

EXTRACTION OF ANTI-IDIOTYPIC ANTIBODIES AT TRYPANOSOMOSIS OF ANIMALS

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Abstract

In Kazakhstan, animal trypanosomiasis harm livestock. The region has recorded two species of trypanosomes: Tr. evansi and Tr. equiperdum. Trypanosomiasis necessarily belong to the group of certified disease when selling animals. However, their diagnosis is difficult because of the lack of a stable and sensitive antigen.

The aim of our research was to study the antigenic spectrum of pathogens trypanosomiasis and receiving anti-idiotypic antibodies against the antigen of trypanosome parasitic mass.

The paper presents the prospects of anti-idiotypic antibodies as an alternative diagnosticum trypanosomiasis in experimental animals.

Key words: trypanosomiasis, anti-idiotypic antibodies, diagnosticum alternative trypanosomiasis

1. TOPICALITY OF THE ISSUE

Blood-parasitic diseases of domestic animals caused by flagellar protozoa, in particular, trypanosomosis, are widely-spread enough in Kazakhstan, Tadzhikistan and cause considerable damage to livestock breeding. There are two kinds of trypanosomes registered in the region: *Trypanosoma evansi* - agent of trypanosomosis and *Tr. equiperdum* - agent of coupling disease Davlatov H.O. et al. (2009), Davlatov H.O. et al. (2010).

Trypanosomosis belongs to the group of transmissible invasions, and this fact conditions a series of biological and epizootic features and grounds a need of integrated method of their control. In case of coupling disease communicated by contact, the chronic disease course and duration of parasitosis, which are hard to diagnose, represent a certain risk in spread of this invasion Davlatov H.O. et al. (2012), Zablocki V.T. et al. (2001), Zablocki V.T. et al. (2003).

One of important biological features of these diseases is continuous, and sometimes lifelong, preservation of parasites in organism of animals have been ill Zablocki V.T. et al. (2003). Such animals represent main reservoir of invasion in nature and continuously infect arthropoda - disease carriers, in which organisms the parasites does not live long relatively. Hence, the role and importance of timely and reliable diagnostics of trypanosomosis are obvious in the complex of disease control measures, especially taking into account the fact that there are rather effective methods and means developed today to clear organism of animals from these parasites even at their single or double use.

It is difficult to diagnose the trypanosomosis, especially if it proceeds in obliterated form. The practical significance of effective diagnostics of trypanosomosis is conditioned by the fact that it is included in the group of diseases to be certified at selling of animals both in the domestic market of our country and in the markets of foreign countries Georgiu Kh. et al. (2004), Georgiu Kh. et al. (2009), Sabanshiev M.S. et al. (1978).

Wide application of modern serologic tests in veterinary practice at parasitic diseases, including trypanosomosis, is difficult because of absence of stable, highly sensitive antigen that is usually prepared using parasitic mass. Preparation of antigens using parasite infected material causes the series of difficulties at maintenance of sufficiently high parasitemia of strain agent in experimental animals, and also the risk of their spreading via bloodsucking arthropoda Suleimenov T.T. (1986), Shabdarbaeva G.S. (1990).

Some researchers attempted to develop diagnosticums using infected material. For this purpose various methods of increasing the parasitic mass of animals were used for production of large amount of target product and for more long use of animals-producers, for example, such as use of immunosuppressants, the preparations removing toxic substances, splenectomy, secreted by parasites, i.e. operative removal of immunocompetent

organ - spleen, combination of splenectomy with introduction of immunosuppressants. However, to this day, there are no developed universal methods of preparation of antigens using parasites and therefore serum diagnostics of many parasites does not find wide application in practice Suleimenov T.T. (1986), Shabdarbaeva G.S. (1990), Kosichenko L.G. et al. (2001), Shabdarbaeva G.S. et al. (1986), Grzych J.M. et al. (1982).

