EXPERIMENTAL ARTICLES

THE TRIAL OF EXPERIMENTAL TEST SYSTEM FOR THE SPECIFIC DIAGNOSTICS OF CATTLE TUBERCULOSIS

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The aim of the research was the experimental investigation of test-system on the panels of blood sera from healthy, leukemic, and experimentally infected with various *Mycobacterium* species cows. The results of serological testing of biological samples from cattle infected with different strains of *Mycobacterium* are presented. The analysis of informative indicators (sensitivity and specificity) of test system for the diagnosis of tuberculosis based on the determination of the immunoglobulins G to bovine tuberculosis pathogen *Mycobacterium bovis*, was done using chimeric recombinant protein MPT83(115-220)-MPT63 as an antigen. Above-mentioned recombinant protein is based on antigens of *M. bovis*/*M. tuberculosis* and achieves high sensitivity and specificity of the test system because it reduces the likelihood of false positive results caused by infection with atypical mycobacteria. The developed test system "IB-Chem Anti-Mycobacterium bovis" was tested at the State Scientific Control Institute of Biotechnology and Strains of Microorganisms and is recommended for use.

Key words: tuberculosis, cattle, recombinant proteins, test system.

Cattle tuberculosis is a serious problem for agriculture of Ukraine and many other countries. This infectious disease annually induces significant economic losses. At the same time, infections caused by *Mycobacterium bovis* not only affect husbandry and food industry, but also are dangerous to public health, because recently *M. bovis* has been frequently isolated as a tuberculosis pathogen (particularly in patients with various immune deficiencies). The situation is complicated by absence of an easy and accessible diagnostics method of cattle tuberculosis. This vastly impedes monitoring of infection and employing relevant preventative measures.

Serological tests due to their relatively cheap cost, quickness, reasonably high sensitivity and specificity are able to compete with a skin test during mass surveys of cows. The test system discussed in this paper is based on indirect ELISA for identification of immunoglobulins G specific to *Mycobacterium bovis*, the pathogen of cattle tuberculosis, using chimeric recombinant protein MPT83(115-220)-MPT63 as an antigen. It has been established that using only one antigen for detection of antibodies to tuberculosis pathogen is ineffective [1]. One of the approaches to increase test system sensitivity is using a combinations of several immunodominant antigens. But this presents serious technological differences because either the serum sample must be tested for specificity to each antigen separately (thus increasing the cost of analysis) or the combination of proteins is used as an antigen, complicating the the reproducibility and standardization of method. There is another, more effective solution: joining sequences of corresponding antigens into a single chimeric protein by combining fragments of their DNA in one reading frame. Combining multiple antigenic structures (epitopes) in one

molecule is an effective approach to creating new antigenic compositions. This allows to simplify the production of antigenic substances and efficiently control the epitope ratio in immunosorbent for the ELISA. The chimeric protein MPT83(115−220)-MPT63 which we previously synthetized [2] fits these parameters splendidly, and the new test system is based upon it.

Gene elements used in construction for the expression of the chimeric protein were chosen based on analysis of literature data. The gene *mpb*63 is found only in the genomes of *Mycobacterium* spp. which belong to the tuberculosis complex (a group of closely related species of mycobacteria with a high degree of homology and identity of 16S rRNA, and which can cause tuberculosis in humans and animals [3]), but is absent in *M. avium*. The genome of *M. leprae* includes a pseudogene *mpt*63, which is not translated. Also, MPB63 is a secretory protein of mycobacteria [4] which is synthetized in large quantities and is a highly potent antigen. MPB83 is characteristic of tuberculosis-inducing mycobacteria and is a homologue of another secretory protein of mycobacteria, MPB70 [5]. It has been known that in cows with tuberculosis, antibodies to MPB83 start occurring at the early stages of disease [6]. The feasibility of using these antigens for diagnostics was also confirmed in our previous studies [7].

