IMMUNOGENICITY AND ANTIGENICITY OF *BRUCELLA* RECOMBINANT OUTER MEMBRANE PROTEINS

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Abstract. Classical serological tests for diagnosing bovine brucellosis are mainly based on the detection of antibodies directed against the smooth lipopolysaccharides (S-LPS) of *Brucella* cell membrane, which could give false positive results because of cross-reactivity with other Gram-negative bacteria. Therefore, there has been an ongoing search for non-LPS candidate antigens for the diagnosis of brucellosis, and several *Brucella* recombinant outer membrane proteins (rOmps) have been identified as targets of antibody response. Here, *Brucella* rOmp25 and rOmp31 were expressed in *E. coli* using prokaryotic pET32 and/or pET28+ expression vectors. BALB/c mice were used to study immunogenicity of these rOmps. The antigenicity of the rOmps, as well as soluble protein preparations (CSP) of *B. abortus* and *B. melitensis* were examined by indirect enzyme-linked immunosorbent assay (i-ELISA) on sera samples from 44 cattle that were positive for brucellosis by agglutination test (AT) and complement fixation test (CFT). The rOmps triggered distinct immune response in mice in the form of antibody production. Antisera to rOmp25 and/or rOmp31 reacted with homologous proteins and also showed mutual cross-reactions, which demonstrate similarity of the epitopes of these antigens. The i-ELISA based on CSP of *B. abortus* and *B. melitensis* indicated the presence of antibodies in 68.2% and 59.5% of seropositive cattle, respectively. Using rOmp25 and rOmp31 as coating antigens indicated the presence of specific antibodies only in 52.3% and 36.4% of cows with positive results by conventional serological tests, respectively. The results of this study suggested that *Brucella* rOmp25 is a promising antigen in serological diagnosis of bovine brucellosis. Further analysis will be necessary to define more precisely the value of this study, as the results of serological tests have not been verified by bacteriological method.

Keywords: brucellosis, recombinant Omp's, serology, ELISA

Introduction. Brucellosis remains one of the most widespread zoonotic diseases that cause not only great economic losses to livestock, but also has a certain public health importance (Corbel et al., 2006, OIE, 2009, Yongqun, 2012). The complicated epizootic and epidemiological situation with brucellosis persists in the Republic of Kazakhstan where the prevalence of infection in some regions is as high as 2.77% (Kisykov, 2009), and the incidence per 100 thousand population makes it 21.8 (Ivanov, 2010). The efficacy of brucellosis control and eradication programs primarily depends on the reliability of serological diagnosis. Classical tests based on the use of *Brucella* cells smooth lipopolysaccharides (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, *Salmonella* spp. and *Escherichia coli* O:157 (Caroff et al., 1984, Baldi et al., 1996, Velasco et al., 1998, Blasco and Molina-Flores, 2011). In addition, traditional methods of serodiagnosis do not allow to differentiate infected animals from vaccinated ones (Godfroid et al., 2010, Nielsen and Yu, 2010, Poester et al., 2010). In this regard, the search continues for non-LPS antigens that are suitable for the diagnosis of brucellosis. Currently, a number of proteins of *Brucella* spp. are described, which can be used as potential antigens in immunoassays to avoid the above-mentioned disadvantages of S-LPS (Vahedi et al., 2011, Lim et al., 2012, Ghasemi et al., 2013, Simborio et al., 2015).

Outer membrane proteins (Omps) of *Brucella* spp. were originally identified on the basis of their molecular weight (mol.wt.) (Moreno and Moriyon, 2006). Major Omps from *Brucella* have been classified in group 2 (36 to 38 kDa) and group 3 (Omp25 and Omp31) (Cloeckaert et al., 2002). It was shown that Omp25 is conserved among all species of *Brucella* in contrast to Omp31, which is absent in *Brucella abortus* (Vizcaino et al., 1997). Nevertheless, the predicted amino acid sequence of Omp31 revealed homology (34% identity) with *Brucella* Omp25 (Vizcaino et al., 1996). It was established that some of the major protein components of *B. abortus,* identified using two-dimensional electrophoresis with matrix-assisted laser desorption ionization - time of flight mass spectrometry and liquid chromatography tandem-mass spectrometry, include not only Omp25, but also Omp31 (Connolly et al., 2006). Moreover, a *B. abortus* membrane protein with an apparent mol.wt. of 31 kDa (Omp31b) has been described; it possess some similarity to Omp31 from other strains of *Brucella* (Kim et al., 2012). Based on this, the nucleotide sequence of *B. ovis* Omp31 was compared with the sequence of Omp31b to obtain identity of 77% (Navarro-Soto et al., 2014).

