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Original Article

MONOCLONAL ANTIBODIES AGAINST THE *BABESIA BOVIS* RAP1 ANTIGEN: OBTAINING AND CHARACTERIZATION

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ABSTRACT

Bovine babesiosis is a disease caused by parasites of the genus *Babesia*. One of the main *Babesia* genera causing disease in cattle, according to the International Office of Epizootics (OIE), is *Babesia bovis*. Recovered cattle become permanent latent carriers and can be a source of tick infestation and spread of infection. Despite susceptibility and specificity, diagnostic methods such as enzyme-linked immunosorbent assays (ELISA) and indirect fluorescent antibody assays (IFA) have some disadvantages. These methods require a long analysis time, the use of specialized equipment and trained specialists. At the same time, immunochromatographic tests (ICA) are widely used in veterinary practice today. ICA is characterized by a short analysis time, ease of performance and interpretation of results. The main components of the ICA test system are monoclonal antibodies. As a result of the studies, three mAbs against the recombinant Rap1 antigen of *B. bovis* were obtained. The resulting mAbs have high diagnostic characteristics. A conjugate of colloidal gold with mAbs is capable of detecting the Rap1 antigen of the causative agent of babesiosis in cattle.

Keywords: monoclonal antibodies, specificity, rhoptry-associated antigen 1, babesiosis, cattle, colloidal gold conjugate.

INTRODUCTION

Intraerythrocytic protozoa of the genus *Babesia* are the causative agents of bovine babesiosis, a tick-borne disease that results in significant financial losses to the global bovine industry. There are two main types of causative agent of bovine babesiosis are identified globally: *Babesia bovis* and *Babesia bigemina.* Nevertheless, *B. bovis* is the most pathogenic species. The infection is transmitted to healthy animals through the bites of ticks that contain parasites in their saliva. Upon entering the bloodstream, the parasites invade red blood cells and proliferate until complete haemolysis occurs. In the absence of immunity, the disease can be fatal. It should be noted that in areas where babesiosis is endemic, calves develop innate immunity [1-3]. Numerous methods are used to diagnose babesiosis in cattle. Serological assays such as indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test (ICA) have been used to study the effects of *Babesia* in cattle and the passive transfer of antibodies in colostrum in calves [4, 5].

The main disadvantages of serological methods for diagnosing bovine babesiosis are labor-intensive and time-consuming laboratory procedures and the need for consumables and equipment. At the same time, the use of ICA has solved some of these problems. ICA is an instrument-free, rapid and sensitive immunoassay based on a nitrocellulose membrane platform. It can be used directly in the field or on farms. The fact that results are available in less than 15 minutes is a clear advantage. Thus, an ICA was developed for the detection of antibodies in serum samples of infected cattle, utilizing the recombinant C-terminal component of *B. bigemina* rhoptry-associated protein-1 and the recombinant *B. bovis* merozoite surface antigen, an ICA was developed for the detection of antibodies in serum samples of infected cattle. The agreement rates of ICA results for identifying antibodies to *B. bigemina* for both assays were 96.8% and 92.5%, respectively, and no antibody cross-reactivity was observed. The concordance rate of ICA for detecting antibodies to *B. bovis* was 92.3% and 90.3% compared with ELISA and IFAT assays, respectively [6–8].

The main components of ICA are monoclonal antibodies (mAbs). mAbs are a highly valuable tool for use in therapeutic, prophylactic, and diagnostic applications. The first method, called hybridoma technology, involved the fusion of B lymphocytes with myeloma cells, thereby enabling the generation of individual mAbs against a specific antigen. In addition to hybridoma technology, a number of other innovative approaches have been developed with the objective of enhancing the production of mAbs. These approaches range from fusion to the identification of entirely new technologies such as B cell immortalization, phage, yeast, bacterial or ribosomal display [9].

This study focused on the diagnostic potential of mAbs. The objective was to obtain hybrid cell strains that produce mAbs with specificity for the recombinant rhoptry-associated protein 1 (rRap1) of *B. bovis*. This study presents the successful generation of murine mAbs against the rRap1 antigen expressed in *Escherichia coli*. The results of research on the diagnostic characteristics of these mAbs underscore their suitability for developing ICA test systems.

MATERIALS AND METHODS

Ethical approval

The research was approved by the LPP «National Center for Biotechnology» Ethics Commission (IRB 00013497).

