TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2019) 43: 486-493

doi:10.3906/vet-1902-75

Serodiagnostic potential of *Brucella* outer membrane and periplasmic proteins

Aitbay BULASHEV[1,*], Orken AKIBEKOV[1], Zhanbolat SURANSHIYEV[1], Bakytkali INGIRBAY[1], Saule ESKENDIROVA[2]

[1]Department of Microbiology and Biotechnology, Faculty of Veterinary and Livestock Technology, S. Seifullin Kazakh Agrotechnical University, Astana, Kazakhstan

[2]Laboratory of Stem Cells, National Center for Biotechnology, Astana, Kazakhstan

Received: 21.02.2019 • **Accepted/Published Online:** 05.07.2019 • **Final Version:** 07.08.2019

Abstract: The aim of this study was to evaluate the serological diagnostic potential of the *Brucella* recombinant outer membrane (rOMP25, rOMP31) and periplasmic proteins (rBP26, rSOD) in a comparative way using an indirect enzyme-linked immunosorbent assay (i-ELISA). Rabbit and/or mouse antibodies to *Brucella* whole cell and/or soluble protein preparations recognized all recombinant proteins used, which confirms the expression of target antigens in *E. coli* in active form. The recombinant proteins showed different antigenicity to antibodies of cattle kept on a brucellosis-affected (endemic) farm and/or a new focus of infection. Thus, the presence of anti-*Brucella* antibodies was confirmed by i-ELISA/rSOD in 79% of cows from endemic conditions with positive results by conventional serological tests (RBPT and/or CFT). However, antibodies specific to this protein were detected in only 14% of seropositive animals kept in the hotbed of a new brucellosis infection. Moreover, rSOD-specific antibodies were not detected in the sera of vaccinated cattle from a brucellosis-free farm, whereas antibodies to other recombinant proteins were found in 2%–8% of animals. Using recombinant proteins in immunoassays significantly reduced the number of cows positive for brucellosis. Furthermore, there was not a single protein among the rOMPs that would show the total positive results of all proteins used. Thus, the development of reliable ELISA tests for the diagnosis of brucellosis requires further comprehensive study of the recombinant proteins in order to design a multiprotein antigen that consists of a combination of several proteins with diagnostic potential.

Key words: Brucellosis, cattle, diagnosis, recombinant proteins, enzyme-linked immunosorbent assay

1. Introduction

Brucellosis is one of the most common zoonotic infections that negatively affects livestock productivity and leads to lifelong disability [1]. More than half a million new cases of human brucellosis are recorded worldwide every year, although this figure is considered to be largely underreported. Kazakhstan, as well as six other former Soviet republics (Kyrgyzstan, Tajikistan, Azerbaijan, Turkmenistan, Armenia, and Uzbekistan), is among the 25 countries with the highest incidence of the disease [2]. An early diagnosis of *Brucella*-infected animals is a key element in eradication programs. For this purpose, conventional serological tests, such as the serum agglutination test (SAT), Rose Bengal plate test (RBPT), complement fixation test (CFT), and enzyme-linked immunosorbent assay (ELISA), have been widely used [3]. In these tests smooth lipopolysaccharides (S-LPS) of *Brucella* spp., the most superficial layer of the cell wall, have been mainly used to identify pathogen-specific antibodies. Therefore, it is very difficult to differentiate animals immunized using live attenuated vaccines from naturally *Brucella*-infected animals [4]. Moreover, traditional tests based on the use of *Brucella* whole cell S-LPS as an antigen do not always give reliable results because of cross-reactivity with other gram-negative bacteria, such as *Yersinia enterocolitica* O:9 [5], *Escherichia coli* O157:H7 [6], *Salmonella* spp., or *Pseudomonas maltophilia* [7]. In this regard, *Brucella* immunogenic proteins have been the focus of attention of researchers engaged in the development of diagnostic kits for brucellosis and vaccine design [8]. At least 67 proteins, including 4 major outer membrane proteins (OMPs), have been described as part of the outer membrane-peptidoglycan complex from *B. abortus* [9]. OMPs were classified according to their apparent molecular weight as 36–38 kDa OMPs or group 2 porin proteins and 31–34 and 25–27 kDa OMPs, which belong to the group 3 proteins [10]. Advances in genetic engineering have made it possible to study the use of recombinant OMPs (rOMPs) as antigens to avoid biological hazards associated with the use of viable *Brucella* strains. Besides, "a test based on recombinant proteins would allow better standardization of the assay, compared with more complex

* Correspondence: aytbay57@mail.ru

whole-cell antigen preparations currently in use, and hence overcome the limitations associated with the use of LPS-based antigens" [11]. Among the known rOMPs, the most studied ones are rOMP25 [12] and rOMP31 [13]. Another group of recombinant proteins, which is of great interest from a diagnostic point of view, are periplasmic proteins: rBP26 or rOMP28 [14] and Cu-Zn superoxide dismutases (rSOD) [15]. However, the comparative diagnostic value of these two groups of *Brucella* proteins is still scarcely explored. The aim of our study was to evaluate the serological diagnostic potential of *Brucella* rOMPs and periplasmic proteins in a comparative way using indirect ELISA (i-ELISA).

