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USING INDIRECT HEMAGGLUTINATION ASSAY FOR THE DIAGNOSIS OF CATTLE BRUCELLOSIS

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Abstract

The possibility of using indirect hemagglutination assay (IHA) with milk (IHA/m) to differentiate post-infectious antibodies from post-vaccination ones in cows immunized with the *Brucella abortus* 82 was studied. Anti-*Brucella* antibodies by IHA/m were detected in the milk of all lactating animals (n=20) culled on the results of tube agglutination test (AT) and/or complement fixation test (CFT), while the milk ring test (MRT) were negative in 20% of cases. IHA/m, as well as IHA with blood serum (IHA/s), confirmed brucellosis in cows with AT- and/or CFT- negative or questionable results. The correlation coefficient between the results of the two IHA variants was very high (r = 793), and the hemagglutinin titers in the blood serum were significantly higher - 1:760 (+13.3%; -11.7%) than in AT-1:260 (+8.7%; -8.0%) (P≤0.01). Vaccination of cows caused increased production of complement-fixing, agglutinating and precipitating antibodies, which tended to weaken during observation of the animals: 30, 60- and 90-days post-vaccination (p.v.). By the end of the experiment, all vaccinated animals showed questionable AT; positive RID/O-PS and IHA/s were noted in 10% and 20% of cases, respectively. However, complement-fixing antibodies remained at diagnostic values in all animals until the end of the experiment. Despite the intense antibody immune response to the vaccine injection, there were no *Brucella*-specific agglutinins detected by IHA/m and MRT in the milk of cows even on the 30th day p.v. The results show the need for further study on a large population to determine the diagnostic value of IHA/m for differentiating infected from vaccinated animals.

Key words: brucellosis; diagnostics; milk; traditional serological test; indirect hemagglutination assay; post-vaccination antibody.

Introduction

Brucellosis is one of the most common zoonoses in the world, causing significant economic losses and public health problems in more than 170 countries. High levels of humans brucellosis are observed in the Middle East, Mediterranean, sub-Saharan Africa, China, India, Peru, Mexico and other countries [1]. The tense brucellosis epizootic and epidemic situation remains in the countries of Eastern Europe and Central Asia, including the Russian Federation (RF) [2,3] and the Republic of Kazakhstan (RK) [4,

5]. As for RF, in the period from 2011 to 2020, 4 490 and 376 brucellosis-affected farms and flocks were registered with seropositive 95668 cattle and 14533 sheep, respectively. A difficult epidemiological situation has developed in the North Caucasus, Transcaucasia, as well as in the Siberia and the Volga regions. Thus, in the Republic of Dagestan (RD) in 2021, 176 cases of human brucellosis were identified (5.64 per 100 000 population), among which there was a high proportion of minors (1.93 per 100 thousand population), which is associated with traditions individual livestock farming in the republic, when children from an early age actively participate in feeding, maintaining and slaughtering livestock [6]. Kazakhstan, as well as other Central Asian countries, are among the 25 countries with the highest brucellosis incidence [7]. In the RK, 63.4% of rural districts are affected by cattle brucellosis with an average incidence rate of 0.45%. The dynamics of sheep brucellosis incidence has tended to decrease over the years and currently amounts to 0.1%, and the incidence rate of people per 100 000 population ranges from 1.9 to 4.91 depending on the regions of the country, although these figures are clearly underestimated [8].

The difficulty of combating brucellosis is, first of all, explained by the lack of a reliable test for the timely detection of an infected animals. Currently widely used serological tests, such as the agglutination test (AT), complement fixation test (CFT), rose Bengal test (RBT) and enzyme-linked immunosorbent assay (ELISA), diagnose brucellosis based on the detection of antibodies against lipopolysaccharides (LPS) - surface immunogenic antigen of the pathogen. *Brucella* and related bacteria have a very similar LPS structure, which often causes false-positive results [9-11].

Kazakhstan has an unsuccessful experience in implementing the “Test-and-Slaughter” policy (2008-2011), when vaccination and classical serological reactions were canceled, and culling of animals was carried out only according to the ELISA kit indications. The innovation has led to a sharp increase in the number of animals reacting positively to brucellosis [12]. This situation forced the Committee for Veterinary Surveillance and Control of the RK to make a decision to return to traditional serological tests and immunoprophylaxis using vaccines registered in the country, as well as in the member states of the Eurasian Economic Union. As would be expected, since 2012 the number of reflectors began to decrease markedly. Practice has shown that the use of ELISA kits can be used in the serodiagnosis of brucellosis only in the availability of *Brucella* specific antigen [13].

