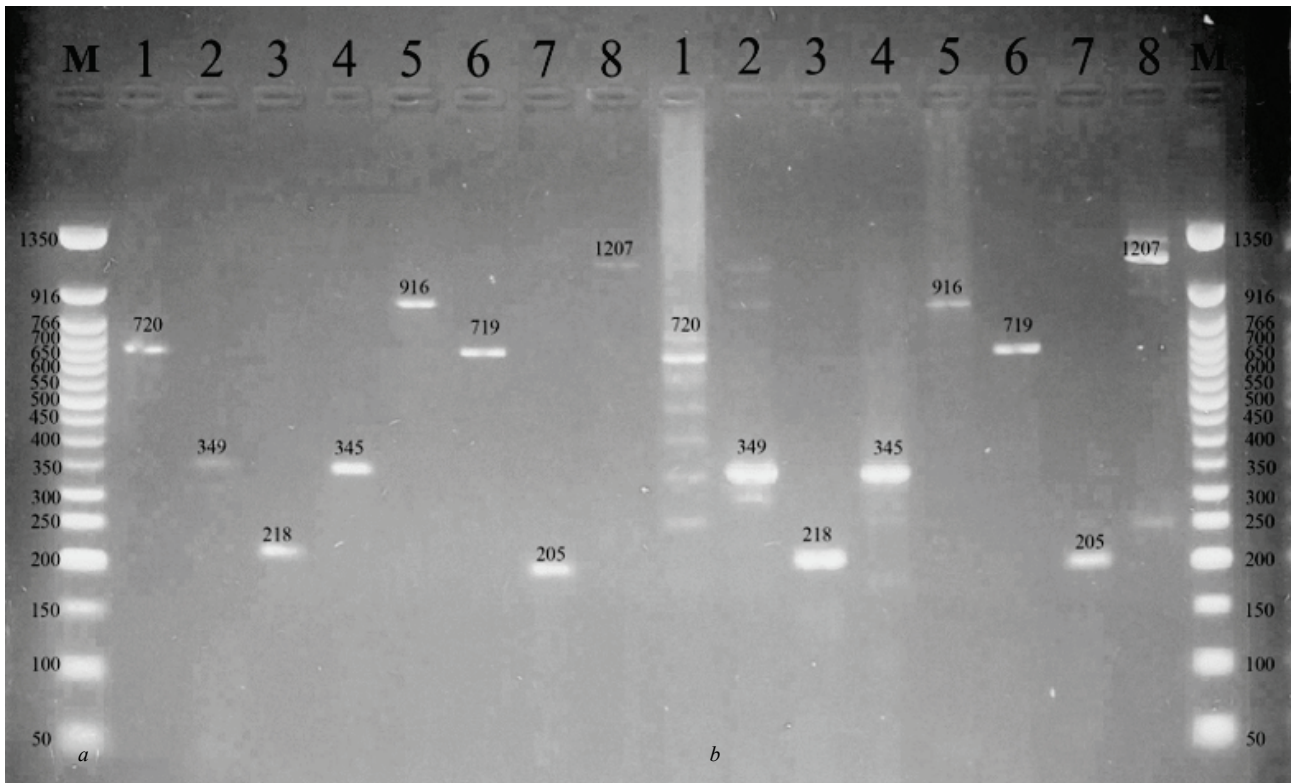
Fig. 4. Results of VNTR genotyping of the initial strain of *M. bovis* (*a*) and culture of *M. bovis* from homogenized beetles on day 30 of the experiment (*b*): *M* – marker of molecular weight, *1–8* amplicon obtained by PCR with primers aimed at VNTR loci ETR-A, ETR-B, ETR-C, ETR-D, ETR-E, QUB-11a, QUB-11b, QUB-3232

According to the results of the study, we identified VNTR profile of the *M. bovis* strain used in the study (Table 2). The data about the strain genotype were introduced into the MIRU-VNTRplus international base: Identification by Similarity Search (www.miru-vntrplus.org). Using on-line tools of the MIRU-VNTRplus base, we analyzed the genetic profile of the examined strain and identified its affinity to other strains of *M. bovis*, recorded in the base of genetic profiles (Fig. 5).

