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Using monoclonal antibodies in developing ELISA for diagnosing bovine tuberculosis

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ABSTRACT

Three stable strains of hybridoma cells (2H10, 3A8, 1B6) producing monoclonal antibodies (Mab) reacted with Protein Purified Derivative (PPD) *Mycobacterium bovis* were obtained. They were incapable of binding to PPD *M. avium* and whole cells *M. bovis, M. kansasii, M. scrofulaceum* as well as to their soluble and sonicated antigens. PPD *M. bovis* was attached to the solid phase via Mab which were directed to its protein with a molecular weight of 66 kDa. The diagnostic efficiency of sandwich ELISA was tested as compared with skin test. Blood samples were obtained from 849 cows belonging to farms unaffected and farms affected by tuberculosis. Ninety cows (10.6%) reacted to PPD *M. bovis*, but among them only 22 cows (2.6%) had a positive ELISA. Average titre of *M. bovis* specific antibodies detected in serum samples by ELISA was equal to 1:920 – 1:6400. Diagnostic value of ELISA and skin test was determined by autopsy of 12 cows. There were *M. bovis* specific antibodies at rather high titres in the blood samples of 9 cows which showed positive to skin test. Three cows developed a response to PPD *M. bovis* but in the blood samples of these animals we failed to detect any specific antibodies. Results of autopsy showed that all cows with positive ELISA and skin test had pathological findings in lungs and lymphatic nodes specific for tuberculosis, but internal organs of cows reacted only to skin test had no alterations. The results of our research testify to the high specificity and efficiency of Mab-based sandwich ELISA in isolating animals infected with tuberculosis and represent an evaluation of the actual epizootic situation.

Key words: *Mycobacterium bovis*, monoclonal antibodies, ELISA, skin test, cows

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Introduction

Cattle tuberculosis remains one of the main problems of veterinary medicine in the countries of Central Asia, including Kazakhstan. Skin (allergic) test is the main method of diagnosis for cattle tuberculosis. Protein Purified Derivative (PPD), or so-called tuberculin - the metabolic product of *Mycobacterium bovis* - is usually used as antigen in skin test.

Unfortunately, it is not specific and often shows false positive results because of cross-reacting atypical *Mycobacterium* sp. which are widespread in the environment and have many common antigenic epitopes with the causative agent of the tuberculosis. Also, skin test cannot identify all cattle infected with tuberculosis. This creates difficulties for veterinarians in evaluating the real epizootic situation by skin test and leads to the unjustified slaughter of healthy animals. This is why it is very important to conduct research directed to the development of new tests which might increase the efficiency of the tuberculosis eradication programme.

ELISA is widely used in developing diagnostic methods for particularly dangerous infectious diseases, including tuberculosis (ENGVALL and PERLMAN, 1971; DANIEL, 1986; HARBOE and WILKER, 1990). However, the whole cells of *M. bovis* and polyclonal antibodies which are used in ELISA reduce its specificity during the period of serological and bacteriological assays. Insufficient specificity is one of the major obstacles for the application of ELISA in practice.

The production of monoclonal antibodies (Mab) against *M. bovis* specific antigens should be an appropriate method in overcoming these difficulties. The series of Mab against the species of genus *Mycobacterium* were obtained by GILLIS and BUCHANAN (1982), BRITTON et al. (1985), SHOU et al. (1985), and ANDERSEN et al. (1986). DANIEL and OLDS (1985) described the properties of Mab to antigen - 5 *M. tuberculosis* with a molecular weight of 35 kDa. Three clones of hybridomas producing Mab to whole cells of *M. bovis* have been described (ANDROSOVA et al., 1996), but their role in diagnosing bovine tuberculosis have not been reported.

This report describes the production and characterization of three Mabs against PPD *M. bovis*, as well as the results of developing serological test employing Mab in a sandwich ELISA for diagnosing bovine tuberculosis.

Materials and methods

Animals. The present study was carried out on herds in tuberculosis affected and unaffected farms located within a radius of 30 km of Astana during the period 1998-2001. Blood samples were collected from 849 cows using sterilised sample bottles, with the usual aseptic precautions being taken. The animals were brought to the Department of Veterinary Medicine, Agrarian University.

