

Development of enzyme-linked immunosorbent assays based on recombinant MSP1a and MSP2 of *Anaplasma marginale*

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Indirect enzyme-linked immunosorbent assays (ELISAs) based on recombinant MSP1a and MSP2 from a Brazilian isolate of Anaplasma marginale were developed to detect antibodies against this rickettsia in cattle. The high sensitivities (99% for both tests) and specificities (100% for both tests) were confirmed with sera from cattle positive or negative for A. marginale antibodies, respectively, by immunofluorescent antibody test. By the analysis of 583 sera from cattle of three regions of the state of Pernambuco, Brazil, the agreement between both tests was high, with a kappa index of 0.89. The similar performances of the ELISAs suggest that both tests can be used in epidemiological surveys for detection of antibodies to A. marginale in cattle.

Key words: *Anaplasma marginale* - indirect enzyme-linked immunosorbent assays - major surface proteins - Brazil

Anaplasmosis is a tick-borne cattle disease caused by the rickettsia *Anaplasma marginale*, which is endemic in tropical and subtropical areas of the world. The disease causes considerable economic loss to both dairy and beef industries worldwide (Kocan et al. 2003), and is characterized by fever, anemia, general weakness, pale mucous membranes, weight loss (Ajayi et al. 1978), abortion (Correa et al. 1978), decreased milk production, and mortality during the acute phase of infection (Palmer et al. 1986).

A variety of serological tests, such as indirect fluorescent antibody test (IFAT) (Lohr et al. 1973), complement fixation (Gainer 1961), agglutination test (Welter & Zuscak 1962), and enzyme-linked immunosorbent assay (ELISA) (Luckins 1977) have been used to detect antibodies to *A. marginale*. Although sensitivities of these assays were considered acceptable, there is potential for false-positive reactions when crude antigens are used, due to the contamination with erythrocyte components (Barry et al. 1986).

Advances in biochemical, immunologic, and molecular technologies during the last decade have been applied to research of *A. marginale* (Kocan et al. 2003). As a result of these studies, the major surface proteins (MSPs) with potential for diagnostics were identified (Palmer et al. 1986, Oberle et al. 1988, Visser et al. 1992).

The objectives of the present study were to develop, evaluate, and compare ELISAs based on recombinant MSP1a and MSP2, two immunodominant membrane proteins of *A. marginale*.

MATERIALS AND METHODS

A. marginale isolate - Infected blood was obtained by the inoculation of a splenectomized Nelore calf with a Brazilian isolate of *A. marginale* (Pernambuco, Zona da Mata). Blood was collected in vacuum tubes with heparin when rickettsemia reached 87%.

DNA purification and PCR - DNA was purified from 350 µl of infected blood by Easy DNA kit (Invitrogen, US). Based on the reported *msp1a* and *msp2* nucleotide sequences (Genbank accession numbers: AY010247 and AF317726, respectively), primers were designed to amplify a fragment of *msp1a* that encodes the hydrophilic domain of MSP1a, and a fragment of *msp2* that encodes part of the N terminal region, the hypervariable region and the C terminal region of MSP2. These primers were *msp1a*F (5' GGGTACGCCACCTATCTCGC 3') and *msp1a*R (5' AACAAGCTGTGTAGTAGTGCCGAAGG 3'); *msp2*F (5' GCAGGCGGCGAGGGTCTATTTTCA 3'), and *msp2*R (5' AGAATCCCCCTAAGCGTTGCCGAACCTCAA 3'). PCR amplification reaction mixture was prepared in a volume of 50 µl, containing 10 mM tris-HCl (pH 8.3), 50 µM KCl, 1.5 mM MgCl₂, 0.2 mM (each) deoxynucleoside triphosphate, 100 ng (each) primer, 100 ng of genomic DNA, and 2.5 U of *Taq* DNA polymerase (Cenbiot, Brazil). Amplification was performed in a PTC-200 thermocycler (MJ Research, US) as follows: 94°C for 1 min (denaturation); the next 35 cycles were 94°C for 1 min (denaturation), 61°C (*msp1a*) or 60°C (*msp2*) for 1 min (annealing), and 72°C for 1 min (*msp1a*) or 1.5 min (*msp2*) (extension), and the last cycle was 72°C for 7 min (extension). PCR products were

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analyzed by electrophoresis in 1% agarose gel, stained with ethidium bromide (0.5 µg/ml).

