

Short communication

Validation of an *Anaplasma marginale* cELISA for use in the diagnosis of *A. ovis* infections in domestic sheep and *Anaplasma* spp. in wild ungulates

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Abstract

A commercially available (cELISA) kit for diagnosing *Anaplasma marginale* infection in cattle was validated for diagnosing *A. ovis* infection in sheep using the bovine serum controls as supplied by the manufacturer (BcELISA) and sheep serum controls from pathogen-free sheep (OcELISA). True positives were identified using two previously established assays, a nested PCR (nPCR) test and an indirect immunofluorescent assay (IFA). The BcELISA was also applied to sera from various species of wild ruminants, comparing the results with the IFA. Receiver operating characteristic (ROC) analysis indicated that the predicted threshold inhibition for the BcELISA was 19.2. The sensitivity for the BcELISA was 98.2% and the specificity was 96.3%. The predicted threshold inhibition decreased to 14.3 for the OcELISA; the sensitivity was 96.5% and the specificity was 98.1%. There was $\geq 90\%$ concordance between IFA and nPCR, as well as between the BcELISA at 19% inhibition cutoff and either IFA or PCR. Concordance between the cELISA and IFA using sera from elk, mule deer, bighorn sheep, pronghorn antelope, and black-tailed deer ranged from 64% to 100%. This commercially available cELISA test kit can be used very effectively to test domestic sheep for infection with *A. ovis* using the kit-supplied controls (i.e. the BcELISA) and a 19% inhibition cutoff; the kit may also be useful for detecting intra-erythrocytic *Anaplasma* infections in wild ruminants.

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1. Introduction

Anaplasma marginale causes a serious hemoparasitic disease of cattle (Ristic, 1968), and related species have been reported to infect wild ruminants (Davidson et al., 2001; Davidson and Goff, 2001; Kuttler, 1984).

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Anaplasma ovis infects domestic sheep without causing severe disease (Splitter et al., 1956), however it can cause severe clinical disease in bighorn sheep (Tibbitts et al., 1992). The epidemiology of *A. ovis* is poorly understood due to lack of a practical diagnostic assay suitable for screening large numbers of animals, serological methods would be well suited for this (Davidson and Goff, 2001). Several screening methods developed for *A. marginale* have been adapted for *A. ovis*, including a complement fixation (CF) test (Splitter et al., 1956; Magonigle et al., 1981; Kuttler and Winward, 1984), and an indirect immunofluorescence assay (IFA) (Tibbitts et al., 1992). Both tests have practical limitations (Goff et al., 1990, 1993; Tibbitts et al., 1992; Jessup et al., 1993; Keel et al., 1995; Zaugg et al., 1996; Crosbie et al., 1997). A competitive enzyme linked immunoassay (cELISA) specific for an epitope of a conserved *A. marginale* major surface protein-5 (MSP-5) antigen (Knowles et al., 1996) is licensed for the detection of *Anaplasma* infection in cattle and although it has been used without validation to detect *A. ovis* infection in goats (Ndung'u et al., 1995) this test has not been validated for use in domestic sheep or wild ruminants. Validation will make the test applicable for epidemiological studies of intra-erythrocytic pathogens in these different host species. The purpose of this study was to validate the MSP-5-based cELISA for use with domestic sheep and to evaluate its usefulness for the detection of *Anaplasma* infection in other wild ruminant species.

2. Materials and methods

2.1. Serum samples

Domestic sheep sera used for test validation were obtained from two sources. The United States Sheep Experiment Station near Dubois, ID is in an area endemic for *A. ovis*. Previous studies suggested an *Anaplasma* prevalence of >70% in this flock.¹ Blood and serum were collected from 402 ewes on 18 March

2003. A flock located at the Agriculture Agri-Food Canada Research Center, Lethbridge, Alberta, Canada served as a source of known negatives since *Anaplasma* infection has never been reported from this flock. Blood was collected from 107 sheep on 6 December 2006.

Serum for cELISA and whole blood for PCR was collected from each animal. Ten microliters of whole blood collected in heparinized tubes and another 10 ml was collected from in non-heparinized tubes for serum. Whole blood was stored in 200 µl aliquots in 1.5 ml microcentrifuge tubes at –70 °C until DNA isolation. Serum aliquots were placed into 1 ml cryotubes and stored frozen at –70 °C until use.

Sera from free-ranging wild ruminants were obtained from several locations in California as previously described (Jessup et al., 1993). See Table 1 for species, numbers and County locations where wildlife was bled. Aliquots of each serum sample were placed into 1 ml cryotubes and stored frozen at –20 °C until used.

