Evaluation of an Enzyme-Linked Immunosorbent Assay Using Recombinant Major Surface Protein 5 for Serological Diagnosis of Bovine Anaplasmosis in Venezuela

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An indirect enzyme-linked immunosorbent assay (ELISA) was developed for the serological diagnosis of bovine anaplasmosis with purified recombinant major surface protein 5 (MSP5) of *Anaplasma marginale* produced in *Escherichia coli*. Serum antibody responses against MSP5 were detected in calves experimentally infected with *A. marginale* as early as 21 days postinfection and reached maximum titers at 28 days postinfection. The MSP5 ELISA performed with serum samples taken from field cattle from different regions of Venezuela showed a seroprevalence of 47%, which seems to be in accordance with the reported epidemiological status of bovine anaplasmosis in Venezuela. Positive results obtained in the MSP5 ELISA were further confirmed by immunoblotting, with the recombinant MSP5 as the antigen. Thus, these results confirmed the importance of MSP5 as a suitable antigen for the serological diagnosis of bovine anaplasmosis.

*Anaplasma marginale* is an arthropod-borne, rickettsial hemoparasite of ruminants. It causes a disease called bovine anaplasmosis, which is characterized by anemia, weight loss, and death. The disease occurs worldwide, especially in tropical and subtropical regions.

Several serological tests have been described for the diagnosis of bovine anaplasmosis, including capillary tube agglutination, card agglutination, indirect immunofluorescence, radioimmunoassay, and enzyme-linked immunosorbent assay (ELISA) (5, 7–10, 16, 18, 19, 21, 23, 28, 30–32, 35). The major drawback of these tests is that the antigens used are crude mixtures of *A. marginale* bodies and erythrocyte material (13, 14). Indeed, *A. marginale* is an obligatory parasite of bovine erythrocytes, and efficacious methods for culturing *A. marginale* are not yet available. Therefore, to produce antigenic preparations, experimental infection of calves is needed, followed by purification of *Anaplasma* bodies from erythrocytes. This implies that antigen production is costly and lacks standardization. The current serological tests also lack acceptable specificity and/or sensitivity, particularly in the detection of carrier cattle (1, 5, 7–9, 16).

To improve the serological diagnosis of bovine anaplasmosis, research has focused on the identification and characterization of *A. marginale* antigens by gene cloning and production of recombinant proteins which would be suitable for use in developing standardized diagnostic tests. Among the antigens of interest, five major surface proteins (MSPs) have been described (1–4, 11, 12, 17, 22, 24, 25, 27, 33). These MSPs have been expressed to high levels and purified from recombinant *Escherichia coli*. MSP1, -2, and -4 have potential for the development of vaccines, and MSP3 and -5 have potential for use in improved diagnostic assays (3, 12, 20, 27).

In the present study, we evaluated MSP5 (19 kDa) as a diagnostic antigen for bovine anaplasmosis in Venezuela, where a high prevalence (about 50%) of the disease has been reported (10). The choice of MSP5 was justified, since this protein has been reported to be conserved in all recognized *Anaplasma* species, and at least one conserved epitope of this protein (defined by monoclonal antibody [MAB] ANAF16Cl) has been found to be immunodominant in infected cattle (3, 11, 12, 22, 33).

**msp5 gene analysis.** *A. marginale* initial bodies were isolated from erythrocytes from a splenectomized calf experimentally infected with a Venezuelan *A. marginale* Táchira strain. Briefly, the calf was inoculated intravenously with 4 ml of cryopreserved infected blood (15) showing about 60% rickettsemia. Sixty days later, blood samples were taken and *A. marginale* bodies were isolated from infected erythrocytes by differential centrifugation as described previously (26). Genomic DNA was isolated from the *A. marginale* bodies by standard procedures (4). For PCR, 20-mer primers were designed to amplify the entire *msp5* gene without its signal sequence (to further produce the mature MSP5) but with its putative transcription terminator sequence according to the reported *msp5* nucleotide sequence (33). These primers were 19A (5′-GGGGTACTCCTA-3′) and 19B (5′-GGGTGTCTCAGCC CAGCT-3′). PCR was performed as described previously (34). Briefly, amplification reaction mixtures were prepared in volumes of 100 μl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mg of gelatin per ml (1× PCR buffer; Appligene, Ilkirch, France), 200 μM (each) deoxynucleoside triphosphate, 1 μM (each) primer, 100 ng of genomic DNA, and 2.5 U of Taq DNA polymerase (Appligene). The temperature cycling for the amplification was performed in a GeneAmp PCR system 9600 thermocycler (Perkin-Elmer) as follows. Cycle 1 was 94°C for 5 min (denaturation); the next 30 cycles were 62°C for 30 s (annealing),
70°C for 30 s (extension), and 94°C for 30 s (denaturation); and the last cycle was 62°C for 30 s (annealing) and 70°C for 10 min (extension). Identity of the PCR-amplified product (714 bp) with msp5 was first controlled by restriction digestion with the following restriction enzymes: A1II, EcoRI, EcoRV, HaeIII, HindIII, SallAI, SphI, SylI, and TaqI (Appligene). The sizes of the restriction products run on agarose gel were as expected according to the A. marginale msp5 published nucleotide sequence (33) (data not shown). DNA sequencing of the PCR product revealed that only 8 bp on the entire nucleotide sequence differed from the msp5 published sequence (data not shown). These results are in accordance with the high level of conservation of MSP5 in Anaplasma spp. reported previously (22, 33).

