**Comparison of the complement fixation test and competitive ELISA for serodiagnosis of *Anaplasma marginale* infection in experimentally infected steers**

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**Objective**—To compare sensitivity of a complement fixation (CF) test and competitive ELISA (cELISA) for detection of *Anaplasma marginale* in experimentally infected steers.

**Materials**—40 crossbred (Angus-Simmental) steers.

**Methods**—Steers were inoculated with $2.6 \times 10^9$ *A. marginale*-infected erythrocytes (day 0). Blood samples were collected on days 9, 13, 20, 28, 34, 41, 61, 96, 126, and 156 days after inoculation. The percentage of parasitized erythrocytes (PPE) was determined by microscopic examination of stained blood films, and sera were evaluated with the CF test and cELISA by use of USDA-approved methods. Sensitivity and agreement (κ statistic) between the 2 methods were determined. Persistent infections were confirmed by inoculation of blood obtained from infected steers into susceptible, splenectomized calves.

**Results**—9 days after inoculation, sensitivity of the cELISA was 47.5%, whereas the CF test failed to identify seropositive steers. After day 13, sensitivity of the cELISA and CF test was 100% and 20%, respectively. During peak parasitemia (day 20), sensitivity of the cELISA and CF test was 100%. Thereafter, sensitivity of the CF test fluctuated between 7.5% and 37.5%, whereas sensitivity of the cELISA remained at 100%. Overall sensitivity of the cELISA and CF test was 94.8% and 76.5%, respectively (κ statistic, 0.003).

**Conclusions and Clinical Relevance**—The cELISA had superior sensitivity for serologic detection of *A. marginale*. The CF test and cELISA each had a high percentage of false-negative results during the prepatent period. These findings are relevant for export certification and anaplasmosis prevention or eradication programs.

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Anaplasmosis, caused by the rickettsial hemoparasite *Anaplasma marginale*, is one of the most prevalent tick-transmitted diseases of livestock throughout the world.\(^1\) Economic loss to the US livestock industry as a result of anaplasmosis is estimated to be $>300 million per annum.\(^1\) Anaplasmosis is currently classified in the OIE Terrestrial Animal Health Code list of notifiable diseases because of its socioeconomic impact and importance in terms of restrictions in the international trade of animals and animal products.\(^4\)

Cattle that recover from acute anaplasmosis, including those treated with recommended doses of tetracyclines, develop persistent infections that range from $10^4$ to $10^7$ infected erythrocytes characterized by cyclic rickettsemia at intervals of approximately 5 weeks.\(^5\)–\(^7\) Although infected erythrocytes are not always detectable in stained blood films during those cycles, persis-
tently infected cattle serve as reservoirs of infection for mechanical and tick-borne transmission. Therefore, serologic tests are needed for identification of *A. marginale*-infected cattle during the incubation period and persistent infections. Historically, the most widely used serologic tests for detecting *A. marginale* infections were the CF and card agglutination tests. The USDA CF method is a serum-dilution test based on hemolysis of erythrocytes as a result of fixation of complement. Titers are expressed in relation to the highest dilution of serum that has <100% hemolysis after addition of antigen. The CF test has a low sensitivity (20%) for identifying persistently infected cattle. Although the CF test is still offered by many accredited veterinary diagnostic laboratories, it has been considered unreliable for use in certifying cattle that are free of *A. marginale* for interstate and international movement of animals.

On the basis of the manufacturer’s information, a cELISA, the development of which was reported elsewhere, has an improved sensitivity of 96% and specificity of 95% when used to identify persistently infected cattle. Results are measured in terms of the percentage of inhibition (typically 30%) relative to a standardized positive control sample. To our knowledge, sensitivity of the cELISA during the incubation period of *A. marginale* infections has not been reported. This information is important for identification of infected cattle and certification of infection-free cattle prior to export. The purpose of the study reported here was to compare sensitivity of the CF test and cELISA for detection of antibodies in experimentally infected steers during the prepatent, acute, recovery, and carrier stages.