The immunogenic and antigenic properties of *Brucella* Omp25 and Omp31 remain insufficiently studied until

now. Immunogenic potential of recombinant Omp25 (rOmp25) and its protective efficacy against virulent *B. abortus* challenge were evaluated in BALB/c mice (Goel and Bhatnagar, 2012, Goel et al., 2013). The results of these studies suggested that intradermal immunization with rOmp25 gave protection comparable to that of *B. abortus* S-19 strain, and immunization with liposome encapsulated protein conferred protection against *B. abortus* challenge in BALB/c mice with a single dose. Immunization with *B. melitensis* recombinant Omp31 (rOmp) did not confer any protective effect against a virulent smooth *B. melitensis*, despite a strong antibody response induced in mice (Guilloteau et al., 1999). However, more recent studies show that rOmp31 is a useful candidate for the development of a subunit vaccine against *B. canis, B. ovis* and *B. melitensis* infection because it elicits antigen-specific responses and creates specific immunity in mice (Estein et al., 2003, Cassataro et al., 2005, Clausse et al., 2014). Immunization of rams with rOmp31 induced IgG specific antibodies in serum able to bind to whole *B. ovis* cells, and immunized animals showed skin reactivity to the protein (Estein et al., 2004). As for antigenicity of recombinant proteins, promising results were obtained in mouse model when Omp25 and Omp31 as well as Omp28 were combined as one coating antigen for indirect ELISA (i-ELISA) (Ahmed et al., 2015). The results of this study suggested that a developed test has the ability to detect specific antibodies due to infection rather than vaccinal or cross-reacting ones. rOmp31 showed reactivity with antibodies of patients and animals with brucellosis. An i-ELISA using rOmp31 allowed to determine brucellosis in 48% of humans, 61% of infected sheep and 87% of infected dogs (Cassataro et al., 2004). rOmp31, which is thought to be absent in *B. abortus* cell membrane, was used in an i-ELISA for the analysis of 10 cattle sera with positive results of bacteriological examination and fluorescence polarization assays, as well as 10 negative sera according to RBT, fluorescence polarization analysis and bacteriology (Navarro-Soto et al., 2014). The results showed not only the antigenicity of the protein, but also significant differences between the optical density (OD) values of positive and negative sera, demonstrating the highest readings with positive sera. Unfortunately, it is difficult to assess the effectiveness of rOmp31 in the diagnosis of bovine brucellosis based on these results because they were obtained in a small number of animals. In addition, the diagnostic value of rOmp25, as well as a comparative evaluation of these two *Brucella* major proteins, in detecting specific antibodies in cattle sera have not been explored yet.

The aim of this research was to study the immunogenicity of *Brucella* rOmp25 and rOmp31 in mice and determine antigenicity of these recombinant proteins in an i-ELISA using serum samples of cattle that are positive for brucellosis by classical serological tests.

Materials and methods

Growth of bacteria and harvesting of cultures. *B. abortus* 19 and *B. melitensis* Rev-1 were grown on hepatic glucose-glycerol agar (GOST 33675-2015) in Roux flask (TECHNOSKLO, Držkov, Czech republic) at 37°C for 48 h. Freeze-dried stock cultures of the strains were maintained as a source of inoculums. They were reconstituted and passaged once on hepatic glucose-glycerol agar before Roux flasks were seeded. *Brucella* spp. were harvested by gentle washing of the agar surface with phosphate buffered saline (PBS) containing 0.5% carbolic acid, and flasks were incubated at 37°C for 48 h in order to inactivate the cells. Bacterial suspension was washed thrice with PBS and centrifuged at 5000×g at 4°C for 30 min.

