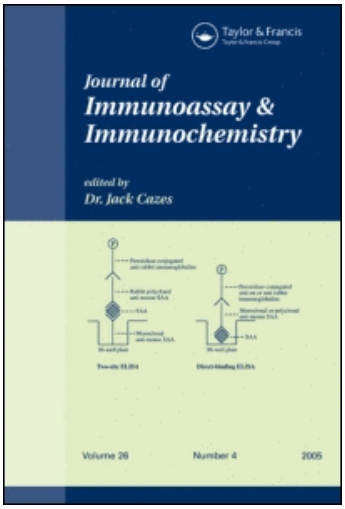

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Development of a Lateral Flow Assay for Rapid Detection of Bovine Antibody to *Anaplasma marginale*

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Abstract: A rapid lateral flow assay for detection of bovine antibody to *Anaplasma marginale* was developed. The assay used a recombinant peptide of major surface protein 5 as the antigen and a monoclonal antibody specific for bovine IgG_1 conjugated with colloidal gold beads for detection.

Serum and anticoagulated blood samples were obtained from cattle in an area where anaplasmosis was endemic. The samples were selected based on positive identification of the organism in blood smears. The unclotted blood samples were used for PCR determination of the presence of *A. marginale* while the sera were tested by a commercial competitive enzyme immunoassay (CELISA) and by the lateral flow assay (LFA). Similar samples, collected at a Canadian sales barn, were tested by the CELISA and LFA and 10% were tested by PCR for the presence of *A. marginale* nucleic acid. In addition, stored serum samples from a second endemic area were tested by CELISA and LFA.

Of the 114 smear positive samples, all were positive by CELISA and LFA. All samples were also positive by PCR. Samples from Canadian sources (n = 524) were negative in the CELISA but 11 sera gave false positive reactions in the LFA. All

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samples tested were PCR negative. Of 113 samples from herds with anaplasmosis, 53 were positive in the CELISA and 50 were LFA positive.

Keywords: *Anaplasma marginale*, Lateral flow test, Rapid diagnosis, Serology, PCR, CELISA

INTRODUCTION

Anaplasmosis is an acute to chronic infection of ruminants in tropical and subtropical regions. It is caused by member of the Genus *Anaplasma*, of which *A. marginale* is the most common.

Although anaplasmosis is exotic to Canada, it is necessary for the Canadian Food Inspection Agency to have the capability to diagnose its presence should an outbreak occur.

The infection is usually diagnosed serologically by a card test, a complement fixation test (CFT), indirect fluorescent antibody test (IFA), or by an ELISA, but it may also be detected directly using blood smears or polymerase chain reaction (PCR) to demonstrate the presence of nucleic acid fragments.[1–6] Initially, a non-modified card test was used,[7] however, because of short comings in sensitivity, a modified version was developed.[8,9] The latter was shown to be more sensitive but less specific than a CFT. The relative lack of sensitivity of the CFT was thought to be partly due to its ability to only detect antibody of the IgG_1 isotype[10] which may attribute to its failure to detect chronically infected animals.[4,9] The IFA was found to correlate well with PCR (4) but lack specificity.[7] Indirect ELISAs have been developed using a variety of formats.[11–19] In general, ELISAs were thought to be more sensitive and specific than the conventional tests, especially for detection of carrier states. In addition, a competitive ELISA was developed. This assay uses a recombinant peptide derived from major surface protein 5 (MSP5) as the antigen and its monoclonal antibody for competition, making it very specific and with good sensitivity.[1,20–22]

Because of the inherent problems with laboratory testing for antibody to *A. marginale*, that is, the delay between sampling and delivery of results, it was thought that an antibody test usable in the field would provide an avenue for rapid provision of remedial measures. A prototype of a rapid lateral flow assay for antibody to *A. marginale* described in this communication may thus be advantageous.

EXPERIMENTAL

Serum Samples

Canadian serum and EDTA treated blood samples ($n = 524$) were collected from cattle at a local sales barn. Canada is considered free from bovine

anaplasmosis (the last case was diagnosed in 1979). These samples were used as the negative population. Serum and blood samples (n = 114) from herds with endemic anaplasmosis were selected based on direct microscopic detection of the microorganism in erythrocytes. Additional serum samples (n = 113), also collected from herds in which anaplasmosis was endemic.

Serological Tests

The commercial CELISA kits were purchased from VMRD, (Pullman, WA, USA) and used as described by the manufacturer. The LFA was performed as described below. All serum samples were tested by CELISA and LFA.

