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Improvement of bovine tuberculosis diagnosis

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SUMMARY

Detection of animals with non-specific reactions to tuberculin is one of the major problems in bovine tuberculosis (TB) diagnosis. There is a need to find and improve methods for detection of the sensitization causes. This paper presents the results of comparative studies of different ways to stabilize red blood cells in order to obtain diagnosticums for indirect hemagglutination (IHA) test. The article describes the stages of red blood cells stabilization and sensitization and demonstrates the diagnostic significance of Fili stabilization method using formaldehyde as a fixative. The highest antibody titers (1:3000 and 1:4000) were received in hyper-immune sera of rabbits immunized with *Mycobacterium bovis* using a homologous diagnosticum. Practical importance of the sensitins homologous to the infection is shown during testing of 1,911 serum samples collected from animals of different categories (diseased; healthy and reacting to tuberculin; healthy and not reacting to tuberculin) with IHA test using diagnosticums produced from *Mycobacterium bovis* and *Mycobacterium fortuitum*. Based on the positive results of the IHA test, TB was diagnosed in 87.5% of animals originating from an infected farm during post-mortem examination. The results of the IHA test agreed with those of the intradermal tuberculin test in 37.7% of cases. Diagnostic antibody titers were found in 206 TB infected animals with no reaction to the intradermal test. However, the post-mortem examination revealed TB changes in internal organs. The obtained data suggest a possibility to use the IHA test to detect TB infected animals with non-specific reactions to tuberculin.

Key words: tuberculosis, indirect hemagglutination test, differentiation, cattle, standardization, antibodies, red blood cells, sensitization.

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К совершенствованию диагностики туберкулеза крупного рогатого скота

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РЕЗЮМЕ

Выявление животных с неспецифическими реакциями на туберкулин – одна из наиболее актуальных проблем в диагностике туберкулеза. Очевидна необходимость поиска и совершенствования методов для выявления причин сенсибилизации. В работе представлены результаты сравнительного изучения различных способов стабилизации эритроцитов с целью получения диагностикума для проведения реакции непрямой гемагглютинации. Отражены этапы стабилизации и сенсибилизации эритроцитов. Показана диагностическая значимость метода стабилизации Фили с использованием формальдегида в качестве фиксатора. Наиболее высокие титры антител (1:3000 и 1:4000) получены в гипериммунных сыворотках крови кроликов, иммунизированных *Mycobacterium bovis*, с гомологичным диагностикумом. Практическая значимость гомологичных заражению сенситинов показана при исследовании 1911 проб сывороток крови животных из хозяйств различных категорий (больные; здоровые, реагирующие на туберкулин; здоровые, не реагирующие на туберкулин) в реакции непрямой гемагглютинации с диагностикумами, изготовленными из *Mycobacterium bovis* и *Mycobacterium fortuitum*. В неблагополучном по заболеванию хозяйстве, на основании полученных в реакции непрямой гемагглютинации позитивных результатов, при проведении патологоанатомического вскрытия диагноз на туберкулез установили у 87,5% животных. Отмечено совпадение результатов реакции непрямой гемагглютинации с показаниями внутрикожной туберкулиновой пробы в 37,7% случаев. У 206 больных туберкулезом животных обнаружены диагностические титры антител при отсутствии реакции на внутрикожную пробу. Однако при проведении патологоанатомического исследования были выявлены изменения туберкулезного характера внутренних органов. Полученные данные указывают на возможность использования реакции непрямой гемагглютинации при выявлении больных туберкулезом животных с неспецифическими реакциями на туберкулин.

Ключевые слова: туберкулез, реакция непрямой гемагглютинации, дифференциация, крупный рогатый скот, стандартизация, антитела, эритроциты, сенсибилизация.

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INTRODUCTION

Often, in TB-infected cattle herds there is a certain number of undetected diseased animals even after a comprehensive diagnosis. There is data (although scattered) on the possibility to detect more TB-infected animals using serodiagnostic methods.

Various serological tests were used to detect specific antibodies in animals: agglutination, precipitation, complement fixation, indirect and direct hemagglutination tests, enzyme immunoassay and others. However, not all of them have found wide practical application due to their low effectiveness and frequent inconsistency with the intradermal tuberculin test results.