**Materials and Methods**

**Cattle**—Forty healthy crossbred (Angus-Simmental) beef steers (from an initial pool of 46 steers) were enrolled in the study. Cattle used in the study reported here were used in another study in which 3 oxytetracycline regimens for the treatment of persistent *A. marginale* infections in beef cattle were tested. Steers were procured from a herd used for teaching and research at Iowa State University and were housed at the Iowa State University Beef Nutrition Farm. Steers were approximately 160 to 230 days old and weighed 214 to 346 kg at the time of the study.

The study was conducted between November 5, 2002, and April 15, 2003, to minimize the risk of vector-borne transmission of *A. marginale* among test cattle. Blood samples were tested for anti-*A. marginale* antibodies by use of a cELISA and slides of stained blood films were examined for evidence of anaplasmosis. The PCV of each steer was determined to provide baseline data. The protocol for this study was approved by the Committee on Animal Care at Iowa State University (No. 5-2-5172-B).

**Housing and husbandry**—Initially, steers were grouped in 8 pens, each of which contained 5 or 6 steers of similar size. Steers were inoculated with an isolate of *A. marginale* and then assigned to randomized treatment groups. Steers were regrouped on the basis of treatment into pens containing 5 steers/pen. Each treatment group of 10 steers was housed in 2 adjacent pens and separated from other treatment groups by an empty pen. Drinking troughs were not shared among treatment groups. Pens were bedded with shredded paper, which was replaced as needed.

**Inoculation of steers**—An isolate of *A. marginale* derived in 1999 from a cattle herd in Oklahoma was used in the study. This isolate has been genotyped and is infective and transmissible by ticks. Infected blood was prepared as a frozen stablate with 10% dimethyl sulfoxide and maintained in liquid nitrogen prior to inoculation of a splenectomized calf at Oklahoma State University. Whole blood (350 mL) was collected in sterile syringes coated with heparin from the inoculated calf when the PCV was 34% and PPE was 21.6%. Each test steer was inoculated IV with 1 mL of whole blood containing approximately $2.6 \times 10^5$ infected erythrocytes. Day of inoculation was designated as day 0.

**Postinoculation monitoring of steers**—After inoculation, steers were monitored daily for clinical signs of anaplasmosis, including anorexia and lethargy. Rectal temperature was also measured at selected time points. Blood samples were collected 9, 13, 20, 28, 34, 41, 61, 96, 126, and 156 days after inoculation for determination of PPE and PCV and serologic testing by use of the CF test and cELISA. Blood samples were collected via jugular venipuncture by use of an 18-gauge, 1-inch needle. To obtain serum, 10-mL sterile glass tubes without additive were used, whereas 7-mL glass tubes containing potassium EDTA were used for collection of blood samples for measurement of PCV and hematologic analysis. Blood in EDTA was refrigerated prior to PCV determination or packaged in insulated material for delivery by overnight courier to Oklahoma State University for determination of the PPE. Serologic testing was conducted at the Iowa State University Veterinary Diagnostic Laboratory.

**Examination of blood films and determination of PPE**—Blood films for determination of the PPE were stained by use of a 30-second, 3-step staining technique, which is comparable to the Wright-Giemsa method. Two slides were prepared for each blood sample and examined for *A. marginale* at 1,000× magnification by use of emersion oil and a grid. A total of 500 cells were counted within the 4 squares of the grid, and the number of infected cells was recorded. The PPE was the number of infected cells divided by the total number of cells counted and the quotient multiplied by 100.

**Measurement of PCV**—The PCV was determined by partially filling heparinized capillary tubes with blood followed by centrifugation (12,600 × g for 10 minutes). The PCV was then determined by manual measurements of the height of the RBCs and total column height.