The aim of this research was testing an experimental test system on panels of blood sera from conventionally healthy, experimentally infected with different types of mycobacteria, and leukemic cows.

Materials and Methods

Panel of referent serum samples, negative, and samples of cow blood serum infected with various mycobacteria were given by National Scientific Centre "Institute of Experimental and Clinical Veterinary Medicine" where the test system was tried serologically. Control studies of cow biological material were performed in State Scientific Control Institute of Biotechnology and Strains of Microorganisms. Industrial samples of immune-enzyme test system for cattle diagnostics tuberculosis were provided by Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine.

The chimeric protein based on recombinant analogues of *M. bovis* MPT63 and MPT83(115-220) was obtained and characterized in previous works [2]. The test system for diagnostics of tuberculosis, based on identification of IgG to *M. bovis* antigen MPT83(115−220)-MPT63, was constructed according to indirect ELISA. Statistical data analysis was performed using conventional methods.

Results and Discussion

According to the results of the study, it was established that test system IB-Chem Anti-Mycobacterium bovis is effective in diagnostics in animals infected with *M. bovis* proper. Aside from that, blood sera of cows infected with other *Mycobacterium* species (*M. intracellulare*, *M. fortuitum*, *M. avium, M. kansasii* and *M. paratuberculosis*) did not test positive by ELISA and according to the positivity index (PI) were identified as negative.

M. intracellulare, M. fortuitum and *M. intracellulare* are widely distributed species of *Mycobacterium* [8]. These strains induce diseases mostly in animals or patients with immune deficiencies. According to phylogenetic analysis, the aforementioned representatives together with *M. bovis* and *M. tuberculosis* are characterized by common antigens, therefore sensibilisation by atypical mycobacteria interferes with diagnostics based on heterogenic mixtures of antigens such as tuberculin.

Antigen components of chimeric protein MPT83(115-220)-MPT63 were identified in representatives of *M. bovis*/*M. tuberculosis*, thus providing the specificity of the test system for the diagnosis of *Mycobacterium tuberculosis complex* in cattle and possibly humans. Aside from that, absence of cross sections of these protein genes in *M. bovis*/*M. tuberculosis* compared to other mycobacteria species excludes false positive results. Thus, studying antibodies of animals infected by non-tuberculosis mycobacteria (NTM) is a necessary step of studying antigen composition in order to ascertain its usability as a component of tuberculosis diagnostics test system.

Not only cow blood serum samples infected with atypical mycobacteria were analyzed but also blood serum samples of cows which negatively react to tuberculin and in immunodiffusion reaction (IDR), and reacted positive to tuberculin for birds whose diagnoses of tuberculosis has not been proven and IDR-positive (leukemic) animals. According to the results obtained in National Scientific Centre "Institute of Experimental and Clinical Veterinary Medicine" these blood serum samples were characterized as negative.

The presence of delayed hypersensitivity reactions in cows to tuberculin for birds confirms the rationality of using serological

methods based on the ELISA to refute false positive reactions. Possible contact of cattle with birds infected with *M. avium* can impact the immunological state of the animal appearing as reaction to avian and cow tuberculins although the animal may not be in fact ill. False positive results of skin test that can be caused by that, negatively affect economic situation of farms, resulting in slaughtered healthy animals and the loss of invested money.

Previously the fusion protein MPB83(115–220)-MPB63 was tested for reaction to sera of animals immunized with vaccine strain *M. bovis BCG*. Exactly this vaccine induces false positive results of skin tests in humans. But these serums when tested with fusion antigen were negative, which supports the effectiveness and feasibility of using serological methods with specific antigen of mycobacteria.

