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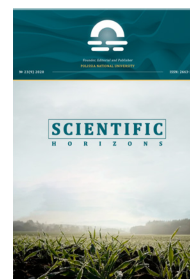

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Epizootological Significance of Rice Weevil as a *Mycobacterium Bovis* Reservoir

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Abstract. This article presents materials on the role of the rice weevil (lat. *Sitophilus oryzae*) in the process of transmission of Mycobacterium of tuberculosis. The relevance of the study is conditioned by the rapid spread of tuberculosis around the world and the need to develop more advanced methods for diagnosing this disease, which, for its part, is impossible without expanding knowledge on all possible reservoirs of the causative agent of infection. Long-term research around the world has proven that insects are carriers of various microflora, including pathogenic ones. Therefore the purpose of study was to establish the epizootological role of the rice weevil as a reservoir for *Mycobacterium bovis* in the process of occurrence and spread of tuberculosis. The study determined the duration of transfer and isolation of Mycobacterium tuberculosis in the external environment by infected beetles. The museum dissociative strain of *Mycobacterium bovis* (118 passage) was used for the study, the subject of the study was rice weevils. For the furtherance of this goal, the culture, microscopic, and statistical studies were conducted, and the viability of mycobacteria was determined after passages through the beetle body by evaluating colony-forming units. The epizootological role of the rice weevil in the development and spread of tuberculosis has been established. It is proved that the beetle can keep mycobacteria in its body for 50 days with a gradual decrease in their number and is then able to secrete the pathogen for another 30 days, contaminating environmental objects. When evaluating colony-forming units of mycobacteria in the dynamics of the experiment, a decrease in their viability after persistence through the body of beetles was revealed from $5.3*10^8$ in 1 g of the original crop up to $1.4*10^8$ in 1 g of crop that grew from beetle homogenisate for 30 days. These study results will allow developing and improving existing measures for the prevention of tuberculosis and prevent the introduction of this infection into safe territory

Keywords: mycobacteria, dissociative strain, infection, beetles-pests of grain stocks, colony-forming units

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INTRODUCTION

Tuberculosis is one of the most common problems among zooanthroponic diseases, and the issue of improving prevention measures is relevant all over the world, including in Ukraine. This disease poses a high epizootological danger and causes great social and economic damage, which include control measures in the affected area, mass destruction and disposal of animals, health and preventive measures [1]. The ability of mycobacteria to adapt to the environment, their variability and existence in various morphological forms complicate the diagnosis of tuberculosis. Moreover, animal and human organisms can carry the causative agent of infection latently, without showing any clinical signs. In particular, *Mycobacterium bovis* has an extremely large range of susceptible animals, which makes it difficult to eliminate tuberculosis.

Despite many years of fruitful work of researchers around the world, there is still no exhaustive data on all possible ways of introducing the pathogen to the farm. And to completely eradicate the disease, the first priority should be to expand and deepen knowledge on the prevention and elimination of tuberculosis. Special attention, according to the authors of the study, deserves the establishment of all reservoirs of mycobacteria, which are a potential threat of outbreaks of infection in previously safe territories.

Numerous studies from researchers around the world indicate that insects are a reservoir of many types of microorganisms. Foreign researchers managed to isolate mycobacteria from cockroaches, beetles, butterflies, ticks, mosquitoes, and other invertebrates [2-4]. According to many researchers, insects are carriers of potentially pathogenic and pathogenic microorganisms, including the causative agent of tuberculosis. In particular, *Mycobacterium intracellulare, Mycobacterium avium ssp., Mycobacterium fortuitum, Esherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella spp., Shigella spp., Pseudomonas aeruginosa* and other types of bacteria were isolated [5-8]. For their part, Fischer et al. have conducted an experimental infection with a suspension of mycobacteria of cockroach nymphs and found that they are able to accumulate and secrete the pathogen into the external environment. Researchers isolated mycobacteria in the excrement of cockroaches after 3 days, and in the homogenisation of infected insects – 10 days after infection [9].

Therefore, existing data indicate the ability of insects to accumulate and secrete mycobacteria into the external environment, that is, to be a potential cause of the active spread of the disease. However, to date, there are no exhaustive data on the duration of the carrier of microorganisms, in particular Mycobacterium of tuberculosis, in the body of insects. And the complete elimination of the disease without perfect knowledge of potential reservoirs and carriers of the causative agent of the disease is impossible.

