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

V. V. Zazharskyi*, K. V. Alifonova*, V. V. Brygadyrenko***, N. M. Zazharska*, V. P. Goncharenko***, V. V. Solomon****

* Dnipro State Agrarian and Economic University, Dnipro, Ukraine  
** Oleks Honchar Dnipro National University, Dnipro, Ukraine  
*** Bila Tserkva National Agrarian University, Bila Tserkva, Ukraine  
**** National University of Life and Environmental Sciences, Kyiv, Ukraine

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, which humans are also susceptible to. Tuberculosis inflicts multiple economic and social losses (Pérez-Morote et al., 2020). To effectively combat this zoonosis, the priorities are 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., 2022). However, there are no data about effect on biological properties of a virulent strain of mycobacteria: prevention of spread of the disease to favourable areas.

Keywords: mycobacteria; tuberculosis; histology; PCR; genotyping.
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 Principles 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 Dniprop 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 (pasage 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 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 beetles 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 4% 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 peroxidase activity by detecting changes in color of the colonies (‘+’ dark-brown color of colonies, ‘++’ brown, ‘+++’ pale-brown, ‘–’ color did not change).

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 rinsed 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 macroscopically 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 ampiclon 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 |
| M. tuberculosis complex | CTCGCGCGGTGCTTCACAGGA | 375 | 63 |
| M. bovis | TGTGCCTGGATGCAACATGC | 500 | 55 |
| ETR-A / 2165 | AAATACGTGCTCAACTTCTTAT | 195 + 75 | 52 |
| ETR-B / 2461 | CGGAACCGGCTGGAAGCTATG | 121 + 57 | 58 |
| ETR-C / 577 | GTGATGCTGCTGACAACTGCGG | 44 + 58 | 60 |
| ETR-D / 350 | GCCAAACGCTCAACAGGAACTAG | 114 + 77 | 55 |
| ETR-E / 3912 | ACTGATTGGCTTCATACGGCTTTA | 492 + 53 | 52 |
| QUB-11a / 2163a | TCTAAGGGGATTGCCAAG | 167 + 69 | 58 |
| QUB-11b / 2163b | CAGAATGGTAAACGGGACAT | 67 + 69 | 49 |
| QUB-3232 / 3232 | CAGGCGCCGTCATCAAC | 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 hydrolyze Tween 80. Catalse and peroxidase activities were identified using the modified Bogen’s technique (Tkachenko et al., 2019). Into test tubes with the culture, we added 1% 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 peroxidase activity by detecting changes in color of the colonies (‘+’ dark-brown color of colonies, ‘++’ brown, ‘+++’ pale-brown, ‘–’ color 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 over laid 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 cloud 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) – 1000.0 cm³, Tween 80 – 0.5 cm³, and the main red 0.1% – 2.0 cm³) 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 alimentary tract, 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 tuberculous.

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 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. The culture of the initial strain and culture from homogenate of 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.
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 online 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

<table>
<thead>
<tr>
<th>Characteristic of PCR product</th>
<th>Names of the VNTR loci</th>
<th>ETR-A</th>
<th>ETR-B</th>
<th>ETR-C</th>
<th>ETR-D</th>
<th>ETR-E</th>
<th>QUB-11a</th>
<th>QUB-11b</th>
<th>QUB-3232</th>
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</thead>
<tbody>
<tr>
<td>Size of repeat, bp</td>
<td>ETR-A</td>
<td>75</td>
<td>57</td>
<td>58</td>
<td>53</td>
<td>69</td>
<td>69</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Fragment size, bp</td>
<td>ETR-B</td>
<td>195</td>
<td>121</td>
<td>44</td>
<td>114</td>
<td>492</td>
<td>167</td>
<td>67</td>
<td>423</td>
</tr>
<tr>
<td>Product of phoresis reaction, bp</td>
<td>ETR-C</td>
<td>729</td>
<td>349</td>
<td>218</td>
<td>345</td>
<td>916</td>
<td>719</td>
<td>205</td>
<td>1207</td>
</tr>
<tr>
<td>Number of repeats</td>
<td>ETR-D</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>14</td>
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</table>

Table 3

<table>
<thead>
<tr>
<th>Experimental culture of M. bovis</th>
<th>Catalase activity</th>
<th>Peroxidase activity</th>
<th>Dehydrogenase activity</th>
<th>Nitrate reductase activity</th>
<th>Hydrolysis of Tween 80</th>
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<tr>
<td></td>
<td>15 min</td>
<td>30 min</td>
<td>2 h</td>
<td>15–30 min</td>
<td>24 h</td>
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<td>Initial culture</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Culture from grain on day 4 of the experiment</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Culture from grain on day 8 of the experiment</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Culture from grain on day 12 of the experiment</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>±</td>
<td>–</td>
</tr>
<tr>
<td>Culture from grain on day 20 of the experiment</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>±</td>
<td>–</td>
</tr>
<tr>
<td>Culture from homogenized beetles on day 30 of the experiment</td>
<td>–</td>
<td>–</td>
<td>±</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

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 hydrolyse 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, III – 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 sensitising 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).

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

Fig. 6. Ulcer in the place of inoculation of weighed amount of the bacteria: *a* – formation, *b* – rupture
Guinea pigs of group I died on the 49–55th days after infection, group II on the 43–49th days, group III on the 45–47th days, group IV on the 40–45th days, group V on the 53–59th 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.

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).

Necrosis 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 infiltr-
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).

![Image](image_url)

**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 infected 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.

![Image](image_url)

**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 caseous 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; l – skin swelling

Discussion

Studies by many authors have confirmed that insects are transmitters of numerous microorganisms (Zarei et al., 2018, Tkachenko et al., 2021a; Zazhurskyi 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 (Bahafii 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 reox enzymes 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 dehydroge

The experimental animals died 17.3% faster, and the index of infection 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.

The authors declare no conflict of interest.

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 achieved 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.

The authors declare no conflict of interest.

References


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