Development of a diagnostic indirect ELISA test for detection of *Brucella* antibody using recombinant outer membrane protein 16 kDa (rOMP16)

Mehdi Golchin[1], Somayye Mollayi[1], Elham Mohammadi[1], Neda Eskandarzade[2]*

[1] Department of Pathobiology, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran; [2] Department of Basic Sciences, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran.

Abstract

Brucellosis is considered as one of the important global zoonotic diseases that causes medical as well as economic problems especially in tropical countries. The illness has no specific pathognomonic signs; therefore, the rapid and accurate diagnosis of the disease has a very important role in preventing the *Brucella* spillover and treatment. The purpose of this study was to design a new indirect ELISA test for detection of human brucellosis based on using recombinant *Brucella abortus* outer membrane protein 16 kDa (rOMP16) as an antigen. OMP16 gene of *B. abortus* was initially synthesized and cloned in pET-21d vector and then expressed in *Escherichia coli* cells. The expression was confirmed by the SDS-PAGE, western blotting and dot blotting. The purified protein was coated in ELISA plates and an indirect ELISA was performed on 70 human serum samples. The results were evaluated with a commercial IgG ELISA kit and Rose Bengal plate agglutination tests as reference tests. Diagnostic performance of designed OMP16 ELISA test in comparison with Rose Bengal plate test revealed 100% of sensitivity, 95.00% of specificity and good Fleiss kappa agreement, whereas, where it was compared to commercial ELISA kit, it revealed very good kappa agreement with 100% of sensitivity and 100% of specificity in cut-off value of 0.13. It was concluded that OMP 16 kDa could be acceptable alternative antigen for detecting *Brucella* IgG antibody with high accuracy.

Introduction

Mediterranean remittent fever or brucellosis is a highly contagious disease that occurs mostly in domestic livestock and contaminates human as an accidental host. Thousands of new cases are being reported worldwide each year and the prevalence rate of brucellosis in humans doubles in some years in tropical communities.[1] The negative impact of this well-known bacterial disease is a significant issue attributing human and animal health, and also impacts animal products and economicity of governments.[2] In countries prone to brucellosis, reducing the incidence of brucellosis through testing and slaughter of suspected animals has become a major prevention program by athorities.[2] *Brucella* infected individuals have some ambiguous symptoms which are similar to other infectious diseases with the symptom of fever and declaring a person or animal as a positive case is not merely based on clinical picture.[3] Slow growth rate of

Brucella spp. in blood culture (about 8 - 10 days) ends up limited isolation of the organism for accurate detection, therefore, serological methods such as Rose Bengal plate test (RBPT), complement fixation test and enzyme-linked immunosorbent assay (ELISA) have become most popular for comprehensive surveillance or eradicating of the brucellosis.[4,5] However, these ways of evaluation are not flawless and applying lipopolysaccharide (LPS) to trace *Brucella* spp. antibodies cannot discriminate immuno-glubolins in response to *Brucella melitensis (B. melitensis) Rev.1* strain vaccine or some other Gram-negative bacteria infection such as *Yersinia enterocolitica* O:9, *Escherichia coli* O157:H7, *Salmonella* group N(O:30).[6-8] Cloeckaert *et al.* dentified seven surface-exposed *Brucella* outer membrane proteins (OMPs) using monoclonal antibodies (mAbs) which later strong evidences indicated that they played a dominant role in host immune responses.[9,10] According to some papers, exposure to *Brucella* outer membrane protein 16 (OMP16) caused cellular and humeral immune responses

*Correspondence:

Neda Eskandarzade. DVM, PhD
Department of Basic Sciences, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran
E-mail: eskandarzade@uk.ac.ir

in the host, thus, a number of *Brucella* OMPs made by recombinant DNA techniques (rOMPs) and specific serological tests including ELISA were developed.[10-16] In this context, the aim of the current study was to develop an indirect ELISA test for detection of human brucellosis based on using recombinant *Brucella* outer membrane protein 16 kDa (rOMP16) as an antigen.

