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https://doi.org/10.29326/2304-196X-2026-15-2-148-154

Science-based set of measures to detect bovine tuberculosis in the Republic of Dagestan

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ABSTRACT

Introduction. Given the current epizootic situation and the specifics of livestock farming in the Republic of Dagestan, it is essential to monitor the implementation of preventive measures and improve methods of bovine tuberculosis (bTB) control to prevent its spread. Within this system of measures, qualified diagnosis is a key element. An integrated differential diagnostic approach using various tuberculosis testing methods in farms with different epizootic statuses is promising.
Objective. To compare the effectiveness of the proposed methods for bTB diagnosis and to summarise data on the circulation of tuberculous and non-tuberculous forms of mycobacteria in nature.
Materials and methods. Allergic tests were conducted using 1,768 cattle; serological tests included 1,634 serum samples in the complement fixation test and 2,127 samples in the indirect haemagglutination test. Bacteriological tests involved 63 biomaterial samples and 97 environmental object samples. The performance of various culture media in mycobacteria isolation was tested using 36 biomaterial samples from tuberculin PPD-reacting cows and heifers.
Results. The practical significance of the palpebral and intravenous tests was confirmed for identifying diseased animals and making initial diagnoses. The intravenous test also proved effective in some cattle. Serum antibody testing revealed high specificity of the complement fixation test for detecting anergic animals in long-term affected herds, whereas the indirect hemagglutination test demonstrated low specificity. Of the 63 biomaterial samples analysed, 46 cultures were isolated: 10 (21.7%) were identified as *Mycobacterium bovis*, and 36 (78.3%) as non-tuberculous species. Of the latter, 32 cultures (88.9%) were classified into group II and 4 cultures (11.1%) – into group III according to Runyon classification. Of the 97 environmental object samples analysed, 64 cultures were isolated: 4 (6.3%) were identified as *Mycobacterium bovis*, 35 (54.7%) belonged to group II and 25 (39.0%) – to group III of non-tuberculous mycobacteria according to Runyon classification. To assess the culture media performance, 36 biomaterial samples from tuberculin PPD reactor cows and heifers were studied. The 8 cultures isolated (22.2%) were identified as *Mycobacterium bovis*; 28 (77.8%) were classified as non-tuberculous mycobacteria of Runyon's groups II (11 – 39.3%) and of group III (17 – 60.7%). As for growth of typical and non-tuberculous forms of mycobacteria, Lowenstein – Jensen medium showed the best growth properties.
Conclusion. A comprehensive study of animals using differential diagnostic methods, including serological tests, improves the effectiveness of differential diagnosis.

Keywords: tuberculosis, cattle, allergy diagnosis, tuberculin PPD, serological tests, biomaterial, environmental objects, culture media, mycobacteria

For citation: Baratov M. O. Science-based set of measures to detect bovine tuberculosis in the Republic of Dagestan. *Veterinary Science Today*. 2026; 15 (2): 148–154. https://doi.org/10.29326/2304-196X-2026-15-2-148-154

Conflict of interests: The author declares no conflict of interests.

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УДК 619:616.98:579.873.21:636.22/.28:616-07

Научно обоснованный комплекс мероприятий для выявления туберкулеза крупного рогатого скота в Республике Дагестан

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РЕЗЮМЕ

Введение. Учитывая сложившуюся эпизоотическую ситуацию и специфику ведения животноводства в Республике Дагестан, в целях недопущения распространения туберкулеза крупного рогатого скота необходимо обеспечить контроль за реализацией мер профилактики и совершенствовать методы борьбы с заболеванием. При этом ключевым элементом системы мер служит квалифицированная диагностика. Перспективным является разработка комплексного подхода к дифференциальной диагностике, а также использование различных методов диагностики туберкулеза в хозяйствах с различным эпизоотическим статусом.
Цель исследования. Сравнение эффективности предложенных методов диагностики туберкулеза крупного рогатого скота и обобщение данных по циркуляции туберкулезных и нетуберкулезных форм микобактерий в природе.
Материалы и методы. Аллергическим исследованиям подвергли 1768 гол. крупного рогатого скота, серологическим – 1634 пробы сыворотки крови

в реакции связывания комплемента и 2127 проб – в реакции непрямой гемагглютинации; бактериологическим исследованиям – 63 пробы биоматериала и 97 образцов объектов внешней среды. Испытание различных питательных сред на высеваемость микобактерий проводили изучением 36 проб биоматериала от реагировавших на ППД-туберкулин коров и нетелей.