Among animals with ascertained tuberculosis status, high response to fusion protein MPB83(115-220)-MPB63 was observed only in cows infected with *M. bovis* (1.083 ± 0.3407, $n = 11$), and not in animals infected with *M. intracellulare* (0.124 ± 0.0077, $n = 1$), *M. avium* (0.084 ± 0.0058, $n = 1$), *M. fortuitum* (0.124 ± 0.0035, $n = 1$), *M. kansasii* (0.123 ± 0.0007, $n = 1$), *M. paratuberculosis* ($0.042 \pm 0,0005$, $n = 1$), and those negative to tuberculin and IDR (0.115 ± 0.011,

$n = 23$), positive to avian tuberculin (0.128 ± 0.019, $n = 10$) and IDR-positive animals (0.119 ± 0.03, $n = 6$) (Fig. 1).

The obtained results indicate a high specificity of the immune-enzyme test system IB-Chem Anti-Mycobacterium bovis that we developed using the fusion protein MPT83(115-220)-MPT63 in diagnostics of cattle tuberculosis (Fig. 2).

According to the State Statistics Service of Ukraine, the number of cattle in Ukraine in January 2016 was 3.750.300 heads. However, at the first stage of implementation of the proposed model in veterinary practice monitoring epizootic situation with TB, it can be expected that ELISA will confirm or disprove the diagnosis established by means of an allergic test with tuberculin. Since a significant share of animals at modern farms can be sensibilized by atypical bacteria, there is a need to differentiate allergic reactions to tuberculin from para-allergic. This part of livestock may need advanced laboratory testing using ELISA.

In addition, ancillary diagnostic methods can be used in the selection of animals for slaughter or diagnostic determination of the causes of allergic reactions, including the anticipated use of ELISA (item 2.15 of the Order N 316 of 03.09.2009 "Instructions for prevention and control of animal tuberculosis",
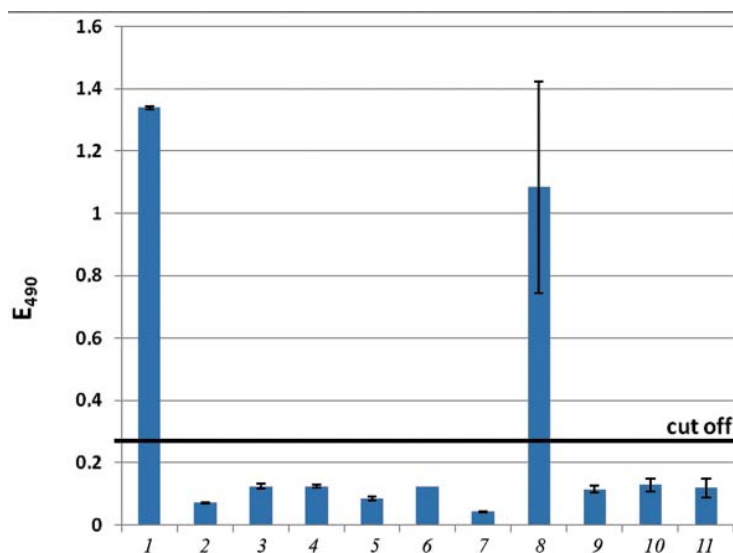

Fig. 1. **Level of IgG to target antigen MPT83(115-220)-MPT63 of the test system IB-Chem Anti-Mycobacterium bovis in control and samples of blood serum of cows with different diagnosed diseases:**
1 — positive control with *M. bovis*-specific cattle; *2* — negative control with negative cattle blood serum; *3* — *M. intracellulare*-infected; *4* — *M. fortuitum*-infected; *5* — *M. avium*-infected; *6* — *M. kansasii*-infected; *7* — *M. paratuberculosis*-infected; *8* — *M. bovis*-infected; *9* — negative to tuberculin and IDR; *10* — positive to avian tuberculin (tuberculosis diagnosis not proved); *11* — IDR-positive animals. Cut off for analyzing the test system is 0.27; limit level calculated by adding 0.2 to the mean negative control value (according to the manufacturer's instructions), thus limit value = OD K-*mean* (mean value of optical density of negative control) + 0.2. All results are significant, $P < 0.05$ compared to controls

Fig. 2. Industrial sample of "IB-Chem Anti-Mycobacterium bovis" test system for diagnostics of cattle tuberculosis:
a — registration certificate of the test system; *b* — the set of components and instructions for the industrial sample

approved by the State Committee for Veterinary Medicine of Ukraine).

