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Expression, purification and immunochemical characterization of recombinant OMP28 protein of *Brucella* species

Y. Manat[1,*], A.V. Shustov[2], E. Evtehova[1] and S.Z. Eskendirova[1]
[1]*Laboratory of Cell Biotechnology, National Centre for Biotechnology, Astana, 010000, Republic of Kazakhstan*
[2]*Laboratory of Genetic Engineering, National Centre for Biotechnology, Astana, 010000, Republic of Kazakhstan*

Abstract
Brucellosis is the lion's share of infectious disease of animals and it has a particular socio-economic importance for the Republic of Kazakhstan. Sixty percent of epizootic outbreaks of brucellosis identified in the Commonwealth of Independent States (CIS) originated from Kazakhstan in recent years. Definitive diagnosis of brucellosis remains a difficult task. Precisely for this reason, we evaluated a purified recombinant out membrane protein 28 (rOMP28) of *Brucella* species (*Brucella* spp.) produced in *Escherichia coli* (*E. coli*) as a diagnostic antigen in an Indirect ELISA (I-ELISA) for bovine brucellosis. The gene encoding OMP28 was synthesized using a two-round PCR procedure. In order to produce the rOMP28, the *de novo* synthesized DNA was cloned into the expression vector pET-22b(+). Then, the rOMP28 was expressed in *E. coli* system and characterized in the present study. We further estimated the diagnostic potential of purified rOMP28 of *Brucella* spp. for screening bovine sera. To determine if rOMP28 has a valuable benefit for use in the serodiagnosis of bovine brucellosis, rOMP28-based I-ELISA was performed. *Brucella* spp. positive (n=62) and *Brucella* spp. negative (n=28) samples from tube agglutination test (TAT) were positive (n=59) and negative (n=27) by I-ELISA, respectively. These findings show that the rOMP28 of *Brucella* spp. could be a good candidate for improving serological diagnostic methods for bovine brucellosis.
Keywords: *Brucella* spp., Brucellosis, I-ELISA, rOMP28, Western blot.

Introduction

Brucellosis caused by Gram-negative, facultative, intracellular bacteria belonging to the genus *Brucella*. It is an emerging zoonosis, and an economically important infection of humans and animals with a worldwide distribution. Owing to its heterogeneous and poorly specific clinical symptomatology, the diagnosis of brucellosis always requires laboratory confirmation, either by isolation of the pathogen or by demonstration of specific antibodies. The slow growth of *Brucella* in culture may delay diagnosis for more than seven days. Furthermore, handling of these microorganisms poses a high risk to laboratory personnel, since *Brucella* spp. are class III pathogens (Christopher *et al*., 2010; Poester *et al*., 2010; Smirnova *et al*., 2013).

The conventional serological tests, of which the most frequently used are the Rose Bengal test (RBT), the tube agglutination test (TAT) and the complement fixation test (CFT), principally measure antibodies against the immunodominant smooth lipopolysaccharide (S-LPS) of the bacterial cell membrane. The traditional serological test for diagnosing brucellosis in cattle in Kazakhstan is TAT. However, agglutination tests sometimes give false-positive results due to cross-reactions with other microorganisms. In addition, serological tests based on anti-LPS antibodies give false positives because of cross-reactivity with other Gram-negative bacteria such as *Yersinia enterocolitica O:9*, *Salmonella* spp. and *Escherichia coli* (Christopher *et al*., 2010; Smirnova *et al*., 2013).

Outer membrane proteins (OMPs) of *Brucella* spp. have been the focus of vaccine development and the diagnosis of brucellosis (Cloeckaert *et al*., 2001; Gupta *et al*., 2010; Ko *et al*., 2012). OMP28 is considered as one of the outer membrane proteins of *Brucella* (Cha *et al*., 2012) and has been identified as an important diagnostic antigen in brucellosis (Seco-Mediavilla *et al*., 2003; Poester *et al*., 2010). OMP28 is highly conserved among *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae* and *B. melitensis*. Recombinant OMP28 was sensitive and specific for diagnosis of *Brucella* infection in animals by indirect enzyme-linked immunosorbent assay (I-ELISA) (Kumar *et al*., 2008; Gupta *et al*., 2010; Thavaselvam *et al*., 2010; Liu *et al*., 2011; Dong-Bao *et al*., 2012; Lim *et al*., 2012; Qiu *et al*., 2012; Azizpour *et al*., 2013; Kim *et al*., 2013; Xin *et al*., 2013).