Antigens. M. bovis 8, *M. bovis* BCG, *M. tuberculosis* H37Rv, *M. avium, M. kansasii, M. phlei, M. scrofulaceum* were grown on Petraniani agar for 48 to 72 hours at 37 °C. Bacteria were inactivated by incubation for one to four hours at 120 °C. The cells were collected by centrifugation (20 minutes, 6.200 rpm) in a table centrifuge and immediately inactivated. Control of inactivation was performed by plating bacteria on the corresponding agar plates and incubating at 37 °C for seven days. After inactivation, cells were washed three times with phosphate-buffered saline (PBS). Thereafter, cells were re-suspended in the same buffer to a concentration of about $1x10^{-6}$ cells/ml.

PPD *M. bovis* was produced by Kursk Biofactory (Russia) and the other tuberculinsm, such as non-albumose tuberculin (NAT) *M. bovis* 8 and culture filtrate protein (CFP) *M. bovis* 8, were kindly donated by Dr. B. Kerimzhanova, of Kazakh Veterinary Research Institute, Almaty, Kazakhstan.

Lipopolysaccharide (LPS) *M. bovis* 8 was prepared from aqueous phenol extract (WESTPHAL and YAN, 1967). The sonicated antigen (SA) *M. bovis* 8 was centrifuged to pellet cell debris and the supernatants removed and stored in aliquots at –70 °C until required.

Production of monoclonal antibodies. 4-8 week-old BALB/c female mice (AMS, Moscow, Russia) immunized with 0.2 ml of the suspension of PPD, LPS and SA separately six times at intervals of one-week duration. The last immunization was three days before cell fusion.

Mab were obtained by fusion (Polyethylene glycol, MW 4000, Merck) X63-Ag8.653 myeloma cells with the spleen cells of immunized mice. Cells were mixed in a ratio of one part of myeloma cells to five parts of spleen cells from immunized mice. Fusion and cloning were performed as

described by OI and HERZENBERG (1980). The fusion mixture was directly transferred to 96-well microtitre plates with feeder cells containing HAT-medium. The RPMI-1640 medium (Flow Laboratories) containing $NaHCO_3$, HEPES, Na pyruvate, glutamine and 10% heat inactivated foetal calf serum was used. Cells were distributed onto the microtitre plates. Between 10 and 20 days post-fusion, supernatants from wells containing hybridomas were tested for their antibody production by ELISA using peroxidase-labelled affinity–purified goat-anti-mouse IgG (Nordic). The OD 488 was determined in a Dynatech photometer (Dynatech, Denkendorf, Microelisa Reader). ELISA were performed on Immunlon microtitre plates (Dynatech). Selected crude clones were sub-cloned by limiting dilution. Cells with positive supernatants were transferred to 24-well macrotitre plates and then to 75 ml-tissue culture flasks.

For the production of ascitic fluid, female BALB/c mice were given an intraperitoneal injection of 0.5 ml 2,5,10,14, tetramethylpentadecane (Pristane, Aldrich Chemical Corp.). Fourteen days prior to the injection of $2x10^{-6}$ viable hybridoma cells and ascite fluids were clarified by centrifugation and Mab were recovered by precipitation with 45% ammonium sulphate and stored at either 4 ^{0}C or -70 ^{0}C in PBS containing 0.01% sodium azide. Protein concentration was determined by the Coomassie blue method (BRADFORD, 1976).

Isotyping and affinity of Mab. Antibody classes of Mab were determined in ELISA using PPD *M. bovis* as antigen and peroxidase-labelled affinity purified goat-anti-mouse IgG (Nordic). Absorbance of the supernatants at 492 nm was measured on Titretek Multiskan Spectrophotometer. Affinity of Mab was established by BEATTY et al. (1987).

Western blot analyses. Sodium dodecilsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by LAEMMLI (1970). The bands of proteins were transferred into nitrocellulose sheets by TOWBIN et al. (1979).

