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EVALUATION OF THE ANAPLASMOSIS RAPID CARD AGGLUTINATION TEST FOR DETECTING EXPERIMENTALLY-INFECTED ELK [1]

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Abstract: Anaplasma marginale was experimentally transmitted from cattle to elk to cattle. Six intact adult elk *(Cervus canadensis canadensis)* inoculated with freshly collected heparinized blood from cattle chronically infected with *A. marginale* became asymptomatic carriers. Although the elk did not develop clinical or hematologic evidence of infection, they became seropositive by the serum (SRCA) and plasma rapid card agglutination (PRCA) tests. Blood from the experimentally-infected elk produced disease in splenectomized bovine calves and the carrier state persisted for at least one year.

Infection did not occur when two elk were inoculated with 0.5 ml of frozen blood from known bovine carriers. The blood had been frozen for four weeks in liquid nitrogen with 6% dimethyl-sulfoxide.

The bovine SRCA and PRCA tests were adapted for use with elk serum. To obtain accurate test results, serum collected from clotted elk blood had to be held for at least 72 h at 21-27 C before performance of the SRCA test. Comparative serologic and infectivity studies indicated that the carrier (reactor) status of elk was accurately identified with the serologic tests in 61 of 68 samples evaluated. Incorrect serologic results with the SRCA and PRCA tests were false-negative readings. In no case were uninfected elk identified as seropositive.

INTRODUCTION

Studies have shown that a number of species of wild ruminants including elk *(Cervus canadensis canadensis),*[10] bighorn sheep *(Ovis canadensis canadensis),*[10] pronghorn antelope *(Antilocapra americana americana),*[10] white-tailed deer *(Odocoileus virginianus),*[19,20] Rocky Mountain mule deer *(Odocoileus hemionus hemionus),*[3,10] and Columbian black-tailed deer *(Odocoileus hemionus columbianus),*[3-5] can be experimentally infected with *A. marginale.* Although elk are susceptible, evidence about the nature of the serologic, hematologic, and clinical response to infection and the carrier status of exposed elk is limited.[10,15] Intact experimentally-infected elk reportedly become asymptomatic carriers and blood from the elk produces disease in splenectomized bovine *(Bos taurus)* calves.[10] Results from several studies indicate that bovine serologic tests for anaplasmosis in elk, in particular the complement fixation (CF) test, may be inadequate.[10,21] The accuracy of the rapid card agglutination

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(RCA) test conducted on serum and plasma from exposed elk has not been evaluated. The only known reliable test for detecting reactor elk is the costly, time-consuming procedure of inoculating elk blood into splenectomized bovine calves. The lack of a reliable serologic test for detecting reactor elk has severely limited attempts to elucidate the role of elk as carriers.

The purpose in the present investigation was to determine the response of intact elk to infection with anaplasmosis and to evaluate the reliability of the RCA test in detecting infected elk.

MATERIALS AND METHODS

Experimental Animals. Eight 1- to 7-year-old male and female elk maintained at the University of Idaho were inoculated intramuscularly with heparinized (10 units/ml) blood from known anaplasmosis carrier cattle. Three 2- to 5-year-old elk served as uninoculated controls. Infected bovine blood was used either freshly-collected or after freezing in liquid nitrogen with 6% dimethyl-sulfoxide (DMSO) for four weeks.[11] Two elk were inoculated with 0.5 ml of frozen blood, two with 0.5 ml and four with 50 ml of freshly-collected heparinized blood. Before inoculation with infected bovine blood the reactor (carrier) status of the eight principal elk was determined by the rapid card agglutination (RCA) test,[4] and inoculation of blood into susceptibile nonsplenectomized 8- to 12-month-old Holstein-Friesian calves. Similarly blood from each of three control elk was inoculated into susceptible nonsplenectomized calves both at the initiation and conclusion of the experimental period to determine that they were not anaplasmosis carriers. Blood from experimentally-exposed elk was inoculated into susceptible splenectomized bovine calves. Inoculations and blood collections were made after elk were sedated with 2.2 mg/100 kg etorphine.[5] Bovine calves were from the anaplasmosis-free experimental dairy herd maintained at the University of Idaho. Before bovine calves were inoculated with elk blood they were each examined to determine there was no serologic, hematologic, or clinical evidence of infection. In each case the RCA test was negative, the packed cell volume (PCV) was in the normal range, and the Wright's-stained blood films showed no evidence of parasitemia.

