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Markers of tuberculosis process in cattle caused by pathogens of different species

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The diagnosis of tuberculosis in animals is a complex process that is crucial for determining the effectiveness of measures to combat this disease. Currently, tuberculosis in animals is not reported in Ukraine; however, each year, 40–60 livestock farms need to assess the epizootic situation regarding this disease due to nonspecific allergic reactions to tuberculin in cows during routine diagnostic tests. This article presents generalized and systematized findings from long-term studies (2000–2024) on comprehensive anti-tuberculosis measures for cattle in various types of Ukrainian farms. This complex included epizootic, clinical, allergic, pathological, bacteriological, and statistical research methods. Based on the experiences gained from scientific and practical work, key criteria (or markers) for establishing a tuberculosis diagnosis in cattle and differentiating this disease within herds have been identified. In addition, the presented work contains original photographs that demonstrate pathological macroscopic changes characteristic of the course of the infectious tuberculosis process in animals, as well as the cultural and morphological features of mycobacterial cultures isolated from biological material. The main indicators (markers) characterizing the course of epizootic and infectious tuberculosis process in cattle herds and used to diagnose this disease were identified: clinical signs, the manifestation of allergy, pathological signs, source of the infectious agent, environmental conditions, biochemical tests, type of laboratory animals.

Keywords: mycobacteria, tuberculosis, cattle, markers, diagnostic complex

Introduction

Infectious diseases of farm animals pose a serious threat not only to productive livestock but also to humans. This is especially true for zoonotic anthroponosis, which is widespread and characterized by emergence [17, 21, 23]. Among these infections, tuberculosis and paratuberculosis occupies a special place as a chronic, difficult-to-diagnose, and difficult-to-treat disease [15, 25, 46].

The ongoing study of tuberculosis keeps this disease at the forefront of concern for both practical and scientific experts in human and veterinary medicine worldwide [1, 26]. This anthropo-zoonotic infection persists despite the high standards of living in countries like Germany, the USA, and France [42, 43]. Tuberculosis requires continuous and careful attention to manage the situation effectively [1, 11, 37]. To address this challenge, comprehensive cooperation among specialists from various fields, such as veterinary medicine, epidemiology, and ecology, is essential.

Tuberculosis in cattle is known to be caused by *Mycobacterium bovis* (*M. bovis*) and *M. tuberculosis* [13, 27]. At the same time, the nature of disease manifestation in animals is highly variable [44]. There is also no consensus among scientists on the role of some species of atypical mycobacteria, as well as the pathogen *M. avium*, in the occurrence of the pathological process in cattle. There are reports that *M. avium* cultures can cause tuberculosis lesions in human bone tissue, especially in children [35, 47]. It is particularly important to pay attention to environmental aspects, as mycobacteria of this species often persist in aquatic ecosystems and can pose a risk to animals in contact with contaminated water bodies [7, 9, 29, 31].

The global concept of the modern system of infectious disease control envisages the principle of "One Health", *i.e.* the maximum eradication of anthropozoonotic diseases to break the vicious circle of animal and human infection [2, 18]. The Global Plan to Fight Human Tuberculosis for 2023–2030 (*Global Health Campus*), presented to the world community, will not be successfully implemented without attention to the elimination of tuberculosis infection in animals. In addition, it is important to raise public and professional awareness of the risks associated with this disease and to improve diagnostic and control methods [30, 39]. Climate change has a significant impact on the biological cycle of development and the ecology of microorganisms, which must be taken into account when forecasting and planning anti-epizootic measures [3, 4, 19].

In Ukraine, the threat of animal tuberculosis is significant, particularly in light of the ongoing full-scale war in the country. Thus, it is crucial to focus on controlling tuberculosis infections in animals, especially among cattle [16, 45]. Additionally, it is vital to emphasize the importance of continuous state oversight regarding the epidemiological situation and the spread of this infection, as well as to improve the regulatory framework and increase funding for veterinary medicine.

This work aimed to investigate and summarize the key characteristic manifestations (markers) of the epizootic and infectious tuberculosis process in cattle, caused by pathogens of various species, to identify priority methods for diagnosing this disease.

