

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

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A competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5 (rMSP5-cELISA) of *Anaplasma marginale* was validated in a naturally infected cattle herd in an area of eastern Oregon where *A. marginale* is endemic. The true positive and negative *A. marginale* infection status of 235 randomly selected cattle was determined by using a nested PCR (nPCR) coupled with *mSP5* sequence analysis and hybridization. Judgment of the reliability of the nPCR and hybridization for detection of persistent infections was based on three observations. First, the nPCR was able to detect as few as 30 infected erythrocytes per ml. Second, the nPCR was able to consistently detect low levels of rickettsemia in seven carrier cattle experimentally infected with *A. marginale*. Third, *mSP5* sequence analysis showed >95% identity among 30 nPCR amplicons from cattle naturally infected with field strains of *A. marginale*. The nPCR and hybridization identified 151 infected and 84 uninfected cattle among the 235 animals tested. With a cutoff point of 28%, the rMSP5-cELISA showed a sensitivity of 96% and a specificity of 95%. These results indicate that the rMSP5-cELISA can sensitively and specifically detect cattle with naturally acquired persistent *A. marginale* infections and suggest that it is an excellent assay for epidemiological studies, eradication programs, and regulation of international cattle movement.

Anaplasma marginale is a rickettsial hemoparasite transmitted to cattle biologically by ticks and mechanically by flies and fomites (2, 18, 36, 45). Following transmission, *A. marginale* invades and multiplies within mature erythrocytes. During acute anaplasmosis, rickettsemia levels exceed 10^6 infected erythrocytes per ml and the resulting disease is characterized by anemia, weight loss, abortion, and death (3, 13, 20). Recovery from acute anaplasmosis results in persistent infection characterized by repetitive cycles of rickettsemia ranging from approximately $10^{2.5}$ to 10^7 infected erythrocytes per ml (11, 14, 20). Persistently infected cattle serve as long-term reservoirs for transmission within herds (11, 22, 45).

Anaplasmosis is an economically important disease affecting dairy and beef cattle in most tropical and subtropical and many temperate countries, including the United States (8, 33, 45). Detection of persistently infected cattle is important to control the movement of infected cattle into and from disease-free regions. Microscopic examination by Giemsa stained blood smears, which is used to confirm acute anaplasmosis, can only detect levels of $>10^6$ infected erythrocytes per ml (1, 15). Subinoculation of *A. marginale*-infected erythrocytes into susceptible, splenectomized calves has been considered the "gold

standard" for detection of persistently infected cattle, but the procedure is not practical for routine testing (25).

Serological tests, including complement fixation and card agglutination, have been the most commonly used methods to detect *A. marginale*-infected cattle in the field (31, 34, 38) and are accepted as the basis for interstate and international movement of animals (43). In addition, the immunofluorescent-antibody test and enzyme-linked immunosorbent assay (ELISA) have been utilized for epidemiological studies (9, 19, 28). All of these current tests for antibody detection use crude antigens obtained from partially purified *A. marginale* and lack the required sensitivity or specificity for a reliable diagnosis (4, 9, 27, 28).

Major surface protein 5 (MSP5) is a 19-kDa surface protein highly conserved among different strains of *A. marginale* and *A. ovis* and in *A. centrale* (30, 37, 41). Both the native protein and a recombinant MSP5 (rMSP5) fused to maltose binding protein (MBP) (21) share an epitope recognized by monoclonal antibody (MAb) AnaF16C1 (41). A competitive ELISA (cELISA) based on serum antibody inhibition of MAb AnaF16C1 binding to rMSP5 has been developed (21). The rMSP5-cELISA has a demonstrated specificity of 100% (99% confidence interval of 98 to 100%) with sera from uninfected cattle in regions where *A. marginale* is not endemic (21). Additionally, under experimental conditions, the cELISA will detect anti-*A. marginale* antibodies early in acute anaplasmosis and during long-term persistence (21). However, the true sensitivity has not been defined with a statistically significant number of animals known to be positive, and neither sensitivity

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nor specificity has been evaluated for cattle from a region where *A. marginale* is endemic (21). Therefore, in this study, we tested the hypothesis that the rMSP5-cELISA will sensitively and specifically identify cattle persistently infected with *A. marginale* in a region where *A. marginale* is endemic. To test this hypothesis required determination of the true infection status of cattle within an area where *A. marginale* is endemic. For this purpose, we optimized a nested PCR (nPCR), coupled with sequence analysis and hybridization, to identify *A. marginale msp-5* DNA in blood. Each of 235 cattle in a naturally *A. marginale*-infected herd was identified as *A. marginale* infected or uninfected by using the nPCR, and sera collected at the same time point was tested for antibodies by using the rMSP5-cELISA.

