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Technology of Preparation and Evaluation of Properties of Brucellosis Hyperimmune Rabbit Serum

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Abstract A bank of rabbit hyper-immune anti-brucellosis sera was created (72 sample variants), including: serum before immunization, after 1, 2, 3, 4 immunizations, 13 days after the last 4th immunization; options for immunization with live attenuated brucellosis vaccine and inactivated culture; without adjuvant and with incomplete Freund's adjuvant.

Keywords Brucellosis, *Brucella abortus* 19, Hyperimmunization, Standard serum, Albumin, Globulin, IgA, IgM, IgG, Heddelson reaction, Wright reaction

1. Introduction

Along with the increase in the number of cases of infectious diseases among the world's population, the number of zoonotic diseases, including brucellosis, is also growing daily. One of the main reasons is the consumption of milk and meat of animals with brucellosis [10].

According to the WHO Joint Committee on Brucellosis, brucellosis has been registered among animals in 155 countries around the world. Brucellosis is most widely spread in the countries of South and Southeast Asia, Africa, Central and South America. Every year, more than 500,000 cases of this disease are registered in the world of people suffering from its negative consequences [7].

In order to effectively control the activity of epizootic and epidemic factors in areas endemic to brucellosis, the use of diagnostic laboratory examination methods for this nosology is carried out starting from the patient's admission to the primary health care unit [1,5]. This is due to the variety of clinical signs of brucellosis, the similarity of its clinical course with other diseases, the absence of strictly specific symptoms, which often causes difficulty in making a preliminary diagnosis of "Brucellosis", leading to an erroneous diagnosis with incorrect therapy. Often such situations arise with incomplete collection of anamneses, the absence of cardinal symptoms of the disease, with super- and reinfection, the development of relapses and latent forms of infection. In such cases, even in non-endemic areas for brucellosis, laboratory research methods become important [11].

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Today, one of the reliable, auxiliary methods in the diagnosis of brucellosis, both in humans and in animals, is the conduct of serological tests. Of the serological methods of research in humans, reactions are currently used: Heddelson agglutination (one of the methods of rapid diagnosis of brucellosis), Wright, passive hemagglutination, Coombs reaction (indirect antiglobulin test), etc. At the same time, the Heddelson reaction is the most sensitive reaction than the Wright reaction, but less specific. Negative results of Heddelson's reaction indicate the absence of brucellosis activity, but positive ones do not always reliably confirm the diagnosis of the disease. The Wright agglutination reaction is a highly specific reaction, with titers of 1:50 and above usually confirming the diagnosis of brucellosis. In animals from serological reactions, the following are used: The Rose Bengal test (RBT), the agglutination and complement binding reaction [2,4,6]. In order to standardize and improve the effectiveness of the above methods, in accordance with the requirements and recommendations of the FAO/WHO Association of Experts on Biological Standardization, each country should use its own individual national standard serum with the appropriate titer, which is expressed in international units (IU/ml) [3,8].

The absence of national standard serums causes problems in quality control of commercial and local antigens, hinders the development of local drugs - brucellosis diagnostics, which reduces the effectiveness of serological diagnostics of brucellosis in humans and animals [9].

Considering the above, it becomes necessary to develop and produce a standard anti-brucellosis serum, check these products for quality.

2. Materials and Methods

To obtain positive standard serums against brucellosis

pathogens, 12 rabbits weighing from 2.2 kg to 4.6 kg aged 6.0 to 12 months were used. The animals were kept in the same conditions on a standard vivarium diet. For the experiment, animals adapted to the conditions of the experiment were taken, which were kept in quarantine for 21 days. To conduct the study, biomodels were anesthetized according to the international rules for the humane treatment of animals (Order No. 755, 1977; Order No. 742, 1984). The rabbits were divided into 4 groups. Each rabbit from group 1 was immunized 4 times by introducing a weakened live *B. abortus* 19 vaccine with a virulence concentration corresponding to 4 billion.