Cloning and expression of *msp1a* and *msp2* - PCR amplicons were cloned in plasmid *pTrcHisTOPO* (Invitrogen), according to the manufacturer's instructions. The recombinant plasmids were used to transform *Escherichia coli* TOP10 cells (Invitrogen). Insert orientation was determined by colony PCR, using the vector forward primer Xpress (Invitrogen) and insert reverse primers. The inserts were also sequenced in both directions by the dideoxide method and analyzed in an ABI 3100 automatic sequencer (Applied Biosystems, US). Only sequences with a PHRED index greater than 20 were considered.

Overnight 50 ml cultures of TOP10 cells carrying the recombinant plasmids (*pTrcHisTOPO-msp1a* and *pTrcHisTOPO-msp2*) grown in liquid selective Luria-Bertani (LB) medium containing 100 µg/ml of ampicillin (LB-amp) were cultured each one in 450 ml of LB-amp for 1 h at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM. The cultures were allowed to grow for 6 h. Aliquots of 2 ml were taken before induction with IPTG and then every 2 h. These aliquots were centrifuged at 18,000 x g and pellets were frozen at -20°C. After a 6-h culture, TOP10 cells were recovered by centrifugation at 10,000 x g for 10 min. Pellets were suspended in 20 ml of 50 mM phosphate buffer and then frozen in liquid nitrogen. After thawing at 4°C, suspensions were sonicated (Branson Sonifier 250, US – output 40) 6 times for 20 sec on ice. The production of recombinant proteins was verified by SDS-PAGE, followed by Coomassie blue staining.

Purification of recombinant proteins - Purifications of recombinant MSP1a and MSP2 (rMSP1a and rMSP2) were performed by affinity chromatography, using agarose-nickel resin (ProBond Purification kit, Invitrogen), following the manufacturer's instructions. A hybrid protocol, with lysis under denaturing conditions and washes and elution steps under native conditions, was used. Purity was verified by SDS-PAGE, followed by Coomassie blue staining.

Western blot - The antigenicity of rMSP1a and rMSP2 was tested by Western blot with sera of immune cattle from different regions of Brazil (states of Pernambuco, Paraná, and Mato Grosso do Sul). A Western blot with recombinant MSP2 and monoclonal antibody (MoAb) ANAO70A2, specific for this protein (McGuire et al. 1984), was also performed.

ELISAs - Optimal dilutions of rMSP1a and rMSP2, sera and conjugate were determined by testing six sera negative for *A. marginale* antibodies, from cattle kept in an isolation area of Embrapa Beef Cattle, Campo Grande, MS, Brazil, and six sera positive for *A. marginale* antibodies, from experimentally-infected cattle.

For determination of the concentrations of rMSP1a and rMSP2, these proteins and known concentrations of human serum albumin were submitted to SDS-PAGE, stained with Coomassie blue, and then analyzed by Alpha-DigiDoc software (Alpha Innotec, US).

For the recombinant ELISAs, 96-well plates (Greiner Bio One, Microlon, Austria, ref. 655001) were adsorbed with 10 ng/well of rMSP1a or 5.7 ng/well of rMSP2, diluted in Dulbecco's phosphate buffer, pH 7.2, for 4 h, at 4°C. Plates were blocked with 5% γ-globulin free equine serum diluted in phosphate buffer saline with 0.1% Tween (PBST), for 60 min, at 37°C. After three washes with PBST, control and test sera, diluted 1:600 in PBST, were incubated for 60 min at 37°C. Plates were then washed five times with PBST, and rabbit anti-bovine IgG horseradish peroxidase conjugate (Sigma, US, ref. A-7414), diluted 1:10,000 in PBST, was added to the wells. Plates were incubated for 30 min at 37°C and after five washes with PBST, the chromogen/substrate *o*-phenylene diamine dihydrochloride (OPD; Sigma)/H₂O₂ was added. The reaction was stopped by addition of H₂SO₄ (2.5 N) and results were read on a Bio-Tek EL-800 ELISA reader (US), with a 490 nm filter.