MATERIALS AND METHODS

nPCR procedure. The nPCR was optimized to identify *A. marginale msp5* DNA from blood. All reagents were handled in a laminar-flow hood by using aerosol-resistant pipette tips (ART, M&P; Molecular Bio-Products, Inc.). Blood samples were thawed, and 300 μ l was used for DNA isolation in accordance with the manufacturer's (Purogene, Gentra Systems, Inc.) recommendations. DNA of each sample was resuspended in 100 μ l of hydration solution to give approximately 100 μ g of DNA per ml. Primers were designed by using the published sequence of *msp5* from *A. marginale* Florida (GenBank accession no. M93392) and were as follows (5'-to-3' sequence and gene location): external forward, 5'-GCATAGCCTCCCCCTCTTTC-3' (*msp5* positions 254 to 273); external reverse, 5'-TCCTCGCCTTGCCCTCAGA-3' (*msp5* positions 710 to 692); internal forward, 5'-TACACGTGCCCTACCGACTTA-3' (*msp5* positions 367 to 387). Bovine lactogen primers (*bPL*) used in the nPCR for *msp5*-negative samples to ensure the presence of amplifiable DNA in the sample were as follows (5'-to-3' sequence and gene location): external forward, 5'-GATACCATTGGC AATATACAAC-3' (*bPL* positions 91 to 111); external reverse, 5'-GAGCCAC TCTGAGATGATG-3' (*bPL* positions 458 to 440); internal forward, 5'-GTTA GCCTGGGGTTAGCAA-3' (*bPL* positions 420 to 438). Two PCR rounds in a final volume of 25 μ l were carried out with a commercial kit (PCR master kit; Boehringer Mannheim) in a Perkin Elmer thermal cycler. The PCR master solution contained 25 U of *Taq* DNA polymerase in 20 mM Tris-HCl, 3 mM MgCl₂, 0.01% (vol/vol) Brij 35, and 0.4 mM each deoxynucleoside triphosphate (pH 8.3) in a volume of 0.5 ml. The first round used 12.5 μ l of the master solution, 1 μ l of 20 μ M *msp5* external primers, 5.5 μ l of water, and 5 μ l of purified DNA. The second round of amplification used 12.5 μ l of the PCR master solution, 1 μ l of 20 μ M *msp5* external reverse and *msp5* internal forward primers, 9.5 μ l of sterile water, and 1 μ l of the PCR product from the first round. Cycling conditions were preheating at 95°C for 3 min and 35 cycles of 95°C for 30 s, 65°C for 58 s, and 72°C for 30 s with final extension at 72°C for 10 min for each round. PCR and nPCR products were visualized in a 2% agarose gel following electrophoresis in 0.4 M (*N*-morpholino)propanesulfonic acid buffer and staining with 0.015% ethidium bromide. A 458-bp band was expected after the PCR, and a 345-bp band was expected after the nPCR. Conditions for the bovine lactogen nPCR were identical to those described for the *msp5* PCR and nPCR. A 347-bp product was expected after the lactogen nPCR.

Sensitivity of nPCR. A calf (B9503) experimentally infected with *A. marginale* Florida was used to determine the minimal rickettsemia detected by the nPCR. Blood samples from calf B9503 and from an uninfected control cow (Z35) were obtained in acid citrate dextrose solution B and washed three times in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). The buffy coat was removed, and erythrocytes were resuspended in PBS at a final concentration of 3×10^9 /ml (Coulter Counter ZM). Standardized erythrocyte samples from calf B9503 were serially diluted in uninfected erythrocytes to maintain a constant total erythrocyte count of 3×10^9 /ml. The rickettsemia of the diluted samples ranged between 3×10^7 (in undiluted samples) and 3×10^{-2} (in 10^{-9} -diluted samples) infected erythrocytes per ml. Samples were maintained frozen at -20°C until processed for nPCR.