2.2. Indirect immunofluorescence

The IFA was performed as described using thin blood films of washed erythrocytes infected with the Idaho isolate of *A. ovis* (Tibbitts et al., 1992). FITC-

Table 1
Species, number, and county location throughout California of wildlife captured for collection of blood samples

| | Wildlife Species | County | Number | |
|--------------------|------------------------|--------------------|-----------|----|
| Pronghorn antelope | <i>Antilocapra</i> | Siskiyou | 8 | |
| | <i>americana</i> | Modoc | 24 | |
| Bighorn sheep | <i>Ovis canadensis</i> | Inyo | 18 | |
| | | San Bernardino | 20 | |
| | | Riverside | 18 | |
| Mule deer | <i>Odocoileus</i> | Nevada | 18 | |
| | | <i>hemionus</i> | Inyo | 18 |
| | | <i>hemionus</i> | Sierra | 11 |
| Black-tailed deer | <i>Odocoileus</i> | Santa Clara | 15 | |
| | | <i>hemionus</i> | Monterey | 15 |
| | | <i>columbianus</i> | Mendacino | 4 |
| | | | Lake | 6 |
| | | | Tehama | 4 |
| | | | Lassen | 22 |
| Elk | <i>Cervus elaphus</i> | Inyo | 9 | |
| | | Solano | 20 | |
| | | Kern | 24 | |
| | | Merced | 7 | |

¹ Stiller, D., Goff, W.L., Shompole, S.P., Johnson, L.W., Glimp, H., Rurangirwa, F.R., Gorham, J., McGuire, T.C., 1989. *Derma-centor andersoni* Stiles: a natural vector of *Anaplasma ovis* Lesto-guard on sheep in Idaho, in: Proceedings of the Eighth National Veterinary Hemoparasitic Disease Conference, St. Louis, MO, April 10–12, pp. 183.

labeled rabbit-anti-sheep IgG was used at a 1:80 dilution for domestic sheep samples. FITC-labeled Protein-G (KPL, Gaithersburg, MD, USA) was used, also at a 1:80 dilution for all wildlife samples. A sample was defined as positive if a 1:100 dilution of serum resulted in a reaction equal to or greater than the weak positive control reaction.

2.3. DNA preparation and polymerase chain reaction

DNA preparations from blood samples were made with the DNeasy[®] Blood and Tissue kit (QIAGEN) following the manufacturer's protocol. Nested polymerase chain reaction (nPCR) assays targeting the single copy MSP-5 gene were conducted on extracted DNA as previously described (Scoles et al., 2005).

2.4. Identification of true positives

All of the 402 Dubois sheep were tested with the cELISA according to the manufacturer's instructions (VMRD Inc., Pullman, WA). All samples and controls were run in duplicate and the mean optical density (OD) at 450 nm was determined. The percent inhibition was determined using the mean ODs of each sample compared to the mean of control wells using the formula: % inhibition = $100 - [(Sample\ OD \times 100) / (negative\ control\ OD)]$, as described in the manufacturer's protocol. Animals with less than 60% inhibition were selected for confirmation with nPCR. A random sample of 10 sheep whose sera produced a higher inhibition was also selected for nPCR confirmation. These sera were also tested with the IFA. Sheep whose blood tested positive by both nPCR and IFA were considered to be true positives.

2.5. Evaluation of cELISA conditions

True positive and true negative samples were evaluated by the cELISA under two conditions: (1) sera were tested using the kit strictly per the manufacturer's protocol (referred to hereafter as BcELISA); (2) bovine negative control serum provided in the kit was replaced by sera from five known negative sheep (hereafter referred to as OcELISA). Negative sheep serum for the OcELISA was collected from pathogen-free sheep reared at

the Animal Disease Research Unit (ADRU) in Pullman, WA.

2.6. Statistical analysis

Diagnostic specificity, sensitivity and predictive values were determined by receiver operating characteristic (ROC) analysis (MedCalc statistical software, version 9.3.0.0). The results from 57 known positive and 107 known negative sheep were analyzed for both the BcELISA and OcELISA. The percent inhibition from each sample was used in the ROC analysis with an estimated prevalence arbitrarily set at 75%. Concordance between IFA and cELISA were determined for the wildlife sera.