Preparation of soluble salt-extractable protein antigens (CSP) from *Brucella* spp. Extraction of CSP from *Brucella* cells was carried out according to the method of Tabatabai and Deyoe (1984).

Serum samples. Forty-four serum samples of cows that were positive for anti-*Brucella* antibodies according to complement fixation test (CFT) and standard agglutination test (AT) were kindly provided by Republican Veterinary Laboratory and National Veterinary Reference Center, the Ministry of Agriculture of the Republic of Kazakhstan. Nineteen serum samples were collected from the heifers of farm "Rodina", Akmola region, Kazakhstan, which has been free of brucellosis for long periods of time.

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study were *E. coli* DH5α and *E. coli* BL21 (DE3). The pET32 and pET28c+ expression vectors were used for cloning and transformation. Nucleotide sequences of the genes encoding Omp25 and Omp31 were synthesized by Life Technologies Company (Carlsbad, USA) and obtained in a plasmid vector pMK-RQ. The cell cultures were grown on LB broth and LB agar media (Thermo Fisher Scientific, Waltham, USA) supplemented with ampicillin or kanamycin (100 µg/ml and 50 µg/ml, respectively) (Sintez, Kurgan, Russia).

Cloning, expression and purification of recombinant proteins. Fragments of DNA encoding Omp25 and Omp31 were digested by restriction enzymes NcoI and XhoI (Thermo Fisher Scientific, Waltham, USA) supplied with 10x Buffer Tango**.** The restriction mixture was analyzed by electrophoresis on 1% agarose gel in Tris-acetate-EDTA buffer and purified from the agarose gel by PureLink Gel Extraction Kit (Thermo Fisher Scientific, Waltham, USA). Then, the purified DNA fragments were ligated to expression vectors using T4 DNA-ligase (Thermo Fisher Scientific, Waltham, USA). This ligated product was then used to transform the expression host, *E. coli* DH5α. The sequence of cloned products was confirmed by Sanger method using automatic DNA analyzer ABI Prism 3100 (Applied Biosystems, USA). Then, *E. coli* BL21 (DE3) cells were transformed with the constructs of rOmp25/pET32 or rOmp25/pET28c+ and rOmp31/pET32 or rOmp31/pET28c+, and incubated at 37°C over night on LB agar plates with ampicillin and kanamycin for pET32 and pET28c+, respectively. A single colony was inoculated into 5 ml of LB broth with appropriate antibiotic and incubated over night at 37°C with shaking.

Four hundred ml of LB broth were inoculated with 5 ml over night culture and Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, USA) was added to a final concentration of 1 mM to induce expression of the rOmps. 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.

To determine protein expression, cells were resuspended in lysis buffer (20 mM Tris pH 7.5; 1 mM EDTA; 100 mM NaCl) at the amount of 10 ml per 1 g wet weight cells, followed by the addition of 1 µl of phenylmethanesulfonyl fluoride (Thermo Fisher Scientific, Waltham, USA) with final concentration 0.2 mM. The bacterial cell suspension was then sonicated and centrifuged at 13000 × g at 4°C for 1 h. The supernatant and cell pellet protein extracts were fractionated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To separate expressed recombinant proteins, cell debris was resuspended in buffer I (20 mM Na_3PO_4 pH 7.4; 500 mM NaCl; 20 mM imidazole, 1 M Urea), sonicated and incubated at room temperature for 30 min, then centrifuged at 13000 × g for 30 min. The supernatant was carefully collected and the pellet was resuspended in buffer II (20 mM Na_3PO_4 pH 7.4; 500 mM NaCl; 20 mM imidazole, 8 M Urea), sonicated and incubated as previously. After that, the cells were centrifuged at 8000 x g for 20 min and the supernatant were collected (discarded the pellet). Each of the collected supernatant samples was analyzed by SDS-PAGE. Recombinant proteins were purified using the His Trap Columns (GE Health care Life Sciences, Cardiff, UK).

