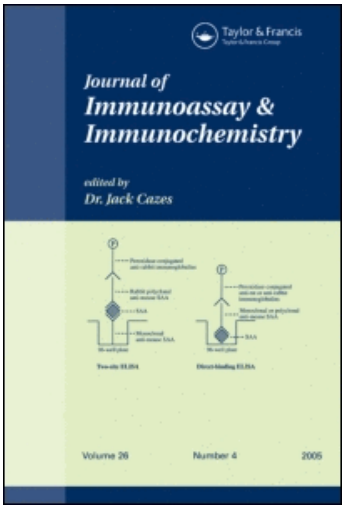

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Validation and Field Assessment of a Rapid Lateral Flow Assay for Detection of Bovine Antibody to *Anaplasma marginale*

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Abstract: The lateral flow assay (LFA) is a rapid diagnostic test which may be performed under most conditions and is especially useful for field applications. This type of assay was applied to the detection of antibody to bovine *Anaplasma marginale* using sera from endemic areas and from areas which have been free from infection for more than 25 years. Briefly, the test uses recombinant *A. marginale* major surface protein 5 peptide (*Msp5*), immobilized on a cellulose acetate membrane. A serum sample is added to a pad containing a monoclonal antibody specific for bovine IgG_1, conjugated with colloidal gold, located at one end of the strip. The sample and gold conjugate are wicked along the membrane and if antibody is present in the serum, a visible line will form between the *Msp5*-antibody – conjugate immune complex in minutes. An additional band of recombinant protein A/G was added to the membrane as a positive control reaction of the monoclonal antibody conjugate. For comparison, direct examination of blood smears and a nested polymerase chain reaction (PCR) were performed on some of the samples.

Using samples from herds in one endemic area, the PCR gave a sensitivity value of 9.2% while a commercial competitive enzyme immunoassay (CELISA)

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gave a sensitivity value of 17.2% and the LFA values of 20.5%. In a second endemic area, selected samples, all positive by direct examination gave a 71.7% sensitivity values with the PCR, 94.5% with the CELISA and 95.5% with the LFA. Using sera from a disease-free area, the specificity values were 100% for the PCR (testing a proportion of randomly selected samples), 99.5% for the CELISA and 98.0% for the LFA.

It is envisaged that the validated LFA will be a useful tool for screening cattle moving from an area with infection to a disease-free area.

Keywords: Anaplasmosis, Competitive ELISA, Diagnosis, Lateral flow assay, PCR, Serology

INTRODUCTION

Anaplasmosis is a common infection of ruminants in tropical and subtropical regions. It is caused by members of the Genus *Anaplasma* of which *A. marginale* is the most common.

The infection can be diagnosed serologically by a card agglutination test, a complement fixation test (CFT), an indirect fluorescent antibody test (IFA) or by an ELISA, or it may also be detected directly using blood smears or polymerase chain reaction (PCR) demonstrating 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 account for its failure to detect chronically infected animals.[4,9] The IFA was found to correlate well with PCR,[4] but to lack specificity.[7] Indirect ELISAs have been developed using a variety of formats.[11–19] In general, ELISAs are thought to be more sensitive and specific than the conventional tests, especially for detection of carrier states. In addition, a competitive ELISA that uses a recombinant peptide derived from major surface protein 5 (*Msp5*) as the antigen and its monoclonal antibody for competition was developed. This test is very specific and has good sensitivity properties. It is commercially available.[1,20–22]

Anaplasmosis is exotic to Canada; however, it is necessary for the Canadian Food Inspection Agency to have the capability to diagnose its presence should an incursion occur. Because of the inherent problems with laboratory testing for antibody to *A. marginale*, that is, the delay between sampling and delivery of results, it is desirable to develop an antibody test usable in the field to provide an avenue for rapid provision of remedial measures. A rapid lateral flow assay for antibody to

A. marginale was developed previously[23] and in this communication its validation is described.

EXPERIMENTAL

Test Samples

Serum and blood samples (n = 470) from cattle in an area with endemic anaplasmosis were selected based on direct microscopic detection of the microorganism in erythrocytes. Additional serum samples (n = 547) were also collected from herds in which anaplasmosis was endemic. Canadian serum and EDTA treated blood samples (n = 1076) 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.

