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Diagnostics Of Crown Cattle Tuberculosis Using An Immuno-Enzy Analysis

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ABSTRACT

The method of enzyme immunoassay in the diagnosis of bovine tuberculosis is promising both for determining the titer of specific antibodies in the blood serum and for detecting the tuberculosis antigen in the tissues of the test animals. One of the important problems in the diagnosis of tuberculosis by intracutaneous tuberculin test is the problem of nonspecific reactions to tuberculin. The urgency of this problem is increasing from year to year. In our studies for the differential diagnosis of bovine tuberculosis, we used enzyme immunoassay using modified DMSO antigens (DMSO-m). The antigens M.bovis, M, avium, M.scotochromogenes, M.nonchromogenes, M.phlei were used. In DMSO-m electrophoregrams, antigens have clearly marked fractions, most of which are in the molecular weight range of 46.5 - 38.5 kD. The results of the immunoblot analysis are shown. That the amount of immunogenic fractions in different antigens is different. Common for all types of mycobacteria seroactive fractions lie in the range of 40-60 kD and 20-35 kD. Also specific fractions come to light. An enzyme immunoassay test system was designed to detect antibodies in serum against mycobacteria of bovine, avian, and some atypical types of mycobacteria. Conducted large-scale production tests proved that the immunoassay test system is suitable and effective for differentiating nonspecific tuberculin reactions of cows and for early diagnosis of tuberculosis. As a result of the research, it was also established that the proposed test system gives positive results in cases of traumatic reticulitis and recarditis and in some invasions. It was established that enzyme-linked immunosorbent assay of blood sera of animals provides additional information for allergic studies, and the effectiveness of diagnostic activities increases.

Keywords: tuberculosis, cattle, immunofermental analysis, diagnosis, antigen, antibody.

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INTRODUCTION

The last decades have been a period of intensive development and introduction of enzyme immunoassay test kits and kits for the immunodiagnostics of infectious animal diseases into a widespread veterinary practice. The conditions for the ELISA are selected for each specific antigen-antibody system, since the results of the analysis are determined mainly by the nature and properties of the antigen. Therefore, starting with the first studies on the possibility of using ELISA in the diagnosis of tuberculosis (12), many scientists are working to find a specific fraction of tuberculosis bacteria suitable for use in analysis as a test antigen and to obtain a highly specific immune serum. For these purposes, extracts from mycobacterial culture filtrates were used [7, 8, 12], ultrasonic extracts of mycobacterial cells were used [6], and PPD-tuberculins were used [1,11]. In recent years, highly purified protein, glycoprotein, glycolipid and recombinant antigens have been used to study the immune status of animals and the differential diagnosis of tuberculosis using immune responses. [4, 16, 17], including antigens extracted with organic solvents [3].

In general, the method of enzyme immunoassay in the diagnosis of bovine tuberculosis is promising both for determining the titer of specific antibodies in the serum and for detecting the tuberculosis antigen in the tissues of the test animals. However, the available literature data indicate the inconsistency of the diagnostic value of ELISA, which is connected, on the one hand, by the difference in the antigens used and the low specificity of the used immune sera on the other [2, 5, 10, 13, 14].

The use of this method for the diagnosis of bacterial infections makes it difficult for a number of objective reasons, which include the extremely complex antigenic structure of bacteria compared to viruses.

In the diagnosis of bovine tuberculosis, the main, mass and generally accepted method is the intradermal test with the use of PPD - tuberculin for mammals. One of the important problems in the diagnosis of tuberculosis by intracutaneous tuberculin test is the problem of nonspecific reactions to tuberculin. The urgency of this problem is increasing from year to year. In our studies for the differential diagnosis of bovine tuberculosis, we used enzyme immunoassay using modified DMSO antigens (DMSO-m). The antigens M.bovis, M, avium, M.scotochromogenes, M.nonchromogenes, M.phlei were used.