Western blotting. The presence of rOmp25 and rOmp31 was confirmed by western blot assay using anti-HisTag monoclonal antibody (mAb) conjugated to horseradish peroxidase (HRP) (Thermo Fisher Scientific, Waltham, USA).

Determination of the immunogenicity of rOmp25, rOmp31 and CSP of *Brucella* in mice. Four groups of BALB/c mice (3 heads per group, males, 8 weeks old) were used in this study. Experimental animals were housed in the vivarium of S. Seifullin Kazakh Agro-Technical University, and the Animal Ethics Committee of Veterinary Medicine Faculty approved their use and care. The mice of the first group were immunized with rOmp25, the second group - rOmp31, the third group - CSP of *B. abortus*. The rOmps used as immunogens were purified from lysates of strains transformed with plasmids rOmp25/pET32 and rOmp31/pET32. On the first day of immunization each mouse was given intraperitoneal administration of 25µg of the appropriate antigen in 100 µl of complete Freund's adjuvant (Sigma-Aldrich, St. Louis, USA). Then, on the 7th day, repeated injection of the antigen was in incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, USA). On days 11, 12 and 13, mice were injected with the antigens in PBS, pH 7.2-7.4. The mice of the fourth group were inoculated by the same scheme, but without antigen as control. Three days after the last immunization, mice were bled from the tail vein into microfuge tubes (Isolab, Wertheim, Germany). Then, serum samples of mice from each group were mixed in equal volume to obtain a pool of antisera.

The titre of antisera against the expressed *Brucella* antigens was determined by i-ELISA. Briefly, microtitre plate wells were separately coated with rOmp25, rOmp31 and/or CSP of *B. abortus* or *B. melitensis* at the concentration of 5.0 µg/ml in bicarbonate buffer (BCB), pH 9.6, overnight at 4°C. To avoid binding of antibodies to thioredoxin, which is present in the expression vector pET32, rOmps obtained from the other producer strains with plasmids pET28c+ were used as coating antigens. Then, the solid phase was washed with PBS and PBS with 0.05% Tween-20 (PBS-T), pH 7.2. Antisera to *Brucella* preparations were diluted in 16 wells pretreated with 1% bovine serum albumin (BSA), starting with 1:100 in PBS-T. After incubation at 37°C for 1 h, the results of immunoassay were developed by peroxidase-conjugated goat anti-mouse IgG (Fc-specific) (Sigma-Aldrich) and its substrate. The pool of sera from BALB/c mice inoculated by the same scheme, but without antigen was used as a control. ELISA result was considered positive if the OD value of the well with antiserum was higher than at least twice the OD value of the control well.

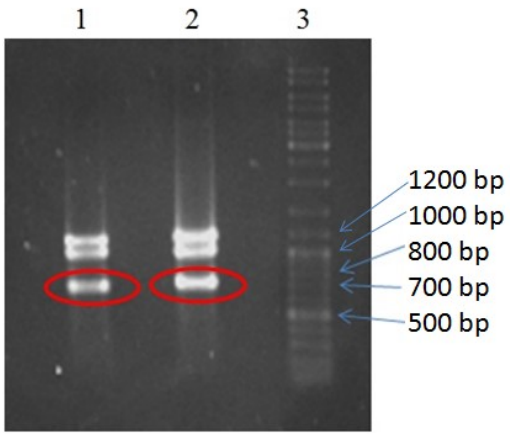
Determination of antigenicity of *Brucella* preparations by i-ELISA. The polystyrene plate wells were separately coated with rOmp25, rOmp31, CSP of *B. abortus* and/or *B. melitensis* (5.0 µg/ml) in BCB (pH 9.6). After overnight incubation at 4°C, the plate was sequentially washed with PBS and PBS-T, blocked by 1% BSA, and incubated in the same mode. After another wash, the dilutions of cattle test serum in PBS-T were prepared starting with 1:100 and the plate was incubated at 37°C for 1 h. After washing, peroxidase-conjugated rabbit anti-bovine IgG (whole molecule) (Sigma-Aldrich) was added and allowed to react with its antigen under the same condition. A cutoff value to distinguish between positive and negative results of i-ELISA was calculated using the mean OD of *B. abortus*-negative sera (Erdenebaatar et al., 2003). Cutoff value was also defined as twice the mean OD of the first four dilutions (1:100-1:800) of negative sera according to the proposed new method. Statistical analysis of the results of serological tests was carried out according to the method described by Sayduldin (1981).