In the 2nd group of rabbits, the 2nd and 3rd immunization was also carried out by administration of a weakened live *B. abortus* 19 vaccine with a virulence concentration corresponding to the 4th billion, and the 1st and 4th immunization was carried out by administration of a live *B. abortus* 19 vaccine with an incomplete Freund adjuvant with a virulence concentration corresponding to the 4th billion (an incomplete Freund adjuvant was prepared by adding two ml of BCG vaccine to 6 ml of Vaseline oil).

Each rabbit from the 3rd group was injected with a suspension of inactivated brucella culture at a concentration of 1 billion (according to the McFarland standard) 2 times, the 3rd immunization was carried out with a concentration of 4 billion, the 4th immunization was carried out with a live *B. abortus* 19 vaccine with a virulence concentration corresponding to 4 billion.

Each rabbit from the 4th group during the 1st immunization was injected with a suspension of inactivated brucella culture at a concentration of 1 billion together with an incomplete Freund adjuvant, the 2nd immunization was carried out by introducing a suspension of inactivated brucella culture at a concentration of 1 billion, the 3rd immunization - at a concentration of 4 billion, and at the 4th immunization was administered a live *B. abortus* 19 vaccine with a virulence corresponding to 4-mm billion.

All rabbits were injected with antigen (live vaccine or suspension of inactivated brucella culture) at a total of 8 points (1-4 points - subcutaneous injection, 5-8 points - intramuscularly) between the muscles.

Bacteriological method. In the process of obtaining serums, we used strains of *B.abortus* 19 vaccines with the lowest virulence. The vaccine was produced in the Shchelkovsky Biocombinat of the Russian Federation, serial number 204, production time - April 2022, expiration date - 12 months. The introduction of the *B.abortus* 19 vaccine was carried out in experimental rabbits by pre-growing an inactivated brucella suspension, according to the McFarland standard, diluted in saline solution.

A feature of the serums was the study of clinical strains of *Enterobacteriaceae spp. 74, Proteus vulgaris 86, Salmonella spp.632, Salmonella spp. 660, Salmonella spp. 662, Salmonella spp. 663, Salmonella spp. 760, Salmonella spp. 766, Klebsiella pneumoniae 97, Salmonella spp. 696, Shigella spp. 5, E. coli 108, E. coli 109, E. coli 110, E. coli 111, E. coli 112, E. coli 114, E. coli 914, Klebsiella pneumoniae 128, Citrobacter spp. 134 Klebsiella pneumoniae 139, Citrobacter spp. 140, Shigella spp. 973, Shigella spp. 1128* isolated in the bacteriological laboratory from patients with acute diarrhea and acute intestinal infection who received inpatient treatment at the clinic on the basis of the Republican Specialized Scientific and Practical Medical Center for Epidemiology, Microbiology, Infectious and Parasitic Diseases.

Serological method: was carried out on the basis of Appendix 12 (methodological instructions for laboratory diagnosis of brucellosis) of the Order of the Minister of Health No. 177 dated May 1, 2015 "On improving laboratory methods conducted in laboratories of bacteriological, virological and especially dangerous infectious diseases".

Immunological studies. Identification of immunoglobulins of classes A, M and G against brucellosis pathogens by the enzyme immunoassay was carried out using a set of reagents Vector BEST, RF.

Determination of the level of total protein, albumin and globulin was established by the enzymatic colorimetric method by using the biochemical analyzer "Mindray" BA-88A on medical equipment of a Chinese company. The results were evaluated based on the manufacturer's instructions.

Statistical method: digital material was processed by the method of variational statistics using the program "Excel-Office" 2013 using the Student's t-test. The mean quadratic error (m) was calculated, as well as the reliability of the differences in the values in the compared groups. The differences were considered significant at $p<0.05$. Nominal data are described with absolute values and percentages. The nominal data were compared using Pearson's $\chi 2$ criterion, Fisher's exact criterion. The differences were considered significant at $p<0.05$.

3. Results and Discussion

Before hyperimmunization of experimental rabbits, their blood serum was subject to serological (using the Wright-Heddelson reaction) and immunological examination for brucellosis (identification of IgA, IgM and IgG by the enzyme immunoassay). In addition, the level of total protein, albumin, globulin and the ratio of albumin to globulin were determined in rabbits.