Materials and Methods

The research was conducted using a set of diagnostic methods provided for by the current regulatory documents of Ukraine to control tuberculosis infection [10]. This complex included epizootic, clinical, allergic, pathological, bacteriological, and statistical research methods. The work was carried out in cattle breeding farms in Kirovohrad, Cherkasy, Sumy, Kyiv, and Chernihiv regions of Ukraine in the period from 2000 to 2024, which allowed us to cover different environmental and epizootic conditions. As a result of the research, a large amount of statistical data was collected (long-term retrospective analysis), which made it possible to assess the prevalence of infection and the effectiveness of preventive measures.

Commercial series of purified mammalian tuberculin in standard solution, allergen from atypical mycobacteria (AAM) and mycobacterial allergen solvent produced by Sumy Bioprocessing Plant (Sumy, Ukraine) were used for allergy studies. Allergic studies were performed by intradermal tuberculin test (ITT) and, as an additional method, by simultaneous allergy test. Mycobacterial allergens were injected intradermally with needleless injectors according to the following scheme: tuberculin on the left side and AAM on the right side in the middle third of the neck on a previously shaved and alcohol-rectified area in a dose of 0.1 cm³. Reactions to allergens in cattle were recorded 72 hours after administration using digital measuring instruments (angle meters) to ensure the accuracy of the reaction assessment.

During the pathological examination of animals slaughtered for diagnostic purposes, the submandibular, pharyngeal, bronchial, mediastinal, portal, mesenteric, forearm, supraorbital, and popliteal lymph nodes, as well as pieces of liver, spleen, lung, and thickened areas of the small and large intestine, were examined and selected for cultural examination. For each sample, the sampling site, anatomic features, and visual changes, such as nodule enlargement, necrotic processes, or changes in tissue structure, were recorded. By using this method, we were able to better determine how the infection spread in the animals and assess its impact on the body.

Experimental studies were conducted in the Laboratory for the Study of Tuberculosis of the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" (NSC "IECVM") (Kharkiv, Ukraine). We also used the materials of state reporting on cattle tuberculosis research of the State Service of Ukraine for Food Safety and Consumer Protection, in particular, provided by the State Research Institute of Laboratory Diagnostics and Veterinary Expertise (Kyiv, Ukraine).

Samples of biological material from cows reacting to mycobacterial allergens were examined by the cultural method in a sterile box. Samples of lymph nodes and pieces of internal organs were pre-decontaminated from foreign microflora. Intestinal samples were ground into pieces of 0.5–1.0 cm² and placed in a 0.9 % cetylpyridinium chloride (CPC) solution for 20 hours. The solution was then removed from the mortars and the pieces were ground with a pestle and sterile sand until a homogeneous mass was obtained. 0.85 % sterile saline in a ratio of 1:5 was added to the mortars, and thoroughly mixed, and after the sand settled, 10.0 cm³ of the sample was taken from the middle layer of the liquid with a sterile pipette, transferred to centrifuge tubes, and centrifuged (3000 rpm) for 15 minutes. After that, the supernatant was poured off, and the resulting precipitate was washed twice with a sterile isotonic solution. The resulting precipitate was resuspended in saline (1:5). Each sample of the biomaterial under study was separately inoculated on an egg nutrient medium for the cultivation of mycobacteria. Intestinal samples were additionally inoculated on a growth factor medium. The tubes with the cultures were cultured in a thermostat at 37.0±0.5 °C for 90 days.

The growth of mycobacteria on the nutrient medium was recorded daily for the first seven days and then once a week for 90 days. When colony growth was detected on the surface of the medium, smears were prepared, and stained by the Ziehl-Nielsen method, and microscopy was performed to determine the presence of acid-fast mycobacteria and their purity. If red acid-fast bacilli were detected in the smears, the primary cultures were inoculated on Pavlovsky's nutrient medium to accumulate bacterial mass. Growth rate, colony morphology, growth at 25, 37, 45 °C, pigmentation, and growth rate on medium with sodium salicylate and 5.0 % sodium chloride were determined in the isolated epizootic cultures. In addition, the catalase activity, the ability to hydrolyze *Tween-80*, and the reaction with potassium tellurite, as well as the reaction of iron accumulation were determined.

The obtained results were systematized and presented in the form of a table, and the photos showed the characteristic lesions of tuberculosis on internal organs and tissues in animals. The generalization and application of the meta-analysis [32] of the research data allowed us to identify the general features of the manifestation of tuberculosis and paratuberculosis in cattle, which made it possible to develop appropriate measures for the prevention and control of these infections.