2. Materials and methods

2.1. Experimental animals

Fifteen white outbred male mice (8–10 weeks, 20–25 g body weight) and a Soviet Chinchilla male rabbit (6 months, 3300 g body weight) were kept under good hygienic conditions in the vivarium of S. Seifullin Kazakh Agrotechnical University (KATU), Astana, and their use and care were approved by the Animal Ethics Committee, Faculty of Veterinary and Livestock Technology, KATU. The animals were provided with food and water ad libitum. A 12-h lighting cycle was maintained in the animal housing. Temperature and humidity were monitored daily. All activities involving animals were carried out according to the Guidance for Accommodation and Care of Animals: Species-Specific Provisions for Laboratory Rodents and Rabbits (Interstate Standard, GOST 33216-2014).

2.2. Microbial cultures

Whole cells of *B. abortus* 19, inactivated with phosphate-buffered saline (PBS) containing 0.5% phenol at 37 °C for 48 h, were kindly provided by the Research and Production Enterprise "Antigen", Almaty, Kazakhstan.

Escherichia coli BL21 strains, producing *Brucella* recombinant proteins, were obtained as described in our previous studies: *B. abortus* rOMP25 and *B. melitensis* rOMP31 [16], rBP26 [14], and rSOD [17].

2.3. Preparation of *Brucella* proteins

Extraction of soluble protein preparations (CSPs) from whole cells of *B. abortus* 19 was based on the elution of the membrane proteins with 0.1 M sodium citrate solution (Sigma-Aldrich, St. Louis, MO, USA) containing 1 M sodium chloride (Fisher Chemical, Loughborough, UK) and 0.1% Triton X-100 (Sigma-Aldrich) [18].

E. coli strains, producing *Brucella* recombinant proteins, were grown in Luria-Bertani liquid medium containing 1% baktotripton, 0.5% yeast extract (all from Thermo Fisher Scientific, Waltham, MA, USA), and 1% NaCl supplemented with ampicillin (Sintez, Kurgan, Russia) at a concentration of 100 μg/mL. In the middle of the logarithmic growth phase of the bacterial mass (absorbance at λ = 600 nm, OD_{600} = 0.6), isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) was added to a final concentration of 1 mM to induce expression of the recombinant proteins. The culture was incubated at room temperature for 16 h with shaking and then bacterial cells were harvested by centrifugation at 5000 × *g* at 4 °C for 10 min. After that, cells were resuspended in a lysis buffer (20 mM Tris, pH 7.5; 1 mM EDTA; 100 mM NaCl) in the amount of 10 mL per 1 g wet weight cells, followed by the addition of 1 μL of phenylmethanesulfonyl fluoride (Thermo Fisher Scientific) with a final concentration of 0.2 mM. The bacterial cell suspension was then sonicated using the ultrasonic homogenizer OMNI-Ruptor 4000 (OMNI International, Kennesaw, GA, USA) in an ice-water bath. The recombinant proteins were purified by metal affinity chromatography using commercial HisTrap columns (GE Healthcare Life Sciences, Cardiff, UK) according to the manufacturer's instructions.

2.4. Immunization of mice with protein antigens

Five groups of 3 mice each were used in this study. The mice were immunized each with 25 μg of the respective proteins as follows: the first group with rOMP25, second group with rOMP31, third group with rBP26, fourth group with rSOD, and fifth group with *B. abortus* CSP. Mice were immunized according to the following scheme: on day 0 the appropriate antigen emulsified in Freund's incomplete adjuvant (Sigma-Aldrich) was injected subcutaneously (s.c.) in an amount of 100 μL. Subsequent injections of antigen in 200 μL of PBS, pH 7.2–7.4 (Amresco, Solon, OH, USA) were performed intraperitoneally on days 7, 14, 21, and 28. Preparation of antisera pools was performed on day 42. Before each immunization blood was taken from the tail vein into microfuge tubes (Isolab, Wertheim, Germany) for the determination of antibody titer by i-ELISA. The control negative sera were sampled on day 0 before the first immunization.