Cut-offs were determined for each plate with 12 negative control sera, according to Frey et al. (1998). A 99% confidence interval was used.

One hundred and seven sera positive for *A. marginale* antibodies by IFAT, from naturally-infected cattle of the states of Mato Grosso do Sul (nr 25) and Goiás (nr 61), Brazil, and sera from experimentally-infected calves (nr 21), were used for determination of sensitivity. Eighty nine sera negative for *A. marginale* antibodies by IFAT, from cattle of the state of Rio Grande do Sul (nr 27), Brazil, and from cattle raised in a tick-free isolation area of Embrapa Beef Cattle (nr 62), were used for determination of specificity. Positive and negative predictive values were also determined with these sera.

Five hundred and eighteen sera from dairy cross-bred cattle, from both sexes, and varying ages, from three regions of the state of Pernambuco, Brazil (Recife, Zona da Mata, and Agreste), were analyzed by both ELISAs for analysis of agreement between tests.

In the quantitative evaluation of the agreement between the ELISAs with rMSP1a and rMSP2, the kappa index was calculated (Kramer & Feinstein 1981). To the samples with disagreements, the Fischer's exact test was applied.

RESULTS

PCRs for *msp2* and *msp1a* - Amplifications of *msp1a* and *msp2* by PCR resulted in fragments of 626 bp and 1,211 bp, respectively (Fig. 1).

DNA sequencing - The cloned fragments were identified (base-calling) using the program PHRED and the consensus sequence was obtained using the program PHRAP. The resulting sequences showed significant alignments, using the program Blastx (nr data base), with sequences of MSP2 (accession nr AAL26275, e-value 10⁻¹⁷⁸) and MSP1a (accession nr AAP49429, e-value 5 × 10⁻⁷⁸) of *A. marginale*, available at the Genbank.

Expression of *msp1a* and *msp2* - The induction of *msp1a* and *msp2* with IPTG resulted in protein bands of approximately 33,0 kDa and 42,2 kDa, respectively, which were purified by affinity chromatography with agarose/nickel. rMSP1a was partially soluble, while rMSP2 was completely insoluble (Figs 2, 3).

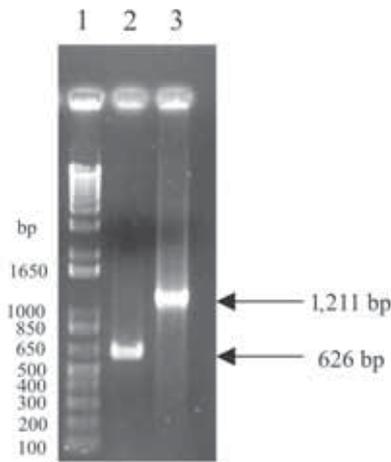


Fig. 1: amplification by PCR of *msp1a* and *msp2* from a Brazilian isolate (Pernambuco-Zona da Mata) of *Anaplasma marginale*. Lanes - 1: 1 kb plus (Invitrogen); 2: *msp1a* fragment; 3: *msp2* fragment.