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

A semi-nested PCR was used to detect the presence of *A. marginale msp5* DNA in blood. The erythrocytes in 100 μL of blood were washed twice with sterile H_2O and centrifuged at 13,200 rpm for 10 min and the supernatant solutions were discarded. The cell pellets were resuspended, then deposited on an FTA Classic cards (Cat No. WB12 0205, Whatman Inc. USA) which were used to extract DNA according to manufacturer's instruction. Primers were designed by using the published sequence of *Msp5* from *A. marginale* Pernambuco-Zona da Mata-Brazil (GenBank accesion no. AY245428) and they also contained *Nde*I and *Not*I enzymes cutting sites for subcloning *Msp5* into the pET30a expression vector. 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 were used. 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 using a template. The second round of PCR was the same as the first except 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 bp. 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* (r*Msp5*)

A PCR product of the *Msp5* gene amplified from the first round of PCR using primers 62 and 65 was subcloned into the *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/mL of kanamycin. 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 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 327 of 470 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 all 547 animals from herds in anaplasmosis endemic areas and as a result, only 97 samples were tested by PCR.

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 r*Msp5* were dialyzed overnight against 0.02 M PBS, pH 7.4. Concentrations of protein A/G (0.15 mg/mL) and r*Msp5* (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.

Assemblage 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 test 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 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.

Table 1. Results obtained when testing cattle sera and blood from animals selected based on direct microscopic observation of the parasite in blood smears, herd samples from an endemic area and Canadian samples, considered as negative

Origin	Number of samples	Direct microscopic examination	PCR Number positive/ number tested	CELISA Number positive/ number tested	LFA Number positive/ number tested
Endemic area	547	ND	9+/97	94+/547	112+/547
	% positive		9.3%	17.2%	20.5%
Endemic area	470	470+/470	327+/456	444+/470	449+/470
	% positive	100%	71.7%	94.5%	95.5%
Canada	1076	ND	0+/140	4+/878	21+/1076
	% negative		100%	99.5%	98.0%

RESULTS

Of 470 serum and EDTA-treated blood samples selected from cattle infected with *Anaplasma* based on direct microscopic observation of the parasite in blood smears, 444 (94.5%) were positive in the commercial CELISA and 449 (95.5%) were positive in the LFA. Of 456 blood samples tested by PCR, 327 samples (71.7%) were positive.

Samples (n = 547) collected from herds in an endemic area were not tested by direct microscopic examination of blood smears. The CELISA resulted in 94 positive reactions (17.2%) and the LFA gave 112 positive reactions (20.5%). The PCR was performed on a subset of 97 samples of which 9 (9.3%) were positive.

Samples collected at a Canadian sales barn (n = 1070) were tested by CELISA, resulting in 10 (1.9%) false positive results, however, on retesting, 6 were negative, for a final specificity value of 99.5%. Similarly, 21 sera gave positive results in the LFA and on retesting, all 21 remained positive, giving a final specificity value of 98.0%. The PCR was performed on 140 randomly selected blood samples. All were negative.

The results are summarized in Table 1.

DISCUSSION

A number of diagnostic tests for anaplasmosis are available, however, all, except the card tests, require samples be submitted to a laboratory for testing, resulting in delays in diagnosis. This delay often results in animals being held in quarantine at costs to the producer. An alternative

