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 $10^9$ 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 rMPS5-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...
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 msp5 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, MBP; Molecular Bio-Products, Inc.). Blood samples were thawed, and 300 μl was used for DNA isolation in accordance with the manufacturer’s (Purogene, Gentra Systems, Inc.) recommendations. DNA of each msp5 was reisolated in 100 μl of hybridization solution to give approximately 100 μg of DNA per ml. Primers were designed by using the published sequence of msp5 from A. marginale Florida (GenBank accession no. M933392) and were 5′-to-3′ sequence of gene located in internal forward, 5′-GCAATAGCCCTCCTTTCTCT-3′ (mp5 positions 254 to 273) and external reverse, 5′-TCTCGCTGTTCCCTTCAAG-3′ (mp5 positions 710 to 692); internal forward, 5′-TACACTGGCTACCCAGCTA-3′ (mp5 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′-GATACATGGC AATATACAAAC-3′ (bPL positions 91 to 111); external reverse, 5′-GAGCCAC TCTCGAGATGATG-3′ (bPL positions 458 to 480); internal forward, 5′-GTTA GCCCTGGGTGTAAC-3′ (bPL positions 420 to 438). Two PCR rounds in a final volume of 25 μl 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 μl of the master solution, 1 μl of 20 μM msp5 external primers, 5.5 μl of water, and 5 μl of purified DNA. The second round of amplification used 12.5 μl of the PCR master solution, 1 μl of 20 μM msp5 external reverse and msp5 internal forward primers, 9.5 μl of sterile water, and 1 μl of the PCR product from the first round. Cycling conditions were preheating at 95°C for 5 min and 35 cycles of 95°C for 30 s, 65°C for 1 min, and 72°C for 2 min with a final extension at 72°C for 2 min. 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% ecdyusmid iodine. A 458-bp band was expected after the PCR and a 294-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 call (B9503) experimentally infected with A. marginale Florida was used to determine the minimum infecting dose determined by dodecyl sulfate. Two blood samples from call 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, 1.5 mM KH₂PO₄, pH 7.4). The buffy coat was removed, and erythrocytes were resuspended in PBS at a final concentration of 3 × 10⁸/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 × 10⁸/ml. The rickettsialism of the diluted samples ranged between 3 × 10⁻² (undiluted samples) and 3 × 10⁻⁷ (in 10⁻⁴-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 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 the 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′-TACACGTGCTGCCACAGCTA-3′ (mp5 positions 367 to 387); internal reverse, 5′-ATACTGGCTTCTCTCCTAG-3′ (mp5 positions 600 to 640). The probe was labeled with digoxigenin-11-deUTP 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. The membranes were hybridized overnight at 50°C with a hybridization solution with the labeled msp5 probe overnight at 50°C. The membrane was washed twice at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–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 μg of amylase resin-purified rMSP5-AT-3 capillary blotting membranes in carbonate 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 μl of PBS per well, test sera adsorbed for 30 min with 5 μg of dried MBP per 100 μl of serum were added in duplicate and incubated for 30 min. After four washes with PBS, 0.08 μg of horseradish peroxidase-conjugated MAb ANA F168C1 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 μl of 0.5 μg/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 μl of 2 N NaOH, and optical density was determined at 492 nm (OD₄₉₂) on the Microscan Plus II reader. Results were expressed as percent inhibition based on the following formula: 100/[(mean OD₄₉₂ of test serum × 100)/mean OD₄₉₂ 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 × 10⁸/ml normal erythrocyte suspension. In-vitro erythrocyte levels ranged from a starting concentration of 3 × 10⁸/ml in undiluted samples to a final concentration of 3 × 10⁻⁷/ml in samples diluted 10⁻⁴. The lowest A. marginale dilution with a detectable primary PCR product of 458 bp was 10⁻⁴, while an nPCR product with the expected 345-bp size was detectable at a 10⁻⁴ dilution (Fig. 1). This sensitivity is equivalent to 30 parasites per ml of blood for the nPCR (approximately 10⁻⁷ rickettsia).

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 10².5 and 10⁻⁷ infected erythrocytes per ml with low levels of rickettsia for about 5 to 8 days of every 5 to 6-week cycle (11, 14, 40). Therefore, the ability of the nPCR to detect A. marginale in persistently infected cattle with daily consistency and over the time period
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).

**mpl5 sequence analysis.** To ensure that hybridization is a valid method to verify the specificity of nPCR-amplified products, mpl5 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 of the 345-bp nPCR product specifically hybridized to the probe. All samples which did not result in a band in the nPCR products from cattle in an 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 mpl5 probe (b). Lanes M contained molecular size markers.

**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* mpl5 probe. An animal was defined as a true negative when there was no amplicon or when the nPCR amplicon did not hybridize to the mpl5 probe. When no amplicon was present with mpl5 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 mpl5 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 mpl5 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 mpl5 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-
TABLE 1. Relationship between A. marginale-infected and uninfected cattle* and rMSP5-cELISA positive and negative results for 235 samples obtained in a naturally infected herd of cattle

<table>
<thead>
<tr>
<th>MSP5-cELISA</th>
<th>Infected</th>
<th>Uninfected</th>
<th>Total no. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>145</td>
<td>6</td>
<td>151</td>
</tr>
<tr>
<td>−</td>
<td>80</td>
<td>84</td>
<td>164</td>
</tr>
<tr>
<td>Total</td>
<td>225</td>
<td>84</td>
<td>235</td>
</tr>
</tbody>
</table>

* Based on nPCR and hybridization.

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 rickettsia in seven persistently infected cattle both on a daily basis and at an interval of 28 days during which the rickettsia 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 rickettsia 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 rickettsia 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 95.2%. 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-
expected 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.

ACKNOWLEDGMENTS

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ERRATA

Multicenter Comparison of Roche COBAS AMPLICOR MONITOR Version 1.5, Organon Teknika NucliSens QT with Extractor, and Bayer Quantiplex Version 3.0 for Quantification of Human Immunodeficiency Virus Type 1 RNA in Plasma

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Volume 38, no. 11, p. 4034–4041, 2000. Page 4036, Table 1: Inter- and intrarun %CV and n at site A for stCA MONITOR 1.5 should read “39.3” and “22,” respectively.

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Genomic and Phylogenetetic Analysis of Hepatitis C Virus Isolates from Argentine Patients: a Six-Year Retrospective Study


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Volume 38, no. 12, p. 4560–4568, 2000. Page 4564, legend to Fig. 3, line 7: The sentence “Numbers in boxes indicate bootstrap values.” should be added.

Page 4565, legend to Fig. 4, line 4: “Shaded boxes” should read “Numbers in boxes.”

Pages 4564 and 4565: Fig. 3 and 4 should appear as shown on the following pages.