Materials and Methods

Reagents and equipment

All primers were synthesized by Invitrogen corporation (Invitrogen, USA). A Bio-Rad T100™ Thermal Cycler was used for PCR. *E.coli* laboratory strain BL21 (DE3) was obtained from Novagen. HisTrap FF crude was sourced from GE Healthcare life sciences. Bovine serum samples [positive (62) and negative (28) well known serum samples of bovine infected with *Brucella*

***Corresponding Author:** Manat Yesbol. Laboratory of Cell Biotechnology, National Centre for Biotechnology, Astana, 010000, Republic of Kazakhstan. Tel: +7(7172) 70 25 27. E-mail: manatyesbol@gmail.com

spp.] were obtained from the RSE "Republican Veterinary Laboratory," the Ministry of Agriculture of RK. All chemicals used in this study were of analytical grade and purchased from Sigma (Str. Louis, MO).

De novo synthesis of OMP28 gene

The gene encoding the OMP28 was synthesized in a constructive PCR using long oligonucleotides as primers. First, amino acid sequences of the OMP28 protein of *Brucella* spp. were downloaded from Genbank and compared in a multiple alignment.

The *in silico* designed sequence for the OMP28 is shown in Fig. 1. This gene was codon-optimized for expression in *E.coli*. The vector NTI suite was used for reverse translation coupled with codon-optimization for heterologous species (*E.coli* K12).

The DNAWorks v3.2.2 was used to calculate sequences of primers for the *de novo* synthesis of DNA fragments. The primers used for the *de novo* gene synthesis are listed in Table 1. These primers were designed for use in PCR with annealing temperature of 62°C in presence of 50 mmol/L Na^+ and 2 mmol/L Mg^{2+}. In the constructive PCR procedure, these primers were divided into two groups and designated as "internal" or "flanking" primers. Each of the internal primers was 100% homologous to the corresponding region in the sequence to be synthesized. Internal primers were interleaved in the sense-antisense-sense-antisense manner. The whole set of internal primers covered the entire length of the DNA fragment to be synthesized except for the very 5′- and 3′-terminal linkers. The terminal linkers with restriction sites for subsequent cloning were included in the flanking primers.

The *de novo* synthesis of OMP28 gene was performed as a two-round PCR. Phusion HotStart DNA polymerase (Thermo Scientific) was used to avoid PCR errors. The mixture of 20 internal primers (each at 0.4 pmol/L final concentration) was subjected to the first round of PCR. The external template was not added to reactions in the first round. A total 30 cycles were carried out as following: denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. The PCR products of the first round were diluted 1:10 with water and 1 µL of the first round PCR product was used in the second round of PCR amplification. For the second round of PCR, a pair of flanking primers (each at 2 pmol/L final concentration) was used. The product of the second round PCR was expected to be 787 bp in length. The PCR products were subjected to electrophoresis in 1% agarose gels, cloned into pGEM-T using the pGEM-T Easy Cloning kit (Promega) and sequenced. Double strand automated sequencing was performed for confirmation of the identity of the cloned fragment to the designed sequence of the gene.

Protein expression and purification

E.coli BL21(DE3) were transformed with the plasmid pET-22b(+) carrying the gene of OMP28 and grown on solid LB/ampicillin (100 µg/mL) plates at 37°C overnight. A single colony was selected to grow a 5mL starter culture overnight at 37°C. The starter culture was inoculated into 500mL LB/ampicillin (100 µg/mL) and incubated at 37°C with shaking until the OD_{600} reached 0.6. Expression of recombinant OMP28 was induced by addition of IPTG to a final concentration of 0.5 mmol/L and expression was continued for 12 h at 25°C. Finally cells were harvested and collected by centrifugation (at 6000xg for 10 min at 4°C) and washed twice with distilled water (20 mL).