cELISA—a commercial cELISA, which was based on recombinant *Anaplasma* MSP-5 antigen produced by plasmid-transformed *Escherichia coli*, was used in accordance with the method described by the OIE and recommended by the manufacturer. Briefly, 70 µL of undiluted serum was added to each well (wells were
coated with MBP) of an adsorption transfer plate that included 2 positive and 3 negative control samples. Adsorption of MBP typically produced by *E coli* prior to plasmid transformation is required to prevent non-specific inhibition of conjugate binding, which thereby improves test specificity. Plates were incubated at 20°C to 25°C for 30 minutes. Fifty microliters of adsorbed serum was then transferred to antigen-coated plates (plastic wells were coated with MSP-5 *Anaplasma* antigen). Contents of wells were mixed, and plates were incubated at 20°C to 25°C for an additional 20 minutes. Plates were then emptied and washed twice with 200 µL of diluted wash solution. Thereafter, 50 µL of diluted LX antibody-peroxidase conjugate was added to each well, and plates were incubated at 20°C to 25°C for an additional 20 minutes. Plates were then emptied and washed 4 times with 200 µL of diluted wash solution. Fifty microliters of substrate solution was added to each test well. Test samples were again incubated at 20°C to 25°C for 20 minutes, which was followed by the addition of a stop solution. Immediately after addition of the stop solution, the OD of each well was measured by use of an ELISA plate reader at a wavelength of 620 nm. Percentage inhibition of each sample was calculated by use of the following equation:

\[
\text{Percentage inhibition} = 100 - \left( \frac{\text{sample OD} \times 100}{\text{mean OD of negative control sample}} \right)
\]

Samples that had inhibition of < 30% were recorded as negative results, whereas samples that had inhibition of ≥ 30% were recorded as positive results.

**CF test**—The CF test was conducted by use of a microtiter technique described by other investigators and detailed in USDA guidelines. Briefly, 0.1 mL of serum, a positive control sample, and a negative control sample were diluted 1:5 in veronal buffered diluent and then heat inactivated by incubation at 58°C for 35 minutes. After heat inactivation, 0.025 mL of diluted serum, 0.025 mL of diluted test antigen, and 0.025 mL of diluted complement containing 5.5 C₅H₅ (ie, 50% hemolytic unit of complement) were added to each well. The hemolytic unit in complement fixation tests is the smallest amount of complement or serum that will produce complete hemolysis of sensitized red cells. Contents of the wells were mixed, and plates were incubated at 37°C for 1 hour. Erythrocytes were sensitized by adding a standardized 2% erythrocyte suspension to an equal volume of optimal hemolysin dilution, which was followed by incubation at 37°C for 10 minutes. Then, 0.05 mL of the sensitized erythrocytes was added to each test well.

Five drops of hemoglobin color standard were added to each well that contained reagent control samples. Plates with these control wells were then shaken and incubated at 37°C for 20 minutes, which was followed by resuspension of cells through shaking and incubation for an additional 23 minutes. Plates were then centrifuged at 300 × g for 5 minutes. Serum antibody titers against *A marginale* were determined as the reciprocal of the highest dilution at which none of the sensitized erythrocytes was hemolyzed. Partial hemolysis (between 25% and 75%) at the 1:5 dilution was interpreted as a suspect result, whereas hemolysis of ≥ 76% was interpreted as a negative result.

**Inoculation of blood into susceptible, splenectomized calves**—Inoculation of blood obtained from suspected carrier cattle into splenectomized calves, which are highly susceptible to infection, is considered to be the criterion-referenced test for detection of *A marginale* infections in cattle. For this test, 46 Holstein calves (6 to 12 weeks old) that weighed between 45 and 112 kg were obtained from a university dairy research facility and a commercial calf-rearing facility. It was known that cattle at each of those facilities had a low prevalence of anaplasmosis. Calves were assessed via clinical examination to ensure that they were healthy, and the cELISA was used to confirm the calves were seronegative and susceptible to infection with *A marginale*. Calves were then splenectomized to increase their susceptibility to *A marginale* infection. Splenectomies were performed in accordance with a technique described elsewhere.

Fifty milliliters of blood was collected from each of the 46 steers in the study into heparinized syringes. Blood from each steer was inoculated IV into splenectomized calves (1 steer for each splenectomized calf). After inoculation, calves were monitored for signs of anaplasmosis by daily observation and weekly determinations of PCV values. Infection with *A marginale* was confirmed by microscopic examination of stained blood films and measurement of cELISA values. When the PCV of an inoculated calf decreased to < 20%, the calf was euthanatized and necropsied.

**Statistical analysis**—The PCV data were entered into a software package for subsequent calculations and manipulation. Mean ± SEM values were calculated for each time point. A software program was used to perform statistical analysis. An ANOVA and the Tukey-Kramer honestly significant difference method were used to conduct multiple comparisons among time points after inoculation.

