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Recombinant Ohr Protein of *Brucella abortus* as a Potential Serological Marker for Bovine Brucellosis Diagnosis

Hop H[1], Reyes AWB[1], Simborio HL[1], Arayan LT[1], Huy TXN[1], Min WG[1], Lee HJ[1], Lee JJ[2], Chang HH[3] and Kim S[1,3]*

[1]*Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Republic of Korea*

[2]*Animal and Plant Quarantine Agency, Anyang, Gyeonggi-do, 430-757, Republic of Korea*

[3]*Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, 660-701, Republic of Korea*

Abstract

To date, detection and segregation of infected animals still be a predominant manner to control brucellosis. However, most of current serodiagnostic tools is problematic because detection of antibodies against the lipopolysaccharide portion pose a risk for false positive reactions related to other pathogens especially that of *Yersinia enterocolitica* 0:9, and difficult to discriminate vaccinated animals and humans from those naturally infected. In this study, we evaluated an immunogenic protein, organic hydroperoxide resistance protein (rOhr) of *B.abortus* as an alternative to LPS. To determine whether rOhr has a potential benefit for use in the serodiagnosis of bovine brucellosis, rOhr-based ELISA was performed and the results were compared with those of tube agglutination test (TAT). In total of 232 samples, rOhr was able to detect anti-Brucella antibodies in positive sera in a dependent manner of TAT values but did not show strongly reaction with most of negative samples. Particularly, the sensitivity, specificity and accuracy of rOhr showed 92.37%, 89.47% and 90.95%, respectively. These findings are very promising and suggest that rOhr might be useful for bovine brucellosis diagnosis.

Keywords: *B.abortus*; rOhr; Serodiagnosis; ELISA

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Correspondence:
Suk Kim, Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, 3Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, 660-701, Republic of Korea.
Tel: *+82–55–772–2359*
E-mail: *kimsuk@gnu.ac.kr*

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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 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]. Vaccination seems to be a predominant manner to eliminate this disease, however, there is no 100% efficacious vaccine for animals and humans.

Hence, detection and segregation of infected animals are most important for controlling brucellosis [2].

Nowadays, most of the serodiagnostic procedures for brucellosis such as rose Bengal plate test, tube agglutination test, ELISA are mainly based on detection of antibodies against LPS that pose a risk for false positive reactions related to other pathogens especially that of *Yersinia enterocolitica* 0:9 which has the most prominent cross reactivity with *Brucella* spp. Furthermore, they are also very difficult to distinguish naturally infected from animals vaccinated with *B.abortus* S19 which is being used to immunize cattle [3-6]. The development of immunoproteomics has paved the way for the identification of immunogenic proteins of *B. abortus* and subsequent application of them will minimize cross reactions in the diagnosis of brucellosis [7]. Thus, several surface or cytoplasmic components of *Brucella* have been used and proven as potential markers for diagnosis of brucellosis including lumazine synthase [8,9], BP26 [10], type IV secretion system protein VirB5 [11], VirB12 [12], outer membrane protein Omp28 [13,14], combination of Omp10, Omp28 and Omp31.

On the other hand, the organic hydroperoxide resistance (Ohr) protein of *B.abortus* which belongs to a family of peroxiredoxins was found to be an immunoreactive protein that strongly reacted with *B.abortus*-positive bovine sera [15]. In addition, the importance of this protein for virulence both in macrophages and mice has been reported in *Francisella,* which further implies that its importance for pathogenesis is conserved in multiple *Francisella* species [16]. These previous studies have provided sufficient data to consider its potential for serodiagnosis. Thus, here we report

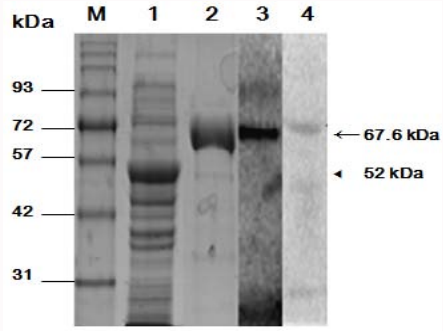

Figure 1: Immunoreactivity of *B. abortus* recombinant Ohr (rOhr). Lysate proteins of pCold TF transformed cells (lane 1) and purified rOhr (lane 2) separated by 10% SDS-PAGE. Immunogenicity of rOhr was determined by WB using *Brucella*-positive (lane 3) or *Brucella*-negative (lane 4) bovine sera. rOhr (arrows) and pCold TF protein (arrowheads) are indicated. M: Marker.

Table 1: Evaluation of diagnostic values of rOhr antigen based enzyme-linked immunosorbent assay (ELISA) compared to a standard tube agglutination test (TAT).