Consistent detection of *A. marginale* in persistently infected cattle by nPCR. Seven persistently infected cattle (no. 803, 807, 808, 810, 811, 813, and B12) experimentally inoculated with *A. marginale* Florida 15 months previously were used to determine the ability of the nPCR to consistently detect low rickettsemia levels within a cycle. Blood samples from these long-term-infected cattle and from control cow Z35 were obtained in two sets on 3 consecutive days each at a 28-day interval. Samples were prepared as described above and kept frozen at -20°C until processed for nPCR.

Evaluation of rMSP5-cELISA in cattle from an *A. marginale*-infected herd. The rMSP5-cELISA was evaluated in a Hereford and Tarantais beef herd located in an area of eastern Oregon where anaplasmosis is endemic. Acute anaplasmosis had been previously observed in the herd, but the current prevalence was unknown. Persistent *A. marginale* infection in the herd was confirmed by an initial random sampling of 52 adult cattle in March 1996. At that time, 38% of the cattle were seropositive (data not shown). Based on this preliminary data

and assuming 90% sensitivity and specificity for the cELISA, 235 randomly selected cattle were sampled in August 1996 for determination of the true sensitivity and specificity with narrow confidence limits (7, 12, 21). Ten milliliters of blood in acid citrate dextrose solution B and 10 ml of blood for serum collection were obtained by jugular venipuncture from each of the 235 cattle. Whole-blood and serum samples were maintained frozen at -20°C until processed by nPCR and rMSP5-cELISA, respectively.

Sequence analysis. To determine the degree of homology among *msp5* sequences of *A. marginale* in naturally infected cattle and with the reference *msp5* gene of the Florida strain, a sample of nPCR products was randomly selected from those that showed a 345-bp DNA size. Expecting that 90% of the 345 bp nPCR product from natural infections would have at least 90% identity to *msp5* of *A. marginale* Florida, sequence information from 30 animals would enable determination of the extent of sequence variation in the population with 95% confidence (7, 14, 41). The selected nPCR products were purified through a silica gel column (Qia Quick; Qiagen) and sequenced. All sequences were compared by using the Pileup system of the Genetics Computer Group package from the University of Wisconsin, version 9.0 (6).

Hybridization assays. A probe designed to hybridize to a 294-bp *msp5* sequence internal to the 345-bp nPCR product was constructed by using the following primers: internal forward, 5'-TACACGTGCCCTACCGACTTA-3' (*msp5* positions 367 to 387); internal reverse, 5'-ATACCTGCCTTCCCATTG AT-3' (*msp5* positions 660 to 640). The probe was labeled with digoxigenin-11-ddUTP in the PCR and was used in Southern blot assays in accordance with the manufacturer's (Genius; Boehringer Mannheim, Indianapolis, Ind.) recommendations. For Southern blot assays, nPCR products were electrophoresed in a 2% agarose gel and transferred to a positively charged nylon membrane by alkaline (0.4 N NaOH) capillary blotting. Membranes were incubated in a prehybridization solution at 50°C for 4 h prior to addition of the probe and then incubated in a hybridization solution with the labeled *msp5* probe overnight at 50°C. The membrane was washed twice at room temperature in 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate. Two high-stringency washes were performed at 70°C for 15 min in 0.5 \times SSC-0.1% sodium dodecyl sulfate. Detection of bound probe was carried out by using chemiluminescence.