The objects of research in this study were beetles-pests of grain stocks. In Ukraine, the most common representatives of this group are weevils of the genus *Sitophilus*, namely, the rice weevil. Numerous foreign research papers indicate that beetles of this genus are end carriers of gamma-proteobacteria of the species *Sodalis pierantonos*. These microorganisms are involved in the formation of the beetle's cuticle. Morphologically *S. pierantonos* is a gram-negative rod with a length of 3-4 to 100 μm, a diameter of 1-2 μm, and a microbial cell surrounded by mucopolysaccharide substances. *S. pierantonos* – an intracellular microorganism that is cultivated exclusively in the body of weevils and is not cultivated on a nutrient medium. The number of these bacteria in the beetle's body varies throughout its life cycle: in the larval stages there are many of them, and in adult insects it decreases sharply or disappears altogether after the final cuticle formation [10-12].

The purpose of the study is to investigate the epizootological significance of the rice weevil *Sitophilus oryzae* as a reservoir for *Mycobacterium bovis* in the process of occurrence and spread of tuberculosis.

Research objectives: to establish the duration of the transmission and secretion of *Mycobacterium bovis* into the external environment with infected beetles.

MATERIALS AND METHODS

The research was conducted in the training laboratory of the Department of epizootology and Infectious Diseases of animals of the Dnipro state agrarian and economic University during 2020. The subject of the study were cultures of the dissociative form of *Mycobacterium bovis* (passage 118, microscopy revealed acid-resistant red grains), which were stored in the museum of the department and beetles-pests of grain stocks, in particular the rice weevil (lat. *Sitophilus oryzae*), from the family *Curculionidae* (Fig. 1).

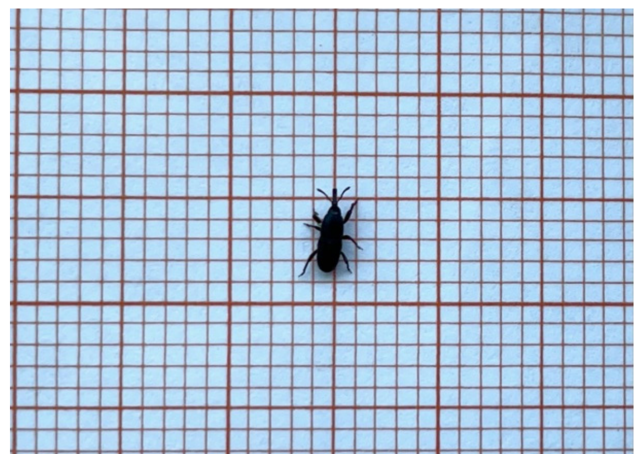


Figure 1. *Rice weevil (lat. Sitophilus oryzae)*

Prior to the start of experimental infection, to exclude the existence of *Mycobacterium bovis* in beetles,

insects were examined by microscopy for the presence of acid-resistant rods. For this purpose, the beetles were washed three times in an isotonic 0.9% sodium chloride solution and smeared on a slide, followed by staining smears according to the Ziehl-Neelsen method.

For the study, an experimental group of beetles was created, which included 50 rice weevils. Experimental beetles were kept in grain treated with a suspension prepared from the culture *Mycobacterium bovis* with subsequent repeated transplantation into sterile grain during the experiment.

For experimental infection of beetles, pre-autoclaved wheat grains were contaminated for 20 minutes at 1.5 atm. For grain contamination with mycobacteria, a suspension from a dissociative strain of *Mycobacterium bovis* (118 passage) was prepared in the proportion of 10 mg of bacterial mass of *Mycobacterium bovis* in 1 cm^3 isotonic 0.9% sodium chloride solution for every 10 g of grain, a total of 20 g of grain and, accordingly, 20 mg of crop per 2 cm^3 solution were used. Adult beetles (50 pieces) were placed in grain contaminated with *M. bovis* for 3 days, on the 4th day the beetles were moved to sterile grain for another 4 days, after which they were again transferred to sterile wheat grain until the 12th day of the experiment, the next transplantation was done on the 20th day of the experiment. Further on, the beetles were moved every 10 days until the experiment was completed to determine the duration of the transfer.

During this time, on days 4, 8, 12, 20 and then every 10 days (up to 90 days inclusive), a microscopic examination of the suspension from grain was carried out after the weevils stayed in it and the beetles themselves were examined directly for the content of mycobacteria. For this purpose, beetles were selected from the experimental group, washed with an isotonic 0.9% sodium chloride solution 3-5 times, depending on the previous results, each time examining the washing samples under an immersion microscope system. After washing, the beetles were smeared on a slide, and the homogenisate was stained according to the Ziehl-Neelsen method.

