Development and Evaluation of a Rapid Dipstick Assay for Serodiagnosis of Bovine Brucellosis

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ABSTRACT

Fast, cheap and sufficient serodiagnostic tools need to be developed for the early detection of brucellosis. Currently the tools cannot differentiate an active infection from vaccinated, nor can it differentiate other bacterial infections with lipopolysaccharides, especially Yersinia infections. In this study, we purified recombinant outer membrane protein 10 and 28 (rOmp10, rOmp28), and a dipstick assay (indirect or sandwich) was constructed with single (rOmp10 or rOmp28) and combined rOmps (rOmp10 and rOmp28) from Brucella (B.) abortus 544 to evaluate bovine Brucella positive serum collected during the beginning of the Korean outbreak from 2006 to 2015. In application with single rOmp, rOmp10 (70%; indirect, 92.11%; sandwich dipstick) and rOmp28 (72.5%; indirect, 86.84%; sandwich dipstick) had comparable results. In addition, results indicated that dipstick with combined rOmps (rOmp10 and rOmp28) were superior in detecting positive serum samples, at 85% indirect and 100% sandwich dipstick. Surprisingly, the results were the same in detecting negative results at 97.78% for both single and combined indirect dipsicks. The dipstick tools with rOmp10 and rOmp28 would be useful for a rapid screen method for bovine brucellosis.

Key words - Brucella abortus, Diagnosis, Portable, Rapid dipstick, Serology

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Introduction

Brucellosis is a zoonotic disease occurs worldwide and affects widest range of animals, both domestic and wild animals, and humans(Acha & Szyfres, 2003). *Brucella* spp. are Gram negative, nonspore-forming, nonflagellar and non-encapsulaed coccobaccili or short rods with rounded ends(Lubroth et al., 2007). In domestic animals, the organism mainly affects the reproductive system causing abortion, infertility and serious economic crisis(Delpino et al., 2007). Transmission for domestic animal to humans in endemic areas caused this disease to be an occupational disease targeting farmers, stockyard and slaughter house workers, butchers, and veterinarians (Corbel, 1997; Acha & Szyfres, 2003).

Clinical diagnosis of brucellosis must be based on symptoms, history and confirmed laboratory tests through the isolation and typing of the casual agents(Acha & Szyfres, 2003). Klaus Nielsen(Nielsen, 2002) has noted many possible serological tests for the diagnosis of brucellosis and OIE Terrestrial Manual 2012 specified that no single serological test is appropriate in all epidemiological situations. Republic of Korea’s eradication program was detection through regular serological herd testing, laboratory confirmation with tube agglutination test (National Veterinary Research and Quarantine Service NVRQS and OIE, Korea) and elimination by rapid removal of infected animals(partial herd depopulation), trade restrictions and increased surveillance of animals both on the infected and farms with confirmed or suspected epidemiological linkages(Wee et al., 2008; Lee et al., 2009). The current serological tools available are based on lipopolysaccharide(LPS) components of the organism that cannot distinguish naturally-infected from vaccinated animals with strain 19(Simborio et al., 2015). Tests are also known to cross react most commonly with *Tersina enterocolita* O:9(Weynants et al., 1996) and less commonly with *Salmonella*, *Afipia*, *Francisella*, and *Vibrio* infections (Mert et al., 2003).

In relation to this, it is important to develop a sufficient portable diagnostic tool that is cheap, easy to use and does not base diagnosis with the LPS. One non-LPS group of immunogens focused on vaccine and diagnostic purposes is the outer membrane protein(Omp) (Lindler et al., 1996). Due to this interest, we considered the combined Omp10 and Omp28 in constructing a sufficient portable diagnostic tool. In our previous study(Simborio et al., 2015), the combined Omp10 and Omp28 ELISA conferred sensitivity, specificity and accuracy of 92.67% and 96.04% and we strongly believe that the combined antigen will be a great dipstick diagnostic kit. For this reason, we developed a simple dipstick assay for the detection of *Brucella* antibodies in bovine serum samples.

Materials and methods

1 Bacteria culture and media

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 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 GAATTCA TGAACGCTTCCGCA-3’(EcoRl) and 5’-ATTA CTGCAG TCAGCGGC GG TTGC-3’(PstI). rOmp28 construction was previously published in earlier studies in our laboratory(Lim et al., 2012).

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 for B. abortus (Genbank NC_006933.1). 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).

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 4,400 x 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(Lim et al., 2012). 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/cm² 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, 1:1,000 dilution) or Y. enterocolitica O:9-positive(1:1,000) 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; 1:5,000 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).