Biochemical assay. During the passage of mycobacteria through the body of the rice weevils, we isolated the following cultures: from grain suspension on the 4th, 8th, 12th, and 20th days of the experiment. In those cultures and the initial strain (that had no contact with the insects), we

identified enzymatic activities: catalase, peroxidase, dehydrogenase, nitrate reductase, and ability to hydrolyze Tween 80. Passage through the insects led to fluctuations in the biochemical activity of the pathogen: changes occurred in dehydrogenase and nitrate reductase activities of mycobacteria (Table 3). We observed no manifestation of activities of catalase and peroxidase in any of the experimental samples. In all the cultures, dehydrogenase activity was negative after 15–30 min after the start of reaction. However, during the monitoring, after 24 h we identified partial loss of colour of methylene blue in the cultures from homogenate of weevils on the 30th day after being infected and from the grain suspension on the 20th day after starting the experiment.

Table 2
VNTR profile of the *M. bovis* strain used in the study

Characteristic of PCR product	Names of the VNTR loci							
	ETR-A	ETR-B	ETR-C	ETR-D	ETR-E	QUB-11a	QUB-11b	QUB-3232
Size of repeat, bp	75	57	58	77	53	69	69	56
Fragment size, bp	195	121	44	114	492	167	67	423
Product of phoresis reaction, bp	720	349	218	345	916	719	205	1207
Number of repeats	7	4	3	3	8	8	2	14

Table 3
Biochemical activity of *M. bovis* in the dynamics of the experiment (n = 3)

Experimental culture of *M. bovis*	Catalase activity		Peroxidase activity	Dehydrogenase activity		Nitrate-reductase activity	Hydrolisis of Tween 80		
	15 min	30 min	2 h	15–30 min	24 h		4 h	5 days	10 days
Initial culture	–	–	–	–	–	–	–	–	–
Culture from grain on day 4 of the experiment	–	–	–	–	–	–	–	–	–
Culture from grain on day 8 of the experiment	–	–	–	–	–	–	–	–	–
Culture from grain on day 12 of the experiment	–	–	–	–	–	±	–	–	–
Culture from grain on day 20 of the experiment	–	–	–	–	±	±	–	–	–
Culture from homogenized beetles on day 30 of the experiment	–	–	–	–	±	±	–	–	–

Note: "+" – positive reaction, "–" – negative reaction, "±" – questionable reaction.

During the study of reduction of nitrates, we determined that the initial form and cultures from the grain suspension on the 4th and 8th days of the experiment exerted no activity. At the same time, cultures from the grain suspension on the 12th and 20th days and from homogenate of the

beetles on the 30th day of the experiment had low activity of nitrate reductase. Also, in the test tubes we saw emergence of light-yellow colour.

The ability of bacteria to hydrolise Tween 80 was negative in all the samples throughout the monitoring period.

Biological study. To identify the influence of rice weevil on pathogenic properties of mycobacteria to infect the laboratory animals (guinea pigs), we used the initial culture and the cultures obtained in the course of the experiment. For subcutaneous infection, the animals were divided into 6 experimental groups and one control (3 guinea pigs in each): group I – infected with a weighed amount of the initial culture, II – infected with the culture from grain on the 4th day of the experiment, II – on the 8th, IV – on the 12th, V – on the 20th, VI – culture isolated from homogenate of the beetles on the 30th day, VII – control (intact) group. Until the 28th day after being infected, the animals of all the groups were active and gained weight. From the 28-35th days, we began noting dynamic weight loss in the experimental groups, but the weight of guinea pigs of the control group increased until the end of the experiment.

Formation of ulcer in the place of inoculation of the weighed amount of bacteria was seen in groups I, II, III on the 20th day, and in groups III, IV, and VI on the 27th day (Fig. 6).