At the same time, the antigen deficit problem and the problem of its production in sufficient amount for practice needs can be solved by application of genetic engineering and immunological approaches - introduction of antigen-specific DNA fragments of parasite into microbial or yeast cells with further cultivation at nutrient mediums and collection of DNA expressed by introduced fragment, antigen-active biopolymer Dissous C. et al. (1982), Dissous C. et al. (1984).

However, the methodology of application of anti-idiotypic antibodies as antigen for the diagnostics purposes is technically easier and much cheaper. Since genetic engineering technology requires trained professional specialists, scarce reactants and equipment, its application seems improbable. But the methodology of application of anti-idiotypic antibodies as diagnosticums is quite practicable and accessible for realization in our conditions.

By specificity, the anti-idiotypic antibodies represent «mirror reflection» of specificity of corresponding antibodies and immunoglobulin receptors of B-lymphocytes. Therefore, anti-idiotypic antibodies can serve as imitator of certain antigen determinant. As for bivalent anti-idiotypic antibodies or artificially aggregated anti-idiotypic antibodies, they can be considered as equivalent of corresponding antigens. Proceeding from these basic provisions, we can conclude that anti-idiotypic antibodies can be used for immunodiagnosis purposes, and in polyvalent form - as vaccine preparations Dissous C. et al. (1982), Dissous C. et al. (1984).

There are data of some researchers on application of anti-idiotypic antibodies (with positive result) for immunization at schistosomiasis and toxoplasmosis Grzych J.M. et al. (1982), Fey M. et al. (1976).

Using available literature, we did not find the case of application of anti-idiotypic antibodies for diagnostic purpose at parasitosis, therefore we made an attempt of scientific and engineering-and-design work out of this problem.

Thus, all above mentioned testifies the need in expansion of theoretical knowledge concerning crucial issues of protozoology, including improvement of the technology of designing of diagnostic test-systems at parasitic diseases of animals. Besides, our approach solves the antigen deficit problem, since the main diagnostic reagent is parasite-specific serum that can be produced relatively easy in large amount.

The goal of our works was to learn the level of prevalence of trypanosomosis in the region, to study the antigen spectrum of trypanosomosis agents, to build up the idiotypic and anti-idiotypic antibodies against trypanosomosis agents, to study the dynamics of idiotypic and anti-idiotypic antibodies and to study the possibilities of their using as alternative antigen in modern serologic tests for diagnostics of trypanosomosis.

2. MATERIALS AND METHODS

The level of prevalence of trypanosomosis in south-east Kazakhstan was studied by examining the «thick» and «hanging» blood drop for the presence of live trypanosomes, the thin blood smears from peripheral vessels, the scrapes from genital organs of animals stained by Romanov-Gimza, prepared from donkeys and horses of different age at different seasons.

At taking the blood smears and scrapes from genital organs, the attention was paid to general state of animals, pulse and respiration rate, temperature reaction, character of staining of visible mucous membranes, presence of hemoglobinuria, thaler plaques, paresis and paralysis. First blood drops from peripheral vessels were studied for the presence of live trypanosomes by clock glass under a microscope in slightly darkened field within first 20 minutes after sampling of material. Thin blood smears were dried at room temperature, soaked during 10 minutes by 96° ethyl alcohol, stained during 45 minutes in thermostat at temperature 37 °C, washed by distilled water, dried in vertical position and studied under immersion system of a microscope. Smears were examined by Meander line in 200 fields of vision of microscope, parasites detected were measured by ocular micrometer, and average infectiousness of animals was expressed as percentage. At examination of blood smears, the attention was paid to changes of morphology of blood cells and their quantities.