Indirect hemagglutination (IHA) test was first used for TB diagnosis by F. I. Awad [1], who used a polysaccharide extract from *Mycobacterium tuberculosis* strain H37Rv as an antigen. Polysaccharide antigen is adsorbed on sheep red blood cells (RBCs), which agglutinate with homologous antibodies of the test serum. The following can be used as an antigen: heated and dialyzed tuberculin [2–8]; tuberculin obtained by ultrasound exposure to mycobacteria [9, 10]; phosphatide fraction of lipids extracted from mycobacteria [11], etc.

Since animal blood serum contains heterogeneous hemagglutinins, various methods of their adsorption by depletion of test sera [12, 13] or by treatment of RBCs with a tannin [14], trypsin or papain [12] solution have been proposed.

There are several ways to stabilize red blood cells, different in the chemical nature of the fixative, and RBC treatment methods. Aldehydes (for example, formaldehyde, glutaraldehyde, acetaldehyde), hydroxal, osmium tetrachloride, and others can be used for RBC fixation. Most often, formaldehyde is used in concentrations from 0.5 to 20%. In some cases, its concentration in RBC suspension is increased gradually by dialysis or stepwise. The duration of RBC fixation with formalin ranges from 1.5–2.0 to 48 hours.

The opinions of researchers regarding the interpretation of diagnostic antibody titres detected in healthy and TB-infected cattle are contradictory. Thus, J. Komura et al. considers blood serum dilution 1: 40 [15] a diagnostic titre, while O. V. Martma – 1:160 [16], G. V. Dunaev – 1:32 [6], E. I. Buryak – 1:64 [17], N. P. Ovdienko – 1:16 [18], etc.

There are various techniques, which, to some extent, can also influence the results.

Thus, the use of different antigens, heterogeneous hemagglutinin adsorption techniques, RBC membrane stabilization and sensitization methods and test techniques leads to contradictory results, which can be clearly seen by different levels of antibody titers considered as diagnostic to distinguish between the diseased and healthy animals. Therefore, the diagnostic value of IHA test for TB diagnosis is debatable.

The aim of this work was to study various RBC membrane stabilization methods used for sensitization with polysaccharide antigen, to determine the practical value of IHA test using different diagnosticums and to compare it with other test methods used on farms with different TB epidemic situations.

MATERIALS AND METHODS

For the purpose of this study, a polysaccharide from *Mycobacterium bovis* and *Mycobacterium fortuitum* was obtained following the procedure: a weight of dry cells of each strain was ground to powder in a grinding mill. Polysaccharide fractions were extracted from the bacterial mass of *Mycobacterium* using an aqueous beta-naphthol alcohol solution with further cooling with cold (0 ... –2 °C) ethyl alcohol. Then the antigen was separated by centrifugation at 3–5 thousand rpm, washed twice with alcohol and ether, dried in a thermostat, rubbed and transferred to vials.

Hyperimmune sera were obtained from rabbits immunized with *M. bovis*, *M. bovis* bacillus Calmette – Guérin, *M. avium*, *M. scrofulaceum* using Freud method.

Chess-border antigen titration was performed using the hyperimmune sera.

1,911 serum samples were collected from healthy and TB-infected cattle, as well as from animals with non-specific reactions to tuberculin, and were tested with IHA test.

The test was performed according to the generally accepted method on Titertek plates, which allowed to reduce the component consumption significantly and to observe their exact dosage.

All the tests were conducted in strict accordance with the interstate standards for the care and keeping of laboratory animals GOST 33216-2014 and GOST 33215-2014, adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the European Union Council as of 22.09.2010 on the protection of animals used for scientific purposes.

RESULTS AND DISCUSSION

Comparative studies of RBC membrane stabilization methods for IHA test is an important element in improving the technology of preparing antigenic erythrocyte diagnosticums. There are several ways to stabilize red blood cells using mainly aldehydes as a fixative. There are no fundamental differences between them, since in all the cases the main criteria determining the quality of stabilization are the concentration of the fixative during treatment, fixative – RBCs ratio, the duration of treatment, and the temperature [19–22]. For the purposes of comparative testing, four methods were used, the details are provided in Table 1.