Materials

To obtain mAbs, rRap1 antigen of *B. bovis*, myeloma cell line X63-Ag8.653, nutrient medium RPMI-1640, secondary antibodies against IgG (H+L) mice, HRP (Thermo Fisher Scientific), incomplete Freund's adjuvant (Gibco), and pristine (2,6,10,14-tetramethylpentadecane) (Sigma, USA) were used. Five Balb/c mice aged 10-12 weeks were used for immunization. Monoclonal antibodies were purified using MAbTrap Kit

columns (GE Healthcare Bio-Sciences AB). The characterization of the purified monoclonal antibodies was performed utilizing the Pierce™ Rapid ELISA mAb Isotyping Kit (Thermo Fisher Scientific).

Obtaining of anti-rRap1 mAbs

Over a two-week period, BALB/c mice were immunized with rRap1 antigen. The first immunization was carried out intraperitoneally by introducing 0.5 ml of a phosphate-buffered saline (PBS) solution containing 0.15 ml of Freund's incomplete adjuvant (Gibco, USA) and 50 μg of rRap1 antigen. The following immunizations were carried out on days 7, 11, 12, and 13 using a 0.5 ml PBS solution containing 50 μg of antigen. The booster dose was administered on day 14 in a 0.5 ml PBS solution containing 0.15 mL of Freund's incomplete adjuvant and 50 μg of rRap1 antigen. The ELISA was used to analyze mouse serum for the presence of anti-rRap1 antibodies. B lymphocytes from immunized mice were hybridized with myeloma cell lines X63-Ag8.653 using a 50% polyethylene glycol (PEG) solution. The cells were transferred into the wells of a 96-well culture plate and incubated in selective medium RPMI-1640, which contained hypoxanthine, aminopterin, and thymidine. Next, hybridoma clones that produced mAbs against rRap1 antigen were transferred into the wells of a 24-well plate. Once the hybridoma concentration reached 5×10^5 cells/ml, the cells were cloned using the limiting dilution method [10, 11].

To produce a preparative amount of monoclonal antibodies, Balb/c mice were intraperitoneally injected with 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane). After 14 days, mice were intraperitoneally injected with hybridomas at a concentration of 2×10^6 cells. The antibodies were isolated from the resulting ascites at the first stage using a 50% ammonium sulfate solution. The mixture was centrifuged for 30 minutes at 4°C and 3000×g. Subsequently, the antibody precipitate was subjected to dialysis against a 2000-fold volume of phosphate-buffered saline (PBS) at a pH of 7.2. At the final stage, monoclonal antibodies were purified using the MAb-Trap™ Kit in accordance with the manufacturer's instructions.

Determination of mAb's constant affinity (Kaff)

The rRap1 antigen at two concentrations - 5 and 10 μg/mL, diluted in bicarbonate buffer, was added to the wells of a 96-well plate (Costar, Cambridge, MA, USA) in a volume of 100 μL. The plates were incubated at 4°C for 24 hours. After each step, the plates were washed three times with PBS-Tween. Blocking was performed by adding 1% BSA solution for 60 minutes at 37°C. Then, two-fold dilutions of antibodies were added in a volume of 100 μL and incubated at 37°C for 60 minutes. Anti-species conjugate (Sigma-Aldrich) was added in 100 μL at a dilution of 1:40,000. Before adding substrate, the plate was washed three times with PBS-Tween and three times with PBS without Tween. After washing, substrate, 3,3',5,5'-tetramethylbenzidine (TMB), was added to the wells and incubated at room temperature for 15 minutes. The reaction was terminated by adding 2M sulfuric acid. The optical density was measured at a wavelength of 450 nm.

The obtained results of 50% of the effective concentration of binding antibodies (EC50) were converted to nmol and substituted into formula 1:

$$Kaff = \frac{1}{2(2[Ab']t-[Ab]t)}; \quad (1)$$

Where: Ab' is antibody concentration reacted with 5 μg/mL proteins at OD-50; Ab is antibody concentration reacted with 10 μg/mL proteins at OD-50.