		TAT positive	TAT negative
		(n=118)	(n=114)
ELISA	Positive	109	12
	Negative	9	102

Sensitivity = (109/118)*100 = 92.37 %; Specificity = (102/114)*100 = 89.47%; Accuracy = (211/232) = 90.95%.

the results obtained with indirect ELISA using immunogenic proteins without cross reaction with *Yersinia enterocolitica* as a supplementary technique that could ensure diagnostic specificity and confirm diagnosis in animals that have been initially screened in reference to TAT.

Materials and Methods

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 *Escherichia (E.) 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* culture was grown at 37°C in LB broth or agar supplemented with 100μg/mL of ampicillin (Sigma, USA).

Protein preparation

The open reading frame of *ohr* gene of *B.abortus* was amplified by PCR and then cloned to pCold TF vector. The recombinant proteins were induced in LB broth supplemented with 100μg/ml ampicillin at IPTG concentrations of 0.2mM at 15°C for 24 hours and Histalon buffer set (Takara) was subsequently 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 by Sodium dedocyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assay as previously described [14,17] in which the *B.abortus*-negative, *Brucella*-positive and *Y.enterocolica* positive-cattle sera were used as primary antibodies.

Tube agglutination test (TAT)

The bovine sera were collected from Korean native cattles and stored at -70°C. They were then primarily differentiated by TAT [18] with sera diluted at 1: 400, 1:200 and 1:100.

Indirect ELISA

The immunoassay plates (Maxibinding, SPL Life Sciences) were coated with 50μl of rOhr (10μg/ml) in phosphate coating buffer (0.1M, pH 9.6) and incubated overnight at 4°C. Following 3 times of washing with 200μl 0.5% PBS-T, wells were blocked with 200μl blocking buffer (5% skim milk in PBS-T) at room temperature for 2 hours. Plates were then washed twice with 200μl 0.5% PBS-T and charged with sera diluted at 1:200 in 100μl blocking buffer. After incubation at 4°C overnight, the plates were washed 4 times with 200μl 0.05% PBS-T and incubated at room temperature for 2 hours after adding 100μl HRP-conjugate protein G. Wells were washed 5 times with 200μl 0.05% PBS-T and added 100μl O-phenylenediamine (OPD). After 15min. at room temperature of incubation, 50μl 3M HCl and 3M H_2SO_4 was added to stop reaction. The results were read at 492nm by ELISA reader (BioTek, Seoul, Korea). A cutoff value was determined as twice of average mean of negative sera.

Statistical analysis

The results of each of the 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 significantly different.

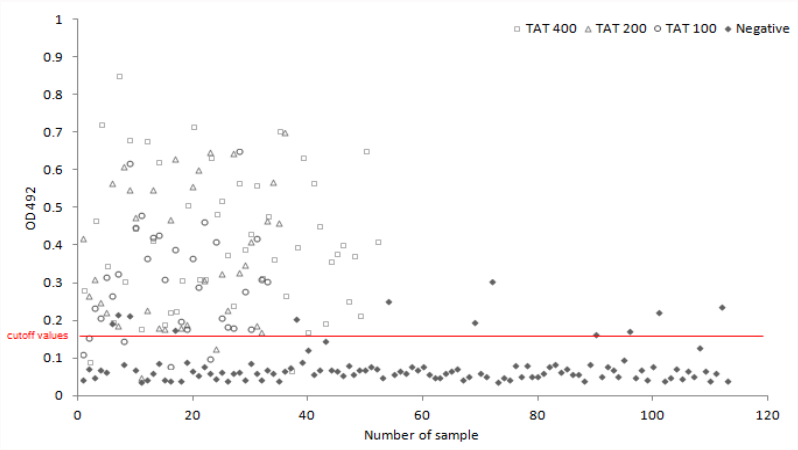

Figure 2: ELISA absorbance values of bovine sera using 10 μg/ml of rOhr. Immunoassay plates were charged with sera at dilution of 1:200.

Results

Expression and immunoreactivity of rOhr

Following transformation of the expression vector pCold TF-*ohr* into *E.coli* DH5α cells, IPTG and Histalon buffer set were used to induce and pufiry Ohr protein, respectively. The molecular mass of purified rOhr protein was approximately 67.6kDa in SDS-PAGE. The immunoreactivity of purified rOhr was evaluated by immunoblotting showing that purified rOhr strongly reacted with Brucella-positive cattle serum but did not react with *Brucella*-negative cattle serum (Figure 1).

Incubation with *Yersinia enterocolica* positive-cattle serum also showed no reaction with rOhr (data not shown).