In addition, on the 30th, 60th, and 90th days of the experiment, beetles pre-washed with sodium chloride solution were pulverised, a suspension was prepared, which was sown on a Mordovsky nutrient medium ("Novaya"). After transplanting the beetles, samples of grain were taken for the study, in which the weevils stayed for a certain time (4-10 days, according to the method). It was added to a mortar with a sterile isotonic 0.9% sodium chloride solution and kept for 30 minutes in a sterile box, after which it was ground. A smear was made from the resulting suspension, stained according to Ziehl-Neelsen method and sown on a Mordovsky nutrient medium ("Novaya"). Grain and beetles that remained after the experiment were decontaminated by autoclaving at 1.5 atm for 3 hours.

The viability of microbial cells was also determined by evaluating colony-forming units (CFU) by serial dilutions [13-15]. For the study, *Eppendorf* microtubes were used, a sterile isotonic 0.9% sodium chloride solution in an amount of 0.5 cm^3 was added in the first test tube and 0.4 cm^3 in the next tubes. The bacterial mass was taken from a test tube using a wire inoculating loop, squeezed between sheets of filter paper, and weighed 50 mg (0.05 g) using a torsion scale. The weighted bacterial mass was placed in micro-tube No. 1 and suspended, thoroughly mixing with an inoculation loop. 0.1 cm^3 of suspension was taken using an insulin syringe, the resulting suspension was transferred to test tube No. 2, thoroughly mixed, and 0.1 cm^3 was taken again and transferred in the next test tube up to the 10th iteration. After that 1 cm^3 of suspension was taken from each microtube using separate insulin syringes, which was introduced into two test tubes with Mordovsky nutrient medium and evenly distributed over the surface. Further on, the test tubes were placed in a thermostat at a temperature of +37 ^{o}C

When determining the viability of microbial cells of the initial culture by evaluating CFU, calculations were made in the 8th dilution in the study of the initial culture and in the 7th dilution in the study of the culture that grew from beetle homogenisate on the 30th day of the experiment. These breeding grounds were chosen because the smaller one showed continuous growth, and the larger one showed no colony growth. After the appearance of colony growth, the number of viable microorganisms in 1 g of the experimental culture was mathematically calculated. Statistical analysis of the findings was performed in the Microsoft Office Excel software application.

RESULTS AND DISCUSSION

Microscopic examination of washing samples and homogenisate from beetles before experimental infection revealed non-acid-resistant cocci (Fig. 2). This means that the beetles were carriers of non-acid-resistant microorganisms before the study began. After conducting an experimental infection of beetles, microscopic examination of the latter revealed blue and red grains, which indicates successful infection of insects with the experimental strain *M. bovis*.

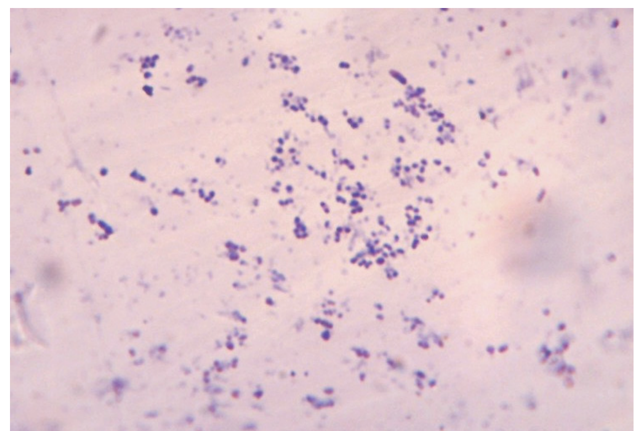


***Figure 2**. Microorganisms from beetle homogenisate before experimental infection*
***Notes**: Ziehl-Neelsen colour scheme*

Washing samples. According to the study results, it was found that during smear microscopy on Days 4 and 8, acid-resistant (red) and non-acid-resistant (blue) cocci were detected up to and including the fourth washing of beetles. On days 12, 20, 30, and 40 up to and including the third wash, microorganisms were detected by the microscopy, and on days 50 and 60 up to the second wash. On day 70, bacteria were detected only in the washing samples after the first rinse. By day 50, acid-resistant and non-acid-resistant cocci were detected, with a gradual decrease in the number of microorganisms, and by day 70, only non-acid-resistant cocci were detected. On days 80 and 90, micro-organisms were absent from the microscope view during the analysis of washing samples (Fig. 3).