2.5. Determination of antibody titer against *Brucella* protein antigens by i-ELISA

The polystyrene plate wells (Thermo Fisher Scientific) were separately coated with *Brucella* protein preparations (4 °C for 18 h) used for immunization of mice at a concentration of 5.0 μg/mL in bicarbonate buffer, pH 9.6. The contents of the wells were then removed and the plate was washed several times with 0.05% Tween-20 (Sigma-Aldrich) in PBS (PBS-T). The unbound sites of the wells were blocked with 1% bovine serum albumin (Sigma-Aldrich), and dilutions of homologous and heterologous antisera as well as negative control sera in PBS-T were prepared in 8 wells, starting at 1:100, and incubated at 37 °C for 1 h. After a washing procedure, horseradish peroxidase (HRP)-labeled antimouse IgG (Sigma-Aldrich) diluted with PBS-T was added to the wells. The plate was incubated at 37 °C for 1 h, and the substrate orthophenylenediamine (Sigma-

Aldrich) was applied. The plate was kept in a dark place at room temperature. After 3–5 min, an equal amount of 2 M sulfuric acid was added to the wells. The absorbance was measured at 492 nm using a plate reader (Bio-Rad 680, Redmond, WA, USA). For the titer of antibodies the dilution of the antiserum was taken, the optical density (OD) of which was two or more times higher than the OD of the negative control blood serum at a dilution of 1:100.

2.6. Production of rabbit antiserum against *B. abortus* 19 whole cells

A rabbit was s.c. immunized with killed *B. abortus* 19 as previously described [19]. Blood samples were taken from the ear's marginal vein under sedation on day 0 as a negative control, and then every 2 weeks to evaluate antibody response. The hyperimmune serum was collected on day 56 and stored at –20 °C until used.

2.7. Serum samples

Sera of 43 cows from a brucellosis-affected (endemic) farm and of 77 unvaccinated cows from a brucellosis-free farm, where an outbreak (a new focus) of infection had been registered during the scheduled serological testing of animals, were kindly provided by the National Reference Center for Veterinary Medicine, Kazakh Ministry of Agriculture, and the Zhetikara Veterinary Laboratory, Kostanay oblast, respectively. Animals of both groups had reacted positively to brucellosis by conventional serological tests (RBPT and/or CFT). Additionally, 48 serum samples were collected from cows kept on a brucellosis-free farm (Bukhar Zhyrau rayon, Karaganda oblast), where the *B. abortus* 19 vaccine (Schelkovsk Biokombinat, Russia) had been used for immunization. It is given to female calves 5–6 months old as a single subcutaneous dose of 8×10^{10} organisms. Revaccination of animals is carried out in 11–12 months with the same vaccine comprising 8×10^{9} CFU per 0.05 mL by administration to the conjunctiva. For calculation of a cutoff value of i-ELISA, serum samples of 20 unvaccinated heifers from the "Rodina" farm, Tselinograd rayon, Akmola oblast, which has been brucellosis-free for long periods of time, were obtained.

2.8. Serological examinations of rabbit and cattle sera by i-ELSA for anti-*Brucella* antibody

Briefly, the wells of a polystyrene plate (Thermo Fisher Scientific) were separately coated with the following *Brucella* antigens: rOMP25, rOMP31, rBP26, rSOD, and/or *B. abortus* CSP. After washing the wells and blocking active sites of the solid phase, dilutions of cattle blood serum sample (1:100 and 1:200) and/or rabbit hyperimmune serum against *B. abortus* 19 whole cells (1:100 to 1:12,800) were prepared in the wells. Then, after 1 h of incubation, antibovine (Sigma-Aldrich) and/or antirabbit IgG antibodies (Jackson ImmunoResearch, West Grove, PA, USA) labeled with HRP were added to the wells. A cutoff value to distinguish between positive and negative results of i-ELISA was calculated using the mean OD of *B. abortus*-negative sera [20]. For the titer of rabbit antibodies the dilution of the hyperimmune serum was taken, whose OD was two or more times higher than the OD of the negative control serum at a dilution of 1:100.

2.9. Serological examinations of cows from brucellosis-free farm by RBPT

The RBPT ("Antigen", Almaty, Kazakhstan) was carried out according to the manufacturer's instructions.

2.10. Statistical analyses

Statistical analysis of i-ELISA readings was performed using MS Excel 2007 for Windows 7. The results were considered significant at a probability level of less than 0.05.

3. Results

The immunogenicity results of *Brucella* protein preparations by i-ELISA are shown in the Figure.

The *Brucella* protein preparations used had a different immunogenicity. For example, a study of mice blood samples on day 7 showed the absence of specific antibodies in animals immunized with rSOD and rBP26. By this time, the immune response in the form of antibody formation began to develop in mice immunized with *B. abortus* CSP, rOMP25, and rOMP31. Antibodies to rSOD were not detected even up to day 14, whereas antibody titers of the analogues injected with rOMP25 and rOMP31 ranged from 1:200 to 1:800. Attention is drawn to a significant increase in antibody titer in animals stimulated with rBP26 at 1:6400 to 1:12,800 by that time. Further injections (third and fourth) of rBP26 into mice did not cause an increase in the concentration of serum antibodies. After the third immunization, antibody formation against rOMP25 and rOMP31 (1:3200 to 1:6400) as well as *B. abortus* CSP (1:6400 to 1:12,800) reached its peak (day 21). However, anti-rSOD antibodies rose to this level only on day 28.