Materials and Methods

Cloning, expression, and verification tests. *Brucella* OMP16 gene sequence (Genebank accession No. AAA59360.1) was synthesized by GenRay Biological Technology Co., Ltd (Guangzhou, China). Then, it was cloned into an expression vector named pET-21d and transformed into *E. coli* cells (DE3) by heat shock (42.00 °C for 90 sec).[17] A single colony was incubated overnight in 10.00 mL of Luria-Bertani (LB) broth containing 100 μg mL^{-1} of ampicillin followed by incubation in a 37.00 °C and shaking at 250 rpm overnight. The overnight culture was added (1:100) to a fresh LB broth and was grown until OD_{600nm} reached to 0.60. The cells were then induced with 1.00 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; Pars Tous Co., Mashhad, Iran) and allowed to grow further for 20 hr at 25.00 °C. After centrifugation (5,000 rpm, 10 min) and discarding the supernatant, the pellet was resuspended in a lysis buffer [NaH_2PO_4 (50.00 mM; Merck KGaA, Darmstadt, Germany), imidazole (10.00 mM; Merck KGaA), Nacl (300 mM; Merck KGaA), pH = 8.00]. The lysate was refrigerated for 30 min and then was sonicated (10-sec pulse on followed by 10-sec pulse off, total time 4 min). After centrifugation (5,000 rpm, 30 min, 4.00 °C), methods including electrophoresis on SDS-PAGE according to Laemmli and blotting on nitrocellulose membranes were applied to analyze the product.[18] Following blocking with 3.00% bovine serum albumin (Sigma, St. Louis, USA)/phosphate buffer saline (Cytomatin Gene Co, Isfahan, Iran), the recombinant protein was detected by monoclonal anti-His-tag antibody conjugated to horse radish peroxidase (Sigma). The reaction was developed with 4-chloro-1-naphtol (Sigma) substrate. Purification of the rOMP16 was performed using Ni-NTA Superflow Cartridge according to the instructions of the manufacturer (Qiagen, Hilden, Germany). Concentration of purified protein was assayed at 280-nm by biophotometer (Eppendorf, Hamburg, Germany).

Samples. The current study was performed on 70 brucellosis suspected human serums which were provided by a laboratory in Qaen County in south Khorasan province in Iran. Additionally, two negative and one positive serum samples also were used to check the procedure. We also used 20 negative serum samples (confirmed by RBPT and anti-IgG *Brucella* ELISA kit) for calculating the cut-off value of our designed kit.

Evaluation of sera with commercial ELISA kits. In this study we tested all serum samples with commercial *Brucella* IgG ELISA kit (Pishtazteb, Tehran, Iran) which used *Brucella* LPS as antigen.

Evaluation of sera with RBPT. The Rose Bengal test was performed on all of the serums according to standard method. The RBPT antigen was purchased from Pasteur Institute, Iran. Equal volume of the serum and RBPT antigen were mixed together on a plate and the results were read within 4 min. Medium to high degree of agglutination was considered as positive.

Recombinant OMP16 ELISA. The immunoassay plate (Nunc, Roskilde, Denmark) was coated with 100 μL purified rOMP16 protein at a concentration of 50.00 μg mL^{-1} and was incubated at 4.00 °C, overnight. The wells were washed three times with PBS and then blocked with 200 μL of BSA 3.00% in PBS. Then, 100 μL diluted sera (1:100 with PBS) were added to each well and were incubated for 1 hr at 37.00 °C. After washing the wells with PBS/Tween 20 (0.10% v/v; Merck KGaA) for three times, the plates were incubated with anti-Human IgG-HRP antibody (Serotec, Kidlington, UK) at dilution of 1:5, 000 for 1 hr at 37.00 °C. In the next step, the wells of immunoassay plates were developed with 3,3',5,5'-Tetra-methylbenzidine (TMB) substrate and the reaction was stopped after 15 min by addition of 1.00 M H_2SO_4 (Ghatran Shimi Co., Tehran, Iran). Absorbance was read at 450 nm by Anthos 2020 ELISA reader (Biochrom, Cambridge, UK).