Experimental animals were hyperimmunized 4 times with an interval of 7 days. The day before each hyperimmunization, the blood serum of experimental animals was examined using the above-mentioned serological and immunological reactions. 13 days after the last 4th hyperimmunization, the experimental rabbits underwent total blood sampling.

In accordance with the "veterinary and sanitary rules for the collection, disposal and neutralization of biological waste", approved by Resolution No. 13, 12 (100%) of the State Committee for the Development of Veterinary

Medicine and Animal Husbandry dated October 14, 2019, rabbits used in the experiment were destroyed by burning at a high temperature of 700oC in a special crematorium furnace located in scientific center of Pharmaco-toxicology and vivarium.

The blood serum from each experimental animal was poured into separate sterile jars with the addition of the highest concentration sodium merthiolate as a preservative in a ratio of 1:10,000, followed by heating the serum in a water bath at 56℃ for 30 minutes, stirring constantly. In order to conduct serological and immunological studies, one part of the serum was placed in refrigerators with a temperature of 2-8℃, and the second part was stored in freezers at a temperature of minus 20℃ for subsequent use.

Evaluation of serum specificity in heterologous microorganisms. The serum obtained as a result of hyperimmunization may react with agglutination, intersecting with antigens of pathogens of other infectious diseases, which in turn may cause erroneous identification of the isolated strain. Therefore, it is important to identify the features and specificity of the serum, check it for the presence of other pathogens of the disease.

Therefore, the specificity of the sera obtained as a result of the experiment was studied using clinical strains of gram-negative bacteria isolated from patients undergoing treatment with diagnoses of acute diarrhea or acute intestinal infection in the bacteriological laboratory of the clinic located on the basis of the Republican Specialized Scientific and Practical Medical Center for Epidemiology, Microbiology, Infectious and Parasitic Diseases. Thus, the blood serum obtained from experimental rabbits in different periods of the study: before pre-immunization, during hyperimmunization, as well as during total blood collection, was subjected to an agglutination reaction on a slide with all clinical strains. As a result of the agglutination reaction with gram-negative bacteria (*Citrobacter spp. Enterobacteriaceae spp*.), a weakly positive response (+) was established on a slide in 16.7% of cases. In a further study, it was these blood serums that were subjected to an agglutination reaction in test tubes with the addition of a suspension of inactivated the same microorganisms (*Citrobacter spp. Enterobacteriaceae spp*.). The results were negative. The results of the study of sera after hyperimmunization are presented in Tables 1-4.

Table 1 provides information on the results of immunization of experimental animals of the first group.

There was a significant increase in the level of total protein after the first and 4th immunization. If, before the first immunization, the level of total protein was 59.57 g/l, then after the 4th immunization it increased three times - 157.17 g/l. An increase in albumin and globulin content (33.2 g/l and 26.37 g/l, respectively) to 79.37 g/l and 77.43 g/l, respectively, was also found.

The IgM level increased 3.5 times during the second immunization, and decreased to 0.24 mg/ml after the 4th immunization. An increase in the level of IgG was revealed after the 2nd and 3rd immunization, and after the 4th immunization, a decrease in its level was found.

Thus, in the process of repeated immunization, an increase in the level of total protein, albumin, globulin, IgM and IgG indicates the formation of an immune response to antigens (an increase in IgM levels in the first week of primary immunization indicates the formation of primary immunity, an increase in IgG levels from the second week indicates the formation of secondary immunity).

Table 2 shows the results of 2 groups of experimental rabbits that were injected with a live *B. abortus* 19 vaccine with an incomplete Freund adjuvant with a virulence concentration corresponding to 4 billion.

In experimental animals of the 2nd group, the results of hyperimmunization showed similar changes in the level of total protein, albumin, globulin and IdA as in the first group.

However, it is worth noting that in this group, the indicators of total protein, albumin, globulin increased in dynamics after 4 immunization and 13 days after the last immunization, the levels of Ig M and IgG compared to the first group correspond in dynamics to the primary and secondary immune response, and IgG indicators were higher than in the representatives of the first group. Consequently, the use of an incomplete Freund adjuvant leads to the formation of a stable immune response.

