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A New ELISA Kit Based on Antigenic Epitopes for Diagnosing *Brucella abortus*

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Brucellosis is one of the most important zoonotic diseases that lead to a great amount of economic losses. Prevention and diagnosis are both necessary to eradicate this disease. The identification and evaluation of different antigens of *Brucella* spp. play a key role in the progress of diagnostic programs. In this study, we designed, produced, and evaluated a 24-kDa polypeptide containing antigenic epitopes of VirB2, 3, and 9 of *Brucella abortus* for use with the ELISA kit. The produced polypeptide is appropriate for diagnosing brucellosis in bovines by a laboratory diagnostic kit, with 100% sensitivity and 97.5% specificity.

Keywords: *Brucella abortus*, protein expression, antigenic epitopes, VirB2.3.9, ELISA

Introduction

Brucella abortus is an optional, coccubacilli-shaped, gram-negative and pathogenic intracellular aerobic bacteria which causes abortion in cows and is the cause of brucellosis in animals [1].

Brucella, which is considered as one of the most serious causes of common diseases among animals and humans in the world, causes short-term and transient fever in humans [2]. The disease remains silent for several months in the absence of treatment and is rarely lethal to humans. Typically, the infection is transmitted through digestive, respiratory, and skin scratches. It often causes infection in humans through milk and dairy products. Following the penetration of the bacteria into the epithelium, it moves toward the lymph glands and proliferates through the macrophages within the lymph nodes, spleen, liver, bone marrow, breast and reproductive organs. *Brucella* is capable of proliferating and increasing the intracellular survival of phagosomes within macrophages by preventing the integration of phagosomes with lysosomes [3, 4]. Accordingly, the key to the infection of *Brucella* is its ability to survive and proliferate in professional and non-professional phagosomes [5]. *Brucella* does not have the type I, II, III secretion systems, but the presence of secretory-dependent and independent secretion genes, and the specificity of the secretion type V, III, IV secretion secretagogues, Type 4 secretion systems (T4SS) are used by many of the gram-negative bacteria to transmit infectious factors to eukaryote cells or as mediators for the transmission of plasmid [6]. T4SS is one of the most important factors for inter-host and pathogenic reactions. *Brucella* survival in different environments such as intracellular compartments of mammals depends on their existence [7]. Removal mutations in T4SS have shown that the survival and proliferation of brucella in macrophages and epithelial cells such as the mouse model will be

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eliminated. T4SS consists of 12 parts ranging from VirB1 to VirB11 along with (VirB12) VirD4. Biochemical, genetic, and cell biology experiments have indicated that VirB2 to VirB11 lead to the formation of a canal which travels from one side to the cytoplasmic space and to the outer space of the cell, involving the internal and external membranes [6]. The role of the VirB3 protein is far less pronounced than the rest, but its function can be related to locating the T4SS components for montage [8]. Complications by the development of Brucellosis result in significant infections in animals and transmission to humans through milk and dairy products. In this study, we have selected Vir2,3 and 9 specific epitopes as antigenic candidates for *Brucella abortus* diagnosis in ELISA kit. This is a sensitive diagnostic laboratory method that can facilitate management of high risk animals during infection. Definitely, control measures are important to prevent disease transmission.

Materials and Methods

Bacterial strains, culture media and reactants

E. coli Top10 strain was nominated as non-expressive host and *E. coli* BL21 strain was designated as the host of the statement. *E. coli* species were cultured in Luria-Bertani Broth (LB Agar). When necessary, kanamycin was added to the liquid or plate medium as much as 30 mg/ml. The pET26b expression vector was used to construct expression systems for the production of recombinant VirB polypeptide. The enzymes were purchased from Fermentas Co. IPTG, agarose, kanamycin and all Western blotting and purification reactors were purchased from the LB Broth & LB Agar mediums of the German Merck Corporation. Finally, the buffers were purchased from Sigma Company.

Samples used for evaluating polypeptide in Enzyme Linked Immuno Assay

A total of 400 animals sera with anti-*Brucella* IgG and 400 animals sera without anti-*Brucella* as well as 100 serum samples with tuberculosis as controls were collected from Veterinary central Laboratory in Tehran.