Studying the sensibitising properties, we determined that guinea pigs of all the experimental groups reacted to intradermal administration of PPD tuberculin for mammals on the 30th day after being infected, and animals of group V also reacted on the 60th day. Twenty four hours after administration of the allergen, we observed swelling and hyperemia, and after 48 days an ulcer formed (Fig. 7).
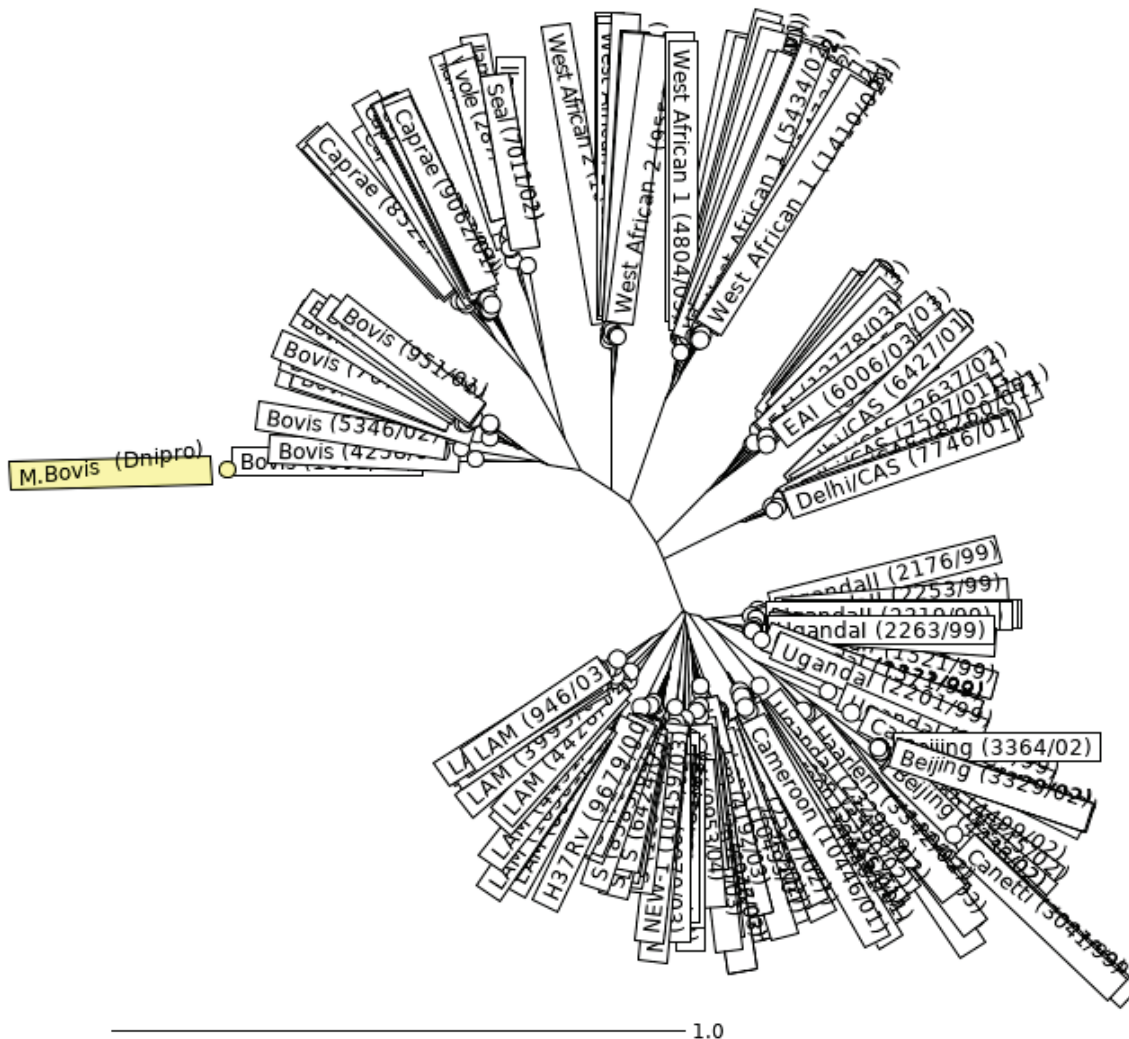

Fig. 5. Radial dendrogram based on the VNTR analysis of affinity of the examined strain of *M. bovis* to strains recorded in the MIRU-VNTRplus base

Fig. 6. Ulcer in the place of inoculation of weighed amount of the bacteria: *a* – formation, *b* – rupture

Fig. 7. Monitoring of tuberculosis skin test after administration of PPD tuberculin:
a – after 24 h, *b* – after 48 h after inoculation; *1* – swelling of the skin, *2* – formation of ulcer

Guinea pigs of group I died on the 49–55th days after infection, group II on the 53–55th days, group III on the 45–49th days, group IV on the 40–45th days, group V on the 62–68th days, and of group VI on the 53–64th days after being infected. Animals of the control group were euthanized on the 90th day.

Necrosis revealed significant exhaustion of animals of groups I–VI. In the guinea pigs, we saw distinctive changes in the internal organs and groin lymph nodes. The liver, lungs, and spleen were enlarged, contained numerous yellowish nodes with caseous content. The lymph nodes contained sites of necrosis, filled with pus. Animals of the control group had no pathoanatomical changes (Fig. 8).

When studying the pathological pathoanatomical changes according to the Trius's scheme, we determined the index of damage to the organism: group I – 19 ± 3, II – 20 ± 2, III – 23 ± 5, IV – 27 ± 1, V – 11 ± 3, VI – 15 ± 3, VII – 0 points.