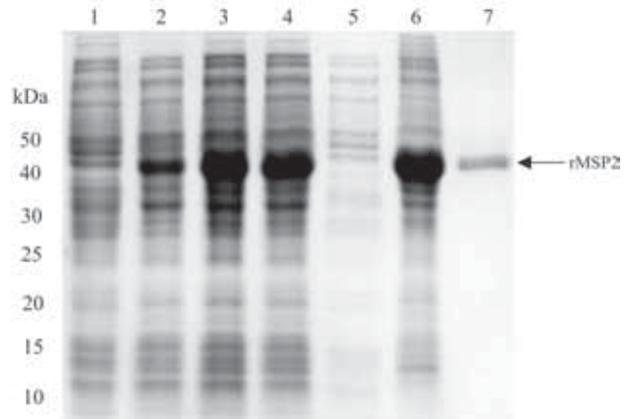


Fig. 3: coomassie blue stained SDS-PAGE profile of *Escherichia coli* TOP10 extracts expressing *msp2* from a Brazilian isolate (Pernambuco-Zona da Mata) of *Anaplasma marginale*. Lanes - 1: extract of *E. coli* TOP10 transformed with pTrcHis-TOPO/*msp2* without induction with IPTG; 2: 2 h induction with IPTG; 3: 4 h induction with IPTG; 4: 6 h induction with IPTG; 5: soluble fraction; 6: insoluble fraction; 7: purified recombinant MSP2.

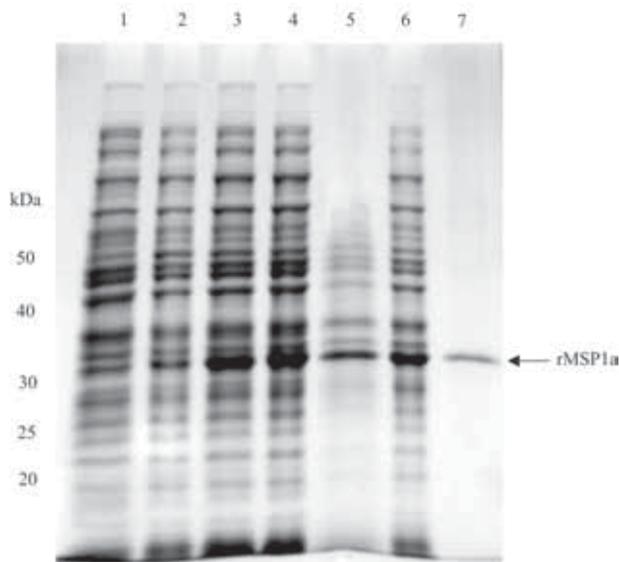


Fig. 2: coomassie blue stained SDS-PAGE profile of *Escherichia coli* TOP10 extracts expressing *msp1a* from a Brazilian isolate (Pernambuco-Zona da Mata) of *Anaplasma marginale*. Lanes - 1: extract of *E. coli* TOP10 transformed with pTrcHis-TOPO/*msp1a* without induction with IPTG; 2: 2 h induction with IPTG; 3: 4 h induction with IPTG; 4: 6 h induction with IPTG; 5: soluble fraction; 6: insoluble fraction; 7: purified recombinant MSP1a.

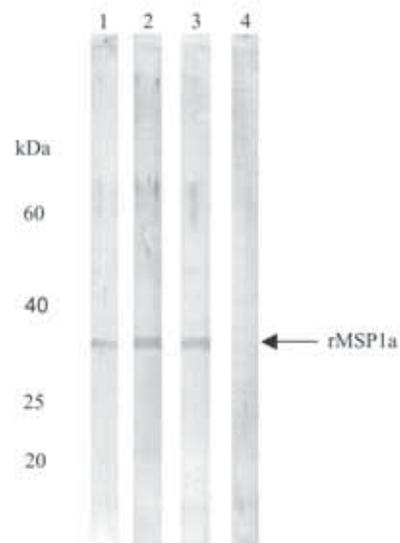


Fig. 4: Western blot with recombinant MSP1a from a Brazilian isolate (Pernambuco-Zona da Mata) of *Anaplasma marginale* and seropositive cattle sera from different regions of Brazil. Lanes - 1: serum of cattle from state of Pernambuco; 2: serum of cattle from state of Mato Grosso do Sul; 3: serum of cattle from state of Paraná; 4: negative control (serum of cattle from state of Mato Grosso do Sul, negative for *A. marginale* antibodies by immunofluorescent antibody test).

Antigenic analysis by Western blot - rMSP1a and rMSP2 were blotted onto nitrocellulose membranes after SDS-PAGE and reacted with sera of *A. marginale* seropositive cattle from different regions of Brazil (Figs 4, 5). Recombinant MSP2 also reacted with monoclonal antibody ANAO70A2 (Fig. 6).