Результаты. Установлена практическая значимость пальпебральной и внутривенной проб при выявлении больных животных и при первичной постановке диагноза, а также эффективность внутривенной пробы при исследовании нескольких голов крупного рогатого скота. Тестирование проб сывороток крови на наличие антител показало высокую специфичность реакции связывания комплемента при выявлении анергичных животных в длительно неблагополучных стадах и низкую – в реакции непрямой гемагглютинации. Из 63 проб биоматериала изолировано 46 культур: 10 (21,7%) идентифицированы как *Mycobacterium bovis*, 36 (78,3%) – как нетуберкулезные виды, из которых 32 культуры (88,9%) отнесены к II группе по классификации Раньона, 4 культуры (11,1%) – к III группе. Из 97 проб объектов внешней среды изолированы 64 культуры, 4 (6,3%) из них отнесены к *Mycobacterium bovis*, 35 (54,7%) – к II и 25 (39,0%) – к III группе нетуберкулезных микобактерий по классификации Раньона. При оценке питательных сред на высеваемость изучено 36 проб биоматериала от реагировавших на ППД-туберкулин коров и нетелей. Восемь выделенных при этом культур (22,2%) были отнесены к *Mycobacterium bovis*, 28 (77,8%) – к нетуберкулезным микобактериям II (11 – 39,3%) и III (17 – 60,7%) групп по Раньону. При выращивании типичных и нетуберкулезных форм микобактерий наилучшие ростовые свойства показала среда Левенштейна – Йенсена.

Заключение. Комплексное исследование животных дифференциально-диагностическими методами с применением серологических тестов повышает эффективность дифференциальной диагностики.

Ключевые слова: туберкулез, крупный рогатый скот, аллергическая диагностика, ППД-туберкулин, серологические реакции, биоматериал, объекты внешней среды, питательные среды, микобактерии

Для цитирования: Баратов М. О. Научно обоснованный комплекс мероприятий для выявления туберкулеза крупного рогатого скота в Республике Дагестан. *Ветеринария сегодня.* 2026; 15 (2): 148–154. https://doi.org/10.29326/2304-196X-2026-15-2-148-154

Конфликт интересов: Автор заявляет об отсутствии конфликта интересов.

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INTRODUCTION

Certain success has been achieved in animal tuberculosis (TB) control in the Republic of Dagestan. At the same time, despite numerous studies describing statistics and characterising the mechanisms of TB development, many issues require further investigation [1, 2, 3].

We believe that the patterns of disease spread and animal health situation improvement in affected areas under different natural and climatic conditions depend on quality and timeliness of implementation of TB prevention and control programs [4, 5].

Experience demonstrates the need for continuous and rigorous monitoring to prevent the introduction of infection into disease-free farms and to improve the disease situation in cattle farms affected by bovine tuberculosis (bTB). Unregulated and haphazard movements of animals, livestock products and feed increase the risk of disease spread to disease-free farms [6, 7].

Today, given the current epizootic situation and the specifics of livestock farming, it is necessary to ensure monitoring of preventive measure implementation aiming at bTB control method improvement [8].

Qualified diagnosis is a key element of the control system. Achieving this goal often requires comprehensive and specialised studies that go beyond the scope of standard regulations [9, 10].

In modern conditions, as bTB incidence declines, the problem of non-specific reactions has become increasingly relevant. The insufficient ability of the proposed diagnostic methods to differentiate non-specific reactions causes significant economic losses, resulting in unjustified culling of healthy cattle and implementation of excessive animal disease prevention measures [11].