rMSP5-cELISA. Serum samples from the 235 cattle and from experimentally infected and uninfected calves used as controls were evaluated by the rMSP5-cELISA. The test was performed as previously described (21). Individual wells of flat-bottom plates (Immulon II; Dynatech Laboratories) were coated with 1 μ g of amylose resin-purified rMSP5-MBP fusion protein in 100 μ l of carbonate-bicarbonate coating buffer, pH 9.6. After overnight storage at 4°C and warming to room temperature, plates were blocked with 0.5 M PBS containing 10 g of fraction V bovine serum albumin, 15 g of glycine, and 40 g of sucrose per liter. Following four washes with 200 μ l of PBS per well, test sera adsorbed for 30 min with 5 μ g of dried MBP per 100 μ l of serum were added in duplicate and incubated for 30 min. After four washes with PBS, 0.08 μ g of horseradish peroxidase-conjugated MAb ANA F16C1 in 0.5 M PBS-1% fraction V bovine serum albumin was added to each well and incubated for 15 min. After an additional four washes with PBS, 50 μ l of 0.5- μ g/ml *o*-phenylenediamine dihydrochloride in substrate buffer (0.2 M Na₂HPO₄, 0.1 M citric acid) was added to each well and incubated for 10 min. Reactions were stopped with 25 μ l of 2 N NH₂SO₄, and optical density at 492 nm (OD₄₉₂) was determined with a Multiskan Plus II reader. Results were expressed as percent inhibition based on the following formula: $100 - [(mean OD_{492} \text{ of test serum} \times 100) / mean OD_{492} \text{ of negative control}]$.

RESULTS

Sensitivity of nPCR. The sensitivity of the nPCR was determined by using 10-fold dilutions of *A. marginale*-infected erythrocytes into a 3×10^9 /ml normal-erythrocyte suspension. Infected-erythrocyte levels ranged from a starting concentration of 3×10^7 /ml in undiluted samples to a final concentration of 3×10^{-2} /ml in samples diluted 10^{-9} . The lowest *A. marginale* dilution with a detectable primary PCR product of 458 bp was 10^{-3} , while an nPCR product with the expected 345-bp size was detectable at a 10^{-6} dilution (Fig. 1). This sensitivity is equivalent to 30 parasites per ml of blood for the nPCR (approximately 10^{-6} % rickettsemia).

Consistent detection of *A. marginale* in persistently infected cattle with nPCR. After acute *A. marginale* infections, cyclical multiplication of the rickettsiae in persistently infected cattle is characterized by fluctuations between $10^{2.5}$ and 10^7 infected erythrocytes per ml with low levels of rickettsemia for about 5 to 8 days of every 5 to 6-week cycle (11, 14, 20). Therefore, the ability of the nPCR to detect *A. marginale* in persistently infected cattle with daily consistency and over the time period

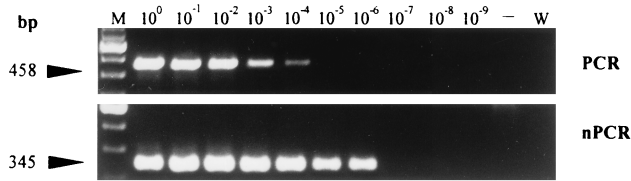


FIG. 1. Minimum *A. marginale* rickettsemia detectable with PCR and nPCR. Tenfold dilutions, starting at 3×10^7 infected erythrocytes per ml, were prepared in a suspension of 3×10^9 normal erythrocytes per ml. W, water; M, molecular size markers; —, uninfected control.

during which rickettsemia levels fluctuate periodically was evaluated. For this experiment, two sets of blood samples, each obtained on 3 consecutive days, were obtained at a 28-day interval from seven cattle experimentally infected 15 months previously with *A. marginale* Florida. The primary PCR detected zero to three infected cattle on each of the 6 days on which they were analyzed, while the nPCR was able to detect all seven persistently infected cattle on each of the 6 days on which they were analyzed (Fig. 2).

msp5 sequence analysis. To ensure that hybridization is a valid method to verify the specificity of nPCR-amplified products, *msp5* sequence variation was determined in a statistically significant number of animals in the study herd. Analysis of the sequences of 30 randomly selected nPCR products with the expected 345-bp size showed over 95% identity of the sequences compared with the Florida strain *msp5* sequence (data not shown). Of the 30 samples, 2 were identical to the Florida strain *msp5* sequence, while the remaining 28 had consistent single-base-pair changes. Based on these results, hybridization was considered a valid method of confirming the specificity of nPCR amplicons.