Statistical analysis

Several statistical methods were employed to analyze data reflecting the patterns of mycobacterial infections in cattle caused by various mycobacterial species. These methods allow us to describe and compare the characteristics of the infectious process between different types of pathogens. A combination of descriptive statistics, comparative analysis, and proportion analysis was used to describe and compare the characteristics of the infection process between mycobacterial species in table. Qualitative data analysis with possible significance

Table. General patterns of mycobacterial infection (or persistence) manifestation in cattle groups caused by different types of mycobacteria

Diagnostic parameters	Infectious agent				
	M. bovis	*M. tuberculosis*	*M. avium*	*M. paratuberculosis* complex	Atypical mycobacteria, nocardia, corynebacteria, rhodococci
Clinical signs in animals	Occur in the context of a prolonged infectious process and are not pathognomonic.	Absent	Absent	Occur in the context of a prolonged infectious process and are not pathognomonic	Absent*
	(76±2,8) %, n=28	n=5	n=18*	(52±1,6) %, n=28	n=127*
Manifestation of allergy	Pronounced manifestation of allergic reactions to mammalian tuberculin, which prevail over reactions to AAM. The reactions are persistent	Weakly pronounced	Pronounced manifestation of allergic reactions to AAM, which prevail over reactions to mammalian tuberculin*	Manifestation of allergic reactions to AAM, which prevail over reactions to tuberculin for mammals	Pronounced manifestation of allergic reactions to AAM, which prevail over reactions to tuberculin in mammals. In this case, allergic reactions disappear after 30–45 days*
	(85±3,6) %, n=350*	(18±0,5) %, n=7	(75±3,4) %, n=118*	(60±2,3) %, n=28	(92±4,0) %, n=281*
Pathological and anatomic changes	Pathologic changes in organs and tissues characteristic of tuberculosis*	Tuberculous changes are predominantly in the bronchial lymph nodes or absent*	Absent*	Pathologic changes in the intestine characteristic of paratuberculosis	Absent*
	(95±2,3) %, n=52*	(31±1,2) %, n=20*	n=37*	(80±3,1) %, n=4	n=74*
The main source of infection or probable routes of introduction of the infectious agent	Cattle with tuberculosis	A person with tuberculosis (farm staff), cattle	Synanthropic birds, cattle	Cattle with paratuberculosis	Synanthropic birds, cattle, green fodder, etc.
Presence of ecological foci	Absent	Absent	Partially available	Partially available	Available
Biochemical tests: Niacin test	Positive*	Positive*	Negative**	Negative*	Negative*
(99±2,7) %, n=120	(100±1,2) %, n=15	(98±2,1) %, n=27)	(100±1,8) %, n=15	(100±2,0) %, n=148	(99±2,7) %, n=120
Biochemical tests: Reaction with carbamide	Positive*	Positive*	Negative*	Negative*	Negative*
(100±1,9) %, n=120	(97±2,4) %, n=15	(100±1,9) %, n=27	(100±2,2) %, n=15	(97±1,6) %, n=148	(100±1,9) %, n=120
Species of laboratory animals for bioassay	Guinea pigs	Guinea pigs	Chickens, rabbits	Rabbits	Guinea pigs

Note. * — statistically significant difference (P≤0.05).

testing to identify differences between groups was also employed. For biochemical tests (niacin test, carbamide reaction), where the result is qualitative (positive or negative), the frequency of positive results was calculated using binary analysis. A significant difference was determined using *Statistica 10.0* software for Windows (*StatSoft*, USA).

Additionally, since the study spanned a long period (2000–2024) and covered different regions of Ukraine, long-term retrospective analysis was employed to summarize data from various sources. This method allowed us to combine results from various farms and studies to obtain an overall assessment of the effectiveness of diagnostic methods.

Results

Based on the results of long-term studies, the main indicators (markers) characterizing the course of epizootic and infectious tuberculosis process in cattle herds and used to diagnose this disease were identified: clinical signs, the manifestation of allergy, pathological signs, source of the infectious agent and ways of its spread in the animal body, environmental conditions, biochemical tests (niacin test, carbamide reaction), type of laboratory animals for biological research (table).