Table 3 shows the results of a blood serum study of animals immunized with a suspension of inactivated brucella culture. Changes in the indicators of total protein, albumin, globulin and IgA in experimental animals of the 3rd group immunized with inactivated brucella culture suspension had the same upward trend as in animals of the 1st and 2nd groups. The IgM and IgG levels were lower compared to groups 1 and 2, but subsequently the IgM level doubled after the 2nd immunization (7-14 days of the primary immune response). At the same time, an increase in IgG levels was found in the second and third weeks (secondary immune response), and after the 4th immunization, a decrease in IgG levels was recorded. Table 4 shows data on the results of hyperimmunization in experimental animals of the 4th group. The analysis of the indicators of total protein, albumin, globulins, IgA, IgM and IgG in experimental animals of group 4 indicated approximately the same trend of changes in their levels as in groups 1, 2, 3. Thus, after hyperimmunization of experimental animals of the 4th group (suspension of inactivated brucella culture + incomplete Freund adjuvant), a formed immune response was observed. However, the IgG level decreased after the 4th immunization and equaled the initial level 13 days after the last immunization.

The results obtained after immunization of experimental animals with the most weakened strain of the live *B. abortus* 19 vaccine and a suspension of a weakened brucella culture showed the development of a persistent and long-lasting immune response (during the observation period). Therefore, to obtain a full-fledged, active hyperimmune serum, it is necessary to use live, weakened bacteria.

Table 1. Results of immunization of experimental animals of the first group

Indicators	Before immunization	After the 1st immunization		After the 2nd immunization		After the 3rd immunization		After the 4th immunization		13 days after the last immunization	
	M±m	M±m	p	M±m	M±m	M±m	p	M±m	M±m	M±m	p
Total protein, g/l	59.57±6.16	84.37±4.4	p=0.04	96.4±4.3	p=0.02	154.57±17.5	p=0.01	157.17±8.7	p=0.002	118.03±4.7	p=0.004
albumin, g/l	33.2±2.37	41.27±3.4	p=0.14	47.37±4.01	p=0.05	62.9±5.05	p=0.01	79.73±4.4	p=0.002	58.95±3.5	p=0.008
globulin, g/l	26.37±4.52	43.10±1.6	p=0.04	51.67±2.1	p=0.01	101.7±10.5	p=0.007	77.43±4.5	p=0.004	58.77±2.6	p=0.008
A/G, g / L	1.27±0.16	0.95±0.07	p=0.16	0.91±0.07	p=0.13	0.62±0.11	p=0.04	1.03±0.03	p=0.23	1.0±0.03	p=0.19
IgA, mg / ml	0.25±0.01	0.2±0.01	p=0.03	0.28±0.05	p=0.59	0.2±0.04	p=0.31	0.16±0.001	p=0.002	0.15±0.01	p=0.005
IgM, mg / ml	0.31±0.001	0.69±0.17	p=0.11	1.12±0.04	p=0.0002	0.58±0.22	p=0.3	0.24±0.09	p=0.4	0.34±0.14	p=0.8
IgG, mg / ml	2.18±0.06	2.06±0.2	p=0.6	4.21±0.04	p=0.0001	4.24±0.4	p=0.01	1.31±0.25	p=0.04	1.28±0.21	p=0.02

Note: p<0.05, significance of differences between pre-and post-immunization values