During the studies of impression smears from the affected organs, the experimental mycobacteria were found in the lungs, liver, spleen, and lymph nodes of animals of the experimental groups. After inoculating the suspension on the growth medium, the initial growth was observed on the 14–26th days. We saw no growth of mycobacteria in the test tubes with inoculated suspensions from the organs of laboratory animals of the control group.
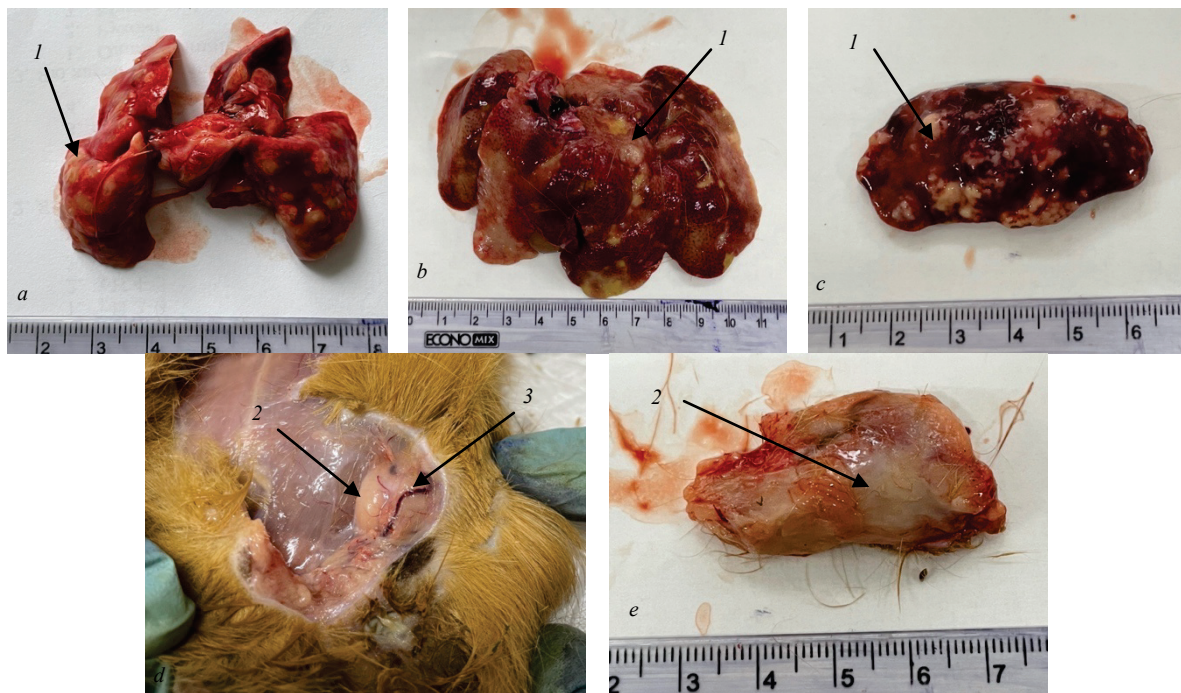

Fig. 8. Pathoanatomical changes in the experimental animals: *a* – lungs, *b* – liver, *c* – spleen, *d, e* – lymph nodes;
1 – tubercles with caseous content, *2* – enlarged lymph node, *3* – hyperemia of the vessels

Histological study. Fragments of the affected internal organs were fixated in 10% solution of formalin and studied histologically. In the lungs of the laboratory animals, we saw massive fused lymphoid-macrophage infiltrates, with presence of perivascular lymphoid infiltrates. Between the infiltrates, the lung tissue had areas of increased and significantly reduced airiness. We observed unevenly expressed intermediate edema, thickening of the interalveolar membranes with lymphoid-macrophage infiltration. In the massive infiltrates, there formed necrotic sites, with lymphoid-

macrophage infiltration around (Fig. 9a, 9b). We saw formation of granulomas around the rounded necrotic sites, with lymphoid-macrophage infiltration around, and presence of giant multinucleated cells (Fig. 9c, 9d).