Performances of the ELISAs - Results concerning the sensitivity, specificity, negative, and positive predictive values and precision of the rMSP1a and rMSP2 ELISAs are shown in Table I. The tests showed identical performances.

Agreement between tests - From the 583 sera analyzed, 571 showed identical results in both tests (MSP1a+/MSP2+ or MSP1a-/MSP2-), corresponding to an agreement of 97.9% (Table II), with a kappa index of 0.89. With regards to the discrepant results, there was a statistically superior number of MSP1a-/MSP2+ sera than MSP2-/MSP1a+ sera (P 0.039).

DISCUSSION

Despite the existence of a central hypervariable region in MSP2, which comprises variable epitopes, including in Brazilian isolates of *A. marginale* (Oliveira et al.

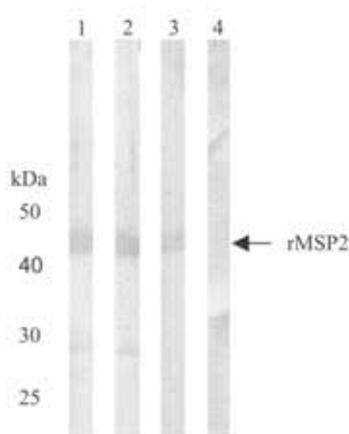


Fig. 5: Western blot with recombinant MSP2 from a Brazilian isolate (Pernambuco-Zona da Mata) of *Anaplasma marginale* and seropositive cattle sera from different regions of Brazil. Lanes - 1: molecular mass marker (BenchMark, Invitrogen); 2: serum of cattle from state of Paraná; 2: serum of cattle from state of Pernambuco; 3: serum of cattle from state of Mato Grosso do Sul; 4: negative control (serum of cattle from state of Mato Grosso do Sul, negative for *A. marginale* antibodies by immunofluorescent antibody test).

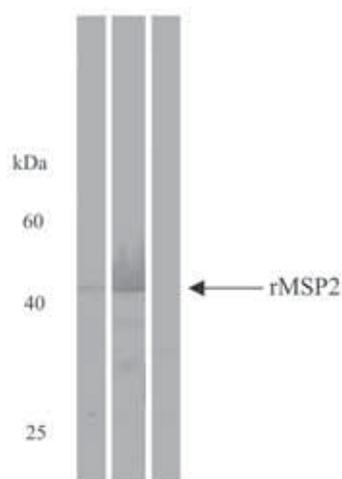


Fig. 6: Western blot with recombinant MSP2 from a Brazilian isolate (Pernambuco-Zona da Mata) of *Anaplasma marginale* and monoclonal antibody (MoAb) ANAO70A2. Lanes - 1: purified recombinant MSP2; 2: *E. coli* lysate of a 6 h induction with IPTG; 3: *E. coli* lysate without induction with IPTG.

2003), this protein exhibits conserved N and C termini (French et al. 1999).

MSP1a is composed of an N-terminal region consisting of 28 or 29 amino acid serine-rich repeats that vary in number and sequence among strains and a highly conserved C-terminal region (Allred et al. 1990). Transmembrane protein prediction analysis of MSP1a from the Virginia strain revealed a large hydrophilic domain (HD), extending from amino acids 1 to 366, and a hydrophobic region extending from amino acid 367 to 593 (Brown et al. 2002).

The *mSP1a* and *mSP2* fragments cloned in this experiment encode for conserved domains, including part of the N-terminal, all the C-terminal regions of MSP2 and the

HD of the C-terminal region of MSP1a. This reflects the recognition of rMSP1a and rMSP2 by sera of cattle from different regions of Brazil in the Western blot and in the ELISAs.

The sensitivities of rMSP1a and rMSP2 ELISAs were high (99% for both tests), situating between an indirect ELISA with crude antigen (100%) (Madruga et al. 2000) and a competitive ELISA with recombinant MSP5 and monoclonal antibody (MoAb) ANAF16C1 (96%) (Torioni de Echaide et al. 1998). The slightly inferior sensitivities of rMSP1a and rMSP2 ELISAs compared to the crude antigen ELISA is probably due to the higher diversity of epitopes in the latter method.