Sandwich ELISA. Microtitre plates were coated with Mab (5 mg/ml) in bicarbonate buffer (pH 9.6) for 1 h at 37 ^{0}C. After washing three times with PBS with 0.05% Tween (PBS/T), a suspension of PPD *M. bovis* was added at a concentration of 10 mg/ml for 1 h at 37 ^{0}C. After the washing

procedure, a serum under test in PBS/T was added at an initial concentration of 1:200 and diluted twofold from well to well up to 1:800 and allowed to incubate for 1 h at 37 ^{0}C. After washing, 0.1 ml of a dilution of peroxidase conjugated rabbit anti-bovine immunoglobulin (Dakopatts, Denmark) was added and incubated for a further hour at 37 ^{0}C for 1 h. Finally, after washing, activated orthophenylendiamin substrate was added and the colour reaction was recorded at 450 nm on a titretek Multiskan.

Results

After fusing the cells of one spleen with an appropriate amount of myeloma cells, the mixture was distributed into 12 microtitre plates. After 10 to 20 days, colonies of fused cells became visible. Supernatants from 624 wells were tested in an ELISA against PPD, LPS or SA *M. bovis*. A total of 52 wells (8.3%) was originally identified as positive (Table 1).

Table 1. Hybridization of immune spleen cells with myeloma

Antigens used for immunization	Number of sowed wells	The wells in which the growth of clones was observed		Clones producing monoclonal antibodies	
		Number	%	Number	%
PPD *M. bovis*	384	236	61.5	21	8.9
LPS *M. bovis* 8	384	187	48.7	12	6.4
SA *M. bovis* 8	384	201	52.3	19	9.5
Total	1152	624	54.2	52	8.3

As shown in Table 1, the best results of cell hybridization were noticed when lymphocytes of mice stimulated with PPD *M. bovis* were used as partner cells. The growth of hybridomas was observed in 236 wells. This means that the percentage of hybridization was rather high (61.5%). In the case of using the lymphocytes of mice immunized with SA *M. bovis* 8 and LPS *M. bovis* 8, the percentages of infusion were equal to 48.7% and 52.3%, respectively. Antibodies directed to PPD *M. bovis* were established with the help of ELISA in cultural supernatants of 21 hybridomas and antibodies specific to SA *M. bovis* 8 and LPS *M. bovis* 8 were detected in supernatants of 12 and 19 hybridomas, respectively.

With the purpose of selection of clones with stable production of Mab their antibody activity was tested by ELISA five times at intervals of 3-4 days. It was established that by the time of the fourth and fifth test in supernatants of 5 clones (1A1, 3H7, 4C10, 2B5, 2Ń10) antibodies against PPD *M. bovis* had not been detected. Antibodies of the clones 1B8, 2H4, 2E2, 2E3 and 1B7 during the period of screening were revealed at low titres (1:2 - 1:4). Immunoglobulins produced by clones 1B6, 3A8 and 2H10 had the highest antibody activity. In the supernatants of these hybridomas the antibodies were tested until titre 1:64. The other 8 clones also showed stable production of specific antibodies with titres from 1:8 to 1:32.

The production of antibodies by the other 12 clones positive with reference to LPS *M. bovis* 8 were observed during three tests. By the fourth test, however, 50% of hybridomas had lost their ability for interaction with the antigen and finally, at the last antibody activity control, all clones proved to be negative. The positive clones obtained in the result of hybridization myeloma cells with lymphocytes stimulated with SA *M. bovis* 8 also had lost their activity by the fifth screening.

Table 2. Cloning of hybridomas producing Mab to PPD *M. bovis*

Designation of clones	Number of subclones	Number of subclones producing Mab	Activity of subclones %	Maximum titres of Mab
3A8	34	30	88.2	1:64
2H10	36	34	94.4	1:64
1B6	30	28	93.3	1:32
Total	100	92	92.0	1:32-1:64

The most active and stable clones (3A8, 2H10, 1B6) were subjected to cloning by limiting dilution (Table 2).

The results of cloning showed a rather high genetic homogeneity of the selected clones, as from 88% up to 94% sub-clones kept the production of immunoglobulins specific to epitopes of PPD *M. bovis*. The maximum titres of sub-clones supernatants were equal to 1:32 - 1:64.

Among sub-clones of above mentioned hybridomas for further research, 3 strains designated as 3A8G6, 2H10B3 and 1B6G6 were selected. They were subjected to a second cloning. It is necessary to stress that all the

secondary sub-clones of the hybridomas demonstrated 100% antibody activity.