Western blot

The separation of homogeneous and heterogeneous antigens was conducted on a 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane using a Mini Trans-Blot® Cell (BioRad, USA). The membrane was incubated in 1% BSA solution for 24 hours at 4°C, after which it was washed three times with PBS-Tween for 5 minutes each. After washing, the membrane was incubated in a monoclonal antibody solution at 37°C for 90 minutes. Next, the membrane was incubated in a solution of anti-species conjugate (Sigma-Aldrich) at a dilution of 1:10000 for 60 minutes at 37°C. After conjugate, the membrane was washed three times with PBS-Tween and three times with PBS without Tween. The reaction was demonstrated by adding 4-chloro-naphthol.

ELISA

Positive control and heterogeneous antigens were diluted in bicarbonate buffer (pH 9.6) at a concentration of 10 μg/mL were added to the wells of 96-well plates. The plates were incubated at 37°C for 24 hours. The plates were washed three times with PBS-Tween, 100 μL of 1% BSA solution was added and incubated for 60 minutes at 37°C. After washing the plate, two-fold dilutions of mAbs were added in a volume of 100 μL and the incubation procedure was repeated. Anti-species conjugate (Sigma-Aldrich) was added in 100 μL at a dilution of 1:40000 according to the instructions. To develop the reaction, TMB substrate was added to the wells and incubated at room temperature for 15 minutes. The reaction was terminated by adding 2 M sulfuric acid and the resulting product was analyzed on a plate spectrophotometer (BioRad) at a wavelength of 450 nm.

Synthesis of colloidal gold conjugate

To 97.5 mL of deionized water, 1.0 mL of a 1% solution of chlorauric acid was added, brought to a boil and with constant stirring, 1.5 ml of a 1% solution of sodium citrate was added. The mixture was boiled for 25 minutes, then cooled and stored at 4–6°C. The pH was adjusted to 6.0 by adding 0.2 M K2CO3. To determine the optimal concentration of mAbs required to saturate colloidal gold, 0.1 mL of serial aqueous dilutions of antibodies were prepared. Next, 1 mL of colloidal gold (pH 6.0) was added to all test tubes. After 10 minutes, 0.1 mL of 10% NaCl was added to each tube. When colloidal gold is not sufficiently saturated, a color change occurs from red to blue. The optical density was determined using a spectrophotometer at a wavelength of 580 nm. For the conjugate, 10 mL of colloidal gold (pH 6.0) was added to 0.2 mL of mAbs solution. Incubate for 10 min at 25°C and add 0.4 mL of 1% PEG to a final concentration of 0.04%. The mixture was incubated for 30 minutes at 25°C, then centrifuged for 45

minutes at 60,000 g. The supernatant was removed, and the sediment was dissolved in 1.5 mL of PBS containing 0.04% PEG. The conjugate was stored at 4°C. Before use, diluted to 1:10-1:20 in PBS containing 0.02% PEG.

RESULTS

Anti-Rap1 *B. bovis* mAbs

To generate mAbs against the RAP1 antigen, BALB/c mice were immunized intraperitoneally. Mouse sera after each immunization and on the third day after the booster dose were tested for the presence of specific antibodies by ELISA. Serum from mice immunized with BSA was used as a negative control. It was found that sera obtained after the second and third vaccines showed a significant increase in binding reactivity to the rRap1 antigen (Figure 1.A). Three days after the final immunization, lymphocytes were extracted from the spleen and hybridized with X63-Ag8.653 myeloma cells to obtain antibody-secreting hybridomas. To select primary clones producing monoclonal antibodies, culture media were analyzed by ELISA (Figure 1.B). It was shown that antibodies produced by seven primary clones H4, B6, B9, A11, F12, F10 and C11, out of 23 clones, were significantly associated with rRap1 antigen. The culture medium from myeloma X63-Ag8.653 was used as a control.

After selecting primary clones, hybridomas were subcloned three times and screened by ELISA. It was found that three subclones A11, F12 and C11 had higher productivity, in contrast to other subclones. For additional studies, monoclonal antibodies A11, F12 and C11 were extracted and purified from the culture medium using MAbTrap™ Kit columns.

Affinity and properties of anti-Rap1 *B. bovis* mAbs.

One of the main indicators characterizing the diagnostic suitability of the obtained mAbs is the affinity constant (Kaff). Kaff is a thermodynamic characteristic illustrating the binding strength of antibodies. Kaff was determined by ELISA using the rRap1 antigen at two concentrations - 10 and 5 µl/mL (Figure 2).