Due to a lack of understanding of the mechanisms behind these reactions, their interpretation remains diverse. Currently, there is no clear understanding of the processes underlying non-specific sensitisation to tuberculin PPD in mammals [12].

According to both domestic and foreign literature, the issue of sensitisation of healthy animals by non-tuberculous mycobacteria and acid-tolerant actinomycetes – which share group-specific similarities (morphological, physiological, cultural, genetic, etc.) with mycobacteria – has been studied in detail [13, 14].

In our previous comprehensive studies we identified the tuberculin-sensitising properties of mycobacterium-like acid-tolerant microorganisms, in particular corynebactera, nocardia and rhodococci. This undoubtedly leads to some confusion in TB differential diagnosis [2, 7]. These findings have also been confirmed in the foreign literature [10].

It is important to note that due to the polyetiological nature of factors that sensitise the macroorganism, controlling the problem of sensitisation to tuberculin and identifying animals with TB based solely on tuberculin test results presents certain difficulties. Positive results for certain tests can often be obtained when using a combination of methods. In our view, this may be related to the multi-stage course of TB and the immunobiological state of the organism under the influence of environmental factors [1, 5, 15].

This undoubtedly complicates diagnosis and requires the combined use of ante-mortem methods, including laboratory ones. It is known that the sensitivity and specificity of the proposed tests (in particular, allergic, serological and immunological) for diagnosing TB are largely determined by the homology of TB-typical antigens. Currently, the use of a range of differential diagnostic methods as part of TB detection efforts is both scientifically grounded and practically effective for improving diagnostic efficiency [16, 17, 18].

Developing a unified algorithm for the use of diagnostic tests to enhance the practical value of the proposed methods represents a promising direction. Classical serological and immunological methods – such as the complement fixation test (CFT), indirect haemagglutination test (IHAT), rosette formation test (RFT), lymphocyte blast transformation test (LBTT) and specific lymphocyte lysis test (SLLT) – are rarely used for serological screening or immunostructure studies as they are time- and labour-consuming [19, 20, 21].

Therefore, it is worthwhile to study the causes of cattle sensitisation to tuberculin PPD, the ubiquity of mycobacteria and group-specific microorganisms in biomaterial and environmental objects, and their potential to sensitise the macroorganism [3, 16, 22].

The aim of this study was to obtain additional data to define a comprehensive approach to differential diagnosis, as well as to assess the capabilities of various TB diagnostic methods in farms with different animal health statuses.

MATERIALS AND METHODS

Allergy testing for TB was conducted in accordance with the "Veterinary rules for preventive, diagnostic, restrictive and other measures, imposing and lifting of quarantine and other restrictions aimed at tuberculosis outbreak spread prevention and eradication"[1], which came into force on 1 March 2021. Mammalian and avian tuberculin PPDs as well as a complex allergen derived from non-tuberculous mycobacteria were used for the study.

The results of allergy testing (intradermal, intravenous, palpebral and ophthalmic) were compared with serological data (CFT using complex TB antigen and SibNIVI antigen; IHAT using erythrocyte diagnosticum), as well as with the results of immunological (RFT, LBTT, SLLT) and bacteriological tests.

Samples of bovine blood, tissues with TB-like lesions and lymph nodes were examined. Pre-inoculation treatment of the material was performed using the modified method of A. P. Alikaeva.

For the study, epizootic strains of mycobacteria isolated from biological material and environmental objects were selectively used: *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium avium* and *Mycobacterium scrofulaceum*.

Preparation of culture media (Lowenstein – Jensen, Finn-2, Petragnani, Gelberg, modified Shkolnikova, synthetic Sauton, etc.), inoculation of material and cultivation were carried out in accordance with current regulatory requirements.

Sampling of environmental objects (hay, straw, soil, manure, feed remains), suspension in sulfuric acid, centrifugation, inoculation into liquid media and incubation were performed in accordance with the veterinary and sanitary regulations.