Hence, serological diagnostic identification of antibodies to cattle tuberculosis pathogen *M. bovis*, particularly using solid-phase ELISA, is an important complementary method of monitoring cattle for tuberculosis since it allows timely detection of *M. bovis*-infected livestock, and latent carrier animals; in households troubled by tuberculosis it enables detection of animals anergic to tuberculin, which are the hidden sources of pathogen; during implementation of health measures it allows for successful control of epizootic situation.

Improvements in antigenic substance are to be proposed later to enhance performance specificity and sensitivity of the diagnostics, and also creation of rapid diagnostic tests for TB prevention.

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ВИПРОБУВАННЯ ЕКСПЕРИМЕНТАЛЬНОЇ ТЕСТ-СИСТЕМИ ДЛЯ СПЕЦИФІЧНОЇ ДІАГНОСТИКИ ТУБЕРКУЛЬОЗУ ВЕЛИКОЇ РОГАТОЇ ХУДОБИ

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Метою роботи було експериментальне випробування тест-системи на панелі дослідних сироваток крові від умовно здорових, експериментально заражених різними видами мікобактерій та лейкозних корів.

Подано результати серологічних досліджень біологічних зразків від інфікованих різними штамами мікобактерій тварин. Проведено аналіз показників інформативності (специфічності та чутливості) тест-системи для діагностики туберкульозу, що ґрунтується на визначенні імуноглобулінів класу G, специфічних до збудника туберкульозу великої рогатої худоби *Mycobacterium bovis*, із використанням як антигену химерного рекомбінантного протеїну МРТ83(115-220)-МРТ63. Рекомбінантний протеїн на основі антигенів *M. bovis*/*M. tuberculosis* дає змогу досягти високої чутливості та специфічності тест-системи, оскільки зменшує ймовірність хибнопозитивних результатів, спричинених інфікуванням атиповими мікобактеріями. Розроблена тест-система «IB-Chem Anti-Mycobacterium bovis» пройшла випробування у Державному науково-контрольному інституті біотехнології і штамів мікроорганізмів та рекомендована до використання.

Ключові слова: туберкульоз, велика рогата худоба, рекомбінантні протеїни, тест-система.

ИСПЫТАНИЕ ЭКСПЕРИМЕНТАЛЬНОЙ ТЕСТ-СИСТЕМЫ ДЛЯ СПЕЦИФИЧЕСКОЙ ДИАГНОСТИКИ ТУБЕРКУЛЕЗА КРУПНОГО РОГАТОГО СКОТА

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Целью работы было экспериментальное испытание тест-системы на панели исследованных сывороток крови от условно здоровых, экспериментально зараженных разными видами микобактерий и лейкозных коров. Представлены результаты серологических исследований биологических образцов от инфицированных различными штаммами микобактерий животных. Проведен анализ показателей информативности (специфичности и чувствительности) тест-системы для диагностики туберкулеза, основанный на определении иммуноглобулинов класса G, специфических к возбудителю туберкулеза крупного рогатого скота *Mycobacterium bovis*, с использованием в качестве антигена химерного рекомбинантного протеина МРТ83(115-220)-МРТ63. Рекомбинантный протеин на основе антигенов *M. bovis*/*M. tuberculosis* позволяет достичь высокой чувствительности и специфичности тест-системы, поскольку уменьшает вероятность ложноположительных результатов, обусловленных инфицированием атипичными микобактериями. Разработанная тест-система «IB-Chem Anti-Mycobacterium bovis» прошла испытания в Государственном научно-контрольном институте биотехнологии и штаммов микроорганизмов и рекомендуется к использованию.

Ключевые слова: туберкулез, крупный рогатый скот, рекомбинантные протеины, тест-система.