Statistical analysis. The SPSS for Windows (version 26.0; IBM Corp., Armonk, USA) was applied to perform statistical analysis. The agreement between the tests was determined by *kappa* index (κ) values with 95.00% confidence intervals and interpreted according to the following classification: Kappa coefficients of < 0.20, 0.21 - 0.40, 0.41 - 0.60, 0.61 - 0.80 and 0.81 - 1.00 indicated the strength of agreement as poor, fair, moderate, good and very good, respectively.[19] Fleiss' kappa coefficient was considered statistically significant when it was different from zero.

Results

The cloning and expression of *B. abortus* OMP16 were achieved using prokaryotic system pET-21d and *E. coli*. The presence of expressed OMP16 was approved in SDS-PAGE as a major band, which is shown in Figure 1A. Because of the expression of OMP16 with 6× His-tag tail, western blotting and dot blotting were performed using a commercial antibody against His-tag on nitrocellulose membrane. Purified rOMP16 was shown in Figures 1B and 2, respectively. After being assured on the purification of aimed protein, assembly of the ELISA test components was started. For calculating the cut-off value of our kit, 20 negative serum samples (confirmed by RBPT and anti-IgG *Brucella* ELISA kit) were used as negative controls.
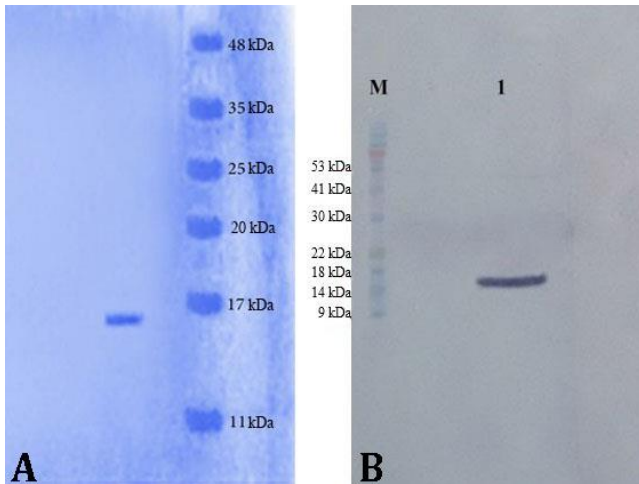
Fig. 1. A) An expected protein product was specified by SDS-PAGE. Right lane: Protein prestained ladder. Left lane: Product (purified *Brucella* rOMP16 kDa). **B)** The rOMP16 protein was determined by commercial anti His-tag peroxidase-conjugated antibody in western blot. M: Protein prestained ladder. Lane 1: Product (purified *Brucella* rOMP16 kDa).
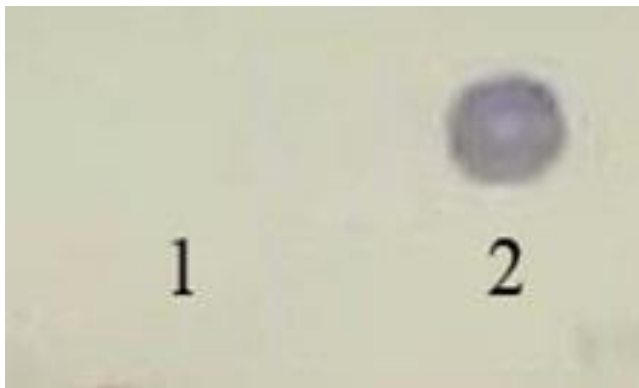

Fig. 2. Purified His-tagged OMP16 protein was chased via dot blot technique using commercial anti-His-tag peroxidase-conjugated antibody. Number 1 indicates negative control (total cell lysate of *E. coli* that not prompted by isopropyl-β-D-1-thiogalacto-pyranoside) and number 2 shows rOMP16 protein.

The mean absorbance value of negative sera plus four standard deviation was considered as cut-off value which meant that samples were considered positive when the absorbance of the measurements was greater than 0.13. Detection of antibodies against OMP16 was carried out by designed test on 70 human serum samples. The results were evaluated using RBPT and a commercial IgG ELISA kit with cut-off point of (0.15) based on the manufacturer's protocol.