Based on the ELISA results, it was established that mAbs A11, F12 and C11 associated with rRap1 antigen at concentrations of 5 and 10 µg/mL have the following EC50: for A11 - 78 and 19 ng/mL, for F12 - 156 and 78 ng/mL, and for C11 – 19 and 4.7 ng/mL. Substituting the results obtained into formula 1, the affinity constant of mAbs was established. Using a commercial ELISA kit, the isotypes of the obtained mAbs were determined (Table 1).

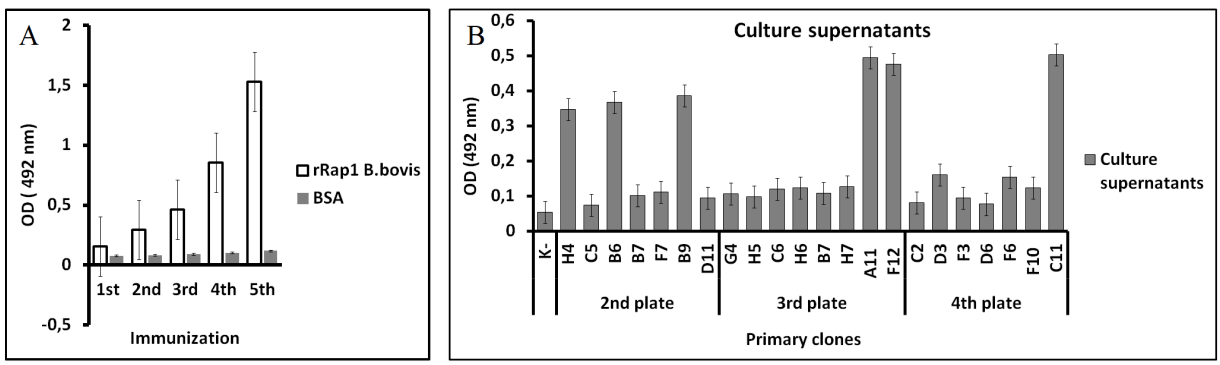

Figure 1. Obtaining of mAb-secreting hybridomas. Levels of Rap1 antigen-specific antibodies were measured in serum samples collected from the mice after each immunization (A) and in culture supernatants of primary clones (B) by ELISA.

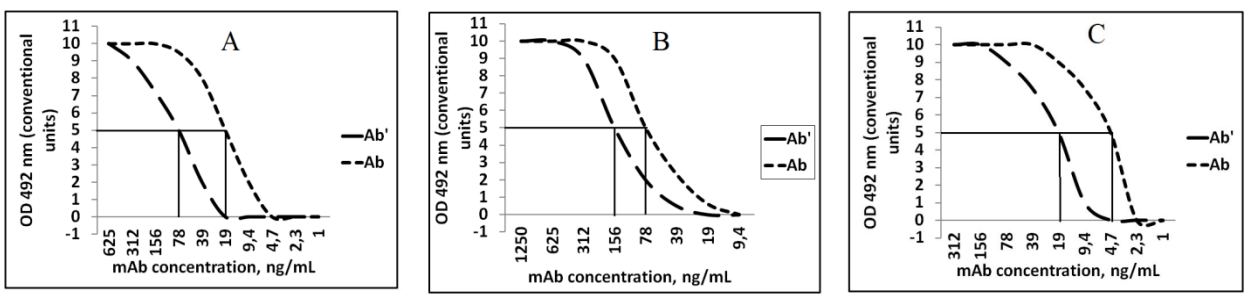

Figure 2. Experimental ELISA curves for mAbs A11 (A), F12 (B) and C11 (C) at different recombinant protein concentrations.

Table 1. Characteristics and affinity constant Kaff (M-1) for anti-Rap1 mAbs

mAb	Isotype	Specificity	[Ab']t, nmole	[Ab]t, nmole	K_{aff}
A11	IgG1	Rap1 *B. bovine*	0.00054	0.000127	$5x10^8M^{-1}$
F12	IgG1	Rap1 *B. bovine*	0.00104	0.00052	$3x10^8M^{-1}$
C11	IgG1	Rap1 *B. bovine*	0.000127	0.00003	$2x10^9M^{-1}$

All mAbs belonged to the IgG1 class and had an affinity constant of A11 – $5x10^8M^{-1}$, F12 – $3x10^8M^{-1}$ and C11 – $2x10^9M^{-1}$. The results demonstrate that the resulting antibodies have a binding strength suitable for diagnostic test systems.