MATERIALS AND METHODS

The protein spectrum of antigens was studied by electrophoresis. Disk electrophoresis was carried out according to the method of Laemli (1970) in a plate-like polyacrylamide gel using sodium dodecyl sulfate detergent and a gap reducing agent by S-S bonds - β meccaptoethanol. The molecular masses of the proteins contained in the antigenic preparations were determined in accordance with the distillation of protein standards by the logarithmic curve of the path length of the markers according to K.Weber, M.Osborn (1969) and N.M. Filipovich (1978), and also instrumentally using optical scanning and densitometry with further computer processing of data using the program Image master. Fractionated gels were stained with 0.25% Coomassie G-250 (Lamb et al., 1976), silver nitrate (Tsai, 1986).

The electric transfer of proteins fractionated in PAAG to the nitrocellulose membrane was carried out according to the method described by Towbin et al. (1989). The polyacrylamide gel was immersed in Tris buffer pH 10.5 for 30 minutes, the nitrocellulose membrane was kept in Tris-glycine buffer pH 8.8 - 5 minutes, after which time the sandwich was collected (filter paper - gel - NCM - filter paper) and placed between the anode and cathode. The transfer took place for 2.5 hours at a voltage of 100 V and a current of 100 mA per plate with cooling using refrigerants (Coolpack MC-3 Iaminarmedica).

Enzyme-linked immunosorbent assay (ELISA) to determine the specific antibodies to mycobacteria was carried out in an indirect variant on polystyrene plates for immunological reactions according to the method described by Woller A. et al. (1976) in a solid non-competitive option. Used polystyrene plates for enzyme immunoassay production "Medpolimer" (St. Petersburg).

100 µl of solutions of antigens in 0.01 M carbonate buffer (pH 9.6) were added to the wells of the plate and incubated for 16–18 hours at room temperature. After washing for 3 times with the washing solution, 100 µl of the test and control sera diluted in phosphate-buffered saline (PBS) with pH 7.3 were added to the wells. Incubated for 1 hour at 37 ° C and washed 3-4 times. After that, 100 µl of the conjugate diluted in

the same buffer was added to the wells. Incubated the plate for 30 minutes at 37 ° C, washed the wells of the plate at least 5 times, and added 100 µl of the substrate solution to each well. After keeping the plate for 15 minutes at 37 ° C, the reaction was stopped by adding 50 µl of 2N sulfuric acid solution to each well.

Recording of the results of the ELISA reaction was performed on a vertical scanning spectrophotometer. To assess the results of the reaction, the specificity coefficient (K) was used, which is equal to the ratio of the optical density of the test (OP0) to the optical density of the control sample (OPC). Samples with K over 2 were considered positively reactive at 1: 200 dilution.

RESULTS AND DISCUSSION

The ultimate goal of this work is to create a diagnostic test that would allow for the differentiation of antibodies to tuberculosis pathogens from antibodies to atypical mycobacteria and to individual structures of mycobacteria.

The initial task was to study the protein profile of the antigens used by electrophoresis. DMSO-m antigens have clearly marked fractions, most of which are located in the molecular weight range of 46.5 - 38.5 kD (Fig. 1)