The activity and specificity of Mab produced by strains 3A8G6, 2H10B3 and 1B6G6 was detected by ELISA against different antigens of the species of genus *Mycobacterium* (Table 3).

Table 3. Specificity of Mab to *Mycobacterium* sp. antigens

Antigens of *Mycobacterium* sp.	Titres of monoclonal antibodies producing by the strains of hybridomas		
	2H10B3	3A8G6	1B6G6
Tuberculins			
PPD *M. bovis*	1:204 800	1:204 800	1:102 400
PPD *M. avium*	1:100	1:100	1:100
NAT *M. bovis* 8	1:1600	1:1600	1:400
CFP *M. bovis* 8	1:51 200	1:102 400	1:3 200
Soluble antigens			
LPS *M. bovis* 8	NR	NR	NR
SA *M. bovis* 8	NR	NR	NR
Whole cells			
M. bovis 8	1:400	1:800	1:400
M. bovis BCG	NR	NR	NR
M. bovis SibNIVI	NR	NR	NR
M. tuberculosis H37 Rv	NR	NR	NR
M. kansasii	NR	NR	NR
M. phlei	NR	NR	NR
M. scrofulaceum	NR	NR	NR

NR - negative result

Data presented in Table 3 testify that Mab 3A8G6 and 2H10B3 proved to be more active when compared with Mab 1B6G6. Antibodies produced by hybridomas 3A8G6 and 2H10B3 also find similar epitopes in the structure of CFP *M. bovis* 8, but Mab 1B6G6 bind with the latter antigen more weakly (1:3200) as compared with PPD *M. bovis* (1:102 400). Epitopes of NAT *M. bovis* proved to be less suitable to the active sections of Mab 3A8G6 and 2H10B3. Antibodies of these hybridomas reacted weakly with the whole cells of *M. bovis* 8 (1:400 - 1:800) and did not bind to the whole cells *M. bovis* SibNIVI and *M. bovis* BCG, as well as *M. tuberculosis* H37Rv. It is important to note that in the structures of PPD *M. avium*, as well as of the whole cells of atypical *Mycobacterium* sp. *(M. phlei, M. scrofulaceum)* there were no epitopes, which were recognized

by antibodies of these hybridomas. Additionally, it is necessary to stress that obtained Mab did not bind to the SA and LPS *M. bovis* 8. These data testify to their specificity to the epitopes, which are located in the structure of PPD *M. bovis*.

All types of Mab belong to subclass b of IgG. The index of affinity Mab 3A8G6 and 2H10B3 were rather high - $1x10^{-9}$ M and $1x10^{-8}$ M, respectively. Mab 1B6G6 have low affinity compared with the above mentioned antibodies - $1x10^{-7}$ M. The samples of PPD *M. bovis* in the SDS-PAGE were separated into 7 bounds of proteins with molecular weights of 13, 116, 95, 80, 66, 45, 28 kDa. Western blot analysis showed the specificity of all three types of Mab to the epitope located in the structure of protein with a molecular weight of 66 kDa (not shown). On the whole, the characteristics of obtained Mab testify to the possibility of using them in developing ELISA for diagnosing bovine tuberculosis.

Mab 3A8G6 were used in a double antibody sandwich ELISA for the detection of specific antibodies in the blood samples of cows infected with tuberculosis.

Sandwich ELISA was tested for the serological assays of the blood samples obtained from cows of tuberculosis affected and unaffected farms as compared with skin test and indirect ELISA (Table 4).

Table 4. Diagnosing bovine tuberculosis by ELISA and skin test

The state of farms	Number of tested cows	Number of cows showed positive results in		
		Indirect ELISA	Sandwich ELISA	Skin test
Affected	50	8 (16,0%)	6 (12,0%)	12 (24,0%)
Affected	312	10 (3,2%)	4 (1,3%)	26 (8,3%)
Affected	93	27 (29,0%)	7 (7,5%)	36 (38,7%)
Affected	69	2 (2,9%)	2 (2,9%)	4 (5,8%)
Affected	270	2 (0,7%)	3 (1,1%)	9 (3,3%)
Unaffected	40	0	0	1 (2,5%)
Unaffected	15	1 (6,7%)	0	2 (13,3%)
Total	849	50 (5,9%)	22 (2,6%)	90 (10,6%)