***A. marginale* status of cattle from the herd in which *A. marginale* is endemic.** To determine the true *A. marginale* infection status of cattle from the selected herd, blood samples from all 235 cattle were analyzed initially by nPCR, followed by hybridization if any ethidium bromide-stained bands were detectable. An animal was defined as a true positive when nPCR resulted in a product of 345 bp that hybridized to the *A. marginale msp5* probe. An animal was defined as a true negative when there was no amplicon or when the nPCR amplicon did not hybridize

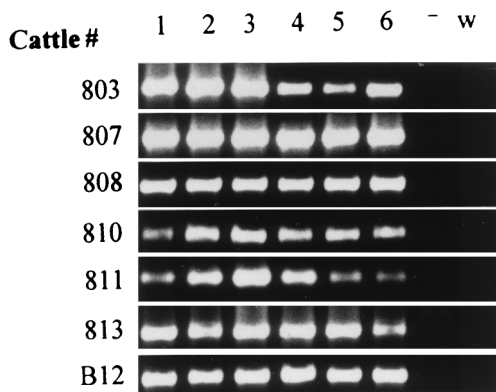


FIG. 2. Consistent detection of *A. marginale* infection in seven persistently infected cattle by nPCR. DNA was obtained from the blood of cattle experimentally inoculated with *A. marginale* Florida 15 months previously. Animal identities are indicated on the left. —, uninfected control; w, water. Lanes 1, 2, and 3 represent samples obtained on 3 consecutive days. Lanes 4, 5, and 6 are samples obtained 28 days later, again on 3 consecutive days.

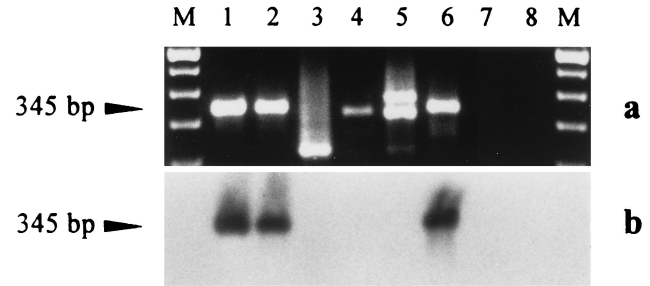


FIG. 3. Southern blot of nPCR products using an *A. marginale msp5* probe. nPCR products from cattle in an *A. marginale*-infected herd (lanes 1 to 5), an infected control (lane 6), an uninfected control (lane 7), and a no-DNA control (lane 8) were electrophoresed in 2% agarose. Gels were stained with ethidium bromide (a), and nPCR products were transferred to nylon membrane and hybridized with an *msp5* probe (b). Lanes M contained molecular size markers.

to the *msp5* probe. When no amplicon was present with *msp5* primers, the presence of amplifiable DNA was confirmed in all of the animals by using primers for the bovine lactogen gene. A 345-bp band in the nPCR hybridized to the *msp5* probe in dot and Southern blots in 151 (64%) of 235 samples. A representative Southern blot is shown in Fig. 3. The 345-bp nPCR product specifically hybridized to the *msp5* probe (Fig. 3, lanes 1 and 2). Occasionally, the nPCR product had multiple bands (Fig. 3, lane 5) or a single band with a smaller apparent molecular size than 345 bp (Fig. 3, lanes 3 and 4), none of which hybridized to the probe. In total, 84 animals (36%) did not have an nPCR product or had a product which did not hybridize to the probe. All samples which did not result in a band in the *msp5* nPCR had amplifiable DNA, as determined by the presence of a 347-bp nPCR product from the bovine lactogen gene (data not shown).

rMSP5-cELISA. Serum samples from all 235 cattle were analyzed by rMSP5-cELISA. Percentages of inhibition by the serum samples were widely distributed between 0 and 99% (Fig. 4). For infected (nPCR-positive) cattle, these values ranged from 16 to 99%, while those for uninfected (nPCR-negative) cattle ranged from 0 to 42%, with the exception of one serum with 75% inhibition. Overlap of the percent inhibition of infected and uninfected cattle ranged from 16 to 42% (Fig. 4). Clearly, the cutoff point selected to discriminate between true-positive and true-negative sera is arbitrary and will affect the calculation of sensitivity and specificity. The cutoff point selected for this study as the threshold which best dis-