Identification of isolated forms, both tuberculous and non-tuberculous, was carried out in accordance with the requirements of GOST 26072-89 (ST SEV 3457-81) "Agricultural animals and poultry. Methods of laboratory diagnostics of tuberculosis"[2] and GOST 27318-87 (ST SEV 5627-86) "Agricultural animals. Methods of identification of non-typical microbacteria"[3].

Identification of L-forms of mycobacteria was performed by examining native preparations obtained from cultures grown on semi-liquid Shkolnikova culture medium using MV 30S microscope (PZO Biolar, Poland), which allows observation of specimens in stereoscopic mode using phase contrast. Upon detection of structures characteristic of L-forms (various granular formations, spherical bodies refracting light and exhibiting different optical densities), sequential inoculations were performed on Shkolnikova medium to assess subculturing performance, and at the same time the inoculations were conducted on Lowenstein – Jensen medium to determine reversion.

The numerical data were processed using the variation statistical method [23] depending on the specific objective, employing the "B-01" and "Correlation" software programs, the sign test method, and determination of the significance of differences between means of dependent and independent samplings.

RESULTS AND DISCUSSION

The results of allergy testing using mammalian tuberculin PPD, avian tuberculin PPD and a complex allergen derived from non-tuberculous mycobacteria showed that among 553 cattle of various ages across all natural and climatic zones of the Republic of Dagestan (mountain, foothill, and lowland), 31.3% of animals were identified as reactors. Of these, 65.7% were identified in disease-free farms, indicating a high level of healthy cattle reacting to tuberculin.

It is worth noting that the proportion of tuberculin reactors in the mountain and foothill areas did not differ significantly from that in the lowland area (Fig. 1).

It should be emphasized that these data differ considerably from previously obtained results, showing an inverse proportion that was generally observed between the number of reactor animals and the altitude of the zone above sea level.

Cartographic analysis and monitoring results of epizootic indicators from the second half of the last century and the beginning of this century indicate that the number of animals reacting to tuberculin PPD and those diagnosed with TB is confined to the lowland area, where the cattle population size and density are significantly higher.

The obtained results indicate the absence of fundamental differences in TB epizootic indicators in the Republic of Dagestan depending on natural and climatic zoning.

It should be noted that in the mountain area, despite the presence of conditions that enhance immune status (effective natural sanitation, green vegetation of alpine and subalpine meadows, more than 300 species of diverse grasses, small farm sizes with limited animal contact, significant volumes of livestock product export, restricted feed import, etc.), a high proportion of tuberculin PPD-positive reactors (46 animals) and animals with confirmed bTB (2 animals) is observed. This is attributed to uncontrolled and haphazard animal movement between farms, large-scale seasonal migrations (spring and autumn) and close links with farms in the lowland area.

It should be emphasized that the absence of identified diseased animals among tuberculin reactor cattle in the foothill area does not reflect the true situation

[1] https://docs.cntd.ru/document/565721619?ysclid=mo13wzj0og324070857 (in Russ.)

[2] https://docs.cntd.ru/document/1200025492?ysclid=moil2eqjzh50057142 (in Russ.)

[3] https://docs.cntd.ru/document/1200025497 (in Russ.)