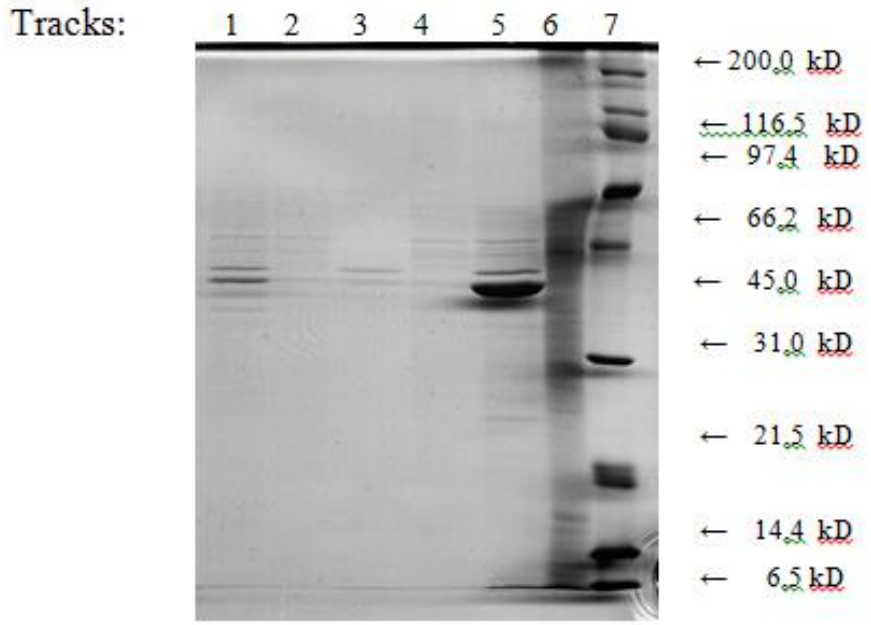

Fig.1. Electrophoretic profile of DMSOm antigens of Mycobacterium.

Legend: Tracks: 1-4, respectively, DMSO-m antigens from M.bovis, M.avium, M.non., M.scot. Tracks: 5-tuberculin; 6-complete cell lysate M.tuberculosis; 7 protein markers.

The detection of the protein spectrum after the fractionation of antigenic preparations in PAAG and the study of the serological activity of electrophoretic components were also carried out after transfer of proteins to membrane filters by immunoblot analysis. Immunoblot analyzes of DMSO antigens were performed with immune sera against homologous antigens (Fig. 2) and with sera from animals with tuberculosis (Fig. 3).

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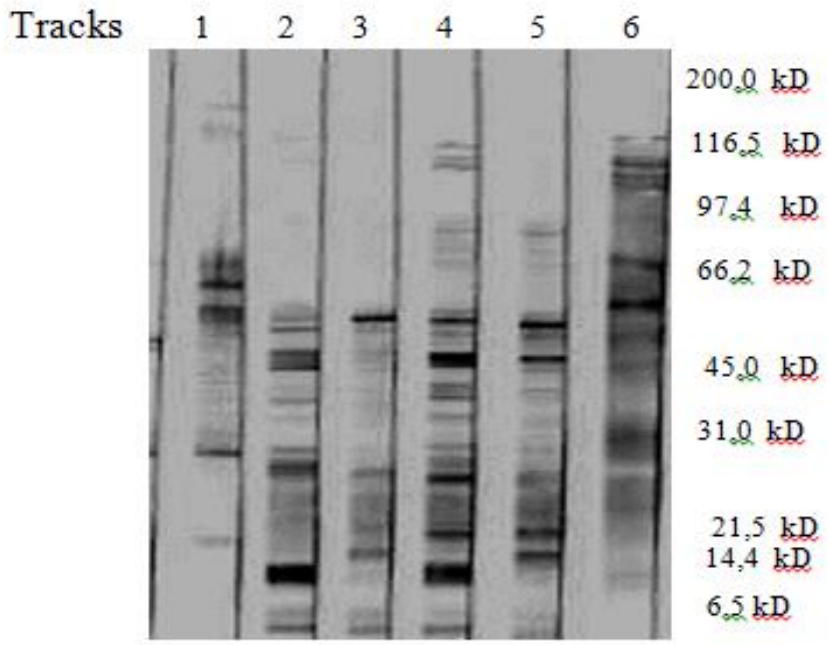

Fig.2. Immunoblots of DMSO antigens obtained from autoclaved killed mycobacteria with immune sera against homologous mycobacterial antigens.

Legend: 1 - M.bovis; 2 - M.avium; 3 - M.scotochromogenes; 4 - M.nonchromogenes; 5 - M.phlei; 6- M. tuberculosis.

As can be seen from the presented figure, the sulfur-active fractions of DMSO antigens mainly range in the range from 20 to 65 kD. The amount of immunogenic fractions in different antigens is different. Common for all types of mycobacteria seroactive fractions lie in the range of 40-60 kD and 20-35 kD. Also specific fractions come to light. In DMSO antigen M. bovis, 5 common fractions for all types with molecular weights of 25. 20, 45, 50, 97 kD are found. The fraction of 60 kD molecular weight is characteristic only for this type of antigen. It can be assumed that the isolation of this fraction in its pure form and its use in serological reactions can increase the specificity of methods for the diagnosis of tuberculosis.