approach is to assume widespread infection and therefore to give each animal a dose of antibiotics. Neither procedure is satisfactory, however, a rapid, accurate field test that could provide a result in minutes would solve these problems. As reported previously,[23] the lateral flow assay (LFA) can be performed in 10–15 minutes, it requires no expensive equipment and it is comparable to laboratory tests in its performance. In order for a diagnostic test to be acceptable, it must be validated according to criteria established by the OIE.[25] These criteria include testing a minimum of 300 positive and 1000 negative reference samples by the 'new' method and comparing the results to accepted 'gold standard' assays. In this case, the direct microscopic observation of the parasite in blood smears was used for positive samples to compare results of a nested PCR, a commercial CELISA and the LFA. Negative samples were collected from cattle at a local sales barn and assumed free from the parasite. Overall, the LFA performed similarly to the CELISA (the performance indices were 193.5 and 194.0, respectively) and both serological tests proved to be more sensitive than the PCR. Based on these results, the LFA is a useful tool for rapid detection of anaplasmosis in cattle.

REFERENCES

1. Torioni de Echaide, S.; Knowles, D.P.; McGuire, T.C.; Palmer, G.H.; Suarez, C.E.; McElwain, T.F. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme linked immunosorbent assay using recombinant major surface protein 5. J. Clin. Microbiol. **1998**, *36*, 777–782.
2. Aboytes-Torres, R.; Buening, G.M. Development of a recombinant *Anaplasma marginale* DNA probe. In P. S. Paul, Ed., Nucleic Acid Probes **1990**, *24*, 391–408.
3. Goff, W.L.; Barbet, A.F.; Stiller, D.; Palmer, G.H.; Kocan, K.M.; Gorham, J.R.; McGuire, T.C. Detection of *Anaplasma marginale*-infected tick vectors by using a cloned DNA probe. Proc. Nat. Acad. Sci. USA **1988**, *85*, 919–125.
4. Goff, W.L.; Stiller, D.; Roeder, R.A.; Johnson, L.W.; Falk, D.; Gorham, J.R.; McGuire, T.C. Comparison of a DNA probe, complement fixation and indirect immunofluorescence tests for diagnosing *Anaplasma marginale* in suspected carrier cattle. Vet. Microbiol. **1990**, *24*, 381–390.
5. Eriks, I.S.; Palmer, G.H.; McGuire, T.C.; Allred, D.R.; Barbet, A.F. Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. J. Clin. Microbiol. **1989**, *27*, 279–284.
6. Shompole, S.; Waghela, S.D.; Rurangirwa, F.R.; McGuire, T.C. Cloned DNA probes identify *Anaplasma ovis* in goats and reveal high prevalence of infection. J. Clin. Microbiol. **1989**, *27*, 2730–2735.
7. Gonzales, E.; Long, R.; Todorovic, R. Comparison of complement fixation, indirect fluorescent antibody and card agglutination tests for the diagnosis of bovine anaplasmosis. Am. J. Vet. Res. **1987**, *39*, 1538–1541.