Recombinant MSP5 production and purification. The PCR-amplified msp5 gene was first cloned in plasmid pCRII (TA cloning kit; Invitrogen, San Diego, Calif.) according to the manufacturer’s instructions, resulting in plasmid pAR1902 (insert noncoding orientation relative to the P lac promoter). E. coli INVαF’ (TA cloning kit; Invitrogen) was used as the host strain. Plasmid isolation and further subcloning procedures were performed as described by Sambrook et al. (29). Insert orientation was determined by the sizes of fragments produced after double digestion with SphI and XhoI. The KpnI-XhoI fragment of plasmid pAR1902, gel purified with the GeneClean kit (Bio 101, La Jolla, Calif.), was further ligated into plasmid pTrcHisC (Xpress system; Invitrogen), cut by KpnI and XhoI, and gel purified with the GeneClean kit, resulting in plasmid pAR1903 [insert coding orientation relative to the P lac promoter]. E. coli JM109 (Promega, Madison, Wis.) was used as host strain. The Xpress system (Invitrogen) allows the production of recombinant proteins fused to six histidine residues at the N-terminal end of the protein, facilitating the purification of the recombinant protein by immobilized metal affinity chromatography. For recombinant MSP5 production and purification, an overnight culture of E. coli JM109 carrying plasmid pAR1903 grown in liquid selective Luria-Bertani medium containing 50 μg of ampicillin per ml was adjusted to an optical density at 600 nm of 0.1 in 50 ml of fresh liquid selective Luria-Bertani medium. After 2 h of growth at 37°C (optical density at 600 nm reached approximately 1.0), isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, Mo.) was added at a final concentration of 1 mM. The cultures were allowed to grow for 6 more h, and the E. coli cells were recovered by centrifugation at 8,000 × g for 20 min. Recombinant protein production in E. coli was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie blue staining, and immunoblotting with an anti-MSP5 MAb as described previously (6). As shown in Fig. 1, recombinant MSP5 (detected in immunoblotting by an anti-MSP5 MAb ANAF16C1) was the most abundant protein in the E. coli (pAR1903) whole-cell lysate. Recombinant protein purification was performed under denaturing conditions by immobilized metal affinity chromatography with the ProBond resin of the Xpress system according to the manufacturer’s instructions. Purity was assessed by SDS-PAGE and Coomassie blue staining. Although the eluate from the affinity column still contained some minor E. coli protein bands (Fig. 1), the degree of purity of recombinant MSP5 appeared to be satisfactory to develop the MSP5 ELISA.

Recombinant MSP5 ELISA. Conditions for optimal specificity and sensitivity were determined by testing negative control sera, sera from calves experimentally infected with the Venezuelan A. marginale Táchira strain, and anti-MSP5 MAb ANAF16C1 on microtiter plates coated with different concentrations of purified recombinant MSP5. The optimal serum dilution was determined as well. Consequently, the recombinant MSP5 ELISA was performed as follows. Ninety-six-well polystyrene plates (Nunc, Roskilde, Denmark) were coated by passive adsorption of recombinant MSP5, used at a concentration of 1 μg/ml (100 μl per well) and diluted in phosphate-buffered saline (pH 7.2), overnight at room temperature. The wells were emptied and washed five times with washing solution (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France), and then nonspecific binding sites of wells were blocked by incubation for 1 h at 37°C with phosphate-buffered saline containing 5% skim milk (100 μl per well). After five additional washings, the serum samples diluted 1/200 in sample dilution buffer (Sanofi Diagnostics Pasteur) were applied (100 μl per well). Following incubation for 1 h at 37°C, the wells were again washed and filled with 100 μl of horseradish peroxidase anti-bovine immunoglobulin conjugate (Sanofi Diagnostics Pasteur) diluted 1/50,000 in washing solution containing 10% horse serum. After incubation for 1 h at 37°C, the conjugate solution was discarded and the plates were washed with washing solution. The wells were filled with 100 μl of substrate solution containing TMB (3,3′,5,5′-tetramethylbenzidine) (Sanofi Diagnostics Pasteur), and the plates were left at room temperature in darkness for 30 min. Color development was stopped by adding 50 μl of stopping solution (H2SO4 [2 M]) (Sanofi Diagnostics Pasteur) per well. A450 values were then recorded with an EL312 plate reader (Bio-Tek Instruments, Highland Park, Vt.) which was interfaced with a computer.