As shown in Table 4, 849 cows were tested by means of ELISA and skin test. Overall, positive skin test showed that 90 cows, among them 50 (5.9%) cows, were positive by indirect ELISA and 22 (2.6%) cows by

sandwich ELISA. It is interesting to note the results of serological and skin tests of cows from unaffected farms. Blood samples of 40 cows showed negative results in both types of ELISA, but one gave a positive skin test. Sandwich ELISA did not detect the specific antibodies to *M. bovis* in the blood samples of 15 cows from the other unaffected farm, while one cow showed positive response both by indirect ELISA and skin test; the other was positive only by skin test. The titres of *M. bovis* specific antibodies detected in the blood samples of cows from affected farms fluctuated from 1:800 up to 1:6400 while the titres of cows from unaffected farms did not exceed 1:400.

The diagnostic value of sandwich ELISA and skin test was determined by autopsy of 12 cows (Table 5).

Table 5. Diagnostic value of sandwich ELISA and skin test

Individual numbers of cows	Skin test		ELISA		Autopsy
	Thickening of skin	Results	Titres	Results	Pathological findings
6	7	+	1:25600	+	+
64	5	+	1:3200	+	+
184	4	+	1:1600	+	+
303	4	+	1:12800	+	+
188	3	+	1:3200	+	+
73	4	+	1:12800	+	+
36	6	+	1:25600	+	+
31	5	+	1:100	-	-
34	3	+	1:100	-	-
211	4	+	1:100	-	-
79	6	+	1:25600	+	+
61	6	+	1:12800	+	+

+ = positive result
- = negative result

There were *M. bovis* specific antibodies at rather high titres in the blood samples of 9 cows which showed positive skin test. Three cows developed a response to PPD *M. bovis* but in blood samples of these animals we failed to detect any specific antibodies. The results of autopsy showed that all cows with positive ELISA and skin test had pathological findings

in lungs and lymphatic nodes specific for tuberculosis, but internal organs of cows reacted only by skin test had no alterations.

Discussion

Developing clinically useful serodiagnostic tests for tuberculosis constitutes one of the real problems in veterinary medicine. There have been numerous attempts to develop effective serological methods for bovine tuberculosis. ELISA appears to be the best choice and can be used for confirmation of skin test results (AVILOV et al., 1998; LIGHTBODY et al., 1998). One advantage of ELISA is its simplicity, but both specificity and sensitivity need to be improved by using specific antigens of the causative agents as well as Mab. There are some difficulties in getting hybridoma clones produced *M. bovis* specific Mab due to the antigenic similarity between mycobacterial species. LYASHCHENKO et al. (1997) described many types of Mab against the culture filtrate′s antigens of *M. bovis*. All of them reacted with the mycobacterial species as well as related genera, but only one showed specificity to *M. bovis*, and its diagnostic value has not been reported.

In the present study, three stable clones (3A8G6, 2H10B3 and 1B6G6) producing Mab directed to the PPD *M. bovis* epitope with a molecular weight of 66 kDa were obtained. Mab did not bind to PPD *M. aviumm* or to the whole cells of *M. phlei* and *M. scrofulaceum.* Moreover, as was to be expected these antibodies did not react with the soluble antigens (LPS and SA) of *M. bovis.*

Mab 3A8G6 were more preferable for the development of ELISA for the diagnosing of bovine tuberculosis because they have higher titre and affinity to PPD tuberculin as compared with the others. Therefore, they were used as the "capture" or first antibodies in sandwich ELISA designated for the detection of *M. bovis* specific antibodies in blood of cows.

Sandwich ELISA, as well as indirect ELISA, was used for the serological assays of 55 and 794 cows from tuberculosis affected and unaffected farms, respectively. 87 cows (10.9%) from tuberculosis-affected farms showed positive skin test. Among them 49 animals (6.1%) gave positive results by indirect ELISA and 22 ones (2.6%) - by sandwich ELISA.

