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An evaluation of ELISA using recombinant *Brucella abortus* bacterioferritin (Bfr) for bovine brucellosis

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ABSTRACT

To date, detection of antibodies against the lipopolysaccharide portion is the backbone of most serodiagnostic methods for brucellosis screening. However this pose a risk for false positive reactions related to other pathogens especially that of *Yersinia enterocolitica* O:9 which has the most prominent cross reactivity with *Brucella* spp. In this study, cloning and expression of *Brucella abortus* bacterioferritin (Bfr) was accomplished by PCR amplification into an expression vector system, and purification of a recombinant *B. abortus* Bfr (rBfr). The immunogenicity of rBfr was confirmed by Western blot with *Brucella*-positive bovine serum. To determine whether rBfr has a potential benefit for use in the serodiagnosis of bovine brucellosis, rBfr-based ELISA was performed. Interestingly, rBfr was able to detect anti-*Brucella* antibodies in positive sera in a dependent manner of TAT values but did not show an immunoreaction with negative samples. Particularly, average OD492 values at the lowest, medium and highest TAT titer levels were 1.4, 2.2 and 2.6-fold increase compared with the cutoff value, respectively. The accuracy, specificity and sensitivity of rBfr showed 89.09%, 93.6% and 85.33%, respectively. These findings suggest that rBfr might be a good candidate for serological diagnosis development of bovine brucellosis.

1. Introduction

Brucella abortus is a Gram-negative cocco-bacilli that are known to be causative agents of brucellosis in animals and humans. They can cause chronic debilitating condition in humans, abortion and infertility in cattle and other animals, leading to severe economic losses as well as public health problems worldwide [1]. To date, there is no effective and safe vaccine for humans, thereby prevention of infection from animals is a predominant manner to control human brucellosis [2]. Different diagnostic approaches have been developed, primarily bacterial culture, serologic assays as well as DNA level detection. Among these approaches, serological assays are considered as the backbone method due to its diagnostic sensitivity, availability and economical advantage. The detection of antibodies against lipopolysaccharide is the principal element in the serodiagnosis of brucellosis. A major drawback however is cross-reactivity with other bacteria such as *Escherichia coli* O157, *Salmonella Typhimurium*, *Vibrio cholerae*, as well as *Yersinia enterocolitica* O:9 which has the most prominent cross reaction due to the high structural similarity of the O-polysaccharide (OPS) structures [3–7].

The development of immunoproteomics has paved the way for the identification of immunogenic proteins of several different pathogens and subsequent application of immunogenic proteins of *B. abortus* has proved to be an effective approach to minimize cross reactions in the diagnosis of brucellosis [6]. Therefore, several surface or cytoplasmic components of *Brucella* have been used and proven as potential markers for diagnosis of brucellosis including lumazine synthase [8,9], type IV secretion system protein VirB5 [10], outer membrane protein Omp28 [11], periplasmic immunogenic protein Bp26 [12].

In particular, bacterioferritin (Bfr), which contains a heme involved in cellular metabolism, was discovered to express strong antigenicity in cattle naturally infected with *B. abortus* S19 strain

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through utilizing (2-D) electrophoresis that allows the analysis of protein expression [7]. This previous study has provided sufficient data to consider the potential of this protein for serodiagnosis.

Diagnosis of brucellosis mainly relies on the detection of specific antibodies for both animals and humans [5,13]. Several serological methods have been evaluated worldwide such as Rose-Bengal test (RBT), serum agglutination test (TAT), enzyme-linked immunosorbent assay (ELISA), complement fixation test (CFT), and *Brucella* fluorescent antibody test (FAT) [4]. To date, the procedure using RBT as a screen test and TAT as a confirmation is considered to be the standard method for the serodiagnosis of brucellosis in Korea [14,15].

The fundamental goal of developing new diagnostic techniques is the ease of rapid detection and efficacy in the diagnosis of brucellosis. In this study, we report the results obtained with indirect ELISA using immunogenic proteins without cross reaction with *Y. enterocolitica* O:9 as a supplementary technique that could ensure diagnostic specificity and confirm diagnosis in animals that have been initially screened in reference to TAT.

2. Materials and methods

2.1. Bacterial strains and growth condition

A smooth, virulent *B. abortus* 544 biovar 1 strain was kindly provided by Animal, Plant and Fisheries Quarantine and Inspection Agency in Korea and *E. coli* DH5α was purchased from Invitrogen (USA). *B. abortus* was routinely cultured overnight in Brucella broth (BD Biosciences, USA) at 37 °C. Solid medium was made by supplementing Brucella broth with 1.5% (w/v) agar (Takara, Japan) as needed. *E. coli* DH5α was used for producing the necessary plasmid constructs. *E. coli* culture was grown at 37 °C in Luria-Bertani broth (LB) or agar supplemented with 100 μg/ml of ampicillin (Sigma, USA).