3. Results

3.1. cELISA, PCR and IFA results on domestic sheep sera

A total of 88 of the 402 Dubois sheep that were tested with the cELISA were selected for confirmation with nPCR based on the % inhibition calculated with the kit negative control. Twenty-eight of the sera were from sheep whose blood was nPCR negative and all of these had inhibition <22%. These 88 sera were also tested with IFA and 28 of the 88 samples were also negative. Only 57 of the sera were from sheep whose blood was positive by both nPCR and IFA, and these sera were considered "true" positives for this analysis. All of the 107 sheep in the Lethbridge flock were negative by nPCR and IFA, and thus were considered "true" negatives for this analysis.

3.2. Sensitivity, specificity and predictive values for domestic sheep sera

The ROC curve and distributional plot for both cELISA conditions are shown in Fig. 1. The predicted threshold inhibition for the BcELISA was 19.2 and the area under the ROC curve was 0.993 with a 95% confidence interval of 0.965–0.999. The sensitivity was 98.2% and the specificity was 96.3% (Fig. 1a). The predicted threshold inhibition decreased to 14.3 for the OcELISA and the area under the ROC curve was 0.995 with a 95% confidence interval of 0.968–0.999. The

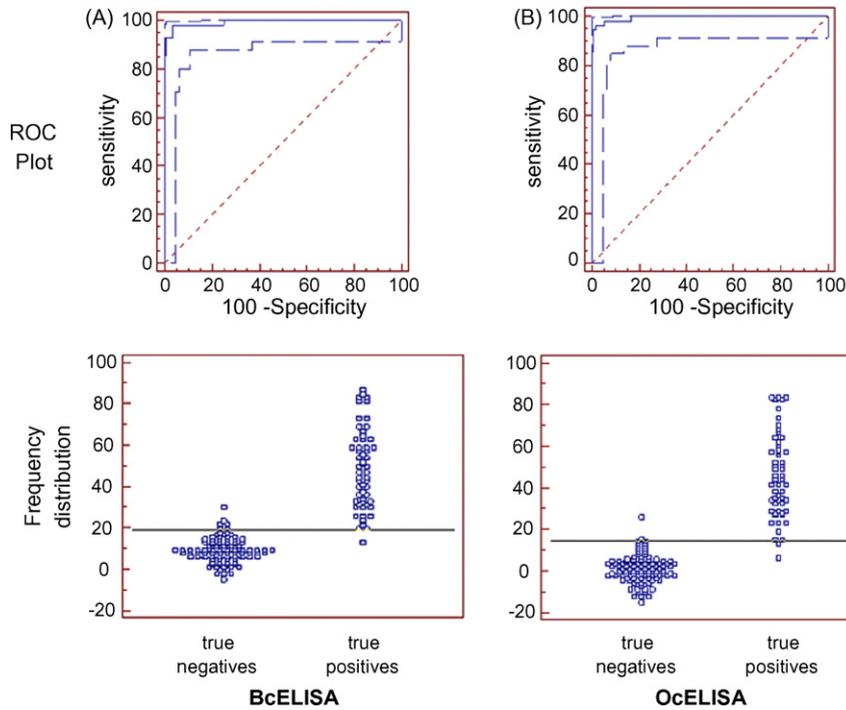


Fig. 1. Upper panels: receiver operating characteristic (ROC) plots (solid line) of sensitivity and specificity with 95% confidence levels (broken lines) calculated from the 57 true positive and 107 true negative serum samples, as established by nPCR and IFA. A random, no discrimination line is shown as the 45° dotted line; (A) plot for BcELISA with 19.2% as the optimum inhibition cutoff, (B) plot for OcELISA with 14% as the optimum inhibition cutoff. Lower panels: frequency distributions of inhibition values for true positive and negative serum samples; BcELISA on the left, OcELISA on the right.

sensitivity was 96.5% and specificity 98.1% (Fig. 1b). The *Anaplasma* prevalence in the population has an effect on the predictive values of the assay, as demonstrated in Fig. 2, however, the values were similar for either assay at the various prevalence rates.

The commercial BcELISA kit validated for use with cattle uses 30% as the threshold inhibition; samples with <30% inhibition are designated as negative. Therefore, we assayed all 88 sheep samples from Dubois using both the BcELISA at 30% and at the ROC level of 19% threshold inhibition and the OcELISA at the ROC level of 14% threshold inhibition. The estimated prevalence of this test group under each of these conditions varied from 61% to 73% (Table 2).