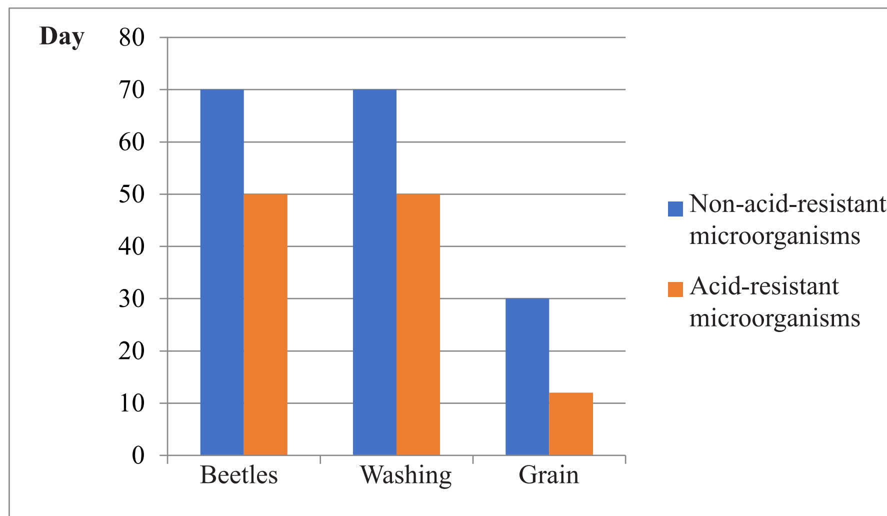


Figure 3. *Duration of isolation of microorganisms from the experimental material (days)*

Bugs. During microscopy of smears of infected beetles, red grains were found in their body up to the 50th day. That is, acid-resistant microorganisms that were contaminated with grain at the beginning of the experiment and blue cocci, non-acid-resistant microorganisms that the beetles were carriers of before infection, also with a gradual decrease in the number of microorganisms, starting from the 20th day of the study (Fig. 4). After the 50th day of the experiment, no bacteria identical to the dissociate Mycobacterium culture that was studied were found in the experimental samples, but by the 70th day, non-acid-resistant cocci were still detected (Fig. 5). On days 80 and 90, no bacteria were present during the microscopic examination.

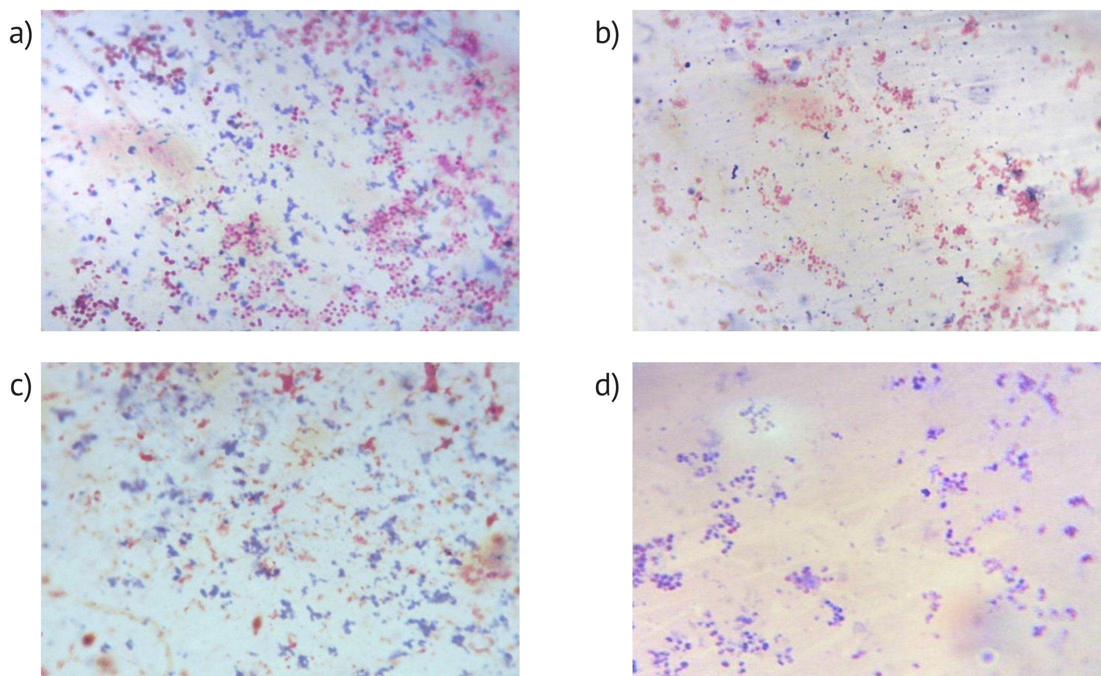


Figure 4. *Microorganisms from beetle homogenisate by day: a) – on the 4th day of the experiment, b) – on the 20th day of the experiment, c) – on the 40th day of the experiment, d) – on the 50th day of the experiment*
Notes: *Ziehl-Neelsen colour scheme*