Data sets consisted of results for the 2 diagnostic tests being investigated and the criterion-referenced test. These diagnostic tests were designed to detect the carrier state of *A marginale* in cattle. Sensitivity estimates for the CF test and cELISA were calculated in a spreadsheet program by use of the following equation:

\[
\text{Sensitivity} = \frac{\text{No. of true positive results}}{\text{No. of true positive results} + \text{No. of true negative results}}
\]

Estimates for the 95% CI for sensitivity were calculated by use of the score CI as recommended in another report. This method of CI calculation yields nearly uniform probabilities for binomial parameters sampled from a uniform distribution, regardless of sample size, whereas the alternative Wald method typically is anticonservative and varies on the basis of sample size. Specificities for the CF test and cELISA could not be determined as part of the study because all steers were infected with *A marginale* as part of a larger project.

Agreement between results for the CF test and cELISA was assessed by calculating a κ statistic. Data were converted to a binary format (0 = no disease and 1 = disease) by use of a cutoff value of 1:5 for the
CF test and 30% inhibition for the cELISA test, as described previously. Thereafter, data were compared by contingency analysis by use of a 2 × 2 table in which \( \kappa \) represented the actual agreement between the tests divided by the potential agreement beyond chance in accordance with the following equation:

\[
\kappa = \frac{2(A - B)}{(C + D)^2}
\]

where \( A \) is the number of steers that had positive results for both the CF test and cELISA multiplied by the number of steers that had negative results for both the CF test and negative results for the cELISA multiplied by the number of steers that had positive results for the CF test and negative results for the cELISA multiplied by the number of steers with positive results for the CF test multiplied by the total number of steers with negative results for the CF test, and \( D \) is the total number of steers with positive results for the cELISA multiplied by the total number of steers with negative results for the cELISA test. The \( \kappa \) statistic measures the agreement between 2 tests on a scale from 0 to 1. When applied to test results, common interpretations of \( \kappa \) values are that < 0.2 is slight agreement, 0.2 to 0.4 is fair agreement, 0.4 to 0.6 is moderate agreement, 0.6 to 0.8 is substantial agreement, and > 0.8 is almost perfect agreement.\(^{23}\) When the \( \kappa \) statistic could not be determined because of a lack of discordant results in 2 or more 2 × 2 cells, an overall proportion of concordance was calculated by dividing the sum of concordant test results (both the CF test and cELISA with positive results and both the CF test and cELISA with negative results) by the number of samples tested.\(^{22}\)

### Results

Prior to inoculation, all steers included in the study were seronegative for anaplasmosis (cELISA, < 30% inhibition), and rickettsiae were not detected in stained blood films. Furthermore, the mean PCV for all steers was 38.03% (95% CI, 37.04% to 39.00%), which was within the reference interval for the Hct (24% to 46%).\(^{24}\) All steers in the study became infected with \textit{A. marginale} as confirmed by clinical signs of anaplasmosis and microscopic identification of organisms on stained blood films obtained from inoculated study steers and inoculated splenectomized calves.

All steers in the study had evidence of \textit{A. marginale}–parasitized erythrocytes by day 13 after inoculation, although the overall mean PPE was < 1% (Table 1). Mean PPE reached a peak of 9.17% (95% CI, 7.58% to 10.75%) on day 20, which was accompanied by a significant reduction in PCV (to 27.58%), although this value was still within the reference interval. The highest mean ± SEM body temperature was 39.68 ± 0.17°C, which was recorded on day 24. The lowest mean PCV was measured on day 28 (ie, 8 days after peak PPE), after which the Hct started to increase (Figure 1). However, evidence of anemia associated with \textit{Anaplasma} infection continued until day 41 as indicated by PCVs that differed significantly from preinoculation values. By day 61, \textit{A. marginale}–infected parasites were not evident in stained blood films, and PCVs had returned to preinoculation values.