In order to ascertain the localization of the expressed recombinant protein, cells were resuspended in 20 mL lysis buffer (20% sucrose; 20mM HEPES pH 7.5; 5mM EDTA; 0.1% Triton X-100), [10mL lysis buffer per gram wet weight cells], followed by the addition of 2mL lysozyme (final concentration is 1mg/mL), 20 µl DNase (final concentration is 0.01mg/mL)and 200 µl

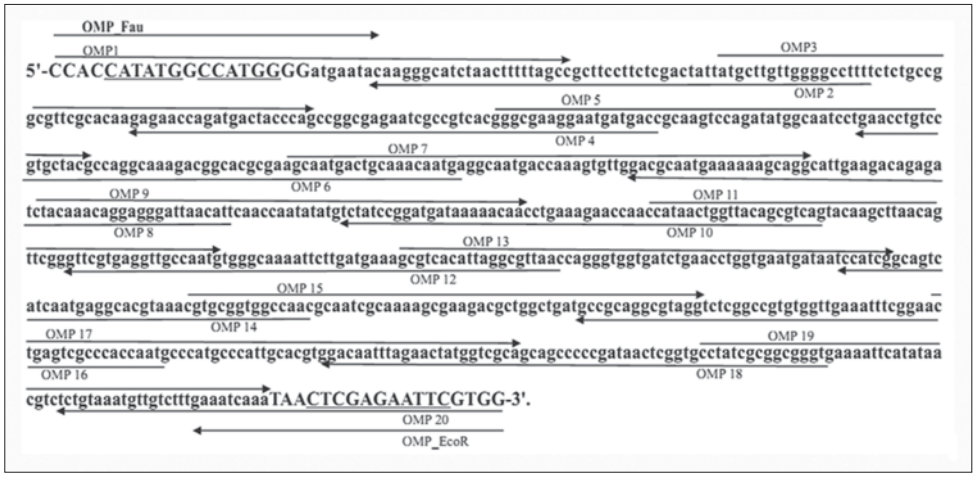

Fig. 1. The nucleotide sequence of OMP28 used for the internal (OMP1-OMP20) and flanking (OMP_Fau and OMP_EcoR) primers. The DNA sequences used for primer design are shown by arrows.

Table 1. Primers for synthesis of OMP28 gene.

Name	*Sequence (5'-3')*	*Internal or flanking*
OMP1	CCACCATATGGCCATGGGGATGAATACAAGGGCAT CTAACTTTTTAGCC	Internal
OMP2	AAAAGGCCCCAACAAGCATAATAGTCGAGAAGGA AGCGGCTAAAAAGTTAGATGCCCTTG	Internal
OMP3	ATGCTTGTTGGGGCCTTTTCTCTGCCGGCGTTCG CACAAGAGAACCAGATGACTACCCAG	Internal
OMP4	GGTCATCATTCCTTCGCCCGTGACGGCGATTCTC GCCGGCTGGGTAGTCATCTGGTTCTC	Internal
OMP5	GGGCGAAGGAATGATGACCGCAAGTCCAGATA TGGCAATCCTGAACCTGTCCGTGCTACG	Internal
OMP6	TCATTGTTTGCAGTCATTGCTTCGCGTGCCGTCT TTGCCTGGCGTAGCACGGACAGGTTC	Internal
OMP7	AGCAATGACTGCAAACAATGAGGCAATGACCAA AGTGTTGGACGCAATGAAAAAAGCAGG	Internal
OMP8	AATGTTAATCCCTCCTGTTTGTAGATCTCTGTCTT CAATGCCTGCTTTTTTCATTGCGTC	Internal
OMP9	CTACAAACAGGAGGGATTAACATTCAACCAATA TATGTCTATCCGGATGATAAAAACAAC	Internal
OMP 10	CTGACGCTGTAACCAGTTATGGTTGGTTCTTTC AGGTTGTTTTTATCATCCGGATAGACA	Internal
OMP 11	CCATAACTGGTTACAGCGTCAGTACAAGCTTAA CAGTTCGGGTTCGTGAGCTTGCCAATG	Internal
OMP 12	GTTAACGCCTAATGTGACGCTTTCATCAAGAAT TTTGCCCACATTGGCAAGCTCACGAAC	Internal
OMP 13	GCGTCACATTAGGCGTTAACCAGGGTGGTGATC TGAACCTGGTGAATGATAATCCATCGG	Internal
OMP 14	GTTGGCCACCGCACGTTTACGTGCCTCATTGAT GACTGCCGATGGATTATCATTCACCAG	Internal
OMP 15	CGTGCGGTGGCCAACGCAATCGCAAAAGCGAAGA CGCTGGCTGATGCCGCAGGCGTAGGT	Internal
OMP 16	CATTGGTGGGCGACTCAGTTCCGAAATTTCAAC CACACGGCCGAGACCTACGCCTGCGGC	Internal
OMP 17	CTGAGTCGCCCACCAATGCCCATGCCCATTGCAC GTGGACAATTTAGAACTATGGTCGCA	Internal
OMP 18	CACCCGCCGCGATAGGCACCGAGTTATCGGGG GCTGCTGCGACCATAGTTCTAAATTGTC	Internal
OMP 19	CCTATCGCGGCGGGTGAAAATTCATATAACGT CTCTGTAAATGTTGTCTTTGAAATCAAA	Internal
OMP 20	CCACGAATTCTCGAGTTATTTGATTTCAAAGA CAACATTTACAGA	Internal
OMP_Fau	CCACCATATGGCCATGGGGATGAATAC	Flanking
OMP_EcoR	CCACGAATTCTCGAGTTATTTGATTTC	Flanking