Both in the RF and in the RK, live attenuated Brucella abortus 19 (S) and Brucella abortus 82 (SR) vaccines have been most used to create immunity in animals against brucellosis. These vaccines, as immunogenic preparations, have given rise to the problem of differentiating infected from vaccinated animals, which remains an important issue of veterinary science to this day. Thus, for intravital brucellosis diagnosis simple methods are needed that are superior to traditional serological tests in specificity and allow distinguishing post-infectious antibodies from post-vaccination ones.

Among the non-traditional serological tests currently used to diagnose brucellosis, the indirect hemagglutination assay (IHA) has the greatest potential for widespread introduction into veterinary practice, which can diagnose brucellosis at an early stage, when conventional tests give questionable or negative results [14, 15]. In previous study, we developed a method for preparing Brucella erythrocyte antigen (EA) for IHA [16], which was used for the serodiagnosis of cattle and sheep brucellosis. IHA based on the new EA, which is an extract of B. abortus 19, obtained by autoclaving cells at 0.5-0.7 atm. within 45 min. (pH 8.0-9.0), significantly exceeded known serological tests in sensitivity and specificity [17,18].

The advantage of IHA is that the analyte for it can be milk, an easily accessible, non-invasive biological material [19]. It has been established that in lactating animals with brucellosis, antibodies can be detected not only in the blood of animals, but also in milk. Moreover, with local damage to the mammary gland, antibodies may be absent in the blood, but detected in milk due to the production of antibodies by B-cells of the udder lymph nodes [20-23]. However, so far the possibility of using milk as a test sample in IHA for screening vaccinated livestock for brucellosis remains unexplored. The purpose of the work was to study the serological potential of IHA with milk (IHA/m) in differentiating post-infectious from post-vaccination antibodies in cows immunized with B. abortus 82.

Materials and methods

Animals. The study used 40 lactating cows, of which 10 were immunized with the live attenuated *B. abortus* 82(SR) vaccine (Shchelkovo Bioplant, Moscow, RF) in accordance with the manufacturer's instructions, 20 were unvaccinated reactors isolated for slaughter according to AT and/or CFT indications, and 10 were healthy unvaccinated animals kept on a brucellosis-free farm.

Biological analytes. Blood serum samples for serological studies were taken from cows on the 30th, 60th and 90th days post-vaccination (p.v.) in vacutainers in a volume of 7-8 ml, milk samples - in sterile tubes in the same days from four udder lobes in a total volume of 20 ml. At the same time, cows kept in a brucellosis-free farm were subjected to serological testing. Similar analytes from seropositive cows were taken once before culling.

Serological tests, namely AT, CFT, radial immunodiffusion with O-polysaccharide antigen (RID/O-PS), milk ring test (MRT), IHA with serum (IHA/s) were carried out in accordance with the Interstate Standard GOST 34105-2017 [24].

The milk and blood serum samples were tested by IHA starting from the dilutions of 1:25 and 1:50, respectively, according to our previously described methods [25]. The IHA results were considered positive in the titer of anti-*Brucella* antibodies 1:100 and 1:200 with a score of no less than four crosses ("#") or three crosses ("+++") when examining milk and/or blood serum of unvaccinated and vaccinated cows, respectively. The results with a score of two crosses ("++"), one cross ("+") and minus ("-") were considered negative.

Control sera. As a control for serological tests, negative brucellosis serum and/or positive brucellosis serum (Scientific-Production Company "Biocenter", Omsk, RF) were used. The latter had titers of 1:200, 1:20#, 1:400# and 1:200# for AT, CFT, IHA/s and IHA/m, respectively.

Control milk samples. Milk from a known healthy cow with negative results by classical serological tests and/or a mixture of milk from a healthy cow with positive brucellosis serum (0.1 ml of serum per 2 ml of milk) were used as negative and/or positive control milk samples.

Testing cows for mastitis. Subclinical mastitis was excluded using the "Al-Test" (Caspian Zonal Research Veterinary Institute, Makhachkala, Russia) before testing milk by IHA and MRT for anti-Brucella antibodies.

Statistical processing of serological tests results was carried out according to the method described by T.S. Sayduldin (1992) [26].

Results

Examination of blood sera from healthy cows (n=10) from a brucellosis-free farm by AT, CFT and IHA/s, as well as milk by MRT and IHA/m showed negative results, which indicates the specificity of the serological tests used.