Necrotization took place against the background of venitis with thrombosis and perivascular lymphoid-macrophage infiltration. We observed thrombosis of small veins, small arteries with presence of near-wall thrombi (Fig. 10a, 10b). In the spleen, we found fields of caseous necrosis, which was demarcated from the tissue of the spleen by macrophage infil-

trate with epithelioid cells and fibroblasts. In the spleen tissues, there were numerous granulomas around the sites of caseous necrosis, with lymphoid-macrophage infiltration, presence of giant multinucleated Pirogov-Langhans cells (Fig. 11a, 11b).
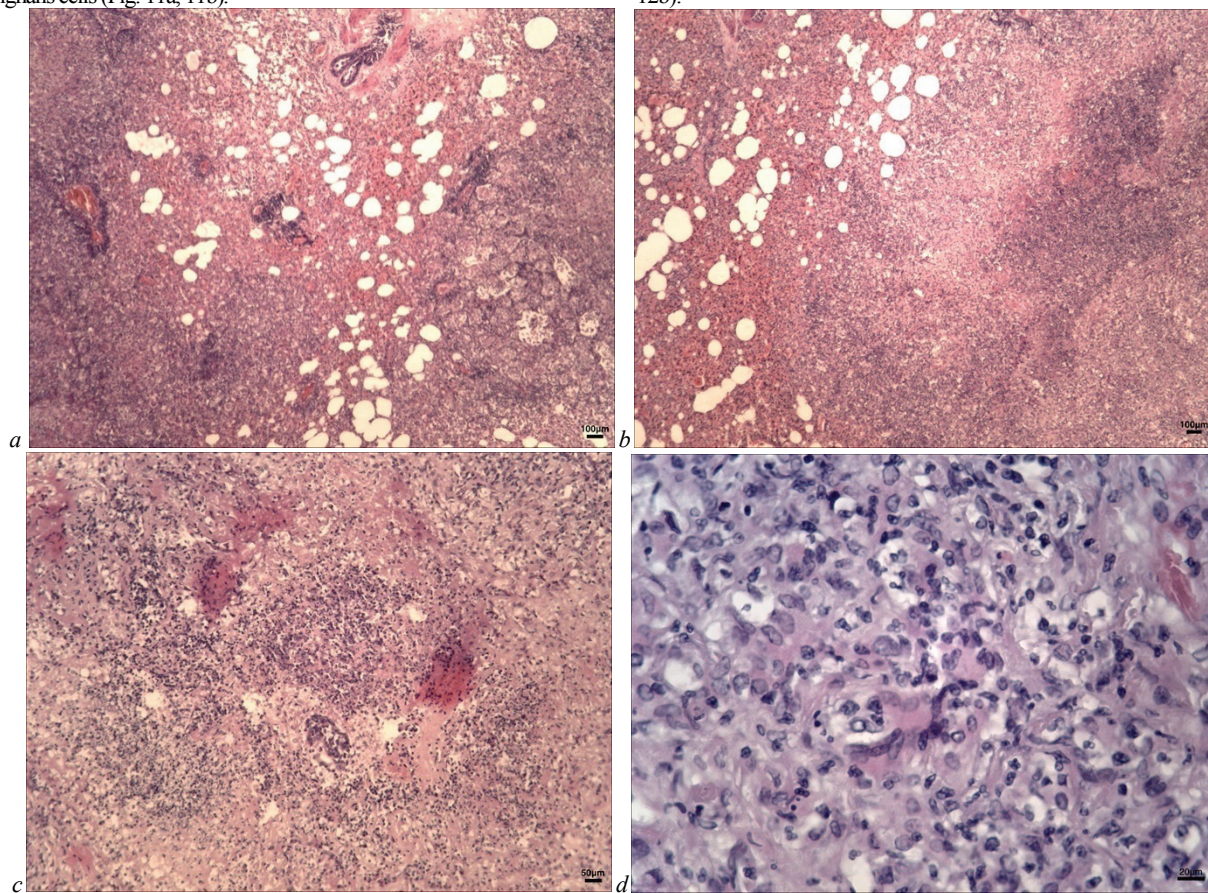
The tissue of the lymph node was completely necrotized. Thickened, infiltrated with lymphocytes and macrophages, capsule of lymph node demarcated necrosis from the surrounding soft tissue (Fig. 12a, 12b).