Results. Digestion of the DNA inserts encoding Omp25 and Omp31, and subsequent electrophoretic analysis, revealed presence of bands with expected sizes of 759 and 795 bp, respectively (Fig. 1).

SDS-PAGE data after fractionation of cell lysates showed that the target proteins are within the inclusion bodies of the cells (Fig. 2).

The maximum amount of rOmps was obtained after treatment of the cell pellets with denaturing buffer containing 8 M Urea. In addition, the SDS-PAGE analysis revealed the presence of the expected recombinant fusion proteins of rOmp25 and rOmp31with approximately mol.wt. of 42 kDa and 48 kDa, respectively. The presence of rOmps in the lysate of the transformed cells after expression was also confirmed by

western blot assay using HRP-conjugated anti-His Tag mAb (Fig. 3).



1 – DNA fragment of Omp25; 2 – DNA fragment of Omp31; 3 – DNA molecular weight marker

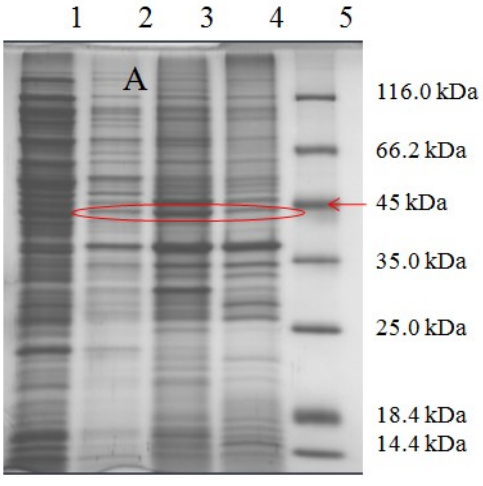
Figure 1. **Restriction fragments of Omp25 and Omp31 genes**

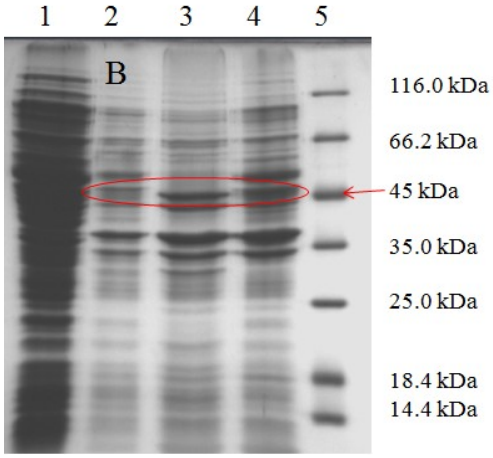
As it can be seen from Fig. 3, anti-HisTag mAb identifies proteins with an apparent mol.wt. of 42 kDa and 48 kDa for rOmp25 and rOmp31, respectively.

The rOmps purified by metal-affinity chromatography possessed distinct immunogenicity in BALB/c mice (Table 1).

As can be seen from Table 1, antibodies to rOmp25 and rOmp31 proved high activity against homologous antigens with the titres of 1:51200 and 1:6400, respectively. Antiserum to rOmp25 cross-reacted with rOmp31 up to titre of 1:12800, whereas serum antibodies to rOmp31 reacted equally with homologous and heterologous antigens. Antibodies of both sera bound to CSP of *B. abortus* with equal activity (1:800; data not shown).