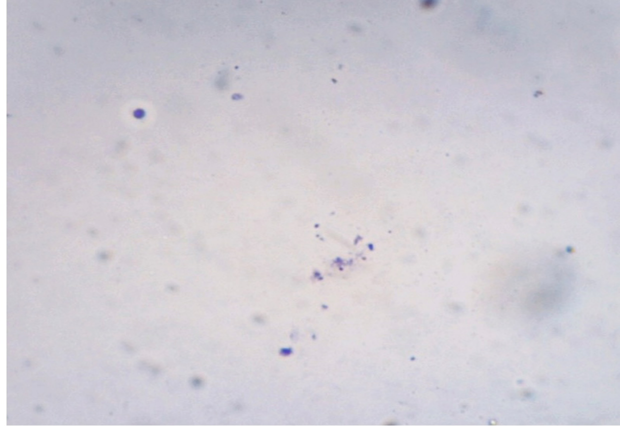


Figure 5. *Microorganisms from beetle homogenisate on the 70th day of the experiment*
Notes: *Ziehl-Neelsen colour scheme*

When sowing homogenisate from beetles on the Mordovsky nutrient medium:

– on day 30 of the experiment: on the nutrient medium (on day 12 after sowing), the growth of single rounded smooth orange colonies with smooth edges was recorded (Fig. 6), identical to the original culture (red grains under microscopy (Fig. 7a)) and the growth (on day 8) of light yellowish rounded S-shaped colonies (blue cocci under microscopy (Fig. 7b));

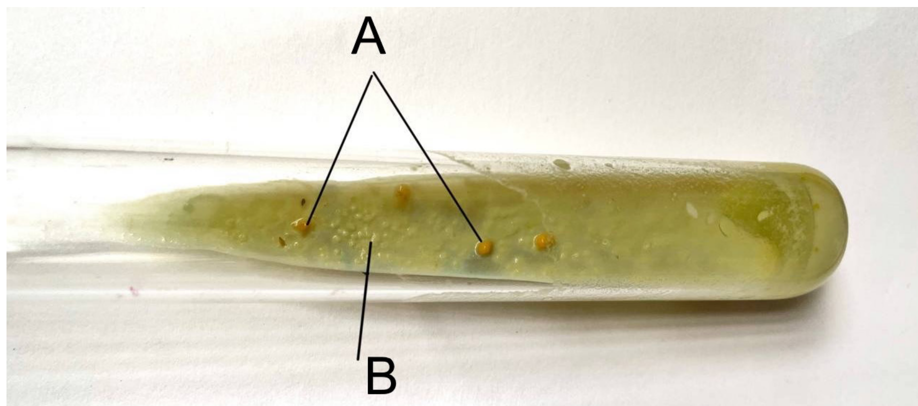


Figure 6. *Colonies of microorganisms from beetle homogenisate on the 30th day of the experiment: A – rounded orange colonies (acid-resistant bacteria); B – yellow rounded colonies (non-acid-resistant bacteria)*

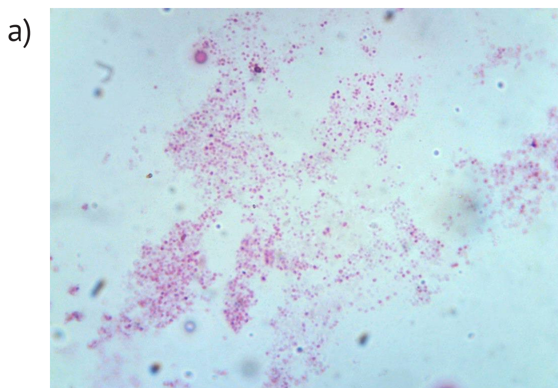
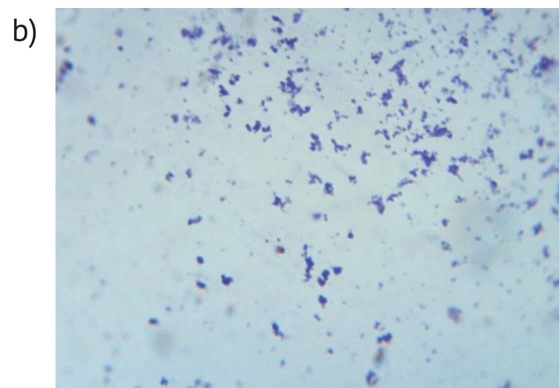
a)

b)


Figure 7. *Microorganisms from colonies from beetle homogenisate on the 30th day of the experiment from the nutrient medium: a) – from orange colonies; b) – from yellow colonies*
Notes: *Ziehl-Neelsen colour scheme*

– on day 60 of the experiment: the growth of light yellowish rounded S-shaped colonies was recorded on the nutrient medium (Fig. 8) on the 13th day of the experiment (blue cocci under microscopy (Fig. 9));