The BcELISA results were compared to IFA and PCR to determine which assay condition would be optimal for domestic sheep samples. A high degree of concordance (93%) occurred between the PCR and IFA results (Table 3). Concordance between the

BcELISA at 30% inhibition cutoff and both the IFA and PCR was 84%. Ten of the 14 disparate results were BcELISA negative and IFA/PCR positive (Table 3) indicating that the use of bovine control sera with 30% inhibition as a cutoff may not be appropriate with sheep sera. Only five samples differed between the BcELISA at 19% cutoff and the OcELISA at 14% cutoff. Two were negative at the 14% cutoff, but IFA and PCR positive; the other three yielded reactions between 14% and 19% inhibition. Of these, one was IFA and PCR positive, one was IFA and PCR negative and one was IFA positive and PCR negative. These results suggested that the current commercial kit provided with control bovine sera would be acceptable as long as the inhibition used as the cutoff was decreased to 19%. This was further substantiated by the high degree of concordance (>90%) between the BcELISA at 19% inhibition cutoff and the IFA and PCR (Table 3).

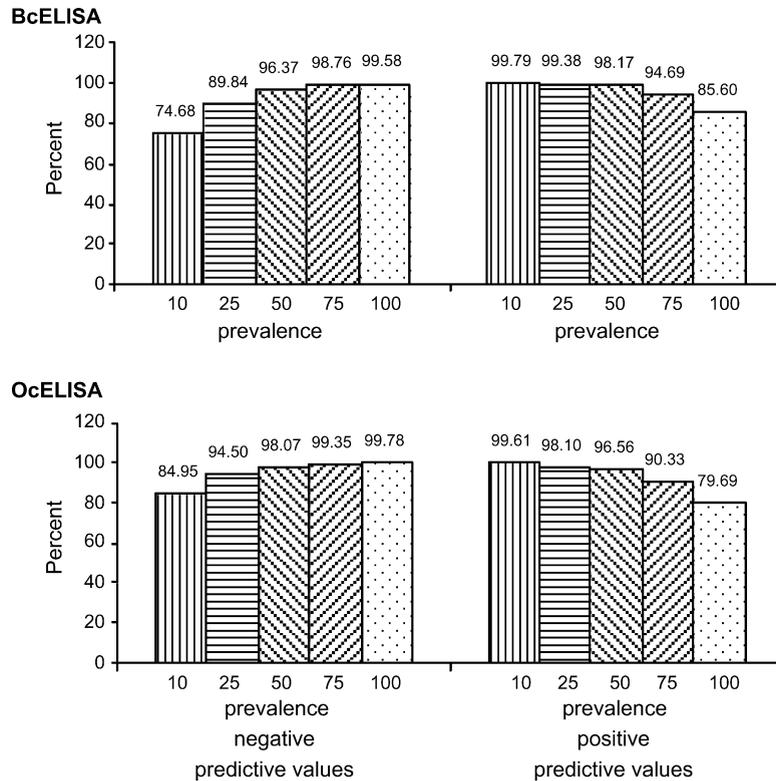


Fig. 2. Positive (right graph) and negative (left graph) predictive values calculated at the indicated prevalence rates for the cELISA with kit supplied bovine control serum (BcELISA; top panel) and for the cELISA with control serum from pathogen free sheep (OcELISA; bottom panel).

3.3. Application to wildlife: concordance with IFA

Sera from various species of wild ungulates had been previously collected but lacked a paired blood sample for PCR. Therefore, the extent of validation of the cELISA for wildlife diagnosis was based solely on concordance between the cELISA and IFA. Samples were evaluated with the commercial cELISA kit (VMRD) using bovine control sera, and positive results were assigned to samples with >19%

inhibition. Concordance of 100% was demonstrated for both pronghorn antelope and bighorn sheep (Table 4) however, the pronghorn antelope samples were uniformly negative and the bighorn sheep had a mixture of positive and negative samples. Concordance between the cELISA and IFA using sera from the other species ranged from 64% to 87% (Table 4).

4. Discussion

Results indicate that this cELISA is a valid and reliable test for diagnosing *A. ovis* infection in domestic sheep. When used as supplied by the manufacturer on domestic sheep sera using an inhibition threshold of 19% this assay has all the attributes necessary for making management decisions in the domestic sheep industry. There was no clear advantage to replacing the negative control

Table 2
MSP-5-based *Anaplasma* cELISA validation for domestic sheep (Dubois herd, n = 88)

| Condition | Prevalence |
|-----------------------------------|-------------|
| Bovine control sera at 30% cutoff | 54/88 = 61% |
| Bovine control sera at 19% cutoff | 63/88 = 72% |
| Ovine control sera at 14% cutoff | 64/88 = 73% |

Prevalence based on three different conditions.