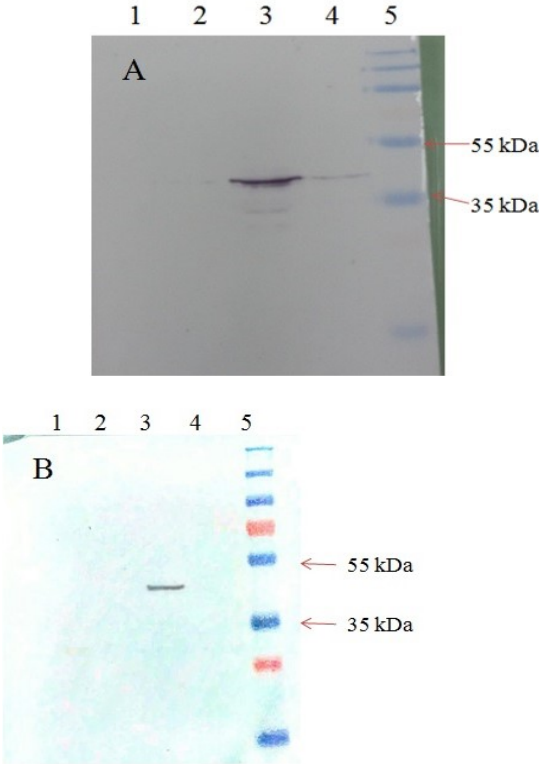
The antigenicity of rOmp25 and rOmp31, as well as CSP of *B. abortus* and *B. melitensis* was studied by i-ELISA using blood serum samples of cows from brucellosis affected farms that use a vaccine *B. abortus* S-19(S) in the framework of brucellosis eradication program (Table 2).





A – rOmp25, B – rOmp31. Lane 1 – supernatant after lysis of an overnight culture; lane 2 – supernatant after treatment with buffer containing 1M Urea; lane 3 – supernatant after treatment with buffer containing 8M Urea; lane 4 – pellet after treatment with buffer containing 8M Urea and dissolved in 1 ml of TNE buffer; lane 5 – protein molecular weight marker

Figure 2. **SDS-PAGE analysis of recombinant antigens**



A – rOmp25, B – rOmp31. Lane 1 – supernatant after lysis of an overnight culture; lane 2 – supernatant after treatment with buffer containing 1M Urea; lane 3 – supernatant after treatment with buffer containing 8M Urea; lane 4 – pellet after treatment with buffer containing 8M Urea and dissolved in 1 ml of TNE buffer; lane 5 – protein molecular weight marker

Figure 3. **Western blot analysis of the recombinant antigens, detected using His Tag Mab**

Table 1. **Activity of mice antisera to rOmp25 and rOmp31**

Dilution of serum	Antisera*			
	rOmp25/pET32	rOmp31/pET32	rOmp25/pET32	rOmp31/pET32
	Antigens			
	rOmp25/pET28c+		rOmp31/pET28c+	
	OD, 492 nm			
1:100	2.025	1.334	2.048	2.099
1:200	2.002	1.411	1.938	2.001
1:400	1.945	1.249	1.690	1.874
1:800	1.948	1.039	1.320	1.512
1:1600	1.822	0.782	1.066	1.017
1:3200	1.543	0.548	0.815	0.596
1:6400	1.100	0.325†	0.546	0.312†
1:12800	0.730	0.185	0.307†	0.179
1:25600	0.414	0.107	0.190	0.106
1:51200	0.240†	0.073	0.113	0.072
1:102400	0.142	0.055	0.077	0.055

* - Each antiserum was tested in triplicate, and Table 1 shows the mean OD.
Mean maximum OD of the control wells with rOmp31/pET28c+ and Omp25/pET28c+ was equal to 0.117
† - OD showing antibody titre
†† - Antisera from dilution 1:102400 and higher reached a plateau of the OD

Table 2. **The results of serological testing cattle for brucellosis**

Antibodies detected in cows (n=44) by					
classic serological tests		i-ELISA using antigens:			
CFT	AT	CSP *B .abortus*	CSP *B. melitensis*	Omp31	Omp25
44 (100%)	25 (56.8%)	30 (68.2%)	25 (59.5%)	16 (36.4%)	23 (52.3%)

Table 2 shows that anti-*Brucella* antibodies were detected in all animals by CFT, and positive results of AT were established in 56.8% of cows. Antibodies against CSP of *B. abortus* and *B. melitensis* by i-ELISA were found in 68.2% and 59.5% of heads, respectively. The correlation coefficient between immunoassay readings using two antigen preparations was equal to 0.5.