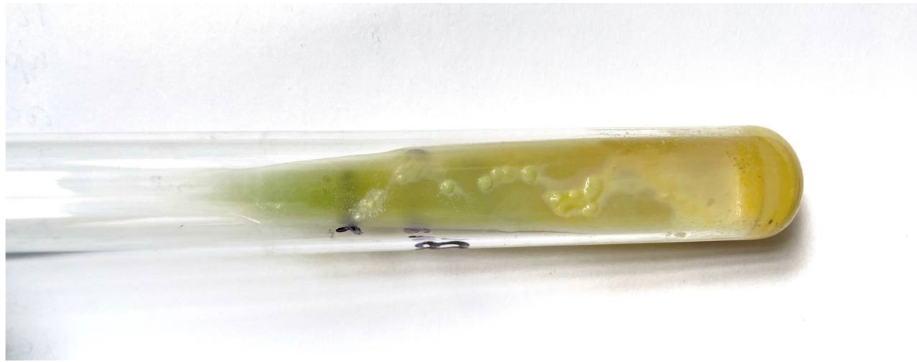


Figure 8. *Colonies of microorganisms from beetle homogenisate on the 60th day of the experiment*

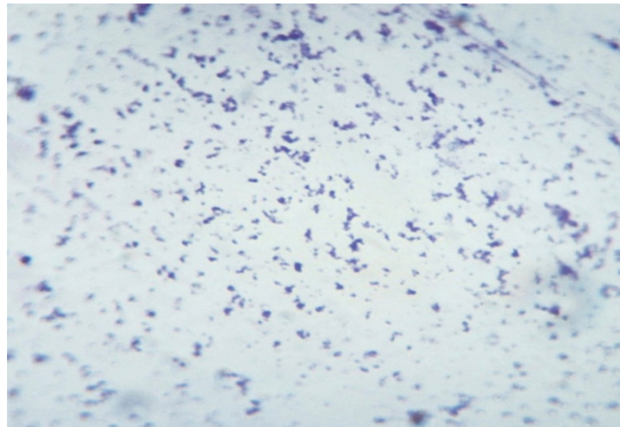


Figure 9. *Microorganisms from beetle homogenisate on the 60th day of the experiment from the nutrient medium*

Notes: *Ziehl-Neelsen colour scheme*

– on day 90 of the experiment: on the medium, the growth of light yellowish rounded S-shaped colonies (Fig. 10) on the 22nd day of the experiment (blue cocci under microscopy) (Fig. 11).

Figure 10. *Colonies of microorganisms from beetle homogenisate on the 90th day of the experiment*

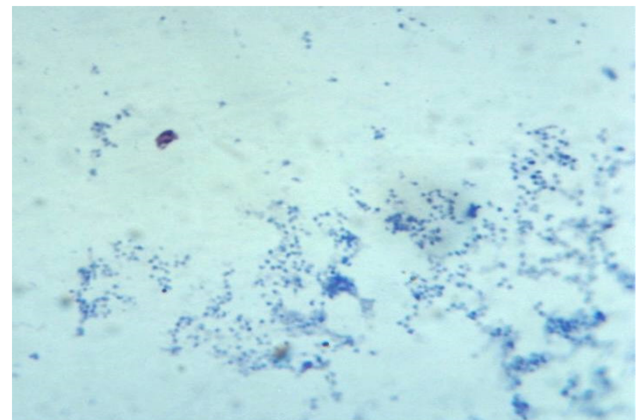


Figure 11. *Microorganisms from colonies from beetle homogenisate on the 90th day of the experiment from the nutrient medium*

Notes: *Ziehl-Neelsen colour scheme*

Thus, the findings indicate the ability of beetles to accumulate microorganisms in their body for quite a long time and be a reservoir of the causative agent of infection.

Grain. Microscopic examination of the grain on which the experimental beetles were kept revealed acid-resistant (red) grains on the 4th day of the experiment, that is, after one beetle transplantation. On days 8 and 12, i.e., after the second and third beetle transplantations, respectively, microscopy of grains revealed acid-resistant (red) and non-acid-resistant (blue) cocci. Further on, no bacteria were found in the grain that would be

identical to the Mycobacterium culture that was studied. However, during microscopic examination of the grain on the 20th and 30th days of the experiment, non-acid-resistant cocci were found. Microscopy on days 40, 50, 60, 70, 80, and 90, i.e., from the sixth to the eleventh beetle transplantation, no microorganisms were detected by the microscope. Consequently, non-acid-resistant microorganisms were detected by the 12th day of the experiment (i.e., by the third beetle transplantation), and acid-resistant microorganisms were detected by the 30th day (i.e., by the fifth transplantation).