By the end of immunization (on day 42) the titers of hyperimmune sera against homologous proteins reached 1:12,800 (data not shown).

The results of studying the cross-reactivity of antisera against heterologous proteins are shown in Table 1.

As can be seen in Table 1, antibodies to *Brucella* CSP in a decreasing degree bound to rOMP25 (1:3200), rOMP31 (1:800), and rBP26 (1:400); however, they did not recognize rSOD. Antiserum to the last antigen was negative for all protein preparations used. As might be expected, rSOD did not react with any antiserum. Cross-reactions of varying intensity were noted between rOMP25, rOMP31, and rBP26.

The hyperimmune serum obtained at the end of the immunization (on day 56) from a rabbit by immunization with killed *B. abortus* 19 whole cells had activity against

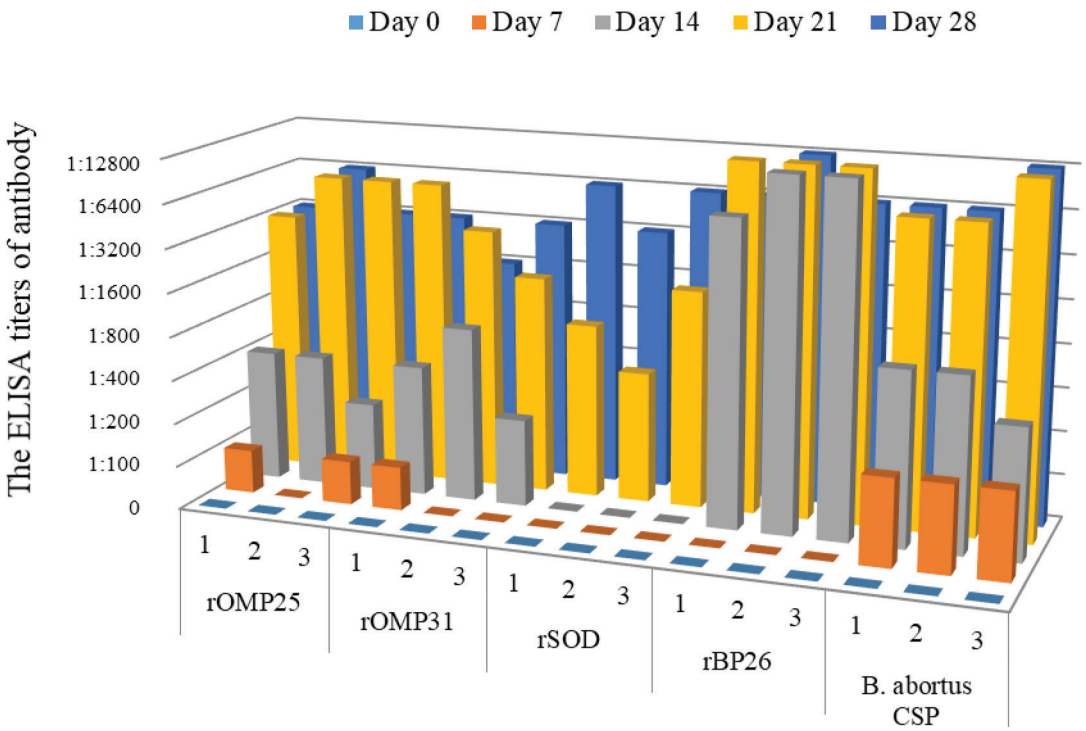

Figure. Immunogenicity of *Brucella* protein antigens in mice. 1, 2, 3 - mice numbers.

Table 1. Cross-reactivity of hyperimmune mice sera to *Brucella* proteins by i-ELISA.

Antisera titers against *Brucella* proteins					
Types of *Brucella* proteins	CSP	rOMP31	rSOD	rBP26	rOMP25
CSP	1:12,800	1:400	NR*	NR	1:200
rOMP31	1:800	1:12,800	NR	1:1600	1:400
rSOD	NR	NR	1:12,800	NR	NR
rBP26	1:400	1:400	NR	1:12,800	1:200
rOMP25	1:3200	1:1600	NR	1:400	1:12,800

* NR: Negative result.

Brucella CSP, showing a titer of 1:51,200, and it was used to determine the antigenicity of *Brucella* recombinant proteins by i-ELISA. The results of the immunoassay showed that hyperimmune serum antibodies bound to recombinant proteins with a high affinity as detected by dilutions to 1: 3200 (rOMP31, rBP26) and 1:12,800 (rOMP25, rSOD), which in turn indicated that these antigens were expressed in *E. coli* BL21 in active form.