The specificities of rMSP1a and rMSP2 ELISAs (100% for both tests) were higher than in the indirect ELISA with crude antigen (94%) (Madruga et al. 2000) and in the competitive ELISA (95%) (Torioni de Echaide et al. 1998). In the first case, the higher specificity of rMSP1a and rMSP2 ELISAs may be due to the absence of contamination of the antigen with erythrocyte proteins, which are associated with false-positive reactions in the ELISA, due to the reaction of anti-erythrocyte antibodies in the sera (Barry et al. 1986).

TABLE I

Performances of recombinant MSP1a and MSP2 ELISAs for *Anaplasma marginale* antibodies

	ELISA infection status of the calves ^a		
	Positive	Negative	Total
rMSP1a			
Positive	106 (a)	0 (b)	106 (a+b)
Negative	1 (c)	89 (d)	90 (c+d)
Total	107 (a+c)	89 (b+d)	196 (a+b+c+d)
rMSP2			
Positive	106 (a)	0 (b)	106 (a+b)
Negative	1 (c)	89 (d)	90 (c+d)
Total	107 (a+c)	89 (b+d)	196 (a+b+c+d)

^a: determined by indirect immunofluorescent antibody test (IFAT); according to Coggon et al. (1993):

Sensitivity: $a / (a+c) \times 100 = 99\%$ for both rMSP1a and rMSP2 ELISAs

Specificity: $d / (b+d) \times 100 = 100\%$ for both rMSP1a and rMSP2 ELISAs

Positive predictive value: $a / (a+b) \times 100 = 100\%$ for both rMSP1a and rMSP2 ELISAs

Negative predictive value: $d / (c+d) \times 100 = 98.9\%$ for both rMSP1a and rMSP2 ELISAs

Precision: $(a+d) / (a+b+c+d) \times 100 = 99.5\%$ for both rMSP1a and rMSP2 ELISAs

TABLE II

Agreement between ELISAs with recombinant MSP1a and MSP2 from a Brazilian isolate (Pernambuco-Zona da Mata) of *Anaplasma marginale*, using sera from three regions of the state of Pernambuco, Brazil

ELISA	MSP1a+	MSP1a -	Total
MSP2+	516	10	526
MSP2-	2	55	57
Total	518	65	583

In the second case, the lower specificity of the competitive ELISA compared to rMSP1a and rMSP2 ELISAs may be due to the Maltose Binding Protein (MBP) fused to the MSP5, which could induce false-positive reactions due to the reaction of anti-MBP antibodies in the sera, blocking the reaction of ANAF16C1 MoAb, even after the adsorption step of test sera with MBP (Torioni de Echaide et al. 1998). This protein is absent in rMSP1a and rMSP2 ELISAs, which showed higher sensitivities and specificities. Another advantage of the rMSP1a and rMSP2 ELISAs is that there is no need for monoclonal antibodies nor for adsorption steps of test sera.

The agreement between the two tests was high, defined by a κ index of 0.89. The κ index was used in the present investigation to determine the concordance of the assays with each other and to assess the reliability of the observed agreement, as this test corrects the agreement expected by chance.

With regards to the discrepant results, the statistically superior number of MSP1a-/MSP2+ sera over the MSP2-/MSP1a+ sera may be attributed to a slightly lower sensitivity or a slightly superior specificity of rMSP1a ELISA. However, this is a low magnitude difference, which doesn't represent a clear advantage of an assay over the other, as the agreement between the ELISAs was high.

The high sensitivities and specificities of rMSP1a and rMSP2 ELISAs, associated with the recognition of both antigens by sera from cattle of different regions of Brazil, suggest that these serological tests can be used for epidemiological studies. The similar performances of both tests, with a high agreement in the results, suggest that both tests can be used indistinctly for the detection of *A. marginale* antibodies.

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