Thus, table, which summarizes the results of long-term studies of tuberculosis in Ukraine, is a conditional scheme for the diagnosis and differential diagnosis of mycobacterial infections in cattle (disease, immunizing subinfection, etc.), both individually and in groups.

Equally important is the ability of veterinarians to detect tuberculosis during diagnostic autopsies of carcasses of animals slaughtered for diagnostic purposes or those that have died. Visually, the pathological lesions, organs, and tissues characteristic of tuberculosis in cattle are shown in fig. 1–2.

As for laboratory animals, guinea pigs are the optimal biological model for tuberculosis research, in particular for reproducing the tuberculosis process caused by the *M. bovis* pathogen (fig. 3), rabbits or chickens are used only for isolation and identification of the *M. avium* pathogen.

A characteristic confirmation of pathological signs (fig. 4) can be obtained by infecting guinea pigs with a suspension of biological material taken from sick cattle or an isolated culture of the tuberculosis pathogen *M. bovis*.

A characteristic cultural feature of mycobacteria is the growth of cultures of these microorganisms on special nutrient media (Pavlovsky's medium, dense egg media, synthetic liquid or semi-liquid media, etc.) (fig. 5a). Another characteristic morphological feature of the tuberculosis pathogen *M. avium* is the arrangement of mycobacterial cells in the form of clusters (fig. 5b).

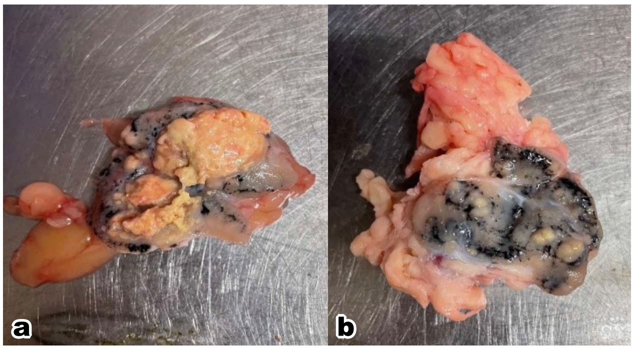

Fig. 1. Tuberculosis lesions in cattle.
a — bronchial lymph node, b — mediastinal lymph node

Fig. 2. Tuberculosis lesions of lung tissue in cattle

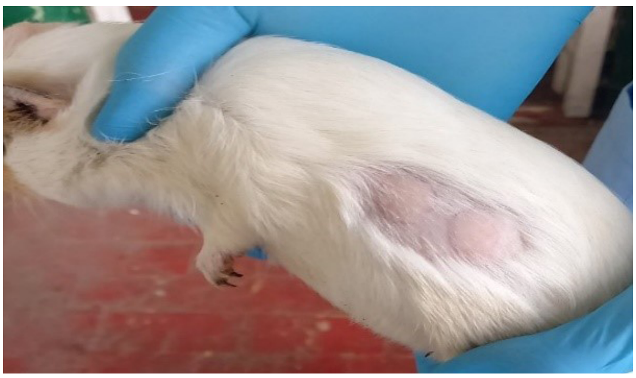

Fig. 3. Allergic reactions to tuberculin and AAM in the form of swelling and erythema at the depilation site in a guinea pig infected with a culture of the tuberculosis pathogen *M. bovis*