2.2. Plasmid preparation

The fully coded sequence of *B. abortus bfr* (bacterioferritin) gene was amplified by PCR using the following primer pair: 5′-AGC GGA TCC ATG AAA GGC GAA CCA AAG GTC-3′ (*Bam*HI site underlined) and 5′-ATC CTG CAG TTA CTC AGC TTC GTC GGC GGG-3′ (*Pst*I site underlined). PCR was performed using the following parameters: heating at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min; and a final elongation at 72 °C for 5 min. The amplified DNA was then digested with appropriate restriction enzymes *Bam*HI and *Pst*I (Takara, Japan) for 4 h at 37 °C and ligated to pCold TF vector (Takara, Japan) at 4 °C overnight, according to the manufacturer's instructions. The recombinant plasmid was transformed into *E. coli* DH5α.

2.3. Induction and purification of recombinant Bfr

The recombinant proteins were induced in LB supplemented with 100 μg/ml ampicillin at isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations of 0.4 mM at 15 °C for 24 h. Then *E. coli* was harvested by centrifugation at 3000 × *g* for 10 min, Histalon buffer set (Takara, Japan) was used for purification, according to the manufacturer's instructions.

2.4. SDS-PAGE and Western blot assays

The lysates of induced cells and the purified protein were identified by sodium dedocyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assay as previously described [16,17], in which the *B. abortus*-positive or -negative, and *Y. enterocolica* O:9 positive-cattle sera were used as primary antibodies. The bovine sera were sourced from a previous study in our laboratory [18], collected from experimentally infected native heifers.

2.5. TAT and indirect ELISA

Standard procedure for evaluation of brucellosis was as previously described [14,15]. Briefly, the bovine sera were collected from Korean native cattle, initially tested with Rose-Bengal test and stored at −70 °C. They were then further confirmed by TAT with sera diluted at 1:200, 1:100 and 1:50. To evaluate indirect ELISA, the immunoassay plates (SPL Life Sciences, USA) were coated with 50 μl of rBfr (6 μg/ml) in phosphate coating buffer (0.1 M, pH 9.6) and incubated overnight at 4 °C. Following 3 times of washing with 200 μl of 0.5% PBS-T, wells were blocked with 200 μl of blocking buffer (5% skim milk in PBS-T) at room temperature for 2 h. Plates were then washed twice with 200 μl of 0.5% PBS-T and charged with sera diluted at 1:200 in 100 μl of blocking buffer. After incubation at 4 °C overnight, the plates were washed 4 times with 200 μl of 0.05% PBS-T and incubated at room temperature for 2 h after adding 100 μl of HRP-conjugate protein G. Wells were washed 5 times with 200 μl of 0.05% PBS-T and added 100 μl of O-phenylenediamine (OPD). After 15 min incubation at room temperature, 50 μl of 3 M HCl and 3 M H_2SO_4 was added to stop reaction. The results were read at 492 nm by ELISA reader (BioRad, USA). A cutoff value was determined as twice of average mean of *B. abortus*-negative sera.

3. Results

3.1. Purification and immunoreactivity of rBfr

The recombinant plasmid pCold-*bfr* were constructed and successfully transformed into *E. coli* DH5α cells. Following induction in IPTG, rBfr was purified by Histalon buffer set. The molecular mass of purified rBfr was approximately 69.98 kDa in SDS-PAGE (Fig. 1A). The immunoreactivity of purified rBfr was evaluated by immunoblotting showing that purified rBfr strongly reacted with *Brucella*-positive cattle serum but did not react with *Brucella*-negative cattle serum (Fig. 1B). Incubation with *Y. enterocolica* O:9 positive-cattle serum also showed no reaction with rBfr (Fig. 1C).

3.2. ELISA

Based on TAT results, different numbers of *Brucella*-positive ($n = 150$) and -negative ($n = 125$) cattle sera were tested by using purified rBfr in indirect ELISA. As shown in Table 1, negative sera almost did not show strong reaction with rBfr protein, leading the cutoff value was 0.164 as determined of average OD492 value of negative samples. Meanwhile, rBfr was able to detect anti-*Brucella* antibodies in positive sera in a dependent manner relative to TAT values. Particularly, average OD492 values at the lowest, medium and highest TAT titer levels were 1.4, 2.2 and 2.6-fold increase compared with the cutoff value, respectively. In addition, analysis of accuracy, specificity and sensitivity showed 89.09%, 93.6% and 85.33%, respectively (Table 2).

4. Discussion

The purpose of this study was to evaluate the immunoreactivity of immunogenic rBfr of *B. abortus* in diagnostic tool for brucellosis through an indirect ELISA relative to the reference method TAT. Diagnosis of brucellosis is conventionally based on the detection of lipopolysaccharide fraction of either smooth lipopolysaccharide or whole cell based. The lipopolysaccharide fraction is known to induce a very strong antibody response however a major