Blood serum and milk samples from unvaccinated cows (n=20), recognized as *Brucella*-infected based on the results of a planned examination of the livestock by the farm's veterinary service, were additionally subjected to serological tests (Table 1).

Table 1 – Titers of agglutinating and complement-fixing antibodies in the blood serum and milk of cows culled due to brucellosis

Animal numbers	Samples examined in serological tests				
	blood serum			milk	
	AT	CFT	IHA/s	IHA/m	MRT
1	1:400#	1:20+++	1:400#	1:400#	+ve
2	1:100+++	1:40#	1:800#	1:400+++	+ve
3	-ve	1:10+++	1:200++	1:200#	+ve
4	1:200#	1:20#	1:400#	1:200+++	-ve
5	1:400#	1:40#	1:800+++	1:800+++	+ve
6	1:200+++	-ve	1:400+++	1:200#	-ve

Continuation of Table 1

7	1:50#	1:10+++	1:200+++	1:200+++	+ve
8	1:100#	1:40#	1:200#	1:400+++	+ve
9	1:200+++	1:20#	1:400+++	1:400#	+ve
10	1:200+++	1:40+++	1:800+++	1:800+++	+ve
11	1:200#	1:10#	1:400+++	1:200#	-ve
12	1:200#	1:20#	1:400#	1:400+++	+ve
13	1:50+++	1:10+++	1:200+++	1:200+++	+ve
14	1;400+++	1:40#	1:400#	1:400#	+ve
15	1:200+++	1:20+++	1:400#	1:400#	+ve
16	1:400#	1:40#	1:800#	1:800+++	+ve
17	1:200#	1:40+++	1:400#	1:400+++	+ve
18	-ve	1:20#	1:400+++	1:400#	+ve
19	1:100+++	-ve	1:200#	1:200+++	-ve
20	-ve	1:10+++	1:100#	1:100#	+ve
Average antibody titer	1:260,0 (+8,7%;-8,0%)	1:17,5 (+8,7%;-8,0%)	1:760,0 (+13,3%;-1,7%)	1:700,0 (+13,3%;-11,7%)	
Notes: (+ve) - positive result; (-ve) - negative result					

Table 1 shows that antibodies in diagnostic titers were detected in all cows by IHA/s, while AT results were negative in 3 and questionable in 5 cows (1:50-1:100). A significant difference was established between the average values of agglutinin titers detected by IHA/s (1:760 (+13.3%; -11.7%) and AT (1:260 (+8.7%; -8.0%) (P ≤0.01). Moreover, the average antibody titer by IHA/s in AT-negative cows - 1:235 (+29.2%; -22.6%) was significantly lower than that of AT-positives - 1:475 (+6.4%; -22.6%). -6.0%) (P≤0.05). CFT was more sensitive than AT, detecting complement-fixing antibodies in 7 sera with negative or equivocal results for anti-*Brucella* agglutinins. It should be noted that among the culled livestock, a cow №19 showed a questionable result by AT (1:100), negative results by CFT and MRT, however, hemagglutinins were detected both in serum and in milk in a titer of 1:200 with an assessment four and three crosses, respectively. In general, both variants of IHA detected anti-*Brucella* antibodies in 45% of cases where AT and/or CFT had equivocal or negative results.

Testing of cows for subclinical mastitis showed the absence of this pathology in culled cows. *Brucella*-specific antibodies by IHA/m were detected in all cows with an average titer of 1:700 (+13.3%; -11.7%), while MRT did not reveal the presence of antibodies in the milk of four cows, two of which were also negative by CFT. The difference between the average titers of IHA/m-antibodies detected in AT-negative and/or positive cows was not significant - 1:235 (+29.2%; -22.6%) and/or 1:400 (+13.3%; -11.7%), respectively. A high direct correlation was established between IHA antibody titers in blood serum and milk (r=0.793), as well as a noticeable direct relationship was noted between the indicators of CFT and IHA/s (r=0.543) and CFT and IHA/m (r=0.564).

The study of the dynamics of post-vaccination antibodies in blood sera by AT, CFT, RID/O-PS and IHA/s, as well as in milk samples by MRT and IHA/m, was carried out during the first three months post vaccination (p.v.) with live attenuated vaccine *B. abortus* 82. Table 2 shows the state of the humoral immune response of cows at the 30th day p.v.