4 Serology

In this study, serum samples were initially tested with TAT and combined rOmps ELISA and further compared with the rOmp dipstick. TAT and rOmp ELISA tests were performed in our previous published study(Simborio et al., 2015). rOmps dipsticks (sandwich or indirect) were performed by previously reported modified method(Paek et al., 2000), made and read with the assistance with Median Diagnostics (Chuncheon, Korea).
In brief, to develop sandwich formed strip, colloidal gold nanoparticle (40 nm diameter; Median diagnostics, Korea) and rOmps rOmp10 and rOmp28 were conjugated and lyophilized (A530 nm=3.0) and dried onto glass fiber sheet (Ahlstrom, USA). The rOmp10 (1 mg/ml) and rOmp28(1 mg/ml) were spread on nitrocellulose membrane (1 Q1/cm, MERCK Millipore, USA) with dispenser (KINEMATIC Automation Inc., USA) and immobilized at 37°C and 20% humidity for overnight. rOmps (rOmp10 and rOmp28) immobilized nitrocellulose membrane, rOmps-gold conjugated pad, sample pad and adsorption pad were laminated, the strip was cut(4 mm) and applied to diagnose brucellosis. To develop indirect formed strip, colloidal gold nanoparticle (40 nm diameter; Median diagnostics, Korea) and protein A were conjugated and lyophilized (A530 nm=10.0) and dried onto glass fiber sheet (Ahlstrom, USA). The rOmp10(1 mg/ml) and rOmp28(1 mg/ml) were spread on nitrocellulose membrane (1 Q1/cm, MERCK Millipore, USA) with dispenser (KINEMATIC Automation Inc., USA) and immobilized at 37°C and 20% humidity for overnight. rOmp10 and rOmp28 immobilized nitrocellulose membrane and protein A-gold conjugated pad, sample pad and adsorption pad were laminated, the strip was cut(4 mm) and applied to diagnose brucellosis.

To apply for diagnosis, 10 Q1 of serum was diluted with 90 Q1 of sample dilution buffer, and loaded on strip, incubated 10 min on RT and read the result.

Over 500 bovine samples were collected from 2006-2014. Randomly, 73(indirect) negative samples and 40 positive samples were selected. Another 44 samples were provided by the NVRQS for further sandwich dipstick assay. The data are expressed in % after being read by a gold reader.

Results and discussion

1 Serology with TAT, ELISA and dipstick

From the previously reported our study, Omp10(70 kDa) and Omp28(88 kDa) were successfully cloned and purified in the pCold(60 kDa) expression system, and immunoreactivity was evaluated by western blot with standard Brucella-positive bovine serum (Lim et al., 2012; Simborio et al., 2015). In addition, all rOmps were also tested for immunogenicity against Y. enterocolitica O: 9-positive and B. abortus-negative cattle serum, and no immunoreactive results were observed (Simborio et al., 2015).

All(indirect and sandwich) dipsticks were generously prepared and read by Median Biotechnology with the B. abortus rOmp10 and rOmp28(Fig. 1). The Brucella positive serum with TAT showed the one line(Fig. 1A), but the negative serum did not show any line on the pad(Fig. 1B).

Brucella negative serum samples were best described with the indirect dipsticks with 97.78% detection compared to TAT either single or combine (Table 1). Positive samples were examined with indirect and sandwich dipsticks. From these results, the sensitivities of indirect and sandwich dipsticks with combined antigen were 85 and 100% reading compared to TAT, respectively. Outcome was excellent,
with combine sandwich dipstick reading 100% of the TAT positive Brucella serum samples. Even sensitivity of dipstick used in the detection of human brucellosis was only at 93.5%, with the range of 83.1% to 100%(Smits et al., 1999). Another similar study conducted had 93.1% sensitivity for the serodiagnosis of human brucellosis(Clavijo et al., 2003).

In conclusion, the dipstick assay described here is a quick method with high sensitivity and specificity and can be recommended highly suitable for performing serodiagnosis for bovine brucellosis. The results suggest that the combined sandwich rOmps had the best results among the tested dipssticks for the diagnosis of Brucella positive serum samples. While the indirect single or combined rOmps dipstick yielded equally good results in detecting Brucella negative cattle serum. Under laboratory conditions, all the factors were considered ideal and the dipstick assay is simple, small, handy, easy to maintain and handle makes it the most suitable application for field conditions. The necessity of further diagnostic test in field conditions with larger number of samples still remains.

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### Table 1. Sensitivity and specificity of dipsticks in comparison to other serological tests

<table>
<thead>
<tr>
<th></th>
<th>Dipstick(^1)(%)</th>
<th>ELISA(^2)(%)</th>
<th>TAT(^3)(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Omp10</td>
<td>Omp28</td>
<td>Combination</td>
</tr>
<tr>
<td>Indirect negative</td>
<td>97.78</td>
<td>97.78</td>
<td>97.78</td>
</tr>
<tr>
<td>Indirect positive</td>
<td>70</td>
<td>72.50</td>
<td>85</td>
</tr>
<tr>
<td>Sandwich positive</td>
<td>92.11</td>
<td>86.84</td>
<td>100</td>
</tr>
</tbody>
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\(^1\)The dipstick was considered positive when the gold reader read \( \geq 35 \).
\(^2\)ELISA results were considered positive at the OD reading of 0.1015±0.004(double the average OD492 negative sera)(Simborio et al., 2015).
\(^3\)TAT samples were done in accordance of the Brucella Serum Agglutination Test Antigen provided by NVRQS and OIE, Korea, and positive was set at 1:50 dilution of serum.

### References