Structural bioinformatics design

Initially, amino acid sequences of the VirB2, VirB3, and VirB9 proteins of *B. abortus* locus were considered (GenBank accession number AF226278) from Expasy and NCBI data Banks. The following sequence was designed with underlined linkers in the pET26b vector.

M K E T A A A K F E R Q H M D S K K S L S R I L P H L L L A L G G S G K T A S P S K K S L S G P G P G P V L G S S G L V V G A A A E I G G S G N G G L D K V N T S G G S G S G Y K M A F R H A G P G P G G A L V V G A A A E G G S S G T F I L P L I V P I V L V M E G G S G L G L K A Q F G P G P G T F R L L G L K A Q F R L I H G G S G V A Y R V Q F R Y P A A Y P S A P D I L V L H R V A K Q M P A A F L V A E D K

The epitope mapping procedure was employed with IEDB analysis Resource (http://tools.immuneepitope.org/main/) and linear epitopes in VirB2.3.9 protein sequences were predicted, where Genpep-prosite and Bcepred were used for Bcell epitope prediction. Antigenicity, solubility and accessibility of the protein were the most important factors for analysis. The designed polypeptide sequences were linked with proper sequences. The polypeptide structure was predicted by PSIpred (http://bioinf.cs.ucl.ac.uk/psipred/) and SSpro (http://scratch.proteomics.ics.uci.edu/). Then the fragment was optimized with *E. coli* codon usage and the sequence of interest with start, stop and S-tag peptide codons was synthesized inside pET 26b vector (Bioneer, Korea) (Fig. 1).

Amplification of the recombinant plasmid and confirmation of its accuracy

In this study, a designed sequence was synthesized in a pET26b vector (Bionner) inserted into *BamHI* and *XhoI* restriction enzyme cut sites of the vector. Initially,

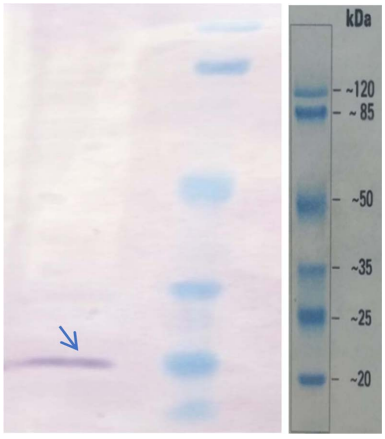

Fig. 1. The 24 kDa VirB2.3.9 protein in western blot analysis. (Right) MW Marker (Left) Sample collected in 4 h after induction.

the product was transferred into predisposed cells of *E. coli* Top10 strains for replication. The plasmid was then extracted by alkaline lysis method and examined by gel electrophoresis. The genetic region encoding VirB2.3.9 polypeptide of *B. abortus* containing 627 bp was confirmed using the universal pET primers with the temperature schedule as below.

Forward (pET) CGCAAATGGGCGGTAGGCGTG

Reverse (pET) GGCCGTTTACGTCGTCGCCGTCC

It was performed in 30 cycles in 3 steps of denaturation at 95℃, at 57℃ annealing temperature and extension step at 72℃.

Expression of the polypeptide VirB2.3.9 in *E. coli BL21* host and its analysis by western blotting

In order to express the recombinant protein of a single colony of *E. coli BL21*, a recombinant plasmid of 100 ml of LB medium containing 30 μg/ml kanamycin was inoculated after 12–16 h incubation at 37℃. At 150 rpm, light absorbance reached about 0.7 at 600 nm. Subsequently, 2 ml of cell suspension was transferred to 50 ml of LB containing 15 μg/ml kanamycin and cultivated under the previous conditions. The expression was induced using 1 mM of IPTG. The expression levels were evaluated two and four hours after induction. The induced cells were analyzed by 12% SDS-PAGE. Next, gel staining was prepared with a coomassie blue G250. The samples were then transferred from the polyacrylamide gel to the nitrocellulose membrane through an electroplating tank using a tris/glycine buffer and 20% methanol. Non-specific areas with non-fat milk 3% were blocked. The membranes were washed with Saline Buffer Tween 20. After two hours, the conjugated anti-S-tag antibody was admixed with alkaline phosphatase. The Tris-containing Triton buffer was washed again and then placed in a NBT-BCIP substrate solution, where observation of the purple strip in the nitrocellulose membrane indicated the successful expression of the polypeptide.