Specificity of anti-Rap1 *B. bovis* mAbs

The specificity of mAbs was determined by ELISA with serial dilutions of mAbs on plates, with rRap1 of *B. bovis*, rSag1 of *Toxoplasma gondii*, and GM6 of *Trypanosoma equiperdum* (Figure 3), as well as using western blot analysis (Figure 4).

From Figure 3 it can be seen that mAbs starting at a concentration of 312 ng/mL for A11, 1250 for F12 and 159 for C11, demonstrated OD values with *B. bovis* rRap1 that were two times higher than the OD obtained with heterogeneous antigens. With an increase in the concentration of mAbs, OD values with rRap1 exceeded 4-5 times compared to OD when using rSag1 and GM6 antigens. According to the results obtained, it can be assumed that the mAbs do not cross-react and exhibit significant specificity for the Rap1 antigen. Western blot with mAb C11 confirmed the specificity of the antibodies and the absence of cross-reactions with heterogeneous antigens.

Evaluation of the colloidal gold-mAbs conjugates

One of the main parameters of ICA test system is the concentration of antibodies required for conjugation with colloidal gold. To plot the adsorption of antibodies on colloidal gold, the optical density was measured at 580 nm after adding 10% sodium chloride solution. Changes in the optical density of a solution of colloidal gold with mAbs indicate the optimal concentration of antibodies (Figure 5).

The resulting graph corresponds to the standard model of antibody adsorption on colloidal gold, where the optical

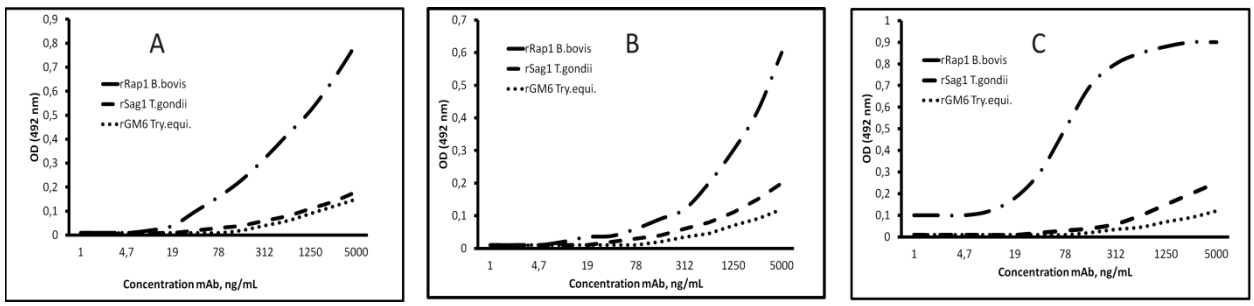

Figure 3. Specificity of anti-Rap1 mAbs A11 (A), F12 (B) and C11 (C)

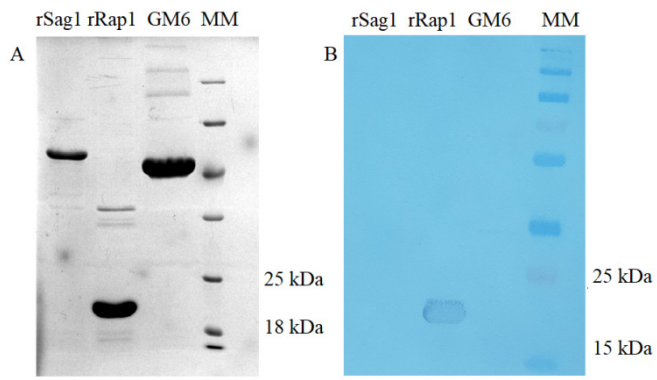

Figure 4. SDS-PAGE (A) and western blot analysis (B) of C11 mAb with rSag1, rRap1 and GM6 antigens.