Table 2 – The results of serological study of milk and blood serum samples of cows at 30th day p.v. with *B. abortus* 82

№	IHA/m (1:50-1:400)	MRT	IHA/s				CFT				AT			RID/O-PS
			1:50	1:100	1:200	1:400	1:5	1:10	1:20	1:40	1:50	1:100	1:200	
1	-ve	-ve	#	#	#	#	#	#	#	+++	#	#	+++	+ve
2	-ve	-ve	#	#	#	#	#	#	+++	+++	#	#	#	+ve
3	-ve	-ve	#	#	#	#	#	#	#	#	#	#	+++	+ve
4	-ve	-ve	#	#	#	#	#	#	#	#	#	#	#	+ve
5	-ve	-ve	#	#	#	#	#	#	#	#	#	#	#	+ve
6	-ve	-ve	#	#	#	#	#	#	#	#	#	#	#	+ve
7	-ve	-ve	#	#	#	#	#	#	#	#	#	#	+++	+ve
8	-ve	-ve	#	#	#	+++	#	#	+++	+++	#	#	#	+ve
9	-ve	-ve	#	#	#	#	#	#	#	+++	#	#	++	+ve
10	-ve	-ve	#	#	#	#	#	#	+++	+++	#	#	#	+ve
Notes: (+ve) - positive result; (-ve) - negative result														

From Table 2 it follows that the live attenuated *B. abortus* 82 vaccine has good immunogenicity. Agglutinins were detected up to the maximum dilutions of blood sera by AT (1:200) and IHA (1:400) in 60% and 90% of cases with a score of four crosses, respectively. With a similar assessment, complement-fixing antibodies were detected up to a dilution of 1:40 in half of the livestock. Moreover, precipitating antibodies were also detected by RID/O-PS in all experimental animals. Despite the intense antibody immune response to the vaccination, anti-*Brucella* agglutinins were absent in the milk of all cows, both by MRT and IHA/m.

Table 3 shows the results of testing the analytes at the 60th day p.v.

Table 3 – The results of serological assay of milk and blood serum samples of cows at 60th day p.v. with *B. abortus* 82

№	IHA/m (1:50-1:400)	MRT	IHA/s				CFT				AT			RID/O-PS
			1:50	1:100	1:200	1:400	1:5	1:10	1:20	1:40	1:50	1:100	1:200	
1	-ve	-ve	#	#	+++	-ve	#	#	#	++	#	#	++	+ve
2	-ve	-ve	#	#	++	-ve	#	#	+++	++	#	#	-ve	+ve
3	-ve	-ve	#	#	+++	++	#	#	#	#	#	#	++	+ve
4	-ve	-ve	#	#	++	-ve	#	#	#	+++	#	+++	-ve	-ve
5	-ve	-ve	#	#	+++	+++	#	#	+++	++	#	#	#	+ve
6	-ve	-ve	#	+++	-ve	-ve	#	#	#	++	#	#	-ve	+ve
7	-ve	-ve	#	#	#	+++	#	#	#	-ve	#	#	+++	-ve
8	-ve	-ve	#	#	+++	++	#	#	+++	+++	#	#	-ve	+ve
9	-ve	-ve	#	#	#	++	#	#	#	++	#	#	-ve	+ve
10	-ve	-ve	#	#	+++	+++	#	#	+++	++	#	#	#	+ve
Notes: (+ve) - positive result; (-ve) - negative result														

As can be seen from Table 3, at the 60th day of immunization of cows, a weakening of antibody formation occurred. For example, precipitating antibodies were not detected in two animals, and the results of IHA/s and AT were negative at maximum dilutions in four and five animals, respectively.

Complement-fixing antibodies were characterized by relative stability. Both analyzes designed to test milk for brucellosis (MRT and IHA/m) gave clear negative results.

The immune status of vaccinated cows at the end of the experiment is shown in Table 4.

Table 4 – The results of serological examination of milk and blood serum samples of cows at 90th day p.v. with *B. abortus* 82