For production of anti-idiotypic antibodies the laboratory animals - rabbits of chinchilla kind weighing not less than 2,5 kg were used. At the first stage of work, the field strain of trypanosomes was educed from

spontaneously infected horses, for this purpose 200 ml of blood with B-Tjrilon was taken from their jugular vein and injected intraperitoneally to dogs by 40-50 ml in the middle one third of abdomen near white line. Increase of parasitemia in blood was controlled daily using microscope and when trypanosomes accumulated in the amount not less than 80 instances in the microscope field of vision, dogs were totally exsanguinated by draining blood from carotid artery into wide jar filled with 2% sodium citrate. Blood was filtered by double cloth filter, put in freezer for 24 hours, and then centrifuged at 3000 r/min during 20 min. After splitting of material into three layers, the upper layer, consisting of plasma and citrate, was poured out, and the middle layer, consisting of trypanosome mass, was collected using glass spatula in special pan. Then trypanosome mass was washed three times in physiological solution within 10 minutes at 3000 r/min. Graduated decomposition of trypanosomes was performed in the field of ultrasound waves of low-frequency disperser - UZDN-2 at oscillation frequency 22-35 kHz and acoustic power 20-50 W/cm² during 10 minutes, primarily twice in physiological solution, then once in distilled water. Suspension of decayed trypanosomes was centrifuged during 15 min at 8000 r/min. Supernatant fluid was removed, and sediment was washed by distilled water by two-threefold centrifuging and repeatedly processed by ultrasound during 15 min at previous mode and then centrifuged again at 8000 r/min. Obtained supernatant - water solution of trypanosome shells is a material for production of specific trypanosomosis antigen.

Further ultrasound isoionic focusing and extraction of specific antigen fragment was performed. In the beginning, the ballast fractions were focused. For this purpose supernatant fluid was exposed to ultrasound during 3 min at 22-35 kHz and 50-75 W/cm² without use of cooling system that results in focusing and separation of the fraction «A». Then material was processed by ultrasound at slow neutralization 0,5-1.0 H by solution of sulfuric acid to 9,0-9,5 pH; lysate was incubated during 30 min and the fraction «B» was separated by centrifuging. Then specific antigen fraction «D» was extracted by analogous isoionic fractioning at 3,5-5,5 pH, and further, after centrifuging, it was dissolved in 30 ml of distilled water at 8,0-8,2 pH.

Obtained solution of antigen fraction k'D" is a specific trypanosomosis antigen. Sensitivity of obtained antigen was tested in the complement binding reaction (CBR) with knowingly positive blood sera received from animals experimentally infected by trypanosomosis. The negative serum received from young animals free from trypanosome was used as control one.

For immunization and obtaining of idiotypes or antibodies of the 1 st order (ATI) we have used rabbits. Immunization of laboratory animals was performed according to three schemes: by the method of Fey et al. (1976) using complete Freund's adjuvant (CFA), injection of antigen into cushions rabbit pads Fey M. et al. (1976); by the method of G.Frimel (1987), combination of intramuscular and intravenous injections of antigens Frimel G. (1987); and by the method of A.M.Safronov (1976), intravenous injection of antigens in two stages with preliminary sensitization of organism by injection of small doses of albumen. In all three schemes the dose of injected antigen was 130ug/ml.

Blood sampling was performed using alcohol-xylol method 3 days after the last injection of antigen with 3 day interval during one month. Dynamics of idiotypes was studied by triggering a quantitative precipitation reaction (QPR) using the method of M.Heidelberger (1938) in each three days Heidelberger M. (1938). The quantity of albumin in each portion of sera received from immunized rabbits was determined by spectrophotometer SP-26 at wave-length 280 ran.

Then portions of anti-sera were combined and sorption of idiotypic antibodies was performed using the method of S. Avrameas (1969). The antibody sorption process was performed in two stages: in the beginning the ballast antibodies, isotypes and allotypes were extracted using immunosorbent prepared from «normal» antigen of blood taken from rabbits before immunization, and then corresponding idiotypic antibodies were extracted using immunosorbent prepared on basis of antigen from trypanosomes. Optical density of obtained cluate was determined using spectrophotometer SP-26 and quantity of albumin in each portion was calculated.