RNase (final concentration is 0.1mg/mL). IPTG was added to a final concentration of 0.2 mM and further incubated at room temperature for 1 hr. The bacterial cell suspension was then sonicated for 10 min with a pulse interval of 5 s (OMNI-Ruptor 4000) in an ice-water bath. The lysate was centrifuged at 6000xg for 30 min at 4°C, supernatant discarded and the pellet was resuspended in 5mL of lysis buffer, then sonicated for 7 min with a pulse interval of 5 s. The sonicated extract was centrifuged at 6000xg for 10 min at 4°C. In this way, inclusion bodies were obtained, followed by resuspention of the inclusion body with buffer A (20mM

Na_3PO_4 pH 7,4; 500mM NaCl; 20mM imidazole, 8 M urea), sonicated 50 % level 4-5, one pulse, incubated for 1 hr at room temperature, then centrifuged at 8000xg for 30 min, carefully collected the supernatant (discarded the pellet). The supernatant and inclusion bodies, with appropriate controls and molecular mass markers, were analyzed by 12% SDS-PAGE, as described by Laemmli (1970). After confirmation of the solubility, purification of the rOMP28 from inclusion bodies was carried out using a HisTrap FF crude with a native purification protocol as specified by the manufacturer: removed the snap-off end at the column outlet, then washed the column with 5 column volumes of distilled water (5mL). Equilibrated the column with 5 column volumes of binding buffer (buffer A containing 8M urea), then applied pretreated sample using a syringe and collected in a separate tube as Flowthrough, followed by washing with buffer A (5mL). Column and buffers (buffer A, buffer B) were then connected to the GE Healthcare chromatography system and equilibration started. The purified protein was checked by 12% SDS-PAGE followed by Coomassie blue staining, and protein concentration was estimated by the Bradford method.

Immunoreactivity of rOMP28 Brucella proteins to bovine sera using Western blot and indirect ELISA

The presence of specific antibodies against rOMP28 in bovine sera was demonstrated by Western blot and indirect ELISA. Briefly, purified rOMP28 preparations were run in 12% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were then blocked with 1% bovine serum for 2 h at 37°C and washed five times with PBS (0.01 mol/L, pH 7.2) containing 0.05% Tween 20 (PBST). Membranes were then incubated with serum samples for 10 h at 4°C (serum: PBST at 1:100). The membranes were washed with PBST and incubated with HRP-conjugated goat anti-bovine IgG antibody (1:5000 dilution) for 1 h at 37°C. Finally, the membranes were washed with PBST and the colors developed by adding 4-chloro-l-naphthol in the presence of hydrogen peroxide.