When sowing a suspension of grain on a Mordovsky nutrient medium on day 4, that is, after one beetle transplantation, the growth of orange rounded colonies with smooth edges was detected, which have the appearance of red grains (identical to the original culture) on day 12. When sowing a suspension from grain on the 8th, 12th, 20th and 30th days of the experiment (i.e., after the second or fifth beetle transplantation), the growth of light yellowish rounded S-shaped colonies (blue grains under microscopy) and orange rounded colonies (identical to the original culture) was detected on the nutrient medium. When the suspension was sown on days 40 and 50 (i.e., after the sixth or seventh beetle transplantation), only light yellowish S-shaped colonies (non-acid-resistant cocci under microscopy) were found to grow, and no growth of dissociative mycobacteria identical to the experimental cultures was detected. No growth was detected on days 60-90.

The presence of microorganisms in the grain that was sterile before the beetles were transplanted suggests that insects play the role of a mechanical and probably biological carrier of the pathogen. Given that the duration of isolation of blue and red grains was not the same, it can be assumed that the period of isolation of non-acid-resistant bacteria was longer than the isolation of acid-resistant microorganisms due to the fact that initially the density of non-acid-resistant bacteria was higher.

It was found that the number of colonies that grew during the eighth dilution of the initial culture was 317 in test tube No. 1 (sample No. 1) and 359 in test tube No. 2 (sample No. 2) (Table 1). According to the CFU assessment methodology, the average value was determined for further calculations, it is equal to 338 (colonies in 0.00064 mg of culture (eighth dilution)), respectively, in 1 g of the original culture 528125000 or $5.3*10^8$ viable microorganisms. And when determining the CFU of the culture that grew after sowing homogenisate from beetles on the 30th day of the experiment, it was found that during the seventh dilution, 419 colonies grew in test tube No. 1 (sample No. 1) and 465 in test tube No. 2 (sample No. 2), the average value was 442 colonies in 0.0032 mg of culture (seventh dilution), respectively, in 1 g of culture 138125000 or $1.4*10^8$ viable microorganisms.

***Table 1**. Assessment of colony-forming units of dissociative form of Mycobacterium bovis (118 passage) in the dynamics of experiment*

	Source culture	**Culture of homogenised experimental beetles on the 30th day of the experiment**
Sample No. 1	317*	419**
Sample No. 2	359*	465**
M±m	338±29.7	442±32.5
Number of CFU in 1g of culture	$5.3*10^8$	$1.4*10^8$

***Notes**: * The number of grown colonies on the nutrient medium for the eighth dilution (0.00064 mg of culture); ** the number of grown colonies on the nutrient medium for the seventh dilution (0.0032 mg of culture)*

When determining the CFU, it was found that the number of viable microorganisms in 1 g of the initial culture was higher than in 1 g of the culture isolated from beetles on the 30th day of the experiment, which may indicate a decrease in the viability of microbial cells after mycobacteria pass through the beetle body. A two-sample t-criterion with different variances was used to assess the reliability of the ratio of the initial culture to the culture obtained from homogenised beetles. The value of p=0.04, which indicates high (96%) statistical reliability.

The problem of the widespread and rapid spread of tuberculosis still requires deepening existing knowledge. Thus, science does not have perfect data on all possible reservoirs of Mycobacterium tuberculosis. After analysing the study results, it was found that these data complement the existing findings of other researchers. The study results confirm that beetles, in particular rice weevils, can be a reservoir of mycobacteria and participate in the active spread of tuberculosis and transmission of the causative agent of infection to susceptible animals. Perhaps it is insects that are one of the main causes of tuberculosis in previously safe places.

After experimental infection of beetles with a dissociative strain *M. bovis*, they were kept on pre-sterilised wheat grain, periodically transplanting the insects into a new grain. Microscopic examination of the grain suspension, after transplanting the experimental beetles, revealed acid-resistant bacteria (identical to those that infected the beetles) for up to 12 days, that is, for three transplantations. Culture studies of grain suspensions revealed the growth of rounded orange colonies with

smooth edges (identical to the original culture) on the nutrient medium for up to 30 days, during which time five beetle transplantations were made into a new sterile grain. These data indicate that the beetles isolated mycobacteria from their bodies during this time and contaminated the grain. The results obtained do not contradict the research by O.A. Fischer et al., which found that microorganisms, in particular mycobacteria, are able to remain viable in the intestines of insects for a certain time and release bacteria into the external environment [5]. Similar data can be traced in other researchers, for example, B.W. Allen and J. Guzman & A. Vilcinskas reported that after passing through the intestines of cockroaches, viable mycobacteria can be released with faeces into the external environment [16; 17].