Hematologic, Serologic, and Clinical Studies of Elk after Inoculation. After inoculation with either freshly collected or frozen blood from known carrier cattle, the elk were observed for hematologic, serologic, and clinical evidence of anaplasmosis. Plasma and serum from the elk were examined with the RCA test to determine their status as anaplasmosis reactors. The RCA test, microhematocrit determination of PCV, and microscopic examination of Wright's-stained blood films were conducted before inoculation of the eight principal elk and at two and four months after inoculation for each and at 12 months for the four elk inoculated with 0.5 ml of blood. Similar studies were conducted on the three control elk at the initiation of the experiment and 12 months later. Inoculated elk were observed every other day through two months for clinical signs of disease. After the two month period, 50 ml of blood from the elk was subinoculated into susceptible splenectomized calves. Twelve months after exposure, 50 ml of blood from each elk inoculated with 0.5 ml of blood was subinoculated into susceptible splenectomized calves. Susceptibility of calves was determined after inoculation of blood from elk, by observing for

[4] Hynson, Westcott and Dunning, Baltimore, Maryland 21200, USA.

[5] M99-Etorphine parenteral solution, 1 mg/ml, D-M Pharmaceuticals Inc., Rockville, Maryland 20800, USA.

clinical, hematologic, and serologic evidence of infection. Calves were observed for four months after inoculation with elk blood. If calves failed to manifest evidence of anaplasmosis during the observation period they were challenged with 10 ml of blood from a known carrier to determine their susceptibility.

Evaluation of the Rapid Card Agglutination Test on Elk Blood. All blood samples collected from control and inoculated elk were evaluated with both the PRCA and SRCA tests.[2] Heparinized (10 units/ml) blood samples were centrifuged at 2,000 × g for 10 min to obtain plasma for use in the PRCA test and serum separated from clotted blood for the SRCA test. Clotted blood samples from elk were held at room temperature (21-27 C) for 12 h before the serum was separated by centrifugation. Serum samples from control and inoculated elk were tested at various time intervals after collection to assess the accuracy of the SRCA test on samples with respect to time. The plasma was used in the PRCA test within 2 h after collection of the heparinized blood samples.

Electron Microscopy of Infected Erythrocytes. Erythrocytes from splenectomized bovine calves with acute anaplasmosis were used for ultrastructural examination. Calves had been inoculated with blood from elk infected with anaplasmosis. Heparinized (10 units/ml) venous blood was washed three times in phosphate-buffered saline (pH 7.3). The cells were centrifuged, fixed for one h in a 2% potassium phosphate-buffered (0.1 M, pH 7.3) glutaraldehyde solution with 1% added sucrose, rinsed in a potassium phosphate-buffered (0.1 M, pH 7.3) solution of 1% sucrose, rinsed in distilled water, and postfixed in potassium phosphate-buffered 1% osmium tetroxide (0.1 M, pH 7.3) for 45 min. The cells were stained *en bloc* with an aqueous solution of 1% uranyl acetate, dehydrated through a graded series of ethanol, and embedded in epon araldite.[13] Thin sections were collected on uncoated 200-mesh grids, stained with lead or uranyl salts,[17,22] and examined in a Philips 200 electron microscope. [6]

RESULTS

The experimental data and their interpretation are given in Table 1. Elk that received either 0.5 or 50 ml of freshly collected heparinized blood from anaplasmosis carrier cattle became SRCA and PRCA test positive; however, clinical signs of anaplasmosis were not observed. When blood from these elk was subinoculated into splenectomized calves two and 12 months after exposure, each calf developed serologic, hematologic, and clinical evidence of infection with *A. marginale*, indicating that each of these elk had become a carrier. Regardless whether the study was terminated at four or 12 months after exposure, each of the six elk infected with freshly collected blood were SRCA and PRCA test positive at the conclusion of the experiment.

However, elk that received 0.5 ml of frozen blood from anaplasmosis carrier cattle did not become SRCA or PRCA test positive, nor was there evidence that their blood was infective for splenectomized calves at either two or 12 months after exposure. There was no clinical, hematologic, or serologic evidence of anaplasmosis during a four month observation period after the splenectomized calves were inoculated with elk blood. When these same calves were subsequently inoculated with blood from a known carrier, they developed clinical signs of anaplasmosis after an average incubation period of 26 days.

A preliminary study using elk serum collected before and two months after

[6] Philips Electron Instruments, Inc., Mount Vernon, New York 10500, USA

TABLE 1. Effect of inoculating blood from anaplasmosis infected cattle into elk and subinoculations into susceptible calves.