Antibody responses were also measured in indirect ELISA against rOMP28 of *Brucella* spp. in bovine sera. Briefly, 96-well microtitre plate (Nunc-Maxisorp, Denmark) was coated overnight with 100 µl of purified rOMP28 antigen (2 µg/mL) in Phosphate-buffered saline (PBS, pH 7.4) at 4°C. Next day, plate was washed three times with PBS-T and blocked with bovine serum albumin (1%) in PBS-T for 1 h at 37°C. After 3 – 4 washings with PBS-T, the plate was incubated with positive sera at a 1/100 dilution, at 37°C for 2 h. After 3 – 4 washings of the plate, anti-bovine HRP conjugate (100 µl/well) was added (1/10000) and incubated at 37°C for 1 h. After incubation, the plate was washed 3 – 4 times and 100 µl of freshly prepared substrate solution (10 mg OPD/10mL substrate buffer with 100 µl of 3% H_2O_2) was added to each well and incubated for 10 - 15 min in the dark. The reaction was stopped by addition of 100 µl of H_2SO_2 (2M) per well. The absorbance was measured using ELISA reader (Bio-Rad) at 490 nm.

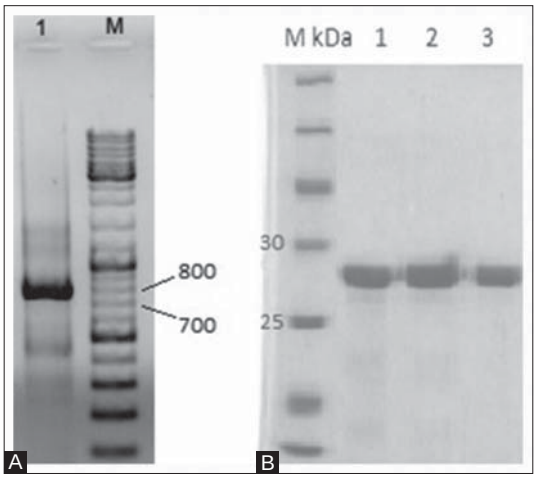

Fig. 2. (A) Agarose electrophoresis of second round PCR product (lane 1), M: DNA molecular weight marker. (B) SDS-PAGE analysis of Purified rOMP28; M: Protein marker; 1, 2, 3: rOMP28. The proteins were separated by 12% SDS-PAGE and stained with Coomassie brilliant blue.

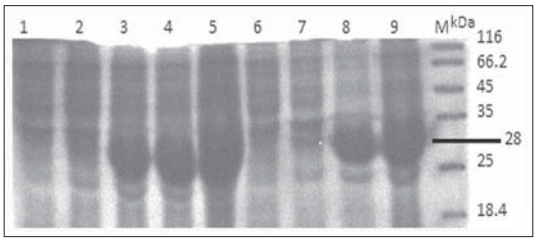

Fig. 3. SDS–PAGE analysis of rOMP28 in pET-22(b+) expression vector with modified buffers under denaturing conditions. 1: Uninduced clone (total); 2: Clear lysate of 2 h induction; 3: Lysate of 4 h induction; 4: Lysate of 6 h induction; 5: Lysate of overnight induction; 6: inclusion body without induction; 7: inclusion body after 2 h induction; 8: inclusion body after 4 h induction; 9: inclusion body induction overnight, M: protein molecular weight marker (Thermo scientific).

Results and Discussion

The outer membrane proteins (OMPs) of *Brucella* spp. were initially identified in the early 1980s and have been extensively characterized as potential immunogenic and protective antigens. However, research about the location of OMP28 has not been consistent so far. Lindler *et al*. (1996) found the OMP28 located in the outer membrane and bleb. Rossetti *et al*. (1996) localized this protein in the periplasm. Contrarily, Cloeckaert *et al*. (2001) considered this protein as a soluble protein by using certain monoclonal antibodies. Making a correct diagnosis of brucellosis in animals is not always possible due to the reduced efficiency of

the bacteriological methods and serological reactions, therefore, these methods need to be further improved. One of the potential attempts to increase the sensitivity and specificity of serologic tests is by using the recombinant analogs of immunodominant proteins of pathogenic *Brucella* which have been extensively studied.