Out of 70 samples, eight samples were positive and 62 were negative by designed OMP16 ELISA. In comparison with the RBPT, its sensitivity and specificity were 100 and 95.00%, respectively, whereas, where it was compared to commercial ELISA, the sensitivity and specificity were 100 and 100%, respectively (Table 1).

Discussion

Brucellosis is recognized as a serious zoonotic disease which impacts broad spectrum of species exposed to *Brucella*-infected animals or their products. Diagnosis of the brucellosis is not based solely on the patient's clinical signs; however, more than one serological test is needed to confirm the disease.[5] Consequently, evaluating the reliability of diagnostic tests is crucial for successful surveillance programs. According to the world organization for animal health (OIE) guideline, isolation and identification of *Brucella* are recommended for definitive diagnosis, however, bacterial culture needs laboratories with high level of biosafety with trained staffs. Additionally, bacterial culture output is associated with false negative probability, which can result in low sensitivity especially in long-term disease situation.[4,20,21] False negative results can also be obtained by serological tests such as RBPT because of prozone phenomenon. Therefore, it is recommended that RBPT data should be confirmed with other recommended screening tests such as ELISA especially in chronic and complicated clinical situations.[4,22] Available *Brucella* ELISA kit recognizes anti-LPS antibodies in sera which is associated with false positive. For introducing more accurate ELISA tests, researchers evaluated diagnostic potentials of multiple recombinant *Brucella* proteins.[12] Some researchers have demonstrated the potential immunogenicity of *Brucella* OMP16 through its ability to induce cellular immune responses in mice.[13,23,24] This has been supported by Mohammadi and Golchin, who showed that *Brucella* OMP16 stimulated the immune system in rabbits by producing high-titer antibodies. As a result, *Brucella* bacteria could be detected by anti-OMP16 antibody in laboratory evaluations.[25] In this study, we attempted to express *Brucella* outer membrane protein (16 kDa) and apply it for designation of an indirect ELISA test capable of specifically recognized antibodies against *Brucella* spp. Despite the fact that the identification of *Brucella* from the culture medium is the gold standard test, the comparison of serological tests with each other seemed more accurate,

Table 1. Reliability comparison of the OMP16 ELISA with other methods for *Brucella* antibodies detection.

Compared tests	Samples	P	N	TP	TN	FP	FN	PPV	NPV	Dse	Dsp	AC	κ	κ-CI at 95.00%	Agreement
Rose Bengal plate test	70	5	65	5	62	3	0	62	100	100	95	95	0.74	0.51 - 0.98	Good
***Brucella* IgG ELISA kit**	70	8	62	8	62	0	0	100	100	100	100	100	1.00	0.76 - 1.23	Very good

P: positive, N: negative, TP: true positive, TN: true negative, FP: false positive, FN: false negative, PPV: positive predictive value, NPV: negative predictive value, Dse: diagnostic sensitivity, Dsp: diagnostic specificity, AC: accuracy, κ: kappa index, and CI: confidence interval.

therefore, we decided to compare OMP16 ELISA to RBPT as a confirmatory test. According to Table 1, designed OMP16 ELISA test and commercial ELISA kit detected 3 more positive samples than RBPT which might be because of prozone phenomenon. This phenomenon in humans was proven to be in patients with chronic latent infection.[26] Designed OMP16 ELISA test showed sensitivity of (100%) and specificity of (95.00%) compared to RBPT (Table 1) and according to Fleiss kappa classification, diagnostic performance of designed OMP16 ELISA test revealed good kappa agreement (κ = 0.74) and accuracy of (95.00%). Infection status of samples which is unknown to researchers could cause sensitivity and specificity variation in the serological tests results. Comparison with commercial ELISA analysis, OMP16 ELISA showed perfect result which was 100% of sensitivity and 100% of specificity (Table 1). It means that specific antibodies against *Brucella* had significant affinity to the OMP16 antigen, therefore, OMP16 ELISA obtained very good kappa agreement (κ = 1.00) and accuracy of (100%) compared to LPS-based ELISA (Table 1). The results of this study expressed efficiency of OMP16 in detecting anti-*Brucella* antibodies that could be applied in serological kits to reduce misdiagnosing of the brucellosis. OMP16 was the same in all of the *Brucella* species, therefore, the same antigen could be used potentially in diagnosing *Brucella*-infected animals if further studies were conducted in the samples.