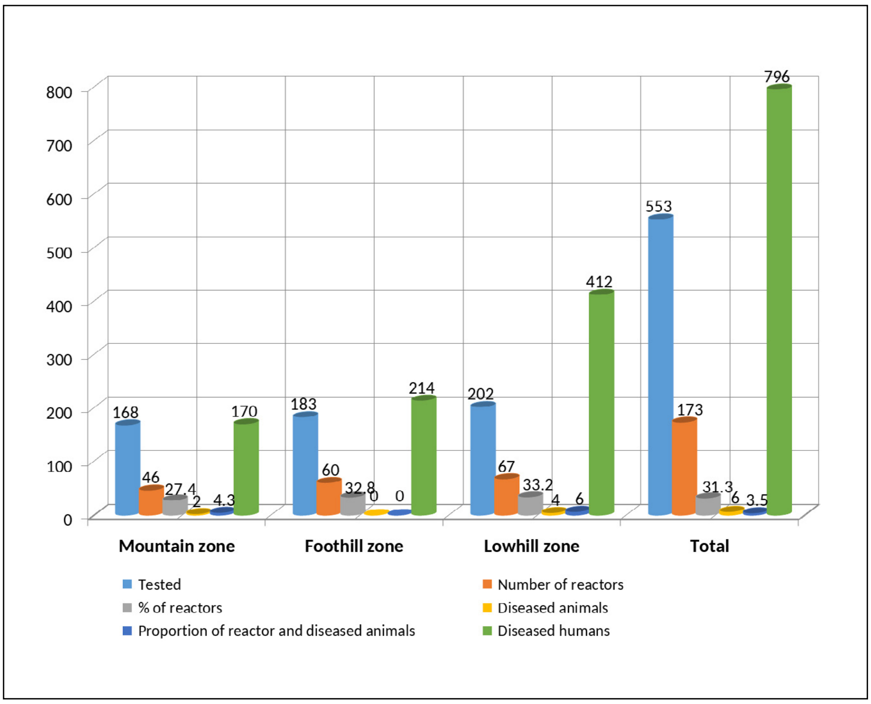

Fig. 1. Means of bovine tuberculosis epizootic indicators in the Republic of Dagestan in 2022–2023

due to the limited scope of farms and animals tested. Comparison with the results of previous systematic studies reveals that the frequency of detecting TB-infected animals within this area was comparable to that in the lowland area. Given that tuberculin reactors and TB-infected animals are registered almost universally across the Republic of Dagestan, annual monitoring of cattle status in this area is necessary.

A significant increase in the number of tuberculin PPD-reactors was observed in spring and autumn, accounting for more than 82% of the total number tested throughout the year.

In a comparative evaluation of the effectiveness of proposed allergic diagnostic tests for detecting TB (intradermal, palpebral, ophthalmic, and intravenous), the simultaneous use of the palpebral and intravenous tests demonstrated dominant efficacy, and their role in differential diagnosis was determined. It was established that their combined application in farms affected by TB allows for the detection of more than 2% of infected animals. The clinical efficacy of this combination was also confirmed for primary TB diagnosis.

When studying the sensitivity and specificity of the simultaneous test using tuberculin PPD and a complex allergen from atypical mycobacteria (CAAM), together with the palpebral test, on 475 age-diverse animals reacting to tuberculin PPD from 3 herds on disease-free farms, inconclusive results were obtained. The proportion of animals reacting to tuberculin PPD was 5.2%, to CAAM – 5.0%, and 0.6% reacted positively to the palpebral test. Given that a substantial share of the Russian small ruminant population (more than 21%) and cattle population (more than 5%) is concentrated in the Republic of Dagestan, most of which are kept in small private holdings, there is an urgent need for an effective method to differentiate non-specific tuberculin reactions.

The officially approved simultaneous test proved ineffective under these conditions. Furthermore, current regulations do not provide for diagnostic slaughter based solely on positive intradermal test results. The obtained results indicate that in small-scale farming operations with limited animal numbers, the intravenous tuberculin test is a more effective method for differentiating non-specific reactions. When evaluating the effectiveness of the intradermal and intravenous tests, it was found that positive results from both tests are confirmed by pathological and laboratory methods in more than 95% of cases. Moreover, positive results from both tests allow for the detection of animals anergic to tuberculin PPD in farms with long-standing TB problems. According to our research, the number of such animals reaches 3%, which is comparable to the published data.

In the foothill area, tests were conducted in two settlements of the Karabudakhkentsky and Buynaksky Raions to assess the practical significance of the intravenous test under stall-and-pasture cattle management conditions. The experimental groups consisted of 96 and 107 animals, respectively.

Similar studies were conducted on farms of different types of ownership located in the lowland area: in an agricultural cooperative farm (213 animals) in the Babayurtovsky Raion and in a small-scale farm (324 animals) in the Kizlyarsky Raion (Fig. 2).