The de novo synthesis of OMP28 gene and purification of recombinant OMP28 antigen

Amino acid sequences of the OMP28 protein of *Brucella* spp. were downloaded from Genbank and compared in a multiple alignment. The gene encodes OMP28 of *Brucella* spp. was *de novo* synthesized. The *de novo* synthesis of OMP28 gene was performed as a two-round PCR. The product of the second round PCR showed a band of the expected length in (787 bp) (Fig. 2A). DNA sequencing results confirmed that the *de novo* synthesized OMP28 gene had the correct orientation to the designed sequence of the gene.

In order to overproduce the 28 kDa outer membrane protein (OMP28) of *Brucella* spp., the synthesized DNA was cloned into the expression vector, pET-22b(+) (Life Technology, USA). Expression of rOMP28 was achieved with *E. coli* BL21(DE3). The SDS-PAGE analysis of the cell lysate and various eluates showed the expression of the expected 28 kDa recombinant protein. Purification of the rOMP28 from inclusion bodies was carried out using a HisTrap FF crude with a native purification protocol as specified by the manufacturer. The different eluates were analysed by SDS-PAGE and the highly purified protein concentration was calculated and was estimated at 3.2 mg/mL (Fig. 2B and Fig. 3).

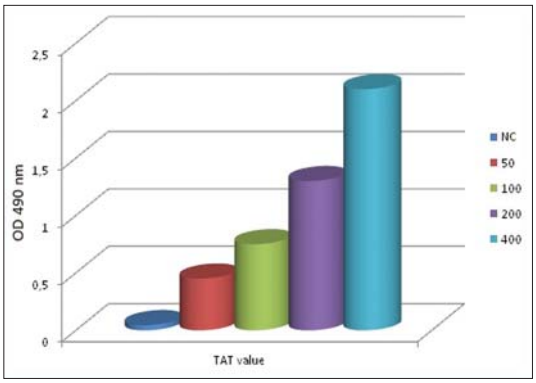

Fig. 4. ELISA absorbance values of bovine sera using rOMP28 compared to a TAT. ELISA absorbance values of *Brucella* positive and negative sera were estimated using 2 µg/mL of rOMP28 antigen. The Brucella positive sera were composed of TAT; 50 (n=5), TAT; 100 (n=23), TAT; 200 (n=24) and TAT; 400 (n=7). Immunoassay plates were charged with sera at a dilution of 1:100.

Immunoreactivity of rOMP28 of Brucella spp.

The diagnostic potential of rOMP28 of Brucella *spp.* was further evaluated for screening positive (n=62) and negative (n=28) bovine serum samples (determined by TAT). The immunochemical reactivity of highly purified rOMP28 was studied in I-ELISA assay compared to a tube agglutination test (TAT). The cut off value for I-ELISA was determined at 0.096 which was double the average OD_{492} value of negative serum 0.042 ±0.003 at a 1:100 dilution. I-ELISA absorbance values of *Brucella* positive sera using rOMP28 had a strong positive reaction in comparison to the TAT value (Fig. 4). The immunoreactivity of rOMP28 based ELISA relative to the reference method (TAT) is shown in Table 2. Totally, 59 (95.1%) and 3 (4.9%) of the 62 TAT-positive sera were rOMP28 antigen based I-ELISA positive and negative, respectively. In addition, it also detected one of the TAT- negative samples as positive (3.6%) and the remaining 27 samples (96.4%) as negative.

The potency of purified rOMP28 was studied in field sera for diagnosis of brucellosis using Western blot (Fig. 5). The immunoreactivity of the expressed protein was confirmed by Western blot. The protein band at 28kDa specifically reacted with bovine brucellosis sera. No reaction was observed with the negative serum samples.