To determine the cutoff value for i-ELISA/rOmp25 and i-ELISA/rOmp31, 19 blood sera obtained from heifers of a farm which is free of brucellosis for long periods of time were used. The average OD_{492} values of *B. abortus*-negative sera were 0.112±0.005 and 0.079±0.003 at 1:200-fold dilution for i-ELISA/rOmp25 and i-ELISA/rOmp31, and the cutoff values were determined at 0.224 and 0.158, which were double the average OD_{492} of negative sera, respectively.

As evident in Table 2, antibodies of 16 animals which were seropositive for brucellosis reacted with both rOmps, but in 7 heads they recognized only rOmp25. The correlation coefficient between two versions of i-ELISA was moderate (0.6). Mean antibody titres against *B. abortus* CSP, Omp25 and Omp31 were equal to 1:250, 1:190 and 1:170 with the same deviations (+3.7%; -3.4%), respectively.

According to the proposed new method, cutoff values for rOmp25 and rOmp31 were determined at 0.360 and 0.120, respectively, which were double the average OD_{492}(0.180×2=0.360; 0.06×2=0.120) of the first four dilutions (1:100-1:800) of negative sera. Antibodies specific to rOmp25 and rOmp31 were detected in the sera of 29 (65.9%) and 24 (54.5%) seropositive cows, respectively. ELISA/rOmp25 confirmed all the positive readings of ELISA/rOmp31 and additionally detected the presence of anti-*Brucella* antibodies in the sera of 5 (11.4%) cows. Comparative antigenicity of *Brucella* rOmps using two methods for calculation of i-ELISA data is given in Figure 4.

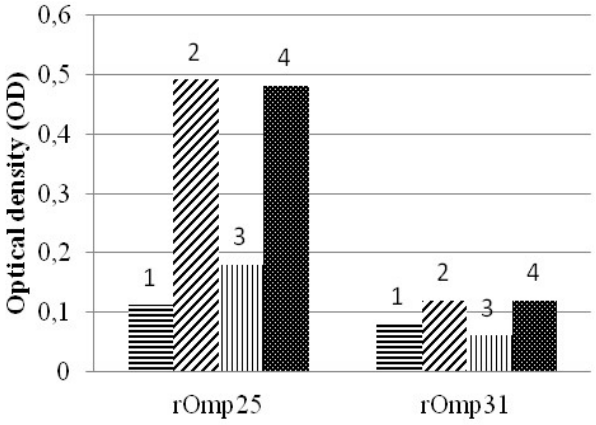


Figure 4. **The antigenicity of recombinant proteins of *Brucella* spp. OD of the negative (1) and positive sera (2) by the method of Erdenebaatar et al. (2003); OD of the negative (3) and positive sera (4) by the proposed method**

The results of both methods showed that rOmp25 was more reactive towards anti-*Brucella* antibodies of seropositive cows than rOmp31.

Discussion

In this study, *Brucella* rOmp25 and rOmp31 have been successfully produced in *E. coli* and their immunogenicity and antigenicity were evaluated in an effort to increase the specificity i-ELISA for serodiagnosis of bovine brucellosis. The genes encoding Omp25 and Omp31 were cloned and expressed in *E. coli* using the pET32 and pET28c+ plasmids. The SDS-PAGE results showed the presence of protein bands of purified rOmp25 and rOmp31 with mol.wt. of 42 and 48 kDa, respectively. These sizes agreed with the mol.wt. of the expressed rOmp25 and rOmp31 fusion proteins, respectively. Moreover, the results of this study agreed with data obtained for rOmp25 (Letesson et al., 1997, de Wergifosse et al., 1995) and for rOmp31 (Vizcaino et al., 1996, Gupta et al., 2007) in previous studies. We also confirmed the expression of the rOmp25 and rOmp31 fusion proteins in *E. coli* by western blot analysis using His Tag MAb.