Purification of recombinant polypeptide by an affinity chromatography method

The bacterial cells were collected from 50 ml of culture medium. Then, 4 grams of bacterial cells were suspended by 4 ml of lysis buffer (Urea 2 M, Tris 50 mM, EDTA 2 mM). The lysate was prepared and the expressed protein was purified by S-tag resin. First, the S-tag resin was washed twice with a washing buffer (Tris 50 mM, EDTA 2 mM). After the chromatography steps, the recombinant polypeptide was purified with 2 ml of eluting solution (sodium thiocyanate 3 M) and dialysed with PBS buffer (pH = 11) at room temperature for 2 h. The quality and quantity of the recombinant protein were verified using western blot method.

Antigen-Antibody interaction in gel diffusion test

For immunological evaluation of the recombinant protein and in order to confirm the antigen and antibody responses in positive sera by gel diffusion protocol, first, to prepare the slides, agarose gel 1% in PBS was poured into a glass to form a thin, uniform layer after drying and punching. The cavities were embedded in the gel in 30 μl of purified protein, while in other cavities 30 μl of different diluted cow serums were added in order to push the protein and serum in the agarose gel into each other. The slides were placed in a 37℃ incubator for 24 h, and the next day they were stained with Commassie Brilliant blue G250 for one hour. After washing, there was an arc between the protein cavity and the positive serum, indicating positive test and response between the antigen and antibody.

Setting up the ELISA (enzyme-linked immunosorbent assay) kit and Brucellosis detection

ELISA analysis was used to study the potential of purified protein as a diagnostic marker of *Brucella* infection. To evaluate the designed polypeptide with sera with Brucella, 400 animals sera with anti-*Brucella* IgG and 400 animals sera without anti-*Brucella* and 100 serum samples with tuberculosis as controls were collected from Veterinary central Laboratory in Tehran. To set up the antibody assay, different concentrations of purified protein in 1x PBS were prepared and coated on a polystyrene 96-well microtiter plate, and the plate was incubated overnight at room temperature. All of procedure steps were performed similar to our previous research [9]. Finally, 100 μl of HRP-conjugated rabbit anti-cow IgG (1:5000 dilution in 1x PBS) was added to each well and the plates were left at room temperature for 1 h. After re-washing the plates, 100 μl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and after 15 min at room temperature, the reactions were

stopped by 50 µl of 2N H_2SO_4. The Optical Density (OD) of each well was measured by ELISA reader at a wavelength of 450 nm with the reference wavelength of 630 nm. The procedures were repeated for all of the 900 serum samples in 1:100 dilution and with 1 µg/ml of purified protein. All of the 400 anti-*Brucella* negative serum samples were considered to set a cutoff value using mean ± 2SD.

Statistical analysis

The sensitivity and specificity of the designed kit were calculated by VassarStats website and using the following formula: Sensitivity = TP/(TP + FN) × 100 and Specificity = TN/(TN + FP) × 100. TP, FN, TN, and FP represent the values of true positive, false negative, true negative, and false positive, respectively.

Results

VirB2.3.9 Construct design in pET26b vector

The plasmid pET26b-VirB2.3.9 was synthesized and transformed in *E. coli* Top10 and was then extracted by alkaline lysis method. The VirB2.3.9 gene with a length of 627 bp was characterized by universal pET primers of a total band of about 1000 base pairs.