Stabilized under the conditions specified in Table 1, sheep RBCs were tested for spontaneous agglutination in inactivated serum and used for polysaccharide sensitization. RBC sensitization was performed by mixing various dilutions (from 1:500 to 1:4000) of polysaccharides with a 10% suspension of stabilized RBCs in 1:4 ratio and incubated at 38 °C for 12 hours with constant shaking in Vibrotherm shaking water bath. RBCs were washed twice and a 2% suspension was prepared in a phosphate-buffered saline solution with the addition of 1% normal inactivated adsorbed bovine serum. Depending on the dilution of polysaccharides intended for RBC sensitization (from 1:500 to 1:4000, at 1:500 interval), 32 variants of diagnosticums were prepared.

For antigen titration, 2 positive and 2 negative sera were used. They were diluted with phosphate-buffered saline solution in 1:4 ratio and inactivated in a water bath at 64 °C for 30 min. To adsorb heterogeneous hemagglutinins, a 5% suspension of sheep RBCs was added to each serum, kept in a water bath at 37–38 °C for one hour and shaken periodically. Then the RBCs were separated from the sera by centrifugation.

The test was performed on ELISA plates in accordance with the generally accepted procedure. Thus, the volume of the components was reduced tenfold. Each diagnosticum sample was tested with sera dilutions of up to 1:2048. Tests were performed both with negative and positive sera. Also, hyperimmune sera were used (from rabbits immunized with *M. avium* and *M. scrofulaceum*, one serum from each).

0.02 ml of a 2% suspension of sensitized red blood cells was added to each plate well containing 0.05 ml of the diluted sera. Serum in a dilution of 1:8 and 0.02 ml of diagnosticum (control of serum adsorption) was added to well 11.

0.05 ml of buffer + 0.02 ml of diagnosticum was added to well 12 (control of spontaneous agglutination of the erythrocyte diagnosticum). An antigen titer was considered a dilution, in which "+++" agglutination was observed with positive serum and "++++" with hyperimmune antiserum.

Testing of the sera collected from control and healthy animals originating from TB-free farms revealed no hemagglutinins reacting with the diagnosticum produced from *M. fortuitum* in any of the antigen dilutions. Hemagglutinins at 1:500 and 1:1000 titers were detected in 1:32 sera dilutions during RBC membrane stabilization by VIEV method (All-Russian Research Institute of Experimental Veterinary Medicine) using a diagnosticum produced from *M. bovis*. In 193 samples of sera obtained from animals known to be healthy, hemagglutinins in higher titers could not be detected. The highest antibody titers were obtained in hyperimmune sera of rabbits immunized with *M. bovis* using a homologous diagnosticum in dilutions of 1:3000 and 1:4000, with RBC membrane stabilization by Fili method. Antibodies were detected in sera dilutions of 1:4096. Hemagglutinins were detected in 1:1024 sera dilutions using diagnosticums prepared by three other methods of RBC membrane stabilization. At the same time, the difference between antibody titers in hyperimmune sera homologous to the *M. bovis* antigen and in heterologous sera was the highest when RBCs were stabilized using Fili method. Thus, when using a diagnosticum produced from *M. bovis* with a homologous serum, hemagglutination titer was 1:4096, while with the sera from rabbits immunized with *M. avium,* – 1:1024, *M. scrofulaceum* – 1:256 and *M. bovis* bacillus Calmette – Guérin – 1:1024.

The situation was opposite in IHA test with a diagnosticum produced from *M. fortuitum*. The highest antibody titers (1:2048) in this case were received in sera of rabbits immunized with *M. scrofulaceim*. Based on these results, a dilution of 1:1500 (double titer) was taken as an antigen titer, when stabilizing RBCs with Fili method.

IHA test using sera with known antibody titers was performed to standardize diagnosticums. Then the diagnosticums were filled in 5 ml ampoules and used for IHA testing, as needed.

The testing was performed in animals originating from four farms with different TB epidemic situation. By the time of the study 75.6% of animals coming from the first farm had demonstrated a positive reaction to the intradermal tuberculin test (tuberculosis isolation unit). On the second farm (which had been infected for a long time), health status was improved based on the systemic study results by isolation and further slaughter of positively reacting animals. 3.9% of animals from that farm resulted positive to the tuberculin test. On the third farm, positive animals were detected during the routine testing (6.5% in this study), but the autopsy and the laboratory testing of the materials obtained from those animals did not confirm the diagnosis. The fourth farm was free from the disease, with no animals reacting to tuberculin.