F. Portaels et al. suggested the theory that animals can be infected with mycobacteria through insect bites, and Marsollier et al. reported that *Mycobacterium ulcerans* are transmitted to rodents through the bites of water bugs from the family *Naucoridae* [18; 19]. J. Kazda notes that mycobacteria, due to their cell wall structure, are resistant to the action of insect gastric enzymes, as a result of which they can be excreted in both saliva and faeces [20]. In addition, the study, L. Durnez et al. reports of isolation of mycobacteria from the body of insectivorous animals and rodents [21]. The ingestion of mycobacteria in their body may have occurred as a result of eating infected insects, which is quite a natural phenomenon, analysing our results. Known data suggest that beetles infected with mycobacteria can be captured by animals or birds susceptible to tuberculosis and digested, while acid-resistant mycobacteria, for their part, remain viable and can persist in the body and be released into the external environment.

Thus, summing up this study and the work of many researchers, it can be argued that insects, in particular the rice weevil, can participate in the process of the emergence and spread of tuberculosis.

CONCLUSIONS

According to the study results, it was found that the rice weevil can accumulate pathogenic microorganisms, in particular *Mycobacterium of tuberculosis,* and able to release them into the external environment, contaminating it, and remain a reservoir of the causative agent of infection for quite a long time. Microscopy revealed acid-resistant grains in the beetle's body for up to 50 days, and non-acid-resistant cocci for up to 70 days of the experiment. Microscopic examination of beetle smear samples revealed microorganisms up to 70 days of the experiment (acid-resistant bacteria – up to 50, and non-acid-resistant ones-up to 70 days inclusive). When sowing homogenisate from beetles on a nutrient medium, the growth of orange colonies (identical to the colonies of the original culture) was detected up to 30 days of the experiment, and the growth of yellow S-shaped colonies (blue cocci under a microscope) up to 90 days of the experiment. At the same time, when evaluating the CFU of a dissociative strain M. bovis a decrease in the viability of mycobacteria after passing through the body of beetles on the 30th day of the experiment was established.

Microscopic examination of the grain suspension revealed acid-resistant bacteria up to 12 days of the experiment, that is, after three beetle transplantations, and non-acid-resistant microorganisms were detected up to 30 days of the experiment. When sowing grain suspensions on a nutrient medium, the growth of orange colonies (identical to the original ones) was detected up to 30 days of the experiment, i.e., after five beetle transplantations, and the growth of yellow S-shaped colonies was detected up to 50 days, i.e., after seven beetle transplantations. The data obtained indicate the ability of the rice weevil to accumulate mycobacteria in its body for 50 days and release them into the external environment for 30 days.

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Епізоотологічне значення рисового довгоносика як резервуара *Mycobacterium bovis*

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Анотація. У даній статті наведені матеріали щодо ролі рисового довгоносика (лат. *Sitophilus oryzae*) у процесі передачі мікобактерій туберкульозу. Актуальність проведеного дослідження обумовлена стрімким поширенням туберкульозу по всьому світу та необхідністю розробки більш досконалих методів діагностики даного захворювання, що, зі свого боку, є неможливим без розширення знань з питання всіх можливих резервуарів збудника інфекції. Багаторічними дослідженнями науковців світу доведено, що комахи є переносниками різної мікрофлори, зокрема й патогенної. Тому метою наукового дослідження було встановлення епізоотологічної ролі рисового довгоносика як резервуару *Mycobacterium bovis* у процесі виникнення та поширення туберкульозу. У науковій статті було визначено тривалість носійства та виділення мікобактерій туберкульозу в зовнішнє середовище зараженими жуками. Для проведення дослідження використовували музейний дисоціативний штам *Mycobacterium bovis* (118 пасаж), предметом дослідження були рисові довгоносики. Для досягнення встановленої мети авторами були проведені культуральні, мікроскопічні та статистичні дослідження, а також визначена життєздатність мікобактерій, після пасажів через організм жуків, шляхом оцінки колонієутворюючих одиниць. Встановлена епізоотологічна роль рисового довгоносика в процесі виникнення та поширення туберкульозу. Доведено, що жук може впродовж 50 діб утримувати мікобактерії у своєму організмі з поступовим зменшенням їх кількості й надалі здатний виділяти збудника ще 30 діб, контамінуючи об'єкти зовнішнього середовища. При оцінці колонієутворюючих одиниць мікобактерій у динаміці досліду виявлено зниження їх життєздатності після персистенції через організм жуків з $5,3*10^8$ в 1 г вихідної культури до $1,4*10^8$ в 1 г культури, яка виросла з гомогенізату жуків на 30 добу. Наведені результати дослідження дозволять розробити та удосконалити вже існуючі правила профілактики туберкульозу та недопустити занесення даної інфекції на благополучну територію

Ключові слова: мікобактерії, дисоціативний штам, інфекція, жуки-шкідники зернових запасів, колонієутворюючі одиниці