Furthermore, the antigenicity of protein preparations was studied using blood sera samples of cows from farms with different epizootic situations regarding brucellosis. To determine the cutoff value for the enzyme immunoassay, 20 *B. abortus*-negative heifers' blood sera were used. The average OD_{492} values of brucellosis-negative sera were 0.101 ± 0.010, 0.126 ± 0.012, 0.109 ± 0.008, 0.148 ± 0.013, and 0.069 ± 0.009 at 1:200-fold dilution for i-ELISA based on *B. abortus* CSP, rOMP25, rOMP31, rBP26, and/or rSOD, and the cutoff values were determined at 0.202, 0.252, 0.218, 0.296, and 0.138, which were double the average OD_{492} of negative sera, respectively .

Below are the results of i-ELISA testing of 43 cows kept on a brucellosis-affected farm (Table 2).

From Table 2 it follows that the use of *B. abortus* CSP and rSOD as antigens gave a relatively high sensitivity to the immunoassay and allowed detection of antibodies in the largest number of animals studied (86% and 79%,

respectively), whereas the other three recombinant proteins detected anti-*Brucella* antibodies in rather low numbers of cattle.

There was a high correlation between i-ELISA/rOMP25 and i-ELISA/rOMP31 (r = 0.72). An appreciable positive correlation was noted between the results of the immunoassay based on rOMP25 and rBP26 (r = 0.52), rOMP31 and rBP26 (r = 0.56), and *B. abortus* CSP and rSOD (r = 0.57).

Table 3 shows the results of studying antigenicity of *Brucella* proteins using blood serum samples of 77 cows from a new focus of infection.

B. abortus CSP showed maximum antigenicity by detecting antibodies in 95% of the cows kept in the hotbed of new brucellosis infection. It should be noted that the periplasmic proteins pBP26 and rSOD turned out to be less antigenic than the OMPs, detecting the presence of specific antibodies in 30% and 14% of the cattle, respectively.

Among the cows kept on the brucellosis-free farm subjected to i-ELISA, 11 and 37 of 48 animals had been vaccinated with *B. abortus* 19 vaccine 6 months and 11 months before the serological examinations, respectively. All but one animal (90%) vaccinated 6 months ago showed a positive result by RBPT, while only 10 animals (27%) vaccinated 11 months ago were seropositive by this test.

Table 4 presents the results of serological testing of cows from the brucellosis-free farm by i-ELISA: *B. abortus* CSP antibodies were detected by i-ELISA in 23% of the cattle, whereas, as mentioned above, among the vaccinated livestock positive RBPT results were obtained in 42% cases.

As for recombinant proteins, antibodies to rOMP25 were only detected in 8% of the animals, and one cow

Table 2. Antigenicity of *Brucella* proteins by i-ELISA to antibodies of seropositive cows from brucellosis-affected farm.

*ODt/**ODc values	*Brucella* protein antigens used in i-ELISA				
	CSP	rOMP25	rOMP31	rBP26	rSOD
	Number of i-ELISA-positive cows				
2.00 – 2.50	2	7	20	12	7
2.51 – 3.00	0	11	3	2	0
3.01 – 3.50	4	1	0	1	1
3.51 – 4.00	0	0	0	3	1
< 4.01	31	0	0	3	25
Total number (%) of seropositive animals	37 (86)	19 (44)	23 (53)	21 (49)	34 (79)
ODt/ODc mean values	8.46 ± 0.54	2.49 ± 0.08	2.31 ± 0.05	2.96 ± 0.28	8.87 ± 1.17

*ODt: OD of test serum, **ODc: mean OD of negative sera.

Table 3. Antigenicity of *Brucella* proteins to antibodies of cows from the focus of the brucellosis outbreak.

*ODt/**ODc values	*Brucella* protein antigens used in i-ELISA				
	CSP	rOMP25	rOMP31	rBP26	rSOD
	Number of i-ELISA-positive cows				
2.00–2.50	3	20	9	12	6
2.51–3.00	2	11	8	5	2
3.01–3.50	0	5	20	5	2
3.51–4.00	3	2	10	0	0
<4.01	65	1	12	1	1
Total number (%) of seropositive animals	73 (95)	39 (51)	59 (77)	23 (30)	11 (14)
ODt/ODc mean values	6.63 ± 0.23	2.55 ± 0.08	3.5 ± 0.12	2.66 ± 0.17	3.5 ± 0.12

*ODt: OD of test serum, **ODc: mean OD of negative sera.

Table 4. Antigenicity of *Brucella* proteins to sera samples of cows from a brucellosis-free farm.