This was in agreement with two relevant studies by Yin *et al.*, who established an indirect ELISA test based on multi-epitope (including rOMP16) protein.[16,27] Human sera were evaluated with their designed test and compared to the plate agglutination test and standard tube agglutination test for verification of brucellosis. They used multiple epitope antigens for brucellosis positive and negative discrimination and the sensitivity and specificity were lower than our single OMP ELISA test.[16,27] Koyuncu *et al.* provided convincing evidence regarding utility of *B. melitensis* rOMP 28 indirect ELISA (I-ELISA) for sero-diagnosing of brucellosis in the clinical human serum samples.[28] They concluded that the rOMP 28 based ELISA yielded high sensitivity (87.80%) and high specificity (96.20%) for detection of anti-*Brucella* antibodies compared to RBPT. Ahmed *et al.* used three different rOMPs (rOMP25, rOMP28 and rOMP31) for developing an I-ELISA that recognized the mice infected with *B. melitensis* strain 0331.[29] They declared that combined OMPs ELISA provided both sensitivity and specificity of 100% and could effectively differentiate the vaccinated mice and mice infected with *Y. enterocolitica* O:9 from actual *Brucella* infection.

In conclusion, in the present study, an indirect ELISA based on rOMP16 was designed and compared to RBPT and commercial IgG I-ELISA kit. Data analysis showed good agreement in comparison with RBPT and perfect agreement with commercial ELISA in cut-off value of 0.13. It was concluded that OMP16 could be a useful protein candidate for precise serodiagnosis of brucellosis.

Conflict of interest

None.

References

1. World animal health information database (WAHIS Interface). Available at: https://wahis.woah.org/#/home. Accessed Aug 04, 2022.
2. Bagheri Nejad R, Krecek RC, Khalaf OH, et al. Brucellosis in the Middle East: Current situation and a pathway forward. PLoS Negl Trop Dis 2020; 14(5): e0008071. doi: 10.1371/journal.pntd.0008071.
3. Gupte S, Kaur T. Diagnosis of human brucellosis. J Trop Dis 2015; 4(1): doi: 10.4172/2329-891X.1000185.
4. World organization for animal health. OIE manual of diagnostic test and vaccines for terrestrial animals. Available at: https://www.oie.int. Accessed Aug 04, 2022.
5. Yagupsky P, Morata P, Colmenero JD. Laboratory diagnosis of human brucellosis. Clin Microbiol Rev 2019; 33(1): e00073-19. doi: 10.1128/CMR.00073-19.
6. Padilla Poester F, Nielsen K, Ernesto Samartino L, et al. Diagnosis of brucellosis. Open Vet Sci J 2010; 4: 46-60.
7. Blasco JM, Molina-Flores B. Control and eradication of *Brucella melitensis* infection in sheep and goats. Vet Clin North Food Anim Pract 2011; 27(1): 95-104.
8. Smirnova EA, Vasin AV, Sandybaev NT, et al. Current methods of human and animal brucellosis diagnostics. Adv Infect Dis 2013; 3(3): 177-184.
9. Cloeckaert A, de Wergifosse P, Dubray G, et al. Identification of seven surface-exposed Brucella outer membrane proteins by use of monoclonal antibodies: immunogold labeling for electron microscopy and enzyme-linked immunosorbent assay. Infect Immun 1990; 58(12): 3980-3987.
10. Tabynov K, Kydyrbayev Z, Ryskeldinova S, et al. Novel influenza virus vectors expressing Brucella L7/L12 or Omp16 proteins in cattle induced a strong T-cell immune response, as well as high protectiveness against *B. abortus* infection. Vaccine 2014; 32(18): 2034-2041.
11. Yang X, He Z, Zhang G, et al. Evaluation of reactivity of monoclonal antibodies against Omp25 of *Brucella* spp.

Front Cell Infect Microbiol 2020; 10: 145. doi: 10.3389/fcimb.2020.00145.