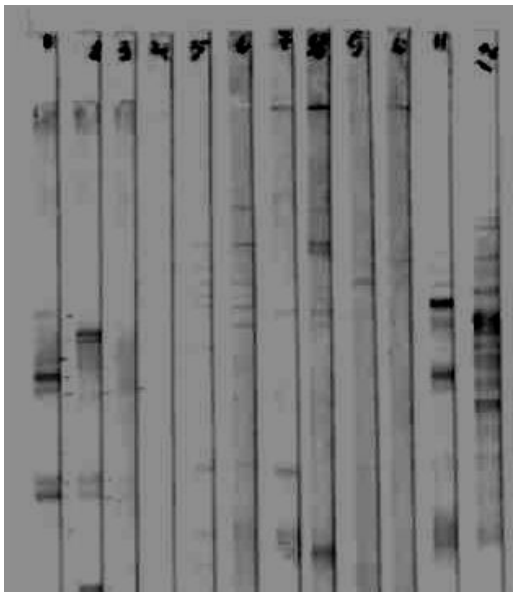

Fig.3. Immunoblots obtained in reactions with sera from cows reacting to PPD tuberculin with DMSO antigen M. bovis.

As it was said, all serum samples were obtained from cows positively responding to PPD-tuberculin, however, in the figure we see that only samples No. 2 and 12 have major and samples No. 6, 8, 11 are minor fractions on the 60 kDa antigen area.

The results of the immunoblot presented in Figure 3 demonstrate the capabilities of the immunoblot analysis in the diagnosis of bovine tuberculosis.

In order to test the obtained antigens in ELISA, samples of blood sera of cows from various farms of the Republic of Tajikistan were examined. A total of about 5,000 samples from farms in 12 districts were investigated. The results of studies in some areas are presented in Table 1 and 2. Table 1 shows the results of allergic studies and ELISA using the M.Bovis antigen of 2539 serum samples from farms in 8 areas. Of these, 600 (23.6%) of the responding, 1939 (76.4%) of non-responding cows.

Table 1 - Results of the study of cattle blood sera from farms of the RT different from the epizootic situation

Enterprises	Total sampling	(+) on PPD	In ELISA		(-) on PPD	In ELISA	
			(+)	(-)		(+)	(-)
Drozhzhanovsky	303	143	131	12	160	41	119
Laishevsky	1269	9	9	-	1260	86	1174
Aznakaevsky	138	38	6	32	100	2	98
V.Uslonsky	160	110	6	104	50	3	47
Kuybeshevsky	110	10	5	5	100	27	73
Alkeyevsky	153	153	59	94	-	-	-
Zelenodolsky	267	25	5	20	242	2	240
Cheremshansky	139	112	97	15	27	5	22
TOTAL:	**2539**	600	318	282	**1939**	**166**	1773
%		23.6%	12.5%	11.1%	**76.4%**	**6.5%**	69.8%

According to the results of research, tuberculosis was established in the farms of the Drozhzhanovsky, Laishevsky and Cheremshan districts. In most cases, in these farms, cattle with tuberculosis did not respond to tuberculin, and the ELISA results of serum samples were positive.

For a more complete picture of the possibilities of ELISA in diagnosing cattle tuberculosis and determining the location of this method in a complex of diagnostic measures, a control slaughter of animals was carried out taking into account the data of an allergic sample and the results of ELISA of blood serums and bacteriological studies in the Republican veterinary laboratory. The results of postmortem studies of 36 cows unresponsive to tuberculin, but positive in ELISA, showed that 18 heads have changes in the lymph nodes that are characteristic of tuberculosis.

The results of the pathoanatomical study and bacterioscopy of the material from 12 cows reacting to tuberculin, but negative in the ELISA confirmed the absence of the causative agent of tuberculosis in 10 of them.