The recombinant proteins were capable of provoking a humoral immune response in mice. It should be noted that antisera containing antibodies against rOmp25 and/or rOmp31 showed mutual cross-reactions with varying intensity. These data suggest the antigenic similarity between these *Brucella* proteins, and they are consistent with the findings of other researchers who have observed the identity of certain amino acid and nucleotide sequences of these two major proteins (Vizcaino et al., 1996, Navarro-Soto et al., 2014). In addition, both antisera recognized related proteins that are present in the composition of *B. abortus* CSP.

The CSP of *Brucella* and rOmps were used as antigens for testing blood sera of 44 cattle from brucellosis infected farms by i-ELISA. These animals were previously vaccinated with *B. abortus* strain S19 and all of them had complement-binding antibodies. However, agglutinins were present in a smaller number of animals (56.8%). Testing sera by i-ELISA using CSP of *B. abortus* and *B. melitensis* as antigen showed the presence of specific antibodies in sera of 68.2% and 59.5% seropositive animals, respectively. In our opinion, these data support the possibility of obtaining false-positive results by classical serological tests that are based on the detection of antibodies directed against S-LPS portion of *Brucella* cell membrane (Caroff et al., 1984, Baldi et al., 1996, Velasco et al., 1998, Blasco and Molina-Flores, 2011).

In this study antigenicity of rOmp25 as compared with rOmp31 has been investigated for the first time by using serum samples of cattle with positive reactions for brucellosis by classical serological tests. Antibodies, specific to these *Brucella* rOmps, are detected only in 52.3% and 36.4% of cows, respectively. In this case, agreement between the results of i-ELISA/rOmp25 and i-ELISA/*B. abortus* CSP was better (29.5%) than that of i-ELISA/*B. melitensis* CSP (15.9%). Definition of border line value of negative sera by the proposed new method somewhat increased the number of positive results as compared with the calculation of cutoff level described by Erdenebaatar et al. (2003). For instance, the new method allowed us to detect antibodies to rOmp25 and rOmp31 in sera of another 6 (13.6%) and 8 (18.2%) cows, respectively. In our view, using the mean OD of not a single but several dilutions of control and test sera allows to obtain a more realistic cutoff value. It should be noted that under both methods rOmp25 showed higher antigenicity to anti-*Brucella* antibodies of seropositive cows than rOmp31. These results contradict previously published data, which described rOmp31 as an antigenic protein for the analysis of cattle sera for brucellosis that provides significant differences between the OD value of positive and negative sera, demonstrating the highest indicators in positive sera (Navarro-Soto et al., 2014), although the authors did not carry out a comparative evaluation of the above two proteins. If one considers rOmp25 to be present in all species of *Brucella* unlike rOmp31, which is absent in *B. abortus* (Vizcaino et al., 1997), then we can conclude that there are epitopes shared between these proteins. This assumption is also confirmed by the results of studies investigating the immunogenicity of rOmps in mice.

The smaller amount of cows with positive i-ELISA/CSP results as compared with the results of CFT probably demonstrates higher specificity of *Brucella* proteins than that of its S-LPS. Nevertheless, CSP of *B. abortus* and *B. melitensis* obtained as described (Tabatabai and Deyoe 1984) are not also devoid of polysaccharides of the cell wall. Therefore, they may react with antibodies formed to S-LPS of the smooth vaccine strains and/or the other Gram-negative bacteria. These findings suggest that *Brucella* rOmps might be a good candidate for increasing ELISA specificity for serological diagnosis of bovine brucellosis.

Conclusions

Brucella rOmp25 may be promising antigen in serological diagnosis of bovine brucellosis. Further analysis will be necessary to define more precisely the value of ELISA based on rOmps of *Brucella* spp. in comparison with the bacteriological investigation.

Acknowledgement

This research was carried out within the framework of the budget program 217 of the Ministry of Education and Science, the Republic of Kazakhstan on the project №0452/GF4 “ELISA test based on recombinant outer membrane proteins of *Brucella* spp.” for the period 2015-2017.

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Received 14 August 2017
Accepted 10 November 2017