Based on the results, tuberculin reactors were identified in all farms. The small number of such animals in the Kizlyarsky Raion of the lowland area is explained by the testing of cattle moved from the mountain area, where the smallest number of reacting animals was revealed based on systematic allergic diagnosis results.

It should be noted that according to the data from the Veterinary Committee of the Republic of Dagestan, there are no tuberculin reactors in these areas.

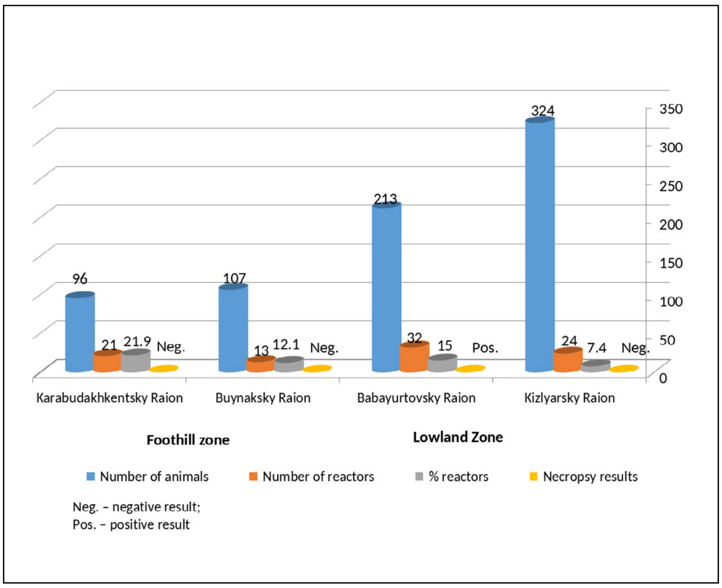

Fig. 2. Number of animals reacting to mammalian tuberculin PPD under stall and grazing conditions

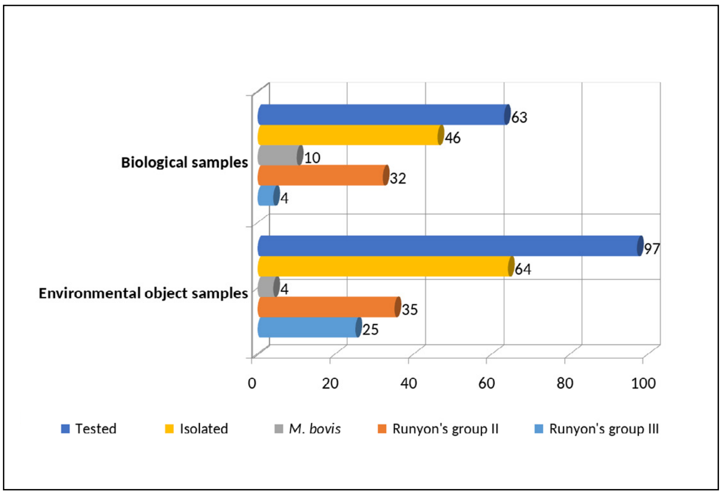

Fig. 3. Number of cultures isolated from environmental object samples and biological material

To expand the possibilities of laboratory verification for differential diagnosis, 1,634 animal serum samples were tested using CFT with complex TB antigen, and 2,127 samples were tested using IHAT with three specific erythrocyte diagnostics: *M. bovis*, *M. avium* and *M. fortuitum*.

The conducted experiments did not reveal a statistically significant correlation between positive IHAT results and the intradermal test. Positive results of sera tests using CFT were consistent with the intradermal test results in 220 cases (13.5%), which, according to the published data, is sufficient for diagnosis confirmation.

According to numerous reports, the CFT method has high specificity (85–100%) and low sensitivity, making it widely used in TB diagnosis. Our data indicate overestimated specificity values.

We believe that in the modern system of epizootological surveillance, CFT can play an important role in detecting cattle anergic to tuberculin PPD in diseased and recovering herds, where, according to various data, the number of such animals can range from 2 to 3%.