Semi-Nested PCR Procedures

The semi-nested PCR was used to identify *A. marginale* Major Surface Protein 5 (MSP5) DNA from blood. Blood samples were thawed and 100 μL was used to extract DNA. The red cells were washed twice with 1 mL of sterile H_2O and centrifuged at 13,200 rpm for 10 min. After the last round washing, the supernatant was discarded. The cell pellets were mixed by vortexing; then deposited on an FTA Classic cards (Cat. No. WB12 0205, Whatman, Inc., USA); and left at room temperature overnight. Then FTA cards were used to extract DNA according to manufacture's instruction. Primers were designed by using the published sequence of *msp5* from *Anaplasma marginale* Pernambuco-Zona da Mata-Brazil (GenBank Accesion No. AY245428) to verify *A. marginale* in blood samples which also contain *Nde*I and *Not*I enzymes cutting sites for subcloning MSP5 into pET30a expression vector. They were as follows: external forward primer P62: ACACATATG AGA ATT TTC AAG ATT GTG TCT AAC; internal forward primer P82: ACACATATG GGC GAT TTT GGC GGC AAG C; external reverse primer P65: ACAGCGGCCGC AAA ACA GCT CCT CGC CTT GG. The two rounds of PCR were performed in a final volume of 50 μL. The first round of PCR used 39 μL of H_2O, 5 μL of 10 × PCR buffer (Invitrogen Inc. USA), 1.5 μL of 50 mM $MgCl_2$, 1 μL of 10 mM dNTP, 1 μL of 25 μM Primers 62, 65, 25 units of *Taq* DNA polymerase and FTA classic cards cut-out as a template. The second round of PCR was the same as the first, except that primer 82 was substituted for primer 62 and 1 μL of PCR products from the first round PCR was used as the template. The programs for the first and second PCRs were the same, composed of 95°C for 3 min, then 35 cycles composed of 95°C for 30 seconds, 65°C for 58 seconds and 72°C for 30 seconds, then with a final extension of 72°C for 10 min. The resulting PCR materials were run in 1.5% agarose gels loaded with 20 μL of product. The PCR product of the

first round was 597 bp; the second round was 405 pb. If no PCR product from the first round was observed under UV light, the semi-nested PCR was performed.

Subcloning and Expression of Recombinant MSP5 (rMSP5)

A PCR product of MSP5 gene amplified from the first round of PCR using primers 62 and 65 was subcloned into *Nde*I and *Not*I sites of expression vector pET30a (Novogen, EMD Bioscience, La Jolla, CA, USA) and transformed into *E. coli* DH5α. One colony was picked and cultured overnight in Luria-Bertani (LB) liquid medium supplemented with 50 μg of kanamycin per mL. Plasmid DNA was extracted and expressed in *E. coli* strain BL21 (DE3) competent cells. *E. coli* strain BL21(DE3) cells containing the expression construct were induced to express the recombinant MSP5 with the addition of 1 mM IPTG. The expressed MSP5 fragment was purified using a Ni-NTA column as previously described.[23] The whole cell and purified proteins were analysed by SDS-PAGE[24] and Western blotting. For Western blotting, the proteins were transferred onto a nitrocellulose membrane using a Bio-Rad Trans-Blot SD (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada) semi-dry transfer cell. The blots were probed with a mouse anti-histidine tag monoclonal antibody. Bound antibodies were detected with horseradish peroxidase (HRPO)-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. USA) and a 4-chloro-1-naphthol-H_2O_2 substrate kit (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada) using the manufacturer's instructions.

Blood samples (EDTA treated) from 114 animals with anaplasmosis based on direct microscopic observation of the organism in erythrocytes were tested by PCR as were 40 randomly selected samples from the Canadian group assumed free from anaplasmosis. Blood samples were not available from 113 animals from herds in anaplasmosis endemic areas.

Labelling Colloidal Gold Particles and Preparing Conjugate Pad

Mouse anti-bovine monoclonal antibody M23, 90 μg, in 10 mL of colloidal gold particles (40 nm) were conjugated following the manufacturer's instructions (Diagnostic Consulting Network LLC, Irvine, CA, USA). After labelling, the beads were stored at 4°C in PBS, pH 7.4, containing 10 mg/mL bovine serum albumin (BSA) and 0.1% sodium azide. Conjugate pads were prepared by loading an aliquot of 20 μL of M23 labelled gold beads onto a membrane (Cat. No. 10537259, Whatman, Clifton, NJ, USA). The bead complex was dried at room temperature overnight, then sealed in plastic film and stored at room temperature until used.