Table 2. Results of immunization of experimental animals of the second group

Indicators	Before immunization	After the 1st immunization		After the 2nd immunization		After the 3rd immunization		After the 4th immunization		13 days after the last immunization	
	M±m	M±m	p	M±m	M±m	M±m	p	M±m	M±m	M±m	p
Total protein, g/l	59.5±7.83	81.3±14.67	p=0.28	75.43±19.31	p=0.50	154.03±11.97	p=0.007	167.77±12.64	p=0.005	134.1±5.88	p=0.004
albumin, g/l	34.67±5.07	41.2±6.74	p=0.49	35.2±6.8	p=0.95	69.3±2.9	p=0.009	82.57±6.08	p=0.009	66.27±2.49	p=0.01
globulin, g/l	24.53±2.76	40.1±8.73	p=0.18	40.23±12.53	p=0.30	84.73±13.39	p=0.02	85.47±6.46	p=0.003	67.83±3.39	p=0.002
A/G, g/L	1.4±0.11	0.91±0.05	p=0.02	0.94±0.17	p=0.1	0.84±0.14	p=0.05	0.96±0.01	p=0.02	0.97±0.01	p=0.03
IgA, mg / ml	0.24±0.001	0.23±0.04	p=0.81	0.26±0.03	p=0.55	0.23±0.03	p=0.76	0.17±0.01	p=0.006	0.16±0.01	p=0.004
IgM, mg / ml	0.37±0.01	1±0.08	p=0.004	1.11±0.01	p=0.0001	0.69±0.16	p=0.13	0.47±0.001	p=0.002	0.35±0.21	p=0.93
IgG, mg/ ml	2.21±0.25	3.45±0.58	p=0.14	4.7±0.18	p=0.05	5.26±0.27	p=0.003	1.94±0.03	p=0.36	1.6±0.35	p=0.25

Note: p<0.05, significance of differences between pre-and post-immunization values

Table 3. Results of immunization of experimental animals of the third group

Indicators	Before im-munization	After the 1st immunization		After the 2nd immunization		After the 3rd immunization		After the 4th immunization		13 days after the last immunization	
	M±m	M±m	p	M±m	M±m	M±m	p	M±m	M±m	M±m	P
Total protein, g/l	57.00±3.47	71.83±4.76	p=0.08	83.4±3.40	p=0.01	134.9±13.63	p=0.01	149.77±13.62	p=0.007	114.03±16.88	p=0.04
albumin, g/l	29.30±1.35	35.63±2.92	p=0.14	36.13±6.45	p=0.37	64.77±5.84	p=0.009	75.25±9.35	p=0.01	59.67±10.13	p=0.05
globulin, g/l	27.7±2.29	36.2±2.29	p=0.07	47.27±7.73	p=0.09	70.3±8.68	p=0.01	76.23±6.7	p=0.006	54.7±6.79	p=0.03
A/G, g/l	1.06±0.6	0.98±0.06	p=0.9	0.51±0.32	p=0.47	0.93±0.08	p=0.8	0.96±0.8	p=0.13	1.08±0.06	p=0.97
IgA, mg / ml	0.18±0.01	0.20±0.02	p=0.43	0.18±0.01	p=1.0	0.18±0.01	p=1.0	0.15±0.01	p=0.12	0.14±0.01	p=0.06
IgM, mg / ml	0.43±0.12	0.97±0.15	p=0.06	0.82±0.18	p=0.16	0.45±0.02	p=0.87	0.49±0.20	p=0.81	0.32±0.02	p=0.43
IgG, mg / ml	1.98±0.19	3.59±0.18	p=0.008	4.44±0.61	p=0.03	4.46±0.42	p=0.01	1.64±0.35	p=0.45	1.45±0.47	p=0.37

Note: p<0.05, significance of differences between pre-and post-immunization values

Table 4. Results of immunization of experimental animals of the fourth group

Indicators	Before immunization	After the 1st immunization		After the 2nd immunization		After the 3rd immunization		After the 4th immunization		13 days after the last immunization	
	M±m	M±m	p	M±m	M±m	M±m	p	M±m	M±m	M±m	P
Total protein, g/l	61.23±1.01	81.53±6.23	p=0.04	99.57±10.64	p=0.03	140.7±4.67	p=0.0004	148.57±6.54	p=0.0009	130.77±1.56	p=0.00004
albumin, g/l	33.03±2.86	39.10±1.90	p=0.17	50.33±4.64	p=0.05	66.7±5.55	p=0.01	70.07±4.43	p=0.005	66.8±1.41	p=0.001
globulin, g/l	28.2±2.45	42.43±4.50	p=0.06	49.23±6.07	p=0.04	73.9±3.36	p=0.001	78.50±3.20	p=0.001	64.07±2.90	p=0.002
A/G, g/ L	1.19±0.19	0.93±0.07	p=0.28	1.02±0.04	p=0.44	0.90±0.1	p=0.26	0.89±0.05	p=0.22	1.04±0.07	p=0.5
IgA, mg / ml	0.16±0.02	0.18±0.04	p=0.68	0.18±0.08	p=0.82	0.14±0.01	p=0.43	0.16±0.01	p=1.0	0.14±0.01	p=0.43
IgM, mg / ml	0.30±0.05	0.93±0.15	p=0.02	0.50±0.10	p=0.17	0.57±0.09	p=0.07	0.33±0.05	p=0.69	0.45±0.15	p=0.41
IgG, mg / ml	1.70±0.06	3.47±0.21	p=0.003	5.70±0.16	p=0.0001	4.31±0.31	p=0.003	1.37±0.23	p=0.25	1.81±0.15	p=0.54