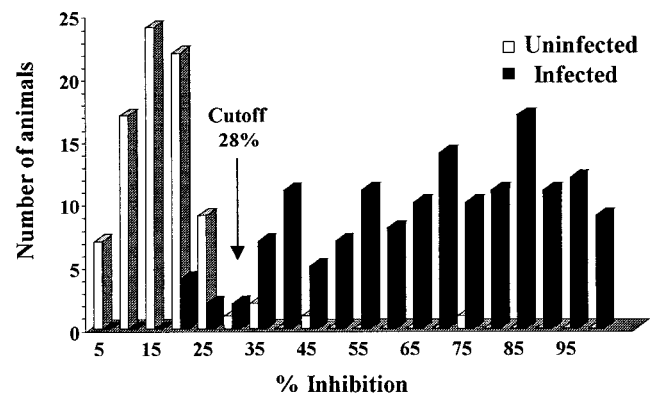


FIG. 4. Frequency distribution of rMSP5-cELISA percent inhibition by sera from cattle in an *A. marginale*-infected herd.

TABLE 1. Relationship between *A. marginale*-infected and uninfected cattle^a and rMSP5-cELISA positive and negative results^b for 235 samples obtained in a naturally infected herd of cattle

MSP5-cELISA	<i>A. marginale</i>		Total no. of samples
	Infected	Uninfected	
+	145	4	149
-	6	80	86
Total	151	84	235

^a Based on nPCR and hybridization.

^b Cutoff, 28% inhibition.

criminated infected from uninfected cattle was 28% inhibition. With this cutoff point, the agreement between the nPCR and rMSP5-cELISA results was 96% with a kappa value of 0.91, indicating excellent agreement (12, 39). Six of 151 true-positive cattle were falsely negative by the cELISA, with 16, 19, 20, 20, 23, and 25% inhibition. Of 84 true-negative cattle, 4 were false positives by the rMSP5-cELISA, with 32, 32, 42, and 75% inhibition (Table 1). Thus, with a cutoff of 28% inhibition, the sensitivity of the rMSP5-cELISA was 96% (95% confidence interval of 91 to 98%) (12) and its specificity was 95% (95% confidence interval of 88 to 98%) (12). The prevalence of *A. marginale* in the study herd, based on nPCR-hybridization, was 64%. Based on this, the rMSP5-cELISA had predictive values for positive and negative results of 96 and 93%, respectively.

Retrospectively, 30 cattle were sampled at both the pre-screening time in March and the sampling in August 1996. Of these 30, 15 were positive by nPCR at both time points, 12 were negative at both samplings, and 3 were negative in March but positive in August. None were positive in March and negative in August. Based on this result, the incidence of anaplasmosis was 20% over this 6-month period. With the exception of nPCR-positive cow 1012, which showed a false-negative rMSP5-cELISA reaction at both sampling dates, the remaining 29 cattle sampled at both dates were correctly detected by the rMSP5-cELISA.

DISCUSSION

In this study an rMSP5-cELISA was validated by using an nPCR coupled with analysis of the amplicon by DNA sequencing and hybridization to determine the true *A. marginale* status of naturally infected cattle. The reliability of the nPCR and hybridization as a gold standard is based on three observations. First, the nPCR was able to detect 30 infected erythrocytes per ml of blood, which is a 10- to 100-fold increase in sensitivity over the previously described quantitative PCR assay, RNA probe assay, PCR-ELISA, and hybridization assay (10, 14–16). Second, nPCR was able to consistently detect low levels of rickettsemia in seven persistently infected cattle both on a daily basis and at an interval of 28 days during which the rickettsemia levels were predicted to cycle. Third, *mSP5* sequence analysis showed high conservation among 30 nPCR amplicon sequences from naturally infected cattle in the herd in which *A. marginale* is endemic. The sequences were over 95% identical to the reference, *A. marginale* Florida, validating the use of hybridization to confirm the specificity of nPCR amplicons. A similar level of *mSP5* sequence conservation has recently been demonstrated at two separate peaks of rickettsemia within one animal (14). Thus, the nPCR with hybridization is a

highly sensitive and specific method of detecting *A. marginale*-infected animals.