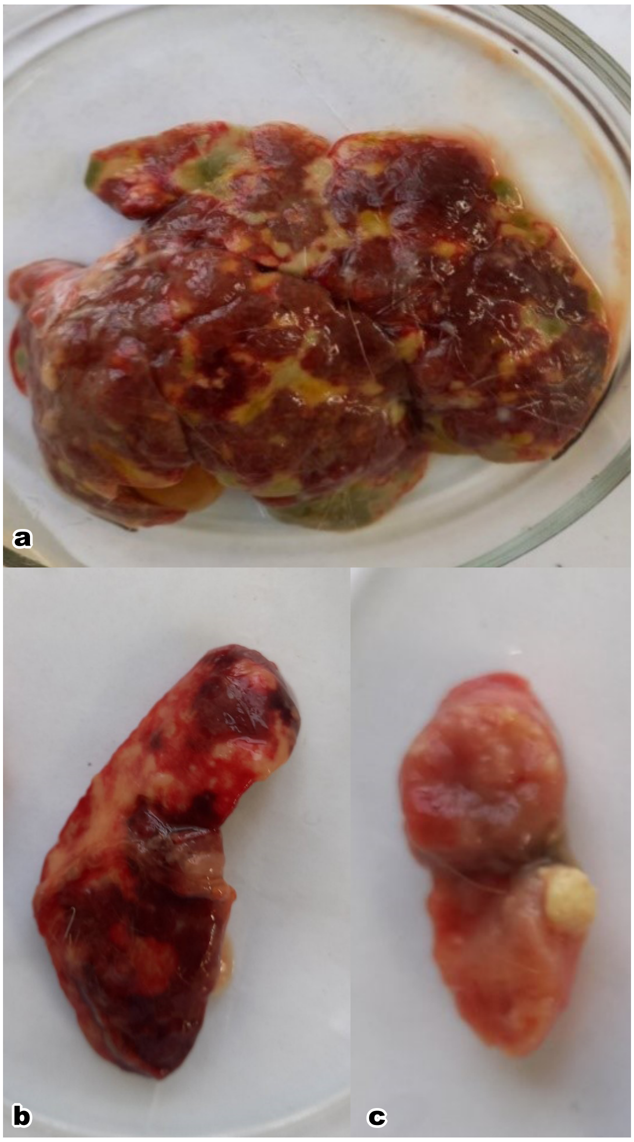

Fig. 4. Lesions characteristic of tuberculosis in guinea pigs. a — liver, b — spleen, c — inguinal lymph node

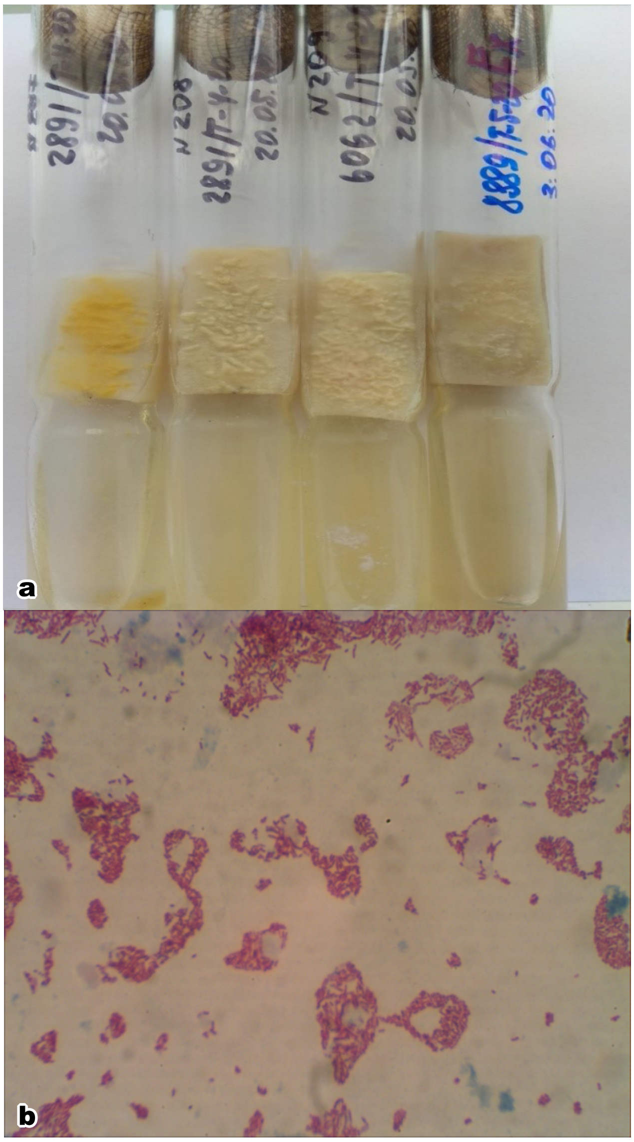

Fig. 5. Cultures of mycobacteria. a — growth on Pavlovsky's glycerol medium, b — culture of *M. avium* (Ziehl-Nlsen staining)