On day 9, the mean ± SEM inhibition for the cELISA was 24.82 ± 2.86%. Nineteen steers were classified as having positive results by use of the 30% inhibition cutoff value recommended by the OIE and the manufacturer. This provided a calculated test sensitivity of 47.5% (95% CI, 32.9% to 62.5%). In contrast, the CF test failed to identify any steers as having positive results at this time point (Figure 1; Table 1). By day 13, the mean inhibition for the cELISA had increased to 66.84 ± 1.59%. At this time point, all cattle were regarded as having positive results for the cELISA (sensitivity, 100% [95% CI, 91.3% to 100%]) by use of the recommended inhibition cutoff value of 30%, whereas the CF test had a calculated sensitivity of only 20% (95% CI, 10.5% to 34.8%). On day 20, mean value for percentage inhibition for the cELISA had increased to 83.92 ± 0.62%. This coincided with a peak mean PPE of 9 ± 1.57% (range, 3% to 24%). At this time point, all steers had positive results for the CF test (sensitivity of 100%).

Table 1—Mean and 95% CI values for several variables at various time points after inoculation of 40 beef steers with \( 2.6 \times 10^5 \) \textit{Anaplasma marginale}–infected erythrocytes.

<table>
<thead>
<tr>
<th>Day after inoculation*</th>
<th>cELISA</th>
<th>CF test</th>
<th>Agreement between CF test and cELISA (κ)</th>
<th>PPE</th>
<th>PCV</th>
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<tr>
<td></td>
<td>Se (%)</td>
<td>95% CI (%)</td>
<td>Se (%)</td>
<td>95% CI (%)</td>
<td>Mean (%)</td>
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<td>9</td>
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<td>20</td>
<td>10.5–34.8</td>
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</tr>
<tr>
<td>20</td>
<td>100</td>
<td>91.3–100</td>
<td>100</td>
<td>91.3–100</td>
<td>0.000</td>
</tr>
<tr>
<td>28</td>
<td>100</td>
<td>91.3–100</td>
<td>37.5</td>
<td>24.2–53</td>
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</tr>
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<td>34</td>
<td>100</td>
<td>91.3–100</td>
<td>12.5</td>
<td>5.5–26.1</td>
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<td>41</td>
<td>100</td>
<td>91.3–100</td>
<td>7.5</td>
<td>2.5–19.9</td>
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<td>61</td>
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<td>15</td>
<td>7.1–29.1</td>
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<td>96</td>
<td>100</td>
<td>91.3–100</td>
<td>17.5</td>
<td>8.7–32</td>
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<td>18.1–45.4</td>
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<td>156</td>
<td>100</td>
<td>91.3–100</td>
<td>25</td>
<td>14.2–40.2</td>
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<td>Overall</td>
<td>94.8</td>
<td>92.1–96.5</td>
<td>26.5</td>
<td>22.4–31.0</td>
<td>0.039</td>
</tr>
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</table>

*Day of inoculation was designated as day 0. †Proportion of concordance analysis.
Se = Sensitivity. NR = Not recorded. ND = Not determined.
*Within a column, values with different superscript letters differ significantly (\( P < 0.05 \)).
After the peak in parasitemia, sensitivity of the CF test fluctuated between 37.5% (95% CI, 24.2% to 53%) on day 28 and 7.5% (95% CI, 2.5% to 19.9%) on day 41, whereas sensitivity of the cELISA remained at 100% (95% CI, 91.3% to 100%) for the remainder of the study. During the period 156 days after inoculation, overall sensitivity for the cELISA was 94.8% (95% CI, 92.1% to 96.5%), whereas overall sensitivity for the CF test was only 26.5% (95% CI, 22.4% to 31.0%). Agreement between the CF test and cELISA at each of the sampling time points could not be determined by use of the κ statistic because of a lack of concordant results in 2 or more $2 \times 2$ cells. The overall proportion of concordance at each time point varied from 0.075 on day 41 to 1 on day 20 (Table 1). Mean ± SEM value for the κ statistic that described the overall agreement between the 2 tests during the course of the study was 0.039 ± 0.009.