The level and cleanness of sorption of idiotypic antibodies was continuously controlled.

For this purpose, eluates with idiotypic antibodies were repeatedly sorbed on magnetic stirrer during 1 hour with immunosorbent received from so-called «normal» antigen, optical density of eluate was preliminarily measured. If optical density did not change before and after sorption with immunosorbent of «normal» antigen, we considered that clean idiotypic antibodies were received as a result of sorption.

The methods of receiving and extraction of anti-idiotypic antibodies using parasitic models are weakly studied. Therefore, at development of the methods of receiving of anti-idiotypic antibodies we have applied two methodological approaches: immunization of laboratory animals by idiotypes using the method of Fey M. et

al. (1976), using different adjuvants - aluminum hydroxide (AH) and complete Freund's adjuvant (CFA) Frimel G. (1987). Rabbits were immunized in cushions of pads. Blood of immunized animals was taken beginning from the 3rd day after the last injection of idiotypic every 3 days during one month. Anti-idiotypic antibodies were separated from each sample by sorption of antibodies according to the method of S.Avrameas and Ternynck (1969) S. Avrameas (1969), using immunosorbent prepared by the method of A.N.Mayanskyi et al. (1976) and with inclusion of idiotypic into matrix of polyacrylamide gel Mayanskyi A.N. et al. (1976). In parallel, we prepared immunosorbent with inclusion of distilled water into matrix of polyacrylamide gel according to the above mentioned method of A.N.Mayanskyi. It was used further for control of sorption of antibodies.

The process of sorption of anti-idiotypic antibodies was performed in two stages. In the beginning, the ballast antibodies were extracted. For this purpose anti-sera were dissolved by buffered physiological solution at a ration 1:3, then it was added with immunosorbent prepared with inclusion of distilled water into matrix of polyacrylamide gel and put on magnetic stirrer for 1 hour. Further the content was centrifuged, supernatant was added with immunosorbent prepared with inclusion of idiotypic into matrix of polyacrylamide gel and put on magnetic stirrer for 1 hour and centrifuged by angle centrifuge. Residual sediment was washed 5 times by buffered physiological solution in angle centrifuge at 3000 r/min. For elution of ballast antibodies we used 4 ml of citric-phosphate acid buffer (pH 2,6). Then eluates were put again on magnetic stirrer for 1 hour for sorption of anti-idiotypic antibodies and centrifuged 5 times during 5 min with buffered physiological solution at 3000 r/min. For elution of anti-idiotypic antibodies we used citric-phosphate acid buffer (pH 2.6).

Titre of anti-idiotypic antibodies was determined individually in each portion by QPR, optical density and quantity of albumin were also determined. Then antibodies were neutralized by tris-HCl bringing pH to 7,0-7,2.

In order to be convinced of the quality of performed sorption of antibodies, we repeatedly performed the sorption using immunosorbent from distilled water. If optical density of supernatant was background, i.e. less than 0,05 after repeated sorption on magnetic stirrer, we considered that sorption of anti-idiotypic antibodies was performed properly.

Then gamma-globulin fraction was extracted by salting-out using 50% ammonium sulfate. Further it was concentrated in dialysis tubes on PEG-600 to the content of albumin 10 mg/ml, subjected to sublimation drying, scaled in vacuum and tested in the indirect hemagglutination reaction (IHGR) with sensitization of erythrocytes by the method of Fili and Vainbah and in immune-enzyme analysis (IEA) by the method of A.Voller et al (1976) to check the possibility of using it for diagnostic purposes at experimental trypanosomiasis Voller A. et al. (1974), Zablocky V.T. (2011), Zablocky V.T. (2001).

3. RESULTS AND DISCUSSION

The study of epizootic situation on blood-parasitic diseases of horses with use of microscopical analyses of blood smears from animals of the south-east of Kazakhstan has demonstrated their significant infectiousness by blood-parasites both in pure form and various associations and the need in improvement of their diagnostics.