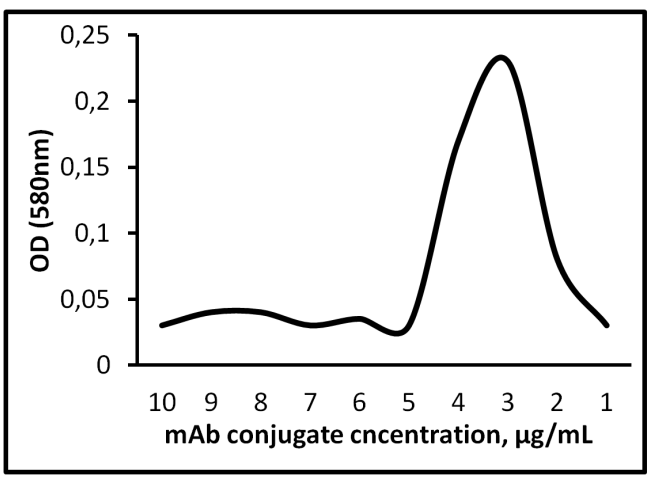

Figure 5. Flocculation dependence of mAb binding on colloidal gold

Figure 6. Testing the specificity of the synthesized conjugate with homogeneous and heterogeneous antigens

density is initially low, then reaches its maximum and subsequently decreases. The maximum OD580 peak upon conjugation of antibodies with colloidal gold is observed at a mAb concentration of 3 μg/mL. Based on the data obtained, a conjugate of colloidal gold and mAb against rRap1 of *B. bovis* was synthesized. Next, the synthesized conjugate was tested with homogeneous and heterogeneous antigens (Figure 6).

The ability of the synthesized conjugate to react with the *B. bovis* rRap1 antigen directly on the membrane was carried out by immunochromatographic testing. Antigens were applied to a nitrocellulose membrane at a concentration of 1 mg/mL. An XYZ 3060 Dispenser system (BioDot, USA) was used to apply the conjugate and antigens to the membranes. As can be seen from the figure, the synthesized conjugate reacts only with a homogeneous antigen.

DISCUSSION

Serological tests such as ELISA and IFA commonly used to diagnose babesiosis are generally time consuming, have problems with sensitivity and specificity, are labor intensive, and require experienced investigators. ICA has emerged as a promising alternative, with advantages such as ease of use, rapid results, and the ability to be used even in the field [12]. This method has shown promising results in the diagnosis of canine leishmaniasis. The diagnosis was completed in 10–15 minutes, with the test being straightforward to use and interpret. In addition, a minimal quantity of sample was utilized, without the necessity for special equipment [13].

Currently, several rhoptry proteins have been discovered and described in *Babesia* parasites. Previous studies have mainly focused on the functional role of rhoptry-associated proteins-1s (RAP-1s), which were later found in all piroplasma such as *Babesia* spp and *Theileria* spp. Due to their immunogenicity, pRAP-1 family proteins can be used to detect neutralizing antibodies, rendering them an attractive candidate for diagnostic studies of babesiosis [14]. It is important to note that the RAP-1 family is a relatively complex and polymorphic protein that performs essential functions, including adhesion, attachment and invasion, as well as communication with the host immune system [15].

Infectious diseases are often complex processes, so antibodies, due to their selectivity and specificity, are ideal molecules for rapid and specific diagnosis. In this context, monoclonal antibodies demonstrate higher target specificity. mAbs specific to a single epitope are the best means of diagnosing infections because they reduce the likelihood of misdiagnosis. Despite some financial constraints, the use of mAbs for diagnostic purposes is attracting considerable attention [16].

In this study, three cell lines producing mAbs against the *B. bovis* Rap1 antigen were obtained. The resulting mAbs were analyzed by ELISA and western blot. Due to the fact that the resulting mAbs were intended for the development of ICA test systems, their ability to bind to colloidal gold was studied. All obtained mAbs reacted only with the rRap1 antigen of *B. bovis*, and did not cross-react with recombinant antigens of other piroplasma. It was established that the resulting mAbs belong to the IgG1 type and have an affinity ranging from $5\times10^{8}M^{-1}$ to $2x10^{9}M^{-1}$. The mAb conjugate with colloidal gold reacted specifically only with a strip containing the rRap1 antigen of *B. bovis* immobilized on a nitrocellulose membrane. However, questions remain regarding the operation of the colloidal gold conjugate with the resulting mAbs directly in an ICA test system. This requires research to refine the parameters for using ICA components and scaling the test system assembly process.