It is necessary to stress that there was no positive result of sandwich ELISA during serological assays of the blood samples obtained from cows of non-affected farms, while 5.5% and 1.8% animals were found to be infected with tuberculosis by means of skin test and indirect ELISA, respectively. Moreover, results of autopsy showed that 3 of 12 cows with only allergic reaction to PPD had no damage in internal organs typical for tuberculosis. The results of our research testify to the high specificity and efficiency of Mab-based sandwich ELISA in isolating animals infected with tuberculosis and evaluating the real epizootic situation.

Higher specificity of sandwich ELISA as compared with its indirect version is in our opinion connected with using "capture" Mab which bind to solid phase only PPD protein with a molecular weight of 66 kDa and removing non-specific antigens during washing procedure. In the case of indirect ELISA the solid phase is coated directly with different antigens of tuberculin, including non-specific ones, and therefore false positive results can be observed. It is necessary to mention that ELISA allows us to determine the presence or absence of *M. bovis* specific antibodies in serum for 3,5 or 4 hours, while skin test used for the identification of sick animals by its allergic response to PPD takes 72 hours. It may also be helpful in detecting anergic cattle. It is important to note that the main reagent of ELISA - Mab can be obtained in an unlimited amount for a short time. Additionally, it is known that periodical injection of PPD *M. bovis* can lead to the formation of the high sensitivity of healthy cows to tuberculin.

In conclusion, this study showed that sandwich ELISA on the bases of Mab directed to PPD *M. bovis* is a more specific and more easily performed method than skin test. We therefore recommend it for the correct isolation of animals infected with tuberculosis. Application of the sandwich ELISA in practice will prevent the spread of infection among cows of affected herds and will therefore accelerate the procedure of National tuberculosis eradication programme.

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SHENZHANOV, K.T., A. K. BULASHEV, G. A. BAKIROVA: Primjena monoklonskih protutijela u razvoju imunoenzimnog testa (ELISA) za dijagnosticiranje tuberkuloze goveda. Vet. arhiv 73, 81-93, 2003.

SAŽETAK

Polučena su tri stabilna soja hibridomskih stanica (2H10, 3A8, 1B6) za proizvodnju monoklonskih protutijela (Mab) koja su reagirala s proteinskim pročišćenim derivatom (PPD) bakterije *Mycobacterium bovis*. Proizvedena monoklonska protutijela nisu se mogla vezati na PPD vrste *M. avium* i cijele stanice vrsta *M. bovis*, *M. kansasii* i *M. scrofulaceum* kao ni za njihove topive i ultrazvukom obrađene antigene. PPD vrste *M. bovis* bio je vezan na čvrstu fazu preko monoklonskih protutijela koja su usmjerena za njen protein molekulske mase 66 kDa. Dijagnostička učinkovitost sendvič imunoenzimnog testa bila je uspoređena s kožnim testom. Krvni uzorci bili su uzeti od 849 krava koje su potjecale iz tuberkulozom zahvaćenih i nezahvaćenih uzgajališta. Devedeset krava (10,6%) reagiralo je na PPD *M. bovis*, ali su među njima samo 22 (2,6%) bile pozitivne imunoenzimnim testom. Prosječni titar specifičnih protutijela za *M. bovis* ustanovljen u uzorcima seruma imunoenzimnim testom bio je 1:920 do 1:6400. Dijagnostička vrijednost imunoenzimnog i kožnog testa bila je određena autopsijom 12 krava. Specifična protutijela za *M. bovis* u visokom titru ustanovljena su u krvnom serumu devet krava koje su bile pozitivne na kožni test. Tri krave su bile pozitivne kožnim testom, ali u njihovim krvnim uzorcima nisu ustanovljena specifična protutijela. Rezultati razudbe pokazali su da je u svih krava s pozitivnim ELISA i kožnim testom, patološki nalaz na plućima i limfnim čvorovima bio specifičan za tuberkulozu. Na unutarnjim organima krava koje su reagirale samo na kožni test nije bilo nikakvih promjena. Rezultati istraživanja potvrđuju visoku specifičnost i učinkovitost sendvič imunoenzimnog testa temeljenog na monoklonskim protutijelima. Test se može rabiti za otkrivanje i izdvajanju tuberkuloznih životinja kao i za prikaz procjene aktualnog epizootiološkog stanja.

Ključne riječi: *Mycobacterium bovis*, monoklonska protutijela, ELISA, kožni test, krave