Prevalence of various blood-parasites in horses according to the results of microscopical analyses is presented in Table 1.

Table 1. Prevalence of blood-parasites in horses of the south-east of Kazakhstan

№	Kind of blood-parasites and their associations	Percentage, %
1	Piroplasma - Piroplasma caballi	24,9 - 31,9
2	Nuttalia-Nuttalia egui	38,2 - 42,2
3	Piroplasma + Nuttalia - P.caballi + N.egui	18,7 – 21,7
4	Trypanosoma - Trypanosoma evansi	2,6 – 3,8
5	Trypanosoma - Trypanosoma equiperdum	15,6 – 18,9

In our studies we frequently detected infectiousness of horses by nuttalliosis - 42,2%, piroplasma was also detected very often - up to 31,9%. In total infectiousness by trypanosome made up 22,7%, at the same time *Trypanosoma evansi* was detected in single instances - up to 3,8%, and *Trypanosoma equiperdum* dominated numerically and made up 18,9%. Among associative invasions we detected only the association consisted of two kinds of blood-parasites: Piroplasma + Nuttalia, which made up 21,7%.

Further, according to the above mentioned scheme we have prepared trypanosomosis antigen from parasitic mass received at exsanguination of dogs experimentally infected by trypanosome, which was used for immunization of the first group of laboratory animals with the purpose of receiving and extraction of idiotypic antibodies or ATI. Rabbits were immunized according to three schemes and the dynamics of idiotypic antibodies at different schemes of immunization was studied in a comparative aspect. The results of tests on receiving and studying of the dynamics of idiotypic antibodies at different schemes of immunization are presented in Table 2.

Table 2 shows that among three schemes of immunization of laboratory animals with trypanosomosis antigen, the immunization performed by the method of Fey et.al. (1976) turned out the most acceptable and it gave the highest titer of antibodies against trypanosome to the 9-12th day after the last injection of antigen. Titer varied within the range 181-210 fig/ml.

The immunization scheme according to G.Frimel (1987) gave high enough titers of antibodies to the 9-12th day within 125-110 ug/ml.

The immunization scheme according to A.M.Safronov (1976) gave in all cases low titers of antibodies, approximately in 2-3 times lower than in two above mentioned immunization schemes and made up only 106-110 Ljg/ml.

Thus, for buildup of idiotypic antibodies it is recommended to perform immunization of laboratory animals by trypanosomosis antigen from parasites according to the method of Fey et.al. (1976) and G.Frimel (1987), and to perform elution of idiotypic antibodies according to the method of S.Avrameas (1969).

Table 2. Dynamics of antibodies - idiotypes to *Tr.equiperdum* at different schemes of immunization of laboratory animals

Immunization	Titer of antibodies - idiotypes, ug/ml									
	3	6	9	12	15	18	21	24	27	30
1. Fey et al.	45	58	66	181	208	210	154	106	78	54
2. G.Frimel	44	72	89	125	110	88	64	28	17	12
3. Safronov	34	68	106	110	103	97	54	28	12	8

The next stage of our work was obtaining of anti-idiotypic antibodies by repeated immunization of another group of laboratory animals by extracted idiotypic antibodies. The results of performed tests on comparative study of two schemes of immunization of laboratory animals and study of the dynamics of anti-idiotypic antibodies are performed in Table 3.

Table 3 shows that titer of anti-idiotypic antibodies was considerably higher at immunization by the method of Fey et.al. (1976) using complete Freund's adjuvant (CFA) than at use of aluminum hydroxide (AH).

During study of titer of anti-idiotypic antibodies to trypanosomosis antigen it was shown that titer of antibodies at immunization with CFA was considerably higher than at immunization with AH, in particular, titer of antibodies reached 254-502 ug/ml to the 9-12 day with further slow decrease to 21-24 day. At immunization with AH the titer of antibodies was in 1,5-2 times lower, i.e. approximately 284-302 ug/ml.

