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

Mehdi Golchin¹, Somayye Mollayi¹, Elham Mohammadi¹, Neda Eskandarzade²*

¹Department of Pathobiology, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran; ²Department 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 an 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 economy 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 authorities.² Brucella infected individuals have some ambigious 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 immunoglobulins 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. identified 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

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in the host, thus, a number of *Brucella* OMPs 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 OD₆₀₀nm 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 [NaH₂PO₄ (30.00 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-napthol (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 serum which were provided by a laboratory in Qen County in south Khorsaran province in Iran. Additionally, two negative and one positive serum samples 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 sera 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’-Tetramethylbenzidine (TMB) substrate and the reaction was stopped after 15 min by addition of 1.00 M H₂SO₄ (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.
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 | k | k-CI at 95.00% | Agreement |
|---------------------------|---------|---|---|----|----|----|----|-----|-----|-----|-----|----|---|----------------|-----------|
| Rose Bengal plate test    | 70      | 5 | 65| 5  | 62 | 3  | 0  | 62  | 100 | 95  | 95  | 0.74| 0.51 - 0.98 | Good      |
| Brucella IgG ELISA kit    | 70      | 8 | 62| 8  | 62 | 0  | 0  | 62  | 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, k: 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 serodiagnosis 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 1-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.

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Conflict of interest

None.

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