The results of the highly sensitive IHAT with erythrocyte diagnosticum (according to published data) in our studies did not reveal an epizootological link with pathologically and laboratory-confirmed diagnoses, which, in our opinion, indicates low specificity and practical significance of this method for TB diagnosis.

When identifying new aspects of the role of specific and sensitive cell-mediated immunity tests (RFT, LBTT, SLLT) in the differential diagnosis of TB, it was found that the complexity of their performance prevents their widespread use. We consider it more appropriate to use these tests for in-depth scientific research.

According to laboratory results, *M. bovis* is identified in pure culture in the vast majority of cases in animals with pronounced pathological changes in internal organs. However, the pathogen is also detected in approximately 7% of cases in animals without visible signs of TB.

Examination of 63 biomaterial samples allowed for the isolation and identification of 46 cultures, of which 10 (21.7%) were identified as *M. bovis* and 36 (78.3%) as non-tuberculous species. Following further classification, 32 of these (88.9%) were assigned to Runyon group II, and 4 cultures (11.1%) to Runyon group III.

During the study, 64 cultures were isolated from 97 environmental object samples. Upon identification, 4 cultures (6.3%) were classified as *M. bovis*, 35 (54.7%) as Runyon group II, and 25 (39.0%) as Runyon group III (Fig. 3).

Analysis of microbiological study data revealed a relationship between the detection of mycobacterial species (typical and non-tuberculous) in bovine biological material and different phases of the epizootic process. When comparing the mycobacterial landscape in the tested samples during the active phase of the epizootic process, the isolation rate of *M. bovis* was 47%, whereas during the declining phase this rate decreased to 16%. The isolation rates of non-tuberculous mycobacteria were comparable to those of typical tuberculous species.

It is important to note that non-tuberculous mycobacteria were detected in both biological material samples and environmental samples with equal frequency and in comparable numbers. Moreover, this ratio remained consistent across all natural-climatic zones by vertical zonation.

To evaluate various culture media in terms of growth yield, 36 biomaterial samples from cows and heifers that reacted positively to tuberculin PPD were tested. Following identification, 8 isolated cultures (22.2%) were classified as *M. bovis*, while 28 (77.8%) were identified as non-tuberculous mycobacteria of Runyon groups II (11 cultures, 39.3%) and III (17 cultures, 60.7%) (Fig. 4).

Growth yield was evaluated based on colony count and culture growth rate on the culture medium (Fig. 5).

The obtained results indicated the influence of culture medium composition and cultivation time on growth properties. Lowenstein – Jensen medium showed abundant growth of both typical (*M. bovis* – 16 colonies without concomitant microflora) and non-tuberculous (*M. avium* – 20 colonies in 9 days; *M. scrofulaceum* – 17 colonies in 8 days) mycobacterial forms in 18–20 days. Finn-2 medium did not support effective microbial growth: *M. bovis* produced 10 small colonies after 18 days, whereas 7, 18, and 14 colonies appeared in other media within 7–12 days. As for the other media, culture growth was slow and characterized by the formation of small colonies.

CONCLUSIONS

1. The obtained data provide a basis for concluding that tuberculosis diagnostic efficacy of the differential diagnostic complex comprising palpebral, intravenous, and intradermal tuberculin tests is high. The conducted studies have made it possible to improve the system of diagnostic measures for bovine tuberculosis. The implementation of this system has led to a significant increase in the detection of infected animals in tuberculosis-affected farms and reliably allows for the differentiation of non-specific reactions to mammalian tuberculin PPD. Annual epizootological monitoring over five years indicates a high frequency of tuberculin reactions, reaching 30% or more in some farms. In most cases, the etiological nature of these reactions remains undetermined.

2. Serological studies have determined the practical significance of CFT (in comparison with other serological tests) in tuberculosis diagnosis. We consider it reasonable to use this test as an additional method for detecting tuberculin-anergic animals in affected farms. The serological (IHAT) and immunological (LBTT, SLLT and RFT) tests studied in comparison have not found widespread application in confirming tuberculosis diagnosis. Further studies using more extensive material are required to determine their practical value.