№	IHA/m (1:50-1:400)	MRT	IHA/s				CFT				AT			RID/ O-PS
			1:50	1:100	1:200	1:400	1:5	1:10	1:20	1:40	1:50	1:100	1:200	
1	-ve	-ve	#	#	+++	-ve	#	#	+++	-ve	#	#	-ve	-ve
2	-ve	-ve	#	#	-ve	-ve	#	#	++	-ve	#	#	-ve	-ve
3	-ve	-ve	#	#	++	-ve	#	#	+++	-ve	#	#	-ve	-ve
4	-ve	-ve	#	#	++	-ve	#	#	+++	-ve	#	-ve	-ve	-ve
5	-ve	-ve	#	#	++	-ve	#	#	++	-ve	#	#	-ve	-ve
6	-ve	-ve	#	++	-ve	-ve	#	#	++	-ve	#	+++	-ve	-ve
7	-ve	-ve	#	#	++	-ve	#	#	++	-ve	#	#	-ve	+ve
8	-ve	-ve	#	#	+++	-ve	#	#	-	-ve	#	+++	-ve	-ve
9	-ve	-ve	#	#	++	-ve	#	#	++	-ve	#	#	-ve	-ve
10	-ve	-ve	#	#	++	-ve	#	#	++	-ve	#	#	-ve	-ve
Notes: (+ve) - positive result; (-ve) - negative result														

At the 90th day p.v. questionable AT results for brucellosis were detected in 9 immunized animals in a titer of 1:100 with a score of one to four crosses, and in one cow (№4) agglutinins were not detected in the diagnostic titer. The precipitating and hemagglutinating antibodies in diagnostic values were found in the blood sera of one (№7) and two cows (№1 and №8; 1:200+++), respectively. It should be noted that complement-fixing antibodies remained in diagnostic titers (1:5-1:10) with a score of four crosses in all animals until the end of the experiment. MRT and IHA/m, as in the previous periods of the observation, did not detect post-vaccination antibodies in immunized cows.

Discussion and conclusion

Milk, as an alternative biological analyte, is of great interest in the intravital diagnosis of brucellosis, since, firstly, in addition to serum antibodies, it may also contain antibodies produced by B - lymphocytes of the mammary lymph nodes due to local brucellosis; secondly, it can be easily collected without the use of special equipment [27, 28]. As a non-invasive biological material milk is preferable to blood serum for many infectious diseases of lactating cattle since they allow to determine the health status of not only an individual animal but also the entire herd with minimal material costs. Today, MRT is used to determine anti-brucellosis antibodies in milk in countries (Russia, Kazakhstan, Armenia, Belarus and Kyrgyzstan) that have adopted the interstate standard GOST 34105-2017 [24]. The test is characterized by sufficient sensitivity, however, sometimes it gives false positive results, but much more often it produces a negative reaction in cows infected with brucellosis, which is associated with the influence of factors such as fat content and/or acidity of milk, size of fat globules, clinical and subclinical forms of mastitis, as well as the state of pregnancy [29-31]. MRT was no more successful in diagnosing sheep and goats brucellosis than in cows. It has been suggested that the small fat globules in sheep and goat milk are less active in absorbing agglutinated stained *Brucella* cells, do not rise to form a typical colored ring, and settle at the bottom of the tube [32]. Antigens absorbed on the surface of the solid phase (erythrocytes) and the IHA protocol neutralize the indicated negative effects of milk factors on the assay results. For example, it has been established that IHA/m is characterized by specificity, and its results do not depend on inflammation of the mammary gland or pregnancy. In cows with milk hemagglutinins, the results of culture isolation and polymerase chain reaction (PCR) were positive in 67 and 83% of cases, respectively [33].

Our results showed that among the serological tests used, the most sensitive were IHA/s and IHA/m, which confirmed the diagnosis of brucellosis when AT and/or CFT gave questionable and negative readings. Moreover, the degree of correlation between the results of the two variants of IHA was very high (r = 793), and hemagglutinin titers in the blood were significantly higher (1:760) than that of agglutinins detected by AT (1:260; P <0.01). In the blood serum of 10% of animals culled due to brucellosis, antibodies were not detected by CFT, while IHA/m in these cows was positive in a titer of 1:200 with a score of three or four crosses.

Vaccination of cows caused increased production of complement-fixing, agglutinating and precipitating antibodies, which tended to weaken during observation of the animals: 30th, 60th and 90th days p.v. By the end of the experiment, all vaccinated animals showed a questionable and/or negative reaction to brucellosis by AT; positive RID/O-PS and IHA/s were noted in 5% and 10% of cases, respectively. However, complement-fixing antibodies remained at diagnostic values in all animals until the end of the experiment. Despite the intense antibody immune response to the vaccine injection, there were no *Brucella*-specific agglutinins in the milk already on the 30th day p.v., both by IHA/m and MRT.

Thus, the data obtained indicate the need for further studies on a large cow population to determine the diagnostic value of IHA/m for differentiating animals immunized against brucellosis with live attenuated vaccines from infected ones in comparison with culture isolation and/or PCR.

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