Expression and confirmation of VirB2.3.9 polypeptide

After transformation of the recombinant plasmid into *E. coli* bacterial host BL21 strain, the expression and its induction with 1 mM IPTG, the 24-kD protein band was obtained on SDS-PAGE gel 12%. The highest expression of VirB2.3.9 polypeptide occurred 4 h after induction with 1 mM IPTG at 37℃ and the expression yield of recombinant protein in the culture media was 3 g/l. After purifying the VirB2.3.9 polypeptide and dialysis with 12 kDa cutoff tube, its amount was 15 µg/µl. The purified protein appeared as a band on the nitrocellulose membrane in the 24 kDa region in western blot test (Fig. 1).

Immuno reactivity assay of VirB2.3.9 polypeptide in gel diffusion method

This designed sequence was selected from different parts of three proteins of *Brucella,* so we checked whether they interact with serum positive antibodies or not. Fortunately the precipitation of antigen-antibody complexes was assessed by Ochtelony test and made an arc between them as shown in Fig. 2. This result suggested that the serum antibodies against protein epitopes of *Brucella* could recognize the bacterial antigens specifically.

ELISA kit setting results for Brucellosis detection

The study of the designed kit in 400 cow serum samples showed the best cut-off OD point was 0.1213. This value was obtained according to negative samples absorbances with SD of 0.01 and CV of 0.1% using the following formula: Cut off = Mean negative serum OD+2SD and CV% = SD/Mean. The comparison of the results of the commercial (vidal test) and recombinant protein ELISA indicated a sensitivity of 100% and a specificity of 97.5% for recombinant ELISA kit.

Hence, from 400 serum samples with *Brucella abortus*, all of them were true positive as anti poly-epitope protein, while from 400 negative sera, 388 cases were true negative. Also none of the 100 *Mycobacterium bovis* positive samples were found to cross react with anti *Brucella abortus* antibodies (Table 1).

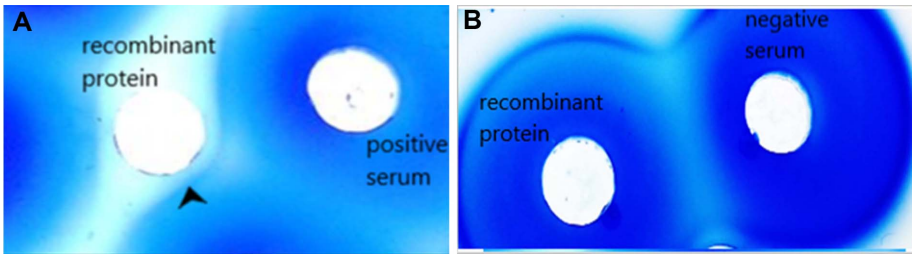

Fig. 2. Vir2.3.9 polypeptide-serum complex precipitated in Gel Diffusion test (A), arrow shows the created arc after reaction between antigen and antibody, Negative control (B), The blue color of commassie Brilliant blue G250 indicate to movement of proteins (antigen and antibody) in the gel agarose. There is not any cross reaction between protein and negative serum.

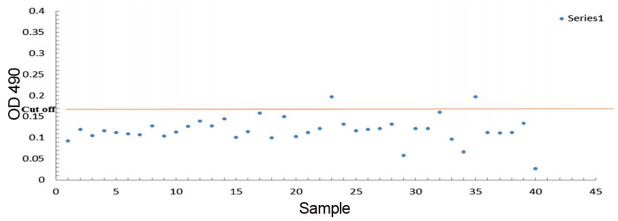

Fig. 3. The ELISA results of Negative sera for cut off determination.