Discussion

It should be noted that the systems of measures for the control and prevention of tuberculosis infection in animals may vary considerably from one country to another. This depends on the specifics of the epizootic situation, the type of livestock production, the economic situation, etc. [6, 8]. For example, in some countries in Western or Northern Europe (Germany, France, Denmark), TB infection is mainly controlled in meat processing plants during the inspection of carcasses and internal organs [12, 20]. This approach is possible due to the high level of livestock development and a stable epidemic and epizootic situation. In other countries, such as Poland, diagnostic allergic testing of cattle for tuberculosis using PPD mammalian tuberculin is carried out every three years. As for Ukraine, given the objective situation, diagnostic TB control measures in livestock are based on systematic allergic tests carried out twice a year (for cattle) in safe farms with a defined epizootic situation. Due to this density of research, the impact of non-specific reactions to tuberculin in animals is of great importance for the entire system of comprehensive diagnosis of tuberculosis infection [45]. Thus, in early November 2024, according to the State Institute of Laboratory Diagnostics and Veterinary Expertise, 264 cattle with non-specific allergy to intradermal injection of mammalian tuberculin were found in Ukrainian farms, of which 177 animals had to be slaughtered for diagnostic purposes. However, none of these animals showed characteristic signs of infectious tuberculosis during pathological examination. These data highlight the need for improved diagnostic approaches, including the introduction of additional methods to exclude non-specific reactions.

Summarizing the available data, we believe that, first of all, attention should be focused on the identification of certain markers that determine the epizootic situation of tuberculosis in cattle herds. The main ones are pathological signs, the nature of allergy to specific diagnostics, epizootological data, individual biochemical tests for selected isolates, and bioassay results. In addition, it is important to take into account clinical signs, as well as data from additional research methods (PCR, ELISA test, gamma interferon test, complement fixation test, latex agglutination test, intravenous tuberculin test, etc.). However, in any case, the diagnostic stage of TB control should end with culture isolation and species identification [33, 38].

The research found that since the beginning of 2024, 47 farms have conducted comprehensive tuberculosis tests to assess the situation regarding this disease. The use of various research methods has allowed us to preserve 97 healthy productive animals (cows) in the cattle herds. These results highlight the importance of a systematic approach to tuberculosis control, which includes both traditional and modern diagnostic methods, such as molecular genetic tests. Therefore, developing a specific set of actions based on identified markers (signs) of the infectious and epizootic tuberculosis process is crucial for effectively managing the situation with animal tuberculosis.

It is important to recognize that, under modern conditions and in light of recent ecological and climatic changes around the world, a significant aspect of diagnosing mycobacterial infections involves considering the eco-geographical component. Specifically, this includes the presence of ecological foci where certain mycobacterial species persist [9, 36]. This is particularly relevant for non-pathogenic or conditionally pathogenic species, often referred to as atypical mycobacteria [40]. Mycobacteria exhibit high ecological plasticity, allowing them to adapt to changing environmental conditions, which is why they can be considered ubiquitous. For instance, certain species, such as *Mycobacterium smegmatis*, can be found in the soil and play an active role in the biodegradation of organic matter. Others, like *Mycobacterium avium*, inhabit both natural and artificial water systems, particularly in wastewater treatment plants, where they may become pathogenic to susceptible organisms. Regarding eco-geographical features, most atypical (saprophytic) mycobacterial species in Ukraine thrive in wetlands, peat bogs, and areas with high humidity. When mycobacteria enter the macroorganism of animals or poultry, only short-term sensitization occurs. This state of hypersensitivity (allergy) to the effects of antigenically related substances does not lead to the development of an infectious pathological process. Synanthropic or wild birds (sparrows, pigeons, crows, ducks, swallows, etc.) are also often mechanical spreaders of mycobacteria. Mycobacteria can form complex relationships with other microorganisms, such as in soil systems where they can coexist with fungi or protozoa, contributing to the breakdown of organic matter and mineralization. In aquatic ecosystems, mycobacteria can colonize the surfaces of biofilms. Under certain environmental conditions, mycobacteria can retain their properties for many years [5, 22]. They are also highly resistant to antimicrobial agents, which increases the risks of cross-species transmission and food contamination [24, 28]. This, in turn, increases their importance in veterinary medicine and food safety.

It has been established that cattle breeding enterprises located near water bodies, where, for example, cattle are regularly grazed in wetlands or green fodder is delivered from such areas, often have cases of persistent manifestations of nonspecific para allergic reactions in animals to mammalian tuberculin, usually caused by the persistence of atypical mycobacterial species that are not pathogenic to cattle. The accidental ingestion of atypical mycobacteria into the body of animals, mainly through the diet, is a key factor in this phenomenon. It is quite difficult to get rid of this phenomenon due to the constant ingestion of mycobacteria into the body of animals, mainly by the alimentary route. The negative consequences of nonspecific reactions are associated with the economic costs of farms to determine the causes of these reactions to exclude the manifestation of tuberculosis infection in herds [2, 34].