In the present study, we selected the outer membrane protein (Omp28) of *Brucella* spp. as a candidate antigen to be further evaluated. The coding gene for *Brucella* spp. OMP28 was *de novo* synthesized, expressed in the *E.coli* system and used to develop rOMP28 I-ELISA in an attempt to increase the sensitivity and specificity for diagnosing bovine brucellosis. Our study has shown that I-ELISA, using rOMP28 protein, yielded high sensitivity and specificity for detection of *Brucella* antibodies in bovine sera, as shown in Table 2. Furthermore, these results contradict previously published data, which described this antigen as of no diagnostic value (Xin *et al*., 2013). However, data from other studies (Cloeckaert *et al*., 2001; Liu *et al*., 2011; Cha *et al*., 2012) showed that the OMP28-based I-ELISA had high sensitivity and specificity in the diagnosis of brucellosis in bovine sera, conforming to our results.

Table 2. Diagnostic values of rOMP28 antigen based I-ELISA compared to a TAT.

Diagnostic values ELISA	*TAT positive (n=62)*	*TAT negative (n=28)*	*Chi-Square distribution*	*Sensitivity*	*Specificity*
ELISA positive	59	1	72.8 (P>0.999)	59:62·100% =95,1%;	27:28·100% =96,4%;
ELISA negative	3	27			

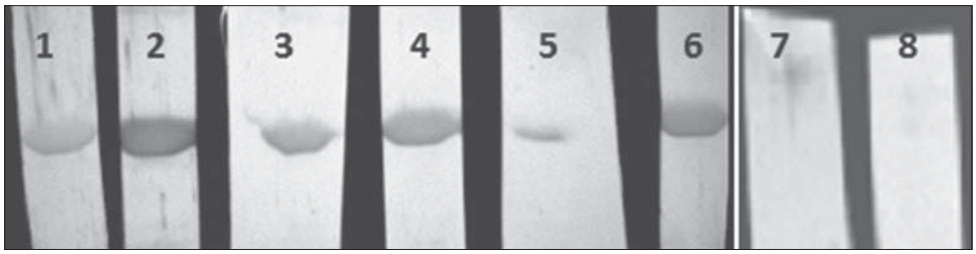

Fig. 5. Analysis of the immunoreactivity of the recombinant protein by Western blot. The immunoreactivity of rOMP28 was elucidated with *Brucella* positive and negative bovine serum. Samples: 1-6 (positive for brucellosis); 7-8 (negative for brucellosis).

In conclusion, the outer membrane protein OMP28 of *Brucella* spp. is identified as a major immunodominant antigen and a potential antigen for developing serological tests for bovine brucellosis.

References

Azizpour, M., Hosseini, S., Akbary, N., Basiri, H., Nezamabadi, M. and Sarikhani, M. 2013. Amplification, cloning, and expression of *Brucella melitensis* BP26 gene isolated from Markazi province in order to produce BP26 recombinant protein. Arak Med. Univ. J. 16, 62-70.

Cha, S.B., Rayamajhi, N., Lee, W.J., Shin, M.K., Jung, M.H., Shin, S.W., Kim, J.W. and Yoo, H.S. 2012. Generation and envelope protein analysis of internalization defective Brucella abortus mutants in professional phagocytes, RAW 264.7. FEMS Immunol. Med. Microbiol. 64, 244-254.

Christopher, S., Umapathy, B.L. and Ravikumar, K.L. 2010. Brucellosis: rewiew on the recent trends in pathogenicity and laboratory. J. Lab. Physicians 2, 55-60.

Cloeckaert, A., Baucheron, S., Vizcaino, N. and Zygmunt, M.S. 2001. Use of Recombinant OMP28 protein in Serological Diagnosis of *Brucella melitensis* infection in Sheep. Clin. Diagn. Lab. Immunol. 8, 772-775.

Dong-Bao, X., Ming-Chun, G., Di-Fei, C., Xiao-Dong, W. and Jun-Wei, W. 2012. Identification of Linear B-cell Epitope of Structural Protein OMP28 of Brucella abortus. Acta Vet. Zoo. Sin. 43, 1444-1448.

Gupta, V.K., Kumari, R., Vohra, J., Singh, S.V. and Vihan, V.S. 2010. Comparative evaluation of recombinant OMP28 protein for serological diagnosis of *Brucella melitensis* infection in goat. Small Ruminant Res. 93, 119-125.