*ODt/**ODc values	*Brucella* protein antigens used in i-ELISA				
	CSP	rOMP25	rOMP31	rBP26	rSOD
	Number of i-ELISA-positive cows				
2.00–2.50	7	2	1	0	0
2.51–3.00	2	1	0	1	0
3.01–3.50	1	0	0	0	0
3.51–4.00	1	0	0	0	0
<4.01	0	1	0	0	0
Total number (%) of seropositive animals	11 (23)	4 (8)	1 (2)	1 (2)	0 (0)
ODt/ODc mean values	1.55 ± 0.68	1.35 ± 0.67	1.16 ± 0.36	1.07 ± 0.25	0.91 ± 0.32

*ODt: OD of test serum, **ODc: mean OD of negative sera.

each was positive for rOMP31 and rBP26, respectively. Moreover, antibodies of these animals simultaneously bound to rOMP25. It should be noted that antibodies specific for rSOD were not detected in any cow from the brucellosis-free farm.

Comparing the ODt/ODc mean values (Tables 2–4), it can be seen that the antigenicity of *Brucella* proteins was significantly higher when testing blood sera of cattle from the brucellosis outbreak and/or endemic foci of infection than animals kept on a brucellosis-free farm. For example, while the ODt/ODc mean value of brucellosis-free cattle was 0.91 ± 0.32 by i-ELISA/rSOD, this indicator reached 3.50 ± 0.12 ($P \leq 0.01$) and 8.87 ± 1.17 ($P \leq 0.001$) for the herd from the brucellosis outbreak and endemic foci, respectively. Significant differences between these groups of animals were noted in the intensity of anti-*Brucella* antibodies binding to other recombinant proteins: rOMP31 and rBP26 ($P \leq 0.01$), rOMP25 ($P \leq 0.05$), and *B. abortus* CSP ($P \leq 0.001$).

4. Discussion

Maximum antibody titers in mice against rOMP25 and rOMP31 were noted after the third injection of antigens on day 21. Antibody production was relatively lower in mice immunized with rSOD. In animals of this group, the highest titers of specific antibodies were established only by day 28 after the fourth injection of antigen. rBP26 had the highest immunogenicity among the protein preparations tested. Thus, maximum production of anti-rBP26 antibodies was already achieved after double immunization of mice on day 15. Our data are consistent with the findings of other researchers who also observed immunological dominance of rBP26 and the possibility of using this antigen in the serological diagnosis of human [21] and bovine brucellosis [22].

Antiserum against *Brucella* CSP recognized rOMP25, rOMP31, and rBP26, confirming the authenticity of the recombinant proteins expressed in *E. coli*. These three recombinant proteins appear to have some similar determinants, as was evident from varying intensities of cross-reactions. The interaction of the anti-*Brucella* whole cell antibodies of hyperimmune rabbit serum with all the recombinant proteins used in i-ELISA also confirms the expression of the target antigens by *E. coli* BL21 in their native states.

B. abortus CSP in i-ELISA was the most antigenic among *Brucella* proteins used, which confirmed positive results of 86.0% and 95% of seropositive cows kept on brucellosis-affected and outbreak farms, respectively.

Using recombinant proteins in immunoassays significantly reduced the number of seropositive cows as compared with the results of RBPT and/or CFT. These results suggest that the standard *Brucella* whole cell antigen that is currently used in the serological diagnosis of brucellosis has low specificity.

The recombinant proteins showed varying degrees of antigenicity to antibodies of cattle kept in endemic and/or new foci of brucellosis infection. Cows of the latter group were better identified by i-ELISA/rOMP25 (51%) and i-ELISA/rOMP31 (77%) than by i-ELISA/rBP26 (30%) and i-ELISA/rSOD (14%). The relatively low antigenicity of rSOD to serum antibodies of cows from a new brucellosis focus can be explained by the low accessibility of superoxide dismutase in the *Brucella* cell. In addition, the SOD identity of mammalian species and *Brucella* may have an inhibitory effect on the humoral immune response of both cows and experimentally immunized mice against this enzyme [23]. Consequently, the production of antibodies against the pathogen's antioxidant enzyme is delayed as compared with the host's

immune response to the OMPs. As for BP26, there are various hypotheses about its location. Some researchers think it is a periplasmic protein [24], while others think it is an intracellular soluble protein [25]. In either of these cases, BP26 is also a less accessible component for the immune system than OMPs. In this regard, antibody production to BP26 is also delayed compared with the immune response to proteins located on the outer cell membrane.

rSOD-specific antibodies were not detected in the sera of cattle from the brucellosis-free farm vaccinated with *B. abortus* 19, while antibodies to rOMPs and rBP26 were detected in 2%–8% of animals. These data are not consistent with the results of researchers who described antiprotein antibody response only in ruminants with active brucellosis [26]. Moreover, in a mouse model, it was found that i-ELISA based on combined rOMPs was able to differentiate infected mice from ones vaccinated with *B. melitensis* Rev.1 [19]. Our results showed that antiprotein antibody response could also be developed by vaccinated animals.