Fig. 9. Lungs: *a* – massive fused lymphoid-macrophage infiltrates, perivascular lymphoid infiltrates; *b* – diffuse-focal lymphoid infiltration, formation of sites of necrosis; *c* – diffuse-focal lymphoid infiltration, formation of granulomas around the rounded necrotic sites, with lymphoid-macrophage infiltration, presence of giant multi-nuclei cells; *d* – diffuse-focal infiltration, giant multi-nuclei cells in the lymphoid-macrophage infiltrate; hematoxylin and eosin staining

For alimentary infection, the laboratory guinea pigs were divided into four experimental groups and one control: I – guinea pigs received grain contaminated with a weighed amount of the initial culture, II – received grain on which the beetles were kept for days 4–8, III – grain infested with beetles during days 8–12 after being infected with mycobacteria, IV – grain infected with the beetles during days 12–20 of the experiment, V – control group, where the beetles were fed with only sterile grain. During the monitoring of the experimental guinea pigs, we saw weight gain up to days 49–56, after which the mammals gradually lost weight, though no

other clinical signs of tuberculosis were observed. During the allergic test, on the 30th day, all the studied groups tested negative, and on the 60th day we saw emergence of hyperemia and thickening of the skin fold in animals of groups I–IV; and on the 90th day guinea pigs of group IV tested positive. The control animals did not react to administration of the allergen (Fig. 13).

Guinea pigs of group I died on the 70–73rd days of the experiment, group II on the 74–82nd days, III on the 86–89th days, and IV and V were euthanized after 90 days.
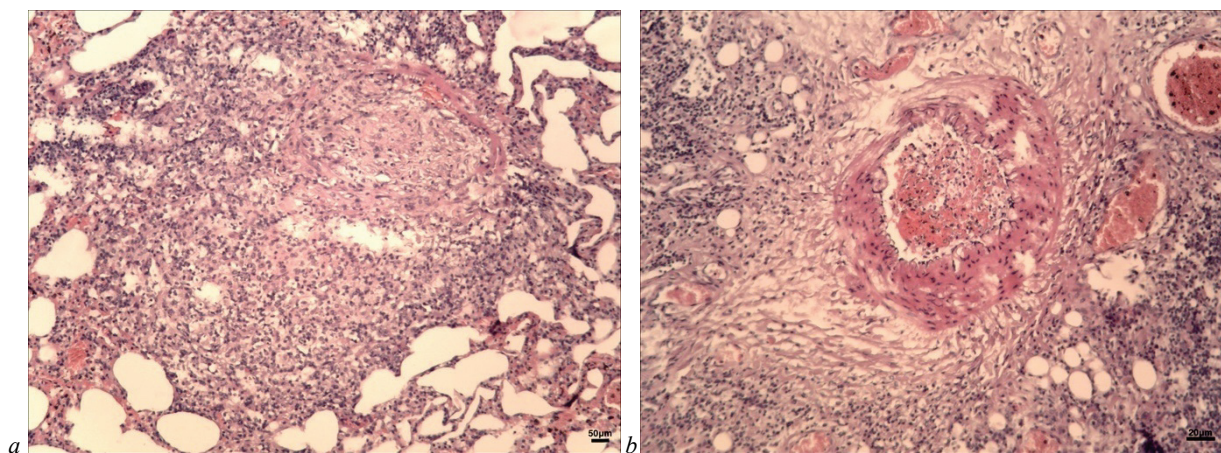

