Evaluation of recombinant AspC of *Brucella abortus* for serological diagnosis of bovine brucellosis in Korea

Lauren Togonon Arayan 1,#, Huynh Tan Hop 1,#, Alisha Wehdnesday Bernardo Reyes 1,2, Hannah Leah Tadeja Simborio 1, WonGi Min 1, Hu Jang Lee 1, Suk Kim 1†

1 Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju 660-701, Republic of Korea
2 Department of Veterinary Paraclinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, College, Laguna, Philippines 4031
# These authors contributed equally to this work

Abstract: To date, most serodiagnostic methods for brucellosis screening are based on antibodies against lipopolysaccharides of *Brucella* spp. However, this approach has the drawback of yielding false-positive results due to cross-reactivity with lipopolysaccharides of other related pathogens, especially *Yersinia enterocolitica* O:9. In this study, *Brucella abortus* AspC was cloned and expressed by PCR amplification into a pCold TF expression system to obtain recombinant AspC (rAspC). The immunogenicity of rAspC was confirmed by western blotting of *Brucella*-positive bovine serum. rAspC-based ELISA was performed to determine whether rAspC could be used in the serodiagnosis of bovine brucellosis. rAspC reacted strongly with anti-*Brucella* antibodies in positive sera in the tube agglutination test (TAT), but did not show strong reaction with most negative samples. In particular, the average OD 492 value at the highest TAT titer showed a 1.4-fold increase with respect to the cutoff value. The accuracy, specificity, and sensitivity of rAspC were 71.88%, 78.33%, and 68%, respectively. These findings suggest that rAspC might be valuable for the serological diagnosis of bovine brucellosis.

Key words: *Brucella abortus*, AspC, ELISA, serodiagnosis

INTRODUCTION

*Brucella abortus* is a Gram-negative coco-bacillus that is known to cause brucellosis in animals and humans. Brucellosis is a chronic debilitating condition in humans, and it causes abortion and infertility in cattle and other animals, leading to severe economic losses as well as public health problems worldwide [16]. To date, there is no effective and safe vaccine for humans. Therefore, the prevention of animal infection is the predominant approach to control human brucellosis [18]. However, current diagnostic protocols for brucellosis in both animals and humans have only a limited effectiveness due to many reasons. Cross-reactivity of the diagnostic tools and overlaps in the clinical profile with other pathogens are the major complications in the diagnosis of brucellosis [19]. Typically, serodiagnostic methods for brucellosis screening rely on the detection of antibodies against the lipopolysaccharide (LPS) fraction of *Brucella* spp. However, this poses a risk for false positive reactions with the LPSs of other pathogens, especially with *Yersinia enterocolitica* O:9 which has an OPS, 1,2-linked 4,6-dideoxy-4-formamido-a-D-mannopyranosyl (u-petrosamine) that is identical to that of *Brucella* spp.[13,14,17,20].

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 as potential markers for diagnosis of brucellosis including lumazine synthase [18,19], type IV secretion system protein VirB5 [13], outer membrane protein Omp28 [14], and periplasmic immunogenic protein Bp26 [17].

In particular, AspC was found to be immunogenic in a study using a 2-DE method in *B. abortus* 2308 infected cattle, which allows the analysis of protein expression (unpublished data). This previous study provided sufficient data to consider the application of this protein for
serodiagnosis. Diagnosis of brucellosis mainly relies on the detection of specific antibodies for both animals and humans. Bacterial isolation is considered the gold standard; however, because bacteria grow slowly, the process is very time consuming. In addition, blood and tissue samples typically show a low rate of sensitivity [15] and isolation of the bacteria could be a potential source of infection for laboratory personnel [19]. The tube agglutination test (TAT) is the standard method for the serodiagnosis of brucellosis. Along with this, other screening tests have also been developed; however, drawbacks with regards to deviations in antigens and respective reactivity, as well as differences in incubation time. TAT is particularly laborious and time-consuming, and is therefore definitely not convenient as a primary screening tool for large samples. Other widely accepted tests include the slide agglutination test, microagglutination test, and the card agglutination test, but these would still require standardization against TAT [12]. The fundamental goal of developing new diagnostic techniques for the diagnosis of brucellosis is to allow for rapid detection at high efficiency. In this study, we report the results obtained for an indirect ELISA using an immunogenic protein showing no cross-reactivity with Yersinia enterocolitica that can be used as a supplementary technique to ensure diagnostic specificity and confirm diagnosis in animals that have been initially screened using TAT.