B. abortus CSP, although it consists of cell wall proteins, contains LPS impurities. Therefore, we postulate that the lower sensitivity of i-ELISA/rOMPs compared to i-ELISA/*B. abortus* CSP is due to the higher specificity of the first variant of the immunoassay. Furthermore, our results show that the use of a single recombinant *Brucella* protein reduced the sensitivity of i-ELISA. Thus, there was not a single protein among the rOMPs that would show the total positive results of all proteins used.

These results allow us to conclude that i-ELISA based on rOMP25 and/or rOMP31 is more sensitive for detecting antibodies in cattle from a new brucellosis focus, whereas the periplasmic proteins (rBP26 and rSOD) are far superior to OMPs for testing animals of a brucellosis-affected farm. We believe that *Brucella* OMPs are useful as antigens for screening cattle of brucellosis-free areas for early detection of infected animals, whereas periplasmic proteins are useful for scheduled serological examinations of animals kept in brucellosis-affected areas. In our view, a more appropriate approach in improving the serological diagnosis of brucellosis is developing ELISA kits based on a multiprotein recombinant antigen, which can be used to test animals regardless of their location area. The efficiency of such tests should be evaluated not only in comparison with serological methods, but also bacteriological analysis, which is considered the gold standard for diagnosis of brucellosis.

Acknowledgment
This study was financially supported by the Ministry of Education and Science of the Republic of Kazakhstan within the framework of Scientific-Technical Program No. BR05236307, "Development of new products and innovative Biotechnologies for Agriculture and Veterinary Medicine", for 2018–2020.

References

1. Franc K, Krecek R, Häsler B, Arenas-Gamboa A. Brucellosis remains a neglected disease in the developing world: a call for interdisciplinary action. BioMed Central Public Health 2018; 18: 125. doi: 10.1186/s12889-017-5016-y
2. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. Lancet Infectious Diseases 2006; 6 (2): 91-99. doi: 10.1016/S1473-3099(06)70382-6
3. Al Dahouk S, Tomaso H, Nockler K, Neubauer H, Frangoulidis D. Laboratory-based diagnosis of brucellosis–a review of the literature. Part II: Serological tests for brucellosis. Clinical Laboratory 2003; 49: 577-89.
4. Mandal SS, Duncombe L, Ganesh NV, Sarkar S, Howells L et al. Novel solutions for vaccines and diagnostics to combat brucellosis. ACS Central Science 2017; 3 (3): 224-231. doi: 10.1021/acscentsci.7b00019
5. Erdenebaatar J, Bayarsaikhan B, Watarai M, Makino S, Shirahata T. Enzyme-linked immunosorbent assay to differentiate the antibody responses of animals infected with *Brucella* species from those of animals infected with *Yersinia enterocolitica* O:9. Clinical and Diagnostic Laboratory Immunology 2003; 10 (4): 710-714.
6. Bonfini B, Chiarenza G, Paci V, Sacchini F, Salini R et al. Cross-reactivity in serological tests for brucellosis: a comparison of immune response of *Escherichia coli* O157:H7 and *Yersinia enterocolitica* O:9 vs *Brucella* spp. Veterinaria Italiana 2018; 54 (2): 107-114. doi: 10.12834/VetIt.1176.6539.2
7. Corbel M, Stuart F, Brewer R. Observations on serological cross-reactions between smooth *Brucella* species and organisms of other genera. Developments in Biological Standardization 1984; 56: 341-348.
8. Ko K, Kim J, Her M, Kang S, Jung S et al. Immunogenic proteins of *Brucella abortus* to minimize cross reactions in brucellosis diagnosis. Veterinary Microbiology 2012; 156 (3-4): 374-380. doi: 10.1016/j.vetmic.2011.11.011
9. Sowa B, Kelly K, Ficht T, Frey M, Adams L. SDS-soluble and peptidoglycan-bound proteins in the outer membrane-peptidoglycan complex of *Brucella abortus*. Veterinary Microbiology 1991; 27 (3-4): 351-369.
10. Cloeckaert A, Vizcaíno N, Paquet J, Bowden R, Elzer P. Major outer membrane proteins of *Brucella* spp.: past, present and future. Veterinary Microbiology 2002; 90 (1-4): 229-247.

11. Navarro-Soto M, Morales-Loredo A, Álvarez-Ojeda G, Ramírez-Pfeiffer C, Tamez-Guerra P et al. Recombinant proteins as antigens in serological diagnosis of brucellosis. In: Baddour MM (editor). Updates on Brucellosis. Rijeka, Croatia: IntechOpen; 2015. pp. 161-169. doi:10.5772/61762.