Agglutination test of sera

The total of 232 clinical serum samples were primarily assessed by tube agglutination test (TAT) that showed 50 samples were positive at 1:400, 36 samples at 1:200 and 32 samples at 1:100 dilution. The remaining 114 samples were negative.

ELISA

Based on TAT results, different numbers of *Brucella*-positive (n=118) and -negative (n=114) cattle sera were tested by using purified rOhr in indirect ELISA. As shown in Figure 2, negative sera almost did not show strong reaction with rOhr protein, leading the cutoff value was 0.168 as determined of average OD_{492} value of negative samples. Meanwhile, rOhr was able to detect anti-*Brucella* antibodies in positive sera in a dependent manner of TAT values. Particularly, average OD492 values at the lowest, medium and highest TAT titer levels were 1.7, 2.28 and 2.45-fold increase compared with the cutoff value, respectively (Figure 3). Futhermore, analysis of sensitivity, specificity and accuracy showed 92.37%, 89.47% and 90.95%, respectively, suggesting a high efficacy of rOhr (Table 1).

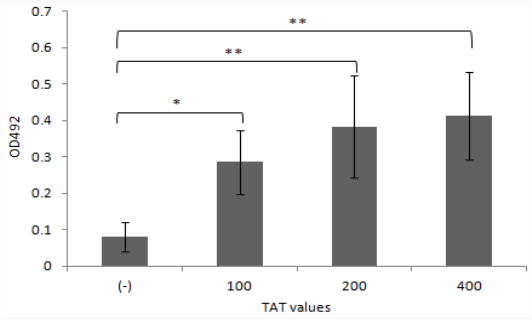

Figure 3: The average absorbance values of bovine sera using rOhr antigen based enzyme-linked immunosorbent assay (ELISA).

Discussion

Vaccination continues to be the most successful procedure for preventing losses in animal due to infectious diseases, however, 100% efficacious vaccine for animal and human brucellosis is nonexistence, resulting in the most importance of serodiagnosis in brucellosis controlling [2].

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 drawback to this would be cross reactivity with other Gram negative pathogens specially that of *Yersinia enterocolitica* 0:9, *Salmonella typhimurium* and many others [19]. Thus there is always the challenge of investigating on a non-LPS candidate antigen for the diagnosis of brucellosis [13].

On the other hand, the organic hydroperoxide resistance (Ohr) protein of *B.abortus*, which belongs to a family of peroxiredoxins has been found to be immunogenicity in bovine brucellosis and likewise, characterized as organic peroxide detoxifier and identified in several bacteria [15,20]. Morever, virulence of *Francisella* Ohr was also reported by Llewellyn *et al.* These earlier studies based on immunoproteomic analyses suggest the potential of immunogenic *B.abortus* Ohr for serodiagnosis of brucellosis. Thus, we particularly evaluated the immunoreactivity of immunogenic rOhr of *B. abortus* in detecting brucellosis through an indirect ELISA relative to the reference method TAT.

The use of single antigen offers no interference with other proteins but one major disadvantage is a likely lower sensitivity because in some cases, a certain population may not be able to recognize a particular antigen [21]. Interestingly, our data showed that application of purified rOhr for ELISA could give high accuracy, sensitivity and specificity (Table 1).

Antigen-antibody interactions involve complex factors. Antibody responses are generally variable and there is no solid evidence of a constant reactivity considering the particular antigen and the specific stage of infection [22]. Thus, this variation in immune responses to specific antigens at different stages of infection is a critical aspect to consider in the serodiagnosis of brucellosis. However there is limited knowledge to this correlation. A previous study conducted by Lee, provides an insight in identifying specific immunogenic proteins present at a given course of infection in a *B. abortus* challenged mice utilizing the MALDI-TOF MS [23]. In the current study however, infected cattle are not clinically evaluated as to when the infection started and as to what clinical signs are being manifested thus the possibility as to what stage of infection they are in could be random. It is likely that most of the cattle sera highly reactive to Ohr protein are at a certain stage of infection at which this particular antigen is expressed. Thus the need to establish this correlation is highly relevant. Previous studies have suggested that efficacy can be maximized by combining more than one immunoreactive antigen. This advantage has been demonstrated in *Brucella* immunoreactive antigens [24]. This is done for the purpose of adding up the potential of an individual antigen with high sensitivity with another antigen with a high specificity. Another reason for doing so is the possibility of covering the variable expanse of protein antibody response expressed by species of interest at whatsoever stage of infection they are in [21]. By doing so, there is a higher possibility of detecting infected animals. Thus, we suggest combining rOhr with other immunoreactive proteins in further study.

Acknowledgments

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