Note: p<0.05, significance of differences between pre-and post-immunization values

Table 5. Results of studies on the geometric mean titer of the Wright reaction

Results of immunization of experimental animals of the first group					
Before immunization	**After the 1st immunization**	After the 2nd immunization	After the 3rd immunization	After the 4th immunization	13 days after the last immunization
0	**1: 800**	1: 2015	1: 2540	1:1600	1:1008
Results of immunization of experimental animals of the second group.					
0	**1: 800**	1: 1270	1: 1270	1:1008	1: 635
Results of immunization of experimental animals of the third group.					
0	**1: 63**	1: 100	1: 79	1:200	1: 200
Results of immunization of experimental animals of the fourth group.					
0	**1: 63**	1: 79	100	1: 126	1: 159

To improve the serological diagnostics of brucellosis for the purpose of hyperimmunization, it is necessary to obtain a standard serum against brucellosis pathogens. To do this, it is important to determine the antibody titer using Wright reactions. The results of studies on the geometric mean titer of the Wright reaction are presented in Table 5. After the first immunization with the live B. abortus 19 vaccine with low virulence, an increase in the antibody titer from the second week with stable levels for 41 days (during the observation period) was found. The titer of antibodies in experimental animals of groups 3 and 4, immunized with a suspension of inactivated brucella culture, was at the same level in dynamics as in the first group, but was significantly reduced hen immunized with a live vaccine *B. abortus* 19 with low virulence. Thus, a sufficient amount of serums against brucellosis pathogens was obtained.

In order to improve the serological diagnostics of brucellosis after further study and standardization, serums will be presented as a reference serum against brucellosis pathogens.

Thanks to the diagnosis of brucellosis in our Republic using the standard national serum:

- it will become easier to evaluate the results of serological analysis;
- it will be possible to compare the results of a serological study of brucellosis obtained by different scientific researchers;
- will allow monitoring the activity and specificity of all commercial diagnostics.;
- the evaluation of the titer of the national standard serum in international units will allow us to achieve compliance of the results obtained by our researchers with the results of a world-class study;
- will create an opportunity to control imported cases of the disease, fully tracking the penetration of this infection into our country from abroad;
- will allow to carry out early diagnostics for timely detection of activation of existing epidemic foci in the republic;
- will create new opportunities in improving and improving methods of diagnosis, treatment and prevention of brucellosis in our country.

In addition, a standard serum is necessary for the correct diagnosis of brucellosis.

Standard brucellosis serum is used to identify and differentiate the isolated brucella culture in the agglutination reaction on a slide during the bacteriological diagnosis of brucellosis.

4. Conclusions

1. In the process of repeated immunization, an increase in the level of total protein, albumin, globulin, IgM and IgG indicates the formation of immunity to antigens (an increase in IgM levels in the first week of primary immunization indicates the formation of primary immunity, an increase in IgG levels from the second week indicates the formation of secondary immunity).
2. The use of an incomplete Freund adjuvant leads to the formation of a stable immune response.
3. Administration of the *B. abortus* 19 vaccine strain and suspension of a weakened brucella culture showed the development of a more stable and long-lasting immune response (during the observation period).
4. The titer of antibodies in experimental animals of groups 3 and 4, immunized with a suspension of inactivated brucella culture, was at the same level in dynamics as in the first group, but was significantly reduced when immunized with a live vaccine B. abortus 19 with low virulence.
5. To obtain a full-fledged, active hyperimmune vaccine serum, it becomes necessary to use a live, weakened bacterium.

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