The diagnosis of tuberculosis infection in animals, particularly in cattle, is a complex process [14, 41] that involves specific characteristic signs. Recognizing these patterns enables veterinary specialists to establish protocols for addressing complications that can arise when assessing the epizootic situation of tuberculosis, especially during preventive tuberculin testing. Implementing regional strategies to combat mycobacterial infections, while considering eco-geographical factors and enhancing veterinary services, can significantly reduce economic losses and improve overall animal health. This, in turn, supports the country's food security. Overall, effectively combating tuberculosis infection requires not only the use of modern diagnostic and treatment methods but also the consideration of environmental, biological, and social factors. An interdisciplinary approach that integrates the efforts of scientists, practitioners, and government regulatory authorities is essential for successfully containing the spread of tuberculosis among animals.

Considering many years of experience in implementing preventive and health-improving measures against tuberculosis, as well as studying the characteristics of tuberculosis pathogens and atypical mycobacterial species, it is important to note that ensuring complete well-being regarding tuberculosis infection in animals can only be achieved through comprehensive diagnostic measures and research. Innovative scientific approaches aimed at combating this infection are essential, particularly due to its insidious and slow progression. The complexity of this approach lies in the application of integrated diagnostic methods, which include clinical, epizootic, allergic, pathological, and bacteriological techniques. Additionally, it is crucial to consider the eco-geographical factors influencing the spread of mycobacteria.

To ensure the well-being of farms regarding tuberculosis, it is essential to carefully implement the preventive and diagnostic measures outlined in current regulatory documents. Additionally, establishing a system for continuous monitoring of environmental sources that could harbor atypical mycobacteria is crucial for the timely identification of potential risks of animal infections.

When diagnosing tuberculosis in cattle, it is important to consider the biological characteristics of all types of tuberculosis and paratuberculosis pathogens in animals, as well as the persistence of atypical mycobacteria such as *M. fortuitum*, *M. intracellularae*, *M. scrofulaceum*, *M. gordonae*, *M. phlei*, and others. Special attention should be given to assessing the sensitivity of these species to standard diagnostic methods used in veterinary medicine. For example, certain species of atypical mycobacteria can provoke nonspecific allergic reactions, which complicates the differentiation from actual tuberculosis infections.

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Маркери туберкульозного процесу у великої рогатої худоби, зумовленого збудниками різних видів

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Діагностика туберкульозу у тварин — це складний комплексний процес, від якого залежить ефективність подальших заходів боротьби. На сьогодні в Україні туберкульоз серед тварин не реєструється, проте щорічно 40–60 тваринницьких господарств потребують з'ясування епізоотичної ситуації щодо цього захворювання з огляду на прояв неспецифічних алергічних реакцій на туберкулін у корів під час проведення планових діагностичних досліджень. Для досліджень використовували комплекс діагностичних методів: епізоотологічні, клінічні, алергічні, патологоанатомічні, бактеріологічні, статистичні. Роботу проводили у скотарських господарствах Кіровоградської, Черкаської, Сумської, Київської, Чернігівської областей України у період з 2000 по 2024 роки, що дозволило охопити різні екологічні та епізоотологічні умови. Представлено матеріали багаторічних досліджень (2000–2024 рр.) у господарствах України. Зібрано великий обсяг статистичних даних (мета-аналіз), що дало змогу визначити основні критерії (маркери) встановлення діагнозу у цього виду тварин, а також диференційної діагностики захворювання. Крім того, робота містить оригінальні фотознімки, які демонструють патологічні макроскопічні зміни, характерні для перебігу інфекційного туберкульозного процесу у тварин, а також культурально-морфологічні особливості ізольованих з біологічного матеріалу культур мікобактерій. На підставі результатів досліджень визначено основні показники (маркери), які характеризують перебіг епізоотичного та інфекційного туберкульозного процесу у стадах великої рогатої худоби і за якими здійснюється діагностика цього захворювання.

Ключові слова: мікобактерії, туберкульоз, велика рогата худоба, маркери, комплексна діагностика

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