Preparation of Strips

Protein A/G and rMSP5 were dialyzed overnight against 0.02M PBS, pH 7.4. Concentrations of protein A/G (0.15 mg/mL) and rMSP5 (0.4 mg/mL) were applied to the nitrocellulose membrane (Hi-flow Plus 120 membrane, Millipore, Danvers, MA, USA) as control and test lines, respectively, using a Bio-Dot air-brush device (Bio-Dot, Irvine, CA, USA). The membranes were dried at 30°C for 30 minutes, left at room temperature overnight, and were then stored in sealed foil sachets until required for use.

Assembly of the Strip Test Device for Lateral Flow Test

The conjugate pad was overlaid onto the base of the nitrocellulose membrane, parallel to the control and test lines. The sample pad (0.48 cm, Surewick CO48 cellulose pad, Millipore, Danvers, MA, USA) was overlaid on the conjugate pad. An absorbent pad (SA3Jo71V04, Surewick cellulose pads, Millipore, Danvers, MA, USA) was applied downstream at the opposite end of the membrane.

Test Procedure

A 3 μL volume of tested serum was added to the sample pad and was allowed to react for about 1 min, followed by the sequential application of 100 μL of running buffer to flow through the conjugate pad, followed by the addition of 100 μL of running buffer at about 90 second intervals. If anti-*A. marginale* antibodies were present in the sample, a dark pink line would form at the test line position. Unbound conjugated gold particles would continue to flow along the membrane to produce a dark pink line with protein A/G at the control line position. The lateral flow time was approximately 5 to 10 minutes. Only control lines were present with samples containing no antibodies. If no control line formed, the test was considered invalid and repeated.

RESULTS

Samples from 114 cattle with anaplasmosis based on microscopic observation of the organism in erythrocytes were tested by PCR, CELISA, and LFA. These samples tested positive in all tests.

Of 524 samples from negative animals, 514 tested negative by CELISA while 10 samples gave low positive reactions (between 31 and 41% inhibition). These samples were retested by CELISA and found negative. The CELISA specificity value was 100%. Eleven of the 524 sera were positive

in the LFA and remained positive on retesting for a specificity value of 97.9%. Forty samples randomly selected from the 524 were all PCR negative.

The sera from 113 animals from herds with endemic anaplasmosis were not prescreened by direct microscopic examination of erythrocytes and no blood was available for PCR testing. However, there was good agreement between the CELISA and the LFA (94.7% agreement, SE: 0.030, IC 95%: 0.887–1.006). Of these samples, 53 tested postitive by CELISA. Fifty of the 53 were also positive by LFA. The three negative samples gave CELISA readings of 36, 39 and 42% inhibition, that is, low reactions. Assuming the sensitivity of the CELISA to be 100%, the sensitivity of the LFA using the same samples was 94.3%.

DISCUSSION

A prototype LFA was developed for rapid assessment of antibody levels in cattle to *A. marginale*. Based on samples from proven cases of anaplasmosis, the LFA performed as well as did a commercially available CELISA. Similarly, the PCR protocol used was 100% sensitive on these samples. The specificity of the LFA was determined to be 97.9% based on 524 Canadian cattle samples obtained from a local sales barn. Thus, 11 sera gave repeatedly positive reactions in the LFA. There were 10 false positive reactions in the CELISA initially, however, retesting of those samples did not confirm the samples as positive. The PCR was performed on 40/524 randomly selected blood samples which all tested negative. Of 113 serum samples from herds with *Anaplasma* infection, 53 were positive in the CELISA while 50 were LFA positive, resulting in a sensitivity value of 94.3% for the LFA, based on the CELISA results.

According to these results, the LFA may be a useful addition to testing for anaplasmosis. It would be particularly useful where screening of cattle from endemic areas into non-endemic areas takes place. Under these conditions, it is a common practice to give each animal a large dose of antibiotics, regardless of infection status. This practice undoubtedly leads to problems with antibiotic resistance of microbes as well as environmental pollution by antibiotics and it should therefore be minimized and used only on infected animals.

Lateral flow tests have been used for detection of a number of infectious and non-infectious agents, including HIV,[25] PRRSV,[26] foodborne pathogens,[27] PSA,[28,29] toxins,[30] pregnancy,[31] and for contaminant detection in animal feed.[32] Highly sensitive and specific LFAs have also been developed for detection of influenza virus in respiratory specimen from children[33–37] and in chickens.[38] These tests provide a very rapid indication of the presence of substances of interest by a technique which requires no equipment and which can be performed under most environmental conditions. While the described LFA appears to provide accurate results, complete validation of the assay remains to be done.

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