It should be noted that in 23 farms, where for the last three years no animals responding to tuberculin were detected, the results of the enzyme immunoassay were negative.

In order to identify the specificity of DMSO antigens, samples of cattle blood serum were tested for the presence of antibodies against various types of mycobacteria.

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Table 2 - IFA for the detection of mycobacterial antibodies in blood serum from tuberculin-responsive cows (summarized by region).

Areas	Total samples	Number of samples reacting with antigens:				
		M.bovis	M.avium	M.scot	M.non	M.phlei
Nizhne-Kamsky	54	-	-	1	8	15
Verkhne-Uslonsky	113	-	21	47	12	-
Bavlinsky	27	-	-	10	4	-
Buinsky	113	37	21	2	13	-
Tyulyachinsky	51	-	33	5	7	-
Zainsky	66	-	2	17	3	1
Kaybitsky	758	121	43	16	37	1
Alkeyevsky	67	-	3	2	27	-
Spassky	53	8	24	-	12	-
Novoshishminsky	493	253	38	1	75	-
Yutazinsky	117	-	-	-	-	11
Kamsko-Ustyinsky	114	4	11	67	7	-
Zelenodolsky	110	-	-	46	22	-
Nurlatsky	620	26	78	21	67	-
Laishevsky	194	62	12	3	8	-
TOTAL	2950	511 17.3%	286 9.7%	238 8.1%	302 10.2%	18 0.6%

Bacteriological methods of research by identifying a pure culture of mycobacteria was established tuberculosis of cattle in farms Buinsky, Novoshishminsky, Kamsko-Ustyinsky, as well as in the farms of Laishevsky and Kaibitsky areas. In the enterprise "Alan" of the Tyulyachinsky district, the cause of the tuberculin reactions of animals was the infection of the mycobacteria of the avian species. In some farms in the Buinsky, Bavlinsky and Zelenodolsky districts, analysis of the studies showed that the basis of tuberculin reactions in animals is a polyetiological factor - helminths, reticulo-peardardites, etc. cattle are not associated with tuberculosis.

In the agrofirm of Nizhnekamsk region, the reaction to PPD tuberculin was established in 54 cows. With the differentiation of a nonspecific reaction to tuberculin, a simultaneous allergic test, the reaction to CAM allergen in 110 heads. Laboratory studies of a biomaterial for isolating a pure culture isolated acid-resistant mycobacteria from the yellow-orange color of the colonies. By identification, they were assigned to the group of rapidly growing atypical mycobacteria, which fully confirms the results of the enzyme immunoassay.

Paraallergic reactions to tuberculin, caused by sensitization of the organism by atypical mycobacteria, were also found in a similar way in the farms of the Kama-Ustyinsky district.

CONCLUSION

Recently, both in veterinary medicine and in medicine, various technologies have been proposed for detecting anti-tuberculosis antibodies using enzyme-linked immunosorbent assay (ELISA). But for the present there is not enough convincing serological methods that could be used for differential diagnosis and determination of the degree of activity of tuberculous changes.

The proposed mycobacterial antigen can be used in ELISA technologies for the in vivo differentiation of nonspecific tuberculin reactions caused by atypical mycobacteria. Using the obtained antigen, an enzyme immunoassay test system was designed to detect antibodies in the serum against mycobacteria of bovine, avian, and some atypical types of mycobacteria. Conducted large-scale production tests proved that the immunofermental test system is suitable and effective for differentiating nonspecific tuberculin reactions of cows, for early diagnosis of tuberculosis and for monitoring the epizootic situation of this infection. A number of domestic and foreign researchers make similar conclusions [11, 9, 15].

As a result of research, it has also been established that the proposed test system gives positive results against all mycobacterial antigens in cases of traumatic reticulitis and perekardita and with some invasions.

Thus, our research has established that an immuno-enzyme analysis of the blood serum of animals provides additional information for allergic studies. The effectiveness of diagnostic measures is increasing, which is reflected in an increase in the reliability of identifying animals with tuberculosis.

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