3. It has been established that over the past decade, a shift in the dominant species of non-tuberculous mycobacteria has occurred in farms of all types of ownership. Representatives of Runyon group II predominated in biological material, while representatives of Runyon groups II and III predominated in environmental object samples.

4. The results showed that in farms of all ownership types, *M. bovis* is often isolated from biological material and environmental object samples in case of non-tuberculous mycobacteria detection.

5. Analysis of the effectiveness of culture media commonly used in laboratory conditions revealed that Lowenstein – Jensen medium provides the best growth yield in terms of growth rate for both typical and non-tuberculous forms of mycobacteria. Finn-2 medium was inferior in terms of growth yield, although in some cases it surpassed Lowenstein – Jensen medium in growth rate.

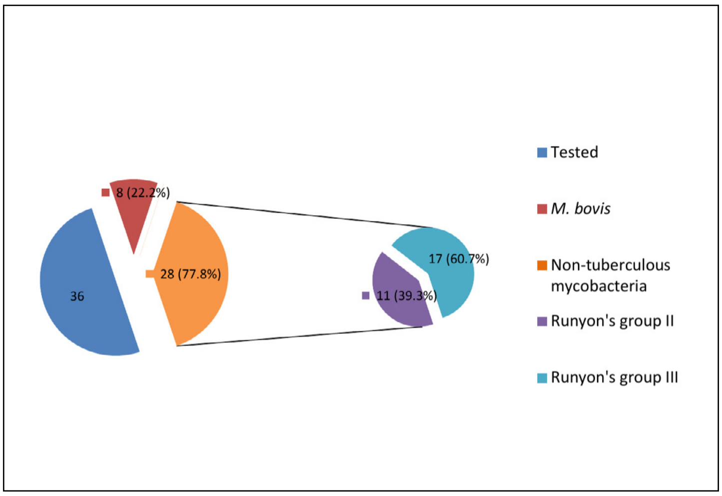

Fig. 4. Testing results for biomaterial samples from tuberculin PPD-positive reactors

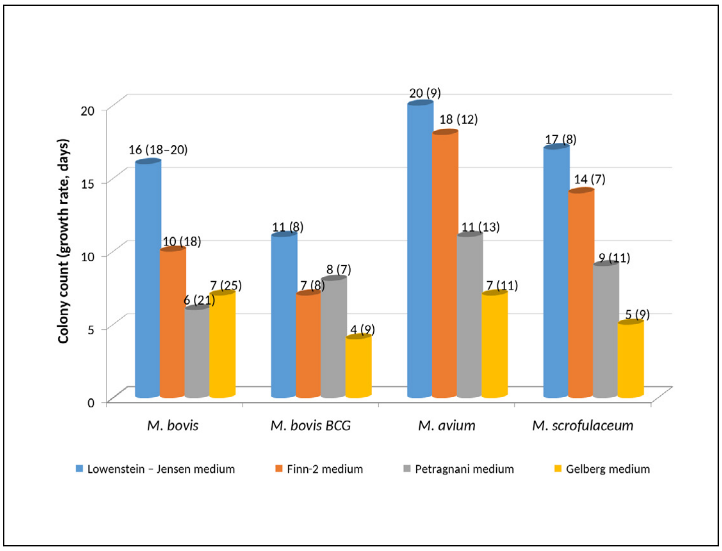

Fig. 5. Mycobacteria growth indicators in various culture media

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Received 17.12.2025
Revised 23.01.2026
Accepted 02.03.2026

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Contribution of the author: Baratov M. O. – formulation of key research objectives and tasks, testing, data collection, analysis and interpretation, design of graphical elements and tables, paper drafting.

Вклад автора: Баратов М. О. – формулировка ключевых целей и задач исследования, проведение исследований, сбор, анализ и интерпретация полученных данных, создание рисунков и таблиц, подготовка рукописи.