8. Todorovic, R.; Long, R.; McCallon, B. Comparison of the rapid card agglutination test with the complement fixation test for the diagnosis of *Anaplasma marginale* infection in cattle in Colombia. Vet. Microbiol. **1977**, *2*, 167–177.
9. Luther, D.G.; Cox, H.U.; Nelson, W.O. Comparison of serotests with calf inoculations for detection of carriers in anaplasmosis-vaccinated cattle. Am. J. Vet. Res. **1980**, *41*, 2085–2086.
10. McGuire, T.C.; Musoke, A.J.; Kurtii, T. Functional properties of bovine IgG_1 and IgG_2, interaction with complement, macrophages, neutrophils and skin. Immunology **1978**, *38*, 249–256.
11. Torioni de Echaide, S.; Bono, M.F.; Lugaresi, C.; Aguirre, N.; Mangold, A.; Moretta, R.; Farber, M.; Mondillo, C. Detection of antibodies against *Anaplasma marginale* in milk using a recombinant *Msp5* antigen indirect ELISA. Vet. Microbiol. **2005**, *106*, 287–292.
12. Nielsen, K.; Smith, P.; Gall, D.; de Echaide, S.T.; Wagner, G.; Dajer, A. Development and validation of an indirect enzyme immunoassay for detection of antibody to *Anaplasma marginale* in bovine sera. Vet. Parasitol. **1996**, *67*, 133–142.
13. Shkap, V.; Bin, H.; Ungar-Waron, H.; Pipano, E. An enzyme linked-immunosorbent assay (ELISA) for detection of antibodies to *Anaplasma centrale* and *Anaplasma marginale.* Vet. Microbiol. **1990**, *25*, 45–53.
14. Montenegro-James, S.; Guillan, A.T.; Ma, S.J.; Tapang, P.; Abdel-Gawad, A.; Toro, M.; Ristic, M. Use of DOT enzyme linked immunosorbent assay with isolated *Anaplasma marginale* initial bodies for the serodiagnosis of anaplasmosis in cattle. Am. J. Vet. Res. **1990**, *51*, 1518–1521.
15. Dugzun, A.; Schuntner, C.A.; Wright, I.; Leatch, G.; Waltisbuhl, D.J. A sensitive ELISA method for the diagnosis of *Anaplasma marginale* infections. Vet. Parasitol. **1988**, *29*, 1–7.
16. Nakamura, Y.; Shimizu, S.; Minami, T.; Ito, S. Enzyme linked immunosorbent assay for detection of antibodies to *Anaplasma marginale.* Jap. J. Vet. Sci. **1988**, *50*, 933–935.
17. Winkler, G.C.; Brown, G.M.; Lutz, H. Detection of antibodies to *Anaplasma marginale* by an improved enzyme linked immunoasorbent assay with sodium dodecyl sulfate disrupted antigen. J. Clin. Microbiol. **1987**, *25*, 633–636.
18. Barry, D.N.; Parker, R.J.; De Vos, A.J.; Dunster, P.; Rodwell, B.J. A microplate enzyme linked immunosorbent assay for measuring antibody to *Anaplasma marginale* in cattle serum. Austral. Vet. J. **1986**, *63*, 76–79.
19. Thoen, C.O.; Blackburn, B.; Millk, K.; Lomme, J.; Hopkins, M.P. Enzyme linked immunosorbent assay for detecting antibodies in cattle in a herd in which anaplasmosis was diagnosed. J. Clin. Microbiol. **1980**, *11*, 499–502.
20. Molad, T.; Mazuz, M.L.; Fleiderovitz, L.; Fish, L.; Savitsky, I.; Krigel, Y.; Leibovitz, B.; Molloy, J.; Jongejan, F.; Shkap, V. Molecular and serological detection of *A. centrale* and *A. marginale* infected cattle grazing within an endemic area. Vet. Microbiol. **2006**, *113*, 55–62.
21. Van Donkersgoed, J.; Gertonson, A.; Bridges, M.; Raths, D.; Dargatz, D.; Wagner, B.; Boughton, A.; Knoop, D.; Walton, T.E. Prevalence of antibodies

to bluetongue virus and *Anaplasma marginale* in Montana yearling cattle entering Alberta feedlots, Fall 2001. Can. Vet. J. **2004**, *45*, 486–492.
22. Knowles, D.; de Echaide, S.T.; Palmer, G.; McGuire, T.C.; Stiller, D.; McElwain, T. Antibody against *Anaplasma marginale MSP5* epitope common to tick and erythrocyte stages identifies persistently infected cattle. J. Clin. Microbiol. **1996**, *34*, 2225–2230.
23. Nielsen, K.; Yu, W.L.; Kelly, L.; Bermudez, R.; Renteria, T.; Dajer, A.; Gutierrez, E.; Williams, J.; Algire, J.; Torioni de Eschaide, S. Development of a lateral flow assay for rapid detection of bovine antibody to *Anaplasma marginale*. J. Immunoassay Immunochem. **2008**, *29*, 10–18.
24. Laemlli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **1970**, *227*, 680–685.
25. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*; Principles of validation of diagnostic assays for infectious diseases. OIE: Paris, 2004; Chapter 1.1.3, 21–29.

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