The cutoff of the MSP5 ELISA was determined by testing 100 negative control serum samples (taken from cattle in regions of France where cases of bovine anaplasmosis have not yet been reported) (data not shown). The mean absorbance value was 0.212, with a standard deviation of 0.116. Animals which presented serum antibody reactivities with absorbance values above 0.560 (i.e., mean absorbance value for negative sera + 3 standard deviations) were scored as positive.

The three calves experimentally infected with the Venezuelan A. marginale Táchira strain developed serum antibodies from the 21st day after experimental infection (Fig. 2). The antibody reactivities reached maximum absorbance values from
Calves experimentally infected with *A. marginale* formed as described previously (6). The amount of MSP5 load-immunoblotting with purified recombinant MSP5 were performed as described previously (6). The antibody kinetics were quite similar among the three experimentally infected calves. Thus, the recombinant MSP5 ELISA appeared to be convenient for the early serological diagnosis of bovine anaplasmosis.

Among serum samples taken from 137 bovines from different regions representative of the bovine population of Venezuela, 64 were found to be positive in the recombinant MSP5 ELISA. The range of absorbance values, from 0.563 to 2.469, showed a certain degree of heterogeneity in the antibody responses against MSP5 (Table 1). Nevertheless, most of these positive animals showed serum antibody reactivities with absorbance values above 1.0. The high number of positive animals detected (46.7%) seems to be in accordance with previously reported seroprevalence (57.7%) in Venezuela by an ELISA. The range of absorbance values, from 0.563 to 2.469, showed a certain degree of heterogeneity in the antibody responses against MSP5 (Table 1). Nevertheless, most of these positive animals showed serum antibody reactivities with absorbance values above 1.0. The high number of positive animals detected (46.7%) seems to be in accordance with previously reported seroprevalence (57.7%) in Venezuela by an indirect immunofluorescence assay (10). Fifty-eight percent of the serum samples tested in the present study were positive by indirect immunofluorescence.

**Recombinant MSP5 immunoblotting.** SDS-PAGE and immunoblotting with purified recombinant MSP5 were performed as described previously (6). The amount of MSP5 loaded per lane on the gel was 1 μg. The dilution used for bovine sera was 1/50. The conjugate used to reveal bound bovine serum antibodies in immunoblotting was peroxidase-conjugated anti-bovine immunoglobulin G (Jackson ImmunoResearch Laboratories, Baltimore, Md.). Anti-MSP5 MAb ANAF16C1 (lane 1), 0 (lane 2), 7 (lane 3), 14 (lane 4), 21 (lane 5), 28 (lane 6), 35 (lane 7), 42 (lane 8), and 63 (lane 9) of infection; lane 10, immunoblotting with anti-MSP5 MAb ANAF16C1.

The sera of the three calves experimentally infected with *A. marginale* showed antibody reactivities against recombinant MSP5 in immunoblotting as early as 21 days postinfection, thus confirming the results of the recombinant MSP5 ELISA (Fig. 2 and 3 and data not shown). Of the 64 serum samples from Venezuelan cattle that were found to be positive in the MSP5 ELISA, 57 reacted positively with the MSP5 band in immunoblotting (data not shown).

In conclusion, both the recombinant MSP5 ELISA and the immunoblotting results confirmed the importance of MSP5 as a suitable antigen for the serological diagnosis of bovine anaplasmosis. However, to obtain a sensitivity close to that obtained in indirect immunofluorescence, further improvement, which might be achieved by the inclusion of MSP3 as a diagnostic antigen, is needed (1, 17). MSP3 has been found to be particularly useful for detecting cattle that are long-term carriers of *A. marginale* (17). However, as MSP3 has recently been reported to be encoded by a polymorphic, multigene family (2), it remains to be determined which *msp3* alleles could be useful for producing recombinant protein which should contain immunodominant conserved epitopes useful for diagnostic purposes.

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