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Evaluation of the combined use of the recombinant *Brucella abortus* Omp10, Omp19 and Omp28 proteins for the clinical diagnosis of bovine brucellosis

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ABSTRACT

Currently, there are several serodiagnostic tools available for brucellosis, however, it is difficult to differentiate an active infection from vaccination. Hence, there is a great need to develop alternative means that can distinguish between these two conditions without utilizing lipopolysaccharide (LPS). This study was an attempt to determine the efficacy of combined recombinant *Brucella* (*B.*) *abortus* outer membrane proteins (rOmps) and individual rOmps in the serodiagnosis of brucellosis by enzyme linked immunosorbent assay (ELISA), utilizing both that standard tube agglutination test (TAT)-positive and -negative serum samples from Korean native cattle. The results are very interesting and promising because the combined rOmp antigens used in the study were highly reactive with the TAT-positive serum samples. The combined rOmps sensitivity, specificity and accuracy were 215/232 (92.67%), 294/298 (98.66%) and 509/530 (96.04%), respectively. While these results are preliminary, the tests performed have very high potential in the serodiagnosis of brucellosis and likewise, the combined rOmps can be used for future vaccine production.

1. Introduction

Brucellosis is a zoonotic disease that occurs worldwide and is caused by bacteria of the genus *Brucella*, which are Gram-negative, non-spore-forming and nonencapsulated coccobaccilli or short rods with rounded ends [1,2]. Brucellosis is transmitted from domestic animals to humans and is endemic in many areas [3]. The organism mainly affects the reproductive system of domesticated animals, causing abortion and infertility, which in turn can cause a serious economic crisis [4].

The definitive diagnosis of brucellosis is isolation and identification of the causative organism [5]. However, diagnosis and disease control are generally based on serological testing. The Rose Bengal plate test and the complement fixation test are the most acceptable diagnostic methods worldwide [6]; however, Korea utilizes the standard tube agglutination test (TAT) as the main serodiagnostic tool for brucellosis [7]. At present, many developed countries have declared that brucellosis is eradicated and no longer present in their countries [3]. Eradication of the disease can be attained through accurate diagnosis. However, current serological tools available are based on lipopolysaccharide (LPS) components of the organism that cannot distinguish naturally-infected from vaccinated animals with *Brucella abortus* S19. Therefore, new serological diagnostic tools that not employ LPS are needed.

Lindler et al. reported that one non-LPS group of immunogens focused on vaccine and diagnostic purposes is the outer membrane protein (Omp) [8]. The major mechanisms of Omp-mediated bacterial adaptive responses to the host environment include iron uptake, antimicrobial peptide resistance, serum resistance, multidrug resistance and bile resistance, among others [9]. However, the

Abbreviations: OMP, outer membrane protein; TAT, standard tube agglutination test; ELISA, enzyme linked immunosorbent assay.

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role of Omp in the survival and virulence during infection remains unknown [10].

Omp28 (BruAb1_1470) is a conserved protein present in 4 or more different *Brucella* species and is well studied both as a vaccine candidate and as an antigen for serodiagnosis [7,11]. Omp19 (BruAb1_1906) and Omp10 (BruAb2_0077) have not been considered as possible antigens for serodiagnosis, but both have been observed in few *Brucella* species [12]. Omp10 is also observed in *B. abortus*, *Brucella melitensis* and *Brucella suis*, while Omp19 is present in all three species including *Brucella microti*. However, Omp10 and Omp19 have no homologs in the sequence database [13]. Consequently, researchers interested in alternative antigens have focused on the outer and inner membrane proteins as well as the cytoplasmic and ribosomal proteins, which have characteristics that make them useful in diagnostic tests for brucellosis [14]. The purpose of this study is to elucidate the individual Omps (Omp10, Omp19 and Omp28) and their associated combinations for a more sensitive and specific serodiagnostic tool for bovine brucellosis using enzyme linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study were *B. abortus* 544 (ATCC23448), a smooth virulent biovar 1 strain, and *Escherichia* (*E.*) *coli* DH5α. *B. abortus* and *E. coli* were cultured in Brucella broth or Luria Bertani (LB) broth (Becton, MD, USA), respectively, overnight at 37 °C in a gyrating shaker. The above media were supplemented with 1.5% (w/v) agar and ampicillin (100 μg/ml) when necessary.