Discussion

Brucella is a germ-free bacterial gland which is capable of inducing infectious disease in many animal and human species. In animals, *Brucella* prefers to settle in reproductive organs and embryonic tissues, resulting in abortion and infertility, and subsequently leads to significant economic losses. In humans, *Brucella* has a tendency to multiply in the reticular ductal system and, through contact with infected animals, aerosol respiration or consumption of infected animal products such as milk, infected milk or infected meat, it develops chronic illness of vaginal fever. Indeed, human brucellosis is one of the most common zoonotic diseases which requires long-term treatment with antibiotics [10]. So, designing and applying sensitive diagnostic tests provides treatment guidance and allows prompt implementation of infection control measures to prevent disease from spreading to humans. Studies on the T4SS system have indicated that the VirB2 protein is present on the surface of bacteria. It is predicted to provide a cellular or peripheral pathway by constructing a Pilus-Like structure and interacting with the VirB1 and VirB3 proteins to link the bacterium to the external environment and, via *Brucella*, to infect macrophages [11]. The VirB9 protein forms the members of the central T4SS portion and the material transfer pathway [12]. This dependence on VirB has been demonstrated both in animal models and in macrophage and Hela cells [13]. According to the above, *Brucella* VirB protein is widely recognized as an immunogenic antigen, and thus can be useful in generating diagnostic and vaccine therapies. Since the preparation of *Brucella*'s natural protein requires mass culture and protein purification, both production processes are very difficult and dangerous, and the level of proteins is not sufficient to purify its natural form. Further, the cloning molecular techniques of this pathway are simple and easily capable of producing high levels of protein. For this purpose, after designing the antigenic and accessible VirB2.3.9 epitopes in a construct, the recombinant protein produced was expressed and purified. According to our expectation, its molecular weight was 24 kDa and the expression yield was 15 mg/ml.

In this study, evaluation of the expressed protein with ELISA technique showed a sensitivity of 100% and a specificity of 97.5% as compared with the commercial results (vidal test). So far, several serological tests have been used to diagnose brucellosis. Since brucellosis is a disease with a long and variable latency period and can become chronic in different states, and due to its nonspecificity of clinical conditions as well as the failure of the culture and to the limited time of the presence of bacteria in the blood and colic, serologic tests play an important role in detecting this disease. Shrivastava *et al.* compared the results of ELISA tests with Wright, Rose Bengal, and supplemental elements, and stated that the sensitivity of other methods is more consistent with the results of the research. In their study, ELISA was 9.87% consistent with Rosebangal and Wright, and the sensitivity of the spot ELISA and Rosenblatt was calculated to be 97.7% and 97.94%, respectively [14]. Although the Wright test is considered as a low-sensitivity test, especially in chronic cases of the disease, and has many deficiencies, it is still used worldwide. In addition to specific antibodies produced by the disease and vaccination against brucellosis, this test also shows specific antibodies. Wright test in staged chronic conditions often results in a negative outcome. Yin *et al.* have established an indirect ELISA based on a polytope recombinant protein with 248 sera obtained from three different groups including patients with brucellosis (146 samples), non-brucellosis patients (82 samples), and healthy individuals (20 samples). Their results presented a sensitivity of

Table 1. Sensitivity and specificity of multi-epitope construct for diagnosis of brucellosis by ELISA.

	N	Mean	Std. Deviation	P-value[1]	P-value[2]
Study group	400	0.155	0.034		
Control	100	0.060	0.004	<0.001	
C-	400	0.078	0.014	<0.001	0.565

P-value[1]: comparison of Study group with Control and C-
P-value[2]: comparison between Control and C-

88.89% and a specificity of 85.54% with a cutoff value of 0.3865 [15]. In another study, Chachra *et al*. compared common tests and ELISA in the gut. Of the samples tested with Rosebangal, Wright, supplementation, indirect hemolysis, and spot ELISA, the highest sample point was positive and the sensitivity of spot ELISA was reported to be 7.5% higher than the rest [16]. Early diagnosis of Brucellosis can reduce unnecessary use of antimicrobial agents thereby reducing antibiotics resistance. Definitely, new technologies in recombinant production should be a target for diagnostic methods. In 2006, Ghorbanpoor *et al*. reviewed the microscopic and microbiological culture and ELISA for diagnosing Brucella in 94 clinical samples. In comparison with the culture, the sensitivity and specificity of the ELISA for diagnosis of brucellosis were 80% and 100% respectively, with 98.9% consistency between these two methods. They reported that ELISA testing can be a sensitive and rapid method for detecting Brucella in clinical specimens [17]. According to all of studies on rapid and good diagnostic performance and our suggestions, ELISA with Vir2,3 and 9 recombinant epitopes polypeptide is the easiest way to detect brucellosis, which has a high sensitivity and specificity, making it a useful tool in the diagnosis of human brucellosis.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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