After primary *A. marginale* infection, cattle which survive acute anaplasmosis remain persistently infected for life, independently of re-exposure to the rickettsia (10, 32), serving as reservoirs for transmission by ticks in the field (11, 22, 45). Accurate immunologic identification of persistently infected animals in areas where *A. marginale* is endemic is difficult. First, persistent infection is characterized by very low-level cyclic multiplication of *A. marginale* with rickettsemia fluctuating between $10^{2.5}$ and 10^7 infected erythrocytes/ml (14, 20). Antibody levels in cattle persistently infected at this low level are difficult to detect with current serological tests (17, 29, 42). Second, cattle in regions where *A. marginale* is endemic can be exposed to multiple rickettsial and ehrlichial agents that may induce antibodies cross-reactive with *A. marginale* proteins (40), causing false-positive serology results (5, 24, 35). This problem has been encountered with the 32-kDa diagnostic antigen of *Cowdria ruminantium* (26). In contrast to sera from animals in areas where *C. ruminantium* is not endemic, false-positive reactions to the *C. ruminantium* 32-kDa antigen were detected in regions where *C. ruminantium* is endemic, presumably due to cross-reactivity with *Ehrlichia* spp. (26).

The previously reported specificity of 100% for the rMSP5-cELISA was obtained by using 261 serum samples from cattle known to be uninfected in *A. marginale*-free regions (21). In this study, with 28% inhibition as the cutoff point, the specificity of the rMSP5-cELISA in the study herd in an area where *A. marginale* is endemic was 95%. As with *C. ruminantium*, the cause of the 5% false positives in this study could be due to cross-reactivity with other, related organisms in the region. False-positive reactions might also be due to recently resolved *A. marginale* infections, with persistence of serum antibodies. However, there are no reports of spontaneous clearance with sterile immunity under natural conditions (23, 44), and antibiotic use was not reported in the herd during the study period. Additionally, in this study, there was no evidence of *A. marginale* clearance in any of 15 infected cattle found to be positive by the nPCR and rMSP5-cELISA in both March and August of the same year. Finally, a false-positive reaction might be a result of a specific reaction between anti-MBP antibodies present in bovine serum and the MBP-rMSP5 antigen, blocking the binding of MAb AnaF16C1 to rMSP5 by steric hindrance. This has previously been demonstrated, requiring an MBP adsorption step prior to addition of sera to the test antigen (21). While the adsorption of sera was performed as previously described to eliminate nonspecific reactions (21), we cannot rule out the possibility that residual MBP antibodies provided some inhibition which would result in false-positive reactions.

The rMSP5-cELISA was able to detect cattle naturally infected with *A. marginale* with a sensitivity of 96%. Other serological tests for anaplasmosis, including card agglutination and complement fixation, have reported sensitivities of 84 and 79%, respectively (17). However, these values were not based on stringently defined true-positive animals, as was done in this study. Most of them used microscopic detection of *A. marginale* or comparison with other serology results as a gold standard (4, 9, 17, 29). A total of six false-negative reactions (4%) were detected by the rMSP5-cELISA. These might occur as a consequence of recent primary infection. Under experimental conditions, the rMSP5-cELISA was not able to consistently detect anti-*A. marginale* antibodies until 16 to 27 days postinoculation, depending on the dose and route of inoculation (21). While the ability of nPCR to detect early infection was not evaluated, early detection of *A. marginale* infection is ex-

pected since an RNA probe with less sensitivity than the nPCR identified the rickettsia 2 days after inoculation (10). Thus, cattle with a recently acquired infection would be identified positively by the nPCR and as falsely negative by the rMSP5-cELISA prior to days 16 to 27. Low responders or nonresponders also should be considered as a possible cause of false-negative reactions. This could explain the results obtained with one infected animal (no. 1012), which was falsely identified as negative by the rMSP5-cELISA at both sampling times.

The rMSP5-cELISA has high sensitivity and specificity when stringent definitions are used to determine true-positive and -negative cattle in a herd in which *A. marginale* is endemic. The results suggest that it is an excellent assay for eradication programs and regulation of interstate and international movement of cattle. Additionally, the ability of the rMSP5-cELISA to accurately detect individually infected animals will facilitate epidemiologic investigations, particularly in areas where the rickettsia is expanding through movement of infected animals into disease-free regions.

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