Table 3
Concordance between IFA and PCR and between IFA or PCR and the BcELISA at different percent inhibitions

| Test 1 | Test 2 | Test 1 + Test 2 + | Test 1 – Test 2 + | Test 1 + Test 2 – | Test 1 – Test 2 – | Concordance (%) |
|--------|---------------|----------------------|----------------------|----------------------|----------------------|-----------------|
| PCR | IFA | 57 | 3 | 3 | 25 | 93 |
| PCR | BcELISA > 30% | 50 | 4 | 10 | 24 | 84 |
| IFA | BcELISA > 30% | 50 | 4 | 10 | 24 | 84 |
| PCR | BcELISA > 19% | 58 | 5 | 2 | 23 | 92 |
| IFA | BcELISA > 19% | 57 | 6 | 3 | 22 | 90 |

bovine serum supplied with the kit with negative control ovine sera. With a specificity of 96.3% and sensitivity of 98.2%, the assay has suitable predictive values for use in epidemiologic studies with positive predictive values ranging from 74.68% at 10% prevalence to 99.58% at 90% prevalence, and negative predictive values of 99.79% at 10% prevalence to 85.60% at 90% prevalence. PCR and IFA were in good agreement and gave similar results when compared with the cELISA. Concordance above 90% when using a threshold of 19% inhibition adds to the validity of using this assay with domestic sheep sera.

The cELISA was also found to be appropriate for use with various wild ungulates. Known positive and negative samples were not available for the wild ungulate samples, consequently concordance with the established IFA test was used as the means of validating the cELISA. Although the concordance for pronghorn samples was 100%, the samples were uniformly negative. Pronghorn antelope are susceptible to experimental infection with *A. ovis* (Zaugg, 1987), thus it is likely that the samples were obtained from truly negative herds, since there was only one sample producing an inhibition close to 19%. The concordance between the cELISA and IFA was also 100% with the bighorn sheep samples although in this

case there were a number of positive and negative samples.

Concordance between the two serologic assays was lower when applied to deer and elk samples. It is difficult to determine why discordance was higher for these samples. Most of the discordant results for elk and mule deer were cELISA positive and IFA negative, while the reverse was true for black-tailed deer. However, the elk samples were apparently from a low prevalence area with 85% of the samples negative on both assays. This, like the pronghorn results, may represent a somewhat artificially high concordance (87%) due to the overwhelming number of negatives. The relatively low concordance of 64% with mule deer samples may be related to a performance issue with the IFA assay since the cELISA percent positive samples for both mule deer and black-tailed deer were similar (73% and 71%, respectively) while the percent positive by IFA was 56% for mule deer and 82% for black-tailed deer. Mule deer and black-tailed deer are considered sub-species; both are susceptible to *Anaplasma* infection, and have been considered as important *A. marginale* reservoirs in California for many years, although direct evidence for reservoir competence is lacking.

Table 4
Concordance between IFA and BcELISA for the detection of *Anaplasma*-specific antibody

| Assay status | Wildlife species | | | | |
|---------------------|--|---|---|---|----------------------------------|
| | Pronghorn antelope (<i>Antilocapra americana</i>) | Bighorn sheep (<i>Ovis canadensis</i>) | Mule deer (<i>Odocoileus hemionus</i>) | Black-tailed deer (<i>Odocoileus hemionus</i>) | Elk (<i>Cervus elaphus</i>) |
| BcELISA pos IFA pos | 0 | 31 | 21 | 43 | 1 |
| BcELISA neg IFA pos | 0 | 0 | 4 | 11 | 1 |
| BcELISA pos IFA neg | 0 | 0 | 12 | 4 | 7 |
| BcELISA neg IFA neg | 32 | 25 | 8 | 8 | 51 |
| Concordance (%) | 100 | 100 | 64 | 77 | 87 |

A standardized diagnostic assay that has the ability to detect erythrocytic *Anaplasma* infections from any host species worldwide would be a valuable research tool. Other assays that rely on the cross-reactivity of *A. ovis*-specific antibodies with *A. marginale* antigens have serious deficiencies in both specificity and sensitivity, or like the IFA, are subjective in nature and not suitable for large-scale sample analysis. This cELISA appears to meet the criteria for use in diagnosing *A. ovis* infection in domestic sheep and for screening wildlife for the presence of erythrocytic *Anaplasma*-specific antibody.

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