Table3. Dynamics of anti-idiotypic antibodies to *Tr.eguiperdum* in hyperimmune sera of rabbits immunized by the method of Fey et.al. (1976)

Immunization	Titer of anti-idiotypic antibodies in fig/ml, days									
	3	6	9	12	15	18	21	24	27	30
1. with CFA	46	108	254	502	440	382	292	124	86	38
2. with AH	41	82	168	284	302	172	127	94	36	12

Thus, we have determined that the best scheme of immunization for buildup of anti-idiotypic antibodies to trypanosomosis antigen is the method of Fey et al. (1976) with use of CFA. The elution of anti-idiotypic antibodies from anti-sera is to be performed according to the method of S.Avrameas and Ternynck (1969), Frimel G. (1987) with use of immunosorbent prepared by the method of A.N. Mayanskyi (1976) with inclusion of idiotype into matrix of polyacrylamide gel.

Adaptation of anti-idiotypic antibodies for serum diagnostics of trypanosomosis

The obtained anti-idiotypic antibodies to trypanosomosis antigen produced from parasitic mass were tested as diagnosticum in some serologic tests, in particular, in the quantitative precipitation reaction (QPR), in the indirect hemagglutination reaction (IHGR) and in the immune-enzyme analysis (IEA) with knowingly negative and positive sera.

QPR was performed according to standard method, titer of complement was 0,080, sera were taken at dilution 1:5, the anti-idiotypic used as diagnosticum was titrated from 1:10 to 1:2560. This reaction did not give any informative results and was not used further.

For conduction of IHGR, gamma-globulin was received from anti-sera by alcohol method. For setting the erythrocytes were used. They were prepared according to methods of Fili and Vainbah and sensitized by rivanol method. Optimal conditions of running of the reaction were determined. The results of studies on adaptation of IHGR with anti-idiotypic antibodies are presented in Table 4.

Table 4. Results of IHGR with anti-idiotypic antibodies

Anti-idiotypic antibodies	Dilutions of positive sera						Control (negative serum)
	1:20	1:40	1:80	1:160	1:320	1:640	1:20
Trypanosome anti-idiotypic antibodies (1:160)	++++	++++	++++	+++	+	-	-

Table 4 shows that trypanosome anti-idiotypic diagnosticum in IHGR gave positive reaction in four crosses at dilution of sera 1:160. Control sera gave negative reaction in all cases. Immune-enzyme analysis was performed according to the method of A.Voller et.al (1976). Anti-idiotypic antibodies and gamma-globulin extracted from anti-sera by the salt method (salting-out by ammonium sulfate at 50% saturation) were used as antigen. Anti-idiotypic antigen was diluted by carbonate buffer, pH 9,0. After administration of anti-idiotypic antibodies, slips were sensitized by albumin. Conjugation of rabbit anti-serum was performed according to the method of P.K.Nakane et.al (1974) with peroxidase from Olain horseradish with index Rz 2,7-3,2 Avrameas S. (1969). Substrate proposed by A.Abraham (1984) was used as substrate Mayanski A.N. et al. (1976). Substrate was prepared from the mixture of orthophenylenediamide and hydrogen peroxide on citrate buffer, pH - 5,0. For washing out of polystyrene plane-tables we used 0,01 M phosphate buffer, pH 7,2 on physiological solution, containing 0,5 g/l of twin-80 (TPB).

The results of the IEA reaction were determined visually. Trypanosomosis anti-idiotypic antibodies gave positive results at dilution 1:320. The results of application of anti-idiotypic antibodies against trypanosomosis antigen.

4. SUMMARY

The technology of extraction of anti-idiotypic antibodies against trypanosomosis antigen using parasitic mass was developed. The possibility of application of anti-idiotypic antibodies as alternative diagnosticum at experimental trypanosomosis of animals was determined

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