Elk No.	Amount of infected blood inoculated (ml)	*Blood preparation	Clinical signs in elk	Elk RCA test** status/month study concluded	Appearance of anaplasmosis in splenectomized calves subinoculated with elk blood: Months since elk were inoculated 2	12
1	.5	Frozen	None	-/12	-	-
2	.5	Frozen	"	-/12	-	-
3	.5	Fresh	"	+/12	+	+
4	.5	Fresh	"	+/12	+	+
5	50	Fresh	"	+/4	+	NT
6	50	Fresh	"	+/4	+	NT
7	50	Fresh	"	+/4	+	NT
8	50	Fresh	"	+/4	+	NT

*Elk were inoculated with either freshly collected heparinized blood (fresh) from cattle chronically infected with *A. marginale* or with blood that had been frozen for four weeks in liquid nitrogen with 6% dimethyl-sulfoxide (frozen).

**RCA test reactions expressed as positive (+) or negative (-) agglutination.

NT = Not tested; + = positive results; - = negative results.

exposure to *A. marginale* was conducted to determine optimal conditions for the SRCA test. It was found that at least 72 h had to elapse between the time serum was collected from clotted elk blood and performance of the SRCA test before accurate test results could be obtained. Pre-exposure serum samples from elk frequently yielded false positive reactions when tested before 72 h. Otherwise, conditions recommended for testing bovine sera also were optimal for testing elk sera. As recommended by the manufacturer of the anaplasmosis card test kit, at least 48 h elapsed between the time of collection and testing of cattle serum samples. As with the elk plasma samples, the bovine plasma samples were used in the PRCA test within 2 h after collection. Utilization of bovine serum factor in both the PRCA and SRCA test was necessary to obtain accurate test results on elk serum. Only once during the course of these studies did the results of the SRCA and PRCA tests not agree on samples taken at the same time from an elk. In that case the SRCA test was positive and the PRCA test was negative for an elk that was shown to be infected with *A. marginale* by calf inoculation. Two other elk that were shown to be carriers by calf inoculation studies were negative by both the PRCA and SRCA tests at two months. One of these animals remained negative to both tests at four months but converted to positive by 12 months. When results of the serologic and calf inoculation studies were compared it was found that the carrier (reactor) status of elk was accurately identified with the RCA test in 61 of 68 samples evaluated. Incorrect serologic results were false-negative readings, i.e., elk were identified as carriers by calf inoculation, but not as reactors by the serologic tests. In no case were uninfected elk found to be seropositive by either the PRCA or SRCA test.

Studies of the morphologic characteristics of the parasitized erythrocytes of splenectomized calves inoculated with elk blood illustrated the marginal bodies diagnostic of anaplasmosis (Fig. 1a). The ultrastructural features of the marginal bodies in the erythrocytes from subinoculated calves were consistent with those reported for *A. marginale* (Fig. 1b).[6,18,19]

DISCUSSION

Anaplasma marginale was experimentally transmitted from cattle to elk and back to cattle. Elk inoculated with either 0.5 or 50 ml of fresh blood from cattle infected with *A. marginale* became carriers and blood from these elk produced disease in splenectomized bovine calves. The carrier state was shown to persist in elk for at least one year. As noted in a previous study, clinical disease was not observed in the intact elk after exposure.[10] This could imply that under appropriate circumstances free-ranging elk could become infected and act as a reservoir of the disease, but that exposure might not constitute a serious threat to their survival. Whereas intact elk apparently do not show clinical evidence of disease following experimental exposure, in splenectomized elk anaplasmosis reportedly has caused severe clinical disease and even death.[10]

The two elk inoculated with 0.5 ml of frozen blood from a known bovine carrier were not infected. The infectivity of a blood sample stored in liquid nitrogen is related to the initial titer and the length of the storage period.[11] Since the infected bovine blood had been mixed with 6% DMSO and frozen for four weeks in liquid nitrogen according to previously described methods,[18] the apparent loss of infectivity following freezing was somewhat unexpected. However, there is some question about the optimal concentration of DMSO. As others have done,[11] we used 6% DMSO, but another investigator has reported that 31.2% is the optimal concentration.[12] Previous studies had shown that *A. marginale*