**Discussion**

In the study reported here, sensitivity of the CF test and cELISA was compared for detection of *A. marginale* in steers from the time of inoculation through the phase of persistent infection for experimentally induced anaplasmosis. To our knowledge, this is the first report evaluating fluctuations in *A. marginale* test sensitivity at sequential time points after onset of infection. Sensitivity of the cELISA was < 50% during the first 10 days after inoculation. These data are clinically relevant because serologic surveys are often conducted in cattle populations in which recent vector-borne or iatrogenic transmission of *A. marginale* could result in some animals having prepatent infections at the time of sample collection. This study revealed the importance of recognizing limitations of these diagnostic tests when applied to cattle during the early stages of anaplasmosis. This finding has important implications for disease classification prior to interstate or international movement, especially when moving cattle from endemic to nonendemic areas or during seasons of the year when tick and biting flies are prevalent.

Sensitivity of the CF test for *A. marginale* in cattle ranges from 20% with a cutoff value of 1:5 (as was used in the study reported here) to 14% with a cutoff value of 1:10.11,25 Serum samples used to determine sensitivity in those other studies11,25 were collected from naturally infected cattle with unknown infection status or duration of infection. The sensitivity values we reported here were contained within the range of values cited in the literature. However, our study revealed that the CF test failed to detect seropositive cattle up to 2 weeks after inoculation and that sensitivity may be as low as 7.5% after acute infection has passed. Sensitivity value for the cELISA test in another study25 was 96%, which is consistent with the sensitivity reported in our study. The κ statistic of 0.039 determined in our study indicated only slight agreement between the tests on the basis of the classification system described elsewhere.23

Variation in the CF test results in the study reported here concurred with a report of poor performance in a posttreatment study.24 The superior sensitivity of the cELISA versus the CF test at all data collection points after inoculation (except for day 20, on which there was equal sensitivity) indicated that the cELISA should be the preferred test for identification of recently infected or persistently infected cattle. However, caution should be used in the direct extrapolation of this information to cattle naturally infected with *A. marginale* because the infective dose used in this study may have been much higher than the dose that would be inoculated through natural vectors (eg, ticks) or mechanical transmission (eg, biting flies or blood-contaminated fomites).

Despite the high infective dose used in the study reported here, the course and duration of infection we detected was consistent with results of another study26 in which the severity of anaplasmosis was found to be directly related to age, with subclinical infections in cattle < 1 year old.27 The PCVs of the calves used in our study were within the reference range, although a significant decrease in Hct was detected after peak parasitemia. The onset of parasitemia in our study was approximately 13 days. This finding is noticeably shorter than the typical prepatent period of 21 days after natural transmission.28

Other investigators29 found that the time of onset of detectable parasitemia was generally shorter and peak parasitemia was often higher with increasing challenge doses of *A. marginale*. These findings were supported in our study and may have led to bias in the study reported here because the time of onset and extent of antibody response detected may be proportional to the challenge dose. This could have resulted in increases in estimates of sensitivity and a shorter incubation period in which test sensitivity may be questionable. The use of naturally infected calves was not feasible for this study because comparison of the 2 tests necessitated the use of a uniform infective dose and knowledge of the exact time of inoculation and subsequent infection. A follow-up
study in which the inoculation doses are comparable to those in vector-borne or fomite-transmitted infection may yield differing results, although to our knowledge, the number of infective organisms transmitted by ticks during the feeding period has not been reported.

Without concurrent calculations for test specificity, the percentage of false-positive results detected by each test could not be determined (ie, 1 – specificity). Because the cost of a false-positive result for a specific animal may be high (exportation restrictions, adverse effects of tetracycline administration, and risk of being culled or euthanized), variations in test specificity during the natural progression of disease may be as enlightening as the sensitivity results reported here. Specificity data would also be important to determine the ideal or optimum cutoff points for the CF test and cELISA in naturally infected animals.

Microscopic examination of stained blood films is commonly used to detect *A. marginale* organisms in erythrocytes of infected animals. However, this diagnostic technique may be unreliable when cattle have minimal infections or in advanced cases of the disease when animals are severely anemic. In the study reported here, the cELISA accurately identified all infected cattle before the number of *A. marginale*-infected erythrocytes exceeded a PPE of 1%. This suggests that the cELISA may be more sensitive than examination of stained blood films for identifying early clinical cases. Furthermore, in instances in which the PPE is low, intraerythrocytic inclusions of *A. marginale* may easily be confused with Howell-Jolly bodies, basophilic stippling of reticulocytes, and stain contamination. This suggests that the cELISA may be a useful alternative to examination of stained blood films for the diagnosis of anaplasmosis, especially in situations in which experience of clinicians or the available facilities are insufficient for interpretation of blood films.