12. Cloeckaert A, Zygmunt M, Bézard G, Dubray G. Purification and antigenic analysis of the major 25-kilodalton outer membrane protein of *Brucella abortus*. Research in Microbiology 1996; 147 (4): 225-235.

13. Gupta V, Verma D, Singh S, Vihan V. Serological diagnostic potential of recombinant outer membrane protein (Omp31) from *Brucella melitensis* in goat and sheep brucellosis. Small Ruminant Research 2007; 70 (2-3): 260-266. doi: 10.1016/j.smallrumres.2006.01.012

14. Manat Y, Shustov A, Evtehova E, Eskendirova S. Expression, purification and immunochemical characterization of recombinant OMP28 protein of *Brucella* species. Open Veterinary Journal 2016; 6 (2): 71-77. doi: 10.4314/ovj.v6i2.1

15. Tsogtbaatar G, Tachibana M, Watanabe K, Kim R, Suzuki H et al. Enzyme-linked immunosorbent assay for screening of canine brucellosis using recombinant Cu-Zn superoxide dismutase. Journal of Veterinary Medical Science 2008; 70 (12): 1387-1389.

16. Bulashev A, Jakubowski T, Tursunov K, Kiyan V, Zhumalin A. Immunogenicity and antigenicity of *Brucella* outer membrane proteins. Veterinarija ir zootechnika 2018; 76 (98): 17-24.

17. Eskendirova S, Shustov A, Evtehova E, Manat Y. Patent №30873 RK, MPK C12N 15/52, C12N1/21 Rekombinantnyy shtamm *Escherichia coli* BL21(DE3)/pET22/SOD, produtsiruyushchiy Cu/Zn-zavisimuyu superoksiddismutazu / Eskendirova S, Shustov A, Evtehova E, Manat Y; zayavitel' i patentoobladatel' Natsional'nyy tsentr po biotekhnologii RK.- № 2014/1335.1; Zayavleno 17.10.2014; Opublikovano 25.12. 2015, Byulleten' № 12.-5 s (in Russian).

18. Tabatabai L, Deyoe B. Biochemical and biological properties of soluble protein preparations from *Brucella abortus.* Developments in Biological Standardization 1984; 56: 199-211.

19. Ahmed I, Khairani-Bejo S, Hassan L, Bahaman A, Omar A. Serological diagnostic potential of recombinant outer membrane proteins (rOMPs) from *Brucella melitensis* in mouse model using indirect enzyme-linked immunosorbent assay. BMC Veterinary Research 2015; 11: 275. doi: 10.1186/s12917-015-0587-2

20. Lim J, Kim D, Lee J, Kim D, Min W et al. Evaluation of recombinant 28 kDa outer membrane protein of *Brucella abortus* for the clinical diagnosis of bovine brucellosis in Korea. Journal of Veterinary Medical Science 2012; 74 (6): 687-691. doi: 10.1292/jvms.11-0512

21. Thavaselvam D, Kumar A, Tiwari S, Mishra M, Prakash A. Cloning and expression of the immunoreactive *Brucella melitensis* 28 kDa outer-membrane protein (Omp28) encoding gene and evaluation of the potential of Omp28 for clinical diagnosis of brucellosis. Journal of Medical Microbiology 2010; 59 (4): 421-428. doi: 10.1099/jmm.0.017566-0

22. Chaudhuri P, Prasad R, Kumar V, Gangaplara A. Recombinant OMP28 antigen-based indirect ELISA for serodiagnosis of bovine brucellosis. Molecular and Cellular Probes 2010; 24 (3): 142-145. doi: 10.1016/j.mcp.2009.12.002

23. Beck B, Tabatabai L, Mayfield J. A protein isolated from *Brucella abortus* is a Cu-Zn superoxide dismutase. Biochemistry 1990; 29 (2): 372-376.

24. Rossetti O, Arese A, Boschiroli M, Cravero S. Cloning of *Brucella abortus* gene and characterization of expressed 26-kilodalton periplasmic protein: potential use for diagnosis. Journal of Clinical Microbiology 1996; 34 (1): 165-169.

25. Cloeckaert A, Debbarh H, Vizcaino N, Saman E, Dubray G et al. Cloning, nucleotide sequence, and expression of the *Brucella melitensis* bp26 gene coding for a protein immunogenic in infected sheep. FEMS Microbiology Letters 1996; 140 (2-3): 139-144. doi: 10.1111/j.1574-6968.1996.tb08327.x

26. Letesson J, Tibor A, van Eynde G, Wansard V, Weynants V et al. Humoral immune responses of *Brucella*-infected cattle, sheep, and goats to eight purified recombinant *Brucella* proteins in an indirect enzyme linked immunosorbent assay. Clinical and Diagnostic Laboratory Immunology 1997; 4 (5): 556-564.