CONCLUSION

As a result of the study, three lines of hybrid cells producing mAbs to the rRap1 antigen of *B. bovis* were obtained. In Western blot analysis, monoclonal antibodies specifically reacted only with rRap1 antigen. All three monoclonal antibodies belong to the class IgG1, and their affinity constant ranges from $5\times10^{8}M^{-1}$ to $2x10^{9}M^{-1}$. Colloidal gold conjugates based on the obtained monoclonal antibodies specifically reacted only with the rRap1 antigen of *B. bovis*. Further studies are required to demonstrate the performance of colloidal gold conjugate and anti-Rap1 mAbs in the diagnosis of babesiosis by ICA.

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Conflict of interest. On behalf of all authors, the corresponding author states that there is no conflict of interest.

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МОНОКЛОНАЛЬНЫЕ АНТИТЕЛА ПРОТИВ RAP1 АНТИГЕНА *BABESIA BOVIS*: ПОЛУЧЕНИЕ И ХАРАКТЕРИСТИКА

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АННОТАЦИЯ

Бабезиоз крупного рогатого скота представляет собой заболевание, вызываемое паразитами рода Babesia. Одним из основных родов Babesia вызывающих заболевание у крупного рогатого скота, по информации Международного эпизоотического бюро (МЭБ), является *Babesia bovis*. Переболевший крупный рогатый скот становится постоянным скрытым носителем, который может быть источником заражения клещей и распространения инфекции. Диагностические методы, такие как иммуноферментный анализ (ИФА) и реакция непрямой иммунофлюоресценции (РнИФ) при высокой чувствительности и специфичности имею некоторые недостатки. Данные методы требуют длительное время анализа, применение специализированного оборудования и подготовленных специалистов. В последнее время в ветеринарной практике нашло широкое применение иммунохроматографических тестов (ИХА) характеризующихся коротким временем анализа, простотой постановки и интерпретации. Основным компонентом ИХА являются моноклональные антитела. В результате проведенных исследований получены три моноклональных антитела против Rap1 антигена *B. bovis*. Полученные моноклональные антитела обладают высокими диагностическими характеристиками и при конъюгации с коллоидным золотом выявляют Rap1 антиген возбудителя бабезиоза крупного рогатого скота.

Ключевые слова: моноклональные антитела, специфичность, роптрий ассоциированный антиген 1, бабезиоз, крупный рогатый скот, конъюгат коллоидного золота

RAP1 BABESIA BOVIS АНТИГЕНІНЕ ҚАРСЫ МОНОКЛОНАЛДЫ АНТИДЕНЕЛЕР: АЛЫНУЫ ЖӘНЕ СИПАТТАМАСЫ

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Ірі қара малдың бабезиозы – *Babesia* тұқымдас паразиттерден болатын ауру. Халықаралық эпизоотиялық бюроның (ХЭБ) мәліметтері бойынша ірі қара малда ауру тудыратын негізгі *Babesia* тұқымының бірі - *Babesia bovis*. Аурып шыққан ірі қара мал тұрақты жасырын тасымалдаушыларға айналады, бұл кенелермен зақымдану және инфекцияның таралуының көзі болуы мүмкін. Диагностикалық әдістер, мысалы, ферменттік иммуносорбентті талдау және жанама иммунофлуоресценция реакциясы (жИФР), жоғары сезімтал және спецификалық болса да, кейбір кемшіліктері бар. Бұл әдістер ұзақ талдау уақытын, арнайы жабдықты және дайындалған мамандарды пайдалануды талап етеді. Соңғы уақытта иммунохроматографиялық талдау (ИХТ) ветеринариялық тәжірибеде кеңінен қолданылуда, талдаудың қысқа уақытымен, орындаудың және түсіндірудің қарапайымдылығымен сипатталады. ИХТ-ның негізгі компоненті - моноклональды антиденелер. Жүргізілген зерттеулер нәтижесінде *B. bovis* Rap1 антигеніне қарсы үш моноклоналды антиденелер алынды. Алынған моноклоналды антиденелер жоғары диагностикалық сипаттамаға ие және коллоидты алтынмен конъюгация кезінде ірі қара малдың бабезиоз қоздырғышының Rap1 антигенін анықтайды.

Кілт сөздер: моноклоналды антиденелер, телімділік, роптримен байланысты антиген 1, бабезиоз, ірі қара мал, коллоидты алтын конъюгаты.