Veterinarians should exercise caution before making a definitive diagnosis of acute anaplasmosis solely on the basis of a positive result for the cELISA and clinical signs such as fever, anemia, and icterus. *Anaplasma marginale* carrier cattle are cELISA positive but not rickettsemic and therefore do not develop anemia and icterus associated with erythropagocytosis of parasitized erythrocytes. Differential diagnoses that should be ruled out on the basis of these clinical signs include acute anthrax, leptospirosis, bacillary hemoglobinuria, oak poisoning, poisoning caused by ingestion of *Brescia* species, multicentric lymphosarcoma, babesiosis, thileriosis, and trypanosomiasis. In such circumstances, it would be advisable to detect intraerythrocytic inclusions of *A. marginale* by examination of blood films to assist in differentiating between anaplasmosis and these other diseases.

The cELISA currently used for the diagnosis of anaplasmosis in cattle is based on the use of monoclonal antibody ANAF16C1 that recognizes MSP5 in *A. marginale*, *Anaplasma centrale*, and *Anaplasma ovis*. Analysis of findings suggests that the commercially available MSP5 cELISA used in the study reported here may also recognize anti-*Anaplasma phagocytophilum* antibodies in infected cattle. The MSP5 sequence is highly conserved among strains of *A. marginale* as well as between *A. marginale*, *A. centrale*, and *A. phagocytophilum*. Cross-reactivity of the MSP5 test with multiple *Anaplasma* spp has been confirmed through the identification of common regions defined to be essential for ANAF16C1 reactivity. Thus, the MSP5 cELISA most likely does not differentiate *Anaplasma* spp when there is coinfection with *A. phagocytophilum* and *A. marginale*.

In an unpublished telephone survey of 34 laboratories accredited by the American Association of Veterinary Laboratory Diagnosticians conducted by the authors, it was found that 16 laboratories still offer the CF test as well as the cELISA. Results of the study reported here, in consideration with results of other published reports, suggest that the continued use of the CF test could result in infected cattle being assigned a negative result, which could thus lead to introduction of persistently infected animals with false-negative results into populations of completely naive cattle. It is estimated that the introduction of anaplasmosis into a naive herd can result in a 3.6% reduction in calf crop, a 30% increase in cull rate, and a 30% mortality rate in clinically infected adult cattle, which can be economically devastating to livestock producers in nonendemic areas.

In addition to the risk of introducing anaplasmosis into naive herds, movement of infected cattle misclassified as seronegative by use of the CF test into areas in which anaplasmosis is endemic may result in the introduction of new *A. marginale* genotypes. A phylogeographic study of *A. marginale* isolates in the United States has revealed that many more genotypes of *A. marginale* exist in nature than have been described. Genetic diversity most likely is attributable to the phenomenon of infection exclusion to *A. marginale* that results in 1 genotype/animal. Once a new genotype is introduced into a particular region, it will be maintained in ticks and cattle by independent transmission events and will likely become endemic. Persistently infected cattle and ticks could both serve as reservoirs of the introduced genotype. This is important because these diverse *A. marginale* genotypes may not provide cross-protection, thus greatly increasing the challenge to use vaccination as a method for control of anaplasmosis. Therefore, correct classification of disease status is essential to restrict movement of infected cattle to prevent diversification of *A. marginale* isolates.

The results reported here support the findings of other studies in which the CELISA has superior sensitivity for detection of anaplasmosis, compared with that for the CF test. However, our study also illustrated the importance of recognizing the limitations of these diagnostic tests when applied to cattle during the early stages of infection. This finding has important implications for disease classification prior to local, interstate, or international movement of cattle and for future disease control and eradication programs. Additional research is necessary to evaluate these findings in populations of naturally infected cattle.

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b. Monoject No Additive sterile glass tubes, Sherwood Medical, St Louis, Mo.

c. BD Vacutainer, Beckton Dickinson Vacutainer Systems, Franklin Lakes, NJ.
References