MATERIALS AND METHODS

Bacterial strains and growth condition

A smooth, virulent B. abortus 544 biovar 1 strain was kindly provided by the Animal, Plant and Fisheries Quarantine and Inspection Agency in Korea, while Escherichia coli DH5α cells were 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 cells were used to produce the necessary plasmid constructs. E. coli cultures were grown at 37°C in LB broth or agar supplemented with 100 µg/ml ampicillin (Sigma, USA).

Plasmid preparation

The fully coded sequence of B. abortus AspC (aspartate aminotransferase) was amplified by PCR using the following primer pair: 5′-AGC GGA TCC ATG GCA TTT CTC GCC GAC GCC-3′ and 5′-ATC CTG CAG TCA GCG CAG GCT GGC GCA GAA-3′ (BamHI and PstI restriction site underlined). Then, the amplified DNA was digested with 2 restriction enzymes (BamHI and PstI, Takara, Japan) and ligated into a pcold vector. The recombinant plasmid was used to transform E. coli DH5α. Positive clones were selected.

Induction and purification of recombinant AspC (rAspC)

Recombinant protein expression was induced by adding 1 mM IPTG to DH5α cells grown in LB broth supplemented with 100 µg/ml ampicillin at 15°C for 24 h. Then, the E. coli cell pellet was harvested by centrifugation at 3,000×g for 10 min. The Histalon buffer set (Takara) was used for purification, according to the manufacturer’s instructions.

SDS-PAGE and western blot assays

The lysates of induced cells and the purified protein were identified after separation via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by western blotting as previously described [9], in which the B. abortus-negative, Brucella-positive and Y. enterocolitica positive-cattle sera were used as primary antibodies.

TAT

The bovine sera were collected from Korean native cattle and stored at -70°C. They were then primarily differentiated by TAT of sera diluted at 1:400, 1:200, and 1:100 [1].

Indirect ELISA

Immunoassay plates (Maxibinding, SPL Life Sciences) were coated with 50 µl rAspC (4 µg/ml), in phosphate coating buffer (0.1 M, pH 9.6) and incubated overnight at 4°C. Following 3 washes with 200 µl 0.5% PBS-T, wells were blocked with 200 µl blocking buffer (5% skim milk in PBS-T) at room temperature for 2 h. Plates were then washed twice with 200 µl 0.5% PBS-T and charged with sera diluted at 1:200 in 100 µl blocking buffer. After incubation at 4°C overnight, the plates were washed 4 times with 200 µl 0.05% PBS-T and incubated at room temperature for 2 h after addition 100 µl HRP-conjugated protein G. Wells were washed 5 times with 200 µl 0.05%
Table 1. Evaluation of diagnostic values of rAspC antigen based enzyme-linked immunosorbent assay (ELISA) compared to a standard tube agglutination test (TAT)

<table>
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<th>TAT positive (n=100)</th>
<th>TAT negative (n=60)</th>
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<tbody>
<tr>
<td>ELISA Positive</td>
<td>68</td>
<td>13</td>
</tr>
<tr>
<td>ELISA Negative</td>
<td>32</td>
<td>47</td>
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Sensitivity=(68/100)*100=68%; Specificity=(47/60)*100=78.33%; Accuracy=(115/160)=71.88%.

PBS-T and 100 µl 0-phenylenediamine (OPD) was added to each well. After an incubation of 15 min at room temperature, 50 µl 3 M HCl and 3 M H₂SO₄ was added to stop the reaction. The results were read at 492 nm using an ELISA reader (BioTek, Seoul, Korea). A cutoff value was set at twice the average mean of negative sera.

**Statistical analysis**

The results of each experiment are expressed as the mean ± SD. One-way ANOVA was used to make statistical comparisons between the groups. Results with $p < 0.05$ were considered significant.

**RESULTS**

**Purification and immunoreactivity of rAspC**

*E. coli* DH5α cells were transformed with the pCold-rAspC plasmid. Following induction with IPTG, rAspC was expressed and subsequently purified using the Histalon buffer set. The molecular mass of purified rAspC was approximately 96.4 kDa based on protein separation via SDS-PAGE. The immunoreactivity of purified rAspC was evaluated by immunoblotting. Purified rAspC reacted strongly with *Brucella*-positive cattle serum but did not react with *Brucella*-negative cattle serum (Fig. 1). Incubation with *Yersinia enterocolitica* O:9 positive-cattle serum also showed no reaction with rAspC (data not shown).