2.2. rOmp expression

Total genomic DNA was prepared from *B. abortus*. Initially, *B. abortus* was cultured in Brucella broth overnight with shaking incubation, and 5 ml of the culture was collected and lysed using the Dokdo-Prep Bacterial Genomic DNA Purification Kit (Elpis Biotech, South Korea). The *B. abortus* Omp10 coding sequence was amplified by PCR with the following primers: 5′-AGCA GAATTC ATGAAACGCTTCCGCA-3′ (*Eco*RI) and 5′-ATTA CTGCAG TCAGCCGGCGTTGC-3′ (*Pst*I). Omp19 was amplified with the following primers: 5′-AGCA GGATCC ATGGGAATTTCAAAAGCAAG-3′ (*Bam*HI) and 5′-ATA CTGCAG TCAGCGCGACAGCG-3′ (*Pst*I). The amplified DNA was digested with the appropriate restriction enzymes and ligated into the pCold vector (Takara, Japan). The ligated product was then used to transform the expression host, *E. coli* DH5α. The sequence of the cloned product was confirmed and found to completely match that of the reported sequence of the Omp10 and Omp19 for *B. abortus* (Genbank NC_006933.1 and NC_006932.1). At the exponential phase of the confirmed rOmp clone cultures, a sample of each culture was spread onto an LB agar plate containing isopropyl-thio-1-D-galactopyranoside (IPTG, 0.5 mM) and ampicillin (100 μg/ml). rOmp28 was already constructed from previous studies conducted in our laboratory [7].

2.3. Purification of rOmp

One liter of LB broth containing ampicillin was inoculated with 10 ml of an overnight bacteria culture containing the fusion plasmid. IPTG was then added to a final concentration of 0.1–0.5 mM, and the culture was further incubated at 15 °C for 8 h. Bacterial cells were harvested by centrifugation at 4400 × *g* for 20 min. The supernatant was then discarded, and the recombinant proteins were purified using the HisTALON™ Gravity Columns Purification Kit (Takara, Japan) according to the manufacturer's instructions. The purified recombinant proteins were stored at −20 °C after a modified Bradford protein assay (Bio-Rad, USA).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using previously described methods [7]. The purified rOmps were diluted with Laemmli sample buffer and boiled for 5 min. Two gels were simultaneously ran, one for Coomassie brilliant blue staining and the other for immunoblotting. After electrophoresis, samples were transferred to Immobilon-P transfer membranes (Millipore, USA) at 2 mA/cm^2 constant current for 1 h using a semi-dry electro blot containing transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membrane was blocked with 5% skim milk in Tris-based saline containing 0.1% Tween-20 (TBS-T) for 30 min at room temperature (RT), washed three times with TBS-T and incubated with *B. abortus*-positive (TAT:400, 1:1000 dilution) or *Y. enterocolitica* O:9-positive (1:1000) sera overnight at 4 °C. The standard *B. abortus*-positive and *Y. enterocolitica* O:9-positive sera were kindly provided by the Animal and Plant Quarantine Agency, Korea. The membrane was washed with TBS-T three times and incubated with horseradish peroxidase (HRP)-labeled protein G (Thermo Scientific, USA; 1:5000 dilution) for 2 h at RT. Finally, the membrane was washed with TBS-T three times, chemiluminescent detection agent (ECL, Japan) was added and immunoreactive protein bands were visualized using a ChemiDoc XRS camera equipped with Quantity Analysis Software (Bio-Rad Laboratories, USA).

2.4. Agglutination test

The bovine sera samples were collected from 530 native Korean cattle from 2006 to 2014. *B. abortus*-positive and -negative sera were selected by Brucella Serum Agglutination Test Antigen, a test currently used and approved by the National Veterinary Research and Quarantine Service (NVRQS) and OIE, Korea. All sera were stored at −70 °C until use.

2.5. ELISA

Clinical bovine sera samples were analyzed by ELISA using the rOmps individually and in combination. The immunoassay plates (Nunc, USA) were coated with 50 μL of antigen (3–9 μg/ml) diluted in carbonated coating buffer (0.1 M, pH 9.6) and incubated overnight at 4 °C. The wells were washed with 0.05% PBS Tween-20 (PBS-T) and blocked in blocking buffer for 1 h at RT. Plates were charged with sera diluted at 1:50–200 and incubated overnight at 4 °C. After washing 3 times, HRP-labeled protein G (Santa Cruz Biotech, USA; 1:5000 dilutions) was added and the plates were further incubated at 37 °C for 2 h. Finally, the plates were washed three times, and the substrate O-phenylenediamin was added. The absorbance was measured at 492 nm using an ELISA reader (Bio Rad, USA). A cutoff value to distinguish between positive and negative samples was calculated using the mean optical density (OD) of *B. abortus*-negative sera multiplied by two. Two sets of ELISAs were designed; one compared single rOmps (3 μg/ml) with combined rOmp (1:1:1 μg/ml) and a second compared combined rOmps at different concentrations (1:1:1, 2:2:2, 3:3:3 μg/ml).

2.6. Statistical analysis

The results obtained were expressed as the mean ± SD for each experiment. The Student's *t*-test was used to make a statistical comparison between the positive and negative serum samples. Comparisons between single and combined rOmps at different concentrations were calculated by ANOVA and post-hoc *t*-test with the Bonferroni correction method. The results with $P < 0.05$ were considered statistically significant.