Fig. 10. Lungs stained with hematoxylin and eosin: *a* – vein thrombosis, perivascular lymphoid-macrophage infiltration; *b* – artery with near-wall thrombosis
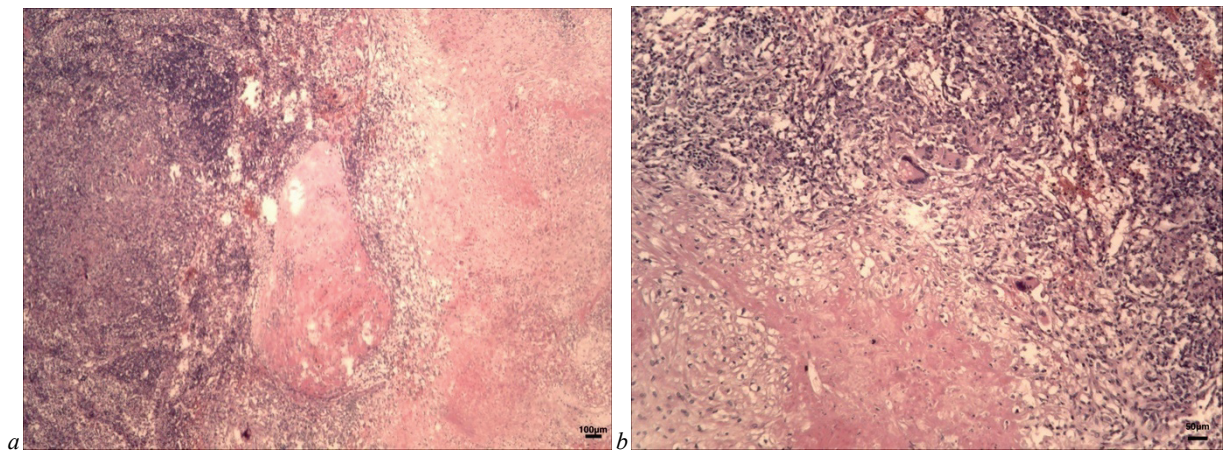
Fig. 11. Spleen: *a* – massive areas of casenous necrosis, *b* – formation of granuloma around sites of caseous necrosis, with lymphoid-macrophage infiltration, and presence of giant multinucleated Pirogov-Langhans cells; hematoxylin and eosin staining
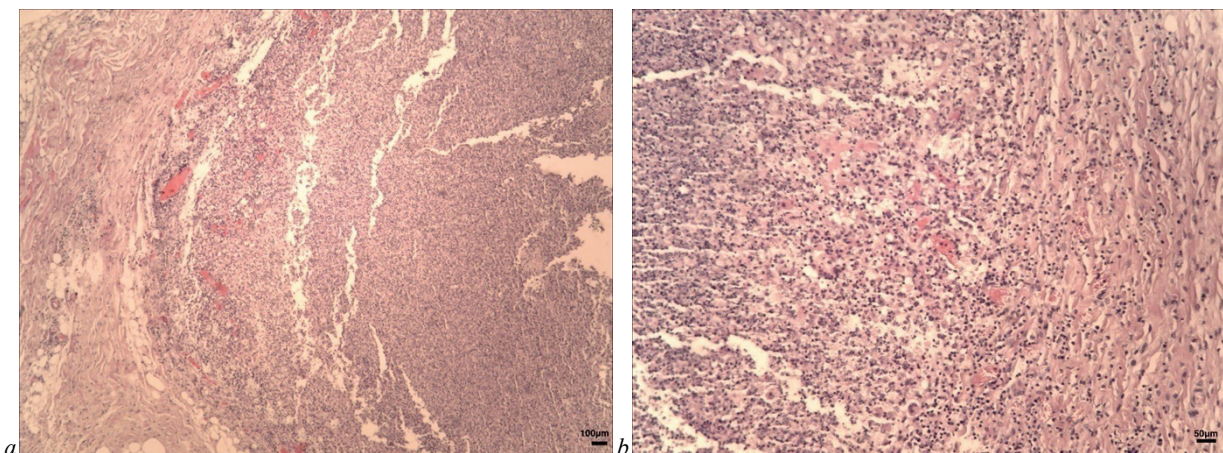

Fig. 12. Lymph node, stained with hematoxylin and eosin: *a* – massive necrosis of the lymph-node tissue, *b* – capsule of the lymph node is thickened and infiltrated by lymphocytes and macrophages

Fig. 13. Monitoring of tuberculosis skin test after administration of PPD tuberculin: *a* – after 24 h, *b* – after 48 h after administration of PPD tuberculin; *1* – skin swelling

Discussion

Studies by many authors have confirmed that insects are transmitters of numerous microorganisms (Zarei et al., 2018, Tkachenko et al., 2021a; Zazharskyi et al., 2022). Sarwar (2015) reported that up to 85% of the cases of infectious diseases have been caused by arthropods that are able to spread pathogens through stings and bites and mechanically via body surface. Our study with experimental infecting of rice weevil produced results that are consistent with the available data. Our results suggest that insects are able to pose a potential threat of tuberculosis and spread this infection across a large distance, releasing viable mycobacteria into the environment. In particular, Sitophilus oryzae, imagoes of which can live up to 6–12 months, can fly quite long distances to find places of grain storage, pasta products, fodders for animals, etc. Those biotopes attract rodents (mice, rats, etc), which can be infected with tuberculosis and spread the infection further. Our results add to the assumptions of Portaels et al. (2001) that animals can be infected by mycobacteria as a result of bites of pathogen-transmitting insects. Kazda (2000) stated that because of the acid-tolerant properties of the cellular wall, mycobacteria are not ruined by action of enzymes of the insects' intestinal tract and can be released into

the environment with insect feces, contaminating environmental objects. Combating the swift spread of tuberculosis is a challenge because of the pathogen's resilience and adaptability to altered surrounding conditions (Bañuls et al., 2015, Gotsulya et al., 2020). The structure of the cellular wall provides mycobacteria with protection from unfavourable living conditions. Moreover, protective functions of bacteria and their adaptation to negative ambient factors rely on the enzymatic-activity parameters.