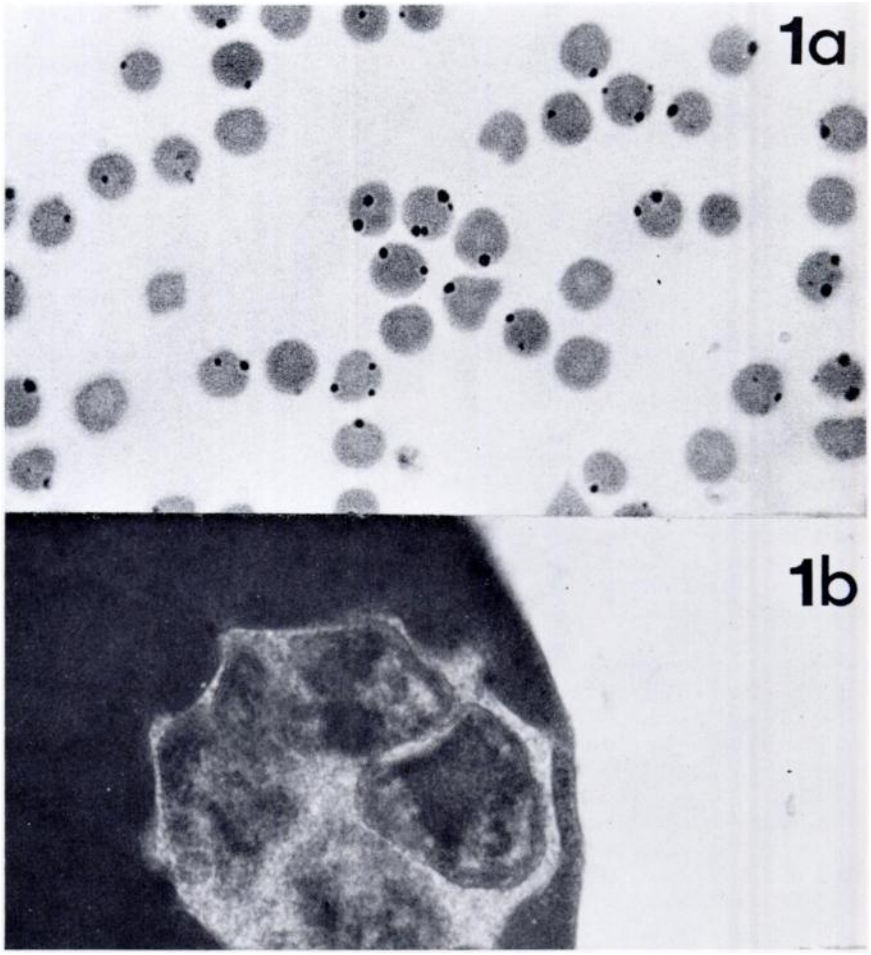

FIGURE 1. Erythrocytes from a splenectomized calf inoculated with infected elk blood. *a* Marginal Anaplasma bodies in parasitized bovine erythrocytes. Wright's stain; × 1,280. *b* Electron photomicrograph of a parasitized erythrocyte, showing an inclusion body of *Anaplasma marginale,* which is composed of several initial bodies. Lead citrate and uranyl acetate stain; × 67,000.

remained viable for long periods of time when blood from acutely infected animals was frozen in liquid nitrogen.[11,12] Blood was collected from a carrier animal in this study and the number of infective units per volume of blood is much less in the carrier than the acutely affected animal. Thus, freezing in liquid nitrogen could cause a loss of infectivity of a blood sample that contains only a few infective units, whereas, it would only slightly decrease the titer of a blood sample from an acutely affected animal.

Studies designed to adapt the bovine SRCA test to elk serum indicated that at

least 72 h had to lapse between the time of serum collection from clotted blood and performance of the test (serum aging) to eliminate false-positive reactions. When a 48 h time span was used as recommended for the test on cattle serum, many false-positive reactions occurred. In a previous study, when serums from free-ranging elk were only aged for 48 h and a different bovine serum factor source was used, false-positive reactions were recorded.[21] The difference between optimal aging periods for elk and cattle serums may be due to differences in the time required for inactivation of nonspecific agglutinins in the respective serums. This and previous studies on deer serums[7,16] suggest that when properly conducted the RCA test may prove to be a valuable tool for investigating anaplasmosis in certain wildlife species.

Carrier elk were not always identified correctly with the PRCA and SRCA tests, but then carrier cattle are not always correctly identified by the RCA test.[1] Approximately 18% of the samples from the infected elk were seronegative. While false-negative readings were occasionally recorded, false-positive readings were not observed. A possible interpretation for these findings could be that production of antibody in elk exposed to *A. marginale* usually is above the levels of sensitivity of the RCA test, but that on occasion antibody levels do not achieve this level and a false-negative reading is recorded. Considering the reported inadequacies of the CF test[10] the RCA test, despite its limitations, appears to be the serologic test of choice for evaluating the role of free-ranging elk as potential reservoirs of anaplasmosis in areas where cattle and elk cohabit an ecosystem.

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