Table 1
Different methods and procedures for red blood cells stabilization

Таблица 1
Различные способы и режимы стабилизации эритроцитов

No.	Method	Fixative	Concentration of the fixative during treatment, %	Ratio of the fixative to red blood cells	Treatment duration, hours	Temperature, °C
1	Fili	Formaldehyde	5.7	0.32:1	2	37
2	Weinbach	Formaldehyde	1.5	0.375:1	18–20	37
3	Ling	Formaldehyde	4.4	0.75:1	24	4
4	VIEV	Glutaric aldehyde	2.5	0.5:1	18–20	37

1,911 blood samples were taken from animals on those farms to obtain sera, which was tested with IHA using diagnosticums produced from *M. bovis* and *M. fortuitum*. The results are presented in Table 2.

The results show an expressed difference between the number of positive animals detected with IHA test using a diagnosticum produced from *M. bovis* and the number of animals tested using a diagnosticum produced from *M. fortuitum* on the TB infected farms. On the first two farms, more serum samples with a diagnostic antibody titer were detected using a *M. bovis*-based diagnosticum than using a *M. fortuitum*-based diagnosticum. On the contrary, when testing sera from the third farm, a greater number of detections were obtained with a diagnosticum produced from *M. fortuitum*. At the same time, the IHA test conducted using a diagnosticum produced from *M. bovis* was positive only for 15% of the tested animals kept in the isolation unit.

On all the four farms, animals with positive and negative IHA results were euthanized for diagnostic purposes. TB was diagnosed in 87.5% of IHA positive animals from the disease-free farms. During the post-mortem examination and the laboratory testing of the biological material collected from animals with non-specific reactions to tuberculin, no changes in internal organs typical for tuberculosis were detected, and the disease was not diagnosed. In 22.1% of cases, cultures of atypical mycobacteria were isolated from the materials.

When comparing the results of studies obtained with the intradermal tuberculin test and IHA test, it was found that the consistency of the results was 37.7%. At the same time, diagnostic antibody titers were detected in 5 animals in the isolation unit and in 201 animals with no reaction to the intradermal tuberculin test on the infected farm. Post-mortem examination of the internal organs of 20 euthanized animals from the infected farm and of 5 animals from the isolation unit, revealed TB in 75 and 100% of cases, respectively. Consequently, IHA test enhances the ability of TB diagnosis.

CONCLUSION

Experimental studies and practical experience in the use of serological methods for TB diagnosis provide additional information on animal immunity. The data available on serological techniques (complement fixation test, indirect hemagglutination test, etc.) with the use of various antigens for the detection of TB-infected animals are contradictory and scattered. The results of this study show that the effectiveness of a serological technique depends on the antigen quality and the method of its use (in this case RBCs sensitization), and are consistent with the data obtained by other researchers. Studies have shown that the best way to sensitize RBCs is Fili method, which allowed higher antibody titers to be obtained with homologous sera as compared to the heterologous ones. It is logical to assume that IHA testing using various mycobacterial diagnosticums will reveal the cause of the macroorganism's sensitization to tuberculin. Testing of sera of animals sensitized with atypical mycobacteria showed that the number of animals positive in IHA test based on *M. fortuitum* diagnosticum was significantly higher than the number of animals positive in IHA test based on *M. bovis* diagnosticum. The opposite was observed in TB-infected farms – a relatively low effectiveness of IHA test of blood sera from TB-infected animals using a diagnosticum prepared from heterologous mycobacteria, which is probably associated with the developed TB process suppressing the immunity.

Thus, the presented results show that diagnosticums prepared from mycobacteria homologous to the infection are effective for the differentiation of non-specific reactions to tuberculin, and allow to expand the understanding of mechanisms for animal TB diagnosis improvement.

Table 2
Results of IHA tests of serum samples collected from animals originating from different farm

Таблица 2
Результаты исследования проб сывороток крови от животных из различных хозяйств в реакции непрямой гемагглютинации

Farm No.	Number of serum samples	Antibodies were detected using diagnosticums produced from:			
		M. bovis		*M. fortuitum*	
		quantity	%	quantity	%
1	180	27	15.0	2	1.1
2	1,016	206	20.3	73	7.2
3	522	43	8.2	79	15.1
4	193	–	–	–	–

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