We carried out molecular-genetic studies in order to control and identify whether the studied cultures were *M. bovis*, which expand the laboratory opportunities of research of passage of mycobacteria through the organism of insects and reduce the risk of wrongful results of microbiological studies. We identified the genotype of the *M. bovis* strain using VNTR analysis with set of primers flanking the loci associated with the most common forms of *M. bovis* variability (Hauer et al., 2016). The data regarding VNTR profile of the studied strain have been introduced to the international data base and are available for further analysis. We confirmed the genetic affinity of the studied strain to other strains isolated from cattle and strains in the MIRU-VNTRplus data base.

In our study, we also found variability of biochemical activity of *M. bovis*. After mycobacteria had travelled through the weevils' body, the activities of dehydrogenase and nitrogenase increased. Identification of the dehydrogenase activity is based on detection of redox enzyme dehydrogenase and metabolism products. Dehydrogenase is responsible for the protective function of bacterial cells and adaptation of bacteria to the conditions of a macroorganism. Also, dehydrogenase takes part in provision of the causative agent with antioxidant protection. Similar data were demonstrated by Tkachenko et al. (2021b), suggesting that the dehydrogenase activity increased after passage through a living organism. Describing BCG on the example of *M. bovis*, Fritz (2002) reported that an important factor for metabolism of bacteria is the nitrogenase activity that partakes in providing energy to the cell and is able to support metabolism of mycobacteria in the absence of oxygen. Numerous authors (Weber et al., 2000; Sohaskey, 2008; Hayashi et al., 2010) have revealed the aspects of the nitrogenase activity that is partially responsible for survival of mycobacteria in macroorganism cells.

Analysis of the results of the biological probe revealed that after subcutaneous infection with a weighed amount of pathogen, mycobacteria isolated during their passage through the beetle organism were characterized by virulence variability: the highest pathogenicity was exerted by the cultures isolated after being in the body of rice weevils for 12 days. The experimental animals died 17.3% faster, and the index of infection grew from 19 to 27, compared with the animals infected with the initial culture. However, after longer stay of mycobacteria in the beetles' organism, pathogenicity of the causative agent decreased.

At the same time, after alimentary infection of the guinea pigs by wheat grain on which the beetles had lived during the experiment, we saw decrease in the index of damage and increase in the life duration of the experimental animals with each following transfer onto new wheat grain. We attribute such a phenomenon to decrease in the number of mycobacteria in wheat after transfer of the weevils onto new grain. Gao et al. (2018) have demonstrated that after alimentary infection, pathoanatomic changes in the experimental animals were similar to those occurring after subcutaneous infection, though in our study we found no confirmation of this, and observed more expressed tropism of the pathogen to the liver, and the changes in the lungs were manifested poorly.

Having analyzed the data, we can state that the pests of barns , namely *Sitophilus oryzae*, pose a potential threat of spreading tuberculosis. They are an optimal environment for the existing mycobacteria because of the adaptive properties of the causative agent. Also, we saw variability of biochemical and pathogenic properties in the dynamics of passage of mycobacteria through the organism of the rice weevil.

Conclusion

The rice weevil is able to retain virulent mycobacteria (particularly *M. bovis*) in its body and release them, thereby contaminating environmental objects. After inoculating homogenate of infected beetles onto the growth medium and microscopy of the weevils, the examined bacteria were found up to the 30th day after the initial infection. Morphological signs and cultural properties were identical to the initial culture.

When studying the enzymatic activity, we saw that after being in the beetles' body, the cultures acquired the ability to reduce the nitrates. We also observed loss of colour of solution of methylene blue (dehydrogenase activity). This was related to the activation of mechanisms of adaptation of the mycobacteria in the altered environment. During the biological study, we determined that passage of the virulent strain of *M. bovis* through rice weevils was accompanied by fluctuations in pathogenicity of the causative agent. In the animals that had been subcutaneously infected by a weighed amount of the culture harvested after a 12-day period in the beetle body, the pathogen attained higher pathogenicity than the initial culture: the guinea pigs died 17.3% faster, the infection index increased from 19 to 27 points. Histological study revealed necrotization of the affected organs, formation of granulomas around the sites of caseous necrosis, with lymphoid-macrophage infiltration, and presence of giant multi-nuclei cells.

At the same time, after the alimentary infection, we saw increase in life duration of the animals with each following transfer of the beetles from one food substrate to another, which was associated with decrease in the number of microbial cells in the grain after the beetle transfer. Therefore, passage of mycobacteria through the rice weevil was accompanied by activation of enzymatic systems in order to increase survival of the causative agent and this caused changes in the virulent properties of the bacteria.

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