**ELISA**

Based on our TAT results, different numbers of *Brucella*-positive (n = 100) and negative (n = 60) cattle sera were tested by using purified rAspC in indirect ELISA. Negative sera showed almost no reaction with the rAspC protein, leading to a cutoff value of 0.204, which is the average OD₄₉₂ value of all negative samples (Fig. 2). Meanwhile, rAspC showed an 1.4-fold increased detection rate of anti-*Brucella* antibodies in positive sera compared with the cutoff value at the highest TAT titer value (Table 1). Furthermore, analysis of accuracy, specificity, and sensitivity showed 71.88%, 78.33%, and 68%, respectively,

![Fig. 1. Immunoreactivity of B. abortus recombinant AspC (rAspC). Maker (lane 1), lysate proteins of pCold-TF transformed cells (lane 2) and purified rAspC (lane 3) separated by 10% SDS-PAGE. Immunogenicity of rAspC were determined by WB using Brucella-negative (lane 4), Brucella-positive (lane 5) bovine sera. rAspC (arrows) and pCold TF protein (arrowheads) are indicated.](image1)

![Fig. 2. ELISA absorbance values of bovine sera using 4 µg ml⁻¹ of rAspC. Immunoassay plates were charged with sera at dilution of 1:200.](image2)
suggesting that rAspC has an average efficacy (Table 1).

**DISCUSSION**

The purpose of this study was to evaluate the immunoreactivity of immunogenic rAspC of *B. abortus* in detecting brucellosis through an indirect ELISA compared to the reference method TAT. Diagnosis of brucellosis is conventionally based on the detection of either the smooth or total LPS fractions of *Brucella* spp. LPSs are known to induce a very strong antibody response; however, a major drawback to this method is possible cross-reactivity with other Gram negative pathogens, especially *Yersinia enterocolitica* 0:9 and *Salmonella typhimurium* [4]. Thus, to circumvent the problem of cross-reactivity, a non-LPS candidate antigen could be used in the diagnostic methods for brucellosis [2]. AspC is an aminotransferase that is responsible for the synthesis of aspartate. An AspC mutant of *B. abortus*, obtained from a mini Tn5 Km2 transposon, could no longer replicate in HeLa cells [5]. In *E. coli*, AspC was found to affect the cell cycle coordination at the level of chromosome replication and cell division. Deregulation of the AspC-mediated aspartate metabolism affects the levels of initiator protein DnaA as well as of the division signal UDP-glucose. The absence of AspC leads to the production of small cells with slower growth rates, and less origins per cell. In contrast, abundant amounts of AspC lead to bigger cells with faster growth rates and more origins per cell [10]. Because of its sequence conservation, AspC is expected to exert essentially comparable functions in other organisms [10]. AspC was previously found to be immunogenic in a study using a 2-DE method in *B. abortus* 2308 infected cattle (unpublished data). These earlier studies validate rAspC as a potential candidate for the serodiagnosis of brucellosis. The use of a single antigen for serodiagnosis minimizes interference with other proteins, but one major potential disadvantage is a lower sensitivity because in some cases, a certain antibody population may not be able to recognize a particular antigen [3]. Interestingly, our data show that application of purified rAspC for ELISA could give average accuracy, sensitivity, and specificity (Table 1), suggesting that the AspC protein represents a valuable target for serodiagnosis of brucellosis.

Antigen-antibody interactions are complex and depend on a variety of factors. Antibody responses are generally variable and can be different depending on the specific stage of infection [11]. This variation is critical in the serodiagnosis of brucellosis; however, information on this correlation is very limited. A previous study conducted by Lee et al. provides more insight by identifying specific immunogenic proteins at a given course of infection in *B. abortus* challenged mice using MALDI-TOF MS [7].

In the current study, infected cattle are not clinically evaluated as to when the infection started or as to what clinical signs are being manifested. Therefore, they could be in any stage of infection. It is possible that most of the cattle sera highly reactive to AspC protein are at a certain stage of infection at which this particular antigen is highly expressed, leading to a suboptimal efficacy. In a next phase, we suggest to test infection stage-specific sera to determine the AspC expression profile during infection. Previous work on *Brucella* immunoreactive antigens has demonstrated that the efficacy can be maximized by combining more than one immunoreactive antigen [8]. As such, the benefits of an individual antigen with high sensitivity can be combined with those of another antigen with a high specificity. Additionally, combinations can target antigens with variable responses in each stage of infection and as such, can improve the overall detection rate of infected animals [3]. Thus, we suggest rAspC as a useful target, which can be combined with other immunoreactive proteins in further studies to improve the detection methods for brucellosis.

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**REFERENCES**