Kim, D., Park, J., Kim, S., Son, Y. and Song, J. 2013. Brucella Immunogenic OMP28 Forms a Channellike Structure. J. Mol. Biol. 425, 1119-1126.

Ko, K.Y., Kim, J.W., Her, M., Kang, S., Jung, S.C., Cho, D.H. and Kim, J.Y. 2012. Immunogenic proteins of Brucella abortus to minimize cross reactions in brucellosis diagnosis. Vet Microbiol. 156, 374-380.

Kumar, S., Tuteja, U., Kumar, A. and Batra, H.V. 2008. Expression and purification of the 26 kDa periplasmic protein of *Brucella abortus*: a reagent for the diagnosis of bovine brucellosis. Biotechnol. Appl. Biochem. 49, 213-218.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lim, J., Kim, D., Lee, J., Min, W., Chang, H. and Kim, S. 2012. Evaluation of recombinant 28kDa outer membrane protein of Brucella abortus for the clinical diagnosis of bovine brucellosis in Korea. J. Vet. Med. Sci. 74, 687-691.

Lindler, L.E., Hadfield, T.L., Tall, B.D., Snellings, N.J., Rubin, F.A., Van De Verg, L.L., Hoover, D. and Warren, R.L. 1996. Cloning of a Brucella melitensis group 3 antigen gene encoding Omp28, a protein recognized by the humoral immune response during human brucellosis. Infect. Immun. 64(7), 2490-2499.

Liu, W.X., Hu, S., Qiao, Z.J., Chen, W.Y., Liu, L.T., Wang, F.K., Hua, R.H., Bu, Z.G. and Li, X.R. 2011. Expression, purification and improved antigenic specificity of a truncated recombinant OMP28 protein of Brucella melitensis M5-90: a potencial antigen for differencial serodiagnosis of brucellosis in sheep and goats. Biotechnol. Appl. Biochem. 58, 32-38.

Poester, F.P., Nielsen, K., Samartino, L.E. and Yu, W.L. 2010. Diagnosis of Brucellosis. Open Vet. Sci. J. 4, 46-60.

Qiu, J., Wang, W., Wu, J., Zhang, H., Wang, Y., Qiao, J., Chen, C., Gao, G.F., Allain, J.P. and Li, C. 2012. Characterization of Periplasmic Protein OMP28 Epitopes of Brucella melitensis Reacting with Murine Monoclonal and Sheep Antibodies. PloS One *7*(3), e34246. http://doi.org/10.1371/journal.pone.0034246.

Rossetti, O.L., Arese, A.I., Boschiroli, M.L. and Cravero, S.L. 1996. Cloning of Brucella abortus gene and characterization of expressed 26-kilodalton periplasmic protein: potential use for diagnosis. J. Clin. Microbiol. 34(1), 165-169.

Seco-Mediavilla, P., Verger, J-M., Grayon, M., Cloeckaert, A., Marín, C.M., Zygmunt, M.S.,

Fernández-Lago, L. and Vizcaíno, N. 2003. Epitope mapping of the Brucella melitensis OMP28 immunogenic protein: usefulness for diagnosis of sheep brucellosis. Clin. Diagn. Lab. Immunol. 10, 647-651.

Smirnova, E.A., Vasin, A.V., Sandybaev, N.T., Klotchenko, S.A., Plotnikova, M.A., Chervyakova, O.V., Sansyzbay, A.R. and Kiselev, O.I. 2013. Current Methods of Human and Animal Brucellosis Diagnostics. Adv. Infec. Dis. 3, 177-184.

Thavaselvam, D., Kumar, A., Tiwari, S., Mishara, M. and Prakash, A. 2010. 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. J. Med. Microbiol. 59, 421-428.

Xin, T., Yang, H., Wang, N., Wang, F., Zhao, P., Wang, H., Mao, K., Zhu, H. and Ding, J. 2013. Limitations of the BP26 protein-based indirect enzyme-linked immunosorbent assay for diagnosis of Brucellosis. Clin. Vaccine Immunol. 20(9), 1410-1417.