12. Tian M, Song M, Yin Y, et al. Characterization of the main immunogenic proteins in Brucella infection for their application in diagnosis of brucellosis. Comp Immunol Microbiol Infect Dis 2020; 70: 101462. doi: 10.1016/j.cimid.2020.101462.

13. Luo D, Ni B, Li P, et al. Protective immunity elicited by a divalent DNA vaccine encoding both the L7/L12 and Omp16 genes of *Brucella abortus* in BALB/c mice. Infect Immun 2006; 74(5): 2734-2741.

14. Pasquevich KA, Estein SM, García Samartino C, et al. Immunization with recombinant Brucella species outer membrane protein Omp16 or Omp19 in adjuvant induces specific CD4+ and CD8+ T cells as well as systemic and oral protection against *Brucella abortus* infection. Infect Immun 2009; 77(1): 436-445.

15. Pasquevich KA, García Samartino C, Coria LM, et al. The protein moiety of *Brucella abortus* outer membrane protein 16 is a new bacterial pathogen-associated molecular pattern that activates dendritic cells in vivo, induces a Th1 immune response, and is a promising self-adjuvanting vaccine against systemic and oral acquired brucellosis. J Immunol 2010; 184(9): 5200-5212.

16. Yin D, Li L, Song X, Li H, et al. A novel multi-epitope recombined protein for diagnosis of human brucellosis. BMC Infect Dis 2016; 16: 219. doi: 10.1186/s12879-016-1552-9.

17. Sambrook, J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. New York, USA: Coldspring-Harbour Laboratory Press 2001; 110-111.

18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227(5259): 680-685.

19. McHugh ML. Interrater reliability: the kappa statistic. Biochem Med (Zagreb) 2012; 22(3): 276-282.

20. O'Grady D, Byrne W, Kelleher P, et al. A comparative assessment of culture and serology in the diagnosis of brucellosis in dairy cattle. Vet J 2014; 199(3): 370-375.

21. Ducrotoy MJ, Muñoz PM, Conde-Álvarez R, et al. A systematic review of current immunological tests for the diagnosis of cattle brucellosis. Prev Vet Med 2018; 151: 57-72.

22. Franco MP, Mulder M, Gilman RH, et al. Human brucellosis. Lancet Infect Dis 2007; 7(12): 775-786.

23. Vitry MA, Hanot Mambres D, De Trez C, et al. Humoral immunity and CD4+ Th1 cells are both necessary for a fully protective immune response upon secondary infection with *Brucella melitensis*. J Immunol 2014; 192(8): 3740-3752.

24. Yingst SL, Izadjoo M, Hoover DL. CD8 knockout mice are protected from challenge by vaccination with WR201, a live attenuated mutant of *Brucella melitensis*. Clin Dev Immunol 2013; 2013: 686919. doi: 10.1155/2013/686919.

25. Mohammadi E, Golchin M. Detection of *Brucella abortus* by immunofluorescence assay using anti-16-kDa outer membrane protein (OMP16) antibody. Comp Clin Path 2017; 26: 1299-1304.

26. Salih SM, Khorsheed HO, Ya`qob JS. Incidence of brucellosis in Kirkuk Province using simple dilution microagglutination rose Bengal test method. Tikrit Med J 2007; 13(1): 70-74.

27. Yin D, Bai Q, Zhang J, et al. A novel recombinant multiepitope protein candidate for the diagnosis of brucellosis: A pilot study. J Microbiol Methods 2020; 174: 105964. doi: 10.1016/j.mimet.2020.105964.

28. Koyuncu I, Kocyigit A, Ozer A, et al. Diagnostic potential of *Brucella melitensis* Rev1 native Omp28 precursor in human brucellosis. Cent Eur J Immunol 2018; 43(1): 81-89.

29. Ahmed IM, Khairani-Bejo S, Hassan L, et al. Serological diagnostic potential of recombinant outer membrane proteins (rOMPs) from *Brucella melitensis* in mouse model using indirect enzyme-linked immunosorbent assay. BMC Vet Res 2015; 11: 275. doi: 10.1186/s12917-015-0587-2.